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Review Article

The predawn dilemma in adeno-associated virus-based gene therapies for hereditary deafness

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Abstract: Hearing loss is a prevalent organ-specific disorder affecting individuals throughout their lifespan, with over 466 million cases reported globally. The conditions can be classified into two broad categories: hereditary and nonhereditary. HHL, caused by genetic mutations or chromosomal abnormalities, can be divided into nonsyndromic (NSHL) and syndromic (SHL) subtypes. NSHL presents as isolated auditory impairment without systemic manifestations, whereas SHL involves concurrent dysfunction in other organ systems. Nonhereditary hearing loss typically results from infections, ototoxic drugs, noise exposure, trauma, or age-related degeneration. Current clinical interventions focus on symptom management through hearing aids and cochlear implants, as no curative treatment exists for genetic forms. Recent studies have shown the therapeutic potential of gene therapy in animal models of genetic deafness, although clinical translation faces challenges, including viral vector safety, transfection efficiency, and target specificity. This systematic review synthesizes current progress in gene therapy for HHL and evaluates barriers to clinical implementation, offering insights for future translational studies.

Keywords: Adeno-associated virus, hereditary hearing loss, hair cell, inner ear

Introduction

Hearing loss is one of the most prevalent sensory disorders in humans, affecting approximately 1 in 500 newborns, with an estimated 466 million cases worldwide. The prevalence of this condition continues to rise, particularly among the elderly population, where the occurrence rate increases significantly with age. It is projected that by 2050, nearly 900 million people (approximately 10% of the global population) will suffer from disabling hearing loss. HHL, caused by gene mutations or chromosomal abnormalities, represents one of the most significant etiological factors in auditory impairment. This condition accounts for a substantial proportion of congenital hearing loss cases and may also manifest as late-onset hearing impairment. More than half of pediatric hearing loss cases are attributed to genetic components. Hearing loss is an extremely heterogeneous disorder with up to approximately 1000 different causal genes. To date, more than 200 genes have been identified (https://hereditaryhearingloss.org/), causing impairments of various degrees of severity and progressivity. Additionally, numerous forms of syndromic hearing loss (SHL) exist, each characterized by distinct genetic bases and corresponding clinical features. Nonsyndromic hereditary hearing loss (NSHL) is defined as isolated auditory dysfunction without abnormalities in other organ systems. This type of hearing loss may follow autosomal dominant, autosomal recessive, X-linked, or mitochondrial inheritance patterns. In contrast, syndromic hereditary hearing loss (SHL) is accompanied by dysfunction in other organs or systems. Examples include Pendred syndrome, which is associated with thyroid abnormalities, and Usher syndrome, which involves synthesis with retinitis pigmentosa, leading to vision loss. Elucidating the genetic basis of HHL is highly

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important for early diagnosis, genetic counseling, and the development of targeted therapies. Advances in genetic testing have significantly enhanced the identification of deafness-causing mutations, enabling more precise diagnoses and personalized treatment plans. Early intervention - whether through hearing aids, cochlear implants, or other assistive devices can substantially improve outcomes for patients with genetic hearing loss, enhancing their quality of life and communication ability. However, current clinical medical or surgical treatments for treating HHL are limited.

The mammalian inner ear is in charge of the sensory organ for hearing (the cochlea) and the organ responsible for balance. There are three major types of functional cells in the inner ear that take part in hearing production and perception: hair cells (HCs), supporting cells (SCs), and spiral ganglion neurons (SGN). The mammalian cochlea contains two types of sensory HCs that play different roles in hearing. Outer hair cells (OHCs) are responsible for amplifying sound and enhancing sensitivity to sound and sharp tuning. Inner hair cells (IHCs) transmit signals to cochlear neurons and communicate sound information to the brain. OHCs mechanically amplify sound-induced vibrations, enabling enhanced sensitivity to sound and sharp tuning [1]. The damage to and death of HCs are the main causes of deafness [2]. Currently, hair cell regeneration is an important therapeutic target for treating deafness.

The concept of gene therapy arose initially during the 1960s and early 1970s. The advantage of this technique is that the continuing expression of genes allows for a cure following a single treatment rather than continuing administration of a drug with a relatively short half-life. To date, there have been over 1800 clinical trials and over $4 billion in capital investment. In the inner ear, the first gene delivery case was reported in 1996, and subsequent studies shed light on the treatment of congenital or later-onset hearing loss and restoring hearing in monogenic disorders in patients [3, 4]. Recently, recombinant adeno-associated viruses (rAAVs) have been shown to be excellent vehicles for the *in vivo* delivery of gene therapies. In the past few decades, adeno-associated virus (AAV) vectors have been successfully used in numerous clinical trials addressing rare genetic diseases diseases (**Figure 1**). In 2012, the first commercial AAV gene therapy product, Glybera, was approved by the European Commission for the treatment of hereditary lipoprotein lipase deficiency (LPLD) [5]. Recently, an increasing number of AAVs have been used to treat hereditary deafness. A typical case of AAV-mediated gene replacement therapy for deafness is the glutamate transporter-3 knockout model. It has been demonstrated that AAV-mediated replacement of Vglut3 can significantly improve the hearing of Vglut3 knockout mice [6]. Similarly, Askew and colleagues reported that delivering AAVs expressing Tmc1 and TMC2 to neonatal Tmc1 mutant mice significantly increased hair cell survival and restored hearing and vestibular function [7]. In addition to targeting sensory hair cells, researchers have also conducted AAV-based gene replacement studies in other structures of the inner ear. For example, the KCNQ1 gene in the inner ear stria vascularis (SV) [8]. In recent years, a good example of AAV treatment for hereditary deafness has been the treatment of OTOF, which encodes the otoferlin protein, which plays an important role in auditory signal transduction. Researchers have achieved very satisfactory results when AAV was used to treat OTOF deficiency in mice [3, 9], macaques [10], and humans [11, 12], with no serious adverse safety events. Recently, breakthroughs in exciting AAV-based gene therapy for GJB2 have been reported [13], which found that co-injection of AAV1 and AAV-ie carrying exogenous Gjb2 effectively restored hearing function in Gjb2-deficient mice. Thus it can be seenadeno-associated viruses (AAVs) have shown great potential in the treatment of hereditary deafness. Here, we summarize and discuss recent advances in inner ear gene therapeutic strategies aimed at restoring or protecting against hereditary deafness and focus on the challenges associated with *in vivo* gene therapy targeting the human inner ear for the treatment of human hereditary deafness.

The anatomy and function of the inner ear

The ears of mammals are composed of the outer, middle and inner ears, which perform different functions in the transmission of sound. For example, the outer ear consists of the earlobe and the external auditory canal. The earlobe is responsible for acoustic wave collection, and the external auditory canal is in charge of

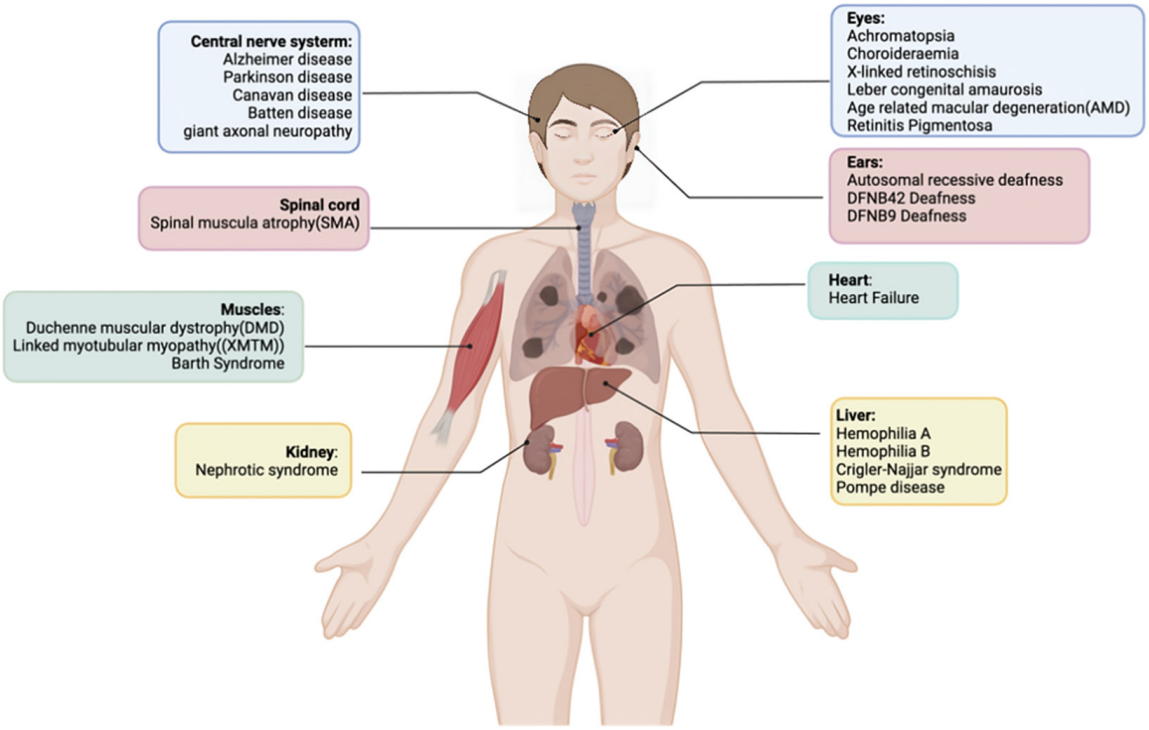

Figure 1. Schematic illustration of the clinical applications of adeno-associated virus (AAV) in human genetic diseases. Clinical applications of AAV across a spectrum of human genetic diseases, including ocular, auditory, neurological, metabolic, hematological, neuromuscular and cardiovascular diseases.

