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Molecular Understanding of Hearing – How Does This Matter to the Hearing Impaired?

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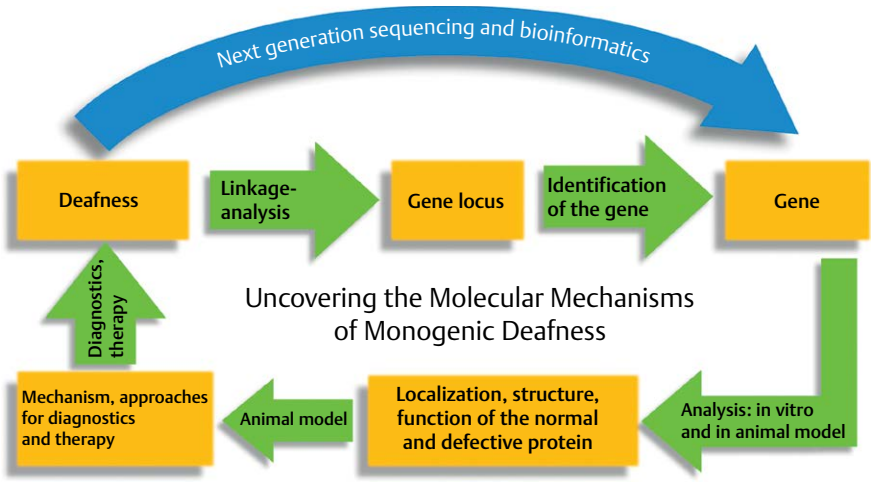
ABSTRACT

This review addresses the advances of our molecular understanding of hearing and how this benefits the hearing impaired. Classical biochemical methods usually fall short in contributing to the analysis of the molecular mechanisms of hearing e. g. in the cochlea, the auditory part of the inner ear, due to the scarcity of the cells of interest. Genetics, molecular cell biology, and physiology, on the other hand, have elucidated the intricate molecular and cellular mechanisms that bring about the outstanding performance of the auditory system. Many of those mechanisms are quite unique and specialized to serve the specific needs of hearing. Hence, their defects often spare other organs and lead to specific non-syndromic deafness. High throughput sequencing can reveal causes of sporadic deafness when combined with careful bioinformatics. Molecular approaches are also helpful for understanding more common forms of hearing impairment such as noise-induced hearing impairment. While molecular therapies are not yet clinically available, careful molecular genetic analysis helps to counsel the hearing impaired subjects.

Contents

1. From human genetics to molecular mechanisms and back to the patient

In the past years, major efforts have been undertaken to discover genes with important function in the inner ear. They spanned from the cloning from cDNA libraries that were created from animal tissue at different time points in inner ear development, over the analysis of the transcriptome of single cells, the proteomics of massive collections of animal inner ears, highly sensitive mass spectrometry of immune-purified subcellular compartments, linkage analyses in large, often consanguine families with genetic hearing impairment, up to the identification of pathogenic mutations associated with sporadic hearing impairment by means of high throughput sequencing technologies. The approaches making use of human genetics had and have a particular significance. Numerous genes with key functions in the cochlea were identified as so-called deafness genes (http://hereditaryhearingloss.org/) by means of human genetic analyses, initially mainly by linkage analyses with subsequent Sanger sequencing of candidate genes within the linked region [1, 2], while nowadays application of high throughput sequencing procedures [3, 4] (next generation sequencing, NGS) is becoming a standard procedure (▶ **Fig. 1**). In contrast to the formerly applied Sanger sequencing that was limited to the identification of mutations in few genes (such as for example the GJB2 gene coding for connexin 26), NGS-based panels with numerous deafness genes can be tested for identification of causal mutations and even sequencing of the entire exome (whole exome sequencing, WES; exons are the entirety of the transcribed gene segments) of the hearing impaired subject or trio-WES (patient and parents) can be performed. The decision,

