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Immunohistochemical localization of Nrf2 in the human cochlea

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Abstract

Oxidative stress plays an important role in several inner ear diseases and normal aging. Nuclear (erythroid-derived 2)-like 2, also known as Nrf2, is a transcription factor encoded by the NFE2L2 gene that controls the expression of genes necessary to reduce oxidative stress. There are no studies to the date on the localization of Nrf2 in the human inner ear in normal or pathological conditions. Therefore, we investigated the immunohistochemical localization of Nrf2 in the human cochlea and vestibule using formalin-fixed celloidin-embedded human temporal bone sections. Nrf2 immunoreactivity (IR) was found in the inner and outer hair cells and supporting cells of the organ of Corti throughout the cochlea. Nfr2-IR was also found in hair cells and supporting cells of the maculae and cristae vestibular sensory epithelia. Nrf2-IR was decreased in the organ of Corti of older age individuals. The immunolocalization of Nrf2 in both auditory and vestibular sensory epithelia suggest that this transcription factor may play a relevant role in protecting sensory hair cells from oxidative stress.

Keywords

Nrf2; Oxidative stress; Cochlea; Sensory hair cells; Supporting cells; Hearing loss

1. Introduction

Oxidative stress can be a causative or an exacerbating factor in aging, metabolic, neurodegenerative or autoimmune diseases (Choung et al., 2009). Persistent oxidative stress caused by excessive noise, exposure to ototoxicants, aging, or Meniere's disease leads to hearing loss (Honkura et al., 2016; Riva et al., 2007; Semaan et al., 2005). Nuclear (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2, is a transcription factor

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encoded by the NFE2L2 gene (Sykiotis et al., 2010). Nrf2 is a central mediator of a prominent cellular defense system (Nguyen et al., 2009). Nrf2 translocates to the nucleus in response to oxidative stress (Katsuoka et al., 2005; Motohashi et al., 2004; Sykiotis et al., 2010). In the nucleus Nrf2 binds to the antioxidant response element (ARE), to activate important phase 2 genes (Katsuoka et al., 2005; Sykiotis et al., 2010). Nrf2-deficient mice showed increased sensitivity to a variety of pharmacological and environmental toxicants (Kensler et al., 2007; Rangasamy et al., 2004).

Nrf2 transcription faction protects against oxidative tissue damage through ARE-mediated transcriptional activation of several phase 2 detoxifying enzymes and antioxidant enzymes. The protective effects of Nrf2 inducers have been tested in a number of models of human diseases, including cancer, neurodegeneration, cardiovascular disease, liver and lung injury (Kensler et al., 2007; Kim et al., 2009; Wakabayashi et al., 2010).

Reactive oxygen species (ROS) produced by oxidative stress are involved in a wide range of cochlear injuries (Riva et al., 2007). The protective role of Nrf2 by the exposure of ototoxic agents has been investigated *in vivo* in the mouse and rat cochlea (Hoshino et al., 2011; Kim et al., 2015; Kong et al., 2009), and *in vitro* using HEI-OC1 cells (House Ear Institute-Organ of Corti 1) (Choi et al., 2011; Choung et al., 2009; Gao et al., 2010; Kalinec et al., 2003; Kong et al., 2009; So et al., 2006).

HEI-OC1 is a conditionally immortalized mouse organ of Corti derived cell line (Kalinec et al., 2003, 2016). This cell line express outer hair cell markers including Math1 Myosin 7a and prestin. As described by Kalinec et al., (2003 and 2016), these cells are extremely sensible to ototoxic drugs and have been shown to be an excellent *in vitro* model system to investigate cytotoxicity (Kalinec et al., 2016) and the effect of several antioxidants as well as screening of pharmacological drugs.

Hoshino et al. (2011) examined the contribution of Nrf2 to cochlear protection using wild type and Nrf2-KO mice. After gentamicin treatment, Nrf2-target genes were upregulated in the wild type, but not in the Nrf2-KO mouse cochlea. Sensory hair cells of the Nrf2-KO mouse cochlea were severely affected after gentamicin treatment (Hoshino et al., 2011). Auditory brainstem response (ABR) and histological analyses in aged Nrf2-KO mouse, showed a significant accelerated impairment of auditory function, when compared with the wild type mouse.

Kong et al., (2009) showed that Nrf2 mRNA expression levels in the tub/tub mice cochlea were reduced. This mouse strain shows progressive hearing loss at 3 weeks of age. The reduction of Nrf2 in the tub/tub mouse cochlea is concomitant to the reduction of thioredoxin and thioredoxin-reductase, proteins relevant in the redox system in auditory cells. Intraperitoneal injection of sulforaphane to the tub mouse showed a partial protective effect on the cochlea (Kong et al., 2009). Bucillamine, a strong antioxidant, increases Nrf2 mRNA levels, induces the expression of phase II detoxification enzymes in HEI-OC1 cells, and prevents cisplatin-induced cell death and hearing loss in wild type mice exposed to cisplatin (Kim et al., 2015). These studies (Hoshino et al., 2011; Kim et al., 2015; Kong et al., 2009) suggest that Nrf2 may protect the inner ear against ototoxic drugs, and age-related

injuries. The *in vitro* studies using HEI-OC1 cells show that the antioxidants kaempferol, phloretin and bucillamine can suppress cisplatin-induced oxidative stress and apoptosis, by induction of hemeoxygenase-1 (HO-1), via JNK pathway, which mediates Nrf2 translocation to the nucleus (Choi et al., 2011; Gao et al., 2010; Kim et al., 2015). Flunarizine (an antagonist of T-type specific calcium channel) stimulates Nrf-2-driven transcriptional activation via PI3K-Akt signaling pathway when HEI-OC1 were exposed to cisplatin (So et al. 2006). It has been recently demonstrated that a single polymorphism (SNP) in the NRF2 promoter is associated with sensory neural hearing loss (Honkura et al., 2016).

A component hypothesis of the present study is that Nrf2 up-regulation could protect the inner ear in chronic diseases, like Meniere's disease, noise exposure, aging and exposure to ototoxicants. Downregulation of Nrf2 in inner ear disease or aging would suggest loss of this protective mechanism. We investigated the localization Nrf2 by immunohistochemistry in formalin fixed, celloidin-embedded human inner ear tissue sections obtained from normal and pathological human temporal bones. Nrf2 was localized mainly in the inner and outer hair cells and supporting cells in both the organ of Corti, and the vestibular sensory epithelia. There was a decline in Nrf2 immunoreactivity in the cochlea of older age individuals. These results suggest that Nrf2 may protect sensory hair cells and supporting cells from oxidative stress. Preliminary results were presented in abstract form (Hosokawa et al., 2014).

