

# TOWARD COCHLEAR THERAPIES

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**Wang J, Puel J-L.** Toward Cochlear Therapies. *Physiol Rev* 98: 2477–2522, 2018. Published August 29, 2018; doi:10.1152/physrev.00053.2017.—Sensorineural hearing impairment is the most common sensory disorder and a major health and socio-economic issue in industrialized countries. It is primarily due to the degeneration of mechanosensory hair cells and spiral ganglion neurons in the cochlea via complex pathophysiological mechanisms.

These occur following acute and/or chronic exposure to harmful extrinsic (e.g., ototoxic drugs, noise...) and intrinsic (e.g., aging, genetic) causative factors. No clinical therapies currently exist to rescue the dying sensorineural cells or regenerate these cells once lost. Recent studies have, however, provided renewed hope, with insights into the therapeutic targets allowing the prevention and treatment of ototoxic drug- and noise-induced, age-related hearing loss as well as cochlear cell degeneration. Moreover, genetic routes involving the replacement or corrective editing of mutant sequences or defected genes are showing promise, as are cell-replacement therapies to repair damaged cells for the future restoration of hearing in deaf people. This review begins by recapitulating our current understanding of the molecular pathways that underlie cochlear sensorineural damage, as well as the survival signaling pathways that can provide endogenous protection and tissue rescue. It then guides the reader through to the recent discoveries in pharmacological, gene and cell therapy research towards hearing protection and restoration as well as their potential clinical application.

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## I. GENERAL INTRODUCTION

Sensorineural hearing loss (SNHL) is one of the most common human sensory deficits and, affecting ~360 million people worldwide and more than half of the population over 60 yr of age, is also a major health problem (130). It is primarily due to the degeneration of mechanosensory hair cells in the cochlea (290) resulting from acute and/or cumulative events of harmful extrinsic (e.g., ototoxic drugs, noise...) and intrinsic causes (e.g., aging, genetic factors). Associated with speech perception disorders, especially in noisy environments, it commonly causes social isolation, depression, and reduction in professional capabilities.

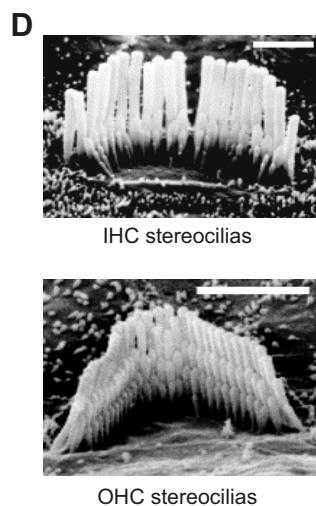
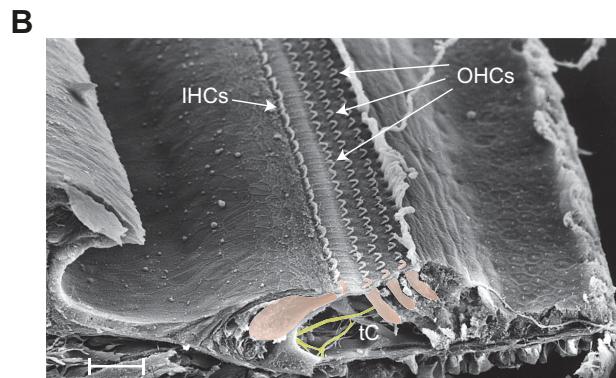
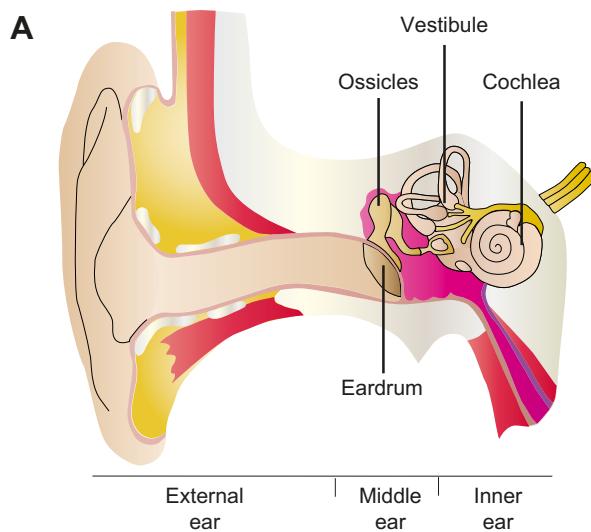
There are two types of cochlear hair cells (FIGURE 1). The outer hair cells (OHCs) amplify the motion of the basilar membrane to improve low-level sensitivity and frequency selectivity (20). The inner hair cells (IHCs) transduce sound-evoked mechanical motion into receptor potentials, leading to transmitter release at their glutamatergic synapses and cochlear afferent fiber action potentials (292). Of the two hair cell types, OHCs are generally more susceptible to damage than IHCs. Their

loss induces a loss of sensitivity and a reduction in frequency selectivity. In such cases, the only possibility to improve hearing is the use of hearing aids that amplify environmental sound according to a patient-specific audiogram (FIGURE 2). One limitation of such devices is their inability to rescue the frequency selectivity that is important for speech intelligibility. The total loss of IHCs leads to profound deafness for which cochlear implantation is the only therapeutic option (FIGURE 2). Such implants use direct electrical stimulation of the auditory nerve, providing to the brain's auditory system a peripheral input, which, however, remains highly unnatural compared with normal sound stimulation. Similar to hearing aids, cochlear implants improve hearing in quiet, but perform poorly in noisy environments.

Until recently, no clinical therapies existed to rescue the dying cochlear sensorineural cells or regenerate these cells once lost. Fortunately, the emergence of new experimental therapies ranging from pharmacological rescuing of the dying cells to newly designed genetic therapies for replacing the mutant sequences or defected genes, as well as cell therapies to graft embryonic or adult stem cells, have provided more promising perspectives for the future restoration of hearing in deaf people. This review starts by recapitulating our current understanding of the molecular pathways that mediate cochlear sensorineural cell death as a consequence of injury (noise, ototoxic drugs), aging, and genetic mutations. Subsequent sections discuss the recent discoveries in pharmacological, gene, and cell therapy research towards hearing restoration.

## II. MAJOR CAUSAL FACTORS OF SENSORINEURAL HEARING LOSS

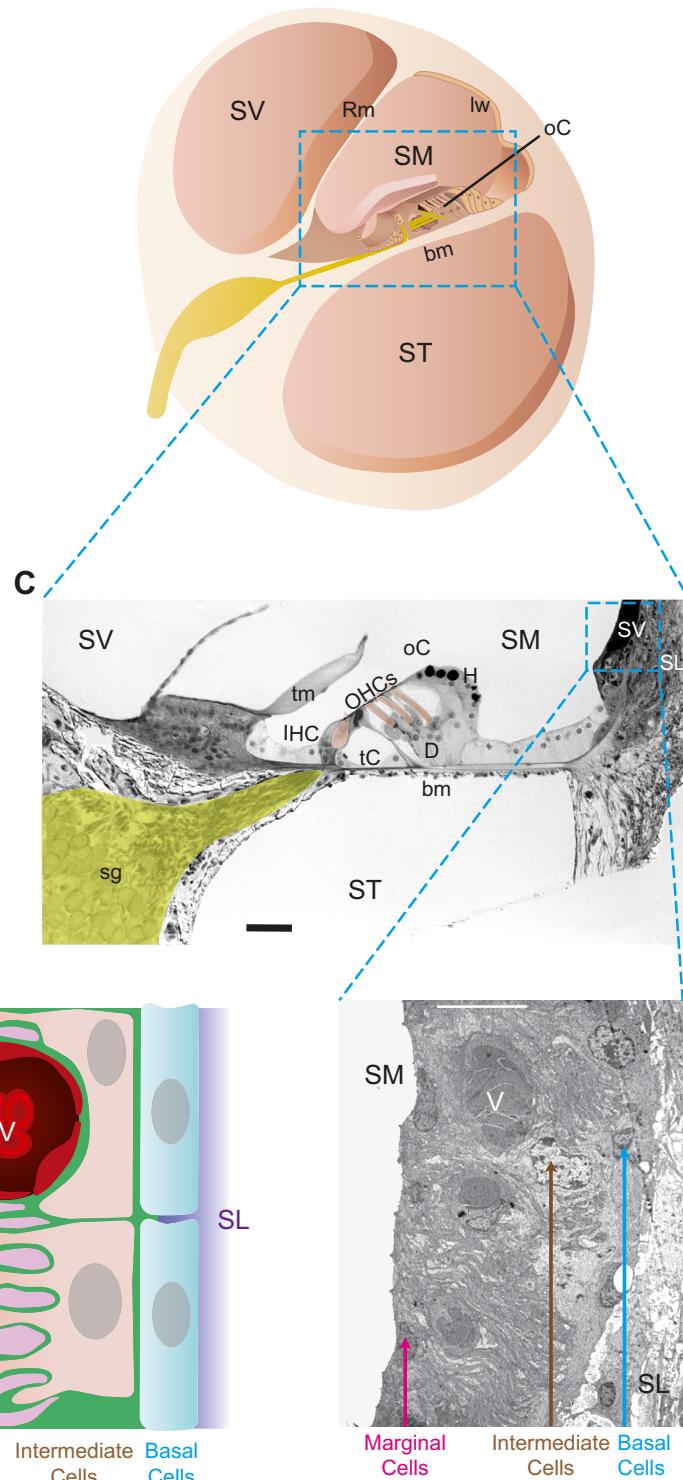
Among all the cases of SNHL, most are acquired forms of impairment due to noise, ototoxicity, or old age. However, susceptibility to the damaging effects of aging or environmental factors differs remarkably among individuals and would thus be expected to be of genetic origin (399).



### A. Environmental Factors

#### 1. Ototoxic drugs

Ototoxicity refers to the property of certain therapeutic agents (e.g., aminoglycoside antibiotics, platinum-based chemotherapeutic drugs, high-ceiling diuretics, and antimalarial drugs) to cause functional impairment and cellular



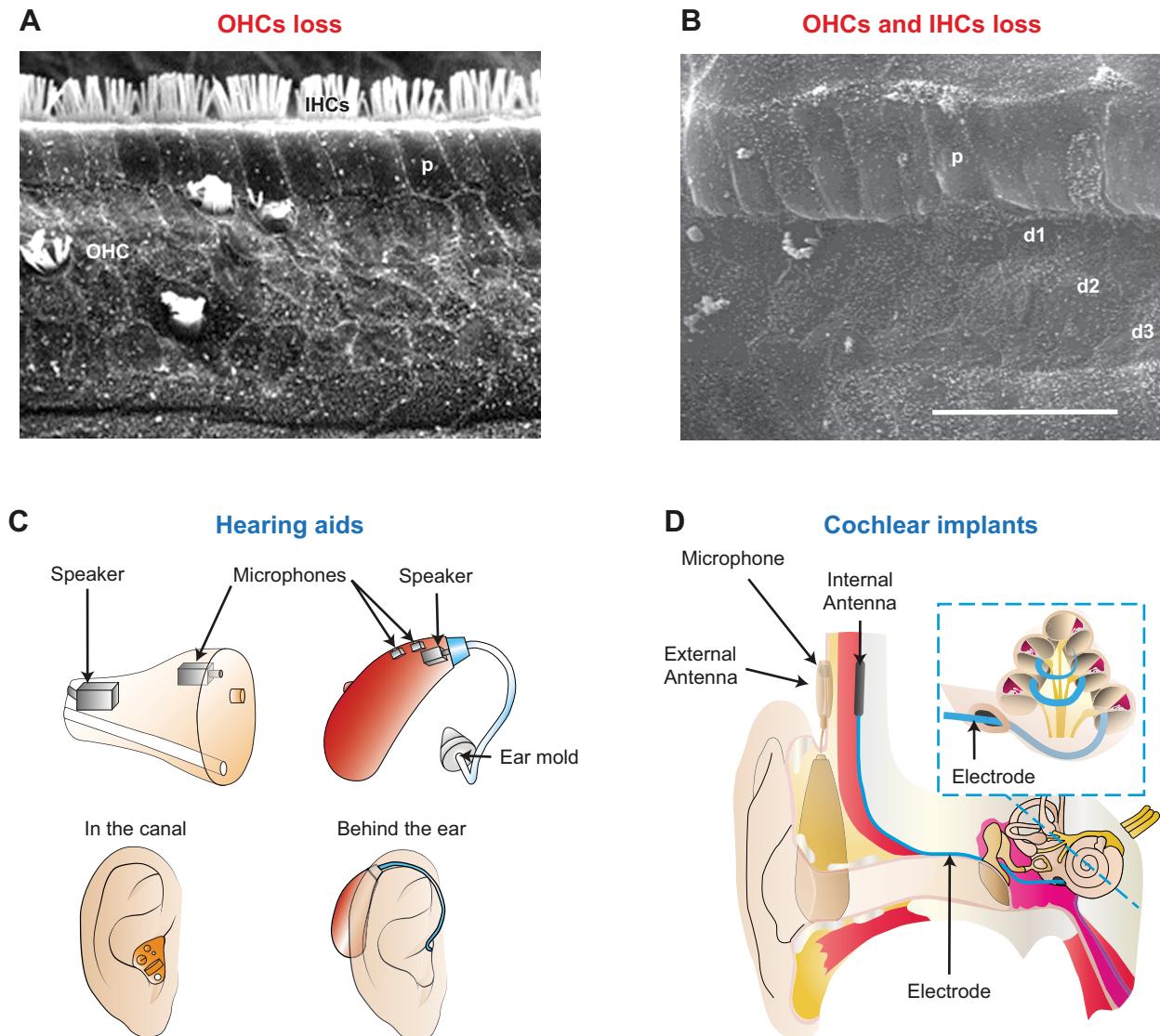
degeneration of the tissues of the inner ear. Here we will focus on the ototoxicity of the aminoglycoside antibiotics and cisplatin (*cis*-diamine-dichloroplatinum II or CDDP).

**A) AMINOGLYCOSIDE ANTIBIOTICS.** Aminoglycoside antibiotics, such as gentamicin, amikacin, neomycin, and tobramycin, are a class of clinically important antibiotics used worldwide in the treatment of infections caused by Gram-positive and Gram-negative bacteria. Their clinical use is, however, limited, due to severe ototoxic side effects (391). Clinical doses of aminoglycoside can lead to hearing loss due to OHC degeneration, which is often profound and irreversible. The hair cell loss progresses with a base (high-frequency sound detection area) to apex (low-frequency sound detection area) gradient. The IHCs are much less vulnerable to aminoglycosides: a secondary loss of the auditory nerve fibers has, however, been observed following the loss of IHCs. Aminoglycoside exposure induces a cumulative, dose-dependent damage to hair cells, to cells of the stria vascularis, and to the spiral ligament. It has been reported that one of the first sites of aminoglycoside movement from the blood into the endolymph is the stria blood-labyrinth barrier (79). A growing body of experimental evidence indicates the potential importance of both mechano-electrical transducer channels and endocytosis in the uptake of aminoglycoside by hair cells (10, 33). In addition, other ion channels, such as the transient receptor potential channels, which are calcium-permeant cationic channels, have also been proposed to be involved in the entrance of aminoglycosides into the hair cells (301, 311). Once in the hair cells, aminoglycosides may interact with many intracellular organelles (e.g., mitochondria and endoplasmic reticulum) and molecules (DNA, RNA, lipid, protein and ribosome) (311). As a result, damage may result from decreased protein synthesis, although no direct evidence for this exists. Increasing evidence suggests that aminoglycoside ototoxicity is mediated by the formation of an aminoglycoside-iron complex. The creation of this complex is a preliminary step

in the generation of free radical species and subsequent hair cell death through apoptosis or necrosis, and mitochondria appear to play an important role in these processes (see sect. III, C1 and E4). Finally, mutations in mitochondrial DNA have been shown to increase the susceptibility to aminoglycoside ototoxicity. These mutations impaired RNA translation within mitochondria, which promotes the binding of aminoglycosides to mutated mitochondrial rRNA (see sect. II B3A). While these predisposing mutations account for a significant proportion of cases, in most cases, the hearing loss is directly related to the toxic dose of the drug.

**B) CISPLATIN.** CDDP is a highly effective and widely used chemotherapeutic agent for the treatment of different types of human tumors, particularly solid tumors (448). Unfortunately, CDDP has a number of side effects, including nephrotoxicity, ototoxicity, and neurotoxicity, which greatly hamper its chemotherapeutic use (148). CDDP has been shown to induce degeneration of the neurosensory epithelium of the cochlea (the organ of Corti), with partial or complete loss of sensory OHCs and sporadic loss of IHCs, resulting in irreversible and severe hearing loss (435, 452). Strial damage secondary to CDDP has also been reported in postmortem studies performed on patients who experienced CDDP-induced ototoxicity (159). Breglio et al. (44) reported in both mice and humans that the cochlea retains CDDP for months to years after treatment, and the accumulation is very high in the stria vascularis. As the vascular tissue of the cochlea, the stria vascularis thus likely represents the entry point for CDDP into the cochlea. In cancer cells, it is generally recognized that CDDP is transported into cells by membrane transporters, such as copper transporter-1 and -2, copper-transporting ATPases (ATP7A, ATP7B), and organic cation transporter-2 (OCT2). In the cochlea, it has been reported that OCT2 is expressed in the hair cells, supporting cells, and type I spiral ganglion cells in the cochlea (67, 152). In addition, OCT1/2 double-knockout mice were protected from CDDP-induced

**FIGURE 1.** Inner ear anatomy. *A*: schematic representation of ear anatomy. The ear is divided into 3 parts (*left*): the external and middle ear transfer the sound waves to the inner ear where they are transduced into neural activity. The external ear is closed off from the middle ear by the eardrum. In the middle ear, the eardrum is mechanically linked, by a chain of three tiny bones (the ossicles), to the oval-window membrane which closes the inner ear. The inner ear comprises the balance organ or vestibule and the hearing organ or cochlea. The cochlea is made up of three canals wrapped around a bony axis, the modiolus. These canals are the scala tympani (ST), the scala vestibuli (SV), and the scala media (SM) (*right*). The ST and SV are filled with perilymph. The SM is filled with endolymph and is surrounded by the reticular lamina, which covers the organ of Corti (oC), by Reissner's membrane (Rm) forming the border to the SV, and by the lateral wall (lw). The oC is situated on the basilar membrane (bm). *B*: scanning electron micrograph of the organ of Corti. The surface of the hair cells (with the stereocilia) and the inside of the organ of Corti are visible along the sectioning plane. The pink color indicates three outer (OHC) and one inner (IHC) hair cell body and their stereocilia. Nerve fibers (in light green) cross the tunnel of Corti (tC). *C*: transverse section of the basal cochlear turn observed by light. Image shows the spiral ganglion (sg) composed of cell bodies of the primary auditory neurons (in green) and the organ of Corti (oC). In the oC, the pink color indicates one IHC and three OHCs. The OHCs, the base of which are seated on Deiters' cells (D), modulate transduction by active mechanical processes. *D*: organization of both IHC and OHC stereocilia. Scanning electron micrographs show a narrow linear shape of IHC stereocilia and a V shape of OHC stereocilia. In both cases, three interlinked rows of stereocilia of graded length are embedded in a glabrous (i.e., bearing no microvilli) cuticular plate. *E*: schematic representation (*left*) and transmission electron micrograph (*right*) of the stria vascularis. The stria vascularis (SV) and spiral ligament (SL) form the lateral wall of the cochlear duct. Rich in capillary vessels (V), the stria is composed of the marginal, intermediate, and basal cells. Marginal cells bordering the SM play a key role in endolymph formation. The endocochlear potential is generated by the intermediate cells. H, Hensen's cells; tm, tectorial membrane. Scale bars: *B* = 20  $\mu$ m, *C* = 40  $\mu$ m, *D* = 3  $\mu$ m, *E* = 10  $\mu$ m. [*B*, *C*, and *D*: micrographs adapted with permission from <http://www.cochlea.eu/> (courtesy of M. Lenoir).]



**FIGURE 2.** Sensory hair cell loss and hearing rehabilitation. *A* and *B*: scanning electron micrographs of aminoglycoside-damaged organ of Corti. Hair cells, specifically outer hair cells (OHCs), are easily damaged; high doses of amikacin eradicate nearly all OHCs, while the inner hair cells (IHCs) remain unaffected. [*A*, micrograph adapted with permission from <http://www.cochlea.eu/> (courtesy of M. Lenoir).] *B*: a further increase in dose leads to the destruction of IHCs. d, Deiters' cells; p, pillar cells. Scale bars = 35  $\mu$ m. *C*: schematic representation of hearing aids. A hearing aid is a small electronic device worn in the ear, in the canal, or behind the ear. The hearing aid receives sound through a microphone, which converts the sound waves to electrical signals and sends them to an amplifier. The amplifier increases the power of the signals and then sends them to the ear through a loudspeaker. *D*: schematic representation of a cochlear implant. A cochlear implant is composed of external parts and internal parts. The external parts include a microphone, speech processor, and antenna. The internal (implanted) parts include a receiver and electrodes. The external speech processor captures sound and converts it to digital signals that are transmitted to the internal implant. There, the signals are converted into electrical energy that is sent to an electrode array inside the cochlea. The electrodes directly stimulate the hearing nerve, thus bypassing damaged hair cells and allowing perception of sound signals in the brain.

ototoxicity and nephrotoxicity (67). However, a recent study on humans reported that patients cotreated with pantothenate, an inhibitor of OCT2, showed no protective effect against CDDP-induced ototoxicity and nephrotoxicity (117). Once inside of the cell, CDDP accumulates and damages different organelles such as mitochondria, lysosomes, endoplasmic reticulum, nucleus, cell membrane, and cytoskeleton lead-

ing to DNA damage, mitochondrial dysfunction, oxidative stress, and cochlear cell apoptosis (382) (see for more detail, see sect. III, C1, E3, and E4). Finally, polymorphisms of genes coding metabolic enzymes such as glutathione-S-transferase (GST), thiopurine S-methyltransferase (TMPT), and catechol-O-methyltransferase (COMT), DNA damage repair protein (XPC), and megalin (an endocytic receptor) are significantly

associated with individual susceptibility to CDDP ototoxicity (see sect. II B3A).

