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Original Article

Expression Patterns of Cav1.3 in the Developing Stria Vascularis of Sprague–Dawley Rats

Zhihui Du[1], Han Liu[2], Hanqi Chu[1], Jin Chen[1]

[1]Department of Otolaryngology-Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[2]Department of Neurosurgery, Hubei Provincial Hospital of Integrative Medicine, Wuhan, China

ORCID IDs of the authors: Z.D. 0000-0002-2560-2134, H.L. 0009-0008-4266-5496, H.C. 0000-0003-0977-622X, J.C. 0000-0001-6076-1141.

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BACKGROUND: Although multiple functions of Cav1.3 in adult rat cochlea have been explored, its role in developing stria vascularis (SV) of Sprague–Dawley (SD) rats has rarely been reported.

METHODS: Sprague–Dawley rats from postnatal 0 to 21 days (p0-p21) were utilized for the experimental model and classified into 4 groups by age randomly: p0, p7, p14, and p21. p3 SD rats were employed to culture primary marginal cells (MCs). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunofluorescence technique were employed to identify Cav1.3 in vivo. Immunocytochemistry was applied to explore the expression of Cav1.3 in vitro.

RESULTS: Quantitative reverse transcription polymerase chain reaction showed that the Cav1.3 calcium channel gene (CACNA1D) was measured in the cochlear lateral wall (STV), which included SV and spiral ligament. Immunofluorescence photographs revealed that Cav1.3 was intensively expressed in the SV, weakly expressed in the spiral ligament and spiral prominence. In the SV, immunofluorescence labeling of Cav1.3 was present in MCs, intermediate cells, and basal cells. Immunocytochemistry confirmed that Cav1.3 was expressed in the cytomembrane of the MCs. Moreover, the expression of Cav1.3 was increased in the developmental STV at both the protein and mRNA levels.

CONCLUSION: Cav1.3 was mainly present in the cytomembrane of MCs in the SV of postnatal SD rats. Additionally, Cav1.3 protein and mRNA increased with development in the cochlear lateral wall of SD rats, including in the SV. The alteration of Cav1.3 expression may influence the homeostasis of ions and benefit the normal function and development of the cochlea.

KEYWORDS: Cav1.3, development, marginal cells, SD rats, stria vascularis

INTRODUCTION

Calcium ion (Ca^{2+}) is a pivotal intracellular signaling molecule implicated in diverse physiological processes, including proliferation, apoptosis, metabolism, neurotransmission, and hormone secretion.[1-3] The suitable level of intracellular Ca^{2+} is necessary for the normal morphology and mechanisms of cells. In the auditory system, L-type calcium ion currents are mainly transported by Cav1.3 channels.[4,5] Ca^{2+} action potentials (APs) fired by inner hair cells (IHCs) in neonatal mice drive afferent synaptic transmission.[6] Moreover, the Cav1.3 calcium channels in MCs of the stria vascularis (SV) are also indispensable for the formation and maintenance of endolymphatic potential (EP).[7,8]

Previous studies have shown that transient asphyxia or intravenous diuretics result in a substantial increase in Ca^{2+} concentration in endolymph, accompanied by a decrease in EP.[9] However, intralymphatic injections of nifedipine or membrane-permeable Ca^{2+} chelators, such as egtazic acid(EGTA)-acethoxymethyl ester (EGTA-AM), inhibited this increase.[10] This process may restrain the increase of calcium ions in EP-generating cells in the SV.[7] Cav1.3 also has been reported to decrease with age in the cochlea, but not in the neurons of the auditory cortex. It is probable that down-regulation of Cav1.3 in the auditory pathway may contribute to hearing loss by enhancing calcium-mediated oxidative stress.[11]

