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**Genes Involved in the Development and Physiology**  
**of both the Peripheral and Central Auditory Systems**

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## **Abstract**

The genetic approach, based on inherited forms of deafness, has proven to be particularly effective for deciphering the molecular mechanisms underlying development of the peripheral auditory system, the cochlea and its afferent auditory neurons, and how it extracts the physical parameters of sound. Although this genetic dissection has provided little information about the central auditory system, scattered data suggest that some genes may have a critical role in both the peripheral and central auditory systems. Here, we review the genes controlling the development and function of the peripheral and central auditory systems, focusing on those with demonstrated intrinsic roles in both systems and highlighting the current underappreciation of these genes. Their encoded products are diverse, from transcription factors to ion channels, as are their roles in the central auditory system, mostly evaluated in brainstem nuclei. We examine the ontogenetic and evolutionary mechanisms that may underlie their expression at different sites.

**Keywords: hair cell, cochlea, auditory hindbrain, auditory cortex, evolution, gene regulatory networks**

## Abbreviations

AC	auditory cortex
cdhr	cadherin-related protein
CN	cochlear nucleus
CSF	cerebrospinal fluid
E	embryonic day
GRN	gene regulatory network
IC	inferior colliculus
IHC	inner hair cell
LL	lateral lemniscus
LNTB	lateral nucleus of the trapezoid body
LSO	lateral superior olive
MGB	medial geniculate body
MNTB	medial nucleus of the trapezoid body
mRNA	messenger RNA
MSO	medial superior olive
OHC	outer hair cell
r	rhombomere
SGN	spiral ganglion neuron
SOC	superior olivary complex
SPON	superior periolivary nucleus

## **Introduction**

In the course of evolution, auditory scene analysis conferred a selective advantage to animals for escaping predators and localizing preys through the detection and localization of sound sources, day and night. Due to its unique ability to process the fine temporal and spectral structures of sound sequences from which sophisticated acoustic characteristics are extracted, hearing has also become increasingly associated with communication between conspecific partners, culminating in the development of language and music. Sound-encoded information reaches the primary auditory cortex (AC) in humans within as little as 20-30 ms (Geisler et al. 1958), despite passing through a larger number of synaptic relays than in any other sensory system before reaching the primary sensory cortex. These amazing characteristics are the basis of low-frequency sound-source localization, speech intelligibility and pitch perception, which is essential for voice identification and the appreciation of music.

Over the last 25 years, a genetic approach based on the study of inherited hearing disorders has proven particularly fruitful for characterizing the development and functioning of the peripheral auditory system, revealing the role of neglected structures and elucidating the molecular networks involved in key cochlear functions (Richardson et al. 2011). Mouse models of various forms of human deafness have been shown to reproduce the impairment of the peripheral auditory system observed in humans faithfully, with only occasional differences. By contrast, information gathered by the genetic approach about the central auditory system has been provided mainly by the study of mouse mutants used to explore embryonic development and is, however, still limited today.

Since the discovery of the first gene responsible for deafness in both humans and mice, the gene encoding myosin-VIIa (Gibson et al. 1995; Weil et al. 1995), 110 genes responsible for non-syndromic forms of deafness (*Hereditary Hearing Loss - Hereditary Hearing loss Homepage*) and about 300 genes responsible for syndromic forms have been

reported in humans and/or mice. These monogenic forms of deafness were found to affect the peripheral auditory system, the cochlea and its afferent innervation. However, the genetic landscape of hearing impairment is much broader. A large number of genes responsible for rare forms of deafness, mostly syndromic (deafness forms associated with other symptoms) and associated with early morphogenesis deficits of the cochlea, remain to be identified. Moreover, the study of sensorineural age-related hearing impairment, presbycusis, a multifactorial disorder considered to result from the interplay between environmental (noise exposure, ototoxic medication) and hereditary factors, is still in its infancy but should lead to the identification of additional genes.

There are some reasons why so little information has been gathered on the central auditory system from the study of inherited hearing impairment. Central hearing disorders in humans are generally unexplored. Moreover, if an intrinsic auditory central deficit is associated with a peripheral one, it may be concealed by the peripheral deficit. Finally, it has been established that both the spontaneous electrical activity of the developing cochlea and acoustically driven cochlear activity trigger and shape central auditory pathway maturation (Levi-Montalcini 1949; Tritsch et al. 2010; Zhang et al. 2001). Distinguishing between intrinsic central auditory deficits and those resulting from peripheral deficits is therefore challenging in humans (Butler & Lomber 2013). In mice, cre-mediated recombination confining gene deletion (conditional knockout) or overexpression (conditional knock-in/overexpression) to a given tissue or cell type, and the use of an inducible cre fusion protein, Cre-ERT2 (tamoxifen-inducible cre) overcome these issues.

*KCNQ4*, encoding a K<sup>+</sup> channel mediating an outwardly rectifying current and underlying the DFNA2 dominant form of human deafness, was the first identified gene playing a crucial role in the peripheral auditory system for which possible involvement in central auditory pathways was suggested (Beisel et al. 2000; Kharkovets et al. 2000; Kubisch

et al. 1999). After the first compulsory central synaptic relay in the cochlear nucleus (CN), sound-encoded information from the peripheral auditory system is dispatched to no fewer than 15 auditory nuclei from the auditory brainstem to the medial geniculate body (MGB) of the thalamus, for parallel processing (Cant & Benson 2003). The five primary central sound processing stations up to the AC are the brainstem nuclei: the hindbrain-derived nuclei of the CN (anteroventral, posteroventral and dorsal nuclei), the superior olivary complex (SOC) (medial and lateral superior olive (MSO and LSO), superior periolivary nucleus (SPON), medial and lateral nucleus of the trapezoid body (MNTB and LNTB)), the lateral lemniscus (LL) complex (ventral, intermediate and dorsal nuclei), the midbrain-derived inferior colliculus (IC) (central nucleus, dorsal and external cortices) and the forebrain-derived MGB (ventral, medial and dorsal nuclei) (Fig. 1c). *Kcnq4* is expressed in the two types of auditory sensory hair cells (the inner hair cells (IHCs), the genuine sensory cells, and the outer hair cells (OHCs), which are mechano-effector cells amplifying and sharpening the frequency response of the IHCs), in spiral ganglion neurons (SGNs) (mainly composed of IHC afferent neurons), and in auditory brainstem nuclei from the CN to the IC (Beisel et al. 2000; Kharkovets et al. 2000; Kubisch et al. 1999). By ensuring shorter membrane time constants, resulting in short action potentials, *Kcnq4* expression is thought to ensure rapid sound processing from the periphery to the IC. Several monogenic forms of syndromic deafness combining peripheral and central auditory deficits have since been described. Most of their causal genes are involved in inner ear morphogenesis from the otic placode and the formation of hindbrain auditory nuclei. Moreover, hereditary forms of deafness known to involve hair cell deficits that fully account for deafness have recently been shown to be associated with abnormalities in AC development, not causally related to the peripheral impairment. In particular, two cadherin-related (cdhr) proteins cdhr23 and cdhr15 (earlier named cadherin 23 and protocadherin 15, respectively) (Hulpiau et al. 2016), which form the hair-bundle tip links

