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REVIEW

The gene regulatory networks underlying formation of the auditory hindbrain

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Abstract Development and evolution of auditory hindbrain nuclei are two major unsolved issues in hearing research. Recent characterization of transgenic mice identified the rhombomeric origins of mammalian auditory nuclei and unraveled genes involved in their formation. Here, we provide an overview on these data by assembling them into rhombomere-specific gene regulatory networks (GRNs), as they underlie developmental and evolutionary processes. To explore evolutionary mechanisms, we compare the GRNs operating in the mammalian auditory hindbrain with data available from the inner ear and other vertebrate groups. Finally, we propose that the availability of genomic sequences from all major vertebrate taxa and novel genetic techniques for non-model organisms provide an unprecedented opportunity to investigate development and evolution of the auditory hindbrain by comparative molecular approaches. The dissection of the molecular mechanisms leading to auditory structures will also provide an important framework for auditory processing disorders, a clinical problem difficult to tackle so far. These data will, therefore, foster basic and clinical hearing research alike.

Keywords Auditory hindbrain · Bird · Development · Evolution · Mammalia · Novelty · Rhombomere · Transcription factor · Signaling · Auditory processing disorders

Abbreviations

A–P	Anterior–posterior
AVCN	Anterior ventral cochlear nucleus
BMP	Bone morphogenetic protein
CNC	Cochlear nucleus complex
DCN	Dorsal cochlear nucleus
E	Embryonic
LSO	Lateral superior olive
MNTB	Medial nucleus of the trapezoid body
NA	Nucleus angularis
NL	Nucleus laminaris
NLL	Nucleus of the lateral lemniscus
NM	Nucleus magnocellularis
P	Postnatal
PVCN	Posterior ventral cochlear nucleus
r	Rhombomere
RA	Retinoic acid
SOC	Superior olivary complex
TF	Transcription factor

Introduction

In mammals, a large number of brainstem nuclei are dedicated to the processing of auditory information. These auditory structures encompass the cochlear nucleus complex (CNC), the superior olivary complex (SOC), the nuclei of the lateral lemniscus (dorsal, intermediate and ventral NLL), and the inferior colliculus (IC) [23, 187,

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213]. Their high number likely reflects the fact that the cochlea lacks explicit representation of auditory space. This feature of the environment, hence, needs reconstruction in the central auditory system. Indeed, many of the auditory hindbrain circuits are involved in sound localization [24, 76, 227]. In addition, auditory hindbrain circuits participate in tasks such as determination of sound duration [26, 93], echo suppression [159], and alignment of acoustic and visual maps [19, 77]. Proper function of the auditory hindbrain, therefore, is pivotal for accurate auditory perception.

Several unique features characterize auditory brainstem circuits. These features include the ability to fire up to several hundred Hz [198, 202, 222], high fidelity neurotransmission [17, 53], and coincidence detection in the submillisecond range [72, 76, 114]. Furthermore, the auditory hindbrain harbors the calyces and the endbulbs of Held, which represent the largest synapses of the central nervous system [17, 69, 76, 117]. How these features develop and evolve is largely unknown.

Biological structures and the functions their morphology endows them are products of gene regulatory networks (GRNs) [39]. GRNs are composed of *cis*-acting DNA control elements such as enhancers and silencers on one hand, and regulatory genes encoding *trans*-acting transcription factors (TFs) and signaling molecules on the other hand [21, 40, 83, 85, 98]. *In toto*, these elements represent the mechanistic basis to generate specific morpho-functional structures, as they directly determine the spatiotemporal expression pattern of genes. In general, GRNs display complex architectures. Each TF affects multiple target genes and the control elements of each regulatory gene respond to multiple inputs, thereby fine tuning expression levels.

Here, we summarize our current knowledge about the GRNs operating in the auditory hindbrain. The major focus is laid on the mammalian auditory hindbrain. It is very well studied and highly relevant to human hearing abnormalities. Mutational changes in auditory GRNs likely contribute to auditory processing disorders, which affect 2–3 % of newborns and 10–20 % of the adult population [28, 151]. Despite their clinical relevance, the lack of criteria to group these disorders into distinct clinical entities precluded genetic insight into disease-causing mechanisms for a long time. The advent of next generation sequencing techniques with their diagnostic application to individual patients will likely overcome this impasse [96, 226]. Nevertheless, a bottleneck of this approach is the identification of the disease-causing variations among the multiple genetic alterations observed in the sequence data. Therefore, assembling the genetic logic operating in the auditory hindbrain will serve as an important framework for research on auditory processing disorders. The information gained by GRNs will be useful to nail down those genetic variations associated with the disorder. In support of this conjecture is the observation that mutations in TFs, which are involved in the patterning and formation of the hindbrain [102, 206], are associated with dysfunctions in the central auditory system [219]. These TFs include Hoxa1 [29], Hoxa2 [47, 66], Hoxb2 [47, 195] and Atoh1 [122].

We start with a short outline of the rhombomeric origin of the mammalian auditory hindbrain and the role, retinoic acid plays therein. Subsequently, the current knowledge on the GRNs, involved in the formation of the different rhombomeres, is summarized. Development represents a progressive succession of new regulatory states, each arising in a particular spatial domain of the embryo. We, therefore, assembled the data according to different time points of development and different rhombomeres. In the second part, we compare the mammalian GRNs with those operating in the peripheral auditory system or in other vertebrate groups. These comparisons provide important insight into the evolutionary processes leading to the formation of the auditory hindbrain during vertebrate radiation.

Development of the mammalian auditory hindbrain

Rhombomeric origin of the auditory hindbrain

The very early mammalian embryonic brain consists of three primary vesicles. The anterior one is the prosencephalon (forebrain), followed by the mesencephalon (midbrain), and the posterior rhombencephalon (hindbrain). The latter gives rise to the cerebellum, the pons (metencephalon) and the medulla oblongata (myelencephalon). The rhombencephalon is separated during early development into structures termed rhombomeres (r) [15, 133]. In total, twelve rhombomere-derived domains exist: the isthmus (r0) [208] and rhombomeres (r) 1 to r11 [5, 125]. The rhombomeres between boundaries r1/2 to r6/r7 are overtly segmented, of transient nature, and display polyclonal cell lineage restriction [57]. The overt inter-rhombomeric boundaries of r2 to r6 correlate well with the anterior boundaries of the *Hox* genes from groups 1–3 [4]. The patterning of the domain anterior of the r1/r2 boundary is not dependent on inter-segmental limits, but relies on the signaling of the isthmic organizer [128, 224]. The hindbrain posterior to the r6/r7 boundary, which gives rise to the medulla oblongata, lacks visible inter-rhombomeric boundaries. It displays a molecular regionalization based mainly on differential expression of *Hox* genes from groups 3–7 [22, 110, 125]. Since the inter-neuromeric limits of the medulla oblongata are not morphologically distinguishable, r7 to r11, together with the isthmic domain, are also called

