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Journal

Annals of Otology, Rhinology and Laryngology, 128(6_suppl)

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Publication Date

2019-06-01

DOI

10.1177/0003489419834952

Peer reviewed

Published in final edited form as:

Ann Otol Rhinol Laryngol. 2019 June ; 128(6 Suppl): 96S–102S. doi:10.1177/0003489419834952.

Otopetrin-2 Immunolocalization in the Human Macula Utricle

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Abstract

Background: In the present study, we investigated the localization of otopetrin-2—a member of the *otopetrin* family that encodes proton-selective ion channels—in the human macula utricle using immunohistochemistry.

Methods: Macula utricle were acquired at surgery from patients who required transmastoid labyrinthectomy for intractable vertigo due to Meniere's disease (MD; n = 3) and/or vestibular drops attacks (VDA; n = 2) and from temporal bones (n = 2) acquired at autopsy from individuals with no balance disorders. Immunofluorescence staining with otopetrin-2 (rabbit affinity purified polyclonal antibody) and GFAP (mouse monoclonal antibody) to identify vestibular supporting cells was made in formalin fixed cryostat sections or whole microdissected utricle (for flat mount preparations). Secondary antibodies against rabbit and mouse were used for the identification of both proteins. Digital fluorescent images were obtained using a high-resolution laser confocal microscope.

Results: Using cryostat sections and flat mount preparations otopetrin-2 immunofluorescence was seen as punctated signal throughout the supporting cells cytoplasm. GFAP immunofluorescence was present in the supporting cell cytoplasm. The distribution of otopetrin-2 was similar in the macula utricle obtained from MD, VDA, or autopsy normative patients.

Conclusions: Otopetrin-2 was localized in supporting cells in a similar fashion that otopetrin-1 previously reported in the mouse macula utricle. The differential expression of otopetrin-2 in the supporting cells of the human macula utricle suggest an important role in the vestibular sensory periphery homeostasis and otolith maintenance.

Keywords

vestibular hair cells; supporting cells; otopetrin-2; balance disorders; otolith; utricle

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Background

The peripheral vestibular system consists of 5 organs: 3 semicircular canals (anterior, posterior, and horizontal) and 2 otoconial organs—the macula saccule and utricle.[1] Studies of individual vestibular end-organs have shown that both semicircular canal and otoconial function seem to decline with age, starting as early as age 40.[2,3] For a recent review on this topic, please refer to Agrawal.[4] The otoconial complex of the utricle and saccule provides inertial mass to generate shearing forces essential for the macular mechanoreceptors to sense gravity and linear acceleration.[5,6] Otoconia are composite crystallites that overlie the macular sensory epithelium of the gravity receptors of most vertebrates and are required for optimal stimulus input of linear acceleration and gravity.[7]

Otoconia-related balance disorders are prevalent.[8,9] Benign paroxysmal positional vertigo (BPPV) is the single most common cause of vertigo seen in the otoneurologic clinic.[10] It has been suggested that BPPV is caused by a lesion of the otolith organs and cupulolithiasis.[9] A variety of damages to the inner ear (trauma, infection, ischemia) could lead to dislodging of the calcium carbonate crystals from the otolith. Vestibular drops attacks (VDA), also known as Tumarkin falls, are sudden violent falls present in a small subset of patients with endolymphatic hydrops. BPPV and VDA have been associated with Meniere's disease (MD).[11] Calzada et al[12] found that the otolithic membrane is consistently damaged in patients with VDA, and a significantly higher incidence of otolithic membrane injury in patients with MD and endolymphatic hydrops.

