Assembly of the Auditory Circuitry by a Hox Genetic Network in the Mouse Brainstem

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Abstract

Rhombomeres (r) contribute to brainstem auditory nuclei during development. Hox genes are determinants of rhombomere-derived fate and neuronal connectivity. Little is known about the contribution of individual rhombomeres and their associated Hox codes to auditory sensorimotor circuitry. Here, we show that r4 contributes to functionally linked sensory and motor components, including the ventral nucleus of lateral lemniscus, posterior ventral cochlear nuclei (VCN), and motor olivocochlear neurons. Assembly of the r4-derived auditory components is involved in sound perception and depends on regulatory interactions between Hoxb1 and Hoxb2. Indeed, in Hoxb1 and Hoxb2 mutant mice the transmission of low-level auditory stimuli is lost, resulting in hearing impairments. On the other hand, Hoxa2 regulates the Rig1 axon guidance receptor and controls contralateral projections from the anterior VCN to the medial nuclei of the trapezoid body, a circuit involved in sound localization. Thus, individual rhombomeres and their associated Hox codes control the assembly of distinct functionally segregated sub-circuits in the developing auditory brainstem.

Introduction

The mammalian brainstem plays a crucial role in the regulation of many vital functions through a complex system of reflex arcs and relays information to higher brain centers through interconnected neuronal circuits. During development, the hindbrain becomes subdivided along the antero-posterior (A-P) axis into serially repeated, spatially segregated, modules of progenitor cells, the rhombomeres (r). Individual rhombomeres give rise to distinct portions of sensory and motor columns depending on the position of progenitors along the dorso-ventral (D-V) axis, respectively [1], thus generating nuclei of multi-segmental origin and topographic patterns of connectivity [2,3,4,5,6,7]. For instance, in the somatosensory system, afferent innervation from mandibular or whisker pad (maxillary) facial dermatomes targets the r2- or r3-derived components of the principal trigeminal sensory nucleus, respectively. In turn, the information is somatotopically relayed to the thalamus and somatosensory cortex, contributing to build a facial somatosensory map [7]. Vestibular nuclei also originate from different rhombomeres and display specific sets of axonal trajectories with distinct targets [3,4,5,9]. Thus, regional patterning along the A-P axis and specific D-V determinants intersect to determine sub-circuit connectivity within functionally related longitudinal neuronal columns.

Topographic connectivity and employment of sensory and motor nuclei are also well described during formation of auditory-dependent circuits [10]. The auditory central pathway consists of sensory nuclei transmitting ascending acoustic information, and efferent motor neurons modulating primary afferent responses. The sensory organ for sound is the cochlea, which contains two types of receptors, namely the inner and outer hair cells. While the inner hair cells (IHCs) are the major detectors of auditory stimuli, the outer hair cells (OHCs) enhance low level sounds by increasing the amplitude and frequency selectivity of basilar membrane vibrations, a process called “cochlear amplification” [11,12]. From the periphery, sound information travels through the VIIIth cranial nerve to the brainstem cochlear nuclear (CN) complex, which is the primary relay station for central auditory processing [13]. This cochlear complex originates from distinct portions of the r2-r5 region, which will give rise to the anteroventral (AVCN), posteroventral (PVCN) and dorsal (DCN) cochlear nuclei, as well as to the cochlear granule cell population of the microneuronal shell [6]. A significant portion of the CN complex derives from a dorsal rim of neuroepithelium referred to as the auditory lip [6,14], which is part of the lower rhombic lip and selectively expresses the transcription factor Atoh1 (also known as Math1) [15,16,17]. Various processed sound-related signals, ultimately leading to qualitative sound perception, travel from the cochlear nuclei through the lateral lemniscus (LL) complex to the inferior
Author Summary

Sound perception and sound localization are controlled by two distinct circuits in the central nervous system. However, the cellular and molecular determinants underlying their development are poorly understood. Here, we show that a spatially restricted region of the brainstem, the rhombomere 4, and two members of the Hox gene family, Hoxb1 and Hoxb2, are directly implicated in the development of the circuit leading to sound perception and sound amplification. In the absence of Hoxb1 and Hoxb2 function, we found severe morphological defects in the hair cell population implicated in transducing the acoustic signal, leading ultimately to severe hearing impairments in adult mutant mice. In contrast, the expression in the cochlear nucleus of another Hox member, Hoxa2, regulates the guidance receptor Rig1 and contralateral connectivity in the sound localization circuit. Some of the auditory dysfunctions described in our mouse models resemble pathological hearing conditions in humans, in which patients have an elevated hearing threshold sensitivity, as recorded in audiograms. Thus, this study provides mechanistic insight into the genetic and functional regulation of Hox genes during development and assembly of the auditory system.

colliculus (IC; midbrain) and medial geniculate nucleus (MG) of the thalamus, which in turn relays auditory information to the auditory cortex. On the other hand, temporal and spatial sound localization are relayed by the cochlear nuclei through a parallel pathway in the ventral brainstem, before reaching high level brain structures [18]. This pathway includes the superior olivary complex (SOC), which is mostly derived from r5 and is partly composed of the corresponding Atoh1 lineage [3,16,19].

Proper hearing function is also controlled by centrifugal (efferent) motor connections, which modulate the incoming afferent sensory auditory information. The major component is represented by the olivocochlear neurons (OC), a subpopulation of the inner ear efferent (IEE) neurons, which are born in ventral r4, cross the midline during early development and segregate from their vestibular counterpart around embryonic day 14.5 of gestation (E14.5) in mice [20,21]. While lateral OC (LOC) motor neurons innervate afferent sensory neurons in contact with the IHCs, modulate cochlear nerve excitability and protect the cochlea from neuronal damage in acute acoustic injury [21,22], medial OC (MOC) motor neurons are innervated by reflex neurons of the PVCN [23,24] and regulate the vibrating OHCs in the cochlea, modulating in this way the “cochlear amplification” process [25,26,27]. This is known as the MOC reflex. Cochlear efferent motor neurons also play a role in the normal maturation of afferent responses, particularly during the early postnatal period [21,28]. Another feedback reflex, the middle-ear muscle reflex (MEM), is closed by facial and trigeminal branchiomotor neurons (FBM, TBM) that activate the stapedius and tensor tympani muscles respectively, thus tensing the chain of tympanic ossicles and reducing the amplitude of sound transmission through the middle ear [29,30]. Thus, the MOC and MEM reflexes represent two parallel sound-evoked feedback mechanisms acting on the auditory periphery to modulate incoming acoustic stimuli [29,31,32].

Little is known about the molecular determinants involved in the assembly of rhombomere-derived auditory sub-circuits. The Hox genes, a large family of homeobox-containing genes, display rhombomere-restricted expression patterns and provide early patterning information to progenitors and their neuronal derivatives [33,34]. In turn, the expression of several Hox genes is maintained through later stages of circuit formation in distinct, rhombomere-derived neuronal subpopulations contributing to portions of hindbrain sensory and motor nuclei [4,7,35,36]. In the developing hindbrain, Hoxb1 selectively expressed in r4 is required to pattern r4-derived ventral efferent neurons, such as IIE and FBM, and to maintain normal levels of Hoxb2 in r4 [37,38]. Hox2 and Hoxa2, unlike Hoxb1, are expressed in r2 to r5 auditory derivatives. Moreover, the expression of Hoxb2 and Hoxa2 is maintained in the ventral CN, ventral nucleus of LL (VLL), and SOC nuclei throughout prenatal and postnatal developmental stages [36]. Thus, Hox genes are prime candidates to be involved in the assembly of sensorimotor functional circuits in the developing hindbrain.

In this study, we find that rostral rhombomeres and their associated Hox genes are required in establishing and maintaining two major functional circuits in the central auditory system, which have different rhombomeric origins. Firstly, we carried out a detailed fate map of r4 derivatives by generating a novel, highly restricted, r4-specific Cre-recombinase driver. We show that cells originating from r4 significantly contribute to the VLL, an important relay station in the sound perception pathway. Furthermore, we found that r4 largely supplies cells to the PVCN and DCN, a finding largely in agreement with previous work [6,16], but not to the granule cells of the microneuronal shell. Second, in Hoxb1 and Hoxb2 mutants, the VLL, PVCN, and MOC motor neurons are selectively affected, though with different severities, leading ultimately to elevated auditory thresholds in adult mutant mice. Thirdly, Hoxb1 negatively modulates Hoxa2 during r4 patterning, whereas Hoxa2 is mainly required in r2/r3 AVCN-derived development. Moreover, early conditional Hoxa2 inactivation in rhombic-lip derivatives selectively perturbs the AVCN axonal pathfinding to the medial nucleus of the trapezoid body (MNTB), resulting in decreased contralateral and increased ipsilateral targeting of MNTB due to the down-regulation of Rig1, the main axon guidance receptor for midline crossing. Altogether, this study provides, for the first time, genetic and functional evidence for a Hox gene network in the establishment and maintenance of proper auditory rhombomere-specific circuitry during hindbrain development.

