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Review

The evolutionary tuning of hearing

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After the transition to life on land, tympanic middle ears emerged separately in different groups of tetrapods, facilitating the efficient detection of airborne sounds and paving the way for high frequency sensitivity. The processes that brought about high-frequency hearing in mammals are tightly linked to the accumulation of coding sequence changes in inner ear genes; many of which were selected during evolution. These include proteins involved in hair bundle morphology, mechanotransduction and high endolymphatic potential, somatic electromotility for sound amplification, ribbon synapses for high-fidelity transmission of sound stimuli, and efferent synapses for the modulation of sound amplification. Here, we review the molecular evolutionary processes behind auditory functional innovation. Overall, the evidence to date supports the hypothesis that changes in inner ear proteins were central to the fine tuning of mammalian hearing.

Highlights

The auditory systems of amniotes (birds, reptiles, and mammals) evolved in parallel after the separate emergence of tympanic middle ears.

Numerous instances of molecular evolution events have been described for inner ear genes in mammals, potentially linking coding sequence changes to the extension of the hearing range to higher frequency sounds.

Changes in the coding sequence of prestin, the driver of somatic electromotility, a sound amplification mechanism present only in mammals, have been linked to the acquisition of voltage sensing capabilities and electromotility.

In mammals, amino acid changes in the α9α10 acetylcholine receptor at the efferent synapse affected the receptor's calcium permeability and channel gating properties, likely in relation to the modulation of electromotile outer hair cells.

Most genes that underwent molecular evolutionary processes in mammals are also deafness loci, underscoring the importance of cross-disciplinary interactions between molecular evolutionary research and the study of deafness.

Evolution of the auditory system

Hearing in mammals is characterised by a greatly expanded hearing frequency range and enhanced frequency tuning. Comparative studies, both within mammals and alongside other vertebrate clades have greatly enhanced our knowledge about its evolutionary and developmental origins, morphological and functional features. The auditory system shares its evolutionary and developmental histories with the vestibular and lateral line systems, all of which rely on sensory hair cells for the detection of mechanical stimuli [1]. Decades of research on vertebrate auditory systems have repeatedly highlighted several features that differentiate hearing, in evolutionary terms, from other sensory systems. First, a dedicated sensory epithelium for the detection of sound is the last major sensory organ to emerge in vertebrates [1–3]. Second, the evolutionary history of vertebrate hearing shows numerous examples of **convergent evolution** (see Glossary), both in the peripheral sensory organ and in central auditory pathways [3–5]. Finally, a high number of genes expressed in inner ear structures have acquired changes in their protein coding regions, predominantly in the mammalian lineage. Using bioinformatics tools, signatures of **positive selection** have been identified for many of these genes. These sequence changes represent starting points for formulating and testing hypotheses on the potential ensuing changes in protein function and how they may have contributed to the exclusive features of mammalian hearing.

Evolutionary processes are ultimately rooted on hereditary random changes at the genome level. Most of these changes are either neutral, without significant effects on the organism, or deleterious, and therefore negatively selected. Sequence changes can also be beneficial, and positively selected, bringing about evolutionary innovation. Whether a nucleotide change will have a deleterious, neutral or beneficial effect will depend on the change itself, where it is on the genome (e.g., protein coding region, regulatory region, etc.), the expression pattern and function of the affected genes, and many other known and unknown factors. Here, we review the molecular evolutionary processes that may have contributed to shaping mammalian hearing, and highlight the few instances for which changes in function have been successfully linked to sequence changes. Overall, the evidence to date supports the notion that, due to their somewhat restricted expression patterns (with a main functional

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role in the inner ear), a major driver for innovation in hearing evolution have been changes in protein sequences of inner ear genes.

A brief evolutionary history of mammalian hearing

During the Carboniferous period, ancestral tetrapods successfully completed the migration to life on land, setting the stage for ensuing solutions to the problem of adapting existing sensory systems, including hearing, from aquatic to terrestrial life (Figure 1). The stem amniote vertebrates are thought to have had a hearing range limited to low frequency, loud sounds [6], as they lacked a tympanic middle ear [2,7–9]. The Triassic period, >100 million years after the separation of the tetrapod lineages, saw the emergence of at least five independent elaborations of tympanic middle ears that operate as impedance matching devices necessary for the efficient detection of airborne sounds [10–12]. Without an efficient middle ear, higher frequency sounds cannot be detected. This demarcated parallel evolutionary histories, along which the hearing systems of tetrapod vertebrates show large differences across clades, while serving the same ultimate function: the detection, transmission, transduction, and encoding of airborne sounds.

Paleontological, morphological, and developmental evidence indicates that lepidosaurs (lizards and snakes) and archosaurs (birds and crocodiles) independently developed single-ossicle middle ears. Separate processes in mammals saw the emergence of the three-ossicle middle ear that is a defining character of the clade [7–9,13]. These changes in the middle ear happened to better transmit higher-frequency sounds and thus contributed to the subsequent evolution of the high-frequency hearing of therian mammals [7–9,14].

Within the inner ear, the auditory sensory epithelium of amniotes developed as a new patch of hair cells between the vestibular sacculus and lagena [1,6]. The consensus to date is that the organ of Corti of mammals and the basilar papilla of reptiles and birds are homologous structures, and that the morphology portrayed by extant turtles and tuataras closely represents the ancestral character state, that is, a small ~1-mm stretch of hair cells on a freely suspended basilar membrane [1,6]. After the emergence of tympanic middle ears, parallel processes of elongation ensued (Figure 1), resulting in the characteristic bent basilar papillae of birds (2–12 mm long) and the coiled cochlea of mammals (6–70 mm long) [6].

All therian mammals (marsupials and placentals) have a coiled cochlea, unlike pretherian mammals (monotremes) that have short and only mildly curved cochlear canals (Figure 1) [6]. Coiling is proposed to have been a space-saving feature, enabling elongation within a confined space [6,9]. Responses to sounds of increasing frequency are localised along the length of the sensory epithelium. Elongation therefore increases the frequency range for sound detection. Coiling and elongation were also likely related to the loss of the lagenar macula, a vestibular sensory epithelium that sits at the apical end of the cochlear duct in nonmammals and monotremes [7]. Early therian mammals also evolved additional bony support around the cochlear ganglion and adjacent to the organ of Corti [14]. This may have initially contributed to more efficient impedance matching with the middle ear and later on to the detection of high frequency sounds [9].