Little is known about the molecular processes involved in otoconial maintenance and pathology in the human vestibule. Slow, progressive degeneration of otoconia is part of the normal aging process and results in significant loss of balance and falls in a large percentage of elderly patients.[2,3] The most conspicuous early sign of demineralization is pitting of the saccular otoconia surface starting at about age 50 to 60 years old. The utricular otoconia are, as a rule, affected much later, at a time when saccular degeneration is already in an advance state.[7] An obvious pathogenic mechanism would be a disturbance in calcium homeostasis associated with systemic disorders, such as osteoporosis and other skeletal diseases.[7,13]

Morphological studies of the otoconial complex in the human maculae at the cellular and subcellular levels have shown that the otoconia begin to deteriorate in the fifth decade.[14] To date, several otoconia-related proteins have been identified mostly in mice and bony fish.[5,6,15–18] To uncover the molecular etiology of otoconial related disorders, it is necessary to determine the protein composition of the otoconia in human utricle and saccule and investigate their changes with age and disease.

Otoconial degeneration can result from ototoxic drugs, infection, trauma, and aging.[16] Abnormal otoconia are also known to occur as a result of genetic mutation as well as ototoxicity.[19] It has long been known that mammalian otoconia contain an organic core consisting predominantly of glycosylated proteins. Oc90 is the principal core protein present in proteinaceous coral-like structures. Pote and Ross[20] demonstrated that the calcite otoconia of mammals contain a principal matrix protein of 90kDA (Oc90) that makes up 90% of the total, with several minor otoconins accounting for the difference. Oc90 is closely related to

secretory phospholipase A2 (PLA2).[7,21] Otolin-1 is present in the otoconia and vestibular supporting cells.[22] Otopetrin-1 is present in otoconial cores and supporting cells.[16,23]

Recently, otopetrin-1, otopetrin-2, and otopetrin-3 have been identified as forming a proton-selective ion channel.[24] To date, there are no studies on the localization of otopetrins in the human maculae. As reported by Hughes et al,[25] the *otopetrin* family consists of 3 genes clustered in 2 chromosomal locations: *Otop 1* and the paralogous tandem genes *Otop2* and *Otop3. Otop1* is expressed in the extrastriolar region of the mouse utricle and saccule, and otopetrin-1 concentrates calcium to allow nucleation, growth, and maintenance of otoconia.[23]

Otopetrin-1 has been shown to modulate purinergic control of intracellular calcium in vestibular supporting cells,[23] and its mutation cause an imbalance phenotype with selective otoconia involvement.[16] It is likely that otopetrin-1 function in a similar fashion in the human utricle. The presence of otopetrin-1, −2, and −3 has not been investigated in the human inner ear. Their role as proton channels[24] suggests that they have an important in role in inner ear homeostasis. In this study, we investigate for the first time the immunolocalization of otopetrin-2 in the normal macula utricle (obtained from autopsy) and the macula utricle from patients diagnosed with MD and VDA. Otopetrin-2 immunoreactivity was found in vestibular supporting cells in the 3 types of the specimens examined. Otopetrin-2 distribution in the human utricle was similar to otopetrin-1 immunolocalization previously reported in the mouse utricle.

Methods

Specimens

Approval was obtained from the University of California at Los Angeles Institutional Review Board (protocol No. 10–001449). Appropriate informed consent for inclusion in the study was obtained from each temporal bone donor. Macula utricle were acquired at surgery from patients who required transmastoid labyrinthectomy for intractable vertigo due Meniere's disease (n = 3; 45-year-old male, 55-year-old female, 65-year-old male) and/or vestibular drops attacks (n = 2; 50-year-old female, 62-year-old male) and from temporal bones (n = 2; 65-year-old male, 67-year-old female) acquired at autopsy from individuals with a documented history of normal auditory and vestibular function.

Inclusion and exclusion criteria

All subjects with MD had stage IV definite MD with profound hearing loss and intractable recurrent vertigo spells despite maximum medical treatment using the criteria developed by the Classification Committee of the Barany Society 2015.[26]

Antibodies

Primary polyclonal affinity purified antibodies against otopetrin-2 raised in rabbit, diluted 1:1000 in phosphate buffered solution (PBS) pH 7.4, 0.1 M (Thermo Fisher Scientific, cat No. PA5–62727, Waltham, Massachusetts, USA) were used in this study. According to the vendor, the immunogen used to generate this antibody was a recombinant protein to human

OTOP2. Monoclonal antibodies against glial fibrillary acidic protein (GFAP) were used to identify supporting cells (Sigma-Aldrich, Cat No. G3893, St. Louis, Missouri, USA). GFAP from pig spinal cord was the immunogen used to generate this antibody.