transmitting sound waves to the eardrum. The middle ear, which contains three ear ossicles (malleus, incus, and stapes) within the tympanic cavity of the middle ear, extends from the tympanic membrane to the lateral surface of the skull [14]. The inner ear is also called the labyrinth and lies between the tympanum and the bottom of the inner ear canal, which consists of the labyrinth of bone and the labyrinth of the membrane [15]. The bony labyrinth, surrounded by dense bone, is a tortuous and irregular bony tunnel located in the temporal bone rock. The membranous labyrinth is a closed membranous sac enclosed within the labyrinth of bone. The lymphatic fluid fills the membranous labyrinth and the space between the osseous labyrinth and the membranous labyrinth. The lymphatic fluid is divided into perilymph and endolymph on the basis of its location. The spaces surrounding the membranous labyrinth within the bony labyrinth are filled with perilymph [16]. The cochlear duct and membranous vestibular apparatus are filled with endolymph (**Figure 2**). Owing to their different locations, the compositions of the perilymph and the endolymph are also quite different. The composition of the perilymph is rich in sodium but poor in potassium, whereas the endolymph is rich in potassium ions but poor in sodium ions, with a pH similar to that of blood plasma. The bony labyrinth is arranged along the long axis of the temporal bone rock, which is divided into the vestibular system and the cochlea, from the posterior to the anterior, medial and inferior parts [17].

Mechanisms of genetic deafness

Deafness is one of the most common birth defects and has a complex etiology in which genetic components play a dominant role. To date, over 150 deafness-associated genes have been identified. However, the strong genotype-phenotype heterogeneity, inconsistencies across cross-species models, and fragmented nature of massive genetic data hinder the elucidation of pathogenic mechanisms and the improvement of clinical diagnostic efficiency. Understanding the pathological mechanisms of HHL is crucial before the implementation of effective AAV-mediated therapies. HHL may follow autosomal dominant, autosomal recessive,

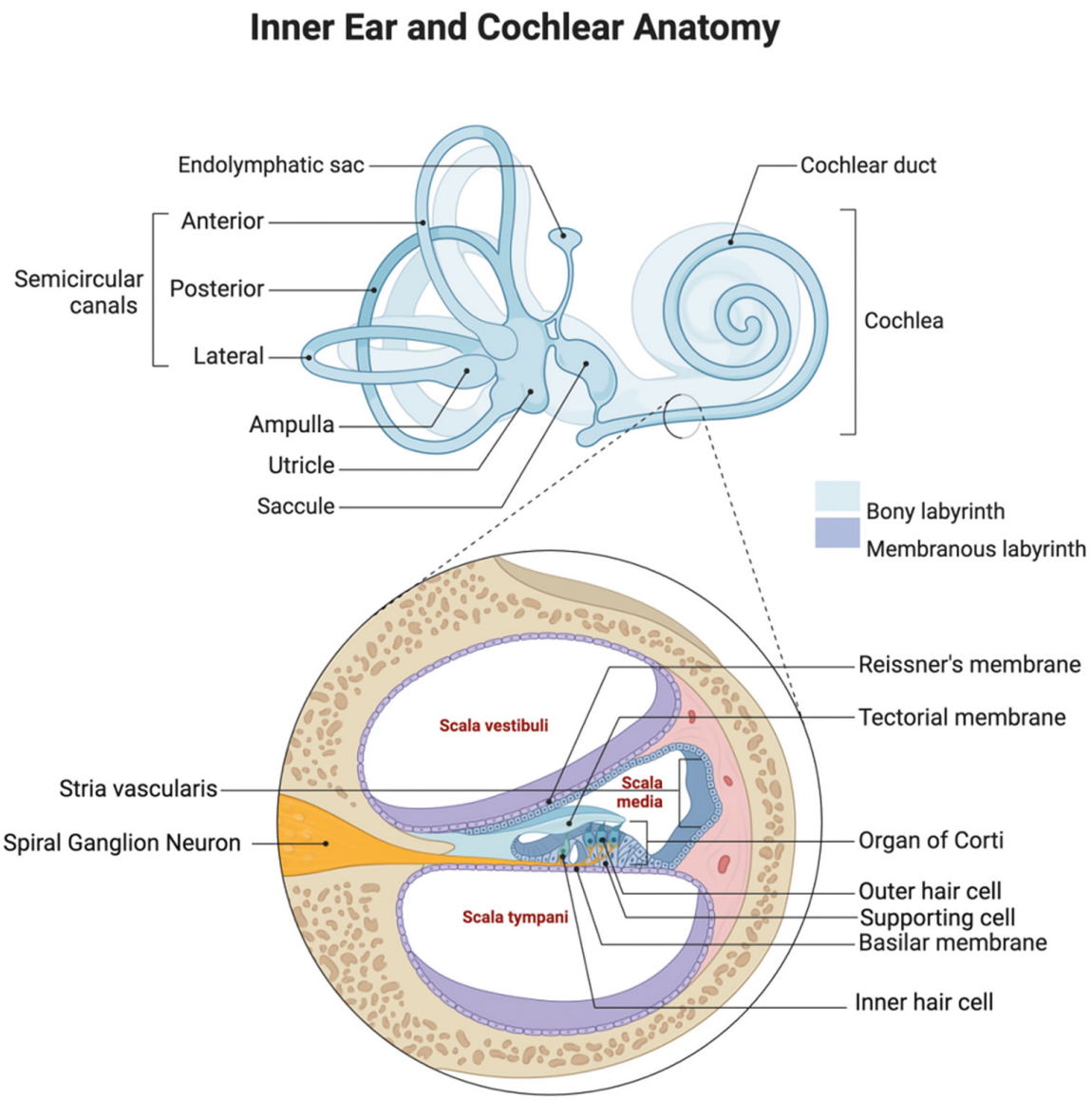

Figure 2. Schematic illustration of the cochlear structure.

X-linked Mendelian, or mitochondrial inheritance patterns [18]. The inheritance patterns are mainly divided into nonsyndromic and syndromic types. Nonsyndromic hereditary hearing loss is caused primarily by mutations in hearing-related genes. For example, mutations in the GJB2 gene, which encodes connexin proteins that connect cochlear hair cells and supporting cells, disrupt potassium ion circulation in the inner ear, impair hair cell function, and lead to hearing loss [19]. Mutations in the SLC26A4 gene are associated with maldevelopment of the inner ear membranous labyrinth, resulting in enlarged vestibular aqueduct syndrome, where changes in endolymphatic pressure within the inner ear can cause hearing loss [20]. Syndromic hereditary hearing loss includes hearing loss as well as abnormalities in other systems or organs. For example, in Waardenburg syndrome, mutations in the PAX3 or MITF gene lead to melanocyte developmental disorders, which not only cause hearing loss but also result in symptoms such as iris heterochromia and white forelock [21]. Genetic factors account for more than 50% of congenital hearing impairment cases in developed countries, of which approximately 70% are nonsyndromic and predominantly monogenic. Owing to its genetic nature, this type of hearing loss is particularly amenable to gene therapy.