Diversification of hair cell types occurred in parallel within the auditory sensory epithelia of amniotes (Figure 1). In archosaurs, there is a gradient of hair cell heights, with tall hair cells receiving mostly afferent innervation, while the shortest short hair cells are contacted solely by efferent terminals, and thought to be exclusively involved in sound amplification [4]. In mammals, the organ of Corti holds inner hair cells (IHCs) on the inside and outer hair cells (OHCs) on the outside of the pillar cells that form the tunnel of Corti. IHCs receive afferent innervation and are the main sound transducers, while OHCs receive direct efferent innervation and are responsible for sound amplification

Glossary

Convergent evolution: refers to similar character states that evolve from different and separate ancestral states. The presence of parallel or convergent evolution is generally interpreted as independent clades arriving at similar solutions as they respond in similar ways to similar selection pressures.

Negative selection: Negative selection, also referred to as purifying selection, refers to the selective removal of deleterious alleles. At the coding sequence level, negative selection is identified as a lower rate of nonsynonymous versus synonymous substitutions.

Nonsynonymous substitution: a change in nucleotide that results in a change in the amino acid encoded by a codon.

Parallel evolution: refers to similar character states that evolve from a different common ancestral state.

Positive selection: evolutionary processes by which beneficial traits become fixed within a population. At the coding sequence level, positive selection is identified as a higher rate of nonsynonymous versus synonymous substitutions.

Synonymous substitution: a change in nucleotide that, as a result of the redundancy of the genetic code, does not result in a change of the amino acid encoded by a codon.

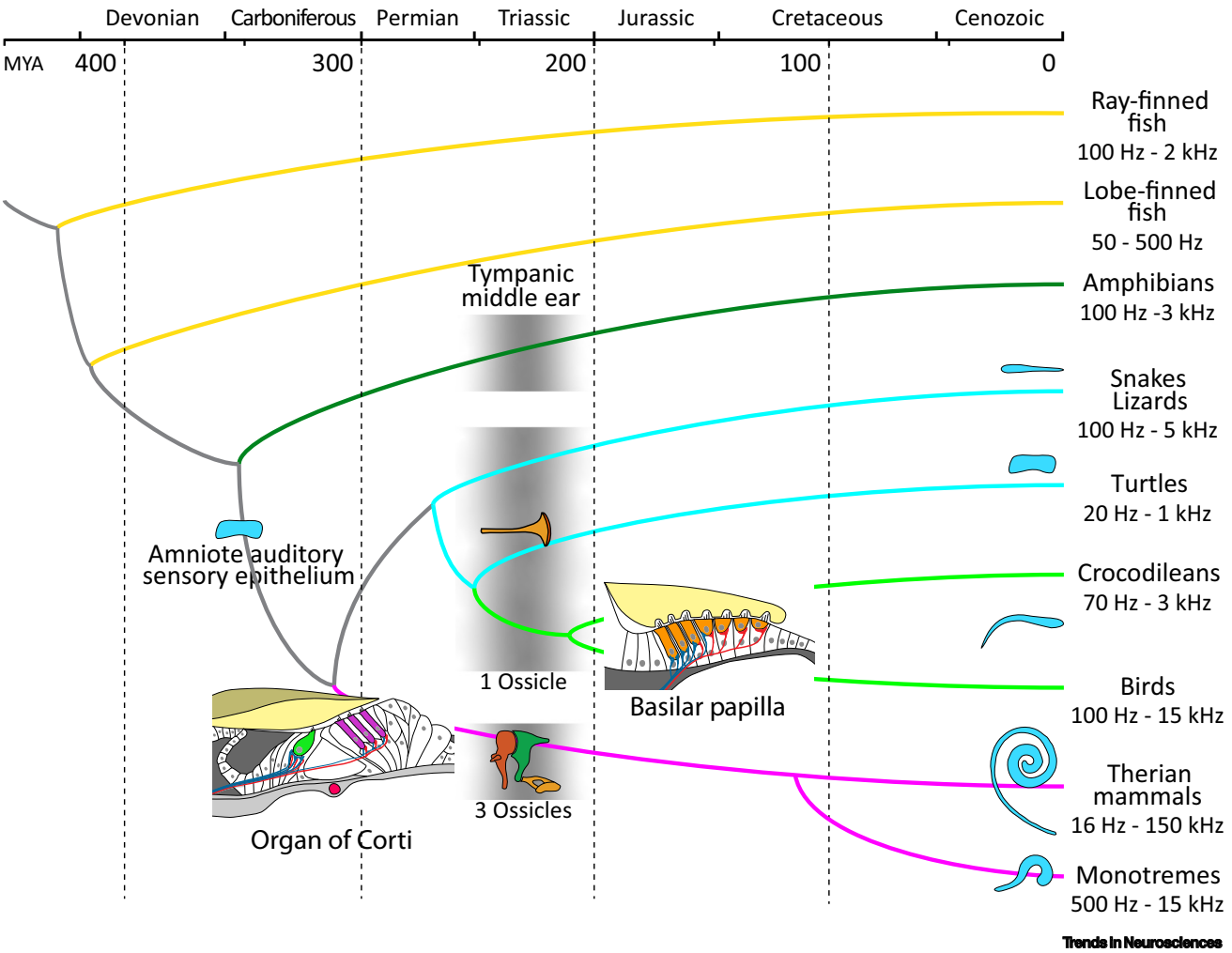

Figure 1. Evolution of the auditory system. Simplified diagram of the vertebrate phylogeny, illustrating the relationships between the major clades, the hearing ranges in extant species and the emergence of novel features in the amniote auditory system. The morphology of the auditory sensory epithelium is schematised in blue for the extant amniote groups. The grey shading denotes the independent emergence of tympanic middle ears in separate groups of tetrapods. The mammalian organ of Corti emerged before the appearance of the three-ossicle middle ear. It is characterised by two distinct hair cell types, inner and outer hair cells. Inner hair cells (green) are the sound transducers and receive afferent innervation. Outer hair cells (purple) drive sound amplification and receive mainly efferent innervation, though they are also contacted by type II afferents. The basilar papilla is the auditory sensory epithelium of birds and crocodilians. It displays a gradient of hair cell morphologies, from tall hair cells in the neural edge to short hair cells in the abneural border, with the tallest hair cells receiving mostly afferent innervation and the shortest hair cells contacted only by efferent innervation. The tectorial membrane overlaying the organ of Corti and the basilar papilla is shown in yellow. Phylogeny diagram compiled from [2,6–9]. Abbreviation: MYA, million years ago.

