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Article

Gap Junction Plaques Fragmented in the Cochlea of a Mouse Model of the Compound Heterozygous p.D50N Variant in Gjb2

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Simple Summary: Individuals carrying variants in the *GJB2* gene may experience either congenital hearing loss or last-onset progressive hearing loss. The underlying reasons for the variability in hearing remain poorly understood. To elucidate the mechanisms by which Cx26 variants contribute to mild to moderate hearing loss, this study established a mouse model, the *Gjb2*$^{\mathrm{D50N/-}}$ mice, which exhibited progressive mild to moderate hearing loss. We hypothesized fragmented gap junction plaques leading to impaired metabolite transfer may be responsible for hearing loss in the Cx26 variants.

Abstract: The *GJB2* gene, which encodes the Connexin26 (Cx26), is the gene most frequently linked to hearing loss. Individuals carrying variants in the *GJB2* gene may experience either congenital hearing loss or last-onset progressive hearing loss. The underlying reasons for the variability in hearing remain poorly understood. Current research primarily focuses on conditional knockout mouse models, which generally exhibit moderate to severe hearing loss. To elucidate the mechanisms by which Cx26 variants contribute to mild to moderate hearing loss, this study established a mouse model, the *Gjb2*$^{\mathrm{D50N/-}}$ mice. Auditory brainstem response (ABR) indicated that the *Gjb2*$^{\mathrm{D50N/-}}$ mice led to a mild to moderate increase in hearing thresholds. Further investigations demonstrated that in the *Gjb2*$^{\mathrm{D50N/-}}$ mice, the gap junction plaques (GJPs) between supporting cells were fragmented, and the capacity of outer hair cells (OHCs) to uptake 2-NBDG (a glucose analog) was reduced. In conclusion, we hypothesize that the mild to moderate hearing loss associated with Cx26 variants may not be attributable to structural abnormalities within the cochlea, but rather to dysfunction of GJPs, which compromises the energy supply to OHCs. In this study, we first established a *Gjb2* compound heterozygous mouse model that causes mild to moderate hearing loss, for subsequent intervention and treatment.

Keywords: *GJB2*; CRISPR-Cas9; hearing loss; metabolic product transport impairment

1. Introduction

Hearing loss is the most prevalent sensory impairment, affecting at least 1.1 out of every 1,000 newborns with permanent hearing loss [1]. In developed countries, genetic factors contribute to 50-60% of hearing loss cases in children [2]. To date, over 120 genes linked to non-syndromic hearing loss have been identified [3], with *GJB2* variants are highly prevalent around the world [4]. The *GJB2* gene encodes gap junction beta-2 protein, as known as Connexin26 (Cx26). Cx26 aggregates on the cell membrane to form gap junction plaques (GJPs) that facilitate intercellular communication and material transfer, playing a crucial role in maintaining the inner ear's homeostasis [5–8].

As gene sequencing increasingly becomes a standard approach for diagnosing congenital hearing loss, the heterogeneity of auditory phenotypes has attracted heightened scrutiny, suggesting that the extent of hearing impairment may be significantly modulated by genetic factors [9]. The

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35delG variant is widely distributed across various populations and is frequently associated with profound congenital hearing loss [4,10,11]. In contrast, the V37I variant exhibits a high carrier frequency and typically presents with mild to moderate hearing loss [12]. The D50N variant is predominantly linked to Keratitis-Icthyosis-Deafness (KID) syndrome, with carriers potentially experiencing late-onset mild to moderate hearing loss or profound hearing loss at birth [13–15]. The substantial variability observed in auditory phenotypes implies that Cx26 may be involved in diverse mechanisms of deafness. Prior research has largely concentrated on Cx26 conditional knockout mice, revealing that early Cx26 deficiency can result in profound hearing loss due to structural abnormalities in the organ of Corti, loss of hair cells (HCs), and degeneration of spiral ganglion neurons (SGNs) [16–19]. Variants in Cx26 can be classified into truncating mutations, frameshift mutations, and missense mutations. Depending on the specific variant types and their locations, Cx26 variants can be categorized into several groups: those that are incapable of forming GJPs, those that produce non-functional GJPs, those exhibiting diminished ion permeability or substance exchange in the formed GJPs, and those that do not display functional abnormalities in GJPs. Consequently, the development of novel models is imperative for a more thorough and comprehensive investigation into the potential mechanisms associated with Cx26-related hearing loss.