Intervention strategies for hereditary deafness

HHL not only affects patients' hearing but also may profoundly impact their language development, social interactions, and quality of life. Consequently, intervention strategies for HHL are critical for mitigating the effects of hearing impairment and enhancing patients' overall life experience. Current clinical interventions for HHL primarily involve cochlear implants or hearing aids, which partially restore auditory function in affected individuals [22]. Hearing aids effectively compensate for mild to moderate hearing impairment by amplifying environmental sounds. Patients with GJB2-related mild or moderate deafness often experience substantial hearing improvement and enhanced speech perception with hearing aid use. Cochlear implants represent the preferred intervention for severe to profound sensorineural hearing loss, bypassing damaged hair cells through direct electrical stimulation of the auditory nerve. Individuals with enlarged vestibular aqueduct syndrome typically achieve functional hearing restoration following cochlear implantation, facilitating social reintegration. Neither of these approaches fully restore damaged inner ear cells or hearing function to their native state. Gene therapy targeting genes associated with deafness may provide a more fundamental solution for hearing restoration [23]. Current strategies include gene replacement, genome editing, and gene suppression. Gene replacement delivers functional copies of defective genes to cochlear cells; for instance, AAV-mediated VGlut3 gene delivery restored auditory function in knockout mice. Clinical trials targeting OTOF mutations have also demonstrated improved speech perception and sound localization in patients. Genome editing directly modifies cellular DNA via precision tools such as CRISPR-Cas13 to correct mutations or introduce functional sequences. This approach has successfully repaired Tmc1 mutations in mice, partially restoring hearing. Alternative methods, such as RNA base editors, have shown efficacy in OTOF mutant mice with premature termination codons [24]. Gene suppression alleviates dominant mutations by blocking their expression, which is particularly valuable for disorders where mutant alleles exert toxic effects. Antisense oligonucleotides have yielded therapeutic benefits in mouse models of Usher syndrome [23]. Collectively, these gene therapy strategies present viable pathways for addressing hereditary deafness.

The biology and vectorology of AAV

AAV is a small (25-nm) virus that belongs to the genus Dependoparvovirus within the family Parvoviridae and was discovered more than 50 years ago [25]. It is composed of an icosaedrical capsid 20-25 nm in diameter and a genome of 4.7 kb flanked by two inverted terminal repeats (ITRs) [26]. AAV is a single-stranded DNA parvovirus that harbors the rep and cap genes, which encode elements that facilitate AAV genome replication and virion assembly [27]. The rep gene encodes four replication-related proteins named Rep78, Rep68, Rep52 and Rep40 [28-30]. The cap gene encodes three capsid proteins, namely, Viral Protein 1, 2 and 3 (VP1, VP2 and VP3), which have overlapping reading frames (**Figure 3**). The ratio of VP1, VP2, and VP3 in the virion is approximately 1:1:10 [31], and these proteins include assembly activating protein (AAP) and a membrane-associated accessory protein (MAAP) [31, 32]. AAVs cannot replicate independently without the presence of helper viruses (such as adenovirus, herpes simplex virus, and vaccinia virus) [33, 34]. AAV has been found in multiple vertebrate species but has not been clearly associated with any clinical pathology or disease at present [35]. Twelve naturally occurring serotypes of human AAV have been identified to date. The cell surface receptors for the recognition and binding of different AAV serotypes vary, which is due to their different capsid protein spatial structures, sequences and tissue specificities. These serotypes have different affinities and transfection efficiencies in different tissues, such as muscle, liver, lung, brain, and visual tissues [36].

The journey of AAV from the cell surface to the nucleus

Once the AAV reaches the surface of cells, the interaction of the AAV with its receptors initiates a cascade resulting in the cellular entry of the AAV and the expression of its transgene. The general process involves the following six steps: AAV attachment to target cells, endocytosis, intracellular trafficking, crossing of the endomembrane membrane, nuclear import and genome release. First, when an AAV reach-

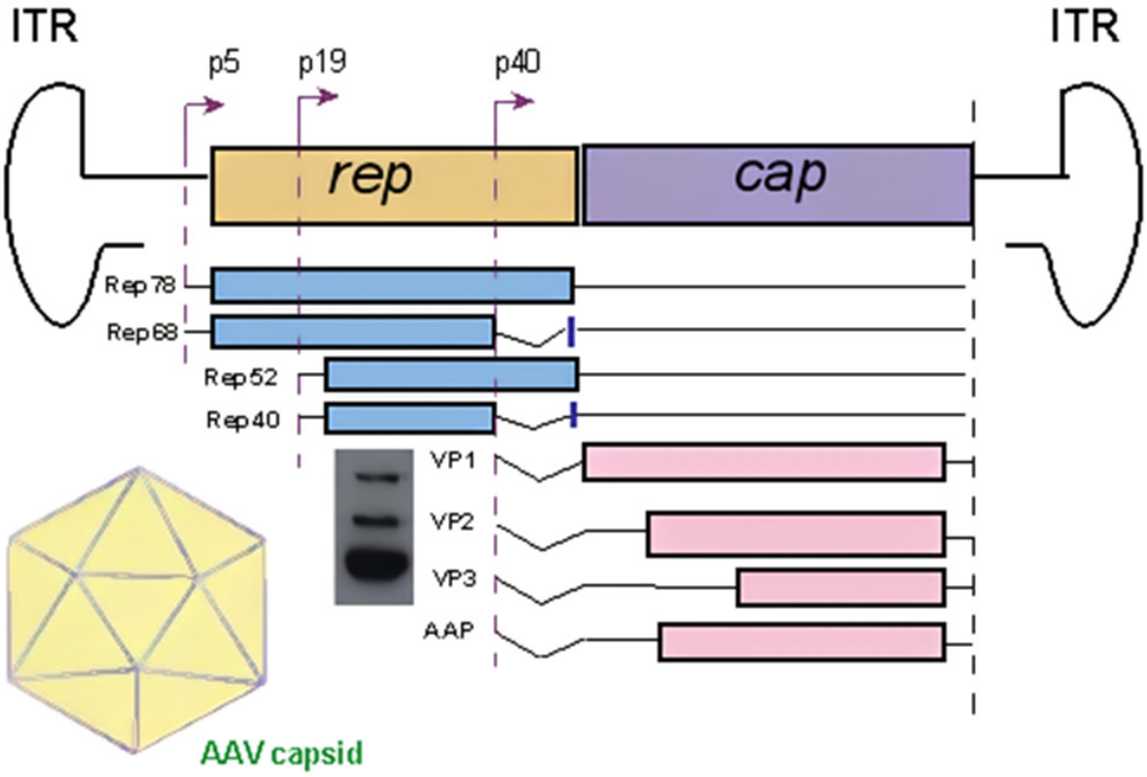

Figure 3. Schematic illustration of the AAV structure. AAVs have an ~4.7 kb single-stranded DNA genome within an ~25 nm capsid. The genome includes three open reading frames bordered by T-shaped inverted terminal repeats (ITRs). It encodes three capsid proteins, four replication proteins, and an assembly activating protein (AAP) that aids in capsid assembly in certain serotypes.

es a cell, it attaches to its receptors/coreceptors and then enters the cell via endocytosis. After entry into cells, the virion is transported to the trans-Golgi network via endocytic vesicles (TGNs). Undergoing a series of trafficking events, the virion escapes into the cytosol, but the exact process of its translocation into the cytosol remains to be elucidated. Following endosomal escape, the virion is imported into the nucleus through the nuclear pore complex (NPC), where the single-stranded genome is released from the capsid. In the nucleus, uncoating of the viral capsid enables genome release. Once released, single-stranded DNA is converted to double-stranded DNA, resulting in the expression of the recombinant transgene [37-39].

Immunological barriers to rAAV gene delivery

Gene therapy with AAV has demonstrated safety and long-term efficacy in many trials in different organs, such as the eye, liver, skeletal muscle, and central nervous system. However, the immune response of the body to AAVs, which is one of the major problems in the application of AAVs, cannot be ignored. The AAV protein capsid, its DNA genome and the protein product of the transgene have complex effects on the host immune response, which includes innate immune and adaptive immune responses.

Innate immune response to rAAV: Host immunity can be divided into innate and adaptive immune responses. The innate immune response is the first line of defense against foreign pathogens and is crucial for transmitting signals to the adaptive immune system. The innate immune response depends on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which are expressed mainly in immune cells such as macrophages, monocytes, granulocytes, natural killer (NK) cells and dendritic cells (DCs) [40]. The molecular recognition of viral nucleic acids, membrane glycoproteins, or even chemical messengers by PRRs activates downstream signaling pathways, such as the

nuclear translocation of nuclear factor kB (NF-kB) and interferon-regulatory factor (IRF), which are transcription factors with central roles in the expression of proinflammatory cytokines and type I interferons (IFNs), respectively [41, 42].

