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Cochlear gene therapy restores hearing and auditory processing in an atypical DFNB9 mouse model

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Abstract

Background The autosomal recessive deafness 9 (DFNB9) is caused by mutations in the otoferlin gene that accounts for 2–8% of all inherited deafness cases. In a previous study, we demonstrated that Adeno-associated virus (AAV) gene therapy restored hearing in a preclinical mouse model of profound DFNB9 deafness caused by a frameshift mutation leading to a complete loss of otoferlin expression. However, it remains to be demonstrated that it can address the full spectrum of DFNB9 deafness severity, while also restoring central auditory processing essential for speech understanding.

Methods Using homologous recombination in mouse embryonic stem cells, we created a knock-in mouse model carrying the E1799del otoferlin mutation, which mirrors the human E1804del variant linked to DFNB9 deafness, characterized by moderate-to-profound deafness during febrile episodes in affected individuals. A mixture of male and female mice was used at P2, P8, and P30. Some were followed for up to 4 months for longevity monitoring and behavioral tests.

Results The mouse model exhibits abnormal otoferlin distribution, failure of synaptic transmission in inner hair cells, and profound hearing loss, all of which is restored to normal through AAV gene therapy. Notably, we conduct objective behavioral testing to provide the first evidence that gene therapy administered to the cochlea, which is part of the peripheral auditory system, can restore frequency discrimination, indicating the recovery of central auditory processing. This is achieved even when treatment is administered late at the end of the critical period.

Conclusions These findings indicate that gene therapy can address the entire spectrum of DFNB9 hearing loss, and that profound deafness during critical period may not impede the restoration of central auditory processing.

In mammals, hearing relies on a complex and precisely regulated mechanotransduction process involving the coordinated function of two types of auditory sensory hair cells: outer hair cells (OHCs), which act as the cochlear amplifier for sound signals, and inner hair cells (IHCs), the genuine sensory cells. When environmental sound waves reach the inner ear, they are first amplified by OHCs and then transformed into electrical signals in the auditory nerve. This conversion occurs through an extremely fast and sustained release of neurotransmitters at the primary auditory synapse, the IHC ribbon synapse. In this process, otoferlin, a protein containing six Ca²⁺ binding C2 domain (C2A-C2F) associated with DFNB9 deafness, functions as the major Ca²⁺ sensor for synaptic vesicle exocytosis and replenishment at the active zone of the IHC ribbon synapse¹⁻³. Congenital hearing loss is the most common human sensory deficit in human, affecting ~1 in 700 newborns. Genetic factors account for up to 80% of these deafness cases⁴. To

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Plain language summary

We investigated gene therapy, a technique that introduces a healthy copy of a gene to restore normal cell function, as a potential treatment for an unusual form of inherited deafness caused by mutations in the otoferlin gene. These mutations result in the production of a defective protein, leading to hearing loss during fever episodes. We developed and studied a mouse model with the same genetic alteration observed in affected individuals and discovered that the deafness resulted from the abnormal protein distribution in the inner ear. We use gene therapy to correct this mislocalization and restored normal hearing. Our findings indicate that gene therapy may be an effective approach for treating all forms of otoferlinrelated hearing loss.

date, 153 genes have been shown to cause hearing loss when mutated⁵. Within the gene encoding otoferlin, about 220 pathogenic and likely pathogenic mutations have been identified, collectively contributing to 2-8% of all cases of genetic congenital deafness⁶. Findings from several preclinical studies indicate that gene therapy shows potential as a treatment for hereditary deafness⁷⁻⁹. Notably, we have proven in a previous work that adeno-associated virus (AAV)-mediated gene replacement therapy can reverse established deafness in a mouse model that faithfully replicates the profound prelingual human DFNB9 deafness caused by frameshift mutations that are predicted to result in truncated forms of otoferlin⁷. This breakthrough led to the initiation of several successful phase II clinical trials for DFNB9 patients¹⁰⁻¹². However, these clinical trials target the typical DFNB9 deafness phenotype, characterized by profound prelingual deafness due to mutations that severely disrupt otoferlin-coding gene, leading to a complete loss of otoferlin function¹³. Nevertheless, in about 15% of cases, pathogenic otoferlin mutations lead to DFNB9 deafness with varying levels of severity, such as mild-to-profound, progressive, or temperature-sensitive all of which are associated with difficulties in understanding speech¹⁴⁻¹⁷. The mutations involved, primarily in-frame mutations, are expected to lead to unstable and/or partially functional protein products, which could impair the ability of IHCs to effectively process or respond to exogenous therapeutic otoferlin.

Our objective is to assess whether gene augmentation therapy could counteract the abnormal otoferlin underlying these various forms of DFNB9 deafness in humans by reinstating normal protein production in the IHCs and their function, thereby resorting both hearing and central auditory processing.

In the present study, we focus on a form of DFNB9 deafness identified by our laboratory in which an in-frame deletion of the glutamic acid residue at position 1804 (E1804del) within the C2F domain is associated with moderate-to-profound thermosensitive hearing loss during febrile episodes in affected individuals¹⁸. We created a mouse model by introducing into the mouse genome the Otof E1799del mutation (Otof E1799del/E1799del) corresponding to the human OTOF E1804del variant. The introduction of this mutation affecting the C2F domain leads to the abnormal distribution of the otoferlin protein within auditory sensory hair cells, causing profound deafness. The Otof E1799del/E1799del mice underwent gene augmentation therapy during the neonatal period or as adults, corresponding to closure of the critical period of central auditory plasticity¹⁹. In the current study, our objective is to assess the effects of gene therapy on various phenotypic aspects, including the restoration of normal otoferlin expression, the biophysical properties of the IHC ribbon synapse, hearing thresholds, and recovery of central auditory processing and hearing perception performance.