The protocol for microdissection of vestibular endorgans,[27] immunofluorescence, and immunohistochemistry in cryostat sections and whole endorgans has been described in detail.[27–29] The vestibular endorgans were immersed in 30% sucrose in PBS for 7 days for cryoprotection. Twenty-micron thick serial sections were obtained using a cryostat (Microm-HN505E).

Immunofluorescence in tissue sections

Tissue sections were incubated at room temperature for 1 hour with a solution containing 1% bovine serum albumin (BSA) fraction-V (Sigma) and 0.5% Triton X-100 (Sigma) in PBS. At the end of the incubation, the blocking solution was removed, and the primary antibodies against otopetrin-2 and GFAP were incubated 48 hours at 4°C in a humidity chamber. The secondary antibodies against rabbit or mouse labeled with Alexa 488 or 594 (1:1000, Molecular Probes, Carlsbad, California, USA) were applied and incubated for 2 hours at room temperature in the dark. At the end of the incubation, sections were washed with PBS (3 × 10 minutes) and covered with Vectashield mounting media containing DAPI (Vector Labs, Burlingame, California, USA) to visualize all cell nuclei.

Controls

Mouse macula utricle cryostat sections were used as positive controls. These sections were subjected to the same protocol as the immunofluorescence protocol of human macula utricle. As negative controls, the primary antibody was omitted or preabsorbed with the antigen as described previously,[28] and the immunoreaction was performed as described previously. No immunoreaction was detected in both types of negative controls.

Immunofluorescence in whole utricles (flat mount preparation)

The utricles were placed in a rotary shaker and incubated for 3 hours in a blocking solution containing 2% bovine serum albumin fraction V (Sigma, SLM), 0.1% Triton X-100 (Sigma, SLM) diluted in PBS at 4°C to 6°C. Subsequently, the blocking solution is removed, and the whole organs are incubated for 72 hours with the primary antibodies, placing the vials in the rotatory shaker in a cold room. At the end of the incubation, the secondary antibodies against rabbit labeled with Alexa 488 and against mouse labeled with Alexa 594 were diluted 1:1000 in PBS (Molecular Probes), applied to the tissue sections, and incubated for 1 hour at room temperature in the dark. At the end of the incubation, the whole endorgans or sections were washed with PBS (20 minutes × 5) and mounted flat on glass slides with Vectashield solution containing DAPI (Vector Labs).

Immunohistochemistry of the macula utricle.—Secondary antibodies against rabbit labeled with HRP and visualized with diaminobenzidine (DAB; Vector Labs) were used to visualize otopetrin-2 immunoreactivity. The immunohistochemistry (IHC) protocol has been described in detail.[28]

Confocal Imaging.—Fluorescent images were acquired using a high-resolution microscope Leica (SP8). All images were prepared using the Adobe Photoshop software program run in a Dell OptiPlex 3020 computer.

Light and transmission electron microscopy imaging

The processing protocol to obtain plastic embedded tissue sections for light and transmission electron microscopy (TEM) was described in detail by our group.[30] Tissue sections counterstained with toluidine blue were viewed and captured using an Olympus BX51 fluorescent microscope (Olympus America Inc., New York, New York, USA) equipped with an Olympus DP70 digital camera. All images were prepared using the Adobe Photoshop software program run in a Dell OptiPlex 3020 computer.