[4]. The origin of the organ of Corti pre-dates the elongation and coiling of the cochlea (Figure 1). It appeared early on in the stem mammals, before the separation of the pretherian branch and the emergence of tympanic middle ears, at a time when high frequency sensitivity was not present [9].

High-frequency sensitivity in mammals, ranging from 16 Hz to 150 kHz, is thought to be largely due to the specialisation of OHCs for sound amplification and tuning [15]. The salient property of OHCs, somatic electromotility, is based on the accumulation of prestin in the basolateral membrane [16–19]. In response to changes in voltage, prestin changes shape, and consequently OHCs length changes [19,20]. The stereocilia of OHCs are in contact with the tectorial membrane

[21], so changes in OHC length boost the sound stimulus to IHCs, improving the mechanical sensibility of the cochlea by ~1000-fold [15,18,22,23].

Mechanotransduction in vertebrate hair cells depends on K^+ influx, down a steep electrochemical gradient, through channels located at the tip of the stereocilia. The gradient stems from a positive endolymphatic potential, driven by high K^+ (and low Na^+) concentration in the endolymph, the fluid that bathes the apical surface of hair cells [24]. The K^+ that enters through mechanotransduction channels is cleared out of the hair cells by passive transport through the basolateral membrane, removed from the extracellular space and pumped back into the endolymph by specialised cells that differ across vertebrates [25–27]. The tegmentum vasculosum and the stria vascularis, evolved independently [25] and maintain the high endolymphatic potential in the auditory organs of birds (~10 mV; ~30 mV in owls) and mammals (~100 mV), respectively. In mammals, the stria vascularis emerged gradually after the split of the monotreme branch pre-dating high-frequency sound sensitivity [14,25].

The evolution of sensory systems dedicated to the detection and processing of sound and motion spans the entire history of multicellular animals [3]. Furthermore, the origin of receptor molecules and sensory cell types pre-dates multicellularity [1,28,29]. Consequently, the molecular processes driving the evolution of the auditory system are likely to encompass numerous examples of changes in protein coding sequences on 'hearing-enriched' genes, as well as changes in gene expression patterns.

Searching for signs of molecular evolutionary processes

Random sequence changes can affect gene expression patterns when they occur in regulatory regions, like promoters or enhancers. If, by contrast, a nucleotide change occurs in a protein coding region, it can be either a **synonymous** or **nonsynonymous substitution**. The former are neutral, as they do not change the amino acid being encoded (although the effect in codon usage can be significant [30]), while the latter change the encoded amino acid. The impact that nonsynonymous nucleotide substitutions can have on protein structure or function will range from negative to positive; that is, be completely deleterious, negligible, or result in a new, and potentially beneficial, function. The prevalence of function-altering changes depends on multiple factors, including the specific protein being affected. For example, amino acid changes that affect the function of a protein occur rarely in genes that are widely expressed because they likely affect the function of many systems, and are often **negatively selected** [31]. By contrast, mutations in genes that have restricted expression patterns are less pleiotropic (i.e., they affect a smaller number of traits) and more common. Nonetheless, it is difficult to identify causal relationships between changes in coding sequences and functional changes at the protein level that directly impact fitness, and examples of one-to-one correspondence are rare. This is both due to the experimental difficulties in establishing this relationship and, perhaps more interestingly, due to the notion that they are also biologically rare [32,33]. In fact, large effect size nonsynonymous mutations may be over-represented in the literature, as they constitute clear and striking examples of evolutionary processes, like the changes in the RNase activity that allowed colobine monkeys to access a different food source [34] and the changes in detection spectra of photoreceptor opsins [35].

Dozens of publications report searches for signatures of positive selection, or **convergent** or **parallel evolution** in inner ear genes (summarised in Tables 1 and 2). The majority of these studies focus on the mammalian lineage, with special emphasis on echolocating mammals. Most of the evidence supporting molecular evolutionary processes to date is limited to *in silico* sequence analysis. Although this does not unequivocally provide a link to the functional and

Table 1. Molecular evolution in inner ear hair bundle proteins[a]

Gene	Location/function	Deafness locus	Evolutionary process	Refs
Adgrv1	Ankle links	USH2B; USH2C	PS (primates, moles) CE (echolocating mammals)	[43,44,50,58]
Cdh23	Tip links	DFNB12	PS (mammals, echolocators, moles) CE, PE (echolocators)	[38,44,49,50,57]
Clic5	Stereocilia base	DFNB103	PS (primates[b])	[43]
Espn	Stereocilia shaft and tip	DFNB36	PS (mammals)	[57]
Fscn2	Stereocilia shaft		PS (chimpanzee)	[43]
Loxhd1	Stereocilia plasma membrane	DFNB77	PS (mammals, echolocating whales, moles[b]) CE (echolocators)	[41,50,57,62]
Myo15A	Stereocilia tip	DFNB3	PS (mammals, primates[b], moles[b]) CE (echolocators)	[43,50,58,61]
Myo3A	Stereocilia tip (transports ESPN)	DFNB30	PS (mammals, moles)	[57–59]
Myo6	Stereocilia shaft and taper (transports PTRPQ)	DFNA22, DFNB37	PS (primates[b])	[43,57]
Pcdh15	Tip links	DFNB23	PS (mammals, bats, echolocating whales, primates[b]) PE (echolocators)	[38,41,43,57]
Pjvk	Stereocilia rootlet	DFNF59	PS (echolocating whales) PE (echolocators)	[40]
Ptprq	Ankle region and stereocilia shafts	DFNA73 DFNB84	PS (primates[b])	[43]
Strc	Stereocilia horizontal top connectors	DFNB16	AS (moles[b])	[44]
Tmc1	Mechanotransduction	DFNA36 DFNB7/11	PS (mammals, echolocating bats) CE (echolocators)	[40,50,57,60]
Ush1C	Tip link upper insertion	DFNB18	PS, AS (moles[b]) CE (echolocators)	[44,50,58]
Ush2A	Ankle links		PS (primates[b]) AS (moles[b])	[43,44]
Whrn	Ankle links and stereocilia tips	DFNB31	PS (mammals)	[57]

