Protection of the cochlear hair cells in adult C57BL/6J mice by T-type calcium channel blockers

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Abstract. The aim of the present study was to investigate the protective effect of T-type calcium channel blockers against presbycusis, using a C57BL/6J mice model. The expression of three T-type calcium channel receptor subunits in the cochlea of 6-8-week-old C57BL/6J mice was evaluated using reverse transcription-quantitative polymerase chain reaction. The results confirmed that the three subunits were expressed in the cochlea. In addition, the capacity of T-type calcium channel blockers to protect the cochlear hair cells of 24-26-week-old C57BL/6J mice was investigated in mice treated with mibefradil, benidipine or saline for 4 weeks. Differences in hearing threshold were detected using auditory brainstem recording (ABR), while differences in amplitudes were measured using a distortion product otoacoustic emission (DPOAE) test. The ABR test results showed that the hearing threshold significantly decreased at 24 kHz in the mibefradil-treated and benidipine-treated groups compared with the saline-treated group. The DPOAE amplitudes in the mibefradil-treated group were increased compared with those in the saline-treated group at the F2 frequencies of 11.3 and 13.4 kHz. Furthermore, the DPOAE amplitudes in the benidipine-treated group were increased compared with those in the saline-treated group at an F2 frequency of 13.4 kHz. The loss of outer hair cells (OHCs) was not evident in the mibefradil-treated group; however, the stereocilia of the inner hair cells (IHCs) were disorganised and sparse. In summary, these results indicate that the administration of a T-type calcium channel blocker for four consecutive weeks may improve the hearing at 24 kHz of 24-26-week-old C57BL/6J mice. The function and morphology of the OHCs of the C57BL/6J mice were significantly altered by the administration of a T-type calcium channel blocker; however, the IHCs were unaffected.

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Introduction

Presbycusis is a major neurodegenerative disease in elderly individuals. Hearing loss and communication difficulty are common social problems associated with the increasing incidence of presbycusis (1). Presbycusis with hearing loss at high frequencies may result in difficulty in understanding speech in a noisy environment (2). If the hearing loss spreads to the frequency range of speech, presbycusis may lead to difficulty in understanding speech in any environment. The loss of hearing and hair cells are the two primary features of presbycusis (3).

Age-related cochlear hair cell loss has been reported in humans (4,5) and animals (6). The loss of inner hair cells (IHCs) and outer hair cells (OHCs) increases with increased age, and the loss of OHCs is particularly pronounced. The molecular mechanisms underlying the age-related loss of hair cells remain unclear. Damage to mitochondrial DNA is speculated to be a cause of presbycusis (7). Mitochondrial DNA damage is associated with intracellular calcium (Ca^{2+}) overload, which induces a cascade of adverse consequences. Excessive amounts of Ca^{2+} ions activate Ca^{2+}-dependent enzymes, which promote the generation of free radicals that cause damage to cells (8,9). The high concentration of Ca^{2+} is hypothesized to cause hair cell degeneration (10).

Preventing the degeneration of hair cells is crucial for the prevention of presbycusis. Research into the regeneration of hair cells in animals has made numerous breakthroughs. For instance, it is known that, in mammals, inner ear stem cells are pluripotent and are capable of differentiating into hair cell-like cells; this implies a possible use of such cells in the replacement of lost inner-ear sensory cells (11). However, regenerative technologies are not yet ready for use in clinical applications. Calcium channel blockers may offer an effective intervention for the prevention of presbycusis, as these blockers inhibit excessive calcium entry and thus protect hair cells against degeneration. T-type and L-type Ca^{2+} channels serve a crucial function in the synaptic release of hair cells during the cochlear development of mammals (12). T-type calcium channels have been found to be present in the organ of Corti and neurons (13,14). Furthermore, T-type calcium channel blockers reportedly protect OHCs from noise damage (14). Similarities between the age-related pathologies of mice and humans indicate that mice may provide a good model for presbycusis (15). The most widely used model is the C57BL/6J mouse, which exhibits marked progressive age-related hearing

loss (16). The results of our previous study indicate that the hearing threshold of C57BL/6J mice is significantly higher at 24-26 weeks of age than in 6-8-week-old mice (17). The aim of the present study was to investigate the protective effect of T-type calcium channel blockers against presbycusis, using the C57BL/6J mice model. Differences in the hearing of the C57BL/6J mice and the function and morphology of their hair cells were analyzed following the administration of T-type calcium channel blockers.