2. Results

2.1. Nrf2-IR in the human cochlea

Nrf2-IR was found predominantly in the human organ of Corti at the apical, medial and basal region (Fig. 1a). In few cases, cells of the spiral prominence and spiral limbus were immunoreactive. The remaining structures of the cochlea: stria vascularis, spiral ligament, and Reissner's membrane were not immunoreactive. Neurons of the spiral ganglia were almost devoid of Nrf2-IR. This Nrf2-IR pattern was consistently seen in all the temporal bones examined (Table 1). Fig. 1b, is a H&E stained adjacent section to show the cells present the human cochlea. These images were taken from a normal cochlea (55-year-old female, Table 1). Fig. 2a, shows a higher magnification view of the organ of Corti (From Fig. 1a, middle level). Nrf2-IR was seen in the cytosol and nuclei of inner and outer hair cells, Deiters, and Hensen cells. Fig. 2b, shows a H&E section of the organ of Corti from the same temporal bone. Inner and outer hair cells as well as adjacent supporting cells are easily identified.

Fig. 3a, shows Nrf2-IR in the organ of Corti from a patient diagnosed with Meniere's disease (92-year-old, female, Table 1). Nrf2-IR was seen in the cytoplasm, but not in the cell nuclei of the remaining epithelial cells. Fig. 3b shows for reference an adjacent H&E section from the same temporal bone. Remaining epithelial cells are visible. Fig. 4a, b, and c, shows Nrf2-IR in the organ of Corti from three different temporal bones. Fig. 4a1, a1, and cl are adjacent H&E stained sections to help the identification of cells cytoplasm and their nuclei. Fig. 4a2, b2, and c2, shows the corresponding spiral ganglia neurons devoid of Nrf2-IR. Fig. 4a, a1, and a2, were taken from a 60-year-old female (decreased hearing, Table 1). Fig. 4b, bl, and b2 were taken from a 67-year-old male (normal hearing, Table 1), and Fig. 4c, c1 and c2, were taken from an 84-year-old male (bilateral Menierés disease, Table 1). Nrf2-IR was

found consistently in the remaining epithelial cells of the organ of Corti and not in the SGNs in the three specimens.

2.2. Qualitative and quantitative analysis of Nrf2-IR in the human cochlea.

2.2.1. Qualitative analysis—We investigated the cellular distribution of Nrf2-IR (nuclear vs. cytoplasmic) in the organ of Corti from normal, young, aged or different inner ear diseases. There was not a distinct pattern of Nrf2-IR cellular distribution in the hair cells and supporting cells throughout the cochlea (Table 1). Twelve temporal bones showed Nrf2-IR in both the cytoplasm and nuclei in hair cells and supporting cells, eighteen specimens showed Nrf2-IR in hair cells and supporting cell cytoplasm, and three specimens showed Nrf2-IR in the nucleus of hair cells and supporting cells.

Quantitative analysis: There was a 10% decrease of NrF2-IR in the disease group, however, there were no statistical significant differences in Nrf2-IR between the two groups ($p = 0.268$). In contrast, there was a significant 20% decrease in the old age group vs the young age group. The differences were statistically significant different ($p = 0.037$). Table 2 summarizes these findings.

2.3. Nrf2-IR in the human vestibular sensory periphery

Nrf2-IR was detected in the macula utricle sensory epithelia (Fig. 5a, 92-year-old female, Table 1). Fig. 5a1, shows a H&E adjacent section to help the identification of cells and nuclei. Fig. 5b, shows NrF2-IR in the macula saccule sensory epithelia from a 47-year-old individual male (Table 1). A similar Nrf2-IR pattern was observed. Fig. 5b1, shows a H&E alternate section from the same temporal bone. Fig. 5c, shows Nrf2-IR the macula utricle epithelia from a different temporal bone (84-year-old female, Table 1). Fig. 5c1 shows a H&E alternate section. In all the immunoreacted sections, the cytoplasm and nuclei of vestibular hair cells and supporting cells were Nrf2-IR (Table 1). Cells in the stroma underneath of the sensory epithelia were non immunoreactive. The neurons of the Scarpa's (vestibular) ganglia were not immunostained (not shown).

2.4. Nrf2-IR in control tissue

Nrf2-IR was found in the cytoplasm of hair cells and supporting cells of the rat organ of Corti (Fig. 6a), and the spiral ganglia neurons cytoplasm (Fig. 6b). In contrast to the human cochlea, cell nuclei in the rat cochlea (normal) were not NrF2-IR. The lateral wall (spiral ligament and stria vascularis) was not immunoreactive. Rat cerebellum sections showed Nrf2-IR in Purkinje cells cytoplasm (Fig. 6c). When the primary antibody was pre-absorbed with the corresponding antigen no immunoreaction was observed in this human cochlea section (Fig. 5d and Fig. 6d).

3. Discussion

In our study Nrf2-IR was consistently localized in hair cells and supporting cells of the organ of Corti and the vestibular sensory epithelium in all temporal bones examined. Our analysis showed a significant decrease of Nrf2-IR in the human organ of Corti with age.

3.1. Cellular localization of Nrf2 in the human inner ear

Nrf2 is ubiquitously expressed in the body, including the brain, retina (Yamazaki et al., 2015; Zong et al., 2013) and the inner ear (Hoshino et al., 2011). Nrf2 is accumulated to the nucleus in response to electrophiles or reactive oxygen species (Calabrese et al., 2010). We found that only three specimens showed Nrf2-IR in the nuclei of cells in the organ of Corti, the rest of specimens showed either cytoplasmic only or both cytoplasmic and nuclei labeling. We can only speculate on the expression of Nrf-2 in both the cytoplasm and nuclei in cells of the organ of Corti. The expression of Nrf2 in the cytoplasm and nuclei of hair cells and supporting cells of the organ of Corti in normal, disease or aged specimens, maybe due to the “hormesis” effect described by Mattson (2008), and recently by Yamazaki (2015). In “hormesis”, a low dose of a toxic substance confers protection against exposure to a subsequent higher amount of the same toxicant.

Sensory and non-sensory cells in the inner ear are exposed to ototoxicants and noise during the life time of an individual. Glutamate is the major neurotransmitter in the cochlea and vestibule (Furness et al., 2009; Sadeghi et al., 2014). Excessive noise or ototoxicants can evoke excessive release of glutamate. Glutamate-induced excitoxicity generates oxidative stress through the accumulation of intracellular Ca^{2+} (Barnham et al., 2004). This may explain the presence of Nrf2 in both the cytoplasm and cell nuclei of sensory and supporting cells in all the specimens examined. Nrf2 controls basal and inducible expression of phase 2 genes (Ma. 2013; Nguyen et al., 2009), suggesting that Nrf2 is a constitutively and functionally active transcription factor. This may explain its presence in the nucleus under homeostatic conditions. Exposure to ROS generated by ototoxicants or noise may induce Nrf2 translocation to the nucleus (Chung et al., 2011).

