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Hindbrain signals in otic regionalization: walk on the wild side

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ABSTRACT The inner ear, the sensory organ responsible for hearing and balance, contains specialized sensory and non-sensory epithelia arranged in a highly complex three-dimensional structure. To achieve this level of complexity, a tight coordination between morphogenesis and cell fate specification is essential during otic development. Tissues surrounding the otic primordium and more particularly the adjacent segmented hindbrain, have been implicated in conferring signals required for inner ear development. In this review, we present the current view on the role of hindbrain signals in axial specification of the inner ear. The functional analysis of mutants of hindbrain segmentation genes, as well as the investigation of signaling pathways potentially involved, all point to an essential role of FGF, Wnt and Hh signaling in otic regionalization. However, these data provide conflicting evidence regarding the involvement of hindbrain signals in otic regionalization in fish and in amniotes. We discuss the possible origin of these differences.

KEY WORDS: *hindbrain, patterning, otic regionalization, FGF signaling*

Basic structure of the adult inner ear

The vertebrate inner ear is a sensory organ responsible for the senses of hearing, balance and detection of acceleration. It consists of a closed epithelial structure, the membranous labyrinth, composed of several sensory and non-sensory structures and surrounded by a bony capsule. The mechanosensory function of the inner ear is provided by the hair cells, which, along with supporting and secretory cells, are contained in the sensory epithelia. Hair cells are innervated by sensory neurons of the vestibular and acoustic ganglia that project to the vestibular and auditory nuclei in the brainstem.

The membranous labyrinth is subdivided into vestibular and auditory regions. The vestibule forms the dorsal part of the labyrinth and is responsible for the senses of motion and position. It comprises the three cristae, the sensory organs located at the basis of three orthogonally arranged semi-circular canals and the utricle and saccule, which contain two additional sensory organs, the maculae. The ventral auditory part is more diverse. In mammals it is composed of the cochlea, a coiled structure whose sensory epithelium is called the organ of Corti. In birds, the auditory region is composed of the basilar papilla, while in fish the saccule and lagena are both involved in hearing (Figure 1). In jawed vertebrates, the adult inner ear is highly regionalised along its three axes. In addition to the dorso-ventral (DV) subdivision into vestibular and auditory regions, an asymmetry along the medio-lateral (ML) axis is also obvious with, for instance, the endolymphatic sac and duct located in the medial part, close to the brain. The whole structure also shows pronounced antero-posterior (AP) asymmetry (Figure 1).

How are these asymmetries established during ear development? What are the signals involved in establishing these asymmetries and where do they come from? Are there similarities and differences in the mechanisms of otic patterning in the different model organisms analysed so far? In the present review we attempt to answer these questions, focusing on the role of the adjacent hindbrain, a highly patterned region of the embryonic brain whose function in otic development has been extensively studied. Although the first observations of the importance of the hindbrain in the development of the inner ear were done by experimental embryologists back in the 1940s, we will focus on the functional data obtained in mice, chick and zebrafish during the last 10 years. Thus, we only describe ear structure and development in these three vertebrate species and we do not discuss evolutionary aspects of ear patterning. For a comprehensive review on ear evolution, see Fritzch, this issue.

Abbreviations used in this paper: AP, antero-posterior; DV, dorso-ventral; FGF, fibroblast growth factor; ML, medio-lateral; ngn, neurogenin.

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Early stages of otic development

The inner ear arises from the otic placode, an ectodermal thickening that forms lateral to the hindbrain during neurulation. At this stage, the hindbrain is subdivided into segments termed rhombomeres (see below). The placode is first visible morphologically at early somite stages, but long before, it is already prefigured by molecular markers, such as transcription factors of the *Pax*, *Eya* and *Dlx* families (Riley and Phillips, 2003). The placode forms lateral to the caudal hindbrain and covers the length of about two rhombomeres (Figure 2). In chick, the otic placode is initially adjacent to r4 and r5 (Figure 2 Aa) and then at the otic cup stage is juxtaposed to r5 and r6. In fish, the placode is centred on r5 and overlaps with both r4 and r6 (Haddon and Lewis, 1996) (Figure 2Ba). Then the placode transforms into the otic vesicle or otocyst, a morphogenetic process that varies among species. In amniotes and amphibians, the placode invaginates, forming the otic cup (Figure 2Ab); then the cup closes and pinches off from the ectoderm to form the otic vesicle (Figure 2Ac-d). In fish (Haddon and Lewis, 1996) and reptiles (see review Barald and Kelley, 2004), the placode first individualises from the ectoderm (Figure 2Ba) and then cavitates (Figure 2Bb-d). Therefore, the intimate relationship between the otic vesicle and the hindbrain differs along the DV axis between amniotes and zebrafish.

The otocyst is a self-contained organ which gives rise not only to sensory and non sensory epithelia, but also to neurons of the statoacoustic ganglion (SAG, VIIIth ganglion, also called cochleovestibular ganglion in mouse). The SAG forms anteroventrally to the otocyst (Figure 2d). The kinetics of neuron production and sensory patches formation is not the same in different species. In amniotes, neurogenesis and neuronal delamination from the otic epithelium begin at cup stage (Figure 2Ab-d). Sensory organs are formed later. In zebrafish, neurogenesis and first hair cell formation occur simultaneously. Cells giving rise to the neurons of the SAG are derived from the ventral floor of the otic vesicle (Haddon and Lewis, 1996). Delamination takes place in the anteroventral region and the SAG is then localised anteroventrally to the otocyst (Figure 2Bc-d). At the same time, the first hair cells -called tether cells- form in two patches, the utricular (anteroventral) and saccular (posteromedial) maculae. These two patches can be distinguished by their position (more medial for the saccular macula) and shape and by the different orientation patterns of their hair cells. Thus, in this species, the SAG and the first sensory patches define anterior and posterior, as well as dorsal and ventral poles as soon as the otic vesicle stage (Figure 2Be). Later, the cristae will form in anterior, posterior and medial positions along the lateral wall of the vesicle (Figure 2Be). In amniotes, the mediodorsal part of the vesicle is marked by the early appearance of the endolymphatic duct (Figure 2Ad-e), while in zebrafish, this duct appears only later in development. After 10.5 dpc in mouse (E3.5 in chick), the vesicle is elongated along the DV axis, with the ventral part prefiguring the cochlear duct (Figure 2Ac) (Cantos *et al.,* 2000).