Clinically, patients frequently present with two distinct types of allele variants. Due to the homozygous lethality linked to the *GJB2* gene, we utilized CRISPR-Cas9 technology to generate the *Gjb2*$^{D50N/+}$ mice, which were subsequently crossed with the *Gjb2*$^{loxP/loxP}$; Rosa26^{CreER} mice to produce the *Gjb2*$^{D50N/-}$ mice. We conducted auditory brainstem response (ABR) assessments and performed pathological examinations on these mice. Our results indicated that the *Gjb2*$^{D50N/-}$ mice exhibited mild to moderate progressive hearing impairment, which may not be attributed to the loss of HCs or SGNs. Importantly, we observed significant fragmentation of GJPs within the supporting cells (SCs), and the notably shortened GJPs were associated with diminished glucose uptake in HCs. We hypothesize that insufficient nutrition supply may further exacerbate HCs dysfunction and contribute to the hearing loss. In our study, we provided an ideal mouse model of mild to moderate hearing loss, which are of great significance for the development of subsequent therapy.

2. Materials and Methods

2.1. Mouse Models

According to the Ensembl database, the mouse *Gjb2*-201 transcript (transcript length 2406 bp, encoding 226 amino acids, spanning 2 exons) was selected for further analysis. Based on the mouse *Gjb2* gene sequence and the requirement for a point mutation, guide RNAs (gRNA) were designed to replace the codon GAT with AAC in Exon 2, corresponding to the *Gjb2* p.D50N variant. Meanwhile, the donor vector was synthesized and verified for accuracy through sequencing. Subsequently, the CRISPR-Cas9 system and the donor vector were mixed at appropriate concentrations and microinjected into C57BL/6JGpt mouse zygotes. The zygotes were then implanted into the uteri of pseudopregnant C57BL/6JGpt female mice for gestation and offspring production. Genomic DNA was extracted from the tails or toes of the mice 5–7 days post-birth using the One Step Mouse Genotyping Kit (Vazyme, China; PD101-01). The target region was amplified by PCR using the following primers: F1: 5'- TTG TCA CCT ATC AGC AGC CTA GAGG-3'; R1: 5'- TTT CAT GTC TCC GGT AGG CCA-3'. Sanger sequencing of the amplified products was then performed to confirm the genotype of the mice. Finally, mice with genotypes *Gjb2*$^{D50N/wt}$ or *Gjb2*$^{D50N/+}$ were obtained through breeding.

Mice were raised in a specific-pathogen-free experimental animal center at Huazhong University of Science and Technology. Due to the homozygous lethality in the GJB2 gene, the *Gjb2*$^{D50N/+}$ mice were crossbred with *Gjb2*$^{loxP/loxP}$; Rosa26^{CreER} mice. Consequently, the *Gjb2*$^{D50N/loxp}$; Rosa26^{CreER} mice were obtained. In our study, the mouse model of *Gjb2*$^{D50N/-}$ were generated by subcutaneous injection of tamoxifen (1.5mg/10g body weight; Sigma-Aldrich, Germany; T5648-1G) diluted in a fat emulsion at P0 and P1.

All experimental procedures were conducted under the policies established by the Committee on Animal Research at Tongji Medical College, Huazhong University of Science and Technology.

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2.2. *Auditory Brainstem Response (ABR)*

The hearing thresholds were measured using Auditory Brainstem Response (ABR) at postnatal days 20 (P20) and 90 (P90). A 1.25% solution of tribromoethanol (0.2ml/10g, intraperitoneally; AibeiBio, China; M2910) was administered to anesthetize the mice. The mice were then placed on a 37°C thermostatic electric blanket to maintain their body temperature within a sound-attenuating chamber. The Tucker-Davis Technology (TDT) System was employed to measure the hearing threshold, as previously published [16]. Tone burst stimuli were generated by the TDT System at frequencies of 8 kHz, 16 kHz, 24 kHz, and 32 Hz, and the responses were amplified and averaged over 1024 times. SigGen32 software (Tucker--Davis Technologies) was used to record the ABR signals. The lowest sound level that elicited a repeatable wave was considered the threshold.

2.3. *Cochlear Tissue Preparation and Immunofluorescent Labeling*

Animals were anesthetized with 1.25% tribromoethanol (0.2ml/10g, intraperitoneally) and perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The cochleas were carefully dissected from the temporal bones and fixed in 4% PFA at 4°C overnight. Following decalcification in 10% ethylenediaminetetraacetic acid disodium salt (disodium EDTA), the cochleas were dehydrated sequentially with 10%, 20%, and 30% sucrose solutions. Tissue-Tek® O.C.T. Compound (Sakura Finetek, America; 4583) was used to embed the processed tissue specimens, allowing for the cutting of sections with a thickness of 10 μm for morphological examination.