Materials and methods

Animals and tissue preparation. A total of 30 male C57BL/6J mice (age, 6-8 weeks) were randomized into three groups for the detection of three calcium channel receptor subunits α1G, α1H and α1I, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, a further 30 C57BL/6J male mice (age, 24-26 weeks) were allocated at random into three treatment groups: Saline, mibefradil and benidipine. Each group was subjected to auditory brainstem recording (ABR) and distortion product otoacoustic emission (DPOAE) tests following treatment. Mibefradil and benidipine were obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in physiological saline solution. A preliminary experiment led to the selection of dosages of 30 mg/kg/day mibefradil and 10 mg/kg/day benidipine. The drugs were administered to the mice by gavage for four consecutive weeks. All experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals, and all studies were approved by the animal care committee of the First Affiliated Hospital of Soochow University (Suzhou, China).

The mice were anesthetized with an intraperitoneal injection of 2.5%, 0.1 ml/10 g chloral hydrate and sacrificed by cervical dislocation following the ABR and DPOAE tests. The cochleae were immediately removed, and the stapes were discarded. The cochleae for RT-qPCR were immersed in ice-cold RNA solution to avoid RNA degradation. The cochleae were rapidly dissected under a microscope in ice-cold 0.01 M phosphate-buffered saline (PBS) and stored at -80˚C. The cochleae for scanning electron microscopy (SEM) were perfused with 2.5% glutaraldehyde via a 10-μm drill hole, created with a needle, in the vestibular and cochlear windows and then immersed in fixative fluid.

RT-qPCR. Total RNA was extracted using TRIzol reagent (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. Reverse transcribed cDNA was synthesized using MMLV reverse transcriptase (Promega Corporation, Madison, WI, USA). PCR was performed using an ABI-7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific) according to the manufacturer's instructions. RT was conducted in a 20-μl reaction mixture containing 4 μl 5X RT buffer, 0.5 μl oligo(dT), 0.5 μl dNTPs, 1 μl MMLV reverse transcriptase, 10 μl diethylpyrocarbonate-treated water and 4 μl RNA template. The reaction conditions to inactivate MMLV were 37˚C for 1 h and 95˚C for 5 min. PCR was performed using a SYBR Green PCR kit (Thermo Fisher Scientific), according to the manufacturer's instructions, in a total volume of 50 μl. The mixture contained 32.5 μl SYBR Green Master mix, 0.5 μl forward primer, 0.5 μl reverse primer, 14.5 μl ddH_2O and 2 μl cDNA template. The PCR cycling conditions were as follows: 95˚C for 30 min, followed by 40 cycles of 95˚C for 30 sec, 58˚C for 30 sec and 73˚C for 90 sec. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for the quantification of the PCR. The relative quantification was based on the Cq (the number of PCR cycles) values.

The DNA sequences of primers (forward and reverse) were as follows: GAPDH, 5'-CCTGGCCAAGGTCATCCATGACAAC-3' and 5'-TGTCATACCAGGAAATGAGCTTGAC-3'; α1G subunit, 5'-AATGGCAAGTCGGCTTCAGG-3' and 5'-TGTCAGAGACCATGGACACCAG-3'; α1H subunit, 5'-ATGTTCCGGCCCTGTGAGGA-3' and 5'-CCATGACGTAGTACATGATGTCC-3'; and α1I subunit, 5'-ATCTGCTCCCTGTCGG-3' and 5'-GAGAACTGGGTCGCTATG-3'. The primers were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized at the Shanghai Institute of Cell Biology (Shanghai, China).

ABR test. Each mouse was anesthetized by an intramuscular injection of 25 mg/kg xylazine and 100 mg/kg ketamine (Sigma-Aldrich). The mice were placed in a soundproof anechoic room with a thermostat prone experimental platform that maintained a body temperature of 37˚C. The ABRs were recorded subcutaneously using specialized needle electrodes (Tucker-Davis Technologies, Alachua, FL, USA) placed at the vertex, mastoid prominence and contralateral mastoid prominence. The speaker was placed in the external auditory meatus. The stimulus signal was generated and the evoked potential was recorded by a System II evoked potential workstation (Tucker-Davis Technologies, Alachua, FL, USA). Tone burst stimuli (duration, 5 msec; rise-fall time, 0.5 msec) were generated, and the average response was determined on the basis of 1,000 repetitive stimuli. A repetition rate of 11 times/sec was applied at frequencies of 8, 16, 24 and 32 kHz. The neuronal activity was amplified (x100,000) and filtered (0.3-3.0 kHz). Recording was initiated at a sound pressure level (SPL) of 100 dB, and 10-dB decrements were applied, followed by 5-dB decrements until a clear wave response was elicited.

