Spatial control of inner ear neurogenesis by retinoic acid, Tbx1 and *her* genes

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Abstract

Sensory neurons are key mediators of the transduction of external stimuli from the ear to the brain essential for the sense of balance and hearing. Understanding when, where and how the sensory nervous system is assembled during development can provide insights on deafness and balance disorders. Here, I show in zebrafish that Her9 transcription factor is a key element in the regulation of otic neurogenesis. Loss of Her9 function leads to the ectopic expression of neurogenic genes *neurod* and *neurod4*. Moreover, I show that Her9 acts downstream of Tbx1, and both genes are activated by retinoic acid signaling emanating from the paraxial mesoderm and negatively regulated by Hedgehog signaling. Altogether, the data demonstrates a role of retinoic acid in axial patterning and the establishment of a neurogenic domain through Tbx1 and Her9. At later stages, retinoic acid has an additional role by regulating neuronal differentiation in the statoacoustic ganglion.

Resum

Les neurones sensorials de l'oïda interna són mediadores claus en la transducció dels estímuls externs des de l'oïda interna al cervell. Entendre a on, quan i com el sistema nerviós sensorial s'organitza durant el desenvolupament embrionari pot ajudar en l'estudi de les malalties neurosensorials. En el present treball, mostro en peix zebra que el factor de transcripció Her9 és un element clau en el control de la neurogènesi òtica i que Her9 es troba sota el control directe del factor Tbx1. A més, ambdos factors estan regulats de manera positva per la via de senyalització de l'àcid retinoic i negativament per la vía de hedgehog. En resum, la tesis demostra un paper de l'àcid retinoic en la regionalització axial del primordi òtic en l'eix anteroposterior i l'establiment d'un domini neurogènic a través de Tbx1 i Her9. En estadis tardans, l'àcid retinoic regula la diferenciació neuronal en el gangli estato-acústic.

Preface

To understand how sensory neurons are precisely generated during development is a crucial task. The correct spatiotemporal control of neurogenesis in the inner ear is essential for ensuring correct functioning of the sense of hearing and balance. During development, otic neurons are generated in the anteromedial domain of the otic anlagen. Previous studies have identified and characterized the intrinsic and extrinsic factors that positively promote otic neurogenesis. However, very little is known on how neurogenesis becomes restricted to only this anteromedial domain, and whether in addition to positive inputs, negative regulators control otic neurogenesis in a spatial manner.

This thesis identifies a new genetic cascade implicated in the axial anteroposterior patterning of the inner ear and explores their function in the establishment of a neurogenic and a nonneurogenic domain. Moreover, the results I have obtained and described here have been integrated and discussed with other data to draw a comprehensive picture on the molecular mechanisms involved in the spatiotemporal control of neurogenesis in the inner ear.

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INTRODUCTION

1. The vertebrate ear

1.1 Anatomy of the vertebrate ear

Hearing loss is one of the main chronic dissabilities of our time, affecting up to one third of the elderly people. It can be classified into conductive (CHL) and sensorineural hearing loss (SNHL), the last one affecting up to 1 in 500 newborns (Smith et al., 2005). CHL results from dysfunction in any of the mechanisms involved in the conduction of sound wave through the outer and middle ear, while SNHL results from the damaged auditory nerve or from the abnormalities in function of the cells that normally sensate the sound stimuli. It can be congenital or acquired, due to the age-related changes caused by drugs that are toxic for the auditory system, viruses, tumors and noise exposure.

The sense of hearing mediates our communication with the exterior world and thus improves the quality of everyday life. However, the sense of balance is the one that is essential, its significance for us probably being the most underestimated among all the senses we dispose of. Moderate dysfunction of sense of balance results in vertigo (the sensation of spinning) and nystagmus (flickering of the eye), while severe cases include disequilibrium, characterized by frequent falls in a specific direction, or a complete loss of the ability to stand (Sando et al., 2001).

Both, sound and equilibrium stimuli, are perceived by the ear. The vertebrate ear is composed of external, middle and inner ear (Fig. 1), the last one being directly responsible for the sensory function.

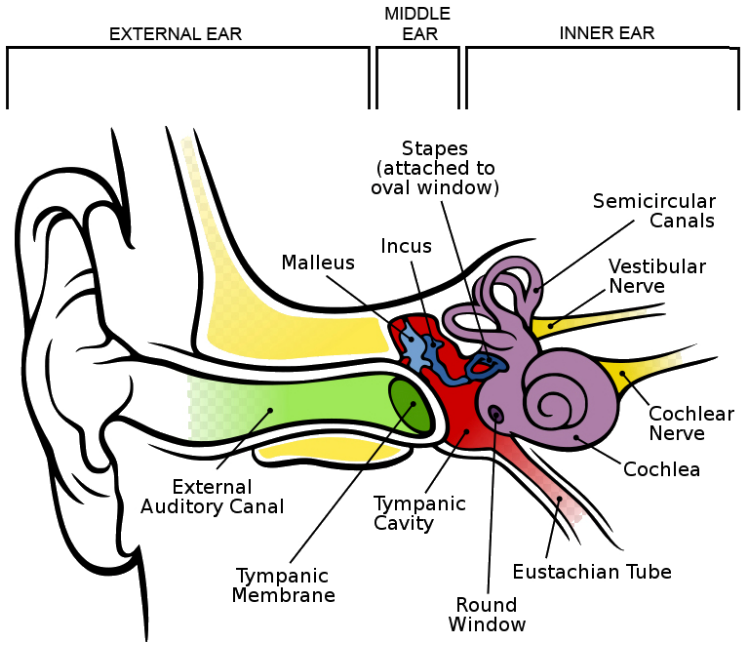


Figure 1. The vertebrate ear.
The vertebrate ear is composed of external, middle and inner ear. The external ear is a sound-collecting tunnel composed of the auricle and the external auditory meatus (light green), and it is bound to the middle ear by tympanic membrane (dark green). The middle ear connects external and inner ear. It is composed of tympanic cavity and Eustachian (auditory) tube (red). Within tympanic cavity, three auditory ossicles of mesenchymal origin: malleus, incus and stapes (blue), mediate transformation of sound waves into mechanical vibrations of the fluid contained within the inner ear (purple). The inner ear converts these mechanical stimuli to the electrical impulses, which are sent to the brain by vestibular and auditory nerves (yellow on the right), where these informations are interpreted and an adequate response is prepared. Modified from Web Images.

From now on I will refer only to the anatomy and development of the inner ear, which is the subject of this thesis.

In addition to the perception of the gravistatic stimuli, the sense of equilibrium requires correct perception of movements in all three dimensions. To acomplish this function, the inner ear evolved as a highly complex three-dimensional structure, called also the membranous labyrinth, with separate domains of vestibular and auditory function. The vestibular portion occupies the dorsal part of the inner ear and is evolutionary conserved among vertebrates (Fig. 2). It is tipically composed of three orthogonally positioned semicircular canals and an utricular pouch. The three cristae, sensory domains responsible for detection of the angular acceleration, are located in ampullar connections of each of the canal with the utricular pouch. The utricular macula, another sensory organ of the vestibular system, is placed in the utricular pouch and is responsible for detection of linear acceleration in the horizontal axis. (Bever and Fekete, 2002).

The ventral portion of the inner ear consists of saccular and lagenar pouches, which contain sensory domains, the maculae, responsible for detection of vestibular stimuli or both vestibular and auditory stimuli, as it is the case for the saccular macula in zebrafish. An additional ventral structure, containing a sensory domain specialized for the perception of the auditory stimuli, appears in birds (basilar papilla) and mammals (organ of Corti).

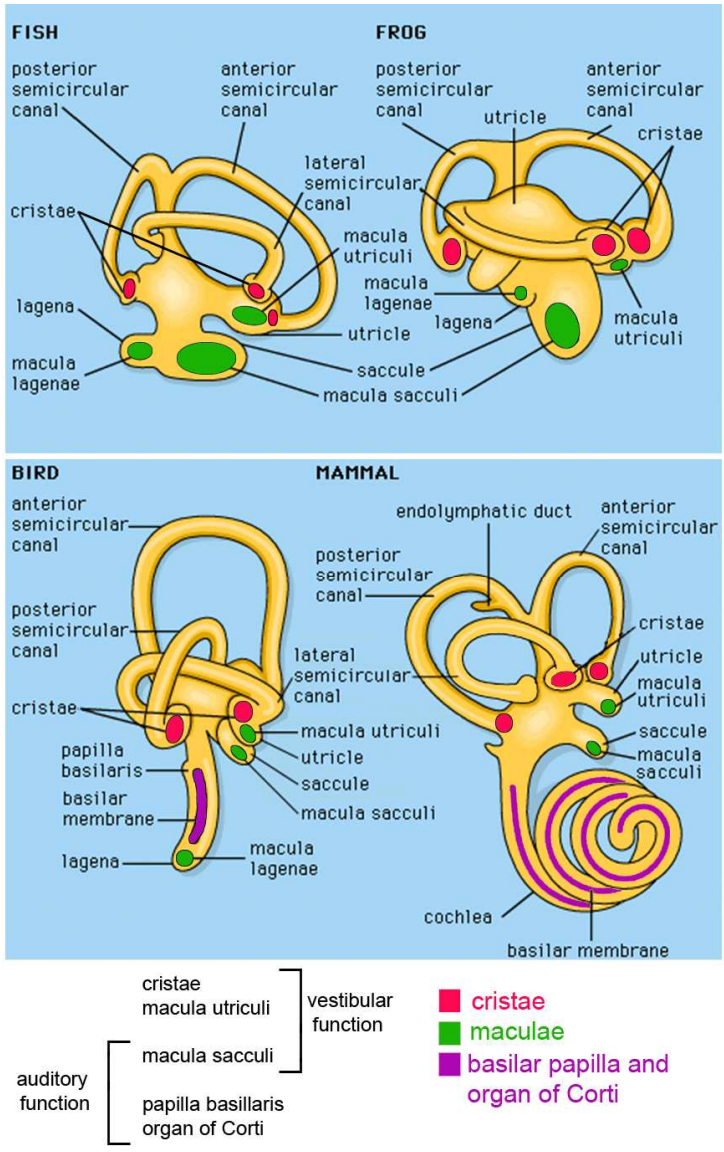


Figure 2. Schematic drawings of the adult inner ear in different vertebrate species.
Dorsal, vestibular part of the inner ear, composed of the semicircular canals and the utricle, is conserved among zebrafish, frog, chick and mouse. Ventral, auditory part shows a prominent evolution, and in mammals it includes a highly complex cochlea. Lateral views, anterior is to the right, dorsal is up. Modified from Web Images.

The capture of sensory stimuli occurs within the specialized sensory epithelia found in discrete regions of the inner ear. These sensory domains, cristae and maculae, have conserved cell composition and organization. In general, two main cell types constitute each of the domains: mechanosensory hair cells and non-sensory supporting cells (Fig. 3).

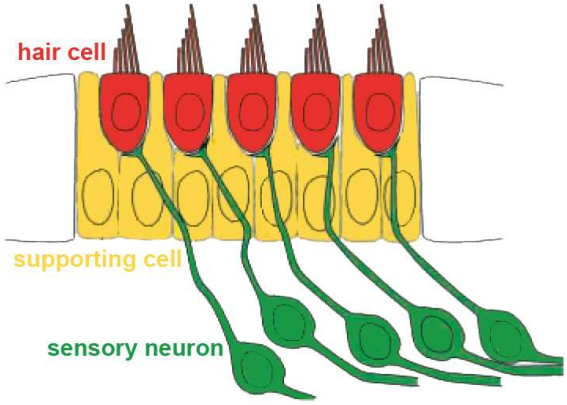


Figure 3. Ear sensory patch.
The functional sensory unit consists of hair cell (red) and supporting cell (yellow), arranged in a mosaic fashion within the sensory epithelia, and of sensory neuron (green), whose soma is located within the SAG positioned adjacent to the inner ear. Sensory neuron dendrites innervate hair cells within the epithelium. Adapted from Adam et al., 1998.

Mechanosensory hair cells act as receptors and transducers of mechanical stimuli. Each hair cell dispose of the stereociliary bundle on its apical surface, and this bundle is attached to the mobile gelatinous structures that overlie each of the cristae (cupulae) and maculae (otolithic membranes). It is the movement of these structures that moves the stereocilliary bundle, and consequently “excitates” hair cell through the opening of

mechanosensitive ion channels. On the other side, hair cells synapse with sensory neurons, whose somae are grouped within the statoacoustic ganglion (SAG), which is closely positioned to the membranous labyrinth. Neuronal axons form the statoacoustic (VIIIth) cranial nerve that branches and projects to the vestibular and cochlear nuclei located in the pons (metencephalon) and medulla (myelencephalon) of the brainstem. Nonsensory supporting cells vary greatly in morphological and functional specialization. In addition to providing mechanical scaffold for the sensory epithelium, these cells are thought to perform a stem cell-like function important for regeneration of hair cells, and are also involved in maintenance and survival of hair cells (Presson et al., 1996, Lanford et al., 1996, Haddon et al., 1999, Eddison et al., 2000, Stone and Rubel, 2000).

All three cell types, as well as all the nonsensory components of the inner ear, originate from a simple inner ear anlagen, called the otic placode.

Using zebrafish as a model for the study of the vertebrate inner ear development, I will next briefly describe the steps that lead to the formation of the mature inner ear. Zebrafish inner ear resembles that of mammals and chick, with the difference referring to the absence of a single sensory epithelia detecting only auditory stimuli. In zebrafish, auditory stimuli is captured by saccular macula that additionally detects linear acceleration, hence contributing to both, auditory and vestibular, function.

1.2 Inner ear development

The inner ear development starts with the specification of the otic field, which progressively develops into the otic placode. In zebrafish, the otic placode appears at 10 ss stage (14 hpf) as a simple ectodermal thickening adjacent to the developing caudal hindbrain. The otic placode cavitates and pinches of from the ectoderm, to finally form the closed epithelial structure, called the otic vesicle or otocyst. It is considered that at this stage the otic field reaches the irreversible state of determination (Waddington, 1937, Jacobson, 1963, Swanson et al., 1990, Gallagher et al., 1996), defined as a property of the otic primordium to develop into the membranous labyrinth independently of the embryonic environment. Once the state of determination is achieved, the otic vesicle undergoes a period of extensive cell proliferation, which is under the control of different growth factors. Coupling extensive cell proliferation with complex morphogenetic changes will transform the otic vesicle into highly organized membranous labyrinth, with all its sensory elements placed at correct positions.

1.2.1 Otic neurogenesis

SAG neurons, mechanosensory hair cells and supporting cells all originate from the otic placode (Fig. 4). The role of positive regulators of the otic neurogenesis was investigated by numerous groups and it is well understood. The generation of otic neurons is a sequential process, which includes steps of neural specification, neuronal determination and neuronal differentiation. Initially,

neuronal progenitors can be detected at the otic placode/vesicle stage in the anterior part of the otic anlagen, as revealed by the expression of *Neurog1* (Ma et al., 1998, Andermann et al., 2002, Alsina et al., 2004). *Neurog1,* together with *NeuroD,* is considered to play essential roles in inner ear neurogenesis. Both genes encode proneural basic helix-loop-helix (bHLH) transcription factors which, upon heterodimerization with other bHLH proteins, activate transcription of the genes that contain a specific DNA sequence, known as the E-box. *Neurog1*, whose expression precedes that of *NeuroD,* is considered as a neuronal determination gene: when overexpressed it drives the formation of ectopic neurons (Ma et al., 1996, Perron et al., 1999, Olson et al., 1998). Mice lacking *Neurog1* lack all sensory neurons in the inner ear (Ma et al., 1998, Ma et al., 2000). *NeuroD* is a potent neuronal differentiation factor, as evidenced by its gain of function phenotypes that include conversion of nonneuronal cell fate into a neuronal fate in *Xenopus* (Lee et al., 1995). Mice lacking *NeuroD* exhibit a near-complete loss of cochlear ganglia and a significant loss of vestibular ganglia (Kim et al., 2001). The loss of neurons occurs at later stages of neuronal maturation, which include neuronal differentiation and survival (Liu et al., 2000, Kim et al 2001).

The selection of neuronal precursors within the neurogenic domain is mediated by Notch signaling pathway (see BOX 1 and Fig. 5). Cells expressing *Neurog1* upregulate the expression of Notch ligand Delta, and the Delta-Notch interaction between neighbouring cells leads to the release of the Notch intracellular domain, leading to the final activation of the proneural repressors in the cells

adjacent to the *Neurog1* positive neuronal precursors. This mechanism is named lateral inhibition.

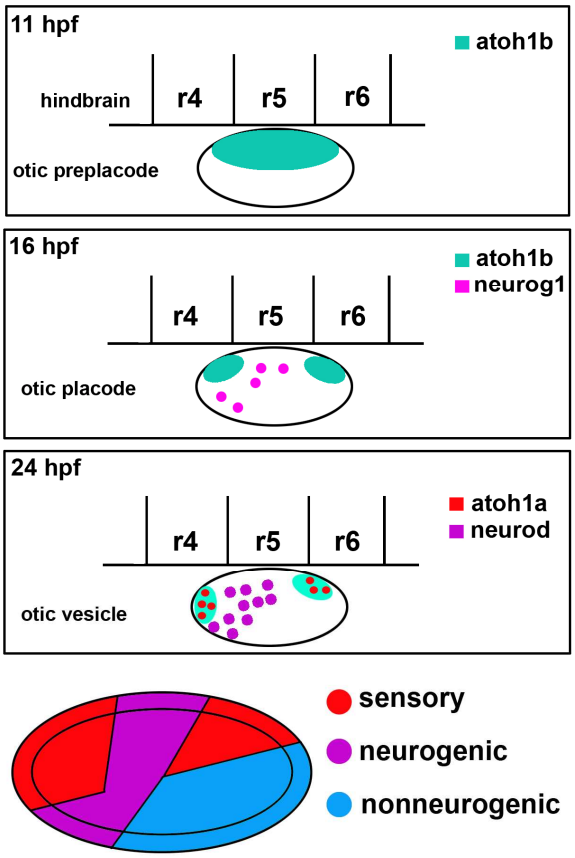


Figure 4. Establishment of the otic neurogenic and sensory domains in zebrafish.
Above: The specification of prosensory anlagen starts with the expression of proneural gene *atoh1b* (dark green) at the otic preplacode stage (11 hpf). This continuous domain resolves into two patches at otic placode stages (here shown at 16 hpf). In parallel, neurogenic domain is specified and first *neurog1* positive cells appear (pink spots at 16 hpf). Further development of the sensory hair cells depends on the activation of *atoh1a* gene in some of the cells within each of the patches, at otic vesicle stages (shown here at 24 hpf, red spots).
Below: The expression patterns of *atoh1b/1a* and *neurog1/neurod* proneural genes permit schematic drawing of the 24 hpf otic vesicle patterned into sensory (red), neurogenic (purple) and nonneurogenic (blue) domains. Anterior is to the left, medial is up.

BOX I: Notch signaling pathway

The Notch signaling pathway regulates a number of developmental processes, being its role in binary cell fate decisions one of the most extensively studied. The core of the pathway consists of DSL (Delta, Serrate, LAG-2) family of ligands, Notch receptors and downstram targets. Notch proteins are single-pass transmembrane receptors for the DSL family of single-pass transmembrane ligands.

The pathway is triggered when the ligands expressed on the surface of neighboring cells interact with Notch receptors. Upon ligand binding, Notch receptor undergoes proteolytic cleavages that lead to the release of the Notch intracellular domain (NICD) from the plasma membrane. The NICD translocates to the nucleus and associates with the DNA-binding transcription factor RBP-J. This converts RBP-J from a transcriptional repressor to an activator. In this process, NICD, RBP-J and Mastermind-like proteins assemble on target DNA and recruit transcriptional coactivators. The Notch targets include members of the *Hairy* and *Enhancer of split* family of transcriptional repressors (Hes in mammals, Her in zebrafish), which subsequently repress the transcription of proneural genes such as *Mash1* (reviewed in Kageyama et al., 2007, Kageyama et al., 2008).

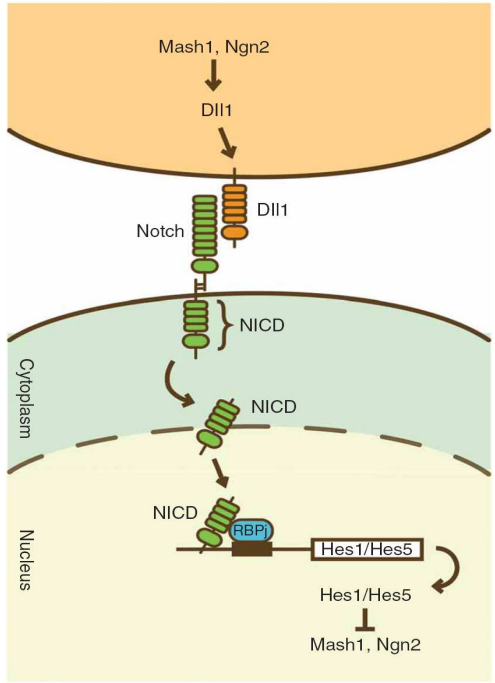


Figure BOX I. Simplified schematic representation of the core of the Notch signaling pathway. Taken from Kageyama et al., 2008.

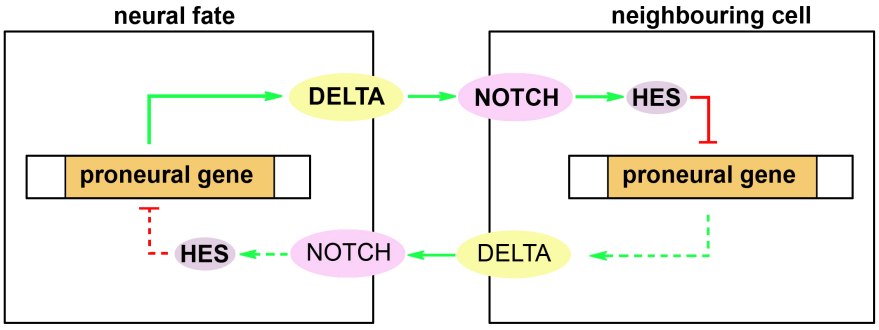


Figure 5. Lateral inhibition by Notch signaling pathway.

Schematic drawing illustrating the mechanism of lateral inhibition. Cell expressing a proneural gene will activate *Hes* target genes in the neighbouring cell, as a result of DSL-Notch binding on the surface of the two cells. The activation of *Hes* prevents the cell to adopt the same fate as its neighbour, by repressing the transcription of proneural gene. Modified from Bertrand et al., 2002.

Among the factors involved in otic neural specification, which precedes neuronal determination step, members of the Fgf family are thought to play the main role. In zebrafish *ace* (Fgf8 null) mutants SAG is smaller and there are less *neurog1* positive cells within the otic epithelium (Léger and Brand). When *ace* mutant embryos were injected with morpholino designed to block *fgf3* translation, *neurog1* positive cells in the otic epithelium could not be detected and, subsequently, SAG did not formed. Similarly, blocking Fgf signaling in chick decreased the number of *Neurog1* and *NeuroD* expressing cells within the otic anlagen (Abelló et al., 2010).

Otic epithelial neuroblasts will delaminate to form the SAG. After a period of cell proliferation, SAG neurons will differentiate to

innervate back sensory organs of the inner ear. Members of the LIM/homeodomain family of transcription factors, Islet1 and Islet3, mark the delaminating neuroblasts and differentiated SAG neurons, respectively.

1.2.2 Otic sensory development

Sensory epithelia of the zebrafish inner ear develop not only in a spacially restricted and well defined otic domains, but also in highly controlled temporal order. The two sensory maculae appear before the sensory cristae, and these appear before the lagenar macula and macula neglecta. As it is the case for otic sensory neurons, the first step in the development of the sensory epithelia is the specification of a prosensory anlagen within the otic epithelium (Fig. 4). For the sensory maculae this specification coincides with the expression of *atoh1b* and *atoh1a* genes at the opposite poles of the otic AP axis, at placodal and early vesicle stages respectively, as shown by Riley and colleagues (Millimaki et al., 2007). The same group placed Fgf signaling upstream of otic *atoh1b* and *atoh1a* expression, implicating this signaling in the otic prosensory specification. They also showed that active Notch signaling is required to resolve the initially continous domain of *atoh1b* expression into the two prosensory patches: when Notch is blocked transiently at relevant stages, the two prosensory domains appear later and are bigger than in control animals.

This role of Notch differs from that one discovered from the analysis of *mindbomb* mutants, in which Notch signaling is impaired (Haddon et al., 1998, Haddon et al., 1999). In these mutants, the two sensory maculae consists of only supernumerary

hair cells, with no supporting cells, suggesting the role of Notch in lateral inhibition-mediated selection of hair cell precursors.

As it happens with the neuronal development, after the period of prosensory domain specification individual cells will develop as either hair cells or supporting cells. Hair cell commitment starts with the upregulation of the *atoh1* expression by some cells within the prosensory group. The first two hair cells to appear are specified by the Atoh1b bHLH transcription factor, while later forming hair cells depend on Atoh1a (Millimaki et al., 2007). Inhibition of both *atoh1a* and *atoh1b* results in a complete loss of macular hair cells, while overexpression of Atoh1a causes appearance of ectopic hair cells (Millimaki et al., 2007), suggesting that this gene acts as a hair cell determination gene. As described above, the hair cell/supporting cell fate decision requires lateral inhibition through the Notch signaling mechanism, which involves the interaction between Delta ligands and Notch receptors among the neighbouring cells (Fig. 5).

Following commitment, developing hair cells begin to express a set of transcription factors that are required for hair cell survival and differentiation. One of the first to be detected is a Pou-domain transcription factor Brn3c (also known as Pou4f3). Deletion of Brn3c leads to the ablation of the inner ear hair cells in mammals (Erkman et al., 1996, Xiang et al., 1997).

2. Patterning of the inner ear

2.1 Otic assymetries

The morphological complexity of the inner ear arose from the necessity to detect movements in all three dimensions. In order to get a fully functional organ, a complex spatiotemporal pattern of cell fates is achieved by coupling morphogenesis and cell fate specification during development. Initially homogenous otic field progressively becomes compartmentalized, such that a group of cells within a single compartment will express the same combination of genes and eventually will develop independently from adjacent territories. The initial compartmentalization of the inner ear is tightly connected with early cell fate decisions and includes the definition of neural, sensory and nonsensory territories.

Molecular studies revealed assymetric patterns of gene expression in the early otic anlagen, and this foreshadows the complexity of the mature inner ear, which displays clear assymetries about all three body axes: anteroposterior (AP), mediolateral (ML) and dorsoventral (DV). For the purpose of this thesis, I will explain in detail what is known about the otic AP patterning, while the further information about the extrinsic patterning signals and intrinsic interpreters of the otic ML and DV assymetries can be found in detailed reviews from Whitfield and Hammond, 2007, and Bok et al., 2007.

2.1.1 Anteroposterior otic patterning

The definition of a neurogenic compartment coincides with the definition of the assymetry along the AP axis in the developing inner ear. Neurogenesis occurs only in the anterior domain of the otic vesicle, and this domain is characterized by the expression of a number of signaling molecules and transcription factors, such as different Fgfs, Neurog1, Hes5, NeuroM and NeuroD, including Notch ligand Delta1 and Notch modulator Lunatic Fringe (Henrique et al., 1995, Adam et al., 1998, Anderman et al., 2002, Alsina et al., 2004, Abelló et al., 2007). A set of genes is transcribed complementary to the neurogenic domain and these define the nonneurogenic posterior otic territory, and these include *Lmx1*, *Irx1*, *Hairy1* and *Tbx1* (Alsina et al., 2004, Raft et al., 2004, Abelló et al., 2007, Abelló et al., 2010).

Although the assymetry along the otic AP axis begins with the expression of the above mentioned neural markers at the otic placode/cup transition stage, this axis in chick is not fixed until mid-cup stage, as shown by otic transplantation experiments (Wu et al., 1998, Bok et al., 2005, Bok et al., 2011). In these experiments either the otic region or the hindbrain were rotated along the AP axis, and the morphology of the inner ear, or expression pattern of a set of genes, was analyzed later on. Rotation of the otocyst had a strong impact on the otic AP axis, while rotations of the hindbrain perturbed mainly DV otic patterning. This is surprising, since it was thought that hindbrain, clearly assymetric along the AP axis, could be the source of signals that pattern the AP axis of the juxtaposed

otic territory. In chick, otic territory is specified and develops adjacent to the rhombomeres 5 and 6 of the hindbrain, and it was postulated that the boundary between the two rhombomeres could impact on the putative boundary between anterior and posterior otic domains. In mouse mutant for *kreisler*, a gene transiently expressed in the r5 and r6 of the developing hindbrain, some anterior markers are expanded posteriorly (*NeuroD*, *Lfng*, *Delta1*), while the posterior marker *Lmx1* was reduced (Vázquez-Echeverría et al., 2008), sugesting that r5 and r6 indeed play a role in the otic AP patterning in mouse.

The posterior otic identity is thought to be carried by T-box transcription factor Tbx1, which thus would be an intrinsic factor that interpretes the positional AP patterning information from the external signals (reviewed in Abelló and Alsina, 2007, Whitfield and Hammond, 2007, Bok et al., 2007). To be considered as an intrinsic interpreter of AP information, and not only as a marker of the position within the vesicle, the deletion of the intrinsic factor should recapitulate the effects that external signals perturbation has on otic patterning (the def. taken from Whitfield and Hammond, 2007). Indeed, in mouse *Tbx1*$^{-/-}$ mutant embryos expression of some anterior markers, such as *Lfng* and *NeuroD*, is expanded posteriorly, and consequently the SAG rudiment is duplicated (Vitelli et al., 2003, Raft et al., 2004, Arnold et al., 2006). *Otx1, Otx2* and *Gsc*, all of them marking the posteroventral otic territory, are lost from these embryos (Vitelli et al., 2003, Raft et al., 2004). Moreover, overexpression of Tbx1 in mouse downregulates otic *NeuroD* expression (Raft et al., 2004), arguing against the idea that defects in *Tbx1*$^{-/-}$ mutants are outcome of a loss of the otic tissue, and that there is no real anteriorisation of the inner ear (Xu et al.,

2007). While the roles of other posterior genes in regulating otic AP patterning need to be analysed, *Tbx1* stands as a master candidate for this function, since its expression is the first obvious manifestation of this assymetry, and it starts before the expression of the other above mentioned posterior genes.

Zebrafish offers more information about the possible roles of the hindbrain in the otic AP patterning than chick and mouse. Initially, zebrafish otic field is specified adjacent to the rhombomere 4 of the hindbrain, but later becomes positioned next to the rhombomere 5, with anterior and posterior poles being adjacent to the r4 and r6, respectively (Kimmel et al., 1995, Riley et al., 1997). In zebrafish mutants for *vhnf1* (encodes for transcription factor expressed in r5 and r6 of the hindbrain) and *valentino* (zebrafish homologue of mouse *kreisler*), anterior markers *pax5, hmx3* and *fgf8* are expanded posteriorly, while some posterior markers, such as *follistatin* and *zp23* are missing (Kwak et al., 2002, Lecaudey et al., 2007). In these mutants, rhombomeres 5 and 6 are misspecified and form a single rhombomere X with joint caracteristics of r4, r5 and r6. Rhombomeric boundaries are absent posterior to r3/r4 boundary, strongly claiming that correct caudal hidbrain patterning is crucial for establishment and/or maintenance of AP polarity in the inner ear.

In addition to the hindbrain and periotic ectoderm, midline tissues (notochord and floorplate) influence otic AP patterning via Hedgehog (Hh) signaling. In *chameleon* (*con*) and *slow muscle omitted* (*smu*) mutants, in which Hh signaling is impaired, or in

embryos treated with cyclopamine A (a potent pharmacological inhibitor of the Hh pathway) posterior otic domain is lost, concomitant with the occasional duplication of the anterior otic structures (Hammond et al., 2003, Sapède and Pujades, 2010).

2.1.2 Dorsoventral otic patterning

The mature inner ear is also assymetric along its DV axis: vestibular structures, including semicircular canals and utricular pouch, are located dorsally to the saccule and auditory organ of Corti/basillar papilla. The otic DV axis is influenced by hindbrain signals, as revealed from the analysis of hindbrain patterning mutants, such as *kreisler* and *Hoxa1*$^{-/-}$ (Choo et al., 2006, Pasqualetti et al., 2001). The role of hindbrain in the otic DV patterning can be explained by differential expression pattern of a set of signaling molecules along the DV hindbrain axis. These include Sonic hedhehog (Shh) signaling from the floor plate and members of the Wnt family expressed in the dorsal part of the hindbrain. Both gain- and loss of function studies confirmed the role of Shh in confering ventral otic identities in mouse, chick and zebrafish (Riccomagno et al., 2002, Riccomagno et al., 2005, Bok et al., 2005, Hammond et al., 2010), while gain- and loss- of Wnt signaling studies revealed its role in promoting the expression of the dorsal otic markers (Riccomagno et al., 2005).

Among the intrinsic otic interpreters of the DV assymetry, *Six1* singled out as a main candidate for confering the ventral otic identity: dorsal otic markers are expanded ventrally in *Six1* mutant mouse , while the expression of ventral markers is reduced or even

abolished (Ozaki et al., 2003, Zheng et al., 2003). On the contrary, several intrinsic factors redundantly regulate dorsal otic patterning: *Dlx5*$^{-/-}$*/Dlx6*$^{-/-}$ double mice mutants lack dorsal vestibular structures (Robledo and Lufkin, 2006), previewed by the reduced expression of dorsal otic markers.

2.1.3 Mediolateral otic patterning

The otic placode/vesicle is positioned adjacent to the developing caudal hindbrain, and it is attractive to think that hindbrain derived signals specify medial identity within the inner ear. This can be achieved in two possible ways. In first, a diffusible molecule from the hindbrain would act as a morphogen with decreasing activity along the otic ML axis, thus assigning different identities to medial and lateral tissues or, alternatively, signals from hindbrain and tissues lateral to otic field may counteract to limit each other´s domains of activity along the otic ML axis.

