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Neuroglobin immunoreactivity in the human cochlea

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Abstract

Neuroglobin (Ngb) is an oxygen-binding protein with a demonstrated role in endogenous neuroprotective mechanisms. It has been shown to function as a scavenger for reactive oxidizing species thereby assisting in cellular defense against oxidative stress. In the present study, we characterized the presence of Ngb in the human cochlea. Immunohistochemical staining was performed on formalin fixed celloidin human cochlea sections obtained from human temporal bones, using affinity purified polyclonal antibodies against Ngb. Thirty-six temporal bones were analyzed, 15 with normal otologic histories and 21 diagnosed with different inner ear pathologies. Ngb immunoreactivity (Ngb-IR) was consistently expressed in the neurons of spiral ganglia (SG) and supporting cells of the organ of Corti. There was a significant decrease of Ngb-IR in SGNs from specimens with inner ear pathologies when compared to normal specimens. In contrast, Ngb-IR in the organ of Corti did not show significant changes between pathological and normal specimens. The differential pattern of Ngb expression in these cochlear structures suggests that Ngb may participate in defense mechanisms in inner ear pathologies where oxidative stress is involved.

Keywords

neuroglobin; human temporal bone; oxidative stress; inner ear; spiral ganglia neurons

1. Introduction

The generation of reactive oxygen species (ROS) by oxidative stress plays a central role in the development of inner ear disease (Calabrese et al., 2010; Schacht et al., 2012). Oxidative

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stress in a physiological setting occurs when there is an imbalance between production and destruction of ROS, resulting in excessive bioavailability of ROS (Lopez et al., 2009). Sensory hair cells of the cochlea are very susceptible to oxidative stress due to excessive production of ROS (Kidd and Bao, 2011) in diseases like Meniere's disease, age-related hearing loss (presbycusis), noise induced hearing loss, and iatrogenic ototoxic damage. Antioxidant defense mechanisms present in the inner ear may prevent further deterioration and exogenous modulation of this defense may be a potential target for therapeutic influence (Kopke et al., 2002).

The modulation of endogenous protective mechanisms is a promising strategy for the development of novel treatments against neurodegenerative and otologic disorders (Abi-Hachem et al., 2010; Shibata and Raphael, 2010; Yu et al., 2013). Neuroglobin (Ngb) is a tissue globin mainly expressed in the central and peripheral nervous systems of mammals with a potential role in regulating oxidative stress pathways (Burmester et al., 2000; Greenberg et al., 2008, Reuss et al., 2015). In the mouse cerebral cortex, Ngb expression is upregulated by neuronal hypoxia *in vitro* and by focal cerebral ischemia *in vivo* (Sun et al., 2001). Neuronal function after hypoxia is worsened when Ngb expression is inhibited with an antisense olygodeoxynucleotide. Conversely, neuronal survival is enhanced by Ngb overexpression (Sun et al., 2001). Ngb is highly expressed in the retina (100-fold higher than in the brain) (Schmidt et al, 2003). The effect of Ngb overexpression in the retina was recently examined (Chan et al., 2015) *in vivo* using a Ngb-transgenic mouse model. The authors found that Ngb overexpression *in vivo* plays a neuroprotective role in retinal ischemia by preventing mitochondrial oxidative stress leading to decreased activated caspase-3 and apoptosis. Altogether these findings suggest that Ngb may be a potential therapeutic option to mitigate neuronal damage from pathologies that produce oxidative stress (Fetoni et al., 2009).

Neuroglobin immunoreactivity and mRNA expression was described in the rat cochlea (Lopez et al., 2010; Reuss et al., 2015) and cerebella (Beltran-Parrazal et al., 2010). In the rat cochlea Ngb, was localized in the spiral ganglia neurons (SGNs), supporting cells of the organ of Corti, and fibrocytes of the spiral ligament (Lopez et al., 2010). Ngb expression (mRNA and protein) was decreased in the rat SGNs after mild carbon monoxide exposure, suggesting an association between Ngb and oxidative stress (Lopez et al., 2010). Using immunohistochemistry, *in situ* hybridization and quantitative real time PCR, Reuss et al., (2015) recently described the expression of Ngb in the rat and mouse peripheral (cochlea) and central auditory system, and in the human superior olivary complex.

