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Differential Expression of Na/K-ATPase in the Human Saccule of Patients with and without Otologic Disease

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Abstract

Hypothesis: Na^+, K^+-ATPase (Na/K-ATPase) α1 subunit expression in the saccule of patients diagnosed with otologic disease is different compared to normal controls.

Background: We have recently characterized changes in the expression of Na/K-ATPase α1 subunit in the normal and pathological cochlea, however, no studies have determined the distribution Na/K-ATPase α1 subunit in the human saccule. The present study utilizes archival temporal bones to study the expression Na/K-ATPase α1 subunit in the human saccule.

Methods: Archival celloidin formalin fixed 20-micron thick sections of the vestibule from patients diagnosed with Meniere's disease (MD) (n=5), otosclerosis (n=5), sensorineural hearing loss (SNHL), and normal hearing and balance (n=5) were analyzed. Sections containing the saccular macula were immunoreacted with mouse monoclonal antibodies against Na/K-ATPase α1 subunit. Micrographs were acquired using a high-resolution digital camera coupled to a light inverted microscope.

Results: In the normal human saccule vestibular sensory epithelium, Na/K-ATPase α1 immunoreactivity (IR) was present in nerve fibers and calyces that surround type I vestibular hair cells and nerve terminals. The transition epithelium cells were also Na/K-ATPase α1 immunoreactive. Comparison between normal and pathological specimens showed that there was a significant reduction of Na/K-ATPase α1 IR in the saccule vestibular sensory epithelium from patients with Meniere's disease, otosclerosis, and SNHL.

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Conclusions: The decrease of Na/K-ATPase-IR α1 in the saccule vestibular sensory epithelium from patients with otopathologies suggests its critical role in inner ear homeostasis and pathology.

INTRODUCTION

The localization and expression of the different Na,K-ATPase subunit isoforms have been investigated in inner ear of human and animal models[1–6]. In a recent study by our group, Stephenson et al[7]demonstrated that the expression of Na,K-ATPase α1 was located in the stria vascularis, in type I, II and IV fibrocytes of the spiral ligament in the cochlea, Deiter's cells and inner phalangeal cells of the organ of Corti, and in satellite cells that surround the neurons of the spiral ganglia[7]. In the inner ear of pathological specimens (those with various inner ear diseases), Na,K-ATPase was decreased (compared to the normal) in the stria vascularis, supporting cells in the organ of Corti and satellite cells of the spiral ganglia. It was concluded that Na,K-ATPase α1 subunit immunoreactivity (IR) is a good marker to identify cellular structures of the human inner ear and may be used to study cellular changes in the cochlea associated with aging and disease. This study is a continuation of the Stephenson study[7], specifically evaluating the expression of Na,K-ATPase α1 in the human saccule of normal and pathologic temporal bone specimens.

It has been shown that there is a decline in cochlear and saccular function with age[8] and this parallel decline may be due to the common embryonic origin of the pars inferior of the labyrinth. We hypothesize that Na,K-ATPase α1 immunoreactivity in the macula saccule decreases in temporal bones diagnoses with Meniere's disease, otosclerosis and sensorineural hearing loss (SNHL).

The aim of this study was to investigate the immunohistochemical distribution of the Na,K-ATPase α1 in the saccule of normal patients and in patients diagnosed with Meniere's disease, otosclerosis and sensorineural hearing loss (SNHL). Celloidin embedded sections containing the human vestibule endorgans from archival temporal bone with documented clinical history were used.

MATERIALS AND METHODS

Human temporal bones

Approval was obtained from the University of California, Los Angeles Institutional Review Board (IRB# 22–001587). The temporal bones used in the present study were part of a National Institute of Health funded Human Temporal Bone Otopathology research collaboration network. Temporal bones were obtained at autopsy from 20 subjects (10 male and 10 female) with a documented history of normal auditory and vestibular function (n=5), Meniere's disease (n=5), otosclerosis (n=5) and SNHL (n=5). Table 1 shows the demographic distribution of temporal bones used in this study. Some celloidin sections of this temporal bones were used previously in Stephenson et al[7].