gating the mechanoelectrical transduction channels and early hair-bundle lateral links (reviewed in (Petit & Richardson 2009)), are also expressed by many parvalbumin-positive interneuron precursors specifically targeted to the AC (Libé-Philippot et al. 2017).

These findings raise questions about the repertoire of genes playing key intrinsic roles in both the peripheral and central auditory systems (Table 1, Figure 1c), and their underlying mechanisms, especially taking into account the different embryonic origins of these two systems, the otic placode and the neural tube, respectively. We provide here an overview of recent results suggesting that the number of genes with developmental and functional roles in both the peripheral and central auditory systems may have been underappreciated. We consider the roles of these genes and their encoded proteins from an ontogenetic perspective. We then examine how the evolutionary origins of the structures and cell types expressing these genes, and their associated gene regulatory networks (GRNs) combining intercellular signaling molecules, transcription factor proteins, and *cis*-regulatory DNA modules (Cheatle Jarvela & Hinman 2015), can enlighten the mechanisms underlying the pleiotropic effects of these genes in the auditory system.

## **I) Genes controlling early patterning in both the inner ear and auditory hindbrain**

### *a) Molecular genetics of early inner ear and auditory hindbrain development*

Early hindbrain patterning is initiated by the segmentation of the developing neural tube along its anterior-posterior axis into spatially segregated modules, the rhombomeres (r) (numbered from r0 to r11), each of them containing restricted domains of progenitor neurons. This process is highly conserved in vertebrates and involves combinations of different early patterning genes, transcription factors and morphogens in GRNs, giving rise to a distinct pool of progenitors to each rhombomere and specifying their fate and migration. The progenitors for auditory hindbrain nuclei (CN, SOC, and ventral LL) lie between the r1/r2 and r5/r6



boundaries. The progenitors for the dorsal and intermediate LL are derived from the isthmus (which separates the hindbrain and the midbrain) and r1, respectively (Fig. 1a).

At the end of gastrulation, on embryonic day 7 (E7) in mice, the homeotic *Hox* genes define the combinatorial code identifying each rhombomere along the anteroposterior axis and its neuronal components. The neuronal subtype identities of the dorsoventral domains of the various rhombomeres are controlled by shared genetic pathways (see for review (Di Bonito & Studer 2017)). Early GRNs of the auditory hindbrain couple *Hox* gene code expression with hindbrain segmentation through at least two proteins: Krox20, a two-zinc finger transcription factor expressed in r3 and r5, and mafB, a leucine zipper transcription factor expressed in r5 and r6. Krox20 controls *Hox2* gene transcription from around E9 and mafB interacts directly or indirectly with several *Hox* genes (McKay et al. 1994; Sham et al. 1993). Later, from E13.5 until at least the neonatal period, *MafB* is expressed in several auditory brainstem nuclei, the ventral CN and the SOC nuclei (Fig.1c) (Farago et al. 2006; Marrs et al. 2013).

The ectoderm-derived otic placode appears ventrally to r5 and r6 around E8, and invaginates to form the otic vesicle, the otocyst, by E9.5. The otic placode is induced by FGF signaling, FGF3 in the ectoderm and FGF10 in the mesoderm (Ohyama et al. 2007). The transcription factors Pax2 and Pax8 are then expressed in the ectoderm, followed by a number of signaling molecules (FGFs, BMP7) and transcription factors (*Eya1*, *Six1*, *Nkx5.1*, *Gbx2*, *Sox9*, *GATA3*, *Foxi1/3*, *Tfap2a*). Multipotent neurosensory progenitors produce both neuronal and sensory precursors. The neuronal precursors of the otocyst anteroventral region then delaminate to form the afferent neurons of the spiral ganglion (Fritzscht & Elliott 2017; Groves & Fekete 2017). Their wiring, predominantly to IHCs, and for about 5% of them to OHCs, is guided by Eph receptors and ephrins (see below). On E11.5, the prosensory domain of the developing cochlea is marked by the expression of *Sox2*, between two regions expressing the notch ligand *Jag1* and BMP4, on the neural and abneural sides of the cochlea,

respectively (Kelley & Wu 2005; Ohyama et al. 2010). Neural cell differentiation begins around E13 in the cochlea, with expression of the following basic helix-loop-helix (bHLH) transcription factors, the mouse homolog of the *Drosophila atonal* gene (*atoh1*), neurogenin 1 (*neurog1*), neurogenic differentiation 1 (*neurod1*), nescient helix loop helix 1 (*nhlh1*), *nhlh2* and *bhlhb5*, a neural-specific bHLH transcription factor related to *atonal*, and the POU-domain transcription factor POU4F3, the promoter activity of which is regulated by *atoh1*. The expression of most of these transcription factors depends on the action of the SWI/SNF chromatin remodeling complex supported by *Eya1*, *Six1*, *Pax2/8*, *Sox2*, *Foxi3* and *Gata3* (see for review (Fritzscht & Elliott 2017)).

We will first consider genes controlling the development and the functioning of both the peripheral and central auditory systems, and highlight those with intrinsic roles in both systems. We will then extend our focus to genes for which there are strong indications for an intrinsic role in both the peripheral and central systems.