Corresponding author: JinChen, e-mail: catkin19832002@163.com
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The SV, a tissue rich in blood vessels, contributes to cochlear homeostasis. Dysfunctions of the SV account for several hearing impairments, including presbycusis and Meniere's disease.[12] Meniere's disease, a form of SV dysfunction due to the dysregulation of calcium homeostasis, shares pathology with space motion sickness.[13] The pathological mechanism involves the loss of many ion channels, especially calcium channels, which affects circulation and perfusion, so that the endolymph cannot be better supported and maintained.[12] Marginal cells (MCs) in the SV, exposed to the endolymph, express different components of the ion transport apparatus, such as K^+ and Ca^{2+} channels, facilitating the generation and maintaining of the EP.[8,14,15] Additionally, the Cav1.3 channel facilitates the evolution of lateral superior olivary nuclei in the central auditory system.[4] Cav1.3 was also a benefit to the growth of the cochlear and vestibular sensory epithelium.[16] Furthermore, Cav1.3 channels are crucial for maintaining the function of spiral ganglion neurons (SGNs) and ribbon synapses.[17] Despite several studies with respect to the Ca^{2+} channels and SV, there has been little research on the relationship between SV and Ca^{2+} channels in the past 10 years. Therefore, it is urgent to study the expression of Cav1.3 in SV, especially in MCs during development. This study attempts to detect the role of Cav1.3 in the SV of SD rats' cochlea and confirmed that Cav1.3 was present in the MCs in vitro. Furthermore, the correlation between the expression pattern of Cav1.3 and the developed SV was also investigated.

METHODS

Animals

Sprague–Dawley rats (p0-p21) were bred and housed in the Animal Research Centre of Tongji Medical College, Huazhong University of Science and Technology. These rats were all without the experience of otitis media, noise exposure, or ototoxic drug application. They were classified into 4 groups by developmental stages randomly (p0, p7, p14, and p21). Additionally, postnatal SD rats (p3) were used for primary culture. The protocol for animal experiments was in accordance with the guidelines of Tongji Medical College, Huazhong University of Science and Technology (approval no.: TJH-202006007, date: March 13, 2024).

Cochleae Sections Preparation

The rats were decapitated after euthanasia. Each cochlea was isolated immediately. Ten rats at each stage were used for cryosection and molecular biological studies. All cochleae of 10 rats were mildly immersed in 4% paraformaldehyde overnight at a temperature of 4°C. The cochleae were placed into 10% ethylenediaminetetraacetic acid sodium (EDTA) for 4 days and then dehydrated in 30% saccharobiose solution overnight. The prepared samples were wrapped in O.C.T. (Sakura, Torrance, CA) the following day. Finally, the embedded specimens were microdissected into 8 μm serial cryosections and placed on glass slides.

Immunofluorescence

The protocol of the immunofluorescence technique was operated as described previously.[18] The cryosections were transferred to blocking solution at room temperature for 40 minutes. Afterward, the primary antibody against Cav1.3 (1 : 200; Santa Cruz Biotechnology) was used to combine with the specimens throughout the night at 4°C. Rinsed 3 times with phosphate-buffered saline (PBS), cochlea tissues were immersed using secondary antibodies (Multi-Sciences, Hangzhou, China) at room temperature for 1 hour. Cochlear sections were then washed with PBS and counterstained by 4′,6-diamidino-2-phenylindole (DAPI) (10 μg/mL, Sigma-Aldrich, USA) for 5 minutes. The cryosections were then rinsed again and covered with coverslips. Finally, they were observed under the Olympus BX 51 microscope (Tokyo, Japan). The images were taken with the same exposure time and magnification.[19]

Primary Culture and Identification of Marginal Cells

The lateral wall (STV) was dissected from postnatal 3-day-old (p3) SD rats and minced into tiny pieces. After digestion with 0.1% collagenase II (Sigma-Aldrich, MO, USA) at 25°C for 30 minutes, the cell suspension was plated in 6-well plates and cultured in animal epithelial medium (EpiCM-animal, ScienCell, USA) with 2% fetal bovine serum. The cell culture medium was replaced every other day. Examine the MCs in primary culture with an antibody of cytokeratin-18.

Immunocytochemistry for Marginal Cells

Cultured MCs were soaked in fixative for 15 minutes and then applied with a mixture of primary antibody incubation at 4°C, a mouse anti-Cav1.3 (1 : 200; Alomone Labs, Israel) and a rabbit anti-CK18 polyclonal antibody (1 : 1000; Abcam, USA), all night. Samples were then stained with DAPI for 5 minutes and observed using an inverted microscope (Leica DMi8).