DPOAE test. Following the ABR test, all mice were prepared for the DPOAE test under anesthesia in the same manner as they were for the ABR test. An acoustic probe was inserted into the external auditory meatus near the tympanic membrane. The DPOAE was measured using an amplifier system that provided two stimulation tones, F1 and F2, which were generated using a dual channel synthesizer (AD3; Tucker-Davis Technologies). The frequency ratio of the F1 and F2 primary stimulation tones was 1.25, and their intensities were L1=65 dB SPL and L2=55 dB SPL. The amplitude of DPOAEs was measured at 2F1-F2. The threshold from low to high frequency was determined when the DPOAE was >5 dB SPL higher compared with the noise floor. In order to measure the frequency-specific responses, the F2 stimulation tone was set between 6 and 40 kHz, and the amplitudes of DPOAEs were recorded at 12 test point frequencies: 6.7, 8.3, 9.5, 11.3, 13.4, 17.9, 21.5, 23.8, 26.7, 33.6, 36.3 and 39.8 kHz.

Table I. Hearing threshold at various frequencies in 24-26-week-old C57BL/6J mice after treatment [sound pressure level (dB)].

Group	8 Hz	16 Hz	24 Hz	32 Hz
Saline-treated	56.5±5.7975	54.5±3.6890	74. 0±3.1620	93.0±2.5820
Mibefradil-treated	58.0±3.4960	54.5±4.9721	69.0±3.9441[a]	90.5±2.8382
Benidipine-treated	57.0±4.2164	53.5±3.3747	68.5±5.2968[a]	91.0±3.9441

[a]P<0.05 vs. the saline-treated group. Data presented as the mean ± standard error of the mean.

SEM. The cochlea was removed and immersed in 2.5% glutaraldehyde for 6 h at 4˚C, followed by two washes in PBS for 10 min each. The volute, spiral ligament, vestibular membranes and covering film were removed from the cochlea under a SGO-45T1 dissecting microscope (Shenzhen Shenshi Guanggu Optical Instrument Co., Ltd, Shenzhen, China) after rinsing in PBS. The basilar membrane and cochlear spiral shaft were then exposed. The samples were post-fixed in 1% osmium tetroxide for 2 h at 4˚C. The samples were dehydrated using a graded series of ethanol (50, 70, 80, 90 and 100% for 10 min each) and incubated in aqueous 90% potassium *tert*-butoxide for 10 min. The samples were critical-point dried in a Leica EM CPD300 dryer (Leica Microsystems, Inc., Buffalo Grove, IL, USA) and coated with gold (Au) using a Hitachi E-1045 ion sputter coater (Hitachi, Ltd., Tokyo, Japan). The hair cells of the base turn of the basilar membrane were visualized using a SU8010 scanning electron microscope (Hitachi, Ltd., Tokyo, Japan).

Statistical analysis. All data are expressed as the mean ± standard error of the mean and were analyzed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used to analyze the RT-qPCR, ABR test, and DPOAE test data sets. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of three T-type channel subunits by RT-qPCR. The expression rate of each subunit was calculated using the formula $2^{-\Delta\Delta Cq}$ (Fig. 1). It was found that all three subunits were expressed in the cochlea of the 6-8-week-old C57BL/6J mice. Among the three subunits, the expression levels of the α1H subunit were lower compared with those of α1G and α1I (P<0.05). The expression levels of α1G and α1I did not significantly differ from each other (P>0.05).

ABR test. The hearing thresholds of the 24-26-week-old C57BL/6J mice differed following the 4-week treatment period. The hearing threshold at 24 kHz was significantly decreased in the mibefradil-treated and benidipine-treated groups compared with the saline-treated group (P<0.05). The hearing threshold was also decreased at 32 kHz in the mibefradil-treated and benidipine-treated groups compared with the saline-treated group; however, this difference was not found to be statistically significant (P>0.05; Table I).