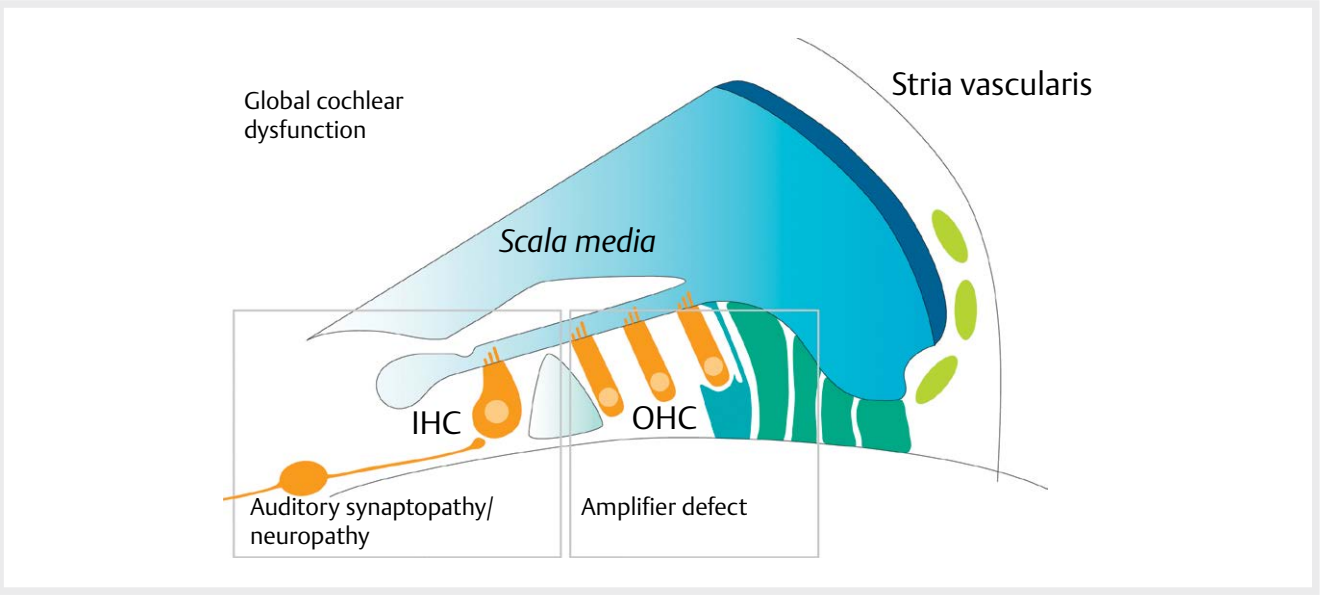


▶ **Fig. 1** Elucidation of the molecular mechanism in cases of monogenic hearing loss. If hereditary hearing loss is suspected, linkage analysis of the gene loci of the affected gene and gene identification may uncover which gene is affected. This procedure can be shortened by next generation sequencing and bioinformatics. In vitro analysis and generation of animal models for newfound mutations may then identify localization, structure, and function not only of the normal but also the defective protein. This allows further assessment of the disease mechanism and the development of diagnostic and therapeutic approaches that may then be applied for treatment of hearing impaired patients.

which type of molecular diagnostics can be performed and how many deafness genes are sequenced, is mainly determined by the context of the examination (scientific evaluation or routinely performed diagnostics).