Results

Mapping of rhombomere 4-specific contribution to the auditory system

Taking advantage of specific r2- and r3/5-Cre-expressing mouse lines [39,40], previous studies have genetically mapped the contribution of the r2- and r5 rhombic lip to distinct portions of the CN and SOC complexes [6,16,19]. However, previous attempts to generate r4-specific lines invariably resulted in additional Cre expression caudal to r4 [7,16,41]. In this study, we generated a novel mouse transgenic line, named b1r4-Cre, which allowed us to exclusively map r4 and its derivatives throughout the mature brainstem (Figure 1A and Figure S1). To this purpose, we used a well characterized enhancer from the Hoxb1 locus [42] to drive the Cre recombinase gene exclusively in r4 (Figure S1A). In b1r4-Cre transgenic animals, onset of Cre expression in presumptive r4 occurs first in a mosaic fashion (Figure S1C), but from E9.0 onwards, Cre is expressed throughout r4 as shown in Figure S1D. To permanently label the polyclonal population of cells derived from r4, the b1r4-Cre transgenic line was mated to the ROs126-TFP reporter mouse [43] and progenies positive for both alleles (herein called b1r4-Cre/TFP)
Figure 1. Rhombomere 4 neuronal derivatives contribute to nuclei involved in auditory perception. (A) Strategy for the Cre-loxP recombinase r4-fate map. Upon Cre-mediated recombination, the loxP sites surrounding the PGKneo cassette of the ROSA26 YY reporter line are excised and YFP is expressed exclusively in r4 and r4 derivatives. (B) Dorsal and lateral views of a E10.5 b1r4-Cre/YFP embryo show restricted expression of YFP in r4 and neural crest-derived cells (ncc) in the second branchial arch (ba2). The white line delineates the level and plane of sagittal section of panels in (C). (C) r4-restricted immunostaining of Cre-recombinase in progenitors of the ventricular zone (vz). YFP+/Cre2 post-mitotic cells at the marginal zone (mz) originate from YFP+/Cre+ progenitors. (D) Sagittal sections of an E12.5 b1r4-Cre/YFP embryo immunostained with a GFP antibody reveal the r4 domain, the caudal migration of facial branchiomotor neurons (mFBM), the ventricular to pial migration of presumptive lateral lemniscus cells (mVLL) and the lateral lemniscus tract (LLt) projecting rostrally. Below, a schematic of an E14.5 sagittal section indicating the position of the various nuclei. The red line delineates the plane of section of panels (E). (E) The olivocochlear (OC) neurons (delimited by a red contour) express choline acetyltransferase (ChAT), Gata3 and Tbx20. (F) Schematic coronal section of a P8 brain illustrating the positions of the various nuclei. The lateral superior olive (LSO) but not the medial superior olive (MSO) nucleus, has an r4 origin, as indicated by YFP and VGlut2 staining. ChAT and Tbx20 are expressed in lateral (LOC) and medial OC (MOC) neurons within the LSO and ventral to the LSO, respectively. (G) Sagittal sections at different ages indicating the YFP+ r4-derivatives: the ventral lateral lemniscus (VLL) (rostrally) and FBM (caudally) nuclei. VLL and cochlear neurons project rostrally to the inferior colliculus (IC) and some fibers continue to the thalamus (arrowheads). (H) Coronal sections of P0 pups indicate high contribution of r4/YFP+ cells to the VLL, positive for Gad67, but not to the dorsal LL (DLL), which is VGlut2+ . Hoxb2 and Hoxa2 are expressed in the VLL and pontine nuclei (PN). A few dispersed YFP+ cells are identified in the PN. (I) Schematic of a P8 brain sagittal section showing the position of the cochlear nuclear complex (CN) and its subdivision into anteroventral (AVCN), posteroverentral (PV CN) and dorsal (DCN) nuclei. Adjacent sagittal sections illustrate a high contribution of YFP+ cells to the PV CN and DCN. The arrowhead indicates the origin of r4-migrating cells. Dots delineate the presumptive boundary between AV CN and PVC N. Only a few YFP-positive cells label the AV CN, which is highly positive for Atoh7. The small intensely basoficial granule cells confined to the microneuronal shell (mnsh and outlined) and identified by Nissl and Pax6 expression (J) are not positive for YFP, indicating that cochlear granule cells do not have an r4-origin. (K) Similarly, YFP+ cells do not co-localize with calbindin- (CB) and calretinin- (CR) expressing cells in the PV CN region. (L) Schematic of the hindbrain in which rhombomeres 2 to 5 and their respective Hox genes are color-coded. The same code refers to (M). (M) Overview of the two main central auditory pathways, the MOC reflex and their rhombomeric origin. While r4-derivatives (in red) contribute mainly to the ascending sound perception pathway, which runs from the CN through the VLL to the IC, r2-, r3- and r5-derivatives (in green) contribute mainly (but not exclusively) to the pathway running through the superior olivary complex (SOC), which function in the localization of the temporal and spatial origins of sounds. The MOC reflex comprising of sensory PV CN interneurons and motor efferent OC neurons is also an r4-derivative. Vn, trigeminal motor nucleus; MNTB, medial nucleus of trapezoid body; Pis principal sensory trigeminal nucleus. Scale bars, 100 μm (C), 200 μm (D–J left), 20 μm (J right, K). See also Figures S1 and S2.

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were analyzed (Figure 1A). Accordingly, at E10.5 activation of YFP was observed exclusively in r4 and its cellular progeny, as shown by double staining of Cre and YFP (Figure 1B, 1C). No ectopic expression of Cre was detected at later stages (data not shown).

We next analyzed the b1r4-Cre/YFP mouse line at different embryonic stages and early postnatal ages, using an anti-GFP antibody that cross-reacts with the YFP protein and labels proliferative and migrating/differentiating cells and their axonal projections. The r4 radial histogenetic territory itself is massively labeled and becomes morphogenetically deformed into a wedge-shaped, dorsally compressed configuration (Figure 1D, 1G). The first cohort of cells migrating out of r4 consists of the well-described caudomedial stream of tangentially migrating FBM neurons, which first move caudolaterally into r6 and then reach their definitive ventrolateral subpial position by radial migration [44,45,46] (Figure 1D, 1G). Another r4-derived effector population is represented by OC neurons [20,21], a subpopulation of embryonic neurons. At E14.5 they form a compact superficial group of cells at the r4/r5 margin and selectively express the cholinergic marker ChAT [21] and the transcription factors Gata3 and Tbx20 [47,48] (Figure 1E). At E8 the OC neurons split into lateral (LOC) and medial (MOC) components [49], which express ChAT and Tbx20 and become located within the lateral superior olive (LSO) and in the medioventral portion of the SOC as scattered cells, respectively (Figure 1F). A part of the LSO, expressing the glutamatergic marker VGlut2 [16], is included within the r4 domain (Figure 1F).

Another sizeable stream of labeled r4-derived cells, which was not previously described, migrates to the basal longitudinal zone and then moves rostrally along the growing lateral lemniscus tract, which courses obliquely through the rostral hindbrain (Figure 1D, 1G). These cells will contribute to the majority of the VLL from E14.5 onwards (Figure 1G). At E16.5, labeled ascending lateral lemniscus fibers, originating from the ipsilateral r4-derived VLL neurons and also from the r4-derived projection neurons of the contralateral CN [50], reach the IC. At E18.5, lateral lemniscus fibers also extend along the brachium of the IC into the medial geniculate nucleus of the thalamus, and collaterals can be distinctly seen in the superior colliculus intermediate layers at P8 (Figure 1G, and data not shown). Transverse sections at P0 clearly show a high density of YFP+ cells in the VLL but not in the dorsal nucleus of LL (DLL), which expresses the glutamatergic marker VGlut2 and originates from the Atoh1+ lineage [19,51]. The majority of VLL neurons express the inhibitory GABAergic/glycinergic marker Gad67, a particular feature of this auditory structure, as previously described [52], and Hoxb2 and Hoxa2 [36]. In summary, our fate map study shows for the first time that r4 massively contributes to the VLL within the LL complex.

Next, we mapped precisely r4 contribution to the plurisegmental CN complex (Figure 1I). Previously, the contribution of r4 was indirectly inferred from the mapping of r3 and r5 derivatives, or from the mapping of the territory posterior to r3 [6,16]. These studies indicated that the AVCN is derived from r2 and r3, the PVCN from r3 and r4, and the DCN from r4 and r5 (summarized in Figure 1I, 1M). A significant proportion of these nuclei were strongly affected in Atoh1 conditional and null mutants, suggesting their origin from the Atoh1+ auditory lip [16,17].

Our results are largely in agreement with previous data and further extend them. First, we show that at E10.5 the YFP+ domain includes Atoh1-expressing cells in the rhombic lip region of dorsal r4 (Figure S2A). However, at E14.5 when Atoh1+ cells migrating from r2 to r3 rhombic lip invade the presumptive cochlear complex [17], only a few r4-derived YFP+ cells overlap with Atoh1 expression domain, whereas no co-localization of YFP with the granule cell marker Barlh1 [6] is found in this region (Figure S2B). Secondly, we show that YFP+ cells contribute to an intermediate sector across the CN complex, which crosses dorsoventrally the magnocellular core portion of the DCN and then gives rise to the majority of the PVCN (Figure 1I and Figure S2C–S2E). Small portions of the DCN remain unlabeled, suggesting additional contribution from r5, as previously reported [6,16], but also a likely contribution from r3 to the region of DCN anterior to the YFP+ domain. Thirdly, we found that only a small number of YFP+ cells are distributed in the AVCN at P8, which, unlike the PVCN, expresses high levels of Atoh1 [also known as Math5] [53] (Figure 1I). Finally, we started to characterize the cellular identity of YFP+ cells and found that at P8, r4-derived cells fail to co-express Pax6 (Figure 1I), a marker for the microneuronal granule cell population [15,54]. Moreover, YFP signal is absent in calbindin- and calretinin-expressing neurons (Figure 1K), corresponding to octopus and globular-bushy cells, respectively [55,56]. These data indicate that subpopulations of glutamatergic neurons, which normally derive from the Atoh1+ neuroepithelial domain [15], do not originate from r4. A full characterization of YFP+ cells in embryonic and adult stages will be reported elsewhere (M.D., L.P. and M.S., in preparation).

In summary, we show that r4 largely contributes to the motor cochlear effenter neurons, to the relay VLL neurons, and within the cochlear nucleus, to the majority of the PVCN, and part of the DCN. Thus, while r4 seems to be required for the structures involved in sound transmission, amplification and protection (i.e. PVCN, VLL, and OC), r2, r3 and r5 are likely contributing to components of the sound localization pathway that runs through the SOC and trapezoid body complex before reaching higher-order auditory structures (Figure 1L, 1M).

Regulation of Hoxb1, Hoxb2, and Hoxa2 in sensory r4 of Hox mutant mice

Previous work dissected the genetic and regulatory network involved in establishing and maintaining the identity of r4 progenitors [34]. Hoxb1 plays a key role in patterning ventral r4 progenitors, partly through transcriptional regulation of Hoxb2 and Hoxa2 [37,38,57]. Maintenance of Hoxb1 expression in ventral r4 requires both Hoxb1 itself and Hoxb2 through auto- and cross-regulatory interactions, respectively [37,58,59,60]; however, it is not known whether a similar mechanism is acting in dorsal/ sensory r4. Unlike Hoxb1, Hoxb2 and Hoxa2 expression is maintained in differentiated r4-derivatives, such as the VLL and VCN [36] and our study). Thus, Hoxb1 may pattern sensory r4-derivatives by regulating dorso-ventral Hoxb2/Hoxa2 expression in r4 progenitor cells, hence controlling post-mitotic specification during the development of the sensory system. To test this hypothesis and investigate the roles of Hoxb1, Hoxb2, and Hoxa2 in the development of auditory pathways, we analyzed the effects of their functional inactivation.

Hoxb1 functional deletion results in an early re-patterning of the ventral r4 territory into a more anterior identity [44,61]. To bypass the early Hoxb1 role in r4 neuroepithelium and investigate its requirement during neurogenesis, we generated a novel Hoxb1 conditional mutant allele, Hoxb1fl/fl (Figures S3 and S4; see Materials and Methods) and mated it to the b1r4-Cre driver. Conditional b1r4-Cre;Hoxb1fl/fl homozygous mutant mice here-
after referred to as $Hoxb^{\text{lateCKO}}$ are viable and fertile. Mutant embryos retain $Hoxb1$ expression in r4 until E8.75–E9.0 (Figure 2A), in accordance with the timing of Cre expression and onset of Cre-mediated excision (Figure 1 and Figure S1). At E9.25, $Hoxb1$ expression is no longer present in mutant r4, while the remainder of its expression is unaltered (Figure 2A).

We also obtained constitutive $Hoxb^{\text{null}}$ homozygous mutants (Figures S3 and S4; see Materials and Methods) that exhibit total loss of $Hoxb1$ expression from its onset (Figure 2A) and lack any selectable cassette that might interfere with adjacent $Hox$ genes [62,63]. To specifically trace r4 derivatives from the control and the two distinct $Hoxb1$ mutants throughout embryonic and postnatal brains, $Hoxb^{\text{flo}}$ and $Hoxb^{\text{null}}$ homozygous mice were mated with the $b1r4$-Cre/YFP reporter line (see Materials and Methods for mating schemes). In addition, we generated a novel, viable and fertile $Hoxb2$ homozygous mutant allele (hereafter referred to as $Hoxb2^{\text{AKO}}$), that, similarly to the $Hoxb^{\text{null}}$ and unlike previously described $Hoxb2$ knockout alleles [37,58,64], has no selectable marker left within the locus (see Materials and Methods). Finally, we made use of the previously described $Hoxa2^{\text{null}}$ and conditional $Hoxa2^{\text{flo}}$ alleles [7,65].