To the best of our knowledge, Ngb expression has not been described in the human cochlea. The purpose of this study was to investigate the distribution of Ngb in the normal human cochlea and changes in Ngb immunoreactivity (Ngb-IR) in the pathological cochlea using immunohistochemistry on archival temporal bones.

2. Results

2.1 Ngb-IR in the normal human cochlea

Ngb-IR was visualized by indirect immunohistochemistry using horseradish peroxidase and the chromogen diaminobenzidine. We used tissue sections from 15 patients with normal hearing (Table 1), and 21 with hearing loss caused by different conditions (Table 2). Figure 1a and 1b shows Ngb-IR in the organ of Corti and SGNs (middle region of the cochlea) from a 50-year-old male (normal hearing). Ngb-IR was seen in the cytoplasm of Deiter's and inner and outer pillar cells, but cell nuclei were not reactive (Fig 1a). The Hensen, Claudius, and inner and outer hair cells were not immunoreactive to Ngb. The stria vascularis and spiral ligament showed no Ngb-IR (not shown). Ngb-IR was seen in the cytoplasm of most of the SGNs, but not in their nuclei (Fig 1b). Large and small sized neurons of the SGN were immunoreactive to Ngb. Satellite cells that surround SGNs were not immunoreactive to Ngb antibodies. This pattern of immunoreactivity in the organ Corti and SGNs was similar at the apical middle and basal portion of the cochlea. Figure 1a′ and b′ shows a consecutive section of the organ of Corti and SGNs (from the same specimen) stained with hematoxylin and eosin to illustrate the different cells present in the human cochlea. The Ngb-IR pattern in the normal specimens (Table 1) was consistent among the different specimens. A low level background was detected in all the specimen's immunoreacted, including the negative controls.

2.2 Ngb-IR in the pathological human cochlea

Fig. 2 shows Ngb-IR in the cochlea of 4 individuals diagnosed with different inner ear pathologies (Table 2). Figs. 2 a-a′, were taken from the organ of Corti and SGNs at the middle region of the cochlea (48-year-old female, diagnosed with mild hearing loss, 5 hours post mortem). Ngb-IR distribution in the Deiter's and pillar cells of the organ of Corti and in the SGNs was similar that of the normal cochlea. Mild Ngb-IR was also seen in the remaining supporting cells. Figs. 2b-b′, were taken from the organ of Corti and SGNs at the basal region of the cochlea (44-year-old male diagnosed with bilateral hearing loss, 16 hours post-mortem). Ngb-IR is present in the remaining supporting cells and the SGNs. Fig 2c-c′ were taken from the organ of Corti and SGNs at the middle region of the cochlea (79-year-old male, diagnosed with Meniere's disease, 10 hours post-mortem). There is an almost complete loss of Ngb-IR in the organ of Corti supporting cells when compared to the Ngb-IR in the normal cochlea (Fig 1a). Ngb-IR also appears to be diminished in the SGNs. Fig 2d-d′ were taken from the organ of Corti and SGNs at the basal portion of the cochlea (76-year-old female, histologically diagnosed with cochlea-sacular degeneration, 9 hours post-mortem). This patient shows an atrophic organ of Corti, the few remaining epithelial cells appear to be immunoreactive. There is also a pronounced loss of SGNs, however, the remaining SGNs were all immunoreactive. The pattern of Ngb-IR in the organ of Corti and SGNs was similar at the apical, middle and basal portions of the cochlea in all the specimens examined.

Ngb-IR stained area in the SGNs located at the apical, middle, or basal turns of the normal and pathological cochlea was not statistically significantly different ($P>0.05$). However, pathological specimens showed statistically significantly lower Ngb-IR in the SGNs

(P<0.05) when compared with the normal specimens. Ngb-IR in the organ of Corti, was not statistically significantly different in the apex, middle or basal turns of the cochlea (P>0.05). There were not statistically significant differences in Ngb-IR in the organ of Corti of normal and pathologic specimens (P>0.05). Because of the small number of specimens, no comparisons of Ngb-IR were made between age, and gender.

