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Extended timeframe for restoring inner ear function through gene therapy in Usher1G preclinical model

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Abstract

Neonatal gene therapy has been shown to prevent inner ear dysfunction in mouse models of Usher syndrome type I (USH1), the most common genetic cause of combined deafness-blindness and vestibular dysfunction. However, hearing onset occurs after birth in mice and *in utero* in humans, making it questionable how to transpose murine gene therapy outcomes to clinical settings. Here, we sought to extend the therapeutic time window in a mouse model for USH1G to periods corresponding to human neonatal stages, more suitable for intervention in patients. Mice defective for *Ush1g* (*Ush1g*−/−) were subjected to gene therapy after the hearing onset. The rescue of inner ear hair-cell structure was evaluated by confocal imaging and electron microscopy. Hearing and vestibular function were assessed by recordings of the auditory brainstem response and vestibulo-ocular reflex, and by locomotor tests. Up to postnatal day 21 (P) 21, gene therapy significantly restored both the hearing and balance deficits of *Ush1g*−/− mice. However, beyond this age and up to P30, vestibular function was restored, but not hearing. Our data shows that effective gene therapy is possible in *Ush1g*−/− mice well beyond neonatal stages, implying that the therapeutic window for USH1G may be wide enough to be transposable to newborn human patients.
**Introduction**

Deafness, with or without associated balance disorders, is the most prevalent inherited sensory disorder in humans, and is a major public health concern. The clinical prevalence of congenital deafness is about 1 in 700 newborns. About 80% of prelingual deafness cases are attributed to a genetic cause (1). Patients with sensorineural hearing loss are currently fitted with auditory hearing aids if they have mild-to-severe hearing loss, and with cochlear implants if they have severe-to-profound deafness. Patients with vestibular disorders currently have limited options, relying on symptomatic medication and vestibular rehabilitation therapy (2), but there is potential for a vestibular implant to become an option in the future, as suggested by Starkov et al. (3). Recent preclinical studies have revealed that gene replacement strategies can have robust and durable therapeutic effects, restoring inner ear function in mouse models of human inner ear diseases (4–9). Such demonstrations have been obtained for several congenital deafness mouse models, including models of User syndrome type I (USH1, Online Mendelian Inheritance in Man no. 276900), the most severe form of Usher’s deafness-blindness syndrome. USH1 is an autosomal recessive disease characterized by bilateral congenital profound deafness, severe vestibular deficits, and bilateral progressive retinitis pigmentosa leading to blindness (10). Patients with USH1 can benefit from early cochlear implantation, but outcomes are variable (11). The associated vestibular defects lead to a delay in motor acquisitions, gaze instability, and a higher rate of accidental falls in older individuals, which worsens with vision impairment (12). Six genes involved in USH1 have been identified, all encoding proteins involved in the mechanoelectrical transduction of sound vibrations and head motion signals, in the auditory and vestibular hair cells, respectively (10). The sensory cells of the auditory organ (the cochlea) and the vestibular organs (utricle, saccule, and the three semi-circular canals, SCC) are the hair cells, which harbor a mechanosensory antenna called the hair bundle, consisting of an array of modified microvilli (stereocilia) forming a staircase pattern at their
apical surface. The deflection of the hair bundle in response to sound or head movements results in the opening of mechanoelectrical transduction channels located at the tips of the stereocilia. This leads to the conversion of the mechanical signals into electrical signals that are conveyed to the central auditory and vestibular nuclei via the primary auditory fibers forming the VIIIth cranial nerve.

Adeno-associated virus (AAV)-mediated gene therapy during the neonatal period has proved effective in several mouse models of USH1, including USH1C and USH1G, caused by defects of the genes encoding the scaffold proteins harmonin and sans, respectively (4, 6). Like USH1G patients, Ush1g−/− mutant mice are profoundly deaf, with no identifiable auditory brainstem responses (ABRs), and vestibular dysfunctions characterized by locomotor, postural and gaze stabilization impairments (4, 13). We have shown that sans gene therapy at an early neonatal stage results in a partial rescue of hearing and a robust restoration of vestibular function in Ush1g−/− mice. However, the neonatal period of mouse inner ear development corresponds approximately in humans to the 19th week of gestation for vestibular function, and the 25th week of gestation for auditory function (14, 15). At birth, the vestibular organs and the cochlea of mice are still immature, with the functional onset occurring at about postnatal day (P)10 and P12, respectively (16, 17), and their central connections continue to develop well after P12. To establish a transposable therapeutic time window for implementing local gene therapy in the clinical setting for patients with USH1G, we investigated the extent to which such a therapy remains effective beyond P12 (i.e. in the mature inner ear) in a USH1G mouse model.

We found that viral gene replacement therapies applied to the inner ear of Ush1g−/− mice, even well into the mature stage, substantially rescued the structure and function of both the cochlea and vestibular organs. While the time window for effective treatment of vestibular defects
appears to be larger than that for the associated deafness, our results suggest the possibility of extending this time frame enough to be transposable to newborn human USH1G patients, laying the groundwork for future clinical applications.