For the flattened cochlear preparation, the cochleae were meticulously dissected from the decalcified tissue using a stereomicroscope. Three segments of the basilar membrane- apical, middle, and basal turn were selected for staining. After permeabilization with 0.1% Triton X-100 in 0.01 M PBS for 20 minutes, the samples were immunolabeled overnight at 4°C with a 1:500 dilution of anti-Cx26 antibody (Rabbit; Invitrogen, America; 71-0500) and anti-CD45 antibody (Goat; R&D system, America; AF114). The secondary antibodies used were Alexa Fluor® 488 donkey anti-rabbit (Antgene, China; ANT024S) and Alexa Fluor® 647 donkey anti-goat (Antgene, China; ANT041S) which were applied at room temperature (22–25°C) for 90 minutes, and nuclei were stained with 4′,6–diamidino-2-phenylindole (DAPI, Antgene, China; ANT165) for 15 minutes. Images were obtained using a laser scanning confocal microscope (Nikon; Japan).

2.4. *Assessment of In Vivo 2-NBDG Uptake in the HCs*

Mice with different genotypes were anesthetized via an injection of 1.25% tribromoethanol (0.2 ml/10g, i.p.). A needle penetrated the left ventricle to introduce solutions into the cardiovascular system of the mice. A 3.8 mM solution of 2-NBDG (Invitrogen, America; N13195) in stroke-physiological saline solution was perfused into the mice for 2 minutes. After 5 minutes, the basilar membranes from the apical turn were rapidly dissected from the cochleae prepared for a flattened cochlear preparation. Finally, images were obtained using a laser scanning confocal microscope, and the fluorescent intensity of the area of interest was measured with ImageJ software.

2.5. *Data Analysis*

Statistical analyses were conducted using GraphPad Prism version 9.5 (GraphPad Software Inc., La Jolla, CA, USA). Data presented in the figures are expressed as mean ± standard deviation (SD). The least significant difference (LSD) post hoc test was employed to compare differences between two of the groups. In all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. *The Gjb2$^{D50N/-}$ Mice Showed Mile to Moderate Hearing Loss*

The model involved a mouse that expresses the *Gjb2* p.D50N variant. First, we generated the *Gjb2*$^{D50N/+}$ mice using CRISPR-Cas9 technology (Figure 1a) which was confirmed by Sanger sequencing (Figure 1b). However, homozygous lethality has been documented in the literature for variants in the *GJB2* gene [20], which is consistent with our inability to obtain homozygous mice through long-term breeding. So, we crossed the *Gjb2*$^{D50N/+}$ mice with the *Gjb2*$^{loxP/loxP}$; *Rosa26*^{CreER} mice,

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resulting in the *Gjb2*$^{D50N/loxp}$; *Rosa26*^{CreER} genotype. Following tamoxifen injection, we successfully obtained the *Gjb2*$^{D50N/-}$ mice (Figure 1c).

