

## Original Article

# Effect of T-type calcium channel blockers on spiral ganglion neurons of aged C57BL/6J mice

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**Abstract:** To explore the expression levels of T-type calcium channel receptors in spiral ganglion neurons of C57BL/6J mice and the effect of T-type calcium channel blockers on the spiral ganglion neurons of 42-44-W C57BL/6J mice. We first quantified the subunits of T-type calcium channel blockers in the spiral ganglion neurons of C57BL/6J mice in three groups (6-8 W, 24-26 W, 42-44 W) according to age via RT-PCR. Next, we administered three drugs (zonisamide, felodipine, saline) to the 42-44-W C57BL/6J mice by gavage for four weeks. We observed the changes in the hearing threshold of 42-44-W C57BL/6J mice after treatment. Meanwhile, we measured the expression of calcium-binding proteins of spiral ganglion neurons after treatment. Our results showed that three receptors were expressed in the spiral ganglion neurons of C57BL/6J mice. The expression level of  $\alpha 1H$  was stronger than that of  $\alpha 1G$  and  $\alpha 1I$ . The expression levels of three receptors especially for  $\alpha 1G$  and  $\alpha 1H$  significantly decreased with age. The hearing threshold at 24 kHz was significantly decreased after zonisamide administration. No significant difference in the expression level of calbindin in spiral ganglion neurons was noted. Interestingly, the expression level of calmodulin in spiral ganglion neurons was lower in the zonisamide-treated groups than in the felodipine- and saline-treated group. We concluded that the administration of T-type calcium channel blocker for four consecutive weeks can improve the hearing by ameliorating calcium overload on spiral ganglion neurons of 42-44-W C57BL/6J mice.

**Keywords:** T-type calcium channel, receptor, calcium channel blocker, spiral ganglion neurons, calcium-binding protein

## Introduction

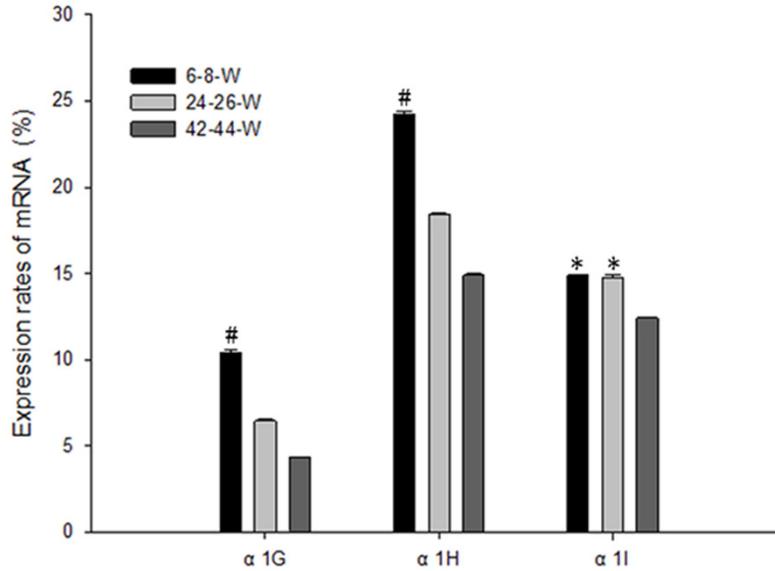
Presbycusis can significantly impact the quality of life of the elderly. Age-related hearing loss involves various parts of the ear and the nervous system. Loss of hair cells and spiral ganglion neurons (SGNs) is a major cause of presbycusis [1]. The molecular mechanisms underlying the age-related loss of hair cells and SGNs remain unclear. Mutations of mitochondrial DNA have been demonstrated to accumulate with aging in several types of neurons, including SGNs [2]. Mitochondrial damage is closely associated with intracellular calcium overload, which induces a cascade of responses. Excessive calcium activates phosphatase A2 and phosphatase C, promoting the hydrolysis of membrane phospholipids, the release of free fatty acids [3], and the peroxidation of lipids [4]. Calcium homeostasis is crucial for the

regulation of a variety of physiological processes, including hearing [5, 6].