Results

Transduction efficiency in mature inner ear hair cells

In the inner ear, the cellular tropism and transduction efficacy of recombinant AAV vectors vary depending on serotype, the promoter used, route of administration, and stage of development (18–21). We have previously observed that there is a positive correlation between the transduction rate of the inner ear sensory cells and the level of functional restoration (4). It is, therefore, essential to target the largest possible number of inner ear cells within a given therapeutic time window in order to optimize gene therapy outcomes. We used the ancestral AAV Anc80L65 vector, which has been shown to efficiently transduce the sensory hair cells of the mature inner ear (18, 22, 23). Due to inter-study variability in transduction efficiency for a given AAV depending on promoters, transgenes, vector doses and mode of purification as well as age of administration, we wanted to check these settings in house for the AAV2/Anc80L65. To this end we assessed the hair-cell transduction efficiency of this serotype expressing the green fluorescent protein (GFP) as reporter gene under the control of the CMV promoter. The viral preparation was administered through the round window membrane (RWM) in C57BL/6 wild-type mice on P20 (Figure 1A). The inner ear organs (organ of Corti, SCC crista ampullaris, utricle and saccule maculae) were micro-dissected and immunolabeled for myosin VI to stain the inner ear hair cells, and for GFP to visualize the transduced cells. AAV2/Anc80L65 transduced both cochlear and vestibular hair cells, but the transduction rate varied with cell type (Figure 1B-D). The mean transduction rate was 97±6% [83-100%] for inner hair cells (IHCs) and 13±24% [0-61%] for outer hair cells (OHCs) (n=7, Figure 1, B and
C). Transduction rates for vestibular hair cells (VHCs) were 45±24% [11-90%] in the SCC crista ampullaris (n=2, Figure 1D), and 57±17% [31-90%] in the utricle macula. The hair cell transduction rates observed in this study confirm that AAV2/Anc80L65 remains one of the most effective variant for transducing sensory cells in the adult inner ear to date, making it the most appropriate serotype for this therapeutic window study.

**Sans gene therapy restores protein targeting and rescues hair cell from degeneration in inner ear of adult Ush1g<sup>−/−</sup> mice**

We first wanted to establish the precise targeting of the virally delivered protein Sans to the top of the stereocilia of the mature cochlea. However, despite numerous attempts to remanufacture the Sans antibody we previously characterized, and the commercial one from Abcam (cat# ab150820), a nonspecific signal persisted in inner ear hair cells of adult *Ush1g<sup>−/−</sup>* mice using these antibodies. This non-specific signal hindered the direct localization of the exogenous Sans protein delivered by the AAV. We tackled this challenge indirectly by creating a viral preparation with an identical configuration, producing Sans fused to GFP (AAV2/Anc80L65-CMV-GFP-Sans-WPRE, 6.2x10<sup>12</sup> gc/mL). The viral preparation was administered through the RWM of P18 *Ush1g<sup>−/−</sup>* mice (n=4) and *Ush1g<sup>+/−</sup>* mice (n=4). Two weeks after the injection, the sensory epithelia of the cochlea and vestibular organs were microdissected and immunolabeled for actin and GFP. Interestingly, GFP shows a primarily cytoplasmic distribution when produced on its own (see Figure 1). However, when fused to Sans, GFP undergoes a relocation to the tips of IHC and OHC stereocilia. This mirrors the typical Sans localization, indicating effective protein targeting after intracochlear injection in *Ush1g<sup>−/−</sup>* mice (Figure 2). In addition, the transduction profile closely matched that obtained with AAV2/Anc80L65-CMV-GFP. As expected, the transduction of OHC was lower compared to that of IHC (respectively 25±12.2% [1-56] and 97±1.9% [93-100], n=4). Transduction of VHC was variable, and lower for cristae
ampullae compared to the utricle maculae (respectively 34±11.7% [8-56] and 49±12.6% [26-85], n=4).

We sought to define a transposable therapeutic time window applicable to USH1G patients, by investigating the outcomes of gene replacement therapy performed during the functional maturation of auditory and vestibular functions, namely between P12 and P30, in Ush1g−/− mice. The structural damage to the inner ear hair cell stereocilia is already severe in these mice at P2.5 (4), and worsens during cochlear development (13). An AAV2/Anc80L65-CMV vector driving Sans expression (AAV2/Anc80L65-CMV-Sans-WPRE 5.15x10^{12} gc/mL, Gene Transfer Vector Core) was engineered to assess its therapeutic effect after injection through the RWM into the inner ear of Ush1g−/− mice at P14 (n=4). The sensory epithelia of the cochlea and vestibular organs were microdissected four weeks after the injection and immunolabeled for actin (Figure 3 and 4). Confocal microscopy imaging showed the stereocilia to be abnormal or absent in the IHCs, OHCs, and VHCs in the untreated Ush1g−/− mice. By contrast, the stereocilia were well organized in the treated mice, in particular in IHCs and VHCs, in which they displayed staircase patterns similar to those characteristic of wild-type hair bundles. Thus, the virally driven expression of the Sans protein resulted in an effective restoration of stereocilia architecture in these cells (Figure 3, A and B, and 4A). Likewise, scanning microscopy examination confirmed that the hair bundles of inner ear hair cells in Ush1g−/− mice underwent fragmentation and degeneration starting from P2.5 and worsening with age (4). At P22, the stereocilia of both IHCs and OHCs are fewer in number and shorter throughout the cochlear spiral than those of their wild-type counterparts, while the VHC stereocilia appear collapsed (4, 13). From P22 onward, the hair-cell stereocilia in both the cochlea and vestibular end organs undergo degeneration in a spreading process, becoming more and more severe throughout the epithelium by P40 (Supplementary Figure 1). On P100, entire areas of the auditory and vestibular sensory epithelia appeared devoid of hair cells, indicating that, at this stage, the
degeneration process also affects the sensory cells. The stereocilia of the few remaining VHCs had a highly abnormal morphostructure (Supplementary Figure 1). Scanning microscopy examinations of the inner ears of treated Ush1g<sup>−/−</sup> mice on P112 showed that gene therapy had prevented cochlear hair-cell degeneration and restored stereocilia to a near-normal shape (Figure 3, B and C). The rescued OHCs had a significantly larger number of stereocilia in the tallest row than in untreated Ush1g<sup>−/−</sup> mice (16±0.4, n=10 cells, vs. 8±0.5, n=8 cells, respectively, p<0.0001, one-way ANOVA). Furthermore, the tallest OHC stereocilia were of almost normal length: 2.1±0.07 μm for treated Ush1g<sup>−/−</sup> mice (n=9 cells) and 1.8±0.08 μm (n=6 cells) for wild-type mice (p=0.02, one-way ANOVA), whereas the tallest OHC stereocilium length was reduced to 1.3±0.08 μm in untreated Ush1g<sup>−/−</sup> mice (n=8 cells; p<0.0001, one-way ANOVA). The number of stereocilia in the tallest row of IHC was also significantly higher in treated Ush1g<sup>−/−</sup> mice than in untreated mice (9±0.4, n=8 cells, vs. 4±0.4, n=6 cells, p<0.0001, one-way ANOVA), and was close to that in wild-type mice (10±0.3, n=8 cells, p=0.01, one-way ANOVA).