## 2. Noise exposure

Noise-induced hearing loss (NIHL) acquired in leisure or occupational settings is a common cause of hearing impairment in industrialized countries, with a prevalence second only to age-related hearing loss (ARHL or presbycusis) (307, 411). After high-intensity exposure (>100 dB sound pressure level, SPL) or repeated overstimulation, an irreversible increase in hearing thresholds can occur, leading to permanent threshold shift (PTS) due to destruction of cochlear hair cells or damage to their mechanosensory hair bundles and loss of spiral ganglion cells (407). Hair-cell damage can be visible within minutes after overexposure, and hair-cell death continues for days after exposure (454), while death of spiral ganglion cells is delayed by months to years (188). Hearing loss associated with punctual- and mild acoustic overexposure is reversible and hearing recovers within 2–3 wk (285). This temporary loss corresponds to a temporary threshold shift (TTS) and is probably due to reversible damage to the stereocilia of hair cells (125). The swelling of cochlear nerve terminals at their hair-cell synapses can be observed in ears with temporary threshold shifts (363), and the recovery of thresholds may also be due to the recovery or regeneration of the cochlear nerve terminals (351). However, Liberman (250, 251) demonstrated that TTS exposure can destroy more than 50% of synapses between cochlear nerve fibers and IHCs without hair-cell damage. They thus introduced the concept of “hidden hearing loss” to describe selective synaptopathy occurring after noise exposure (219), because the damage is not detectable in the routine threshold audiogram (250). The threshold recovery to normal levels was attributed to the recovery of OHC function, together with the unexpected resistance of the high-spontaneous rate auditory fibers encoding the best thresholds. Nevertheless, the fragility of the low-spontaneous rate fibers is not yet understood. The loss of hair-cell synapses and auditory peripheral terminals is rapid (within hours post exposure), while the loss of spiral ganglion neurons (SGNs) is slow (months to years) (180). Acoustic trauma can also initiate edema of the stria vascularis and thus compromise the blood supply to the cochlea (401). Injuries to the stria vascularis and spiral ligament result in damage to type II and type IV fibrocytes that are important for maintenance of the endocochlear potential, and such damage can thus lead to permanent hearing loss (160).

## B. Genetic Factors

Certain genetic factors play a crucial role in congenital or later-onset hearing loss in monogenic disorders that can be broadly classified by the presence of associated phenotypic features (i.e., syndromic and nonsyndromic). Nonsyndromic hearing loss is a partial- or total loss of hearing that

is not associated with other signs and symptoms. In contrast, syndromic hearing loss occurs with signs and symptoms affecting other parts of the body. A genetic predisposition (also called genetic susceptibility) results from specific genetic variants that also contribute to the susceptibility of the individual to noise- and ototoxic drug-induced, as well as age-related, hearing loss. It is estimated that mutations in hundreds of genes cause or predispose people to congenital, progressive, noise-induced, and age-related forms of hearing loss.

### 1. Nonsyndromic deafness

Nonsyndromic deafness is commonly classified according to the pattern of inheritance: autosomal dominant (DFNA), autosomal recessive (DFNB), X-linked (DFNX), or mitochondrial (which does not have a special designation), with each type being numbered in the order in which they were first described. It shows strong genetic heterogeneity, currently with 130 loci mapped and 97 genes identified, including 55 DFNB genes (autosomal recessive), 30 DFNA genes (autosomal dominant), 4 X-linked genes, and 7 mitochondrial genes (see Hereditary Hearing Loss Homepage, <http://hereditaryhearingloss.org>). One more gene (*AUNA1*) has been found to be responsible for autosomal-dominant auditory neuropathy. Among the genes identified are those encoding transcription factors (POU3F4, EYA4), ion channels (KCNQ4, several gap-junction proteins), extracellular matrix components (COCH), cytoskeletal proteins (several unconventional myosins), and proteins of unknown function (DFNA5, TMC1). Nonsyndromic deafness is, in most (~79%) cases, inherited in an autosomal-recessive fashion (389). Autosomal, dominantly inherited, nonsyndromic deafness accounts for most of the remaining cases (20%), with just 1% of the total being due to X-linked inheritance.

### 2. Syndromic deafness

Syndromic deafness includes nearly 600 forms of deafness combined with other clinical signs and symptoms (see Hereditary Hearing Loss Homepage, available at <http://hereditaryhearingloss.org>). In this review, we highlight the Usher and Pendred syndromes for their relatively higher frequency and recent pioneering and successful efforts to restore hearing with novel therapeutic approaches.

Usher syndrome is a genetic disease affecting both hearing and vision. There are three clinical types: USH1, USH2, and USH3. These types are differentiated by the degree of hearing loss, balance problems, and the age at which hearing, vestibular, and visual symptoms occur. To date, 16 loci on different chromosomes have been reported to be involved in the occurrence of Usher syndrome. Among them, 12 genes were identified as causative and 1 as a modifier gene (see for review, see Refs. 205, 275). In the inner ear, USH proteins

are integrated into a protein network that is critical for the development and maintenance of the sensorineural cells, and for hair-bundle morphogenesis (483).

Pendred syndrome is an autosomal recessive disorder that is classically defined as the combination of SNHL, goiter, and abnormal iodide organification with or without hypothyroidism. The hallmark of this syndrome is impaired hearing, which is associated with inner-ear malformations. Pendred syndrome is provoked by mutations in the *SLC26A4* gene encoding pendrin, an anion exchanger protein expressed in the inner ear (105, 387, 466). The mutations in the *SLC26A4* gene can also cause nonsyndromic SNHL (350).

### 3. Genetic susceptibility

A) SUSCEPTIBILITY TO DRUG OTOTOXICITY. Mutations in the mitochondrial genome have also been shown to favor aminoglycoside-induced hearing loss. Patients carrying an A1555G mutation in the 12S ribosomal RNA are at increased risk of aminoglycoside-induced profound deafness (348). This mutation may account for ~20% of aminoglycoside ototoxicity-induced hearing loss. Worthy of note is that increased susceptibility to aminoglycoside-induced hearing loss appears to be associated with an altered mitochondrial 12S ribosomal subunit morphology and function, ribosome hypermethylation, and defective translation, leading to excessive generation of reactive oxygen species (ROS) and cell death (75, 164, 276, 353).

Polymorphisms of genes coding for GST protein, primarily *GSTM1*, *GSTP1*, and *GSTT1*, have been involved in modifying the susceptibility of the patients to CDDP ototoxicity (323). An increasing number of risk alleles in the *TMPT* gene encoding TMPT and *COMT* gene encoding COMT correlates with earlier onset and severity of CDDP-induced ototoxicity in children (365). Both of these enzymes are methyltransferases that utilize S-adenosylmethionine as a methyl donor in methionine synthesis. Mice receiving CDDP in combination with S-adenosylmethionine (SAM) display increased CDDP-induced renal dysfunction (312). Thus higher levels of SAM upon reduced activity of TMPT and COMT may also potentiate CDDP ototoxicity (312). In addition, polymorphisms in the *LRP2* gene encoding megalin, expressed within the marginal cells of the stria vascularis, and *XPC* gene encoding XPC, a component of the nucleotide excision repair pathway, were also reported to link to CDDP ototoxicity (296). Altogether, these results point to the potential contribution of polymorphisms of genes coding metabolic enzymes such as GST, TMPT, and COMT, DNA damage repair protein (XPC), and megalin to individual susceptibility to CDDP ototoxicity.

B) SUSCEPTIBILITY TO NOISE. Genetic evidence points to a link between mutations found in both mitochondrial genes and endogenous, antioxidant defense-related genes and individ-

ual susceptibility to NIHL (3). Factory workers exposed to occupational noise are at higher risk for hearing loss when they carry a specific single nucleotide polymorphism in the mitochondrial Mn-superoxide dismutase (*MnSOD*) gene (260). Human studies utilizing a candidate gene approach identified NIHL-susceptibility genes encoding potassium ion channels (*KCNQ4* and *KCNE1*), catalase (*CAT*), protocadherin 15 (*PCDH15*), myosin 14 (*MYH14*), and heat-shock protein (*HSP70*) (reviewed in Ref. 399).

The best-studied genetic basis of NIHL in animals is *Cdh23<sup>ahl</sup>*, which promotes noise injury and ARHL in B6 mice, and presumably many other strains (101, 187). Yet even when the influence of *Cdh23<sup>ahl</sup>* is removed from B6 mice, they remain more susceptible to noise when compared with CBA/J or CBA/CaJ mouse strains (316). The discovery of another pro-NIHL locus, *Ahl3* on chromosome 17 of B6, may also help explain the susceptibility to noise (291, 320). Therefore, the larger susceptibility for NIHL in B6 mice may be due to a combination of several genetic factors such as *Cdh23<sup>ahl</sup>* and *Ahl3*. Indeed, alleles that promote noise-induced TTS have been made from knockout experiments in mice for NF-κB (226), estrogen receptor β (*ERβ*) and aromatase (280), and orphan glutamate receptor δ1subunit (*Glur81*) (124). In mice, the deletion of genes encoding plasma membrane Ca<sup>2+</sup>-ATPase isoform 2 (*PMCA2*) (215), copper/zinc superoxide dismutase (*SOD1*), selenium-dependent glutathione peroxidase (*GPx1*) (277, 317), heat shock protein HSP70 isoforms (486), the antioxidants paraoxonase and manganese superoxide dismutase (*SOD2*) (116), KCNE1 potassium channel regulatory subunit (438), and BK calcium-activated K<sup>+</sup> channel expressed by basal OHCs (99) are more sensitive to noise than their wild-type littermates. Taken together, these human and animal reports provided strong support for a relationship between mutations of genes coding antioxidant enzymes, *KCNQ4*, or the cadherin superfamily on the one hand, and susceptibility to noise-induced cochlear tissue injury on the other.

C) SUSCEPTIBILITY TO AGING. ARHL shows a clear familial aggregation. Heritability, reflecting the percentage of phenotypic variance due to genetic factors, has been estimated as 35–55% for sensory presbycusis and greater still for the strial phenotype, with stronger aggregations in women than in men (129). To identify human susceptibility genes, linkage-based and association-based approaches have been developed. The region 11p, which overlaps with genes known to cause monogenic deafness, and the locus DFNA18 have been identified via the linkage-based approach. Single nucleotide polymorphisms in the grainyhead-like 2 gene (*GRHL2*), *DFNA5* and *KCNQ4* genes, whose mutations are responsible for DFNA28, DFNA2, and DFNA5, respectively, are significantly associated with individual susceptibility to ARHL (436, 439). The results from a whole-genome association study for ARHL showed that single nucleotide polymorphisms (SNPs) at the gluta-

mate metabotropic receptor 7 (*GRM7*, e.g., OMIM ID: 604101) are associated with higher risk for ARHL (119, 309). In addition, polymorphisms in the genes encoding detoxification enzymes, such as glutathione transferase (*GSTM1* and the *GSTT1* null genotypes) and N-acetyltransferase 2 (*NAT2\*6A*) (23, 439), have been reported to link to ARHL. Finally, a common mtDNA 4,977-bp deletion was frequently found in ARHL patients (436).

Some genes associated with ARHL have also been found in mice, including *Ahl1* localized in chromosome 10, *Ahl2* (186) on chromosome 5 (associated with earlier-onset hearing loss when combined with a homozygous disease allele at the *Ahl1* locus), and *Ahl3* on chromosome 17 (291). The Ahl candidate region contains several interesting candidate genes, including genes encoding gap-junction proteins and several collagens. Mouse strains exhibiting ARHL are also more sensitive to NIHL than are other strains. Collectively, polymorphisms in some monogenic deafness-causing genes, neurotransmitter-related genes, and genes involved in detoxification of oxidative stress and mitochondrial function are clearly associated with ARHL.

## C. Aging

ARHL is the third most prevalent chronic medical condition affecting older adults (245) and results from a complex, multifactorial disorder attributable to confounding intrinsic and extrinsic factors (130, 318). ARHL is symmetric, progressive, and sensorineural; it begins in the high-frequency region of the hearing spectrum and spreads toward the low-frequency regions with age. The age-dependent reduction in threshold sensitivity is generally associated with difficulty in speech discrimination as well as sound detection and localization, particularly in noise. Males are generally more severely affected than females. According to Schuknecht (386), based on temporal bone analyses correlating the patterns of hearing loss with defect location, three major forms of ARHL can be distinguished: 1) sensory presbycusis characterized by an abrupt pure-tone threshold elevation in the high frequencies and hair-cell loss at the basal end of the cochlea; 2) stria presbycusis found in patients with a flat- or slightly descending pure-tone audiogram, correlated with atrophy of the stria vascularis; and 3) neural presbycusis, characterized by a loss of cochlear neurons throughout the entire cochlea. The precise mechanisms underlying the age-related degeneration of the different cochlear structures remain unclear. This is in part due to the complexity of each causal factor, but more importantly to the interaction of the different mechanistic pathways that can cause ARHL. Indeed, sensory hair cells are susceptible to an accumulation of injuries inflicted over time from a number of different sources, including direct mechanical, mitochondrial oxidative injury from noise, ototoxic drugs such as aminoglycosides, CDDP, or other unknown factors (315). The degeneration of SGNs can be triggered by cumu-

lative exposure to loud noise, leading to glutamate excitotoxicity and loss of the afferent dendrites (110).

## III. MOLECULAR MECHANISMS OF CELL DEATH AND SURVIVAL

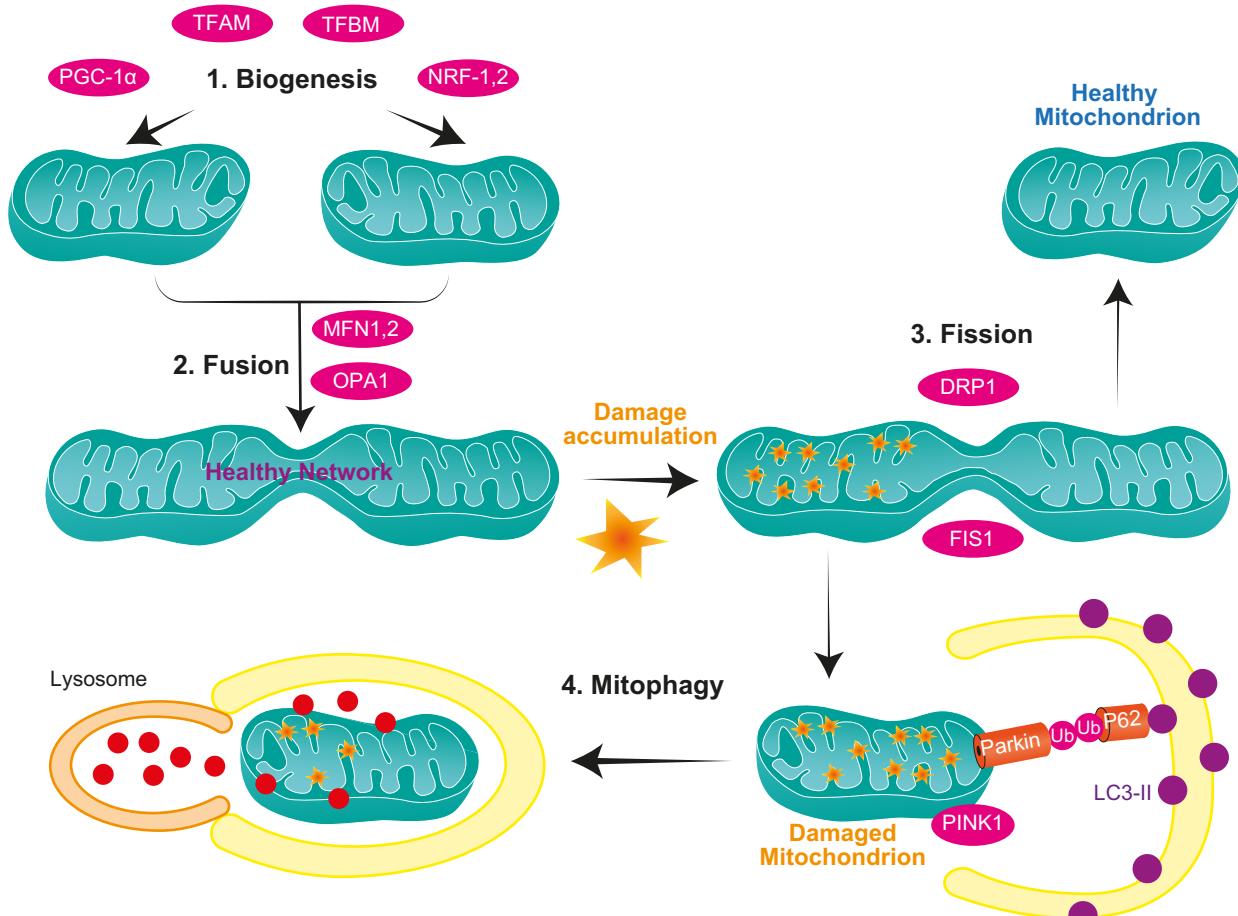
Several important advances have been made in our understanding of the pathways leading to dysfunction and death of cochlear cells in acquired deafness. The triad of age-related, noise-induced, and drug-induced hearing loss displays intriguing similarities in terms of particular cellular responses of the cochlear sensory neural cells. Such responses would suggest the potential involvement of impaired mitochondrial quality control, mitochondrial DNA mutation and dysfunction, oxidative stress, inflammation, apoptosis, autophagy, and/or necrosis.

### A. Impaired Mitochondrial Quality Control

The mitochondrion represents a critical organelle for cell function and survival. Its principal roles are provision of chemical energy through ATP production, control of cellular metabolism, and regulation of programmed cell death. Mitochondrial quality control and turnover is of particular importance to cochlear sensory and neural cells, because of their constant need for high levels of energy supply. The production of ATP through oxidative phosphorylation is the primary source of ROS, which contribute to mitochondrial dysfunction, cochlear cell death, and hearing loss. To protect cells against the potential detrimental effects of mitochondrial damage, cells develop well-coordinated quality-control mechanisms that maintain overall mitochondrial health through mitochondrial biogenesis, mitochondrial dynamics (fusion and fission events), and mitochondrial autophagy (mitophagy) (FIGURE 3) (503).

#### 1. Mitochondrial biogenesis

Mitochondrial biogenesis is the process by which cells increase their individual mitochondrial mass and copy number to increase the production of ATP as a response to greater energy expenditure, or during times of cellular stress (381). This process is involved in cochlear cell responses to gentamicin and acoustic trauma (174). By measuring the ratio of mtDNA/nuclear DNA and activity of citrate synthase, we showed an overactive mitochondrial biogenesis in the cochlea of the senescence-accelerated mouse prone 8 strain (SAMP8) at a young age that decreased in old age; the opposite was observed in the senescence-accelerated mouse resistant 1 strain (281). The key regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), whose age-related reductions in itself as well as in its tissue-specific function might represent important contributing factors of mito-



**FIGURE 3.** Mitochondrial dynamics and mitophagy leading to quality control. The mitochondrial life cycle starts with growth and division of preexisting organelles through a mechanism called biogenesis (1). Biogenesis is regulated by peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), which activates nuclear respiratory factor (NRF)-1 and NRF-2, mitochondrial transcription factor A (TFAM), and mitochondrial transcription factor B (TFBM). During their life cycle, mitochondria undergo frequent cycles of fusion (2) to form elongated mitochondrial networks and fission (3) into smaller individual organelles. Fusion is mediated by mitofusin (MFN) 1, MFN2, and optic atrophy protein 1 (OPA1). Fission is mediated by dynamin-related protein-1 (DRP1) and fission 1 (FIS1). Fission provides a mechanism to isolate damaged components (yellow stars) for elimination. The mitochondrial life cycle ends with the degradation of impaired or surplus organelles by mitophagy (4). Mitophagy involves mitochondrial depolarization, retention of phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1) in the mitochondrial membrane, and recruitment and activation of Parkin. Active Parkin, as an E3 ubiquitin ligase, is then able to promote the ubiquitination of many proteins on the mitochondrial surface. These ubiquitinated proteins are recognized by p62, an ubiquitin- and LC3-binding adaptor protein. The p62 then plays a role in targeting cargo to the autophagosome. Finally, the autophagosome fuses with the lysosome forming an autolysosome in which the mitochondrial cargo is degraded.

chondrial function in age-related diseases (467). The over-expression of PGC-1 $\alpha$ , with its consequential increase in the transcription factors nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), significantly decreased the accumulation of damaged mtDNA and the number of apoptotic cells in the strial marginal cells senescence model (501).