Quantitative Reverse Transcription Polymerase Chain Reaction

The tissues of the lateral wall (STV) were dissected, and total RNA from them was extracted with TRIzol Reagent (Invitrogen, California, USA). Subsequently, the extracted RNA (1 μg) was pipetted and reversely transcribed into cDNA according to the manufacturer's instructions. The nested primers of Cav1.3 were: 5′-CATCATGCTCAACACGCTCT-3′ for forward and 5′-TATCAACGACGCTACCGAC-3′ for reverse. As a housekeeping gene, GAPDH was applied for internal comparison, and the sequences of its primers were as follows: 5′-GTCGGTGTGAACGGATTTGG-3′ for forward and 5′-GACTGTGCCGTTGAACTTGC-3′ for reverse. The cDNA samples' amplification was performed on a LightCycler 480II (Roche, Switzerland) using SYBR (TaKaRa Biotechnology, Dalian, China). The amplification parameters were: 95°C for 3 minutes, then 40 cycles: 95°C (15 seconds), 60°C (1 minute), and 72°C (30 seconds). Each sample was operated on 3 times and the average was taken. The relative Cav1.3 mRNA was estimated with the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The data were shown as mean ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) test. If $P < 0.05$, it was statistically significant.

MAIN POINTS

- This study confirmed that Cav1.3 was expressed in the marginal cells of the stria vascularis in postnatal SD rats.
- This study demonstrated that the levels of Cav1.3 mRNA and protein increased during the development of the cochlear lateral wall.
- This study suggested that the expression patterns of Cav1.3 may influence the homeostasis of ions, and contributes greatly to proper tissue development and function of the cochlea.

RESULTS

Expression of Cav1.3 mRNA in the Developing Lateral Wall of Cochlea

Quantitative reverse transcription polymerase chain reaction (Figure 1) showed that the Cav1.3 mRNA in the lateral wall (STV) was as follows: p0 group, 0.965 ± 0.218; p7 group, 2.225 ± 0.614; p14 group, 6.442 ± 0.964; and p21 group, 10.129 ± 0.960. The level of Cav1.3 mRNA in the p21 group was much higher than that of the other 3 developmental stages (one-way ANOVA, $F = 26.45$, $df = 3$, $P < 0.001$). Compared with the p0 and p7 groups, Cav1.3 mRNA was also increased in the p14 group (SNK test: p0 group, $q = 6.510$, $P < 0.001$; p7 group, $q = 5.414$, $P < 0.001$). However, the difference in Cav1.3 mRNA expression between the p0 group and the p7 group was not statistically significant (SNK test, $q = 1.497$, $P > 0.05$). Therefore, it can be concluded that Cav1.3 was located in the lateral wall of the cochlea and increased gradually with development.

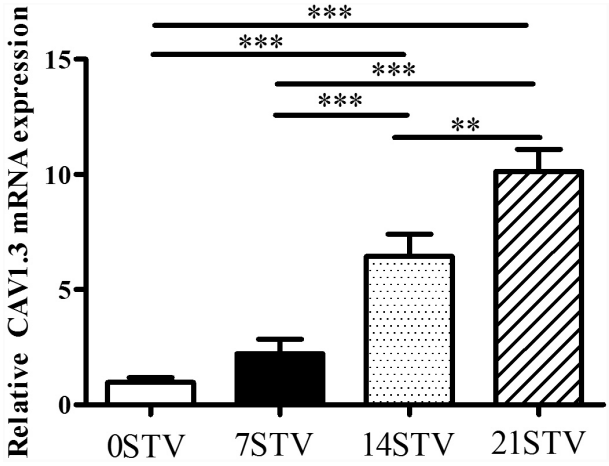

Figure 1. Expression of Cav1.3 mRNA in the lateral wall of the cochlea. The relative mRNA expression of Cav1.3 in the lateral wall. $^{**}P < 0.01$, $^{***}P < 0.001$.