Results

Otopetrin-2 immunofluorescence and GFAP immunofluorescence in cryostat sections of the human macula utricle

Figure 1a shows otopetrin-2 immunofluorescence (IF; green color) and GFAP-IF (red) colocalization in supporting cells from a macula utricle harvested at surgery from a patient diagnosed with MD, Figure 2b shows IF in a patient diagnosed with VDA (Tumarkin crises), and Figure 1c shows IF in a normal utricle dissected from a temporal bone obtained at autopsy. DAPI in blue shows cell nuclei. Otopetrin-2-IF was similar in the 3 types of specimens examined, namely, confined mainly at the apical portion of the supporting cells. GFAP-IF shows the location of the supporting cells (from base to apex) in the epithelia.

Otopetrin-2-IF in whole mount preparations of the human macula utricle

Figure 2a shows the distribution of otopetrin-2-IF in the macula utricle from a patient diagnosed with Meniere's disease (only), and Figure 2b shows IF in the utricle from a patient diagnosed with VDA. Figure 2c shows IF in the macula utricle sensory epithelia (normal autopsy). The 3 samples showed similar otopetrin-2-IF (punctate distribution) in the vestibular supporting cells. Quantification of otopetrin-2-IF was not performed in cryostat sections or whole mount preparations immunostained due to the small number of specimens used in this study.

Figure 3a shows a whole mount preparation in another Meniere's specimen stained with antibodies against otopetrin-2 and GFAP. Otopetrin-2-IF (green) was seen at the apical and basolateral portion of the supporting cells (red color). To corroborate otopetrin-2-IF, another macula utricle from a Meniere's patient was immunohistochemical stained as described in whole mount IF but with secondary polyclonal antibodies against rabbit labeled with HRB-DAB (Figure 3b). A punctated distribution of otopetrin-2 (dark amber color) was observed at the apical portion of the macula utricle sensory epithelia.

Morphology of the macula utricle sensory epithelia

Figure 4a shows a 1 micron–thick plastic section of the macula utricle from a patient with VDA (stained with toluidine blue); the vestibular sensory epithelia were composed by normal-look alike hair cells and supporting cells. Figure 4b shows an ultrathin section from

the same specimen to illustrate the presence of supporting cells and hair cells. The apical portion of the supporting cells cytoplasm is full of vesicles.

Discussion

We present for the first time the distribution of otopetrin-2 in the human macula utricle. Otopetrin-2 was mainly localized in the apical portion of GFAP-IF supporting cells, although in some cases, otopetrin-2-IF was seen in the entire supporting cells cytoplasm. Otopetrin-2 distribution was similar in Meniere's, VDA, and normal utricles. Otopetrin-2-IF was similar to that observed for Otopetrin-1 in mouse models[16]; however, the distribution of otopetrin-1, −2, or −3 has not been investigated in the human macula utricle.

In this study, we did not detect qualitative changes in otopetrin-2 in Meniere's, VDA, or normal utricles. This suggest that otopetrin-2 may not be directly involved in otoconia regulation as it has been demonstrated to be the case for otopetrin-1[16] in mouse models. In addition, the limited number specimens to investigate did not allowed quantitative changes. However, it is important to mention that the localization of otopetrin-2 is very similar to the localization of otopetrin-1 in mouse models. Further analysis is required on the distribution of otopetrin-1 in the human macula utricle and saccule.

We used the macula utricle of Meniere's and VDAs as it is easy to remove the saccule during the ablative surgery. It is important to mention that in BPPV and VDA, it has been suggested that the saccule[7] is more damaged, thus the utricle maybe less susceptible to changes in the expression of otoconia-related proteins. Otopetrins and other protein localization need to be investigated in the saccule.

Otopetrin-1 is an important protein essential for mineralization of otoconia.[16] This protein is expressed in supporting cells.[23] In the mouse animal models and zebra fish, it has been recently suggested that otopetrin-1 acts as a sensor of the extracellular calcium concentration near supporting cells and responds to ATP in the endolymph to increase intracellular calcium levels during otoconia mineralization.[16] Tu et al[24] recently demonstrated that otopetrin-1, otopetrin-2, and otopetrin-3 may function as proton channels in the mouse and human vestibular hair cells. Whether or not this is the case for otopetrin-2 in the human macula utricle remains to be investigated.

