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Original Article

Expression patterns of $Ca_V1.3$ channels in the rat cochlea

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Although $Ca_V1.3$ channels are known to be essential for neuronal excitation and signal transduction in the auditory system, their expression patterns in the cochlea are still not fully understood, particularly in the regions where non-sensory cells are located. We performed immunohistochemistry, western blotting and reverse transcription-polymerase chain reaction (RT-PCR) to identify the expression and distribution of $Ca_V1.3$ channels in the rat cochlea. Immunohistochemistry revealed that $Ca_V1.3$ channels were localized in the outer hair cells (OHCs), inner hair cells (IHCs), limbus laminae spiralis, spiral ganglion cell, spiral ligament (SL), and stria vascularis (STV). The results of RT-PCR and western blotting demonstrated $Ca_V1.3$ channels had a tissue-specific expression pattern. $Ca_V1.3$ mRNA and protein were intensively expressed in the basilar membrane and spiral ganglion while moderate level of $Ca_V1.3$ channels was observed in SL and STV. Our study preliminarily revealed the expression patterns of $Ca_V1.3$ channels in the rat cochlea, providing a theoretical basis for further research on the role of $Ca_V1.3$ channels in the periphery auditory system.

Keywords calcium; $Ca_V1.3$ channels; cochlea; rat; endocochlear potential

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Introduction

Ca^{2+}, a key second messenger involved in cell signaling as well as a cytotoxin, regulates virtually all cellular processes, including proliferation, differentiation, growth, apoptosis, and cell death [1]. The resting cytosolic Ca^{2+} concentration of ~100 nM is about 10,000 folds lower than the interstitial Ca^{2+} concentration. This enormous gradient is rigorously controlled to prevent ambiguity in cell signaling and cell death due to Ca^{2+} overload [2]. An increase in the cytosolic Ca^{2+} concentration could translate mechanical signals such as cellular deformation and chemical signals (e.g. hormones, neurotransmitters, and growth factors) into a variety of cellular actions such as regulation of enzyme activities, neurotransmitter release, salt and water secretion, contraction, proliferation, and cell death [1,2]. Therefore, maintaining the balance of intracellular Ca^{2+} level could ensure the survival and function of a cell.

In the inner ear, prominent expression of Ca^{2+} has been observed in the cochlear tissues, such as inner hair cells (IHCs) and outer hair cells (OHCs), stria vascularis (STV), Reissner's membrane, and interdental cells. IHCs and OHCs sequester Ca^{2+} into cytosolic stores. Ca^{2+} stores in IHCs and OHCs express ryanodine receptors and inositol-1,4,5-trisphosphate receptors, which permit highly localized releases of Ca^{2+} into the cytosol and thereby can function as amplification mechanisms for Ca^{2+}-mediated cell signaling [3,4]. Homeostasis of intracellular Ca^{2+} is crucial for normal hair cells' development and functions. Recent data indicated that transmitter release from hair cells requires Ca^{2+} influx through voltage-gated Ca^{2+} channels [5,6]. In auditory hair cells, most of the voltage-gated Ca^{2+} current appears to be carried by L-type channels [7]. The data obtained from $Ca_V1.3$ knockout mice also demonstrated the importance of this channel for mature IHCs function after the onset of the hearing [7]. In view of the above findings, it is reasonable to speculate that L-type calcium channels (Ca_V1) play an important role in balancing intracellular Ca^{2+} concentration. Ca_V1 are present in four forms, referred to as $Ca_V1.1$ (α_{1S}), $Ca_V1.2$ (α_{1C}), $Ca_V1.3$ (α_{1D}), and $Ca_V1.4$ (α_{1F}) [8].

As one main Ca_V1, the $Ca_V1.3$ gene has been found in a variety of cells including ventricular cardiac muscle, smooth muscle, neuroendocrine cells, photoreceptors, amacrine cells, and hair cells of the inner ear where it mediates synaptic transmission [8]. Although $Ca_V1.3$ channels were

first cloned in the early 1990s, low-level expression in heterologous systems limited the study of $Ca_V1.3$ channels. The study of this L-type calcium channel was mainly focused on the property of electrophysiology. Much less is known about the expression and distribution of $Ca_V1.3$ channels in the whole cochlea. In the present study, therefore, we attempted to evaluate the distribution of $Ca_V1.3$ channels in rat cochlear tissues and identify the expression of $Ca_V1.3$ channels in basilar membrane (BM) including the organ of corti, spiral ganglion cell (SGC), STV including spiral ligament (SL) at both mRNA and protein levels.