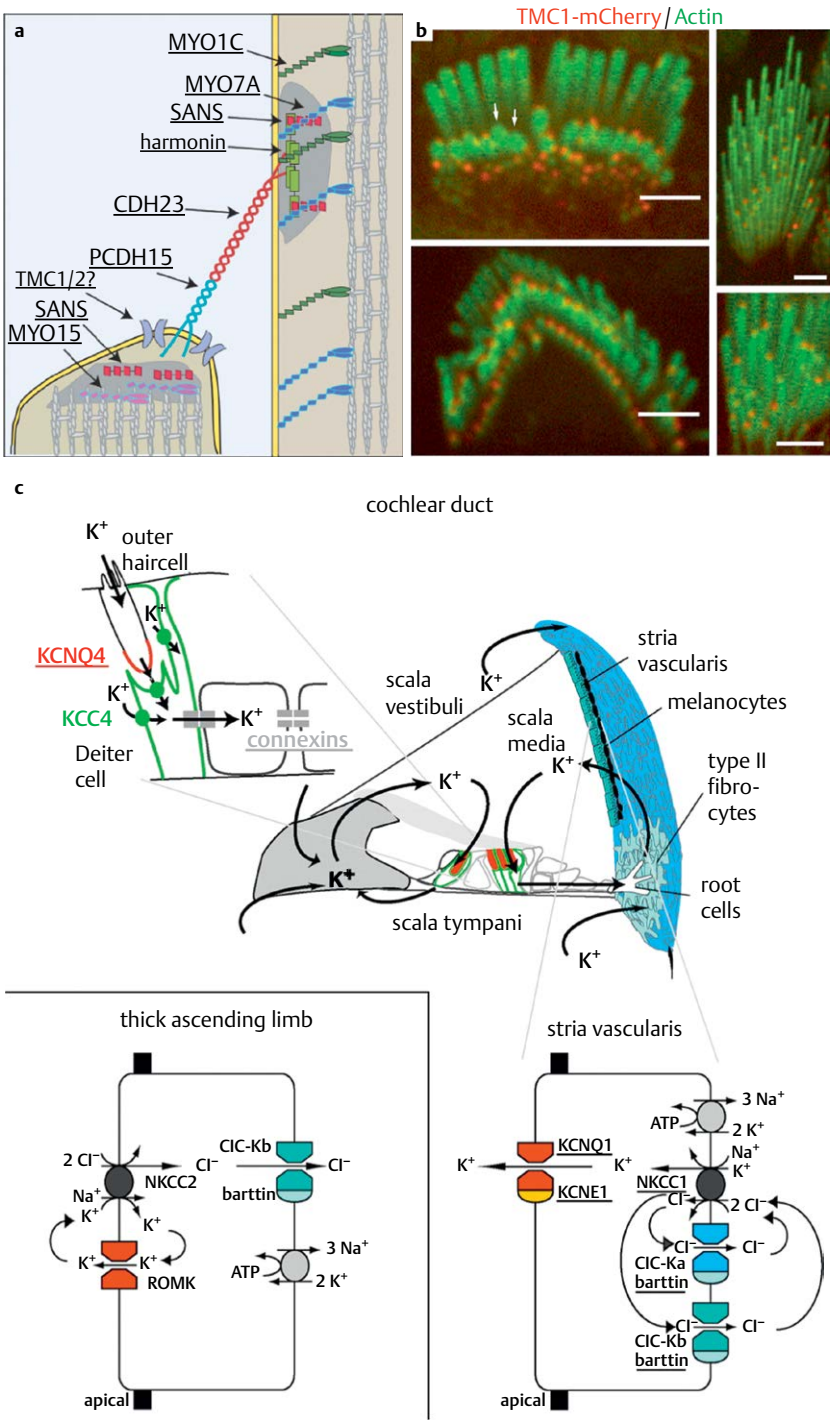
In conjunction with audiometric phenotyping and pedigree analysis, NGS-based sequencing then also identifies the pattern of inheritance; e. g. autosomal recessive (gene/disease is marked with DFNB, both parents are carriers of the mutation but healthy), autosomal dominant (DFNA, depending on penetrance one of the parents may be hearing impaired), and X-chromosomal (DFNX, the mother is carrier of the mutation but usually healthy, male descendants are affected). The sequences generated by NGS-based methods (so-called "reads") require a careful bioinformatic analysis as well as professional interpretation of the detected variants by a human geneticist in order to identify potentially pathogenic mutations. Even if the sequencing is available for well-informed otolaryngologists, the interpretation of the variants from the NGS data requires experience and is performed e. g. in the Institute of Human Genetics of the University Medical Center Göttingen by an entire team of experts. Since July 2016, there are now for the first time items in the German physicians' fee schedule (Einheitlicher Bewertungsmaßstab, EBM) that allow application of NGS-based routine diagnostics. However, the number of genes to be analyzed is restricted so that large panels with many genes or even exome sequencing require prior declaration to bear costs by the responsible health insurer. Despite those restrictions, the possibility to invoice NGS-based services represents a great success and will facilitate the identification of pathogenic mutations in cases of sporadic sensorineural hearing impairment. Already today, NGS-based analysis is applied in human genetics in university medical departments but also in practices and so it is recommended to establish intensive cooperation in this field.