Methods

The Otof E1799del/E1799del (MGI:1891247) mice were generated by homologous recombination (Geneoway, Lyon, France). A targeting vector carrying the Otof E1799del mutation in exon 43 along with a neomycin resistance (NeoR) cassette flanked by loxP sites serving as a selectable marker in intron 43, was introduced, by electroporation, into embryonic stem cells derived from the129Sv mouse strain, and positive embryonic stem (ES) cells (n = 372) were selected based on their resistance to G418. Two clones of ES cells carrying the targeted construct were injected into blastocysts from C57BL/6J mice to create chimeric animals. Mice receiving the construct through germline transmission were then crossed with Pgk-1-cre mice in a mixed C57BL/6-129/Sv background to remove the neo cassette. The heterozygous animals were crossed to generate Otof^{E1799del/E1799del} and Otof^{+/EI799del} mice. Animals were genotyped with the p1 primer (5' CTGAC CACTTCTCGGCTGACGACT 3') and the p2 primer (5' CCCTC GCTTAGCTAGACATCTTCATCC3'), to distinguish between the wildtype (380 bp amplicon) and knock-in (487 bp amplicon) alleles. Both male and female mice were used. The animals were housed in a controlledtemperature room under a 12 h/12 h light/dark cycle. Food and water were available ad libitum and experiments were conducted in accordance with European guidelines for the care and use of laboratory animals, with the approval of the ethics committee of Institut Pasteur under agreement no. APAFIS #18368–019010910106739 (France). The number of male and female subjects used in each experiment and the age of the animals are detailed in the figure legends. We did not observe any differences in gene therapy outcome between males and females.

Recombinant AAV construct

The full-length coding sequence of the murine otoferlin cDNA (*Otof1* isoform 1; NM_001100395.1) was split in half to obtain a 5' fragment (nucleotides 1–2448) and a 3' fragment (nucleotides 2449–5979). These fragments were synthesized by GenScript. The 5' construct contained the smCBA promoter followed by the 5' fragment of the *Otof* cDNA (encoding amino acids 1–816) and a splice donor site. The 3' construct contained a splice acceptor site and the 3' fragment of the *Otof* cDNA (encoding amino acids 817–1992). Both constructs contained the alkaline phosphatase recombinogenic bridging sequence and were inserted into AAV-vector plasmids to generate a pair of recombinant vectors referred to as the "Dual AAV-Otof vector". The recombinant vector was packaged into the AAV8 capsid and recombinant viruses were purified and their titers were determined by fluorometric assay at the ETH Vector Core Facility (Zurich). The viral titers were 1.5×10^{13} vg/mL for the 5' vector and 1.5×10^{13} vg/mL for the 3' vector.

AAV delivery

The protocols were approved by the Animal Care and Use Committee of Institut Pasteur. Gene therapy was administered to two groups of Otof E1799del/E1799del mice: one group was treated during the neonatal period, before the onset of hearing (at P2), and the other group was treated in adulthood, after the onset of hearing (at P30) as previously described^{8,9,20,21}. On P2 and P30, mice were placed under isoflurane anesthesia and positioned on a warming pad. For P2 mice, 2% lidocaine (5 mg/kg) was injected locally under the skin before incision. For P30 mice, this local injection of 2% lidocaine was combined with a subcutaneous injection of meloxicam (1 mg/kg) 30 min before surgery. Animals were shaved (P30), the skin was disinfected with Vetedine, and the eyes were protected with Ocry-gel. A left postauricular incision was made, and the cochlear basal turn, stapedial artery, and facial nerve were used as landmarks to access the round window membrane of P2 mice. A micropipette (10 µm diameter) was used to inject 1-2 μL of AAV viral vectors through the round window membrane. For P30 mice, the vectors were injected into the posterior semicircular canal (SCC). After the incision and identification of the facial nerve, the muscles on the SCC were dissected. A small hole in the posterior SCC was created to induce perilymph flow. A catheter linked to a micropipette was then inserted into the SCC hole, pointing towards the facial nerve, for injection of the AAV viral vectors. The hole was then plugged with muscle and connective tissue, and the skin was sealed with biological glue (3M Vetbond). Mice were kept on the warming pad until they woke up and were then placed in their home cage.

Auditory brainstem response (ABR) recording

The mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed on a warming pad in an attenuated-sound box. Three electrodes were placed on the vertex, the ipsilateral mastoid, and the lower background electrode. Pure-tone stimuli were used at frequencies of 5, 10, 15, 20, 32, and 40 kHz. Sound intensities of 20–110 dB, in 10 dB steps, were tested. The hearing threshold was determined as the lowest stimulus level resulting in visually recognizable ABR waves. ABR analysis was performed with MATLAB software. Mice body temperature was monitored and maintained within physiological ranges (36–37 °C) during the time of recording. The mice were then kept on the thermopad until they woke up and placed back in their home cage.

Distortion product otoacoustic emission analysis

Distortion product otoacoustic emissions (DPOAEs) were measured to evaluate OHC function. Mice were anesthetized with ketamine and xylazine

as previously described, and body temperature was maintained within physiological ranges with a thermopad. DPOAEs were recorded with Otophylab (Echodia) and analyzed with RT-Lab software or with the CubeDis system (Mimosa Acoustics, ER10B microphone, Etymotic Research). The DPOAE at a frequency 2f1-f2 was recorded in response to two simultaneously presented primary tones of equal energy levels at different frequencies, f1 and f2, with f2/f1 = 1.20. Frequency f2 was swept at 12 kHz and 16 kHz and responses were recorded in response to an 80 dB SPL stimulation. The DPOAE threshold was plotted against frequency f2. The DPOAE threshold was defined as the smallest primary tone energy level leading to a detectable DPOAE.

Immunofluorescence

The cochleas were dislodged from the skulls of $Otof^{+/E1799del}$ mice, untreated control $Otof^{E1799del/E1799del}$ mice or treated $Otof^{E1799del/E1799del}$ mice. Cochleas were first gently and slowly perfused with 4% paraformaldehyde in PBS through the round window until the solution emerged from small hole at the apex. They were then postfixed with 4% paraformaldehyde in PBS for 40 min at room temperature. The cochleas were processed for whole-mount dissection of the organs of Corti followed by immunofluorescence analysis. The organs of Corti were then washed (3 × 10 min) with PBS and incubated for 1 h at room temperature in PBS containing 20% normal horse serum and 0.3% Triton X-100.