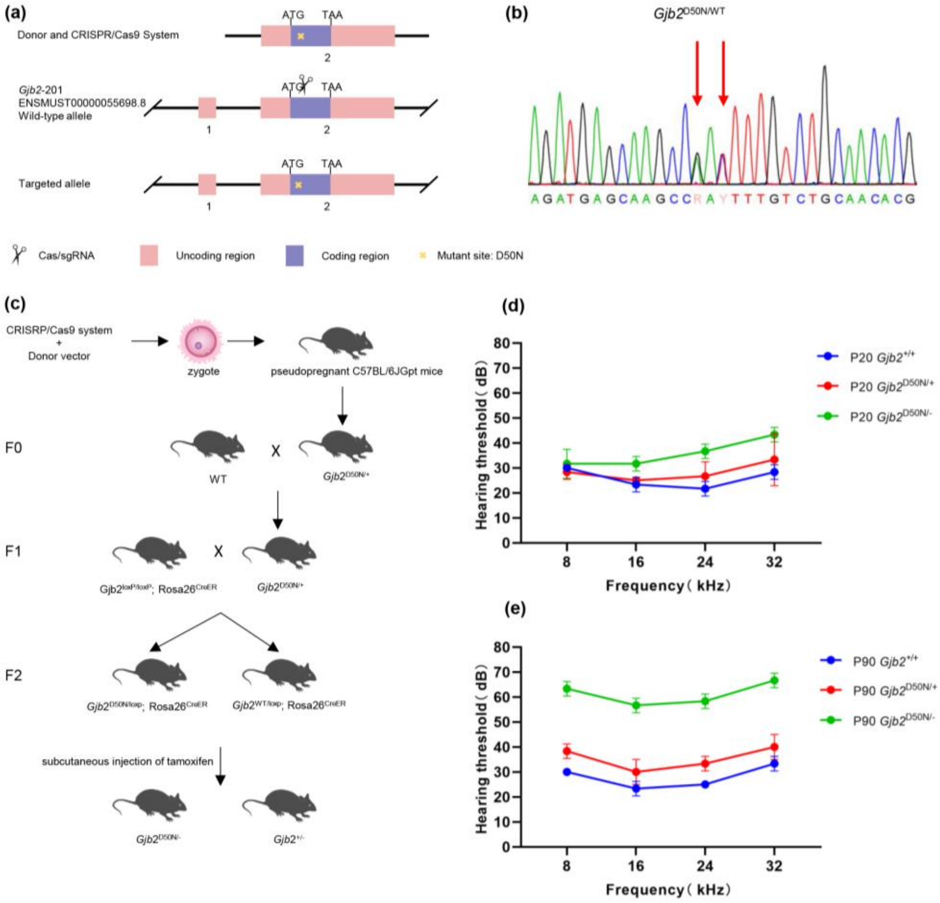

Figure 1. Mouse models and hearing loss in different mouse models. (a, b) *Gjb2* p.D50N variant site and Sanger sequencing result. (c) The process of constructing different mouse models. (d, e) The averaged ABR thresholds of the 20-days-old and 90-days-old *Gjb2*$^{+/+}$, *Gjb2*$^{D50N/+}$ and *Gjb2*$^{D50N/-}$ mice (n=3 in each group) at 8, 16, 24, and 32 kHz.

To evaluate if there was hearing loss of the *Gjb2*$^{D50N/-}$ mice, the ABRs of all the mice were measured at postnatal days 20 and 90 (P20 and P90). At P20, the ABR thresholds evoked by tone-bursts across a range of frequencies (8, 16, 24, 32kHz) in the *Gjb2*$^{+/+}$ mice were 30±0, 23.33±2.89, 21.76±2.89, 28.33±2.89 dB SPL, while the ABR thresholds in the *Gjb2*$^{D50N/-}$ mice were 31.67±5.58, 31.67±2.89, 36.67±2.89, 43.33±2.89 dB SPL (Figure 1d). Compared to age-matched *Gjb2*$^{+/+}$ mice, the *Gjb2*$^{D50N/-}$ mice showed 8.33, 15 and 15 dB increase in hearing thresholds at 16, 24, and 32 kHz at P20, respectively. The *Gjb2*$^{D50N/+}$ mice did not show significant hearing loss. At P90, there were no significant hearing loss in the *Gjb2*$^{+/+}$ mice. While, the ABR thresholds in the *Gjb2*$^{D50N/-}$ mice were 63.33±2.89, 56.67±2.89, 58.33±2.89, 66.67±2.89 dB SPL, which mean there were about 30 dB increase in hearing thresholds at all frequencies we measured (Figure 1e). The *Gjb2*$^{D50N/+}$ mice showed 5-10 dB increase. To summarize, the *Gjb2*$^{D50N/-}$ mice have progressive hearing loss that gradually progresses to full frequency, and the *Gjb2*$^{D50N/+}$ mice showed last-onset hearing loss.

3.2. The Gjb2$^{D50N/-}$ *Mice Showed no Significant HCs and SGNs Loss*

Whole-mount preparations were utilized to investigate HCs counting (Figure 2a). At P20, the *Gjb2* $^{D50N/-}$ mice exhibited sporadic OHCs loss in the apical, middle and basal turns, and we did not observe loss of OHCs in the *Gjb2* $^{D50N/+}$ mice(Figure 2b). Compared to other mouse models, *Gjb2* V37I knock-in mice [21] and *Gjb2*$^{V37M/V37M}$ mice [22] showed similar results. There was also no significant

HCs loss in *Gjb2*$^{35delG/delG}$ mice at P14, while significant degeneration of the OHCs had occurred, especially in the basal turn at P35 [23]. In R75 W+ mice, microtubules of inner pilar cells (IPCs) were poorly formed and hypoplasia, tunnels of Corti did not open and even collapse. Interestingly, the supporting cells were incompletely developed, but HCs were not affected [24]. SGNs degeneration was quantified through SGNs counting (Figure 2c). In all groups, we did not observe significant losses of SGNs (Figure 2d). Therefore, we speculate that early in hearing formation, the D50N variant may not yet affect the survival of SGNs. In conclusion, we speculated the loss of HCs and SGNs is not the primary factor contributing to the elevation of hearing thresholds.