The molecular underpinning of vestibular diseases, among them BPPV, is currently unknown; however, otolin-1 is a significant candidate because its physical association with otoconial matrix proteins and distribution throughout the otoconial matrix suggest that it has an important role in embedding otoconial crystals.[16] Otopetrin-1 gene is one of a handful that when mutated causes an imbalance phenotype with selective otoconial involvement in mouse models.[16,23,31] The presence of otopetrin-2 in the human macula utricle suggests that an alteration in its expression may contribute to balance disorders. mRNA studies and/or DNA next generation sequencing in human utricle and saccule will help to identify changes in the expression of otopetrins and other related proteins in normal and disease conditions.

Supporting cells of the inner ear are recognized as essential players in inner ear homeostasis.[32] In this respect, we have recently reported a decrease in aquaporin-4 and redistribution of

aquaporin-6 in vestibular supporting cells from the macula utricle of patients diagnosed with MD[33] as well as morphological deterioration of vestibular and supporting cells in vestibular endorgans obtained from ablative surgery diagnosed with MD[34] and utricular hair cells loss with age in normal aging.[35]

Falls are a highly morbid and costly health condition affecting older individuals.[3] A recent analysis showed that vestibular dysfunction is common in the US population and that the prevalence of vestibular dysfunction increases steeply with age.[2,10,11] The molecular pathology of otoneurologic entities, such as vestibular fall attacks in Meniere's disease, BPPV, or senile otoconial degeneration and therapy, remains to be investigated.[7,33,36–40]

Limitations of our study and future directions

Given the limited availability of human tissue specimens, we did not perform quantitative immunohistochemistry of otopetrin-2 to investigate changes with age, disease condition, or gender. Immunoelectron microscopy of otopetrin-2 will be needed to determine the subcellular localization of the punctated immunofluorescent signal detected. However, we did immunohistochemistry and immunofluorescence staining and obtained a similar otopetrin-2 immunoreactive pattern in our specimens; in addition, we found a similar pattern of immunostaining as that reported for otopetrin-1 in the mouse utricle.

There is the need to immunolocalize otopetrin-1 and 3 as well as other otoconial proteins in the macula utricle and saccule using celloidin embedded sections from patients diagnosed with BPPV and other vestibular disorders. There is also the need to study a systematic differential distribution of otopetrin-1, −2, and −3 in the whole utricle and saccule to determine whether the striola and extra-striola region changed with age and disease. Our study suggests that human inner ear tissue could be used to compare and contrast findings in animal models to design better therapies for vestibular and auditory disorders.

Conclusions

Otopetrin-2 immunofluorescence was detected in all the human macula utricle examined. Otopetrin-2 was localized mainly at the apical portion of supporting cells; in some cases, otopetrin-2 was also seen throughout the entire supporting cells cytoplasm. The immunolocalization of otopetrin-2 was similar to otopetrin-1 previously described by other groups in the mouse macula utricle.

Acknowledgments

We appreciate Dr Matthew Schibler from the Advanced Microscopy Laboratory and Spectroscopy (AMLS) of California Nanosystems Institute at UCLA (CNSI) for allowing the use of SP8 Leica laser confocal high-resolution microscope and Mr Ivo Atasanov for the use of the T12 (FEI) transmission electron microscope located at the Electron Imaging Center for NanoMachines (EICN)-CLMS-UCLA.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by a NIDCD (Grant No. 1U24DC015910-01).