2.3 Ngb-IR in positive and controls

Figure 3a shows human SGNs incubated with no Ngb antibody and with the Ngb antibody pre-absorbed with Ngb protein (Fig 3b) (described in methods section). No Ngb specific immunoreaction was detected in either case. Figure 3c shows Ngb-IR in the SGNs and organ of Corti of the rat where SGNs and supporting cells in the organ of Corti were immunoreactive to Ngb antibodies. A similar Ngb-IR localization in the rat cochlea was previously reported by Lopez et al., (2010), and Reuss et al., (2015).

3. Discussion

Ngb-IR was detected in the organ of Corti supporting cells (Deiter's, Claudius, Hensen, inner border and inner sulcus cells) and SGNs of the human cochlea. There was a significant decrease of Ngb-IR in the SGNs in the pathological specimens when compared to the normal.

Ngb-IR and mRNA expression in the mammalian inner ear was recently investigated in the rat (Lopez et al., 2010; Reuss et al., 2015) and mouse cochlea (Reuss et al., 2015). In the rat exposed to mild carbon monoxide (Lopez et al., 2010), Ngb expression was reduced in the rat SGNs, a consistent finding at different perinatal time points. After carbon monoxide exposure in the prenatal period, Ngb expression was significantly decreased in the spiral ligament and SGN but essentially unchanged in the organ of Corti, suggesting that Ngb may play a role in SGNs oxidative stress responses. Reuss et al., (2015) demonstrated the expression of Ngb in the rat and mouse cochlea using immunohistochemistry, *in situ* hybridization and quantitative PCR.

Ngb-IR in the rat and mouse cochlea was, to some extent, similar to the human cochlea. Ngb-IR was consistently found in the SGNs, throughout the cochlea in the rat, mouse and human cochlea. Ngb-IR was also found in the organ of Corti, the spiral ligament (Lopez et al 2010), and the stria vascularis (Reuss, 2015), but not in the lateral wall of the human cochlea. These findings suggest that some species differences may occur, however, different tissue processing i.e. embedding media, and reagents (primary antibodies source) may contribute to the different immunoreactive patterns. Altogether, these results suggest that Ngb may have an important role in inner ear homeostasis.

Ngb has been the focus of avid research efforts for its neuroprotective property that may hold the key to improving outcomes or possibly curing neurological disorders, including stroke and Alzheimer's disease (Yu et al., 2013). Ngb overexpression in stroke animal models decreased the severity of functional and histological deficits (Khan et al., 2006; Sun et al., 2003; Wang et al., 2008). Studies in the Ngb knockout mouse model show that genetic elimination of Ngb does not affect core clock function (Hundal et al., 2012), and has a minor

effect on light–induced retinal gene expression response (Ilmajarv et al., 2014). A recent report on the expression of Ngb in hippocampal sections obtained at autopsy from patients with Alzheimer's disease shows that Ngb levels were increased in early and moderately advanced Alzheimer's disease compared to controls, but declined to control levels when disease progressed to advanced stages, suggesting that Ngb levels may influence the progression of neurodegeneration (Sun et al., 2013). This neuroprotective function may stem from its intimate association with mitochondrial function in ATP production, programmed cell death and reactive oxygen species scavenging. The Ngb role in these processes implicates its importance in cellular defense against oxidative stress (Antao et al., 2010; Brunori et al., 2005; Yu et al., 2013). Studies *in vitro* have shown that human Ngb changed structurally during oxidative stress and functions as an oxidative stress-responsive sensor for neuroprotection (Watanabe et al., 2012).