Table 1 Origin and birth time of auditory brainstem nuclei

Mouse			Chick		
Nucleus	Rhombomere	Birth date	Nucleus	Rhombomere	Birth date
AVCN	r2 + r3	E11–E14	NM	r5 − r8	E2.5–E3
PVCN	r4	E11–E14	NA	r3 − r6	nd
DCN	r5	E9–E17	NL	r5 + r6	E3.5–E4
LSO	r5	E9–E14	SON	r5	nd
MSO	r5	E9–E12	dNLL	r1	nd
MNTB	r3 + r5	E11–E12	iNLL	r2	nd
LNTB	r5	E9–E12	vNLL	r3	nd
VNTB	r5	E10–E12			
OCN	r4	nd			
dNLL	isthmus	nd			
iNLL	r1	nd			
vNLL	r4	nd			

AVCN anterior ventral cochlear nucleus, *DCN* dorsal cochlear nucleus, *dNLL* dorsal nucleus of the lateral lemniscus *iNLL* intermediate nucleus of the lateral lemniscus, *LNTB* lateral nucleus of the trapezoid body, *LSO* lateral superior olive, *MNTB* medial nucleus of the trapezoid body, *MSO* medial superior olive, *PVCN* posterior ventral cochlear nucleus, *vNLL* ventral nucleus of the lateral lemniscus, *VNTB* ventral nucleus of the trapezoid body, *nd* not determined

crypto-rhombomeres referring to their "hidden" (cryptic) nature [5].

Fate map analysis revealed that most mammalian auditory hindbrain nuclei originate from specific compositions of rhombomeres between the r1/2 to r5/6 boundaries (Table 1). The lower lip of rhombomeres r2 to r5 gives rise to the three subdivisions of the cochlear nucleus complex (CNC) [55]. The anteroventral nucleus (AVCN) is largely generated from r2 and r3, the posteroventral cochlear nucleus (PVCN) from r4, and the dorsal cochlear nucleus (DVCN) mainly from r5 [55] (Table 1). The majority of the superior olivary complex (SOC) is generated from r5, with r3 contributing to the medial nucleus of the trapezoid body (MNTB) [55, 122, 127, 170]. The olivocochlear neurons, which serve as an efferent feedback system and originate within the SOC, are derived from r4 [20, 47]. Recent data indicate that the dorsal NLL derives from the isthmus [138], the intermediate NLL from r1 [138], and the ventral NLL from r4 [47]. This is in agreement with the observation that a r3- and r5-specific Cre driver line does not label the NLL [170]. Finally, a fate mapping analysis revealed that the mesencephalic domain gives rise to the inferior colliculus [229]. Based on these findings, we limit our review to the GRNs operating in r2 to r5 and their derived auditory structures, i.e., the CNC, the SOC, and the NLL.

Retinoic acid

Retinoic acid (RA), generated from vitamin A (retinol), is necessary for the formation of rhombomeres [65, 116]. RA binds to nuclear receptors [126] and controls expression of various genes including those encoding TFs of the *Hox* gene family and vHnf1 [70]. In the mouse, RA synthesis starts at embryonic day (E) 7.5 in the trunk paraxial mesoderm, which flanks the future r7, r8, and the spinal cord [143, 136]. E7.5 is shortly before the development of the hindbrain sets in [171]. RA synthesis is controlled by retinaldehyde dehydrogenase 2, which is encoded by the gene *Aldh1a2*. RA degradation is in part dependent on *Cyp26* genes expressed in the anterior neural plate (Fig. 1) [51, 176, 215]. RA acts either directly by RA receptors or via retinoid X receptors, which bind to RA response elements. These *cis*-regulatory elements are necessary for the hindbrain-specific expression of TFs such as Hoxa1, Hoxb1, Hoxb4 and Hoxd4 [56, 73, 144, 189].

The boundaries of RA activity shift during development. The most anterior extension is observed at E7.6–E7.8, reaching the presumptive r2/r3 boundary. At E8.0, RA has regressed to the r4/r5 boundary, caused by the RA-initiated expression of *Cyp26c1* in r4 [185]. This boundary is then maintained up to E8.5 [185]. Thus, RA activity extends temporarily rostrally up to the supposed r3 of the presumptive rhombencephalon and transcriptionally activates *Hoxa1* and *Hoxb1* (Fig. 1) [67, 87, 140, 189].

Rhombomere 2

The identities of the rhombomeres along the A–P axis are generated by specific combinations of *Hox* gene expression patterns in conjunction with other TFs [4]. *Hoxa2* is the only *Hox* gene expressed in r2 [66, 102], starting at E8.5 [145]. Its expression is likely activated by TFs of the SOX family (Fig. 2) [204]. Hoxa2 is essential for identity specification of r2 [66, 150, 168] and participates in transcriptional activation of *EphA7* between E8.5 and E9.0 (Fig. 2) [197], *Phox2b* at E9.25, and *Pax6* at E10.5 (Fig. 2) [38]. Due to its important role in r2, Hoxa2 is required for proper formation of the AVCN [47].

Rhombomeres 3, 5, and 6

r3 and r5 as well as r5 and r6 share key factors such as Krox20 or MafB, respectively (Fig. 3). Krox20 is crucial for r3 and r5 formation, as they vanish or increase due to loss or misexpression of this zinc-finger type TF [180]. *Krox20* is expressed first in r3 at E8.0 [217], probably

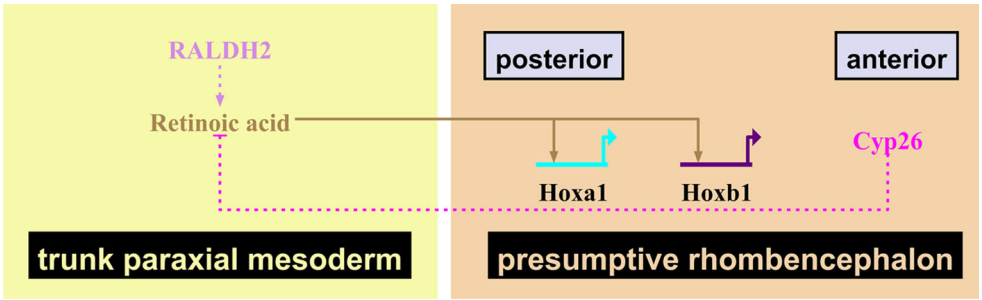

Fig. 1 Retinoic acid (RA) signaling prior rhombomeric boundary formation from E7.5 till E8.0. *Dotted line with* arrow indicates the synthesis of RA by RALDH2, whereas the *dotted line with vertical bar* illustrates the degradation of RA by Cyp26 proteins. *Solid lines with arrows* describe positive regulations. Anterior is to the *right*