Next, we assessed $Hox$ cross-regulatory interactions within r4 in $Hoxb1$, $Hoxb2$ and $Hoxa2$ mutant alleles. Flat-mounted preparations and sagittal sections of control embryos show high $Hoxb2$ expression levels in r4 to r6 and low levels in r3 (Figure 2B). In E10.5 $Hoxb^{\text{null}}$ and $Hoxb^{\text{lateCKO}}$ hindbrain preparations, $Hoxb2$ expression is down-regulated throughout r4 to similar levels as in r3 (asterisks in Figure 2B), resulting in a duplication of r3-features (“r3” in Figure 2B). This is confirmed in mid-sagittal sections showing a decrease of $Hoxb2$ expression in r4 progenitors of $Hoxb^{\text{null}}$ and $Hoxb^{\text{lateCKO}}$. Expression is still maintained in early differentiating cells, similarly to r3 (arrowheads in Figure 2B). In contrast, $Hoxa2$ is normally expressed at low levels in r2 and r4, and at high levels in r3, particularly in a wide intermediate stripe of the dorsal sensory column (horizontal bracket in Figure 2C) and in a thinner stripe laterally (vertical bracket in Figure 2C), the presumptive auditory column. In E10.5 $Hoxb1$ mutant embryos, expression of $Hoxa2$ is abnormally up-regulated in r4, predominantly in the two sensory stripes, resulting in a duplication of r3-typical features in r4 (Figure 2C). Sagittal sections at different alar plate levels of $Hoxb^{\text{null}}$ mutant embryos confirm up-regulation of $Hoxa2$ in the ventricular zone, and, strikingly, also in the mantle zone of r4 (arrows in Figure 2D and inset), which normally expresses low levels of $Hoxb1$ and $Hoxb2$ (arrowheads in Figure 2D and inset). In mutant r4, $Hoxa2$ is maintained at high levels in the post-mitotic neurons, similarly as in r3. Thus, our data show that in the absence of $Hoxb1$, r4 acquires $Hox$ features typical of r3, such as low levels of $Hoxb2$ and high levels of $Hoxa2$, indicating a re-patterning of r4 into r3. In addition, $Hoxb1$ differentially regulates $Hoxb2$ and $Hoxa2$ expression levels in r4, further supporting a complex regulatory interaction between $Hoxb1$ and $Hoxb2$/Hoxa2 in specifying r4 identity [37,38,57,38,59].

To investigate whether $Hoxb2$ acts similarly to $Hoxb1$ in r4 patterning, we assessed $Hoxb1$ and $Hoxb2$ expression in WT and $Hoxb^{\text{null}}$ hindbrains from E8.75 to E10.5. $Hoxb1$ protein is lost in $Hoxb^{\text{null}}$ at E10.5, though it is present at earlier embryonic stages (Figure 2E), indicating that $Hoxb2$ is required for maintaining $Hoxb1$ in r4. Similarly to $Hoxb1$ mutants, $Hoxa2$ expression is dramatically up-regulated throughout r4, with particular emphasis in the intermediate and lateral columns (Figure 2F), and in post mitotic neurons (arrows in Figure 2G and inset). In contrast, no changes are observed in $Hoxa2^{\text{null}}$ embryos (Figure 2H), apart from the r2/r3 alar defects previously described [66]. Taken together, our data show that within r4, $Hoxb2$ acts mainly in maintaining high expression of $Hoxb1$ and that in its absence, r4 acquires r3-typical features, similarly to $Hoxb1$ mutant hindbrains. Importantly, we found increased $Hoxa2$ expression, at levels similar to r3, in post-mitotic r4 neurons of $Hoxb1$ and $Hoxb2$ mutants, implying that r4-derived sensory cells normally maintain $Hoxa2$ expression throughout hindbrain development (Figure 2F).

Finally, we investigated whether $Hoxb1$ and $Hoxb2$ are required in the differentiation process of the IEE and OC motor neurons, which normally differentiate in ventral r4 and interact with dorsally-derived sensory structures during the development of the auditory sensorimotor circuitry. At E10.5, IEE are located in the r4 mantle zone next to the floor plate and normally express Gata2/1, Isl1 and Phox2b [48,59,67] (Figure S5A, S5B). No IEE neurons are identified in E10.5 $Hoxb^{\text{null}}$ and $Hoxb^{\text{lateCKO}}$ embryos, as seen by the absence of Gata2/3 expression within the pool of Phox2b+/Isl1+ motor neurons (Figure S5B). This is due not to a delay in specification, since no OC neurons, positive for ChAT and Gata3, located in ventral peri-olivary positions can be distinguished in E14.5 $Hoxb1$ mutant embryos (Figure S5E). On the contrary, a few Gata3+ and Tbx20+ cells are present in $Hoxb^{\text{null}}$ mutant embryos at E10.5 and E14.5, even if they are located in a slightly more dorsal position than in control embryos (Figure S5C, S5F).

These data indicate that early IEE and late OC specification are maintained in $Hoxb^{\text{null}}$ embryos despite the late absence of $Hoxb1$ in r4.

$Hoxb1$ is a key determinant gene in r4-derived VLL development

To investigate the requirement of $Hox$ genes in the generation of sensory auditory structures, we first analyzed the size and position of the VLL with various markers on adjacent coronal sections at E18.5 and sagittal sections of P8 WT and mutant brains (Figure S6 and Figure 3). Very few cells contributing to the VLL are identified in E18.5 $Hoxb^{\text{null}}$ mutant hindbrains, as shown by YFP and Gad67 staining (Figure S6B). Only some YFP+ cells with mesencephalic projections, scattered rostral to the r4 wedge, are still maintained in $Hoxb^{\text{null}}$ brains at P8 (Figure 3A). No detectable $Hoxb2$ or $Hoxa2$ expression can be found in sagittal sections at all levels, whereas reduced expression of Gata3 and Gad67, which label the GABAergic/glycinergic cellular cohort of the VLL [52,68], is still present in the remaining VLL, which is reduced by almost 90% in area (91.9±0.02) when compared to control VLL (Figure 3A, 3D). This indicates that the early absence of $Hoxb1$ function prevents normal r4-derived VLL specification and/or migration, and supports a major contribution of r4 to the formation of the VLL, particularly to its GABAergic cohort, which contributes to the majority of the VLL [52]. Moreover, absence of VGlut2 expression observed at E18.5, and confirmed at P8 (Figure S6B and Figure 3A), rules out any inhibitory to excitatory cell fate transformation within the VLL.

A less severe reduction of the VLL was observed in $Hoxb^{\text{lateCKO}}$ mutants (n = 3; 49.2±0.1% in area as compared to WT; Figure 3D and legend). This was mainly supported by the maintenance of Gata3+, Gad67-, $Hoxb2$- and $Hoxb2$-expressing cells at E18.5 and P8, and by the presence, although reduced, of YFP+ VLL projections to the IC (Figure 3A and Figure S6B). Similarly, the VLL area is reduced by 41.7±0.3% in $Hoxb^{\text{null}}$ (n = 3) when compared to WT (n = 3) brains (Figure 3D and legend), whereas no size reduction is measured in the VLL of $Hoxa2^{\text{null}}$ mutants (n = 3; 105±5.7%) at E18.5 before $Hoxa2^{\text{null}}$ perinatal death [69] (Figure 3C, 3E and legend). Together, these data indicate that $Hoxb1$ is an important determinant gene in r4-derived VLL development, because r4 to r3 change of identity occurring in
Figure 2. Regulatory interactions between Hoxb1, Hoxb2, and Hoxa2 in r4. (A) The diagrams above the panels indicate the interactions between Hoxb1 and Hoxb2. While Hoxb1 auto-regulates its own expression in r4, it also binds to an Hoxb2 r4 enhancer to maintain Hoxb2 expression in r4. Hoxb2 maintains expression of Hoxb1 in r4. Crosses indicate loss of Hoxb1 protein in Hoxb1null embryos and loss of the auto- and cross-
regulatory loops in Hoxb1\textsuperscript{lateCKO} mutants. Lateral views of E8.5 to E9.25 embryos indicate that while Hoxb1 expression is still maintained in r4 (although at lower levels) of E8.75 Hoxb1\textsuperscript{lateCKO} mutants, r4 expression is completely abolished in E9.25 mutant embryos (arrowheads). Expression in the posterior region is still maintained at both ages (arrow). B) Ventricular views of flat-mount preparations of E10.5 WT, Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} hindbrains hybridized with Hoxb2. Expression of Hoxb2 is strongly decreased (but not abolished) in r4 of Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} embryos, at similar levels to r3 (asterisks). R4 acquires an expression pattern of r3, as indicated by "r3". Down-regulation of Hoxb2 in r4 can also be appreciated in mid-sagittal sections of mutant embryos. The line of cells expressing high levels of Hoxb2 denotes early post-mitotic cells (arrowhead). C) Ventricular views of flat-mount preparations of E10.5 WT, Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} hindbrains hybridized with Hoxa2. Expression of Hoxa2 is increased in r4 and the characteristic Hoxa2 expression profile of r3 is now duplicated in r4 of Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} embryos supporting an r4 to r3 change of identity. The horizontal and vertical brackets indicate higher expression domains of the characteristic Hoxa2 expression. In mutant embryos, the expression of Hoxa2 is maintained at levels comparable to r3 in the r4 mantle zone (m2) (i.e. post mitotic neurons) with respect to WT (arrows, see also insets). E) In Hoxb2\textsuperscript{null} mutants, lack of Hoxb2 (indicated with a cross) results in failure to maintain Hoxb1 expression in r4. Sagittal and coronal views show that Hoxb1 protein is present in r4 of E8.75 embryos, but not maintained in E10.5 Hoxb2\textsuperscript{null} mutant embryos. R4 acquires expression levels to r3 (asterisks). R4 acquires an expression pattern of r3, as indicated by "r3". Down-regulation of Hoxb2 in r4 can also be appreciated in mid-sagittal sections of mutant embryos. R4 acquires an expression pattern of r3, as indicated by "r3". Down-regulation of Hoxb2 in r4 can also be appreciated in mid-sagittal sections of mutant embryos. 