The gene identification is then followed by generation of molecular probes such as antibodies, in vitro structural, biochemical, and biophysical analyses of the recombinant protein and the creation of genetically modified organisms such as fruit flies, zebrafish, and/or mice for further functional characterization of the gene [5]. For this, different genetic approaches like chemical mutagenesis, homologous recombination, and genome editing are applied. In the last years, numerous mouse mutagenesis programs have been conducted and in some cases the genetically modified embryonic stem cells or even finalized mouse mutants were provided for scientific purposes at reasonable expense (e. g. https://www.komp.org/; http://www.mousephenotype.org/; https://www.infrafrontier.eu/search). However, the path from embryonic stem cells to homozygous mouse mutants is challenging and typically even university otolaryngology departments have to rely on cooperation with experienced partners to successfully complete it. If the function of one specific gene is in the focus of interest, it is recommended to start researching which other group(s) are working on this gene even or in particular when those groups do not themselves perform research in the auditory system. Additionally, there are now companies which offer the production of genetically modified animals for research purposes, but the costs are substantial. Finally, the mouse holding capacities are limited in many medical universities and/or the expenses for animal holding are extremely high such that an intensive gene analysis in the mouse model is limited. Similar repositories exist for mutant fruit flies and zebrafish and the costs for keeping them are significantly lower. Based on this and also on other advantages such as the ease of acute expression of a specific gene, the zebrafish may represent an important intermediate stage with the hair cells of its lateral line organ and the inner ear (e. g. [6]). However, one must bear in mind that the anatomy and physiology of the inner ears of fish and mammals are markedly different so that statements on human hearing impairment based on findings in zebrafish have to be made with great caution.



▶ **Fig. 2** Pathomechanisms of sensorineural hearing loss. Damage of the outer hair cells leads to a reduction or the complete loss of non-linear cochlear amplification. This impairs the sensitivity and frequency discrimination of hearing. Impaired sound coding and signal propagation by inner hair cells and spiral ganglion neurons lead to auditory synaptopathy or neuropathy, where in particular the temporal processing of registered sound stimuli is impaired. In cases of global cochlear disorders such as e. g. disturbed endolymphatic secretion or defective mechanoelectrical transduction, the amplifying mechanisms as well as sound coding are impaired. (OHC: outer hair cells, IHC: inner hair cells). Taken from [9].

The morphological and functional characterization of a gene requires the availability of several methods such as immunohistochemistry, light and electron microscopy with different resolutions, systemic and cellular auditory physiology, and behavioral analyses. Some of the applied methods will be discussed in the following chapters. The observed mutant phenotypes allow understanding the role of the gene of interest in hearing [5, 7]. The expertise available in otolaryngology departments regarding the morphological and functional analysis of hearing is readily used by research groups working in other disciplines. Among others, the measurements of auditory brainstem responses (ABR) and otoacoustic emissions in rodents [8] as well as immunohistochemistry and confocal microscopy can be realized here and are appropriate work for doctoral theses and research rotations of residents in training. Furthermore, in particular findings regarding systems physiology and behavioral biology in a mouse model for genetic hearing impairment may be very helpful for clinical diagnostics. Findings in research of molecular physiology in mice, for example, have led to a pathophysiological classification of sensorineural hearing loss (▶ **Fig. 2**, [7, 9]). The specificity of the determined phenotypes can be controlled by re-insertion of a healthy copy of the gene, e. g. with the help of viral vectors. Such "rescue experiments" and their methodical implementation in the mouse model may further serve as feasibility trial for the development of possible gene therapeutic approaches [10–14]. Genetic trials that investigate the role of candidate genes in the inner ear because of an expected analogy to other systems have often found interesting deviations in the molecular profile of inner hair cells and helped to consider alternative and unconventional molecular mechanisms (see chapter on synaptic sound coding). In addition, those studies were helpful for the establishment of new methods for examination of the inner ear. Finally, molecular and cell physiological understanding of hearing is an important precondition for novel approaches to hair cell regeneration [15] and other innovative therapy approaches like the optical cochlear implant [16]. Based on this, a clinical trial on transdifferentiation of supporting cells into hair cells is at the moment conducted in the USA [17] in which virus-mediated expression of the transcription factor Atoh1 shall be induced in these cells [18]. With regard to strategies for cell replacement therapy making use of exogenous cells obtained from stem cells, further progress has been made, for example in attempts to regenerate spiral ganglion neurons [19]. The possibility to generate inner ear organoids [20–22] promises a clearly improved availability of sensory cells and neurons for pharmacological and genetic analyses and progress in future cell replacement therapy. However, its development and potential clinical translation will likely need more than another decade. Up to then, hearing aids and cochlear implants will remain the most important options for hearing rehabilitation and their further improvement is a relevant focus of research and development. One future-oriented example for those activities is the field of optic stimulation of the cochlea where research in Germany is currently in a leading position. On one hand, the Medical University of Hannover and the Saarland University Medical Center initiated the development of a new generation of hearing aids that use the possibility of optomechanically stimulating the cochlea [23, 24]. On the other hand, the University Medical Center Göttingen in cooperation with the University of Freiburg and the Technical University of Chemnitz works on the development of an optical cochlear implant for optogenetic stimulation of virus-transduced spiral ganglion neurons [25, 26]. Since light can be confined in space better than electric stimuli, the optical cochlear implant promises a finer frequency resolution of coding and thus an improved speech understanding in noise.