The role hindbrain plays in otic ML patterning was analysed using mouse mutant for retinoic acid (RA) synthesizing enzyme Raldh2. This mutant has impaired hindbrain segmentation, such that caudal hindbrain is shorter, *Krox20* expression is detected only in a single domain corresponding to the r2-r3, while the expression of r5-r8 markers, such as *kreisler* and *Hox3/4* group of genes, is missing (Niederreither et al., 2000). The otic vesicle of Raldh2$^{-/-}$ mutant embryos is smaller and lateralized, as revealed by the expanded expression domain of the otic lateral marker *Hmx3*, while the expression of *Pax2*, a medial otic marker, is lost (Niederreither et

al., 2000). The observed phenotype could be explained by the disturbed, hindbrain derived, Fgf signaling, proposed to specify medial otic domain. For example, in *kreisler* mutants, in which the Fgf signaling is attenuated in the caudal hindbrain, the medial otic expression of *Gbx2* is completely abolished, while lateral marker *Otx2* expanded medially (Choo et al., 2006). Consequently, endolymphatic duct is lost from these embryos, as it is the case for single *Fgf3*$^{-/-}$ mutants, pointing out to an important role of Fgf signaling in establishment of ML axis within the inner ear (Mansour et al., 1993, McKay et al., 1996, Choo et al., 2006). Here, one has to be careful, since Fgf ligands are also expressed in the otic epithelium, and the described phenotype could be an outcome of the abolished otic Fgfs exression.

The intrinsic factors shown, by loss of function studies, to regulate the development of medial and lateral otic domains are Gbx2 and Otx1/Otx2, respectively (Morsli et al., 1999, Lin et al., 2005).

In zebrafish, the first assymetrical expression of the otic genes appears along ML axis, and this includes the medial expression of *delta* and *atoh1b* genes at 14 hpf (Haddon et al., 1998, Millimaki et al., 2007). Soon after, the AP assymetry starts with the anterior expression of neurogenic markers *hmx3* and *neurog*1 at 16 hpf (Adamska et al., 2000, Andermann et al., 2002), while the DV assymetry seems to be the last one to appear in the inner ear.

When dealing with the otic axes, one has to bear two things in mind. First, otic axes do not correspond strictly to the vertebrate body axes, due to an angular displacement of the otic placode

relative to the body midline. However, to simplify the explanation of the results, this will be ignored in the following Chapters.

Second, the assymetries along the otic axes not always appear strictly along the only one axis, but instead the expression patterns are more complex and at the same time abutte different axes. In this sense, it would be more correct to say that neurogenic domain, for example, is established in anteromedial and not only in the anterior otic domain. This fact will not be ignored in the following Chapters, hoping not to contribute to potential confusions.

3. *Hes* gene family

3.1 Basic helix-loop-helix proteins

The basic helix-loop-helix (bHLH) proteins form a group of more than 130 transcriptional regulators, found in organisms ranging from yeast to humans (reviewed in Atchley and Fitch, 1997, Massari and Murre, 2000). They play key roles in many developmental processes, including neurogenesis, somitogenesis, pancreatic development and sex determination, acting on cell proliferation, cell differentiation and lineage commitment. Highly conserved HLH domain contains mainly hidrophobic residues and allows these proteins to form homo- and/or heterodimers. The dimerization motif consists of around 50 amino acids and forms two amphipatic α-helices separated by a loop of variable length. The basic domain, rich in basic residues, permits dimers to bind to the

conserved DNA hexanucleotide sequence CANNTG, called the E-box, found in the regulatory regions of the target genes (Fig. 6).

Based on the structure and DNA binding properties, bHLH proteins are further classified into several groups (Murre et al., 1994, Atchley and Fitch 1997, Fisher and Caudy, 1998, Kageyama et al., 2007). Briefly, transcriptional activators such as MyoD and Mash1 bind preferentially to the class A site (CANCTG), while bHLH-leucine zipper type proteins such as Myc and Max, bind to class B site (CANGTG). Class A and B sites both belong to E-box sequences (reviewed in Massari and Murre, 2000, Bertrand et al., 2002).

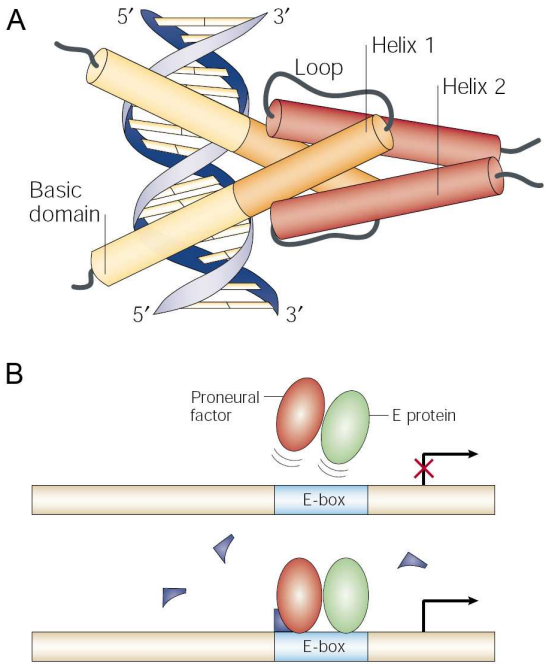


Figure 6. bHLH proteins: mode of action.
(A) Schematic representation of the structure of a bHLH dimer that is complexed to the DNA domain. **(B)** Proneural bHLH transription factors bind to the E-box DNA sequence and activate the transcription of the downstream gene. Modified from Bertrand et al., 2002

3.2 bHLH transcriptional repressors

3.2.1 bHLH repressors: the structure and mode of action

One class of bHLH proteins is not able to bind to the E-box, but instead binds to the N-box sequence (CACNAG) or class C site CACG(C/A)G. These bHLH genes encode nuclear proteins that act as transcriptional repressors, rather than as transcriptional activators. They belong to the family of *Drosophila hairy* and *Enhancer of split (E(spl))* genes and their homologues. In *Drosophila melanogaster*, there is only one *hairy* gene and seven *Enhancer of splits* (Fischer and Gessler, 2007). In these proteins, the bHLH domain is followed by two additional α-helical stretches, called the Orange domain (Fig. 7A). This domain serves for the regulation of the selection of the partner for dimerization, and it acts as a repressor when fused to DNA binding domain (Dawson et al., 1995, Taelman et al., 2004, Kageyama et al., 2007).

The specificity to bind to the N box or class C sequence is thought to arise from the presence of proline residue within the basic domain of these proteins. The acquisiton of proline in this domain led to the loss of the specificity for the binding to the E-box (Davis et al., 1990). Another conserved feature of this bHLH subfamily is the presence of C-terminal WRPW tetrapeptide (Trp-Arg-Pro-Trp), able to recruit the co-repressor Groucho (Fisher and Caudy, 1998), leading to the repression of the transcription (Fig. 7B).

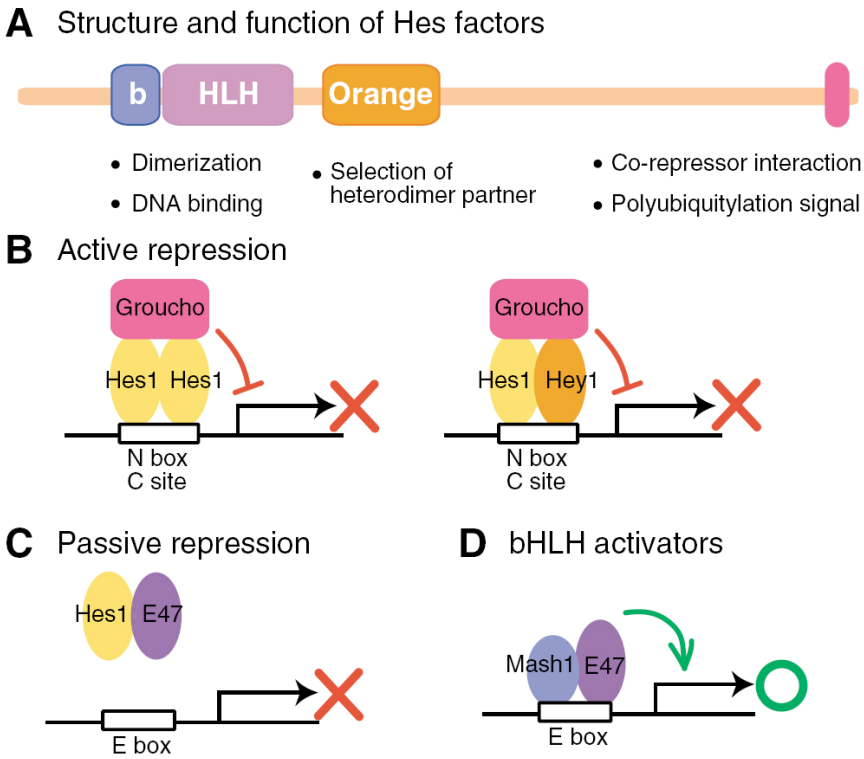


Figure 7. bHLH repressors: structure and mode of action.
(A) Schematic representation of the bHLH repressor proteins, showing the conserved bHLH (blue, purple), Orange (orange) and WRPW (pink) domains. **(B)** Active repression by bHLH proteins assumes recruitment of corepressor upon homo- or heterodimer binding to the N-box or class C site sequence in the regulatory region of the downstream gene. **(C)** Passive repression by Hes proteins occurs through the formation of inactive heterodimers with proneural bHLH activators or their heterodimeric partners, such as E47. **(D)** Proneural Mash1 protein activates the transcription of downstream gene by binding to the E-box, upon heterodimerization with E47. Modified from Kageyama et al., 2007.

In *Drosophila*, *Enhancer of split* genes are activated by Notch, and together with *hairy* negatively regulate neuronal differentiation by inhibiting proneural bHLH activators like Atonal, Daughterless and those of Achaete-Scute complex (Ohsako et al., 1994, Van Doren et al., 1994). Notch-independent *hairy* represses proneural function in the *Drosophila* ectoderm, acting as a prepatterning gene to promote a nonneural fate over a large territory, whereas the inhibition of proneural function by *Enhancer of split* [*E(spl)*] regulates the number of neuronal precursors in the neuroectoderm (Campos-Ortega and Jan, 1991, Campos-Ortega, 1993).

Hes/her genes are mammalian/zebrafish homologues of *Drosophila* genes *hairy* and *E(spl).* There are 7 members of the family in mammals (with only Hes1 being Hairy-like, while other members are E(spl)-like) and at least 15 members in fish (Fig. 8) (Akazawa et al., 1992, Sasai et al., 1992, Ishibashi et al., 1993, Pissarra et al., 2000, Bessho et al., 2001, Sieger et al., 2004, Gajewski et al., 2006, Kageyama et al., 2007). They share the same basic structure with *Drosophila*'s homologues, having conserved bHLH, Orange and WRPW domains. Another group of *Hes*-related genes are found in vertebrates. These are named *Hey* genes, and their protein products differ from HES/Her in two aspects: a glycine residue instead of the proline in the basic domain, and a YRPW motif instead of the WRPW. In addition, they have another conserved carboxy-terminal motif, TE(V/I)GAF, which is absent in HES proteins (reviewed in Iso et al., 2003). There are 3 *Hey* genes in both mammals and zebrafish.

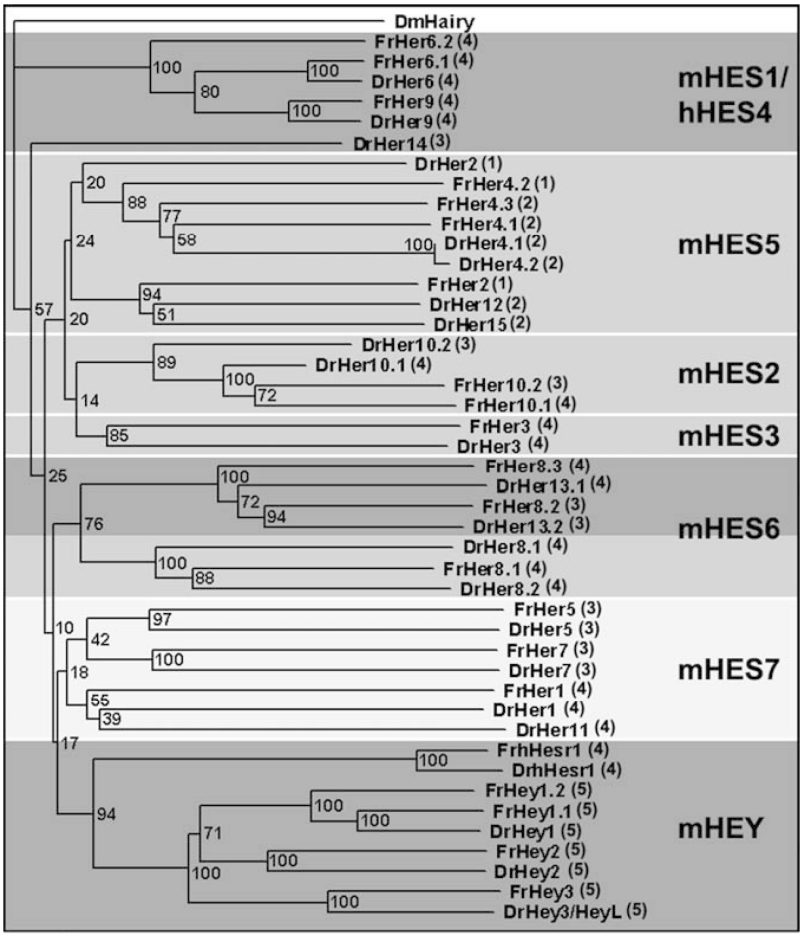


Figure 8. Phylogram of the different teleost Hairy and Enhancer of Split proteins and *Drosophila*'s hairy.
Dm, *Drosophila melanogaster,* Dr, *Danio rerio*, Fr, *Takifugu rubripes*. The different grey shadings reflect the extent of similarity to the respective mouse Hes proteins, with dark grey being the highest similarity and white being the lowest. Taken from Sieger et al., 2004.

All *Hes* and *Hey* genes studied so far were shown to act as repressors, except *Hes6*, which was shown to inhibit the repressor activity of other HES proteins, by forming nonfunctional heterodimers with them (Sasai et al., 1992, Bae et al., 2000,

Koyano-Nakagawa et al., 2000, Gratton et al., 2003). Hes factors repress transcription by two different mechanisms: actively and passively (Fig. 7B,C) (Sasai et al., 1992, Kageyama et al., 2007). Active repression assumes that upon binding to the N-box or class C sequence, the WRPW domain of the bHLH factor interacts with corepressors encoded by *Transducin-like E (spl)* (TLE) genes/*Groucho-related* gene (*Grg*), which are homologues of *Drosopila*'s Groucho, known to repress transcription by recruiting histone deacetylases, that inactivate chromatin (Fisher et al., 1996,Grbavec and Stifani, 1996, Grbavec et al., 1998, McLarren et al.,, 2001, Nuthall et al., 2002).

Passive repression is possible because repressor bHLH proteins can form heterodimers with proneural bHLH activators, being these dimers unable to bind to the E-box (Fig. 7D) (Sasai et al., 1992, Akazawa et al., 1992).

3.2.2 Hes roles and targets

Hes/Hey proteins play multiple roles in diverse developmental processes, such as somitogenesis, hematopoiesis and pancreatic development (reviewed in Massari and Murre 2000, Iso et al., 2003, Fischer and Gessler, 2007, Kageyama et al., 2007), but their role in neural development is one of the best understood. Basically, their function was associated with the Notch signaling to regulate binary cell fate decisions and maintenance of progenitor cells. Mouse mutants for *Hes1* gene have severely disrupted brain development and eye anomalies, due to the premature neuronal

differentiation in these organs (Ishibashi et al., 1995, Tomita et al., 1996, Hatekayama et al., 2004). During neural development, neuroepithelial cells, which form the neural plate, will become radial glial cells, which will divide assymetrically to give rise to another radial glial cell and a neuron or neuronal precursor (reviewed in Fishell and Kriegstein, 2003, Götz and Huttner, 2005, Kageyama et al., 2007). The maintenance of radial glial cells during a prolonged period of time is important, since from these cells different types of neurons will be born at different times. Both neuroepithelial and radial glial cells are considered to be embryonic neural stem cells, and express *Hes* genes (Hatakeyama et al., 2004). Ectopic overexpression of *Hes1* or *Hes5* inhibits neuronal differentiation and maintains the radial glial cell identity (Ishibashi et al., 1994, Ohtsuka et al., 2001). Vice versa, these neural stem cells prematurely differentiate into early born neurons in double *Hes1/Hes5* and triple *Hes1/Hes3/Hes5* mutant mice (Ohtsuka et al., 1999, Ohtsuka et al., 2001, Hatakeyama et al., 2004).

The central nervous system (CNS) is organized in compartments separated by boundaries, which are the places of restricted cell mixing and delayed or absent neurogenesis. *Hes* genes are expressed in both compartments and boundaries, but specifically it was shown that *Hes1* levels are higher in boundaries (Baek et al., 2006). In the absence of *Hes1* and *Hes3* genes in mouse, or *her3/5/9/11* in zebrafish, proneural genes are prematurely expressed in the midbrain/hindbrain boundary (Hirata et al., 2001, Geling et al., 2003, Geling et al., 2004, Ninkovic et al., 2005, Baek et al., 2006), arguing for the idea that the function of the *Hes* genes is important for the maintenance of this nonneurogenic organizer (Fig. 9).

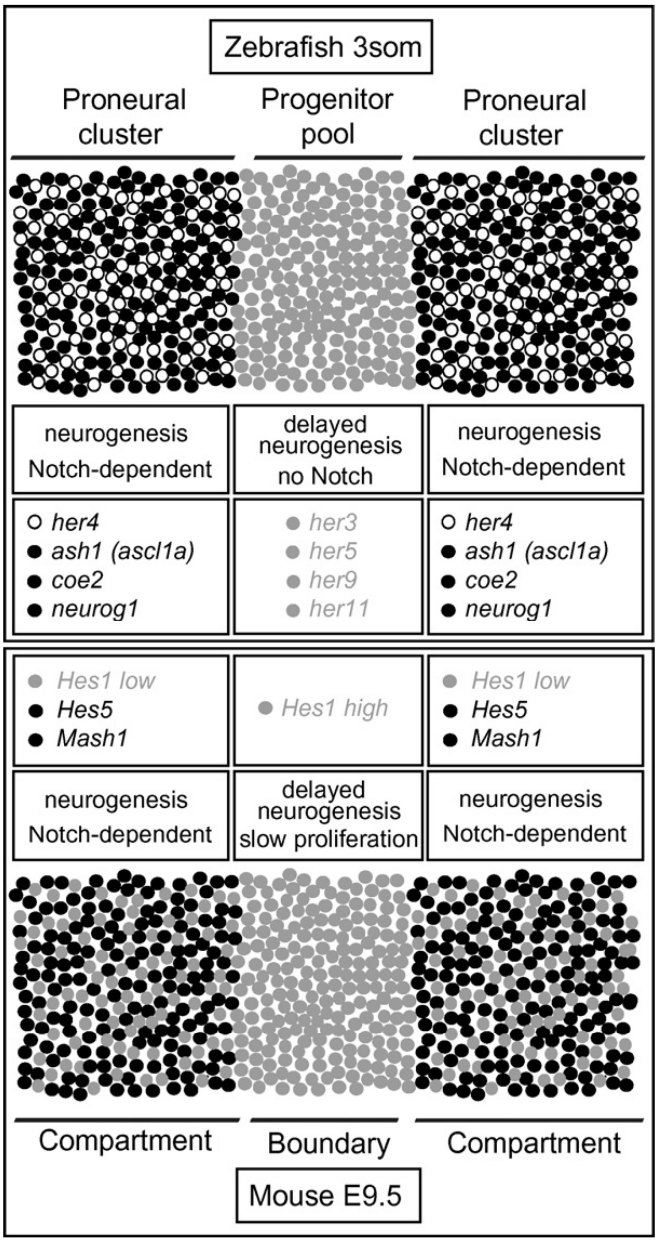


Figure 9. The differential roles of different *Hes/her* genes in the nonneurogenic boundaries and neurogenic compartments of the CNS are conserved in mouse and zebrafish.
High Hes1 levels, in mouse, or combined expression of several *her* genes in zebrafih midbrain/hindbrain boundary define a slow dividing progenitor

cells, with repressed proneural gene expression. Low Hes1 levels (in mouse) and Her4 (in zebrafish) within the neurogenic CNS compartments mediate selection of proneural progenitors by the lateral inhibition mechanism. Taken from Stigloher et al., 2008.

Targets of Hes proteins include Mash1, Math1 and E47, all of them being bHLH activators, homologous to the *Drosophila*'s proteins encoded by *achaete-scute* complex, *atonal* and *daughterless*, respectively (Johnson et al., 1990, Sasai et al., 1992, Ishibashi et al., 1994, Akazawa et al., 1995). Mash1 forms heterodimers with E47 and activates neuronal-specific gene expression by binding to the E-box, thus it positively regulates neuronal differentiation (Johnson et al., 1992). HES proteins repress *Mash1* through the active mechanism, by binding to the N-box sequence in *Mash1* promoter (Sasai et al., 1992), but also can passively inhibit the transcription from the E-boxes by forming heterodimers with E47 (Sasai et al., 1992, Akazawa et al., 1992). Direct suppresion of human achaete-scute homolog-1 (hASH1) by Hes1 was found in lung cancer cell line, and this repression goes through the binding to the class C site in hASH1 promoter (Chen et al., 1997). In conclusion, *Hes* genes regulate the maintenance of stem cell and progenitors, and thus regulate the timing of differentiation by antagonizing the effects of proneural bHLH activators. In addition to the role in neuronal development, Notch-Hes pathway also regulates the maintenance of stem cells and progenitors in digestive organs, such as pancreas. In developing pancreas, *Hes1* represses *Ngn3* (homolog of *Drosophila*'s *atonal*) and *Ptf1a*, genes that promote the differentiation of the endocrine and exocrine pancreatic cells (Jensen et al., 2000, Esni et al., 2004).

Hes proteins appear to have two other main characteristics: first they have short half-lives, and second it was shown that Hes1 and Hes7 negatively regulate their own expression by binding to the N-box within their own regulatory sequences (Takebayashi et al., 1994, Hirata et al., 2002, Zeiser et al., 2008). This is the molecular basis of the periodical appearance of the somites during somitogenesis. Each pair of somites appears with a regular periodicity, and this time period correlates with the oscillatory expression of *Hes1* and *Hes7* in mouse, *Hairy1* (chick homolog of Hes1) and *her4* and *her6* genes in zebrafish (Palmeirim et al., 1997, Hirata et al., 2002, Pasini et al., 2004, Masamizu et al.,2006) making them molecular determinants of this biological clock.

Oscillatory expression of *Hes1* was also discovered in pluripotent embryonic stem cells (Kobayashi et al., 2009, Kobayasi and Kageyama, 2010). These cells can give rise to a different types of differentiated cells, the mechanism in part being is controlled by *Hes1*. Oscillatory expression of *Hes1* means that some cells within the same group will have low Hes1 levels, while others will have high Hes1 levels. Those cells expressing low levels of Hes1 tend to differentiate into neural cells, while the cells expressing high levels of Hes1 tend to increase the expression of the mesodermal marker *Brachyury* (Kobayashi et al., 2009).

Different levels of Hes1 within the single cell can determine which fate cell will adopt, as described above, but also this can be a main factor in regulating cell proliferation versus cell differentiation decision. Low levels of *Hes1* were shown to promote cell

proliferation through the downregulation of cell cycle inhibitor $p27^{Kip1}$ (Murata et al., 2005, Murata et al., 2009), while high levels of Hes1 inhibit cell cycle progression probably through the regulation of other cell cycle regulators, such as E2F-1 (Hartman et al., 2004, Baek et al., 2006).

3.2.3 Regulators of *Hes/her* genes

The classical view of *Hes* genes as targets of Notch signaling (Jarriault et al., 1995., Schroeter et al., 1998, Struhl and Adachi, 1998, Artavanis-Tsakonas et al., 1999, Yoon and Gaiano, 2005) does not stand anymore for all the members of the *Hes* family. *Hes2, Hes3* and *Hes6* were found to be Notch-independent (Nishimura et al., 1998, Koyano-Nakagawa, 2000). Moreover, *Hes1* can respond to Sonic hedgehog, in addition to the Notch signaling (Ingram et al., 2008). In the chick retina, *Hairy1* depends on Wnt signaling, while in zebrafish floor plate and interproneuronal stripes within the developing neural plate, *her9* (zabrafish orthologue of *Hes1*) is regulated by Nodal and BMP signaling, respectively (Bae et al., 2005, Latimer et al., 2005, Kubo and Nakagawa, 2009). In conclusion, it seems that *Hes* genes can change the competence to respond to different signals during time, or alternatively they can respond to different signals depending on the tissue where they are expressed.

3.3 *Hes* genes in the inner ear development

Several Hes genes are expressed within the developing inner ear. In the early chick otic cup, *Hes5* and *Hairy1* (chick ortholog of mouse *Hes1*) are expressed in the neurogenic and nonneurogenic regions, respectively (Abelló et al., 2007). The expression of *Hes5* is complementary and mosaic to that of Notch ligand *Delta1*, suggesting a role of Hes5 in selection of the neuronal precursors within the neurogenic domain by the lateral inhibition mechanism. Later on, the expression of both *Hes5* and *Hes1* is detected in the supporting cells of the sensory epithelia (Shailam et al., 1999, Lanford et al., 2000, Zheng et al., 2000, Zine et al., 2001, Hartman et al., 2009), while *Hes6* is coexpressed with *Math1* in developing hair cells of the cochlea and vestibular sensory organs (Qian et al., 2006). The function of all three genes in the inner ear hair cell formation was investigated, and both *Hes1* and *Hes5* were shown to be negative regulators of inner ear hair cell differentiation. Mouse mutants for *Hes1* or *Hes5* have increased hair cell number in developing cochlea and vestibular epithelia of the saccule and utricle, as confirmed by the increased and ectopic expression of *Math1*, a hair cell marker (Zheng et al., 2000, Zine et al., 2001, Zine et al., 2002, Li et al., 2008, Tateya et al., 2011). This phenotype resembles that of the loss of Notch function, suggesting that during sensory development both genes are regulated by Notch signaling (Lanford et al., 1999, Lanford et al., 2000, Kiernan et al., 2001). Establishment and maintenance of both genes is Notch-dependent, during both otic neurogenesis and hair cell differentiation stages (Abelló et al., 2007).

Recently, it was reported that *Hes1* has an additional role in the development of the organ of Corti, before the process of cellular differentiation starts there. A cell cycle inhibitor $p27^{Kip1}$ demarcate the prosensory region in developing cochlear primordium, which consists of the sensory progenitors that have completed their terminal mitosis. Its expression is upregulated in *Hes1*$^{-/-}$ mice (Murata et al., 2009), suggesting that Hes1 regulates correct proliferation of the sensory precursors before the overall onset of hair cell differentiation.

However, the role of *Hes* genes in otic neuronal development was not investigated so far, although increased neuronal number in embryos with impaired Notch signaling (Haddon et al., 1998, Abelló et al., 2007, Raft et al., 2007) indicates that *Hes* genes may repress neurogenesis in the inner ear. So far, the only gene discovered to repress the otic neurogenesis belongs to another family of genes, and is called *Tbx1.*

4. Tbx1 transcription factor

Tbx1 belongs to the T-box family of genes, found in the genomes ranging form nematodes to humans. The name of the family came from the member called Brachyury or T (tailless), whose haploinsufficiency in mouse and zebrafish affects, among other features, the elongation of the tail (Herrmann et al., 1990, Wilkinson et al., 1990, Schulte-Merker et al., 1994). These genes encode transription factors, that range in size from 50 kDa to 78 kDa, and are characterized by the presence of the conserved DNA

binding domain, called the T-box (Fig. 10). The T-box is defined as the minimal region within the T-box protein that is both necessary and sufficient for sequence-specific DNA binding. Despite the sequence variations within the T-box between different family members, it seems that all T-box proteins can bind to the consensus DNA sequence TCACACCT (reviewed in Wilson and Conlon, 2002).

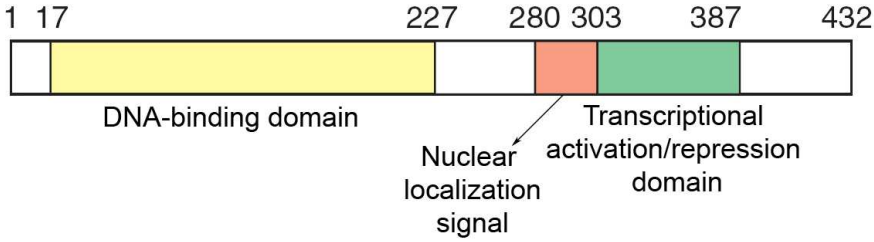


Figure 10. Schematic representation of T-box transcription factors. T-box proteins have conserved DNA-binding domain (yellow), in addition to the nuclear localization signal (red) and transcriptional acitvation/repression domain (green). Modified from Wilson and Conlon, 2002.

T-box genes are implicated in a number of developmental processes, including cardiogenesis, hematopoiesis, limb outgrowth and patterning, pituitary gland development and others, acting either as transcriptional activators or repressors (reviewed in Tada and Smith, 2001, Wilson and Conlon, 2002, Ryan and Chin, 2003, Kiefer, 2004, Naiche et al., 2005). Mutations in these genes lead to the severe developmental abnormalities in all species tested so far, including humans. For example, human Holt-Oram syndrome is a developmental disorder associated with haploinsufficiency of *TBX5*, while human ulnar-mammary syndrome, caused by the

haploinsufficiency of *TBX3* includes defects in limb, tooth and genital development (Li et al., 1997, Bamshad et al., 1997).

Another human syndrome, the DiGeorge syndrome, was associated with the mutation in *TBX1* gene. Patients having chromosomal 22q11 deletion (chromosome 22) develop DiGeorge syndrome, velocardiofacial syndrome or conotruncal anomaly face, all of them including endocrine, immunological, craniofacial and cardiac anomalies (reviewed in Scambler, 2000). This deletion includes around 30 genes, and the experiments carried out in mouse showed that one of the main candidates for the most of the abnormalities seen in DiGeorge syndrome is *TBX1* (Merscher et al., 2001, Jerome and Papaioannou, 2001, Lindsay et al., 2001).

Part of the spectrum of craniofacial defects seen in DiGeorge syndrome includes external, middle and inner ear abnormalities (Ford et al., 2000). Hearing impairment occurs in more than 50% of DiGeorge/velocardiofacial cases, and from these around 10% is of sensorineural type (Digilio et al., 1999, Reyes et al., 1999). In mouse and chick, *Tbx1* is expressed early in developing otocyst, complementary to the neurogenic domain, and in the periotic mesenchyme (Vitelli et al., 2003, Abelló et al., 2010). In addition to the defects in external and middle ear development, *Tbx1*$^{-/-}$ mouse mutants have small otic vesicles, with impaired morphogenesis, such that cochlea and semicircular canals, as well as their associated sensory epithelia, do not form (Whitfield et al., 1996, Piotrowski and Nusslein-Volhard, 2000, Vitelli et al., 2003, Raft et al., 2004, Arnold et al., 2006). Gain- and loss of function studies,

performed in mouse, have shown that *Tbx1* negatively regulates otic neurogenesis, through the suppression of proneural *Ngn1* and *NeuroD* genes (Raft et al., 2004, Xu et al., 2007), and subsequently the development of the SAG is affected.

Despite the importance Tbx1 has in the ear development, the regulators of its expression within the otic epithelium, as well as the genetic framework in which Tbx1 operates, are poorly understood. Retinoic acid singled out as a candidate for regulating otic *Tbx1* expression in the avian embryos, where the implantation of the RA-coated beads within the otic vesicle abolished the *Tbx1* expression (Roberts et al., 2005). Similarly, *tbx1* expression is lost from the pharyngeal arches when zebrafish embryos were treated with exogenous RA (Zhang et al., 2006), altogether suggesting an inhibitory influence of RA over Tbx1.

5. Retinoic acid

5.1 Retinoic acid signaling pathway

Retinoic acid, a derivative of vitamin A (see BOX II), is a signaling molecule that has received much scientific attention during the last century. Studies performed in a wide variety of vertebrate models revealed that vitamin A deficiency during embryonic development results in complex congenital malformations, affecting nervous system, vision, fertility, limb development and other organs. (reviewed in Maden, 2002, Maden, 2007, Niederreither and Dollé, 2008). Developmental roles of retinoic acid are many, but essential ones are associated with the early axial patterning, the regional

patterning of the central nervous system and the regulation of the neurogenesis.

BOX II: Retinoic acid signaling pathway

Retinoic acid is metabolic derivative of vitamin A (retinol). Animals cannot synthesize vitamin A, thus they must extract it from their diet in form of carotenoids, of plant origin, or retinyl esters, of animal origin. These dietary components are stored as retinyl esters mainly in liver, but also in lung, kidney and bone marrow. The canonical RA synthesis is nicely reviewed in Maden, 2007 and Niederreither and Dollé, 2008. The final step in RA production, the oxidation of retinaldehyde into RA, is carried out by three retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3, called also Aldh1a1-3), being Aldh1a2 the enzyme responsible for the majority of RA production in the early embryo (Niederreither et al., 1999, Niederreither et al., 2000).