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

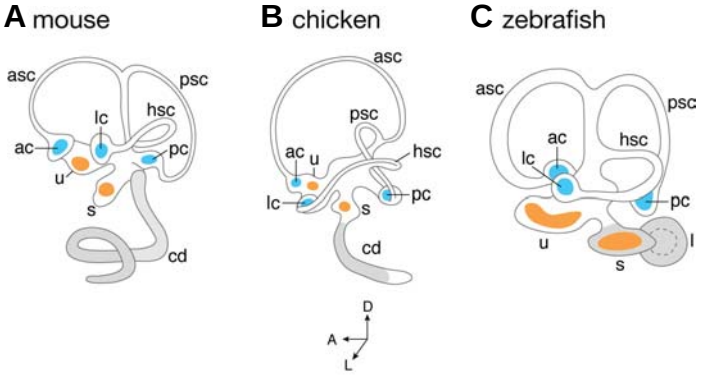


Fig. 1. Structure of the adult inner ear. *Morphology of the membranous labyrinth in three vertebrate species: mouse* **(A)**, *chicken* **(B)** *and zebrafish* **(C)**. *The vestibular (dorsal) part of the membranous labyrinth contains five sensory organs: the three cristae (blue) located at the basis of the three semicircular canals and the utricular and saccular maculae (orange), surrounded by otoliths. The ventral, auditory part of the inner ear (grey) is highly variable in morphology and complexity in different vertebrates. In the mouse, the cochlear duct, a coiled structure, contains a finely patterned sensory organ, the organ of Corti. In chicken, the auditory organ, the basilar papilla, is also contained in the cochlear duct. In zebrafish, there is no ventral cochlear duct and the auditory function is carried by the saccular and lagenar maculae. The endolymphatic sac and duct (not represented) produce the endolymph, which circulates in the membranous labyrinth. ac: anterior crista; asc: anterior semicircular canal; cd: cochlear duct; hsc: horizontal semicircular canal; l: lagena; lc: lateral crista; pc: posterior crista; psc: posterior semicircular canal; s: saccule; u: utricle. The embryonic axes are indicated at the bottom.*

Axial specification in the inner ear and its relationship to cell fate

As mentioned above, the cells of the developing inner ear undergo a sequence of cell fate decisions to generate all different cell types from a single otic epithelium and the cell types must arise in the correct spatial position with respect to one another. It is tempting to speculate that this occurs similarly to the adjacent hindbrain, where compartment formation and establishment of positional identity direct cell fate specification (Schneider-Maunoury *et al.,* 1998; and see below). A compartment based model has indeed been proposed for cell specification in the inner ear (Brigande *et al.,* 2000a; Brigande *et al.,* 2000b). However, how the axes of the otocyst are established and where the positional cues come from is a field under current investigation. We summarize below several lines of evidence suggesting that, early on, the otic placode and vesicle are already regionalised along their different axes.

Early molecular asymmetries

Molecular asymmetries precede morphological asymmetries; by the otic vesicle stage, several gene expression domains compartmentalize the otocyst along its three axes. Genes that are expressed within the early otocyst are thought to specify the future regions of the ear (reviewed by Fekete and Wu, 2002) and typically encode transcription factors. For some of these genes, functional studies have been performed, thus allowing to corre-

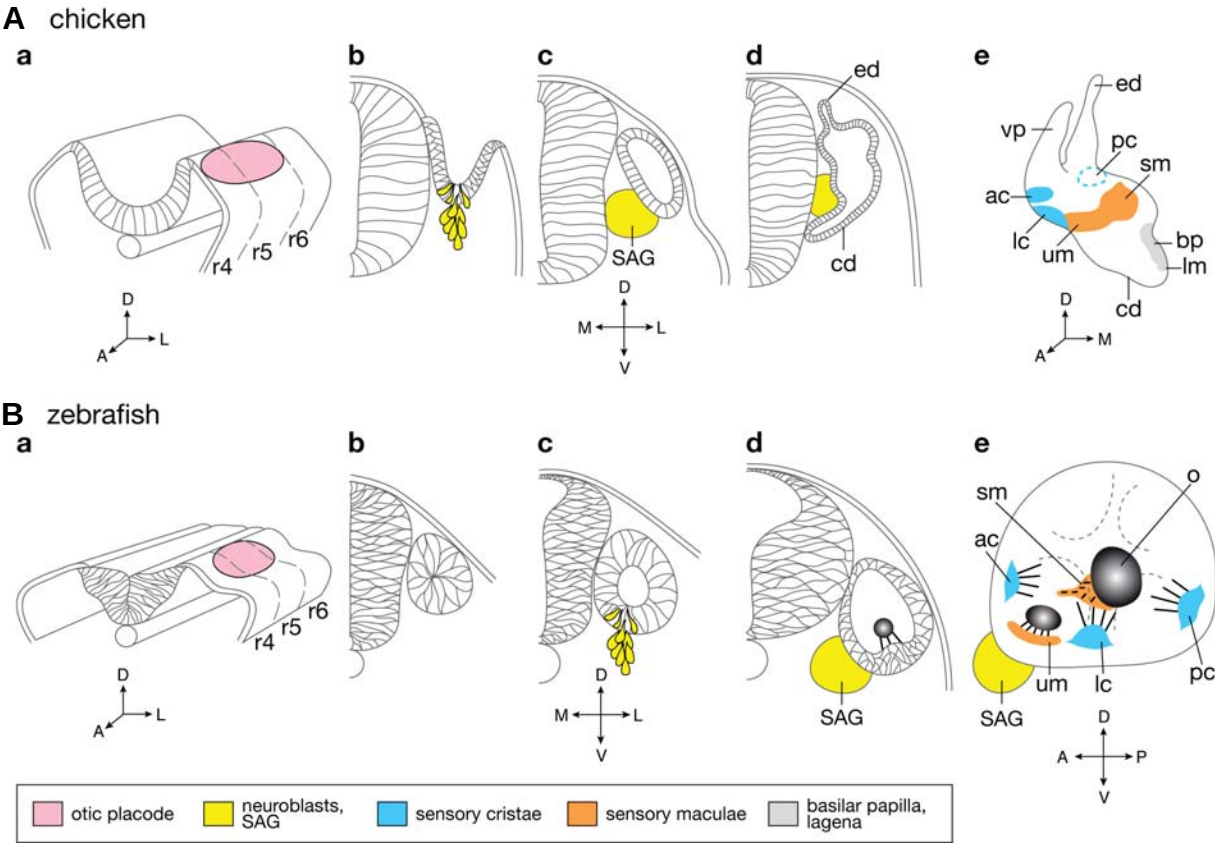


Fig. 2. Early development of the inner ear.*Schematic drawings of the development of the inner ear in chicken* **(A)** *and zebrafish* **(B)**. *The development of the mouse inner ear is similar to that of chicken.* **(a)** *3D representations of a transverse segment of the head region.* **(b-d)** *Transverse sections at otic level.* **(e)** *Diagrammatic representations of the chick inner ear at E5 and of the zebrafish larval ear. The otic placode is pink, the neuroblasts and SAG are yellow, the sensory cristae are blue, the sensory maculae are orange and the basilar papilla and lagena are grey. Abbreviations: ac, anterior crista; bp, basilar papilla; cd, cochlear duct; ed, endolymphatic duct; lc, lateral crista; lm, lagenar macula; o, otolith; pc, posterior crista; r, rhombomere; SAG, statoacoustic ganglion; sm, saccular macula; um, utricular macula; vp, vertical pouch (semicircular canal primordium). The dotted lines in (Be) represent the epithelial protrusions that will form the semicircular canals; Stages in (A) are: 10 s (a), 19 s (b), 30 s (c), E3.5 (d) and E5 (e). Stages in (B) are: 10s (14 hpf) (a); 14 s (16 hpf) (b); 26s (22 hpf) (c); 30 hpf in (d), 120 hpf in (e). hpf means hours post-fertilization at 28°C. The embryonic axes are indicated underneath each drawing. Axes in A and B are the same.*

late the early gene expression domains with the functions of these genes in otic patterning, cell fate, and/or cell survival and proliferation.