Among the enzymes activated by the Nrf2 transcription factor are heme-oxygenase-I (HO-1), quinine oxidoreductase gene (NQO1), two superoxidase dismutase genes (SOD1 and SOD2), and two glutathione peroxidase genes (Kim et al., 2015; Motohashi et al., 2004). SOD2 expression in the human spiral ganglia neurons has been well characterized and it has been suggested as a protective mechanism (Ying et al., 2009). What mechanisms of defense may be upregulated by Nrf2 activation in the human inner ear in response to noise, aging or disease remains to be determined.

3.2. Nrf2-IR in the cochlea of older age specimens

We found a statistically significant decrease in Nrf2-IR in the old age group. A decrease of Nrf2-IR in the organ of Corti is likely due to loss of sensory hair cells or supporting cells during normal aging and disease (Nadol, 2010). Particularly a gradual loss of cells in the organ of Corti from the base to apical portion. We have detected a decrease in GLAST (glutamate transporter-1), and a translocase located in the outer membrane of the mitochondria (Tom20) in the cochlea of aged individuals (Ahmed et al., 2013; Balaker et al., 2013). There is also the possibility that Nrf2 mRNA and/or protein expression decrease with age. In this respect Hosino (2011), examined the cochlea of Nrf2-KO mice and found deterioration of cochlear hair cells after gentamicin exposure and the lack of expression of several Nrf2 driven antioxidant enzymes.

3.3. Nrf2-IR in inner ear disease

The activation of Nrf2 pathway may be a promising novel strategy for the prevention and modification of chronic diseases of the inner ear (Hoshino et al., 2011; Kim et al., 2015). We found no statistical difference in Nrf2-IR between the normal group and inner ear disease group. This may be due to the small number of specimens analyzed in our study. However, we have previously detected changes in the expression of other proteins (aquaporins, cochlin and basement membrane proteins) in vestibular endorgans from patients diagnosed with Meniere's syndrome obtained from ablative surgery (Calzada et al., 2012; Ishiyama et al., 2010). Recently we demonstrated a decrease in expression of Tom20 in the cochlea of individuals diagnosed with Meniere's disease (Balaker et al., 2013). Increased mRNA expression of cochlin and Nrf2 has been detected in a mouse model of usher syndrome type 1F (Chance et al., 2010), and in human glaucomatous trabecular meshwork (Picciani et al., 2008). Cochlin expression is also altered in patients diagnosed with DFNA9 (Robertson et al., 2006). These suggests that pharmacological modifications on Nrf2 expression may protect sensory hair cells from oxidative stress.

3.4. Nrf2-IR in the rat cochlea

It is important to mention that in the normal rat cochlea, Nrf2-IR was detected only in supporting cells cytoplasm but not in the inner and outer hair cells. In the spiral ganglia, Nrf2 immunolocalization was confined to the cytoplasm of the spiral ganglia neurons, their nuclei were not immunoreactive. In the rat cerebellum, Purkinje cells cytoplasm was Nrf2-IR. To the best of our knowledge Nrf2-IR has not been demonstrated in the rat cochlea. The difference of Nrf2-IR in the human and rat cochlea may be due to the difference in tissue processing and acquisition. The normal rat cochlea is formalin fixed, frozen and processed for immunohistochemistry, the human temporal bones were formalin fixed, decalcified and embedded in celloidin before sections are obtained.

3.5. Limitations on the use of human cochlea celloidin embedded sections for immunohistochemistry

There are technical considerations in the interpretation of the cellular localization of Nrf2 by immunohistochemical techniques using celloidin embedded human inner ear sections (Lopez et al., 2016). The cochlea and vestibular endorgans pathology is at different stages, when the donors expire. Some patients may have advanced inner ear diseases while others may only be in their early stages. The celloidin embedding protocol includes the use of decalcifying agents like EDTA and solvents like ethanol and ether. The cyto-architecture of the neuro-epithelia in the internal ear is compromised by the postmortem time before the temporal bones are harvested, however, the morphology of the organ of Corti spiral ganglia neurons is relatively well preserved up to 20 hrs postmortem. In contrast there are several advantages on the use of inner ear celloidin embedded sections for immunohistochemistry, among them, is the availability of the clinical history. In many cases the clinical history of the temporal bone donors was followed for more than 20 years. There is an existing archive of remaining celloidin sections for each temporal bone because only one in every tenth section is stained with hematoxylin and eosin. This allows the histopathological evaluation

and the identification and classification of the inner ear pathology, leaving the rest of the sections useful for modern molecular biological investigations.

In conclusion, Nrf2-IR was detected in the cytoplasm and nuclei of hair cells and supporting cells thorough the human cochlea and the vestibular sensory epithelia. Nrf2-IR decreases significantly the cochlea of older individuals, but no in disease specimens. These findings suggest that Nrf2 expression may be a good candidate to prevent and or protect from hearing loss due to noise, exposure to ototoxic drugs and normal and pathological aging.

4. Experimental procedure

4.1. Human tissue

Approval was obtained from the University of California, Los Angeles Institutional Review Board (protocol # 10-001449). 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 subjects with a documented history of normal auditory and vestibular function, Meniere's disease, and other diseases. Temporal bones from 13 males and 17 female), ranging from 18 to 92 years of age, with an average age of 67 years were used in this study (Table 1). Post-mortem time for temporal bone collection was between 5 and 20 h (averaged 11 h).

4.2. Immunohistochemistry

The methodology for celloidin removal and antigen retrieval has been described previously in detail (Ahmed et al., 2013; Balaker et al., 2013; Lopez et al., 2016; Nguyen et al., 2014). In brief, celloidin sections were immersed in 100% acetone (2 × 15 min), sodium-ethoxide-100% ethanol (1:3, 30 min), 100% and 50% ethanol, and distilled water (5 min each), and 3% hydrogen peroxide in 100% methanol for 10 min. Sections were immersed in antigen retrieval solution (Vector antigen unmasking acidic solution, Vector Labs, Burlingame CA) diluted 1:500 in double distilled water. Sections were heated in the microwave using two intermittent heating 2-minutes cycles. Sections were allowed to cool for 20 min and washed with phosphate buffered saline (PBS) before immunohistochemistry. Sections were incubated for one hour with a blocking solution containing 1% normal goat serum/1% bovine serum albumin (BSA) fraction-V (Sigma, St. Louis, MO) and 0.5% Triton X-100 (Sigma) in PBS. Followed by the incubation with antibodies against Nrf2 (1:1000) diluted in PBS for 48 h at 4 °C in a humid chamber. Nrf2 rabbit polyclonal antibody (IgG) is affinity purified, raised against a peptide mapping at the C-terminus of Nrf2 of human origin (Cat. #sc-722, Lot # 2807, Santa Cruz Biotechnology, Santa Cruz, CA). At the end of the incubation the sections were washed with PBS (3 × 15 min), incubated for one hour with biotinylated goat anti-rabbit polyclonal IgG (1:1000, Vector Labs, Burlingame, CA), and then washed with PBS (3 × 5 min). Next, one-hour incubation was performed with Vectastain Elite ABC reagent (Vector Labs) followed by PBS washes (3 × 15 min). Immunoperoxidase staining was performed using Immpact DAB solution (Vector Labs). The reaction was stopped with distilled water washes (5 min × 10). Slides were mounted with Polymount mounting media (Fisher Scientific).