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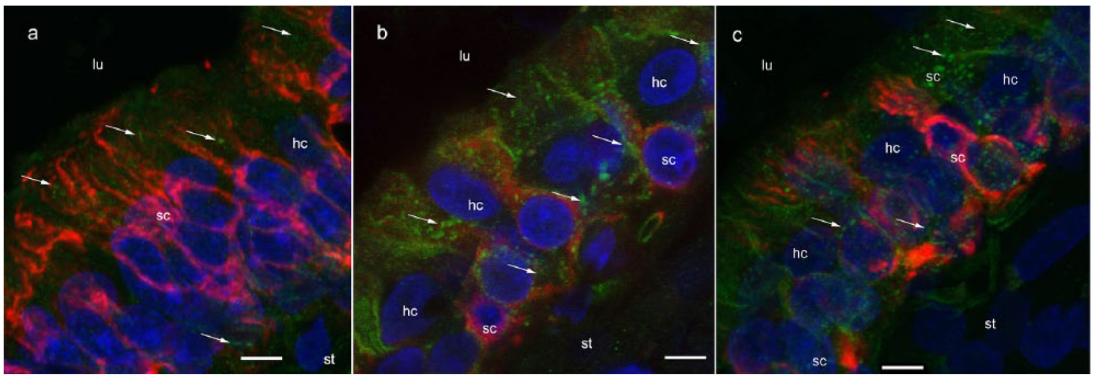

Figure 1.

Otopetrin-2 immunofluorescence (IF; green color) and GFAP-IF (red color) in cryostat sections of the human macula utricle. (a) Patient diagnose with Meniere's disease (MD). (b) Patient diagnosed with vestibular drops attacks (VDA; a and b are surgical specimens). (c) Normal utricle obtained at autopsy. Arrows point to otopetrin-2-IF. DAPI in blue shows cell nuclei.

Abbreviations: hc, hair cells; lu, epithelial lumen; sc, supporting cells; st, stroma. Magnification bar is 5 μm.

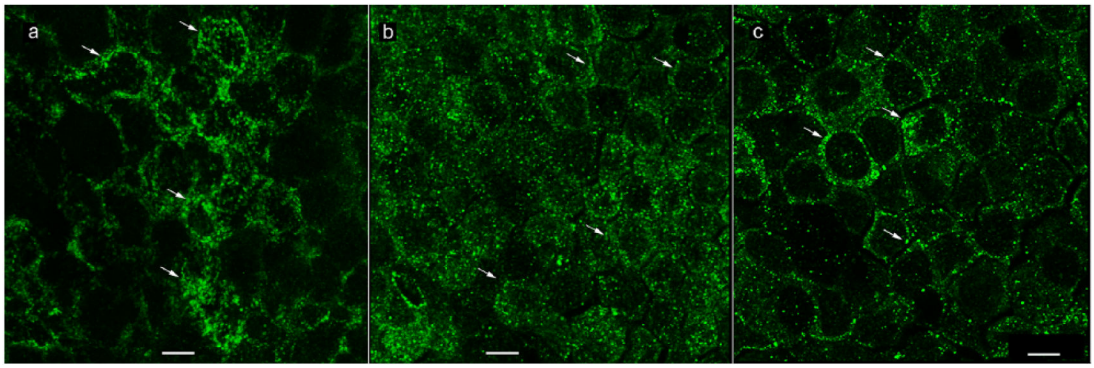

Figure 2.

Otopetrin-2 immunofluorescence (IF) and GFAP-IF in whole mount preparation the human utricle. (a) Meniere's disease (MD) specimen. (b) Patient diagnosed with vestibular drops attacks (VDA; a and b are surgical specimens). (c) Normal utricle obtained at autopsy. Arrows point to otopetrin-2-IF. Bar is 5 μm.