In mouse and chicken, molecular asymmetries along the DV axis are observed early on (Figure 3Aa). The ventral cells of the otic vesicle are marked by expression of *Otx1*, *Otx2* and *Pax2*, while the dorsal cells express *Hmx2, Hmx3* (formerly named *Nkx5.1*), *Dlx5* and *Gbx2* (Lin *et al.,* 2005; for review see Brigande *et al.,* 2000). Inactivation of these genes in the mouse confirms that they participate in establishing the dorsal (vestibular) and the ventral (cochlea) components, mainly through the control of cell survival and proliferation during ear morphogenesis. For example, *Pax2* mutants show agenesis of the cochlea and of the spiral (auditory) ganglion (Torres *et al.,* 1996) due to a reduction of cell proliferation (Burton *et al.,* 2004). The loss of *Otx1* and *Otx2* also affects the cochlea, as well as the development and the final positioning of some of the sensory organs within the mouse inner ear (Morsli *et al.,* 1999). In contrast, the dorsally expressed genes *Dlx5*, *Hmx2* and *Hmx3* are essential for vestibular morphogenesis (Wang *et al.,* 1998; Wang *et al.,* 2001; Merlo *et al.,* 2002). *Gbx2* has a cell-autonomous effect in the development of the endolymphatic duct and vestibular structures (Lin *et al.,* 2005).

In zebrafish, molecular asymmetries along the three axes are apparent before 20 hpf (Figure 3B). *nkx5.1/hmx3*, the first known asymmetrically expressed gene, is induced in a large anterior region of the placode and vesicle from 14 ss (16 hpf) onwards (Adamska *et al.,* 2000). Later, several markers are specifically expressed in anterior (*pax5*, *fgf8*), posterior (*follistatin*, *bmp7*), medial (*pax2a*), dorsal (*dacha* and *dlx3b*) and ventral (*otx1, eya1*, *six1*) regions of the vesicle (Krauss *et al.,* 1991; Akimenko *et al.,* 1994; Bauer and Goetz, 1998; Pfeffer *et al.,* 1998; Reifers *et al.,* 1998; Sahly *et al.,* 1999; Mowbray *et al.,* 2001; Hammond *et al.,* 2002; Bricaud and Collazo, 2006). The function of several of these genes has been addressed using morpholino injection and/or mutants. Interestingly, they are not only involved in ear patterning but, also, in regulating cell proliferation and apoptosis. Loss of function of *pax5* leads to impairment of vestibular functions due to hair cell death in the utricular macula (Kwak *et al.,* 2006). Analysis of the *dog-eared* mutants (*dog*) shows that *eya1* is involved in survival of sensory hair cells, particularly in the cristae

(Kozlowski *et al.,* 2005). *six1* expression in the ventral otic vesicle has contrasting roles on neuronal and hair cell lineages: it promotes hair cell formation by increasing cell proliferation and inhibits neuronal formation by inducing apoptosis (Bricaud and Collazo, 2006).

Not surprisingly, genes involved in cell fate specification are also expressed in region-specific patterns. In amniotes, the specification of some otocyst cells as neuroblasts at the otic cup stage is perhaps the earliest cell fate decision that takes place in the ear (Figure 3Ab) (Ma *et al.,* 1998). In chick, *Fgf10* precedes the expression of the proneural genes *ngn1* and *neuroD* in the neurogenic domain, which corresponds to the ventral anteromedial quadrant of the otic primordium. *Fgf10* expression in this region is required for neuronal formation (Alsina *et al.,* 2004) although it is not the only player, since *Fgf10* null mice have normal initial

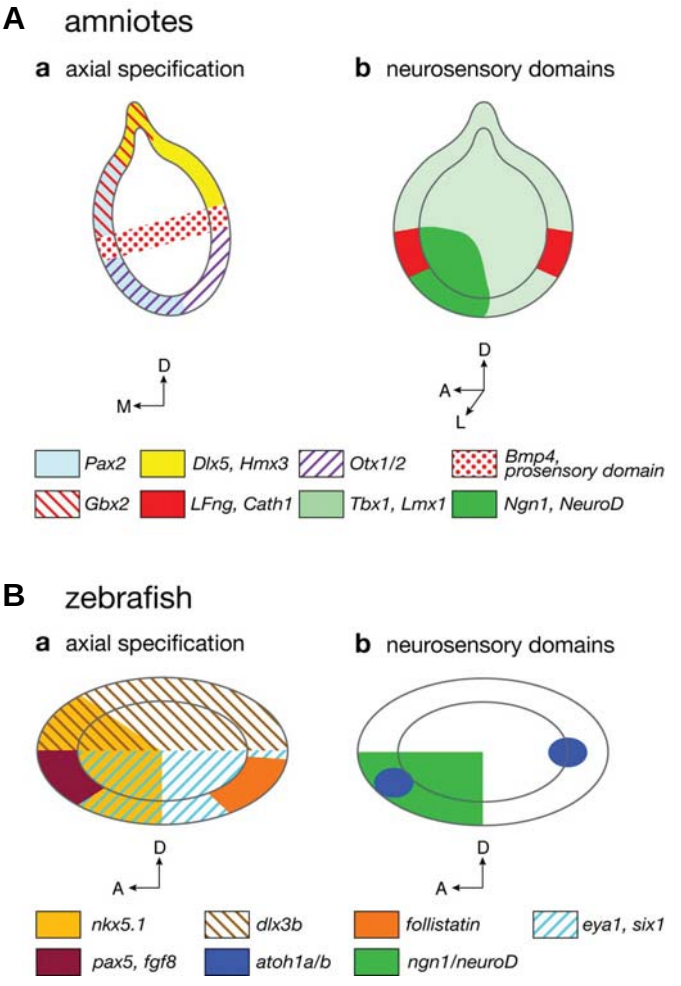


Fig. 3. Early regionalization and cell fate specification markers in the otocyst of amniotes and zebrafish. (A) *Schematic drawings of amniotes otic vesicles. (Aa) represents a transverse section at E2-3 in chick and (Ab) shows a lateral view at E2-3.* **(B)** *Schematic lateral views of the zebrafish otic vesicle at 24 hpf. (Ba) represents gene expression domains regionalized along the AP and DV axes; (Bb) shows early markers of neurogenic and sensory specification. Gene expression domains that prefigure different regions of the inner ear, as well as cell fate markers are color-coded. Embryonic axes are indicated.*

neurogenesis (Pauley *et al.,* 2003). In the mouse, *Tbx1* is expressed in the posterior region of the otic cup and vesicle and is required to delimit the neurogenic domain (Raft *et al.,* 2004; Xu *et al.,* 2007). Markers of sensory cell fate in amniotes appear later than those of neuronal fate and are marked by the expression of neurotrophins *BDNF* and *NT-3*, *Bmp4*, *LFng, Ser1* and *Cath1* (Figure 3A, Figure 2Ae) (Wu *et al.,* 1998; Cole *et al.,* 2000; Fariñas *et al.,* 2001; Pujades *et al.,* 2006).