Immunohistochemistry (IHC) in celloidin embedded inner ear sections

The methodology for celloidin removal, antigen retrieval and immunohistochemistry has been described previously[7,9]. In brief: celloidin sections were immersed a saturated solution

of sodium hydroxide in 100% ethylic alcohol (EtOH prepared one hour before) diluted 1:3 with EtOH for one hour, 100% EtOH (3 times x 5 min), and distilled water (3 × 5 min). Sections were immersed in heated antigen retrieval solution −100°C- (diluted 1:500 in double-distilled water, Vector antigen unmasking acidic solution, Vector Labs, Burlingame, CA). Sections were allowed to reach room temperature for 30 min, washed with phosphate-buffered saline –PBS- (3× 10 min PBS) and immediately incubated for 8 minutes in a diluted trypsin solution (1:3, Abcam Trypsin Kit) and washed 4×10 minutes in PBS before immunohistochemistry. Sections were incubated for 2 h with a blocking solution containing 0.1 normal horse serum and 0.5% Triton X-100 (Sigma) in PBS. Followed by the incubation with the primary monoclonal antibodies against Na, K-ATPase α1 at 1:1000 dilution in PBS (α1 subunit, Hybridoma Bank), for 48 h at 4 °C in a humid chamber. Secondary antibodies against mouse labelled with HRP (ABC kit, Vector Labs) were used, the antigen-antibody reaction was visualized with diaminobenzidine (ImmPact™ DAB Chromogen, Vector Labs).

IHC controls

As a positive control, cryostat sections from formalin fixed human vestibular endorgans were incubated with the Na,K-ATPase α1 antibodies as described above[7]. These sections were subjected to the same immunohistochemistry protocol. As negative control cochlea celloidin embedded sections were processed for immunohistochemistry as described above, except that the anti-Na,K-ATPase α1 antibody was omitted.

Microscopic observation and documentation

Immunohistochemical stained tissue sections were viewed and imaged with a Leica inverted microscope equipped with a high-resolution color camera (Leica DFC7000T). Panoramic digital micrographs of the vestibular sensory epithelium were made using the 10x objective using the Leica LAX software. Differential interference contrast (DIC) filter was used to identify cellular components. The region of interest (ROI) was created using the rectangle command, and 10 × 10 tiles were collected and then merged, the images were saved as TIFF files. Micrographs were prepared using the Power point software program run in a MacBook Pro computer.

Quantitative immunohistochemical analyses

Quantitative analysis of Na,K-ATPase α1 immunoreactivity (IR) in each sample was made as described by Stephenson et al 2021[7], using *ImageJ2* software (https://imagej.net/Contributors1.53s). Each digital micrograph was opened using the ImageJ2 program and converted to gray scale (image/type 8 bit). The threshold for IR detection was set (image/adjust/threshold), and the threshold level was adjusted (same for all images). Background IR was measured in a small area located apart from the Na,K-ATPase IR and was subtracted from the Na,K-ATPase α1 IR area values. The image was converted to black and white, and the IR area was selected using the drawing tool. To determine the Na,K-ATPase α1 IR area within the region of interest the command (analyze/analyze particles was selected), and the “mask tool” was selected. The resulting measurements represent the area fraction, which is the proportion of the region of interest that was Na,K-ATPase α1 IR. Averaged IR values obtained the normal saccule sensory epithelium were considered 100%.

Statistical analysis

Statistical comparisons between groups were made using a nonparametric Mann-Whitney U test. A value of $p < 0.05$ was denoted as a statistically significant difference. The IBM SPSS statistics software program version 25 (IBM Corporation, Armonk, NY, United States). Na,K-ATPase α1 IR comparisons between groups were made as follows: 1. Normal versus Meniere's. 2. Normal versus otosclerosis and 3. Normal versus sensorineural hearing loss (SNHL).