The samples were then incubated overnight with various primary antibodies in PBS: rabbit anti-otoferlin (1:200) (Homemade; for more information see the reference Roux et al.²), mouse anti-CTBP2 (1:100) (Cat#612044; RRID:AB_399431; Thermo Fisher Scientific), and/or antimyosin 7a-biotin (1:50) (Cat#25-6790; RRID:AB_10015251; Proteus Biosciences). The samples were washed $(3 \times 10 \text{ min})$ with PBS and incubated for 1 h with various secondary antibodies (1:500 dilution): Alexa-488 goat anti-rabbit IgG antibody (Cat#18772; RRI-D:AB_1137637; Sigma-Aldrich), Alexa-633 goat anti-mouse IgG1 antibody (Cat#A-21126; RRID:AB 2535768; Thermo Fisher Scientific), and streptavidin-555 antibody. Nuclei were labeled by incubation with 4',6diamidino-2-phenylindole (DAPI) (1:1000) (Cat#MBD0015; Sigma-Aldrich) for 10 min. The samples were washed $(3 \times 10 \text{ min})$ with PBS and then mounted in Fluorsave medium. Images were captured with a Zeiss LSM-900 confocal microscope equipped with a Plan Apochromat 63x/1.4 N.A. oil immersion lens (Carl Zeiss). Quantification of ribbons per IHC was performed manually by counting CtBP2-positively stained cells using z-stack images acquired under a confocal microscope encompassing the entire volume of IHCs. The results were obtained from a total of 4 animals per group.

Patch-clamp recording and capacitance measurement

All IHC capacitance measurements were performed with an EPC10 amplifier controlled by Patchmaster software in the mid-apical region of the cochlea, 20-40% of the normalized distance from the apex. This area encodes frequencies ranging from 8 to 16 kHz. IHCs were selected at random from the tissue preparation for recordings. Patch pipettes were pulled with a micropipette puller (P-97 Flaming/Brown; Sutter Instrument) and fire-polished with an MF-830 microforge (Narishige) to obtain a resistance range of 2-3 MΩ. They were filled with an intracellular cesium-based solution containing the following: 145 mM CsCl, 1 mM MgCl₂, 5 mM HEPES, 1 mM EGTA, 20 mM tetraethylammonium chloride, 2 mM ATP, and 0.3 mM GTP, pH 7.2, 300 mOsm. Changes in cell membrane capacitance (ΔC_m) were used to monitor the fusion of synaptic vesicles during exocytosis. ΔC_m was measured with the Lindau and Neher (1988) technique²², using Patchmaster lock-in amplifier software (HEKA) and applying a 1 kHz command sine wave (20 mV amplitude) at a holding potential of -80 mV before and after the pulse experiment. As recording conditions can greatly influence capacitance measurements, only IHC patch-clamp recordings with low series resistance, below 10 M Ω , and a maximum leak current of 25 pA (at $V_h = -80 \text{ mV}$) were considered in this study.

Startle reflex/prepulse inhibition

The ASR and PPI were measured with the SR-Lab system (San Diego Instruments, San Diego, CA, USA). Mice were placed in a restraining cylinder on a platform measuring the vibrations produced by the mouse movements with a piezoelectric accelerometer. The system was calibrated each day, before experiments, with the SR-LAB Standardization Unit: the mean response of the motion generator had to remain at about 700 ± 15 arbitrary units. Each SR and PPI procedure began with a 3-min habituation period with a background noise of 50 dB. Startle responses were elicited during random trials with a short acoustic stimulus of an intensity of 60–110 dB. Each trial was repeated 40 times, with a randomly selected inter-trial interval time of 1–4 s. For the PPI, mice were subjected to a combination of four trials repeated 30 times each. Each combination consisted of a prepulse of 0, 60, 70, or 80 dB followed 100 ms later by a 120 dB pulse. Only trials with plausible startle latencies between 5 ms and 70 ms were retained.

Audiobox

The Audiobox (TSE, Bad Homburg, Germany) is designed for auditory behavior procedures based on Go/NoGo tasks such as sound frequency discrimination and has been extensively described in previous study^{23,24}. The system consists of two chambers connected by a Perspex tube. One chamber contains food and bedding. The other has a corner providing access to water in an attenuated-sound box. Each mouse can be detected individually via an implanted transponder, making it possible to detect specific behaviors (nose-poking and licking) via a sensor at the entrance to the drinking corner. Mice were first subjected to a period of habituation with no sound and free access to water. Then, during a learning period, they were trained to lick a drop of liquid in response to a 6.8 kHz pure-tone frequency corresponding to the Go tone. The sound discrimination testing phase began with a 100% difference between the Go and NoGo tones (13.3 kHz). When the mouse nosepoked during the NoGo tone, it received a gentle puff of air. During the testing phase, the difference between the NoGo and Go tone frequencies was progressively decreased until the mice could no longer distinguish between the two frequencies. The Go tone was kept at 6.8 kHz and the NoGo tone was progressively decreased from 13.6 to 7 kHz, corresponding to frequency intervals decreasing from 100% to 4% of the Go tone.

Statistics and reproducibility

All statistical analyses were performed using GraphPad InStat software version 10.2.1.

The normality of each dataset was tested using the D'Agostino–Pearson normality test to ensure the appropriate statistical methods were applied. According to the data structure, for two-group comparisons, we used a twotailed Student's *t*-test when the data followed a normal distribution. For multiple group comparisons, we applied either a parametric one-way ANOVA followed by Tukey's post hoc test for normally distributed data or a non-parametric Kruskal–Wallis test followed by Dunn's post hoc test for non-normally distributed data and two-way ANOVA test followed by a Bonferroni's multiple comparison post hoc test. This approach ensures robust and appropriate statistical analysis tailored to the characteristics of each dataset.

Statistical significance is indicated in the figures as follows: n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ***p < 0.001. All the data are expressed as means ± SEM. Sample size and additional details are provided in the figure legends.