In zebrafish, the sensory and neuronal lineages both arise from the ventral part of the vesicle and genes involved in the formation of sensory and neuronal progenitors are expressed very early in the ventral region of the otocyst (Figure 3Bb). The proneural genes *ngn1* and *neuroD*, which mark neuronal progenitor cells and are necessary for formation of the SAG, are expressed in the anteroventral region of the otocyst (Andermann *et al.,* 2002), while *atoh1a/b*, markers of sensory progenitor cells, are expressed as soon as the placode stage in groups of cells, one anterior and one posterior, prefiguring the utricular and saccular maculae, respectively. *atoh1a/b* are necessary for hair cell formation in all sensory patches, thereby playing a classic role of proneural genes in the ear (Millimaki *et al.,* 2007). The cristae arise later and are marked by the expression of *bmp* pathway members (Mowbray *et al.,* 2001).

Temporal programme of otic patterning

It is tempting to assume that early molecular asymmetries prefigure later ear patterning and in some cases this is confirmed by the analysis of mouse mutants. However, the existence of an asymmetry (morphological or molecular) at early stages does not imply that the regional fate of the cells is already determined. First, cell dispersion and/or active migration may prevent this regional specification or may change the position of cell groups relative to the axes. Second, definitive regional specification may depend on different signals acting over a large temporal window. Therefore, two important questions to ask are: 1) where and when is cell dispersion and migration restricted in the otocyst and 2) when do the axes of the inner ear become fixed during development?

In an attempt to answer the first question, fate maps of the otic primordium have been performed in different species and at different stages (Brigande *et al.,* 2000; Lang and Fekete, 2001; Kil and Collazo, 2001; Streit, 2002; Kozlowski *et al.,* 1997; Satoh and Fekete, 2005; Abello *et al.,* 2007; reviewed in Kil and Collazo, 2002). However, the results obtained so far fail to give a clear picture. For instance, in *Xenopus*, a fate map of the sensory organs performed at otocyst stage has shown a high degree of cell dispersion along the AP and DV axes (Kil and Collazo, 2001), while in chick, a fate map of the rim of the otic cup has identified lineage boundaries, leading to a compartment model for inner ear patterning (Brigande *et al.,* 2000b; Satoh and Fekete, 2005). More experiments will be required to determine, first, at what stage cell dispersion becomes restricted in the otocyst in different species and second, if the establishment of lineage boundaries is an important event in inner ear regionalization, as it happens during segmentation of the adjacent hindbrain.

The timing of otic axis specification was addressed in chick by axial rotations of otic cups and vesicles. These works suggested that the DV axis is established later than the AP axis (Wu *et al.,* 1998; Bok *et al.,* 2005). Moreover, the AP axis seems to be established earlier for sensory structures than for non-sensory

structures. Although these data need to be extended by using more and/or earlier markers of different ear structures, they argue in favour of a multistep process of axis establishment acting on a relatively large temporal window and probably involving many different signals.

Caudal hindbrain patterning

During neurulation in vertebrates, the hindbrain is transiently subdivided along its AP axis into a series of 7/8 segments termed rhombomeres, which are compartments of cell lineage restriction. This segmentation process sets up the stereotyped pattern of neuronal specification in the brainstem. It is also involved in neural crest cell migration in three streams towards the pharyngeal arches, thereby influencing craniofacial morphogenesis. A number of regulatory genes are expressed in specific rhombomeres or groups of rhombomeres and many of them have been implicated in different steps of the segmentation process (for review Schneider-Maunoury *et al.,* 1998). Here we review the molecular mechanisms of formation of rhombomeres 4 to 6, the hindbrain region directly adjacent to the developing otocyst (Figure 4), with greater interest for the genes whose functional studies have indicated a role of the hindbrain in inner ear patterning.

The hindbrain is patterned under the influence of two major signaling pathways, the FGF and retinoid pathways. FGFs are synthesized at the midbrain-hindbrain boundary region and also in different rhombomeres and play a role in the specification of adjacent domains (see below). Retinoic acid (RA) is synthesized in the anterior somitic mesoderm and plays and essential role in setting up AP positional information in the hindbrain (reviewed in Glover *et al.,* 2006). RA controls, directly and indirectly, the expression of several genes in the hindbrain, among which several *Hox* genes, *vhnf1* and *MafB*. In particular, they directly control *Hox* genes of the paralogous groups 1 and 4 (Marshall *et al.,* 1994; Gould *et al.,* 1998).

During the last 15 years, the molecular mechanisms of hindbrain patterning have been extensively studied in the mouse by the means of knock-out mutants and transgenesis. These studies have identified *Hox* genes as major actors in the control of AP identity in the hindbrain and pharyngeal arches (for review Schneider-Maunoury *et al.,* 1998; Trainor and Krumlauf, 2001). The 4 first groups of *Hox* paralogues (1-4) are expressed in the hindbrain, with temporal and spatial patterns that depend on their position in the clusters. One of the *Hox* genes, *Hoxa1*, plays an early and central role in formation of the caudal hindbrain illustrated by the strong reduction of r4 and r5 in *Hoxa1* mutants (Dolle *et al.,* 1993; Helmbacher *et al.,* 1998). Another essential gene for caudal hindbrain patterning is *MafB*, which codes for a bZIP transcription factor and is expressed in prospective r5 and r6. The phenotype of *MafB* loss-of-function has first been studied in the mouse mutant *kreisler*, named accordingly to its circling behavior. In the hindbrain, the *kreisler* mutant displays a loss of r5 and a misspecification of r6 (McKay *et al.,* 1994; Sadl *et al.,* 2003). *kreisler* is not a null mutant for *MafB* but rather a regulation mutant, which abolishes *MafB* expression in r5/r6 and activates it in r3 (Eichmann *et al.,* 1997; Giudicelli *et al.,* 2003). More recently however, the essential function of *MafB* in regulating the maintenance and expansion of r5 and specification of r6 has been confirmed using a mouse mutant obtained by ENU mutagenesis (Sadl *et al.,* 2003) and in zebrafish, where several mutant alleles of *valentino* (*val*), the zebrafish *MafB* gene, have been obtained (Moens *et al.,* 1998; Prince *et al.,* 1998). Among other regulatory functions, *MafB* is necessary for the expression in r5 of *krox20*, a gene required for the formation and specification of r3 and r5 (Schneider-Maunoury *et al.,* 1993; Schneider-Maunoury *et al.,* 1997; Voiculescu *et al.,* 2001).