Abnormal specification of r4-derived ventral cochlear structures in Hox mutants

We next investigated the involvement of Hoxb1, Hoxb2 and Hoxa2 in the development of the CN complex (Figure 4). In Hoxb1 mutant mice, no considerable changes in the overall size of the CN were observed at P9. However, careful analysis showed that r4-derived YFP\textsuperscript{+} cells massively invade the granule shell layer, normally derived from the Atoh7\textsuperscript{+} lineage [15,17,19], and ectopically expressed Pax6, as confirmed by the abnormal presence of double YFP\textsuperscript{+}/Pax6\textsuperscript{+} cells in the microneuronal shell (Figure 4A). This strongly indicates that r4-derived mutant cells have now acquired a granule cell identity, which r4 does not normally contribute to (Figure 1I–1J and Figure 4A). In addition, while Hoxb2 expression is maintained in the increased microneuronal shell of the Hoxb1 mutants, Hoxb2 and Hoxa2 expression levels are slightly affected in the ventral CN (Figure 4B). Namely, Hoxb2 is down-regulated in the PVCN (asterisks in Figure 4B), whereas Hoxa2 expression is slightly up-regulated in PVCN regions ventral to the microneuronal shell layer (arrows in Figure 4B), in line with their respective altered expression levels observed at E10.5 (Figure 2B–2D). Notably, Atoh7, predominantly expressed in the Atoh7\textsuperscript{+} lineage, is now strongly up-regulated in the PVCN of Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} mutants, indicating that the PVCN has acquired features of the r2/3/5-derived AVCN [6] (summarized in Figure 4E). Next, we asked whether Hoxb2 and Hoxa2 are also required in the specification of VN components. Interestingly, Atoh7 and Hoxa2 expressions are also increased in the PVCN of Hoxb2\textsuperscript{null} (arrows in Figure 4C). In contrast, Atoh7 and Hoxb2 expressions are strongly reduced in the AVCN of E10.5 Hoxb1\textsuperscript{null} mutants (arrows in Figure 4D), in accordance with the early patterning defect previously described in the rostral hindbrain of these mutants [66]. The dramatic increase of cells that express high levels of Atoh7 in the PVCN and the ectopic formation of YFP\textsuperscript{+} granule cells observed in Hoxb1 mutants (both are Atoh1\textsuperscript{+} lineage derivatives), led us to hypothesize that an increase of Atoh1 in r4 is the cause of the ectopic expression of glutamatergic neurons. Normally, the Atoh1\textsuperscript{+} domain in dorsal r4 (i.e. YFP\textsuperscript{+}) of E10.5 embryos is smaller than in adjacent rhombomeres, such as r3 (Figure 4F). In contrast, in Hoxb1\textsuperscript{null} mutant animals this domain is enlarged to a similar extent as in control r3, suggesting an up-regulation of Atoh1 in r4, which may contribute to the acquisition of r3-like fate and hence to the ectopic generation of glutamatergic cell types (summarized in Figure 4G). This is even more pronounced at E14.5, when cells from the r2 to r5 rhombic lip contribute to the CN primordium. In WT embryos, the majority of YFP\textsuperscript{+} cells is identified lateral to the Atoh1\textsuperscript{+} region, whereas YFP\textsuperscript{+} cells abnormally express Atoh1 and clearly invade the Atoh1\textsuperscript{+} domain in Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} mutants, as demonstrated by the sizeable overlap of both domains (Figure 4G). Hence, Hoxb1 normally restricts the Atoh1\textsuperscript{+} domain in r4, impinging in this way on a specific r4 dorsal fate, which is different from the Atoh1\textsuperscript{+} lineage-related ones of adjacent rhombomeres. In summary, these data show that Hoxa2 is involved in the formation of the r2/3/5-derived AVCN, whereas Hoxb1 and Hoxb2 are required in the specification of the r4-derived PVCN by imposing an r4-specific identity during auditory development.

Axon pathfinding defects of cochlear nuclei in Hox mutant mice

We next assessed whether deletion of specific Hox genes may have direct consequences on the VN connectivity pattern at postnatal stages. In P8 Hoxb1\textsuperscript{null} and Hoxb1\textsuperscript{lateCKO} mutants, we identified ectopic r4-derived YFP\textsuperscript{+} projections crossing the ventral midline and innervating the medial nucleus of the trapezoid body (MNTB), a normal contralateral target of r2/3 AVCN-derived fibers. These projections are never labeled by YFP in control mice, since they do not originate from the PVCN, the major source of r4-derived YFP\textsuperscript{+} CN projections (Figure 5A). Similarly, dextran injections in the PVCN of Hoxb2\textsuperscript{null} label ectopic projections to the contralateral MNTB (cMNTB) (arrowhead in Figure 5B), whereas in control mice the very few axons projecting ventrally normally innervate contralateral MOC neurons as part of the
Figure 3. The r4-derived VLL is affected in Hoxb1 and Hoxb2 mutant mice. (A) Schematic view of a sagittal brain section indicating the YFP⁺ r4-derived nuclei and projections. A strong reduction of the YFP⁺ VLL nucleus (arrowhead) and projections (arrow) in Hoxb1 mutants is observed. In constitutive mutants the reduction is much more severe than in conditional mutants, as quantified in (D). Adjacent sagittal sections show no Hoxb2 and Hoxa2-expressing cells in Hoxb1lateKO mutants, whereas cells in the reduced VLL of Hoxb1lateKO still express Hoxb2 and Hoxa2. Adjacent sections of another P8 pup confirm reduction of the VLL and indicate persistence of Gata3- and Gad67-expressing cells in both Hoxb1 mutants. No ectopic expression of VGlut2 is detected in the VLL region. (B) The VLL is reduced in Hoxb2ΔKO mutant pups, similarly to Hoxb1lateKO mutants, as indicated by expression of Gata3, Gad67, Hoxa2 and quantification in (D). (C) In contrast, Hoxa2null mutants show no significant changes in the VLL position and size quantified in (E). The apparently bigger shape is due to the slightly oblique sections in mutant compared to WT brains. (D) Histogram showing the percentage of the VLL area size in WT (set up to 100%) and in the different genotypes as indicated on the y-axis. Mutants show statistically significant differences when compared to WT, or when Hoxb1lateKO and Hoxb2ΔKO are compared to Hoxb1null (inter-genotype comparison, ANOVA p<0.001; Hoxb1null versus WT: p = 0.001; Hoxb1lateKO versus WT: p = 0.01; Hoxb2ΔKO versus WT: p = 0.04; Hoxb1lateKO versus Hoxb2ΔKO: p<0.001;
MOC reflex (arrow in Figure 5B). This is in keeping with the finding that Atoh7 is increased in the PVCN of Hoxb1null, Hoxb1lateCKO and Hoxb24KO mutants (Figure 4B, 4C), and the notion that AVCN Atoh7+ neurons normally target the MNTB [10,53]. Thus, the molecular identity transformation of PVCN to AVCN observed in Hoxb1 and Hoxb2 mutant mice is further supported by abnormal connectivity to their respective targets (Figure 5C).

To directly investigate the role played by Hox2 in PVCN connectivity, we crossed the Hoxa2flox allele with a Wnt1::Cre driver [70] that allowed cell-autonomous inactivation of Hox2 at early stages in rhombic lip (and neural crest) derivatives. Wnt1::Cre;Hoxa2flox/flox mutants die around birth due to impaired neural crest development [71], thus preventing postnatal analysis of cochlear nuclei axon connectivity and functional impact on auditory function. Nonetheless, anterograde tracing by dextran injection allowed cell-autonomous inactivation of Hox2 at early stages in rhombic lip (and neural crest) derivatives. Wnt1::Cre;Hoxa2flox/flox brains at E18.5 reveal a neuronal population aberrantly innervating the ipsilateral MNTB (mMNTB) in mutant brains (arrowhead in Figure 5D). This phenotype is reminiscent, though less prominent, of the defects observed in the Robo3/Robl mutant mice, in which PVCN projections are prevented from crossing the midline and accumulate ipsilaterally [72]. Analysis in E13.5 control and Hoxa2 conditional mutant mice revealed that Robl expression is indeed selectively down-regulated, though not completely abolished, in the cochlear column (arrow, Figure 5D; and data not shown). Interestingly, Robl expression is not affected in the anterior extramural migratory stream (Figure 5D), derived from the precrerebellar Wnt7a lineages domain, where Hoxa2 regulates the expression of the slit receptor Robo2 [35]. Furthermore, Robl expression is not affected in Hoxb24KO mutants (data not shown). Thus, our data show that Hoxa2 selectively regulates Robl expression during the guidance of contralateral PVCN projections.

Abnormal specification and innervation of olivocochlear neurons in Hoxb1 and Hoxb2 mutant mice

The OC neurons become subdivided into medial and lateral components (MOC, LOC). The MOC neurons support OHC maturation at early postnatal stages and regulate the pre-synaptic-inhibited vibration of OHCs in the cochlea [12,21], and the LOC neurons, jointly with the MOC neurons, protect the cochlea from acoustic damage [22,31,32]. We next assessed whether the axonal behavior of efferent MOC neurons and synaptic MOC terminals on OHCs are affected in P8 and adult Hoxb1 mutant mice.

From E18.5 onwards, MOC motor axons have reached the contralateral cochlea. We thus injected Dil or dextran in Hoxb1null, Hoxb1lateCKO, Hoxb24KO or Hoxb24KO mutant mice, and in their respective controls, to retrogradely label the fluorescent bundle of MOC efferent fibers crossing the midline (Figure 6A). No crossing axons are identified in P8 Hoxb1null and Hoxb1lateCKO mutants (Figure 6B), in keeping with the lack of OC molecular markers observed at earlier stages (Figure S3). While the MOC bundle develops normally in E18.5 Hoxb14KO mutant brains, no axons cross the midline in Hoxb24KO mutants (Figure 6C), albeit a few presumptive OC Gata3+ cells are detected at earlier stages (Figure S3C, S3F). This suggests that, either the few Hoxb24KO mutant MOC neurons fail to target the cochlea, or that our axonal tracing procedure is not sufficiently sensitive to label just a few crossing axons.

To further ascertain whether a few MOC axons may nonetheless reach the cochlea and establish synaptic contact in Hoxb1 and Hoxb2 mutant animals, we used transmission electron microscopy and looked for MOC terminals contacting OHCs in the organ of Corti (Figure 6D). In contrast to WT animals, in which 1 to 2 MOC terminals are normally seen in synaptic contact with individual OHCs (n = 4; 54 MOC terminals on 32 OHCs; Figure 6E, 6F), almost no MOC terminals are found in adult Hoxb1null cochleae (n = 4; 1 MOC terminal on 40 OHCs; Figure 6E, 6F). Similarly, a few residual synaptic contacts are identified in adult Hoxb1lateCKO (n = 6; 12 MOC terminals on 64 OHCs; Figure 6E, 6F) and Hoxb24KO mutant cochleae (data not shown), indicating that, although in highly reduced number, some MOC neurons are able to innervate OHCs in these mutant mice (Figure 6F).

Next, we investigated whether LOC neurons were properly specified in P8 Hoxb1 mutant mice. While ChAT and Th20 label the cholinergic population of LOC neurons, vGlut2 labels the glutamatergic population of the LSO, which is primarily derived from the Atoh7 lineage (Figure S7) [16]. No ChAT+ or Th20+ cells are found in the LSO of Hoxb1null brains, whereas very few positive cells can be identified in Hoxb1lateCKO mutant brains (Figure S7B). On the contrary, vGlut2 expression is only slightly decreased, particularly in Hoxb1null mutants, possibly because the LSO is only partially derived from r4, as previously shown [3,16]. Thus, the populations primarily affected in Hoxb1 and Hoxb2 mutants are the cholinergic LOC neurons. Since the LSO largely forms within r5, this implies a previously unnoticed migration of some r4-derived cholinergic LOC cells into r5, possibly accompanying in part the migration of FBM neurons.

In summary, our molecular and cellular data confirm the absence of MOC and LOC efferent neurons in Hoxb1null mutants, but reveal the residual presence of a few LOC connections and some LOC neurons in Hoxb1lateCKO and Hoxb24KO adult animals. This suggests that specification of OC neurons is primarily dependent on Hoxb1 expression in progenitor cells and on Hoxb2 expression in early post-mitotic neurons for their normal migratory and connectivity properties.