Results

Generation of Otof E1799del/E1799del knock-in mouse

We investigated the impact of gene therapy on DFNB9 auditory synaptopathies by creating a mouse model that expresses the *E1799del Otof* mutation from the endogenous otoferlin genomic locus, mirroring the thermosensitive human *OTOF E1804del* variant. We used homologous recombination in mouse embryonic stem cells to introduce the E1799del mutation into exon 43 (Supplementary Fig. 1). Correctly targeted stem cell



clones were identified through PCR and Southern blot analyses and used to generate chimeric mice that transmitted the targeted mutation to their offspring. The neomycin cassette flanked by loxP site was deleted by crossing the heterozygous mice with PGK-Cre mice, which express Cre recombinase driven by the PGK promoter. Subsequent interbreeding between heterozygous ($Otof^{+/E1799del}$) mice resulted in wild-type, heterozygous, and homozygous ($Otof^{E1799del/E1799del}$) knock-in pups in the anticipated Mendelian ratios with no apparent morphological or behavioral abnormalities.

Otof ^{E1799del/E1799del} mice are profoundly deaf

We first assessed hearing using ABR recordings in response to brief sound stimuli at frequencies from 5 to 40 kHz, comparing $Otof^{+/E1799del}$ and $Otof^{E1799del/E1799del}$ mice with wild-type mice. At 1 month of age, $Otof^{+/E1799del}$ mice (n = 7) exhibited ABR waveforms comparable to those of wild-type mice (n = 5) at all frequencies tested. Surprisingly, regardless of body temperature the $Otof^{E1799del/E1799del}$ mice (n = 8) showed flat ABR traces, indicating profound deafness relative to $Otof^{+/E1799del}$ and wild-type mice (p < 0.0001; two-way ANOVA test) (Fig. 1A). The morphological structure

Fig. 1 | *Otof*^{E1799del/E1799del} mice are profoundly deaf. A Mean ABR thresholds of P30 $Otof^{+l+}$ (gray, n = 5, 2 females and 3 males), $Otof^{+l/E1799del}$ (black, n = 7, 4 females and 3 males) and $Otof^{E1799del/E1799del}$ (purple, n = 8, 4 females and 4 males) mice for pure-tone frequencies between 5 kHz and 40 kHz. **B** Mean DPOAE at 12 and 16 kHz increasing stepwise from 20 to 70 dB SPL in $Otof^{+l+}$ (gray, n = 5), $Otof^{+l/E1799del}$ (black, n = 7), and $Otof^{E1799del/E1799del}$ (purple, n = 8) mice. In (**A**, **B**) data are presented as the mean \pm SEM. ****p < 0.0001, ns not significant (two-way-ANOVA test followed by a Bonferroni's multiple comparison post hoc test). Confocal microscopy images of IHCs and OHCs from whole-mount preparations of the organ of Corti from P30 mice: $Otof^{+l+1}$ (**C**) $Otof^{+l/E1799del}$ (**D**) and $Otof^{E1799del/E1799del/E1799del}$ (E) immunolabeled for otoferlin (blue), myosin 7a (red), and ribeye (green). The insets show higher magnifications of the boxed IHCs and "n" indicates the nucleus. Scale bar: 10 µm. Inset scale bar: 5 µm, the dotted line outlines IHCs wherein the star marks the active zone lacking otoferlin in IHC of an $Otof^{E1799del/E1799del}$ mouse. **F** Histogram depicting ribbon quantification per IHC of $Otof^{+l/E1799del}$ (black, n = 121 IHCs from 4 mice, 2 females and 2 males) and $Otof^{E1799del/E1799del}$ (purple, n = 106 IHCs from 4 mice, 2 females and 2 males) mice. Data are presented as the mean ± SEM. ****p < 0.0001 (unpaired *t*-test). **G** Mean Ca²⁺ current amplitudes (ICa²⁺) of P30 IHCs from $Otof^{+/E1799del}$ (black, n = 8 IHCs from 3 mice, 1 female and 2 males) and $Otof^{E1799del/E1799del}$ (purple, n = 12 IHCs from 5 mice, 2 females and 3 males) obtained from patch-clamp recordings after 20 ms depolarization to potentials between -60 mV and +40 mV. **H** Mean corresponding capacitance changes ($\Delta C_{\rm m}$) evoked by voltage-steps from -80 to -10 mV with an increasing duration of depolarization steps from 5 to 100 ms, for $Otof^{+/E1799del}$ (black) and $Otof^{E1799del/E1799del}$ (purple) IHCs. The fusion of the readily releasable pool (RRP) and the sustained release pool (SRP) vesicles was evaluated for durations of 5–20 ms and 30–100 ms, respectively. In (**A**, **B**, **F**, **G**, **H**), data are presented as the mean ± SEM. **** p < 0.0001, *** p < 0.001, ns not significant (two-way-ANOVA test followed by a Bonferroni's multiple comparison post hoc test).

of the organ of Corti in *Otof* ^{EI799del/EI799del} mice was normal, as were the distortion product otoacoustic emissions (DPOAE), indicating normal functioning of OHC (Fig. 1B, C–E). These observations are consistent with otoferlin not being required for either cochlear development or OHC function. The deafness of these mutant mice is therefore very likely to be due to IHC synapse dysfunction, as previously demonstrated in other *Otof* mutant mouse models^{1,2}.

The E1799del mutation disrupts otoferlin distribution and reduces the ribbon number

We next studied otoferlin expression and cellular distribution within the cochlear hair cells using immunohistofluorescence and confocal imaging on organs of Corti before (on P5, Supplementary Fig. 2A) and after (on P30, Fig. 1C-E) the onset of hearing function in mice. The immunohistofluorescence analyses showed otoferlin expression in both OHCs and IHCs of the neonatal cochleas (Supplementary Fig. 2A). Interestingly, in the adult cochlea (Fig. 1C-E), otoferlin was no longer expressed in the OHCs of the mutant similarly to the wild-type mouse. This result implies that the posttranscriptional regulation of otoferlin expression during cochlear development was unaffected by the E1799del mutation. However, at the adult stage, otoferlin clusters around the nuclei of IHCs, while the presynaptic active zone, where synaptic ribbons are located, was devoid of otoferlin (Figs. 1E and 2B). Furthermore, cochleas from P8 and P30 mice immunostained for ribeye, otoferlin, and myosin 7a showed that Otof E1799del/E1799del IHCs had significantly fewer ribbons at both ages (P8: 12.33 ± 0.31 , P30: 11.25 \pm 0.11) compared with $Otof^{+/E1799del}$ IHCs (P8: 16.33 \pm 0.51, P30: 18.02 ± 0.40 ; p < 0.0001 for two comparisons) (Figs. 1F and S2D). This suggest that proper subcellular expression and distribution of otoferlin are critical for IHC ribbon maintenance.