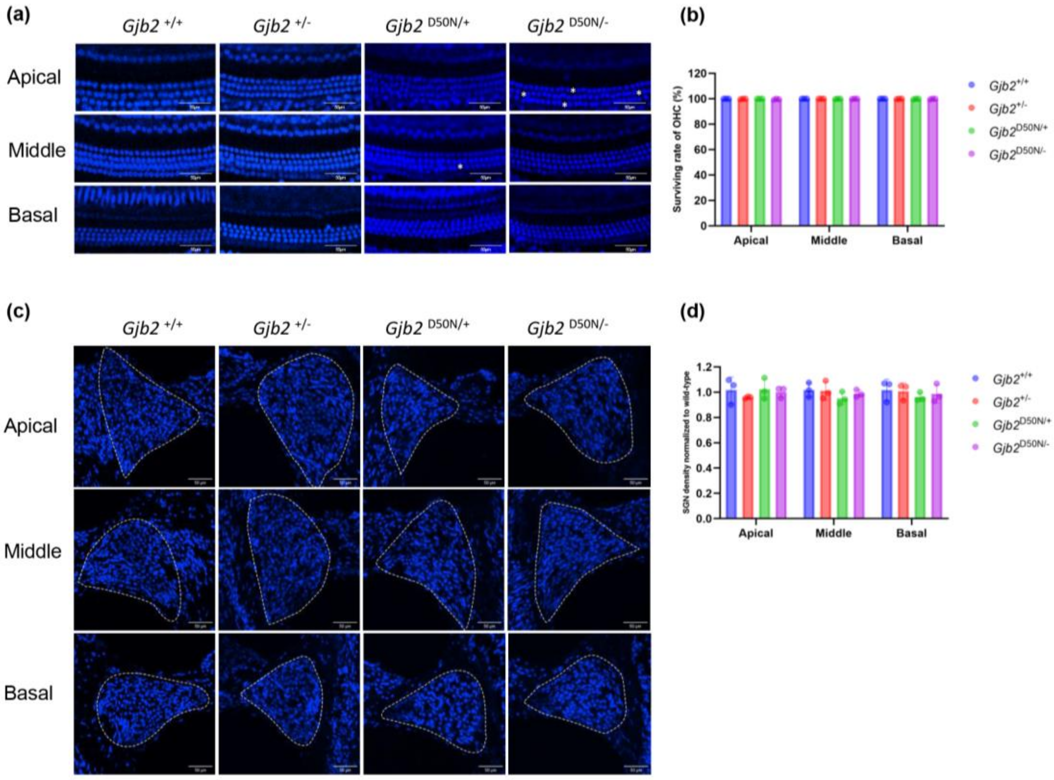

Figure 2. Hair cells count and SGNs count in different mouse models. (a) Representative images of HCs (DAPI, blue) of different turns in the *Gjb2*$^{+/+}$, *Gjb2*$^{D50N/+}$ and *Gjb2*$^{D50N/-}$ mice. Scale bars: 50μm. (b) OHCs count in each group. P>0.05. (c) Representative images of SGNs (DAPI, blue) of different turns in the *Gjb2*$^{+/+}$, *Gjb2*$^{+/-}$ *Gjb2*$^{D50N/+}$ and *Gjb2*$^{D50N/-}$ mice. Scale bars: 50μm. (d) SGNs count in each group. P>0.05.

3.3. CD45^{+}Cells in the Organ of Corti in the Gjb2$^{D50N/-}$ Mice Underwent Morphological Changes

In our preliminary observations in the Cx26-null mice, we found that inflammatory cells in the cochlea may play a role in the mechanism of hair cell damage [25]. To further validate this mechanism in the *Gjb2*$^{D50N/-}$ mice, we labeled cochlear macrophages with CD45. The CD45^{+}cells located at the cochlear basilar membrane in the *Gjb2*$^{+/+}$ mice (Figure 3a), the *Gjb2*$^{+/-}$ mice (Figure 3b), and the *Gjb2* $^{D50N/+}$ mice (Figure 3c) showed irregular appearance, while the *Gjb2*$^{D50N/-}$ mice (Figure 3d) exhibited morphological changes. Therefore, we speculate that the sporadic loss of HCs in the *Gjb2*$^{D50N/-}$ mice may be associated with the activation of CD45^{+}cells.

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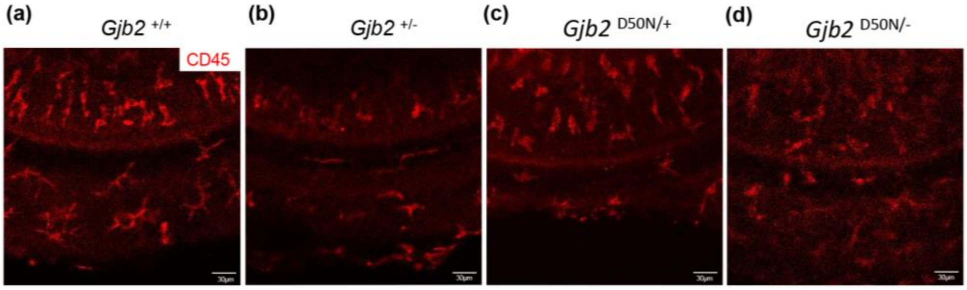

Figure 3. CD45^{+}cells in the organ of Corti. (a-d) Representative images of CD45^{+}cells (CD45, red) in the organ of Corti in the *Gjb2*$^{+/+}$, *Gjb2*$^{+/-}$ *Gjb2*$^{D50N/+}$ and *Gjb2*$^{D50N/-}$ mice. Scale bars: 30μm.