Abnormal morphology of cochlear hair cells in Hoxb1 and Hoxb2 mutant mice

The failure of MOC neurons to innervate OHCs and the absence of LOC neurons innervating IHCs might affect the correct development of cochlear hair cells and/or render them more susceptible to degenerative acoustic trauma [22,25,28,32,73]. To assess hair cell morphology in Hox mutant cochleae, we used scanning electron microscopy on the apical and basal turns of WT, Hoxb1null, Hoxb1lateCKO and Hoxb24KO cochleae. Since the medullary efferent system is mature prior to the onset of hearing [21], we performed this analysis at P8, when the interactions of MOC fibers with OHCs become established, and in 3-month-old animals, when the centrifugal cochlear connections are fully functional (Figure 7 and Figure S8). In this way, we could discriminate a developmental intrinsic defect of OHCs from a defect due to the absence of MOC/OHC interactions.
Figure 4. The cochlear nuclear complex is differently affected in Hoxb1, Hoxb2, and Hoxa2 mutants. (A) Ectopic YFP+ r4-derived cells (arrows) are observed in the cochlear microneuronal shell (msh) (limited by solid line) of P8 Hoxb1null and Hoxb1lateCKO mutant sagittal sections. These cells now express Pax6 indicating that they are granule cells (white arrowheads). (B) Hoxb2 expression is decreased in the PVCN of Hoxb1null and in Hoxb1lateCKO mutants (asterisks). On the contrary, Hoxa2 expression is increased and Atoh7, normally expressed at high levels only in the AVCN, is dramatically up-regulated in the PVCN of P8 Hoxb1 (B) and Hoxb2DKO mutant pups (C) (arrows). Arrowheads in WT indicate Hoxa2 and Atoh7 low-
expressing regions. (D) Formation of the AVCN is strongly affected in E18.5 Hoxa2-null brains, as seen by decreased expression of Atoh7 and Hoxb2 (arrows). (E) Summary schematic indicating that in the absence of Hoxb1 and Hoxb2, the PVCN (r4-derived in brown) has acquired AVCN-like features (r2/3-derived in yellow) and YFP+ cell (brown) contribute to the shell. (F) The dorsal-most regions of WT and Hoxb1null hindbrains at r3 and r4 levels on adjacent coronal sections hybridized with Atoh1 and revealed by YFP epifluorescence (indicate r4 levels). Atoh1 is expressed in progenitors and differentiating cells migrating along the lateral ridge. The Atoh1-expressing domain is reduced in r4 compared to r3 in WT, whereas an enlarged Atoh1-expressing domain (arrowhead) is identified in r4 of Hoxb1null embryos. (G) On adjacent coronal sections at E14.5, YFP+ (r4-derived) cells located more laterally, do not overlap with Atoh1+ cells in the presumptive cochlear nucleus (CN), which originates from the r2–r5 auditory pit. In the absence of Hoxb1, YFP+ cells invade the Atoh1+ domain, thus acquiring the Atoh1 fate of adjacent rhombomeres. Cb, cerebellum; CES, caudal extramigratory stream. Scale bars, 200 μm (A up panels, B, C, D), 50 μm (A bottom panels), 100 μm (E, G). See also Figure S2.

Normally, three rows of OHCs and one row of IHCs are orderly arrayed along the entire organ of Corti at both ages. High magnification images of the hair bundles of individual WT OHCs illustrate the normal three rows of stereocilia of increasing height arranged in the characteristic V-shaped morphology; the latter appears slightly wider at the basal than apical turn (Figure 7 and Figure S8; n = 8). We found no obvious differences in the shape and organization of OHCs at the apical and basal cochlear turns in Hoxb1null (n = 6) and Hoxb1-paseCKO (n = 4) pups at P8 (Figure 7A and Figure S8A), indicating a normal morphological development of the cochlea in young Hoxb1 mutant pups. However, when the architecture of the OHC area was assessed in 3-month-old animals, once the MOC/OHCs functional interactions are established and the cochlea has become fully responsive to sound, severe morphological defects in ciliar shape and organization of OHCs at the apical and basal cochlear turns appear (Figure 7B, 7C; Figure S8B; n = 6), indicating that the major defects occur predominantly at the cochlear apex. In contrast, in Hoxb1-paseCKO and Hoxb2nullOHCs, morphological defects of OHCs are less severe compared to those in Hoxb1null mutants, although a loss of OHCs is still statistically significant, with occasionally missing cells (Hoxb1-paseCKO, n = 6: average of 3.1/100.4 OHCs missing; Hoxb2null, n = 3: 1.3/100.1 OHCs missing; Figure 7D) and moderate OHC and IHC cilar malformations (Figure 7B, 7C). In any case, the major abnormalities observed in Hoxb1 and Hoxb2 mutants are the severe morphological defects in cilar shape and organization, rather than the OHC loss, which was negligible, even if statistically different between the genotypes. Finally, no abnormalities are observed in basal regions of Hoxb1-paseCKO and Hoxb2nullOHCs (Figure S8B; and data not shown).

Taken together, we found strong late postnatal morphological defects of outer hair cells in Hoxb1null mutants, particularly in the apical region of the cochlea where low-frequency sounds are normally perceived [11]. In addition, minor defects were detected in the cilar shape and number of hair cells in Hoxb1-paseCKO and Hoxb2nullOHCs, indicating that abnormal development of r4-derivatives in the auditory brainstem can affect, with different severities, the long-term survival and organization of cochlear hair cells.

Hearing loss in Hoxb1 and Hoxb2 mutant mice

The observed defects of the different components of the central auditory pathway leading to sound perception prompted us to test general auditory function in Hoxb1 and Hoxb2 mutant mice. We measured the auditory brainstem response (ABR), in which a series of electrical potentials evoked by auditory stimuli ranging from 110 to 40 dB SPL (Sound Pressure Level) are analyzed, determining the lowest decibel level, or threshold, at which a response peak is reproducibly present [74]. The sequence of waves of the ABR reflects the synchronous short-latency synaptic activity of many neurons in successive nuclei along the central auditory pathway. We first analyzed the ABR response in control, Hoxb1null and Hoxb1-paseCKO mice from 1 to 12 months of age (n = 30). While control mice have a normal 40 dB SPL threshold, the threshold is elevated to 90 dB SPL in Hoxb1null mice representative ABRs of 3-month-old mice (Figure 8A). Hoxb1-paseCKO mutant mice also show a pathologically elevated average threshold, although lower than that of Hoxb1null mice (Figure 8A, 8C). In all ages examined, the threshold values of the responding Hoxb1 mutant mice are significantly higher than those of control mice (Figure 8C). Analyzing all data with regard to age, we observed a progressive increase of the hearing threshold for all three groups, although with different severities (doubled for Hoxb1null and 1.6 times for Hoxb1-paseCKO with respect to WT), most likely due to a secondary degeneration of cochlear hair cells and/or related afferent neural structures [75]. Interestingly, our data do not show any differences in the latencies of the evoked waves (Figure 8B), suggesting that the auditory stimuli can seemingly travel normally along the successive nuclei of the central auditory pathway, once the decibel levels surpass the elevated threshold. Furthermore, we analyzed a group of Hoxb2null 3-month-old mutant mice (n = 5), together with their respective controls, and found an average threshold similar to that of Hoxb1-paseCKO mice at the same age (Figure 8D).

Taken together, our data demonstrate that in the absence of Hoxb1 or Hoxb2 function, low-amplitude sounds are not perceived in young and adult mice.

Environmental auditory stimuli are not the primary cause of auditory impairment in Hoxb1null mice

Previous studies have shown that the MOC and LOC neurons are required in protecting the cochlea against acoustic injury [22,28,31,32]. Thus, the lack of OC neurons observed in Hoxb1null mutant mice may render these mice particularly hypersensitive to environmental sounds and lead to severe OHC damage and hearing defects. To directly address this issue, a group of WT and Hoxb1null pups (n = 5) were placed in an acoustically isolated environment at birth, and their hearing capacity was tested after one month of age. We found that acoustically isolated Hoxb1null mice have the same threshold shift as non-acoustically isolated mice (Figure 8E). This suggests that the environment is not the primary cause of the auditory threshold defects observed in Hoxb1null mice. However, the absence of protection from acoustic injury might determine secondarily the progressively more drastic increase of threshold observed with age in mutants compared to WT mice [76].

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Discussion

Rhombomere 4 contribution to the auditory system

Our present r4-restricted fate map has confirmed previous studies, but also highlighted specific aspects that were not recognized before [6,16]. In particular, we found that r4 contributes primarily to the generation and specification of auditory nuclei involved in sound transmission and amplification, as well as in the establishment of specific sensorimotor auditory circuitry during development (Figure 1M). Previous studies showed that ventral r4 is responsible for the specification of distinct subtypes of motor neurons, such as facial branchiomotor and inner ear efferent neurons [21,44,61,77]; here, we show that dorsal r4 contributes to alar-plate-derived sensory components, such as the VLL, PVCN and DCN. In particular, cells migrating rostrally from r4 along the lateral lemmiscus tract form the VLL nucleus, whereas dorsal sensory cells remaining within r4 contribute massively to the CN complex (PVCN and DCN), as well as to the vestibular and trigeminal sensory columns (M.D., L.P., M.S., unpublished). Interestingly, we find that r4-derived cochlear sensory neurons form jointly with basal-plate-derived motor structures two distinct auditory sensorimotor feedback sub-circuits essential for proper hearing. Some PVCN neurons together with auditory efferent MOC neurons generated within r4 support the sound-evoked MOC reflex, which terminates directly on the OHCs of the cochlea and modulates within r4/r3 the gain of the cochlear amplifier [23,24,25,26]. Interestingly, VCN neurons are also involved in the MEM reflex loop [29] through the action of the facial motor nucleus, originated in ventral r4 and strongly affected in Hoxb1 mutant mice [44,61] (data not shown). This implies that a single rhombomere, in this case r4, contributes to various derivatives of the auditory pathway; these are distributed across several rhombomeres via selective migrations and structurally linked into functional circuits essential for proper hearing.

We also found that the majority of r4-derivatives do not overlap with the r3/r5-derived Atoh1+ lineage, which contributes to the AVCN and to the nuclei of the superior olive complex involved in the spatial localization of sounds [16,19]. Although further analyses are necessary to characterize the single populations derived from r4, our study suggests that r4 contributes more to inhibitory neurons (GABAergic and glycinergic) of the VLL and CN than to excitatory glutamatergic sub-populations. Firstly, we found that the VLL nucleus, which contains a majority of inhibitory Gad67+ neurons, is mainly an r4-derivative, differentially from the VGlut2+ DLL, which is excitatory and originates primarily from the Atoh1+ lineage [17,19,51]. Secondly, r4-derived rhombic lip cells do not contribute to the cochlear granule cell populations, nor to octopus and globular bushy cells, all of which are glutamatergic Atoh1-derivatives [15,53]. Thirdly, the change of r4 to r3 identity as a result of Hoxb1 inactivation in Hoxb1 and Hoxb2 mutants, leads to an increase of the excitatory populations (such as the cochlear granule cell population) and decrease of the inhibitory cell types (such as the Gad67- and Gata3-expressing populations in the VLL). Fourthly, the Atoh7+ glutamatergic neurons, which derive from the Atoh1-expressing neuroepithelial regions, are massively increased in the PVCN of Hoxb1 mutant mice. Accordingly, mutant r4/YFP neurons ectopically project to the MNTB nucleus, as normally done by AVCN cells originating from the Atoh1+ lineage in r3 [16,72]. Finally, we observed an increased dorsal r4 Atoh1+ domain in Hoxb1+ mutants; this may be correlated to the ectopic production of glutamatergic granule cells and Atoh7+ neurons in the mutant r4-derived CN.