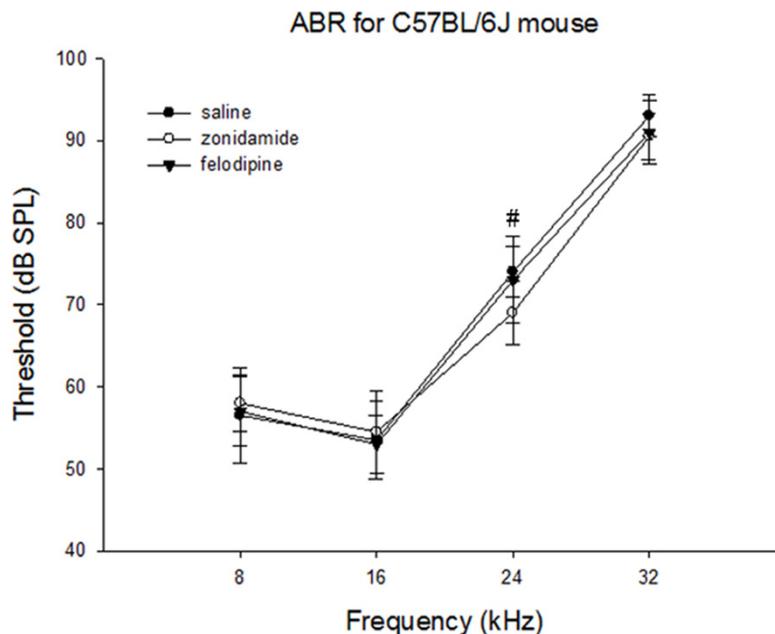
Presently, there are no drugs that prevent or treat presbycusis, but calcium channel blockers may represent a potential therapeutic strategy. Voltage-gated calcium channels are classified into low-voltage-gated and high-voltage-gated channels. Low-voltage-gated calcium channels primarily include T-type calcium channels, whereas high-voltage-gated calcium channels include L-, P-/Q-, N-, and R-type calcium channels. T-type calcium channels are further categorized into three subtypes, Cav3.1, Cav3.2, and Cav3.3, based on their encoding genes. The receptor subtypes Cav3.1, Cav3.2, and Cav3.3 correspond to the  $\alpha 1G$ ,  $\alpha 1H$ , and  $\alpha 1I$  subunits, respectively.

It has been suggested that T-type calcium channels contribute to the cell death of glia and neu-

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**Figure 1.** The quantified expression of three T-type channel subunits via real-time quantitative RT-PCR analysis revealed that the expression level of  $\alpha 1H$  was the highest, whereas that of  $\alpha 1G$  and that of  $\alpha 1I$  were the lowest at the age of 6-8 W. The expression levels of three receptors especially for  $\alpha 1G$  and  $\alpha 1H$  significantly decreased with age. # $P < 0.05$ , \* $P > 0.05$ .



**Figure 2.** The hearing threshold of C57BL/6J mice at 42-44 weeks of age after treatment. The hearing threshold at 24 kHz significantly decreased in the zonisamide-treated group compared to threshold of the felodipine-treated group and the saline-treated group. # $P < 0.05$ .

rons under ischemic conditions [7]. Inhibition of T-type calcium channels can protect neurons after stroke [8]. It has been reported that T-type calcium channel blockers can provide protection for the inner ear against cisplatin-mediated toxicity [9]. A T-type calcium channel blocker

can ameliorate noise-induced hearing loss by preserving the outer hair cells [10]. However, few studies have explored the effect of T-type calcium channel blocker on auditory organ especially SGNs of aged mice. SGNs are the primary auditory neurons as the bridge connecting hair cells with cochlear nucleus. C57BL/6J mice are often used as a model of presbycusis because they exhibit marked progressive age-related hearing loss [11]. The hearing of C57BL/6J mice was profound loss at 42-44 weeks of age (42-44-W) in our previous study [12]. The objective of this study is to explore the effect on the SGNs of 42-26-W C57BL/6J mice by T-type calcium channel blockers. First, we quantified the subunits of T-type calcium channel blockers in the spiral ganglion neurons of C57BL/6J mice in three groups (6-8-W, 24-26-W, 42-44-W) according to age via RT-PCR. Next, we administered three drugs (zonisamide, felodipine, saline) to the 42-44-W C57BL/6J mice by gavage for four weeks. We observed the changes in the hearing threshold of 42-44-W C57BL/6J mice after treatment. Meanwhile, we measured the expression of calcium-binding proteins of spiral ganglion neurons after treatment.