It is well known that different inner ear cell populations have cell-specific sensitivity to oxidative stress. The outer and inner hair cells are more vulnerable to oxidative stress damage due to the exposure of ototoxic drugs, while the supporting cells are the least susceptible (Wan et al., 2013). This increased tolerance to oxidative stress may be related to Ngb's presence in the supporting cells. However, our study, showed no statistically significant changes in Ngb-IR in supporting cells of pathological cochlea when compared to the normal group (no hearing loss). The lower Ngb-IR in temporal bones with inner ear pathology may be due to loss of sensory and non sensory epithelial cells in the organ of Corti, and loss of neurons in the spiral ganglia, an inherent result of the inner ear pathology (acute or chronic condition), and/or the advanced age of the individual (Kidd and Bao, 2012). However, the small number of individuals at different ages did not allowed us to make comparisons of Ngb-IR in normal aged individuals. We examined the Ngb-IR distribution in the SGNs located in the basal, middle and apical regions and found no difference in Ngb expression. This distribution was similar to the one found in the rat cochlea (Lopez et al., 2010). This is in contrast with the differential expression of the enzyme superoxide dismutase II (SOD-2), which is highly expressed in the apical and middle region of the human cochlea, and less abundant in the basal region (Ying and Balaban, 2009).

In our study Ngb-IR, was present in most of the SGNs, however, we could not determined whether small and large sized neurons were all immunoreactive. In the mouse and rat cochlea Ngb-IR was not detected in type II SGNs (Reuss et al., 2015). Colocalization studies in the human cochlea using Ngb and peripherin, a specific marker that stains small sized neurons (Liu et al., 2010), could help to determine whether all type II (small sized neurons) SGNs have Ngb-IR.

There are several inherent limitations in using temporal bones for immunohistochemical studies. Temporal bones are collected postmortem after patients expire at different stages of their inner ear pathology (Table 1 and 2). Some patients may have far advanced inner ear disease while some 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. These treatments likely affect the antigens present in the tissue section. The integrity of sensory and supporting cells in the organ of Corti is compromised by prolonged postmortem time

before temporal bone harvesting; however, the SGN morphology is consistently well preserved. In addition, the sample size is small, limiting our ability to comparatively make claims between different types of patients.

There are, in contrast, several advantages on the use of celloidin embedded sections for immunoreactivity. Among them, the availability of the clinical history for each temporal bone collected. In addition, for each temporal bone, one in every tenth section is always stained with hematoxylin and eosin, which allows the histological evaluation and identification of the inner ear pathology, leaving the rest of the sections available for analysis such as immunohistochemistry and proteomic analysis.

3.1. Conclusions

Ngb is an oxidative stress marker with neuroprotective properties and has been detected in the human cochlea with consistent expression in supporting cells of the organ of Corti and neurons of the spiral ganglia. Ngb-IR pattern in the human cochlea resembles those found in the mouse and rat cochlea. There was a significant decrease of Ngb in pathological cochleas when compared to the normal controls. The differences in Ngb-IR between the normal and pathological cochlea may suggest a protective role in the organ of Corti and SGNs from oxidative stress pathways.

4. Experimental Procedures

4.1. Human specimens

The University of California, Los Angeles Institutional Review Board (IRB) approved the use of archival human temporal bones in this study (Protocol # 14-001753). The temporal bones used in this study were part of a National Institute of Health-funded Human Temporal Bone Consortium for Research Resource Enhancement through the National Institute on Deafness and Other Communication Disorders. Before death, donors provided informed consent for inclusion of their temporal bone into such studies. The medical history for each of the patients who donated their temporal bones was maintained and preserved in a secured electronic database. Celloidin embedded sections from archival temporal bones from 36 patients (18 male and 18 female) were used for Ngb immunohistochemistry, there were 15 specimens with normal otologic histories (Table 1) with post-mortem time ranging from 8–19 hours (12 hours average) and 21 specimens diagnosed with different inner ear pathologies (Table 2) with postmortem time ranging from 4 to 18 hours (11 hours average). The quality of the cochlear tissue sections to be immunostained was determined by the examination of adjacent sections stained with hematoxylin and eosin.