Recently, studies performed in the zebrafish embryo have allowed identifying early events of caudal hindbrain formation and patterning. In particular, they have uncovered the essential functions of the transcription factor *vhnf1* and of FGF signaling in

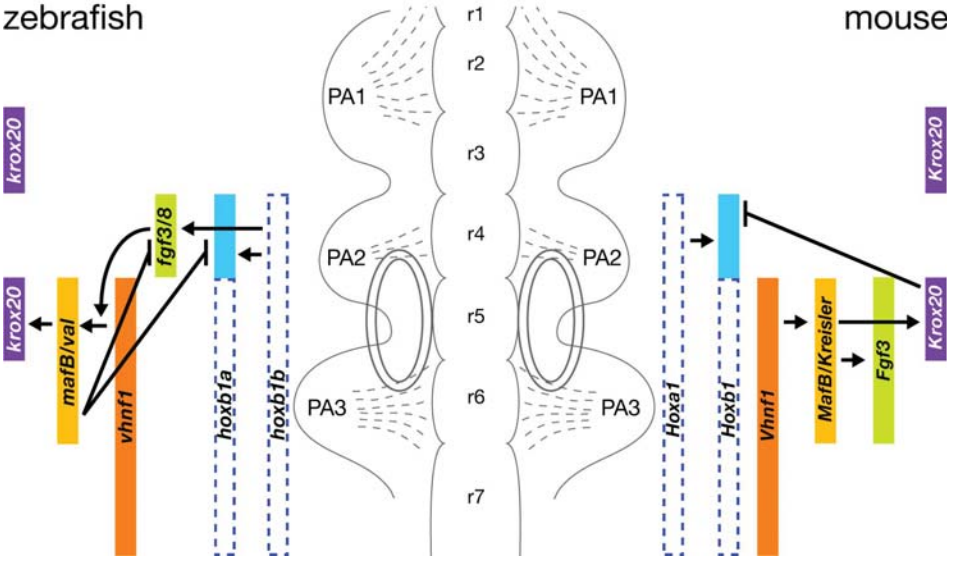


Fig. 4. Caudal hindbrain patterning. *A schematic vertebrate hindbrain region is presented, with rhombomeres (r1-r7), the three first pharyngeal arches (PA1-3), the three streams of migrating neural crest cells (dotted lines) and the bilateral otic vesicles. Crest cells from r1-r2 migrate into PA1, crest cells from r4 migrate into PA2 and crest cells from r5 and r6 migrate into PA3. The expression patterns of regulatory genes are indicated and color-coded on the left for zebrafish and on the right for the mouse. The dotted lines for* Hox *genes indicate a transient expression, which is downregulated before the onset of morphologic segmentation:* Hoxa1 (hoxb1b *in zebrafish*) *and its paralogue* Hoxb1 (hoxb1a *in zebrafish*) *are activated at mid-gastrulation and present an anterior limit in the neural plate in the prospective hindbrain region, which later coincides with the r3/r4 boundary. Contrary to* Hoxa1, Hoxb1 *is maintained in r4 after the end of gastrulation and this expression in r4 requires* Hoxa1 *function. Regulatory interactions between the different genes are indicated. Arrows represent positive regulations and vertical bars represent negative regulations. Some of these regulatory interactions, but not all, have been shown to be direct. Note that some expression patterns (e.g. for* Fgf *genes) and regulatory interactions are different in these two species. Anterior is to the top.*

caudal hindbrain specification. The *vhnf1* (*Hnf1β*/*Tcf2*) gene codes for a homeodomain transcription factor. In the neural plate, *vhnf1* is expressed from the end of gastrulation onward, with an anterior expression limit at the prospective r4/r5 boundary. The study of a *vhnf1* insertional mutant in zebrafish has shown the involvement of this factor in caudal hindbrain formation (Sun and Hopkins, 2001). In *vhnf1* homozygous embryos, *val* expression in r5-r6 and *krox20* expression in r5 are severely reduced or absent. Notably, r4 markers such as *hoxb1a* and *fgf3* are expanded caudally as in *val* mutants. Therefore, *vhnf1* is involved in specification of r5-r6 and in the repression of r4 identity (Wiellette and Sive, 2003; Hernandez *et al.,* 2004).

FGF signaling is essential for caudal hindbrain patterning. In zebrafish, two *fgf* genes, *fgf3* and *fgf8*, are expressed in prospective r4. Loss-of-function studies in this species showed that *fgf3* and *fgf8* are required for the activation of *val* expression in r5-r6 and *krox20* expression in r5 (Maves *et al.,* 2002; Walshe *et al.,* 2002). Moreover, FGF signals from r4 have been shown to synergize with *vhnf1* for this activation (Wiellette and Sive, 2003; Hernandez *et al.,* 2004). Thus, by secreting FGF signals, r4 forms a signaling centre involved in caudal hindbrain patterning, in particular in *val* and *krox20* activation. Although *Fgf* gene expression patterns in the hindbrain of amniotes differ from that of zebrafish (see below), FGF signaling in chick embryos has also been involved in hindbrain patterning, particularly in the regulation of *MafB* and *Krox20* expression (Marin and Charnay, 2000; Aragon *et al.,* 2005).

The functional data obtained in different vertebrates can be combined into a regulatory hierarchy involved in caudal hindbrain formation (Figure 4). However, it should be mentioned that several data have been obtained in only one vertebrate species. Moreover, in some cases, differences have been found in the gene expression patterns and/or in the functional data in amniotes and in zebrafish. One important difference lies in the expression of *fgf3* and its regulation by segmentation genes. In the mouse, *Fgf3* is expressed at early somite stages in r4 and in the surface ectoderm including the prospective otic placode region. Later, its high level expression domain in the hindbrain restricts to r5 and r6 (Mahmood *et al.,* 1996). *MafB* is necessary for *Fgf3* expression in the r5-r6 domain (McKay *et al.,* 1996). In contrast, in fish *fgf3* is expressed in r4 (Maves *et al.,* 2002; Walshe *et al.,* 2002) and its expression in the *val* and *vhnf1* mutants extends posteriorly (Kwak *et al.,* 2002; Wiellette and Sive, 2003; Hernandez *et al.,* 2004; Lecaudey *et al.,* 2007). Thus, the caudal hindbrain in zebrafish *MafB/val* mutants expresses *fgf3* ectopically, whereas in *MafB/kreisler* mutant mice, the caudal hindbrain shows reduced *Fgf3* levels (McKay *et al.,* 1996).

In addition to AP patterning, the hindbrain is also regionalized along its DV axis. The mechanisms of neural tube DV patterning have been extensively studied in the spinal cord (for review Lee and Jessell, 1999; Jessell, 2000) and similar mechanisms are thought to function in the hindbrain. In particular, Hh signaling from the notochord and floor plate and Wnt and BMP signaling from the ectoderm, dorsal neural tube and roof plate, control the nested expression of a series of transcription factors along the DV axis of the spinal cord. These transcription factors, through complex regulatory interactions, establish distinct neuronal progenitor domains. Recent data have shown that in the hindbrain, the integration of AP and DV molecular inputs provides a two-dimensional grid of coordinates for motor neuron progenitor specification (Samad *et al.,* 2004).

Hindbrain segmentation and otic development: insights from functional data

Tissues surrounding the inner ear, such as the hindbrain, mesoderm and endoderm, have been implicated in conferring signals required for inner ear development (Giraldez, 1998; Fekete, 1999; Kiernan *et al.,* 2002). An essential function of the hindbrain has been demonstrated by the analysis of mutants of regulatory genes expressed in this tissue -but not in the otic tissue- and involved in caudal hindbrain segmentation, such as *MafB*, *vHnf1* and *Hoxa1*. The contribution of these functional studies is summarized below (Figure 5). In all cases, the inner ear defects are attributed to defects in rhombomeres 4 to 6, the region of the hindbrain juxtaposing the developing otocyst (Figure 2). While there are several discrepancies between the results obtained in different species, all the data point to an essential role of signaling from the hindbrain and in particular FGF signaling, in otic regionalization.