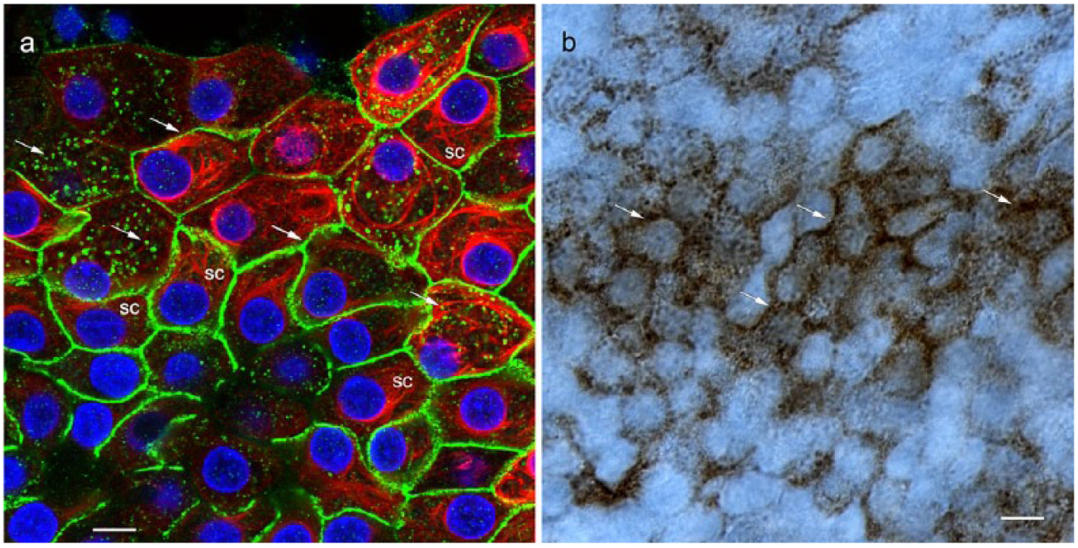

Figure 3.

(a) Colocalization of otopetrin-2 immunofluorescence (IF) and GFAP-IF in a whole mount preparation for a Meniere's disease (MD) patient. (b) Immunohistochemical visualization of otopetrin-2 using HRP-DAB (dark amber color) in another MD patient. Magnification bar is 5 μm in both figures.

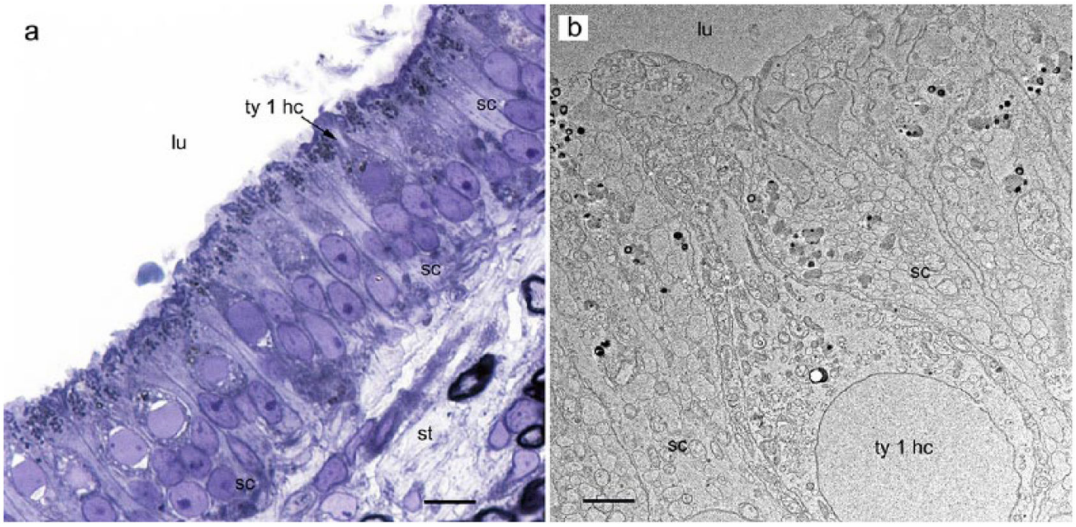

Figure 4.

(a) Light microscopy image from a patient diagnose with vestibular drops attacks (VDA). Two micron–thick plastic section stained with toluidine blue. (b) Transmission electron microscopy image from an ultrathin plastic section, from the same specimen as Figure 4a. Abbreviations: lu, lumen; st, stroma; ty 1 hc, type I hair cells. Magnification bar is 8 μm in Figure 4a and 1 μm in Figure 4b.