#### *b) Genes involved in both cochlea and auditory hindbrain development*

Mutations of the *Hox* genes expressed in r2-r6 — *Hoxa1*, *Hoxa2*, *Hoxb1*, and *Hoxb2* — have deleterious effects on rhombomere identity leading to severe auditory brainstem abnormalities associated with major developmental abnormalities of the ear (see for review (Di Bonito & Studer 2017)). These ear abnormalities are thought to result from indirect effects of the brainstem development abnormalities, such as the smaller r4 and the absence of r5 in *Hoxa1* knockout mice, leading to diverse deficits, including an almost complete absence of the SOC and major defects of external, middle, and inner ear formation (Chisaka et al. 1992). As far as *MafB* is concerned, Kreisler mice carrying a hypomorphic *MafB* allele lack r5 (Cordes & Barsh 1994), have altered r6 identity, and a misplaced otocyst, with highly impaired ear patterning resulting from alterations to the inductive signals derived from the

hindbrain (Cordes & Barsh 1994; Choo et al. 2006). Nevertheless, *MafB* also plays an intrinsic role in the peripheral auditory system. Upon conditional *MafB* inactivation in the SGNs (using the transgenic *Neurog1*-cre driver line), postsynaptic densities fail to form in the synaptic contacts they make with the IHCs, resulting in smaller synapse numbers (Yu et al. 2013b). Along the same line, *MAFB* loss of function causes Duane retraction syndrome, the most common congenital disorder of cranial dysinnervation (Park et al. 2016). *Krox20* knocking-out markedly decreases or eliminates r3 and r5 and also has indirect effects on the development of the cochlea, including abnormal juxtaposition of the cochlear VIII<sup>th</sup> nerve and trigeminal V<sup>th</sup> nerve (Schneider-Maunoury et al. 1993). These findings highlight the importance of indirect effects of key hindbrain early patterning transcription factors on cochlear development, and the need to disentangle their possible association with direct effects by conditional gene inactivation.

Two Wnt (‘Wingless/Integrated’) proteins, Wnt1 and Wnt3a, are essential for hindbrain dorsoventral patterning. Wnts, secreted glycoproteins with major roles in development, including cell proliferation control and cell fate decision, are expressed dorsally in the neural tube and form a countergradient with Sonic Hedgehog (Shh), which is ventrally expressed and derived from the notochord and floorplate. In the hindbrain, Wnt1-expressing progenitors from the lower part of the rhombic lip (the dorsal region of the developing hindbrain) in the anterior rhombencephalon and the mesencephalon contribute to neurons throughout the CN and the IC, respectively (Brown & Zervas 2017), and Wnt-3a-expressing progenitors from the rhombic lip and its neighboring caudal and rostral regions contribute to the CN (ventral and dorsal), the SOC, the LL, the IC and the MGB (Louvi et al. 2007). *Wnt1* and *Wnt3a* are not expressed in the otocyst. However, inner ear morphogenesis is severely disrupted in *Wnt1*<sup>-/-</sup> *Wnt3a*<sup>-/-</sup> double-mutant embryos, showing that they play indirect roles in the formation of the cochlea (Riccomagno et al. 2005).

Genes involved in the formation of tonotopic maps, spatially ordered frequency representations that emerge in the cochlea, are maintained, from nucleus to nucleus, until the AC. As in other sensory systems displaying a topographic organization of sensory parameters, Eph transmembrane receptors of the receptor tyrosine kinase family and their membrane-bound ligands, ephrins, play major signaling roles in neuronal wiring and establishing coarse tonotopic maps. Three Ephrins (Ephrin-B1, Ephrin-B2, and Ephrin-A5) and five Eph receptors (EPHB1, EPHB2, EPHB3, EPHA4, and EPHA7) are directly involved in both the formation of synaptic contacts between SGNs and hair cells, and in the wiring of central auditory nuclei throughout the auditory pathway, with some of them displaying gradients of expression along the frequency axis (Cramer & Gabriele 2014; Kim et al. 2016; Wallace et al. 2016). Other Ephs/ephrins may also be involved in the formation of tonotopic maps, particularly in the MGB and AC, which have yet to be explored in depth. Of note, *Hoxa2* and *Hoxb2* have been implicated in the fine-scale refinement of tonotopic organization in the anteroventral CN (Karmakar et al. 2017) through the conditional double inactivation of *Hoxa2* and *Hoxb2* (with the *Atoh1-cre* driver line, which expresses cre in auditory hindbrain nuclei from E10.5 onwards), demonstrating a role for *Hox* genes beyond early patterning.

Although broadly expressed outside the nervous system, three other genes underlying syndromic forms of deafness have also intrinsic roles in both the cochlea and the auditory hindbrain nuclei: *GATA3*, *CHD7* and the gene encoding insulin-like growth factor I (*IGF1*). *Gata3*, a GATA DNA sequence-binding transcription factor, is defective in Barakat syndrome, a developmental disorder characterized by hypoparathyroidism, renal disease and sensorineural deafness. Because of the early lethality resulting from *Gata3* inactivation and its broad expression in the auditory system from E9 onwards, *Gata3* roles have been investigated with various cre driver lines (Karis et al. 2001). Conditional *Gata3* inactivation in the cochlea (with the *Foxg1-cre*, *Pax2-cre*, and *Bhlhb5-cre* driver lines, driving cre expression in the otic

placode from E8.5, in the otic placode from E9.5, and in SGNs from E9.5, respectively) demonstrated an essential role of *Gata3* in hair cell differentiation and maturation, and in the tonotopic patterned wiring of SGNs to hair cells upstream from *mafB* (Appler et al. 2013; Duncan & Fritsch 2013; Yu et al. 2013b). Moreover, *Gata3* plays a major role in activating the hair cell prosensory genes *Atoh1* and *Sox2* (Duncan & Fritsch 2013). In the central auditory system, *Gata3* is expressed in a few cells in the most posterior part of r3, r4, and the rostral region of r5. Later in development, it is expressed principally in the olivo-cochlear efferent system consisting of cholinergic neurons originating from the SOC and projecting to the cochlea. The absence of *Gata3* does not affect the initial differentiation of these neurons, but leads to an unusual routing of their axons (Karis et al. 2001). Additional conditional *Gata3* inactivations are required to clarify the roles of *Gata3* in other central auditory nuclei (LSO, SPON, LL and IC) in which it is expressed. Both *CHD7* and *IGF1* are broadly involved in cell processes. Haploinsufficiency of *CHD7* encoding *chd7*, an ATP-dependent chromatin remodeling enzyme, underlies CHARGE syndrome, an epigenetic disorder with multiple malformations including major morphological defects of the inner ear (Choo et al. 2017). Conditional early *Chd7* inactivation (with the *Foxg1*-cre driver line) leads to severe vestibule and cochlea deficits (Hurd et al. 2010). Defects in *IGF1* underlie a very rare syndrome combining intrauterine and postnatal growth retardation with sensorineural deafness and intellectual deficit. Mice carrying *Igf1* mutations display morphological abnormalities of several cochlea structures (stria vascularis, tectorial membrane, and SGNs) (Varela-Nieto et al. 2013). Detailed studies of the central auditory pathway are lacking, but several lines of evidence indicate critical roles for *chd7* and *Igf1* there, too (Alavizadeh et al. 2001; Cedié et al. 2006; Yu et al. 2013a).