Similarly, the stereocilia of the VHCs in the utricular macula of the treated mice had a near-normal structure with a typical staircase pattern (Figure 4, B and C). At P40, the length of the tallest VHC stereocilia in treated Ush1g<sup>−/−</sup> mice was similar to that in wild-type mice (7.5±0.15 μm, n=13 cells, vs. 7±1.39 μm, n=8 cells, respectively, p>0.05, one-way ANOVA), whereas the VHC stereocilia in untreated Ush1g<sup>−/−</sup> mice were significantly shorter (3.7±0.35 μm, p<0.0001, one-way ANOVA, n=16). At P112, a near complete restoration of stereocilium diameter relative to that in untreated Ush1g<sup>−/−</sup> mice was observed (0.29±0.01 μm, n=9 cells, vs. 0.6±0.08 μm, n=13 cells, in treated and untreated Ush1g<sup>−/−</sup> mice, respectively, p=0.009, one-way ANOVA). However, the hair cell stereocilia had similar abnormal appearance in the crista ampullaris of treated and untreated mice. This is not a surprising finding, since the transduction rate of hair cells in the SCC sensory epithelia is lower than that observed in the utricular macula.
Of note, the hair bundle morphological aspect of the contralateral inner ear was similar to the untreated $Ush1g^{-/-}$ mice indicating little to no diffusion of the viral particles to the right ear. Additional morphological and structural analyses are provided in Supplementary Table 1.

**Time window for gene therapy to restore hearing in $Ush1g$ mice**

To assess the window for effective restoration of hearing function by gene therapy in $Ush1g^{-/-}$ mice, ABR recordings were performed at adult stages (P40 and P60) in response to tone-bursts at frequencies of 5, 10, 15, 20, 32, and 40 kHz, on untreated mice and on mice treated between P12 and P21 ($n=36$), or at a later stage (P22-P30, $n=11$). While the untreated $Ush1g^{-/-}$ mice were all found to be profoundly deaf, the mice treated between P12 and P21 showed a significant restoration of auditory function on P40 in about one third of them ($n=12$) with clearly identifiable ABR waves for frequencies between 10 and 20 kHz and sound levels of 70 dB SPL or greater ($p<0.0001$, two-way ANOVA; Figure 5, A and B). Notably, this partial rescue persisted up to P60 (Supplementary Figure 2). However, no hearing restoration was achieved when gene therapy was administered after P21 (0 out of 9 treated ears after P21), suggesting that P21 may constitute an upper limit for the restoration of hearing with this recombinant viral vector configuration. Of note, there was no correlation between the level of auditory threshold restored and the age of mice treated between P12 and P21, (Supplementary Figure 3A).

**Time window for gene therapy to restore vestibular function in $Ush1g$ mice**

We evaluated the vestibular phenotype of untreated and treated $Ush1g^{-/-}$ mice with various behavioral tests performed at least 15 days post-treatment (24). We previously showed that restoration of vestibular function to wild-type levels was obtained when unilateral gene therapy was administered at the neonatal stage (4). By contrast, unilateral treatment at later stages (P12-P30) led only to a partial and rather modest recovery (Figure 6, A-C). We hypothesized that
this partial rescue was due to a lower rate of VHC transduction, and to the lack of diffusion of
the recombinant vector to the contralateral ear in adult mice, as it occurs in neonatal mice
through the cochlear aqueduct and the cerebrospinal fluid (18, 25) (Supplementary Figure 4).
To verify this hypothesis, we assessed the restoration of vestibular function in Ush1g+/−
subjected to a bilateral injection at mature stage (P13-P25) using the behavioral tests mentioned
above. The results of all these tests indicated a significantly improved rescue of locomotor and
vestibular balance functions in these mice (Figure 6). No correlation was observed between the
rescue effect and the age of injection in mice treated either unilaterally or bilaterally
(Supplementary Figure 3, B and C).
In balance platform tests performed on P40 (Figure 6C), the untreated Ush1g−/− mice spent
much shorter times on the platform (mean: 12±2.3 s, range: [0-22s]; n=10) than mice that had
received a unilateral injection (mean: 30±5.9 s, range: [1-55s]; n=9; p=0.01, one-way ANOVA),
but those subjected to bilateral injections sustained their balance on the platform for the longest
times (mean: 43±4.8 s, range: [13-60s]; n=11; p<0.0001, one-way ANOVA). In the contact
righting test, none of the untreated Ush1g−/− mice tested (n=0/10) managed to roll over onto their
feet, whereas the majority of mice that had received injections of the gene therapy agent
(unilateral, n=7/9, p=0.002; bilateral, n=9/11, p=0.0008, Chi-squared test) displayed almost
wild-type behavior. Similarly, all the untreated Ush1g−/− mice (n=10) curled their trunks toward
their tails, whereas most of the treated mice reached or tended to reach the horizontal surface
(unilateral, n=8/9, p=0.0005; bilateral, n=9/11, p=0.0008, Chi-squared test). All behavioral
analysis is detailed in supplementary table 2.
In addition, while all of the untreated Ush1g−/− mice displayed head bobbing and circling (10/10 mice), such behaviors were observed significantly less frequently in the treated mice (unilateral injection, n=4/9, p=0.01; bilateral injection, n=4/11, p=0.004, Fisher’s exact test). Circling was
seen in 5/9 and 5/11 mice after unilateral and bilateral injections, respectively (p=0.03 and
11

$p=0.01$, respectively, Fisher’s exact test). Video-tracking on P40 showed that the number of rotations over a period of 180s was significantly lower in mice that had received bilateral injections (mean: 22±4.7, range: [10-33], $n=5$) than in untreated mice (mean: 55±8.2, range: [33-104], $n=8$, $p=0.001$ – one-way ANOVA; Figure 6A), or in mice that had unilateral injections (mean : 46±3.7, range [36-56], $n=6$, $p=0.03$, one-way ANOVA).