Materials and Methods

Animals

Six adult Sprague-Dawley rats (200−250 g) and 20 neonatal Sprague-Dawley rats (P3−P5) were purchased from Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). The care and experimental treatment of animals was approved by the Animal Research Committee, Tongji Medical College, Huazhong University of Science and Technology.

Immunohistochemistry

The temporal bones of six mature rats were removed immediately after euthanasia and were fixed by injection with 4% paraformaldehyde in 0.1 mM phosphate-buffered saline (PBS, pH 7.4) into the round and oval window and were kept in this fixative overnight at 4°C. The cochleae were then washed with PBS and decalcified in 10% sodium ethylenediaminetetraacetic acid (EDTA) (adjusted pH to 7.4) for 7 days, then immersed in gradient sucrose solutions: 2 h in 10% sucrose, 2 h in 20% sucrose as well as 12 h in 30% sucrose and then frozen in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, USA). Serial mid-modiolar cryosections of 10 μm were cut and mounted on glass slides. Representative sections were used for immunofluorescence.

Cochlea sections were incubated in 0.5% Triton X-100 for 15 min at 37°C. The sections were then washed thrice with PBS and blocked for 1 h by 5% bovine albumin serum (BSA; Boster, Wuhan, China) at room temperature. Antibodies were diluted in 5% BSA. The primary antibody was rabbit anti-$Ca_V1.3$ channels polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, USA). After incubation at 4°C for 24 h, sections were washed thrice in PBS and afterwards incubated with DyLight™ 488 conjugated goat anti-rabbit immunoglobulin G (IgG) (1:600; Multi-Sciences Biotech Co. Ltd, Hangzhou, China) at 37°C for 2 h in the dark. After three washes in PBS, the sections were incubated with 10 μg/ml propidium iodide (PI) (Sigma, St Louis, USA) for 10 min at 37°C. After a final wash with PBS, the slides were covered with glass cover slips. Control incubations were routinely processed without primary antibody. Immunolabeling was observed and imaged with an Olympus Fluoview 500 IX 71 confocal microscope (Tokyo, Japan). Images were digitally recorded at the same magnification and time of exposure.

Western blot analysis

The tissues of BM, SGC, and STV were, respectively, separated by microdissection from 10 neonatal rats and the kidney of a rat was obtained as a positive control. The samples were pooled to obtain equalized protein (20 μg) as follows: total protein extracts were lysed with 50 mM Tris−HCl (pH 7.4), 300 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche, Basel, Switzerland). All the procedures were performed on ice. The cochleae homogenate were centrifuged (13,800 *g*, 4°C, 30 min), and protein concentration in the supernatant was determined by BCA protein assay. Equal amounts of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Millipore, Billerica, USA). Membranes were probed with rabbit anti-$Ca_V1.3$ channels polyclonal antibody (1:1000; Santa Cruz Biotechnology) overnight at 4°C and also incubated with β-actin antibody (1:2000; Sigma) as internal control. After being rinsed in Tris-buffered saline with 0.02% Tween (TBS-T), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:3000; Sigma) for 2 h at room temperature and finally developed using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, USA). All western blots were performed in duplicate and visualized using a chemiluminescence system (PTC-200; Bio-Rad Laboratories, Hercules, USA).