## 2. Mitochondrial dynamics

Within the cell, a family of dynamin-related proteins regulates the constant division and elongation of mitochondrial

networks by respectively fission and fusion (FIGURE 3). While fission plays a role in segregating dysfunctional mitochondria that contain damaged proteins, destabilized membranes, and mutated or damaged mtDNA (165), fusion, in contrast, has been shown to aid in equilibration of matrix metabolites, intact mtDNA, and even membrane components (14). The master mediator of fission is dynamin-related protein 1 (Drp1) and fission 1 (FIS1). Three different GTPases mediate fusion: optic atrophy 1 (Opa1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) (355). Both Mfn1 and Mfn2 mediate fusion of the outer mitochondrial membranes, while Opa1 mediates the fusion of the

inner mitochondrial membrane (102). In addition to its role in the fusion of the inner mitochondrial membrane, Opa1 is involved in the maintenance of the respiratory chain and membrane potential, cristae organization, control of apoptosis, and mitochondrial DNA maintenance (238). Mutations in *Opa1* lead to dominant optic atrophy (DOA) and hearing loss, starting from childhood or early adulthood (238). Although the majority of studies broadly qualify the hearing disorder as “SNHL,” some authors have proposed this auditory neuropathy to be the pathophysiological mechanism underlying the hearing impairment in OPA1-DOA (171). In addition, mitofusin-2 gene mutations cause Charcot-Marie-Tooth type 2A, sometimes complicated by additional features such as optic atrophy and SNHL (310).

### 3. Autophagy

Autophagy is the catabolic mechanism by which intracellular cytosolic components, including proteins, organelles, aggregates, and many other intracellular materials, are delivered to lysosomes for degradation. Autophagic proteins Atg5, Atg4b, Atg9a, Beclin-1, and LC3B are expressed in the mouse and chicken cochlea and vestibule from developmental stages through to adulthood (4). The inner ear autophagy flux, illustrated by decreased SQSTM1/p62 and increased relative levels of LC3-II, is developmentally regulated and reaches a plateau at the age of 2 mo (83). In the adult mouse cochlea, LC3II expression was found primarily associated with SGNs (83), and its up-regulation occurred concomitantly with an accumulation of lipofuscin, specifically in SGNs, but not in the organ of Corti or in the stria vascularis of SAMP8 mice during the aging process (281). Together, these results suggest that autophagy may be a housekeeping mechanism necessary for SGN activity.

Autophagic morphological features were found in adult hair cells exposed to ototoxic insult (429). The LC3II expression responses appeared to depend on noise exposure, clearly increasing after TTS-noise exposure, increasing only slightly following PTS-noise, and remaining unaltered by severe PTS-noise exposure. Furthermore, treatment with rapamycin, an autophagy activator, significantly increased LC3B expression, while diminishing noise-induced hair-cell and hearing loss. In contrast, treatment with either the autophagy inhibitor 3-methyladenine (3MA) or with LC3B siRNA-reduced LC3B expression exacerbated TTS to PTS (496). Rapamycin treatment also led to an upregulation of the LC3-II/GAPDH ratio, to increased Beclin-1 expression together with decreased oxidative stress, and to hair-cell and hearing loss in CDDP-treated rats (108).

### 4. Mitochondrial autophagy

Mitochondrial autophagy (hereafter referred to as mitophagy) is an important cellular process facilitating the

removal of damaged or unwanted mitochondria (**FIGURE 3**). In the cochlea, the only study relied on hybrid cell lines derived from symptomatic members of a Chinese family carrying a m.1494C.T mutation. Under gentamicin treatment, mutant cells displayed a greater population of dysfunctional mitochondria forced into fusion and mitophagy to improve mitochondria function (493). Nevertheless, the role of mitophagy in the cochlea under physiopathological conditions remains obscure.

## B. Mitochondrial DNA Mutations and Dysfunction

Thirteen of the mitochondrial proteins are encoded by mitochondrial DNA (mtDNA), which is particularly vulnerable to mutations, due to their less efficient machinery for DNA damage repair when compared with nuclear DNA. Several multisystem syndromes that are associated with deafness and due to inherited mtDNA point mutations have been well characterized, such as maternally inherited diabetes and deafness (224).

### 1. Noise- and ototoxicity-induced hearing loss

In support of impaired mitochondrial function underlining the detrimental effect of noise-induced cochlear injury, acetyl-L-carnitine, carbamathione, and D-methionine, known to enhance mitochondrial bioenergetics, protected chinchillas against NIHL (211). In addition, mitochondria swelling and localized destruction of mitochondrial cristae in cochlear hair cells has been demonstrated following sound exposure (211). Mitochondria also play a role in aminoglycoside-induced hair-cell injury, since inhibition of mitochondrial homeostatic mechanisms accentuated gentamicin-induced auditory hair-cell death (174). Streptomycin ototoxicity is also mediated by mistranslation of mRNAs at the mitochondrial ribosome, which in turn lead to complex I dysfunction (172). Altogether, these data are consistent with earlier observations that noise trauma and aminoglycoside ototoxicity can decrease inner-ear mitochondrial respiration and cause degeneration of mitochondria in cochlear hair cells (449, 452).

### 2. Age-related hearing loss

It has long been speculated that cochlear mitochondria are involved in ARHL (53). One specific deletion (mtDNA4977) is frequently observed in the temporal bone of patients with ARHL, at levels that are strongly correlated with the severity of ARHL (272). Mutations in *POLG* encoding DNA polymerase  $\gamma$  that maintains mtDNA replication fidelity (115), or *OPA1* encoding the mitochondrial dynamin-like GTPase (252), impact mtDNA genomic stability. These mutations are also known to cause premature hearing loss in patients and mice (383). Finally, the incidence and frequency of mtDNA mutations increase expo-

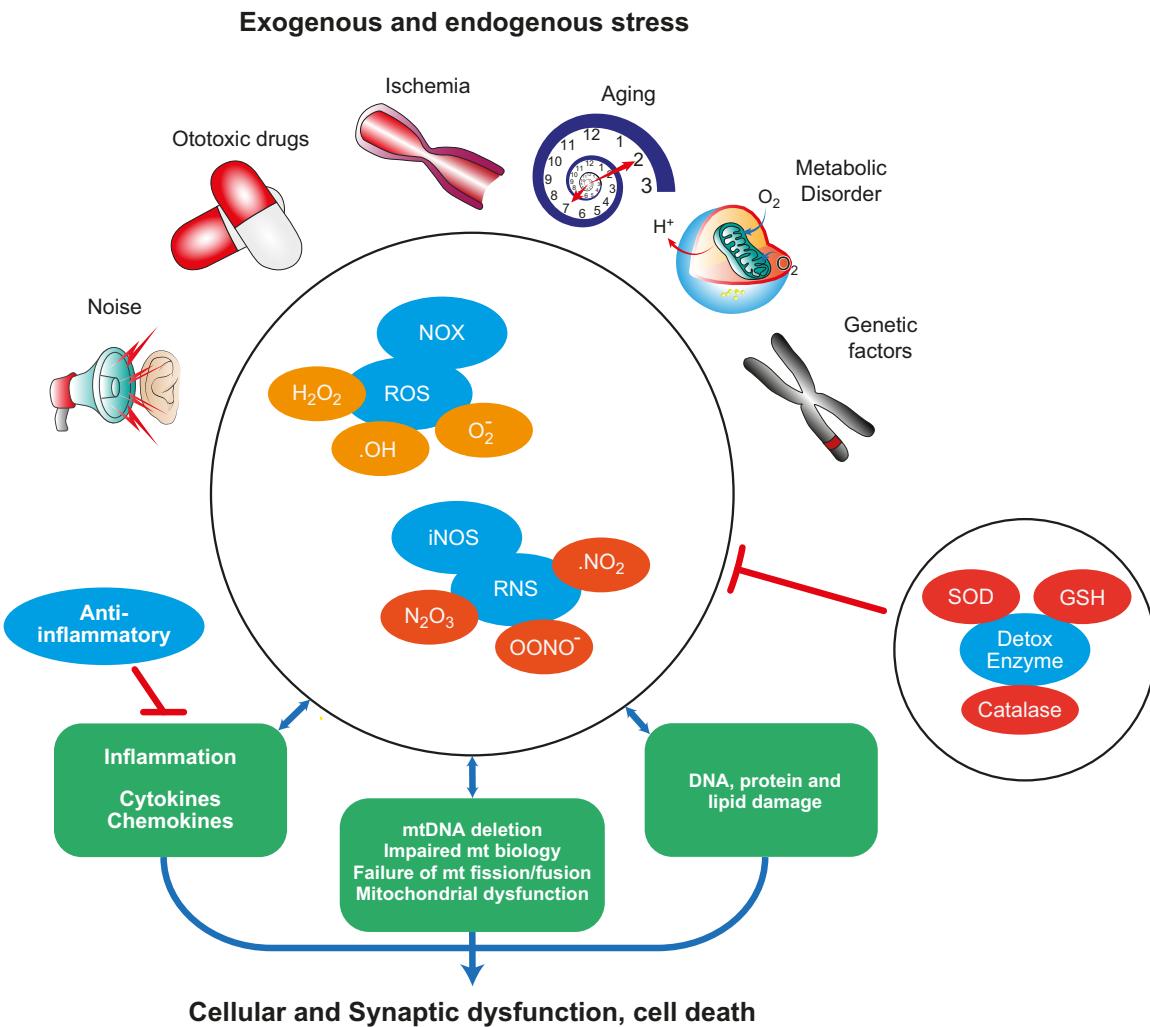
nentially with age and contribute to cellular senescence (434).

The mitochondrial theory of aging was substantiated by studies in mice expressing error-prone mitochondrial DNA polymerase  $\gamma$  (PolgD257A) defective for proofreading activity. Increased mutation frequency, resulting from a loss of DNA proofreading activity, led to the expression of defective respiratory chain proteins and premature aging (433). These Polg knockin mice also displayed early-onset ARHL, with severe loss of SGNs and degeneration of the stria vascularis (220). A decrease in the activity and of the expression of complex IV was observed in cochlear tissue from 9-month-old SAMP8 mice, which present senescence-accelerated hear-

ing loss (281). The decreased expression of the complex IV subunit was also observed postmortem in SGNs from the temporal bone of elderly patients with ARHL (271).

### C. Oxidative Stress

Oxidative stress is a cellular condition induced by the deregulated production of ROS and reactive nitrogen species (RNS), which are highly reactive molecules generated by several biochemical and physiological processes of cellular metabolism under both physiological and pathological conditions (FIGURE 4). ROS are produced when single electrons are transferred to oxygen, leading to the generation of the rela-



**FIGURE 4.** Reactive oxygen species (ROS)/reactive nitrogen species (RNS) generation and scavenging balance in cochlear cell degeneration. Various exogenous and endogenous cochlear cell stresses (e.g., noise, ototoxic drugs, ischemia, aging, cochlear cell metabolic disorders, and genetic factors) lead to increased levels of oxidative stress mediators [e.g., NADPH oxidase (NOX), ROS, inducible nitric oxide synthase (iNOS), and RNS]. Cochlear cells are endowed with robust antioxidant defenses to counteract excessive oxidative stress via the activity of superoxide dismutase (SOD), glutathione (GSH), and catalase. Under disease conditions, this balance is shifted towards oxidative stress, leading to an imbalance between mediators of inflammation (proinflammatory cytokines and chemokines) and anti-inflammation, deletion of mtDNA, impaired mitochondrial quality control and mitochondrial dysfunction, and damage to DNA, proteins, and lipids, altogether resulting in cochlear cell death.

tively inert superoxide radical ( $O_2^{\cdot-}$ ) (300), the more reactive hydrogen peroxide ( $H_2O_2$ ), and the highly reactive and damaging hydroxyl radical ( $\cdot OH$ ). RNS result from the reaction of nitric oxide ( $NO\cdot$ ) with ( $O_2^{\cdot-}$ ), generating the highly reactive peroxynitrite ( $ONOO^{\cdot-}$ ) (FIGURE 4) (352). ROS and RNS are mainly generated by mitochondria, endoplasmic reticulum (ER; particularly in the setting of ER stress), and peroxisomes. In addition, various enzymes, including NADPH oxidase and nitric oxide synthase (NOS), generate ROS as part of their enzymatic reaction cycles. An excess of ROS and RNS can damage lipids, proteins, or DNA within cells and thus inhibit their normal function (35). Oxidative stress has thus been implicated in a number of human diseases as well as in the aging process, including ARHL. Fortunately, cells are armed against oxidative stress and are endowed with robust antioxidant defenses to counteract excessive ROS/RNS production via the activity of antioxidant enzymes such as manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), catalase, glutathione peroxidase (GPX), and glutathione reductase (GSR), as well as a variety of small-molecule antioxidants such as glutathione (GSH) and thioredoxin (327) (FIGURE 4).

### *1. Reactive oxygen and nitrogen species in damaged cochlear tissue*

Oxidative stress has been proposed as the main mechanism responsible for CDDP-induced cochlear cell apoptosis (344). Increased ROS/RNS and lipid-peroxidation levels were observed in CDDP-intoxicated cochlear explants in culture (202). In addition, products of increased lipid peroxidation, such as 4-hydroxynonenal and malondialdehyde, were also found *in vivo* in CDDP-treated rat and mouse cochleae (232). The consequences of this oxidative stress can include hair-cell loss and permanent deafness (FIGURE 4).

Aminoglycosides are considered to be redox-inactive compounds meaning that they can only induce ROS formation after conversion into a redox-active form. To generate a ROS, aminoglycosides form a complex with iron, which then catalyzes the oxidation of unsaturated fatty acids located within the inner leaflet of the plasma membrane (244). In the absence of iron, arachidonic acid enriched in phosphoinositides can serve as an electron donor (243). In support of these observations, ROS have been found in hair cells after aminoglycoside exposure *in vivo*, and methods of blocking ROS in the cochlea have been attempted with some success in animal models (173, 372).

Following noise exposure, the emergence of  $O_2^{\cdot-}$ , nitrotyrosine (NT), 4-hydroxy-2-noneal (4-HNE), and/or  $\cdot OH$  is almost immediate and may persist for several days, causing a widening of the area of morphological damage (319, 482). Increased levels of the oxidative DNA damage marker 8-hy-

droxy-2'-deoxyguanosine (8OHdG), as well as the lipid peroxidation product 8-isoprostanate, were observed in noise-damaged cochlear tissues (314, 437) (FIGURE 4).

### *2. Reactive oxygen and nitrogen species in aging cochlear tissue*

Oxidative stress is an important causal factor in ARHL (404) and occurs in various forms such as lipid peroxidation (281), oxidative mitochondrial and nuclear DNA damage, and glutathione-conjugated proteins (183, 404). Consistently, low serum levels of the ROS scavenger melatonin is significant in the development of high-frequency hearing loss in the elderly (227). In addition, cellular antioxidant defense systems such as catalase, apoptosis inducing factor (AIF), SOD1, and SOD2 are reduced during cochlear aging in mice (183, 281, 404). Mice lacking the antioxidant enzyme SOD1 display enhanced age-related cochlear hair cell loss, reduced thickness of the stria vascularis, and severe degeneration of SGNs (194). Moreover, an increase in SOD2 expression gradient in ganglion cells has been reported along the basal-to-apical turns of rodent and primate cochlea (489), consistent with the decrease in vulnerability from high- to low-frequency regions in most ARHL.

### *3. Source of ROS in cochlear cells*

Mitochondria are the primary source of ROS generation. A study using zebrafish demonstrated that aminoglycosides may disrupt ER-mitochondrial calcium regulation, leading to increased ROS production and enhanced mitochondrial membrane permeability (104). The involvement of mitochondrial ROS in hearing loss is also supported by a marked decrease in the levels of mitochondrial ATP and membrane potential, with a concomitant increase in the production of ROS in the immortalized lymphoblastoid cell lines derived from patients carrying the deafness-associated mitochondrial tRNA His mutation (135) or 12S rRNA A839G mutation (494).

A second source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), which generates ROS and consumes oxygen. As opposed to mitochondria, which generate ROS as a byproduct of their metabolism, NOX enzymes are professional ROS generators. These transmembrane proteins consist of seven isoforms in humans, and the ROS they produce directly regulate major physiological events in healthy cells (27). However, excessive NOX activation has also been implicated in oxidative, stress-mediated neurodegeneration (123). Among NOX enzymes, NOX3 is highly expressed in both the vestibular and the cochlear compartments of the inner ear (22), making it a prime suspect in the search for sources of ROS that damage the inner ear after noise or ototoxic drug exposure or during aging (366). In support of this scenario, NOX3-dependent ROS generation was observed after CDDP ex-

posure (22). Elsewhere, noise exposure induced an upregulation of NOX1 and Dual oxidase 2 (DUOX2) and a downregulation of NOX3, indicating the involvement of NOX enzymes in noise damage (442). While the involvement of NOX enzymes in several age-associated diseases is widely supported (218), their role in cochlear aging and ARHL has not yet been investigated.

## D. Proinflammatory Cytokines

Even in the absence of infection, cochlear damage from a variety of causes (noise, ototoxic drugs, aging...) can initiate the inflammatory cascade. This inflammatory response involves an upregulation of inflammatory mediators generated primarily by various resident cochlear cells, followed by the rapid recruitment and infiltration of inflammatory cells from the endolymphatic sac (384) or from the systemic circulation through the spiral modiolar vein (195). The resident cochlear cells involved in inflammatory cytokine production would appear to be mainly the spiral ligament fibrocytes (402), the hair cells in the organ of Corti, and neighboring supporting cells. The intrusion of inflammatory cells may include lymphocytes, polymorphonuclear leukocytes, monocytes, and macrophages (223, 432).

In noise-exposed cochleae and in the cochlear nucleus, the first relay station of the auditory pathway, interleukin (IL)-1 $\beta$ , and tumor necrosis factor (TNF)- $\alpha$  are upregulated by reactive microglia, fibrocytes, and neurons. This noise-induced, inflammatory-related reaction may be implicated in regulating the initiation and progression of NIHL (120). High levels of IL-1 $\beta$  have also been detected in the cochlea following gentamicin exposure or electrode insertion trauma (25). This cytokine can promote caspase-1-dependent cell death via pyroptosis, which is a highly inflammatory form of programmed cell death (47). In addition, high levels of IL-1 $\beta$  and TNF have been observed in aging cochleae (281). Furthermore, an association between systemic inflammation and ARHL has recently been noted in human studies (306, 440). The polymorphisms of genes encoding inflammatory mediators (TNF- $\alpha$  rs1800630 and TNFRSF1B rs1061624) have also been reported to contribute to the incremental risk of hearing impairment in elderly Japanese (436).

## E. Cell Signaling Pathways for Survival or Death

Evidence from experimental models favors apoptotic cell death as representing the common final stage responsible for irreversible loss of cochlear sensory and neural cells. Signal transduction research is beginning to unravel the complex mechanisms governing the stages leading up to cochlear cell survival or death after noise- or ototoxic-drug challenge or during the aging process.

### 1. Stress-activated protein kinase

Mitogen-activated protein kinases (MAPKs) are the major components of multiple pathways controlling fundamental cellular processes (221). Among MAPK family members, c-Jun NH<sub>2</sub>-terminal kinase (JNK), and p38 MAPK are mainly activated by stress stimuli, while the extracellular signal-regulated kinase 1/2 (ERK1/2) markedly responds to growth factors and is implicated in tissue growth and survival.

A) JNK PATHWAY. JNK is composed of three isoforms, JNK1/2/3, with ubiquitous expression of JNK1/2 and a more specific expression of JNK3 in the brain, heart, and testis (484). JNKs are strongly activated by environmental stress of various sources, including cochlear cell stress (221). In the steady state, JNKs exhibit cytoplasmic localization. Following activation, they translocate to the nucleus where they control gene expression through the phosphorylation of diverse transcription factors, including c-Jun, c-Myc, Elk1, Jun B, NFAT, and p53, thereby regulating multiple cellular processes (373). JNK also plays an important role in the extrinsic and intrinsic apoptotic pathways (86). In the cochlea, it can be activated by various forms of insult, such as loss of neurotrophic support, drug ototoxicity, and intense sound exposure (455). A predominant nuclear JNK substrate is c-Jun, a transcription factor of the activator protein 1 complex (69). Phosphorylation of c-Jun NH<sub>2</sub>-terminal serines 63 and 73 by JNKs is linked to trauma and apoptotic death; it can be detected by antibodies and is thus widely used as an indicator of stress-induced JNK/c-Jun signaling (43).

Ototoxic aminoglycosides have been shown to activate the JNK signaling pathway and induce cochlear cell death both in vitro and in vivo (341, 458). In contrast, activation of JNK upon CDDP exposure seems to protect cochlear cells from CDDP ototoxicity, as inhibition of the JNK pathway rather increased cochlear damage (451). In the noise-exposed cochlea, the JNK signaling pathway has been linked to hair cell-intrinsic death pathways, including activation of Bax, release of cytochrome *c*, and cleavage of alpha fodrin by activated effector caspases (278, 458), and may function as a stress-induced regulator of apoptosis (458). The protection of inner-ear function offered by JNK inhibitors against ischemic inner ear damage and NIHL have raised the question of their possible therapeutic use for acute SNHL (324, 456).