IHC Ca²⁺-dependent synaptic exocytosis is defective in *Otof*^{E1799del/E1799del} mutant mice

We assessed the effect of the E1799del mutation on otoferlin function by investigating Ca²⁺-dependent exocytosis in IHCs on P8 (Supplementary Fig. 2B, C) and P30 (Fig. 1G, H), corresponding to timepoints before and after hearing onset, respectively. IHC exocytosis was examined through a proxy: change in cell membrane capacitance (Δ Cm) following stepwise voltage depolarizations resulting in an opening of presynaptic voltage-gated calcium channels^{22,25}. In both genotypes, the magnitude of the Ca²⁺ current (I_{Ca}^{2+}) induced by step depolarizations from a holding potential of -80 mVwas similar in IHCs from control Otof +/E1799del and mutant Otof E1799del/E1799del mice (P8: p = 0.84 and P30: p = 0.28; two-way ANOVA). At both stages, the amplitude and voltage dependence for activation of the IHC Ca²⁺ current were comparable in both groups (Figs. 1G and S2B). We characterized the kinetics of the Ca²⁺-evoked exocytosis of the readily releasable pool (RRP) and the sustained release pool (SRP) of vesicles by depolarizing IHCs from -80 to -10 mV for periods of increasing duration, from 2 to 100 ms. The plot of ΔC_m as a function of depolarization duration indicated that on P8, only the SRP fusion was significantly reduced in Otof E1799del/E1799del/ IHCs compared to control IHCs (p < 0.0001; two-way ANOVA) (Supplementary Fig. 2C). By contrast, on P30, both the RRP and SRP were almost completely abolished in *Otof* ^{E1799del/E1799del} IHCs compared to *Otof* ^{+/E1799del} mice (RRP: p = 0.0003 and SRP: p < 0.0001; two-way ANOVA) (Fig. 1H). This indicates that otoferlin with the E1799del mutation is incapable of fulfilling its function as the main calcium sensor for IHCs synaptic exocytosis, resulting in profound deafness^{1,2}.

Gene augmentation therapy corrects the expression abnormalities of otoferlin in the IHCs of *Otof* ^{E1799del/E1799del} mice

We first investigated whether the dual AAV therapeutic approach known to be effective for gene replacement therapy in Otof null mice, as demonstrated by Akil et al.7, could also reinstate normal otoferlin production and distribution within the IHCs of Otof E1799del/E1799del mice. A single unilateral injection of the Dual AAV-Otof vector into the cochlea was administered to Otof E1799del/E1799del mice on either P2 or P30, before and after hearing onset, respectively. Three weeks after the injection, cochleas were extracted from and fixed and the organs of Corti were microdissected and immunolabeled for otoferlin and ribeye (Fig. 2A-D). Regardless of whether the gene therapy was administered on P2 (Fig. 2C) or P30 (Fig. 2D), normal otoferlin production and cellular distribution were reinstated throughout the entire cochlear spiral in a large number of IHCs from Otof^{E1799del/E1799del} mice (on P2: $70.32 \pm 4.87\%$, n = 4 mice and on P30: $93.64 \pm 1.34\%$, n = 4 mice). By contrast to IHCs from untreated mutant mice (Fig. 2B), in IHCs from treated mice, otoferlin was found to be widely distributed in the presynaptic active zone, the location of the synaptic ribbons (Fig. 2C, D). Notably, the average number of ribbons per IHC in the entire cochlea of Otof E1799del/ treated mice on P30 (12.25 ± 0.19 , n = 302 cells from 4 mice) were significantly higher than in untreated mice $(9.38 \pm 0.14, n = 311 \text{ IHCs};$ p < 0.0001; one-way ANOVA), though still lower than in wild-type mice $(14.68 \pm 0.12, n = 299 \text{ IHCs from 4 mice; test, } p < 0.0001; \text{ one-way}$ ANOVA) (Fig. 2E, panel 4). This shows that gene therapy significantly increased the number of ribbon synapses, as previously reported in Otof null mutant mice⁷.

Thus, dual AAV gene therapy administered to the cochlea of *Otof* ^{E1799del/E1799del} mice at neonatal and mature stages not only restores the production of otoferlin when it is completely absent but also effectively overcomes the expression abnormalities caused by the E1799del mutation.

Gene therapy restores IHC synaptic exocytosis and hearing function in *Otof*^{E1799del/E1799del} mice

We then investigated whether the re-establishment of both otoferlin production and normal cellular distribution restored IHC synaptic exocytosis. No differences in voltage-gated Ca²⁺ current in IHCs were observed regardless of the genotype of the mice or the stage at which they were treated (p > 0.99, two-way ANOVA; Fig. 3A, C). Nearly all IHCs from treated $Otof^{E1799del/E1799del}$ mice, assessed randomly for synaptic exocytosis, displayed a full restoration of the RRP and SRP exocytosis regardless the age of treatment, reflecting the high transduction rates observed in these mice





Fig. 2 | AAV gene therapy in *Otof*^{E1799del/E1799del} mice restores the normal production and subcellular distribution of otoferlin and IHC ribbons. Confocal microscopy images of IHCs from whole-mount preparations of the organ of Corti immunolabeled for otoferlin (blue) and ribeye (green) in the apical, middle, and basal cochlear turns of a P30 *Otof*^{+/E1799del} mouse (**A**), an untreated P30 *Otof*^{E1799del/E1799del} mouse (**B**) a P30 *Otof*^{E1799del/E1799del} mouse treated on P2 (**C**) and a P50 *Otof*^{E1799del/E1799del} mouse treated on P30 (**D**). Scale bar: 10 µm, the dotted line outlines two IHCs in the organ of Corti of an *Otof*^{E1799del/E1799del} mouse wherein the stars mark the active zone lacking otoferlin. Arrowheads indicate non-transduced