▶ **Fig. 3** The cochlear potassium cycle. **a** Tip-links between the stereocilia of hair cells are generated and anchored by a complex molecular mechanism. The proteins involved are entirely coded by sequences identified as deafness genes. **b** TMC1 is a candidate for the potassium-permeable mechanotransduction channel of the stereocilia which is opened by tension within the tip-links. Immunofluorescence labeling of TMC1 (red) localizes it at the tips of the stereocilia, which are immunolabeled against actin (green). The figures show the stereocilia of inner hair cells (top left) and vestibular hair cells (top and bottom right) of the mouse cochlea. Scale bar: 2 µm. Modified from [31]. **c** The potassium cycle in the cochlea enables the activity of the hair cells. The high potassium concentration in the scala media allows passive influx into the hair cells from which potassium in turn flows out via potassium channels, among others KCNQ4. There, it is taken up by supporting cells and forwarded to the stria vascularis where it is taken up into the marginal cells via NKCC1 (bottom right). From there, it is then released back into the scala media by KCNQ1 channels. This process, too, involves numerous deafness genes (underlined). For comparison: similar functioning of the potassium recycling of the kidneys (bottom left). Modified from [50].

2. The potassium cycle, transduction, and division of labor in the cochlea

The so-called "potassium cycle" of the inner ear is a "hotspot" of genetic hearing impairment, including the connexin 26 gap junction channels that are defective in the frequently observed autosomal recessive hearing loss DFNB1A. The physiological significance is with the use of potassium ions for mechanoelectrical transduction by hair cells, which is a "genius invention" of evolution allowing depolarization (receptor potential generation) of the hair cells without them consuming metabolic energy for the restoration of the sodium and potassium homeostasis. In contrast to neurons that are depolarized by influx of sodium which afterwards has to be pumped out into the extracellular space against the ion gradient in exchange with potassium, the hair cell "simply" opens voltage-gated potassium channels of the basolateral membrane and passively releases potassium into the perilymph. One of the involved potassium channels is $K_V7.4$ (or KCNQ4), which is defective in autosomal dominant hearing loss DFNA2 [27, 28]. Influx of potassium from the endolymph into the hair cell during mechanoelectrical transduction requires an appropriate electrochemical gradient (driving force) that is permanently generated by the stria vascularis by providing a high potassium concentration (about 150 mM) and a positive potential (80–120 mV, endolymphatic potential) in the endolymph.

This way, mechanoelectrical transduction of the hair cell can pursue despite poor oxygen supply due to the minimal blood perfusion in the organ of Corti, allowing maximal acoustic sensitivity, as the metabolic burden is mainly picked up by the stria vascularis: division of labor. The hair cell uses highly specialized mechanosensitive ion channels for mechanoelectrical transduction that are opened by a fine "thread" ("tip-link", ▶ **Fig. 3a**) during deflection of the stereocilia. Currently so-called transmembrane like channels (TMC 1 and 2) are considered as promising candidates for the hair cell transduction channel (▶ **Fig. 3b** [29–31]). TMC 1 is affected in the human hearing impairment types DFNB7/11 and DFNA36 and recently the general feasibility of gene therapy could be shown in corresponding mouse models by means of viral gene transfer [12].