3.4. The Gjb2$^{D50N/-}$ *Mice Show Shortened GJPs*

According to the results of in vitro experiments, Cx26 variants can be classified into the following categories: those that cannot form GJPs on the cell membrane, those that form non-functional GJPs, those formed GJPs exhibit reduced ion permeability or material exchange capabilities, and those that form functionally GJPs. In our study, at P20, large and regular GJPs were observed on the cell membrane of the inner sulcus cells (ISCs) of the *Gjb2*$^{+/+}$ mice (Figure 4a) and the *Gjb2*$^{+/-}$ mice (Figure 4b), with maximum lengths were 11.73±1.93μm and 10.87±2.14μm. The longest GJPs in the *Gjb2* $^{D50N/+}$ mice (Figure 4c) and the *Gjb2* $^{D50N/-}$ mice (Figure 4d) measured 5.15±1.19μm and 2.73±0.60μm respectively, which were shortened 56% and 77% compared to the *Gjb2*$^{+/+}$ mice (Figure 4e). Consequently, we propose that fragmented GJPs impair their function, resulting in diminished energy supply between sensory epithelial cells in the inner ear. Interestingly, the D50N variant had a perinuclear localization in NEB1 keratinocytes and corneal epithelial cells, which is different from our findings [26,27]. However, plasma membrane localization can be observed in the sweat gland cells for a KID patient who carried D50N variant[26]. This suggests that D50N variant may exhibit inconsistent behavior in different cells or in vitro versus in vivo.

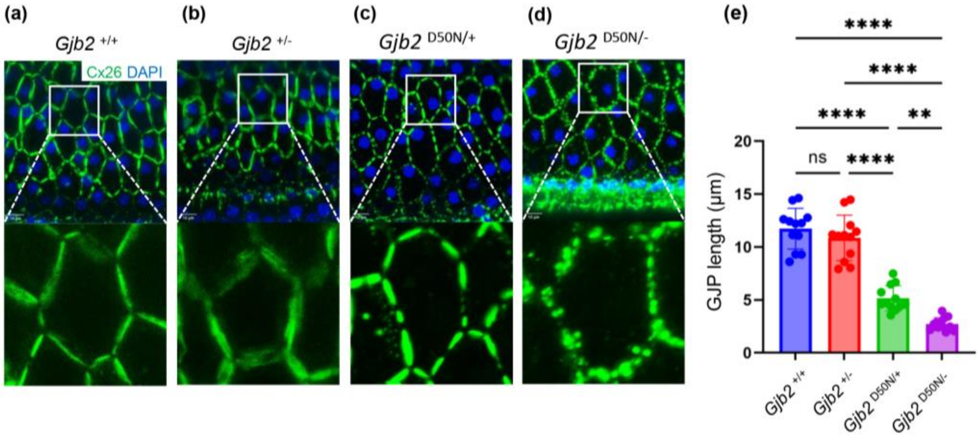

Figure 4. Cx26 expression patterns. (a-d) Representative confocal images of connexin 26 (Cx26, green; DAPI, blue) from apical turns of the basilar membrane. Scale bars: 10μm. (e) Histograms presenting the average lengths of the largest GJPs along a single cell border (mean±SD, n=12 for each group). ns, no significant difference; $^{**}p \le 0.01$; $^{****}p \le 0.0001$.

3.5. The Gjb2$^{D50N/-}$ *Mice Decreased Intake of 2-NBDG*

The sensory epithelium of the inner ear is avascular; consequently, it is strongly believed that these GJPs play a crucial role in the transfer of cellular signals and metabolites. In vitro experiments demonstrated that the D50N variant has abnormally high hemichannel activity and may lead to aberrant Ca^{2+} regulation[28–30]. Previous studies have demonstrated that gap junction mediated intercellular metabolite transfer in the cochlea was compromised in the Cx30-null mice [31]. Therefore, we investigated if there were differences in glucose uptake among OHCs, focusing on the glucose analog 2-NBDG. We conducted fluorescence quantification of 2-NBDG in the OHCs and observed that compared to the *Gjb2* $^{+/+}$ mice (Figure 5a), the fluorescence intensity of the *Gjb2* $^{+/-}$ mice (Figure 5b), the *Gjb2* $^{D50N/+}$ mice (Figure 5c), and the *Gjb2* $^{D50N/-}$ mice (Figure 5d) had decreased by 21.88%, 42.52%, and 61.42% (Figure 5e). Our study observed the Cx26 variant reduces glucose uptake, and OHCs thus suffer from impaired energy supply, which may lead to decreased OHCs function.