We therefore propose that Hoxb1 is indirectly involved in regulating and/or modulating the ratio between inhibitory and excitatory neurons in the r4-derived auditory circuits. Since r4 is changed to a more rostral identity (r3) in absence of Hoxb1 function, the rhombomere-specific ratio between GABAergic/ glycinergic and glutamatergic neuronal fates might be consequently altered. Previous studies have shown that Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord and cerebellum [78,79,80], and is required for inhibitory GABAergic and glycinergic fate in the cochlear nucleus [15]; hence, it is plausible to speculate that Ptf1a expression in the r4 ventricular zone may act downstream of, or together with Hoxb1 in the determination of r4-specific inhibitory features; altered Ptf1a expression may be responsible for the loss of GABAergic neurons in the VLL and/or for the ectopic expression of Atoh1, production of glutamatergic granule cells and Atoh7 neurons in the r4-derived CN.

However, some Atoh1+ cells do derive normally from r4, even if the corresponding progenitor domain is reduced compared to that of other rhombomeres, and might thus also partially contribute to the glutamatergic lineage. Intersectional long-term fate mapping between r4 and neuronal subtype-specific mouse lines together with careful characterization of individual sub-populations will be required to fully elucidate this aspect.

Distinct regulation of Hoxb2 and Hoxa2 expression by Hoxb1 during patterning of sensory r4-derived neuronal structures

Previous studies reported that Hoxb1 together with Hoxb2 are crucial in impinging on an r4 identity during rhombomere patterning, and that ventral r4 is changed into a more anterior identity in the absence of Hoxb1, based on ectopic expression of markers and abnormal behavior of FBM neurons [34,37,38,44,61]. Here, we show that a similar regulation occurs also in dorsal r4 during the specification of auditory sensory derivatives. We found a re-patterning of r4 into r3 identity in Hoxb1 mutants, in which Hoxb1 is either constitutive (Hoxb1+), or conditionally (Hoxb1+baso) eliminated in r4, and in Hoxb2+baso.
mice, in which Hoxb1 expression fails to be maintained in r4. In the absence of Hoxb1, Hoxb2 expression is reduced and Hoxa2 expression is increased in r4 at levels similar to those in r3, leading ultimately to the loss of specific r4-derived auditory nuclei (VLL and PVCN) and the ectopic formation of r3-like derived structures (AVCN and cochlear granule cells).

We thus conclude that within this sensory system Hoxb1 and Hoxb2 are critically required during the specification of r4-derived structures, and that in their absence, r4 alar and rhombic lip derivatives largely acquire r3-like features. This is based on our data and is consistent with previous intersectional fate mapping.
Figure 7. Late degeneration of OHCs in the apical turn of Hoxb1 and Hoxb2 mutant cochleae. (A) Scanning electron microscopy (SEM) views of the cochlea at P8: an overview of the apical turns of WT, Hoxb1null and Hoxb1lateCKO cochleae showing three orderly arrayed rows of outer hair cells (OHCs) and one row of inner hair cells (IHCs). Representative high magnification images illustrate stereocilia of hair bundles of single OHCs arranged according to their different lengths. Shape and organization of OHCs in apical regions are normal at this stage in both mutants. (B) SEM views of 3-month-old WT, Hoxb1null and Hoxb1lateCKO cochleae and representative higher magnification images of OHCs. In Hoxb1null and Hoxb1lateCKO cochleae, OHCs have lost their regular organization and fail to develop in some areas (white arrowheads). Moreover, in Hoxb1null cochleae most stereocilia have completely lost their typical V-shaped morphology and their characteristic differences in lengths (arrows). OHCs are less severely affected in Hoxb1lateCKO cochleae. IHC cilia appeared weakly disarranged (red arrowheads). (C) SEM views of 3-months-old WT and Hoxb2ΔKO mutant cochleae and higher magnifications of representative OHCs. Note that, similarly to Hoxb1lateCKO cochleae, Hoxb2 mutants have occasional missing OHCs (white arrowheads), disarranged IHC cilia (red arrowheads) and disorganized OHC stereocilia (arrows). (D) Histogram quantifying the percentage of OHC loss in controls, Hoxb1null, Hoxb1lateCKO and Hoxb2ΔKO cochleae. While controls (n = 8) showed no OHC loss, in Hoxb1null mutants (n = 6) 7.2 ± 0.8% of OHCs were absent, whereas 3.1 ± 0.9% and 1.3 ± 0.5% were lost in Hoxb1lateCKO (n = 6) and Hoxb2ΔKO (n = 3) cochleae, respectively. Inter-genotype ANOVA p < 0.001; Hoxb1null versus WT: p < 0.001; Hoxb1lateCKO versus WT: p < 0.001; Hoxb2ΔKO versus WT: p = 0.02. Scale bars, 10 μm (A, B, C, left panels), 1 μm (A, B, C, right panels). See also Figure S8.

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with r2 and r3/r5-specific enhancers together with the selective inactivation of Atoh1 in r3 and r5 [6,16].

In addition, we observed that Hoxb2\textsuperscript{DKO} and Hoxb1\textsuperscript{lateCKO} mutants reproduce a similar phenotype. Indeed, although early Hoxb1 expression is able to partially specify r4 identity in Hoxb1\textsuperscript{lateCKO} and Hoxb2\textsuperscript{DKO} mutants, failure to maintain Hoxb1 at later stages inhibits further development of r4-derived structures, leading ultimately to a milder phenotype when compared to constitutive Hoxb1 null mutants. We observed slight differences in the phenotypic severity of these two mutant lines, which might essentially be due to the differences in timing of Hoxb1 inactivation in r4.

Importantly, our data show that in r4 Hoxb2, besides being involved in maintaining high levels of Hoxb1 in progenitor cells (our study and [37,38]), also plays a key role in relaying r4-dependent regional fate to post-mitotic cells. In support of this, we show that Hoxb2 is expressed in both r4-derived progenitors and post-mitotic neurons, and that its expression is maintained in r4-derived structures, such as the VLL, VCN and OC.

A different situation applies for Hoxa2, which plays crucial roles in cell migration and axonal connectivity during hindbrain development, and whose expression is also maintained in the auditory sensory nuclei [36,66]. Hoxb1 expression is not affected in Hoxa2\textsuperscript{null} mutants, as previously reported [66] and, accordingly, no defects were observed in VLL and MOC development, two r4-derived structures. However, we found that Hoxa2 expression in r4 is increased in Hoxb1 and Hoxb2\textsuperscript{DKO} embryos, both in progenitors and, importantly, in post-mitotic cells. This ectopic expression is maintained in the postnatal PVCN of these mutants together with...
higher expression of Atoh7, ultimately leading to a change of PVCN to AVCN identity, as confirmed by a corresponding abnormal connectivity pattern. Thus, we propose that Hoxb1 negatively modulates Hoxa2 expression levels in r4. In the absence of Hoxb1, ectopic up-regulation of Hoxa2 in PVCN changes r4-specific neuronal properties and drives cochlear neurons to innervate an inappropriate target, the MN TB cells, normally innervated by r3-derived AVCN axons [72].

Taken together, we show that while Hoxb1 and Hoxb2 establish and maintain the regional identity of r4 progenitors (Hoxb1 and Hoxb2) and possibly of their derivatives (Hoxb2), Hoxa2 is normally expressed at low levels in differentiating cells of r4 and thus plays only a minor role in patterning r4-derived sensory structures.

**Hoxa2 regulates axon guidance receptor Rig1 expression in the auditory lip**

In this study, we found that in AVCN, Hoxa2 controls the expression of the Slit receptor Rig1/Robo3, known to regulate crossing of the midline by commissural axons in the hindbrain [72]. In Wnt1::Cre;Hoxa2flox/flox mutants, in which Hoxa2 is conditionally inactivated solely in rhombic lip and neural crest cells, the CN complex is properly formed but many axonal projections from the AVCN fail to reach the contralateral MN TB, indicating that Rig1 is directly involved in these axonal defects.

Concerning possible ways by which Hoxa2 may regulate Rig1 expression, it is noteworthy to mention that Rig1 expression is not affected in the anterior extramural migratory stream derived from the posterior precerebellar Wnt1 domain, where Hoxa2, instead, directly regulates the expression of another Slit receptor, Robo2 [35]. Thus, Hoxa2-mediated regulation of Rig1 is mostly evident in the most rostral domain of Hoxa2 activity (i.e., in the r2–r5-derived auditory lip), whereas in the r6–r8-derived precerebellar lip, the role of Hoxa2 may be functionally compensated by other Hox factors of the paralogue groups 3–5. Alternatively, Hoxa2-dependent regulation of Rig1 might require a specific co-factor only present in r2–r5 rhombic lip derivatives. While Hoxa2 is required for the expression of Rig1 throughout the r2–r5 auditory lip column, Rig1 is normally expressed in Hoxa2KO mutants (data not shown), strongly suggesting that Hoxa2 expression in Hoxb2 (and, likely, Hoxb1) knockout cells is sufficient to support normal Rig1 transcriptional regulation and thus, drive the contralateral ectopic projections of PVCN mutant neurons. However, based on the available Rig1 functional data [72], we predict that the Rig1 function by itself is not sufficient to switch PVCN-to-AVCN target specificity (i.e. to target the MNTB), holding that Rig1 expression only confers the ability of axons to cross the midline. Thus, it is unlikely that the Hoxa2-mediated regulation of Rig1 alone could explain the target connectivity switch observed in Hoxb1 and Hoxb2 knockouts. Hoxa2 likely controls a larger downstream transcriptional program to provide r2/3 AVCN neurons with their proper regional identity and connectivity.

**Assembly of a sensorimotor auditory sub-circuit by Hoxb1 and Hoxb2**

We show that Hoxb1 and Hoxb2 mutants have increased auditory thresholds leading to severe hearing impairments. This phenotype is often associated with affected CN function [16] and/or alterations in the cochlear amplification mechanism executed by the OHCs [11,12,81]. Accordingly, we found defects in the CN complex and additional strong morphological damage of the OHCs. We exclude a direct role of Hoxb1 and Hoxb2 on hair cell development, since they are not expressed in presumptive hair cells [41,82]. We also exclude that a defect in satellite glial cells, surrounding the spiral ganglion neurons and originating from r4-derived neural crest (Figure S9), can affect OHC development and/or contribute to the altered auditory threshold. Even if we observed a decrease of double YFP+/Sox10+ cells in Hoxb1null cochleae, spiral ganglion neurons seem to differentiate properly and appropriately express Gani3 (Figure S9). Moreover, type II ganglion fibers innervating the OHCs are unmyelinated, different from type I fibers innervating the HHCs, indicating that the r4-neural crest-derived YFP Schwann cells myelinate mainly type I fibers. Furthermore, we rule out abnormalities of the second arch-derived middle ear ossicles potentially contributing to the auditory phenotype observed in this study, since they are unaffected in both Hoxb1 and Hoxb2 mutant mice [30,61]. Finally, we also exclude a involvement of LOC efferent neurons, which, even if affected in our mutants, appear to have no direct effect on cochlear thresholds measured by ABR [22].