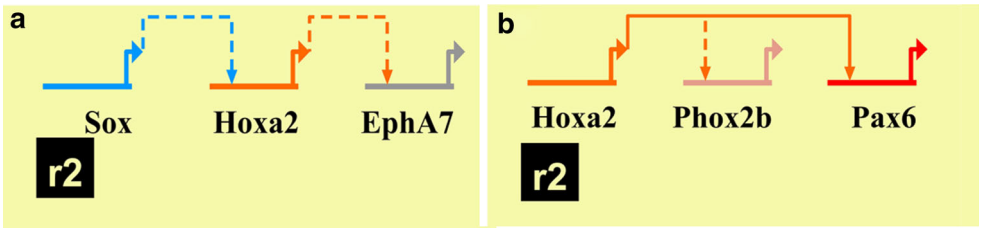

Fig. 2 Gene regulatory networks in r2. Expression pattern in r2 from E8.5 till E9.0 (**a**) and from E9.25 onwards (**b**). *Arrows* describe positive regulations, whereas *vertical bars* are standing for negative regulations. *Dashed lines* indicate indirect or not yet confirmed direct regulations. These genetic logics apply to all subsequent figures

activated by Fgf signaling (Fig. 3a) [123, 210], whereas it is repressed in r5 by Hoxa1 and Hoxb1 till E8.5 (Fig. 3c) [11, 134, 172]. At E8.5, expression of *Hoxa1* and *Hoxb1* has diminished in r5, and *Krox20* expression starts in this rhombomere. This transcriptional activation likely requires MafB and Fgf3 signaling (Fig. 3d) [8, 120, 214]. Krox20, in turn, activates *EphA4* (Fig. 3b, d) [200], *Hoxa2* (Fig. 3b, d) [145, 197], and *Hoxb2 (*Fig. 3b, d) [182]. Furthermore, Krox20 represses *follistatin* (Fig. 3b), an inhibitor of bone morphogenetic protein (BMP) signaling [11, 181] and *Hoxb1* (Fig. 3b) [97]. Thus, Krox20 is a key TF involved in the formation of hindbrain segments, specification of their A–P identity, and prevention of cell mixing by expression of the cell–cell signaling molecules *EphA7* and *EphA4* in odd-numbered rhombomeres.

Expression of *Krox20* is regulated by a positive autoregulatory loop [18] and by a negative cross-regulatory loop, involving *Nab1* and *Nab2* (Fig. 3b, d) [46, 63]. These two genes are positively regulated by Krox20 and act in turn as repressors of *Krox20* expression [174, 194]. *Nab1* and *Nab2* expression is, furthermore, stimulated by the paired-box TF Pax6 (Fig. 3b, d) [97]. Pax6 expression begins in r3 and r5 at E8.5 [97]. At E9.0–E9.5, the TF is present in all rhombomeres with highest expression in r3 and r5. Hoxa2 is necessary for an upkeep of *Pax6* expression in r2 and r3 (Figs. 2b, 3b). In r3, Hoxb2 likely participates in maintaining *Pax6* expression (Fig. 3b) [38]. Krox20 has a negative effect on *Pax6* expression and both TFs are, therefore, linked by a bi-directional negative regulatory crosstalk (Fig. 3b, d) [97]. Pax6 dysfunction results in the elimination of the strict borders of *Hoxa2* expression as well as in the loss of clearly defined r3 and r5 segments [97].

Central to the GRNs operating in r5 and r6 is the basic leucine zipper domain-type TF MafB. Its disturbed expression underlies the *kreisler* mouse phenotype. *Kreisler* mice lack r5 [36, 44] and display altered r6 regional identity [121, 134, 175]. In addition, r3 and r4 show abnormalities, although both rhombomeres do not express *MafB* during development. Hence, these abnormalities are likely secondary effects [44, 121].

MafB is expressed as a stripe in the caudal hindbrain at E8.0. At E8.5, it exhibits a distinct border at the r4/r5 boundary, whereas its posterior expression ends at a blurred border roughly in the middle of r6 [36]. At E9.0, *MafB* is expressed in r5 and r6 at equal levels, whereas at E9.5, down-regulation commences in r5 and later in r6 [36, 199]. The initiation of *MafB* expression in the hindbrain is dependent on RA [143, 215] and the homeobox TF vHnf1 (Fig. 3d, f) [100]. Prior to E8.5, Hoxa1 is additionally required for *MafB* expression in r5 (Fig. 3d) [11, 172]. Pax6 has an inhibiting effect on *MafB* expression in r5 through its positive regulation of *Nab1* and *Nab2*, which act as transcriptional repressors of *MafB* (Fig. 3d) [97].