### Materials and methods

#### Animals and tissue preparation

Forty five C57BL/6J male mice consisted of three groups (6-8-W, 24-26-W, 42-44-W) according to age. Each group was separated into three subgroups (n=5 in each subgroup) to

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**Table 1.** The expression levels of calmodulin and calbindin in the SGNs of 42-44-W C57BL/6J mice after treatment (mean optical density  $\pm$  S.E.M)

	Zonisamide	Felodipine	Saline
Calmodulin	0.4877 $\pm$ 0.0262 <sup>#</sup>	0.5744 $\pm$ 0.0254	0.5851 $\pm$ 0.0196 <sup>#</sup>
Calbindin	0.4813 $\pm$ 0.0168 <sup>*</sup>	0.4812 $\pm$ 0.0220 <sup>*</sup>	0.4792 $\pm$ 0.0281 <sup>*</sup>

<sup>#</sup>P<0.05, <sup>\*</sup>P>0.05.

detect the three T-type calcium channel subunits ( $\alpha$ 1G,  $\alpha$ 1H,  $\alpha$ 1I) via RT-PCR. Another thirty 42-44-W C57BL/6J male mice were separated into three treatment groups (saline, zonisamide, and felodipine). Each group was subjected to an auditory brainstem response (ABR) test after treatment. Zonisamide and felodipine were obtained from Sigma Chemical Co. (St. Louis, USA). Preliminary experiments using mice led us to select dosages of 160 mg/d/kg for zonisamide and 10 mg/d/kg for felodipine. The drugs were administered to the animals via gavage for four consecutive weeks. All experiments were performed in compliance with Chinese legislation on the care and use of laboratory animals, and all studies were approved by the Soochow University Institutional Animal Care and Use Committee.

The mice were sacrificed via cervical dislocation after the ABR test. The SGNs were isolated using a dissecting microscope. The tissues used for *in situ* hybridization and immunohistochemistry were fixed using 4% paraformaldehyde and embedded in paraffin after complete fixation. The tissues used for RT-PCR were immersed in ice-cold RNA solution to prevent RNA degradation.

### Real-time quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was extracted using Trizol Reagent (Gibco, USA). The concentration and quality of the extracted RNA were determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280). The A260/A280 ratio of each sample was 1.8 to 2.0. RT cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, USA), and PCR was performed according to the manufacturer's instructions (ABI-7500, USA) using a total volume of 20  $\mu$ L of a reaction mixture containing 4  $\mu$ L of 5 $\times$  RT buffer, 0.5  $\mu$ L of Oligo (dT), 0.5  $\mu$ L of dNTPs, 1  $\mu$ L of MMLV reverse transcriptase, 10  $\mu$ L of DEPC-treated water, and 4  $\mu$ L of the RNA template. The reaction conditions for inactivating MMLV

were 37°C for one hour followed by 95°C for 5 minutes.