B) P38 MAPK PATHWAY. p38 family members are strongly activated by various environmental stresses and inflammatory cytokines (221). p38 MAPK activation is also a major component deciding cell fate, primarily by inducing apoptosis in response to CDDP or gentamicin (49, 465). In a cochlear cell line, pharmacological inhibition of p38 before radiation exposure prevented radiation ototoxicity (398). In animals exposed to intense noise, a change in MAPK phosphoryla-

tion in the cochlea, as well as the activation of p38 MAPKs in the SGNs, has been observed (179, 267).

**C) ERK1/2 PATHWAY.** Canonical ERKs include two highly homologous members, ERK1 and ERK2, and are activated by growth factors, hormones, proinflammatory cytokines, and osmotic stress. The molecular mechanism behind ERK1/2 activation by receptor tyrosine kinase (RTK) ligands has been thoroughly investigated, and it was proposed to mediate tissue growth and survival (221). In the cochlea, ERK1/2 has been found activated in the sensory and support cells of the cochlear sensory epithelium after intense noise exposure (267).

## 2. NF- $\kappa$ B signaling pathway

NF- $\kappa$ B is one of the redox-sensitive transcription factors and an important central mediator of inflammatory processes. Activation of NF- $\kappa$ B by aminoglycoside in damaged OHCs of the organ of Corti can rescue the sensory hair cells of the cochlea (182, 228). In contrast, the activation of NF- $\kappa$ B in CDDP-intoxicated cochlea mediates hair cell death through NO generation, due to the expression of inducible NOS in both the stria vascularis and the stria ligament (463).

Noise trauma leads to a significantly enhanced NF- $\kappa$ B DNA binding in nuclear extracts of the cochlea 2–5 h after exposure, with a return to basal levels 12 h later (303). This was at least in part due to nuclear translocation of NF- $\kappa$ B subunits p65 and p50 in the lateral wall of the noise-overexposed cochlea (303). The activation of NF- $\kappa$ B appears to play an important role in the modulation of iNOS expression after the noise exposure (480), thereby protecting cochlear cells against iNOS-triggered oxidative stress and caspase-3-mediated apoptosis that occur following NIHL (426). In addition, the activation of NF- $\kappa$ B protects against noise-induced and age-related hearing loss, as well as against the degeneration of auditory afferent dendrites under IHCs and the subsequent loss of SGNs (226, 422).

## 3. p53 pathway

p53 is a transcription factor that activates or represses the expression of multiple genes (361), but is also found in the cytosol and mitochondria, eliciting a great number of extranuclear and nontranscriptional functions. In the cochlea, we and others have shown that p53 accumulation and activation play a critical role in CDDP-induced IHC and OHC death (30, 475). Moreover, we demonstrated that genetic deletion or pharmacological inhibition of p53 attenuated the loss of both types of hair cell, thus preserving hearing during CDDP treatments. Inhibition of mitochondrial-specific p53 activity also confers significant hair cell protection from damage from gentamicin or neomycin intoxication in zebrafish lateral line (70). The activation of p53 through phosphorylation (Ser15) was briefly (4–24 h)

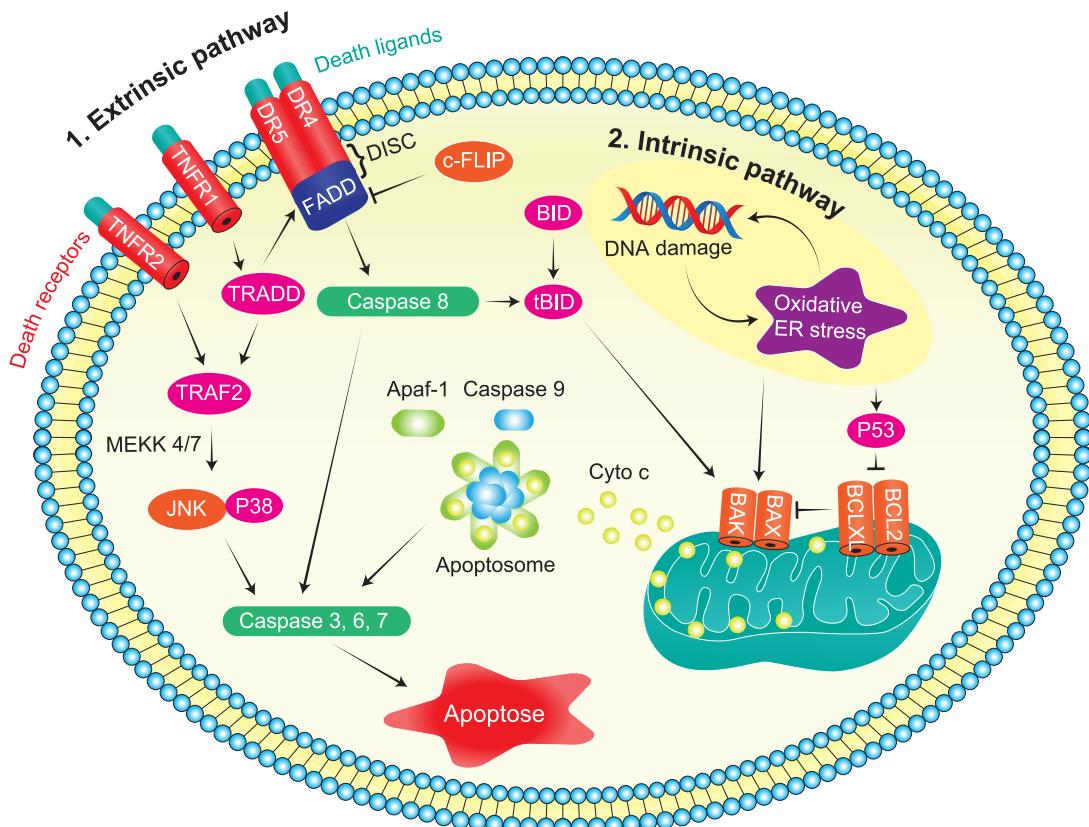
upregulated in the hair cells of the organ of Corti in adult chinchillas after impulse noise exposure, and reversible p53 inhibition with pifithrin- $\alpha$  (PFT- $\alpha$ ) decreased threshold shift and reduced the number of missing OHCs (111). p53 expression and acetylation is also increased in the cochlea of ARHL mouse models (421, 477).

## 4. Intrinsic and extrinsic pathways

In a normally functioning cell, a delicate balance exists between apoptosis-inducing and -inhibiting factors to ensure the cell is able to live and proliferate. However, this balance is disturbed in stress situations which may lead an internal messaging system to instruct the cell to enter the apoptotic death program. Apoptosis can be stimulated by two different pathways: 1) the intrinsic pathway (or mitochondria), that mainly occurs via the release from the mitochondria of cytochrome *c*, which then activates different caspases as downstream signals; and 2) the extrinsic pathway, in which death receptors are activated by a signal coming from outside of the cell.

The intrinsic (mitochondrial) pathway is activated by both exogenous and endogenous death stimuli, such as DNA damage, hypoxia, loss of survival signals, ototoxic drugs, intense sound stimulation, oxidants, and Ca<sup>2+</sup> overload (78, 456). The central step of this pathway is formation of the apoptosome (5), which is a molecular complex of cytochrome *c* (cyt.*c*), 20-deoxyadenosine 50-triphosphate (dATP), and the apoptotic protease activation factor-1 (Apaf-1). The formation of the apoptosome leads to activation of caspase-9. Once activated, caspase-9 itself activates effector caspases (e.g., procaspase-3, -6, and -7) (158). Another group of molecules released by mitochondria, including endonuclease G or AIF, is also proapoptotic. These proteins are translocated into the nucleus, where they first cause an elementarily DNA fragmentation and chromatin condensation (418). All the intrinsic apoptosis events are primarily controlled by the Bcl-2 family of proteins and their activator, p53 tumor suppressor protein. Members of this family are either pro-apoptotic (Bax, Bak, Bid, Bim, Puma, Noxa, Bad, and Blk) or anti-apoptotic (Bcl-2, Bcl-XL, Bcl-X, and BAG); they determine the membrane integrity of mitochondria and are involved in the process of cytochrome *c* release (74) (**FIGURE 5**).

The death-receptor (extrinsic) pathway (**FIGURE 5**) is initiated by the interactions between extracellular death ligands such as the TNF superfamily of proteins and Fas, and their cell-membrane death receptors. Binding of Fas ligand to its receptor induces the binding of the adaptor protein Fas-associated death domain (FADD), while interaction between TNF and their receptor TNFR causes the binding of TNFR-associated death domain (TRADD), either of which results in pro-caspase-8 and -10 activation. Active caspase-8 either induces Bid, thus involving the intrinsic pathway, or downstream effector caspases-3, -6, and -7 (see Ref. 203; **FIGURE 5**). The



**FIGURE 5.** Extrinsic and intrinsic cell-death pathways. The extrinsic pathway (1) is activated by binding of the tumor necrosis factor-related apoptosis-inducing ligand death receptors DR4 and DR5, and tumor necrosis factor receptors (TNFR1/2) to their ligands. Activated death receptors recruit an adaptor protein called Fas-associated death domain (FADD). FADD binds directly, or via another adaptor such as TNFR1-associated death-domain protein (TRADD), to the death receptor and to pro-caspase 8, to form a complex called the death-inducing signaling complex (DISC). Recruitment of caspase 8 leads to its activation. Active caspase 8, in turn, activates effector caspases such as caspase 3, 6, and 7, causing apoptosis. The activation of this pathway can be inhibited by c-FLIP, an endogenous inhibitor. The BH3-only protein BID is cleaved by caspase 8 and is then translocated to the mitochondria to activate the intrinsic pathway. The activation of this pathway can induce, via distinct mitogen-activated protein kinase kinases (MKK), the activation of the stress-activated kinases p38 mitogen-activated protein kinase (MAPK) and JNK, which can both induce gene transcription and cell apoptosis. The intrinsic pathway (2) can be initiated by DNA damage, oxidative stress, or endoplasmic reticulum (ER) stress. Mitochondrial protein release often occurs after BH3-only members of the BCL2 family bind to and neutralize anti-apoptotic members of the BCL2 family. This promotes release of mitochondrial proteins including cytochrome *c* [Cyt *c*]. Released Cyt *c* interacts with Apaf-1, pro-caspase 9, and dATP to form a complex called the apoptosome. This complex activates caspase 9, which then activates effector caspases to induce apoptosis. Finally, in response to a stress signal, cytoplasmic p53 may rapidly translocate to mitochondria, where it interacts with multi-domain members of the anti- and proapoptotic BCL2 family to either inhibit or activate them. This direct action of p53 results in robust mitochondrial outer membrane permeabilization and activation of the intrinsic apoptotic pathway.

extrinsic activation of apoptosis can also be inhibited by the binding of FLICE-like inhibitory protein (cFLIP) to either FADD or pro-capase-8, thereby blocking their activity (203). The cell-death receptor and mitochondrial pathways converge at the level of effector caspases activation, which cleaves different proteins such as kinases, DNA control proteins, cytoskeletal proteins, or inhibitors of endonucleases. DNA condensation, membrane blebbing, and all the morphological changes relating to apoptosis are regulated by these effector caspases via common mechanisms for both intrinsic and extrinsic triggers (431).

A) OTOTOXIC, DRUG-INDUCED HEARING LOSS. Aminoglycoside-induced cochlear cell damage occurs mainly through an apoptotic process due to the generation of ROS. The ROS activate various pathways including the E2F1-cyclin dependent kinase 1 (CDK1) pathway, MAPK-JNK, NF- $\kappa$ B, and Fas-Fas ligand signaling pathways, resulting in intrinsic and extrinsic apoptotic cell death (21, 61, 455). Similar to aminoglycosides, CDDP induces DNA damage and free radical production in the cochlea and triggers the activation of intrinsic and extrinsic, caspase-dependent and also caspase-independent apoptotic pathways (30, 455). Caspase-3 was

also detected in the stria vascularis, the spiral ligament, and the supporting cells of the organ of Corti from CDDP-treated guinea pigs (462). Other potential apoptotic pathways in the stria vascularis of the lateral wall or in the spiral ganglion include the activation of NF- $\kappa$ B and the formation of NO, 4-hydroxynonenal (4-HNE), and iNOS (232, 463).

**B) NOISE-INDUCED HEARING LOSS.** Several members of apoptosis-inducing gene families are activated in the immediate hours following noise insult. These include the TNFR family, the family of BCL2, the TNFR-associated factor, the inhibitor of the apoptosis protein family (169), and the BCL-2-associated death (BAD) promoter (441). Moreover, the activation of the extrinsic pathway (caspase-8) and/or the intrinsic pathway (caspase-9) has been observed in noise-damaged cochleae (111, 455, 457).

**C) AGE-RELATED HEARING LOSS.** Both intrinsic and extrinsic pathways are involved in ARHL (421). Deletion of the mitochondrial proapoptotic gene encoding brassinosteroid insensitive-1-associated receptor kinase (*Bak*) can protect against ARHL (404). Our results in the SAMP8 mouse model concordantly showed an increase in Bax expression and a decrease in cytochrome *c* oxidase expression and activity (281). Moreover, the ratio of the expression of two genes, proapoptotic *BAK1*/anti-apoptotic *BCL2*, was statistically significantly upregulated in the peripheral blood samples from ARHL and was also positively correlated with the results of the audiometric tests (107).

### 5. Calcium, calpains, and cathepsins.

An increase in the intracellular concentration of calcium is lethal to cells. In the cochlea, thapsigargin blocks the transport of calcium into intracellular storage sites, thus increasing levels of free intracellular calcium, which causes hair-cell loss (36). The mechanism involved appears to be the activation of at least three proteases including caspases, calpains, and cathepsins, all induced by increased intracellular levels of calcium (336).

**A) OTOTOXIC DRUG-INDUCED HEARING LOSS.** Several studies suggest that calpains and cathepsins may have a role in ototoxic drug-induced cell death in the cochlea. An increase in cytosolic  $Ca^{2+}$  levels is found in aminoglycoside-damaged hair cells (161, 249), a condition that is required for calpain activation (185). Ladrech et al. (222) showed an abnormal accumulation of fodrin breakdown products, specifically produced by calpain activity, in amikacin-damaged OHCs and IHCs. Mandic et al. (269) reported on the possible involvement of calpains in CDDP toxicity in a human melanoma cell line, leading to questions about their potential role in CDDP ototoxicity.

**B) NOISE-INDUCED HEARING LOSS.** Noise exposure induces an increase in intracellular calcium concentrations in sensory hair cells in vitro (118) and calpain immunoreactivity in

vivo (450). In addition, the accumulation of calpain-cleaved fodrin was observed in noise-damaged hair cells in guinea pigs (456). Taken together, these results suggest an involvement of calpains in NIHL.

**C) AGE-RELATED HEARING LOSS.** During both premature and normal cochlear cell aging in mice, one of the common features is the accumulation of lipofuscin and other abnormal proteins in the cytoplasm of the cochlear cells, particularly in the SGNs (46, 281). It has been suggested that this phenomenon is due to the impaired function of the proteolytic systems, such as the lysosome, the calpains, and the proteasome. A downregulation of calpain 2 has been observed with age and hearing loss in CBA mice (421), although the exact roles of these pathways in cochlear aging processes remains to be elucidated.

## IV. FROM EXPERIMENTAL PHARMACOTHERAPIES TO CLINICAL TRIALS

Our knowledge regarding the mechanisms of cell death provides deeper insight into various cochlear diseases, and thus boosts the development of pharmacological therapies. This section aims to emphasize the potential importance of using recently developed mitochondrial metabolic regulators, autophagy modulators, antioxidants, or inhibitors of kinases and apoptosis to protect cochlear cells against auditory stress-induced (through noise or ototoxic drug exposure) or age-related hearing loss (see **FIGURE 6**).

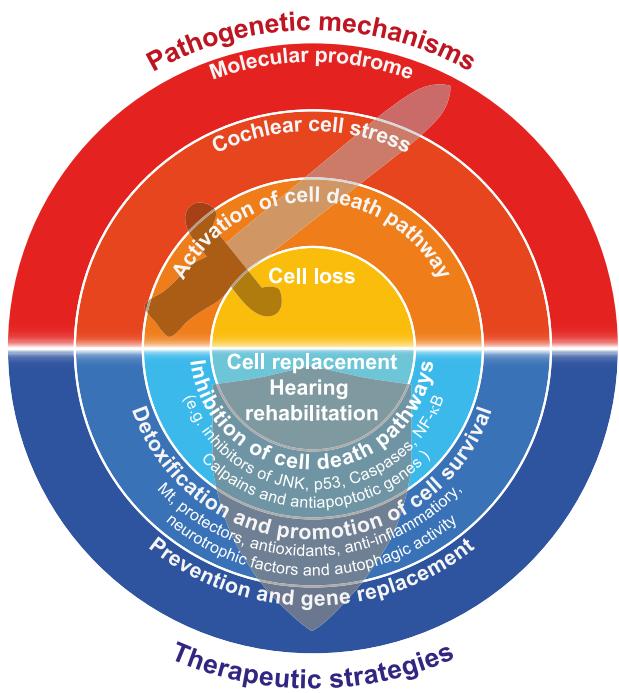
### A. Regulators of Mitochondrial Metabolism

Given the important role of mitochondrial dysfunction in cochlear injuries, therapeutic strategies targeting mechanisms of mitochondrial biogenesis, ROS production and respiration to restore mitochondrial function have recently been developed in an attempt to restore hearing (**FIGURE 6**).

#### 1. Modulators of sirtuins

Sirtuins are evolutionarily conserved nicotinamide adenine dinucleotide (NAD) $^{+}$ -dependent acetyl-lysine deacetylases and ADP ribosyltransferases, i.e., dual-function enzymes involved in the regulation of metabolism and life span. They are also implicated in determining the balance between apoptosis, cell survival, and cell proliferation. In mammals, the sirtuin family has seven members (SIRT1–7), which diverge in tissue distribution, subcellular localization, enzymatic activity, and targets. SIRT1, SIRT2, and SIRT3 have deacetylase activity and show dependence on NAD $^{+}$ , thereby directly linking their activity to the metabolic status of the cell (385).

**A) MODULATORS OF SIRT1.** Resveratrol, an ingredient of red wine, is a polyphenolic sirtuin 1 activator with antiaging effects in



**FIGURE 6.** Evaluation of molecular pathways in sensorineural hearing loss (SNHL) and therapeutic strategies. Schematic illustration summarizing recent advances in understanding of the pathogenetic mechanisms responsible for SNHL and potential targeted therapeutic strategies. The genetic composition and environment of cochlear sensorineural cells determines their biochemical signature and their predisposition for degeneration, called the molecular prodrome. These disease-causing factors trigger cochlear cell stress and lead to the activation of cell-death pathways, ultimately resulting in cell death. The identification of these pathogenetic mechanisms has led to the development of candidate interventions at different molecular targets, designed to slow down or reverse cochlear cell death and rescue hearing. Primary prevention of disease-causing factors (noise, ototoxic...) or replacement of defective genes will be appropriate at the molecular prodrome level. Promotion of cellular compensatory mechanisms can be useful for reversing cellular stress and can be achieved with mitochondrial protectors, antioxidants, anti-inflammatories, neurotrophic factors, and improved autophagic activity. Inhibition of cell-death pathways with specific inhibitors of JNK, p53, caspases, or calpains, or with the overexpression of antiapoptotic genes, may block cell apoptosis. Finally, cochlear cell regeneration with gene- or stem-cell therapies, or biotherapies in combination with cochlear implantation, would be appropriate for potential cell replacement, hearing rescue, and rehabilitation.

lower organisms such as *Caenorhabditis elegans* (26). In the cochlea, pretreating the cochlear explants with resveratrol that, via activation of sirtuin 1 (SIRT1), prevented CoCl<sub>2</sub>-induced hair cell loss, which deacetylates NF-κB (459). Concordantly, dietary supplements of resveratrol significantly reduced ARHL and hair-cell loss in C57BL/6 mice (477). In addition, some indirect evidence concerning microRNAs (miRs) also supports the potential protective effect of SIRT1 activation on cochlear hair cell survival. miRs are noncoding RNAs of ~20–24 nucleotides that act posttranscriptionally to regulate messenger RNA (mRNA) stability and, ultimately, translation (2). The overexpression of miR-29b or miR-34a, via the transfection of miRs mimics, inhibits SIRT1 and PGC-1α expression, leading to

an increase in mitochondrial dysfunction and apoptosis. In contrast, the inhibition of miR-29b or miR-34a with their inhibitors increased SIRT1 and PGC-1α expression, while it decreased apoptosis (255, 477). Conversely, another study showed that SIRT1 deficiency reduced age-related oxidative damage of cochlear hair cells and SGNs and delayed the early onset of ARHL (144).