4.2. Immunohistochemistry

Celloidin was removed from the temporal bone sections and then subjected to the antigen retrieval technique (Ahmed et al., 2013; Balaker et al., 2013). In brief, celloidin embedded sections containing the cochlea were mounted on Super frost plus slides (Fisher Scientific) and air dried, then slides were immersed in acetone for 30 minutes followed by sodium ethoxide (1:3 in ethanol) for 30 minutes. Tissue sections were rehydrated and covered with phosphate buffered saline (PBS, 0.1M pH 7.2), and then immersed in an antigen retrieval

solution (Vector) and microwaved 2 times for 2 minutes each time. The protocol for immunohistochemistry was described previously (Balaker et al. 2013). Tissue sections were incubated for 3 hours with a blocking solution (1% bovine serum albumin fraction V, in PBS plus 0.5% Triton x100) and subsequently for 48 hours at 4°C with the Ngb chicken polyclonal antibodies directed against recombinant human Ngb (1:1000 in PBS). This antibody recognized human, dog and rat Ngb (Biovendor Laboratory Medicine Inc; Cat # RD-1345).

Immunohistochemical controls: To provide negative controls, the primary antibody was omitted from the immunoreaction or pre-absorbed with the Ngb protein (human Ngb, recombinant protein cat # RD-952). Pre-absorption of the antibody was made by mixing the Ngb antibody with the recombinant Ngb protein (1μg/1μ each). The mixture was placed at 37°C for 1 hour, and the immunoreaction was performed on human cochlear tissue as described above using this mixture. No immunoreactivity was detected in any of the negative controls (Fig. 3a and 3b respectively). For positive controls, rat and mouse cochlear sections were immunoreacted with the same Ngb antibodies as previously described (Lopez et al., 2010). In the normal rat cochlea, Ngb-IR was present in the SGNs, and supporting cells of the Organ of Corti (Fig 3c).

4.3. Microscopic observation and documentation

The immunoreacted cochlear sections were viewed and imaged with an Olympus BX51 fluorescent microscope (Olympus America Inc, NY, USA) equipped with an Olympus DP70 digital camera. To provide unbiased comparisons of the immunoreactive signal between each specimen, all images were captured using the same camera settings. Images were acquired using MicroSuite™ Five software (Olympus America Inc).

4.4. Quantification of Ngb immunoreactive area

Quantitative immunohistochemical analysis was made as described by Rangan and Tesch (2007), and adapted to immunostained human cochlear sections by Balaker et al. (2013), using ImageJ software (http://imagej.nih.gov/ij/download.html version 1.50b). 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. To determine possible regional variations in the immunoreactive area, quantification was made from each section at the basal, middle and apical region of the cochlea. The immunoreacted area was measured in the region of interest (ROI) and includes the organ of Corti, and the SGNs with their surrounding neuropil. In brief: The digital image was open with the *ImageJ* program and converted to grayscale (*Image/Type/8 bit*). The threshold for immunoreactivity detection was set by selecting (*Image/Adjust/Threshold*), and the threshold level was adjuste to be the same for all images. Background immunoreactivity was measured in a small region located away from the specific immunoreactive signal, and was subtracted from the Ngb-IR values of the immunostained cells. The image was then converted to binary (black and white). The immunoreacted area to be quantified was selected using the “rectangular” tool. To determine the area stained within the ROI, *Analyze/Analyze particles* was selected in the tool bar, and the “masks” tool was selected from the menu. A summary of results appeared showing the area fraction, which is the proportion of the ROI that was immunoreactive.

4.5. Statistical Analysis

For each specimen (Table 1 and 2), mean values of the immunoreactive area were averaged and subjected to one way repeated measures analysis of variance (ANOVA). Comparisons were made between the three cochlear regions: basal versus apical, basal versus middle, and middle versus apical, and between the normal (Table 1) and inner ear disease group (Table 2). Data were entered into a Microsoft Excel Worksheet (Excel 2011; Microsoft Corp, USA) and then examined for statistical analysis using Sigma Stat 3.1 software program (sigma Stat, Ashburn, VA). Values were considered statistically significant at a p-value of 0.05.