4.3. Immunohistochemistry controls

As a positive control, cryostat sections from rat cochlea were incubated with the Nrf-2 antibodies described above. Rats were handled and cared for in accordance with the Animal Welfare Act and in strict compliance with the National Institute of Health Guidelines. Rat cochlea and cerebellum sections used for this study were taken from a frozen pool available for a study made before in the rat cochlea (4 weeks-old, male). Tissue sections were subjected to the same immunohistochemistry protocol. As negative control celloidin embedded sections were processed for immunohistochemistry as described above, except that the Nrf2 antibody was pre-absorbed with the corresponding peptide used to generate the primary antibody (Nrf2 (C-20) P, Cat # sc-772P, Santa Cruz, CA). Pre-absorbed antibody was prepared by mixing the Nrf2 antibody with the Nrf2 peptide (1 μg/1 μl). The mixture was incubated for 1 h at 37 °C. Additional negative control included the omission of the Nrf2-IR during the immunoreaction. No specific immunoreaction was detected in both cases.

4.4. Microscopic documentation

Immunoreacted and hematoxylin eosin stained sections were viewed and imaged with an Olympus BX51 fluorescent microscope (Olympus America Inc., NY, USA) equipped with an Olympus DP70 digital camera. Images were acquired using MicroSuiteTM Five software (Olympus America Inc.). All images were prepared using the Adobe Photoshop software program run in a Dell Precision 380 computer.

4.5. Qualitative assessment of Nrf2-IR

Assessment of intensity/immunoreactive signal was made visually and was qualitative as a result. Nrf2-IR was assessed using a scale ranging from: (1) low immunoreactivity, (2) mild immunoreactivity, (3) strong immunoreactivity. Two independent and blinded observers assessed each stained slide for Nrf2-IR and scored the intensity of the signal, and a third person, not blinded to sample intensity, observed and then tabulated the results (Table 1).

4.5.1. Quantification of the Nrf2-IR area—For quantitative immunohistochemistry, cochlea sections from normal, and inner ear disease temporal bones were simultaneously immunoreacted, using the same lot of Nrf2 antibodies, other reagents and solutions. The HRP-DAB staining was stopped at the same time. To avoid bias in the tissue analysis, one researcher was blinded to the identity of each analyzed sample (coded). A second researcher not blinded to the samples coded each sample.

Nrf2-IR area measurements were made in the organ of Corti at the base, medial and apical region (Fig. 1a). Quantitative analysis was made as described by our group (Ahmed et al., 2013; Balaker et al., 2013; Ishiyama et al., 2010), using *ImageJ* software (https://imagej.nih.gov/ij/download.html version 1.50 g). In brief: The image captured was open using the *ImageJ* 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 Nrf2-IR and was subtracted from the Nrf2-IR area values. The image was converted to black and white, and the IR area was selected using the rectangular tool. To determine the Nrf-2 IR

area within the region of interest (Organ of Corti) the command (analyze/analyze particles was selected), and the “mask tool” was selected. The resulting measurements represent the are fraction, which is the proportion of the region of interest (Organ of Corti) that was Nrf2-IR. Average values obtained in the normal specimens, or young specimens were considered 100%. This value was compared accordingly: normal vs disease, and young-age vs. old-age specimens. Results are summarized in Table 2.

4.6. Statistical analysis

We tested whether the Nrf2-IR area in the organ of Corti of the normal specimens was statistically different from Nrf2-IR in the disease specimens. We also tested whether Nrf2-IR area in the organ of Corti was statistically different between the young age group (<70 years-old) and old age group (greater than70 years-old). For each specimen, mean values of the immunoreacted area were averaged and subjected to one-way repeated measurements analysis of (ANOVA). Post-hoc individual univariate group comparisons were made for any significant results. A p-value of ≤0.05 was considered to be statistically significant different. The Sigma Stat 3.1 software program (Sigma Stat, Ashburn, Va., USA) was used for statistical analysis.

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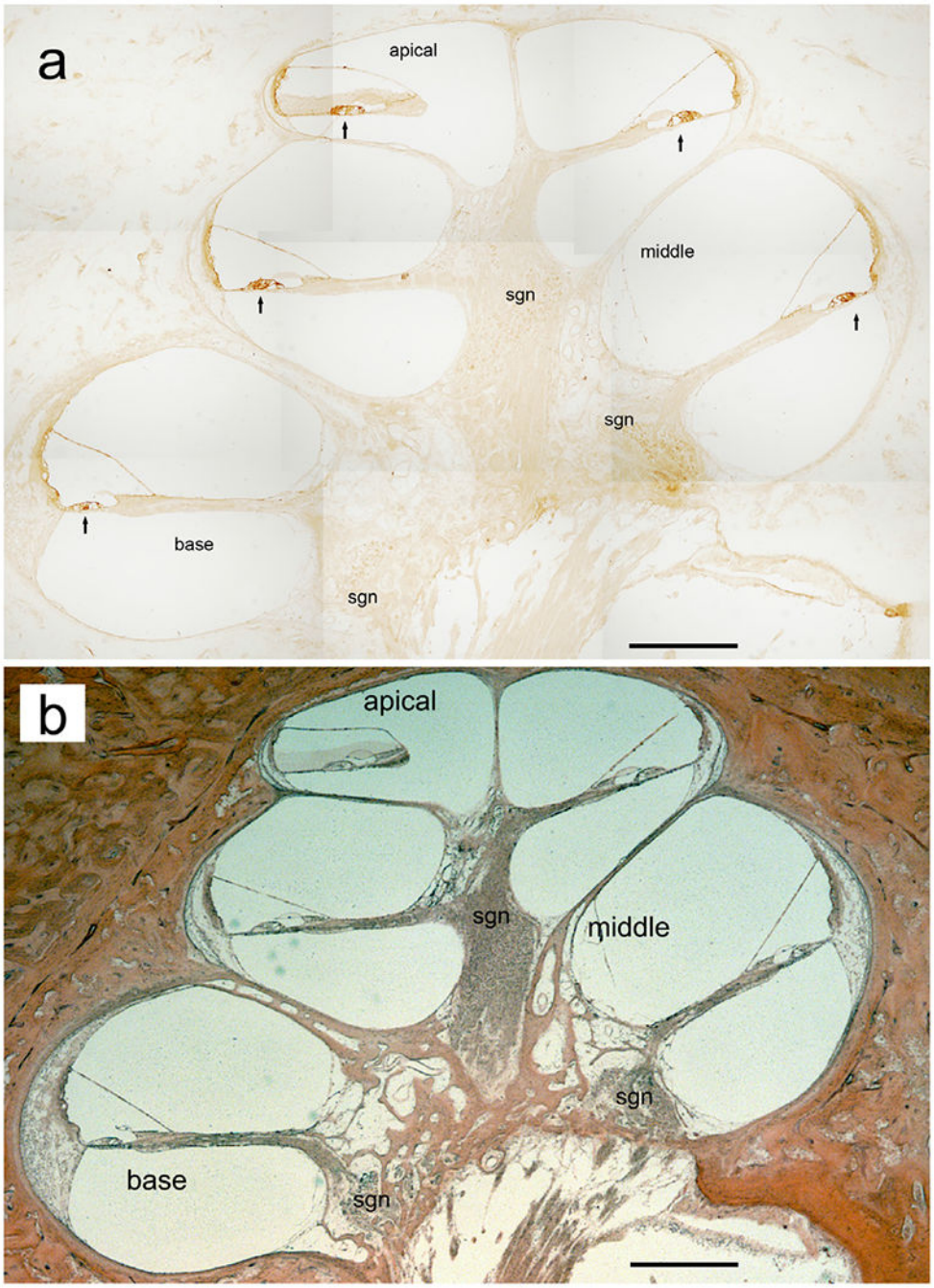