Reverse transcription-polymerase chain reaction

The tissues of BM, SGC, and STV from 10 neonatal rats and a kidney were separately dissected under the dissecting microscope. Total RNA was isolated by using TRIzol® reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol and was used for first-strand cDNA synthesis using ReverTra-Plus-TM (Toyobo, Osaka, Japan) in the presence of oligo-dT, dNTPs, and RNase inhibitor. The sequence of primers used was as follows: glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*), forward: 5′-GTCGGTGTGAACG GATTTGG-3′, reverse: 5′-GACTGTGCCGTTGAACTTGC-3′ and $Ca_V1.3$ (*CACNA1D*), forward: 5′-CATCATGCTCAA CACGCTCT-3′, reverse: 5′-TATCAACGACGCTACCGAC A-3′. The PCR was carried out by pre-incubation at 95°C for 2 min and then was cycled 40 times at 95°C (1 min), 58°C (30 s), 72°C (45 s), followed by a 10-min extension step at 72°C. *GAPDH* was used as a housekeeping gene. The PCR products were separated by electrophoresis on a

2% agarose gel and visualized by ethidium bromide staining.

Statistical analyses

All the data were presented as the mean ± standard error of mean (SEM). One-way analysis of variance (one-way ANOVA) with Tukey's correction was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

Results

Distribution of $Ca_V1.3$ channels

Several lines of evidence have suggested that $Ca_V1.3$ channels play an essential role in Ca^{2+} homeostasis. Therefore, immunohistochemistry was used to determine whether $Ca_V1.3$ channels were present in the inner ear. The immunoreactivity of $Ca_V1.3$ channels in the cochlea is presented in **Fig. 1** . In the cochlear sections, $Ca_V1.3$ channels were prominently localized in the hair cells [**Fig. 1(A,C,G,I)**] and SL including the limbus laminae spiralis (LLS) [**Fig. 1(A,C,J,L)**], and the immunoreactivity of $Ca_V1.3$ channels in IHCs was more stronger than that in OHCs [**Fig. 1(A,C,G,I)**]. Intense labeling of $Ca_V1.3$ channels were also observed in the cytomembrane and neurite of SGCs [**Fig. 1(D,F)**]. Medium intense labeling was occasionally detectable in supporting cells [**Fig. 1(G,I)**]. In the STV, a positive staining reaction for these channels was detected in the marginal cells, intermediate cells, and basal cells [**Fig. 1 (A,C,J,L)**].

$Ca_V1.3$ channels expression at protein level in cochlea

Western blotting was performed to determine the expression of $Ca_V1.3$ channels at protein level in the cochlea. We detected a 199-kDa band corresponding to $Ca_V1.3$ channels while a 43-kDa band corresponding to β-actin in BM, STV, and SGC [**Fig. 2(A)**]. Normalized to the densitometry values of β-actin in each sample, the relative levels of $Ca_V1.3$ channels protein were presented in **Fig. 2(B)**. Data obtained here indicated that there was a different expression of $Ca_V1.3$ channels protein in the rat cochlear tissues (one-way ANOVA, $F = 18.425$, df = 3, $P < 0.001$). There was remarkable difference in BM compared with STV (Tukey's test, $q = 9.746$, $P < 0.001$). Meanwhile, we noticed that the expression of $Ca_V1.3$ channels displayed difference between STV and SGC (Tukey's test, $q =$