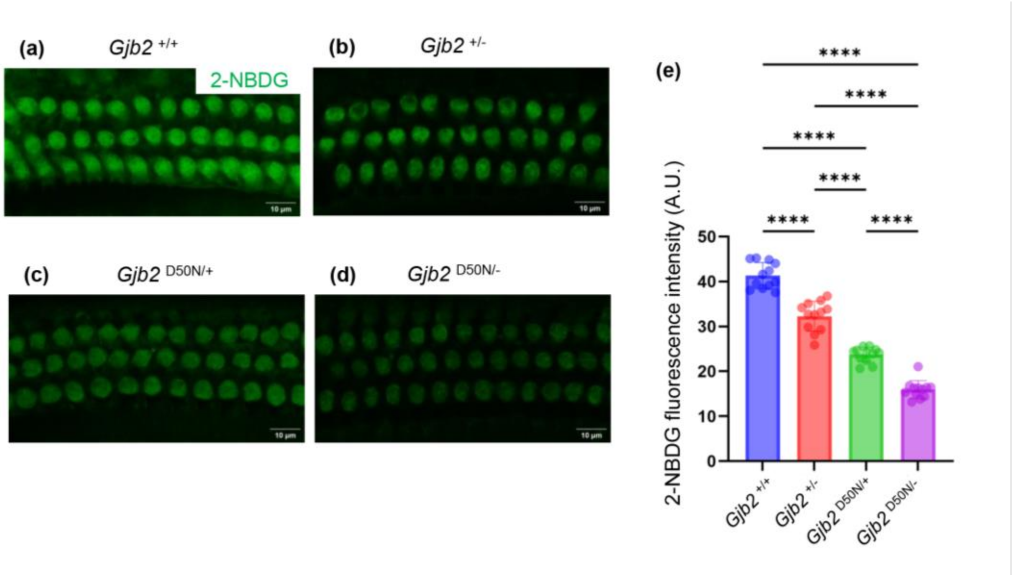

Figure 5. 2-NBDG uptake of OHCs. (a-d) Representative confocal images of 2-NBDG (green) as a fluorescent tracer of glucose from apical turns of the basilar membrane. Scale bars: 10 μm. (e) Statistics of 2-NBDG fluorescence intensity of OHCs (mean±SD, n=12 for each group). ****$p \leqslant 0.0001$.

4. Discussion

The *GJB2* gene is recognized as a significant contributor to deafness [4,32]. The ClinVar database catalogs over 500 variants of the *GJB2* gene (https://www.ncbi.nlm.nih.gov/clinvar), encompassing a range of variants and variant sites that result in considerable variability in the hearing phenotypes of affected individuals [4]. Presently, the primary interventions for hearing loss include the hearing aids and cochlear implants. Research suggests that early intervention with hearing aid devices is correlated with improved outcomes in language development for patients [33,34]. However, for individuals experiencing late-onset progressive hearing loss, the early adoption of cochlear implants may not be feasible due to the substantial costs associated with the devices and their upkeep, as well as the potential for unpredictable complications that could adversely affect patients' adherence to treatment. Given the heterogeneity of hearing phenotypes and the unique circumstances of each patient, a blanket approach to cochlear implantation may not represent the most effective or singular solution. Consequently, it is imperative to pursue or develop effective and cost-efficient pharmacological treatments tailored to individual needs.

Targeted therapy necessitates a deeper investigation into the underlying mechanisms, as the presence of various hearing phenotypes may indicate multiple pathogenic mechanisms linked to the *GJB2* gene. The currently hypotheses struggle to fully explain the pathogenesis of Cx26-related hearing loss. The hemichannels, or gap junction channels, formed by Cx26 appear to play multiple roles in the structure and function of the inner ear [35,36]. Current in vivo studies primarily focus on various conditional knockout mice, which frequently display severe to profound hearing loss and exhibit significant pathological defects. The integrity of the organ of Corti is crucial for the cochlea's active amplification process [37–39]. In Cx26-null mice, the developmental arrest of pillar cells results in the abnormal morphology and the Corti tunnel failure to open properly, which may contribute to hearing loss [18,19,40]. Mice with Cx26 knocked-out early after birth show a progressive loss of HCs beginning in the middle turn, accompanied by a reduction in the density of SGNs [16,41,42]. Conditional knockout of Cx26 in supporting cells leads to an early loss of Deiters' cells [42]. However, these pathological changes were not observed in the *Gjb2* $^{D50N/-}$ mice in our study. The *Gjb2* $^{D50N/-}$ mice exhibited mild to moderate progressive hearing loss. These differing findings underscore the importance of developing various Cx26 variant mouse models, as they may more accurately reflect the conditions experienced by certain patients.