**B) MODULATORS OF SIRT3.** Mitochondrial sirtuin 3 (SIRT3) has received much attention through its role in metabolism and aging. Specific small nucleotide polymorphisms in *Sirt3* are linked to increased human life span. In addition, SIRT3 prevents apoptosis by reducing levels of ROS and inhibiting components of the mitochondrial permeability transition pore. In the cochlea, a recent study demonstrated that caloric restriction induces upregulation of the SIRT3 gene, which, in turn, promotes the glutathione-mediated mitochondrial antioxidant defense system and delays the onset of ARHL in mice (403). Activation of SIRT3 by the NAD<sup>+</sup> precursor nicotinamide riboside also protects cochleae from NIHL and spiral ganglia neurite degeneration in mice (48).

## 2. Mitochondria targeted protective compounds

Acetylcarnitine is naturally produced by the body. In the blood, acetylcarnitine is broken down by plasma esterases to carnitine, which is used to transport fatty acids into the mitochondria. In CDDP-intoxicated Wistar albino rats, acetylcarnitine attenuated hair-cell damage (141). This protective effect was driven by the induction of anti-apoptotic gene expression and by attenuating levels of proinflammatory cytokines (12, 141). Systemic administration of acetyl-L-carnitine significantly reduced NIHL and OHC loss in a chinchilla model (65).

Coenzyme Q10, or its analog, coenzyme Q10-Ter, are important molecules implicated in mitochondrial energy production and protection of mitochondrial membrane proteins, lipids, and DNA from oxidative damage. The systemic administration of coenzyme Q10-Ter or coenzyme Q10 in guinea pigs and Wistar rats restores neuronal morphology in the cochlea and auditory cortex as well as restoring hearing function, by reducing the noise-induced redox imbalance in these auditory pathways (112, 162). Both compounds protected OHCs against gentamycin-induced hair-cell and hearing loss in guinea pigs (113). Coenzyme Q10 has also been shown to protect sensory hair cells against neomycin-induced death in the mammalian vestibular epithelium (416). The protective effect of coenzyme Q10 and coenzyme Q10-Ter against aminoglycoside ototoxicity may result from their potent antioxidative capacity and protection of mitochondria. Since then, the mitochondrial and cytoplasmic ribosome was identified as an intracellular target of aminoglycoside (163, 311). Finally, oral supplements of coenzyme Q10 and alpha-lipoic acid also

suppress Bak expression in the mouse cochlea, reduce cochlear cell death, and prevent ARHL (404).

Lecithin is a polyunsaturated phosphatidylcholine, which is a major component of biological membranes. Polyunsaturated phosphatidylcholine plays a rate-limiting role in the activation of numerous membrane-located enzymes, including superoxide dismutase and glutathione, which are important antioxidants protecting cell membranes from damage by ROS. Administration of lecithin delayed the progression of ARHL in rats by protecting cochlear mitochondrial DNA (390).

### 3. Clinical trials

An orally administered food supplement containing coenzyme Q10-Ter for 30 days in volunteers reduced recovery time after exposure to noise (410). The clinical trial data from 60 patients with ARHL and treated via systemic administration of water-soluble coenzyme Q10 formula (Q-TER, 160 mg, once a day for 30 days) showed a significant improvement of air- and bone-conduction thresholds at 1, 2, 4, and 8 kHz frequencies when compared with values from vitamin E (50 mg) or placebo groups (378) (FIGURE 7).

## B. Chemical Modulators of Autophagy to Rescue Cochlear Dysfunction

Autophagy malfunction is a common feature observed in almost all neurodegenerative diseases. Consequently, the pharmacological activation of autophagy offers an attrac-

tive therapeutic approach to combating these pathologies. Autophagic processes can be modulated at several stages within different autophagic pathways. Here, we focus on the use of rapamycin and metformin, which are known to inhibit mammalian target of rapamycin (mTOR) and protect the cochlea against noise, drug injuries, and ARHL (FIGURE 6). Yuan et al. (496) showed that systemic administration of rapamycin before and immediately after noise exposure increased LC3B expression, while diminishing 4-HNE and 3-NT levels, which in turn reduced noise-induced and subsequent hair cell loss (496). Similar protective action was obtained in CDDP-intoxicated rats (108). Inhibition of mTOR with rapamycin also protected hair cells against gentamicin damage in cochlear explants *in vitro* (96). While rapamycin clearly offers good levels of cochlear protection, it has deleterious effects at higher doses (50–100  $\mu$ M), with dose-dependent hair-cell loss; low-dose concentrations of rapamycin (10  $\mu$ M) had, however, no toxic effect on cochlear explants (237).

One other mTOR inhibitor and autophagy inducer evaluated for potential cochlear protection is the antidiabetic drug metformin, an adenosine monophosphate-activated protein kinase (AMPK). However, even though metformin prevented gentamicin-induced OHC loss *in vitro*, no hearing preservation was observed in CBA/J mice treated with low doses of metformin in combination with gentamicin. Moreover, higher doses potentiated gentamicin ototoxicity (321). These results underline the necessity of *in vivo* confirmation of all protective drugs screened *in vitro*.

## C. Antioxidants and Free Radical Scavengers

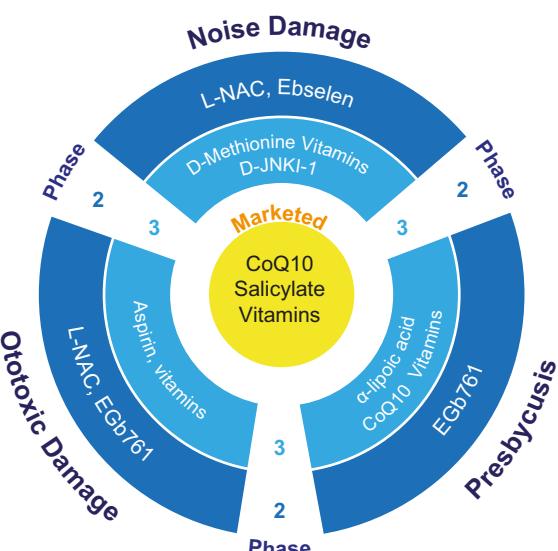
Physiologically, excess ROS in the body can be eliminated by endogenous antioxidant enzymes and nonenzymatic scavengers, such as vitamins E, C, and D and beta-carotene (that is metabolized to form vitamin A). Under oxidative stress conditions, however, the endogenous antioxidant systems are believed to be insufficiently effective. In such cases, supplementation with exogenous antioxidants can play a role in maintaining homeostasis (375).

### 1. Thiol-based antioxidant compounds

The major thiol antioxidant is tripeptide glutathione (GSH), a multifunctional, intracellular, nonenzymatic antioxidant considered to be the major cellular thiol-disulfide redox buffer (274). Exogenous thiols have successfully been used in cell cultures, animal models, and humans to increase cell- and tissue-thiol levels. Increased levels of GSH and other thiols have been associated with increased tolerance to oxidant stresses in all these systems and, in some cases, with disease prevention or treatment in humans (85).

A) N-ACETYL CYSTEINE. N-acetylcysteine (NAC) is a precursor of glutathione and a potent antioxidant. NAC and L-NAC

**FIGURE 7.** A brief note on some of the drugs in clinical trials. Schematic representation of the latest investigational antioxidants, mitochondrial protective drugs, and cell-death inhibitors encompassing a variety of causes of human sensorineural hearing loss. L-NAC, L-N-acetylcysteine.



have been used both in vitro and in vivo to reduce aminoglycoside-, CDDP-, and noise-induced hearing loss, but not ARHL (9, 82, 212, 297). Some negative findings offset these potentially positive results. Hamernick et al. (143) exposed three groups of chinchillas to a continuous broadband noise (105 dBA, 8 h/day for 5 days) and showed that no statistically significant difference was found between L-NAC-treated, saline-treated, and untreated groups. Davis et al. (81) confirmed these negative results in B6 mice exposed to 104-dB broadband noise and treated with L-NAC 1 h before, and immediately after, the exposure.

**B) D-METHIONINE.** D-Methionine (D-Met) is the dextro-isomer of the essential amino acid L-methionine (L-Met). Biochemically, methionine acts as a synthetic substrate and may enhance the intracellular production of the key antioxidants reduced GSH (263), superoxide dismutase, and catalase (443). D-Met has been proposed to alter the activity of the ion-transport mechanisms within the cell (63). It can prevent oxidative stress-induced apoptosis (132) and protect the cochlea from ototoxicity following CDDP-, aminoglycoside-, or noise-induced injury in a number of animal models (51, 68, 136).

**C) ALPHA-LIPOIDIC ACID.** Alpha-lipoic acid is an endogenous disulfide compound synthesized de novo in mitochondria, where it is an essential enzymatic cofactor. Besides its important role in mitochondrial energy metabolism, it also has powerful antioxidative effects. Alpha-lipoic acid effectively attenuated kanamycin- (447) and CDDP-induced (201, 209) ototoxicity as well as noise-induced cochlear cell loss (478). This compound has also successfully provided cochlear protection against ARHL (389). Controversially, another study showed that an antioxidant-enriched diet, with vitamins A, C, and E, L-carnitine, and alpha-lipoic acid significantly increased the antioxidant capacity of inner ear tissues, but did not delay the progression of ARHL in CBA/J mice (392).

**D) SODIUM THIOSULFATE.** Sodium thiosulfate, an antidote for cyanide poisoning and nitroprusside overdose, is among the most widely studied thiol compounds and has repeatedly demonstrated reduction of CDDP ototoxicity in both animal and clinical studies (412, 452). In our previous study in guinea pigs, intracochlear administration of sodium thiosulfate (10 mM) completely prevented CDDP-induced hearing loss and hair-cell loss (452). In contrast, Wimmer et al. (469) found no significant protective benefit after administering sodium thiosulfate to the middle ear of guinea pigs. The variation between the different groups may be resulted from the application methods (380).

## 2. Antioxidant extracts from *Ginkgo biloba* leaves or *Curcuma longa*

Antioxidant extracts from *Ginkgo biloba* leaves (EGb761, Renexin) effected a dose-dependent attenuation of CDDP-,

aminoglycoside-, and noise-induced hearing loss and hair cell death via the reduction of ROS and NO-related apoptosis in rats, C57BL/6 mice, and guinea pig models (66, 487). In the case of ARHL, experimental data showed that EGb761 prevented aging-related caspase-mediated apoptosis in rat cochlea (308).

**Curcumin** is a polyphenol isolated from turmeric (*Curcuma longa*) that has a variety of antioxidant, anti-inflammatory, and antineoplastic properties. The numerous effects of curcumin are dependent on interactions with multiple molecular targets, including growth factors, kinases, transcription factors, inflammatory cytokines, apoptosis-related proteins, adhesion molecules, and enzymes associated with inflammation and cellular proliferation (111, 379). Systemic administration of curcumin protected the cochlea against CDDP-induced ototoxicity in rats (111, 379).

## 3. Vitamins

Vitamins E, C, D, and beta-carotene (metabolized to form vitamin A) act as nonenzymatic ROS scavengers. Dietary supplements of vitamins like A, C, and E can act synergistically with minerals like magnesium to prevent ROS-induced damage to the inner ear. For example, systemic administration of vitamin E in combination with curcumin, or a combination of vitamins A, C, and E prevented CDDP or gentamicin ototoxicity, respectively (189, 230, 406). Le Prell et al. (229) found also that a diet supplemented with a combination of beta-carotene, vitamins C and E, and magnesium protected CBA/J mice against noise-induced PTS. Similarly, a combination of beta-carotene; vitamins C, E, and magnesium; or L-NAC, effectively reduced TTS and PTS in chinchillas (212). In addition, a recent review of synthesis of the different epidemiological, genetic, and experimental model studies suggested that increased total plasma homocysteine, a protein amino acid, and vitamin deficiencies may play an important role in the development of sensorineural hearing loss. Moreover, long-term dietary supplementation with omega-3 fatty acids improved homocysteine metabolism and reduced sensorineural hearing loss (335).

## 4. Iron chelators

Iron is an important factor in ROS-mediated tissue injury (155), and in gentamicin-induced cochlear damage, ROS are generated by a redox-active, iron-gentamicin complex (349). Iron chelators such as 2,2'-dipyridyl, dihydroxybenzoate, and deferoxamine have generally been investigated regarding their protection against aminoglycoside- (84, 293) and CDDP-induced ototoxicity (461). Despite their success in laboratory animals, no positive results have been reported from their use in humans.

## 5. Other antioxidants

**A) ACETYLSALICYLIC ACID.** Acetylsalicylic acid (aspirin), a derivative of salicylic acid, is one of the most widely used drugs

worldwide. In addition to blocking the conversion of arachidonic acid to prostanooids and inhibiting the activity of isolated cyclooxygenase, aspirin also displays anti-inflammatory and antioxidant properties (103). In the cochlea, salicylate reduced by 80% the gentamicin-induced OHC loss in guinea pigs (394). In rats, based on auditory brain stem responses (ABRs) measurements, its subcutaneous injection offered otoprotection (246). Similar otoprotection against CDDP ototoxicity was observed in guinea pigs (175). In combination with NAC, sodium salicylate was also effective in reducing noise damage to the cochlea of chinchillas (71).

B) EBSELEN. Ebselen, an organoselenium compound, mimics glutathione peroxidase activity. It is a multifunctional compound, which catalyzes several essential reactions and protects cellular components from oxidative- and free-radical damage. High doses of ebselen (16 mg/kg) were shown to reduce CDDP ototoxicity in Wistar rats (371), and also in Fisher 344 rats when administered either alone or in combination with allopurinol (264). However, only a narrow range of ebselen doses for otoprotection has been documented in guinea pigs exposed to acoustic trauma (347). Kil et al. (199) reported that oral ebselen given to Fisher 344 rats before and immediately after noise exposure reduced both OHC loss and acute swelling of stria vascularis by scavenging ROS and other free radicals and by stimulating glutathione peroxide expression and activity.

#### *6. Limitation of systemic administration of antioxidants*

The main limitation in using systemic thiol compounds for cancer patients is the marked decrease in the oncologic effectiveness of CDDP (88). To prevent this problem, local middle- or inner ear administration of thiol compounds has been performed in animal models. Due to their relatively low molecular weights (163.2 Da for L-NAC and 149.21 Da for L-Met and D-Met), these compounds can be administered via the intratympanic route into the middle ear (214, 452). Concerning the systemic administration of vitamin E, the dose described in animal models (4 g/kg) is extremely high. In humans, high doses of vitamin E can cause an increase in mortality due to subarachnoid hemorrhage (242). Furthermore, such high doses of vitamin E can depress both leukocyte oxidative and bactericidal activity, and mitogen-induced lymphocyte transformation, making such treatment inappropriate for patients undergoing CDDP treatment.

#### *7. Clinical trials*

A) L-NAC. One clinical trial of transtympanic administration of L-NAC in 11 patients receiving CDDP chemotherapy showed a significantly improved hearing in the L-NAC-treated ear of only two of the patients (18.2%), with the difference in hearing preservation across the whole group

not reaching significance (492). In another trial testing the potential for NAC to effectively prevent aminoglycoside-induced ototoxicity in peritoneal dialysis patients, no improvement in hearing was observed after 1 wk, although a significant improvement was found after 4 wk of NAC treatment (294). The greatest otoprotective effect of NAC was noticed in the high audiometric tone frequencies of gentamicin-treated hemodialysis patients (109) (**FIGURE 7**).

Lindblad et al. (256) explored hearing loss before and after a shooting session in a bunker-like room. A control group of 23 military officers was exposed without NAC, and 11 officers received oral NAC directly after the shooting. The results showed that early effects of noise trauma can be prevented by NAC. Doosti et al. (93) studied the effect of NAC in preventing NIHL in male textile workers. Oral administration of NAC at 1,200 mg/day for 14 days to 16 subjects led to significantly reduced noise-induced TTS in workers exposed to occupational noise. Similar results were reported by Lin et al. (254) using the same dose and same period of NAC administration to male workers employed for at least 1 yr in a steel manufacturing company. In contrast, in a randomized, double-blind, placebo-controlled study, Kramer et al. (217) observed no positive effect of NAC on NIHL in 31 normal-hearing humans exposed to 2 h of live music in a nightclub. The average music level was 98.1 dBA, ranging from 92.5 to 102.8 dBA. They found no statistically significant differences in pure-tone thresholds and distortion-product otoacoustic emissions between participants who received NAC versus placebo. The limitation of this study was the very slight threshold shift, making any differences in threshold difficult to evaluate.

B) D-METHIONINE. The protection from NIHL offered by oral administration of D-methionine tablets has also been investigated in clinical studies (**FIGURE 7**). In one such study by Ge et al. (131), 113 of 203 volunteers received oral administration of D-methionine tablets before noise exposure. Routine audiometric evaluation and ABR testing led to the authors to conclude that it has a protective effect against NIHL.

C) ANTIOXIDANT EXTRACTS FROM GINKGO BILOBA LEAVES (GBE 761). The protective effect of *Ginkgo biloba* extract on CDDP-induced ototoxicity was tested in a pilot study, in which cancer patients were randomly assigned to receive drug (120 mg GBE 761 twice a day along with maximal cumulative CDDP dosage of 300 mg/m<sup>2</sup>, n = 7) or placebo (maximal cumulative CDDP dosage of 300 mg/m<sup>2</sup>, n = 8). Treatment was double-blinded. Hearing was evaluated by distortion-product otoacoustic emissions assessed for 90 days. The results showed otoprotective effects of GBE 761 against CDDP ototoxicity in these patients (87). However, no protective effect against ARHL was observed in a double-blind, randomized clinical trial with this drug in 120 elderly patients (345).

D) VITAMINS. One randomized, double-blind clinical trial evaluated the efficacy of a combination of vitamins A, C, and E and mineral magnesium at preventing NIHL in Swedish military personnel exposed during military weapons training (**FIGURE 7**). The noise exposure resulted in no significant TTS in either the placebo or the treatment groups (229). In a more recent population-based study including 1,910 participants aged between 50 and 80 yr, Kang et al. (190) did find a protective role of dietary vitamin intake against ARHL. In particular, dietary supplements of vitamin C significantly preserved hearing at all frequencies. Vitamin E, on the other hand, while it offered protection against aminoglycoside damage in guinea pigs, was ineffective in a clinical setting (197).

E) ACETYLSALICYLIC ACID. The efficiency of aspirin against gentamicin ototoxicity was tested in a randomized, double-blind, placebo-controlled trial on patients receiving gentamicin for acute infections in combination with aspirin (89 patients) or with placebo (106 patients) (393). Their results showed that cotreatments with aspirin reduced the number of patients suffering from gentamicin-induced hearing loss (3% vs. 13% for aspirin and placebo groups, respectively). In addition, aspirin did not affect the efficacy of the gentamicin therapy. Another trial gave similar results (29).

F) EBSELEN AND ALPHA-LIPOID ACID. Phase II clinical trials of ebselen (SPI-1005) for prevention of TTS induced by listening to music through an iPod or personal music player is still ongoing, and no clinical data are yet available (294). Alpha-lipoic acid plus vitamin C treatment for 6 mo in ARHL patients had no effect on hearing when compared with placebo data (345).

## D. Inhibition of JNK/MAPK Signaling Pathway

In the cochlea, pharmacological inhibition of mixed-lineage kinases (MLKs), or JNK activation, conferred protection against cochlear cell death (341, 456, 491). In this section, we will focus on inhibitors of MLK or JNK activation that have been tested in the cochlea (**FIGURE 6**).

### 1. CEP-1347

CEP-1347 is a potent, semi-synthetic inhibitor of the MLKs, a distinct family of mitogen-activated protein kinase kinase kinases (MAPKKK; Ref. 334). CEP-1347 blocks the activation of JNKs through ATP-competitive inhibition of the upstream mixed-lineage kinase (MLK) family (273). CEP-1347 is a highly otoprotective agent for preventing neomycin-induced loss of sensory hair cells from neonatal rodent organ of Corti explants. Its systemic administration partially protected auditory sensory hair cells from the ototoxic effects of gentamicin (341, 491), while its subcutaneous delivery attenuated noise-induced hearing and sensory hair-cell loss (341).

### 2. D-JNKI-1

D-JNKI-1 is a cell-permeable and protease-resistant peptide developed to selectively interrupt JNK signaling (38). We have shown that d-JNK-1 provided complete protection of the cochlea against neomycin ototoxicity in vitro and in vivo. Inclusion of D-JNKI-1 in the culture medium protected sensory hair cells in P-3 mouse organ of Corti explants against neomycin ototoxicity. In addition, the intracochlear perfusion of D-JNKI-1 effectively prevented both loss of sensory hair cells and the development of PTS induced by neomycin ototoxicity or by sound trauma in guinea pigs (458). The therapeutic window for protection of the cochlea from sound trauma with round window membrane delivery of D-JNKI-1 extended to 12 h post-sound exposure (456). Conversely, D-JNKI-1 exacerbated the ototoxicity of CDDP (451). This suggests that the JNK pathway is not involved in CDDP-induced hair cell death, but instead may have a role in DNA repair and maintenance of CDDP-damaged sensory cells.

### 3. SB202190 and SB203580

SB202190 and SB203580 are selective inhibitors of p38 MAPK. Pharmacological inhibition of p38 with SB203580 lessened radiation-induced apoptosis and mitochondrial injury in HEI-OC1 cells by reducing the activation of JNK, p38, cytochrome *c*, and the cleavage of caspase-3 and PARP (398). Scanning electron microscopy showed that SB203580 also prevented radiation-induced destruction of both kinocilia and stereocilia in zebrafish neuromasts (398).