Distribution and Developmental Alteration of Cav1.3 in the Lateral Wall of Cochlea

Figure 2A-D shows that strong immunoreactivity was observed in the SV. Moderate positive labeling for Cav1.3 was also visible in spiral ligaments and spiral prominence. Additionally, the fluorescence intensity of Cav1.3 gradually became stronger with increasing age (p0 group, 115.3 ± 1.386; p7 group, 145.0 ± 2.687; p14 group, 156.0 ± 2.369; p21 group, 158.2 ± 3.435) (Figure 2E), indicating a gradual increase in the expression of Cav1.3 during development.

Detection of Marginal Cells In Vitro

Figure 3A shows that MCs can be recognized from fibroblasts based on their characteristic shape using an inverted microscope. The MCs grow polymorphically, with dark and clear boundaries. They are tightly packed and can grow into monolayers with the appearance of "pebbled-like." As shown in Figure 3B, a strong CK18 (marker for epithelial cells) fluorescent signal could be observed in primary cultured MCs.

The Expression of Cav1.3 in Marginal Cells In Vitro

Immunocytology techniques (Figure 4) showed that the Cav1.3 (green fluorescence) and CK18 (red fluorescence) were co-expressed in the cytoplasm of the primary cultured MCs in vitro.

DISCUSSION

The cochlea possesses acute auditory receptors that respond to the slightest sound stimulus. Calcium channels are involved in almost all pathways of the signaling.[15] L-type calcium channels are components

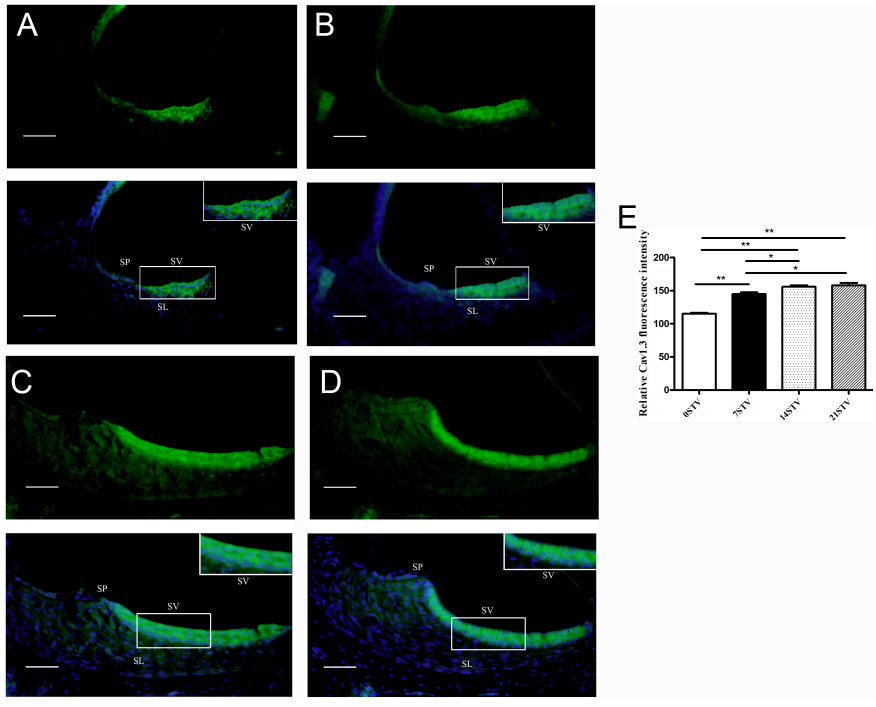

Figure 2. The expression of Cav1.3 in the lateral wall of developing cochlear rat (magnification, ×400): (A) p0 group; (B) p7 group; (C) p14 group; and (D) p21 group. The Cav1.3 protein is stained with green fluorescence and the nuclei are labeled by blue fluorescence (A, B, C, and D under panel). (E) Quantitative analysis of Cav1.3 in STV. Scale bar: 50 μm, SV, stria vascularis; SL, spiral ligament; SP, spiral prominence.