IHCs in treated mice. E Histograms illustrating the corresponding number of ribbons per IHC in the apical, middle, basal cochlear turns and the entire cochlea spiral for each case: $Otof^{+/E1799del}$ (black; apex (n = 98), middle (n = 96), base (n = 105), all cochlea (n = 299)), untreated $Otof^{E1799del/E1799del}$ (purple; apex (n = 108), middle (n = 104), base (n = 99), all cochlea (n = 311)), P2-treated $Otof^{E1799del/E1799del}$ (orange; apex (n = 97), middle (n = 98), base (n = 63), all cochlea (n = 248)) and P30-treated $Otof^{E1799del/E1799del}$ (teal; apex (n = 108), middle (n = 91), base (n = 103), all cochlea (n = 302)) mice. Data are presented as the mea $n \pm$ SEM. ****p < 0.0001, *p < 0.05 (one-way-ANOVA followed by Tukey's post hoc test).

(Fig. 3B, D). Ca²⁺-dependent exocytosis in IHCs was rescued to near-wild-type levels in treated *Otof* ^{E1799del/E1799del} mice (p = 0.99 for treated groups relative to wild-type mice, p < 0.0001 relative to untreated mice; two-way ANOVA) (Fig. 3B, D).

Interestingly, ABRs recorded 3 weeks after injection in treated mice at P2 and P30 revealed hearing thresholds similar to those in control mice (p = 0.10 and p = 0.14 for both treated groups versus wild-type mice and p < 0.0001 versus untreated mice; two-way ANOVA; Fig. 3E). Nevertheless, ABR wave I amplitude, reflecting the synchronous firing of the cochlear spiral ganglion neurons synapsing onto the IHC, showed robust improvement but did not reach the wild-type levels in mutant mice treated at P2 ($1.29 \pm 0.14 \mu$ V) or P30 ($1.27 \pm 0.12 \mu$ V) compared with wild-type mice

 $(2.45 \pm 0.14 \,\mu\text{V})$ (p = 0.0006 and p < 0.0001 respectively; one-way ANOVA), confirming the observed reduction in the number of ribbon synapses. Whereas ABR wave I latencies in mice treated on P2 $(1.44 \pm 0.13 \text{ ms})$ and P30 $(1.58 \pm 0.06 \text{ ms})$ were not significantly different from that of wild-type mice $(1.42 \pm 0.08 \text{ ms})$ (p > 0.99 and p = 0.96 respectively; one-way ANOVA) (Fig. 3E). This implies that the presence of abnormal or partially functional otoferlin in IHCs does not impede the effectiveness of the wild-type version of the protein when delivered to the deaf ear *Otof* ^{E1799del/E1799del} mice using dual AAV gene therapy approach.

It is noteworthy that the restored hearing thresholds of treated *Otof*^{E1799dd/E1799dd} mice remained normal for at least 3 months post-injection,



the last time point at which they were determined (p > 0.99 for both treated groups relative to wild-type mice and p < 0.0001 relative to untreated mice; two-way ANOVA) (Fig. 3F).

Cochlear gene therapy restores normal cortical auditory processing in *Otof*^{E1799del/E1799del} mice

The gold standard for the evaluation of peripheral hearing is widely acknowledged to be ABRs²⁶. However, it is important to note that normal ABR responses, which reflect the synchronized electrical activity of nerve fibers along the auditory pathway, do not necessarily ensure full recovery of sound perception. Furthermore, it is not known how depriving the auditory centers of sensory information during the critical period for establishing

normal tonotopy impacts gene therapy outcomes. Therefore, it is crucial to address these issues, as they are essential for speech understanding, especially when considering clinical applications in human patients. We addressed these issues by evaluating sound encoding in the central nervous system following gene therapy administration, both before the onset of hearing (P2) and as the critical period was ending (P30). We first investigated the acoustic startle response (ASR), which is mediated by a brainstem circuit linking cochlear root neurons to spinal motoneurons²⁷. We found that the ASR amplitudes of *Otof* ^{EL799del/EL799del} mice treated on P2 (n = 8) or P30 (n = 12) were similar to those of control mice (n = 10) for all sound intensities tested, from 60 to 110 dB, demonstrating a restoration of acoustic startle reactivity with respect to untreated mice (n = 12), with no significant

Fig. 3 | AAV gene therapy in Otof^{E1799del/E1799del} mice rescues IHC Ca²⁺-dependent exocytosis and restores hearing function. Mean IHC Ca²⁺ current amplitudes (ICa^{2+}) (A) and the corresponding mean capacitance changes (B) in control $Otof^{+/E1799del}$ (black, n = 15 IHCs from 3 mice, 1 female and 2 males), untreated $Otof^{E1799del/E1799del}$ mice (purple, n = 12 IHCs from 6 mice, 3 females and 3 males) and the treated mice on P2 (orange, n = 21 IHCs from 6 mice, 3 females and 3 males). Mean IHC Ca^{2+} current amplitudes (IC a^{2+}) (C) and the corresponding mean capacitance changes (**D**) in control $Otof^{+/E1799del}$ (black, n = 8 from 3 mice, 1 female and 2 males), untreated $Otof^{E1799del/E1799del}$ mice (purple, n = 12 IHCs from 3 mice, 2 females and 1 male) and the treated mice on P30 (teal, n = 15 IHCs from 4 mice). Patch-clamp recordings were performed on IHC from P20-25 mice when treated on P2 (A, B) and P45-50 mice when treated on P30 (C, D). The fusion of the readily releasable pool (RRP) and the sustained release pool (SRP) vesicles was evaluated for durations of 5–20 ms and 30–100 ms, respectively. Data are presented as the mean \pm SEM. A, C ns not significant for all groups tested and B, D ****p < 0.0001 for the wildtype and treated groups relative to untreated Otof E1799del/E1799del mice (two-way-ANOVA test followed by a Bonferroni's multiple comparison post hoc test). E, left Mean auditory brainstem response (ABR) thresholds 3 weeks post-injection for Otof +/E1799del