[a]Abbreviations: AS, adaptive selection; CE, convergent evolution; PE, parallel evolution; PS, positive selection.
[b]Denotes molecular evolutionary processes within specific branches of the clade.

morphological changes that characterise mammalian hearing evolution, the repeated and independent identification of the same individual genes, which code for proteins crucial for inner ear function, lends support to their likely roles. Crucially, these bioinformatics analyses provide valuable starting points for experimental hypothesis testing.

Protein coding changes in inner ear genes

The evolutionary processes leading to mammalian-specific features of the auditory system at the molecular level have started to be unravelled. Early work described signatures of positive selection in the mammalian lineage, in two hair cell proteins, the motor protein prestin and the α10 nicotinic acetylcholine receptor subunit [36]. Since then, a number of studies have pointed to a high prevalence of protein-coding sequence changes during the evolution of the mammalian inner ear. For example, a high-throughput phylogenetic analysis reported that 13% of inner ear expressed genes showed signatures of positive selection in mammals, compared to nonmammalian vertebrates, both in sensory and nonsensory inner ear cells [37]. Moreover, positive selection in inner-ear-expressed genes has also been reported within mammalian branches [38–44]. In addition, instances of parallel and/or convergent evolution between echolocating bats and whales have been identified in hearing-related genes [38,40,45–51], potentially reflecting distinct environmental demands. Thus, the coding sequences of inner ear genes can be proposed as hotspots for evolutionary innovation in mammals.

Table 2. Molecular evolution in inner ear proteins involved in sound tuning and amplification, endolymphatic potential and synaptic transmission[a]

Gene	Location/function	Deafness locus	Evolutionary process	Refs
Frequency tuning and sound amplification				
Otoa	Attachment of the TM to spiral limbus	DFNB22	CE (echolocators)	[51]
Otog	Attachment crowns	DFN18B	PS (mammals, echolocating whales)	[41,61]
Slc26a5	Basolateral membrane	DFNB61	PS (tetrapods, mammals, therians, placentals, primates) CE, PE (echolocators)	[39,43,46,50,51,57,98,99]
Sptbn5	Cortical lattice?		PS (mammals)	[77]
Tecta	Tectorial membrane	DFNA8/12DFNB21	PS (mammals, moles[b])	[61,62]
Tectb	Tectorial membrane		PS (moles[b])	[58]
Endocochlear potential				
Cld14	HCs-SCs tight junctions	DFNB29	PS (whales[b])	[82]
Gjb2	Conexin26, K^+ recirculation	DFNA3 DFNB1	CE (echolocators)	[50,51]
Gjb6	Conexin30, K^+ recirculation	DFNA3 DFNB1B	CE (echolocators)	[51]
Kcnq4	Basolateral membrane	DFNA2A	PS (mammals, primates[b]) PE (echolocating bats)	[43,45,57]
Slc12a2	K^+ secretion	DFNA78	PS (moles[b])	[62]
Slc4a11	Spiral ligament fibroblasts	CHED2	PS (echolocating bats)	[42]
Ribbon synapse				
Homer2	Presynaptic scaffold	DFNA68	CE (echolocators)	[51]
Otof	Ribbon synapse Ca^{2+} sensor		PS (mammals) CE, PE (echolocators) RE (marine mammals)	[38,48,49,51,57]
Slc17a8	VGLUT 3 – glutamate vesicle loading	DFNA25	CE (echolocators)	[50,51]
Efferent synapse				
Chrna9	Efferent synapse		FS (mammals)	[93,94]
Chrna10	Efferent synapse		PS, FS (mammals) AE (human, mouse, opossum)	[36,92,93,105]

[a]Abbreviations: AE, accelerated evolution; CE, convergent evolution; FS, functional shift; PE, parallel evolution; PS, positive selection; RE, relaxed evolution.
[b]Denotes molecular evolutionary processes within specific branches of the clade.

Hair bundles and mechanotransduction

The hair bundles of inner ear sensory cells are staircase-like arrangements of actin-filled microvilli, termed stereocilia. They are composed of dozens of proteins, including actin-binding proteins, links, and connectors, supporting the mechanotransduction machinery that sits at the top of the shorter stereocilia [52–55]. Variations in hair bundle morphology and mechanotransduction functional properties are crucial contributors to frequency selectivity [15,54,56], and therefore potential substrates for molecular changes driving the expansion of the hearing range. Numerous instances of molecular evolutionary processes have been described for hair bundle proteins (Table 1). Genes encoding proteins constitutive of stereocilia bundles have been described under positive selection in the lineage leading to mammals [37,57], as well as in different mammalian branches (primates [43]; echolocating whales [38,41]; echolocating bats [38]; and mole rats [44,58,59]). These include proteins localised at ankle links and stereocilia rootlets, along the stereocilia shaft, at the stereocilia tips and on the tip links (Figure 2). Additionally, convergent evolution has been reported for stereocilia proteins in echolocating mammals, leading to the hypothesis that these were likely driven by selection for the detection of high frequency sounds [38,40,49–51].