Mice that had received bilateral injections also covered a much shorter distance in 180 s (mean: 1430±244.8 cm, range: [840-2260 cm]) than untreated mice (mean: 2590±131.8 cm, range: [1984-3064 cm], $p<0.0001$, one-way ANOVA; Figure 6B, and Supplementary Figure 5), or mice that had received unilateral injections (mean: 2255±165.6 cm, range: [1767-2883 cm], $p=0.003$, one-way ANOVA.

Finally, during the swimming test, all the untreated $Ush1g^{-/-}$ mice ($n=10$) started to drown and had to be immediately rescued, whereas a few (2/9) of the mice receiving unilateral injections and the majority (6/11) of those receiving bilateral injections were able to swim. The recovery of vestibular function after bilateral injections persisted at least until P100 (Supplementary Figure 6).

In order to assess specific vestibular recovery and distinguished between canal- and otolith-dependent functions, we used video-oculography on P70 to investigate the vestibulo-ocular reflex (VOR) in untreated $Ush1g^{-/-}$ mice ($n=4$), $Ush1g^{-/-}$ mice receiving bilateral injections ($n=9$) between P15 and P17, and in wild-type mice ($n=11$). Due to the modest recovery obtained after unilateral injection, only 3 $Ush1g^{-/-}$ mice receiving unilateral injection were tested, and all showed a modest non-significant recovery in VOR (Figure 6, D and E). During spontaneous nystagmography (in absence of vestibular stimulation) on P70, gaze was completely stable in all wild-type mice, whereas pupil flutter was observed in all of the untreated $Ush1g^{-/-}$ mice and unilaterally treated mice. However, most (8/9) of the treated $Ush1g^{-/-}$ mice had unilateral
spontaneous nystagmus, suggesting that the restoration of vestibular function was likely asymmetric in most individuals. Of note no eye fluttering was observed in one bilateral treated mouse, indicating that complete gaze stability can be recovered following bilateral injection at mature stage.

In agreement with the observed low hair cell transduction rate in the SCC sensory epithelia, angular VOR gain, which is used to explore horizontal canal function, was absent at all frequencies studied (0.2, 0.5, 0.8, 1 and 1.5 Hz), in both treated and untreated $Ush1g^{-/-}$ mice (Figure 6D).

Remarkably, static utricular function was partially restored after bilateral gene therapy, with a significant increase in static ocular counter roll (OCR) gain in the bilaterally treated $Ush1g^{-/-}$ mice relative to untreated $Ush1g^{-/-}$ mice (0.21±0.03 [0.11-0.39], n=9, vs. 0.03±0.03 [0.00-0.10], n=4, $p=0.0063$ – two-way ANOVA), although the wild-type OCR gain of 0.61±0.09 [0.44-0.75] (n=11) was not reached (Figure 6E). The increase in OCR gain remained stable at P140 (Supplementary Figure 7).

These data, together with the structural improvement in VHC, demonstrate a significant rescue of utricle-dependent vestibular function by bilateral injections, associated with a substantial recovery of otolith-dependent gaze stabilization, improvements in balance and locomotor capacities.

**Discussion**

Despite ongoing exciting advances in applying the translational gene therapy approach to mouse models of human deafness, many challenges lie ahead before this approach can be used to treat inner ear diseases in humans (26). The preclinical investigations performed so far demonstrate the feasibility and efficacy of gene therapy for restoring hearing and balance in several mouse models of genetic inner ear defects (4–9, 27, 28). In most of these studies, the
gene therapy was carried out during the neonatal period in mice. This would correspond in humans to a time window falling between 18 and 25 weeks of gestation, implying interventions in utero. To be feasible, the clinical application of gene therapy for hearing and vestibular defects in humans will require the development of treatments that are effective when administered to mouse models at a stage that corresponds to human neonatal period. Here, we assessed the efficacy of gene therapy performed after the onset of hearing in mice (P12), in a mouse model of USH1G. We previously showed that virus vector-mediated gene therapy in neonatal $Ush1g^{-/-}$ mice results in a partial restoration of hearing and a long-lasting, almost complete recovery of vestibular function (both otolithic and semicircular canal function). The partial restoration of hearing was presumably due to the very low transduction rate of OHCs (4). Our strategy here was to deliver the Sans-encoding cDNA with AAV2/Anc80L65, a serotype which has been shown to efficiently transduce mature inner ear hair cells (23). We found that unilateral viral gene therapy in $Ush1g^{-/-}$ mice at mature stage prevented cochlear and vestibular hair cells from degeneration, and re-established the characteristic staircase organization of their stereociliary hair bundles. Interestingly, the rescue of morphostructure was more robust for IHCs than OHCs in the cochlea, whereas it was more evident in utricular than in ampullary vestibular hair cells. This finding is consistent with the differences in hair cell transduction rate observed after injection through the RWM, resulting in high transduction rates of IHCs, but a lower and variable transduction rate of OHCs and VHCs, especially in the SCC crista ampullae, at mature stages.