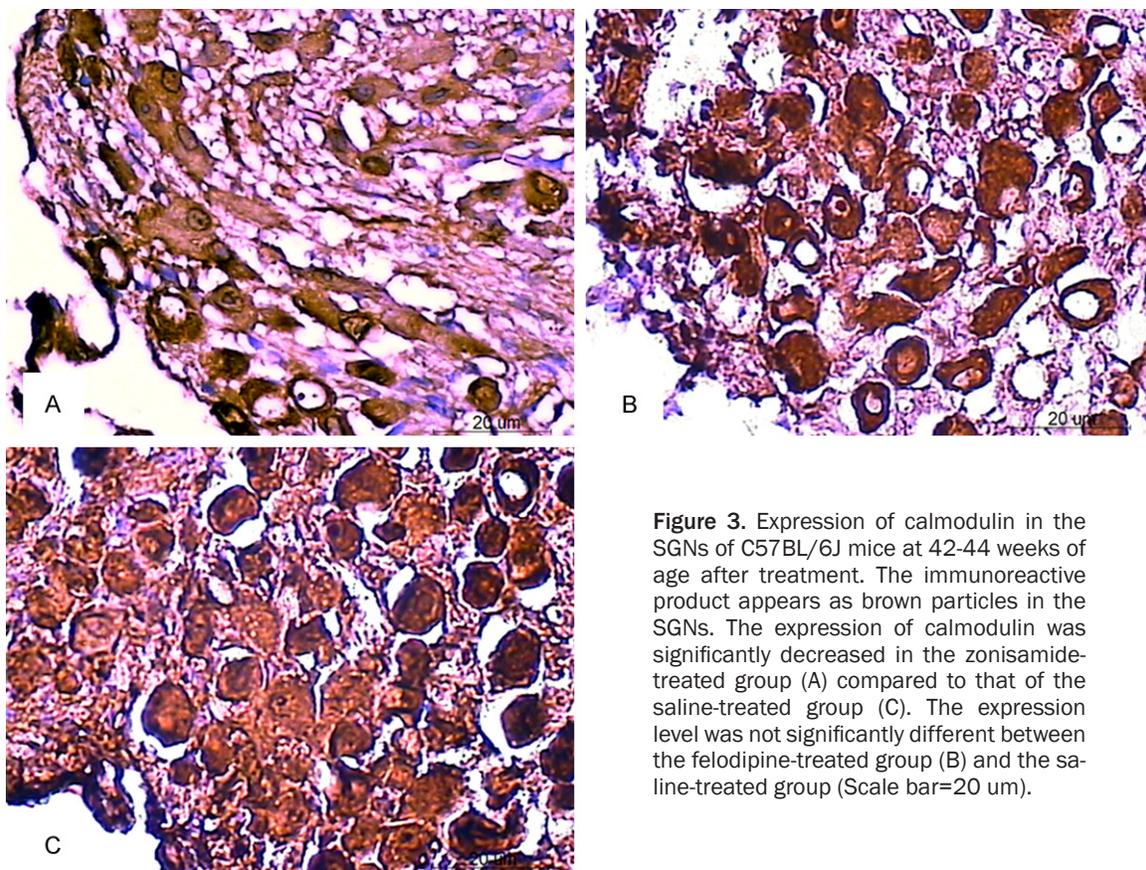
PCR (SYBRGreen PCR kit, Thermo, USA) was performed using a total volume of 50  $\mu$ L. The mixture contained 32.5  $\mu$ L of SYBRGreen Mix, 0.5  $\mu$ L of the upstream primer, 0.5  $\mu$ L of the downstream primer, 14.5  $\mu$ L of ddH<sub>2</sub>O, and 2  $\mu$ L of the cDNA template. The PCR conditions were as follows: 95°C for 30 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 73°C for 90 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control for the quantification of the PCR results. Relative quantification (RQ) was performed based on the number of PCR cycles necessary to obtain the threshold signal of fluorescence (Ct).

The DNA sequences of the primers (forward and reverse primers, respectively) were as follows: GAPDH (5-AACGGATTTGGTCGATTG-3 and 5-GGAAGATGGTGTGGGATT-3),  $\alpha$ 1G subunit (5-ACCTGCCTGACACTCTGCAG-3 and 5-GCTGGCCTCAGCGCAGTCGG-3),  $\alpha$ 1H subunit (5-GGCGTGGTGGTGGAGAACTT-3 and 5-GATGATGGTGGGATTGAT-3),  $\alpha$ 1I subunit (5-ATCTGCTCCCTGTCGG-3 and 5-GAGAACTGGGTCGCTATG-3). The primers were synthesized using the Primer Premier 5.0 software program (Invitrogen, USA) at by the Shanghai Linyan Institute (Shanghai, China).

