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Immunohistochemical localization of glucocorticoid receptors in the human cochlea

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Abstract

In the present study we investigated the localization of glucocorticoid receptors (GCR) in the human inner ear using immunohistochemistry. Celloidin-embedded cochlear sections of patients with normal hearing (n = 5), patients diagnosed with MD (n = 5), and noise induced hearing loss (n = 5) were immunostained using GCR rabbit affinity-purified polyclonal antibodies and secondary fluorescent or HRP labeled antibodies. Digital fluorescent images were acquired using a light sheet laser confocal microscope. In celloidin-embedded sections GCR-IF was present in the cell nuclei of hair cells and supporting cells of the organ of Corti. GCR-IF was detected in cell nuclei of the Reisner's membrane. GCR-IF was seen in cell nuclei of the stria vascularis and the spiral ligament. GCR-IF was found in the spiral ganglia cell nuclei, however, spiral ganglia neurons showed no GCR-IF. Although GCRs were found in most cell nuclei of the cochlea, the intensity of IF was differential among the different cell types being more intense in supporting cells than in sensory hair cells. The differential expression of GCR receptors found in the human cochlea may help to understand the site of action of glucocorticoids in different ear diseases.

Keywords

Human inner ear; Glucocorticoid receptors; Celloidin-embedded sections; Cochlea

1. Introduction

The glucocorticoid receptor (GCR) is a nuclear receptor that functions as a ligand-activated transcription factor mediating the diverse physiologic effects of glucocorticoids. Glucocorticoid receptors are classic steroid hormone receptors that belong to the nuclear receptor subfamily 3 (NR3). The mineralocorticoid receptor, androgen receptor,

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Declaration of Competing Interest

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progesterone receptor and estrogen receptor also belong to this family (Grossman et al., 2021).

Glucocorticoids (a type of corticosteroid) bind to GCRs and up-regulate the expression of anti-inflammatory proteins in the nuclei and repress the expression of pro-inflammatory proteins in the cytoplasm (Goppelt-Struebe et al., 1989; Jun et al., 1999; Horowitz et al., 2020; Yeager et al., 2018). Glucocorticoids interact with mineralocorticoid receptors (MCRs) (Nethathe et al., 2020).

Corticosteroids in the form of dexamethasone and prednisone are administered into the middle ear via the tympanic membrane or systemically to treat Meniere's disease, idiopathic sudden hearing loss, tinnitus, and autoimmune inner ear disease (Lavigne et al., 2016; Molnar et al., 2021; Sakano and Harris, 2019; Tong et al., 2021, Trune and Canlon, 2012, Trune et al., 2019). Corticosteroids are administered to prevent inflammation due to cochlear implantation and acoustic trauma (Gotamco et al., 2020; Shaul et al., 2019; Skarzynska et al., 2018; Trune et al., 2006). Corticosteroids are also used to prevent hearing loss when ototoxic chemotherapeutics are administered (Marshak et al., 2014).

The protective effect of GCRs activation in the inner ear has been investigated in mouse and guinea pigs (Kil and Kalinec, 2013; Heinrich et al., 2016; Tahera et al., 2006). In mouse restraint stress models, the spiral ganglion neurons (SGN) are protected from acoustic trauma by increasing corticosterone and activating GCRs (Tahera et al., 2006). Intratympanic injection of dexamethasone in guinea pigs increases the expression of GCRs in the spiral ligament, (Heinrich et al., 2016). Dexamethasone administration differentially modulates cochlear GCRs mRNA expression and cytokine expression in a mouse model of noise-induced hearing loss (Lee et al., 2019). In the guinea pig cochlea, GCRs are expressed in the cytoplasm and cell nuclei of inner and outer hair cells, as well as supporting cells in the organ of Corti, SGN, and spiral ligament cells (Kil and Kalinec, 2013). Trune et al., (2019) demonstrate that intratympanic delivery of steroids affects more inner ear genes than systemic delivery.

There are just a few reports about the localization of GCRs in the human inner ear (Rarey and Curtis, 1996). In an enzyme-linked immunosorbent assay on the human cochlea, the highest concentration of GCRs protein was detected in the spiral ligament tissues and the lowest concentration of GCRs was detected in the macula of the saccule (Rarey and Curtis, 1996). GCRs immunofluorescence in paraffin embedded sections of the human inner ear obtained from autopsy was found in the cytoplasm of vestibular hair cells, but not in their nuclei. (Kumagami et al., 2013).

In this study, we investigated the localization GCRs by immunofluorescence and immunohistochemistry in celloidin-embedded human inner ear tissue sections obtained from normal and pathological human temporal bones. GCRs were differentially expressed in the normal and pathological human cochlea.