The improvement in cochlear hair-cell structure, mainly IHCs, led to a partial rescue of hearing, which persisted until at least P60 when AAV2/Anc80L65-CMV-$Sans$-WPRE was administered between P12 and P20. No hearing restoration was observed for treatment performed later than P21. This stage thus indicates an upper limit for the therapeutic time window beyond which
gene therapy would be ineffective, in the Ush1g−/− mouse model. The partial improvement of
hearing observed probably indicates that the number of transduced OHCs — the functions of
which are essential for cochlear amplification and normal auditory function — remains below
that required to fully rescue hearing. Indeed, mouse models of DFNA25 and DFNB9, which
are both profoundly deaf, and where the IHCs are defective but the OHCs are functional, gene
therapy administered at a mature stage restored hearing to near-normal thresholds (5, 28). This
finding suggests that improvements in the efficiency of OHC transduction are paramount for
the restoration of normal hearing thresholds in deafness affecting both IHCs and OHCs
function. This conclusion is consistent with the reported positive correlation between the rescue
of hearing threshold and the percentage of sensory cells transduced within the inner ear (4). It
may, therefore, be possible to rescue the inner ear function of Ush1g−/− mice to wild-type levels
by using an AAV serotype that transduces OHCs more efficiently. Unfortunately, the OHCs of
the mature cochlea have proved resistant to transduction with all AAV serotypes tested to date
(18, 22, 23, 29, 30).
Our results also show that the bilateral delivery of AAV2/Anc80L65-CMV-Sans-WPRE to the
inner ears of Ush1g−/− mice at a mature stage induces significantly greater improvements in
vestibular behavior than the unilateral delivery (Figure 6, A-C). Gene therapy administered at
neonatal stages resulted in an improvement in vestibular behavior to wild-type levels (4). The
long-term restoration of locomotor function to near-normal levels observed in mice treated at
neonatal stages is due to the high rate of VHC transduction in both ears, probably reflecting
contralateral leakage of the viral preparation (Supplementary Figure 4). By contrast, very few
of the contralateral inner ear cells were transduced following unilateral gene therapy at a later
stage (18), at which VHC transduction rates were also much lower.
Remarkably, bilateral gene therapy carried out as late as P30 led to significant utricular recovery in \textit{Ush1g} \textsuperscript{\textminus/\textminus} mice, suggesting that the time window for gene therapy is broader for vestibular deficits linked to USH1G than for the associated deafness. Interestingly, a significant restoration of many locomotor-related behaviors was observed, despite static head tilt analysis showing restoration of only a third of the OCR gain. Since there was no recovery of canal-related function, our results confirm that otolithic organs, encoding the head-in-space position relative to gravity, provide an essential contribution for balance, postural control and locomotion (17). We have previously shown that mice with only two-thirds of the normal OCR gain conserve normal swimming ability, while animal without otolith function are unable to swim (31). This suggests that even with partial restoration, the redundancy of hair-cell directions in the macula, taking into account hair cells with opposite axes on either side of the curved striola (in both ears), could compensate for hair-cell loss. Our results seem to confirm this, as performance in balance and locomotion tests exceeded expectations given the absence of improvements in angular VOR gain in mice treated with bilateral injections. This finding is very encouraging for gene therapy in humans, as even if transduction rates are low, a partial restoration of otolithic organ function may be of great benefit for the development of children with USH1.

In conclusion, our study has established the upper limit of the time window during which gene therapy can effectively restore hearing and vestibular functions in \textit{Ush1g} mice, with extensions up to P21 and P30, respectively. It is essential to emphasize that the observed restoration of auditory and vestibular functions in \textit{Ush1g} mice suggests that the exogenous Sans protein, administered to these mice during mature stages, has the capability to integrate into the Usher1 network, crucial for hearing and balance functions (32). Therefore, AAV vector-based gene therapy strategies targeting the near-mature inner ear can significantly rescue the structure and function of both auditory and vestibular organs. Considering that a 21-day-old mouse represents
a therapeutic window in humans similar to that of a young individual aged 3 to 4 years, these findings establish a transposable therapeutic time window to newborn human patients and laying the foundations for future clinical applications (33). Indeed, today, systematic screening for hearing loss in newborns which is widely adopted throughout the world, improved the early diagnosis and intervention in neonatal and time-to-intervention.

**Methods**

**Viral vector construct and packaging**

The p0101_CMV-SV40-Sans-bGH plasmid was generated by amplifying the murine *Sans* cDNA sequence (GenBank accession no. NM_176847) by PCR (1401 bp amplicon, with 5’-GGCGGCGCCGCACCAGTATCACC-3’ as the forward primer and 5’-GGAAGCTTACA TAGCTCCGTGTCCTCCA-3’ as the reverse primer) from the pCMV-Tag 3B mSans plasmid (Snapgene). It was then inserted into the pAAV.CMV.PI.EGFP.WPRE.bGH vector (Addgene) and packaged into the Anc80L65 capsid. The Sans construct fused to GFP (CMV-GFP-Sans-WPRE) was generated using gene synthesis and plasmid subcloning (pAAV.CMV.PLEGFP.WPRE.bGH) methods (Genscript, Europe). Two recombinant viruses, AAV2/Anc80L65-CMV-Sans-WPRE and AAV2/Anc80L65-CMV-GFP, were produced at the Gene Transfer Vector Core facility (Grousbeck Gene Therapy Center, Schepens Eye Research Institute / Mass. Eye and Ear, Boston, USA), at a titer of 5.15×10^{12} genome copies per milliliter (gc/mL) and 5.5×10^{12} gc/mL, respectively. The AAV2/Anc80L65-CMV-GFP-Sans-WPRE recombinant virus was produced by ETH Vector Core facility (Zurich) at a titer of 6.2×10^{12} gc/mL.
Animals

All studies were performed in mice (both male and female) with a mixed C57BL/6j genetic background. The generation and characterization of Ush1g-knockout mice has been described elsewhere (13). Animals were housed in the Institut Pasteur animal facilities accredited by the French Ministry of Agriculture for experiments on live mice.

Ush1g recombinant animals were genotyped by means of two PCR amplifications, with the oligo-fw1 (5’-GGCCTCGAAGAAGATCCTG-3’) and oligo-rev (5’-GGCAAGTCAAAGGATCAGAT-3’) primers used to detect the wild-type allele (460 bp amplicon), and the oligo-fw2 (5’-CAGTTTCCCCATGTTGATCACCAAC-3’) and oligo-rev primers used to detect the presence of an allele with a deletion of the Ush1g exon 2 (332 bp amplicon) and the wild-type (1964 bp amplicon) allele.