Fig. 1.

(a) Nrf2 immunoreactivity (-IR) distribution in the human cochlea. Low magnification view of a cochlea section (mid-modiolar area), showing Nrf2-IR visualized with diaminobenzidine (amber color). Nrf2-IR was localized in hair cells and supporting cells of the organ of Corti throughout the cochlea (arrowheads). Nrf2-IR was almost absent in the spiral ganglia neurons (sgn) (55-year-old female, no inner ear disease, Table 1). (b) H&E adjacent temporal bone section. Bar is 500 μm.

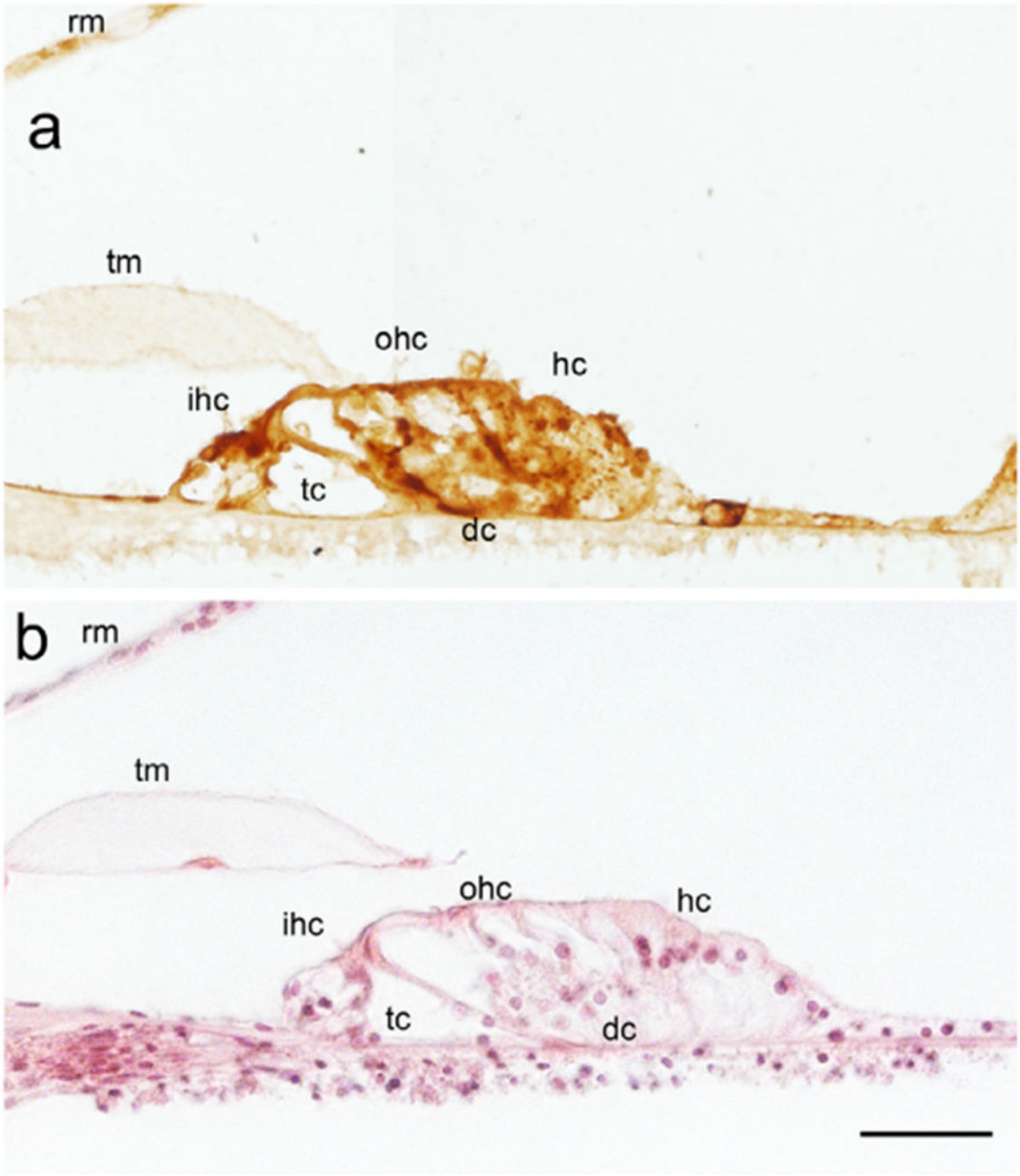

Fig. 2.

(a) Higher magnification view of the human organ of Corti (same specimen from Fig. 1). (a) Nrf2-IR was present in inner hair cells (ihc) and outer hair cells (ohc), Deiters' cells (dc), Hensen cells (hc), (b) Hematoxylin and eosin (H&E) adjacent section form the same specimen (mid-apical portion), to illustrate the sensory and supporting cells in the organ of Corti. rm: Reissner's membrane, tm: tectorial membrane, tc: tunnel of Corti. Bar is 150 μm.