### Auditory brainstem response (ABR)

Each mouse was anesthetized via intramuscular injection of a mixture of xylazine (25 mg/kg) and ketamine (100 mg/kg) and placed in a soundproof anechoic chamber containing a thermostabilized prone experimental platform that maintained the body temperature at 37°C. ABRs were recorded subcutaneously using needle electrodes placed at the vertex, the ipsilateral mastoid process and the contralateral mastoid process. The speaker was placed in the ear canal. Generation of stimulus signals and recording of evoked potentials were performed using an ABR workstation (Tucker-Davis Technologies AD3, USA). Tone burst stimuli (5-ms duration, 0.5-ms rise-fall time) were generated, and each average response was based on 1000 repeated stimuli at a rate of 11 stimuli/s applied at frequencies of 8, 16, 24 and 32 KHz. Neuronal activity was amplified

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**Figure 3.** Expression of calmodulin in the SGNs of C57BL/6J mice at 42-44 weeks of age after treatment. The immunoreactive product appears as brown particles in the SGNs. The expression of calmodulin was significantly decreased in the zonisamide-treated group (A) compared to that of the saline-treated group (C). The expression level was not significantly different between the felodipine-treated group (B) and the saline-treated group (Scale bar=20  $\mu$ m).

(100,000U) and filtered (0.3-3.0 kHz). The hearing threshold at each frequency was determined based on a clear wave when reducing the stimulus intensity from the suprathreshold level. Beginning at a sound pressure level (SPL) of 100 dB, decreasing steps in 10-dB SPL increments were applied, after which 5-dB increments were applied until a clear wave response occurred. The mean threshold value was determined after repeating the ABR test at least two times.

### *Immunohistochemistry*

The paraffin-embedded slices were sectioned (4  $\mu$ m thick) and mounted on poly-lysine-coated glass slides. The sections were washed 3 $\times$  with 0.01 M PBS (5 min each) and blocked using 1% BSA for 30 min at room temperature. The sections were incubated overnight in a primary antibody (calmodulin 1:300; calbindin 1:200; Abcam Company). The sections were rinsed 3 $\times$  with 0.01 M PBS (5 min each) and incubated for 2 h in biotinylated goat anti-rabbit antibody (1:200). The sections were incubated for another 2 h in ABC solutions after identical rinsing steps. After washing with PBS as indicated

above, the sections were stained using diaminobenzidine (DAB) and counterstained using hematoxylin. The slides were coverslipped using neural gum mounting medium. The data were quantified by measuring the mean optical density.