Vector delivery to the inner ear

All surgical procedures and viral injections were performed in a biosafety level 2 laboratory. Mice were anesthetized with isoflurane (4% for induction and 2% for maintenance). At the start of surgery, a subcutaneous injection of an analgesic, meloxicam (Metacam®, 0.2 mg/kg/day) was administered for pain relief, together with a subcutaneous injection of local anesthetic (lidocaine, Laocaïne®, 5 mg/kg) in the retro-auricular region. The anesthetized animal was placed on a heating pad throughout the procedure and until the mouse was fully awake.

Intracochlear injections were performed as described by Akil et al. (5). The left and/or right ear were approached via a retro-auricular incision. After dissection of the cervical muscles, the otic bulla was exposed and punctured with a 25G needle. The opening was expanded as necessary with forceps to visualize the stapedial artery and the RWM. A glass pipette was used to puncture the center of the RWM and the viral solution (2 µl of viral vector) was then injected through the RWM with a pump system coupled to a glass micropipette. The RWM niche was rapidly
sealed after removal of the pipette, with a small plug of muscle secured with a small drop of biological glue (Vetbond® 3M) placed on the muscle to prevent leakage from the round window, and with a small plug of fat to close the opening of the bulla.

**Immunofluorescence**

The inner ear was perfused with 4% paraformaldehyde in PBS for 45 min at 4°C, and the cochlea and vestibular organs were then microdissected, and rinsed three times in PBS for 10 min each. The samples were incubated for 1 h at room temperature in PBS supplemented with 20% normal horse serum and 0.3% Triton X-100, and were then incubated overnight in PBS with primary antibodies: chicken anti-GFP (1/500; Cat# 13970, Abcam), rabbit anti-myosin VI (1/200) (34). The next day, the samples were rinsed three times in PBS for 10 min each, and incubated for one hour at room temperature with the following secondary antibodies: ATTO-488-conjugated goat anti-chicken IgG antibody (1:500 dilution; Cat# A11039, Thermo Fisher Scientific) and ATTO-550-conjugated goat anti-rabbit IgG antibody (1:500 dilution; Cat# 43328, Sigma-Aldrich). The organ of Corti and vestibular organs were mounted in Fluorsave (Calbiochem, USA). Images were captured with a Zeiss LSM-700 confocal microscope equipped with a Plan Apo-chromat 63×/1.4 N.A. oil immersion lens (Carl Zeiss).

**Hair-cell counting**

Transduction rates in the treated ears were calculated by dividing the number of hair cells immunolabeled for GFP by the total number of IHCs, OHCs, and VHCs immunolabeled for myosin VI. Counts were obtained for the apical, middle, and basal regions of the cochlea.
Scanning electron microscopy

The inner ear was fixed by incubation in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 2 hours at room temperature. The cochlea and vestibular organs were microdissected. The samples were then incubated in five alternating baths (OTOTO) of 1% osmium tetraoxide (O) and 0.1 M thiocarbohydrazide (T). The samples were rinsed and dehydrated in a graduated series of ethanol solutions (35, 50, 70, 85, 95 and 100%), then dried to critical point with hexamethyldisilazane. The samples were analyzed in a Jeol JSM6700F-type field emission scanning electron microscope operating at 5 kV. Images were captured with a charge-coupled camera (SIS Megaview3, Surface Imaging Systems).

Hair-bundle analysis

Scanning electron microscopy images were analyzed with Photoshop CS6. The length of the stereocilia was determined for the tallest row of IHCs, OHCs, and VHCs. The stereocilia in the tallest row in IHCs and OHCs, and the middle row in VHCs were counted. Finally, the diameter of the largest stereocilium in the hair bundle was measured for VHCs.

Audiological tests

ABRs to sound stimuli were recorded and analyzed as previously described (4). ABRs to sound stimuli were recorded at least 15 days post-injection. Briefly, the mice were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg) and 2% xylazine hydrochloride (10 mg/kg) and placed in a sound-proofed chamber. Subdermal needle electrodes were placed at the vertex, the ipsilateral mastoid (reference), and on the back (ground). Pure-tone stimuli (bursts) were used at frequencies of 5, 10, 15, 20, 32 and 40 kHz. The hearing threshold was defined as the lowest-level stimulus for which ABR peaks for waves I-V were clearly defined and repeatedly present upon visual inspection.
Behavioral analysis

Various behavioral tests were performed to assess vestibular deficits (24). We first carefully observed the mouse to look for circling behavior or head tossing. The trunk curl test was then performed, with the mouse held by its tail: the test was considered successful if the mouse reached a horizontal landing surface (score of 1), partially successful if the mouse almost reached the horizontal surface (score of 0.5), and unsuccessful if the mouse curled its trunk toward its tail (score of 0). The contact righting test was performed by placing the mouse in a transparent tube and determining whether it could right itself rapidly when the tube was rotated 180° (score of 1), whether the rollover occurred but more slowly (score of 0.5), or whether the mouse failed to right itself (score of 0). In the platform test, the time spent by the mouse on a platform (7 cm×7 cm wide and at a height of 29 cm) before falling off was determined over a period of 1 min. The test was repeated three times and the mean time spent on the platform over the monitoring period was determined. We also scored the swimming ability of each mouse in a container (at least 15 cm deep) filled with water at 22–23°C over a period of 1 min: score of 0 if the mouse swam correctly, with the body elongated and the tail propelling in a flagella-like motion; 1 if swimming was irregular (vertical swimming, swimming in a circle, swimming on the side, swimming in an unbalanced manner); 2 if the mouse remained in an immobile floating position; 3 if the mouse was drowning.