2. Methods

2.1. Human temporal bones

University of California, Los Angeles Institutional Review Board (IRB) approved the use of archival human temporal bones (Protocol # 10–001499 and # 22–001587). The temporal bones used in this study were part of a National Institute of Health-funded Human Temporal Bone Network for Research Resource Enhancement through the National Institute on Deafness and Other Communication Disorders. We used celloidin-embedded sections from 15 temporal bones. Table 1 shows the demographics of specimens used in this study: age, gender, diagnosis, and duration of disease. Meniere's disease was active at the time of death. Post-mortem time for the temporal bone collection was between 12 and 18 h.

2.2. Immunofluorescence in celloidin embedded inner ear sections

Celloidin removal and antigen retrieval: The methodology for celloidin removal and antigen retrieval has been described in detail (Ahmed et al., 2013; Balaker et al., 2013; Lopez et al., 2016). In brief, celloidin sections were immersed in sodium-ethoxide (saturated solution) diluted in 100% ethyl alcohol (1:3, 60 min), 100% ethanol (2 times, 5 min), and distilled water (3x10 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 cool for 30 min, washed with phosphate-buffered saline (3x 5 min, PBS) and immediately incubated for 8 min in a diluted trypsin solution (1:3, Abcam Trypsin Kit) and washed 4x10 minutes in PBS before immunofluorescence.

Immunofluorescence (IF): Sections were incubated for 2 h with a blocking solution containing 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 the antibodies against GCR (1:500) diluted in PBS for 72 h at 4 °C in a humid chamber. GCR rabbit polyclonal antibody (IgG) is affinity purified, raised against a peptide mapping of GCRs of human origin (Cat. #PA1-511A, Lot # SE253368, Invitrogen, Carlsbad, CA, USA). These GCRs antibodies have been tested in other tissues (Lien et al., 2008; Lu et al., 2006; Yakirevich et al., 2011). Acetylated tubulin mouse monoclonal antibodies 1:1000 (Cat # T7452, Sigma SLM, USA) were used to identify supporting cells in the organ of Corti, marginal cells in the stria vascularis, and SGN (Liu et al., 2018; Christov et al., 2020). After the 72 h of incubation, primary antibodies were removed by 4 × 15 min PBS washing step. The sections were incubated in goat anti-rabbit antibody labeled with Alexa 488 (1:1000 in PBS, Invitrogen) for 2 h followed by 2 h incubation with goat anti-mouse antibodies labeled with Alex 594 (1:1000 in PBS, Invitrogen). The tissue sections were then washed with PBS (3 × 15 min) and coverslip with aqua soluble mounting media containing DAPI to visualize cell nuclei (Vecta-shield, Vector).

Immunohistochemistry (IHC) staining was used to corroborate the GCR-IF staining. Secondary antibodies against rabbit labelled with HRP (ABC kit, Vector Labs) were used, the antigen–antibody reaction was visualized with diaminobenzidine (ImmPact™ DAB

Chromogen, Vector Labs). The IHC protocol has been described in detail (Lopez et al., 2016).

2.3. Immunohistochemical controls

As a positive control, cryostat sections from mouse cochlea were incubated with antibodies against GCRs, these sections were subjected to the same protocol, mouse cochlea cell nuclei were immunoreactive. As a negative control, the primary antibodies against GCRs were omitted and the immunoreaction was performed in the human cochlea sections as described above, no immunoreaction was observed.

2.4. Microscopic observation and documentation

Digital fluorescent and light microscopic images were obtained using a Leica (SP8) high-resolution light-sheet laser confocal microscope, located in the Advanced Microscopy Laboratory and Spectroscopy of California Nanosystems Institute at UCLA, and a Leica inverted microscope (Thunder system) coupled to a high-resolution light and a fluorescence camera.

2.5. GCR-IF quantification

To minimize bias in the analysis quantification was made double blinded, i.e., the researcher performing the immunostaining and the quantification was only given the specimen number. A second person not blinded to the sample identity coded each sample. GCR-IF levels in the different samples were evaluated using *ImageJ* free software (https://imagej.nih.gov/ij/download.html, version 1.52 s) as previously described (Ahmed et al., 2013; Balaker et al., 2013; Ishiyama et al., 2018). Each digital micrograph was opened using the *ImageJ* software program and converted to gray scale (image/type 8 bit). The threshold for IF detection was set (image/adjust/threshold), and the threshold level was adjusted (same for all images). Background IF was measured in a small area located apart from the GCR-IF and was subtracted from the GCR-IF area values. The image was converted to black and white, and the IF area was selected using the drawing tool. To determine the GCR-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 GCR-IF. GCR-IF area measurements were made GCR-IF in the organ of Corti, stria vascularis, spiral ligament and spiral ganglia at the apical, middle, basal-hook region of the cochleas used in the present study (Table 1). For each specimen IF area was averaged and standard error of the mean (SE) was calculated (See Table 2).

2.5.1. 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). GCR-IF comparisons between the groups at the different regions of the cochlea were made as follows: Normal vs. Meniere's disease, and normal vs. noise induced hearing loss. Results were summarized in Table 2.