In the search for intrinsic critical proteins common to the peripheral and central auditory pathways, such broadly expressed transcription factors can become more

informative, if the GRN to which they belong includes another shared regulatory gene, their coexpression leading to a more restricted profile. Interestingly, proteins or regulatory RNAs with very limited expression outside the auditory system may be immediately informative, as illustrated by the microRNA miR-96. MicroRNAs, single-stranded non-coding short RNAs (19-25 nucleotides), mediate post-transcriptional regulation of gene expression through an RNA-induced silencing complex, involving the complementary binding of their seed region to their target messenger RNAs (mRNAs), mainly in their 3'UTR. miR-96, is required for the development of both the cochlea and central auditory system. It belongs to the miR-183 cluster, which is transcribed as a polycistronic transcript containing miR-183, miR-96, and miR-182 (Friedman & Avraham 2009). Dominant mutations of miR-96 lead to progressive hearing loss in humans (DFNA50) (Mencía et al. 2009) and mice (Lewis et al. 2009). In zebrafish, miR-96 overexpression induces ectopic patches of sensory hair cells and its knockdown reduces the number of hair cells and associated statoacoustic ganglion neurons (Li et al. 2010). In *Dmdo/Dmdo* mice, a mutation in the seed region of miR-96 leads to loss of functions by suppressing the recognition of miR-96 target mRNAs, accompanied by gain of functions due to the recognition of new target mRNAs. Embryonic hair cells seem to develop normally in *Dmdo/Dmdo* mice. However, they have an abnormally immature morphological and functional state from birth onwards. Adult hair cells are abnormally small, lack several  $K^+$  conductances, display abnormal spontaneous  $Ca^{2+}$ -dependent action potentials, and have immature hair bundles and  $Ca^{2+}$ -dependent synaptic exocytosis (Kuhn et al. 2011; Lewis et al. 2009). The CN and SOC nuclei of *Dmdo/Dmdo* mice are small. The calyces of Held, the giant synaptic terminals between the globular bushy cells of the anteroventral CN and the principal cells of the MNTB (see Figure 1c), display growth arrest from P4 onwards, with abnormal morphofunctional features, reminiscent of the hair cell phenotype (Schlüter et al. 2018). These findings suggest that some GRNs involving miR-96 operate in both the peripheral auditory

system and in at least some hindbrain auditory nuclei. Conditional knockouts should provide more information about the intrinsic role of miR-96 in central auditory nuclei. So far, miR-96 seems to affect only auditory structures.

Finally, four HLH transcription factors appear to be involved in cell fate decision in both the cochlea and auditory hindbrain. The proneural gene *Atoh1* is essential for the genesis of several neural cell types, including cerebellular granule cells (Ben-Arie et al. 1997), Merkel cells (Ben-Arie et al. 2000), hair cells (Bermingham et al. 1999), and several neuronal populations in the auditory brainstem (Maricich et al. 2009; Wang et al. 2005). In the inner ear, *Atoh1* is required for hair cell formation (Bermingham et al. 1999), maturation, and survival after differentiation (Cai et al. 2013; Chonko et al. 2013). During development, the canonical Wnt pathway mediator,  $\beta$ -catenin, regulates *Atoh1* expression in hair cells (Shi et al. 2014) and interacts with the Notch pathway. In the central nervous system, *Atoh1* is expressed as early as E9.5 in the rhombic lip (Akazawa et al. 1995; Wang et al. 2005). *Atoh1* knockouts and conditional inactivations (with *Krox20*- and *Hox-b1*-cre driver lines) disrupt the formation of the ventral CN, the dorsal CN, and of a glutamatergic population of neurons in the MSO and LSO, due to the disruption of an *Atoh1*-dependent migratory stream of cells derived from the rhombic lip (Maricich et al. 2009; Wang et al. 2005). Hair cell and SGN fates are also dependent on *neurog1* and *neurod1*. *Neurog1* is required for SGN formation (Ma et al. 1998). *Neurod1* expression is upregulated by *atoh1* and *neurog1* in hair cells and SGNs, respectively. In turn, it provides negative feedback on *Atoh1* and *Neurog1* expression (Jahan et al. 2010). In the absence of *neurod1*, very few SGNs form (due to defective delamination and the apoptosis of spiral ganglion neuroblast precursors of the otic vesicle), and ectopic OHCs and IHCs develop in the organ of Corti (Liu 2000). It also plays an intrinsic role in the central auditory system, as *Neurod1* knockout mice do not develop the DCN, whereas mice with a conditional knockout (with the *Pax2*-cre driver line) in the cochlea do (Jahan et al. 2010; Liu et al. 2000).

Spatiotemporal fate mapping for *Neurog1*-expressing cells with *Neurog1*-Cre BAC (bacterial artificial chromosome) transgenic mice has shown *Neurog1* to be expressed in the CN, LL, and IC, and throughout the cortex and most of the thalamus (Kim et al. 2011), but the mode of action of *neurog1* in these brainstem auditory nuclei has yet to be investigated. Finally, the gene encoding the proneural bHLH transcription factor *bhlhb5* is expressed in SGNs from E10.5 (Brunelli et al. 2003), in the dorsal CN from E12 (Cai et al. 2016), and in several primary sensory cortices, including the AC, soon after birth (Joshi et al. 2008). *Bhlhb5* knockout mice have very small numbers of dorsal CN neurons, such as cartwheel cells and unipolar brush cells (Cai et al. 2016). However, the functional roles of *Bhlhb5* in the SGNs and the AC remain to be determined.

## **II) Genes involved in the development and functioning of hair cells and the central auditory system**

The second major category of genes with roles in both the peripheral and central auditory systems are those involved in the late differentiation and functioning of hair cells. However, other proteins playing key roles in two highly specialized hair cell structures — the apical hair bundle, which is responsible for mechano-electrical transduction, and the IHC afferent synapse, an atypical synapse equipped with a ribbon-shaped osmiophilic structure to which synaptic vesicles are tethered — also have roles in the central auditory system.