Finally, mouse behavior was recorded and analyzed with EthoVision XT video tracking software (Noldus Information Technology®, Wageningen, The Netherlands). The mouse was placed in its cage and tracked by video over a period of three minutes. The location and movement of the tip of the noise, the central point of the animal and the tail base were
determined. The distance covered (in cm) and the number of rotations during three minutes were recorded.

Vestibulo-ocular reflex analysis

The experimental set-up, apparatus and data acquisition mechanisms were as previously described (35, 36). For pupil position recording with the head fixed, a head post was implanted in the skull of the animal at least 48 h before vestibular exploration, following the methodology previously described (37). All eye movements were recorded in the dark with an infrared video system (ETL-200, ISCAN, Burlington Massachusetts, USA), using non-invasive video-oculography methods to record pupil and corneal reflection (CR) position. Mice were head-fixed in a ~30° nose-down position to align the horizontal canals with the yaw plane. Myosis was induced by the topical application of 2% pilocarpine 10 minutes before the experiment. The recorded eye and head position signals were sampled at 1 kHz, digitally recorded (CED power1401 MkII, Cambridge, United Kingdom) with Spike 2 software and exported into the Matlab programming environment for off-line analysis (Matlab, The MathWorks, Natick, Massachusetts, USA). Videonystagmography was performed to record spontaneous eye movements without vestibular stimulation and eye movements during sinusoidal rotation for the horizontal angular vestibulo-ocular reflex (aVOR) and static head tilt roll for the ocular counter-roll (OCR) or utriculo-ocular reflex. Briefly, aVOR was tested during horizontal sinusoidal rotation of the turntable (at 0.2; 0.5; 0.8; 1 and 1.5Hz; peak velocity 30°/s), with gain and phase analyzed. The gain was defined as the amplitude of the eye (response) rotation over the amplitude of the head (stimulus) rotation. As the head of the animal was fixed to the rotating table, the movements of the head and the table were identical. The phase was the temporal shift between the eye and table rotations, expressed in degrees as a ratio of the sinusoidal cycle (2 pi) (38). The static OCR tests specifically tested the static utriculo-ocular reflex. Vertical pupil
position was measured as a function of head tilt angle with the platform tilted to different roll positions, at 10°, 20°, 30° and 40° to the right and left alternately. Measurements were made in a static position over at least 15 seconds, to identify the stable pupil position. The vertical eye angle was then calculated from the raw vertical pupil position (39). The OCR gain was calculated as the slope of a linear regression line for both variables (vertical eye angle and head tilt in degrees).

**Statistics**

Values are presented as n (%) or mean ± SEM. All statistical analyses were performed with Prism 6 (GraphPad Software, San Diego, California, USA) after use of the D’Agostino & Pearson omnibus normality test to determine whether the data were normally distributed. For quantitative data, Mann-Whitney U tests or Student’s t-tests were performed for two-group comparisons, and one- or two-way ANOVA was used for comparisons of more than two groups, with systematic correction for repeated measures. Two-tailed Fisher’s exact tests and Chi-squared tests were used for qualitative data. Differences were considered to be significant if p<0.05.

**Study approval**

Animal experiments were performed in accordance with INSERM and Institut Pasteur welfare guidelines. VOR measurements were specifically approved by the ethics committee of Université Paris Cité.

**Data availability**

All data reported in this paper are available from the corresponding author upon request.

Data point values for all graphs are found in the Supporting Data Values file.
Author contributions

GL designed and performed experiments on mice (injections, auditory and vestibular assessments, immunofluorescence, scanning electron microscopy), analyzed data, and wrote the manuscript.
CC performed experiments on mice (injections, auditory and vestibular assessments).
FS conducted experiments (vestibulo-ocular reflex recordings), analyzed data, and wrote the manuscript.
VM conducted experiments (scanning electron microscopy).
LA performed experiments on mice (auditory and vestibular assessments).
BP performed experiments on mice (auditory and vestibular assessments, immunofluorescence).
JB analyzed data.
MJL reviewed the manuscript.
MB designed VOR experiments, secured funding and ethical authorizations, analyzed data and wrote the manuscript.
CP reviewed the manuscript.
SS designed the research study, analyzed data, and wrote the manuscript.
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References