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Highlights

- Ngb-IR was detected in the human organ of Corti, and spiral ganglion neurons (SGNs)
- SGNs Ngb-IR was significantly reduced in cochleas with otologic pathologies
- Neuroglobin (Ngb) may protect the inner ear from oxidative stress
- Ngb-IR localization in the human and rodent cochlea was similar

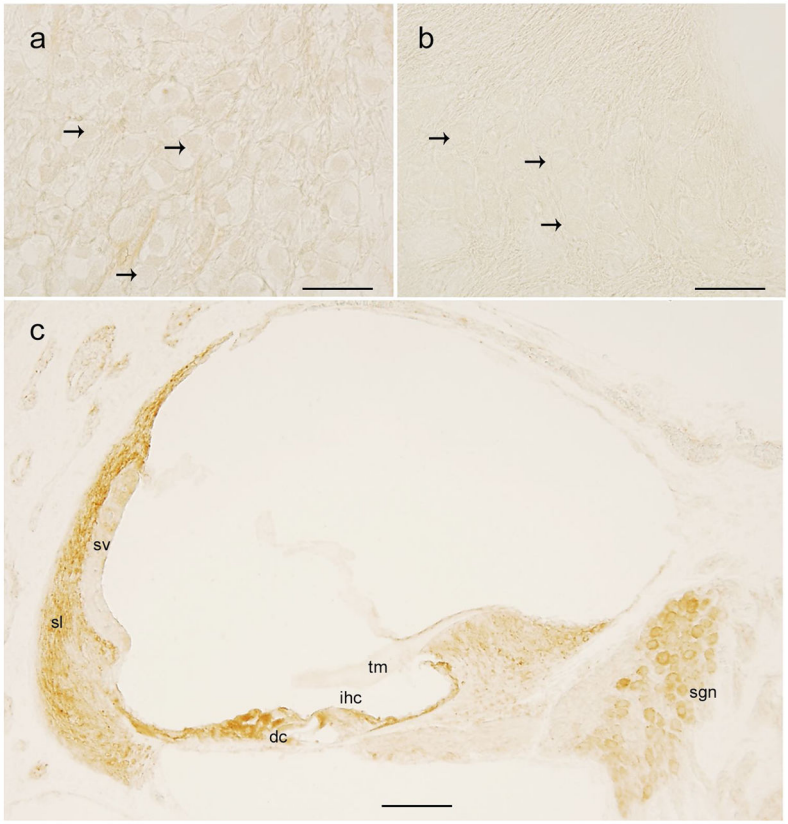

Fig 1.

Ngb-IR in the normal human cochlea. (a) Ngb-IR in the organ of Corti. Immunoreactivity was present mainly in Deiter's cells (dc) and pillar cells (pc). (a′) Alternate section stained with hematoxylin and eosin, to illustrate the cells present in the human organ of Corti. (b) Ngb-IR in the SGNs. Ngb-IR was located in the cytoplasm of the neurons of the spiral ganglia. (b′) Alternate section stained with hematoxylin and eosin to illustrate the morphology of SGN's. All images were taken from a 50-year-old (male) cochlea (middle region) with normal hearing. Abbreviations: cc: Claudius cells, hc: Hensen cells; ohc: outer hair cells; ihc: inner hair cells, bm: basement membrane; tc: tunnel of Corti; tm: tectorial membrane; sgn: spiral ganglia neurons. Bar in (a) and (a′) = 50 μm, in (b) and (b′) = 100 μm.