### *Statistical analysis*

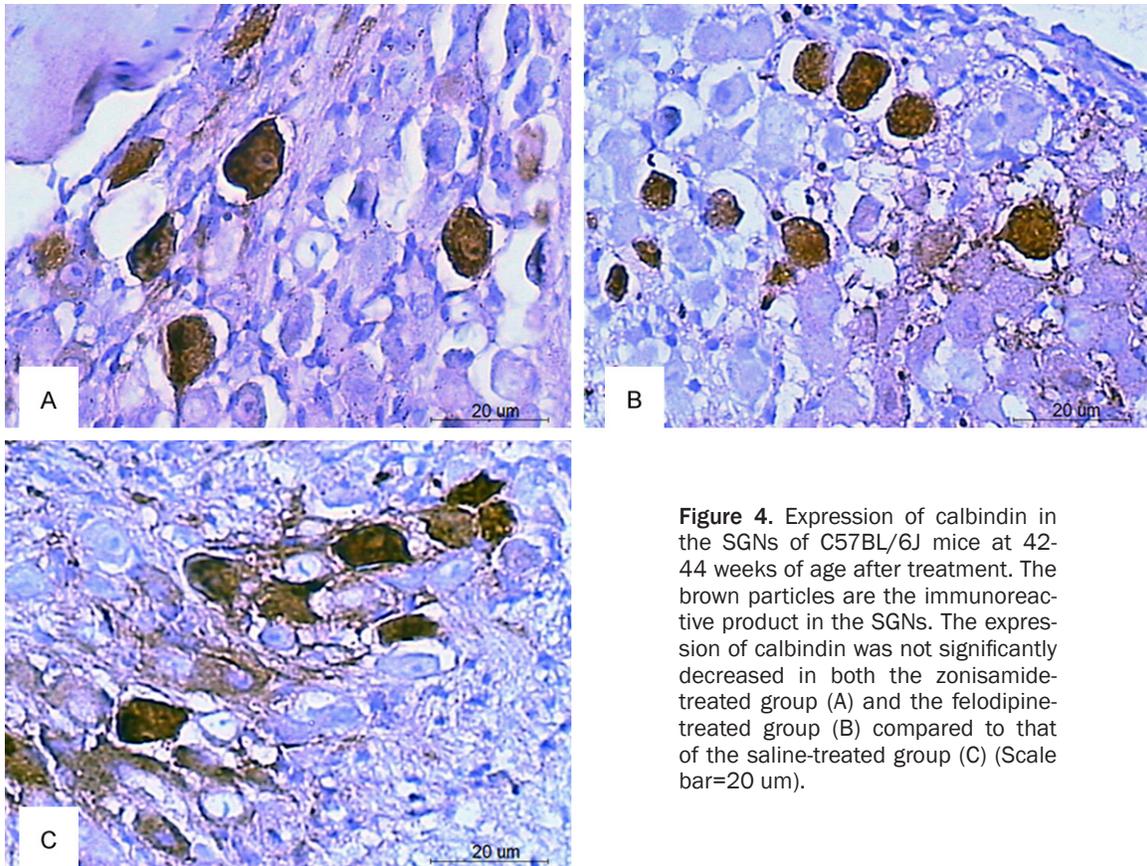
All data were expressed as means  $\pm$  SD and were analyzed using SPSS software, version 17.0 (SPSS, USA). One-way analysis of variance (ANOVA) with the LSD post hoc test was used to analyze the ABR test, RT-PCR and immunohistochemistry data sets. Differences between the groups were considered significant for  $P \leq 0.05$ .

## **Results**

### *Expression of three T-type channel subunits by real-time quantitative RT-PCR*

The expression level of each receptor was calculated using the formula  $2^{-\Delta\Delta CT}$  (Figure 1). Compared to the other two subunits, the expression level of  $\alpha 1H$  in the SGNs was the greatest at the age of 6-8 W ( $P < 0.05$ ). The

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**Figure 4.** Expression of calbindin in the SGNs of C57BL/6J mice at 42-44 weeks of age after treatment. The brown particles are the immunoreactive product in the SGNs. The expression of calbindin was not significantly decreased in both the zonisamide-treated group (A) and the felodipine-treated group (B) compared to that of the saline-treated group (C) (Scale bar=20 µm).

expression levels of  $\alpha 1G$  and  $\alpha 1I$  were lower and there was no significant difference between them ( $P > 0.05$ ). The expression levels of three receptors decreased with age. The expression levels of  $\alpha 1G$  and  $\alpha 1H$  significantly decreased at the age of 42-44 W compared to the age of 6-8 W ( $P < 0.05$ ).

### ABR test

The hearing loss was profound at 42-44 weeks of age of C57BL/6J mice. Compared to the saline-treated group, the hearing threshold at 24 kHz significantly decreased in the zonisamide-treated group ( $P < 0.05$ ). The hearing threshold at 32 kHz decreased in the zonisamide- and felodipine-treated groups compared to the saline-treated group, but these differences were not significant (**Figure 2**).

### Expression of calmodulin and calbindin based on immunohistochemistry

In the SGNs of 42-46-W C57BL/6J mice (**Table 1**), the expression level of calmodulin was significantly lower in the zonisamide-treated group (**Figure 3A**) than in the saline-treated group (**Figure 3C**) ( $P < 0.05$ ). However, the expression

level of calmodulin was not significantly different between the saline-treated group and the felodipine-treated group (**Figure 3B**) ( $P > 0.05$ ). The expression level of calbindin was not significantly different in the zonisamide-treated group (**Figure 4A**) or the felodipine-treated group (**Figure 4B**) than in the saline-treated group (**Figure 4C**) ( $P > 0.05$ ).