DPOAE amplitudes. The DPOAE amplitudes were measured in the 24-26-week-old C57BL/6J mice at F2 frequencies between 6 and 40 kHz (Fig. 2). The DPOAE amplitudes in the mibefradil-treated group were increased compared with those in the saline-treated group at the F2 frequencies of 11.3 and 13.4 kHz (P<0.05). The DPOAE amplitudes in the

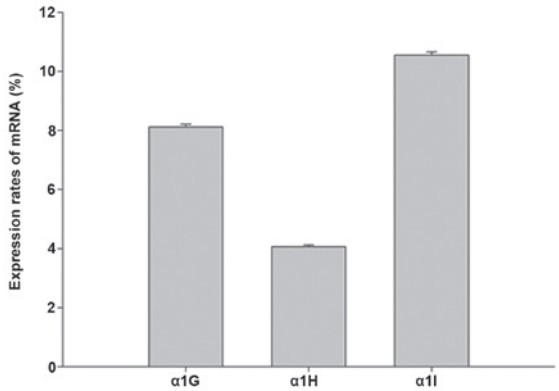

Figure 1. Quantified expression of three T-type channel subunits in the cochlea of 6-8-week-old C57BL/6J mice via reverse transcription-quantitative polymerase chain reaction revealed that the expression levels of the three subunits were low, and that α1H had the lowest level of expression.

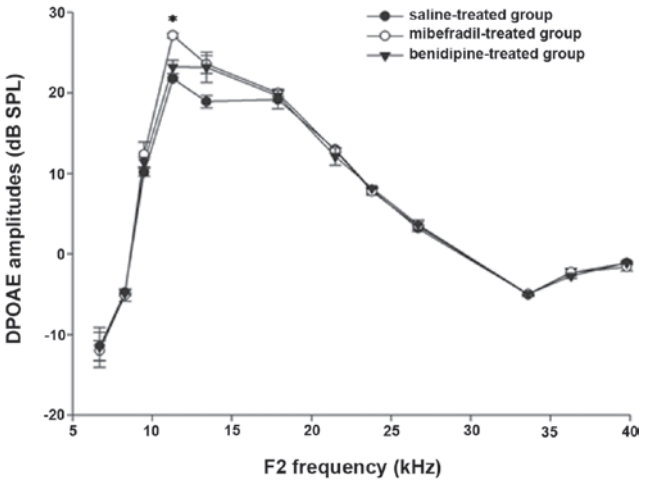

Figure 2. DPOAE amplitudes at the F2 frequency in 3 treatment groups of 24-26-week-old C57BL/6J mice. The DPOAE amplitudes in the mibefradil-treated group at F2 frequencies of 11.3 and 13.4 kHz were significantly increased compared with those in the saline-treated group. In addition, the DPOAE amplitudes in the benidipine-treated group at an F2 frequency 13.4 kHz were significantly increased compared with those in the saline-treated group. The DPOAE amplitudes at other F2 frequencies did not significantly differ between the mibefradil- or benidipine-treated and the saline-treated group. *P<0.05 between the saline- and mibefradil-treated groups. DPOAE, distortion product otoacoustic emission; SPL, sound pressure level.