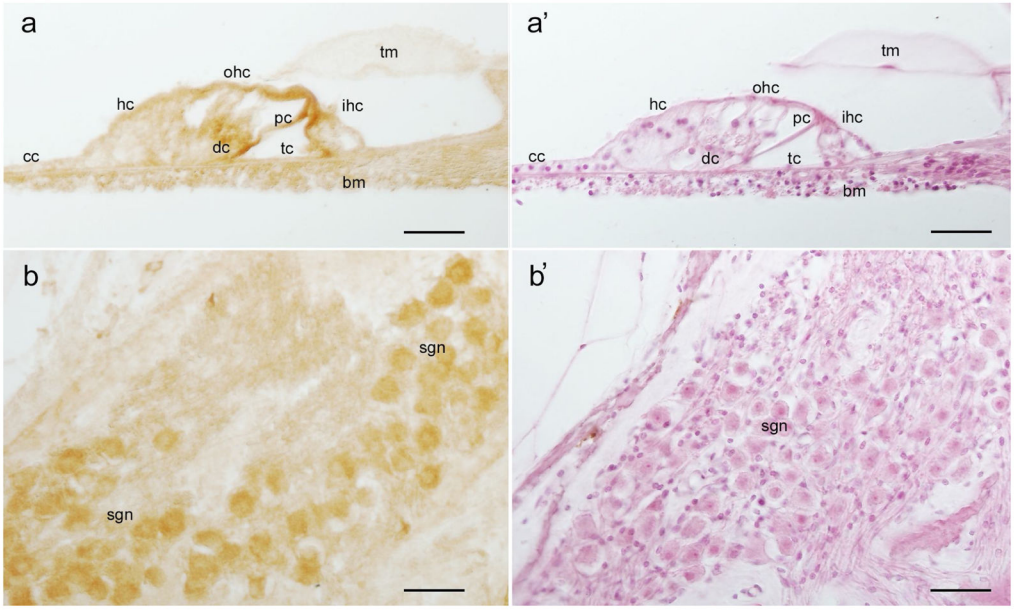

Fig 2.

Ngb-IR in the pathological human cochlea. (a-a′). Ngb-IR in the organ of Corti and SGNs from a 42-year-old female, diagnosed with mild hearing loss. (b-b′) Ngb-IR in the organ of Corti and SGNs (arrowheads) from a 44-year-old male, diagnosed with bilateral hearing loss. (c-c′) Ngb-IR in the organ of Corti and SGNs from a 79-year-old male, diagnosed with Meniere's disease. (d-d′) Ngb-IR in the organ of Corti and SGNs from a 76-year-old male, diagnosed with Meniere's disease. Abbreviations: cc: Claudius cells. hc: Hensen cells, dc: Deiter's cells, tc: tunnel of Corti, bm: basilar membrane. Bar in (a) to (d) = 50 μm, in (a′) to (d′) = 65 μm.