### 4. Clinical trials

Among all the JNK inhibitors tested in animals, only D-JNKI-1 was entered into clinical trials. The results from a phase I/II clinical trial in 11 patients after acute acoustic trauma indicated that intratympanic treatment could have beneficial effects (413). Another multicenter, randomized clinical trial on 210 patients, to test the efficacy and safety of D-JNKI-1 against acute SNHL, showed a significant improvement in hearing and speech discrimination compared with placebo (414) (**FIGURE 7**).

## E. Targeting the NF- $\kappa$ B Signaling Pathway

The complex NF- $\kappa$ B signaling pathway provides drug targets at several levels. Modulation of NF- $\kappa$ B signaling has the potential to interrupt multiple inflammatory and apoptotic mechanisms through one specific molecular target. To date, over 800 NF- $\kappa$ B inhibitors have been reported, and the number continues to rise (133). These inhibitors can be broadly divided into three categories including 1) proteins and peptides, 2) small molecules, and 3) nucleic acids. Here we will only summarize those tested in the cochlea. Dexamethasone, a drug, inhibits NF- $\kappa$ B signaling as part of its

therapeutic effects. Because of their dual anti-inflammatory and antiapoptotic actions, corticosteroids have long been used to protect against several types of acute SNHL (91). Resveratrol is known for its roles of MAPK inhibition and SIRT1 activation, which in turn suppress NF- $\kappa$ B signaling (55, 92). Resveratrol treatment prevented CoCl<sub>2</sub> (an inducer of hypoxia)-induced activation of NF- $\kappa$ B and loss of hair cells in cochlear explants *in vitro* (459). Dietary supplements of resveratrol significantly reduced age-related hearing and hair-cell loss in mice (477).

## F. p53 Inhibitors

PFT- $\alpha$ , a reversible p53 inhibitor blocking p53 transcription-dependent apoptosis, was initially proposed by Komarova and Gudkov (206) to reduce the side effects induced by anticancer therapies (140). We showed that targeting the p53 pathway in mice bearing patient-derived triple negative breast cancer, using PFT- $\alpha$ , preserves hearing function without compromising the anti-tumor effect of CDDP (30). The activation of p53 was found to be involved in impulse NIHL and OHC degeneration in chinchillas, and its reversible inhibition, using PFT- $\alpha$ , protected cochleae against such injury (111).

## G. Caspase Inhibitors

A number of specific and broad-spectrum peptide caspase inhibitors have been developed to elucidate the role of each specific caspase in cell death. Some of them have already been shown to be effective in decreasing both cell death and inflammatory processes in animal models of human diseases.

### 1. Pan-caspase inhibitors and specific caspase inhibitors

When mouse utricles were treated with neomycin, z-LEHD-FMK (caspase-9-inhibitor) provided significant protection of hair cells, whereas z-IETD-FMK (caspase-8-inhibitor) was not effective (77). Treatment with either z-VAD-FMK (pan-caspase inhibitor) or z-LEHD-FMK also prevented gentamicin-induced hearing loss and sensory hair-cell damage in guinea pigs (322). In addition, we and others (257, 451) showed that inner ear local administration of z-DEVD-FMK (caspase-3 inhibitor) and z-LEHD-FMK dramatically reduces the ototoxic effects of CDDP, as demonstrated by a lack of DNA fragmentation, with almost no apoptotic cochlear cell death or loss of hearing in the CDDP-treated animals. In the case of noise damage, an intracochlear perfusion of the caspase-3 inhibitor z-DEVD-FMK prevented noise-induced F-actin cleavage in the OHCs of chinchilla cochleae (170). In addition, the pan-caspase inhibitor z-VAD-FMK prevented cochlear cell apoptosis resulting from gunshot noise trauma in guinea pigs

(1). Finally, intraperitoneal injection of Z-VAD-FMK for 8 wk starting at 1 wk of age in DBA/2J and A/J mice, which display early-onset ARHL, preserved hearing by >10 dB SPL in the ABR thresholds and significantly reduced OHC loss in the basal turns of the cochleae (146, 485).

### 2. Overexpression of XIAP

Among the inhibitor of apoptotic protein family members, the X-linked inhibitor of apoptosis (XIAP) is considered to be the most potent, because of its ability to strongly suppress caspase-3, -7, and -9 activity (97). In the cochlea, transgenic mice with C57BL/6J genetic background that overexpressed human XIAP under control of the ubiquitin promoter (ubXIAP) delayed both the development of ARHL and the loss of cochlear hair cells, afferent dendrites, efferent axons, and SGNs when compared with wild-type littermates (369, 453). Moreover, these transgenic mice were more resistant to noise-induced hearing loss; to the loss of IHCs, OHCs, SGNs, and auditory nerve fibers (457); and to neomycin-induced hearing and hair-cell loss (417). Jie et al. (184) showed that round-window membrane AAV-XIAP delivery resulted in the transduction of >90% of IHCs and ~50% of OHCs in the basal cochlear turn, and thus protected cochleae against CDDP ototoxicity.

## H. Calpain Inhibitors

Regulation of calpain activity is tightly controlled by the intracellular concentration of calcium and also by the natural endogenous inhibitor calpastatin (76). However, calpastatin has large active polypeptide fragments that prevent it from crossing membrane barriers, which strongly reduces its therapeutic potential (231). To overcome this issue, various cell-permeable calpain inhibitors have been synthesized for pharmacological inhibition of calpain activity. Among all synthesized calpain inhibitors, we will discuss those tested in the inner ear (**FIGURE 6**).

### 1. Leupeptin

Leupeptin, a prototypic aldehyde inhibitor, exhibits low cell permeability and inhibits calpain, cathepsins, trypsin, and the proteasome (405). It has been shown to inhibit programmed cell death in gentamicin- and neomycin-damaged cochlear and vestibular sensory hair cells (89, 289, 397). Infusion of leupeptin into the basal turn of chinchilla cochleae reduced the sensory hair-cell loss observed following a 105 dB SPL exposure by as much as 60% (450). However, cochlear infusion of leupeptin via a mini-osmotic pump after a gunshot noise-induced trauma did not improve hearing nor did it reduce hair-cell loss in guinea pigs (1). Moreover, the calpain inhibitors are not effective against CDDP- or carboplatin ototoxicity on sensory hair cells and auditory neurons *in vitro* or on sensory hair cells *in vivo* (62, 450).

## 2. Other inhibitors of calpains

BN82270 is a membrane-permeable prodrug of a chimeric compound (BN 82204) possessing both calpain-cathepsin L inhibitory and antioxidant properties (339). Intracochlear infusion of BN82270 in guinea pigs prevented hair cell degeneration and hearing loss caused by sound trauma, with a therapeutic window of 24 h (454). PD 150606, a non-peptide cell-permeable selective calpain inhibitor, attenuated glutamate-induced SGN apoptosis through the apoptosis-inducing factor pathway in vitro (90).

## I. Trophic Factors

Trophic factors are proteins that act on membrane receptors to activate protective signals. Among these trophic factors, insulin-like growth factor I (IGF-I) plays a central role in the regulation of cochlear development, growth, and differentiation, and its mutations are associated with hearing loss in mice and humans (299). In addition, treatment with IGF-I maintained hair-cell numbers in the postnatal mammalian cochlea after various types of hair cell injuries, including ischemia and toxic drug treatment (268, 481). IGF-I treatment promoted the cell cycle in supporting cells and inhibited hair-cell apoptosis in an aminoglycoside-treated neonatal mouse cochlear explant culture. This was achieved via the activation of PI3K/Akt or MEK/ERK pathways, leading to the survival of both IHCs and OHCs (149). Local IGF-I application was successfully used in the treatment of both noise-induced and ischemia-related hearing loss in animals (121, 233). Its topical use via gelatine hydrogels is well tolerated and may be efficacious for hearing recovery in patients with sudden SNHL that is resistant to systemic glucocorticoids (305). These recent data provide us with new perspectives for IGF-I in the treatment of deafness.

Neurotrophins are another class of trophic factors that act by preventing a particular neuron from initiating programmed cell death. Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) are the two neurotrophins essential for normal auditory neural development (342) and play an important role in the survival and maintenance of auditory neurons in the mature mammalian cochlea (122). Many studies have indeed demonstrated that treatment of deafened ears with exogenous BDNF or NT3 can significantly enhance SGN survival to near-normal levels (11, 100). Suzuki et al. (419) showed that in mice, round window membrane administration of NT3, even 24 h after a synaptopatic noise exposure, induced the regeneration of presynaptic and postsynaptic components and the recovery of ABR suprathreshold amplitude. In addition to neurotrophins, other molecules, such as fibroblast growth factor and glial cell line-derived neurotrophic factor (GDNF), have been shown to influence the maintenance and growth of the auditory nerve (134, 191). Exogenous neurotrophins may also promote neuritogenesis both in vitro and in vivo (286, 471) (**FIGURE 6**).

## V. TOWARD GENE AND CELL THERAPY

Results from recent studies have raised hopes that gene- and cell-therapy approaches may one day treat congenital or later-onset hearing loss in monogenic disorders and/or enable the regeneration or replacement of lost sensory hair cells. The transfer of exogenous genetic material using viral or nonviral vectors and the transplantation of exogenous stem cells into the mammalian inner ear have been examined over the last decade. In the following section, we summarize the current state of inner-ear gene and cell therapies, including vectors, therapeutic genes, cellular material, delivery routes, and various targets.

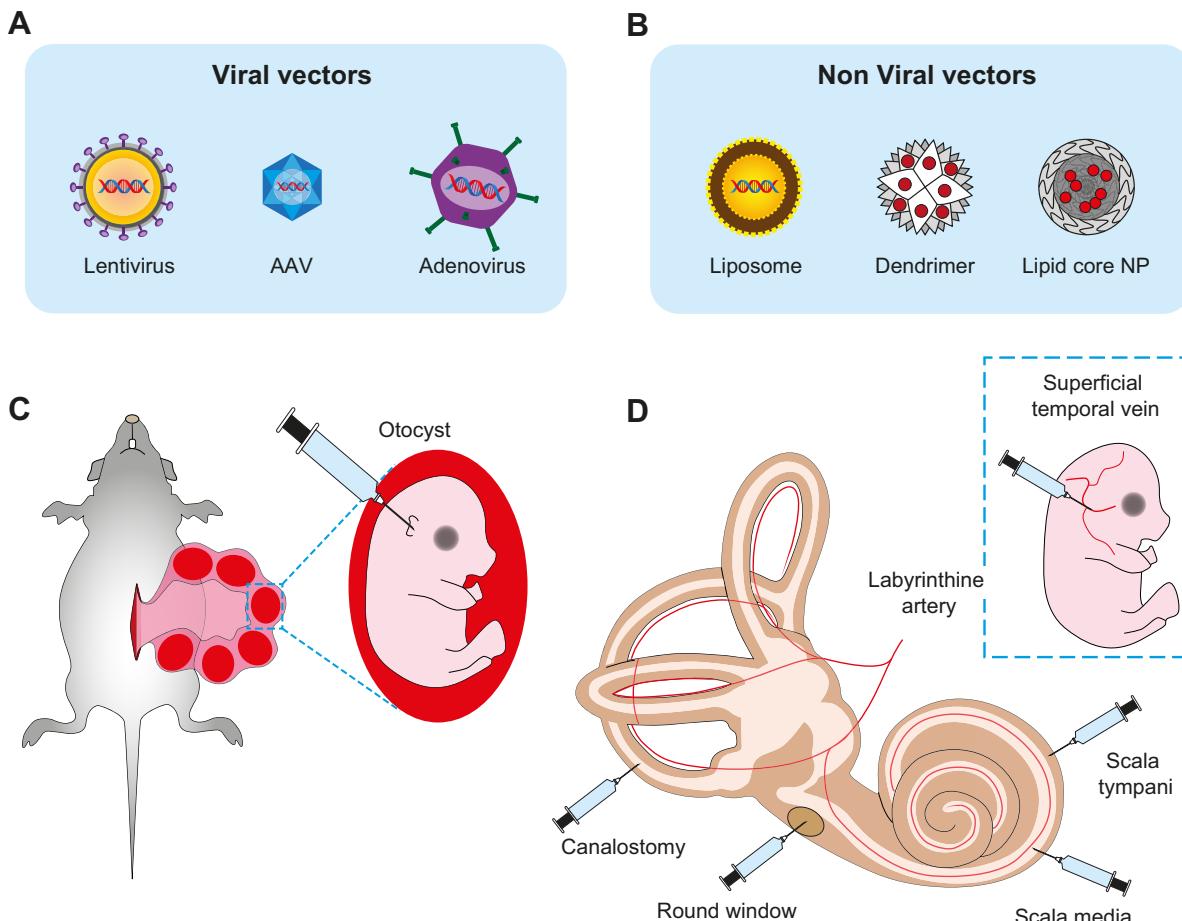
### A. Gene Therapy

Gene therapy is an experimental technique that uses genes to treat or prevent disease by introducing a desired foreign gene or gene-regulatory element, such as RNA interference, into the target cells to replace or fix the defective gene (298). Different factors, such as vector types, administration method, and promoter, have an impact both on the type of inner-ear cells targeted, as well as on transgene expression. There are currently two main technical approaches for gene transfer: viral and nonviral vectors. Gene transfer using viruses is named transduction, whereas gene transfer using nonviral vectors is referred to as transfection.

#### 1. Viral vectors

To date, a number of different viral vectors have already been used to transduce the inner ear, including adenovirus, adeno-associated virus (AAV), lentivirus, herpes simplex virus, vaccinia virus, and newly developed synthetic AAV vectors (**FIGURE 8**) (see for review, see Ref. 374). The most recent studies have focused on optimizing Ad- and AAV-based vector systems, since the other virus types did not transduce cells of the sensory epithelium efficiently enough.

A) ADENOVIRUS. The adenovirus (Ad) vector is a promising gene delivery vehicle for human gene therapy, as illustrated by its predominant use in clinical trials for human disease (253). The Ad vectors are nonenveloped virions with a double-stranded DNA genome of 26–45 kb. The viral linear DNA genome-coding region contains five early transcription regions (E1A, E1B, E2, E3, and E4), which have regulatory functions, and one late transcription region encoding structural proteins (210). Adenoviruses exist as over 50 different serotypes, but only a few of them have been modified to generate vectors. Most emphasis has been placed on serotype 5 (45). Adenoviral vectors have several advantages for cochlear gene therapy, including their large potential insert size, which greatly expands the number of target genes, and their effective transduction of many cochlear cell types, including stria vascularis, spiral ligament, hair cells and supporting cells of organ of Corti, and SGNs (196, 368).



**FIGURE 8.** Schematic illustration of the main vectors and delivery approaches tested in the cochlea. *A* and *B*: vectors. Schematic illustration summarizing the main viral vectors [e.g., lentiviruses, adeno-associated viruses (AAVs), adenoviruses...] and nonviral vectors [e.g., liposomes, dendrimer and lipid core nanoparticles...] used for delivering genetic material or drugs into the inner ear cells. *C* and *D*: route of administration. Shown are established routes for gene delivery in the developing ear (*in utero*, *C*) and in postnatal or adult ear using local (*D*) delivery approaches. Note that the vectors can be delivered into the perilymph via a cochleostomy or trans-round-window membrane route; into the endolymph via cochleostomy of the scala media space and canalostomy. *Inset*: shown is the systemic delivery of AAV9 via the superficial temporal vein.

**B) LENTIVIRUS.** Lentiviruses belong to a subclass of retroviruses that offer the possibility of inserting long DNA fragments and the ability to transduce both mitotic and postmitotic cells. The one used for transduction is derived from human immunodeficiency virus (HIV)-1. Another unique feature of lentiviruses is their capacity to integrate into the host genome with minimal inflammatory response, thus allowing long-term transgene expression. Lentiviruses were considered as suitable candidates for inner-ear gene therapy. *In vitro* infection of cochlear explants from neonatal rats allowed successful transduction of SGNs and some supporting cells, but not of hair cells (145). One report demonstrated that lentivirus injected into the rat scala tympani remained within the cochlea, with no evidence of its spread to the central nervous system; this is an important feature with regards to minimizing toxicity to tissues outside the cochlea and provides further support for the safety of inner-ear, viral-mediated gene delivery (95).

**C) HERPES SIMPLEX VIRUS.** Herpes simplex virus-1 (HSV-1) has a double-stranded DNA genome enclosed within a protein capsid, itself surrounded by a lipid membrane envelope. The capsid and envelope are separated by a tegument containing viral and cellular proteins. The 152-kb genome of HSV-1 is the largest and most complex of all the viruses being developed for gene therapy. HSV-1 is neurotrophic and can establish a lifelong presence in sensory neurons. Due to this natural tropism, the majority of gene transfer applications of HSV-1 vector have been directed toward the nervous system (253). Two types of HSV-1 vector systems have been developed: recombinant viruses and amplicons. The advantages of using amplicon vectors are the reduced cellular toxicity and low immune response compared with recombinant HSV-1 vectors (313), as well as their capacity to carry large fragments of foreign DNA. In the cochlea, HSV-1 amplicon vectors harboring brain-derived neurotrophic factor, NT3, or bcl-2 cDNA have successfully

been transduced into hair cells and SGNs in vitro, where they protected both cell types against CDDP- and neomycin-induced injuries (58, 409). Similar amplicons were injected in vivo through the round window or via the lateral wall of the scala vestibuli. Although very few cells of the organ of Corti were transduced, SGNs were highly infected and protected from both direct neomycin ototoxic insult or indirect insult following hair-cell destruction (41, 408).

D) AAV. The AAV is a 25-nm, nonenveloped, icosahedral capsid virus carrying a 4.7-kb single-stranded DNA genome flanked by inverted terminal repeats. It is unique among viruses that are being developed for gene therapy in that the wild-type virus is not associated with any known human disease. To date, a total of 12 human serotypes (AAV-1 to AAV-12) and more than 100 serotypes from non-human primates have been discovered. AAVs are able to transduce a wide range of cells and tissues and are devoid of adverse effects. One major drawback of AAV is that the vector capacity is limited to 4–5 kb, making it unsuitable for the transfer of large genes. A number of different AAV subtypes have been successfully used for the delivery of genes into cochlear hair cells, supporting cells, and the auditory nerve and spiral ligament, with little, if any, damage to the organ of Corti (200, 208). Delivery of AAVs into scala media resulted in a wide distribution of the reporter gene within hair cells and supporting cells in guinea pigs, and within the spiral ligament, Reissner's membrane, and SGNs in mice (200).

E) DESIGNED SYNTHETIC AAV VECTORS. To improve transduction efficiency and specificity, and alter tropism, synthetic vectors designed via ancestral sequence reconstruction have allowed substantial improvements over conventional AAV vectors. To date, nine functional ancestral AAVs have been generated (505). Among them, Anc80L65, a synthetic AAV approximating the ancestral state of AAV 1, 2, 8, and 9, is a potent in vivo gene-therapy vector for targeting cochlear cells (225, 420). Intracochlear injection of Anc80L65 through the posterior semicircular canal or round window membrane resulted in successful transduction of all IHCs and the majority of OHCs in adult mouse cochleae (225, 420).

## 2. Nonviral vectors

The main limits in using virus vectors are its immunogenicity and cytotoxicity. The first death from a gene therapy was related to the inflammatory reaction to the adenoviral vector (236). In addition to immune responses, virus vectors can cause insertional mutagenesis and the activation of oncogenes and the formation of malignant cells (354). Therefore, nonviral vectors have drawn significant attention due to their lower immunotoxicity. These nonviral vector systems are based on entrapment or electrostatic interactions of anionic genetic material and cationic carriers, which provide protection from enzymatic degradation. Nonviral gene

transfection carriers can be categorized into lipid-based and polymer-based systems (**FIGURE 8**).

A) LIPOSOMES. Liposomes are nanocarriers comprised of lipid bilayers encapsulating an aqueous core. Positively charged (cationic) liposomes coupled with a negatively charged (nucleic acids) integrated target gene are able to bind to the plasma membrane of target cells and release the gene into the cytoplasm (476). Neonatal organs of Corti have been successfully transfected in vitro using Lipofectamine 2000 or Lipofectin with plasmids containing *Math1* (497) or with antisense oligonucleotides (504). However, the transfection yield was low (~3%) as compared with viral delivery approaches. The genes delivered by liposomes have been shown to be incorporated into the genome of the host, with transgene expression persisting up to 14 days in the neurosensory epithelia and surrounding tissues of the cochlea in guinea pigs (460). Similarly, delivery of liposome-transgene complex or lipid core nanocapsules to the mouse or rat inner ear resulted in transgene expression in Reissner's membrane, spiral limbus, spiral ligament, spiral ganglion cells, and nerve fibers (506).