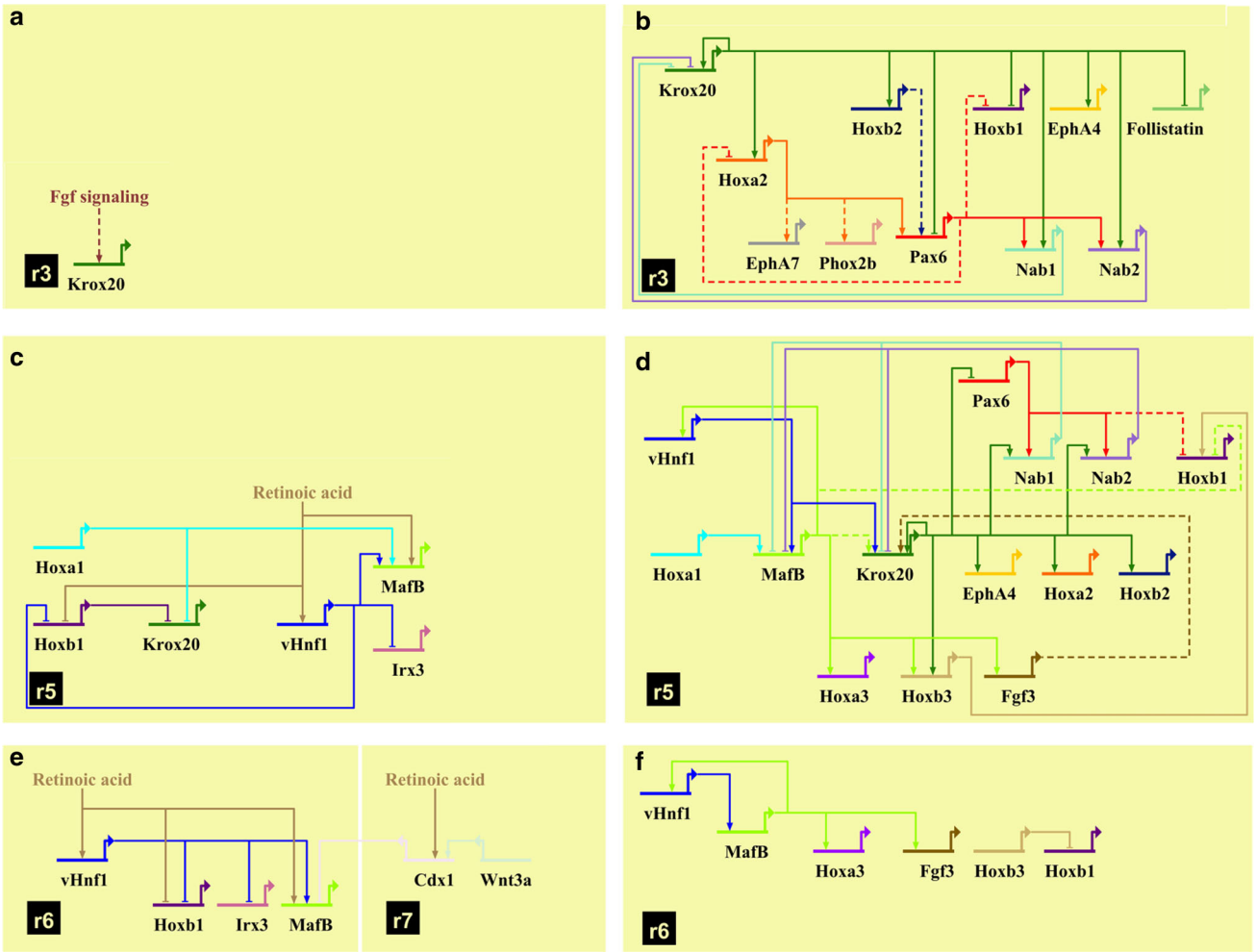

Fig. 3 Gene regulatory networks in r3, r5, and r6. **a–c** Expression patterns in r3 (**a**, **b**), r5 (**c**, **d**), and r6 (**e**, **f**) at E8.0–E8.5 (**a**, **c**, **e**) or from E8.5 onwards (**b**, **d**, **f**)

Posterior to the r6/r7 boundary, early *MafB* expression is directly inhibited by Cdx1 till around E8.5 (Fig. 3e) [191]. *Cdx1* expression is initiated by RA [84] and later maintained by Wnt3a (Fig. 3c) [163].

MafB directly regulates *Hoxa3* in r5 and r6 (Fig. 3d, f) and, in concert with Krox20, *Hoxb3* in r5 (Fig. 3d) [119, 120]. Thus, *Hoxb3* expression is dependent on MafB and Krox20, whereas *Hoxa3* is independent of Krox20 [120]. In addition, MafB activates the expression of *Fgf3* in r5 and r6 between E9.0 and E9.5 (Fig. 3d, f) [133, 199] and presumably partakes in restricting the auto-regulated *Hoxb1* expression to r4 (Fig. 3d) [134]. Double knockout of *Hoxa3* and *Hoxb3* causes loss of all somatic motoneurons in r5, whereas *Hoxb1* expression was de-repressed in r6, leading to a r4-like differentiation and migration of facial branchiomotor neurons [64]. Subsequent analysis revealed that Hoxb3 directly initiates suppression of *Hoxb1* in r5 and in the posterior hindbrain from E8.5 till around E10.5 (Fig. 3d,f) [220]. Thus, *Hox3* genes are required in the posterior part of the hindbrain for rhombomere-specific neurogenesis by suppression of the *Hoxb1* gene.

An important target of MafB is the gene *vHnf1*. The encoded TF plays a crucial role in hindbrain development. *vHnf1* expression starts at E7.8 in the caudal hindbrain and exhibits at E8.0 its most anterior expression up to the r4/r5 boundary [35, 185]. This expression pattern is strictly dependent on RA till around E8.25–E8.5 (Fig. 3c, e) [161, 185]. Later on, MafB is required for transcriptional up-regulation of the TF in r5 and r6 (Fig. 3d, f) [161]. The neural expression of *vHnf1* is conserved among vertebrates [9, 106, 185]. The protein is involved in setting the r4/r5 border by repressing a r4 fate posterior to this boundary [30, 80]. Furthermore, it plays a vital role in the identity determination of r5 and r6 by activating *Krox20* in r5 (Fig. 3d) [30, 80], and *MafB* in r5 and r6 (Fig. 3d, f). Finally, vHnf1 represses *Hoxb1* and possibly acts as a repressor of *Irx3* in the posterior hindbrain between E8.25 and E8.5 (Fig. 3c, e) [185]. *Irx3*, which is expressed in the

anterior hindbrain up to the r4/r5 border, represses in turn the expression of *vHnf1*, thus further sharpening the r4/r5 boundary (Fig. 4a) [185].

Rhombomere 4

Hoxa1 and *Hoxb1* are the first *Hox* genes expressed in the hindbrain at E7.75, just before rhombomeric boundary formation (Fig. 1) [11, 140]. Their expression is dependent on RA (Fig. 1) and is required for correct initiation of the identity specification program of r4 [71, 172, 189, 190]. Absence of *Hoxa1* or *Hoxb1* causes transformation of r4 to a r2 identity [172, 189, 190], whereas their ectopic expression leads to a transformation of r2 to r4 [230]. The establishment of *Hoxb1* expression in r4 requires both Hoxa1 and Hoxb1 function prior E8.0 and involves a positive auto-regulatory loop for *Hoxb1* (Figs. 1, 4a) [11, 189]. During rhombomere boundary formation, *Hoxa1* expression regresses and vanishes completely from the hindbrain by E9.5 [139, 193]. Its role for *Hoxb1* expression is then assumed by *Hoxb2*, which maintains *Hoxb1* expression in a positive cross-regulatory loop (Fig. 4a, b) [38, 67, 158]. The sharp expression borders of *Hoxb1* are, at least in part, dependent on its negative regulation by Pax6 in r3 and r5 (Fig. 3b, d) [97]. Hoxb1 maintains *Hoxa2* expression in this rhombomere, likely supported by a positive auto-regulatory *Hoxa2* loop (Fig. 4a, b) [203]. Hoxb1 is also involved in the positive regulation of *Hoxb2* (Fig. 4a, b) [115].