Newly synthesized retinoic acid binds to the cellular retinoic acid binding proteins (CRABPs), and enters the nucleus, where it binds to a transcription complex, that includes a pair of ligand-inducible transcriptional activators comprising RA receptor (RAR)-retinoic X receptor (RXR) heterodimer. In mammals there are three RARs (RARα, RARβ and RARγ) and three RXRs (RXRα, RXRβ and RXRγ) Without ligand, the heterodimer, with recruited corepressors, is constitutively bound to a DNA sequence called retinoic acid-response element (RARE) and represses the transcription of the target genes. Upon ligand binding, RARs suffer conformational change, which leads to the release of the corepressors and enables recruitment of coactivators. More than 500 genes were described to be RA-responsive, but only around 25 were undoubtly shown to have RARE elements in their promoters (Balmer and Blomhoff, 2002). Once all-trans RA has activated the RARs, it exits the nucleus and is catabolized in cytoplasm into more polar derivatives (4-OH-RA and 4-oxo-RA) by CYP26 class of Cytochrome P450 enzymes (CYP26A1, B1 and C1) (White et al., 1996, MacLean et al., 2001, Tahayoto et al., 2003).

RA is also able to act in paracrine manner, but the mechanism of its transport to the neighbouring cells is not clear (Molotkov et al., 2006).

Endogenous RA levels can be manipulated either dietarily, pharmacologically or genetically. DEAB (4-(diethylamino)-benzaldehyde) and citral are pharmacological inhibitors of retinaldehyde dehydrogenases (Russo et al., 1988, Kikonyogo et al., 1999) and their application abolishes RA synthesis. R115866 is chemical inhibitor of Cyp26 enzymes, and its application increases the endogenous levels of RA (Stoppie et al., 2000). BMS493 and AGN193109 are pan-RAR synthetic retinoid antagonist (Johnson et al., 1999, Dupé and Lumsden, 2001), while RARa receptor can be specifically blocked by Ro 41-5253 (Apfel et al., 1992). All-*trans*-RA is also commercially available

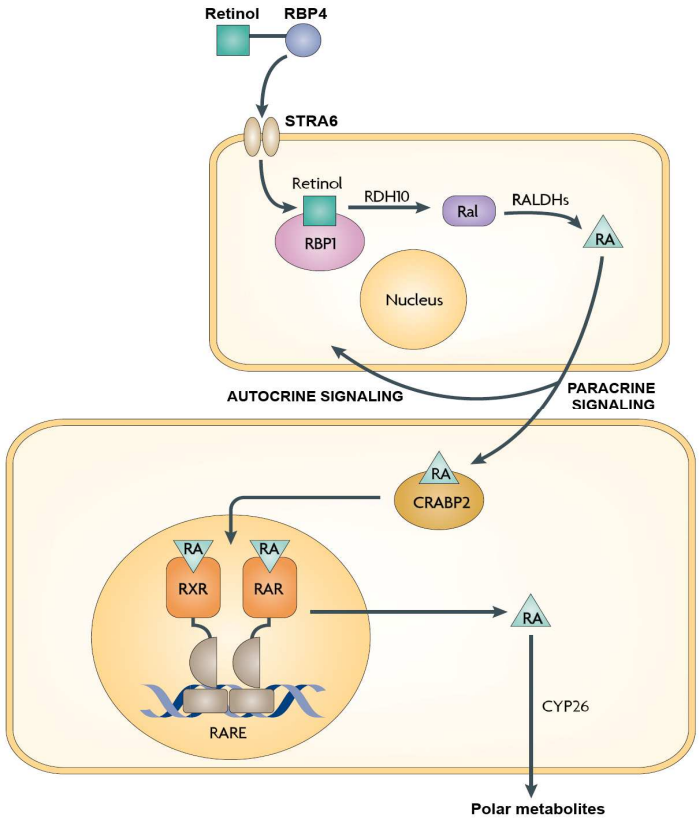


Figure BOX II. Retinoic acid signaling pathway. Taken from Maden, 2007.

5.2 The role of retinoic acid in the anteroposterior patterning of the nervous system

During the anteroposterior axial patterning of the vertebrate embryo, retinoic acid acts to promote posterior and suppress anterior fates (Durston et al., 1989, Conlon, 1995, Maden et al., 1996, Blumberg et al., 1997, Kudoh et al., 2002, Linville et al.,

2004, Linville et al., 2009). Depletion of RA signaling either dietarily, pharmacologically or genetically, leads to the expansion of the anterior hindbrain at the expense of the posterior hindbrain (Niederreither et al., 1999, Gale et al., 1999, White et al., 2000, Dupé and Lumsden, 2001, Grandel et al., 2002, Maves and Kimmel, 2005). In amniote embryos, complete absence of RA results in complete loss of r5-r7 of the hindbrain, accompanied by an expansion of r3-r4. Surprisingly, in zebrafish mutant for Aldh1a2, the enzyme thought to be the major producer of RA in embryos, the anteriorization of the hindbrain is less pronounced (Begemann et al., 2001, Grandel et al., 2002), suggesting the existence of additional RA producer in zebrafish embryos. Indeed, the complete pharmacological blockade of RA signaling in zebrafish embryos recapitulates the defects described for amniotes: r5-7 are lost, and rhombomeres 3 and 4 are expanded posteriorly (Grandel et al., 2002, Maves and Kimmel, 2005). Conversely, treatments of embryos with excess of RA posteriorizes anterior hindbrain, such that r2-r3 changes to r4-r5 identity, and in more severe cases forebrain and eyes are missing (Sive et al., 1990, Marshall et al., 1992, Kudoh et al., 2002).

The effects of RA on the hindbrain patterning are mediated by the *Hox* genes. The *Hox* genes contain a conserved homeobox that encodes a DNA binding homeodomain within the protein, and these transcription factors are molecular interpreters of the positional identity of embryonic tissues (reviewed in Marshall et al., 1996, Trainor and Krumlauf, 2001, Alexander et al., 2009). Specifically, it was proposed that RA, synthesized in paraxial mesoderm, diffuse to the neural tube where it acts as a morphogen, with decreasing concentration from posterior to

anterior (Fig. 11A). *Hox-1* family of genes is more sensitive,than Hox-4 family to the RA signaling and, thus, is expressed in the anterior hindbran where the levels of RA are lower, while Hox-4 genes are expressed in the posterior hindbrain (Simeone et al., 1995, Dupé and Lumsden, 2001, Maves and Kimmel, 2005). In this way, retinoic acid gradient is differently interpreted and enables anteroposterior patterning of the embryonic neural tube.

However, the existence of such a gradient was only recently proved to exist, and it can be visualized using transgenic zebrafish line that reports yellow fluorescet protein under the promoter of RARE element (White et al., 2007). The gradient of RA activity is thought to be established by the combined action of Aldh1as and Cyp26s enzymes, involved in RA synthesis and metabolism, respectively (McCaffery et al., 1999, Blentic et al., 2003, Reijntjes et al., 2004, White et al., 2007, White and Schilling, 2008).

5.3 The role of retinoic acid in neuronal differentiation

In cultured embryonic stem cells, carcinoma or neuroblastoma cells, addition of exogenous RA increases the proportion of neuronal and glial cell types (Jones-Villeneuve et al., 1982, Sidell et al., 1983, Andrews, 1984, Stavridis et al., 2010). This fact can be used to guide stem cells to differentiate to a specific cell type, and potentially can be used to replace lost neurons in vivo. Some advances are already made in this direction, in experiments in which neurons, obtained from cell cultures treated with different

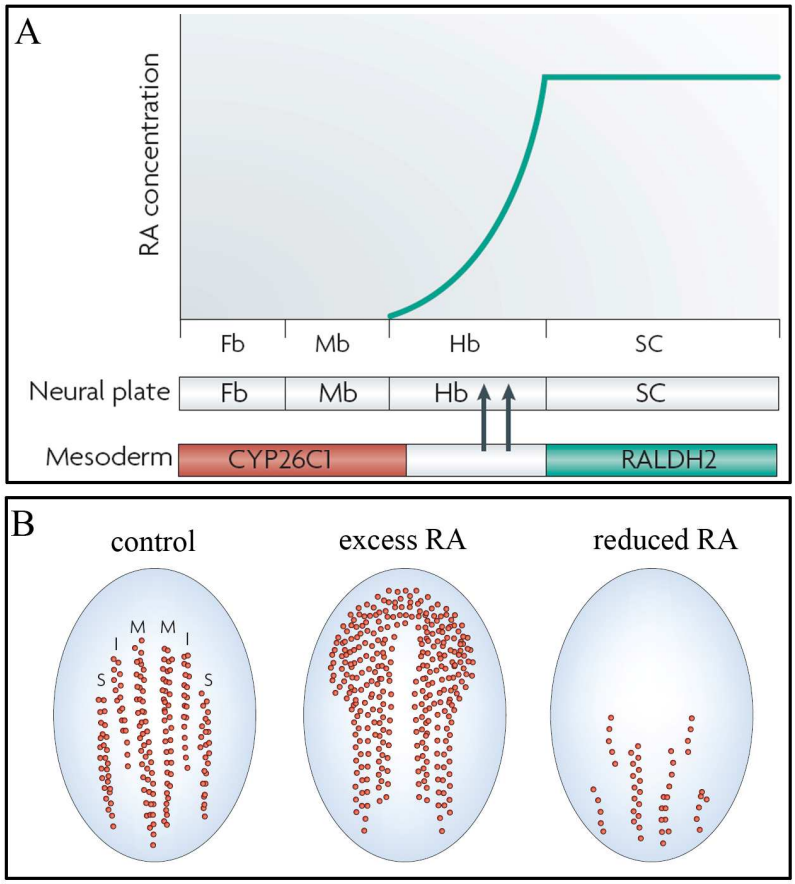


Figure 11. Different roles of RA during the AP patterning of the hindbrain and in neuronal differentiation.
(A) A gradient of RA activity is established by the joint action of RA synthesis and degradation enzymes. In the developing hindbrain, the gradient decreases from posterior to anterior, suggesting that RA acts as a morphogen to assign different identites depending on its concentration. **(B)** During primary neurogenesis in *Xenopus*, excess RA induces neuronal differentiation, while reduced RA signaling has the opposite effect. M, motor neurons, I, interneurons, S, sensory neurons. Modified from Maden, 2007.

combinations of RA and growth factors or neurotrophins, were transplanted to a specific zones of the rat brain, and survived there (Maden, 2007 and references therein).

In vivo, RA was shown to have a role in regulating differentiation of primary neurons in *Xenopus* (Fig. 11B) (Sharpe and Goldstone, 1997, Franco et al., 1999), branchiomotor neurons in zebrafish (Linville et al., 2004) and specific subtypes of motor neurons in chick caudal hindbrain and spinal cord (Sockanathan and Jessel, 1998, Guidato et al., 2003, Novitch et al., 2003, Wilson et al., 2004). This control is achieved through the regulation of expression of markers for specific neuronal subtypes, such as *Lim1*, *Isl2 Nkx6.1, Pax6, Irx3*, *Dbx1* and *Dbx2*.

Thus, RA affects CNS neurogenesis by regulating the patterning of the neural tube and differentiation of specific neuronal subtypes.

5.4 The roles of retinoic acid during the inner ear development

5.4.1 The role of retinoic acid in otic induction

Retinoic acid signaling affects otocyst induction/formation, inner ear patterning/morphogenesis and cell fate specification/differentiation, acting thus on multiple points during otic development. Since changes in endogenous RA levels affect hindbrain patterning, and hindbrain signals are known to regulate many aspects of the otic development (Gavalas and Krumlauf, 2000, Romand, 2003, Romand, 2006), the effects of perturbed RA signaling on inner ear development are mostly considered as secondary consequences of the hindbrain misspatterning. Specifically, reduced RA levels in quail and rat, or increased RA levels in zebrafish, result in

supernumerary or enlarged otic vesicles, and this correlates with the caudal expansion of hindbrain *FGF3* (and *fgf8* in zebrafish) expression (White et al., 2000, Phillips et al., 2001, Kil et al., 2005). Members of the Fgf signaling pathway are the primary factors involved in the otic induction. In mouse, hindbrain Fgf3 and mesodermal Fgf10 act as redundant signals during the otic induction (Mansour et al., 1993, McKay et al., 1996, Mahmood et al., 1995, Alvarez et al., 2003), while in avian embryos this function is performed by Fgf3, Fgf8 and Fgf19 (Represa et al., 1991. Mahmood et al., 1995, Ladher et al., 2000, Vendrell et al., 2000, Ladher et al., 2005, Martin and Groves, 2006). Hindbrain derived Fgf3 and Fgf8 act redundantly to specify otic field in zebrafish (Phillips et al., 2001, Maroon et al., 2002, Léger and Brand, 2002, Liu et al., 2003, Phillips et al., 2004). Blockade of RA in zebrafish leads to the smaller otic vesicles, and this correlates with reduced (although expanded) *fgf3* expression in the r4 primordium of the hindbrain at the stages of the otic placode induction (Hans and Westerfield, 2007).

However, in zebrafish embryos treated with FGF inhibitor, the otic competence factor Foxi1 was able to induce *Pax8*, an early marker of otic primordium, suggesting that factors other than Fgfs may also regulate the otic induction (Solomon et al., 2004). Moreover, enlarged otic vesicles are seen in zebrafish embryos treated with low dosis of RA that does not significantly alter embryonic anteroposterior patterning (neither the FGF signaling within the neural tube), and this effect goes through the RA-dependent ectopic activation of Foxi1 (Hans and Westerfield, 2007).

5.4.2 The role of retinoic acid in otic patterning

The role of RA signaling in regional patterning of the otic vesicle was investigated in chick embryos treated with exogenous RA (Choo et al., 1998) and in mouse mutants for RA synthesizing enzyme RALDH2 (Niederreither et al., 2000). Experiments in which avian embryos were treated with increasing concentrations of RA, by bead implantation (Choo et al., 1998), revealed that dorsal ear structures (such as semicircular canals) are more sensitive to lower RA concentrations than ventral ear structures (e.g. basillar papilla). These experiments elegantly demonstrated that exogenous retinoic acid can regulate otic patterning autonomously (independently of RA influence on hindbrain), the molecular targets responsible for the phenotype remaining unknown.

The role of RA in mediolateral otic patterning, deduced from the analysis of the Raldh2 mouse mutants, is desribed in details in the part 2.1.3 of the Introduction Chapter.

5.4.3 The role of retinoic acid in cell fate specification

Almost all experiments done so far to address the role of RA in otic cell fate specification/differentiation were done in cell or otic explant cultures. It was observed that chick otic explants, incubated with RA, tend to enter into differentiation earlier than the control explants. Thus, it was proposed that this happens due to the RA dependent inhibition of cell proliferation (Sidell et al., 1983, Represa et al., 1990).

Exogenous RA promoted the regeneration of the sensory hair cells in the cultured mammalian organ of Corti, after they were damaged by ototoxin application. However, direct transformation of supporting cell into hair cell type was excluded as a possible mechanism of the hair cell reappearance, since the regeneration was conditioned by the intact cell division process (Lefebvre et al., 1993). Moreover, when the otic explants were treated with RA without previous ototoxin application, no new extra hair cell appeared in the cultured organ of Corti, suggesting that transdifferentiation of the supporting cells into hair cell type indeed does not occur under these conditions. However, these experiments were done much after the onset of the overall cell differentiation processes within the organ of Corti, and the possible role of supporting cells as a stem cells for the hair cell type could not be truly deduced.

When organ of Corti explants were treated with RA before the onset of overall cellular differentiation, supernumerary hair cells appeared in distinct patches within the sensory epithelia (Kelley et al., 1993). However, mosaic arrangement of hair cells and supporting cells was not changed in these expanded sensory domains, raising the question of the possible role of RA in prosensory specification, rather than in supporting cell respecification, at least concerning the auditory sensory epithelium. Conversely, the blockade of RA signaling using citral or synthetic RARα antagonist Ro 41-5253, led to the decrease in hair cell number in the cultured organ of Corti (Raz and Kelley, 1999). Blockade of RARα receptor alone did not change the length of the sensory epithelia, but hair cell density within the epithelia

decreased, and cells were disorganized and malformed. This suggests that RARα receptor affects hair cell differentiation/survival, rather than specification of the prosensory epithelia. Instead, when RA synthesis was blocked completely by citral, sensory epithelia shortened, hair cell number and density within the epithelia decreased even more, suggesting that RA might act to specify the size of the prosensory domain.

The role of RA in differentiation of the otic neurons was also investigated (Martinez-Monedero et al., 2008). Inner ear stem cells can be isolated from the vestibular organs of the inner ear, organ of Corti or spiral (cochlear) ganglion. When isolated from utricle, these stem cells express *Sox2*, and after 10 days in culture start expressing neuronal markers, such as *TuJ* (b-III tubulin), glial markers (such as *GFAP*) or hair cell markers (*Math1*, MyoVIIa). The percentage of neurons obtained from these stem cells increased if they were cultured with RA, but also the percentage of hair cells and glial cells, without changing the total number of stem cells, suggesting that function of RA would be a general stimulation of cell differentiation. The completely differentiated neurons obtained under these conditions had character of sensory neurons, as confirmed by marker expression and physiological analysis.

AIMS OF THE THESIS

Aims of the thesis

The role of proneural genes in driving otic neurogenesis was previously investigated by numerous groups, including our. Extrinsic signals, providing initial establishment of the neurogenic domain in the anteromedial part of the otic placode, include different members of the FGF family of secreted molecules and Hh signaling pathway. In the complementary posterolateral region, a number of transcription factors is expressed. The potential role of any of these factors in the spatial regulation of the otic neurogenesis was, so far, assessed only for the mouse Tbx1 transcription factor. However, the role of other posterolateral genes, as well as the extrinsic regulators of their otic establishment have not been fully investigated.

Using zebrafish as a model system for the vertebrate inner ear development, the aims of this thesis are:

1. To search for the genes expressed in the posterolateral, nonneurogenic otic domain.

2. To investigate the role of one such factor, Her9, in the regulation of the otic neurogenesis.

3. To analyse other possible roles of Her9 during the otic development, including sensory development and cell proliferation.

4. To analyse if Tbx1 has conserved role in mouse and zebrafish as a negative regulator of the otic neurogenesis.

5. To investigate the epistatic relationship between Her9 and Tbx1 transcription factors.

6. To determine if otic *her* genes are Notch targets, as classically viewed.

7. To search for the extrinsic signals that establish the expression of the posterolateral otic genes, such as *tbx1* and *her9.*

8. To position the source of such signaling.

9. To analyse the possible cross-interactions between positive and negative extrinsic regulators of the otic neurogenesis.

10. To analyse the possible later role of RA signaling in the otic neurosensory development.

RESULTS

1. *Her* genes in the inner ear

1.1 Screening for the expression of *her* genes in the inner ear

her genes are zebrafish orthologs of *Drosophila*'s *Hairy* and *Enhancer of split* genes. Their expression and function was analyzed during somitogenesis (Takke et al., 1999, Henry et al., 2002, Pasini et al., 2004, Sieger et al., 2004, Gajewski et al., 2006, Sieger et al., 2006), the development of the central nervous system (Geling et al., 2003, Geling et al., 2004, Bae et al., 2005, Ninkovic et al., 2005, Scholpp et al., 2009) and in the adult brain (Stigloher et al., 2008, Chapouton et al., 2011). These studies revealed that *her* genes mainly have conserved roles with their mammalian orthologs. As mentioned in the Introduction Chapter, in chick otic placode, *Hes5* and *Hes1* are expressed complementary in neurogenic and nonneurogenic domains. In mouse, the roles of Hes1 and Hes5 had been assessed during the otic sensory development, where it was shown that both transcription factors negatively regulate the number of hair cells. In order to better understand the role of *Hes* genes in inner ear development, we have decided to study them in zebrafish, where neither the expression pattern nor the role of any of the *her* genes was studied unil now.

In order to address which *her* genes might be present in the developing inner ear, I did a screening for their expression at

different developmental stages by in situ hybridization. Zebrafish otic placode becomes morphologically visible at 14 hpf (10 ss). Then placode cavitates (starting at 16 hpf (14 ss) stage) to finally form the otic vesicle (the lumen of the vesicle is visible from 18 hpf (18 ss) stage onwards). The screening was done at 16, 18 and 24 hpf. There are at least 15 *her* and 3 *hey* genes in zebrafish, out of which I analyzed the expression of 10 *her* and 3 *hey* genes: *her1, her3, her4, her5, her6, her7, her8a, her9, her11, her15, hey1, hey2* and *hey3 (heyL)*. I could detect the expression of three *her* and one *hey* gene in the otic anlagen at these stages. At 16 hpf, *her4*, a zebrafish ortholog of *Hes5*, is expressed in two patches at the opposite poles of anteroposterior axis of the otic placode, that correspond to the future anterior (utricular) and posterior (saccular) sensory maculae (Fig. 12A). At 18 hpf, *her4* expression is detectable in the whole anteromedial half of the otic vesicle, including the neurogenic domain in addition to the sensory region. This pattern of expression is maintained at 24 hpf (Fig. 12B,C). *her6* and *her9,* two orthologs of mouse *Hes1,* are also expressed in the zebrafish inner ear. *her6* starts to be expressed in the medial wall of the otic vesicle at 18 hpf (Fig. 12G), with higher expression in the presumptive sensory maculae, and this expression pattern is maintained at 24 hpf (Fig. 12H). *her9* starts to be expressed at 16 hpf in a posterolateral otic domain and this expression pattern, complementary to that of *her4*, is maintained at 18 and 24 hpf (Fig. 12D-F). From 3 *hey* genes, only one was found to be expressed in the inner ear: at 24 hpf *hey1* is expressed in the anterior and posteromedial group of cells, that judged by the position correspond to the anterior and posterior maculae (Fig. 12I).

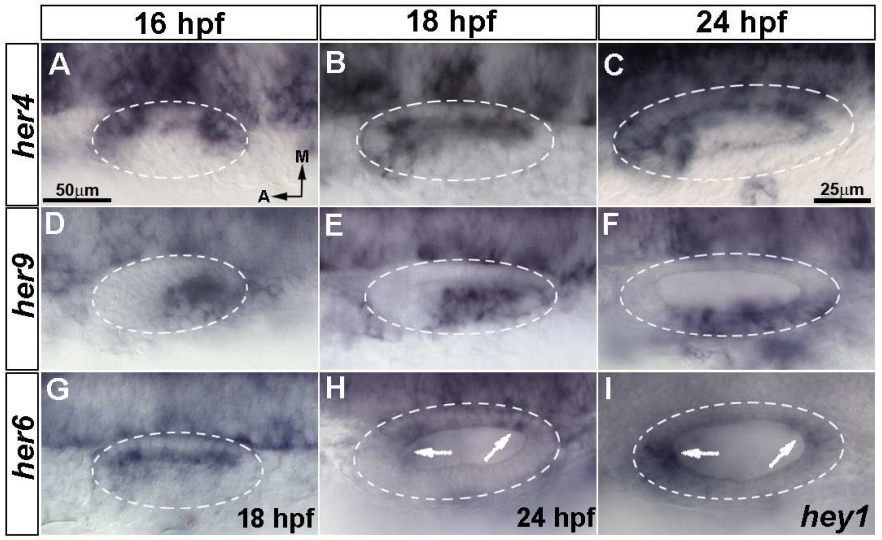


Figure 12. Screening for the presence of the *her* genes expression in the inner ear.
(A-I) Dorsal views of whole mount zebrafish otic placodes/vesicles, with anterior to the left and medial to the top. Dashed circles delineate the otic anlagen. In situ hybridization was done for *her4*, *her9*, *her6* and *hey1* genes. (A-C) *her4* expression initiates at the anteromedial and the posteromedial poles of the otic placode (A) expanding later to include whole anterior and medial otic walls at otic vesicle stages (B and C). (D-F) *her9* posterolateral otic expression does not change during placodal (D) and vesicle stages (E and F). (G and H) *her6* is expressed in the medial part of the otic territory, with higher expression in presumptive sensory maculae, starting from 18 hpf onwards. (I) *hey1* expression is detected in anteroventral and posteromedial otic domains at 24 hpf (white arrows in I). The same magnification in A,B,D,E,G,H and C,F,I.

1.2 The role and regulation of the otic *her4* expression

As it is the case for mouse and chick *Hes5* gene, its zebrafish ortholog *her4* is activated by Notch within the neurogenic zones of the embryonic neuroectoderm, in response to the lateral inhibition

mechanism of selection of neuronal precursors (Takke et al., 1999, Pasini et al., 2004, Bae et al., 2005, Yeo et al., 2007). The lateral inhibition mechanism also operates during the development of the inner ear, and correspondingly, in chick and mouse, the otic *Hes5* expression is established by Notch signaling (Lanford et al., 2000, Yamamoto et al., 2006, Abelló et al., 2007).

To test whether Notch establishes *her4* expression in zebrafish otic territory, the activity of Notch was blocked by exposing embryos to the γ-secretase inhibitor DAPT (N-[N-(3,5-diuorophenacetyl)-l-alanyl]-S- phenylglicine t-butyl ester) (Geling et al., 2002). The activity of γ-secretase is required for the cleavage of the Notch intracellular domain that results in a target gene activation. Embryos were treated with 150 μM DAPT, or with 1.5% DMSO as a control, from 9 hpf, prior to the establishment of the otic *her4* expression, to 18 or 24 hpf, when the expression of *her4* was analyzed by in situ hybridization (Fig. 13). At 18 hpf *her4* was expressed in the whole anteromedial half of the otic vesicle in DMSO-treated embryos, but the most medial expression was lost in Notch-depleted embryos, leaving *her4* expression limited only to the anterior and posterior patches (Fig. 13A,B). At 24 hpf *her4* expression is completely lost from the neurogenic part of the otic vesicle and is maintained in the anterior and posteromedial sensory patches (Fig. 13C,D), suggesting that Notch signaling regulates *her4* expression in the otic neurogenic domain, while the establishment of *her4* expression in the presumptive sensory maculae is Notch-independent.

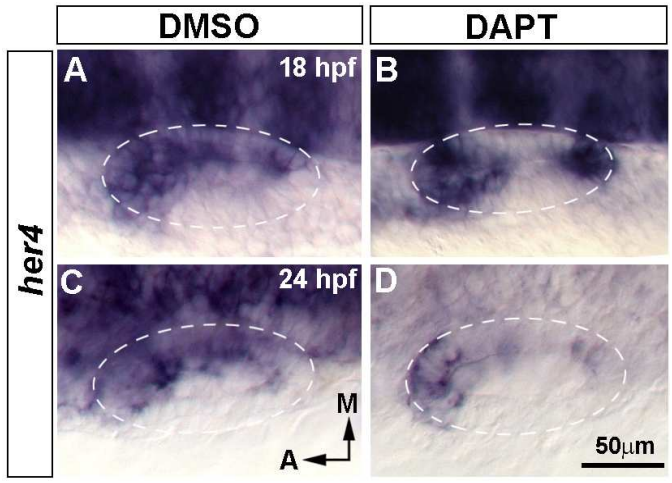


Figure 13. Otic *her4* expression is only partially established by Notch.
(A-D) Whole mount in situ hybridization for *her4* in 18 (A,B) and 24 hpf embryos (C,D) treated with DMSO or Notch inhibitor DAPT from 9 hpf. *her4* expression is maintained in sensory regions in Notch-depleted embryos (B and D). Dashed circles delineate otic vesicles. Anterior is to the left, medial is up. All images are at the same magnification.

Selection of neuronal precursors in the otic neurogenic domain involves Notch-dependent lateral inhibition mechanism triggered by the proneural determination transcription factor Neurog1. Cells expressing *neurog1* will express Notch ligand Delta and Delta-Notch interaction will activate downstream Notch targets in neighbouring cell, preventing them from adopting the neuronal fate.To test if *her4* mediates this role of Notch in the zebrafish inner ear, I have analyzed the expression of *her4* in zebrafish mutants for proneural gene *neurog1* (Golling et al., 2002, Madelaine and Blader, 2011). If proneural function is impaired, one could expect that cells will not express *delta*, and subsequently Notch signaling

and its downstream targets will not be activated. Indeed, at 24 hpf *her4* expression was abolished from the presumptive neurogenic

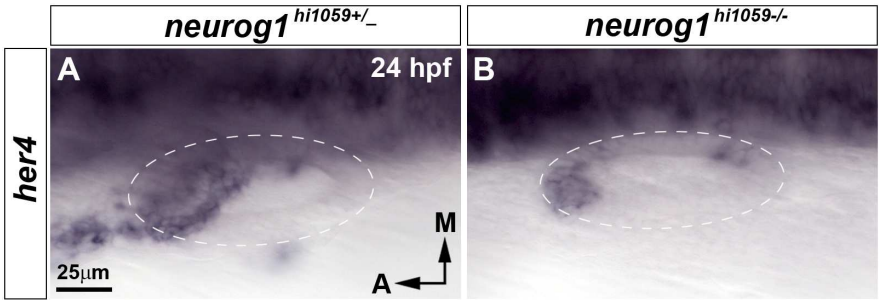


Figure 14. Neurog1 regulates the expression of *her4* within the otic neurogenic domain.
(A-B) Whole mount in situ hybridization for *her4* in 24 hpf zebrafish embryos obtained from heterozygotic *neurog1*$^{hi1059+/-}$ crosses. Dashed circle delineates the otic vesicle. *her4* expression is lost from the otic neurogenic domain in *neurog1*$^{hi1059+/-}$ mutant (B). Anterior is to the left, medial is up. Both images are at the same magnification.

domain in a quarter of the embryos obtained from the heterozygotic *neurog1*$^{hi1059+/-}$ crosses (Fig. 14A,B; n=5/21), this number corresponding to the statistical prediction of the proportion of the homozygotic mutants.

These results strongly suggested that Her4 indeed might negatively regulate otic neurogenesis within the neurogenic domain. To test this hypothesis, a morpholino designed to block *her4* translation (So et al., 2009) (referred to here as her4-MO) was injected into 1-cell stage zebrafish embryos, and the phenotype was analysed at 24 hpf, by in situ hybridization, to detect *neurog1* and *neurod* mRNA transcripts. From the lateral inhibition model, blocking Notch target would result in a higher density of *neurog1*/*delta*/*neurod* expressing cells within the neurogenic domain, due to the absence of the inhibition from the neighbouring cells. Surprisingly, neurogenic domain seemed to have the same

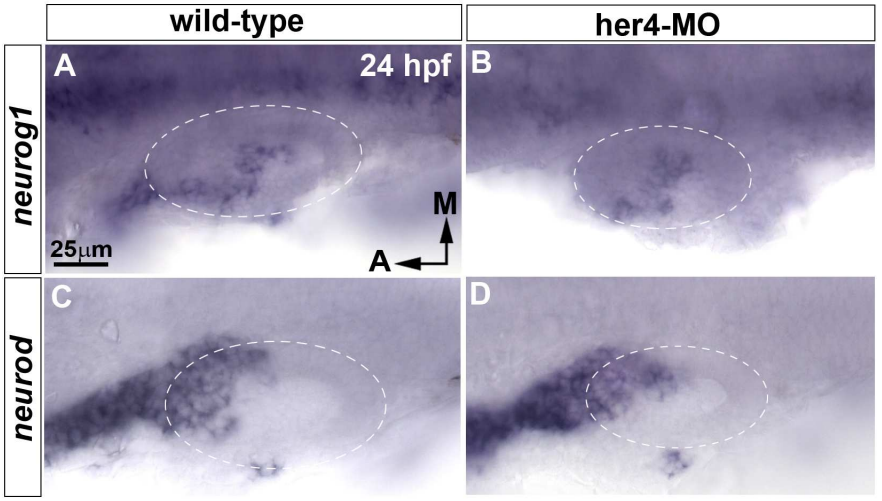


Figure 15. *neurog1* and *neurod* expression is unchanged in Her4-depleted embryos
(A-D) Whole mount in situ hybridization performed at 24 hpf to detect *neurog1* (A,B) and *neurod* (C,D) transcripts in uninjected (A,C) and her4-MO injected (B,D) embryos. No change in the density of the cells expressing *neurog1* or *neurod* could be observed in Her4-depleted embryos, when compared to uninjected control. Dashed circle delineates the otic vesicle. Anterior is to the left, medial is up. All images are at the same magnification.

density of *neurog1* and *neurod* positive cells (Fig. 15A-D), as judged by the analysis of the whole mount embryos. However, thedouble staining for *neurog1/delta/neurod* and *her4*, although not done for this thesis, should give more information and enable the final conclusion.

I have also started to study the possible role of Her4 in the sensory maculae development. Notch-independent macular expression of *her4* roughly coincides with the expression of hair cell specification markers *atoh1b* and *atoh1a* (Millimaki et al., 2007) at 16 and 24 hpf, respectively. The otic *atoh1a* expression is established after

her4, suggesting that Her4 might be upstream regulator of *atoh1a* in the inner ear. At 24 hpf, *atoh1a* expression labels the anterior macula, and this is not changed in embryos injected with her4-MO at 1-cell stage (Fig. 16C,D).

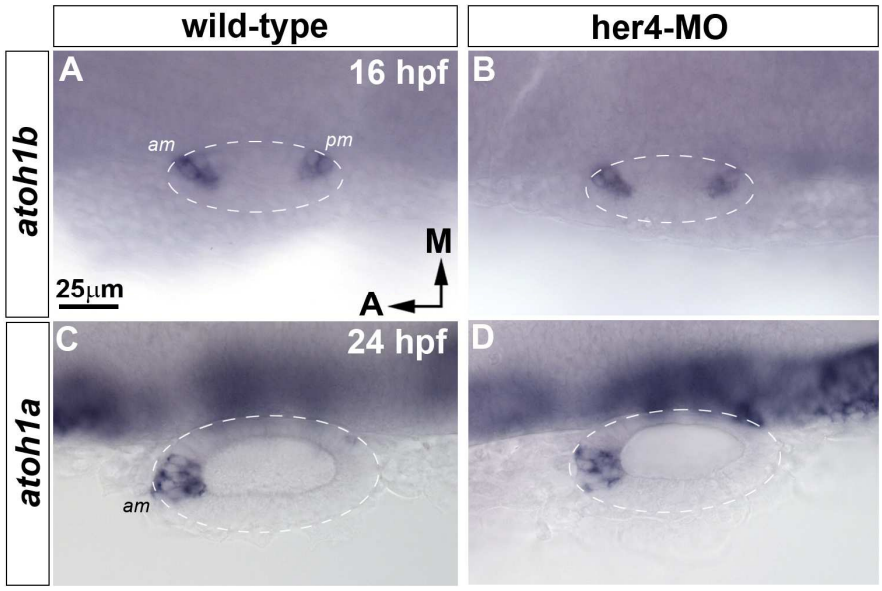


Figure 16. Her4 does not regulate the expression of the hair cell specification markers *atoh1b* and *atoh1a*
(A-D) Dorsal views of whole mount embryos probed by in situ hybridization for *atoh1b* (A,B) and *atoh1a* (C,D). No change in expression of any of the two genes could be observed after blocking Her4 translation (B,D) when compared to wild-type embryos (A,C). Dashed circle delineates the otic vesicle. Anterior is to the left, medial is up. All images are at the same magnification.