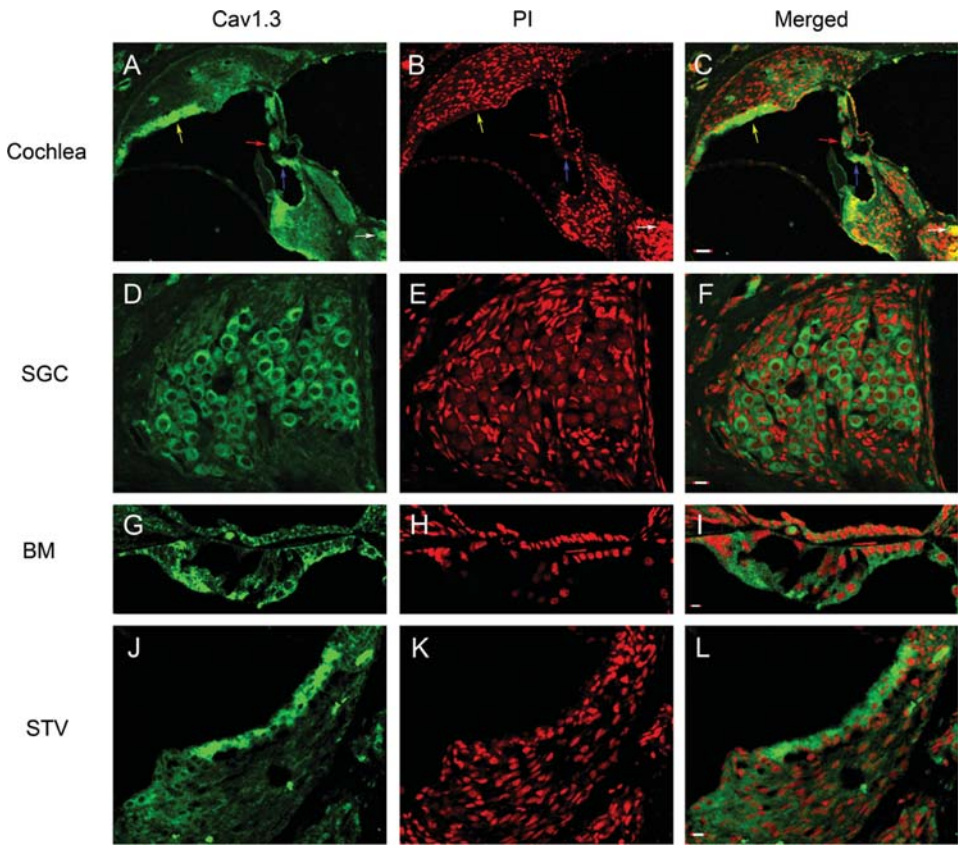

Figure 1 Expression of $Ca_V1.3$ channels in the cochlea Expression of $Ca_V1.3$ channels (A,D,G,J) can be seen in green. The nuclei are stained with PI in red (B,E,H,K). (C,F,I,L) show an overlap of $Ca_V1.3$ channels (green) and PI (red). Note $Ca_V1.3$ channels are mainly expressed in hair cells (both OHCs and IHCs; A,C,G,I), LLS (A,C), SGC (D,F), SL (A,C,J,L) and STV (A,C,J,L). OHCs: red arrow; IHCs: blue arrow; SGC: white arrow; STV: yellow arrow. Scale bar: A–C, 50 μm; D–L, 20 μm.

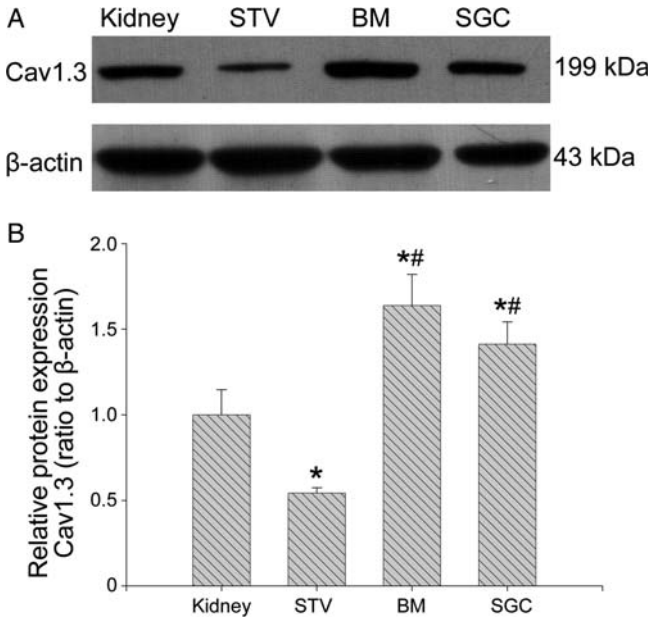

Figure 2 Expression of $Ca_V1.3$ channels at protein level in the cochlea and kidney (A) Protein level of $Ca_V1.3$ channel as detected by western blotting. (B) The abundance of expression of the calcium channel in protein level in different tissue of rats' cochlea and kidney. $^*P = 0.021$ comparing STV with kidney, $P = 0.010$ comparing BM with kidney, $P = 0.032$ comparing SGC with kidney; $^{\#}P < 0.001$ comparing BM with STV, $P = 0.002$ comparing SGC with STV.