Toll-like receptors (TLRs) are a family of innate immune sensors that are conserved across mammalian species and are found on endosomal or plasma membranes of immune cells. The rAAV capsid and vector genome are sensed after delivery by the innate immune system through Toll-like receptor 2 (TLR2) and TLR9, respectively [43, 44]. TLR9 commonly senses AAV vector genomes, which contain unmethylated cytosine-phosphate-guanine (CpG) motifs. When its CpG group binds to TLR9, it promotes its dimerization and activates TLR9 signaling via the signaling adaptor MyD88, leading to the induction of IFNs and proinflammatory cytokines [45]. Recently, Ying et al. reported that the use of a TLR9-inhibitory approach in the AAV vector genome inhibits immunogenicity and enhances transgene expression in multiple animal models [46]. Furthermore, the vector DNA genome and dsRNA also contribute to the induction of innate immunity to AAV. Research from clinical trials for hemophilia B using AAV vectors has shown decreased transgenic coagulation factor IX (hFIX) expression after the administration of a high vector dose. Mechanistic analysis indicated that dsRNA may lead to the production of type I IFN-β by stimulating the MDA5 sensor in human hepatocytes transduced with AAV [47]. In contrast, TLR2 reportedly senses the AAV capsid to activate innate immunity in primary human liver cells [48]. Both the rAAV capsid and the vector genome can activate the innate immune response, which is important for the subsequent adaptive immune response.

Adaptive immune response to AAV: The adaptive immune response is considered the second barrier of organs, and it occurs following the innate immune response. In general, adaptive immune responses are antigen-specific and generate long-lived immunological memory [49]. Several preclinical and clinical studies have shown that adaptive immune responses pose a significant obstacle to the clinical application of AAV vectors.

Humoral adaptive immune responses: Humoral immunity is mediated by antibodies produced by B lymphocytes. Wild-type AAV (wtAAV) infection is a common phenomenon, and anti-capsid antibodies (NAbs) are generated from natural exposure to WT AAV in a large portion of the human population (ranging from 30-60%) [50, 51]. NAbs can effectively bind and neutralize rAAV to block gene delivery and persistent gene expression. AAV1 and AAV2 have higher seroprevalence rates than other AAV serotypes in populations [52]. Typically, subjects receiving gene transfer from an AAV vector develop anti-AAV antibodies from all four IgG subclasses, with IgG1 being the predominant subclass [53, 54]. In the treatment of certain diseases, the re-administration of AAVs can be used to circumvent the reduction in therapeutic transgene expression and maximize the clinical benefit. An additional question of concern is the secondary injection of AAV: does AAV induce powerful NAbs that limit the clinical utility of gene therapy? To overcome the obstacle of NAbs, several approaches have been developed, such as plasmapheresis [55], capsid decoys [56], and rAAV capsid engineering [57]. Recently, IgG-cleaving endopeptidases or strategies for the removal of anti-AAV antibodies have also been developed [58].

Cellular adaptive immune responses: In addition to a humoral immune response, the T-cell-mediated immune response also plays an important role in terms of both the safety and efficacy of AAV gene transfer in humans. Effector CD4+ T cells induce inflammation and immune activation through cytokine production. However, cytotoxic CD8+ T cells mediate targeted killing of infected cells by secreting granzyme, perforin, and inflammatory cytokines [59]. Furthermore, Tregs prevent hyperimmune activation and return the immune response to homeostasis by directly interacting with immune cells or producing immunosuppression [60]. Some early clinical trials of gene transfer with rAAV have indicated that T-cell-mediated immunity hinders the efficiency of gene transfer. The first examples of a cytotoxic T-cell response to AAV gene therapy were observed in a clinical study of AAV2-mediated liver gene transfer of human coagulation factor IX in hemophilia B patients. The transgene expression reached levels approximately 10% of those of healthy controls after an initial intravenous infusion of AAV2 carrying a functional copy of the factor IX (FIX) gene. While the FIX levels decreased to baseline levels after 4 weeks, an

anti-AAV2 capsid T-cell response was observed [61].

Limited transport capacity

One of the major challenges of AAV-mediated gene delivery is the limited capacity to deliver large genes over 4.7 kb. In fact, more than 6% of human protein coding sequences(CDS) exceed 4 kb, which makes it impossible to fit expression cassettes for these cDNAs into a single AAV vector [62]. To overcome the packaging limit of AAV genomes, dual AAV and triple vector approaches and mini-gene strategies for the delivery of larger transgenes have been developed. The dual AAV and triple vector approaches require two or three AAV vectors. In dual AAV vectors, one AAV vector carries the promoter element and upstream portion of a given coding sequence flanked by ITRs. The other AAV vector carries the 3' downstream portion of a given CDS and poly A, which is also flanked by ITRs. The two transgenes are packaged separately. The dual AAV vector strategy has been applied in several mouse models [3, 9, 63]. Furthermore, split-AAV strategies can be divided into overlapping, trans-splicing and hybrid dual vectors to separate AAV vectors to deliver the target gene [9, 64, 65]. When dual AVV vectors cannot be used to transduce larger genes, such as cadherin-23 (CDS, 10.1 kb) [66] and ALMS1 (CDS, 12.5 kb) [67], a triple-AAV approach was developed. Mini-gene strategies also contribute to the delivery of large genes. For example, a truncated cystic fibrosis transmembrane conductance regulator (CFTR) cDNA allows the incorporation of an effective promoter with the CFTR gene into AAV vectors to rescue Cl conductance in airway epithelia [68, 69].

AAV vectors used for inner ear gene delivery

More than 100 AAV serotypes have been identified to date, but effective AAVs that can be distributed to specific inner ear cell types are still lacking. Different serotypes of AAV are more favorable to different cell types in the inner ear. For example, AAVs 1-4, 7, and 8 have been shown to transduce the spiral ligament, spiral limbus, and spiral ganglion cells. AAV5 was identified to be more suitable for transducing Claudius cells, sulcus cells, and spiral ganglion neurons. In addition, AAV1 is the most effective transducer of OHCs and supporting cells. Recently, AAV serotype 1 carrying a human OTOF transgene (AAV1-hOTOF) has been used to treat children with autosomal recessive deafness 9 [11]. In addition to these natural AAV serotypes, an increasing number of variants have been isolated from different animal species for application in the inner ear. Several new serotypes are described in **Table 1**.

Anc80L65

Anc80L65 is a novel synthetic AAV that was generated via in silico reconstruction of ancestral AAVs [70]. It was shown to be a potent gene transfer agent in the liver, retina, and muscle in previous studies. Anc80L65 was found to transduce both cochlear and vestibular sensory organs via injection through a round window membrane. *Lukas D. Landegger* et al. reported that Anc80L65 transduces outer hair cells with high efficiency to restore auditory function [71]. Anc80L65 has been applied in animal models of deafness, and preliminary results have been obtained. Anc80L65 has also shown promise for the treatment of genetic deafness, including that induced by Usch1c or Tmc1 mutations, in the postnatal stage. Usher syndrome is a rare genetic condition, a devastating genetic disorder that causes blindness, balance disorders and profound deafness. *Ush1c c.216G>A* is a mouse model for human Usher I syndrome that reproduces both auditory and retinal deficits [72, 73]. Early postnatal round window membrane injection of Anc80L65 encoding harmonin successfully transduced larger numbers of IHCs and OHCs and effectively restored auditory and vestibular function to near wild-type levels in otherwise deaf and dizzy c.216AA mice [4]. TMC1 is a pore-forming component of mechanosensory transduction channels in auditory and vestibular hair cells, and mutations in *Tmc1/Tmc1* can lead to both dominant and recessive forms of deafness in mice and humans [74, 75]. A synthetic AAV2/Anc80L65 encoding Tmc1 led to the restoration of function in inner and outer hair cells, enhanced hair cell survival, restoration of cochlear and vestibular function, restoration of neural responses in the auditory cortex and recovery of behavioral responses to auditory and vestibular stimulation by round window membrane injections [76]. Recent research has indicated that AAV2/Anc80L65 is highly expressed in the inner ears of neonatal mice [77]. The transduc-

Table 1. A summary of the AAV vectors recently reported in the literature in the last 10 years for *in vivo* gene delivery