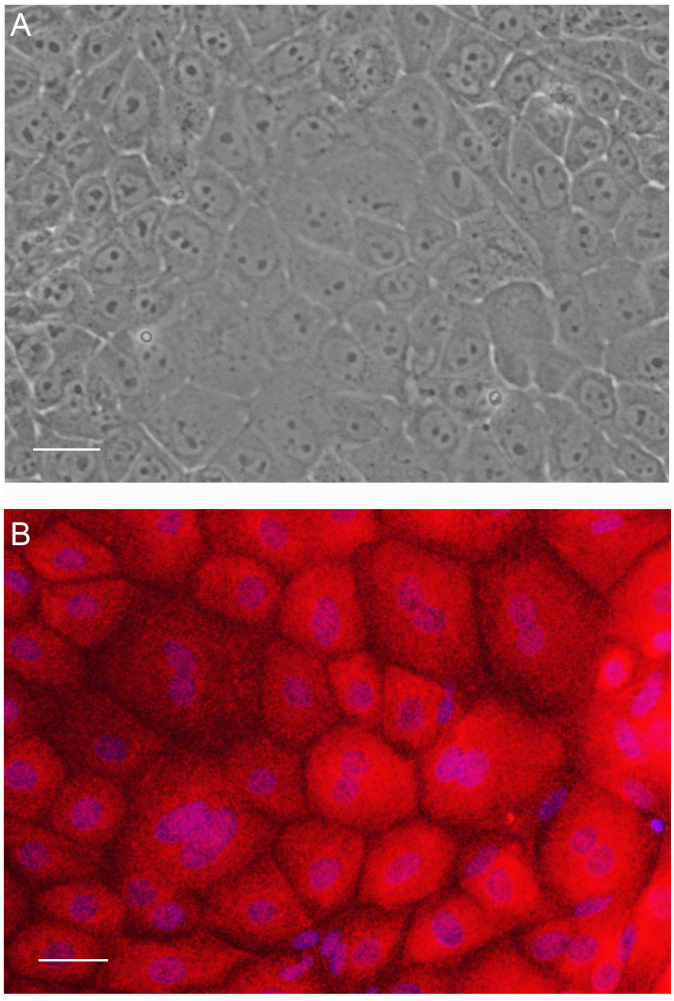

Figure 3. The identification of MCs. (A) The morphology of MCs in primary culture. This figure shows the marginal cells, which exist in a polymorphic cellular morphology growth pattern with darker color and well-defined boundaries. Due to their close arrangement, the monocellular layer may exhibit a "pebbles-like" appearance. (B) The detection of CK18 in the MCs by immunofluorescence. Since CK18 is mainly distributed in the cytoplasm of epithelial cells, the red fluorescence of CK18 is expressed in the cytoplasm of MCs. The nucleus was counterstained with DAPI (blue fluorescence). Scale bar: 50 μm.

of the voltage-gated calcium channel family and are highly sensitive to dihydropyridine calcium channel blockers, among which the Cav1.3 calcium channels are essential for the auditory process. Cav1.3$^{-/-}$ KO mice develop auditory and vestibular variability, which is identical with the disturbance of calcium ions in the inner ear environment.[20]

In this study, the expression characteristics of the Cav1.3 subunit channel in the cochlear SV of SD rats were described in detail. Like previous studies on mice, Cav1.3 mRNA was also detected in the cochlear lateral wall as early as birth,[16] and its expression increased during development (Figure 1). Furthermore, immunofluorescence results demonstrated that Cav1.3 channels were prominently located in the SV, and moderate expression was also detected in the spiral ligaments and spiral prominence (Figure 2), which is consistent with other research.[21] Besides this, the stable method for primary culture of marginal cells in neonatal rat cochleae was successfully established (Figure 3) and Cav1.3 calcium channel protein was demonstrated to be expressed in the plasma membrane of the MCs in neonatal rats for the first time (Figure 4). It was similar to the research that Cav1.3 resided on the plasma membrane of marginal cells rather than the intermediate cells or basal cells in guinea pigs.[7] In addition, we also found that Cav1.3 mRNA and protein reached adult-like levels by the third week of life (Figures 1 and 2E). This expression pattern was the same as in mice.[16]