The three TFs Hoxb1, Hoxa2, and Hoxb2 initiate the expression of r4 target genes. The expression of *EphA2* in r4 is partially controlled by Hoxa1 and Hoxb1 (Fig. 4a) [27]. Furthermore, Hoxb1 and Hoxb2 positively regulate *Phox2b* (Fig. 4b) [177]. Hoxb1 also activates *Gata2* which in turn targets *Gata3* (Fig. 4b) [156]. The zinc-finger type TF Gata3 is involved in the correct routing of r4-specific facial and vestibuloacoustic efferent neurons [95, 190]. In addition, Hoxb1 acts as a transcriptional activator of *CRABPI* and *CRABPII* (Fig. 4b) [10], which both act as regulators of RA activity [49]. Finally, Hoxb1 transcriptionally represses the LIM-homeodomain TFs Lhx5 and Lhx9 (Fig. 4b) [10], which promote neuronal differentiation [43]. Due to their important role in r4, Hoxb1 and Hoxb2 participate in the generation of the PVCN [47].

GRNs in late embryonic and postnatal development of the auditory system

As neurons mature, primary fate regulator genes as well as downstream TFs called secondary selector genes are induced [118]. Later on, their expression is stopped and subtype-specific genes, such as those associated with neurotransmitter phenotypes, become activated by the secondary selector genes. Currently, this part of the GRNs

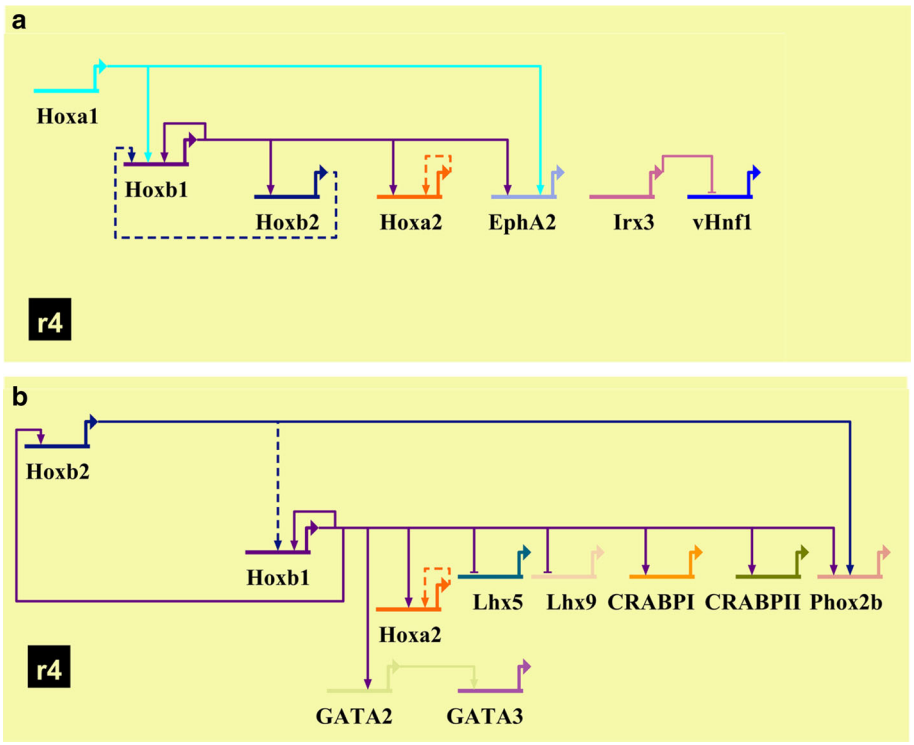

Fig. 4 Gene regulatory networks in r4. Expression pattern in r4 at around E8.0 till E8.75 (**a**) or from E9.0 onwards (**b**)

has been poorly resolved. The *Wnt1*-, *Atoh1*- and *En1*-expressing area of the rhombic lip, the most lateral portion of the developing hindbrain, is assumed to contribute to the CNC and SOC [61, 122, 127, 142]. *Wnt3a*-expressing domains, which are mostly located in the roofplate, likely contribute as well [111]. These expression patterns point to a function of *Wnt1*, *Wnt3a*, *Atoh1* and *En1* during development of the auditory hindbrain. Recent analyses in transgenic mice confirmed the importance of *Atoh1* and *En1*. Early embryonic ablation of *Atoh1* in r3 and r5 results in severe disruption of the CNC, whereas the SOC forms normally [122]. Furthermore, Atoh1 is essential for Lhx9 expression [14] in glutamatergic neurons of the SOC [169]. *En1* is expressed in embryonic cells of the medial, ventral, and lateral nuclei of the trapezoid body of the SOC between E12.5 and E13.5 and this expression persists in all three nuclei till postnatal stages [127]. Ablation of *En1* in r3 and r5 results in the absence of the MNTB and the ventral nucleus of the trapezoid body, whereas the lateral nucleus of the trapezoid body still forms [90].

The precursor cells of the lateral and medial superior olive (LSO and MSO), two major nuclei of the SOC, are Wnt1 and Atoh1 positive [122, 127] and do also express *MafB* from E13.5 onwards [127]. Of note, the VCN, which projects to the LSO and MSO, expresses the same three genes [55, 86, 104, 211]. This implicates that these structures share GRN components. This might be advantageous for coordinated development of pre- and postsynaptic neuronal populations in sound localization circuits. MNTB neurons, furthermore, express *FoxP1* at E14.5 till at least P0, whereas the lateral nucleus of the trapezoid body shows at P0 separated expression areas for *FoxP1* and *MafB* [127]. The authors speculate that MafB induces a glycinergic fate in neurons, whereas FoxP1 is promoting a mixed glycinergic and GABAergic fate [127].

Hox genes, which are required for setting up the hindbrain, also play important roles thereafter. Hoxa2 is essential for the formation of correct contralateral projections from the AVCN by regulating the expression of Rig1, the main axon guidance receptor for crossing the midline [47]. Furthermore, Hoxb1 seems to be required for specification and Hoxb2 for maturation of olivocochlear neurons [47]. GATA3 is another TF, likely important for these neurons. Its constitutive deletion results in rerouting of the efferent fibers as they join the facial branchial motor nerve [95].