### *a) The auditory ribbon synapse*

Studies of deafness genes have revealed the unconventional nature of the IHC ribbon synapse exocytotic machinery, echoing its unusual physiological characteristics enabling its sustained and highly temporally precise neurotransmitter release (reviewed in (Safieddine et al. 2012)). Mature auditory ribbon synapses lack many classical synaptic proteins, including the  $\text{Ca}^{2+}$ -sensing  $\text{C}_2$ -domain-containing proteins synaptotagmins 1 and 2, complexins,



synaptophysin, and other synaptic proteins (Safieddine et al. 2012); even the neuronal SNARE proteins have been proposed to be dispensable for IHC exocytosis (Nouvian et al. 2011). Several proteins are associated with the ribbon, including ribeye, which contains an N-terminal A domain forming the ribbon scaffold, and two structurally related multidomain scaffolding proteins, piccolo (a *piccolo* isoform) and bassoon. Inactivation of the ribbon-specific ribeye isoform leads to a lack of synaptic ribbons in hair cells, with a surprisingly modest dysfunction (Becker et al. 2018; Jean et al. 2018), and bassoon inactivation results in impaired ribbon anchoring to the active zones in hair cells (Khimich et al. 2005). Piccolo and bassoon play many roles in the organization of central auditory synapse active zones in the auditory system, and in several other brain areas (reviewed in (Gundelfinger et al. 2015)). At the endbulb of Held synapse formed between spiral ganglion neurons and bushy cells of the anteroventral CN, bassoon and piccolo have additive roles in the replenishment of synaptic vesicles (Butola et al. 2017). At calyx of Held terminals, piccolo or bassoon knockdown results in slowed synaptic vesicle replenishment in the active zone (Parthier et al. 2018). Glutamate uptake in IHC synaptic vesicles is mediated by the vesicular glutamate transporter vglut3. *VGLUT3* mutations lead to an autosomal dominant form of progressive deafness (DFNA25) (Ruel et al. 2008) and *Vglut3* inactivation in mice causes profound deafness (Seal et al. 2008). Vglut3 is found in several discrete populations across the brain (Herzog et al. 2004) and its encoding gene is expressed in several types of inhibitory neurons that co-release glutamate (Stensrud et al. 2015). In the auditory system, it is expressed by inhibitory glycinergic MNTB neurons, which receive glutamatergic inputs from CN spherical bushy cells of the contralateral ear and project to LSO principal neurons. The latter extract interaural sound intensity differences, a core element of the circuit for localizing the source of high-frequency sounds.

Only two major players in vesicle fusion at the mature IHC ribbon synapse are

currently known — the presynaptic  $\text{Ca}_v1.3$  L-type  $\text{Ca}^{2+}$  channels, responsible for rapid, focalized voltage-gated  $\text{Ca}^{2+}$  entry (Brandt et al. 2005; Frank et al. 2009), and otoferlin, a six- $\text{C}_2$  domain transmembrane vesicular protein that acts as a  $\text{Ca}^{2+}$  sensor for synaptic vesicle fusion and replenishment in the IHC active zones (Michalski et al. 2017; Pangrsic et al. 2010; Roux et al. 2006). Inactivation of either of these genes results in profound deafness (Platzer et al. 2000; Roux et al. 2006). Otoferlin has been detected in several brain regions, including hippocampal neurons expressing GAD65, a marker of inhibitory neurons (Roux et al. 2006; Wu et al. 2015), but no functional deficit has yet been characterized in the brains of any of the *Otof* mutant mice. By contrast,  $\text{Ca}_v1.3$  channels play a key role in central auditory pathway development. In *Ca\_v1.3* knockout and conditional knockout mice (with the *Krox20*-cre driver line), all the auditory nuclei between the CN and IC are extremely small, due to their abnormally small numbers of neurons. More specifically, *Ca\_v1.3* plays essential roles in the maturation of the MNTB to LSO synapse and in the developmental switch between mixed GABA/glycinergic to purely glycinergic transmission of MNTB neurons (Hirtz et al. 2011, 2012; Satheesh et al. 2012).

#### *b) The hair bundle*

The hair bundle is a highly specialized hair cell structure that can detect sound-induced mechanical movements in the nanometer range. In mammals, cochlear hair bundles consist of three rows of actin-filled protrusions called stereocilia, organized in a staircase pattern of increasing height. Upon sound stimulation, deflection of the hair bundle increases tension in tip-links, apical links connecting the tip of each stereocilia to the wall of the adjacent taller stereocilium, inducing the opening of mechano-electrical transduction channels at the apex of the shortest and middle-sized stereocilia rows. We recently showed that several protein components of different hair-bundle link types also play intrinsic roles in the central auditory pathway. In hair cells, *cdhr15* and *cdhr23* compose the lower and upper parts of the tip-link,

respectively (Kazmierczak et al. 2007). In the mouse embryonic brain, an additional role for *cdhr15* and *cdhr23* has been described, based on the susceptibility of heterozygous *Cdhr15*<sup>+/-</sup> and *Cdhr23*<sup>+/-</sup> mutant mice to audiogenic seizures, reflex seizures elicited by loud sounds. Many GABAergic interneuron precursors express these two cadherin-related proteins, from their generation in the medial ganglionic eminence until their settlement exclusively in the AC, where they give rise to parvalbumin-positive interneurons (Fig. 1b-c) (Libé-Philippot et al. 2017). In the absence of either of these cadherins, in *Cdhr15*<sup>-/-</sup> or *Cdhr23*<sup>-/-</sup> mutant mice, these precursors fail to enter the neocortex. Conditional *Cdhr15* inactivation in cortical GABAergic interneuron precursors derived from the medial ganglionic eminence, with the *Nkx2.1-cre* driver line, produce the same effect, demonstrating the intrinsic role of *cdhr15* and *cdhr23* in the embryonic cortex. *Cdhr15*- and *Cdhr23*-expressing interneuron precursors display cell polarity abnormalities *in vitro*. These two observations strongly suggest a role for *cdhr15* and *cdhr23* in interneuron migration. The precise role of *cdhr15* and *cdhr23* in specific targeting to the AC is unclear. *Adgrv1*, encoding the adhesion G protein-coupled receptor V1 protein (or VLGR1) forms another type of hair-bundle link, the ankle links, which are critically involved in hair-bundle maturation (McGee et al. 2006; Michalski et al. 2007). *Adgrv1* inactivation also results in susceptibility to audiogenic seizures associated with a strong decrease in the number of parvalbumin interneurons in the AC. The entry of *Cdhr15*- and *Cdhr23*-expressing interneuron precursors into the embryonic cortex is also impaired in the absence of *adgrv1*. In the telencephalon, *Adgrv1* is not expressed in interneuron precursors but in radial glial cells required for interneuron precursor migration to the neocortex (Libé-Philippot et al. 2017). Other roles of *adgrv1* have been described elsewhere in the auditory system, such as in IC oligodendrocytes, in which it is thought to regulate the stability of the myelin-associated glycoprotein (Shin et al. 2013).