AAV vector and transgenes	Animal model	Injection time	Injection route	Targeted cells	Reference
AAV1	Vglut3^{KO} mice	P1-3; P10-12; 5w, 8w, and 20w	RWM (Round window membrane)	IHCs	[6, 132]
	Cx26^{KO} mice	P0, P42	RWM	SCs, SLFs	[133]
	Kcnq1^{KO} mice	P0-P2	SM (Scala media)	SV marginal cells	[8]
	Otof-/- mice/*Macaca fascicularis*	P0-P2 and P30 mice; 5-7 years old *Macaca fascicularis*	RWM	IHCs	[10]
	Lhfpl5-/- mice	P0-P1	RWM, Cochleostomy	IHCs, OHCs	[134]
	Children with autosomal recessive deafness 9	1-18 years	RWM	-	[11]
AAV2	Otof-/- mice	P10, P17, and P30	RWM	IHCs	[9]
AAV2/1	cCx26 mice	P0-P1	SM	IHCs, OHCs, Spindle-shaped cells and Marginal cells	[91]
	Tmc1 mutant mice	P0-P2	RWM	IHCs, OHCs	[7]
	MsrB3-/-	E12.5	Otocysts	IHCs, OHCs	[135]
	TMC-/- mice	P0-P2	RWM	IHCs, OHCs	[7]
Dual AAV2/6 half-vector	Otof-/- mice	P6-P7	RWM	IHCs	[3]
AAV2.7m8	CBA/J mice	P0-P5	PSCC (Posterior semicircular canal)	IHCs, OHCs; Pillar cells; Phalangeal cells	[82]
AAV2/8	Mouse (FVB/N)	5-6 Weeks	PSCC, RWM	IHCs	[125]
	Pjvk-/- mice	P3	RWM	IHCs, OHCs	[136]
	Clrn1ex4-/- and Clrn1ex4fl/fl Myo15-Cre+/- mice	P1-P3	RWM	IHCs, OHCs	[137]
AAV2/9	Tmc1$^{Bth/+}$	P0-P2	RWM	IHCs, OHCs	[138]
AAV3	Mouse (C57BL/6J and ICR)	4 Weeks/2 months	RWM	IHCs	[139]
AAV5	Gjb2cKO mice Cx26fl/flP0-Cre	P0 and P42	RWM	IHCs, OHCs, and SCs	[133]
	CD1, CBA/CaJ mice	P1-P2	SM	IHCs, SCs	[140]
AAV6	CD1, CBA/CaJ mice	P1-P2; 6 Weeks	SM	SCs	[140]
AAV7	CD1, CBA/CaJ mice	P1-P2	SM	SCs	[140]
AAV8	Vglut3^{KO} mice	5 weeks; 8 weeks; and 20 weeks	PSCC	IHCs	[6]
	CBA/J mice	P0-P5	PSCC	IHCs, OHCs	[77]
AAV9	ICR mice	P1	RWM	IHCs, OHCs, SCs	[141]
Bovine adeno-associated virus (BAAV)	Guinea pigs	-	ST (Scala Tympani) and SM	SCs, Interdental, inner sulcus and Hensen cells	[142]

AAV9-PHP.B	Tmc1-KO mice	P1, P7, P28-30	Utricle	IHCs, OHCs	[65]
	Wild-type mice	P1, P7, P16.	RWM, Utricle	IHCs, OHCs, VGN	[85]
	Crln1-/- mice	P1	RWM	IHCs, OHCs	[87]
	Syne4-/- mice	P0-P1.5	PSCC	IHCs, OHCs	[88]
	Strc$^{\Delta/\Delta}$ mice	P0-P1	Utricle	IHCs, OHCs	[143]
AAV-PHP.eB	*Myo6*$^{c442Y/+}$ mice	P0-P2	SM	IHCs, OHCs	[144]
	DFNB9 mouse (OTOF)	P0-P2	RWM	IHCs	[145]
	Myo6$^{WT/C442Y}$	P0-P2	SM	IHCs, OHCs	[146]
	Tmc1$^{Bth/+}$ mice	P1-P2	RWM	IHCs, OHCs	[147]
	ICR neomycin-induced mice	P1	SM	IHCs, OHCs	[148]
	Tmc1$^{\Delta/\Delta}$ mice	P1-P2	Utricle	IHCs, OHCs	[149]
	Pcdh15$^{R245X/R245X}$ mice	P0-P1	RWM	IHCs, OHCs	[150]
Anc80L65	Ush1c c.216G>A mice	P10-P12	RWM	IHCs, OHCs	[4]
	Noise-induced CBA/CaJ mice	6 weeks or 9 weeks	PSCC	IHCs	[151]
	Tprn-/- mice	P0-P2	RWM	OHCs	[152]
	ICR mice	P1	SM	IHCs, OHCs	[153]
AAVAnc80	iCKO mice (Sox10iCreERT2-mediated GJB2)	P28	RWM	IHCs, OHCs, SCs	[154]
AAV-S	TgAC1+/Clrn1KO mice	Mice: P1, P21, P60, P150, P90; NHPs: 1-3 years	RWM, PSC	IHCs, OHCs, SCs, VGN	[93]
AAV-i.e.,	C57BL/6 mice	P0, P3	RWM	IHCs, OHCs, SCs	[92]
	Cisplatin-induced C57BL/6J mice	P16	RWM	IHCs, OHCs	[155]
	C57/B6 mice	P2-P3; 4 weeks	RWM	IHCs, OHCs	[156]
	FVB mice	P2	RWM	SCs	[157]
AAV-DJ	*Tmprss3*$^{tm1/tm1}$ mice	P1	PSCC	IHCs, OHCs, SCs	[158]

tion efficiency of AAV2/Anc80L65-eGFP was high in the hair cells of the vestibules and semicircular canals and in spiral ganglion neurons after microinjection into otocysts *in utero* [78]. Recently, Anc80L65 was shown to allow efficient cochlear gene transfer in nonhuman primates [79].

AAV2.7m8

The AAV2.7m8 vector is a synthetic capsid with a 10-amino acid insertion in the AAV surface variable region VIII (VR-VIII) to change an antigenic region of AAV2 [80]. AAV2.7m8 was first applied in the transduction of retina cells and efficiently transduced retina cells after intravitreal administration [81]. The IHCs and OHCs were infected with high efficiency throughout the entire cochlea, through which AAV2.7m8-GFP was injected into the neonatal mouse inner ear via the posterior semicircular canal approach [82]. Unlike conventional AAVs, AAV2.7m8-GFP showed superior cochlear hair cell transduction efficiency, particularly with respect to OHCs. Interestingly, AAV2.7m8 also preferentially targeted cochlear hair cells rather than vestibular hair cells [83]. Research from *Kevin Isgrig* shows that AAV2.7m8 is an excellent viral vector for inner ear gene therapy, and it will likely greatly expand the potential applications for inner ear gene therapy.

AAV9-PHP. B

AAV9-PHP. B is an AAV9 capsid variant that was originally selected for the transduction of mouse neurons within the central nervous system [84]. Recently, AAV9-PHP. B exhibits the highest transduction efficiency in both inner and outer hair cells and robust transduction in spiral and vestibular ganglion neurons compared with Anc80L65 and AAV2.7m8 in mice and nonhuman primates [85]. AAV9-PHP. B has also been applied for the treatment of recessive deafness and blindness disorders, such as Usher syndrome type 3A (Usher 3A; caused by mutations in *CLRN1*), DFNB76 recessive deafness and mutations in Tmc1, which cause dominant DFNA36, recessive DFNB7/11, and deafness [86]. *Bence György* et al. used AAV9-PHP. B to deliver a normal copy of the *Clrn1* coding sequence to cochleas in a mouse model of Usher 3A and found significant rescue of hearing [87]. *SYNE4* is a gene encoding the protein nesprin-4, a member of the linker of the nucleoskeleton and cytoskeleton (LINC), and its variants can lead to DFNB76-induced human deafness. In the latest research, Syne4-/- mice were used as a model of DFNB76-induced recessive deafness to explore whether AAV9-PHP. B is suitable for genetic therapy as a vector. After delivery of the coding sequence of *Syne4* by AAV9-PHP. B In the inner ears of neonatal Syne4-/- mice, the rescue of hair cell morphology and survival, nearly complete recovery of auditory function, and restoration of auditory-associated behaviors were observed [88]. In addition, *Jason Wu* et al demonstrated that AAV9-PHP. B gene therapy can promote hair cell survival and successfully rescue hearing in three distinct dominant and recessive hearing loss models caused by Tmc1 [65]. These results revealed that Tmc1-targeted gene therapy via single or dual AAV9-PHP. B vectors offer potent and versatile approaches for treating dominant and recessive deafness. In addition to studies in mouse models, researchers have identified the transduction potential of AAV9-PHP. In primates, cynomolgus monkeys were taken to receive the ssAAV9-PHP. B-CBA-GFP through the round window membrane (RWM) via a transmastoid surgical approach. The results revealed that AAV9-PHP. B efficiently transduces the IHCs and OHCs of cynomolgus monkeys in a dose-dependent manner [89]. The studies above revealed that AAV9-PHP. The B capsid is a promising clinical delivery vehicle for treating hereditary deafness.