In mice, important information about hindbrain signals was brought by analysis of the *kreisler* mutant, in which *MafB* gene expression in r5 and r6 is abolished. *kreisler* mice are deaf, present a circling behavior and show many defects in otic development. Since *MafB* is not expressed in the otocyst, it has been proposed that the deficit in FGF3 signaling was a main cause of the otic defects seen in *kreisler* (McKay *et al.,* 1996). In these mice, dorsomedial markers such as *Gbx2* and *Wnt2b* are lost, while the ventral *Otx2* domain is expanded. Later, the cochlea is expanded and the endolymphatic duct and sac are absent, suggesting a role of the hindbrain in specifying dorsomedial structures of the inner ear (Choo *et al.,* 2006). The role of FGF3 as a hindbrain signal in ear patterning is consistent with the analysis of the *Hoxa1* mutant, in which the appearance of ear patterning defects is also correlated to the loss of *Fgf3* expression in the hindbrain (Pasqualetti *et al.,* 2001).

In contrast to the situation in mouse, the *MafB/val* mutation in zebrafish results mainly in AP patterning defects (Kwak *et al.,* 2002). Anterior markers such as *hmx3* are expanded posteriorly, while caudal markers are reduced or absent. *val* mutants also present an excess of hair cells, ectopically produced between the anterior and posterior maculae (Kwak *et al.,* 2002). Surprisingly, although the *val* mutation clearly affects AP patterning of the ear, it does not lead to an increase in the size of the SAG, suggesting that SAG formation is under a tight, hindbrain-independent, control. As mentioned above, the *val* mutation results in a posterior expansion of *fgf3* expression in the hindbrain. Reduction of *fgf3* RNA levels in *val* mutants using morpholinos rescues some of the otic defects, strongly suggesting that, in zebrafish as well as in mouse, FGF3 is a major signal involved in ear patterning downstream of *MafB*.

The analysis of the zebrafish *vhnf1* mutant adds more complexity to the picture. As shown earlier, *vhnf1* positively controls *val* expression in the hindbrain. As expected, AP patterning phenotypes are observed in the inner ear of *vhnf1* mutants, which display an expansion or a duplication of anterior otic markers such as *hmx3, fgf8* and *pax5*. However, *vhnf1* mutants also show DV

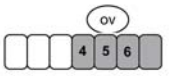
GENE EXPRESSION PROFILE	NEURAL TUBE PHENOTYPE	OTIC PHENOTYPE
Transcription factors		
 *Hoxa1* up to r3/r4	**Mouse mutant:** Reduction of r4 and absence of r5 Loss of *Fgf3* expression in caudal hindbrain	Small otic vesicles displaced from the hindbrain Defects in vestibular and cochlear structures
 *vHnf1* up to r4/r5	**Conditional mouse mutant:** Posterior expansion of *Hoxb1* expression ***Zebrafish mutants:*** *Defects in caudal hindbrain specification* *Posterior expansion of fgf3*	ND *Expansion/duplication of anterior domain* *Dorsal shift of sensory cell groups* *Presence of hair cells at ectopic positions*
 *MafB/kreisler* r5, r6	***kreisler* mouse mutant:** Loss of r5 and defects in r6 specification Posterior expansion of r4 Loss of *Fgf3* expression in caudal hindbrain ***Zebrafish val mutants:*** *Misspecification of r5 and r6* *Posterior expansion of fgf3 expression*	Absence of dorsomedial, vestibular structures Expansion of ventral, cochlear structures *Expansion of anterior domain* *Presence of hair cells at ectopic locations*
Secreted molecules		
 *zfgf8/ace* r1, r2, r3 *zfgf3* r4	***Loss-of-function of fgf3 and fgf8 in zebrafish:*** *Defects in r5/r6 specification* *Loss of val (r5-r6) and krx20 (r5) expression*	*Smaller ears* *Abnormal semicircular canals* *Smaller neurogenic and sensory domains*
 *mFGF3/10* r5, r6	***Fgf3/Fgf10* compound mice mutants:** No changes in caudal hindbrain patterning	Defects in otic induction
 *Shh* fp+not	**Mouse mutant:** Loss of ventral cell fates in the neural tube ***Loss-of-function of the Hh pathway in zebrafish:*** *Defects in ventral cell fates in the neural tube*	Loss of neuronal progenitors and SAG ***Loss-of-function of the Hh pathway:*** *Loss of anterior structures* ***Gain-of-function of the Hh pathway:*** *Mirror image duplications of anterior structures*
 *Wnt* rp+dnr	**Compound *Wnt1/Wnt3a* mutants:** Reduction of dorsal neural tube and neural crest derivatives	Loss of dorsal, vestibular structures Defects in the cochlea

Fig. 5. Neural tube mutations affecting otic development. *The first column shows gene expression profiles in the neural tube of mice and zebrafish embryos. The second column indicates the hindbrain phenotype observed in the corresponding mutants. The third column summarizes the otic phenotypes described for those mutations. Genes are organized depending if they code for transcription factors or secreted molecules. Results obtained in zebrafish are indicated in italics. All the summarized data correspond to references quoted all along the review except for Sirbu* et al., *2005. In schemes in the first column, anterior is to the left.*

patterning defects, with an expansion of ventral markers at the expense of dorsal markers and a dorsal shift of intermediate markers such as *atoh1a,* which marks the future maculae. Most probably as a consequence of these patterning defects, hair cells form at ectopic positions in the center of the vesicle (Lecaudey *et al.,* 2007). Following this study, we reinvestigated DV patterning in *val* mutants and found defects similar to those found in *vhnf1* mutants (Lecaudey *et al.,* 2007; S. Schneider-Maunoury and C. Pujades, unpublished results).

In conclusion, analyses of mutants for different hindbrain segmentation genes in both zebrafish and mice point to a role of hindbrain signaling downstream of these genes. However, there is a striking difference between the two species. The main defects seen in mice are along the DV axis, while in zebrafish, defects have been found primarily along the AP axis. However, this difference may be only apparent, since a closer examination of the mutants has allowed to detect DV patterning defects in both *vhnf1* and *val* zebrafish mutants. Further insight into the role of hindbrain in otic patterning is brought by the functional analysis of signaling pathways potentially involved.

The hindbrain as a source of instructing molecules for otic regionalization

Three main signaling pathways, the Hedgehog (Hh), Wnt and FGF pathways, have been involved in otic patterning from the adjacent hindbrain. *Hh* genes are expressed in the floor plate and underlying notochord. *Wnt* genes, in particular *Wnt1* and *Wnt3a*, are present in the dorsal neural tube in all vertebrates. As mentioned before, several *Fgf* genes are expressed in the hindbrain, with species-specific patterns.