The tip-links are built from the extracellular matrix proteins cadherin 23 and protocadherin 15 [32] and anchored in the membrane of the stereocilia by means of refined protein complexes [5] (▶ **Fig. 3a**).

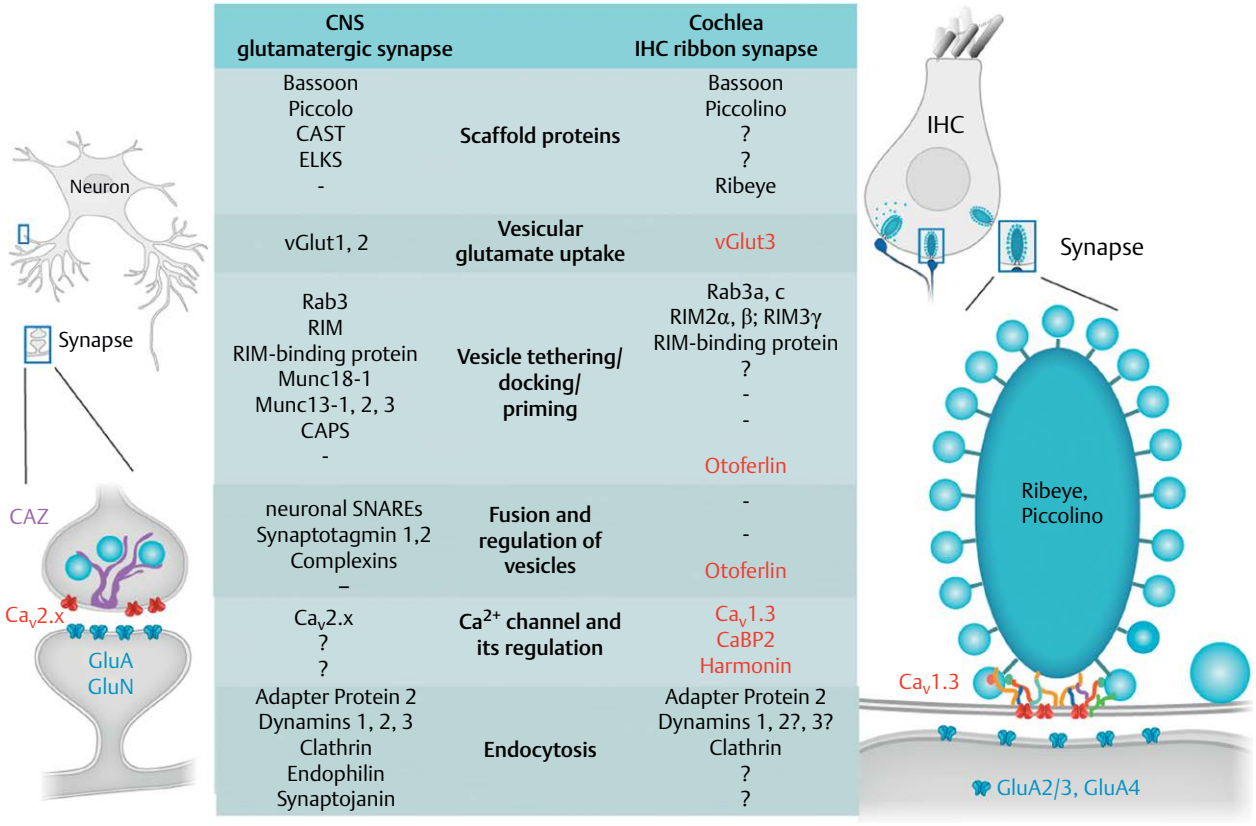
Mutations of some genes coding for the proteins of the tip-links and the anchoring proteins lead to Usher syndrome (sensorineural hearing loss, retinitis pigmentosa, depending on severity in combination with a loss of vestibular function) [5] because these proteins are also relevant for the function of photoreceptors of the eye.