AAV-ie

The majority of cases of SNHL are due to genetic mutations in HCs and SCs [90]. At present, all newly discovered AAV serotypes target hair cells and spiral ganglion neurons. Once, the AAV1 serotype was used to deliver wild-type *GJB2* into the cochlear SCs of *GJB2*-knockout mice, but it could not restore hearing function because of its low transduction efficiency in SCs [91]. Three different cell-penetrating peptides (CPPs) and one CPP-like peptide (DGTLAVPFK) were inserted into the VP1 capsid of AAV-DJ to obtain the novel serotype AAV-ie. AAV-ie is able to highly transduce not only HCs but also SCs and SGNs in both animal models and human utricle samples and has no negative effect on auditory function. To assess the potential of the AAV-ie vector for HC regeneration, AAV-ie,-Atoh1-NLS-mNeonGreen (AAV-ie,

-Atoh1) was used to deliver mouse Atoh1 into the cochlea. The immunofluorescence and SEM results also indicated that new hair-like cells were generated in the AAV-ie-*Atoh1* group. In conclusion, AAV-ie can be used not only for correcting genetic hearing impairment but also for HC regeneration under environmental damage [92].

AAV-S

AAV-S, a novel AAV9 variant isolated from a random AAV9 capsid library, is capable of transducing nearly all cell types of the cochlea with high efficiency in the inner ears of mice and cynomolgus monkeys after RWM injection [93]. To identify potential treatments for a deafness model, AAV-S was used to deliver an optimized Clrn1 construct to TgAC1-Clrn1-KO mice, a model of CLRN1-related Usher syndrome type 3A. The results revealed robust rescue of hearing at low and middle frequencies and preservation of hair bundle morphology across all frequencies up to at least 5 months postinjection, whereas it did not rescue hearing at the highest frequencies. To further investigate whether AAV-S can transduce the cochleae of NHPs, cynomolgus monkeys, which are a much more relevant model for transgene delivery to the human inner ear, received AAV-S-EGFP via RMW injection. The results showed that AAV-S-EGFP had very high transduction efficiency in all cell types of the cochlea at high doses but greatly reduced transduction efficiency in SGNs [93]. In conclusion, AAV-S is capable of transducing the majority of cells in the cochlea in mice and NHPs; thus, it should serve as a promising vector for inner ear gene therapy.

The target cells of AAV gene delivery

HHL is caused by gene mutations in various types of cells in the inner ear and leads to auditory dysfunction. According to the cellular types affected by mutations in the cochlea, mutations in genes may occur in hair cells (HCs), supporting cells, spiral ganglion neurons (SGNs) and the stria vascularis. Therefore, identifying the functions of different cell types in the inner ear and gene mutations in different cell types is highly important for understanding the pathogenesis of hereditary deafness and treatment strategies. Clarifying the precise spatial and temporal patterns of expression of a wild-type gene can help us select the appropriate time for intervention, and studying cell types with gene mutations can help us select the appropriate AAV vector types for intervention treatment. Thus, here, we discuss the major inner ear cell types and their application to be targeted by gene therapy approaches.

Hair cells

Hair cells constitute the sensory epithelium of the mammalian inner ear and include two types of mechanosensory cells, OHCs and IHCs, which are capable of transducing the mechanical force generated by sound waves into electrical signals. More than 50% of the gene mutations that cause hereditary deafness are expressed in hair cells [76, 94-97]. The death of hair cells caused by gene mutations always leads to permanent hearing loss and vestibular dysfunction because of their limited regenerative capacity, and hair cells are particularly susceptible to damage and death owing to the fragile cytoarchitecture of hair bundles. In contrast, lower vertebrates such as birds, zebrafish and reptiles are able to spontaneously regenerate lost hair cells from existing supporting cells, leading to full functional recovery [98-100]. Recently, studies have shown that supporting cells have the potential to transform and regenerate into hair cells under certain conditions [101-103]. Overall, maintaining the number and function of hair cells is important for auditory function. In addition, hair cells in the cochlear sensory epithelial region are the most frequently transduced cell type by AAV serotypes. Therefore, recent progress has led to the development of a number of novel AAV vectors, such as AAV9-PHP, to improve infection efficiency in both inner and outer hair cells. B, AAV2.7m8 and AAV-ie.

Supporting cells

Supporting cells constitute one of the sensory epithelia of the inner ear; are distributed throughout the entire depth of the epithelium, from the basal lamina to the lumen; and are involved in development, survival, phagocytosis, death and regeneration in the inner ear. In addition to their multiple functions, supporting cells present various morphological and molecular features in mature sensory epithelia. There are five different cell types: Hensen's cells,

Deiters' cells, pillar cells, inner phalangeal cells and border cells, which are organized in rows along the organ of Corti. To date, more than 150 nonsyndromic hearing loss genes have been identified. Some of these genes, such as *Cx26, Cx30, Sox2* [104]*, Lgr5* [105]*, and Atoh1*, are expressed in supporting cells. In transgenic mouse models, knockout of *Cldn9, Cldn14* or *vezatin* causes hair cell death and hearing loss [106-108]. Furthermore, mutations in *Cx26* are the most common cause of hereditary deafness (DFNB1/DFNA3). Recently, great progress has been made in AAV-mediated gene therapies for hearing loss in animal models; however, most studies have focused on HCs and SGNs. Notably, SCs are significant targets for gene therapy. An increasing number of studies have focused on SCs. AAV1 was once used to deliver wild-type *GJB2* into the cochlear SCs of *GJB2*-knockout mice, but it could not rescue hearing loss because of its low transduction efficiency in SCs [91]. Compared with conventional AAV serotypes, Fangzhi Tan et al. identified an AAV variant, the AAV-inner ear (AAV-ie), which transduces cochlear supporting cells (SCs) with high efficiency [92].

Spiral ganglion neurons

SGNs act as a bridge for auditory information between hair cells and the central nervous system, and the loss of SGNs causes irreversible hearing impairment because they cannot regenerate. SGNs act as a bridge for auditory information between hair cells and the central nervous system, and the loss of SGNs causes irreversible hearing impairment because they cannot regenerate. The loss of the SGN includes the following aspects. On the one hand, the loss of SG neurons may underlie the debilitating decline in hearing-in-noise ability with aging or noise trauma. On the other hand, the gene mutation that occurs in SG neurons also causes the loss of SG neurons. For example, the mutation of the MAP1B gene, which is expressed in spiral ganglion neurons and encodes a highly conserved microtubule-associated protein, is related to nonsyndromic SNHL [109]. Currently, cochlear implantation (CI) is a therapeutic strategy for profound hearing loss. Moreover, the success of contemporary CIs depends partly upon the survival and condition of cochlear spiral ganglion (SG) neurons. Recently, AAV-based gene therapy for the survival of SGNs has become a promising approach to ameliorate hearing loss. AAV2 and AAV5 were transduced into SG neurons after injection through the round window. Furthermore, AAV-neurotrophin gene therapy can elicit the expression of physiological concentrations of neurotrophins in the cochlea, supporting improved SG neuronal and radial nerve fiber survival [110]. Overall, SGNs are vital target cells for gene therapy.

Stria vascularis

The stria vascularis (SV) is a highly vascularized epithelium located on the lateral wall of the cochlear duct. It consists of three distinct cell types: marginal cells (MCs), intermediate cells (ICs), and basal cells (BCs) [111]. The stria vascularis is capable of producing endolymph, and the endocochlear potential and high potassium content of the endolymph of the cochlear duct are necessary for normal hair cell function [112]. K+ circulates between the endolymph and phlymph via the stria vasularis to provide endocochlear potential. Some K+ channels and transporters, such as Na+, K+-ATPase [113], Na+, K+, 2Cl - cotransporter (NKCC) [114], the inwardly rectifying channel Kir4.1 [115] and KCNQ1/KCNE1 [116], are expressed in the stria vasularis and are involved in EP formation. Research has shown that genetic defects in genes that mediate K+ recycling in the inner ear cause inherited deafness. For example, mutations in the gene encoding the basolateral Na-K-Cl cotransporter Slc12a2 (Nkcc1, mBSC2) cause the deafness observed in Shaker-with-syndactylism (sy) and sy (ns) mice [117]. Another study revealed that autosomal recessive Jervell and Lange-Nielsen (JLN) syndrome may be caused by mutations in the KCNQ1 and KCNE1 genes, which are expressed in marginal cells of the SV [118]. Gene therapies to target and correct mutations in SVs are still a major challenge since efficient AAV vectors that can transduce SVs are lacking. The Kcnq1-expressing AAV1 viral construct was injected into the endolymph of Kcnq1-/- postnatal mice (P0-P2), which resulted in Kcnq1 expression in most cochlear marginal cells, and auditory brainstem responses also showed significant hearing preservation in the injected ears. This is the first time that a gene therapy approach has been applied in a mouse model of JLN syndrome to successfully treat gene defects that specifically affect the functions of the SV [8].