mice (black, n = 11, 7 females and 4 males), $Otof^{E1799del/E1799del}$ mice treated on P2 (orange, n = 15, 9 females and 6 males), Otof E1799del/E1799del mice treated on P30 (teal, n = 12, 5 females and 7 males) and untreated Otof E1799del/E1799del mice (purple, n = 9, 4females and 5 males) for pure-tone frequencies between 5 kHz and 40 kHz. Data are presented as the mean \pm SEM. ****p < 0.0001 for each group relative to untreated Otof E1799del/E1799del mice (two-way ANOVA). E right Bar graph showing the amplitude and latency of the ABR wave I for 80 dB SPL stimuli at 15 kHz in control mice (black, n = 11, 7 females and 4 males), Otof E1799del/E1799del mice treated on P2 (orange, n = 15, 9females and 6 males) and P30 (teal, n = 9, 4 females and 5 males). Data are presented as the mean \pm SEM. ****p < 0.0001, ***p < 0.001, ns not significant (Kruskal–Wallis test followed by Dunn's post hoc test). F Mean ABR thresholds 3 months post-injection for $Otof^{+/E1799del}$ (black, n = 7, 4 females and 3 males), $Otof^{E1799del/E1799del}$ mice treated on P2 (orange, n = 18, 10 females and 8 males), Otof ^{E1799del/E1799del} mice treated on P30 (teal, n = 4, 2 females and 2 males) and untreated Otof E1799del/E1799del mice (purple, n = 9, 4 females and 5 males), for pure-tone frequencies between 5 kHz and 40 kHz. Data are presented as the mean \pm SEM. ****p < 0.0001 for each group relative to untreated Otof E1799del/E1799del mice (two-way ANOVA test followed by a Bonferroni's multiple comparison post hoc test).

Fig. 4 | Gene augmentation therapy in Otof^{E1799del/E1799del} mice restores acoustic startle responses. A Acoustic startle responses were assessed with acoustic pulse-alone stimulations (60–110 dB) in P60 control mice (black, *n* = 10, 6 females and 4 males), Otof E1799del/E1799del mice treated on P2 (orange, n = 8, 4 females and 4 males) or mice treated on P30 (teal, n = 12, 5 females and 7 males) and untreated $Otof^{E1799del/E1799del}$ mice (purple, n = 12, 8 females and 4 males). B Prepulse inhibition responses of P60 control mice (black, n = 10), $Otof^{E1799del/E1799del}$ mice treated on P2 (orange, n = 8) or on P30 (teal, n = 12), and untreated *Otof* $^{E1799del/E1799del}$ mice (purple, n = 12). Pulses were presented alone or with a 60 dB SPL prepulse. Note the decrease in the response to the pulse in the presence of the prepulse for control and treated mice. The data are presented as the mean \pm SEM. ****p < 0.0001, ***p < 0.001, *p < 0.05 (two-way ANOVA test followed by a Bonferroni's multiple comparison post hoc test).



difference in the reaction to the acoustic stimuli at 105 and 110 dB between the control and the two treated groups (p > 0.99; two-way ANOVA; Fig. 4A). Furthermore, we assessed complex auditory discrimination behavior by exposing control mice, and untreated and treated Otof E1799del/E1799del mice to prepulse inhibition (PPI). This paradigm involves inhibiting the ASR by presenting an acoustic stimulus (60 dB SPL) shortly before the startling sound (120 dB SPL). The circuit mediating a prepulse in the startle reflex includes central structures of the auditory pathway, such as the inferior colliculus and the auditory cortex²⁷. Interestingly, the prepulse significantly inhibited the startle response in both control (p < 0.0001; twoway ANOVA) and treated Otof E1799del/E1799del mice, whether treated on P2 (p = 0.0006; two-way ANOVA) or P30 (p = 0.04; two-way ANOVA), but not in untreated mice (p = 0.93; two-way ANOVA) (Fig. 4B). Thus, the ASR and PPI analyses indicate that peripheral gene therapy improves central acoustic behavior in treated Otof E1799del/E1799del mice, for administrations at either the neonatal or the mature stage.

We then conducted additional behavior tests using an autonomous and automated behavioral system to evaluate the impact of gene therapy on the restoration of sound processing. Mice from the untreated, treated, and control groups were trained in a Go/NoGo task to discriminate between two pure-tone frequencies. Throughout the learning phase, mice were conditioned to lick water in response to the pure tone (Go tone) and received air puff if they licked at a different frequency (NoGo tone). Subsequently, during the test phase, the ability of the mice to differentiate between two frequencies was evaluated by gradually adjusting the frequency of the NoGo tone to bring it closer to that of the Go tone. The Go tone was kept at 6.8 kHz and the NoGo tone was progressively shifted from 13.6 to 7 kHz, corresponding to intervals between the two frequencies of 100% to 4% of the Go tone (Fig. 5A). For control mice (n = 11), the percentage of visits without licking behavior during the NoGo tone (correct rejection) was significantly higher than the percentage of visits with licking behavior (error: false alarm), for frequency intervals from 100% to 40% (p < 0.0001; two-way-ANOVA). We found no significant differences for the various frequency intervals tested in the untreated Otof E1799del/E1799del mice (n = 6, p = 0.99 at 100%, *p* > 0.99 at 40%, *p* = 0.93 at 20%, *p* = 0.99 at 10%, *p* = 0.99 at 4%; two-way-ANOVA). In contrast, the proportion of correct rejections during the NoGo tone was significantly higher in Otof E1799del/E1799del mice that received gene therapy at the neonatal (n = 10) or mature stage (n = 10), for frequency intervals of 100% (p < 0.0001 for both; two-way-ANOVA) and 40% (p = 0.0003 and p = 0.0041; two-way-ANOVA) (Fig. 5B). Those data reveal that Otof E1799del/E1799del mice treated on P2 or P30 improved their pure tonediscrimination performance and displayed a frequency-dependent response similar to the control mice.