MicroRNAs are essential components of the auditory hindbrain GRNs. Dicer1 is an enzyme required for generation of these small RNAs. Its deletion in r3 and r5 causes severe volume reduction of the CNC and complete absence of the SOC [170]. Elimination of Dicer1 in the AVCN at later embryonic stages does not affect the development of this auditory structure. These data suggest that microRNAs are required during early embryonic stages of the auditory hindbrain [170], similar to other brain areas. The precise nature of these microRNAs is unknown.

A recent study demonstrated the importance of BMP signaling for postnatal maturation of the calyx of Held. Microarray-based gene expression studies revealed higher expression of BMP4 and BMP5 in the MNTB compared to the LSO. Lack of BMP signaling in a BMP receptor 1a/1b double knockout mouse line results in impaired function of the calyx of Held [225]. Another microarray analysis compared the expression pattern of the developing SOC with that of the entire brain. This revealed increased expression of genes encoding the TFs Peg3, Gata3, Phox2a, Dbx2, NKx6.2, Hoxa2, Pbx3, Mitf, Olig1, Esrrb, and Sox10 in the SOC before (P4) and after (P25) hearing onset, compared to the entire brain [52]. At P4, the SOC highly expresses genes encoding the TFs Nkx6.1, Onecut1, Meis2, Irx2, Hoxa2, Mab21l2, MafB, Etv4, Zfmp2 and Znf503, and the serotonin signaling components Tph2, Slc6a4, and Gchfr [52, 94]. The precise role of these TFs and signaling molecules in the auditory hindbrain as well as their spatial expression patterns have not yet been investigated.

The L-type channel $Ca_v1.3$, encoded by *Cacna1d*, plays a crucial role in signaling during development of the auditory brainstem. Its deletion causes a reduction in volume and neuron number of auditory hindbrain nuclei [81]. Furthermore, refinement of the inhibitory projection between MNTB and LSO neurons and the shift from a mixed GABAergic/glycinergic release to a purely glycinergic neurotransmission are abrogated [82]. The importance of $Ca_v1.3$-mediated signaling is underlined by abnormal auditory hindbrain responses after conditional ablation of *Cacna1d* in r3 and r5 [178].

GRNs and the evolution of the central auditory system

The evolutionary origin of the central auditory system

The emergence of the tympanic ear around a 100 million years after the water–land transition [32] presumably entailed evolution of auditory structures for high-frequency sound and directional hearing [31]. These auditory structures can either represent true novelties similar to the tympanic ears or reflect transformations from evolutionary older neuronal populations. An attractive hypothesis was based on transformations within the octavolateralis system. This evolutionary old system, already present in Devonian gnathostomes [149, 223], embraces sensory systems with hair cell receptors, that is the lateral line mechanosensory and electrosensory systems, the membranous labyrinth or inner ear, and their terminal fields in the dorsolateral area

of the medulla [16]. Based on the analysis of anuran metamorphosis, a transformation within the octavolateralis system through recruitment of the orphaned lateralis system by the auditory nerve was proposed [105]. However, several findings argue against such a functional replacement theory. Some frogs and bony fishes retain the lateral line system and yet possess auditory nuclei [58, 60, 131]. In addition, analysis in the bullfrog revealed that the mechanoreceptive lateral line nucleus and the auditory nuclei coexist during early developmental stages prior metamorphosis [88]. Finally, the lateral line and the octaval systems display hodological differences in anurans. The auditory nucleus dorsolateralis (the frog equivalent to the amniote CNC) projects to the superior olive/secondary octaval nucleus (SO) but not to the tectum. In contrast, the nucleus medialis of the lateral line system projects to the tectum and not to the SO [58, 218, 231].

Alternatively, transformation of electroreceptive nuclei into auditory nuclei has been proposed. This idea was based on the observation that the emergence of auditory nuclei in anurans parallels the loss of the ampullary organs, which represent the electrosensory end organ [58, 60]. However, similar to the ascending mechanoreceptive lateral line circuits, the electroreceptive lateral line pathway differs in its connections from the auditory system [131]. These difficulties with the theory of transformation resulted in the proposal that auditory nuclei evolved concomitantly with the inner ear and likewise represent novelties [59].

This hypothesis was sharpened by the suggestion of a genetically interlinked evolution of the mammalian peripheral and central auditory system by recruiting the same TFs [60]. This idea was initially put forward by the discovery that TFs such as Atoh1 and NeuroD1 are expressed both in the peripheral and central auditory system and are required for both the inner ear [13, 108] and the auditory hindbrain [108, 122]. The three genes *Atoh1*, *Neurog1* and *Neurod1* are involved in an intricate GRN important for the development of the inner ear [13, 101, 112]. Neurog1 and Neurod1 are required for the specification of neurons in the otic vesicle [12, 101, 112], whereas commitment of hair cells to a sensory fate occurs after neurogenesis and relies on Atoh1 [13, 141, 153]. The expression of *Neurod1* is positively regulated in neurons by Neurog1 [112] and by Atoh1 in hair cells [129, 152]. Neurod1 in turn suppresses *Neurog1* and *Atoh1* [89] to inhibit precursor proliferation and hair cell differentiation in neurons. Furthermore, a mutual inhibition of Neurog1 and Atoh1 may exist [129, 166], with Neurod1 as a possible negative link [89]. This GRN is thought to be key for neuronal and hair cell development in the inner ear. A similar GRN may be deployed in the auditory hindbrain for defining different neuronal fates, as *Neurog1* seems to be expressed in the developing auditory hindbrain [99]. Further evidence for a partially similar GRN between the peripheral and central auditory system is summarized in the following.

Comparison of the GRNs underlying the development of the mammalian peripheral and central auditory system

The dual function of Hox proteins, Atoh1, GATA3, BMPs, and $Ca_v1.3$ in the ear and the auditory hindbrain were reviewed recently [219] and their role in the auditory hindbrain has been detailed above. We will, therefore, only shortly portray their function in the ear. Hoxa2 is required for inner ear formation [3], whereas Atoh1, Hoxb1 and Hoxb2 are important for development, organization and maintenance of cochlear hair cells [47]. BMPs are involved in the development of the cochlea [109, 148, 164], and Gata3 is essential for the differentiation of hair cells as well as spiral ganglion neurons of the organ of corti [7, 50]. Finally, $Ca_v1.3$ participates in neurotransmission at the inner hair cell synapse and in hair cell development [137, 160].