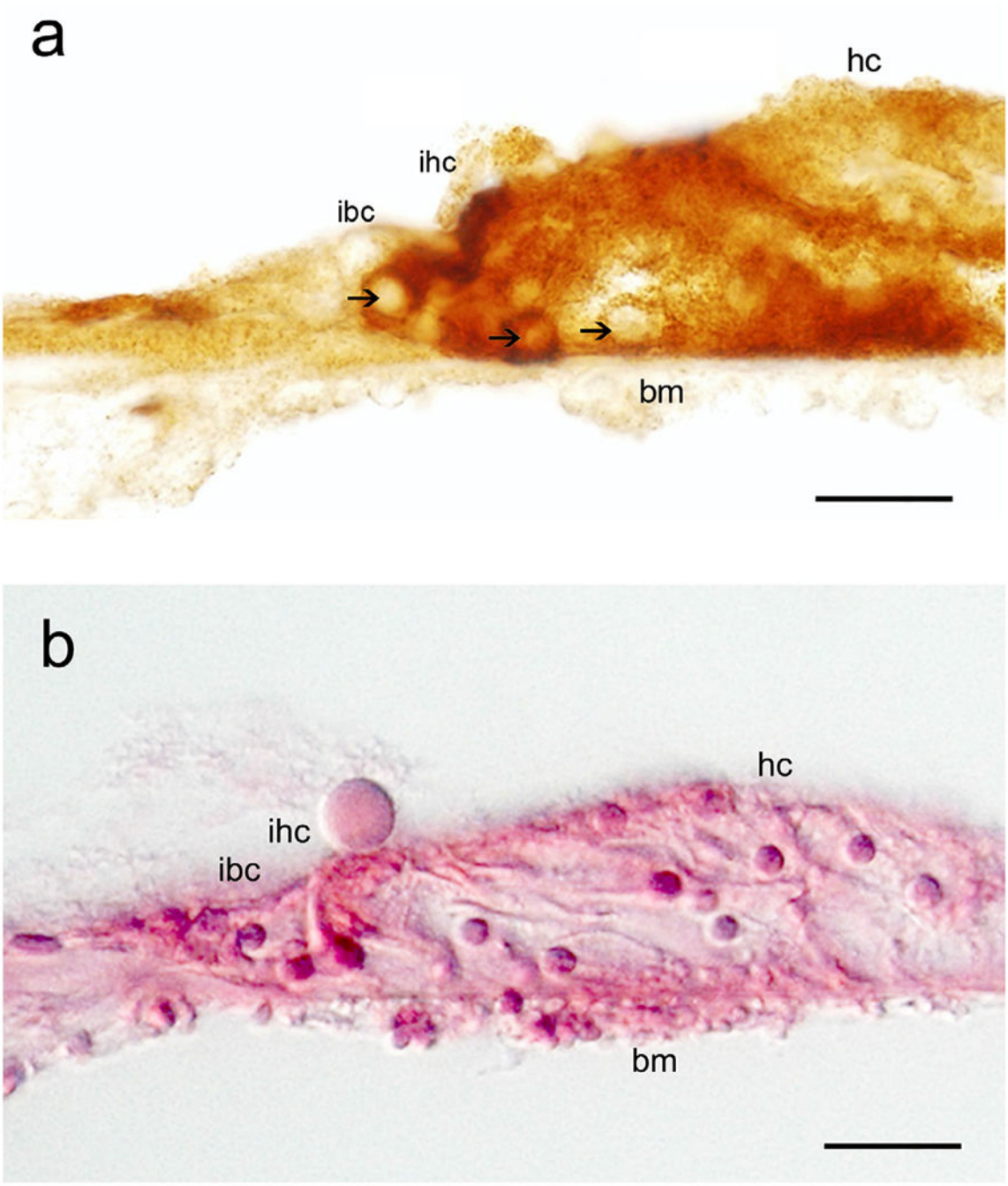

Fig. 3.

(a) Nrf2-IR in the organ of Corti of a Meniere's disease patient (92-year-old female). Remaining cells were Nrf2-IR. Nuclei were non-immunoreactive (arrowhead), the cells underneath the basilar membrane (bm) were non-immunoreactive. (b) H&E stained adjacent section of the organ of Corti. Remaining cells in the epithelium were present. ibc: inner border cells, ihc: inner hair cells, hc: Hensen cells. Bar in 25 μm.

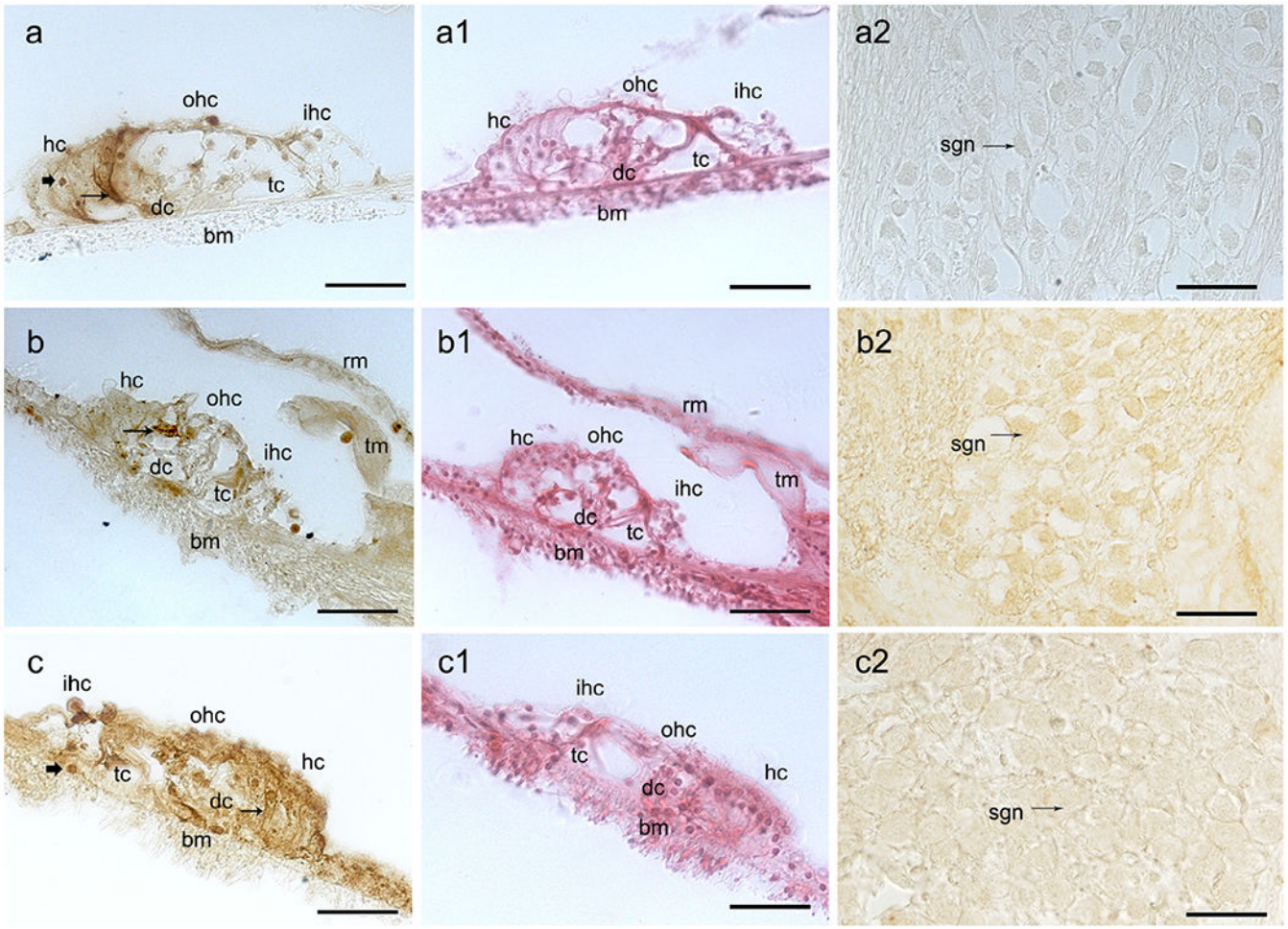

Fig. 4.