The routes of AAV gene delivery

The routes of administration are generally divided into systemic and local routes. These two delivery methods are suitable for the treatment of different diseases. Systemic administration is a simple and traumatic route to deliver genetic material to cochlear cells, but the issue of increased probabilities of off-target delivery, toxicity, or BLB-restricted transportation needs to be addressed. Compared with systemic administration, local delivery methods are more commonly used in gene therapy for deafness. Local delivery methods are supposed to cause fewer side effects than systemic methods. In addition, local administration in the inner ear is more precise.

Systemic route of administration

Intravenous administration is the main approach used to systemically deliver virus vectors to the cochlea. Although systemic administration is quick, convenient and noninvasive, the systemic delivery of viral vectors still faces numerous challenges. The key factor is the appropriate AAV serotype, which is able to cross the blood-brain barrier to reach target cells. Recently, the AAV9 and Anc80L65 capsids were identified as serotypes that can cross the blood-brain barrier and reach the cochlea after intravenous injection [119]. In addition, the greatest adverse effects of systemic AAV administration are off-target effects. Systemic delivery requires a high titer and a high volume of virus, and the ability to quantify the viral dose that simultaneously reaches the target cell needs to be considered.

Local routes of administration

Round window: A round window membrane (RWM), a three-layered membranous opening leading to the perilymphatic space of the scala tympani, is the most common and successful way to deliver agents to the inner ear to date (**Figure 4**). Round window injection is generally divided into the following steps: first, the bulla is opened via a postauricular incision; second, the virus vectors are injected through the round window membrane, which directly access the perilymph of the scala tympani via microinjection; finally, the viral vectors are in contact with the basilar membrane and then reach the targeted cells via the endolymphatic space. This process can cause some injuries, while research from *Li, X* et al. indicated that RWM injection results in only a small risk of residual hearing damage in mice [120]. Furthermore, the RWM approach in nonhuman primates has also shown highly efficient transgene transfer into the cochlea [87]. Currently, RWM appears to be a promising means of gene delivery for treating deafness in humans. However, this approach still has significant adverse effects, such as perilymphatic fluid leakage, virus transportation to the cerebellum, and cross-transfer to the contralateral inner ear through the cochlear aqueduct, hematogenous, or systematic spread via the temporal bone marrow. In addition to these safety factors, the major disadvantage is that the viral vector injected through the window into the scala tympani is not uniformly distributed throughout the cochlear duct.

Oval window: The oval window is an oval hole on the inner wall of the tympanum of the middle ear. Unlike the round window, the oval window is covered by a footplate of the stapes instead of a membrane. The delivery approach requires a transcanal or transmastoid microsurgical procedure for access in humans, and oval window injection is more complicated and riskier than round window injection from a technical point of view.

Canalostomy: Canalostomy is a useful approach to deliver drugs into the inner ear via injection through semicircular canals (canalostomy). Since 2001, canalostomy has been used to deliver various reagents, such as viral vectors, siRNAs, stem cells, and aminoglycoside, into the murine inner ear [121-124]. Recently, AAV has also been delivered into the inner ear to infect target cells such as HCs and SGN cells via canalostomy [122]. When various reagents, including fast green dye and AAV serotype 8 (AAV8), together with the green fluorescent protein (GFP) gene (AAV8-GFP) and streptomycin, were injected into adult and neonatal mouse inner ears, the results revealed a broad distribution of reagents in the cochlea and vestibule with minimal disturbance of hearing and vestibular function [125]. Xuewen Wu et al. conducted gene therapy by injecting viral vectors via the canalostomy approach in *Kcne1*$^{-/-}$ mice to treat both hearing and vestibular symptoms, and their results revealed that early treatment

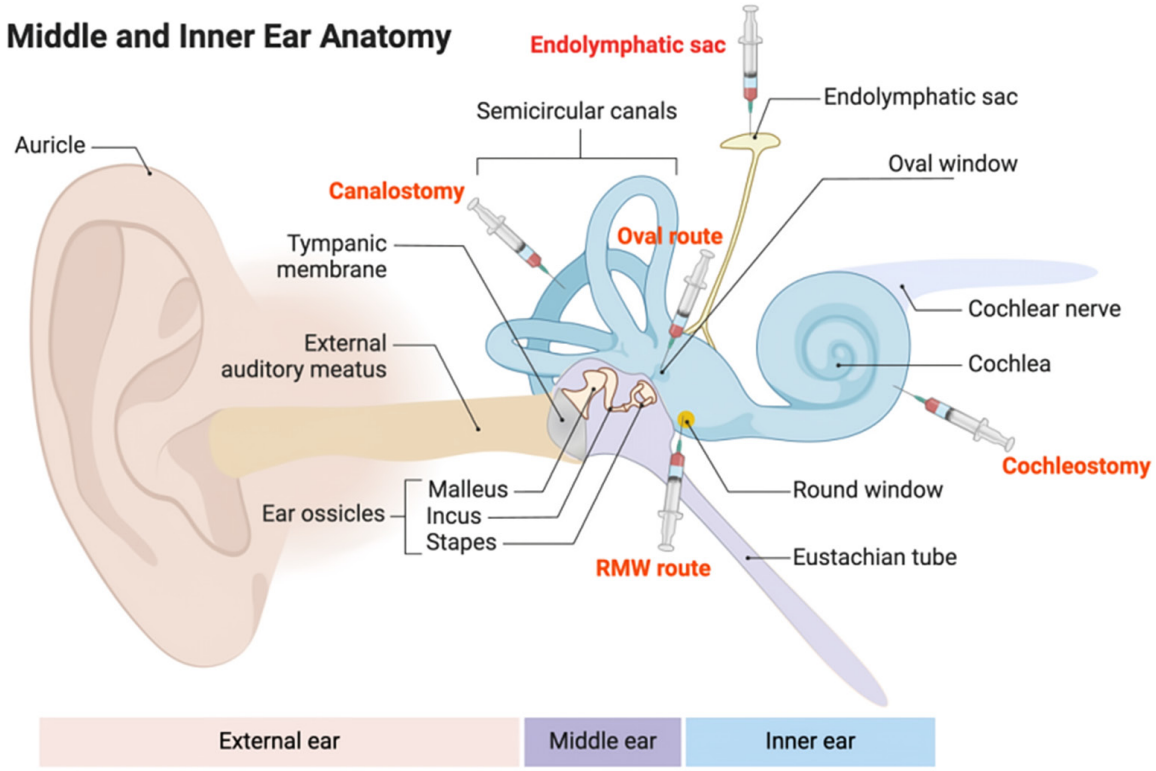

Figure 4. Schematic illustration of methods for delivering therapeutics to the human ear. Viruses introduced into the inner ear are indicated in red and encompass both indirect and direct methodologies. The indirect methodology involves administration through the tympanic membrane (either transtympanic or intratympanic), facilitating the deposition of the therapeutic agent in the middle ear, from which it diffuses into the inner ear via the oval and round windows. Direct methodologies entail delivery into the cochlea through application over or through the round window membrane, via a surgically created cochleostomy adjacent to the round window, through a fenestration in the bony oval window, or through a semicircular canal.

prevented collapse of the Reissner's membrane and vestibular wall, retained the normal size of the semicircular canals, and prevented the degeneration of inner ear cells [126]. In conclusion, canalostomy is an effective and safe approach for drug delivery into the inner ears of adult and neonatal mice and may be one of the more feasible delivery methods for human inner ear gene therapy in the future.