The cochlear basilar membrane is characterized by the absence of a direct blood supply, with GJPs playing a critical role in the signal transduction and substances transportation. In vitro investigations have demonstrated that various variants in the Cx26 gene can result in a range of effects, including the failure to establish GJPs between cells, the production of non-functional GJPs, or a reduction in ion permeability or substance exchange; it is also possible that some variants do not lead to any functional abnormalities[43–47]. A lot of researches indicated that variants in Cx26 can modify the microenvironment of the inner ear. The elevated concentration of potassium (K^+) in the endolymph is essential for mechanical-electrical transduction processes within the HCs [48,49]. Mice with a knockout of Cx26 at an early developmental stage exhibited a diminished endolymphatic potential (EP), suggesting that the proper functioning of Cx26 in the sensory epithelium and the lateral wall is crucial for the establishment of EP [24,50]. Previously, researchers introduced the hypothesis of metabolic product transport impairment in Cx30 knock-out mice [31], and we have also noted a decrease in glucose uptake in OHCs in the *Gjb2* $^{D50N/-}$ mice. A compromised glucose supply can lead to increased ATP consumption and the subsequent production of elevated levels of ROS [51]. Although various connexin family proteins are expressed within the cochlea, a defect in Cx26 may not entirely inhibit the transport of energy substrates; however, the accumulation of ROS may exacerbate cochlear dysfunction and contribute to hearing loss [52]. Functional impairments, rather than structural abnormalities, may contribute to those mild to moderate progressive hearing loss.

In GJPs, newly synthesized connexins continuously replace older connexins to maintain a dynamic equilibrium [53,54]. However, under pathological conditions, the synthesis and degradation of connexins may become imbalanced [55]. Previous studies have shown that the expression of Cx26 decreases in aged rats [56], and a partial deficiency of Cx26 accelerates age-related hearing loss by inducing redox imbalance and dysregulation of the Nrf2 pathway [52]. Furthermore, Cx26 haploinsufficiency has been shown to heighten sensitivity to noise through enhancing cochlear amplification [57].

The success of *OTOF* gene therapy has enhanced researchers' confidence in the potential of gene therapy for hereditary hearing loss [58,59]. Research on the mechanisms and treatment of other deafness-causing genes is also in full swing[60–63]. Adeno-associated viral (AAV) vectors mediated gene transfer of *GJB2* had made some progress [64]. However, in mature variant cochlear of the mice with inducible Sox10iCre^{ERT2} -mediated loss of *Gjb2*, AAV-mediated gene transfer of *Gjb2* did not show hearing improvement and even exacerbated hearing loss and resulted in HCs loss in some mice [65]. Consequently, further investigation is required to assess the safety and efficacy of *GJB2* gene therapy. Our previous studies demonstrated that dexamethasone and PARP inhibitors exhibit a protective effect against hearing loss in mice with Cx26 defects [25,66]. Besides, protection of HCs and SGNs by a number of small molecule drugs has been demonstrated in drug-induced hearing loss[67,68]. It is posited that for individuals experiencing mild to moderate progressive hearing loss, it is both feasible and beneficial to investigate or develop suitable pharmacological interventions aimed at preventing further deterioration or mitigating the progression of their condition. Such pharmacological agents should exhibit: i) high permeability, allowing for direct action within the inner ear by traversing the blood-labyrinth barrier; ii) possess high targeting capability to minimize off-target effects on other tissues and organs through localized administration; iii) demonstrate a prolonged half-life to decrease the frequency of dosing and improve patient adherence; iv) be economically accessible to reduce the financial burden on patients.

5. Conclusions

In conclusion, our study established a *Gjb2* compound heterozygous variants mouse model that exhibit mild to moderate hearing loss, which is associated with compromised glucose supply. Additionally, we propose further research directions for the exploration or development of therapeutic interventions aimed at addressing mild to moderate progressive hearing loss attributable to *GJB2* variants.

Author Contributions: X.S. and X.L. designed the study. X.S., X.L., Y.X. and X.X. were involved in the experiments. X.S. and X.L. wrote the manuscript. Y.S. provided technical support and modified the manuscript. All authors critically reviewed the final version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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