The potential role of Her4 in the regulation of the otic *atoh1b* expression was analysed (Fig. 16A,B), because soon after we first detected *her4* transcripts in the inner ear (at 16 hpf) *atoh1b* expression ceases. Since Her4 acts as a transcriptional repressor, the hypothesis was that Her4 inhibits *atoh1b* expression within the sensory maculae. At 16 hpf, *atoh1b* is still detected in only a few of cells within the anterior and posterior prosensory regions, and

surprisingly this expression pattern is not changed in Her4-inhibited embryos (Fig. 16A,B). In conclusion, my preliminary results suggest that *her4* has no role in early specification of the macular hair cells, as revealed by no change in *atoh1a* and *atoh1b* expression after the Her4 inhibition.

1.3 Her9 as a candidate for neurogenic versus nonneurogenic otic patterning

1.3.1 The early otic vesicle is regionalized into neurogenic and nonneurogenic domains

The *her9* gene is a zebrafish ortholog of mouse *Hes1* gene, described to have a dual role in regulating neurogenic processes: a role in lateral inhibition, mediating selection of neuronal precursors within the neurogenic zones, and a role in definition of the nonneurogenic territories such as midbrain-hindbrain and rhombomeric boundaries within CNS (Kageyama and Nakanishi, 1997, Baek et al., 2006, Kageyama et al., 2008). Similarly, in zebrafish, *her9* together with *her3*, *her5* and *her11* contributes to the inhibition of neurogenesis in the midbrain-hindbrain boundary by the maintenance of an undifferentiated pool of progenitors (Ninkovic et al., 2005), and with *her3* defines nonneurogenic interproneuronal stripes during primary neurogenesis in the neural plate (Bae et al., 2005), thus acting as a patterning gene to define nonneurogenic, and restrict neurogenic, territories.

Previous analysis of *Hairy1* expression in the chick otic placode revealed that this gene is expressed complementary to the neurogenic domain (Abelló et al., 2007). To test if *her9* has a role in a regulation of the otic neurogenic patterning in zebrafish, I compared its expression pattern with that of neurogenic genes *neurog1* and *neurod* and with *tbx1*, the only gene described so far to have a role in regulating the extent of the otic neurogenic domain, in mouse (Raft et al., 2004). Otic neurogenesis at 24 hpf, as depicted by the expression of the proneural genes *neurog1* and *neurod*, is observed in a band that runs from anterolateral to medioposterior (Fig. 17A,B). At the same stage *atoh1a*, a proneural gene required for the specification and maintenance of sensory hair cells in the otic vesicle (Millimaki et al., 2007), is detected in the anterior macula (Fig. 17G).

By contrast, *her9* expression is detected in the posterolateral region of the otic vesicle (Fig. 17C), overlapping with *tbx1* expression (Fig. 17D,H). Double staining for *neurod* and *tbx1* or for *neurod* and *her9* transcripts reveals that in the ventral otic region *neurod* and *tbx1/her9* exhibit an exactly complementary pattern of expression (Fig. 17E,F and see Fig. 18B,B',D,D'). However, in the dorsal part of the otic vesicle, *neurod* was absent and *tbx1* and *her9* displayed a more anterior limit of expression (Fig. 18A,A',C,C'). These results suggest that *tbx1* and *her9* might together contribute to the definition of the nonneurogenic otic territory (Fig. 17I). To add support to this hypothesis, I explored whether *tbx1* or *her9* was expressed before the onset of neurogenesis, revealed by *neurog1* expression (starting at 16 hpf; Fig. 17J). I found that *tbx1* is already transcribed in the posterior

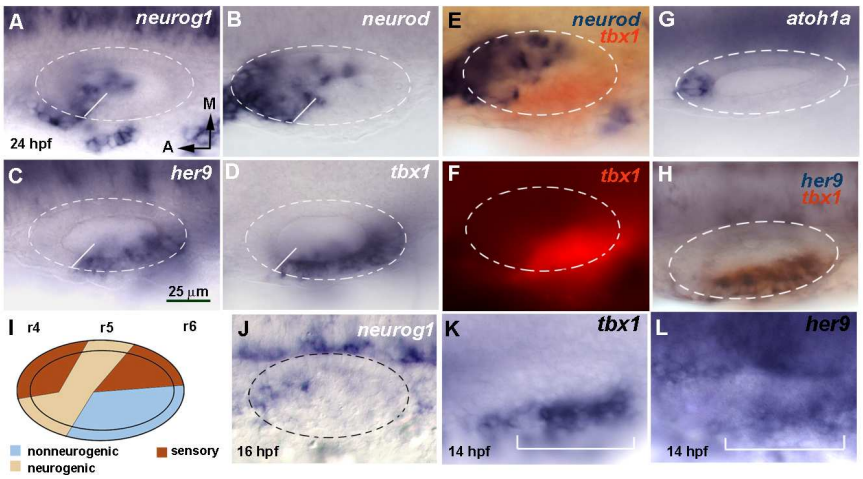


Figure 17. *her9* and *tbx1* expression is complementary to the neurogenic domain.
(A-H,J-L) Dorsal views of flat-mounted zebrafish embryos at 24 hpf, with anterior to the left and medial to the top. Dashed circles delineate the otic vesicle. In situ hybridization was done for *neurog1* (A), *neurod* (B), *her9* (C), *tbx1* (D) and *atoh1a* (G). White lines in A-D indicate the boundary between the neurogenic and non-neurogenic domains. Double in situ hybridization is shown for *tbx1* (red chromogen in E,H, red fluorescence in F) and *neurod* (blue, E) or *her9* (blue, H). (J) Otic *neurog1* expression starts at 16 hpf. (K,L) Expression of *tbx1* and *her9* in the otic placode at 14 hpf. The white bracket indicates the extent of the placodal domain.
(I) Schematic representation of the neurogenic, sensory and nonneurogenic territories. r, rhombomere. All images are at the same magnification.

otic placodal domain at 14 hpf and that *her9* is also expressed at this stage, although with a more diffuse pattern (Fig. 17K,L). Thus, the expression of *her9* and *tbx1* precedes the initiation of neurogenesis, suggesting that these genes indeed may act as prepatterning genes.

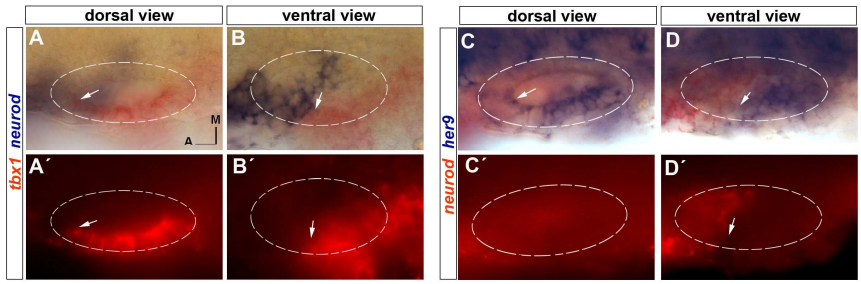


Figure 18. Complementary expression of *neurod* and *tbx1/her9* in 24 hpf wild-type embryos.
(A-B') Double in situ hybridization for *neurod* (blue) and *tbx1* (red) at two different focal planes. White arrows point to the anterior limit of *tbx1* expression. **(C-D')** Double in situ hybridization for *neurod* (red) and *her9* (blue) at two different focal planes. White arrows point to the anterior limit of *her9* expression (C,D) and to posterior limit of *neurod* expression (D'). Anterior is to the left, medial is up.

1.3.2 Her9 negatively regulates otic neurogenesis

A prepatterning function would imply that Her9 and/or Tbx1 sets the limits of the neurogenic domain in the inner ear. To test this hypothesis, I injected into 1-cell stage zebrafish embryos morpholino oligonucleotides directed against the splice donor site of the first intron of *her9* pre-mRNA transcripts (referred to here as her9-MO). This morpholino was previously shown to inhibit the development of the interproneuronal stripes in the posterior zebrafish neuroectoderm and to decrease the number of *atoh1a* positive neuronal precursors in the hindbrain (Bae et al., 2005). Both phenotypes were recapitulated (Fig. 19A-D), arguing for the efficiency of morpholino. *neurod4* expression at 10 hpf marks three longitudinal proneuronal stripes (black arrows in Fig. 19A) which in

control embryos are separated by *neurod4*-negative, nonneurogenic interproneuronal stripes (Fig. 19A). In Her9-depleted embryos *neurod4* expression is expanded into interproneuronal stripes (Fig. 19B). *atoh1a* expression in 30 hpf embryos is observed in the progenitor cells of the brainstem, and this domain of *atoh1a* expression is reduced in her9-MO injected embryos. (Fig. 19C,D).

A temporal study of efficiency of the morpholino was assessed by reverse-transcriptase PCR performed to amplify sequences that flank the first intron of *her9* pre-mRNA (Fig. 19E). At 24 hpf *her9* splicing was blocked completely. The normally spliced *her9* mRNA, 125nt smaller than unspliced form, could be amplified at 77 hpf. For this reason, the analysis of the effects of Her9 inhibition was done at 24 and 48 hpf, when the Her9 inhibition was complete.

Loss of function of Her9 led to the ectopic expression of proneural genes *neurod* (n=21/41) and *neurod4* (n=22/45) in the non-neurogenic posterolateral domain at 24hpf (Fig. 20E,F,I,J). The change in cell fate was observed from cells ectopically expressing *neurod* inside the posterolateral wall of the otic vesicle, as visualized by double in situ hybridization with *tbx1* (Fig. 20G-H'). *neurog1* expression pattern was not significantly changed in morphant embryos (expansion was observed only in 2/21 embryos, Fig. 20A-D'), suggesting a differential response of proneural genes to Her9 function in the otic vesicle. In addition to *neurod* and *neurod4*, *her4* expression was also expanded in the posterolateral otic territory (Fig. 20K,L; n=8/21).

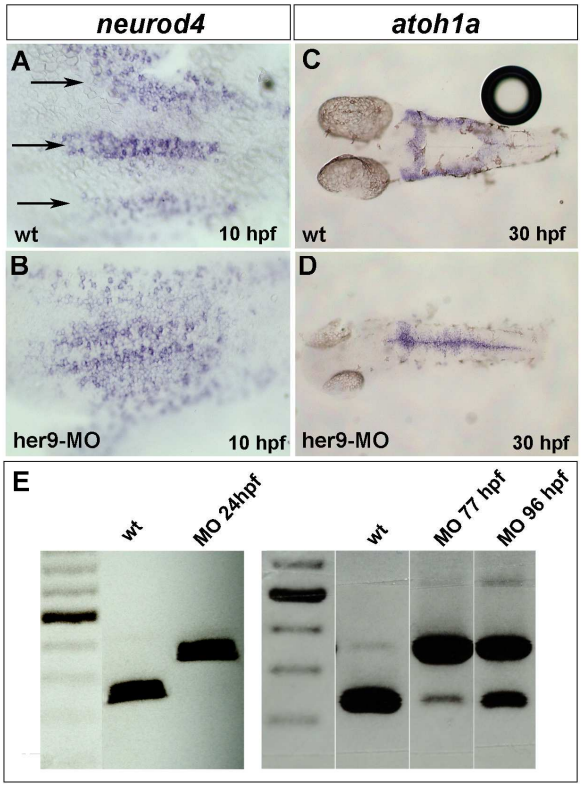


Figure 19. Depletion of *her9* by injection of a splice donor morpholino (her9-MO) at 1-cell stage expands neurogenic stripes and affects her9 mRNA splicing until 77hpf.
(A-D) Expression of *neurod4* and *atoh1a* in wild-type and her9-MO injected embryos at 10 hpf (A,B) and 30 hpf (C,D). *neurod4* marks proneuronal stripes black arrows)in A. *atoh1a* marks neuronal precursors in the fourth ventricle in C. Dorsal views of whole mount embryos, anterior is to the left. **(E)** Temporal analysis of *her9* mRNA splicing by RT-PCR in wild-type and embryos injected with her9-MO. Unspliced form of *her9* pre-mRNA is 125nt longer than spliced one.

Immature otic neurons delaminate through the anteroventral wall of the otic vesicle from 22 hpf to 48 hpf, and coalesce to form the SAG. SAG neurons can be visualized in vivo using the *islet3:GFP* line, that emits green fluorescence under the promoter of *islet3* gene (Pittman et al., 2008). Confocal images of 48 hpf wild-type and her9-MO injected *islet3:GFP* embryos show that morphant SAG displays a change in morphology, such that its two branches, the anterior and the medial, are less segregated than in control embryos (Fig. 21A,B).

In order to test whether the ectopic *neurod*-positive cells delaminate from the otic epithelium and increase the size of the SAG, ganglionar neurons were counted from z stack images of coronal sections in uninjected and her9-MO injected *islet3:GFP* embryos (Fig. 22A-A'''). The number of neuronal cells in the SAG as a whole or in each of its two branches was not significantly changed in morhants when compared to wild-type embryos (Fig. 22B,C). This suggests that depletion of Her9 results mainly in morphological patterning, but not in gross quantitative changes in SAG neuronal number.

However, as the otic vesicle of Her9-depleted embryos was overall smaller (reduction in size was 16% when compared to control otic vesicles, as measured by pixel numbers in selected imaged otic areas), the maintenance of the number of sensory neurons might indicate an increase in neuronal population within the otic epithelia, relative to that of nonneuronal cells.

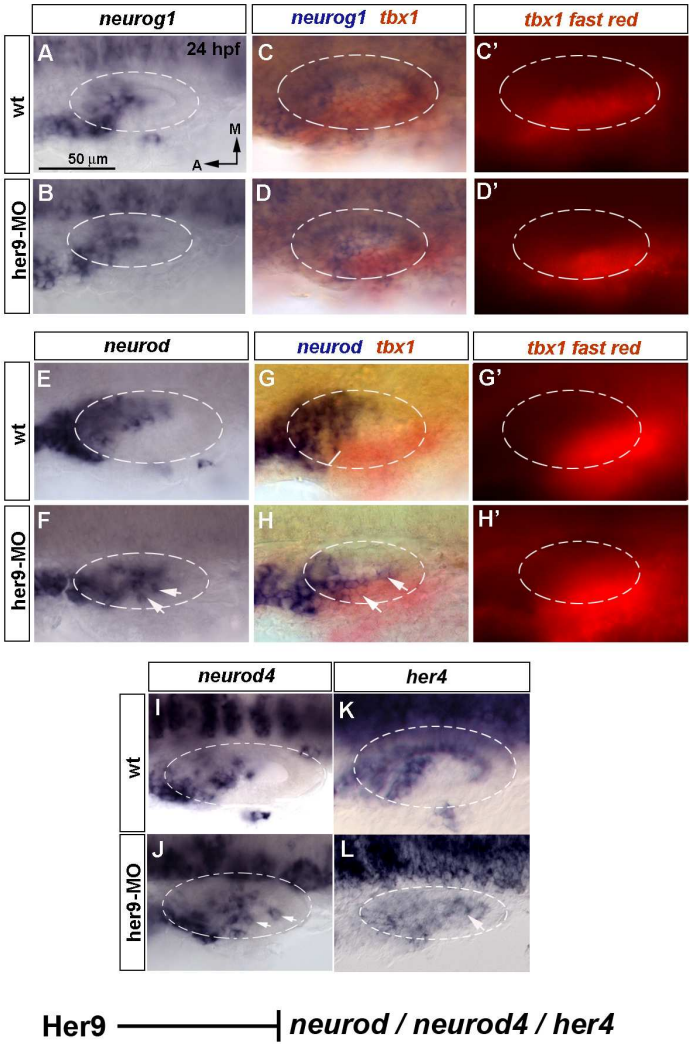


Figure 20. Blocking *her9* function results in ectopic expression of the proneural genes *neurod* and *neurod4.*
(A-L) In situ hybridization in 24 hpf wild-type and Her9 morphant zebrafish embryos performed to detect changes in *neurog1* (A-D'), *neurod* (E-H'), *neurod4* (I,J) and *her4* (K,L) expression in the inner ear. (C-D',G-H') Double in situ hybridization for *tbx*1 (red chromogen in C,D,G,H and red fluorescence in C',D',G',H') and *neurog1* (blue) or *neurod* (blue). Dashed circles delineate the otic vesicle. Arrows indicate ectopic neurogenesis. All images are at the same magnification.

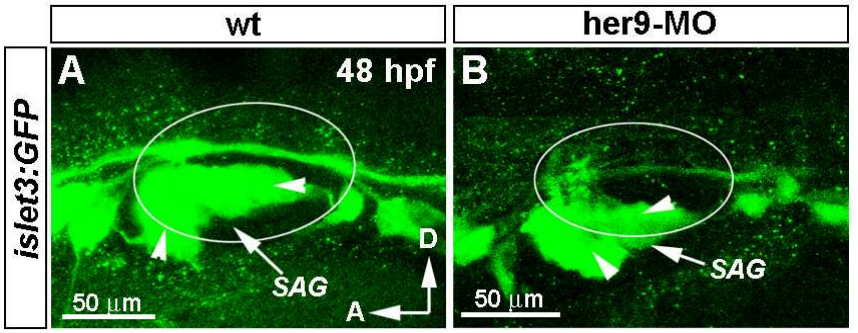
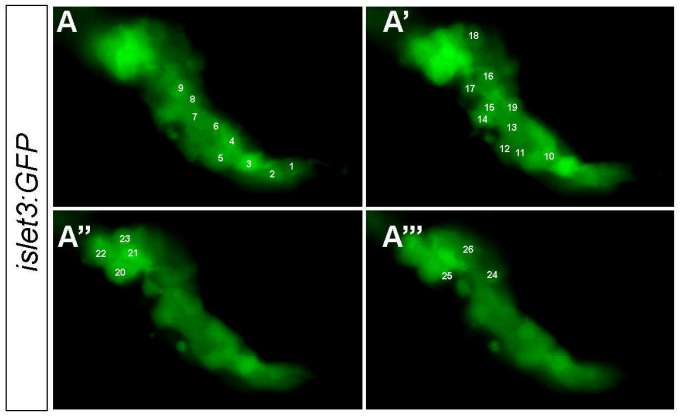
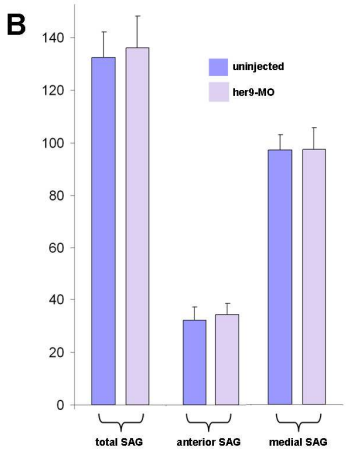


Figure 21. Depletion of *her9* distorts the development of the SAG.
(A,B) Three-dimensional reconstruction of the SAG at 48 hpf visualized in wild-type and her9-MO-injected zebrafish embryos carrying the *islet3:GFP* transgene. Arrowheads point to anterior and medial SAG branches. Arrows point to the region where SAG morphology is changed. SAG, statoacoustic ganglion.

1.3.3 Her9 function in otic sensory development

Hes1, the mouse ortholog of Her9, negatively regulates inner ear hair cell differentiation in the cochlea and utricle (Zheng et al., 2000, Zine et al., 2001, Zine et al., 2002, Tateya et al., 2011). To test whether this is the case in zebrafish, after injecting her9-MO, embryos were probed by in situ hybridization for *atoh1a* expression at 24 hpf. At this stage *atoh1a* labels anterior macula. In morphant embryos neither the expansion nor misspecification of *atoh1a*-expressing cells was apparent at 24 hpf (Fig. 23A,B; n=0/8). Also, her9-MO was injected into Tg(*brn3c:GFP*) embryos to image and count the number of hair cells that developed at later stages (Xiao et al., 2005). In these embryos, GFP was visualized in vivo in the sensory maculae and the three sensory cristae at 48 and 96 hpf, respectively.





C

	total SAG		anterior SAG		medial SAG	
	Uninjected	Her9-MO	Uninjected	Her9-MO	Uninjected	Her9-MO
mean	132,2	136	32,2	34,2	97	97,3
SD	9,75	12,2	4,9	4,3	5,9	8
p value	0,56		0,47		0,8	

Figure 22. Number of SAG neurons is not significantly changed upon Her9 depletion.

(A-B) Counting of SAG neurons in wild-type and her9-MO injected embryos. (A-A''') Individual SAG neurons were counted on z stack series (step approx. 1µM) on coronal sections of 48 hpf wild-type or her9-MO injected embryos. Anterior is to the left, medial is down. (B,C) Bar diagram and table show the mean number of neurons in whole SAG, and separately in its anterior and medial parts.

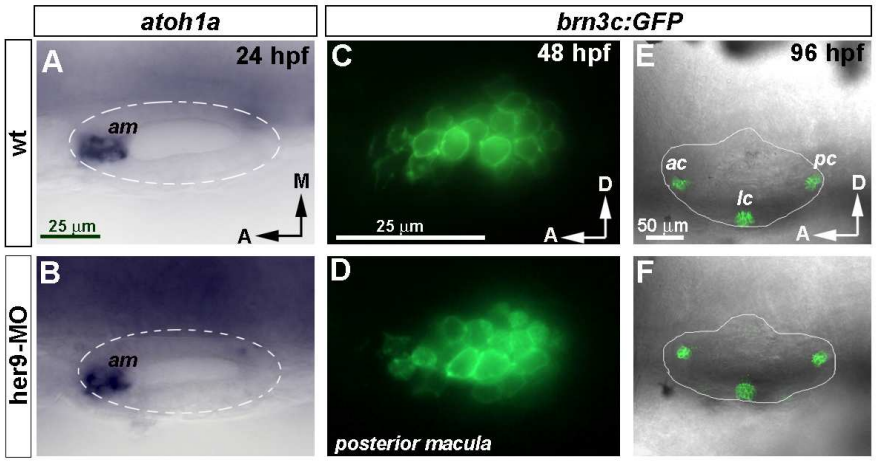


Figure 23. Otic sensory development proceeds normally in the absence of her9 function.
(A,B) Expression of *atoh1a* in wild-type and her9-MO injected embryos at 24 hpf. No change in *atoh1a* expression is observed. **(C,D)** Sagittal sections of posterior macula showing GFP fluorescence under the control of *brn3c* promotor in a wild-type and her9-MO injected embryo. **(E,F)** Three-dimensional reconstruction of selected confocal sections showing the sensory cristae in wild-type and her9-MO injected embryos. GFP in adjacent neuromasts of the lateral line has been removed for clarity. A,B, C,D and E,F are at the same magnification. SAG, statoacoustic ganglion; am, anterior macula; ac, anterior crista; lc, lateral crista; pc, posterior crista.

The anterior and posterior macula of Her9 morphants at 48 hpf contained between 16 and 20 GFP-positive cells (n=8) each of them, as counted from transverse and sagittal sections. The same number of cells was found in wild-type embryos (n=10) (Fig. 23C,D). At 96 hpf, approximately 8-10 cells were counted in each of the three cristae in both morphants and wild-type embryos (Fig. 23E,F). The conclusion obtained from this analysis is that *her9*

regulates otic neuronal development as observed by ectopic expression of proneural genes *neurod* and *neurod4* and improper SAG morphology in Her9 morphants, while Her9 does not control the expression of other proneural genes involved in otic sensory development, such as *atoh1a* or *brn3c*.

Otoliths, small gelatinous structures located in a close proximity to saccular and utricular maculae, are important for proper stimulation of hair cells when the head moves. At 48 and 72 hpf Her9 depleted embryos appear to have otoliths of the same size and at the same positions as otoliths in wild type embryos (Fig. 24A-D). Morphogenesis of the Her9 morphant otic vesicles also proceeds normally, as judged by the presence of endolymphatic duct and semicircular canals anlagens at 72 hpf (Fig. 24C,D).

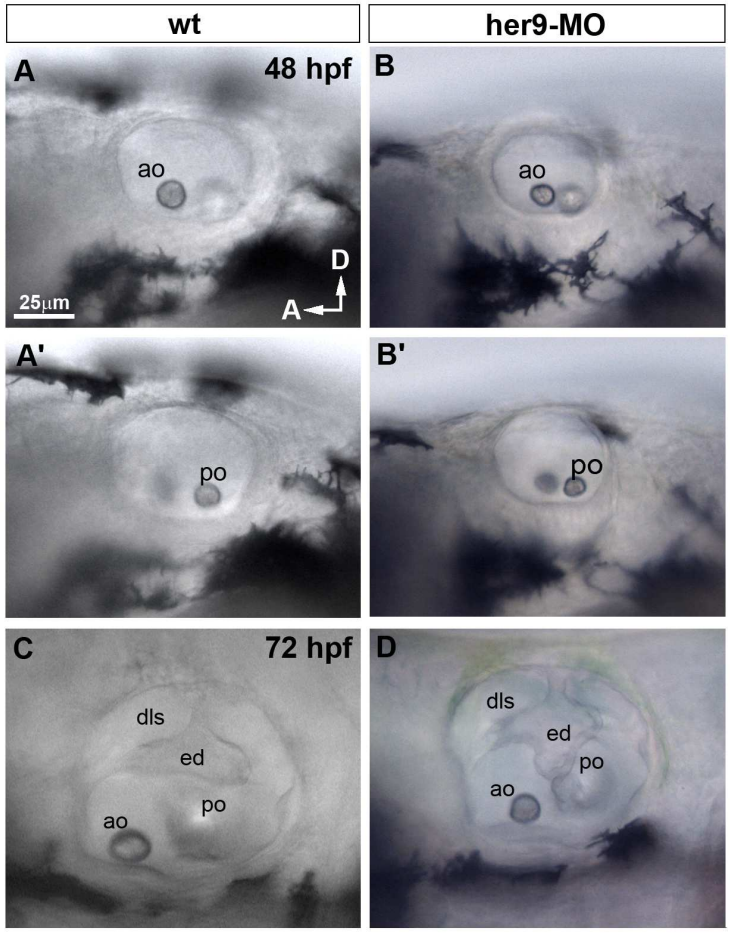


Figure 24. Her9 loss-of-function does not impair otolith development, neither normal otic morphogenesis.
(A-D) Bright field images of whole mount otic vesicles in wild-type and her9-MO injected embryos at 48hpf (A-B') and 72hpf (C,D). Anterior is to the left, dorsal is up. ao, anterior otolith; po, posterior otolith; dls, dorsolateral septum; ed, endolymphatic duct. All images are at the same magnification.

1.3.4 The relation of *her9* and other posterolateral genes

To test if *her9* has a role in the establishment and/or maintenance of the posterolateral domain, in situ hybridization for *tbx1, her9* and *lmx1b1*, normally coexpressed in this domain was done after injecting her9-MO. No change in expression pattern was observed for any of the genes when compared to the control embryos (Fig. 25A-F). However, increased levels of unspliced *her9* pre-mRNA could be observed in the nucleus of the morphant cells (Fig. 25F'), tentatively suggesting that Her9 might negatively autoregulate itself, the mechanism already described for its mouse ortholog *Hes1* (Takebayashi et al., 1994, Hirata et al., 2002).

her9 was also present in the hindbrain, with highest levels at rhombomeric boundaries (Fig. 26A, white arrowheads), where it is reported that neurogenesis is absent (Amoyel et al., 2005). In morphant embryos, ectopic neurogenesis in the hindbrain could be observed, concomitant with a reduction of nonneurogenic domains (Fig. 26C,D), similarly to our observation in the inner ear. Since *her9* is present in the hindbrain and hindbrain is known to impact otic induction and patterning, the effect of Her9 loss of function on otic neurogenesis could be indirect outcome of possible hindbrain misspatterning. To test if this is the case *krox20* expression, normally observed in rhombomeres 3 and 5 of the developing hindbrain, was assessed in wild-type and Her9 morphant embryos. No change in pattern of *krox20* expression was observed (Fig. 26E,F), suggesting that hindbrain patterning proceeds normally in Her9-depleted embryos and that effect Her9 has on the otic neurogenesis is otic-autonomous.

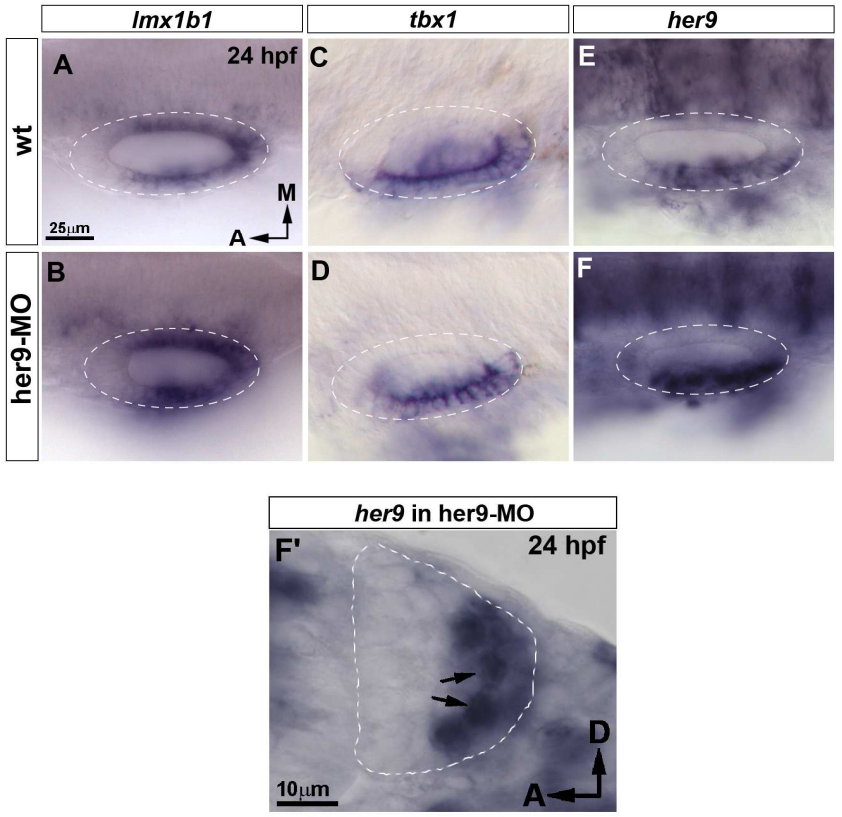


Figure 25. Expression of some posterolateral otic markers is unchanged in her9-MO injected embryos.
(A-F) Otic expression of *lmx1b1, tbx1* and *her9* in wild-type (A,C,E) and her9-MO injected (B,D,F) embryos at 24 hpf reveals no change in pattern of expression of these genes. **(F')** Transversal section of her9-MO injected embryo probed by in situ hybridization for *her9.* Arrows point to increased nuclear staining for *her9,* an outcome of improper splicing. A-F are at the same magnification.

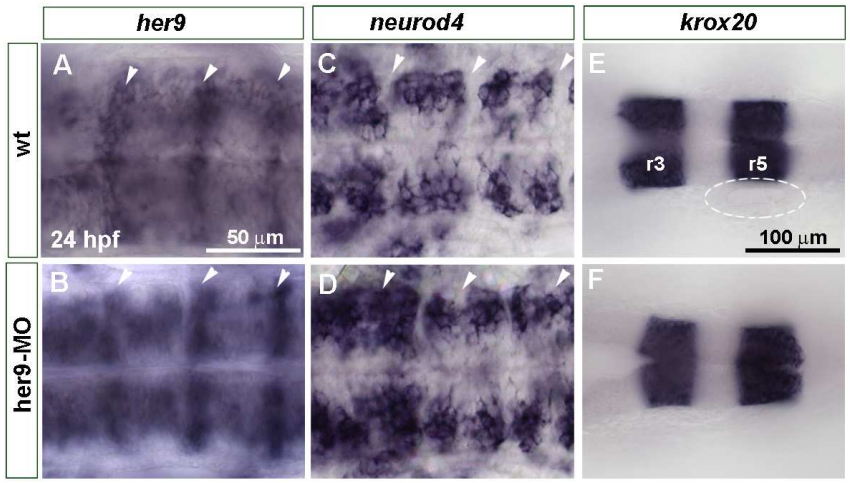


Figure 26. Hindbrain patterning and neurogenesis in Her9-depleted embryos.
(A,B) Expression of high levels of *her9* in hindbrain rhombomeric boundaries (arrowheads) in wild-type and her9-MO injected embryos. **(C,D)** *neurod4* expression is expanded in the hindbrain of Her9 morphants. **(E,F)** *krox20* expression in rhombomeres 3 and 5 is unchanged in Her9-depleted embryos. Dashed circles delineate the otic vesicle. r3, rhombomere 3; r5, rhombomere 5. A-D and O,P are at the same magnification. Anterior is to the left in all images.