MafB and Wnt3a are two other factors in support of an overlapping genetic program between the peripheral and central auditory system. MafB is required not only for rhombomere formation, but also for the development of auditory ribbon synapses at the inner hair cell—spiral ganglion neuron interface [228]. The Wnt3a—expressing domain is assumed to be a source for the SOC nuclei [111, 113], and likewise plays a role in the patterning of the otic placode [78]. These data reveal shared features between the GRNs underlying development of the mammalian peripheral and central auditory system. The significance of this observation is currently unknown. In the future, it will therefore be important to determine whether these factors were recruited to the GRNs of the peripheral and central auditory system at a similar time during evolution, and whether they represent unique combinations only found in the ear and associated nuclei.

Distinct as well as shared features of central auditory structures in vertebrate groups

Detailed analyses of fossil ear structures revealed that tympanic ears evolved independently in at least five major tetrapod groups—the anurans, turtles, lepidosaurs, archosaurs and mammals [32–34]. Since no common ancestor existed capable of detecting air-borne sound, central auditory pathways dedicated to high-frequency sounds and directional hearing likely evolved independently in the different tetrapods as well. Current knowledge holds that the amniote CNC and the anuaran dorsolateral nucleus arose independently and that fish lack this first-order

nucleus at all [131]. Differences in the bauplan for sound localization circuits and in electrophysiological properties of neurons in equivalent auditory nuclei between mammals and birds ([25, 74] support the view that these auditory nuclei represent homoplasious structures in the different tetrapods.

However, there are also striking similarities in the central auditory system of different tetrapods [23, 24, 75, 114, 146, 201]. These similarities include the presence of first- and second-order auditory nuclei in the hindbrain [23, 75] and the channeling of low- and high-frequency acoustic cues into two different sound localization pathways [31, 76]. Furthermore, the laminar architecture of the coincidence detector [MSO in mammals and nucleus laminaris (NL) in birds] [186, 188] and the presence of giant synapses such as the endbulb of Held [79, 117, 155] at the interface between the auditory nerve and its target neurons are shared characters. On the molecular level, fast AMPA receptors [68, 167] and potassium channels including K_v1 and K_v3 [92, 107, 147, 154] were recruited to auditory neurons to ensure temporal precise, fast, and high-frequency neurotransmission [72]. These observations raise two questions: (1) what is the origin and evolutionary relation of the central auditory system in the various tetrapods, and (2) what genetic mechanisms secure similar molecular and cellular properties across different vertebrate groups. Answers to these questions will require comparisons between GRNs and we will, therefore, review our current knowledge on this topic as well.

Comparison of GRNs for auditory structures across vertebrate groups

Next to mammals, the auditory system and its development are best studied in birds. In birds, the auditory nerve terminates in the nucleus angularis (NA) and the nucleus magnocellularis (NM). These first-order nuclei are followed by the NL, the superior olivary nucleus (SON), and the NLL [23, 75]. The NM and the NL are widely assumed to be functional equivalent to the spherical bushy cells of the AVCN and the MSO, respectively [75]. The correspondence of the NA is less clear and its heterogeneous cell population might fulfill functions of both the DCN and PVCN [75]. Fate mapping revealed that r1 to r8 are involved in the development of the chicken auditory hindbrain (Table 1). Each of them gives rise to one or more of the nuclei [22, 37, 124]. r3 and r4 give rise to the rostral NA [22, 124], while r5 is important for the development of the medial NA, the rostral parts of the NL and NM, and the SON [22, 37, 124]. r6 contributes to the caudal NA and NL, as well as to the medial NM [22, 37, 124]. The caudal NM arises from r7 and r8 [22]. Finally, the avian NLL is derived mainly from r1 to r3 [22]. Thus, the rhombomeric origins of auditory nuclei vary considerably between birds and mammals (Table 1). Only the origin of the secondary avian nucleus SON in r5 corresponds well to the mammalian SOC. Of note, the second-order octaval nucleus of teleost fish likely originates from r5 as well [48].

Due to differences in developmental timing [179], direct comparison of birth dates of auditory neurons between mammals and birds is not possible. However, we might estimate the conservation of timing by analyzing the order of birth. Again, differences become apparent. In chicken, birth of the NM precedes the NL, as NM neurons are born between E2.5 and E3, whereas NL neurons are born between E3.5 and E4 [173]. This order is reversed for the AVCN and MSO, which are assumed to represent the equivalent mammalian structures (Table 1). Thus, both the origin and order of birth differ between the mammalian and the avian auditory brainstem.

The different origin of mammalian and avian central auditory structures likely entails differences in the underlying GRNs as well, making it a central challenge to explain the numerous shared features. Indeed, comparison of the GRNs operating in the different vertebrate rhombencephalon reveals both similarities as well as notable differences. The negative feedback loop between Krox20 and its antagonists Nab1 and Nab2 appears to be conserved from fish to mammals [135]. Likewise, *Pax6* demonstrates a similar segmental and dynamic expression pattern in the developing hindbrain of zebrafish, frog, chicken, and mouse [45, 165, 196]. Furthermore, its activity is required in both mice and chicken for precise expression of *Krox20*, *MafB*, *Hoxa2*, *Hoxb1*, and *EphA4* [97]. *vHnf1* demonstrates a conserved expression pattern among vertebrates as well [9, 106, 185, 192], and an essential vHnf1-binding site in the *MafB* gene is conserved between mouse and chicken [100]. Finally, the segmental organization of *Hox* expression is highly conserved among vertebrates [6, 41, 206], and their anterior expression boundaries are identical in the hindbrain of mouse and chicken [206]. Still, in view of the differing origins of auditory hindbrain structures across vertebrates, these similarities in GRNs at early development are likely not sufficient to explain the emergence of shared features in the adult auditory systems.