RESULTS

In the normal human saccule sensory epithelium, robust Na/K-ATPase α1 IR was present in nerve fibers and calyces that surround type I vestibular hair cells and nerve terminals. DIC illumination micrographs allowed identification of nerve fibers, calyces and stereocilia. Figure 1A shows a low magnification view of the macula saccule sensory epithelia from a 67-year-old male (with normal hearing and vestibular function). Na/K-ATPase α1 IR was seen in the nerve fibers within the stroma and the vestibular sensory epithelium. Fig 1A1 shows a higher magnification view from Fig 1A, Na/K-ATPase α1 IR was seen in calyceal nerve terminals. Figure 1B shows a low magnification view of the macula saccule from a 65-year-old male with Meniere's disease, immunoreacted with antibodies against Na/K-ATPase α1. Fig 1B1 shows a higher magnification view from Fig 1B, Na/K-ATPase α1 IR was seen in calyceal nerve terminals. Na/K-ATPase α1 IR signal is significantly reduced in both the sensory epithelium and the nerve fibers within the stroma in Meniere's saccule as compared with the normal individual of a similar age.

Additionally, Na/K-ATPase α1 IR decreases significantly in the saccule sensory epithelium from temporal bones diagnosed with otosclerosis and SNHL. Figure 2A, shows the macula saccule from a patient diagnosed with otosclerosis (65-year-old-male) and Figure 2B shows the macula saccule from a patient diagnosed with SNHL (73-year-old female).

Comparisons of normal vs Meniere's disease, normal vs otosclerosis, and normal vs SNHL showed statistically significant differences (Table 2). Na/K-ATPase α1 IR decreases 63% for Meniere's disease, 70% for otosclerosis and SNHL respectively, when compared with the IR in the normal specimens. The decreases in IR were statistically significant (Table 2).

Na/K-ATPase α1 IR distribution in the normal utricle and cristae vestibular sensory epithelium showed a similar distribution to the normal macula saccule (Figure 3A, 3B and 3C, 67-year-old female). Figure 3A shows a low magnification view of the lateral crista and macula utricle. Figure 3B and 3C shows higher magnification view of the crista and utricle sensory epithelium Na/K-ATPase α1 IR was present in nerve fibers and calyces that surround type I vestibular hair cells and nerve terminals.

DISCUSSION

Our study shows that in the normal human saccule, robust Na/K-ATPase immunoreactivity (IR) was present in nerve fibers and calyces that surround type I vestibular hair cells and nerve terminals. The basolateral membrane of extramacular saccular epithelium was

also Na/K-ATPase immunoreactive. In the present study we found that the saccule sensory epithelium from patients with Meniere's disease, otosclerosis and SNHL, had a significant reduction in Na/K-ATPase α1 IR.

There are two basic cell types within the vestibular sensory epithelium: supporting cells and hair cells[10]. Supporting cells form a ring around each individual hair cell and form tight junctions and desmosomes with their respective hair cell. Vestibular hair cells can be divided into type I and type II hair cells, which are differentiated by the presence of a calyx, which is an afferent nerve ending enveloping the entire sub-apical portion of type I hair cells. Simple calyceal endings envelope only one type I hair cell, while complex calyceal endings, found in the striola, envelopes two or more type I hair cells. As opposed to the flask like shape of type I hair cells, type II hair cells are cylindrical and are contacted on the basal surface by multiple afferent and efferent nerve endings, called synaptic boutons[10].