### III) The origin and evolution of the otic placode and hair cells

The growing number of genes shown to have roles in both the peripheral and central auditory systems, mostly in several brainstem nuclei of the central auditory system, raises questions about the mechanisms underlying the diversity of expression sites in tissues of different origins. The genes shown to have key roles in both the peripheral auditory system and the auditory brainstem nuclei encode highly diverse proteins, including transcription factors (*Hoxa1*, *Hoxa2*, *Hoxb1*, *Hoxb2*, *mafB*, *Krox20*, *Gata3*, *atoh1*, *neurog1*, *neurod1*), chromatin remodeling proteins (*chd7*), microRNA (*miR-96*), morphogens (*Wnt1*, *Wnt3a*), axon guidance molecules (ephrin signaling proteins) and effector proteins (*Kcnq4*, *piccolo*, *bassoon*, *vglut3*, *Ca<sub>v</sub>1.3*). In addition, three genes encoding components of the hair-bundle links in hair cells (*cdhr15*, *cdhr23*, and *adgrv1*) have also intrinsic roles in the AC. Below, we consider these genes from an evolutionary perspective. We focus on the origin of the otic placode and hair cells, current knowledge about the evolution of the central auditory system being rudimentary, except for the SOC (Nothwang 2016).

The otic placode is thought to have emerged in the common ancestor of tunicates and vertebrates. Several aspects of ectodermal patterning are common to vertebrates and tunicates, which are considered to be their closest living relatives. Atrial siphons in ascidians, which are tunicates, are thought to be homologous to the otic placode on the basis of their anatomical position and siphon expression of *HrPax-258*, the ascidian homolog of the vertebrate otic placode genes *Pax2* and *Pax8* (Schlosser et al. 2014; Wada et al. 1998). In amphioxys, which diverged before tunicates (Delsuc et al. 2006), there is currently no evidence for the presence of an ectodermal structure homologous to the otic placode. However, most if not all of the genes known to be involved in early patterning in both the cochlea and the auditory hindbrain predate this transition, and several of the corresponding transcription factor families were

already present in the last common ancestor of eukaryotes (de Mendoza et al. 2013) and these gene families expanded in metazoans.

The surprising finding that the hair-bundle proteins *cdhr15*, *cdhr23*, and *adgrv1* are also expressed in interneuron precursors of the AC calls for a tentative tracing of the evolution of these two cell types. An evolutionary definition of cell types based on GRNs has recently been proposed (Arendt et al. 2016). Choanoflagellates, the closest single-celled living relatives of metazoans, have many of the features of hair cells. They display apico-basal polarization with, at their apex, a structure consisting of a primary cilium surrounded by a collar of actin-filled microvilli. Many of the genes essential for multicellularity are already present in choanoflagellates, including, for example, genes encoding various cadherins (Abedin & King 2008). Remarkably, in sea anemone, a species of the cnidarian phylum that is the closest to bilaterians, mechano-electrical transduction currents have been recorded in hair cells (Mire & Watson 1997) and a *cdhr23*-like protein has been localized to the hair bundles (Watson et al. 2008). This suggests that the molecular complex for mechano-electrical transduction had already evolved by the time this group of organisms diverged and that the machinery involved may resemble that of vertebrates. In addition to *cdhr15* and *cdhr23*, three other proteins, myosin-VIIa, harmonin, and sans are encoded by genes underlying Usher syndrome type 1, an autosomal recessive disorder combining congenital hearing impairment with delayed-onset sight loss. The proteins encoded by these genes belong to a molecular complex forming part of the mechano-electrical transduction machinery within the hair bundle of hair cells. A similar complex is present in the calyceal processes (protrusions resembling stereocilia) of some vertebrate retinal photoreceptor cells (Sahly et al. 2012). This complex might thus be co-opted *ad integrum* in various cell types. It is currently unknown whether this is the case in the interneuron precursors of the AC. The observed sharing of molecules between hair cells and interneuron precursors suggests that there is a GNR operating in the

two cell types and highlights the lack of information about hair cell GRNs, which will need to be rectified to address this issue. It is tempting to include the GABAergic cerebrospinal fluid (CSF)-contacting neurons cell type in this comparison. In the zebrafish tail, these neurons have an apical bulbous dendritic extension bearing microvilli bathing in the CSF of the spinal cord central canal and they extend a GABAergic ascending axon within the spinal cord, contacting several classes of interneurons and motoneurons. These neurons are both chemo- and mechanosensitive, responding to changes in extracellular pH and to mechanical deflection of their CSF-bathed microvilli, elicited by CSF movement during spinal bending (Djenoune & Wyart 2017). In the absence of transcriptomic analyses of CSF-contacting sensorineurons, it is unknown whether *cdh15/cdhr23*, and other key hair cell genes are expressed in these cells. However, they are known to express *Gata3* during their development (Petracca et al. 2016).

Understanding the evolutionary mechanisms involved will require reconstitution of the precise sequence of gene expression and identification of the underlying GRNs in both the cochlea and along the central auditory pathway. *Kcnq4* is of particular interest for studies investigating how evolutionary pressure has maintained its expression, for functional reasons, from the hair cells to the IC, including all the auditory brainstem nuclei. However, the GRN with which it is associated is unknown. One or several active GRNs are probably common to the cochlea and the CN-SOC, as suggested by the expression of the hair-cell ribbon-synapse proteins, Ca<sub>v</sub>1.3 channels, piccolo, bassoon, and vglut3 in several hindbrain auditory nuclei. In addition to ribbon-synapse proteins, the K<sup>+</sup>-Cl<sup>-</sup> cotransporter KCC2 and the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> cotransporter NKCC1, which are expressed in different areas of the cochlea, are also both involved in the excitability of inhibitory neurons in the SOC through the regulation of their intracellular chloride content, further highlighting the existence of GRNs common to the cochlea and hindbrain (Balakrishnan et al. 2003; Pace et al. 2001). One promising entry point