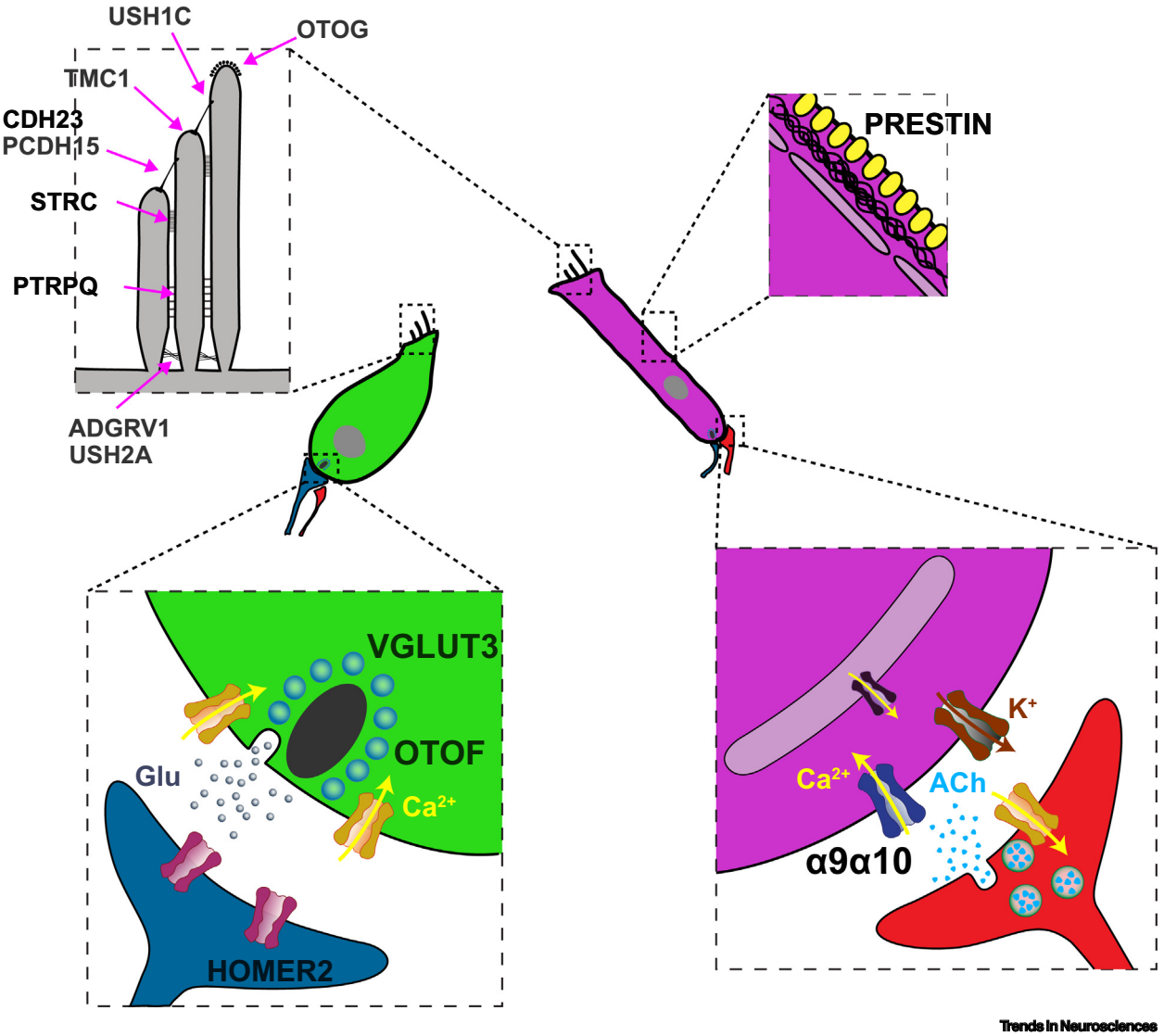

Figure 2. Molecular actors of mammalian hearing evolution. Diagram of mammalian inner and outer hair cell showing the location of key proteins identified to have undergone molecular evolutionary processes in mammals. Zoomed in diagrams show the location of hair bundle proteins (top left), prestin in the lateral membrane of outer hair cells (top right), as well as components of the ribbon synapse (bottom left) and of the efferent synapse (bottom right). See Tables 1 and 2 for further details and references.

It was initially thought that the subterranean lifestyle of mole rats, which shifted their auditory environment to lower frequency sounds (125 Hz to 8 kHz in naked mole rats) had led to relaxed evolutionary pressure on hair bundle proteins that had been fine-tuned to higher frequencies [44]. However, careful analysis identified adaptive amino acid changes in stereocilia proteins in mole rats that, through altered hair bundle morphology, may have resulted in the elevated auditory thresholds and lack of cochlear amplification in extant mole rats [44].

Finally, the mechanotransduction complex is formed by multiple subunits, with TMC1 lining the channel pore [52,53]. Signatures of positive selection have been identified in Tmc1 in the lineage

leading to mammals [57,60] and in clades of echolocating bats [40] and whales [41]. In addition, convergent evolution has been described between echolocating mammals [40,50]. Overall, the numerous examples of molecular evolutionary processes in hair cell stereocilia proteins suggest a crucial role for hair bundles in the expansion to high-frequency hearing.

Sound frequency tuning and amplification

The frequency components of sound are separated along the auditory sensory epithelium. Frequency discrimination is based on electrical and/or mechanical, passive or active resonance behaviour [15]. In vertebrates, there are three main frequency tuning mechanisms: electrical resonance, mechanical resonance (and active force generation by hair bundles) and somatic electromotility. Electrical resonance operates at sound frequencies under 1 kHz, and is based on the properties of potassium channels. It has been described in amphibians, reptiles, and birds, but is absent in mammals [15]. Mechanical resonance relies on the morphological properties of the basilar membrane, sensory epithelium, hair bundles and overlaying tectorial membrane. The latter is composed mainly of α and β tectorins and type II collagen, and sits above the auditory sensory epithelium. Signatures of positive selection have been identified in the coding region of α-tectorin in mammals [61] and mole rats [62], and β-tectorin in the star-nosed mole [58]. Convergent evolution in echolocating mammals has been described for otoancorin, a protein involved in the attachment of the tectorial membrane to the spiral limbus (Table 2) [51,63]. This identifies proteins involved in mechanical resonance as potential drivers of evolutionary change in mammalian hearing.