Various cells in the cochlear SV act synergistically to generate endolymphatic fluid with a high concentration of K^+, which promotes the formation of the positive EP, creating a large electrochemical gradient to drive receptor currents through the hair cells.[22] Deterioration of the SV and imbalance of EP are the main reason of age-related hearing loss, which is referred to as metabolic or stria presbycusis by Schuknecht. He also proposed that the loss or dysfunction of stria marginal cells contributed to presbycusis.[23,24] Marginal cells with multiple ion channels on the plasma membrane are believed to primarily contribute to the homeostasis and maintenance of the endolymph, which plays an important role in the conduction of sound through hair cells.[25] It has been reported that Nifedipine inhibits transient asphyxia-induced EP decline (TAID), whereas L-type Ca^{2+} channels in the MCs may contribute to EP regulation by maintaining cytosolic Ca^{2+} at an appropriate level.[7] It has also been reported that Ca^{2+} pumps and Ca^{2+} permeable channels are expressed more in the SV than in the SL and OC (organ of Corti), and they may play an important role in EP maintenance by regulating Ca^{2+} concentration in MCs.[26] In addition, MCs are an important part of the tight junctions which maintain EP through strictly regulated ion channel functions, such as Cav1.3.[27] Together with our experimental results that Cav1.3 was expressed in the plasma membrane of MCs, we suggest that L-type Ca^{2+} channels in marginal cells may regulate and maintain the EP required for sound transduction. Moreover, the expression of Cav1.3 increased after birth, which is identical to the changes in Ca^{2+} current recorded in postnatal mice in other studies.[28,29] At p6, the magnitude of the EP in mice was approximately +16 mV. At p14, 2 days after the onset of hearing, the amplitude was more than +60 mV. After p14, the amplitude continued to increase, reaching +80 mV at p21.[30] The growth trend of EP was consistent with the expression pattern of Cav1.3 between p0 and p21 observed in our present studies in SD rats. Additionally, the auditory function of rats matures at 21 days after birth, and the high expression of the Cav1.3 calcium channel at this time may participate in the formation and maintenance of hearing by regulating calcium homeostasis.[7]

Taking all the findings into consideration, we suggest that Cav1.3 might be beneficial for regulating EP. The following discussion may also attempt to explain this opinion. The early expression (p0) of Cav1.3 in the SV implies that this channel plays a key role in developing cochlea. Cav1.3 currents present in cochlear OHCs and IHCs are required for synaptic transmitter transmission and normal hair cell development.[28,3132] Other findings demonstrated that Cav1.3 channels are crucial for brainstem development and particularly important for the auditory system.[4] In terms of long-term structural changes and development, Ca^{2+} influx through L-type channels benefits the survival of SGN in vitro.[33] Cav1.3 is essential for postpartum normal cochlear development and maintenance of intact morphology, as demonstrated by typical deformation patterns observed with electron microscopy.[31] Deletion of the L-type Ca^{2+}