for deciphering the GRNs operating in the SOC is miR-96, which seems to be specific to the auditory system and regulates the gene encoding  $Ca_v1.3$  channels (Lewis et al. 2016). The best candidate members of these GRNs are the transcription factors and morphogens with roles in both the cochlea and the central auditory pathway. The *Hox* and *Wnt* genes are probably the key initiators of the GRNs common to the cochlea and hindbrain, and *mafB*, *Gata3*, and *atoh1* the central elements of these GRNs. Remarkably, *atoh1*, *neurog1* and *neurod1* belong to the same GRN active within the cochlea and the central auditory system, but also outside the auditory system (Willaredt et al. 2015). One plausible explanation for this reuse of GRNs in the cochlea and hindbrain is that each of the many hindbrain auditory nuclei has functional properties and requirements similar to those of the cochlea: they each analyze one aspect of the physical characteristics of sound, such as sound duration, sound offset, or binaural intensity differences, for example, whereas the cochlea analyzes the frequency content of sounds. Likewise, the cochlea and the hindbrain auditory nuclei are both faced with a need for indefatigability. This requirement may have led, through common GRNs, to the selection of similar molecular mechanisms for neuronal protection in the inner ear and central auditory pathways. One such mechanism is likely mediated by parvalbumin, which is abundant in the auditory system, in hair cells and in different types of neurons throughout the auditory system, including glutamatergic, glycinergic and GABAergic neurons (Hackney et al. 2005; Lohmann & Friauf 1996). It has been suggested that parvalbumin, with its slow  $Ca^{2+}$ -buffering properties, may protect neurons against deleterious high free  $Ca^{2+}$  concentrations (Sloviter 1989).

One issue linked to this sharing of GRNs is that it may be difficult for a single auditory structure to evolve independently from the others. Indeed, transcription factors are pleiotropic, so a mutation beneficial in one context is highly likely to be deleterious in other contexts within the same organism. However, several mechanisms for minimizing the

potential pleiotropic effects of GRN evolution have been identified. The transcription of a given gene is not dependent on a single transcription factor, but on a complex of several such factors. In addition, a large proportion of the genes in the genome are regulated by *cis* regulatory sequences containing multiple independent binding sites for transcription factors. Transcription factors are themselves also thought to participate in modular evolution of this type, through several different mechanisms, including the expression of different isoforms, post-transcriptional modifications, variations of expression levels to alter activity levels and, in more extreme cases, the duplication of an ancestral gene, such as *atoh7*, expressed in the VCN with *atoh1*, both these genes being derived from the same *atonal* ancestral gene (see for review (Cheatle Jarvela & Hinman 2015; Fritzscht & Elliott 2017; Willaredt et al. 2015)).

### **Concluding remarks**

Here, we have pinpointed 34 genes with a critical role in both the peripheral and central auditory systems. These genes most likely only represent the tip of the iceberg. Indeed, unless systematically sought, most central auditory deficits will remain unnoticed, masked by peripheral deficits. Moreover, many genes underlying monogenic forms of hearing impairment are yet to be identified and embryonic lethal mutations, as well as functional redundancy, likely hide several genes key to the peripheral and central auditory systems. A strategy to uncover genes common to the peripheral and central auditory systems is to screen heterozygous and moderately deaf mutant mice carrying mutations in deafness genes for susceptibility to audiogenic seizures. At least two other deafness genes are responsible for audiogenic seizure susceptibility, *Tecta* encoding alpha-tectorin, a major component of the tectorial membrane (Legan et al. 2014) and *Gipc3* encoding a PDZ-domain containing protein of hair cells, GAIP-interacting protein C terminus 3 (Charizopoulou et al. 2011). A high proportion of the genes analyzed here have essential roles in the CN and SOC. The possibility



of a strong bias towards the CN and SOC resulting from the already substantial molecular deciphering of these structures, contrary to the LL, IC, MGB and AC, cannot be excluded.

The main challenge ahead is to elucidate the roles of these genes in both the peripheral and central auditory systems. In addition, the characterization of the GRNs common to the two systems is essential. The present review brings to light the limited existing knowledge of GRNs, even in the hair cells. Efforts to characterize them at the transcriptomic, post-transcriptomic, and epigenetic levels, have focused on a limited number of genes, including *Atoh1*, *Pou4f3*, and a few hair cell genes, such as *Myo7a* (reviewed in (Ryan et al. 2015)). Ongoing work to decipher the pathways involved in hair cell regeneration, in a therapeutic perspective, should contribute to filling this gap (Kwan et al. 2009). Moreover, recent technological leaps in transcriptomics and epigenetics, including at the single-cell level, should also provide important new insights into the GRNs of the cochlea and the many auditory nuclei of the central auditory system (Friedman & Rando 2015; Tasic et al. 2016), and bring to light co-evolutionary profiles.

Auditory rehabilitation, currently through the use of prosthetics, but hopefully also by gene therapy in the near future, depends on the ability to revive maturation of the central auditory pathway by neuronal plasticity for optimal processing of the restored sensory stimulation. Identifying and further characterizing genes with essential roles in the peripheral and central auditory systems should provide clues to the reasons for the unusual speech-recognition difficulties faced by some patients after prosthetic intervention and help to improve hearing rehabilitation.