Although we cannot ascertain the major structure responsible for the increased auditory threshold, we propose that abnormal development of MOC neurons, which are required for proper postnatal survival and functioning of OHCs during the hearing process [20], are involved in the hearing impairments of both mutants. In support of this, the strongest morphological hair cell abnormalities is found towards the apical region of the cochlea, where normally low frequency sounds are perceived [11]. Furthermore, early development of hair cells proceeds normally in the absence of efferent neurons but become affected at later stages when OHCs are dependent on proper MOC innervation [21]. Hence, degeneration of OHCs and consequently, altered hearing thresholds, might be caused by the absence of synaptic/trophic stimulation of cochlear hair cells from the centrifugal OC fibers during a postnatal critical period, which is essential for accurate maturation of OHCs [29]. More support comes from the observations that persistence of some MOC neurons innervating the OHCs in Hoxb1mutant and Hoxb2mutant mutant cochleae is sufficient to partially “rescue” the auditory threshold and OHC morphological defects. This occurs in the presence of seemingly comparable patterning and connectivity defects observed in the CN complex of Hoxb1 and Hoxb2 mutants. In addition, no differences in the latencies of the evoked responses are found in our ABR analysis, indicating that the auditory stimuli, when perceived, can travel normally along the successive nuclei of the central auditory pathway, even in the presence of abnormal CN and VLL.

Finally, we found altered hearing thresholds already in one-month-old Hoxb1mutant mice that were acoustically isolated at birth, and thus not exposed to noise. This rules out that the observed increased threshold is due to reduced function of the MOC and MEM reflexes, as well as LOC neurons that cannot protect the organ of Corti from noise-induced hearing damage. Nevertheless, this does not exclude that deficiencies in the efferent feedback systems are involved in the progressive age-related degeneration of hearing [76], which is more pronounced in mutant mice than in WT. Hence, the minor increase of threshold with age observed in the Hoxb1mutant compared to Hoxb1mutant might be due to the presence of a few efferent neurons in the conditional mice.

Thus, our data suggest that efferent innervations play an important postnatal role in the hearing impairment observed in Hoxb1 and Hoxb2 mutants, although we cannot completely rule out that altered development of other auditory structures might also contribute. Selective deletion of MOC efferents during development may further support this hypothesis.
Selective involvement of individual rhombomeres and Hox genes in patterning auditory circuits

Our data unravel a novel function for r4 and its crucial patterning genes, Hoxb1 and Hoxb2, in the ascending sound transmission pathway involving CN and VLL, as well as in the establishment of a sensorimotor reflex circuit formed by PVCN and MOC neurons. We found that within the cochlear nuclear complex, Hoxa2 expression is primarily maintained in the r2/r3-derived AVCN, whereas Hoxb2 is highly expressed in r4-derived structures, such as the PVCN, in the r2/r3-derived portion of AVCN and in r5/r6-derived granule cochlear cells. Both genes are expressed in the DGC, which is a r3/r4/r5 derivative ([6,36] and our study). Such partially overlapping and complementary expression patterns, already observed at early stages, might reflect distinct functional rhombomere-specific pathways within the auditory circuit. In this study we show that the absence of Hoxa2 mainly affects development of the r2/3-derived AVCN and their respective projections to the contralateral MNTB nucleus, [6,16]. This phenotype might alter a pathway crucial for sound localization [18]. In contrast, in the absence of Hoxb1 or Hoxb2 functions, PVCN acquires an r3-derived AVCN identity and the resultant functions fail to reach their normal targets, i.e. r4-derived VLL and MOC neurons, which are also affected. As a consequence, the efferent reflexes and the innervation of OHCs by MOC neurons are impaired and Hoxb1 and Hoxb2 mutants display hearing problems, although with different severities. Thus, Hoxb1 and Hoxb2 appear to act primarily upon r4-derived structures, contributing to the main pathway of sound perception, protection and amplification, whereas Hoxa2 seems to contribute to the sound localization circuitry centered in r3 and r5. In summary, our data support a model whereby rhombomere-specific (thus A-P) and alar- to basal-restricted pools of neurons (thus D-V) contribute to distinct functional pathways and circuits by maintaining differential expression levels of specific Hox gene combinations that, in turn, will continuously refine regional identity within the multi-segmental neuronal columns of the hindbrain (see also Model in Figure 1L, 1M).

Materials and Methods

Generation of Hox mouse mutant lines and matings

For the detailed generation of the b1r4-Cre transgenic line, the Hoxb1 fos and Hoxb1mol mutant mice, and the Hoxb2floxed line see the Protocol S1 section. Generation of the Hoxa2mol and Hoxa2flos alleles are described in other studies [65,83]. The b1r4-Cre mice were crossed with the ROSA26YY reporter line [43] to obtain double knockin and the b1r4-Cre/TFP transgenic line to obtain Hoxb1 flox/flox in which Hoxb1 is inactivated exclusively in r4 at around E9.5. Similarly, Hoxb1 flos mice were mated to the b1r4-Cre/TFP transgenic line to obtain Hoxb1 flox/flox in which Hoxb1 is inactivated exclusively in r4 at around E9.5. Similarly, Hoxb1 flos mice were mated to the b1r4-Cre/TFP line to permanently label r4 and r4-derivatives in a null background. All experiments were conducted following guidelines of the Institutional Animal Care and Use Committee of the Cardarelli Hospital, Naples, Italy, the University of Nice Sophia-Antipolis, Nice, France and the Friedrich Miescher Institute, Basel, Switzerland.

Tissue preparation

Adult and P8 mice were perfused with 4% paraformaldehyde (PFA). Embryos, brains and cochleae were fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline, pH 7.4 (PBS). Tissues were cryoprotected with 10, 20 and 30% sucrose in PBS and frozen in OCT embedding matrix (Kaltek) and sectioned at 14 μm (E10.5 brains; transversal plane), 16 μm (E10.5 embryos sagittal plane; E14.5 to E18.5 brains), or 20 μm (P8 and adult brains).

Immunohistochemistry and Nissl staining

Tissue cryosections or whole mount dissected cochleae were incubated overnight at 4°C with primary antibodies diluted in blocking buffer (5% goat serum, 1% BSA, 0.3% Tween 20 in PBS): rabbit anti-GFP (1:500, Chemicon), mouse anti-Cre (1:200, BABCO), rabbit anti-Pax6 (1:100, Chemicon), rabbit anti-Hoxb1 (1:200, Covance), rabbit anti-Phox2b (Pattyn et al., 1997) (1:750, gift from C.Goridis), mouse anti-Gata3 (1:50, Santa Cruz), mouse anti-Isole (1:300 clone 39.4D5; Developmental Studies Hybridoma Bank), rabbit anti-calbindin (1:2500, Swant), guinea pig anti-Sox10 (1:1000, gift from M. Wegner [84]), rabbit anti-Atoh1 (1:500, gift from J. Johnson). For Atoh1 antibody immunofluorescence, a particular blocking buffer was used (1% goat serum, 0.1% Triton-X). Tissue was washed in PBS 0.1% Triton-X and incubated for an hour at room temperature with secondary antibodies: Alexa Fluor 488 (green) goat anti-mouse and goat anti-rabbit, Alexa Fluor 568 (red) goat anti-mouse, goat anti-rabbit and goat anti-carboxyanine dye DiI (Molecular Probes) was placed unilaterally in the cochlea (exposed through the base of the cranium) and allowed to diffuse for 1 to 3 months (the first two months at 37°C; afterwards at RT) in PBS containing 0.025% sodium azide. Rhodamine-conjugated dextran (Molecular Probes, Eugene,
Oregon) was employed for tracing the MOC, AVCN and PVCN projections of WT (n = 5), Hoxb2 (n = 3) and Hoxa2 (n = 2) mutants. E18.5 and P8 heads (MOC tracing) and brains (AVCN and PVCN tracing) were dissected and dextran crystals were inserted unilaterally into cochlea, AVCN and PVCN regions, respectively. The embryos were incubated for 8–12 hours as described in [7]. All brains were fixed in 4% PFA, embedded in 3% agarose in PBS and vibratome-sectioned (100 µm-thick).

**Auditory brainstem response (ABR) and statistical analysis**

ABR recordings were performed as previously reported [86] and described in the Protocol S1. The graph plot mean ± SE (standard error) statistics for dual comparisons and were generated using Student’s t-tests, whereas statistics for multiple comparisons were generated using one-way analysis of variance (ANOVA) followed by a suitable post hoc t-test; 0.01 ≤ p < 0.05, **0.001 ≤ p < 0.01, ***p < 0.001 for all statistics herein.

**Scanning electron microscopy (SEM)**

For SEM, cochleae were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4 (19 ml of 0.2 M sodium phosphate monobasic NaH2PO4 and 81 ml of 0.2 M sodium phosphate dibasic Na2HPO4) for 4 h at 4°C and rinsed in PBS over night. The organs of Corti were isolated, rinsed in PBS and post-fixed in 1% OsO4 in the same buffer for 1 h at 4°C. After several rinses in PBS, the cochleae were separated in apical and basal turn and then the specimens were subjected to serial dehydration followed by critical point drying. The samples were mounted on aluminum stubs and sputter coated with gold. The processed specimens were investigated and photographed using a JEOL 6700F SEM operated at 5 kV and at a 8.3 mm working distance. SEM images were collected digitally.

**Transmission electron microscopy (TEM)**

For TEM, cochleae were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4 for 4 h at 4°C and rinsed in PBS over night. The organs of Corti were isolated, rinsed in PBS and post-fixation was based on 1% osmium tetroxide solution (Electron Microscopy Sciences) and dehydrated in a series of ethanol (Electron Microscopy Sciences) for 10 min at room temperature and 2.66% lead citrate (Electron Microscopy Sciences) for 8 min at room temperature. Grids were examined using a Philips EM 208 S transmission electron microscope (Philips) operating at 80 kV.

**Quantification and statistical analysis**

The VLL area was quantified on adjacent sections of P8 WT, Hoxb1null and Hoxb2null brains and Hoxa2null and Hoxa2null brains hybridized with Gad67. The area of the tissue was measured using the MacBiophotonics Image J software. The sum of the area of all sections was expressed as a percentage of the VLL area in wt.

The loss of outer hair cells (OHCs) on SEM sections was quantified on 10 non-overlapping areas of 3060 µ², considering at least one area per sample. Quantitative data are depicted as mean with standard error (SE) of the mean obtained from at least 3 pups or animals tested for significance by the unpaired Student’s t-test or for multiple comparisons by the one-way analysis of variance (ANOVA) followed by a suitable post hoc t-test; 0.01 ≤ p < 0.05, **0.001 ≤ p < 0.01, ***p < 0.001 for all statistics herein.