The function of FGFs in otic development has been extensively

studied. Both in amniotes and in fish, the loss of function of *Fgf* genes leads to smaller and malformed otic vesicles, demonstrating a role for this signaling pathway in otic induction (Represa *et al.,* 1991; Vendrell *et al.,* 2000; Adamska *et al.,* 2000; Phillips *et al.,* 2001; Leger and Brand, 2002; Maroon *et al.,* 2002; Alvarez *et al.,* 2003; Wright and Mansour, 2003; Liu *et al.,* 2003; Hans *et al.,* 2004; Phillips *et al.,* 2004; Millimaki *et al.,* 2007). This function is attributed mainly to FGF signals from the hindbrain in zebrafish, while in amniotes, other surrounding tissues such as the mesenchyme and endoderm are also a source of FGFs (Ladher *et al.,* 2006). FGF target genes are expressed in the otic epithelium, suggesting a direct effect of this signaling pathway (Chambers *et al.,* 2000; Raible and Brand, 2001; F. Aragon and C. Pujades, unpublished results). The redundancy between different FGFs and their role in otic induction have hampered the analysis of their role in otocyst regionalization. Thus, the best indication so far of FGF function in otic patterning comes from the analysis of mutants of hindbrain segmentation genes such as *MafB*, *Hoxa1* and *vHnf1*. As mentioned above, in these mutants, there is a strong correlation between loss-of-function or gain-of-function of FGF in the hindbrain and otic patterning defects. Moreover, in *val* mutants, part of the defects appears to be rescued by depletion of *fgf3* function.

Shh signaling from the notochord and floor plate is essential for ear patterning in mice (Riccomagno *et al.,* 2002). The study of *Shh* mutants shows that this signaling pathway is required for the formation of the cochlea, a ventral otocyst-derived structure. In *Shh* mutant embryos, ventral *Otx1/2* expression is reduced and dorsal *Dlx5* expression is expanded ventrally. Medial *Pax2* expression is also lost. While sensory specification is not affected, proneural gene expression is strongly reduced and the SAG is absent. The reverse phenotype is seen after missexpression of *Shh* in the otocyst using transgenic mice: dorsal, vestibular structures are lost and ventral, auditory cell fates are expanded. Accordingly, *Pax2* is expanded laterally and dorsal *Dlx5* and *Hmx3* expression is lost. Interestingly, neurogenesis appears increased and the SAG is larger. These results led the authors to propose that Shh instructs ventral fates, but differently along the AP axis: anteriorly it activates *Ngn1* and *NeuroD* promoting neurogenesis, while posteriorly it activates *Pax2* and *Otx1/2* and promotes cochlear fate (Riccomagno *et al.,* 2002). The Hh signaling targets *Gli1* and *Ptc1* are expressed broadly in the otic epithelium suggesting that Hh signaling may act directly. A recent paper suggests that different levels of Shh activity mediate the formation of inner ear structures, with Gli3 repressor required dorsally for vestibular formation and Gli activators functioning ventrally to form the cochlear duct (Bok *et al.,* 2007).

Surprisingly, manipulating the Hh signaling pathway in zebrafish results, not in DV or ML, but in AP patterning defects (Hammond *et al.,* 2003). Two strong Hh pathway mutants exhibit a striking partial mirror image duplication of some anterior otic markers and of anterior sensory structures such as the utricular macula, concomitant with a loss of posterior otic domains. The SAG, however, is not duplicated. Hh signaling from both floor plate and notochord needs to be abolished to obtain this phenotype. The reverse phenotype, namely expansion of posterior structures at the expense of anterior ones, is obtained when the Hh pathway is constitutively activated by overexpression of Shh or by injection of a dominant negative form of PKA. Based on the expression patterns of Hh target genes in these experimental contexts, a direct effect of Hh signaling on posterior otic cells is proposed (Hammond *et al.,* 2003).

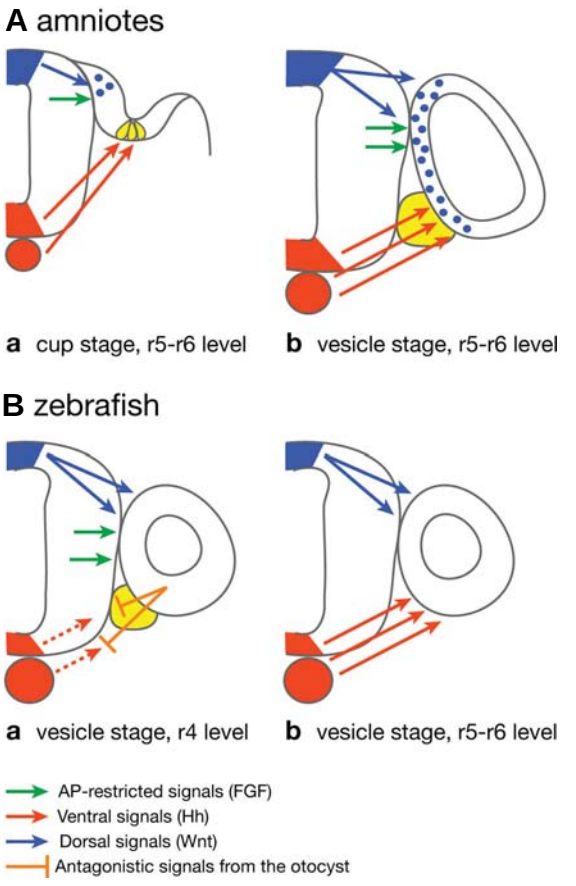


Fig. 6. Hindbrain signals involved in inner ear patterning in zebrafish and amniotes: current models. (A) *In amniotes, the otic cup is positioned close to the dorsal half of the neural tube. At early stages of otic development, only the dorsomedial half of the cup makes intimate contact with the hindbrain (Aa). As invagination proceeds, medial otic cells that at otic cup stage were in contact with the dorsal part of the hindbrain and were Wnt-responsive cells (blue dots in Aa), are now located in more ventral position (blue dots in Ab) and receive signals from the ventral aspect of the neural tube. Hh signals from the notochord and floorplate (red arrows) are received in the ventral part of the otic cup, directly, or indirectly through a relay mechanism involving the mesenchyme and are essential for cochlear specification and SAG formation. Putative FGF signals (green arrows) downstream of r5-r6 segmentation genes are received in dorsomedial otic tissue and are essential for vestibular fate.* **(B)** *In zebrafish, the otic vesicle receives dorsalising signals from the neural tube (presumably Wnt signals, blue arrows). Hh signals from the notochord and floorplate are received in the posteroventral otic vesicle (facing r5-r6) (Bb), where they specify posterior fate. In the anterior otocyst (facing r4) (Ba) FGF signaling is active and specifies anterior fate. Possibly as a consequence, Shh signal is antagonized (orange).*

The role of canonical Wnt signaling from the dorsal neural tube has been studied in mouse (Riccomagno *et al.,* 2005). Surprisingly, while Wnt-responsive cells are distributed along the dorsomedial otic cup and later confined to the dorsal aspect of the

otic vesicle, both vestibular and cochlear structures are reduced in double *Wnt1/Wnt3a* mutants (Riccomagno *et al.,* 2005). To explain these conflicting observations, the authors performed lineage studies using an inducible genetic marker of Wnt-responsive cells. They show that progenitors of the cochlea received Wnt signaling, demonstrating that these ventral cells originate from the dorsomedial part of the otic cup. This study underlines the contribution of cell migration and morphogenetic movements to otic patterning processes: otic cell groups originally located close to the dorsal neural tube will end up being ventral after otic invagination. Gain-of-function studies confirmed the role of canonical Wnt pathway in vestibular formation and showed a mutual repression between Wnt and Shh pathways in ear DV patterning. However, Wnt signals cannot be the only cues involved since ventral otic determinants are appropriately expressed in double mutants for *Wnt1* and *Wnt3a* (Riccomagno *et al.,* 2005). Other dorsal secreted cues, such as BMPs, could play a role in this process.