Nrf2-IR in the organ of Corti of three different temporal bones. (a). Nrf2-IR was detected in the cytoplasm of Hensen cells (hc) (arrow), and nuclei (arrowhead). (a1), shows a H&E adjacent section, (a2) cells in the spiral ganglia (long arrow) were devoid of immunoreactivity (60-year-old female, Table 1). (b) Nrf2-IR was present in the cytoplasm of Hensen cells (arrow), (b1) H&E adjacent section, (b2) cells of the spiral ganglia (long arrow) were devoid of Nrf2 immunoreactivity (67-year-old male, Table 1), (c). Nrf2-IR was preset in the cytoplasm (arrow) and nuclei of sensory and supporting cells (arrowhead). (c1) H&E adjacent section, (c2) cells of the spiral ganglia (long arrow) were devoid of immunoreactivity (84-year-old male, Table 1). Tc, tunnel of Corti; tm: tectorial membrane. Bar in all figures = 200 μm.

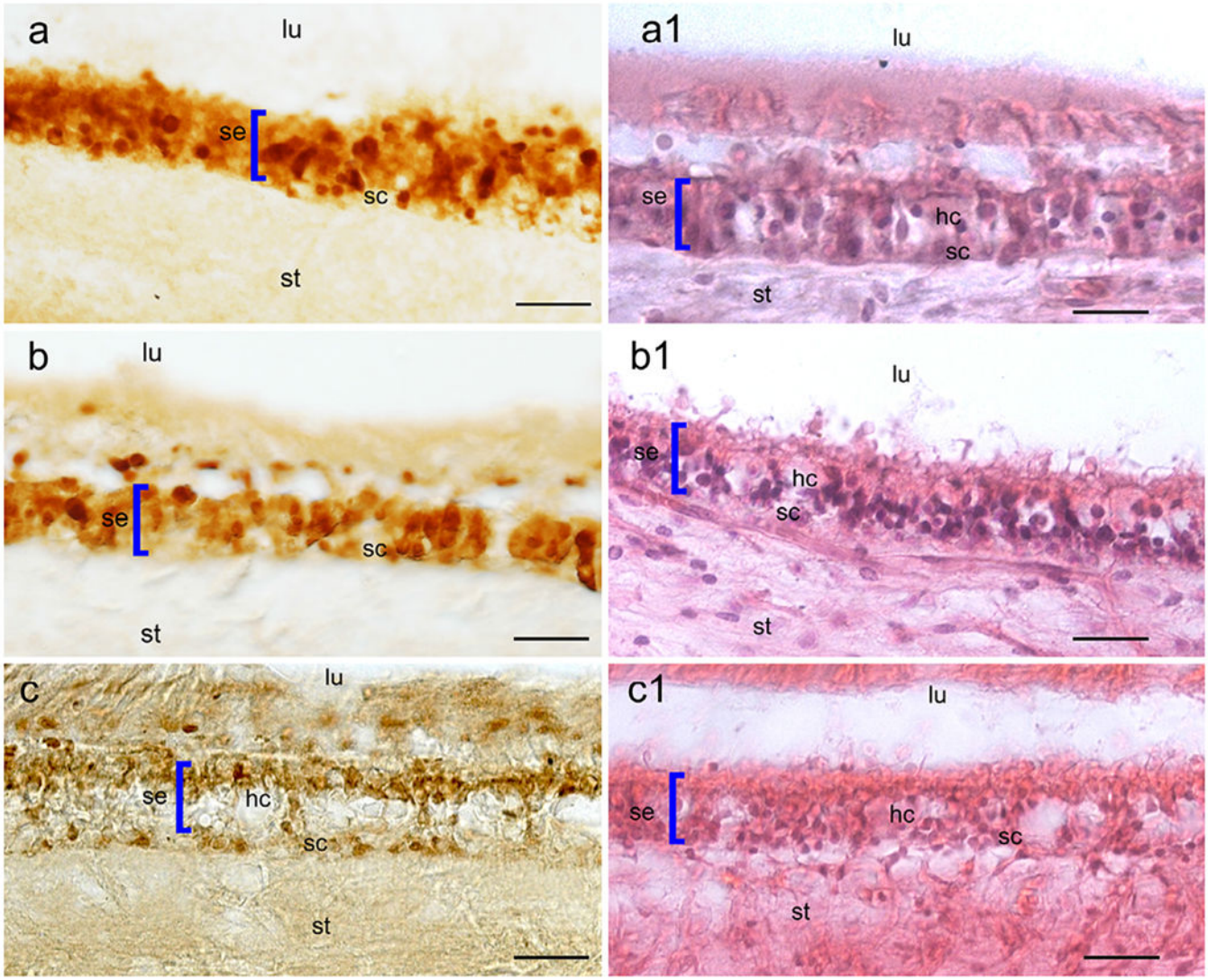

Fig. 5.

Nrf2-IR in the macula utricle and saccule in three different specimens. (a) Nrf2-IR was confined to the sensory epithelium ([in blue color). Note the lack of Nrf2-IR in the stroma underneath the sensory epithelium (92-year-old female, same patient from Fig. 3a), (a1) H&E stained adjacent section to help the identification of cells present in the macula utricle. (b) Nrf2-IR was confined to the sensory epithelia hair cells and supporting cells of the saccule (47-year-old male, Table 1), (b1) H&E adjacent section from the same temporal bone. (c) Similar Nrf2-IR pattern was seen in the macula utricle (84-year-old female, Table 1), (c1) H&E from an adjacent section. se: sensory epithelia, hc: hair cells, sc: supporting cells; lu: lumen; st: stroma. Bar = 100 μm.