After potassium has served mechanoelectrical transduction of the hair cells and has been released into the perilymph, it is thought to return into the stria vascularis via several pathways (potassium cycle, ▶ **Fig. 3c**). It is assumed that supporting cells in the organ of Corti, similar to the brain's glial cells, take up potassium via ion pumps such as KCC4. The potassium then diffuses through the network of the "coupled" supporting cells, probably via gap junction channels generated from connexin 26 and 30 (both coding genes are deafness genes). It reaches the fibrocytes of the cochlea's lateral wall that are also coupled by gap junctions until it is finally "re-used" by the stria vascularis. The stria vascularis, a kidney-like epithelium in the lateral wall of the cochlea with very efficient blood circulation ("vascularis") is equipped with a sophisticated system of ion channels and ion pumps (▶ **Fig. 3c**). For some of the genes coding for these proteins, pathogenic mutations are known that lead to deafness and sometimes also to renal or cardiac diseases (syndromic hearing loss). Defects of the potassium cycle or of transduction lead to a global cochlear dysfunction (▶ **Fig. 2**) because the driving force for outer as well as inner hair cells and their output function is missing (see below). Finally, this often results in a degeneration of the sensory elements of the cochlea.

3. Cochlear amplification and synaptic sound coding

The receptor potential resulting from mechanoelectrical transduction primarily leads to electromotility in outer hair cells and glutamate release in the inner hair cells at their synapses with type 1 spiral ganglion neurons. The electromotility of outer hair cells (voltage-controlled changes of the cellular length) is mediated by the so-called "motor protein" prestin (named after the musical notation "presto") and probably forms the mechanism that is responsible for the cochlear amplifier [33, 34]. Prestin is a protein that changes its conformation with the membrane potential and may be described in a simplified way as a type of piezo crystal. Similar to the voltage in quartz watches, the receptor potential leads to an expansion of prestin in the lateral hair cell membrane. Since a high number of prestin molecules do the same "in series", a microscopically visible change of the length of the hair cell can be observed and the hair cell introduces the mechanical energy into the oscillation of the organ of Corti. Selective dysfunction by mutations in the prestin gene [35] or loss of the electromotility or the outer hair cells themselves [27, 28] lead to a type of hearing loss that can be called a physiological "amplifier defect" (▶ **Fig. 2**). Beside the loss of otoacoustic emissions due to the lacking electromotility of the outer hair cells, a hearing loss of maximally 50–60 dB [36] can be expected along with deteriorated frequency discrimination [37].

Within the sensory inner hair cells, the depolarizing receptor potential opens voltage-gated $Ca_V1.3$ Ca^{2+} channels at the active zones of transmitter release. The incoming Ca^{2+} ions serve as signal that couples the mechanoelectrical transduction to the release of the transmitter glutamate, which leads to stimulation of the postsynaptic auditory neuron and finally to the generation of an action potential that "informs" the brain about the acoustic stimulus. The active zones of the inner hair cells are highly specialized in order to meet the high functional requirements of sound coding with regard to rate, temporal precision and duration of synaptic transmission (▶ **Fig. 4**). This specialization is readily illustrated by the presence of the synaptic ribbon generated from the protein ribeye and the molecular machinery that is different from the "typical" glutamatergic synapse of the CNS. The individual proteins, however, are less in the focus of this review (overviews can be found in [38–40]), which will rather concentrate on the extent of deviation from the "conventional construction design" and the fact that several genes coding for proteins of the hair cell synapse are defective in the context of hereditary hearing loss [7, 41]. Beside the Cav1.3 Ca^{2+} channel and its regulator CaBP2, the protein otoferlin is to be mentioned which plays a role as



CNS glutamatergic synapse		Cochlea IHC ribbon synapse
Bassoon Piccolo CAST ELKS -	**Scaffold proteins**	Bassoon Piccolino ? ? Ribeye
vGlut1, 2	**Vesicular glutamate uptake**	vGlut3
Rab3 RIM RIM-binding protein Munc18-1 Munc13-1, 2, 3 CAPS -	**Vesicle tethering/ docking/ priming**	Rab3a, c RIM2α, β; RIM3γ RIM-binding protein ? - - Otoferlin
neuronal SNAREs Synaptotagmin 1,2 Complexins –	**Fusion and regulation of vesicles**	- - Otoferlin
$Ca_V2.x$? ?	**Ca^{2+} channel and its regulation**	$Ca_V1.3$ CaBP2 Harmonin
Adapter Protein 2 Dynamins 1, 2, 3 Clathrin Endophilin Synaptojanin	**Endocytosis**	Adapter Protein 2 Dynamins 1, 2?, 3? Clathrin ? ?