There are also notable differences among vertebrates. The Fgf3-related subcircuit (Fig. 5) provides a remarkable example. In zebrafish, *Fgf3* is expressed in r4 [103], whereas in chicken, it is first expressed in r4 and r5 and expands later to r6 [8, 9, 123]. In mammals, *Fgf3* is expressed in r5 and r6 [134]. In addition, the position of *Fgf3* within the network architecture varies. In zebrafish, *Fgf3* expression is activated by Hoxb1a [80] and repressed by MafB in r5 and r6, resulting in its r4 restricted expression pattern (Fig. 5a) [103]. In the chicken, *Fgf3* is a direct transcriptional target of vHnf1 and operates via the

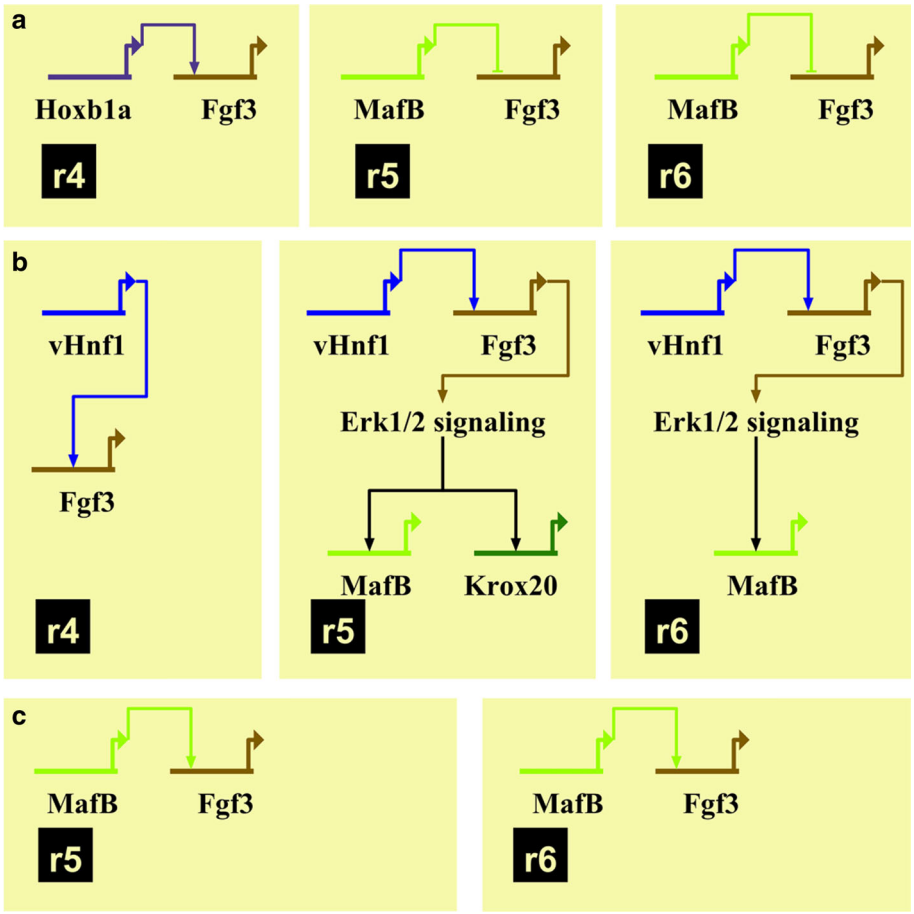

Fig. 5 *Fgf3* subcircuit in different vertebrate species in the developing rhombencephalon. **a** zebrafish, **b** chicken, **c** mouse

Erk1/2 pathway to induce *MafB* in r5 and r6 and *Krox20* in r5 (Fig. 5b) [8, 214]. In mammals, the functional link between *Fgf3* and *MafB* is inversed, as *Fgf3* is a downstream target of MafB in r5 and r6 (Fig. 5c) [134]. Further differences are found in the function of these subcircuits. In zebrafish, Fgf3 and Fgf8 are important in organizing the hindbrain [130, 210, 216], while mouse mutants show no hindbrain segmentation defects [221].

Despite the high degree of conservation in the expression pattern of *Hox* genes, species-specific differences exist as well. In the mouse, *Hoxa2* becomes up-regulated specifically in r3 and r5 and *Hoxb2* in r3 through r5 [182, 207]. In chicken, *Hoxa2* is strongly expressed from r2 to r6, whereas *Hoxb2* is only faintly expressed in the hindbrain [162, 205]. Studies for *Hoxa2* revealed that this difference in expression across tetrapod groups is due to a different organization of enhancer elements [205].

These ontogenetic differences in network architecture as well as origin and timing of birth are in line with the assumption that auditory hindbrain nuclei represent homoplasious and not homologous structures in the different tetrapod groups. Consequently, shared features had evolved by convergent evolution, due to similar requirements for sound processing after evolution of sound pressure sensitive receivers [75]. Yet, it is important to remember that homologous structures can have different genetic and developmental basis [1, 2, 209]. The body segments in insects provide an instructive example. These are clearly homologous structures, yet genes important for segmentation in the fruit fly exhibit other expression patterns and functions than in the grasshopper [42, 157]. Based on this and other findings, it was suggested that the most conservative parts of the developmental process are the GRNs that specify the identity of the character, the so-called character identity networks. In contrast, other aspects of development, from early patterning to the execution of the developmental program, appear more variable [209]. Homology can, therefore, be expressed at different hierarchical levels of biological organization such as genes, gene expression patterns, embryonic origin, and morphology. In summary, determination of the precise evolutionary mechanisms leading to tetrapod auditory brainstem structures requires further studies. They should be directed towards identification of the later developmentally operating GRNs and a better characterization of auditory hindbrain characters in other vertebrate groups such as fish and amphibia [1, 2, 91].

Conclusion

Currently, a wealth of information is present concerning the GRNs required for the formation of those hindbrain structures, giving rise to the mammalian auditory hindbrain. Furthermore, TFs and signaling molecules involved in maturation of auditory neurons became recently evident. They provide a promising basis to build upon. However, a large gap exists in our knowledge of genes specifying the auditory lineages in the rhombomeres. Furthermore, GRNs are at the heart of the twin phenomena of development and evolution [39, 54] and developmental studies should, therefore, include comparative expression analyses across vertebrate groups. These studies are key to understanding evolution of the auditory hindbrain. As a corollary, they will reveal whether the currently operating GRNs in the different vertebrate groups contain components of an ancestral GRN already deployed in a pre-existing development system. In this respect, it is noteworthy that TFs of the *ato, sal* and *dll* families partake in the development of the auditory organ of both vertebrates and *Drosophila* 15704117. Molecular data of wing and eye formation revealed a striking conservation of genes and circuits across vertebrates and invertebrates, as distant as flies and mammals, resulting in the concept of deep homology [183, 184]. It will therefore be exciting to analyze whether the developmental program of the auditory system demonstrates ancient regulatory circuits as well, which would make it another instructive example of the deep homology seen in genetic programs. The availability of genome sequences from all major vertebrate radiations combined with the availability of high-throughput transcriptome profiling methods [132, 212] provides unprecedented resources for such an approach. The emergence of techniques such as TALENS or CRISPR/Cas for genetic manipulations of model organisms as well as non-model organisms will assist in defining the role of the identified candidate genes [62]. Thus, it is now a favorable time to dissect the development and evolution of the auditory system, a sense so closely linked to the evolution of *Homo sapiens.*

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