Na,K-ATPase is a membrane bound enzyme whose structure is typically made up of three subunit isoforms: α, β, and FXYD. The enzyme actively utilizes energy from hydrolysis of adenosine-triphosphate (ATP) molecules to translocate 3 Na+ and 2 K+ molecules. In the case of the scala media, K+ is translocated into the endolymph while Na+ is translocated out of the endolymph at a 2 to 3 ratio, creating what is known as the endocochlear potential, which is a gradient across the cell membrane and high K+ concentration. This active transport process is necessary to maintain the positive voltage of 80–100 mV in the endolymphatic compartment[11–12]. The endocochlear potential is then the force that drives cations through the mechanotransduction channels of inner hair cells (IHC)[12–13]. Mechanotransduction is achieved when the stereocilia are deflected by mechanical energy leading to the influx of K+ ions into hair cells and subsequently hair cell depolarization[13]. Displacement of the stereocilia (commonly referred to as the "hair bundle") in the saccule results from the movement of the overlaying otoconial membrane during accelerations forward, backward, upward, or downward[14]. The resting potential of hair cells is moderately depolarized, lying in the range of −70 to −50mV[15]. The apical extracellular fluid filled space of the vestibular hair cell in which the stereocilia lay is the endolymph. As noted previously, the endolymph is positively charged, and is continuous with the scala media and overlies hair cells in the other macular epithelia. Once depolarized, there is increased conductance to K+ in the basolateral membrane of the hair cell by opening voltage-gated K+ channels[16]. In most hair cells, this then leads to an efficient return of the negative K+ gradient in the hair cell, and the K+ can diffuse through the perilymph and cell-to-cell gap junctions and recycle back to the various other regions of the inner ear, such as the stria vascularis, to be used to re-establish the endo cochlear potential[14]. However, type 1 vestibular hair cells are surrounding by a calyx, which constrains outward diffusion of K+ from the basolateral membrane, leading to accumulation of K+ in the synaptic cleft. This is theorized to directly depolarize the afferent bouton by the resulting charge accumulation, which is supported by the fact that the calyceal afferent membrane has voltage sensitive KCNQ-type potassium channels[16].

Is it possible that the Na/K-ATPase plays a role in re-establishment of the K+ homeostasis in the calyceal synaptic cleft, though future analysis will be needed to clearly define its role in this region of the vestibular system. Na/K-ATPase IR reduction in Meniere's patients could

potentially explain the progressive loss of vestibular function seen in this population, and future studies may be able to elucidate this.

Limitations to this study include: the processing of embedding celloidin-embedded sections includes long fixation and decalcification. Although it is possible analyze the expression of proteins of interest, including Na,K-ATPase, some proteins which have been detected using other tissue embedding methods (paraffin embedded tissue, or formalin frozen tissue), have not been observed on celloidin-embedded specimens[9], possibly because of the type of tissue processing and post-mortem time. DIC illumination used to analyze the immunostained sections also shows that nerve calyces are swollen in some cases; this is very likely due to postmortem time of collection. Our study was also limited by the number of normal specimens, making it difficult to generalize our findings and ensure real statistical significance, as it could not be adequately powered due to limitations in temporal bone specimens. Future directions includes the use of Na/K-ATPase-IF using myosin VIIa to identify vestibular hair cells in both the maculae and cristae, as well as comparison on pathological changes between the different diseases and endorgans. In this respect we have shown Na/K-ATPase-IF in formalin fixed cryostat sections from the human macula utricle obtained from autopsy (normal) and surgically from patients diagnosed with Meniere's disease, IF comparison between the two types of specimens showed no significant changes in IF.

CONCLUSIONS

In conclusion Na,K-ATPase α1 IR was found throughout the normal human saccule and in diseased patients. Na,K- ATPase α1 IR area was influenced by inner ear pathology including endolymphatic hydrops, otosclerosis, and SNHL. The present results suggest an important role of Na,K-ATPase in the normal and pathological saccule and its utility as a cellular marker to identify inner ear structures.