B) CATIONIC POLYMERS. Another class of DNA carriers suitable for gene delivery are synthetic and natural cationic polymers, such as polyamidoamine, polypropyleneimine dendrimers, cationic dextran, chitosan, and poly (lactic-co-glycolic) acid (126). PEI-mediated cochlear gene transfer via cochleostomy and osmotic minipump has been tested in guinea pig cochleae (427). In this study, mesothelial cells lining the scala vestibuli and scala tympani, mesenchymal and epithelial cells of the Reissner's membrane and fibrocytes in the suprastriatal zone of the spiral ligament were successfully targeted. In addition, round-window membrane delivery of dendriplices-*Atoh1* construct to rat cochleae enabled *Atoh1* expression mainly in the auditory hair cells, as indicated by green fluorescence (474). Application of a gelatin sponge immersed in hyperbranched poly-L-lysine nanoparticles onto the rat round-window membrane in vivo favored the targeting of hair cells and supporting cells of the organ of Corti, cells of the stria vascularis, the spiral ligament, and SGNs (498). One novel nanocarrier polyethylenimine-polyethylene glycol (PEI-PEG) was transfected into in vitro cultured cochlear spiral ganglion cells with an efficiency of 16.5% (52). More interestingly, infusion of this PEI-PEG nanocarrier in complex with XIAP via the scala tympani in rats resulted in XIAP protein expression in the cytoplasm of cells in the spiral ganglion, the organ of Corti, and the stria vascularis, which allowed the prevention of CDDP-induced cochlear spiral ganglion cell damage and improved hearing (52). Unfortunately, all these polymer-based vehicles for DNA delivery lack selectivity toward cells and show significant cytotoxicity that results in cell apoptosis. The molecular basis of their cytotoxicity is poorly understood, but a mitochondrially mediated apoptotic program resulting from PEI-induced channel forma-

tion in the outer mitochondrial membrane may account for PEI cytotoxicity (288).

C) OTHER NONVIRAL CARRIERS. Among the nonviral vectors, cell-penetrating peptides have been extensively used for the delivery of nucleic acids and other bioactive molecules both in vitro and in vivo (13). Cell-penetrating peptides are short, cationic, and/or amphipathic peptides. They gain access to the cell interior mainly by endocytosis, and thus have the capacity to promote intracellular delivery of conjugated bioactive cargo (151). Dash-Wagh et al. (80) tested the efficiency of a novel cell-penetrating peptide, PepFect6 (PF6), to deliver connexin 26 siRNA into the cochlear cells in vitro. They showed that PF6 was internalized by all cells in cochlear cultures without inducing cytotoxicity, and led to the knockdown of both connexin 26 and 30 mRNA in cochlear cells.

### *3. Route of administration*

In addition to the need for vectors for cellular transduction, inner-ear gene therapies require a safe and efficient delivery route without damaging the delicate structures of the inner ear. To date, the most common routes of administration into the inner ear in adult or postnatal animals are through 1) round-window membrane injection; 2) a hole drilled through the cochlear capsule into the cochlear fluid spaces (tympani scala or scala media), also called cochleostomy; 3) a hole drilled into the posterior semicircular canal where it runs near the external surface of the skull, called canalostomy (see **FIGURE 8**) (64, 128, 200); or 4) coated electrodes of cochlear implants (340, 357).

A number of studies have also been performed in the developing ear using in utero delivery of viral vectors (28, 139). Until recently, the systemic route of foreign gene administration was believed infeasible due to the potential for systemic toxicity and the two physiological barriers: the blood-brain barrier and blood-labyrinth barrier. In a pioneer study, Lentz et al. (241) showed correct splicing and restoration of low-frequency hearing following intraperitoneal injections of antisens oligonucleotides in adult Ush1c.216AA mice. In 2017, an interesting study (396) reported that intravenous injection of rAAV2/9 carrying an eGFP-reporter gene resulted in binaural transduction of IHCs, SGNs, and vestibular hair cells. In addition, hearing acuity in treated animals was unaltered at postnatal day 30. Altogether, these results suggest that nonsurgical methods of transducing the inner ear may in the future be used in clinical practice.

### *4. Potential clinical targets*

Molecular gene therapy for inner-ear disorders has developed along three major lines: 1) preservation of nondamaged or damaged hair cells and auditory neurons, 2) regen-

eration of hair cells and auditory sensory neurons, and 3) gene therapy for genetic deafness. Concerning this latter therapeutic approach, recent advances in human genomics have led to the identification of numerous defective genes causing deafness (see [hereditaryhearingloss.org](http://hereditaryhearingloss.org)), which represent novel putative therapeutic targets.

A) HAIR CELL PRESERVATION. Protection of the inner ear can be achieved via the overexpression of several types of molecule. Adenovirus- (192) and AAV-mediated (259) delivery of GDNF in the rat cochlea prevented aminoglycoside-induced hair-cell and hearing loss. Rat cochleae injected with AAV encoding XIAP showed significant protection from CDDP-induced ABR-threshold shift and hair-cell loss (72). siRNA against transient receptor potential vanilloid 1 attenuated CDDP-induced hearing loss in the rat (295). Adenovirus-mediated overexpression of heat-shock proteins (HSP70) prevented IHC but not OHC loss and hearing loss induced by kanamycin and furosemide treatment in guinea pigs (423). Pretreated mice with adenovirus-mediated overexpression of human *bcl-2* prevented aminoglycoside-induced degeneration of auditory and vestibular hair cells (337).

B) PRESERVATION OF THE AUDITORY SENSORY NEURONS. Auditory sensory neuron degeneration occurs slowly following noise injury or during aging, offering a therapeutic window (302) in which to realize treatment. The most studied treatments for auditory sensory neuron and neurite protection and regeneration in animal models comprise neurotrophic factors, such as nerve growth factor, fibroblast growth factor, ciliary neurotrophic factor, BDNF or NT3 (50, 338, 446), and electric stimulation or magnetic radiation (156). Using cell-specific inducible gene recombination in mice to modulate neurotrophin expression in supporting cells or hair cells of the postnatal inner ear, Wan et al. (446) demonstrated that the supporting cells of the sensory epithelia are the key source of BDNF or NT3 for the formation and/or maintenance of ribbon synapses in the postnatal inner ear. NT3 has major effects only in the cochlea, while postnatal *bdnf* appears to act only in the vestibular organs. Furthermore, *Ntf3* overexpression elicits regeneration of the synaptic contacts between cochlear nerve terminals and inner hair cells, and recovery of cochlear function after noise-induced synaptopathy (446). Interestingly, scala tympani injection of AAV8-NT3 via cochleostomy leads to the transduction of IHCs of the basal cochlear turn, but not to the adjacent supporting cells, and prevents noise-induced synaptopathy in adult albino guinea pigs (54), opening a potential new perspective to the treatment of noise-induced synaptopathy.

Wise et al. (470) demonstrated a significant increase in SGN survival in the entire basal turn of cochleae of deafened guinea pigs that received gene therapy with AAV-mediated transfection of BDNF and NT3. Adenovirus-mediated gene

transfer of GDNF also prevented SGN degeneration for up to 4 wk after a glycoside/diuretic-induced insult in guinea pigs (479). HSV-1 expressing NT3 also prevented SGN loss after CDDP-induced ototoxicity in mice (41, 58). Adenovirus-mediated human  $\beta$ -nerve growth factor gene transfer prevented SGN loss and hearing loss in rats exposed to blast waves (473). Interestingly, forced expression of BDNF or NT3 in epithelial or mesothelial cells in deafened guinea pigs induced regrowth of nerve fibers towards both the cells secreting the neurotrophin and the basilar membrane area in animals that had no remaining hair cells (395, 470). Persistence of gene expression and significantly greater neuronal survival were seen 6 mo after adenovirus injection. However, the eventual degeneration of the transduced cells as a result of the original ototoxic insult limited the clinical effectiveness (19). Moreover, *Ntf3* overexpression may exert detrimental effects on hearing. Actually, Lee et al. (234) showed that Ad or AAV vector-mediated overexpression of *Ntf3* in normal guinea pig cochleae led to disruption of synaptic connections between inner hair cells and peripheral nerve endings and induced hearing threshold shifts. These features were especially prominent after Adv.*Ntf3* injection in perilymph, where the auditory nerve endings grew far away from inner hair cells. Finally, an important problem before going to clinical practice is the lack of diagnostic tools for the detection of synaptopathy.

**C) HAIR-CELL REGENERATION.** Cochlear gene therapy has, to date, been mainly focused on hair-cell regeneration, which would be applicable to the most common forms of hearing loss, including ARHL, NIHL, and that due to infection or ototoxicity. The two main strategies used to regenerate hair cells are 1) direct transdifferentiation of supporting cells into hair cells via exogenous expression of *atonal homolog 1* (*Atoh1*) and 2) inactivation of the cell-cycle inhibitors such as cyclin-dependent kinase inhibitor p27Kip1 (also known as *Cdkn1b*) and retinoblastoma protein (Rb) to promote the reentry of supporting cells into mitosis.

**I) Reexpression of *Atoh1*.** The most promising work on hair-cell regeneration involves studies on the atonal gene *Atoh1* (*Math1* in the mouse) that encodes a proneural basic helix-loop-helix transcription factor involved in inducing the development of sensory hair cells from supporting cells in the cochlea (32, 472). Ablation of this transcription factor in mice results in a complete disruption of cochlear sensory epithelium formation, including the development of both hair cells and associated supporting cells (472). The overexpression of *Atoh1* in the distal cells of the greater epithelial ridge (GER) in postnatal cochlear explant cultures generated hair-cell-like cells (502). In addition, cotransfection of Pax2 to promote proliferation of supporting cells before hair cell regeneration with *Atoh1* in neomycin-damaged neonatal mouse organs of Corti in culture promoted supporting cells to proliferate and differentiate into functional hair cells (59).

Functional hair cells were also produced in the mouse cochlea by in utero *Atoh1* transfer via microinjection of an expression plasmid. The ectopic hair cells attracted neuronal fibers that terminated at their base and ~50% expressed correctly localized ribbon synapse proteins (Ctbp2), suggesting the formation of synapses with the nerve fibers. The new hair cells were also found to display mechanotransduction properties that were similar to normally developing hair cells (139). Adenovector-mediated forced expression of *Atoh1* in the nonsensory cells of mature, deaf guinea pig cochleae induced regeneration of new hair cells and substantially improved hearing thresholds, although a great variability in the hearing thresholds was observed (177). In contrast, while Atkinson et al. (18) observed a significantly greater number of cells expressing hair cell markers 3 wk post-treatment with *Atoh1* gene therapy, the hearing thresholds were not improved. This inconsistency may be explained by the latter studies not extending beyond 3 wk after *Atoh1* inoculation (18). Indeed, other studies indicate that 3 wk is the minimum time needed for supporting cells to convert to hair cells and that hearing recovery takes place between 1 and 2 mo post transfection (216, 261).

These findings have implications for a clinical trial now underway that uses gene therapy to restart expression of human atonal transcription (*HAT1*) factor to regenerate hair cells for treatment of hearing loss. Dr. Hinrich Staecker, in collaboration with Novartis, focused on developing a translational study using a recombinant adenovirus 5 (Ad5) vector containing a cDNA encoding HAT1 (called CGF166). The study, which started in 2014, is evaluating the safety, tolerability, and potential efficacy of CGF166 and the associated delivery procedures in patients with severe-to-profound bilateral hearing loss (Clinical Trials.gov Identifier: NCT02132130). However, the need for differentiated supporting cells may limit the clinical efficacy of *HAT1* gene therapy for hair cell regeneration. Indeed, after sensory hair cell damage, supporting cells die, leading to a rapid degeneration and flattening of the epithelium that greatly limits the time frame during which the *HAT1* transgene can be introduced. In addition, the direct transdifferentiation of supporting cells into hair cells depletes the supporting cell population and could compromise the stability and function of the sensory epithelium. Does gene therapy that worked in animals restore hearing in humans? Will hearing restoration be complete or partial, permanent or not? The current clinical trial should start to answer these very exciting questions.

**II) Inhibition of cell-cycle inhibitors.** Quiescence of hair cells and supporting cells occurs via CDK inhibition and subsequent hypophosphorylation of Rb (346). The second strategy used to regenerate the hair cells is thus inhibition of the CDK inhibitor (p27Kip1) and inactivation of Rb.

**A) Inhibition of P27Kip1.** P27Kip1, a cyclin-dependent kinase inhibitor (CDKI), is expressed in the developing organ of Corti from embryonic days 12–14 of mice. Its expression coincides with the arrest of cell division of the otic epithelial progenitors that give rise to hair cells and supporting cells. Thereafter, the expression of P27Kip1 is downregulated in the hair cells but continues in all types of supporting cells in the mature cochlea.

Neonatal mouse supporting cells have been observed to generate sensory hair cells by both transdifferentiation and mitotic regeneration after p27Kip1 downregulation (468). However, when the same cells are purified from 14-day-old mice, they are unable to downregulate p27Kip1 protein and do not reenter the cell cycle (468). Knockdown of *p27Kip1* with short hairpin RNA-expressing vectors in p3 ICR mouse cochlear explants *in vitro* did result in the cell-cycle reentry of postmitotic supporting cells; activation of the apoptotic pathway was, however, observed in some supporting cells (325). Comanipulation of *Atoh1* and *p27Kip1* allows the creation of new cochlear hair cells in adult mature mouse cochleae, even after noise damage (444). Walters et al. (445) showed that hair-cell specific, conditional deletion of *p27Kip1* at neonatal stages in mice results in hair-cell proliferation and survival of supernumerary hair cells to adulthood without any loss of hearing. These studies further suggest that *p27Kip1* inhibition may be a viable therapeutic strategy for hair-cell regeneration.

**B) Inactivation of retinoblastoma protein.** Retinoblastoma (Rb) is a member of the pocket protein family that is crucially involved in regulation of cell-cycle exit, differentiation, and survival. Its upregulation in embryonic and postnatal hair cells would suggest its functional requirement in the maintenance of hair-cell quiescence, and that its manipulation could lead to the production of functional hair cells. Deletion of the tumor suppressor gene encoding Rb (*Rb1*) in mice readily promotes proliferation of cochlear hair cells and supporting cells (270, 377, 495). However, some pathological changes are also observed, both within and outside the organ of Corti in Rb-null mice, including apoptosis and polyploidy in Rb-null hair cells (270). At 3 mo of age, the Rb-null mice show total hair-cell loss and profound deafness, as assessed by ABR recordings, suggesting that Rb plays further roles in hair-cell maturation and survival (377). Weber et al. (464) inactivated Rb in postnatal cochlear hair cells using an inducible *Cre* under *Atoh1* regulatory control. They showed that ~40% of the Rb-null hair cells had reentered the cell cycle by P4, although they did not progress through to cell division. Rapid loss began at P4–P15, leading to profound deafness. These postnatal studies indicate that permanent inactivation of Rb function alone will not permit functional mitotic regeneration in the diseased inner ear.

**III) Notch signaling disruption with  $\gamma$ -secretase inhibitor.** Notch signaling has two key developmental roles in the inner ear. The first occurs early in inner-ear development and defines the prosensory domains that will develop into the inner-ear sensory organs used to enable hearing and balance. The second role occurs later in development and establishes the mosaic-like pattern of the mechanosensory hair cells and their surrounding supporting cells through the better-characterized process of lateral inhibition. In the mammalian organ of Corti, Notch signaling is still active in newborn mice. Its inhibition with  $\gamma$ -secretase inhibitors, in embryonic and neonatal organ of Corti explants, results in robust production of supernumerary hair cells (213, 425). Prolonged pharmacological  $\gamma$ -secretase inhibition also seems to induce mitogenic proliferation of supporting cells in embryonic organ of Corti cultures (425).

Notch ligands are downregulated within the first few days after birth (147), and the organ of Corti loses its ability to generate supernumerary hair cells in response to Notch inhibition (400). Nevertheless, in mature cochleae, some reports suggest that the Notch pathway can be upregulated in response to damage. Mizutari et al. (287) reported that hair-cell damage from exposure to 8–16 kHz octave-band noise induced expression of the Notch target gene *Hes5*, as shown by RT-qPCR. More importantly, they showed that Notch inhibition, with round-window membrane delivery of a  $\gamma$ -secretase inhibitor LY411575, causes an increase in OHCs derived from support cells. Generation of new hair cells *in vivo* was sufficient to restore partial auditory function, as determined by ABR recordings. While systemic administration of the  $\gamma$ -secretase inhibitor caused severe side effects, local delivery to the inner ear appeared to overcome these effects (287). Finally, an increase in expression of the Notch ligand Jagged1 was observed in inner sulcus cells in adult cochleae deafened using kanamycin and ethacrynic acid, and local application of a  $\gamma$ -secretase inhibitor MDL28170 generated ectopic hair cells (166).

**D) DEFECTIVE GENE REPAIR.** The optimal therapeutic strategy for genetic disorders leading to congenital or early-onset hearing loss could be gene replacement therapy for the defective gene(s) (FIGURE 7). However, rescuing genetic deafness would require lifelong correction of the defective gene expression, and thus presents a considerable challenge. Cochlear gene therapy for hair-cell regeneration requires only short-term expression of the appropriate gene(s). Here, we document some successful results on the replacement of defective genes, or on supplementation therapies aimed at providing the wild-type gene product needed for function.

**I) Restoration of connexin function.** Connexins are structurally related transmembrane proteins that assemble to form gap junctions between adjacent cells. In the inner ear, they are essential for fluid homeostasis and/or intercellular signaling (198, 500). Mutations in the connexin genes

*GJB2* and *GJB6* (encoding CX26 and CX30, respectively) result in syndromic and nonsyndromic deafness (178). Interestingly, connexin (Cx) 30 null and deaf mice had their hearing successfully restored by genetically overexpressing the *Cx26* gene (6). This suggested that upregulation of *Cx26* or slowing down its protein degradation might be a therapeutic strategy to prevent deafness that result from *Cx30* mutations. Maeda et al. (266) used an RNA interference (RNAi) approach to specifically silence a dominant-negative mutant allele (R75W) of *GJB2* (Gap junction protein, beta2) gene, achieving efficient hearing restoration in mice that became deaf after introduction of a mutated sequence-liposome complex into the round-window niche. These preliminary results represent a very encouraging breakthrough, since connexin-related deficiencies account for the majority of cases of genetic deafness.

*II) Restoration of mechanotransduction.* Recent studies have suggested that transmembrane, channel-like protein isoforms 1 (TMC1) and 2 (TMC2) are possible pore-forming subunits of the mechanotransduction channel (193, 330). Autosomal recessive (DFNB7/11) and dominant (DFNA36) forms of deafness are both caused by mutations in *TMC1*. Mice that carry either a targeted deletion of *Tmc1* or a dominant *Tmc1* point mutation, known as Beethoven (Bth), are good models for human DFNB7/11 or DFNA36. Kawashima et al. (193) used adenoviral vectors in vitro to introduce the coding sequence for *Tmc1* or *Tmc2* into hair cells excised from mice deficient in *Tmc1* and *Tmc2*. Their results demonstrated partial restoration of sensory transduction in cultured hair cells in vitro. Askew et al. (16) demonstrated that postnatal, round-window membrane delivery of AAV2/1 driving either the expression of exogenous *Tmc1* or *Tmc2* in deaf mice lacking TMC1 and TMC2 restored sensory transduction and partially restored ABRs and acoustic startle reflexes. Furthermore, mice carrying dominant Bth mutations in *Tmc1* also responded to *Tmc2* gene therapy, with recovered ABR responses but no improvement in startle response, suggesting that exogenous *Tmc2* expression may not be sufficient to overcome the dominant Bth mutation in a behaviorally relevant assay. More recently, a pioneer study demonstrated the successful reduction of progressive hearing loss and IHC death using novel genome-editing techniques in a Beethoven mouse model (*Tmc1*<sup>Bth/+</sup>) carrying the orthologous missense mutation (p.M412K, c.T1235A) in the mouse *Tmc1* gene (127). These authors demonstrated that neonatal, scala-media delivery of cationic, lipid-mediated Cas9-sgRNA complex in *Tmc1*<sup>Bth/+</sup> mouse successfully disrupted mutant alleles, increased hair-cell survival, reduced ABR threshold shift, and enhanced acoustic startle responses in injected ears when compared with uninjected ears. This work convincingly demonstrated the possibility of using newly developed genome-editing strategies to restore genetically produced hearing loss.

*III) Hearing restoration in Usher syndrome.* The USH1C. 216G>A (216A) mutation accounts for the most severe form: Usher 1 syndrome (240). This mutation creates a cryptic 5' splice site, resulting in a frameshift and truncated harmonin protein (239). *Ush1c* c.216G>A mice reproduce both auditory and retinal deficits typical of human Usher I. In this mouse model, Lentz et al. (241) used an antisense oligonucleotide designed to redirect cryptic splicing of 216A RNA to the authentic site, to correct defective pre-mRNA splicing of transcripts from the mutated *USH1C.216G>A* gene. Their results showed that treatment of neonatal mice with a single systemic dose of the antisense oligonucleotides partially corrected *USH1C.216G>A* splicing, increased protein expression, improved stereociliary organization in the cochlea, and rescued cochlear hair cells, vestibular function, and hearing in these mice.