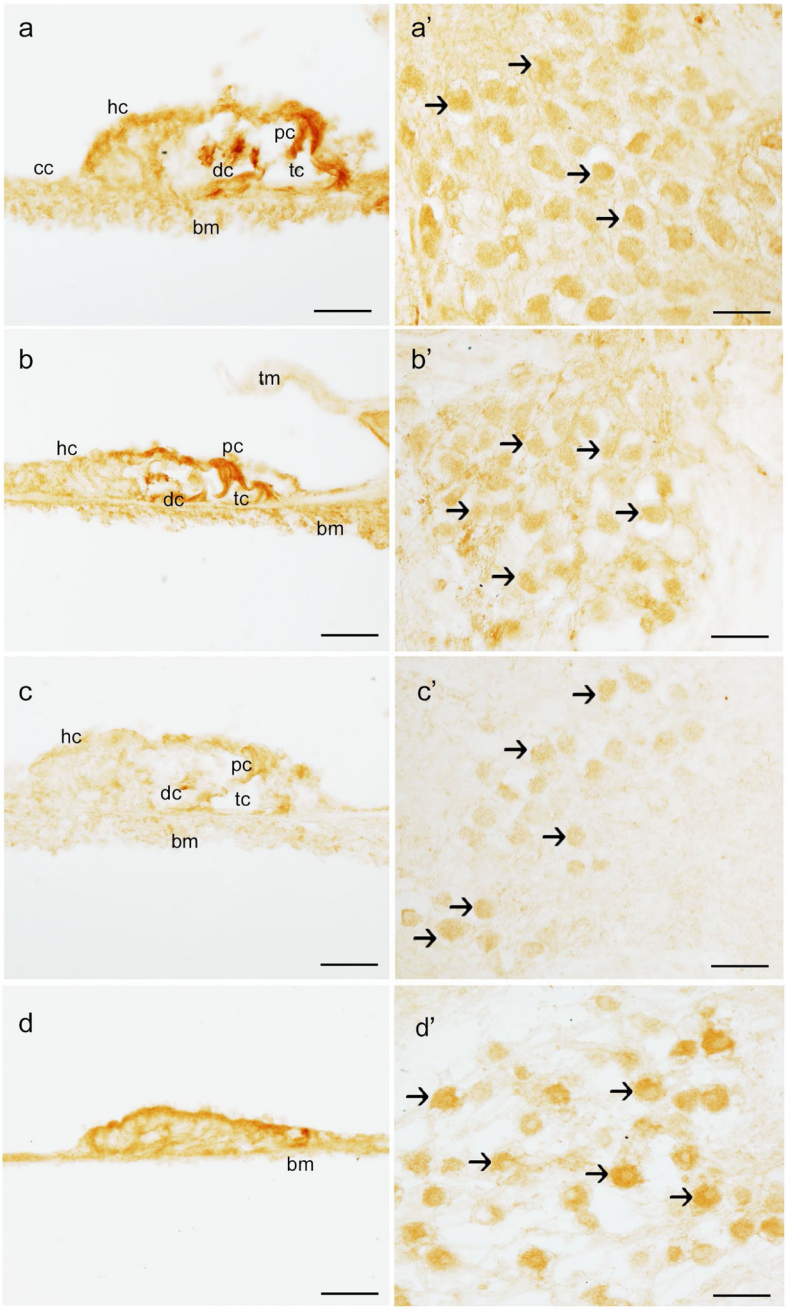

Fig 3.

Ngb-IR controls. (a) Negative control: In this human cochlea section, the Ngb antibody was omitted during the immunoreaction. No specific immunoreactivity was detected in the SGNs. Background unspecific staining was observed. (b) Absorption control: This human cochlear section was incubated with the Ngb antibody pre-absorbed with Ngb-protein. No positive reaction was detected in the SGNs (arrows) and surrounding tissue (neurophil). (c) Positive control: In this rat cochlear section, Ngb-IR was present in the spiral ligament,

organ of Corti (OC) and SGNs. sl: spiral ligament, SV: stria vascularis, SpL: spiral limbus. Bar in (a) and (b) = 75 μm, (c) = 50 μm.

Table 1

Temporal bones used in this study (normal hearing).

TB side	Age	Gender	PMT	Type of hearing reported
1L	8.5	F	10	Normal
2L	10	M	18	Normal
3R	18	M	9	Normal
4L	32	M	12	Normal
5R	50	M	9	Normal
6L	52	F	11	Normal
7L	55	M	10	Normal
8L	55	M	12	Normal
9L	55	F	14	Normal
10R	59	F	16	Normal
11R	67	M	17	Normal
12R	67	F	10	Normal
13L	67	F	8	Normal
14R	71	F	13	Normal
15R/L	72	F	18	Normal

Table abbreviations.

TB: temporal bone; R: right side; L: left side; Age: in years, M: male; F: female; PMT: Post mortem time in hours. Normal: indicate normal hearing and balance.

Table 2

Temporal bones used in this study (inner ear disease).

TB side	Age	Gender	PMT	Type of hearing loss (HL) reported
1L	10	M	6	Moderate HL
2L	10	F	4	Pronounced HL (OM)
3L	42	F	5	Moderated HL (vertigo)
4R	44	M	16	Bilateral HL
5L	46	M	12	Bilateral HL
6L	52	M	17	HL (Meniere's disease)
7L	62	M	12	Decreased hearing
8L	63	F	18	Moderated HL (OM)
9L	64	M	14	Moderated HL
10L	65	M	12	HL (inner ear degeneration)
11L	69	F	9	HL (Meniere's disease, otosclerosis)
12R	73	M	5	Bilateral HL (Tinnitus)
13L	74	F	12	Severe HL
14R	75	M	4	Nerve deafness
15L	76	F	9	Cochlea and saccule degeneration
16R	79	M	10	HL (Meniere's disease)
17R	80	F	10	HL (Kanamycin ototoxicity)
18R	81	F	9	Bilateral HL (vertigo)
19L	82	F	12	Bilateral Sensory neural HL
20R	85	M	18	Decreased hearing
21R	95	F	10	Pronounced HL (Meniere's disease)

Table abbreviations.

TB: temporal bone; R: right side; L: left side; Age: in years, M: male; F: female; PMT: Post mortem time in hours. Normal: indicate normal hearing and balance. OM: otitis media.