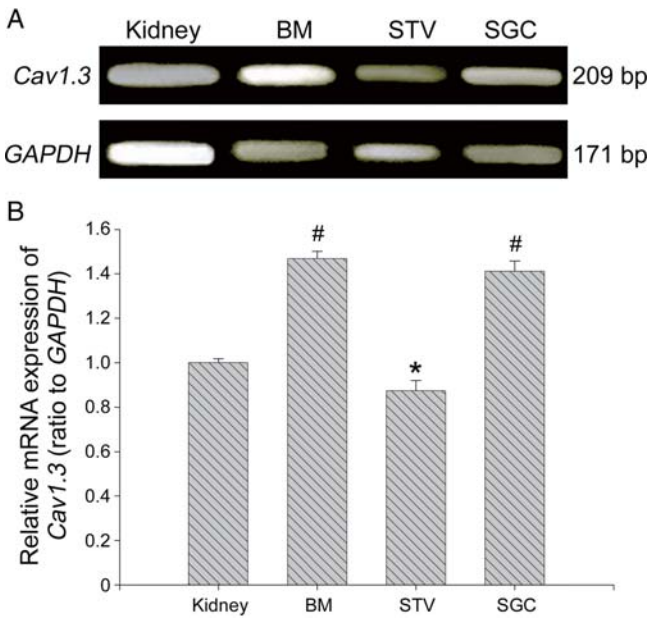

Figure 3 Expression of $Ca_V1.3$ channels and GAPDH at mRNA level in the cochlea and kidney (A) The results of RT-PCR showed the presence of a unique 209-bp band corresponding to $Ca_V1.3$ channels and a unique 171-bp band corresponding to GAPDH in BM, SGC, STV, and kidney. (B) The abundance of expression of the calcium channels at mRNA level in different tissue. $^*P = 0.04$ comparing STV with kidney, $^{\#}P < 0.001$ comparing BM or SGC with STV, $^{\#}P < 0.001$ comparing BM or SGC with kidney.

7.732, $P = 0.002$). However, there was no significant difference in the expression of $Ca_V1.3$ channels between BM and SGC (Tukey's test, $q = 2.014$, $P = 0.192$). These results suggested that the expression of $Ca_V1.3$ channels in individual rat cochlear tissue is different at protein level.

$Ca_V1.3$ channels expression at mRNA level in cochlea

The relative mRNA expression of $Ca_V1.3$ channels in different tissue of cochlea was also compared using RT-PCR. The results of RT-PCR showed the presence of a unique 209-bp band corresponding to $Ca_V1.3$ channels and a unique 171-bp band corresponding to *GAPDH* in BM, SGC, STV, and kidney [**Fig. 3(A)**]. As demonstrated in the figure, the expression of $Ca_V1.3$ channels mRNA presented more in BM than in SGC, and it was weakly expressed in STV [**Fig. 3(B)**]. Normalized to *GAPDH*, there was a different expression of $Ca_V1.3$ channels at mRNA level in the rat cochlea and kidney (one-way ANOVA, $F = 185.247$, $df = 3$, $P < 0.001$) [**Fig. 3(B)**]. There was a remarkable difference in BM (Tukey's test, $q = 27.376$, $P < 0.001$) and SGC (Tukey's test, $q = 24.719$, $P < 0.001$) compared with STV. Meanwhile, we noticed that the expression of $Ca_V1.3$ channels displayed difference in cochlear tissues compared with kidney (Tukey's test, $P < 0.05$). In addition, there was no significant difference in the expression of $Ca_V1.3$ channels between BM and SGC (Tukey's test, $q = 2.657$, $P = 0.097$). The results indicated that the expression of $Ca_V1.3$ channels mRNA was detected both in cochlea tissues and kidney. The distribution of the calcium channels was characterized by tissue specificity.