Somatic electromotility is a mammalian-exclusive feature that plays a crucial role in sound amplification and tuning even at frequencies exceeding 20 kHz [23,64,65]. It is based on the motor function of prestin (Slc26a5); a member of a family of anion transporters that in mammals is present at the basolateral membrane of OHCs [19]. Prestin expression has been reported in many vertebrate hair cells [66], including in the chicken basilar papilla [67]. A role for prestin in sound amplification in birds has been proposed [15,67]. However, no direct evidence supports the presence of prestin-driven electromotility in chicken [68,69].

The ancestral character state of prestin is that of an electrogenic anion exchanger, similar to that of other members of the family [70]. However, although binding of anions into a central core domain binding site is mechanistically and structurally conserved in comparison to other SLC26 transporters [71,72], and fundamental for the electromotile function of prestin [20,73,74], mammalian prestin lost transport capacity and gained voltage-driven motor capabilities [20,73]. The molecular evolutionary history of this remarkable functional metamorphosis has been studied in detail (Box 1).

In addition to changes in prestin, a complete analysis of the evolutionary history of somatic electromotility would need to look further into the origin of OHCs as a distinct cell type. For example, the tips of the OHC stereocilia are attached to the tectorial membrane, via attachment crowns formed of otogelin, otogelin-like, and stereocilin [21,75]. For otogelin, signatures of positive selection have been identified in the mammalian lineage [61]. Also, a specialised submembrane cortical network supports the lateral wall of OHCs. This network is fundamental for electromotility and formed by a trilaminated structure comprising the plasma membrane (containing highly packed prestin molecules), the cortical lattice, and subsurface cisternae [17,76]. The cortical lattice consists of circumferential actin filaments cross-linked by longitudinal spectrin. It has been suggested that the giant spectrin βV is a crucial component of the cortical lattice, necessary for electromotility and that the coding region of the *Sptbn5* gene has been under positive selection in the mammalian lineage potentially linking it to a novel function in sound amplification [77]. However, recent evidence contests the expression of spectrin βV in OHCs and suggests that OHC function is normal in

Box 1. Molecular evolution of prestin electromotility

The electromotile properties of prestin first appeared in the stem lineage of all mammals [69,98] and where refined in the therian and placental branches [98] and in echolocating mammals [39,46,99]. Instances of positive selection highlight amino acid changes [36,37,57,98] that are linked to the acquisition of electromotility [98]. The ancestral character state of prestin is that of an electrogenic anion exchanger [70]. In extant mammals, prestin directly converts voltage changes to force [20,73].

The voltage-sensing properties of prestin are experimentally measured as nonlinear capacitance (NLC), with a characteristic bell-shaped dependence on membrane potential [100]. The latest cryoelectron microscopy structures of prestin support a model for an intrinsic voltage sensor; a mechanism that is fundamentally different from membrane transporter movements [73]. In contrast to voltage-gated ion channels, prestin lacks an extensively charged region embedded in the membrane that can act as the voltage sensor, and residues that affect prestin NLC show a wide dispersion, mainly in the transmembrane domains [20,73]. Some of these transmembrane sites have been identified as positively selected: C426, in mammals; M236, in therians; and C207, in placentals (numbering from Figure S2 in [98]). Furthermore, mutation of three mammalian-exclusive glycine residues in the TM6 domain back to their nonmammalian counterparts affects NLC [74], strongly suggesting they constitute crucial steps during the acquisition of electromotility.

Cl^-, although not transported by mammalian prestin, binds to it [20] at a domain similar to the one identified for the SLC26A9 transporter [20,72,73]. Here, residue 140 (numbering from Figure S2 in [98]) at the TM3 domain is a conserved threonine in SLC26A9 and in nonmammalian prestin, but a proline in mammalian prestin. Since nonmammalian prestin orthologs are electrogenic divalent/Cl^- transporters [70], it can be proposed that this substitution may be part of the evolutionary transitions that led to the loss of anion transport in mammalian prestin. Moreover, the reverse mutation in gerbil prestin does not alter NLC, indicating that this Cl^- binding site is not part of the voltage sensor, but is, however, necessary for electromotility, likely via an allosteric mechanism [73].

While loss of transport capabilities and the emergence of electromotility involve different sites within the protein [101], parallels have been drawn between these two processes [68,98]. Three adaptive events have been proposed for early mammalian evolution in this context: gain of nonlinear capacitance and electromotility in stem mammals; improvement in electromotility in therians; and further enhancements related to prestin loss of solute transport in placental mammals [98]. The voltage dependence of prestin was further refined in echolocating mammals, seemingly driven by convergent amino acid changes and in relation to high frequency hearing [46,51,99].

Spnb5 knockout mice [78]. Further research on the spectrin protein family, alongside the many other genes that define OHCs is needed to unravel the evolutionary origins of somatic electromotility.

Endolymphatic potential

A positive endolymphatic potential, and the concentration gradient of K^+ and Ca^{2+} ions between the endolymph and cytosol of hair cells, are the electrochemical driving force for hair cell mechanotransduction currents. The analogous mammalian stria vascularis and avian tegmentum vasculosum generate and maintain the endolymphatic potential, using a conserved molecular tool kit for ion transport, albeit with some exceptions [25,26]. The K^+ channels kir4.1 and 5.1, are expressed in the stria vascularis and absent from the avian analogue [26]. The incorporation of the kir4.1 current may have been a major driver for the increase in K^+ concentration, and endolymphatic potential, in the mammalian endolymph a [25]. A higher endolymphatic potential has been linked to higher frequency hearing, even within the mammalian cochlea, where the endolymphatic potential increases alongside the tonotopic gradient [25].