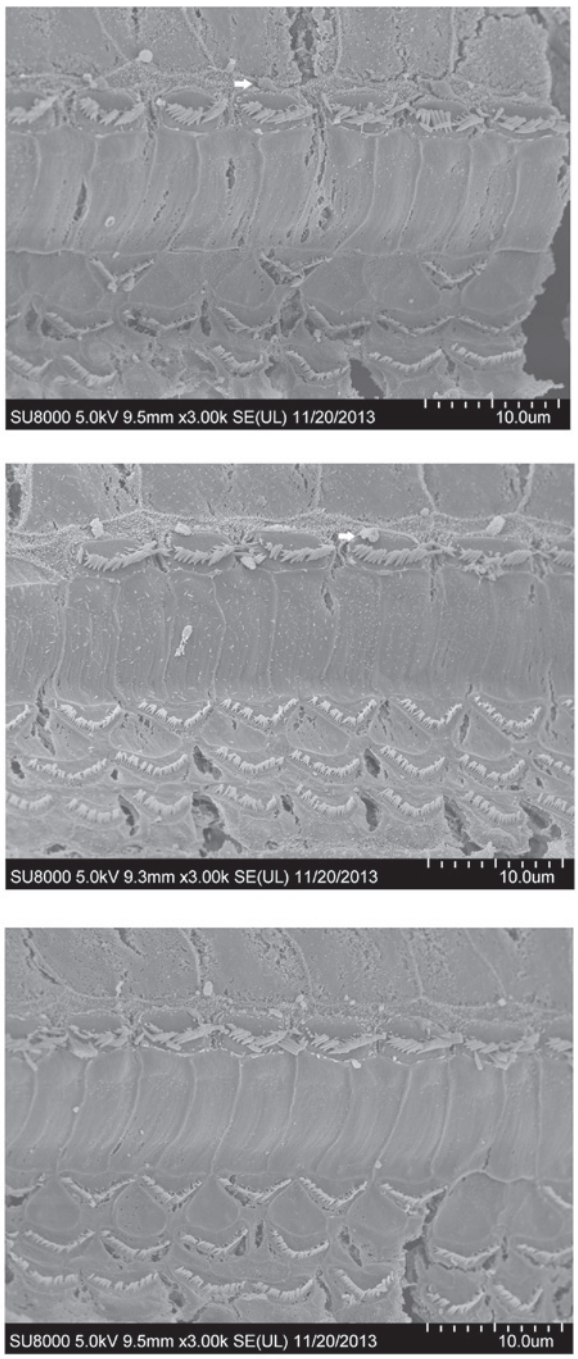

Figure 3. Morphological analysis of cochlea hair cells observed by scanning electron microscopy in the three treatment groups of 24-26-week-old C57BL/6J mice. (A) Morphology of hair cells in the saline-treated group indicated the rupture of the cuticular plate, lost outer hair cells (OHCs) and sparse stereocilia bundles on inner hair cells (IHCs). Spherical extrusions appeared on the outside of the stereocilia of inner hair cells. (B) Morphology of hair cells in the mibefradil-treated group showed no obvious loss of OHCs; however, the stereocilia of the IHCs were disorganised and sparse. (C) Morphology of hair cells in the benidipine-treated group indicated a small quantity of missing OHCs, and disorganised or sparse hair cells. White arrows indicate spherical extrusion (scale bar, 10 μm).

benidipine-treated group were increased compared with those in the saline-treated group at a F2 frequency of 13.4 kHz ($P<0.05$). The DPOAE amplitudes did not significantly differ at other F2 frequencies between the mibefradil-treated or benidipine-treated groups and the saline-treated group.

Morphology of hair cells observed using SEM. The hair cells of the cochleae in the 24-26-week-old C57BL/6J mice observed by SEM showed differences following the administration of treatment for four consecutive weeks. Degeneration of hair cells was evident in the saline-treated group (Fig. 3A). The rupture of the cuticular plate, loss of OHCs, and dispersed stereociliary bundles on IHCs were observed. Spherical extrusions appeared on the outside of the stereocilia of the IHCs. The loss of OHCs was not obvious in the mibefradil-treated group (Fig. 3B); however, the stereocilia of IHCs were disorganised and sparse. A limited number of the OHCs were lost in the benidipine-treated group (Fig. 3C).

Discussion

The mechanisms underlying the pathogenesis of presbycusis, including mechanisms associated with the auditory system, remain unclear. This ambiguity has prevented scientists from discovering improved treatments for age-related hearing loss. Mutations of mitochondrial DNA are known to accumulate with aging (18). These mutations have been associated with Ca^{2+} overload (19). The homeostasis of Ca^{2+} is crucial for cell survival and for numerous physiological processes, including hearing (20,21). An elevated concentration of intracellular Ca^{2+} may induce the release of neurotrophins (22), attenuate action potentials in hair cells and improve neuronal connections (23).

Calcium channel blockers may provide a novel intervention for the protection of hair cells against degeneration during presbycusis. Calcium channels are divided into L, N, P/Q, R and T-types, according to their electrophysiological and pharmacological properties (24). In the present study, the effects of T-type calcium channels blockers were investigated, as the protective effects of L-type calcium channels are controversial (25,26).