**Supporting Information**

**Figure S1** Generation of a novel r4-restricted Cre driver line and expression of the Cre-recombinase. (A) Schematic diagram of the b1r4-Cre-recombinase construct. The gene of the Cre-recombinase is cloned downstream of the Wnt1 basic promoter [87] and under the control of the Hoxb1 r4 enhancer [42]. (B) Genotyping of the b1r4-Cre mice by PCR using internal primers for Cre and primers for actin as an internal PCR control. (C) Lateral and dorsal views of E8.0 to E8.25 embryos hybridized with Cre-recombinase. Note that Cre-recombinase starts to be expressed around E8.0 in a patchy way. (D) At E9.0 Cre is expressed in all cells and restricted solely to r4. (TIF)

**Figure S2** Dorsal r4-derivatives are complementary to the Atoh1- and Barhl1-positive rhombic lip regions and contribute to the cochlear nucleus. (A) Schematic of an E10.5 coronal section indicating the position of the rhombic lip region, from which the adjacent pictures are taken. Details of the dorsal region of r4 indicate that Atoh1+ cells express YFP, as seen by the merging of the two images. (B) Schematic of an E14.5 coronal section through the rhombic flexure, in which the position of upper and lower rhombic lip cells (URL, LRL) appears colored in brown. The boxed area indicates the region shown in the adjacent panels. Only a few r4/YFP+ cells co-localize with Atoh1-expressing cells, but no Barhl1-expressing cells are positive for YFP, as also seen in high magnification details. (C) Schematics illustrating the position of the cochlear area in a lateral parasagittal plane. The boxed area indicates the magnified region shown in D and E. (D, E) Immunodetection of YFP protein at E14.5 and E16.5 illustrates progressive migration of the r4 lower rhombic lip (LRL)-derived YFP-positive cells to the cochlear nuclear complex (CN), cp, choroid plexus; cb, cerebellum. Scale bars, 20 µm (A), 200 µm in (B), 50 µm insets in (B), 100 µm in (D, E). (TIF)

**Figure S3** Targeting strategy to generate Hoxb1floxneo embryonic stem (ES) cells. (A) Schematic diagram of the Hoxb1 locus, the targeting construct and the Hoxb1floxneo targeted allele. The construct contains a loxp site upstream of the r4 enhancer and two loxP sites flanking the positive selector, the neomycin gene, in the intron; a negative selector, the Diphtheria Toxin subunit A (DTA), is located downstream of the 3′ homology region. Two heterospecific FRT sites are inserted internal to the two first lox sites; these sites can be used to knock-in any putative target gene into the Hoxb1 locus with the help of the recombinase-mediated cassette exchange (RMCE) technology [88]. The Hoxb1floxneo allele was obtained by homologous recombination between the 5′ 2.3 kb EcoRV-Apal and the 3′ 6.6 kb ScaI-SacI Hoxb1 genomic regions. (B–E) Identification of Hoxb1floxneo ES cells by PCR (B, C) and Southern blot (D, E). PCR specific primers (arrows in A) discern wt and recombinant alleles (B) and amplify the three loxP sites (C). Southern blotting confirms proper homologous recombination after digestion genomic DNA with BamHI and using a 5′ internal probe (D), resulting in a 6.7 kb-long fragment for the wt allele and a 3.5 kb-long fragment for the recombined Hoxb1floxneo allele, and after digestion with SmaI-Ndel and using a 3′ internal probe (E) giving 8.0 kb and 9.6 kb fragments for the wt and Hoxb1floxneo alleles, respectively. The probes and the restriction fragments are indicated in A. The asterisks indicate non-homologous recombi-
nant clones. The arrow indicates the clone used to generate Hoxb<sup>fl</sup> and Hoxb<sup>fl</sup> ES cells.

**Figure S4** Targeting strategy to generate Hoxb<sup>fl</sup> and Hoxb<sup>fl</sup> mice. (A) Diagram of Hoxb<sup>l</sup> locus and Hoxb<sup>fl</sup>, Hoxb<sup>fl</sup> and Hoxb<sup>l</sup> targeting alleles. To obtain “floxed” and “null” alleles, a Hoxb<sup>Fl</sup> clone was electroporated with a plasmid expressing Cre-recombinase, which excises the regions between the different combinations of two lox sites, generating in this way distinct types of alleles. The Hoxb<sup>fl</sup> allele, obtained by excision of the neomycin (neo) gene, contains the FRT/lox sites flanking the Hoxb<sup>l</sup> genomic region that was conditionally ablated after mating with the r4-Cre-recombinase line. In the Hoxb<sup>l</sup> allele the region flanked by the FRT/loxP sites is also excised. (B, C) Identification of Hoxb<sup>fl</sup> and Hoxb<sup>l</sup> ES clones by PCR (B) and Southern blot (C). Three different PCR reactions (B) were used to identify the wt, Hoxb<sup>fl</sup>, and Hoxb<sup>l</sup> ES clones by PCR (B) and Southern blot (C). Southern blot analysis (C) confirms the presence of the recombined clones. 2.2 kb- (wt), 3.4 kb- (Hoxb<sup>l</sup>), 5.9 kb- (Hoxb<sup>fl</sup>) and 7.5 kb- (Hoxb<sup>l</sup>) long fragments were obtained after ScaI-EcoRV genomic digestion and using an internal probe. The probe and the restriction fragments are indicated in A. (D) Genotyping of Hoxb<sup>fl</sup> and Hoxb<sup>l</sup> mice by PCR to identify wt, homozygous and heterozygous mutant mice.

**Figure S5** Abnormal development of olivocochlear (OC) efferent neurons in Hoxb<sup>l</sup> and Hoxb<sup>2</sup> mutant embryos. (A) Lateral view of the E10.5 r4/YFP<sup>+</sup> embryo; the line indicates the plane of section. To the right, schematic representation of a coronal section illustrating the areas shown in B. (B) No inner ear efferent (IEE) neurons, identified as double Gata3/Phox2b- or double Gata2/Isl1-expressing cells, are detected in E10.5 Hoxb<sup>l</sup> and Hoxb<sup>l</sup> mutant embryos. In addition, the population of Isl1<sup>+</sup> cells is reduced in both mutants. The asterisks indicate absence of Phox2b in r4 progenitors, as previously described [59]. (C) In ventral r4 of E10.5 Hoxb<sup>2</sup> mice, Isl1<sup>+</sup> visceral motor neurons (VMN) (which include FBM and IEE) and Gata3<sup>+</sup> IEE are present, but reduced. (D) Schematic representation of an E14.5 sagittal section indicating the plane of section and the corresponding coronal section. (E) The small group of OC neurons, normally positioned at the r4/r5 margin and positive for YFP, ChAT and Gata3<sup>+</sup> cannot be identified in Hoxb<sup>l</sup> and Hoxb<sup>l</sup> mutant embryos. (F) In contrast, a tiny but compact group of cells positive for Gata3 and Tbx20 can be identified in E14.5 Hoxb<sup>2</sup> mutant embryos, although in a more dorsal location than normal and close to the abnormally positioned ‘FBM’ nucleus, previously described [64]. fp, floor plate; vz, ventricular zone; mz, marginal zone; FBM, facial branchiomotor neurons; VLLa, nucleus of lateral lemniscus. Scale bars, 100 μm (B, C); 200 μm (E, F).

**Figure S6** Reduced VLL in Hoxb<sup>l</sup> mutant brains at E18.5. (A) Schematic view of a brain; the red line shows the plane of sections. (B) Adjacent coronal sections of E18.5 WT, Hoxb<sup>l</sup> and Hoxb<sup>l</sup> mutant brains stained for Nissl and YFP, and hybridized with Gata3, GABA<sub>ergic</sub>/glycinergic Gad67 and glutamatergic vGlut2 markers. Only a few YFP<sup>+</sup> and Gad67<sup>+</sup> scattered cells are identified in Hoxb<sup>l</sup> YLL at this stage, whereas no Gata3<sup>+</sup> (though some cells are recovered at P8) and vGlut2<sup>+</sup> neurons are detected (arrows). The reduction of the VLL and relative expression of its markers is less severe in Hoxb<sup>l</sup> mutants (arrowheads), as also confirmed postnatally (see Figure 3). No ectopic expression of vGlut2 is detected in the VLL region of mutant mice. The DLL and the PN are not affected. VLL, ventral nucleus of lateral lemniscus; DLL, dorsal nucleus of lateral lemniscus; PN, pontine nucleus; IC inferior colliculus; FBM, facial branchiomotor neurons; MG, medial geniculate nucleus. Scale bars, 200 μm.

**Figure S7** Cholinergic LOC neurons are strongly affected in Hoxb<sup>l</sup> mutant brains. (A) Adjacent coronal sections of ventral P8 WT brains stained with the cholinergic marker ChAT and the transcription factor Tbx20, labeling LOC and MOC motor neurons (red arrowhead), and the glutamatergic marker vGlut2 expressed by LSO neurons. The boxes in A indicate the area where the high magnifications of (B) are taken. (B) High magnifications of WT, Hoxb<sup>l</sup> and Hoxb<sup>l</sup> mutant brains. The cholinergic population is the most severely affected population in Hoxb<sup>l</sup> mutant brains, as seen by complete absence of ChAT- and Tbx20-expressing MOC and LOC neurons in Hoxb<sup>l</sup> and the presence of only few LOC neurons in Hoxb<sup>l</sup> mice (black arrowhead). On the contrary, the glutamatergic population in the LSO is almost preserved, although slightly less vGlut2<sup>+</sup> neurons are found, particularly in Hoxb<sup>l</sup>, in the region derived from r4 (shown in Figure 1F). LSO, lateral superior olive nucleus; LOC, lateral olivocochlear neurons; MOC, medial olivocochlear neurons; Vn trigeminal motor nucleus. Scale bars, 400 μm (A), 200 μm (B).

**Figure S8** Moderate outer hair cell abnormalities in basal turns of Hoxb<sup>l</sup> cochleae. (A) Scanning electron microscopy (SEM) views of the cochlea at P8: an overview of basal turns of WT, Hoxb<sup>l</sup> and Hoxb<sup>l</sup> cochleae and representative high magnifications of OHCs. In basal turns the typical V-shaped morphology of OHCs is slightly enlarged compared to their counterparts in the apical turn. Shape and organization of OHCs in the basal cochlear region are not affected in Hoxb<sup>l</sup> mutants. (B) SEM views of 3-month-old cochleae: an overview of basal turns of WT, Hoxb<sup>l</sup> and Hoxb<sup>l</sup> cochleae and representative high magnifications of OHCs. In Hoxb<sup>l</sup> and Hoxb<sup>l</sup> cochleae OHCs maintain their regular organization. Slight abnormalities in stereocilia organization and orientation are present only in basal turns of Hoxb<sup>l</sup> (arrows), but not of Hoxb<sup>l</sup> cochleae. OHCs, outer hair cells; IHCs, inner hair cells. Scale bars, 10 μm (A, B left panels), 1 μm (A, B right panels).

**Figure S9** Spiral ganglion glial cells originate from r4. (A) Dissected whole-mount cochleae of WT and Hoxb<sup>l</sup> P8 pups immunostained with an anti-GFP antibody (which cross-reacts with the endogenous YFP) indicate that r4 neural crest cells contribute to glial cells required for myelination of spiral ganglion (spg) neurons and their projections. Note that in Hoxb<sup>l</sup> cochleae YFP<sup>+</sup> cells are still present although in reduced number. (B) Details of cochleae on adjacent sagittal sections of E18.5 heads immunostained with an anti-GFP antibody and hybridized with Gata3, a reliable marker for spiral ganglion neurons and hair cells in the organ of Corti (c) [81]. Gata3 expression is not changed in spg neurons of Hoxb<sup>l</sup> cochleae despite decreased YFP labeling. This indicates that spiral ganglion neuron differentiation is not affected in the absence of Hoxb<sup>l</sup>. (C) Adjacent sections of (B) immunostained with anti-GFP and the glial lineage marker Sox10 [84]. The merge YFP/Sox10 indicates that YFP<sup>+</sup> cells express Sox10 in the spiral ganglion (arrowheads in high magnification views of the area indicated in the boxes on the left). A reduced number of double YFP<sup>+</sup>/Sox10<sup>+</sup> (arrowheads) cells are present in Hoxb<sup>l</sup> mutants. Scale bars, 100 μm (A, C), 200 μm (B).

**Protocol S1**
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Author Contributions

Conceived and designed the experiments: MDB LP FMR MS. Performed the experiments: MDB VN LS BA GA. Analyzed the data: MDB VN BA LS AMF LP FMR MS. Contributed reagents/materials/analysis tools: BA LS AMF FMR. Wrote the paper: MS MDB FMR LP.

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