Cochleostomy: In 1993, Lenhardt proposed a new soft surgery, cochleostomy, which performs a minimal cochleostomy anteriorly and inferiorly to the round window to apply for cochlear implants [127]. Research from Wade W. Chien revealed that the cochleostomy approach caused more severe cochlear damage than did the round window approach via a comparison of the patterns of cochlear infection and effects on hearing between these two surgical approaches using AAV serotype 2/8 (AAV8) as the gene delivery vehicle [128]. However, the two surgical approaches resulted in comparable viral infection efficiencies. In conclusion, cochleostomy is riskier than round window injection is and is not the optimal choice for drug delivery.

Endolymphatic sac: The endolymphatic sac is a nonsensory organ of the inner ear that is connected to the endolymphatic compartment. The main functions of the endolymphatic sac are the regulation of the volume and pressure of the endolymph and the protection of the inner ear from pathogen invasion [129]. The endolymphatic sac is thought to contribute to the pathophysiology of Ménière's disease. Endolymphatic sac surgery is considered an effective method for treating Ménière's disease [130]. The endolymphatic sac communicates directly with the endolymphatic fluid, so the virus can enter the endolymphatic fluid through the opening of the endolymphatic sac to reach its target cells. Research from Tatsuya

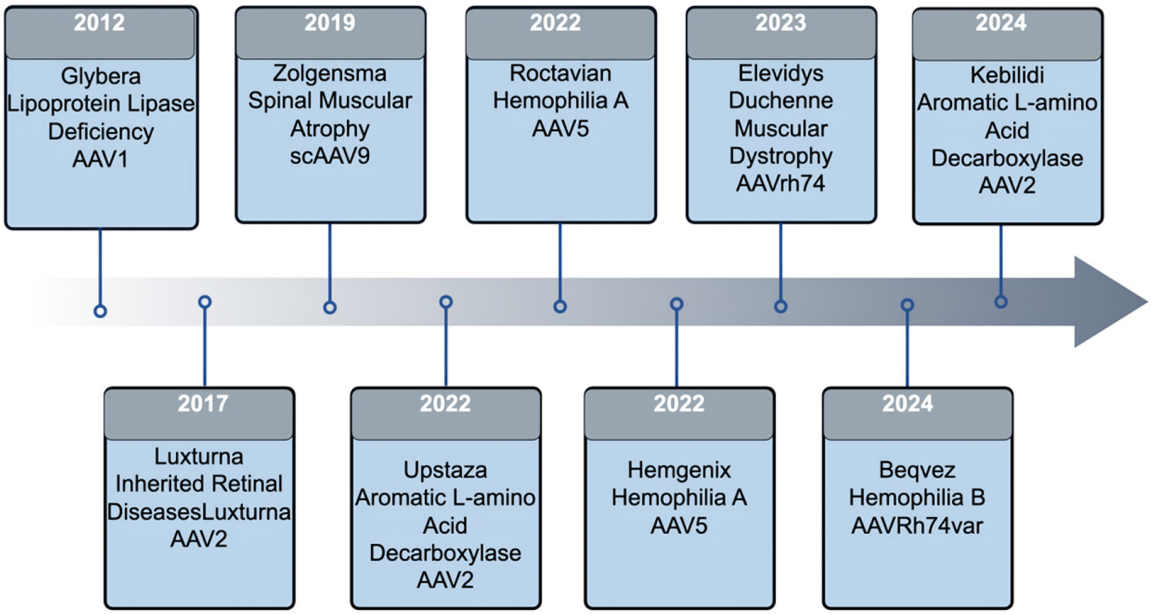

Figure 5. A timeline of AAV-based gene therapy approvals to date on the market. The first AAV-based gene therapy drug, Glybera, was approved by the European Medicines Agency (EMA) in 2012, with Luxturna becoming the first AAV gene therapy product to receive US Food and Drug Administration (FDA) approval 5 years later. With the advancement of gene therapy, the time it takes for AAV-vectored drugs to hit the market has significantly decreased. Currently, 9 AAV-vectored drugs have entered the market.

Yamasoba et al. indicated that the injection of an application-deficient adenoviral vector, Ad.RSVntlacZ, into the guinea pig endolymphatic sac resulted in many blue (LacZ-positive) cells in the endolymphatic sac and duct, vestibule, and ampulla [131]. However, the surgical approach is accompanied by greater surgical risk than the other routes are. Therefore, further consideration is needed in practical applications.

Clinical trials of AAVs in inner ear gene therapy

Since the development of gene therapy in the 1970s, several virus-based vector gene therapy drugs have been approved on the market (**Figure 5**). Previously, only two AAV-mediated gene therapies for hearing loss were evaluated in clinical trials. The first trial is CGF166 aimed at unilateral severe to profound hearing loss or bilateral severe to profound hearing loss, which was sponsored by Novartis and is currently in clinical phase I and phase II. CGF166 is a recombinant adenovirus 5 (Ad5) vector containing cDNA encoding the human atrial transcription factor Hath1. (clinicaltrials.gov/ct2/show/NCT02132130). The second trial is ongoing at the Institute Pasteur and aims to study *in vitro* viral transduction of AAV in human inner ear cells. (clinicaltrials.gov/ct2/show/study/NCT03996824). A preclinical research study is ongoing by Decibel Therapeutics called DB-OTO. DB-OTO is an AAV-based dual-vector gene therapy product designed to selectively express functional OTOF in the inner hair cells of individuals with OTOF deficiency with the goal of enabling the ear to transmit sound to the brain and facilitate hearing. In 2023, the first clinical trial of two-vector gene compensation therapy based on an AAV for DFNB9 was conducted in the clinic [11].

Prospects and challenges for inner ear gene therapy

To date, nine AAV-based gene therapy drugs - namely, Kebilidi, Beqvez, Roctavian, Elevidys, Hemgenix, Upstaza, Zolgensma, Luxturna, and Glybera - have received approval from the European Medicines Agency and the United States Food and Drug Administration for the treatment of various genetic disorders. These disorders include hereditary LPLD, inherited retinal disease (IRD), aromatic L-amino acid decarboxylase (AADC) deficiency, hemophilia

A, hemophilia B, and spinal muscular atrophy (SMA). Owing to its superior safety profile with a relatively low risk of genotoxicity from the insertion of transgenes into the genome, AAV has been regarded as the leading gene delivery platform for gene therapy, and AAV-based gene therapy also shows great promise in the treatment of hereditary diseases. Despite these notable therapeutic advancements, many challenges and considerations still warrant attention. Adenoviral-associated virus (AAV) vectors may provoke immune responses in the host, including the generation of neutralizing antibodies, which have the potential to neutralize the viral vector and diminish therapeutic efficacy. For example, neutralizing antibodies against AAV are commonly present in the majority of the human population, thereby limiting the systemic application of AAV. The genomic size limitation of AAV vectors, approximately 4.7 kilobases (kb), implies that the size of the therapeutic genes they can carry is constrained, possibly precluding the accommodation of larger genes, which may limit the treatment of certain genetic disorders. Although AAV vectors can target specific tissues and cell types, variations in infection efficiency and specificity among different AAV serotypes for cells within the cochlea exist, restricting their broadness and effectiveness in clinical applications. Effective gene delivery to a target is crucial for the success of gene therapy. While AAV gene therapy has been well tolerated in a range of diseases thus far, the regulation of expression remains a significant safety consideration for target and disease selection. Furthermore, the durability of gene therapy has yet to be determined, necessitating lifelong follow-up for assessment.

Conclusion

The adeno-associated virus (AAV) offers distinct advantages as a gene therapy vector because of its low immunogenicity, sustained gene expression, and broad cellular tropism. These characteristics make it particularly promising for treating HHL. Functional gene delivery via AAV vectors could address the genetic defects responsible for hearing impairment and the recovery of auditory function. However, clinical implementation faces several obstacles. Neutralizing antibodies against AAV vectors may diminish transduction efficiency and therapeutic outcomes. The ~4.7 kb packaging constraint further limits the incorporation of larger therapeutic genes. Additionally, transduction efficiency and cell-type specificity remain suboptimal, as existing AAV serotypes show inconsistent tropism for inner ear cell populations. Future efforts should prioritize engineered AAV variants with improved functionality, refined therapeutic strategies, and combination therapies. Parallel innovations in gene editing, synthetic biology, and nanotechnology are expected to advance the field. Although challenges remain, next-generation AAV vectors, optimized delivery methods, and multimodal approaches - supported by technological advances and clinical validation - could provide safer, longer-lasting treatments for HHL. Successful translation would significantly benefit patients while addressing broader clinical and societal needs. Closing the gap between research and clinical practice will be essential for realizing the therapeutic potential of AAV-based interventions in auditory medicine.

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Disclosure of conflict of interest

None.

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