Discussion

We explored the potential of AAV-mediated gene augmentation therapy for the treatment of forms of human DFNB9 deafness by restoring not only ABR thresholds reflecting the electrical activity of the neural fibers along the



Fig. 5 | **Peripheral gene augmentation therapy in** *Otof* ^{ECTYMERT/SM}

13340, 9334, 8004, 7337 and 7137 Hz for the NoGo tone, corresponding to frequency intervals (deltaFs) of 100, 40, 20, 10, and 4%. In a Go/NoGo task, responses are categorized as hit (correct response to Go stimuli), miss (failure to respond to Go stimuli), false alarm (response to NoGo stimuli), and correct rejection (withholding response to NoGo stimuli). **B** Performance during the NoGo tone (%) for correct rejection (closed circle) and error or false alarm (open circle) at deltaFs of 100, 40, 20, 10, and 4% for P60 control (black, n = 11, 6 females and 5 males), $Otof^{E1799del/E1799del}$ mice (purple, n = 6, 3 females and 3 males) and $Otof^{E1799del/E1799del}$ mice treated on P2 (orange, n = 10, 5 females and 5 males) and on P30 (teal, n = 10, 4 females and 6 males). The data are presented as the mean ± SEM. ****p < 0.0001, ***p < 0.001, ***p < 0.001 (two-way ANOVA test followed by a Bonferroni's multiple comparison post hoc test).

auditory pathway, but also normal auditory processing. We focused on the in-frame deletion of the glutamic acid residue at position 1804 (E1804del) within the C2F domain, which is associated with severe-to-profound thermosensitive deafness in individuals affected whilst in a febrile state¹⁸. We created and studied a mouse model by introducing the *Otof* E1799del mutation, aligning with human *OTOF* E1804del variant.

We found that although IHC expressed an abnormal form of otoferlin, which could retain partial functionality, $Otof^{E1799delE1799del}$ mice exhibit profound deafness and, as expected, a complete lack of central auditory processing. These abnormal phenotypic features are attributed to severe defects of Ca²⁺-dependent synaptic exocytosis in IHCs, arising from disruptions in both expression and subcellular distribution of the otoferlin protein and ribbon degeneration in these cells. Notably, the E1799del mutation of otoferlin leads to the complete exclusion of the protein from the presynaptic active zone of IHCs, where exocytosis occurs.

Interestingly, the profound deafness associated with *Otof* E1799del mutation is not influenced by body temperature, implying that the E1799del otoferlin protein is inactive even at the normal physiological

temperature of mice. The most plausible explanation for the absence of a temperature effect is that murine otoferlin, unlike human OTOF, lacks the arginine-rich motif responsible for the temperature-dependent expression of various membrane-associated proteins¹⁴. A similar observation was made in Otof 1515T/1515T mice, which mimic the human thermosensitive I515T mutation in the otoferlin C2C domain, leading to progressive deafness. This suggests that the severity of deafness can range from mild-to-profound, depending not only on which otoferlin C2 domain is impacted by the in-frame mutations but also on which amino acid is deleted or substituted. This variability might explain the wide range in deafness severity and the phenotypes observed in several DFNB9 forms^{5,11}. It also highlights a pattern where in-del mutations within or near the C2F domain led to profound deafness, emphasizing the crucial role of this domain in otoferlin function. This statement is consistent with findings that "mini-otoferlins" lacking the C2F domain were unable to rescue synaptic exocytosis in IHCs of Otof-knockout mice and in zebrafish Otof-knockdown models^{28,29}. Notably, the C2F domain, located near the transmembrane domain, not only binds calcium and interacts

with SNARE proteins, but also anchors itself to lipid membranes, probably facilitating the docking of synaptic vesicles to the membrane in the synaptic active zone of $IHCs^{30-32}$.

Remarkably, AAV-mediated local gene augmentation therapy in neonatal and adult Otof E1799delE1799del mice successfully erased the abnormal subcellular distribution of otoferlin, preserved the existing ribbons from degeneration, and regenerated additional ribbons, as previously reported in *Otof* null mutant mice⁷. This led to the restoration of IHC Ca²⁺-dependent exocytosis to normal levels and a sustained reinstatement of normal hearing thresholds despite reduced wave I amplitude, suggesting central gain compensation^{29,30}. Finally, our results provide key evidence that were previously missing regarding the restoration of normal auditory perception and the impact of the critical period (P10-P30), during which the plasticity of the central auditory networks gradually decreases^{19,33,34}. This decline in plasticity could potentially hinder the re-establishment of functional auditory circuits and the normal central processing of sound signals. Here, we provided the proof of concept that peripheral gene therapy is beneficial, with specific auditory functions restored even when treatment is administered at the end of the critical period¹⁹.

These findings suggest that peripheral gene therapy, whether based on gene replacement or gene augmentation, and whether administered before, during, or at the end of the critical period, has the potential to restore normal function of the peripheral hearing organ, together with central auditory sound processing and hearing perception. Furthermore, our data indicate that the current therapeutic strategy can also effectively broadened to target the various forms of otoferlin mutation and their associated deafness, thereby covering the entire spectrum of hearing loss linked to DFNB9 deafness. Finally, these findings constitute a significant milestone, further emphasizing the potential of gene therapy for tackling genetic deafness, even when administered at the end of the critical period during which the central auditory pathways have been deprived of the acoustic experience needed for their proper functional connectivity³⁵.

Data availability

The numerical data plotted (source data) in the graphs in Figs. 1–5, and Supplementary Figs. 1 and 2 are in Supplementary Data. Other data used in this publication will be made available to qualified researchers who provide a valid research question within the scope of the studies and may be subject to a data use agreement. Please direct inquiries to the corresponding author (S.S., saaid.safieddine@pasteur.fr).

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Author contributions

N.B. carried out experiments, analyzed the data, and wrote the manuscript. H.L., C.F., M.G., O.P., M.S., and B.P. carried out experiments and analyzed data. N.M., O.A., and B.G. carried out experiments and reviewed the manuscript. C.P., M.-J.L., and Y.N. provided significant feedback, contributed to data interpretation, and helped refine the manuscript. S.S. funded and coordinated the project, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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