Discussion

Previous studies showed that the voltage-dependent Ca^{2+} channels (VDCCs) regulate neurotransmitters release and cellular excitability, which are essential for excitable cells [9,10] and $Ca_V1.3$ channels, as the major VDCC of auditory hair cells, are essential for normal hair cell development and synaptic transmission [7,11]. The $Ca_V1.3$ knockout mice showed congenital deafness and consequent degeneration of auditory hair cells due to nearly complete absence of $Ca_V1.3$ channels in hair cells [7,12]. Furthermore, the recent study showed that $Ca_V1.3$ channels are required for normal hearing and cardiac pace-making in human, and that loss-of-function in only a subset of channels is sufficient to cause a human channelopathy (termed SANDD syndrome, sinoatrial node dysfunction and deafness) with a cardiac and auditory phenotype that closely resembles that of Cacna1d$^{-/-}$ mice [13]. In view of these, $Ca_V1.3$ channels contribute greatly to auditory functions and the expression pattern of these calcium channels in the cochlea might be expected to correlate with the homeostasis of Ca^{2+} signaling in the inner ear.

Although numerous studies have investigated $Ca_V1.3$ channels in the mammalian inner ear focusing on the property of electrophysiology in the earlier years, there is still an absence of information regarding the differential expression in whole cochlea tissue and the significance of that. The results in this study suggested that $Ca_V1.3$ channels were expressed in the rat cochlea, with a selective localization among cell types. As reported in previous studies, we also detected the expression of $Ca_V1.3$ channels both in IHCs and OHCs [11,12] and spiral ganglion neurons[14]. Besides this, immunohistochemistry results also revealed that $Ca_V1.3$ channels were not only localized in STV, but expressed in SL, and LLS. Furthermore, RT-PCR and western blot analysis also indicated that $Ca_V1.3$ channel gene (*CACNA1D*) had tissue specificity. The expression of $Ca_V1.3$ channels were mainly in the BM and SGC and moderate expression was observed in SL and STV. Why is the expression of this calcium channel different in cochlear tissues? We speculate that the differential and specific expression of $Ca_V1.3$ channel potentially aims to maintain the Ca^{2+} concentration in cochlea.

In the inner ear, the endolymph is an unusual extracellular fluid for its high K^+, low Na^+, and low Ca^{2+} concentration. Endolymph contains 20 μM Ca^{2+}, which is very low compared with other extracellular fluids such as perilymph or plasma which contain 1−2 mM Ca^{2+} [15–18]. For normal auditory function, the endolymphatic Ca^{2+} concentration can neither be too low nor too high. Elevated Ca^{2+} concentration blocks transduction and the generation of microphonic potentials but reduced Ca^{2+} concentration suppresses microphonic potentials as well [19–21]. In addition, a large increase in the Ca^{2+} concentration in the endolymph concomitant with a fall in the endocochlear potential (EP) was induced by transient asphyxia or the intravenous administration of diuretics, and the increase of Ca^{2+} concentration was inhibited by the endolymphatic application of a membrane-permeable Ca^{2+} chelator, such as egtazic acid (EGTA)-acetoxymethy-lester [22]. Meanwhile, there was significant inhibition of the transient asphyxia-induced decrease in the EP by the application of nifedipine through the endolymph or a vertebral artery, but not through the perilymph [23]. On the contrary, there was much evidence confirming that Ca^{2+} absorption from endolymph appears to be driven at least in part by the EP, since the endolymphatic Ca^{2+} concentration is somewhat correlated with the magnitude of the EP [17,24].

In summary, our research indicated differential expression pattern of $Ca_V1.3$ channels in the rat cochlea. $Ca_V1.3$ channels were mainly present in the hair cells, SGC, marginal cells in the STV, SL, and LLS in the cochlea. The differential distribution of $Ca_V1.3$ channels may be determined by the demands of Ca^{2+} homeostasis, EP regulation and normal hair cells function in the cochlear tissues. However, the major mechanism responsible for $Ca_V1.3$ channels in the regulation of the positive EP needs further identification. Meanwhile, further experiments are required to clarify the role of $Ca_V1.3$ channel in the inner ear, especially in the regions where those non-sensory cells are located. It may provide a new way for us to get a better understanding of the property and the role of $Ca_V1.3$ channels in auditory physiology and pathology.

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