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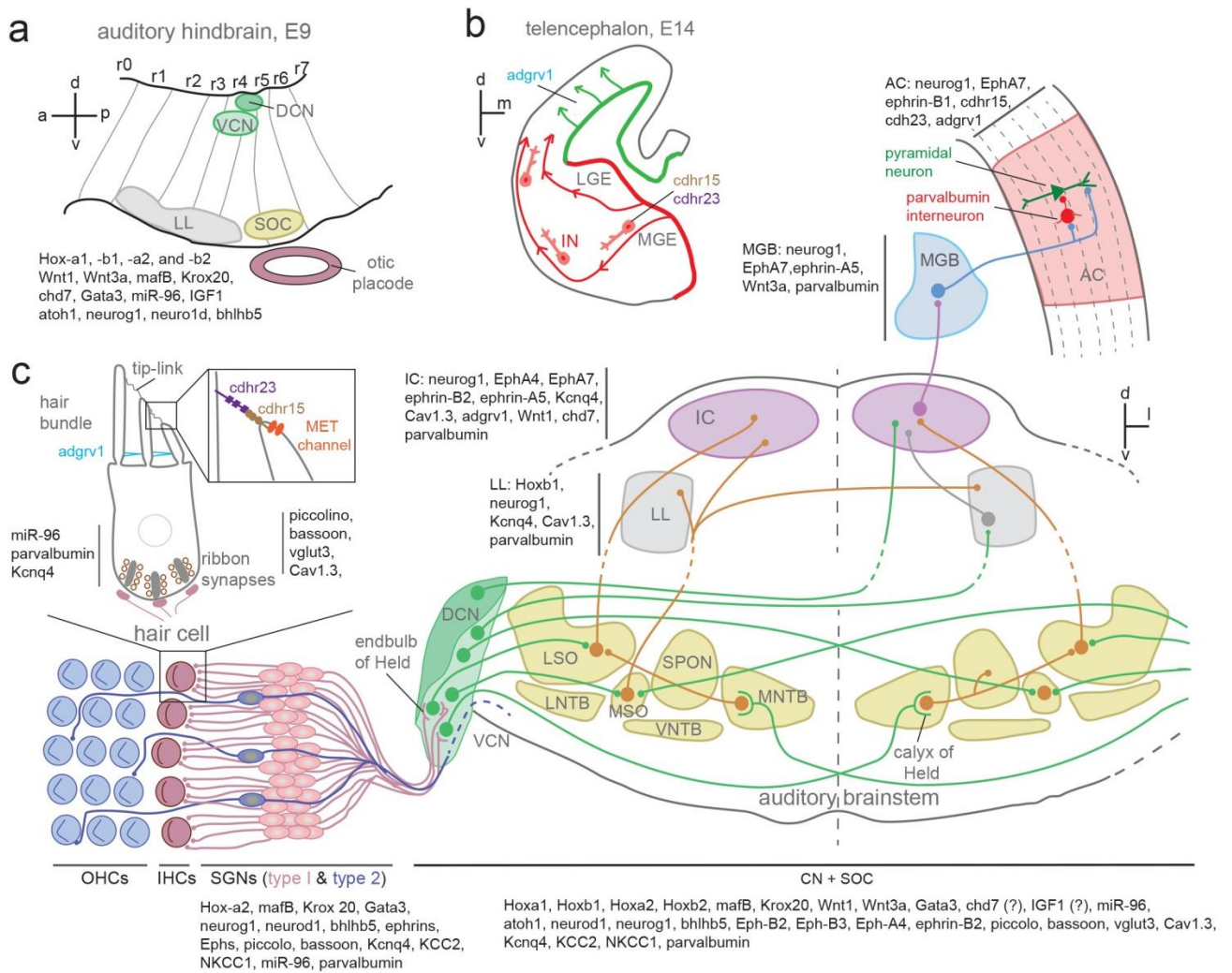
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**Figure 1: Anatomy of the auditory system and genes common to the peripheral and central auditory systems**

(a) Diagram showing the otic placode and the rhombomeric origin of the hindbrain auditory nuclei (adapted from (Di Bonito & Studer 2017)). Genes involved in the development of the hindbrain and the otic placode are listed.

(b) Diagram showing the migration pathway of a population of embryonic MGE-derived interneuron precursors co-expressing *Cdhr15* and *Cdhr23* specifically targeted to the AC.

(c) Simplified diagram of the peripheral auditory system and ascending central auditory pathways, with magnification of an IHC. Genes associated with the development of each structure are listed.

Abbreviations: r, rhombomere, DCN, dorsal cochlear nucleus; VCN, ventral cochlear nucleus; LL, lateral lemniscus; SOC, superior olivary complex; IN, interneuron; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MET, mechanoelectrical transduction channels; OHCs, outer hair cells; IHCs, inner hair cells; SGNs, spiral ganglion neurons; AN, auditory nerve; LSO, lateral superior olive; MSO, medial superior olive; LNTB, lateral nucleus of the trapezoid body; VNTB, ventral nucleus of the trapezoid body; MNTB, medial nucleus of the trapezoid body; SPON, superior periolivary nucleus; IC, inferior colliculus; MGB, medial geniculate body; AC, auditory cortex; a, anterior; d, dorsal; v, ventral; p, posterior; m, medial; l, lateral.

<b>Protein</b>	<b>Function in the cochlea</b>	<b>Function in the central auditory system</b>
Hox-a1,-b1,-a2,-b2	indirect effects	anteroventral rhombomeric patterning
Wnt1, Wnt3a	indirect effects	dorsoventral rhombomeric patterning
mafB	indirect effects & direct roles in the formation of the postsynaptic densities in SGNs	r5 & r6 patterning
Krox20	indirect effects, expression in Schwann cells	r3 & r5 patterning
Gata3	signaling of prosensory genes, differentiation and maturation of hair cells, wiring of SGNs	formation of the efferent olivo-cochlear system & expression in the auditory brainstem
chd7	early patterning of the inner ear	early patterning of rhombomeres, IC formation
IGF1	development of the stria vascularis, tectorial membrane, and SGNs	role in the auditory brainstem, to be confirmed
miR-96	formation and maturation of hair cells and SGNs	maturation of the CN and SOC
atoh1	hair cell differentiation, maturation and survival	formation of the VCN, DCN, and a glutamatergic cell type of the MSO and LSO
neurog1	SGN differentiation	expressed in the CN, LL, IC, cortex and most of the thalamus
neurod1	SGN differentiation	formation of the DCN
bhlhb5	expressed in SGNs	expressed in the AC
Eph-A7,-B1,-B2,-B3, ephrin-A4,-A5,-B1	wiring of the SGNs	wiring & tonotopy of all the auditory nuclei
piccolino & piccolo	ribbon morphology (photoreceptors)	synaptic vesicle replenishment
bassoon	anchoring of ribbons to the plasma membrane	synaptic vesicle replenishment
vglut3	ribbon synapse vesicular glutamate transporter	maturation of the MNTB to LSO synapse
Cav1.3	ribbon synapse presynaptic Ca <sup>2+</sup> channel	maturation of auditory brainstem nuclei
cdhr15	hair bundle tip-link	interneuron precursors of the AC
cdhr23	hair bundle tip-link	interneuron precursors of the AC
adgrv1	hair bundle ankle link	expressed in radial glia of the AC and in oligodendrocytes of the IC
Kcnq4	K <sup>+</sup> inward rectifier (hair cells and SGNs)	K <sup>+</sup> inward rectifier channel expressed from the CN to the IC
KCC2	expressed in SGNs	excitability of the LSO inhibitory neurons
NKCC1	expressed in the stria vascularis	excitability of the LSO inhibitory neurons
parvalbumin	Ca <sup>2+</sup> buffering properties (hair cells & SGNs)	Ca <sup>2+</sup> buffering (from the CN to the MGB, PV interneurons in the AC)

**Table 1: List of genes common to the peripheral and central auditory systems**