1.3.5 Her9 function in cell proliferation

A feature of the otic vesicles of her9-MO injected embryos was their reduction in size (16% reduction is size). *Hes1* has been reported to prevent differentiation and to maintain cells as precursors or stem cells. In liver, thymus, brain and, as shown recently, in cochlear epithelia, *Hes1* promotes precursor cell proliferation through the transcriptional downregulation of the cyclin-dependent kinase inhibitor (Cdkn) $p27^{kip1}$ (Murata et al., 2005, Murata et al., 2009). To assess the possible role of *her9* in

cell proliferation in the inner ear, first I searched for the otic expression of different *cdkn* genes, by in situ hybridization. Among seven *cdkn* genes screened, two were expressed in the early zebrafish inner ear. *cdkn1c* (*p57*) was expressed throughout the entire neurosensory domain (Fig. 27C), whereas *cdkn1bl* (*p27-like*) was expressed in only its posterior part (Fig. 27A). None of the genes was expressed in the *her9*-positive territory in wild type embryos at 24 hpf, suggesting that Her9 might inhibit their expression there. Indeed, in *her9*-MO injected embryos, ectopic expression of *cdkn1bl* was apparent in the posterior epithelium (Fig. 27B, n=8/12), whereas *cdkn1c* expression did not change significantly (Fig. 27D, n=3/12).

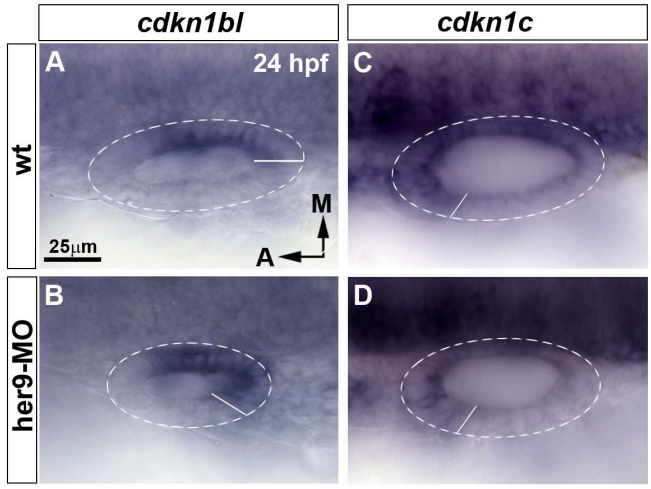


Figure 27. *cdkn1bl* and *cdkn1c* are expressed in the inner ear and negatively regulated by *her9.*
(A-D) In situ hybridization for *cdkn1bl* (A,B) and *cdkn1c* (C,D) in wild-type (A,C) and her9-MO injected (B,D) zebrafish embryos at 24 hpf. Dashed circles delineate the otic vesicle. White lines indicate limits of posterolateral and anterolateral expression of *cdkn1bl* and *cdkn1c,* respectively. Anterior is to the left, medial is up. All images are at the same magnification.

To assess the proliferative status of the cells within the otic vesicle, cells in M phase of the cell cycle were immunostained with anti-phospho-Histone H3 (anti-pH3) (Fig. 28A-E). Counting the number of mitotic cells inside and outside the *her9*-positive domain in wild-type and morphant otic vesicles (Fig. 28F) showed that the subfraction of mitotic cells in the *her9* domain strongly decreased after Her9 depletion, when normalized with number of mitotic cells in *her9*-negative domain (control, n=16, 0.43±0.22; her9-MO, *n*=15, 0.15±0.17; *P*=0.001).

Taking into account that (1) no *cdkn* gene is endogenously expressed in *her9*-positive domain, (2) *cdkn1bl* is ectopically induced in the otic vesicles of Her9 morphant embryos and that (3) there is a loss of mitotic cells in the *her9* territory, we conclude that Her9 has a role in maintaining cells in proliferative state in the nonneurogenic domain.

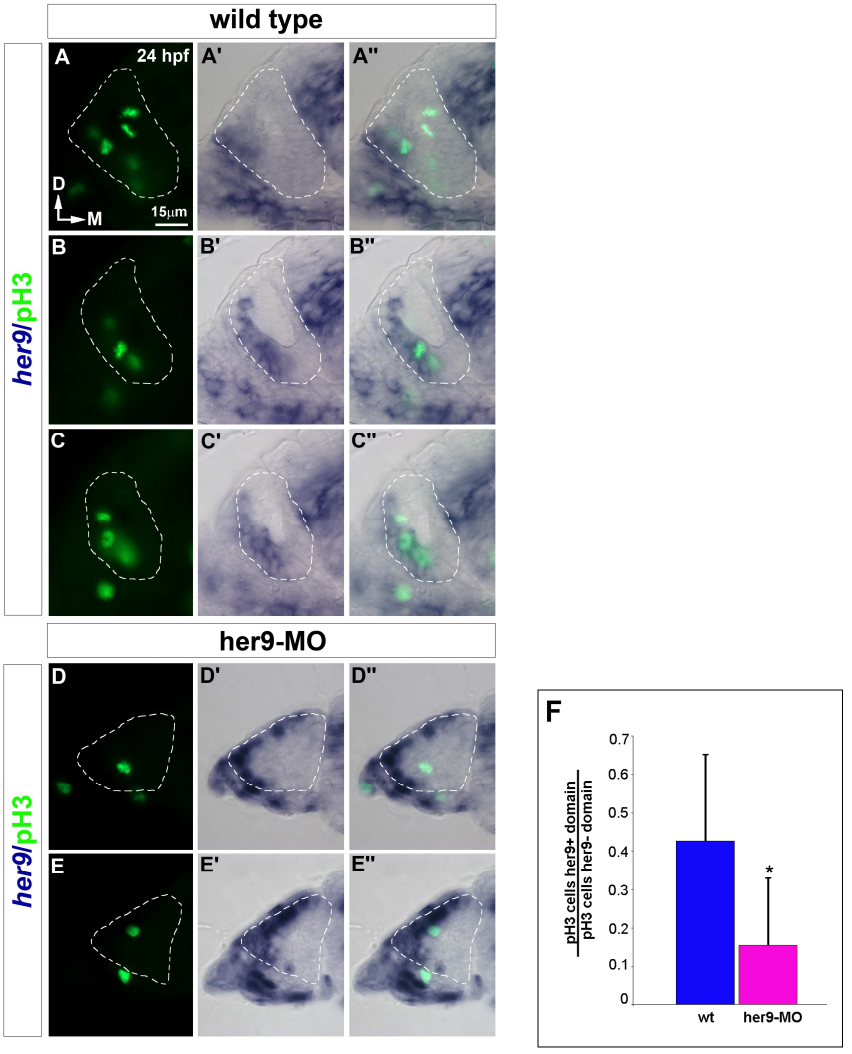


Figure 28. *her9* regulates cell proliferation within the otic vesicle. **(A-E'')** Transversal sections of wild-type and Her9 morphant otic vesicles stained by in situ hybridization for *her9* (blue) and immunohistochemistry for anti-pH3 (green) at 24 hpf. Dashed circles delineate the otic field. Sections are arranged from anterior (A and D) to posterior (C and E). All images are at the same magnification. Medial is to the right, dorsal is up. **(F)** Proportion of pH3 positive cells counted inside and outside the *her9*-expression domain in wild-type and Her9-depleted embryos. Error bars indicate mean + s.e.m. **P*=0.001.

1.3.6 Epistatic relationship between *her9* and *tbx1*

The loss of Tbx1 function in mouse leads to the increased otic neurogenesis and duplication of SAG (Raft et al., 2004). However, such role for zebrafish Tbx1 was not yet demonstrated. To analyze this, loss of Tbx1 function studies were done using zebrafish *van gogh* mutant (*vgo*^{tm208}) which carry a null mutation for *tbx1* gene (Piotrowski et al., 2003). *tbx1* mRNA staining in *vgo* mutants was still visible in the non-neurogenic territory, enabling performance of double in situ hybridization for proneural genes *neurog1/neurod* and *tbx1* at 24 hpf (Fig. 29A-F). The ectopic *neurog1* and *neurod* positive cells could be observed in posterolateral otic domain (Fig. 29B,C,E,F), confirming that in zebrafish Tbx1 has a role in the regulation of the otic neurogenesis, homologous to that one in mouse. In addition, *her4* expression also expanded posterolaterally (Fig. 29G-I). These phenotypes, although stronger, resemble to the phenotypes observed in Her9 morphant embryos. This, together with the fact that *tbx1* is coexpressed with *her9* in the same domain within the otic vesicle, led to the analysis of potential epistatic relationship between them. As shown above, Her9 blockade does not suppress *tbx1* expression (Fig. 25C,D), most probably positioning Tbx1 upstream of Her9. Therefore *her9* expression was

analysed in *van gogh* (*vgo*) mutant embryos. From descendants of *vgo*^{tm208} heterozygotic crosses, in a quarter of embryos it was observed that otic *her9* expression was abolished at 16 hpf (data not shown), 18 hpf (Fig. 30A,B; *n*=9/40) and 24 hpf (Fig. 30C,D; *n*=9/32). The capacity of Tbx1 to activate *her9* was further demonstrated by the ectopic induction of *her9* in the anterior neurogenic domain at 24 hpf (Fig. 30E,F; n=8/24) after injection of *tbx1* capped mRNA into 1- to 2-cell stage embryos. Concomitantly, in Tbx1 overexpressing embryos, *neurod* expression within the otic epithelium was reduced in about a third of embryos (Fig. 30G,H; n=10/28). Taken together, these results reveal that both *tbx1* and *her9* are required for the establishment of the nonneurogenic compartment during inner ear development, and that Tbx1 is necessary and sufficient to induce and maintain *her9* expression in the otic vesicle.

In *van gogh* mutant, hindbrain segmentation is unnafected (checked by *krox20*, *hoxb2*, *hoxb3*, *ephrin-B2*, Piotrowski and Nusslein-Volhard, 2000), suggesting hindbrain-independent regulation of the extent of the otic neurogenesis.

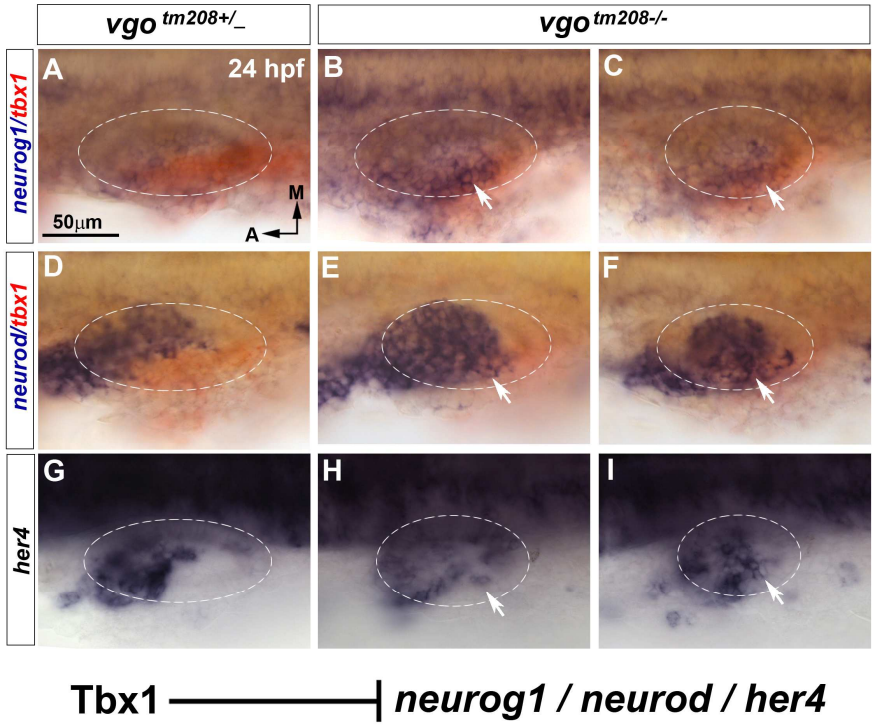


Figure 29. *tbx1* represses proneural genes in the inner ear.
(A-F) Double in situ hybridization for *tbx1* (red) and *neurog1* (blue, A-C) or *neurod* (blue, D-F) in 24 hpf embryos obtained from heterozygotic *vgo*^{tm208} crosses. **(G-I)** In situ hybridization for *her4* in 24hpf embryos obtained from heterozygotic *vgo*^{tm208} crosses. Two examples of *vgo*^{tm208} mutants are given for each gene in A-I. Arrows point to ectopic gene expression. Anterior is to the left, medial is up. All images are at the same magnification.

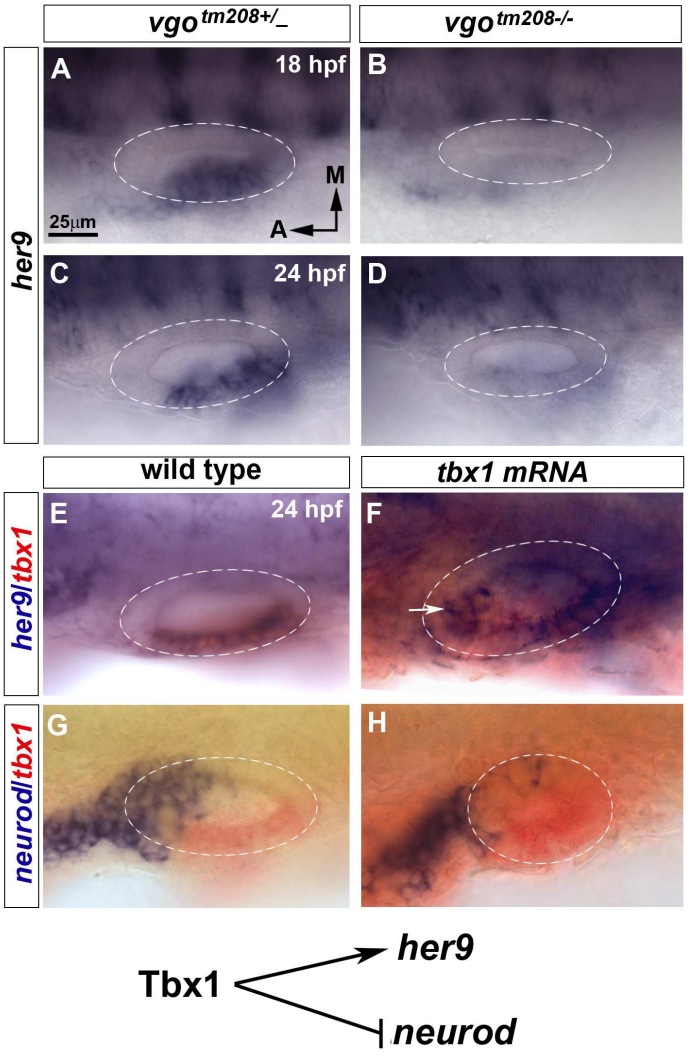
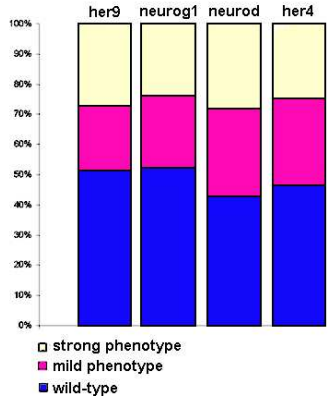


Figure 30. *tbx1* is genetically upstream of *her9*.
(A-D) Expression of *her9* in 18 and 24 hpf zebrafish embryos obtained from heterozygotic *vgo*^{tm208} crosses. **(E-F)** Double in situ hybridization for *tbx1* (red) and *her9* (blue) shows ectopic expression of *her9* (white arrow) after injection of *tbx1* capped mRNA. **(G-H)** Double in situ hybridization for *tbx1* (red) and *neurod* (blue) at 24 hpf shows downregulation of *neurod* after overexpression of capped *tbx1* mRNA. Dashed circles delineate otic vesicle. Anterior is to the left, medial is up. All images are at the same magnification.



Graphic with proportion of progeny with *her9/ neurog1/ neurod/ her4* phenotype. Proportion of descendants fromheterozygotic *vgo*^{tm208} crosses showing a stron phenotype (complete abrogation of *her9* expression, substantial expansion of *neurog1* and *neurod*), mild phenotype or wild-type phenotype at 24 hpf.

2. Regulators of the inner ear anteroposterior patterning

2.1 Retinoic acid, and not Notch signaling, acts to establish posterolateral otic genes

Members of the *Hes/her* gene family are classicaly considered as targets of Notch receptors. To assess whether *her9* is regulated by this pathway, I blocked Notch activity by incubating embryos with the γ-secretase inhibitor DAPT starting from 10.5 hpf, 3.5 hours before *her9* expression could be detected in the otic placode. Embryos treated from 10.5 hpr to 24 hpf displayed no inhibition nor induction of *her9* or *tbx1* expression (Fig. 31A-D, *her9*, n=11/11; *tbx1*, n=9/9), indicating that restricted posterolateral expression of these genes is established independently of Notch. As expected from the reported role of Notch signaling in hair cell development (Haddon et al., 1999; Millimaki et al., 2007), I found increased hair cell specification in DAPT-treated embryos, revealed by in situ hybridization for *atoh1a* (Fig. 31E-H) at 18 and 24 hpf. In addition, *neurog1* expression increased inside the neurogenic domain in DAPT-treated embryos (Fig. 31I,J), due to the impaired lateral inhibition, which normally controls the number of neuronal precursors within the neurogenic zones.

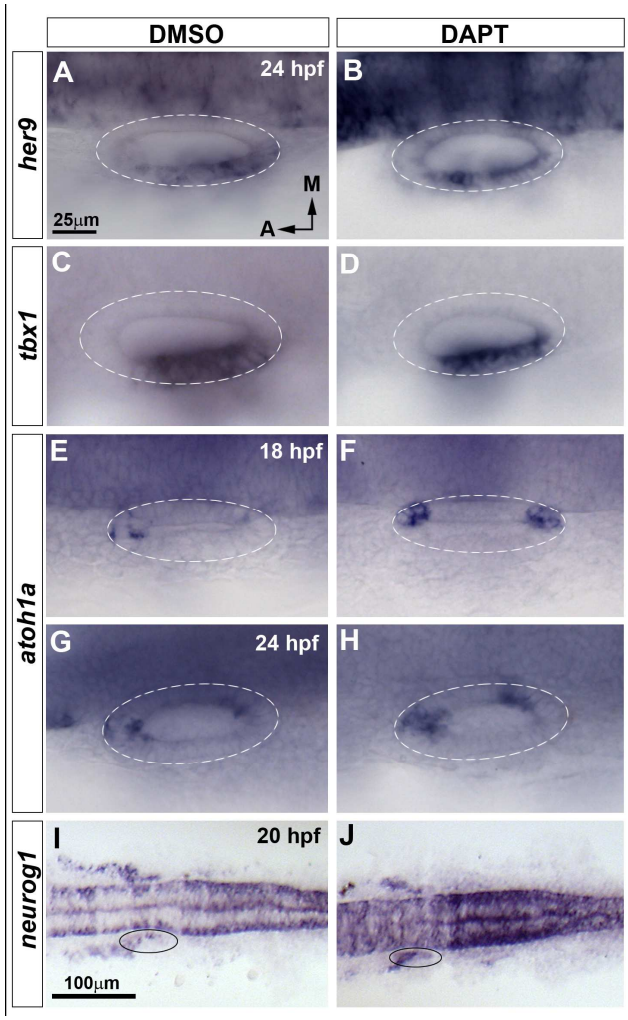


Figure 31. Notch signaling does not establish *her9* and *tbx1* expression in the inner ear.
(A-D) In situ hybridization for *her9* and *tbx1* at 24 hpf in control (DMSO) and Notch-inhibited (DAPT-treated) zebrafish embryos shows no change in their expression patterns. **(E-H)** In situ hybridization for *atoh1a* at 18 hpf (E,F) and 24 hpf (G,H) in control (DMSO) and DAPT treated embryos shows increased hair cell specification in sensory maculae. **(I,J)** In situ hybridization for *neurog1* in DMSO and DAPT treated 20 hpf embryos argues for the efficiency of DAPT treatments. Dashed circles delineate otic vesicles. Anterior is to the left, medial is up. A-H and I,J are at the same magnification.

Several lines of evidence pointed to retinoic acid (RA) signaling as a good candidate for controlling the expression of posterolateral genes in the otic placode. Loss of RA signaling affects craniofacial patterning (Niederreither et al., 1999; Niederreither et al., 2000) and phenocopies some of the features associated with DiGeorge syndrome (Begemann et al., 2001, Vermot et al., 2003), linked also to the mutation in *Tbx1* gene.

To assess RA activity over the otic territory, I have analysed the expression of Aldh1as and Cyp26s enzymes, involved in RA synthesis and metabolism, at the stages when the otic expression of posterolateral genes, such as *tbx1* and *her9*, is established. At 15 hpf, RA is synthesized by Aldh1a2 in the mesoderm located posterolaterally to the otic placode (Fig. 32A) and in somites (data not shown). Genes encoding for RA degrading enzymes, *cyp26b1* and *cyp26c1* are expressed in the rhombomeres 2-4 and rhombomeres 5-6 respectively at this stage (Fig. 32B,D), suggesting the possible existence of a gradient of retinoic acid activity that decreases across the otic placode from posterolateral to anteromedial.

To test whether RA is responsable for the establishment and/or maintenance of the posterolateral otic genes, embryos were exposed to 20μM DEAB (4-(diethylamino)-benzaldehyde), a potent pharmacological inhibitor of retinaldehyde dehydrogenases (Raldhs/Aldhs), from 10.5 to 24 hpf. Efficiency of treatments was assessed by in situ hybridization for *hoxb4*, a neural tube target of RA signaling (Fig. 42A,B). Interestingly, blocking RA signaling

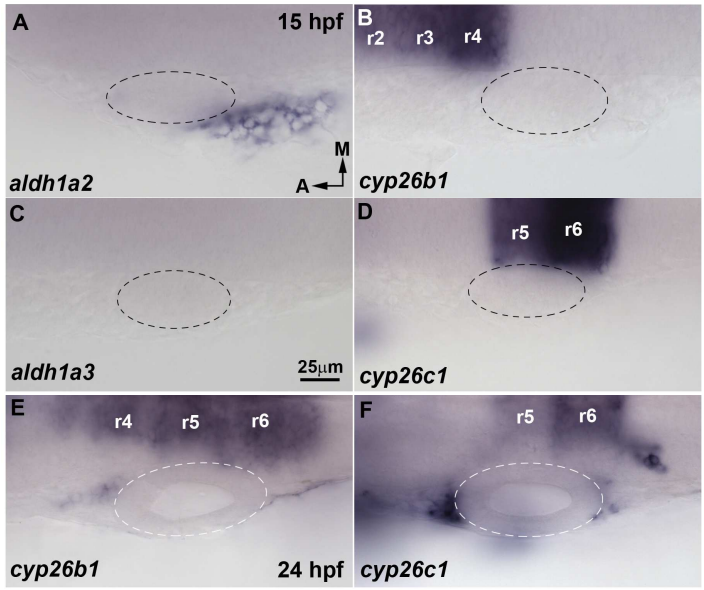


Figure 32. Retinoic acid synthesizing and degrading enzymes are expressed adjacent to the otic placode.
(A-D) Dorsal views of 15 hpf zebrafish embryos, probed by in situ hybridization for *aldh1a2*, *aldh1a3, cyp26b1* and cyp26c1. Dashed circles delineate the otic placode. **(E-F)** In situ hybridization for *cyp26b1* and *cyp26c1* at 24hpf. Dashed circles delineate the otic vesicle. r, rhombomere. Anterior is to the left, medial is up. All images are at the same magnification.

abolished the expression of *her9* and *tbx1* (Fig. 33A,B,D,E; *her9*, n=9/9; *tbx1*, n=6/7) and in parallel shifted the boundary between neurogenic and nonneurogenic domains, which was revealed by posterior expansion of the *neurog1*, *neurod* and *her4* expression (Fig. 33G,H,J,K,M,N; *neurog1*, n=6/7; *neurod*, n=4/6; *her4*, n=6/6). Conversely, when embryos were incubated with nonteratogenic doses of RA (20nM RA, Hans and Westerfield, 2007) from 10.5 to 12 hpf and left to develop until 24 hpf, the limit of *her9* and *tbx1* expression shifted anteriorly (Fig. 33C,F; *her9*, n=20/24; *tbx1*, n=12/13), concomitant with a medial shift of *neurog1*, *neurod* and

her4 expression (Fig. 33I,L,O; *neurog1*, n=9/11; *neurod,* n=5/5; *her4*, n=11/13). Together, these results further confirm the cross-interaction between the *neurog1/neurod* and *tbx1/her9* expression domains, and demonstrate the influence of RA in setting the anteroposterior (AP) boundary between neurogenic and nonneurogenic domains in the otic vesicle.

Since RA activity influences AP patterning of the hindbrain and hindbrain signals pattern the inner ear, the change in *tbx1* and *her9* expression in DEAB- and RA-treated embryos might be an indirect result of hindbrain misspecification. To assess hindbrain patterning at the level of the otic vesicle, *krox20* expression was analyzed in 24 hpf embryos in which RA was up- or downregulated from 10.5 hpf. *krox20* expression was unchanged in both conditions, as compared to wild type embryos (Fig. 34A-C), thereby excluding gross hindbrain patterning defects in our manipulations.

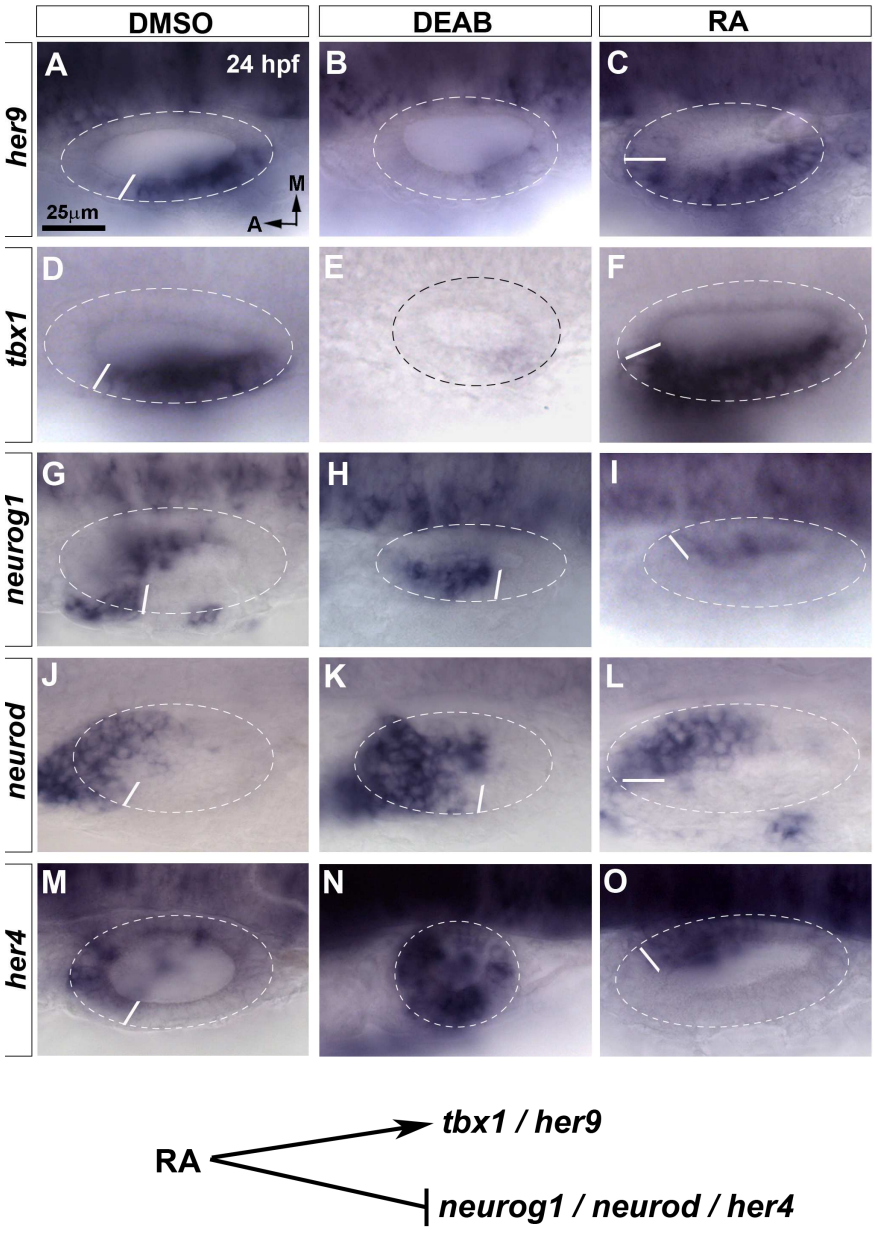


Figure 33. Retinoic acid regulates *her9* and *tbx1* expression in the inner ear, and correspondingly the boundary between neurogenic and nonneurogenic otic territories.

(A-O) Expression of *her9* (A-C), *tbx1* (D-F), *neurog1* (G-I), *neurod* (J-L) and *her4* (M-O) in 24 hpf embryos treated with DMSO (A,D,G,J and M), the retinaldehyde dehydrogenase inhibitor DEAB (B,E,H,K and N) or retinoic acid (RA) (C,F,I,L and O) from 10.5 hpf. *her9* and *tbx1* expression

is lost in the absence of RA signaling (B and E). Dashed circles delineate otic vesicles. Boundary between neurogenic and non-neurogenic otic domains is depicted by the white line. Anterior is to the left, medial is up. All images are at the same magnification.

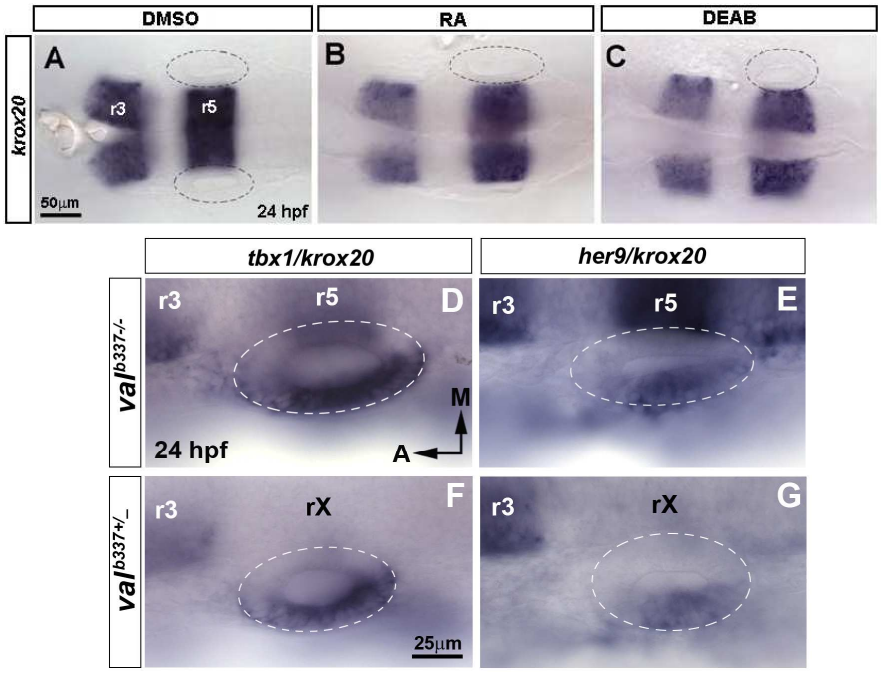


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Figure 34. Hindbrain is dispensable for establishment of posterolateral otic genes.
(A-C) *krox20* expression in embryos treated with DMSO (A), 20nM RA (B) and 20μM DEAB (C), showing no hindbrain patterning defects. **(D-G)** In situ hybridization for *krox20* and *tbx1* (D,F) or *krox20* and *her9* (E,G) in embryos obtained from heterozygotic *val*$^{b337+/-}$ crosses. Dashed circles delineate otic vesicles. r, rhombomere. Anterior is to the left. A-C and D-G are at the same magnification.

Moreover, I analyzed the *tbx1* and *her9* expression in *valentino* mutants (*val*^{b337}), which exhibit misspecification of rhombomeres 5 and 6 (Moens et al., 1996), and no change in the expression pattern for any of the two genes was observed when compared to

wild type embryos (Fig. 34D-G). This further confirms that posterior hindbrain patterning is dispensable in establishing the otic *tbx1/her9* expression domain.

In conclusion, RA has a crucial role in early otic regionalization, acting to establish the expression of posterolateral genes such as *tbx1* and *her9*, which define the non-neurogenic otic compartment, and this occurs independently of hindbrain patterning.

2.2 The role of FGF signaling in otic anteroposterior patterning and its interaction with retinoic acid signaling

As shown above, increasing the levels of RA shifted the anterior limit of *tbx1* expression, but a small anteromedial domain was always devoid of *tbx1* and was still expressing *neurod*. The following question arose: Does an anterior signal also exist to limit *tbx1* expression? Fgf3 and Fgf8 signals from rhombomere 4 are required for the specification of the otic sensory territory and for otic neuronal production (Léger and Brand, 2002, Phillips et al., 2001). To test whether Fgfs might inhibit *tbx1* expression in the anterior otic domain first I analyzed the expression of *tbx1* and *neurod* in *acerebellar* (*ace*) mutants, in which *fgf8* gene is mutated and thus encode nonfunctional Fgf8 product (Reifers et al., 1998). Although transversal sections showed that there are less *neurod*-positive cells in mutant embryos, no change in expression pattern for any of the two genes analysed was observed (Fig. 35A-P). In these mutants Fgf3 signaling is still operative, probably compensating for the loss of Fgf8 function. To overcome this problem I treated embryos with SU5402, a potent pharmacological

inhibitor of Fgf receptors (Mohammadi et al., 1997). Efficiency of the treatments was confirmed by downregulation of otic *pea3* expression in SU5402-treated embryos (Fig. 42C,D). To characterize the temporal requirement for Fgfs in the formation of the otic vesicle, neurogenesis and the expression of *tbx1*, treatments of the embryos with 60 μM SU5402 were done starting at different time points. Otic induction was abolished in Fgf-depleted embryos treated with SU5402 from 4.3 to 30 hpf, judging by the lack of any morphological sign of otic vesicles (Fig. 36A,B; n=12/12), and this is in accordance with previously reported early role of Fgfs in the otic induction (Léger and Brand, 2002).