### Discussion

Research on the etiology of presbycusis has generated much significant progress, but the ideal method for the prevention and treatment of presbycusis has yet to be discovered. Some studies on the relationship between calcium channels and deafness have been reported recently. A missing gene, CACNA1D, closely associated with congenital deafness and heart disease was reported [13]. A variety of calcium channel blockers have been broadly used to treat diseases of the cardiovascular system and other organ systems. There is evidence that these drugs can reduce the cytotoxicity of calcium ions in ischemic heart disease [14]. There is controversy, especially with respect to L-type calcium channels, over whether calcium

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channel blockers exert a protective effect on the inner ear. The L-type calcium channel blocker nifedipine was observed to decrease noise damage to the guinea pig cochlea [15]. In contrast, nimodipine, another L-type calcium channel blocker, did not protect the cochlea from hearing loss and even worsened the damage to the organ of Corti when administered in combination with pentoxifylline [16]. However, the T-type calcium channel blocker flunarizine prevented cisplatin-induced cochlear hair cell death in an in vitro study [9].

We chose 42-44-W C57BL/6J mice experiencing profound hearing loss as our animal model mimic old people to determine effect of T-type calcium channel blockers. Age-related progressive retrograde degeneration of SGNs had been demonstrated in C57BL/6J mice [17]. Therefore, we observed the effect of T-type calcium channel blockers on the SGNs of 42-44-W C57BL/6J mice. First, we confirmed that three T-type calcium channel subtypes were distributed in the SGNs of C57BL/6J mice. Among these three subunits,  $\alpha 1H$  was highly expressed and the other two subunits were weakly expressed based on RT-PCR. The expression levels of three receptors especially for  $\alpha 1G$  and  $\alpha 1H$  significantly decreased with age. We chose the T-type calcium channel blocker zonisamide and the L-type blocker felodipine, saline as control group. Zonisamide is an antiepileptic drug that also reduces the symptoms of Parkinson's disease [18]. The possible mechanism of action of this drug is related to its inhibition of T-type calcium channels in neurons [19, 20]. Felodipine is a preferred drug to reduce high blood pressure by inhibiting L-type calcium channel [21]. The amelioration of hearing is associated with the peripheral or central auditory system or both. SGNs may represent one neuron type that benefits from this treatment. The finding that the zonisamide-treated group exhibited better hearing than the felodipine-treated group may be due the effect of zonisamide on T-type calcium channel.

Calcium homeostasis is maintained in hair cells and SGNs by regulating proteins such as calcium-binding proteins [22, 23]. Calcium-binding proteins play important roles in intracellular  $Ca^{2+}$  signaling, modulating neuronal excitability and plasticity [24]. To determine the changes in SGNs after the administration of T-type calcium channel blockers, we measured the expression

levels of two calcium-binding proteins, calbindin and calmodulin. Calbindin is a calcium-binding protein belonging to the troponin C superfamily. Calmodulin is known to modulate the calcium-dependent inactivation of calcium channels [25]. Calmodulin and calbindin are both EF hand-containing calcium-binding proteins that are widely expressed in neurons and act as  $Ca^{2+}$  buffers to regulate calcium signal transduction [26, 27]. Increased calcium-binding protein immunoreactivity in the cochlea nucleus of old mice has been observed to reflect an endogenous protective strategy to counteract calcium overload [28]. Interestingly, our results revealed that the expression level of calmodulin decreased after administration of the T-type blocker zonisamide. The results indicate that T-type blocker can alleviate calcium overload in the SGNs of 42-44-W C57BL/6J mice. Our results demonstrate that the more pronounced effects of T-type channel blockers may be due to the larger dose of the drugs provided by administration via gavage rather than via drinking water for four consecutive weeks. One benefit of this route of administration is that the dosage of the drugs could be accurately controlled. However, the side effects must be considered if a larger dose is administered to humans.

Nevertheless, these drugs cannot be immediately used clinically for presbycusis because the results of this study were obtained using a limited number of mice. Many factors may affect these results, such as the sex of the animals, the type of calcium channel blockers used, use of these drugs alone or in combination, and the route of administration. Future studies will be focused on the manner in which to select and administer these calcium channel blockers and the reduction of their side effects.

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### Disclosure of conflict of interest

None.

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