The efflux of K^+ from hairs cells and the subsequent recirculation involve ion channels, transporters and pumps localised in hair cells, supporting cells and nonsensory epithelial cells. In mammals, K^+ leaves the hair cells mainly via kir4.7 channels (encoded by Kcnq4) and via BK Ca^{2+}-dependent K^+ channels. In chicken hair cells, Kcnq1 and Kcne1 are also involved [27]. Signatures of positive selection in mammals [43,57] and of parallel evolution in echolocating bats [45,79] have been identified in the coding region of Kcnq4 (Table 2). Moreover, in echolocating bats, positively selected and parallel sites have been identified in Slc4a11 [42], a solute carrier involved in osmotic balance present in the fibroblasts of the spiral ligament and essential for the generation of the endolymphatic potential in the cochlea [80]. Also relevant for K^+ homeostasis is

claudin-14, a tight-junction protein expressed in hair cells and supporting cells that may be involved in maintaining the ionic composition of the fluid surrounding the basolateral surface of OHCs [81]. Signatures of positive selection have been identified in the coding region of the *Cldn14* gene in echolocating toothed whales [82]. Additionally, Slc12a2, which encodes the Na^+/K^+ transporter NKCC1 involved in K^+ secretion to the endolymph [26] and in K^+ reuptake by supporting cells in chickens [27], has been under positive selection in naked mole rats [62]. Finally, *Gjb2* and *Gjb6*, the genes coding for connexin 26 and connexin 30 that participate in K^+ recirculation [83], have been described under convergent evolution in echolocating mammals [50,51]. Overall, these lines of evidence identify a high endolymphatic potential and K^+ recirculation as crucial factors in the refinement of amniote hearing, with changes in both coding sequences and gene expression patterns potentially contributing to the extension of the hearing range in mammals.

Ribbon synapse

Information transfer from hair cells to the auditory nerve occurs at a specialised ribbon synapse that transmits acoustic information with high fidelity and precision to the afferent dendrites of the spiral ganglion neurons. This synapse differs from those typically encountered in the central nervous system, and is present in cochlear, vestibular, and lateral line hair cells, photoreceptors and bipolar neurons that respond to sustained and graded stimuli. In ribbon synapses, vesicles are tethered to electron dense ribbons and use a unique combination of molecules and mechanisms for neurotransmission [84]. Vesicle release is triggered by the activation of L-type ($Ca_V1.3$) voltage-gated Ca^{2+} channels located at the presynaptic active zones (Figure 2). Moreover, hair cell ribbon synapses do not express neuronal SNARE proteins, nor classical Ca^{2+}-sensing proteins such as synaptotagmins. Instead, in cochlear hair cells otoferlin is the primary Ca^{2+} sensor for exocytosis [85]. *Otof*, the gene encoding otoferlin, has been described to be under positive selection in the mammalian lineage [57], with additional signatures of parallel and convergent evolution in echolocating bats and dolphins [38,49,51]. Additionally, glutamate loading into vesicles in the hair cell ribbon synapses is exclusively mediated by Vglut3 (Slc17a8), also reported to be under convergent evolution in echolocating mammals [50,51]. Finally, a convergent amino acid substitution has been reported in the postsynaptic scaffold gene *Homer2* between echolocating bats and dolphin [51]. Overall, the unique combination of ribbon synapse proteins, alongside the accumulation of individual amino acid changes in several crucial actors (Table 2), may relate to more reliable high frequency transmission, therefore facilitating the acquisition of high frequency hearing in mammals.

Efferent synapse

An ancestral feature of vertebrate inner ear hair cells is the presence of efferent innervation from (mainly) cholinergic neurons located in the ventral hindbrain [86]. In the auditory system, efferent innervation modulates sound amplification and the innervation patterns have followed hair cell diversification, mainly targeting short hair cells in birds and OHCs in mammals [4]. The postsynaptic acetylcholine receptor in hair cells is composed of α9 and α10 nicotinic subunits [87,88]. The α9α10 nicotinic acetylcholine receptor (nAChR) is a nonselective cation channel. However, the efferent synapse to hair cells is inhibitory, due to the coupling to Ca^{2+} activated K^+ channels (Figure 2) [89–91], indicating the functional relevance of the extent of Ca^{2+} permeability of the α9α10 nAChR for shaping the postsynaptic responses and hair cells inhibition.

The coding sequences of the α9 and α10 subunits have changed significantly within the mammalian lineage, with several sites under positive selection in the α10 subunit [36,92], and functionally divergent sites identified in mammalian α9 and α10 subunits [93]. The functional implications of these changes have been extensively studied in recombinant α9α10 nAChRs, describing the loss of choline agonism and an increase in calcium permeability in mammalian α9α10 nAChRs [94,95] (Box 2).

Box 2. Molecular evolution of the α9α10 nAChR

The hair cell nAChR is composed of α9 and α10 subunits. These were the latest members identified of the family of nicotinic acetylcholine ligand-gated ion channels [87,88] that also includes neuronal and muscle nAChRs [102]. They are also the most divergent subunits, in terms of their sequence identity with other nicotinic subunits [92,93,102] and within the amino acid sequences of vertebrate α9 [93,94] or α10 [92,93] subunits. Signatures of positive selection have been identified for the α10 subunit, specifically in the branch leading to mammals [36,92] and functional amino acid shifts have been pinpointed, again for mammals, in the α9 and α10 subunits [93]. Within the α9 subunit, three amino acid changes have resulted in an increase in relative calcium permeability of the mammalian α9α10 receptor [94]. Two changes, N110D and F127S (see [94] for numbering) sit at the vestibule region of the extracellular domain. Molecular dynamics simulations and homology modelling showed that the residues interact with permeating Ca^{2+} and that they contribute to the electrostatic potential of the vestibule region, potentially affecting the entry of Ca^{2+} into the transmembrane pore region [94]. The third amino acid change, A264D, is located at a well characterised amino acid ring important for ion permeation in nAChRs [103] and may affect Ca^{2+} exit from the channel pore [94].