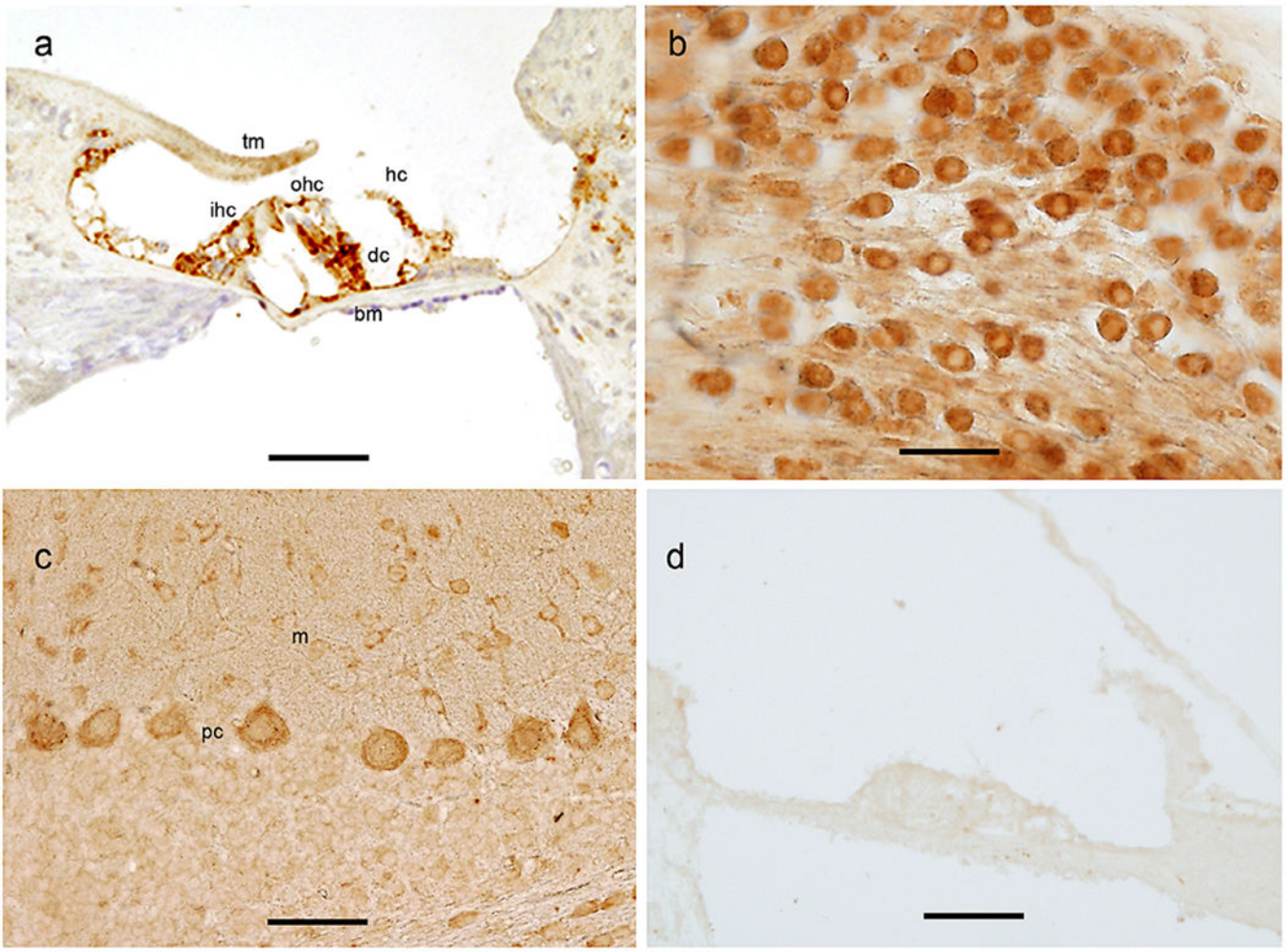

Fig. 6.

Nrf2-IR in control tissue sections. (a) Nrf2-IR was present in the rat organ of Corti. Nrf2-IR was seen in hc and dc cytoplasm, tm: tectorial membrane, bm: basilar membrane. (b) The rat spiral ganglia neurons were also Nrf2-IR, their nuclei were non-immunoreactive. (c) Nrf2-IR in the rat cerebellum. Purkinje cells and cells in the molecular layer (m) were Nrf2-IR. (d) Nrf2 Negative control. The antibody was absorbed with the corresponding antigen. No immunoreactivity was detected. Bar = 100 μm (a, b, c), (d) =200 μm.

Table 1

Temporal bones used in this study.

Specimen	Age/Gender	PMT	Localization	Intensity	Hearing problem
1/R	44/M	16	N, C	3	Amikacin ototoxicity
2/L	46/M	8	N, C	3	Bilateral HL
3/L	52/F	8	C	1	Chronic otitis media
4/L	52/M	10	C	3	BMD
5/L	60/f	19	C	3	Mild hearing loss
6/R	62/M	9	C	3	Mild hearing loss
7/R	67/M	17	C	2	Gradual hearing loss
8/R	67/F	8	C	3	Bilateral otosclerosis
9/L	69/F	14	C	3	COM
10/R	73/M	5	N, C	3	Bilateral HL, tinnitus
11/L	74/F	12	C	3	SNHL
12/R	75/M	4	N, C	3	SNHL, nerve deafness
13/L	76/F	7	N, C	3	COM/cochlea sac deg
14/L	79/M	6	N, C	3	BMD
15/L	80/m	10	C	3	COM
16/R	81/F	12	C	1	Bilateral SNHL, BPPV
17/R	83/F	11	N, C	3	Bilateral HL
18/R	84/F	20	C	2	BMD
19/R	84/M	12	C	2	Bilateral deafness
20/R *	92/F	14	N, C	3	BMD
21/L *	92/F	14	C	3	BMD
22/R	18/M	13	N	3	Normal
23/L	23/M	6	N	3	Normal
24/R	50/m	10	C	3	Normal
25/L	55/F	16	N, C	3	Normal
26/R	59/F	20	C	1	Normal
27/R *	67/F	12	N, C	3	Normal
28/L *	67/F	10	N, C	3	Normal
29/R	71/F	8	N, C	3	Normal
30/L	72/F	13	C	3	Normal
31/R	76/F	12	N	3	Normal
32/R *	84/F	6	C	3	Normal
33/L *	84/F	6	C	3	Normal

Abbreviations. R: right side, L: Left side; F: female; M: male. COM: chronic otitis media, HL: hearing loss, BMD: bilateral Meniere's disease. PMT: post mortem time. N: nuclear immunoreactivity, C: cytoplasmic immunoreactivity, sac: saccule, deg: degeneration. Age in years. Nrf2-IR level: 1. Low-IR, 2. Mild-IR, (3) Strong -IR.

* Indicates that R and L temporal bones are from the same patient.

Table 2

Statistical analysis of Nrf2-IR area measurements.

Group	p value	% fold change
Normal vs Disease	0.268	8*
Young age vs Old-age	0.037	20*

A p-value ≤ 0.05 represents statistically significant difference between Nrf2-IR areas, a p-value ≥ 0.05 represents no statistically significant difference.

*% of decrease.