C57BL/6J mice, which have an age-related hearing loss gene (Ahl), were selected as our experimental model, as the degeneration of cochlear hair cells begins early in adulthood and progresses with advancing age. To clarify the impact of T-type calcium channel blockers on the cochlear hair cells, the distribution of calcium channels in the cochlea was determined. CaV3.1 is a T-type calcium channel subunit that has been shown to be involved in intracellular Ca^{2+} regulation in mature rat OHCs (27). In the present study, the three receptor subunits α1G, α1H and α1I corresponding to calcium channels CaV3.1, CaV3.2 and CaV3.3, respectively, were confirmed to be expressed in the cochlea, although the expression levels were low. These results provided a theoretical basis for the use of T-type calcium channel blockers. The calcium channel blockers mibefradil and benidipine were selected, and saline was administered to the animals in the control group. Mibefradil selectively blocks T-type calcium channels and has been demonstrated to exert a cardioprotective effect in rats with acute myocardial infarction (28). Benidipine has been widely used for hypertension therapy as it is able to block the L-type and T-type calcium channels in various cell types (29).

To determine whether hair cells are protected by T-type calcium channel blockers, the hearing level, function, and morphology of hair cells were observed following treatment of C57BL/6J mice with mibefradil and benidipine. The results of the ABR analysis showed that the hearing threshold

decreased at 24 and 32 kHz, particularly at 32 kHz, both in the mibefradil-treated and benidipine-treated groups compared with the saline-treated group. The hearing threshold reduction at high-frequencies indicated the status of the hair cells of the base turn. The improvement in hearing may be associated with the cochlea, the spiral ganglion neurons, the auditory cortex or a combination of these. Future studies are required to confirm whether the hair cells are affected. To understand the function of hair cells, the DPOAE test was conducted immediately after the ABR test. The DPOAE measurement is recognized as an effective method of investigating the function of OHCs (30). The results indicated that the DPOAE amplitudes in the mibefradil-treated group were higher at the F2 frequencies of 11.3 and 13.4 kHz compared with those in the saline-treated group, and in the benidipine-treated group. the DPOAE amplitudes were higher at an F2 frequency of 13.4 kHz. The results of the ABR and DPOAE tests suggested that the function of OHCs was significantly improved, particularly in the base turn. However, the present results cannot verify the effect of calcium channel blockers on IHCs. Morphological changes are typically associated with a change of function. The SEM images clearly display the morphology of hair cells, including the IHCs. The progressive loss of hair cells has been previously observed in the C57BL/6J mouse strain (31). The present results showed that degeneration of OHCs and IHCs at the base turn of the cochlea was evident after the 4-week administration of saline in 24-26-week-old C57BL/6J mice, which indicated that the saline did not affect the hair cells of 24-26-week-old C57BL/6J mice. The loss of OHCS was reduced after 4 weeks of T-type calcium channel blocker administration, particularly in the mibefradil-treated group. However, the stereocilia of IHCs continued to be disorganised and sparse.

Collectively, the present results indicate that hearing thresholds and DPOAE amplitudes improve at high frequencies following the administration of T-type calcium blockers. This result suggests that T-type calcium blockers, such as mibefradil or benidipine, can protect the OHCs of the base turn in 24-26-week-old C57BL/6J mice, and this result was confirmed by SEM. In addition, the SEM images showed that IHCs were not protected following the administration of T-type calcium blockers. We hypothesize that the distribution of T-type calcium channel differed between OHCs and IHCs, which requires confirmation in future studies.

The improvements observed in the present study may be due to the manner and dose with which T-type calcium channel blockers were administered. However, these results are experimental and insufficient to recommend the clinical application of T-type calcium channel blockers. The underlying mechanisms involved in the function of calcium ions of the cochlea remain to be further elucidated in future studies. The use of calcium channel blockers is required to be specific and individualized. The treatment of presbycusis may require interventions beyond calcium channel blockers, as presbycusis is, like aging, an irreversible natural process. The two calcium channel blockers used in the present study may affect L-type or other ion channels in addition to exerting an effect on T-type calcium channels.

In conclusion, the results of the present study demonstrated that three T-type calcium channel subunits were expressed in the cochlea of 6-8-week-old C57BL/6J mice. The expression levels of the α1H subunit were lower compared with those of α1 G and α1I. The hearing threshold and DPOAE amplitudes of the 24-26-week-old C57BL/6J mice were significantly improved at high frequencies following the administration of mibefradil or benidipine for four consecutive weeks. The degeneration of OHCs was not evident following the treatment with mibefradil, although the stereocilia of the IHCs continued to be disorganised and sparse. Therefore, the administration of a T-type calcium channel blocker for four consecutive weeks appears to protect OHCs, but not IHCs, against presbycusis-associated damage.

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