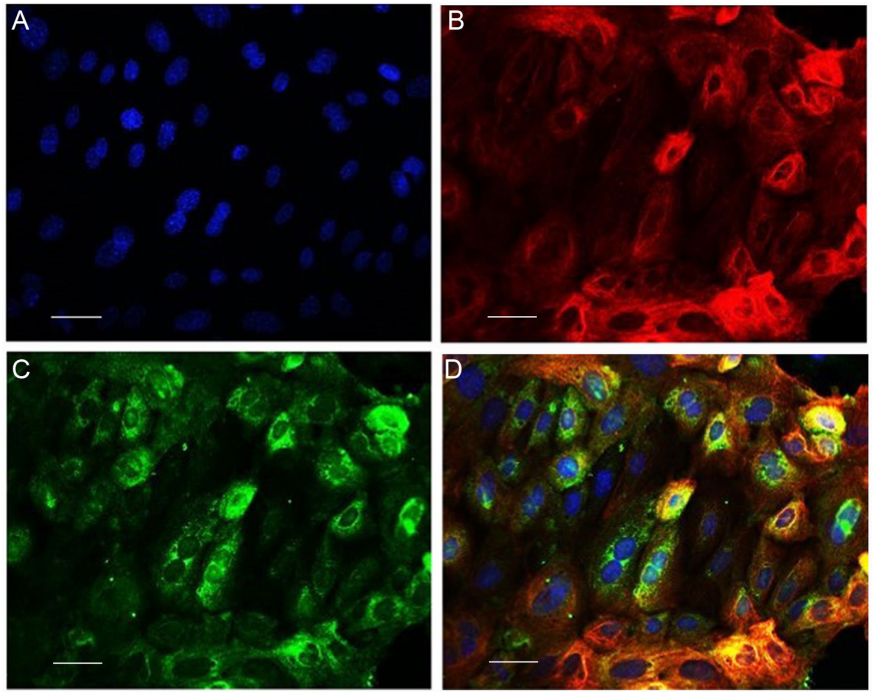

Figure 4. The location of Cav1.3 in MCs. We labeled the nuclei with DAPI (blue, 4A), CK18 with red fluorescence (4B), and stained the target protein Cav1.3 with specific antibodies (green, 4C). The figure showed Cav1.3 is mainly localized in the cytoplasm (4D). Scale bar: 50 μm.

channel only leads to a reduction in synaptosomes before p3, and does not inhibit other morphological development of the cochlea. However, it may result in degenerative changes in the spiral ganglion, OHCs, and IHCs after p3.[31] All degenerative changes may result from the deficiency of Ca^{2+} channels in spiral ganglion cells or in IHCs. Previous findings have demonstrated that Cav1.3 channels are responsible for the development of the central auditory system.[4] The lack of Ca^{2+} APs may be the underlying pathological mechanism of those observed developmental defects. Therefore, we suppose that the expression of Cav1.3 in SV and MCs may contribute to their development and differentiation. On the contrary, excessive Ca^{2+} hinders neuronal growth, raising the possibility that an appropriate concentration of intracellular Ca^{2+} is required for the plasticity of auditory neurons.[34]

The Cav1.3 channels are activated at more negative voltages, allowing Ca^{2+} to flow in under relatively weak depolarizing pulses, and this prolonged inflow of Ca^{2+} may also have implications for SV development.[35]

Our study is one of the few to explore Cav1.3 during developmental stage. Moreover, we confirmed the presence of Cav1.3 in MCs of the SV using primary culture techniques. However, there are several drawbacks. First, protein-level experiments were insufficient, and the gene and immunofluorescence detection techniques could not adequately explain the expression pattern of Cav1.3. Secondly, functional assays would make the results more convincing if they had been done, such as the Cav1.3 knockout experiment. Therefore, we intend to apply gene-targeting techniques to generate Ca^{2+} channel-deficient SD rats to explore the role of these Ca^{2+} channels in MCs in vivo or in vitro. Furthermore, the potential off-target effects in the qRT-PCR primers of Cav1.3 will be fully utilized in this experiment. Meanwhile, the nonlinear capacitance and membrane potential of MCs can be recorded using the whole-cell patch-clamp technique in voltage clamp mode. In short, more research is needed to elucidate the function of Cav1.3 in SV.

CONCLUSION

The present study demonstrated that Cav1.3 is expressed early during cochlear development and is widely expressed in the MCs of the SV. Overall, it is reasonable to conclude that intracellular Ca^{2+} homeostasis may be crucially involved in the regulation of EP and hearing, as well as in the morphogenesis of marginal cells. Our findings perhaps provide a new research direction to reveal the function of Cav1.3 and suggest that voltage-gated calcium channels may be involved in the homeostasis of endolymph, and are associated with normal inner auditory system development.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: This study was approved by the Ethics Committee of Huazhong University of Science and Technology (approval no.: TJH-202006007; date: March 13, 2024).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Z.D.; Design – Z.D., H.L.; Supervision – J.C.; Resources – J.C.; Materials – H.C.; Data Collection and/or Processing – Z.D., H.L.; Analysis and/or Interpretation – H.C.; Literature Search – J.C.; Writing – Z.D., H.L.; Critical Review – J.C., H.C.

Declaration of Interests: The authors have no conflicts of interest to declare.

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