Otic patterning in amniotes and zebrafish: similarity or specificity?

Current data provide contrasting evidence regarding the role of hindbrain signals in otic axis specification in different vertebrates. Is this due to incomplete experimental data or to true differences in the mechanisms of ear patterning? There are arguments in favour of the second hypothesis: for instance, the main structure specified by Shh signaling in the mouse, the cochlea, does not have any counterpart in zebrafish. It could be argued, however, that the structures specified by Hh signaling in zebrafish and amniotes are functionally similar: the posterior (saccular) macula in zebrafish and the ventral cochlea in amniotes are both involved in hearing. Similarly, dorsal structures in amniotes and the anterior (utricular) macula in zebrafish have vestibular function. Therefore, similar patterning mechanisms could be used in different vertebrate species to specify similar functional parts of the inner ear.

Figure 6 summarizes the signals proposed to pattern the otocyst in fish and amniotes and the different structures specified by these signals. In amniotes, the available data show that the hindbrain is involved in DV patterning of the ear. In chicken, ablation of both the floor plate and notochord result in loss of ventral structures, while ablation of the neural tube results in loss of vestibular structures (Bok *et al.,* 2005). On the other hand, rotation of the r4-r7 region of the hindbrain along the AP axis at a stage when the AP orientation of the otocyst was still plastic did not affect the establishment of the AP axis of the ear. This strongly suggests that, in this species, the rhombomeric identity is not an essential cue for ear AP patterning. However, it is interesting to note that this rotation does not change the position of the hindbrain FGF3 source relative to the otic vesicle. Together, these results suggest that hindbrain (and notochord) signals are mainly involved in DV patterning of the inner ear, consistent with functional analyses in mice. They also support the hypothesis that the spatial distribution of the signaling molecules along the AP axis of the hindbrain is not essential for proper patterning. This means that, if FGF signaling from r5-r6 has a role in ear patterning, its global level rather than its distribution may be important. Accordingly, the level of *Fgf3* gene expression in the caudal hindbrain is under the control of segmentation genes such as *MafB* and *Hoxa1* (Figure 4). It cannot be totally excluded, however, that hindbrain signals also affect the early subdivision of neurogenic (anterior) versus non-neurogenic (posterior) domains. Indeed, an ongoing study (C. Vázquez-Echeverría and C. Pujades, in preparation) shows that *kreisler* mutants display an expansion of the otic neurogenic region as a result of changes in the early expression of otic patterning genes such as *Lfng* and *Lmx1*. Thus, in addition to the published DV and ML patterning defects, *kreisler* mutants display an early AP patterning defect, affecting mainly the neurogenic/non-neurogenic fate decision or other aspects of AP patterning of the ear, which may not have been carefully analyzed so far.

The situation is quite different in zebrafish: the study of the *vhnf1* and *val* mutants has clearly shown an involvement of hindbrain cues in AP patterning. FGF has been proposed as the main signal involved in otic AP patterning downstream of these hindbrain segmentation genes, but Hh signals also play a role in this process. An intriguing question is how a ventral signal such as Hh, which shows an even distribution along the AP axis, can specify AP patterning of the otic vesicle. Several hypotheses can be made. First, the posterior part of the ear may receive more signal than its anterior part. This could be achieved by tilting the early axes of the otic vesicles relative to that of the neural tube. Second, the Shh signal may interact with AP restricted cues. FGFs signals from the hindbrain are good candidates to locally restrict the differential response to Hh signaling of the ventral otic cells. The control of FGF and Hh production is independent: the production of the FGF signal is not affected in *Hh* mutants (Hammond *et al.,* 2003) and *hh* expression in hindbrain segmentation mutants is not affected (our unpublished data). Therefore, the interaction may operate at the level of receiving cells in the ear (Riccomagno *et al.,* 2002). More recently, DV patterning has also been shown to be affected in *vhnf1* and *val* mutants. However, the signaling pathways involved in this DV patterning process have not been identified so far. *Wnt1* and *Wnt3a* expression is reduced in r5 and r6 in *vhnf1* mutants, suggesting that Wnt signals may be involved in some aspects of zebrafish ear DV patterning (Lecaudey *et al.,* 2007).

Future directions

In this review we have discussed the current view on the role of hindbrain signals in axial specification of the inner ear. Essential information has been brought by the analysis of mutants of hindbrain segmentation genes, as well as by the functional analysis of some signaling pathways. As we mentioned, the available information is still fragmentary. In particular, more complete fate maps and lineage studies must be performed in different vertebrate species and at different stages of development. It would also be helpful to know with better precision when the different structures, sensory and non-sensory, of the inner ear are specified in each species.

Hindbrain signals may have additional functions in otic patterning. For instance, it is intriguing that both in zebrafish and amniotes, sensory markers appear at anterior and posterior extremities of the otic vesicle. In zebrafish *val* and *vhnf1* mutants, where r5 is absent, tether cells of the maculae are specified at ectopic positions along the otic AP axis, correlating with the extent

of *krox20* reduction (Lecaudey *et al.,* 2007). These data suggest that a signal from r5 restricts sensory cell specification. To test this hypothesis, it would be useful to analyze the inner ear of mice and fish with a loss of function of *krox20*, a gene involved in the formation of r3 and r5.

To gain a better understanding of the issue, the involvement of different signaling pathways has to be studied in greater detail. In that respect, the potential function of BMP signals is an interesting question to address. Indeed, BMP pathway genes are expressed in the dorsal neural tube and surrounding dorsal ectoderm and BMP antagonists display a rhombomere-specific pattern (Seitanidou *et al.,* 1997).

Finally, signals from surrounding tissues may be involved in other aspects of ear patterning. As an example, none of the hindbrain mutants examined so far display changes in AP position of the SAG, suggesting that it is under the tight control of hindbrain-independent signals. Another signaling pathway involved in ear patterning is the retinoid pathway. Both in mice and humans, variations in retinoid levels have profound effects on ear formation. Furthermore, the defects seen in ear formation in mouse *Hoxa1* mutants are rescued by treatment with non teratogenic doses of RA (Pasqualetti *et al.,* 2001). At placode and early otocyst stages, RA is synthesized in the somitic mesoderm and may act, not only by controlling segmental gene expression in the hindbrain and, in consequence, FGF3 signaling, but also directly on the otocyst (for review Romand, 2003; Romand *et al.,* 2006). The identification of other signals and their integration with hindbrain-derived signaling pathways will lead to a more complete picture of the mechanisms of inner ear patterning.

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