The amino acid changes in the α10 subunit are more numerous. Overall, they affected the role of the α10 subunit during channel gating. The mammalian α10 subunit is unable to contribute the complementary side to the ligand binding site, which sits at the interphase between consecutive subunits [104]. Moreover, the changes in the mammalian α10 subunit resulted in the loss of agonism by choline, the degradation product of ACh in the synaptic cleft [95]. The increase in relative Ca^{2+} permeability, alongside the loss of choline agonism, of the mammalian α9α10 nAChR may relate to the specific demands for efferent modulation of electromotile OHCs [102].

It is likely that the evolutionary changes in the α9α10 nAChRs of mammals accompanied the specialisation of the efferent system for the modulation of high-frequency hearing and active somatic electromotility.

Experimental evidence for evolutionary changes in inner ear genes

The accumulation of changes in the protein coding regions of genes expressed in the inner ear, and especially the accumulation of those changes in key structural proteins, suggests that a higher-than-expected prevalence of positively selected changes, affecting the function of such proteins, characterises the evolution of mammalian hearing. However, experimental evidence supporting this proposal is limited. The functional consequences of changes in coding sequences have only been experimentally tested in prestin and the nAChR (Boxes 1 and 2 and references therein). For the majority of evolutionary processes identified in inner ear proteins (Tables 1 and 2), only correlations have been described between amino acid changes and system level changes (e.g., stereocilia morphology or high frequency hearing). Nonetheless, the recurrent, and independent, identification of genes under positive selection (often highlighting specific amino acid changes), provides a strong foundation for building testable hypotheses (see Outstanding questions).

Genetics of deafness and molecular evolution of mammalian hearing

The expression pattern of genes greatly influences their evolutionary histories. Genes may be more likely to go through positive selection if they have reduced or tissue-specific expression [31]. Numerous examples of inner ear genes fall under such description. In addition, an overwhelming majority of the genes for which evidence of molecular evolutionary processes at the coding sequence levels has been described are also hereditary deafness loci (Tables 1 and 2). These includes genes related to syndromic and nonsyndromic hearing loss, with the latter more abundantly represented.

A study of 11 deafness-related genes showed that the disease-associated mutations are predominantly located in conserved regions of the coding sequence of these genes [61], suggesting that the individual amino acid changes driving evolutionary or disease processes are largely unrelated. This is perhaps unsurprising, given that amino acid changes fixed during evolution tend to be neutral or beneficial, while disease-associated amino acid changes greatly

impair protein function. Nonetheless, both processes are localised to the same gene highlighting the importance of bringing in evolutionary insights to the study of deafness and vice versa.

Concluding remarks and future perspectives

From an evolutionary perspective, the most salient property of mammalian hearing is the extension of the frequency range to high frequency sounds. However, many of the mammalian-exclusive features of the inner ear pre-date high frequency hearing and some occurred before the emergence of a tympanic middle ear (Figure 1), raising the question of what the selection pressures behind them were (see Outstanding questions). The study of the molecular evolutionary histories of genes involved in the development, morphology and function of mammalian auditory structures can contribute new insights into their emergence along mammalian phylogeny. Here, we reviewed evidence on coding sequence changes that may have affected the function of inner ear proteins. To date, the functional consequences of those changes have only been analysed in detail for prestin (Box 1) and the α9α10 nAChR (Box 2). At the whole-cell level, amino acid changes in stereocilia proteins likely resulted in the altered hair bundle morphology of mole rats [44]. The functional impact of the remaining molecular evolution events listed in Tables 1 and 2 remains to be elucidated (see Outstanding questions). Additionally, comparative functional genomics, including the analysis of changes in gene expression patterns of orthologous genes across species [96] holds the potential to complement studies on protein sequence change, by identifying the gene regulatory networks and signalling pathways behind the emergence of novel cell types (e.g., IHCs and OHCs) and tissue level structures (e.g., stria vascularis). These studies are so far scarce, but the implementation of single cell transcriptomics as a routine tool for analysing cell-specific gene expression profiles holds the promise to quickly advance current knowledge of the phylogenetic origin of inner ear cell types (see Outstanding questions). Finally, several comparative studies have described clade-specific features of the neuronal circuits dedicated to sound processing [5,97]. However, the molecular evolutionary events behind them are yet to be fully explored. Such studies may provide an interesting contrast against inner ear evolution. The neurons of the hindbrain nuclei lack the extensive collection of near-exclusive genes that are a hallmark of inner ear cells. This leads to the hypothesis that sequence changes linked to the evolution of hindbrain auditory nuclei may be predominantly located in regulatory regions, leading to changes in gene expression patterns within the component neurons, whereas sequence changes linked to the evolution of inner ear structures, as reviewed here, can be localised to protein coding regions (see Outstanding questions). The decades of research summarised here highlight the relevance, for both basic and clinical research, of embracing comparative approaches for the study of sensory systems.

Outstanding questions

Bioinformatics analyses have identified hundreds of positively selected sites in inner ear proteins within the mammalian phylogeny. Beyond the well characterised prestin and α9α10 nAChR, what are the functional consequences of the amino acid changes at the protein level for inner ear genes?

Identifying genes that have changed along a phylogeny is informative to framing evolutionary adaptations. Equally important is the identification of genes that have remained conserved at the sequence level. Which are the inner genes under negative selection in the mammalian lineage?

What are the evolutionary changes in gene expression patterns in the inner ear? How do they relate to the emergence of new cell types or structures? How do they relate to widely conserved features present in all mechanosensory hair cells?

What were the evolutionary drivers behind the emergence of the organ of Corti?

What are the molecular evolutionary processes behind the emergence of novel neural circuits for sound processing?

Regeneration of lost sensory hair cells does not occur in the mammalian organ of Corti. By contrast, there is prevalent hair cell regeneration in non-mammalian auditory epithelia like the avian basilar papilla. Can expanding our knowledge of the evolutionary history of mammalian hearing help address the unsolved puzzle of hair cell regeneration in the mammalian organ of Corti?

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Declaration of interests

The authors declare no conflicts of interest.

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