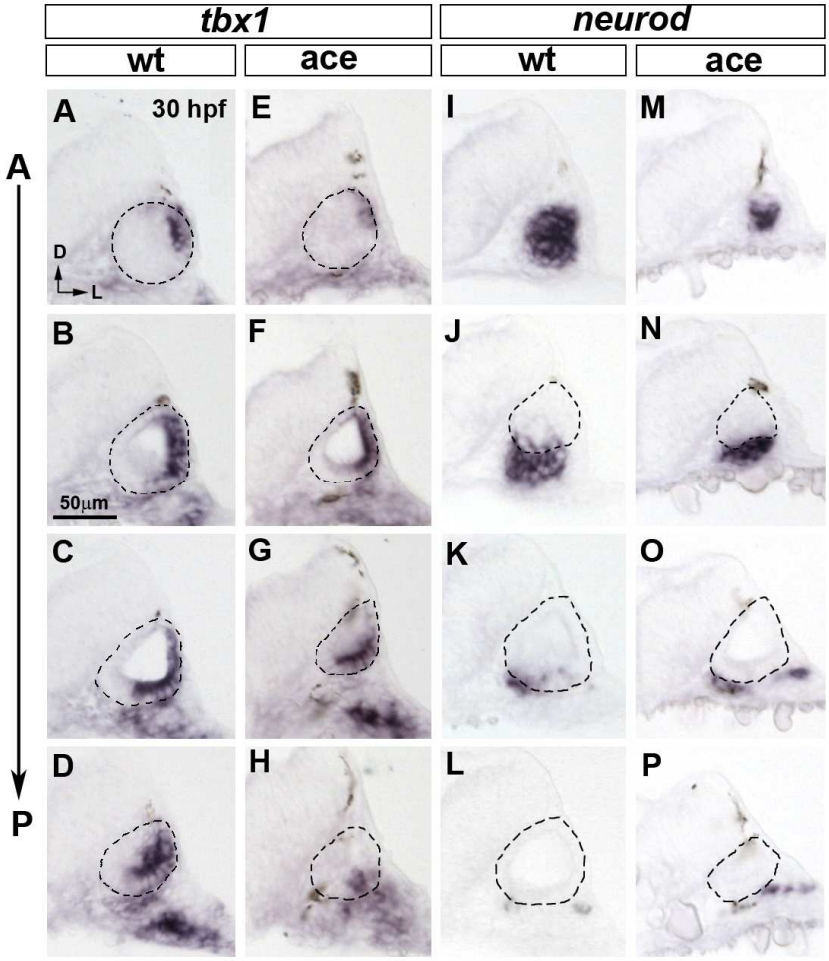

Figure 35. *tbx1* and *neurod* expression patterns are unchanged in ace mutants.
(A-H) Transversal sections of 30 hpf wild-type (A-D) and ace mutant embryos (E-H) stained for *tbx1* by in situ hybridization, showing no change in expression pattern. **(I-P)** Transversal sections of wild-type (I-L) and ace mutant embryos (M-P) stained for *neurod* expression, showing no change in spatial distribution of expression, although there are less *neurod* positive cells in ace mutant compared to wt. All sections are arranged from anterior to posterior. Dashed circles delineate sectioned otic vesicles. Dorsal is up, lateral to the right. All images are at the same magnification.

When Fgf blockade was performed from 50% epiboly (5.3 hpf) to 24 hpf, otic induction was not abrogated but the treatment led to the very tiny otic vesicles, without neurogenesis and with reduced levels of *tbx1* expression (Fig. 36D,H; n=7/7). Treatment from 7 hpf onwards resulted in no change in the overall pattern of expression of *tbx1* and *neurod*, although both domains were smaller due to an effect on otic growth (Fig. 36E,F,I,J). These data show that FGF signaling is required for otic induction before the gastrula stage, for neural commitment at the early gastrula and to maintain the otic growth after 7 hpf. Thus, Fgf signal does not seem to have a role in limiting *tbx1* expression anteriorly in the inner ear anlagen.

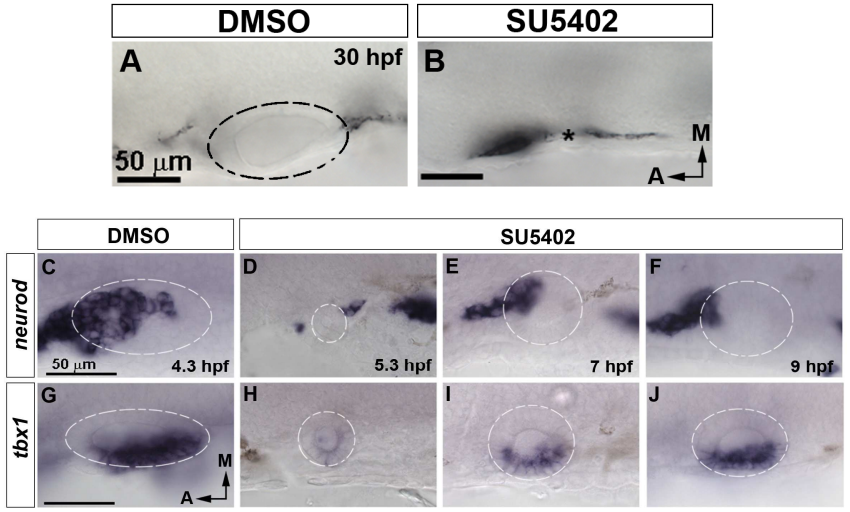

Figure 36. Temporal requirement for Fgf signals in otic development.
(**A-B)** Bright-field images of 30 hpf embryos treated with DMSO or with Fgf inhibitor SU5402 starting from 4.3 hpf reveals a requirement for Fgf signaling at this stage for otic placode induction. Asterisk in B indicates putative position of otic vesicle. **(C-J)** Stage dependent influence of Fgf signaling on *neurod* and *tbx1* expression. Dashed circles delineate the otic vesicle. Numbers indicate the stage when the treatment begun. Anterior is to the left, medial is up. A,B and C-J are at the same magnification.

Moreover, inhibition of Fgf signaling at 50% epiboly resulted in a loss or severe downregulation of *tbx1* expression, suggesting that at this stage Fgf might interfere with RA activity. To assess this, we examined the expression of *aldh1a2* and *cyp26b1/c1*, after blocking Fgf signaling. At 13 hpf, the expression of *aldh1a2* is initiated in the cranial mesoderm adjacent to the otic placode (Fig. 37A). Inhibition of Fgf signaling from 50% epiboly suppressed *aldh1a2* expression in the cranial mesoderm at 13 hpf and also partially suppressed *cyp26c1* expression in the hindbrain (Fig. 37A). Since the patterning of the hindbrain is severely disrupted in these embryos, the loss of *aldh1a2* expression might be a direct effect of Fgf signaling or an indirect effect of hindbrain mispatterning. However, blocking Fgf signaling at 10.5 hpf did not have a strong impact on the expression of RA pathway components (Fig. 37B), in agreement with the lack of the effect on the otic *tbx1* expression at these stages. Together, these data suggest that Fgf influences RA levels at gastrula stages, whereas RA activity in the otic territory is Fgf-independent at later stages.

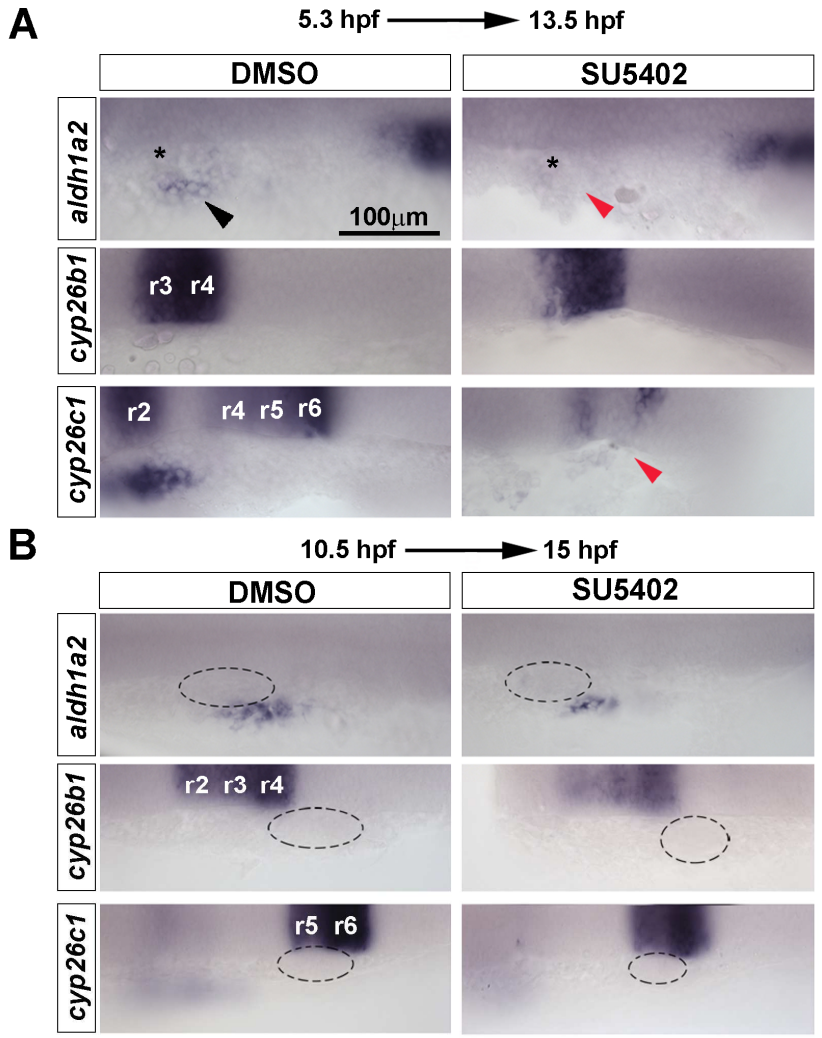

Figure 37. Fgf signaling at early gastrula stages, but not later, is requiered for *aldh1a2* establishment in the vicinity of the otic placode.

(A-B) In situ hybridization for *aldh1a2*, *cyp26b1* and *cyp26c1* in embryos treated with DMSO or SU5402 starting from 5.3 hpf (A) or 10.5 hpf (B). Black arrowhead indicates mesodermal expression of *aldh1a2.* Red arrowheads indicate suppression of *aldh1a2* and *cyp26c1* expression. Asterisk in A indicates position of the otic placode. Dashed circles delineate otic placode. Anterior is to the left, medial is up. r, rhombomere. All images are at the same magnification.

2.3 The role of Hedgehog signaling in the otic anteroposterior patterning

Hedgehog signaling (Hh) was previously shown to be required for correct anteroposterior patterning of the zebrafish otic vesicle (Hammond et al., 2003), but this role was not directly correlated to the otic neurogenic patterning. To test the role of Hh signaling in the otic neurogenic vs nonneurogenic patterning, zebrafish embryos were treated with a 100μM Hh blockator cyclopamine A (CyA) (Chen et al., 2002) for consecutive 3-hour periods starting at 9, 12, 15, 18 or 21 hpf. The expression analysis was done afterwards, at 24 hpf stage. Inhibition of Hh signaling between 12 and 18 hpf caused a strong posteromedial induction of *tbx1* and *her9* (Fig. 38A,B,D,E and 39B,C; *tbx1, n*=10/12; *her9*, n=3/4), and consequent retraction of *neurod* expression domain (Fig. 38C,F), indicating that Hh has a role in limiting the expression of nonneurogenic genes in the posteromedial domain of the otic vesicle. This inhibitory function is limited to the 12-18 hpf time window, since blockade of Hh signaling before or after these stages had no effect on otic *tbx1* expression (Fig. 39A and D), indicating that at 18 hpf the posteromedial domain is already determined.

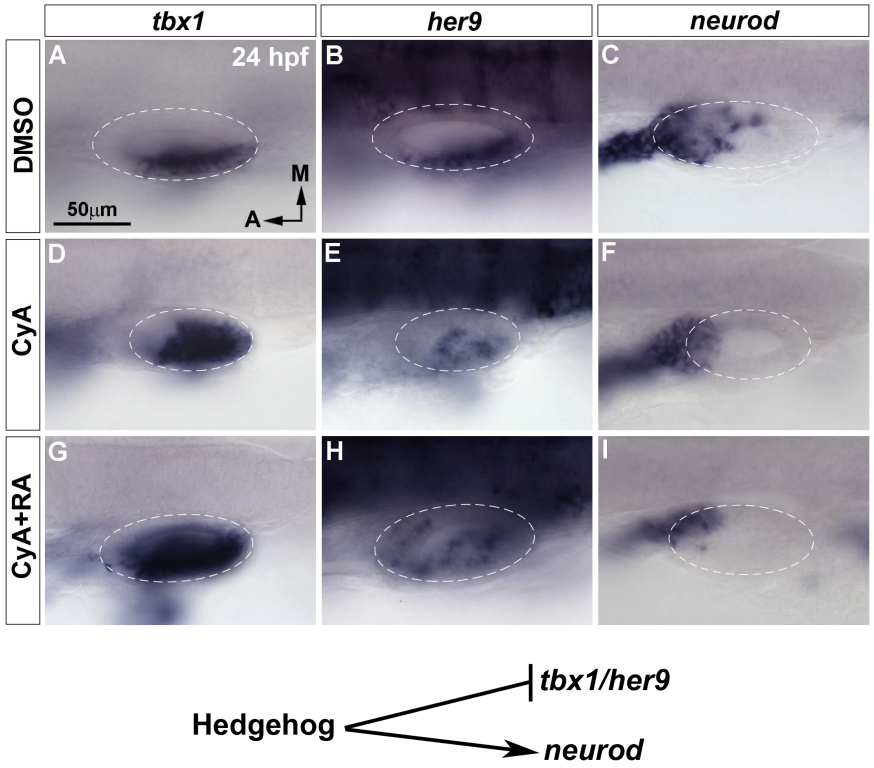

Figure 38. Hedgehog and retinoic acid signaling together regulate otic *tbx1*, *her9* and *neurod* expression.
(A-I) Whole mount in situ hybridization for *tbx1* (A,D,G), *her9* (B,E,H) and *neurod* (C,F,I) in 24 hpf embryos treated with DMSO (A-C), the Hh inhibitor cyclopamine A (CyA), and CyA+RA starting from 10.5 hpf. CyA and RA act additivily to expand *tbx1* and *her9* expression (G,H). Dashed circles delineate otic vesicles. Anterior is to the left, medial is up. All images are at the same magnification.

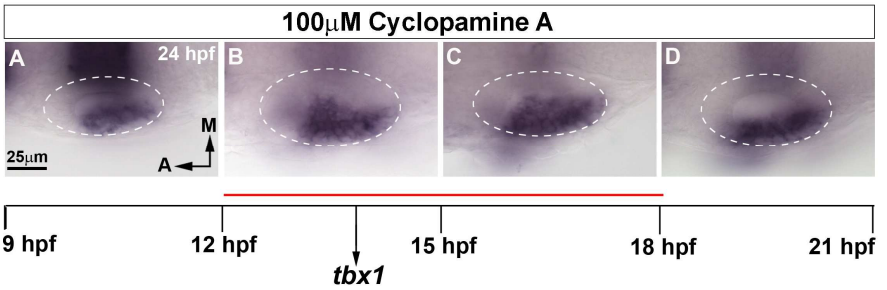

Figure 39. Hedgehog signaling affects otic tbx1 expression in a 12-18 hpf time window, but not before nor later.
(A-D) Whole mount in situ hybridization performed at 24 hpf to detect tbx1 (and krox20) expression after blocking Hh signaling with 100 □M CyA from 9-12 hpf (A), 12-15 hpf (B), 15-18 (C), 18-21 (D). tbx1 expression is expanded only in embryos treated with CyA from 12-18 hpf, while earlier (A) and later (D) treatments do not change tbx1 expression pattern. Anterior is to the left, medial is up. All images are at the same magnification.

In the limb/wing buds and forebrain development in mouse and chick, RA signaling cross-talks with Shh (Riddle et al., 1993, Helms et al., 1994, Schneider et al., 2001, Ribes et al., 2006). To test if this also happens in the otic territory, since the same otic genes are affected in experiments with manipulated levels of RA or Hh, embryos were probed for *aldh1a*, *aldh1a3*, *cyp26b1* and *cyp26c1* expression by in situ hybridization after incubation with 100μM CyA from 10.5 to 15 hpf. No change in expression pattern for any of these genes was observed in CyA-treated embryos compared to control, EtOH-treated embryos (Fig. 40A-H)

However, incubation of 100µM CyA together with 20nM RA at 10.5 hpf had an additive effect and almost the entire vesicle expressed *tbx1 and her9* (Fig. 38G,H; *tbx1*, *n*=3/3; *her9*, n=2/3). These data, although not suggesting that Hh and RA directly regulate each other, point to an interplay of positive and negative regulators of the *tbx1/her9* expression domain within the otic vesicle: RA signaling induces *tbx1/her9* expression, which is limited to the posterolateral wall by the inhibitory Hh signal. Finally, this effects are independent of Notch signaling, since co-treatments of RA and DAPT or CyA and DAPT did not modulate the main effect RA and CyA had alone on *tbx1* expression (Fig. 41A-C).

Efficiency of CyA treatments was analysed by in situ hybridization for *ptc1*, a target of Hh signaling. *ptc1* expression was strongly downregulated in CyA-treated embryo (Fig. 42E,F).

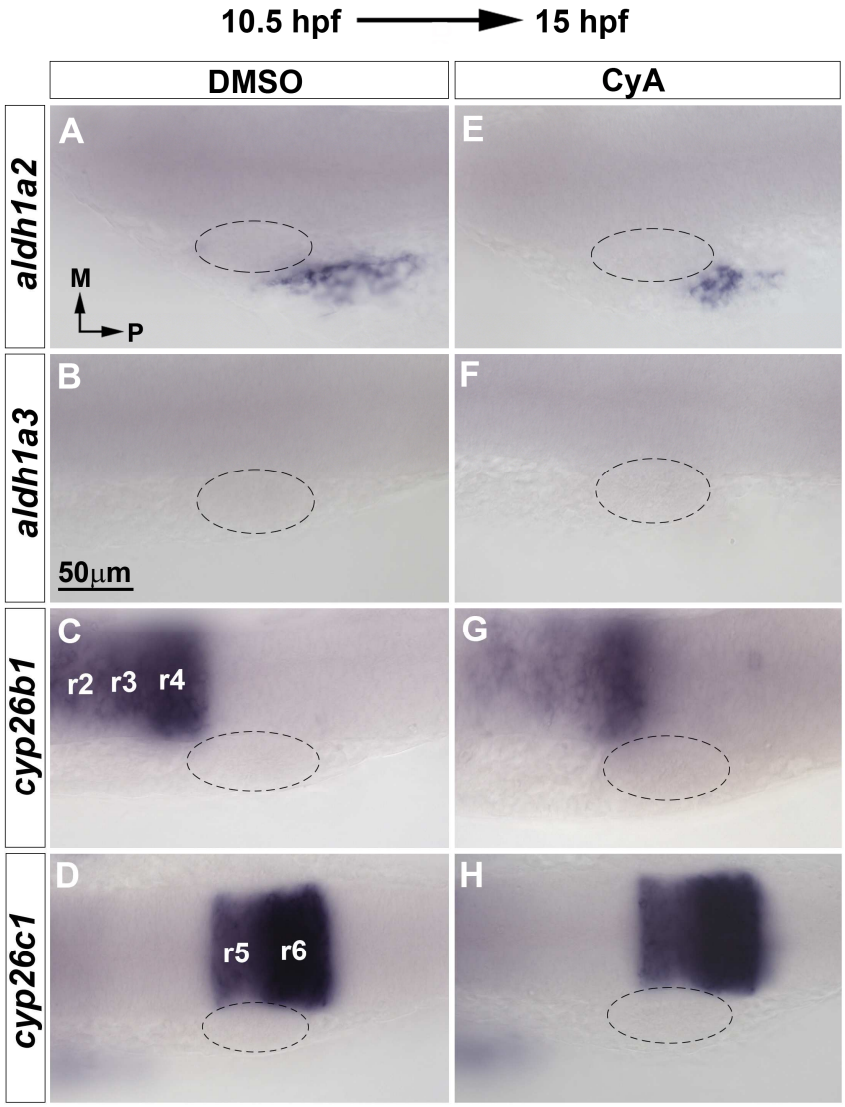

Figure 40. Hedgehog signaling does not affect retinoic acid levels at the stages when RA impacts expression of posterolateral otic genes. (A-H) Expression of *aldh1a2*, *aldh1a3*, *cyp26b1* and *cyp26c1* in 15 hpf embryos treated with DMSO (A-D) or CyA (E-H) from 10.5 hpf. No change in expression pattern could be observed for any of the genes tested. Dashed circle delineates otic placode. Posterior is to the right, medial is up. r, rhombomere. All images are at the same magnification.

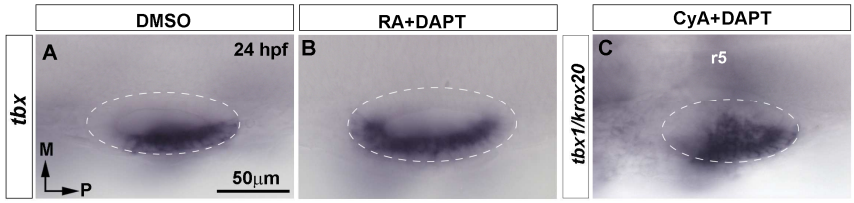

Figure 41. Tbx1 expression is modulated by RA and CyA, independently of Notch.
(A-C) In situ hybridization for tbx1 in 24 hpf embryos treated with DMSO, 20nM RA +150μM DAPT or 100μM CyA + 150μM DAPT from 10.5 hpf. Posterior is to the right, medial is up. r, rhombomere. All images are at the same magnification.

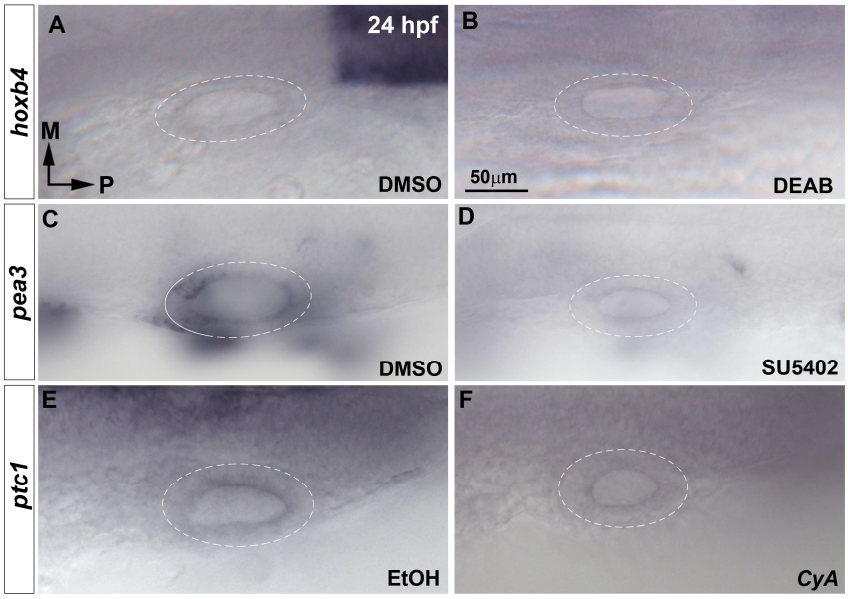

Figure 42. Efficiency of the treatments.
(A,B) In situ hybridization for *hoxb4* in 24 hpf embryos treated with DMSO or DEAB from 10.5hpf. Expression is lost from the neural tube in DEAB-treated embryos. **(C,D)** Expression of *pea3* in 24 hpf embryos treated with DMSO or SU5402 from 10.5 hpf. Expression is lost from the otic vesicle in SU5402-treated embryos. **(E.F)** Expression of *ptc1* in embryos treated with DMSO or CyA from 10.5hpf. *ptc1* expression is lost from the hindbrain in CyA-treated embryos. Anterior is to the left, medial is up. All images are at the same magnification.

The results described above permit modeling the complex genetic cascade that underlies neural patterning of the otic vesicle (Fig. 43). After the initial, Fgf-dependent, induction of the neural competence within the future otic territory, the neurogenic domain is restricted to only the anteromedial portion of the inner ear. This is achieved through the negative regulation of the otic neurogenesis by RA-Tbx1-Her9 genetic network. RA, synthesized by Aldh1a in paraxial mesoderm, establishes otic *tbx1* expression at 14 hpf (otic placode stage), before the onset of *neurog1* in the anteromedial domain. Tbx1 then activates the bHLH transcriptional repressor Her9 which will inhibit the expression of proneural *neurod/neurpod4* genes in the posterolateral otic domain. At the same time, otic *tbx1/her9* expression is inhibited by active Hh signaling in the posteromedial otic territory, pointing to the interplay between different signaling pathways during the establishment of the neurogenic and nonneurogenic otic territories.

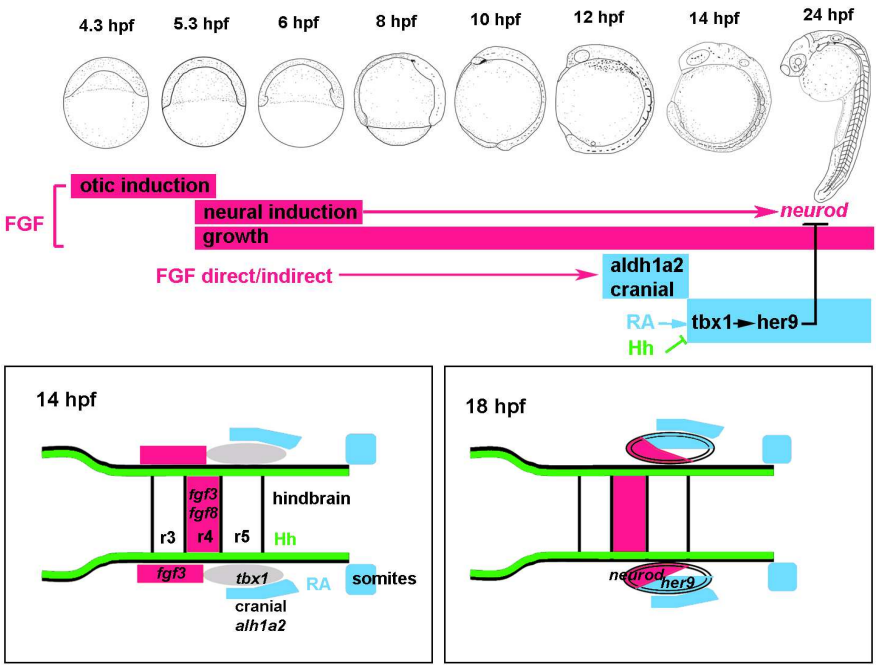

Figure 43. Integrative model of neurogenic versus non-neurogenic patterning by Fgf/RA/Hh-Tbx1/Her9-Neurod cascade.
(Top) The temporal requirements of Fgf (pink bars), Hh (green) and RA (blue bars) signaling during the otic development are illustrated. Before 5.3 hpf (50% epiboly), Fgf signaling is required for the otic induction, whereas from 5.3 until 7 hpf it is required for otic neural induction and for the establishment of the *aldh1a2* expression (at 13 hpf) in the cranial mesoderm After 7 hpf, Fgf signaling is required for otic growth but not for the establishment of *aldh1a2* nor for the otic *tbx1* expression. RA from the cranial mesoderm is required for *tbx1* induction at 14 hpf, leading to the activation of its downstream target: the proneural repressor Her9. In parallel, medial sources of Hh restrict otic *tbx1* expression to the posterolateral domain, acting from 12-18 hpf.
(Bottom) The extrinsic signals and intrinsic regulators of the otic neurogenic vs nonneurogenic patterning. From an initially uniform otic field, the pattern of *tbx1* and *her9* expression established by the opposing actions of RA and Hh at 14 hpf finally will result in regionalized *neurod* expression within the otic vesicle at 18 hpf.

3. Late roles of retinoic acid in the inner ear development

3.1 Late expression patterns of *aldh1a* and *cyp26 genes*

Retinoic acid plays multiple roles during otic development (see: Introduction: The roles of RA in the otic development). Briefly, the excess of RA was shown to misspattern hindbrain signals involved in otic induction process, such as Fgf signaling (White et al., 2000, Phillips et al., 2001, Kil et al., 2005), or to expand the expression of otic competence gene *foxi1* (Hans and Westerfield, 2007). As we have demonstrated, during the early axial patterning RA is required for the establishment of posterolateral otic genes, which through Her9 and Tbx1 define nonneurogenic otic domain. However, once early patterning events are disclosed, RA is still synthesized by retinaldehyde dehydrogenases (Aldh1as), expressed adjacent to or within the otic vesicle. This expression patterns are revealed by in situ hybridization for *aldh1a2* and *aldh1a3* at 30 and 48 hpf. At these stages otic vesicle undergoes gross cytodifferentiation processes, which lead to the production of the otic neurons and hair cells of the sensory maculae. At 30 hpf, *aldh1a2* is detected in the mesoderm lying below the anterior otic domain, while at 48 hpf its expression lies posteroventrally to the otic vesicle (Fig. 44A,B). Coronal sections revealed another, medial to otic, domain of *aldh1a2* expression (Fig. 45A,B), which was masked by pigmentation in whole mount embryos. *aldh1a3* was expressed in

future endolymphatic duct at 30 hpf, but later appears in distinct patches throughout anterior and lateral otic walls (Fig.44C,D). To better localize the expression of the two genes relative to the sensory patches and SAG, in situ hybridization was done in 48 hpf *brn3c:GFP* and *islet3:GFP* embryos. Coronal sections show that none of the genes is expressed in the hair cells of the sensory maculae nor in the SAG neurons (Fig. 45).

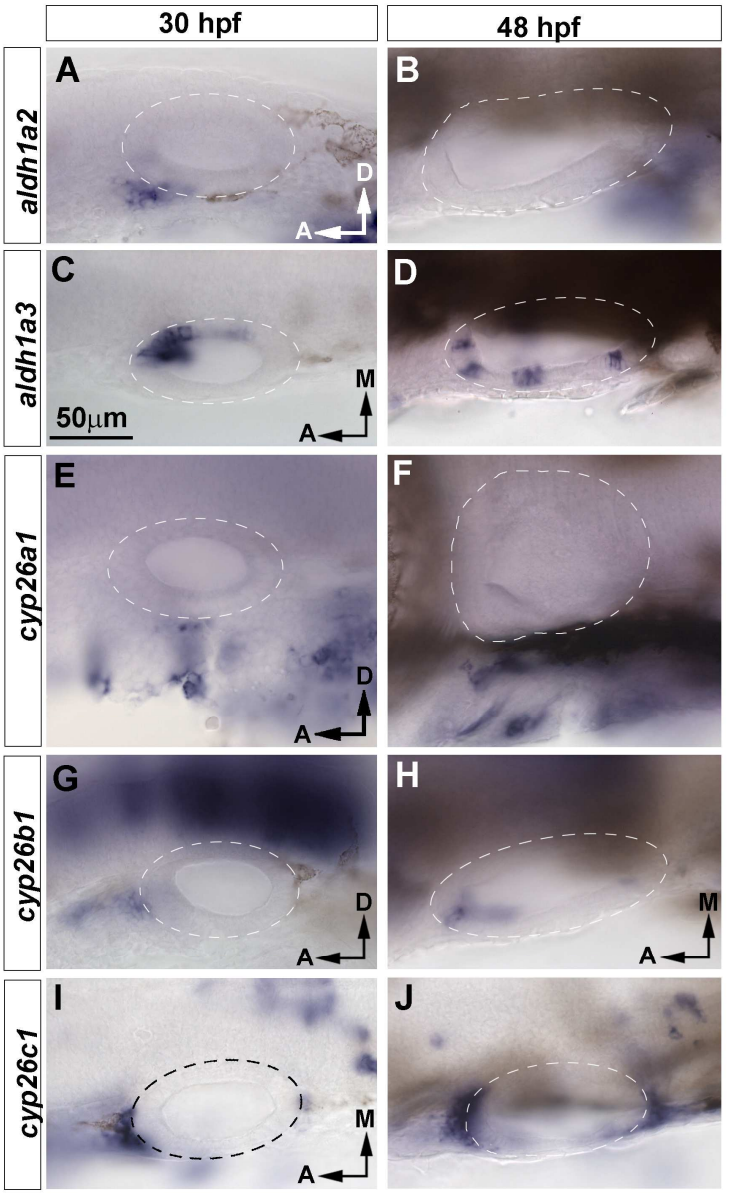

Figure 44. Late expression patterns of *aldh1as* and *cyp26s* genes in the otic vesicles.
(A-B) At 30hpf *aldh1a2* expression is detected in mesoderm below anterior otic domain, while at 48 hpf it is expressed posteroventrally to the otic vesicle. **(C-D)** In situ hybridization for *aldh1a3* reveals complex pattern of expression which at 30 hpf is limited to the nascent endolymphatic duct and later on appears in distinct patches within anterior and lateral otic walls. **(E-F)** *cyp26a1* is expressed in endodermal pouches,

below otic vesicle, at both 30 and 48 hpf. **(G-J)** At 30 hpf *cyp26b1* and *cyp26c1* are expressed in cells adjacent to the anterior otic domain. At 48 hpf a group of cell adjacent to posterior otic domain express *cyp26c1*, while *cyp26b1* stays limited to the anterior field. Dashed circles delineate otic veiscles. All images are at the same magnification.