Figure 1. AAV2/Anc80L65 efficiently transduces mature inner ear hair cells. (A) The upper panel shows a schematic representation of the inner ear (the cochlea and vestibular sensory epithelia in purple), illustrating viral vector injection through the round window membrane (RWM) of the cochlea. The vestibular hair cells (VHC) are located in the crista ampullae (AC) of each semi-circular canal, and in the utricular and saccular macula (UM and SM) (left inset), whereas the sensory hair cells of the cochlea (inner hair cells (IHCs) and outer hair cells, (OHCs) are harbored in the organ of Corti (right inset). (B) Organ of Corti, spanning from the middle to apical turns of the cochlea in a wild-type mouse, underwent AAV2/Anc80L65-CMV-GFP injection on P20 and immunostained for myosin 6 (in red) and GFP (in green) on P25. (C, D) Maximum-intensity projections of confocal z-sections of IHCs from the cochlear middle turn (C) and SM (D). Almost all IHCs and most VHC were transduced with AAV2/Anc80L65. Scale bars: 50 µm for B; 10 µm for C and D.
Figure 2. AAV2/Anc80L65 vector-mediated transfer of Sans cDNA fused to GFP restores protein expression and targeting. (A) Schematic representation of the mechanoelectrical transduction (MET) machinery in the hair bundle of inner ear hair cells. The Ush1g protein (Sans protein) is located in the upper tip-link density, together with the Myosin7A (MYO7A) and the Harmonin. The tip-link connecting two stereocilia is composed of the Cadherin23 (CDH23) and the Protocadherin15 (PCDH15) proteins. (B, C) Confocal images of auditory (B) and vestibular (C) hair cells from P32 Ush1g−/− mice following intracochlear injection of AAV2/Anc80L65-CMV-GFP-Sans-WPRE at P18, immunolabeled for actin (in orange), and GFP (in green), showing that the GFP-Sans exhibits a localization pattern mirroring the typical distribution of the protein Sans in the transduced IHC, OHC and VHC. GFP-Sans is localized at the tip of the stereocilia (white arrow). The dotted circle delineate transduced (green) and non-transduced (white) VHCs. Scale bars: 5 µm for low magnification and 1 µm for high magnification.
Figure 3. Viral vector-mediated transfer of the Sans cDNA at a mature stage restores cochlear hair-cell architecture. (A) Confocal images of auditory hair cells from P45 wild-type (at left), untreated Ush1g−/− (at middle) and treated Ush1g−/− (at right) mice, immunolabeled for actin (in orange), and DAPI staining the nuclei (in blue). Scale bars: 10 µm. The insets are
a close-up view of the OHC and IHC at high magnification showcasing the recovery of a near-normal stereocilia shape in the treated mice. Scale bars: 3 µm. (B) Low- and intermediate-magnification scanning electron micrographs of the organ of Corti of wild-type (at left), untreated *Ush1g*<sup>−/−</sup> (at middle) and treated (at right) *Ush1g*<sup>−/−</sup> mice, showing a partial restoration of hair-bundle architecture in IHCs and OHCs. Scale bars: 50 µm; inset: 1 µm. (C) Comparative analyses of stereocilia number and length in the tallest row in both OHCs and IHCs showing a significant improvement in *Ush1g*<sup>−/−</sup> mice after gene therapy treatment (blue) relative to untreated mice (red), the measurements for wild-type mice are shown in black (one-way ANOVA). Number of stereocilia in the higher row of OHC, IHC, and length of OHC stereocilia were assessed respectively in 10, 8, and 9 treated *Ush1g*<sup>−/−</sup> mice; 8, 6 and 8 untreated *Ush1g*<sup>−/−</sup> mice; and 10, 8, and 6 wild-type mice.
Figure 4. Viral vector-mediated transfer of Sans cDNA restores vestibular hair-cell architecture. (A) Low- and inset at high-magnification confocal microscopy images of utricular hair cells from P45 wild-type (at left), untreated $Ush1g^\text{-}$ (at middle) and treated (at right) $Ush1g^\text{+}$ mice, immunolabeled for actin (in orange), demonstrating that the vestibular sensory epithelium of the treated $Ush1g^\text{-}$ mouse is populated with hair cells with stereocilia of nearly normal shape, similar to those in the wild-type mouse. In contrast, the vestibular epithelium of the untreated $Ush1g^\text{-}$ mouse is
entirely devoid of hair cells with stereocilia. Scale bars for low- and high-magnification images: 10 µm and 5 µm, respectively. (B) Low- and high-magnification scanning electron micrographs of the utricular sensory epithelium of P112 wild-type (upper panel), untreated Ush1g−/− (middle panel) and treated Ush1g−/− (lower panel) mice. These results show that gene therapy treatment prevents vestibular hair cell (VHC) degeneration and restores the staircase pattern of hair bundles. (C) Comparative analyses of the hair-cell stereocilia on P40, between treated Ush1g−/− mice displaying a full recovery of stereocilia length (blue), wild-type mice (black), and untreated Ush1g−/− mice (red) (upper graph, p<0.0001, one-way ANOVA; n=13, 16, and 8 respectively for treated Ush1g−/−, untreated Ush1g−/−, and wild-type mice). On P112, considerable heterogeneity in VHC stereocilium diameter is observed in untreated Ush1g−/− mice; this heterogeneity was significantly decreased after gene therapy treatment (lower graph, p=0.009, one-way ANOVA; n=9, 13, and 4 respectively for treated Ush1g−/−, untreated Ush1g−/−, and wild-type mice).
Figure 5. AAV2/Anc80L65-Sans gene therapy at the P12-P21 stage restores hearing in Ush1g⁻/⁻ mice. (A) ABR traces for 15 kHz stimulation in wild-type, treated Ush1g⁻/⁻, and untreated Ush1g⁻/⁻ mice. (B) ABR thresholds in P40 wild-type (n=3), untreated Ush1g⁻/⁻ (n=5), and treated Ush1g⁻/⁻ mice (n=12), showing a partial recovery for the 10, 15, and 20 kHz frequencies (two-way ANOVA).
Figure 6. AAV2/Anc80L65-Sans gene therapy at the P12-P30 stage restores the balance function in Ush1g−/− mice. (A, B, C) Locomotor tests performed at P40, comparing wild-type (black), untreated Ush1g−/− (red), and treated Ush1g−/− mice after unilateral (orange) or bilateral (blue) AAV2/Anc80L65-Sans injection. (A) Videotracking, performed respectively on 5, 6, 8 and 10 bilaterally, unilaterally treated, untreated Ush1g−/−, and wild-type mice, showed a significant improvement after bilateral injection in circling behavior ($p=0.001$, one-way ANOVA). (B) Distance traveled ($p<0.0001$, one-way ANOVA) relative to untreated Ush1g−/− mice. (C) In the platform test, Ush1g−/− mice subjected to unilateral ($p=0.019$, one-way ANOVA, n=9) or bilateral ($p<0.0001$, one-way ANOVA, n=11) injections of gene therapy agent spent longer time on the platform than untreated mice (n=10); 9 wild-type mice were analyzed. (D, E) Angular vestibulo-ocular reflex gain (aVOR, D) and static ocular counter-roll gain (OCR, E), determined by video-oculography after sinusoidal horizontal rotation (at different frequencies) and static head tilt roll, respectively, at P70 respectively on 9, 3, 4, and 11 bilaterally treated (blue), unilaterally treated (orange), untreated Ush1g−/− (red), and wild-type mice (black). Static OCR gain was significantly greater after bilateral injection than in untreated Ush1g−/− mice ($p=0.0063$, one-way ANOVA).