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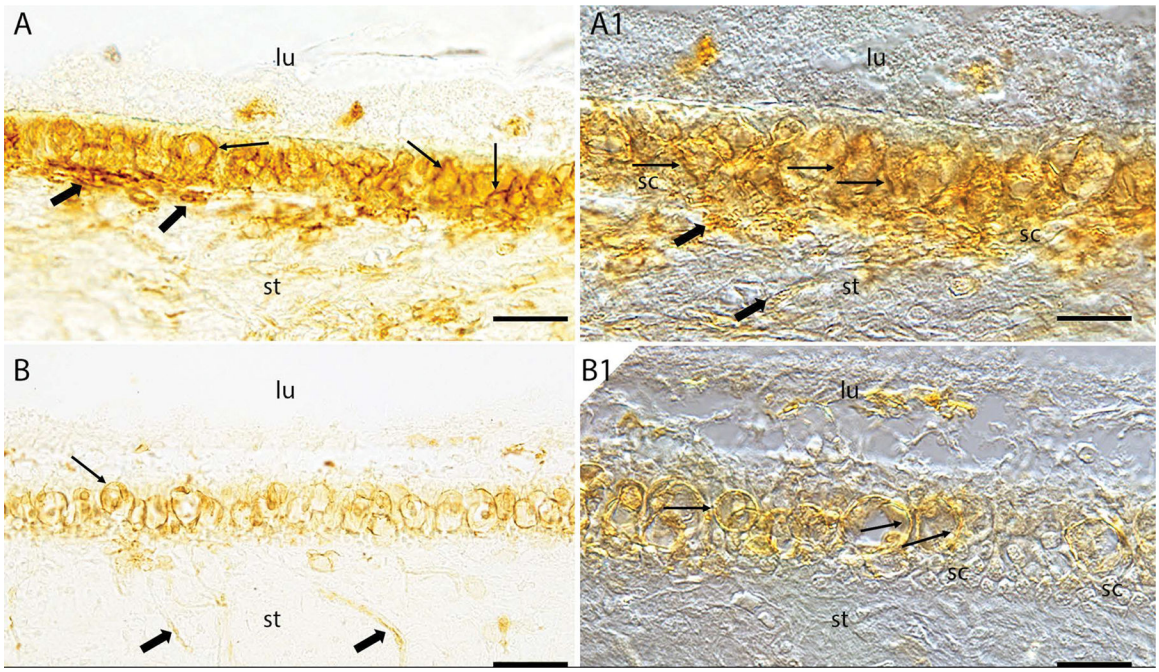

Fig 1.

(A) Na/K-ATPase α1 IR in the normal macula saccule, (A1) Higher magnification view from image A. (B) Na/K-ATPase α1 IR in the macula saccule from a Meniere's patient. (B1) Higher magnification view from image B.

Arrow points to a type I vestibular hair cell, thick arrowhead points to calyceal terminal. sc: supporting cells, lu: lumen, st: stroma. Bar in A and B is 30 microns, in A1 and B1 15 microns.