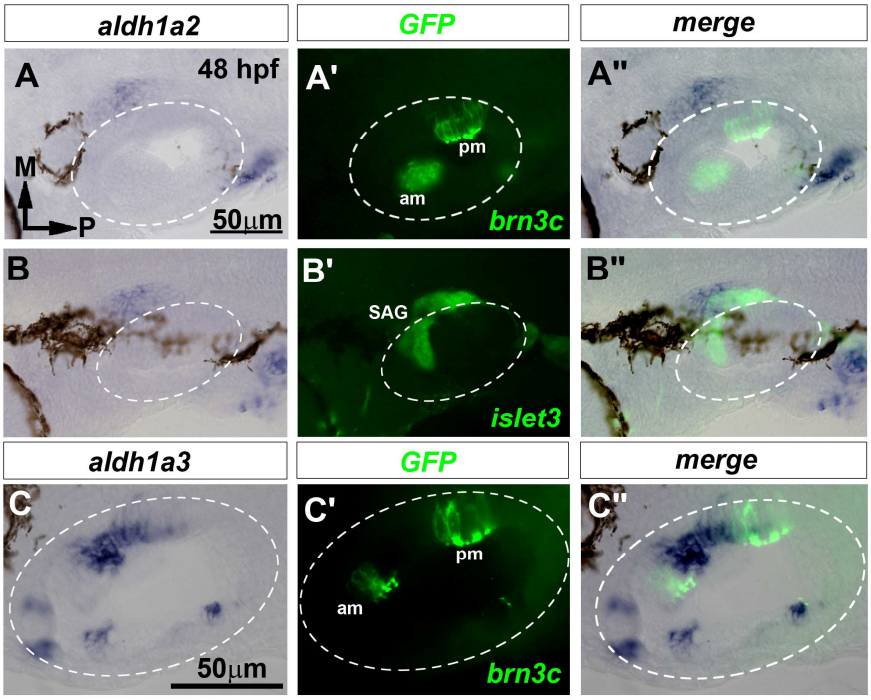

Figure 45. *aldh1a2/a3* genes are not expressed in sensory hair cells nor in SAG neurons.
(A-C") Coronal sections of 48 hpf *brn3c:GFP* (A-A", C-C") and *islet3:GFP* (B-B") embryos stained by in situ hybridization for *aldh1a2* (A-B") or *aldh1a3* (C-C"). None of the *aldh1a* genes is co-expressed with *islet3* or *brn3c*. Dashed circles delineate otic vesicles. Posterior is to the right, medial is up. am, anterior macula; pm, posterior macula; SAG, statoacoustic ganglion. A-B" and C-C" are at same magnification.

The same analysis was done for RA degrading enzymes, *cyp26a1*, *cyp26b1* and *cyp26c1*. *cyp26a1* was expressed in the endodermal pouches underlying the otic vesicle, at both 30 and 48 hpf (Fig. 44E,F), and thus was excluded from further analysis for co-expression with *brn3c* or *islet3*. However, *cyp26b1* and *cyp26c1* showed more interesting patterns of expression. *cyp26b1* was expressed in a group of cells lying anterior to the otic vesicle at 30 and 48 hpf, corresponding to the putative position of the anterior part of the SAG (Fig. 44G,H). *cyp26c1* was also expressed in a group of cells located adjacent to the anterior otic wall at 30 hpf, and at 48 hpf could be detected also in a group of cells adjacent to the posterior otic wall (Fig. 44I,J). Coronal sections of 48 hpf *brn3c:GFP* and *islet3:GFP* embryos stained by in situ hybridization for *cyp26b1* or *cyp26c1* confirmed that *cyp26b1* is expressed in a subset of SAG neurons (Fig. 46B'', C-C'', red arrowheads), while *cyp26c1* is not (Fig. 46E-E'').

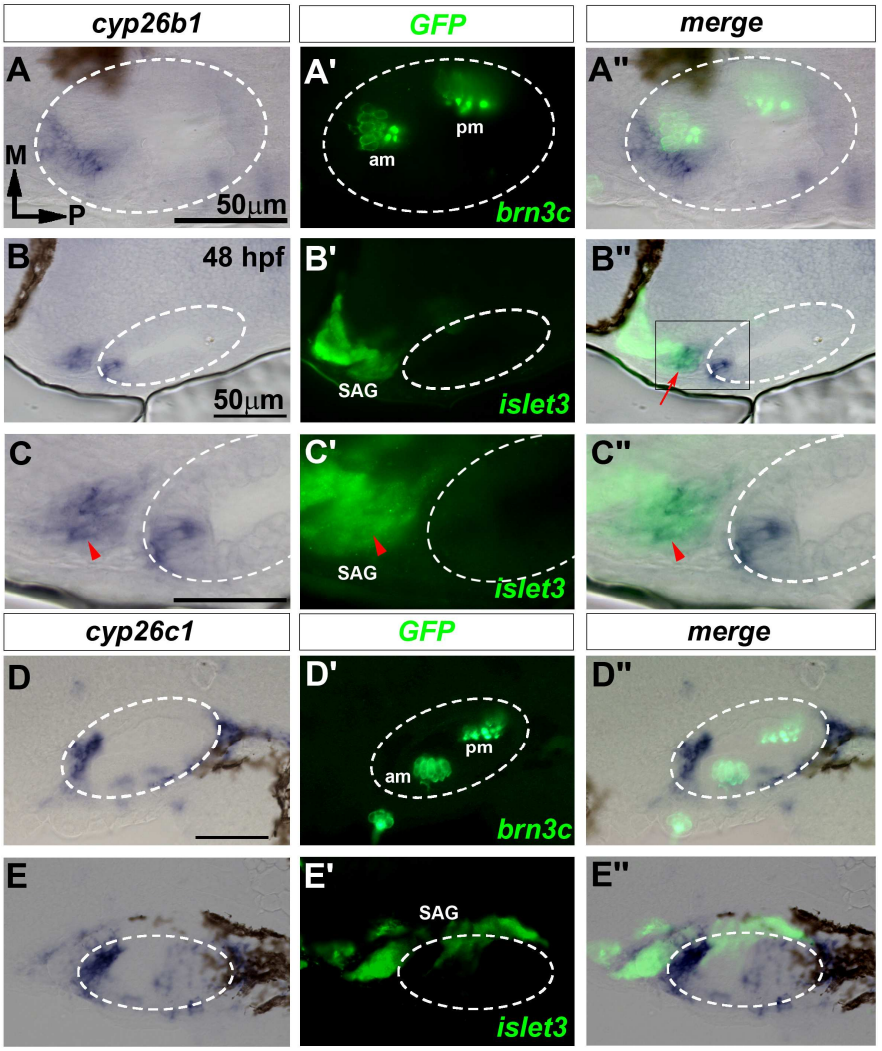

Figure 46. *cyp26b1* is expressed in a subset of the SAG neurons.
(A-E") Coronal sections of 48 hpf *brn3c:GFP* (A-A", D-D") and is*let3:GFP* (B-B", E-E") embryos stained by in situ hybridization for *cyp26b1* and *cyp26c1*. Dashed circles delineate otic vesicles. The black quadrant in B" labels the area shown enlarged in C-C". Note the co-expression of *cyp26b1* and GFP from *islet3* promoter (red arrow in B" and red arrowheads in C-C"). am, anterior macula; pm, posterior macula; SAG, statoacoustic ganglion. A-A", C-C" and B-B", D-E" are at same magnification.

3.2 The late role of retinoic acid in otic neurosensory development

The co-expression of *cyp26b1* and *islet3* raised the question of whether downregulation of RA is necessary to allow neurons to differentiate? This would be in contrast to the described roles of RA in the promotion of neuronal differentiation in cultured embryonic carcinoma or neuroblastoma cells (Jones-Villeneuve et al., 1982, Sidell et al., 1983, Andrews, 1984, Stavridis et al., 2010), or during the primary neurogenesis in *Xenopus* (Sharpe and Goldstone, 1997, Franco et al., 1999). To test this hypothesis, I manipulated RA levels in *islet3:GFP* embryos by treatments with 20μM DEAB or with 20nM *all-trans* RA starting from 16 hpf, when the otic *tbx1/her9* expression is already established and the first *neurog1* positive cells appeared in the neurogenic otic domain. The SAG size was analysed on coronal sections at 48 hpf (Fig. 47). The results show that the excess of RA leads to the decrease in total size of the SAG when compared with DMSO-treated controls (Fig. 47A-D''), while the inhibition of RA synthesis by DEAB leads to the increase in the SAG size (Fig. 47E-F'').

The specification of the sensory maculae of the utricle and saccule occurs around 10 hpf (Millimaki et al., 2007), but the first hair cells start to accumulate after 22 hpf (Riley et al., 1997, Millimaki et al., 2007, Sapéde and Pujades, 2010). To test the possible role of RA in cell differentiation within the sensory maculae, *brn3c:GFP* embryos were treated with exogenous 20nM RA from 20 hpf, and sensory maculae development was analysed at 72 hpf. Neither

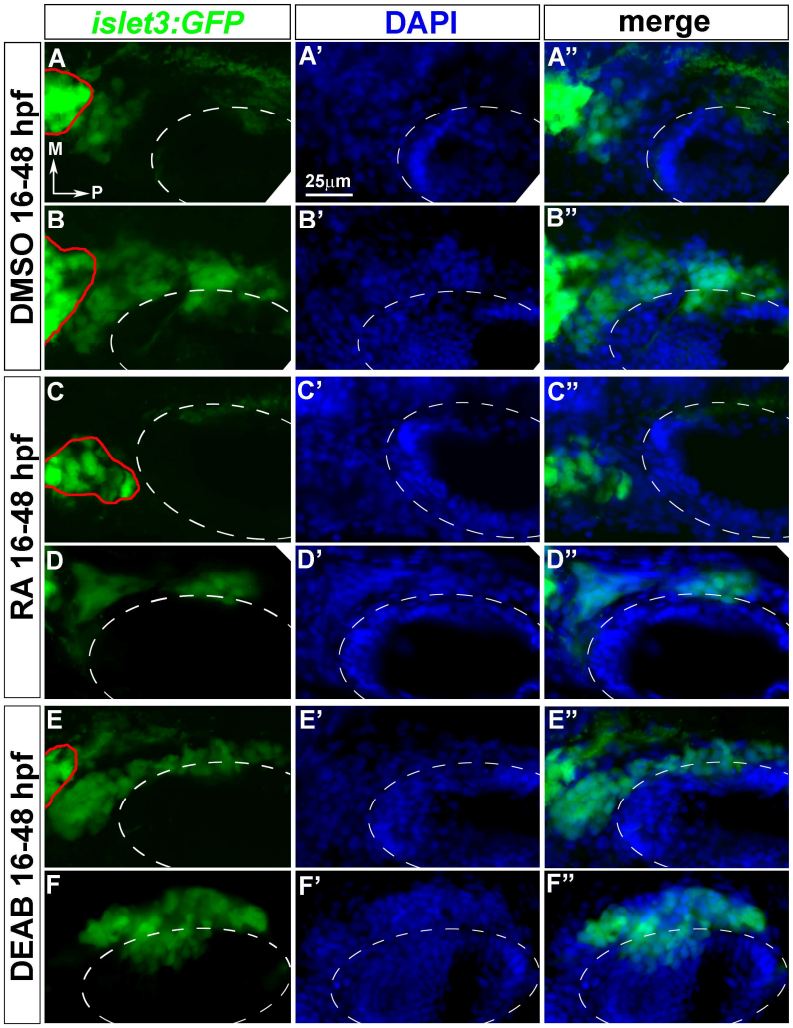

Figure 47. Perturbation in RA levels leads to the abnormal SAG development.
(A-F'') Coronal sections of DAPI stained *islet3:GFP* zebrafish embryos treated with 20nM RA (C-D''), 30μM DEAB (E-F'') or DMSO (A-B'') from 16-48 hpf. Two section, abutting whole ganglion, are shown for each treatment. Exogenous RA downregulated SAG neuronal development, and consequently the SAG is smaller (C and D) than in DMSO-treated controls (A and B), while DEAB induces the opposite phenotype (E and F). Red line in A, B, C and E delineates trigeminal ganglion, easy recognizable by the stronger GFP fluorescence. Dashed white circle delineates the otic vesicle epithelium. Anterior is to the left, medial is up.

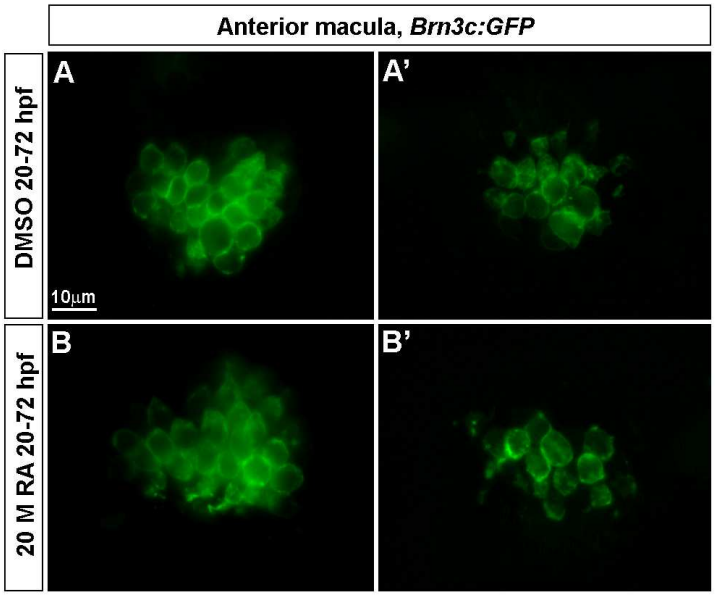

Figure 48. The development of the anterior macula is not affected by increased RA levels.
(A-B') Sagittal sections of posterior macula showing GFP fluorescence under the control of *brn3c* promotor in 72 hpf embryos treated with DMSO (A,A') or 20nM RA from 20 hpf. No change in the size of anterior macula, nor in the number of hair cells, could be observed. All images are at the same magnification.

macular size nor hair cell number within the two maculae were changed (Fig. 48A-B' for anterior macula, and data not shown for posterior macula), suggesting that RA does not play a late role in promotion or inhibiton of hair cell differentiation in the sensory maculae. However, the expression pattern of *aldh1a3* within the presumptive sensory cristae at 48 hpf suggests that RA function may be specific for these sensory epithelia, and analysis of the RA gain and loss of activity phenotypes should be done at later stages to make final conclusions.

DISCUSSION

Discussion

The precise innervation of the mechanotransducing hair cells of the inner ear is the basis of the correct perception and transduction of the vestibular and auditory stimuli. This is accomplished through the spatiotemporal control of the development of the inner ear neurosensory elements. The neurogenic domain is established in the anteromedial part of the otic anlagen, and previous studies have investigated in detail the external signals and intrinsic factors involved in THE positive regulation of otic neurogenesis (Léger and Brand, 2002, Andermann et al., 2002, Abelló et al., 2007, Abelló et al., 2010). However, it is not known if neural competence is acquired only by cells within the anteromedial otic domain, or if repression of neural fate in the posterolateral domain is required to restrict the extent of the otic neurogenesis. The expression of a number of transcription factors complementary to the neurogenic domain (Abelló et al., 2007, Abelló et al, 2010) suggested that the second hypothesis might be true.

In the present study, I identify *her9*, a zebrafish ortholog of mouse *Hes1*, as a key gene in regulating otic neurogenesis through the definition of the posterolateral nonneurogenic otic field. First, *her9* emerges as a novel otic patterning gene that represses proneural function and regulates the extent of the neurogenic domain. Second, Her9 acts downstream of Tbx1, linking these two families of transcription factors for the first time in the inner ear and suggesting that the reported role of Tbx1 in repression of the otic neurogenesis is in part mediated by the bHLH transcriptional repressor Her9. Third, I have identified retinoic acid (RA) signaling from the paraxial mesoderm as the upstream patterning signal of

the otic posterolateral genes *tbx1* and *her9*. The results show that at the level of the cranial otic field, opposing RA and Hedgehog signaling position the boundary between the neurogenic and non-neurogenic compartments. Finally, the preliminary results suggest that RA has the later inhibitory role over the otic neurogenesis, the function probably connected to the necessity to degrade RA cell autonomously in order to allow neuronal differentiation to proceed.

1. *Her* genes in the inner ear development

The role of Her9 in otic neurosensory development

I have analyzed the expression pattern of different members of the *her/hey* family at the stages of the early otic neurogenesis. Out of four *her/hey* genes expressed in the inner ear, only *her9* was expressed complementary to the neurogenic domain, suggesting its role as a patterning gene in the definition of the nonneurogenic otic territory. Indeed, loss of Her9 function leads to the ectopic neurogenesis, as revealed by the ectopic expression of the proneural genes *neurod* and *neurod4* in the nonneurogenic domain. Surprisingly, the expression pattern of *neurog1* did not change after Her9 blockade, suggesting a differential response of the proneural genes to the Her9 function, which is probably connected to the timing of the neuronal differentiation.

At 48 hpf, when the otic neurogenesis ceases, statoacoustic ganglion (SAG) contains two morphologically distinguishable branches: one located anterior to the otocyst and the other located medially, between the otocyst and the hindbrain. Concomitant with

the change in *neurod/d4* expression, the morphology of the statoacoustic ganglion (SAG) in Her9-depleted embryos was aberrant, displaying a ventral shift of its medial portion. If this affects, and how, the innervation of the hair cells within the sensory patches was not investigated in this study, but it deserves further attention.

The loss of Her9 function did not significantly increase the number of *islet3:GFP* positive SAG neurons neither in the SAG as a whole, nor in each of its two main branches, suggesting that the ectopic *neurod/d4*-positive cells either do not delaminate from the otic epithelia, or they do not survive. The finding that ectopic *neurod/d4*-expressing cells do not become supernumerary *islet3*-positive SAG neurons is in agreement with the reported phenotype of Her9 loss of function in the zebrafish neuroectoderm (Bae et al., 2005). There, in the absence of Her9 function, *neurod4* expands to the nonneurogenic interproneuronal stripes, but this does not result in major changes in the expression of later neuronal subpopulation-specific differentiation markers, such as *islet1, tlx3, lim1* or *pax2.1*.

The function of *Hes1*, the mammalian ortholog of *her9*, was analysed during the development of the sensory epithelia of the cochlea, utricle and saccule. There, *Hes1* is expressed in supporting cells and negatively regulates hair cell differentiation by repressing proneural gene *Math1* (Zheng et al., 2000, Zine et al., 2001, Zine et al., 2002, Li et al., 2008, Tateya et al., 2011). Although zebrafish does not develop cochlea, the loss of Her9 function does not affect the hair cell number in any of the sensory

patches, including the three cristae. However, due to the genomic duplication in the teleosts (Amores et al., 1998, Postlethwait et al., 2000), this function might be performed by another *Hes1* ortholog, namely *her6*. The onset and maintenance of *her6* expression in two sensory maculae from 18 hpf onwards correlates with the onset of hair cell differentiation processes in these sensory epithelia. Her6 was proposed, together with Her4, to direct the maintenance of cyclic gene expression coordination among adjacent cells in the presomitic mesoderm during somitogenesis, the role that is conserved with that of the Hes1 in mouse. On the contrary, *her9* is not expressed in the presomitic mesoderm, neither in developing somites at relevant stages, strongly suggesting that also in the inner ear Her6, and not Her9, might function to regulate the hair cell number in the sensory epithelia, like Hes1 does in mouse. The part of my future plans is to block Her6 function by morpholino injection approach and to test this hypothesis.

The regulation and role of Her4 during inner ear development

Another candidate for the negative regulation of the hair cell differentiation within the sensory epithelia is *her4*. Its mouse ortholog Hes5 was shown to inhibit hair cell differentiation in the developing cochlea, saccule and utricle (Zine et al., 2001). Although I found that *her4* is expressed in the sensory maculae at the time of hair cells differentiation, blocking Her4 function by morpholino injections did not affect the expression of hair cell specification markers *atoh1b* and *atoh1a*. However, this does not exclude the possibility that Her4 regulates some later aspects of hair cell maturation, or their survival, and further studies will be

performed to answer this question. In addition, the upstream activator/s of *her4* expression within these sensory domains remained unknown, and it will be an exciting task to search for such molecule/s and to explain why and how *her4* expression is differentially established in otic neurogenic and sensory domains.

In the chick otic placode, Notch target *Hes5* was proposed to negatively regulate neurogenesis within the neurogenic domain through the mechanism of lateral inhibition, due to its mosaic expression with *delta1*-positive neuroblasts (Abelló et al., 2007). In zebrafish, *her4* expression was lost from the otic neurogenic domain after Notch signaling blockade and in *neurog1*$^{-/-}$ mutant embryos, suggesting that *her4* is involved in the selection of neuronal precursors via lateral inhibition mechanism. However, the initial experiments showed that blockade of Her4 function by morpholino injection did not cause any obvious change in the density of *neurog1*- and *neurod*-positive cells within the neurogenic domain, the phenotype that would be expected when the mechanism of lateral inhibition is blocked. Although more detailed analysis of the phenotype must be done to make final conclusion, it is also possible that other *Hes5* orthologs, not analyzed in this study, such as *her2* and *her12*, are expressed in the otic neurogenic domain and could compensate for the loss of Her4.

In the developing trigeminal ganglion, *her4* is coexpressed with *islet1/2* in differentiating neurons that once were *neurog1*-positive and loss of Her4 function does not affect the neuronal number, but the formation of periferal projections from the ganglion (So et al.,

2009). This opens the door to a new area of the study, since occasionally I could detect *her4*-expressing cells outside the otic epihelium, at the location that corresponds to the presumptive SAG rudiment at 24 hpf.

The next interesting question is: does the loss of Her9 or Her4 function affect the hearing and/or equilibrium of the fishes and if it does, how? In a pilot auditory test in which Her9-depleted zebrafish embryos were stimulated at 8 dpf with a short 300Hz sound stimuli, the time fishes needed to generate the escape response was longer than in control animals and they moved less far away (data not shown). Unfortunately, since *her9* is also expressed in the central nervous system, one cannot exclude that this phenotype is a result of the possible impairment in the motor neuron development, and not merely the effect of the inner ear neuronal misspatterning. To exclude the possible role of CNS in this case, it is reccomendable to perform spatially restricted loss of Her9 function, such that Her9 activity stays intact in the CNS. This could be achieved through the construction and injection of a vector which will express a dominant-negative form of Her9 under the control of promoter sequences of pan-otic markers, such as *cldna* (*claudin a*) or *stm* (*starmaker*). The dominant-negative form of Her9 should be generated such that it can form dimers but cannot bind to the DNA. In this way it would sequester endogenous, functional Her9 proteins, and this would finally lead to the reduced otic Her9 function.

Her9 as the mediator of Tbx1 activity during otic neurogenesis

In the mouse inner ear, Tbx1 acts to restrict the neurogenesis by inhibiting proneural function of *NeuroD* (Raft et al., 2004, Xu et al., 2007). Here, I validate this result in zebrafish and we have postulated that this function is mediated by Her9 transcriptional repressor. In zebrafish, the genetic relation between *T-box* and *Her* gene families was demonstrated so far only in the anterior presomitic mesoderm, where Tbx24 activates the expression of *her1* during somitogenesis (Nikaido et al., 2002, Brend and Holley, 2009). Recently it was shown that *Tbx1*-null cells isolated from mouse embryos downregulate *Hes1* and that *Hes1* mutation phenocopies some of the aspects of the DiGeorge syndrome related to the development of the pharyngeal arches (van Bueren et al., 2010). This suggested that Hes1 might function downstream of Tbx1 during the development of these embryonic structures. However, the inner ear defects seen in *Tbx1-/-* mouse mutants were never connected with *Hes1* loss of function. Our data indicate that Tbx1 acts as an activator of *her9* transcription in the inner ear: abrogation of Tbx1 leads to a complete loss of otic *her9* expression and overexpression of Tbx1 leads to the ectopic expression of *her9* concomitant with the inhibition of *neurod*. The activation of *her9* in the inner ear by Tbx1 is probably direct, since both genes are established at the same time in the otic placode and *her9* abolishment in *van gogh* mutants is observed already at 15.5 hpf, the earliest stage analyzed in the work, only 1.5h after the normal onset of *her9* expression in the otic placode. Moreover, the

analysis of the genome sequences upstream of *her9* coding region indicated the presence of T-box binding site, strongly arguing for the possibility of direct regulation by Tbx1. T-box genes act mainly as transcriptional activators, with only Tbx2, Tbx3 and Tbx20 reported to act as repressors (Carreira et al., 1998, He et al., 1999, Prince et al., 2004, Stennard et al., 2005).

The surprising fact that in *van gogh* mutants otic *neurog1* expression is expanded, the effect not attributable to the Her9 function, might mean that Tbx1 act as a direct transcriptional repressor of *neurog1* or that Tbx1 activates other downstream targets to additionaly control the otic neurogenesis. Along with these lines, *van gogh* embryos display a severely disrupted semicircular canal formation, with a resulting failure to develop sensory cristae (Whitfield et al., 1996, Piotrowski et al., 2003). Defects in semicircular canal formation were not found in Her9-depleted embryos, which suggests that Tbx1 activates targets other than *her9* that mediate its role in the otic morphogenesis.

Fgf signals are implicated in Tbx1-mediated control of pharyngeal development (Vitelli et al., 2002, Xu et al., 2004, Hu et al., 2004). It is tentative to postulate that Tbx1 also regulates otic Fgf signals, known to be a key players in the development of the otic neurosensory elements. In the otic epithelium of *Tbx1-/-* mouse mutants, *Fgf3* expression is expanded along with *Neurog1* and *NeuroD* (Raft et al., 2004), while *Fgf10* expression, labeling the presumptive vestibular sensory epithelium, is lost from the lateral otic epithelium, but not from the SAG (Vitelli et al., 2003). This makes *Fgf10* a good candidate for Tbx1 downstream target in the regulation of the development of the inner ear vestibular structures.

The other proposed Tbx1 targets for this function include *Bmp4* and *Otx1*, both regulating the development of the semicircular canals and their associated cristae (Vitelli et al., 2003, Raft et al., 2004, Whitfield and colleagues, 2009-oral talk in the 18th CDB meeting, Kobe, Japan).

RA-Tbx1-Her9 cascade in otic proliferation

Blockade of RA signaling by DEAB or ablation of Tbx1/Her9 function both led to the decrease in the size of the otic vesicle, while treatments with exogenous RA and occasionally Tbx1 overexpression led to the otic size enlargement. In mouse, Tbx1 regulates the proliferation of the otic epithelial cells, as shown by decreased staining for a mitotic marker phospho-Histone H3 within the *Tbx1*-expressing domain in *Tbx1*$^{-/-}$ embryos (Xu et al., 2007). Moreover, the cells that were Tbx1-positive once in their lives populate most of the mature otocyst, and vesicles with impaired Tbx1 function do not develop semicircular canals and their associated cristae, nor the cochlea, pointing to the importance of the cell proliferation within the *Tbx1*-positive domain (Vitelli et al., 2003, Raft et al., 2004, Arnold et al., 2006). Similar phenotype is observed in zebrafish *van gogh* mutants (Whitfield et al., 1996, Piotrowski and Nusslein-Volhard, 2000), although at the stages we analyzed the size of the otic vesicles was variable, and few embryos with no decrease in the otic size could also be found.

In Her9 loss of function study, the decrease in otic vesicle size was quantified and was approximately 16% when compared to the

controls. *Hes* genes were reported to regulate cell proliferation through the transcriptional downregulation of cell cycle inhibitors, such as $p27^{Kip1}$, in mouse cochlea (Murata et al., 2009), and here I validate this finding for the early posterolater otic epithelia by showing that *cdkn1bl* (zebrafish ortholog of $p27^{Kip1}$) and anti-pH3 are up- and downregulated, respectively, within the *her9*-positive otic epithelium in Her9-depleted embryos. However, the structures thought to derive from the *tbx1*/*her9*-expressing domain, such as lateral and posterior semicircular canals, do develop in Her9-depleted zebrafish, arguing that some other target mediates the role of Tbx1 in expanding that part of the mature inner ear. Moreover, lateral and posterior sensory cristae develop normally in Her9-inhibited embryos, and this is in agreement with Murata and colleagues, who show that dysregulation of $p27^{Kip1}$ in mouse cochlea did not lead to a precocious differentiation of hair cells.

Retinoic acid is repeatedly reported to have growth-inhibitory and differentiation-inducing properties, for example in different carcinoma cell lines (see for example: Ozeki and Shively, 2008) and in chick otic vesicle explants, where the addition of RA inhibits vesicular growth and causes the precocious development of the endolymphatic sac and sensory epithelia (Represa et al., 1990). Interestingly, this paper shows that, in the presence of RA, vesicles growed inwardly, with external side exposed to higher RA concentration and internal side exposed to lower RA concentration. This might mean that lower RA concentrations still permitted cell proliferation on the internal vesicle surface. In agreement with this, I treated zebrafish embryos with very low RA concentrations (20nM) and observed the enlargement of the otic vesicles. Treatments with higher RA concentration (50nM) during the same

time window led to the decrease in the otic vesicle size (data not shown), pointing to the concentration-dependent response of the otic vesicle to the RA. Intriguingly, the smaller otic vesicle from these treatments expressed *tbx1* allover otic epithelium, suggesting that inhibition of cell proliferation by high RA levels can overcome Tbx1 mediated supression of cell cycle inhibitors, probably acting directly on other cell cycle regulators. However, low levels of RA probably promote otic cell proliferation through the transcriptional activation of the Tbx1, which in turn activates *her9*, implicating this genetic network in the control of the cell proliferation within the nonneurogenic otic epithelium.

Since the expansion of the otic neurogensis, seen in *van gogh* and Her9-depleted embryos, may be due to the decreased cell poliferation in the posterolateral otic domain and not to the real neuronal misspecification, double staining was performed to detect *neurod*-positive cells within the *tbx1*-positive domain in *van gogh* and Her9-depleted embryos, confirming that *tbx1*-positive cells switched their cell fate. Thus, the role Tbx1 has in otic cell proliferation is probably connected to the development of the later forming semicircular canals, and not to the spatial restriction of the neurogenesis which is mediated by Her9. This means that RA-Tbx1-Her9 genetic network plays dual role during the inner ear development: a role in negative regulation of the otic neurogenesis, and a role in the control of the otic vesicle growth.

2. Extrinsic regulators of the inner ear anteroposterior patterning

The role of retinoic acid in the otic neurogenic patterning

The evidence for a Notch-independent regulation of *Hes/her* genes is accumulating. In particular, in the floor plate, *her9* expression requires active Nodal signaling (Latimer et al., 2005), whereas in the nonneurogenic interproneuronal stripes *her3* and *her9* are controlled by BMP signaling (Bae et al., 2005). I investigated which positional cues regulate the expression of posterolateral otic genes *her9* and *tbx1* and found that their establishment and maintenance is also Notch-independent. Surprisingly, the expression of *her4* in the presumptive sensory maculae was also Notch-independent, and this, together with Notch-independent regulation of *her4* expression in the trigeminal ganglion (Yeo et al., 2007), opens the question of the regulators of the *her4* expression in these sensory territories.

RA signaling is one of the major patterning signals during embryonic development and it has strong influence on the development of craniofacial structures and the hindbrain (Grandel et al., 2002, Linville et al., 2004, Maves and Kimmel, 2005, Linville et al., 2009). In embryonic tissues, RA is produced by the activity of Aldh1a2 and Aldh1a3 enzymes, which oxidize retinaldehyde into RA. At 14 hpf, when assymetric expression of *tbx1* and *her9* along the otic AP axis is established in zebrafish, *aldh1a2* is expressed in somites and paraxial mesoderm posterolateral to the otic placode.

Mouse mutants for Aldh1a2 display lateralization of the otic vesicle (Niederreither et al., 2000), but the role of RA in anteroposterior otic patterning was not investigated so far, neither in mouse nor in zebrafish. At earlier stages RA is required for the otic induction: in zebrafish the excess of RA induces supernumerary or enlarged otic vesicles, while blocking RA signaling decreased the otic induction (Phillips et al., 2001, Hans and Westerfield, 2007). Hans and Westerfield have suggested that precise levels of RA are required to determine the extent of the otic competence domain independently of hindbrain signals involved in otic induction. Thus, I manipulated RA levels after the period of otic specification to address the role of this signaling pathway in the otic AP patterning. Consequently, no supernumerary otic vesicles could be observed in experiments in which RA levels were elevated, nor the hindbrain was misspaterned. I found that exogenous RA induced *tbx1* and *her9* ectopically, while loss of RA signaling suppressed the otic expression of both genes. Consequently, these manipulations affected the development of the nonneurogenic domain and the expression of proneural genes, such as *neurog1* and *neurod*. These results support a genetic network in which RA from the cranial mesoderm activates Tbx1 in the posterolateral part of the otic placode, which in turn activates *her9* to restrict the otic neurogenesis to the anterior otic domain. Recent report from Wu and colleagues demonstrates the relation between RA and Tbx1, and the report place this network upstream of the *NeuroD* expression in the chick inner ear (Bok et al., 2011). These and our results suggest that the RA-Tbx1 network has conserved role in negative regulation of the otic neurogenesis, in zebrafish and chick,

and it would be interesting to analyse if this network also exists in mouse, where the repressive role of Tbx1 was first demonstrated (Raft et al., 2004).

Several lines of evidence demonstrate that the induction of posterolateral otic genes is independent of hindbrain patterning. First, decreasing RA signaling from 10.5 hpf onwards completely abolished *tbx1* and *her9* expression, while AP patterning of the hindbrain, revealed by *krox20* expression, did not changed. Second, in *val*^{b337} mutants, in which rhombomeres 5 and 6 are misspecified, otic *tbx1* and *her9* expression patterns are unaffected. In the abovementioned study in chick, local source of RA was provided by implantation of the RA-coated beads next to the otic placode, avoiding thus the possible effects on hindbrain patterning (Bok et al., 2011). The results obtained from the study confirmed our finding that RA activates otic *tbx1* expression independently of the hindbrain signals. In summary, the data from chick and our results strengthen the relevance of hindbrain-independent RA signaling in the AP patterning of the inner ear and highlight RA as a novel extrinsic factor controlling the establishment of the posterior, nonneurogenic otic compartment.