▶ **Fig. 4** Comparison of synaptic proteins found in "conventional" glutamatergic synapses of the central neural system and ribbon synapses of the cochlea. Numerous proteins found in conventional synapses (left) are not present in the hair cells' ribbon synapses (right) where special proteins are found, of which many are encoded by deafness genes (marked in red). Modified from [38].

"master protein" of the afferent hair cell synapse [7, 38]. Genetic defects of genes coding for proteins of the hair cell synapse lead to a type of hearing loss that is called auditory synaptopathy (▶ **Fig. 2**). For example, defects of the otoferlin gene lead to auditory synaptopathy (DFNB9 and temperature-sensitive hearing loss) where the cochlear function may be intact upstream of the hair cell synapse (at least initially) but the temporally precise sound coding is limited or even impossible [7, 42]. Accordingly, otoacoustic emissions and/or microphone/summation potential (electrocochleography) are found but no – or a pathological – compound action potential or ABR. Subjectively the sound threshold may be preserved, however speech understanding especially in noise is very poor [7, 42]. Also in the field of genetic auditory synaptopathy, first studies revealed a restoration of the hearing function by viral gene transfer in an animal model [10, 43]. However, many questions remain to be addressed before this approach may be clinically implemented. Still, already now it can be safely stated that in patients with auditory synaptopathy (e. g. DFNB9 [7]) the results of hearing rehabilitation with cochlear implants are very promising while hearing aids only provide insufficient benefit. In contrast, the results regarding different types of auditory neuropathy are less clear [44]. The efforts on the correlation of molecular genetic findings and the outcome of cochlear implantation that are already underway (e. g. [45]) promise improved possibilities for otolaryngologists regarding counseling of hearing impaired patients that might be candidates for cochlear implantation.

Understanding auditory synaptopathy or loss of synapses, however, is of general significance for clinical otolaryngology. Animal experiments and investigations of the human temporal bone lead to the expectation that the damage and the loss of hair cell synapses is causal for frequent types of hearing impairment such as noise-induced hearing loss, age-related hearing loss (complex genetic diseases: genetic predisposition and exogenous influences) and hearing loss after application of ototoxic medications as well as Menière's disease [7, 46, 47]. The classic experiment conducted by Kujawa and Liberman [48] on temporary threshold shift in the mouse indicates that noise trauma, even in cases of complete remission of the otoacoustic emissions and the hearing threshold, may lead to an irreversible loss of up to half of all hair cell synapses: "hidden hearing loss". Immunohistochemistry could confirm this for the synaptic ribbon and ABR measurements show a linear relation between the amplitude of the spiral ganglion compound action potential and the number of hair cell synapses. Meanwhile comparable findings were assessed also in other species and electrocochleography in adolescents with a history of increased noise exposure revealed a relative reduction of the compound action potential [46]. Further clinical studies as well as analyses of human temporal bones will have to verify the transferability of those animal experimental findings.

4. Summary

Progress in the fields of genetics and molecular physiology of hearing allows for a better understanding of molecular mechanisms of hearing and sensorineural hearing loss. In order to turn this progress into clinical use, updates on current research, on differential audiometric diagnostics, and the cooperation with NGS-experienced human geneticists are required. First, the focus must be on improved diagnostics, especially in cases of suspected genetic hearing impairment. This helps to counsel patients and their families adequately and allows the informed choice of the most appropriate means of hearing rehabilitation, e. g. hearing aid versus cochlear implant in cases of auditory synaptopathy. Similar aspects also apply for differential diagnostics of frequent types of sensorineural hearing loss such as noise-induced deafness. The translation of innovative pharmacological or gene-therapeutic procedures for sensorineural hearing loss will need several years of intensive research, but major progress is expected in this field. Therefore, it is recommended to "stay tuned" for future developments.

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Conflict of Interest

The author states that there is no conflict of interest.

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