The *Ush1c* c.216G>A mutation affects expression of all conventional harmonin isoforms due to a point mutation (240). The harmonin-b splice form is found at the tips of stereocilia near the tip-link insertion point in mouse hair cells (37, 138, 235), where it has a structural role and is likely required for sensory transduction in both auditory and vestibular hair cells (37, 284). On the other hand, harmonin-a splice form is localized at the synapse, where it associates with Cav1.3 Ca<sup>2+</sup> channels and limits channel availability through a ubiquitin-dependent pathway (137). Homozygous *Ush1c* c.216G>A mice (c.216AA) suffer from severe hearing loss, with disorganized hair bundles and loss of both IHCs and OHCs in the middle and basal turns of the cochlea by 1 mo of age (240). To restore hearing function in these mice, Pan et al. (329) used Anc80L65, a synthetic adeno-associated viral vector that needs to be efficiently transduced in both auditory and vestibular hair cells in vivo (225). They thus designed Anc80L65 vectors encoding harmonin-a1 or harmonin-b1, which successfully transduced large numbers of IHCs, OHCs, and vestibular cells and drove harmonin expression and correct protein localization. Early postnatal round-window membrane injection of the harmonin vectors successfully restored auditory and vestibular function to near wild-type levels in otherwise deaf and dizzy c.216AA mice. The latest experiments using the deaf whirler mouse, a model of human Usher syndrome type 2D (manifested by hearing loss, dizziness, and blindness) (176), demonstrated that unilateral, posterior semicircular canal injection of AAV 2/8 driving wild-type whirlin into neonatal deaf whirler mice was able to restore balance function as well as improve hearing in these mice for at least 4 mo (176). Finally, a single delivery of cDNA encoding the scaffold protein sens by AAV 8 to the inner ear of a newborn mouse model of Usher syndrome type 1G efficiently restored the structure and function of inner-ear hair cells and prevented the balance deficit and hearing loss only in the low frequencies (98).

**IV) Restoration of neurotransmission.** In the cochlea, the vesicular glutamate transporter VGLUT3 accumulates glutamate in the synaptic vesicles of the sensory IHCs before it is released onto receptors of auditory-nerve terminals. In humans, mutations in the coding gene *VGLUT3* cause autosomal dominant deafness linked to auditory synaptopathy. Null mice, with a targeted deletion of exon 2 of *Slc17a8* gene encoding Vglut3, lacked ABRs to acoustic stimuli, although ABRs could be elicited by electrical stimuli, and robust otoacoustic emissions were recorded (370, 388). A successful restoration of hearing was demonstrated in this *Slc17a8*-null mouse model by reinstating the expression of Vglut3 via postnatal AAV-mediated delivery, as shown by the restoration of synaptic transmission and hearing (8). Although the vectors carried the broad activity promoter chicken-beta actin, the *Vglut3* transgene was only expressed in the targeted auditory IHCs. The authors suggested that endogenous regulatory mechanisms may govern expression level and thus may help limit off-target expression.

Other mutations also impair transmitter release at the first auditory synapse (292). For instance, mutations in the gene encoding otoferlin are responsible for the nonsyndromic form of deafness DFNB9 (488). Because of its multiple C2 domains, which bind phospholipids in a calcium-dependent manner, otoferlin was originally thought to be critical for exocytosis. This is like synaptotagmin 1–2, which harbor two C2 domains and are the putative calcium sensor for transmitter release in most of the synapses (415). Accordingly, deletion of otoferlin in the mouse results in severe deafness due to the lack of calcium-triggered exocytosis (367). However, further investigation revealed that the role of otoferlin is not limited to the calcium-sensor hypothesis (283). Indeed, vesicular reformation, resupply, and tethering at the active zone have been shown to be otoferlin-dependent, making otoferlin a multitasking protein (331). AAV-mediated gene transfer of the gene encoding otoferlin has not been yet attempted, because the coding sequence is too large to be packaged into AAV vectors. However, it has been reported that synaptic exocytosis operates in the otoferlin knockout mice at early stages of development. While synaptotagmin1/2 are not expressed in adult IHCs (376), calcium-triggered exocytosis depends on synaptotagmin 1 up to the fourth postnatal day in the mouse (34). Thus one attractive strategy to rescue the loss of otoferlin in DFNB9 would be to extend the synaptotagmin 1 expression over a long-term period. In this framework, Reisinger et al. (356) investigated the possibility that synaptotagmin1 might replace otoferlin and used AAV-mediated gene transfer of the synaptotagmin1 gene into mouse hair cells deficient in otoferlin. Unfortunately, the strategy failed to restore the  $\text{Ca}^{2+}$  influx-triggered exocytosis in IHCs of *Otof*−/− mice. This calls for an alternative strategy, such as the dual AAV viral-vector approach to mediate gene transfer (7), or the transfer of short forms of otoferlin (430).

## B. Cell Therapy

Cell therapy consists of using live cells to repair damaged or injured cells and to replace lost cells. For this purpose, stem cells, including embryonic, adult tissue-specific or induced pluripotent (iPSCs), and differentiated cells can be used (332). iPSCs were first generated in 2006 from adult cell types by forcing the expression of only four genes, Sox2, Pou5f1, Klf4, and Myc, via retroviral transduction (424). They offer several advantages over ESCs. In particular, ethical concerns are significantly reduced, since iPSCs can technically be created from any individual, and iPSC-derived cells can be transplanted back into the same person without concern about immunological rejection.

### 1. Hair-cell regeneration with exogenous stem cells

Theoretically, activation of local somatic stem cells would be the best strategy for the repair or replacement of damaged cells within the cochlea. Unfortunately, it appears that there are only low numbers of these endogenous somatic stem cells in the adult cochlea. Consequently, recent studies have focused on the transplantation of exogenous stem cells (ESCs) into the inner ear. There are many studies on the use of ESCs to regenerate hair cells in vitro (204, 364); their use, however, may cause the generation of teratomas and immunological rejection (106). Encouragingly, inner-ear progenitors created from murine embryonic stem cells in vitro (248) and adult stem cells isolated from vestibular and cochlear sensory epithelia or the spinal ganglia zone (247) gave rise to functional, hair-cell-like cells in vitro (326).

Some studies have already shown in vitro that stem cells may be directed towards a desired cell fate (hair cell or neurons) by certain chemical agents or culture conditions. Indeed, the addition of IGF or epidermal growth factor, together with the inhibition of bone morphogenetic protein 4, in embryonic rat or human stem cells, can lead to the production of otic progenitors and to the formation of hair-cell-like cells expressing hair-cell-specific markers such as Atoh1, Myosin 7a or Brn3c in culture (204, 247, 326, 362, 364). These hair-cell-like cells displayed bundles and tip links, and mechanical deflection of the bundles evoked mechanosensitive transduction current. However, the amplitudes of the currents were small and lacked directional sensitivity, but in this did resemble those of immature vestibular hair cells (326). Finally, grafted progenitor cells integrate into the developing inner ear, differentiate into hair cells at sites of epithelial injury, and express hair-cell markers and display hair-cell bundles when situated in cochlear or vestibular sensory epithelia in vivo (248).

While these results offer definitive proof of the ability to drive stem cells to develop into functional hair cells in vitro, introduction of these new hair cells into the mammalian inner ear has rarely been reported. In addition, the results obtained to date concerning the delivery of stem cells into

the mammalian inner ear have been inconsistent (157, 333). Whereas some of the transplanted cells have been shown to develop as potentially relevant cell types, including neurons, glia, and possibly hair cells and supporting cells, a far greater number of cells cannot be accounted for after several weeks of a transplant. One of the major challenges is the microenvironment in the host organ of Corti, which is significantly different from the culture conditions in which the new hair cells were generated *in vitro*. Generation of new hair cells will also require proper integration and orientation within the sensory epithelium. In addition, the differentiation into hair cells is a major challenge. As seen after Atho1 gene therapy (177), the neo-differentiated hair cells remain in the immature state, and therefore do not adopt a specific phenotype of OHC or IHC. To transduce functionally relevant stimuli, new hair bundles must be sensitive to the appropriate sound-evoked stimulus, which means that their bundles must be oriented with the same polarity as native cells. Another difficulty is targeting the damaged cochlear regions and producing sufficient, but not too many, sensory cells (139, 262). Finally, the last step will be to ensure the correct innervation of new sensory cells.

### *2. Auditory neuron regeneration with exogenous stem cells*

Several groups have identified factors capable of promoting stem-cell differentiation into glutamatergic neurons with otic-like, SGN phenotypes (207, 358). To investigate the integration of neural progenitors into the damaged inner ear, Corrales et al. (73) used ouabain-deafened gerbils. They showed that transplanted otic-like neural progenitors successfully engrafted into the modiolus (central axis of the cochlea containing all the auditory nerve fibers) forming ectopic ganglia with differentiated neuronal-type cells that projected to the sensory cells in the organ of Corti. Some fibers were observed at the point where they exited the modiolus and projected toward the brain stem. Finally, Chen et al. (57) demonstrated a restoration of auditory function after transplantation of neural progenitors in adult ouabain-deafened animals.

### *3. Patient-derived hiPSCs for drug screening and correction of defective genes*

In the cochlea, several patient-derived hiPSCs have been generated for modeling genetic diseases, drug screening, and correction of defective genes. Hosoya et al. (167) established an *in vitro* cochlear cell model by inducing otic progenitor-cell-like cell lines derived from Pendred syndrome-specific hiPSCs and revealed a degenerative phenotype characterized by intracellular aggregations and increased susceptibility to cellular stress. They showed that these degenerative phenotypes could be rescued by site-specific gene corrections or by the activation of autophagy with low-dose rapamycin and metformin. The Pendred syndrome iPSC

model has thereby provided an approach for a rational treatment strategy for progressive hearing loss (167).

Another interesting study (428) demonstrated that genetic correction of a mutation in MYO7A, which plays an important role in the assembly of stereocilia into stereociliary bundles of hair cells, via the CRISPR/Cas9 technique, resulted in morphological and functional recovery in hair-cell-like cells differentiated from iPSCs of a deaf patient. With the use of the same approach, the same laboratory rescued the morphology and function of hair-cell-like cells differentiated from the hiPSCs from members of a Chinese family carrying MYO15A c.4642G>A and c.8374G>A mutations, following the genetic correction of these mutations (56). These results demonstrate the feasibility of generating IHCs from hiPSCs and of functionally rescuing gene-mutation-based deafness using genetic correction.

## VI. LINKING THERAPIES TO COCHLEAR IMPLANTATION

Cochlear implants work by direct electrical stimulation of the remnant auditory neural structures within the deafened cochlea, allowing for a restoration of hearing in people suffering from severe to profound hearing loss. The remarkably improved speech discrimination observed in patients who have residual low-frequency hearing has led to the consideration of extending the implantation selection criteria to patients displaying useful residual low-frequency hearing. During recent years, increasing evidence favors the combination of cochlear implants with drug, gene, or cell treatments to reduce insertion trauma and residual sensory-neural element degeneration, prevent fibrous- and bony-tissue encapsulation formation, and reduce immune activation (153, 340).

### A. Procedures to Reduce Insertion Damage, Cell Loss, and Fibrotic Scarring

The introduction of the electrode array into the cochlea can cause damage to sensorineural structures and compromise residual hearing. Although cochlear implant manufacturers provide softer and shorter electrodes to minimize the acute electrode-insertion trauma, this does not reduce the occurrence of fibrotic scar tissue and new bone formation around the electrode array. Therefore, the coating of electrodes with drugs aimed at preventing acute inflammation, cell death, and fibrosis has recently been tested in both experimental and clinical settings.

#### 1. Corticoid-coated electrodes

The beneficial effects of glucocorticosteroids in reducing acute inflammation and preventing fibrotic scar formation have been clearly documented in the literature (279). Coat-

ing electrodes with dexamethasone significantly reduces the hearing loss caused by insertion trauma (17, 94, 258), limits fibrotic scars, and avoids electrical impedance increases (24, 94). In one pilot study, the safety of dexamethasone-eluting electrodes was demonstrated in a small patient group, and lower impedances were measured among the group of patients with dexamethasone-eluting cochlear implant electrodes (343).

## 2. Antimitotic agents

Jia et al. (181) investigated the efficiency of an antimitotic agent cytosine arabinoside (Ara-C, or cytarabine) in preventing fibrosis in rat cochlear slices in vitro and in keyhole limpet hemocyanin (KLH)-induced immune labyrinthitis and platinum-wire cochlear implantation-induced fibrosis in vivo. They found that Ara-C was more efficient in preventing fibrosis with fewer side effects on hair cells and neurons than dexamethasone.

## B. To Improve Frequency Resolution of the Cochlear Implants

### 1. Electrodes coated with bioactive substrates

To improve spatial resolution, frequency discrimination, and speech perception in cochlear implant patients, research efforts initially focused on the application of bioactive substrates such as collagen, polymer, or neurotrophin to coat electrodes to reduce the degeneration of SGNs and guide growth of afferent nerve fibers towards the implant electrodes (142, 359, 499). Richardson et al. (359) used the electro-active polymer polypyrrole into which the growth factor NT3 was incorporated to protect auditory neurons from degeneration after SNHL and to stimulate the growth of neurites towards the electrode in vitro. They also discovered that this NT3-polymer-coating had an additional protective effect on ganglion cells against aminoglycosides (360).

### 2. Neurotrophin gene therapy

Pinyon et al. (340) used a cochlear implant electrode array to generate localized high electric fields for electroporation-mediated BDNF gene delivery in a deafened adult guinea pig. Their results showed that mesenchymal cells lining scala tympani and scala vestibuli were efficiently transfected, resulting in regenerated spiral ganglion neurites extending to close to the cochlear implant electrodes, with localized ectopic branching. This neural remodeling enabled bipolar stimulation via the cochlear implant array, with low stimulus thresholds and an expanded dynamic range of the cochlear nerve, as determined via electrically evoked ABRs. Pfingst et al. (338) examined the long-term effects over 5–14 mo of a AAV2-Ntf3 neurotrophin gene-therapy delivery method on SGN preservation and function

in mature guinea pigs with cochlear implants. They found that inoculation with AAV.Ntf3 after deafening was effective in preserving higher SGN densities. They also showed that AAV.BDNF was more effective than AAV.Ntf3 in preserving SGNs in deafened adult guinea pigs (50). It should, however, be kept in mind that overexpression of NT3 may alter the pattern of innervation in the organ of Corti (234), thus limiting the use of neurotrophins in the context of cochlear implantation with preservation of hearing.

### 3. Gene therapy for cochlear implants based on light stimulation

Cochlear-implant technology is limited in terms of music appreciation and speech understanding in noisy environments. This is due to the wide spatial spread of electrical current from the stimulation contacts, which limits frequency resolution. Indeed, the number of independently usable stimulation channels is limited to <20. An alternative approach to overcome this problem would consist of activating the afferent nerve fibers over a very limited area, in a reversible manner together with a rapid onset and offset rate. Such an approach is now affordable with the development of optogenetic tools. This method relies on the protein channelrhodopsin 2 (ChR2) isolated from the green alga *Chlamydomonas reinhardtii*. ChR2 is a member of a family related to opsins that shows two peculiar features: it is a light-sensitive protein and it forms a cation channel (304). Thus blue-light excitation opens the ChR2, allowing cation entry that quickly depolarizes the cells in which ChR2 is expressed (42). Light can be applied over a restricted spatial area so that expressing ChR2 in a neuronal assembly would enable control of the excitation to a limited number of neurons on a millisecond scale (150, 328).

Working with mice has allowed the use of available transgenic strains (15) that provide expression of the light-gated ion channel ChR2 with little variability along the tonotopic axis and among mice (154). Combining conditional alleles (265) with appropriate Cre-lines has enabled cell-specific expression and gene transfer into the spiral ganglion via viruses such as adeno-associated virus, a standard approach in optogenetics (490). Using genetically engineering to express ChR2, Hernandez et al. (154) showed that optogenetic stimulation of SGNs activated the auditory pathway and restored auditory activity in deaf mice, achieving better frequency resolution than monopolar electrical stimulation. On the basis of these results, it appears likely that optogenetic stimulation of the auditory pathway will contribute greatly to auditory research and, in the future, to cochlear prosthetics.

## VII. FUTURE DIRECTIONS

This review of the literature has presented cochlear cell death mechanisms multidirectionally and the molecular,

cellular, and genetic therapies that are currently in development to treat the consequent deafness. Pharmacological therapy, cell therapy, gene therapy and biotherapies, in combination with cochlear implants, are all promising. New discoveries are continuing to drive innovation in the development of new treatments for auditory stress-induced, age-related, as well as genetic deafness. In terms of pharmacological therapy, progress made over the last few years in understanding the molecular mechanisms involved in mammalian cochlear cell degeneration and repair capacity has raised the possibility that targeted therapies might prevent the loss of these cells and thus preserve hearing. Numerous studies have demonstrated that administration of pharmacological compounds can prevent sensory hair-cell death and restore auditory function in animal models. These discoveries boost hopes for developing clinically applicable therapies, although obviously ongoing basic research remains necessary to fine-tune the therapeutic agents. In addition, targeted delivery of drugs still remains an important challenge, and research is ongoing on the use of nanocarriers to improve the efficacy of commonly used drugs in cochlear therapies. The appropriate enveloping of nanoparticle drugs will allow their increased stability and enable the careful control of the characteristics of their membrane passage and kinetics. The development of multifunction nanoparticles with a porous matrix, allowing encapsulation of a large range of molecules including chemicals, proteins, or gene products, is providing a promising perspective for future cochlear treatments (see <http://www.sciencedirect.com/science/journal/17732247/39?sdc=1>). In the meantime, the first clinical trials using pharmacological compounds such as NAC or d-JNKI-1 attest to the fact that treatment of the inner ear is becoming a clinical reality. The tolerability, toxicity, and efficacy of these compounds now need further testing in large populations of patients with well-characterized cochlear pathologies.

With regards to gene therapy, substantial progress has been made in recent years to accumulate tools that could potentially be used, either alone or in combination, to regenerate hair cells and auditory neurons and replace defective genes using gene therapies. Viral and nonviral gene delivery and manipulations that affect *Atoh1* and cell cycle genes or correct genes causing deafness have reached the proof-of-concept stage. The safety of AAV has been confirmed and designed synthetic AAV vectors such as *Anc80* seem promising for their performance as potent and broadly applicable gene-therapy vectors with unique properties (505). Clinical trials are now needed to confirm their efficiency in a large number of patients. Nevertheless, whatever the progresses in gene-carrier capacity, the complex nature of regeneration and repair processes and the huge range of molecular and cellular targets for regeneration-inducing drugs highlight the need for precisely controlled systems capable of delivering a broad range of biomaterials such as genes, siRNA, RNA, and DNA. Other exciting approaches rely on the use

of genome-editing technologies based on programmable nucleases, including clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated nuclease Cas9 (168). These new technologies bring us one step closer to achieving therapeutic genome editing in diseased cells and tissues, which may result in the removal or correction of deleterious mutations or the insertion of protective mutations. The CRISPR-Cas9 genome-editing technique has recently been investigated in patient-derived hair-cell-like cells differentiated from iPSCs of deaf patients carrying *MYO7A* or *MYO15A* mutations. Interestingly, it allowed the successful correction of these mutations together with morphological and functional recovery in the hiPSCs from the deafened patients (56, 428). The recent demonstration that the injection of CRISPR-Cas9 complexes inside the ears of neonatal mice can reduce hearing loss in "Beethoven" mutants (127) lays out a potential pathway for genome editing in deafened patients carrying monogenic mutations. Another new approach to correct gene mutations is using spliceosome-mediated RNA *trans-splicing*, or SMaRT. This technique relies on the correction of mutations at the posttranscriptional level by modifying the mRNA sequence. The proof of concept of SMaRT has already been established in several models of genetic diseases caused by recessive mutations in which the size of the cDNA that would correct the phenotype is not compatible with the currently available gene-transfer vectors (31). This new technology has not yet been investigated in the inner ear, but offers hope of a single treatment for restoring hearing in patients carrying recessive gene mutations.

In terms of cell therapy, iPSC technology is promising. The ability to isolate, expand, and then direct stem cells toward a specific cell fate holds great potential for both clinical and fundamental auditory research. As a transplant source, autologous neurons from patient-derived iPSCs are ideal for the replacement of neurons in the injured cochlea. Such patient-derived iPSCs are also useful for drug discovery and fundamental research into the mechanisms involved.

Finally, recent encouraging results obtained from the combination of cochlear implants with continuous neuroprotective drug delivery or virus-mediated neurotrophic factor expression has demonstrated the feasibility of improving the survival of SGNs and promoting the proximity of their peripheral neurites to the electrode contacts. Cochlear implantation in combination with long-term drug delivery could be achieved with progress in developing totally implantable and programmable inner-ear drug-delivery systems. Progress towards safe and efficacious inner-ear delivery systems will benefit from microsystems-based approaches, where multiple therapeutic compounds can be introduced in a highly controlled and time-sequenced manner over periods of months to years, enabling precise control over the drug concentration and infusion kinetics. Microsystems-based drug delivery systems are emerging for a

range of clinical applications and have been exploited in some inner ear applications (39, 40, 60, 114, 282). These implantable mini-pump systems will need to be coupled with cochlear implants to preserve residual hearing, and thus improve the performance of the implants.

Future therapies to rescue auditory stress-induced, age-related, or genetic deafness must consider multiple targets that account for the complexity of cochlear cell-degeneration mechanisms and disease-causing factors. This is especially true for genetic deafness and profound deafness. Looking back on the historical, functional, and molecular achievements made in this field, each was made possible by technological developments defining a new epoch. To overcome the somewhat static current status in terms of clinical trials, we now need to refine the protocol of these trials and search for more predictive animal models of deafness on which they are based, to allow careful retrospective analyses. Efforts should continue towards the development of regenerative and personalized medicine for treating deafness.

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