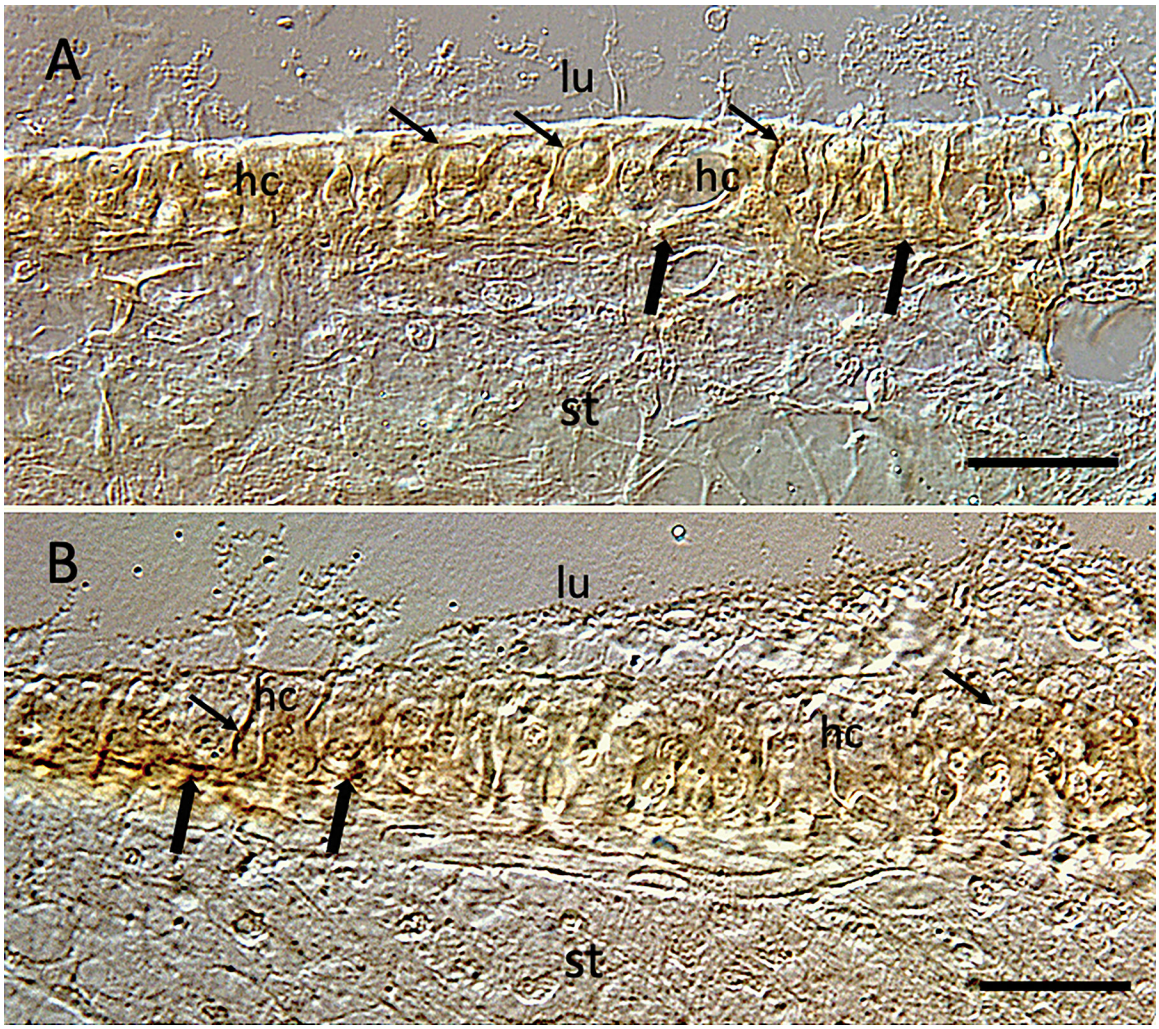

Fig 2.

(A) Na/K-ATPase α1 IR in the macula saccule from an otosclerotic patient, (B) Na/K-ATPase in the macula saccule from a SNHL patient.

Arrow points to a type I vestibular hair cell, thick arrowhead point to calyceal terminal. hc: hair cells, sc: supporting cells, lu: lumen, st: stroma. Bar in A and B is 40 microns.