Hh signaling pathway in the otic neurogenic patterning

In mouse, expression of *Tbx1* in the pharyngeal endoderm is directed by a specific enhancer located 14.3 kb upstream of the coding sequence (Yamagishi et al., 2003). This regulatory region is responsive to Sonic hedgehog (Shh), an action mediated by the Foxc1 and Foxc2 transription factors, but it remains to be explored whether it also regulates *Tbx1* expression in the inner ear.

In zebrafish otic territory, rather than a loss, I detected an expansion of *tbx1* expression upon Hh inhibition. This suggests either that independent enhancer elements control the ear and mesenchyme *tbx1* expression or that downstream effectors of the Hh pathway, together with ear context specific transcription factors, inhibit rather than activate *tbx1* transcription. The mechanism through which Hh signaling regulates otic *tbx1* expression seems not to involve modulation of the RA levels, suggesting that cross-talk between the two pathways is not direct, although the analysis of the Hh activity upon RA level modulation needs to be done to confirm this. Retinoic acid response element (RARE) sequence was found in upstream region of the zebrafish *shh* gene, and in HeLa cell system retinoic acid receptors and retinoic X receptors are able to activate the transcription from this regulatory sequence (Chang et al., 1997). In vivo, RA was shown to induce Shh during pectoral fin bud development (Hoffman et al., 2002). This permits modeling a scenario in which RA could activate Hh in the midline structures to restrict its own activity, or the expression of *tbx1*,within the otic territory. This hypothesis, although very speculative, deserves further investigation.

In accordance with our results obtained from the loss of Hh function experiments, Hammond and colleagues reported the downregulation of the otic *tbx1* expression when Hh signaling was overactivated within the otic epithelium (Hammond et al., 2010). Recent report implicated the Hedgehog signaling in the positive regulation of otic neurogenesis (Sàpede and Pujades, 2010), and here we refine these data by providing evidence that Hh acts first

on *tbx1*, which then regulates the expression of the proneural genes.

Fgf signaling pathway in the otic neurogenesis

Fgf signals are well established regulators of the otic induction and development. In agreement with the published data that suggest a sequential requirement of Fgfs in the inner ear development (Leger and Brand, 2002), our data demonstrate that a role of Fgf signals in otic induction could be dissociated from a subsequent role in neural specification at 50% epiboly. Otic induction was completely abrogated in Fgf-depleted embryos from 4.3 hpf, whereas otic growth and neural specification were impaired in SU5402-treated embryos from 50% epiboly (5.3 hpf). Once the anteromedial otic field is neural specified at 7 hpf, Fgf signaling is only required for otic growth and the *tbx1* expression pattern does not change. This indicates that the establishment of *tbx1* expression by RA activity is independent of Fgf signaling, but that the anterior limit of *tbx1* expression is set up by the previous step of specification of the neurogenic domain. By contrast, the posteromedial domain is not fully determined to a neurogenic fate until 18 hpf, as *tbx1* can be ectopically induced after Hh inhibition until this stage. Interestingly, this reveals spatiotemporal control of the determination of the neurogenic domain: anterior field is specified before the posterior, in agreement with the results published for the chick otic neurogenesis (Bell et al., 2008).

Do Fgf and RA signaling cross-talk to regulate the otic patterning?

Surprisingly, otic *tbx1* expression was reduced or abolished in embryos treated with Fgf signaling inhibitor SU5402 from 5.3 hpf. RA and Fgf signaling counteract each other during somitogenesis, posterior spinal cord neurogenesis and hindbrain development (Diez del Corral et al., 2003, White et al., 2007). The results show that at early gastrula stages, but not later, Fgf activity is necessary for the establishment of the mesodermal *aldh1a2* expression in zebrafish embryos, as shown previously for *Xenopus* embryos (Shiotsugu et al., 2004). The fact that *tbx1* expression is reduced in the otic vesicle when *ald1ha2* from cranial mesoderm, but not from somites, is lost in embryos treated with SU5402 from 50% epiboly onwards, favors a major role of cranial mesodermal Aldh1a2 activity in setting up *tbx1* expression.

On the other hand, we saw that short treatments with exogenous RA, prior to the establishment of the otic *tbx1/her9* expression, led to the medial shift of *fgf8* and *fgf3* expression at 24 hpf (data not shown). In zebrafish, both genes act redundantly to drive the otic neurogenesis (Leger and Brand, 2002). It would be interesting to test if the early mesodermal *fgf3* and endodermal *fgf8* expression (Maves et al., 2007, Nechiporuk et al., 2007) expands to include the preotic ectodermal territory, where these genes would specify the neurogenic territory like it is the case for Fgf8 in chick (Abelló et al., 2010). If yes, I would like to analyse the role of RA in the establishment of this Fgf-positive ectodermal domain. Our

laboratory currently dispose of *aldh1a2*+/- mutant zebrafish adults, which in combination with temporally controlled manipulations of RA levels (by treatments with RA or DEAB) will contribute to the more comprehensive understanding of the possible RA-Fgf interaction. In addition, *tbx1* was reported to regulate the expression of different Fgf signals during the development of the mouse pharyngeal endoderm and the otic vesicle (Vitelli et al., 2002, Xu et al., 2004, Raft et al., 2004, Hu et al., 2004) and might mediate the potential role RA has over the otic Fgf signaling. Thus, I would also like to analyse what happens with the expression of different otic Fgf ligands after Tbx1 overexpression, or in *van gogh* mutant embryos.

3. The role of RA in the late development of the otic vesicle

RA signaling affects the inner ear development at multiple points. After the role in the otic induction and the early otic neurogenic vs nonneurogenic patterning, RA starts to be synthesized endogenously, within the otic anlagen, by Aldh1a3 enzyme. The putative RA activity within the otic territory is the best assessed through the comparison of the expression patterns of Aldh1as and Cyp26s, its synthesizing and metabolizing enzymes. This is particularly truth for the zebrafish embryos, where the existing RARE:GFP or YFP lines do not report endogenous RA activity within the inner ear. Although CRBPs and CRABPs expression could also be detected in the otic epithelium in mammalian embryos (Ruberte et al., 1992, Kelley et al., 1993, Ylikoski et al., 1994, Romand et al., 2000), loss of function studies have revealed that these proteins are dispensable for inner ear development and function (Lampron et al., 1995, Romand et al., 2000). On the other hand, expression patterns of RAR and RXR receptors in the inner ear (Dollé et al., 1994, Raz and Kelley, 1999, Romand et al., 2002) does not necessarily reflect where and when RA binds to these molecules and affects downstream targets.

In mouse there are three Aldh1a enzymes involved in RA synthesis, but only two orthologs exist in zebrafish. a*ldh1a2* seems not to be expressed within the otic epithelium (Pittlik et al., 2008 and our results), while a*ldh1a3* have dynamic pattern of expression. At early otic vesicle stage it is expressed in a narrow

anteromedial band, proposed to correspond to the future sensory epithelia of the utricular macula (Pittlik et al., 2008), while at 48 hpf it is reported that *aldh1a3* expression domain coincides with the position of the utricular macula, all three sensory cristae and nascent endolymphatic duct (Pittlik et al., 2008, Dutton et al., 2009). However, our results did not confirm coexpression of *aldh1a3* and *brn3c* (hair cell marker) within the utricular macula, and instead placed *aldh1a3* in the zone between the two maculae, probable corresponding to the future endolymphatic duct rudiment. Since *aldh1a3* expression was analyzed at 48 hpf, when hair cell maturation within the sensory cristae is just starting we could not detect expression of *brn3c* within the sensory epithelia, but the expression of *aldh1a3* within the thickenned otic epithelium suggests that indeed *aldh1a3* is expressed in the sensory cristae. Analysis at later stages needs to be done to conclude if this expression is confined to the hair cells or supporting cells or to the both.

In each prosensory region, the first hair cell appears in a specific region of the epithelium, and in the case of the utricular macula it is the centre of the patch where first differentiated hair cell can be identified. As development continues, additional hair cells appear in a stereotyped manner, depending on the sensory patch (reviewed in Whitfield et al., 2002, Kelley, 2006). The expression of *aldh1a3* first in a single domain (at 45 hpf, data not shown) and later only at the borders of the putative anterior crista suggests that RA signaling might promote hair cell differentiation within the sensory territories. RA and FGF signaling together are reported to regulate cell differentiation during, for example, the extension of the body axis or in the embryonic stem cell cultures (Diez del Corral et al.,

2003, Stavridis et al., 2010). In the latter work, RA initially activates Fgf signals to promote loss of self renewal properties of the embryonic stem cell, but later attenuates Fgfs to allow the onset of cell differentiation. *fgf10,* a gene essential for the development of the sensory cristae in mouse (Pauley et al., 2003), is also expressed in the three cristae in 48 hpf zebrafish embryos. Retinoic acid is reported to positively regulate *fgf10* expression during the development of the pectoral fin (Gibert et al., 2006) and it would be interesting to analyse if such interaction also occurs in the inner ear. In this scenario, RA synthesized within the otic epithelium by Aldh1a3 would activate or attenuate *fgf10* expression within the sensory cristae, and this would provide the correct subsequent hair cell differentiation. I will test this hypothesis by analysing what happens with hair cell formation in *aldh1a3-/-* mutant zebrafish embryos. In a way, this will be a temporally controlled experiment and the possible effects will be otic autonomous, since *aldh1a3* expression is initiated quite late and only in the otic epithelium, while the surrounding tissues do not activate the expression of this gene. Why nascent hair cells have to downregulate *aldh1a3* expression in the centre of the sensory epithelium and through which mechanism this is acomplished is another interesting question, and it deserves further attention.

Another intriguing question arises from the observed coexpression of RA metabolizing enzyme *cyp26b1* and neuronal marker *islet3* within the part of the SAG. In the hindbrain nonneurogenic rhombomere centres, Cyp26b1 is identified as a main inhibitor of neuronal differentiation, a result that contrasts our findings (Gonzalez-Quevedo et al., 2010). The blockade of RA synthesis after the *tbx1/her9*-expressing domain is established, leads to the

increase in the number of SAG neurons. This result suggests that RA indeed needs to be downregulated to allow neuronal differentiation to proceed, a hypothesis already postulated above for the hair cell development within the sensory cristae. To really involve *cyp26b1* in this loss of RA phenotype I will inject cyp26b1 morpholino, to specifically block only the function of this gene, and I will analyse how the formation of the SAG neurons is affected.

Alternatively, Islet3-positive SAG neurons may activate *cyp26b1* expression to promote the formation of the putative RA gradient at the level of the otic vesicle, which in turns would provide the correct spatiotemporal appearance of the later forming otic structures, such as semicircular canals, sensory cristae, and endolymphatic duct, all of them being affected when RA levels are exogenously manipulated. The existence of such a gradient was proven at the caudal hindbrain level, but the information lacks for when the otic field is concern. The putative RA gradient over otic territory probably would be complex and dynamic, since the expression patterns of RA synthesizing and degrading enzymes within and adjacent to the otic field are changing in space and time. The RA gradient dynamics may further account for the complex RA contribution to the final three-dimensional organization of the fully functional, mature inner ear.

CONCLUSIONS

Conclusions

1. In zebrafish, members of the hairy and Enhancer of split family of transcriptional repressors are differentially expressed within the otic epithelium, being *her4*, *her6* and *hey1* expressed in neurogenic and/or prosensory domains, while *her9* is transcribed in the posterolateral, nonneurogenic territory.

2. *her4* expression in the neurogenic domain of the otic placode is positively regulated by Notch signaling and depends on Neurog1 function, while the establishment and/or maintenance of the posterolateral otic genes, such as *tbx1* and *her9* is Notch-independent.

3. The expression pattern of *her9* coincides with the expression of *tbx1* and both genes are established in the placode before the onset of the expression of proneural genes, such as *neurog1*, suggesting they may act as prepatterning genes.

4. Her9 is the otic repressor of proneural function in the posterolateral otic domain, as revealed by ectopic *neurod* and *neurod4* expression in Her9 loss of function study.

5. Her9, in contrast to its mouse ortholog Hes1, does not regulate the development of the sensory maculae, neither the early development of the sensory cristae.

6. Her9 does not regulate the expression of other posterolateral otic genes, such as *lmx1b1* and *tbx1.*
7. Tbx1 represses otic neurogenesis in zebrafish, being this role conserved with that of Tbx1 in mouse and chick.

8. Her9 is a downstream target of Tbx1.

9. Her9 and Tbx1 are established in the posterolateral otic domain by positive action of retinoic acid signaling emanating from the paraxial mesoderm.

10. The expression of RA synthesis enzyme Aldh1a2 in paraxial mesoderm is positively regulated by early, gastrula stages, Fgf signaling, while later Fgf signaling does not influence neither *aldh1a2* expression nor the establishment of the posterolateral otic genes.

11. Tbx1 null mutants, Her9-depleted and DEAB-treated embryos all develop smaller vesicles, suggesting the role of RA-Tbx1-Her9 genetic cascade in the regulation of otic cell proliferation. Her9 negatively regulates the expression of cell cycle inhibitor *cdkn1bl* and promotes mitosis in the posterolateral otic territory.

12. Perturbation in RA levels, after the posterolater genes were established in the otic placode, affect SAG development, while the same manipulations do not affect sensory developmental.

Conclusions

13. *cyp26b1*, a gene encoding RA degrading enzyme, is expressed in a subset of the *islet3* positive SAG neurons.

MATERIALS AND METHODS

Embryos and staging

All the experiments performed for this thesis were done using zebrafish embryos. Embryos from wild-type (AB) strain, transgenic lines *Tg(isl2:gfp)zc7* (also called *islet3:GFP,* Pittman et al., 2008) and *Tg(Brn3c:GAP43-GFP)s356t* (also called *brn3c:GFP,* Xiao et al., 2005) were grown at 28℃, staged according to standard protocols (Kimmel et al., 1995) and fixed at the indicated timepoints. Mutant homozygotic *vgo*^{tm208} (Piotrowski et al., 2003), *val*^{b337} (Moens et al., 1996) and *neurog1*^{hi1059} (Golling et al., 2002, Madelaine and Blader, 2011) embryos were obtained by pairwise mating of heterozygous adult carriers.

Genotyping *vgo*^{tm208} and *val*^{b337} mutants

The A to T transition mutation in the *vgo*^{tm208} mutant allele leads to the loss of an *AlwN1* restriction site in the *tbx1* gene. Primers for genotyping this mutant are designed to flank the sequence in which the mutation occurs. Val mutation disrupts the PvuII restriction site within the *valentino* gene and primers for genotyping are designed to amplify this sequence.

Genomic DNA extraction: XNAT2 Extract-N-Amp kit (Sigma):

1. anesthetize the adult fish in Tricaine solution (Sigma, A5040) and cut the fin (approx. 1/4)

2. put the fin into clean 1.5ml eppendorf tube with the identification number of the fish
3. mix 4 volumes (25 µl) extraction buffer and 1 volume (6,25 µl) Tissue Preparation solution, vortex and spin down
4. add 31.25 µl of the prepared solution to each sample, vortex 15 sec and spin down, then incubate 10 min at 55ºC
5. incubate 3 min at 95ºC to inactivate proteinase K
6. add 25 µl of Neutralization solution to each sample, vortex 15 sec and spin downstream
7. use 1 µl of obtained DNA for PCR genotyping

Polymerase chain reaction (PCR)

The following PCR mix was prepared on ice:

5µl 10x PCR buffer	(final conc. 1X, EUROBIO)
2µl 50mM $MgCl_2$	(final conc. 2mM, EUROBIO)
1µl 10mM dNTPs	(final conc. 0.2mM)
1µl cDNA template	
0.8µl 10µM 5' primer	(final conc. 0.8µM)
0.8µl 10µM 3' primer	(final conc. 0.8µM)
0.2µl 6U/µl Taq Pol	(final conc. 0.024U/µl, EUROBIO)
39.2µl ddH_2O	

Primers used for *vgo*^{tm208} genotyping:
5'-vgo: 5'-GCTCTGGAGTGAACTTGATTACCTG-3'
3'-vgo:5'-AACGGTCAAGTAGGCCTGTAGCTAC-3'

Primers used for *val*^{b337} genotyping:

5'-val: 5' GAT CGC GCC GTA CTG GTG TT 3'

3'-val:5' GAT CGC GCC GTA CTG GTG TT 3'

PCR program for *vgo*^{tm208} mutants

94ºC	4 min	
94ºC	60 sec	
57ºC	40 sec	32x
72	80 sec	
72ºC	7 min	
4ºC	hold	

PCR program for *val*^{b337} mutants

94ºC	5 min	
94ºC	1 min	
61ºC	1 min	32x
72	2 min	
72ºC	7 min	
4ºC	hold	

Digestion of the products was done 3h at 37ºC:

10µl PCR reaction mixture

2µl restriction buffer

1µl restriction enzyme (*AlwN1* for *vgo*^{tm208} , *PvuII* for *val*^{b337})

7µl ddH_2O

The products were run on 3% agarose gel. Homozygotic mutants give only one band.

Morpholino and capped mRNA injections

The her4-MO (Gene Tools) (So et al., 2009) was injected at concentration 0.02 mM. This morpholino is designed to block the translation of her4 mRNA transcript and its sequence is: 5'-ATT GCT GTG TGT CTT GTG TTC AGT T-3'. The efficiency of the her4-MO injections was assessed by the specific loss of GFP signal from the *Tg(her4:EGFP)*^{y83} transgenic line (Yeo et al., 2007).

The her9-MO (Gene Tools) (Bae et al., 2005) was injected at concentration 0.5 mM. This morpholino is directed against the splice donor site of the first intron in her9 pre-mRNA transcript, and its sequence is 5′-GTG ATT TTT ACC TTT CTA TGC TCG C-3′. Efficiency of the her9-MO was assessed by reverse transcriptase PCR using primers designed to amplify sequences flanking the first intron of the her9 pre-mRNA transcript (125 bp long, Ensembl RefSeq peptide, accession number NP_571948):
5'-her9: 5'-AGG GAC TCA CAC TCT CTC TCG T-3'
3'-her9: 5'-CTG CCC AAG GCT CTC GTT-3'.

RNA isolation:

RNA isolation was done using Trizol (Invitrogen, 15596-026) extraction protocol:

- add 800µl Trizol to 35 dechorionated uninjected or her9-MO injected zebrafish embryos and incubate 5 minutes (min) at room temperature
- add 160µl chloroform (Fluka, 25690) and vortex 15 seconds (sec), incubate 3 min at room temperature and centrifuge 15 min at 4ºC, 12000 rcf (g)
- keep upper phase and add 500µl phenol (Sigma, P4557) and 100µl chloroform, then centrifuge 15 min at 4ºC, 12000 rcf
- keep upper phase and add 400µl 2-propanol (Fluka, 59304), incubate 10 min at room temperature and centrifuge 10 min at 4ºC, 12000 rcf
- keep the pellet and add the 800µl 70% EtOH (Merck), centrifuge 5 min at 4ºC, 7600 rcf
- dry the pellet and dilute in 25µl ddH_2O

Reverse transcription

Reverse transcription of obtained RNA was done using SuperScript III Reverse Transcriptase Kit from Invitrogen:

- add 1µl of 50µM oligodT to 10µl of RNA obtained from Trizol extraction, incubate 5 min at 65ºC

add 1µl 0.1M dTT

4µl 5x First Strand Buffer
2µl 10mM dNTPs
1µl SS III RT-enzyme
1µl RNAs inhibitors

and incubate 1h at 50ºC

Inactivate reaction by incubation at 70ºC for 10 min
use 1µl for PCR, the rest store at -20ºC

Polymerase chain reaction (PCR)

Preparation of PCR mix was done on ice and it contained:

5µl 10x PCR buffer (final conc. 1X, EUROBIO)
2µl 50mM $MgCl_2$ (final conc. 2mM, EUROBIO)
1µl 10mM dNTPs (final conc. 0.2mM)
1µl cDNA template
0.8µl 10µM 5'-her9 primer (final conc. 0.8µM)
0.8µl 10µM 3'-her9 primer (final conc. 0.8µM)
0.2µl 6U/µl Taq Pol (final conc. 0.024U/µl, EUROBIO)
39.2µl ddH_2O

PCR program:

94ºC	2 min	
94ºC	35 sec	
58ºC	30 sec	35x

72ºC	1 min
72ºC	5 min
4ºC	hold

The products of PCR reaction were run on 2% agarose gel.

tbx1 capped mRNA was synthesized from *tbx1* full-length cDNA (Piotrowski et al., 2003). First, the following mix was prepared:

2µl 3µg/µl *tbx1* full-length cDNA
2.5µl Not1
4µl Buffer H
31.5µl ddH_2O

-incubation O/N at 37ºC
-run the gel
-purify the band from the gel using DNA Gel Extraction Kit

mRNA was transcribed from the purified linearized DNA using the mMESSAGE mMACHINE Kit (Applied Biosystems) and SP6 RNA polymerase:

6µl linearized DNA
10µl 2x NTP/CAP
2µl 10x reaction buffer
2µl SP& enzyme mix

-incubation 2h at 37ºC

-add 1µl Turbo DNAse, mix well and incubate 15 min at 37ºC
-precipitate using Kit reagents
-quantify by nanodrop and dilute to 500ng/µl

tbx1 mRNA was injected in 1 cell-stage zebrafish embryos at 250 ng/µl.

In situ hybridization and immunohistochemistry

Antisense RNA probe synthesis

Antisense RNA probe synthesis was done by in vitro transcription of the linearized DNA vectors. The vectors carried the sequence of interest, flanked by T3, T7 or SP6 RNA polymerases sequence. The restriction enzymes and polymerases used to synthesize each of the probes are given in Table 1.

DNA linearization, protein degradation and DNA purification:

1. incubate 2h at 37ºC the following mix
   5µl 2-3µg/µl DNA (final ammount 10-15µg)
   3µl restriction enzyme (final conc. 0.015U/µl)
   10µl 10x buffer (final conc 1x)
   82µl ddH_2O
2. spin down and add 5µl 10mg/ml proteinase K and 5µl 10%SDS, vortex, spin down and incubate 30 min at 37ºC

3. add 100µl phenol, vortex and centrifuge 5 min at 4ºC at max velocity
4. keep upper layer and add 50µl phenol and 50µl chloroform, vortex and centrifuge 5 min at 4ºC at max velocity
5. keep upper layer and add 225µl 100%EtOH, vortex, spin down and incubate 2h at -20ºC
6. centrifuge 15 min at 4ºC
7. remove the supernatant and add 500µl 70% EtOH to the pellet, centrifuge 15 min at 4ºC
8. dry the pellet 10 min at room temperature and dilute in 10µl ddH_2O

Zebrafish gene	**Restriction enzyme**	***RNA polymerase***
aldh1a2	KpnI	T7
aldh1a3	NotI	T3
atoh1a	HindIII	T7
atoh1b	BamHI	T7
cdkn1bl	Not1	T3
cdkn1c	Not1	T3
cyp26a1	SalI	T7
cyp26b1	EcoRI	SP6
cyp26c1	XbaI	SP&
her4	XhoI	T3
her6	HindIII	T7
her9	SalI	SP6
hey1	NotI	T7
hoxb4	KpnI	T3

Zebrafish gene	Restriction enzyme	*RNA polymerase*
krox20	XbaI	T3
lmx1b1	EcoRI	T7
neurog1	XhoI	T7
neurod	EcoRI	T7
neurod4	BamHI	T7
pea3	NotI	T7
ptc1	BamHI	T3
tbx1	HindIII	T7

Table 1. Restriction enzymes and RNA polymerases used for generation of the probes for in situ hybridization

In vitro transcription, DNA degradation, RNA precipitation and RNA purification:

1. incubate 3h at 37ºC the following mix
   1µl of linearized DNA (approx. 1µg)
   2µl 10x transcription buffer (final conc. 1x)
   4µl Digoxigenin-UTP mix (Dig-UTP, UTP, ATP, CTP and GTP), or Fluorescein NTP mix
   2µl RNA polymerase (final conc. 2U/µl)
   1µl RNAs inhibitor (final conc. 2U7µl)
   10µl ddH_2O
2. spin down, degrade DNA by adding 2µl DNAseI-RNAse free (approx. 20U) and incubate 30 min at 37ºC, then spin down
3. precipitate RNA with: 70µl ddH_2O, 10µl 4M LiCl and 300µl 100%EtOH, vortex, spin down and incubate 1h at -20ºC
4. centrifuge 10 min at 4ºC

5. keep the pellet, add 400µl 70%EtOH, centrifuge 10 min at 4ºC
6. dry the pellet and resuspend in 20µl ddH_2O
7. store at -20ºC

Whole-mount in situ hybridization (ISH) in zebrafish embryos

Single whole mount in situ hybridization was carried out with DIG-labeled RNA probes and alkaline-phosphatase coupled anti-DIG antibody (anti-DIG-AP), which was then detected with NBT/BCIP according to Thisse et al., 2004.

ISH protocol:

1. dechorionate embryos
2. fix the embryos at desired stages with 4% paraformaldehyde (PFA) in PBS overnight at 4ºC
3. wash the embryos 2 x 5 min with PBS-0.1%Tween-20 solution (PBT)

Pre-treatments

4. dehydrate embryos with 100% MeOH at least 1h at -20ºC
5. rehydrate embryos gradually by rinsing them at room temperature 10 min with 75% MeOH, 10 min 50% MeOH, 10 min 25% MeOH and 2 x 10 min PBT

6. incubate embryos with proteinase K (10mg/ml, 1/1000 in PBT) for 10 min if they are younger than 48 hpf, or for 20 min if they are 48 hpf or older
7. wash 5 min with PBT
8. postfic with 4% PFA for 40 min

Hybridization

9. prehybridize the embryos with the hybridization buffer, 1h at 70ºC

Hybridization buffer: 25ml 100% formaldehyde (FAD)
12,5ml 20x SSC pH 4.5
250µl 10mg/ml heparin
1ml 25mg/ml tRNA
500µl 10% Tween-20
125µl 1M citric acid pH 6.0
10,5ml ddH_2O

10. hybridize the embryos with probes diluted in hybridization buffer 1/200, overnignht at 70ºC

Washes

11. incubate embryos with:
    Wash 1, 10 min at 70ºC
    Wash 2, 10 min at 70ºC
    Wash 3, 10 min at 70ºC
    Wash 4, 10 min at 70ºC
    Wash 5, 2x30 min at 70ºC

PBT 2x10 min at room temperature

HYB- buffer: 32,5ml 100% FAD
12,5ml 20x SSC
500µl 10% Tween-20
4,5ml ddH_2O

Wash 1: 75% HYB- / 25% 2xSSC
Wash 2: 50% HYB- / 50% 2xSSC
Wash 3: 25% HYB- / 75% 2xSSC
Wash 4: 2xSSC
Wash 5: 0.5xSSC

Immunohistochemistry

12. incubate embryos 4h at room temperature with blocking solution (2% Bovine serum albumin (BSA), 10% heat inactivated goat serum (NGS), 0,1%PBT)
13. incubate embryos overnight at 4ºC with anti-DIG-AP diluted in blocking solution 1:4000
14. wash 10x10 min with PBT
15. incubate the embryos 3x 10 min with alkaline phosphatase buffer:

Alkaline phosphatase buffer
5ml 1M Tris-HCl pH 9.5 (final conc. 100mM)
2,5ml 1M $MgCl_2$ (final conc. 50mM)
1ml 5M NaCl (final conc. 100mM)
1ml 10% Tween-20 (final conc. 0,1%)

100µl Triton X-100 (final conc. 0,2%)

16. Develop the reaction by incubating embryos in the dark with the following solution:
    45µl NBT
    35µl BCIP
    10ml alkaline phosphatase buffer
17. stop reaction with PBT
18. wash overnight with PBT and postfix with 4%PFA, 2h at room temperature

DISH-chromogenic double in situ hybridization:

-the same as single ISH
-after developing the first reaction with NBT/BCIP, stop the reaction by washing embryos 2-4x with ddH_2O
-incubate embryos with 500µl 0.1M glycine (pH2.2) 10 min at RT
-wash 4x5 min with o.1%PBT
-incubate with anti-fluorescein-AP antibody (1:4000 in blocking solution) overnight at 4ºC
-wash 5x20 min with 0.1% PBT
-develop the reaction in dark with 30µl INT/BCIP mix (stock solution: 33mg/ml NBT and 33mg/ml BCIP) in 5ml NTMT solution
-stop the reaction by incubation with 0.1%PBT.

DISH using Fast Red tablets (Roche):

The protocol is the same as normal chromogenic DISH, except that the second developing solution is:
-one tablet of Fast Red in 2ml 0.1M Tris-Hcl (pH5.2), dissolved by vortexing.

Anti-GFP and anti-pH3 immunostaining

-after ISH is developed, postfix embryos fith 4%PFA for 30 min
-incubate with blocking solution (2% BSA, 10% NGS, 0.1% PBT) 1h at RT
-incubate overnight at 4ºCwith primary antibody (mouse anti-GFP or rabbit anti-phospho-histone H3, Millipore) diluted 1/400 in blocking solution,
-wash 10x10 min with 0.1% PBT
-incubate overnight at 4ºC with secondary antibody (anti-mouse or anti-rabbit Alexa Fluor 488, Invitrogen) diluted 1/400 in blocking solution
-wash 10x10 min with 0.1% PBT
-detect fluorescence under the fluorescent scope

Pharmacological treatments

DAPT treatments

In order to asses the role of Notch pathway in the establishment of *her* genes in the inner ear, dechorionated zebrafish embryos were incubated at 28.5ºC, in dark, with y-secretase inhibitor DAPT (N-[N-(3,5 - diuorophenacetyl) - l - alanyl] - S - phenylglicine t-butyl ester) (Calbiochem, 565770). The stock solution was 50 mM DAPT, in DMSO (dimethyl sulfoxide, Sigma)) and it was stored at -20ºC. The final solution, 150 μM DAPT, was prepared by diluting the stock solution in system water 3:1000. For control treatments DMSO was diluted in system water at 3:1000 proportion, and applied over dechorionated embryos.

Retinoic acid and DEAB treatments

Stock solutions, 10 μM for RA (Sigma, R 2625)) and 10 mM for 4-(diethylamino)-benzaldehyde (DEAB, Sigma, D86256) were made in DMSO and kept at -20ºC. The final solutions were made just before the treatments, and were 20 nM RA (dilution: 1/500 in system water) and 20 μM DEAB (1/500 in system water). For control treatments, DMSO was diluted in system water at 1/500. Embryos were dechorionated, and after the addition of the treatment solution were incubated at 28.5ºC, in dark.

Treatments with DEAB were done from 10.5 hpf stage until the sacrification of the embryos, while treatments with RA were done from 10.5-12 hpf, then embryos were washed several times with system water and incubated, until the sacrification, at 28.5ºC.

SU5402 treatments

Dechorionated zebrafish embryos were incubated with 60 μM SU5402 (Calbiochem, 572630), a potent pharmacological inhibitor of Fgf signaling. Incubations were done in dark, at 28.5ºC, starting from different embryonic stages until the sacrification of the animals (at 24 hpf or 30 hpf). The final solution was made in system water, from the 5mM SU5402 stock solution (kept at -20ºC, in DMSO). Dilution was 6/500. Corresponding control DMSO solution was made by diluting DMSO in system water in proportion 6/500.

Cyclopamine A treatments

Cyclopamine A (CyA, LC Laboratories, C-8700) was kept at -20ºC as a 10mM stock solution (in ethanol, EtOH, Merck). The final dilution in system water was 1/100. For control treatments corresponding EtOH dilution was made (1/100 in system water). Embryos were dechorionated before the treatments, and the incubation with CyA or EtOH was performed for consecutive 3-hour periods starting from different time points, after that embryos were washed with system water and let to grow until sacrification. All the incubations were done in dar, at 28.5ºC.

Cotreatment with 100 μM CyA and 20 nM RA was done from 10.5-14.5 hfp, then embryos were washed with system water and let to grow until sacrification, in dark, at 28.5ºC. As a control, embryos were treated either with corresponding dilution of EtOH or with DMSO.

Cotreatments with 20 nM RA and 150 μM DAPT, or with 100 μM CyA and 150 μM DAPT were done from 10.5 hpf until the sacrification of the embryos, and DMSO was used in control treatments.

Neuronal and hair cell number countings

Statoacoustic ganglionar neurons were counted from the z stack (average step was 1μm) images of the coronal sections of 48 hpf uninjected or her9-MO injected *islet3:GFP* embryos. The images were taken at 40x magnification using a Leica DM6000B fluorescence microscope with DFC300KX camera under the control of LAS-AF (Leica Application Suite Advanced Fluorescence 1.8) software.
Hair cells were counted from images taken from sagittal and transversal sections of 48 and 96 hpf *brn3c:GFP* embryos.

Cryostat sectioning

1. fix embryos with 4% PFA overnight at 4ºC
2. wash 3x 10 min with PBT
3. transfer embryos to 15% sucrose in PBS until the embryos sink (approx. 1h)
4. incubate embryos with 15%s sucrose / 7.5% gelatin in PBS for 30 min at 37ºC
5. place embryos in cryomold under the scope to obtained the desired section orientation
6. cool isopentane (2-methylbutane) at -80ºC

7. immerse the block with embryo into pre-cooled isopentane for 30-45 sec
8. keep the frozen blocks at -20ºC until sectioning

9. section with cryostat at 20 μm step and collect the sections on Superfrost slides
10. mount sections with mowiol

Student t-test was used for the analysis of neuronal and anti-pH3-postive cell countings.

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ANNEX

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