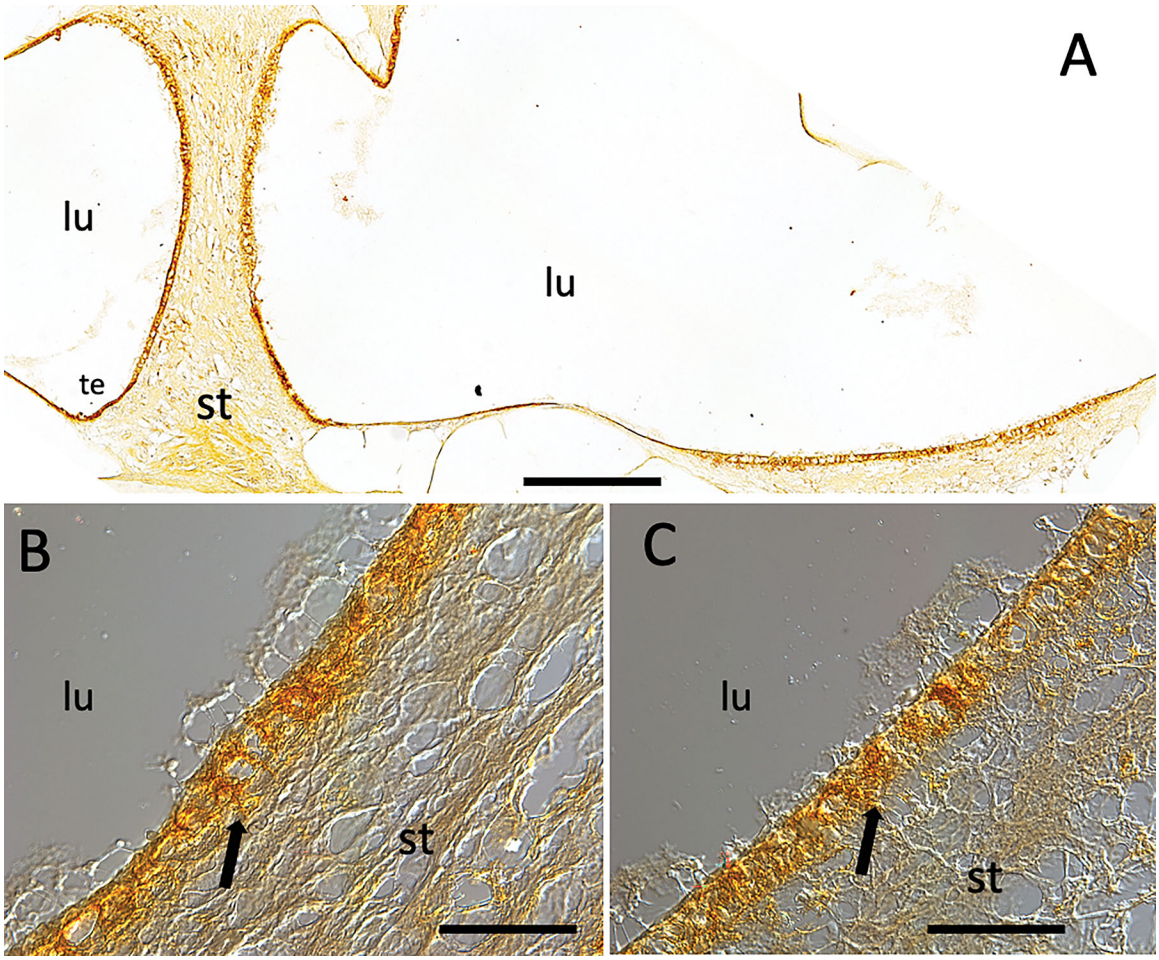

Fig 3.

Na/K-ATPase α1 IR in the normal macula utricle and crista ampullaris. (A) Low magnification views of the lateral crista and macula utricle. (B) High magnification view of the lateral crista ampullaris epithelium Na/K-ATPase α1 IR was seen in nerve calyces that surround type I vestibular hair cells, (C) In the macula utricle sensory epithelium similar Na/K-ATPase α1 IR was also seen. The transition epithelium was also IR. Thick arrowhead point to calyceal terminal. lu: lumen, st: stroma, te: transition epithelium. Bar in A, B, and C is 20 microns.

Table 1.

Temporal bones used in this study. Abbreviations. F: female, M: male, SNHL: sensory neural hearing loss, MD: Meniere's disease.

Specimen	Age (years)	Gender	Diagnosis
1	92	F	Meniere's
2	65	M	Meniere's
3	51	M	Meniere's
4	77	M	Meniere's
5	80	F	Meniere's
6	91	F	Otosclerosis
7	76	F	Otosclerosis
8	92	F	Otosclerosis
9	72	M	Otosclerosis
10	65	M	Otosclerosis
11	84	F	SNHL
12	73	F	SNHL
13	67	M	SNHL
14	75	M	SNHL
15	75	M	SNHL
16	42	F	Normal
17	67	F	Normal
18	55	M	Normal
19	84	F	Normal
20	72	M	Normal

Table 2:

Comparisons of Na/K-ATPase α1 subunit IR in normal vs Meniere's disease, normal vs otosclerosis and normal vs SNHL. All pathologic specimens showed significant decrease from normal ($p<0.05$).

Comparison Group	% Decrease of IR area from normal	*p*
Normal vs MD	68.2	0.01
Normal vs otosclerosis	59	0.01
Normal vs SNHL	71.5	0.012

Abbreviations MD: Meniere's disease, SNHL: sensory neural hearing loss, IR: immunoreactivity.