3. Results

For anatomical reference a hematoxylin and eosin (H&E) stained section of the cochlea from a 65-year-old male with normal hearing is shown in Fig. 1a. Fig. 1b,1c and 1d shows the organ of Corti, the spiral ganglia and the stria vascularis. Each cell type is easily identified.

3.1. GCR-IF in the normal organ of Corti, spiral ganglia, stria vascularis and spiral ligament of the cochlea.

Double IF labeling of GCRs-IF and acetylated tubulin (AT) IF allows the identification of cell types in the organ of Corti (apical level). Fig. 2a shows GCR-IF in cell nuclei of inner and outer hair cells and supporting cells, GCR-IF was also present in mesenchymal cells underneath the basilar membrane. Fig. 2b shows AT-IF in pilar and Deiters' cells in the organ of Corti. Fig. 2c, shows a merged image form 2a and 2b the different cell types are easily identified. For reference Fig. 2d shows a diagram of different type of cells present in the organ of Corti. GCR-IF intensity was higher in mesenchymal cell nuclei located underneath the basal lamina, followed by supporting cells and inner and outer hair cells nuclei. Fig. 2e, shows GCR-IF in the spiral ganglia, GCR-IF was seen in cell nuclei intermingle with SGN identified by AT-IF. The SGN were devoid of GCR-IF. Fig. 2f, shows GCR-IF in marginal, intermediate, and basal cells of the stria vascularis, AT-IF allowed the identification of marginal cells. GCR-IF was seen in cell nuclei of the spiral ligament. Fig. 3 shows GCR-IF and AT-IF in the middle and basal region of the cochlea (same specimen from Fig. 2). Fig. 3a, 3c and 3e shows GCR-IF and AT-IF in the organ of Corti, spiral ganglia, stria vascularis and spiral ligament respectively at the middle portion of the cochlea, and Fig. 3b, 3d and 3f shows GCR-IF and AT-I at the basal portion of the cochlea. A similar pattern of GCR-IF was seen in the apical, middle, and basal region of the normal cochlea.

3.2. GCR-IF in the cochlea from patients diagnosed with Meniere s disease.

GCR-IF cell nuclei distribution in Meniere's disease cochlea was similar the distribution in the normal cochlea (Fig. 4). Differences in GCR-IF in Meniere's specimens were due to the histopathological changes in the cochlea, rather than changes in GCR-IF. A cochlea section from a 65-years-old male patient affected with Meniere's disease is seen in Fig. 4. Fig 4a and 4b shows the organ of Corti at the mid-apical and mid-basal region of the cochlea. GCR-IF was present in cell nuclei of remaining hair cells, supporting cells and mesenchymal cells underneath the basal lamina. AT-IF allowed the identification of pilar and Deiters' cells. Like in the normal organ of corti, GCR-IF was intense in mesenchymal cell nuclei. In the spiral ganglia, intense GCR-IF cell nuclei were present at the mid-apical, middle, and base of the cochlea. AT-IF allowed the identification of SGNs which showed no-GCR-IF (Fig. 4c, 4d, and 4e). GCR-IF was present in cell nuclei of the marginal, intermediate, and basal cells of the stria vascularis of Meniere's cochlea (Fig. 4f, 4g and 4h). AT-IF allowed the identification of marginal cells. GCR-IF was also present in cell nuclei of the spiral ligament.

Fig. 5 shows GCR-IF in another Meniere's disease cochlea from a 75-year-old male. In the organ of Corti remaining cells showed GCR-IF (5a). GCR-IF and AT-IF were present

cell nuclei of the spiral limbus. Fig. 5b and 5c shows GCR-IF in the marginal (AT-IF), intermediate, and basal cell nuclei, and the spiral ligament cell nuclei. Reisner's membrane (5d, oblique section) cell nuclei were GCR-IF.

GCR-IF was also present in the spiral ganglia (Fig. 5e), however, SGN were non-GCR-IF. GCR-IF was also seen in cell nuclei of blood vessels (Fig. 5f). GCR-IF distribution in this Meniere's cochlea was like the 65-years old specimen diagnosed with Meniere's disease (Fig. 4).

3.3. GCR distribution in the cochlea from patients diagnosed with noise induced hearing loss (NIHL).

Histopathological changes in NIHL patients showed almost complete loss of the hair cells in the organ of corti and differential loss of spiral ganglia neurons. To corroborate the GCR-IF signal, the same section was incubated with secondary goat anti rabbit antibodies labeled with HRP and the immunoreactivity visualized using HRP-DAB. Both IF and IHC give similar GCR immunoreactive pattern.

Fig 6a, shows H&E stained the cochlea of an 80 years-old-male diagnosed with NIHL. There was a pronounced loss of hair cells and supporting cells form the apical to the basal portion. There was also significant loss of SGN at the three levels of the cochlea. Fig. 6b shows GCR immunohistochemistry (IHC) in the adjacent section. The antigen–antibody reaction was visualized using secondary antibodies labeled with HRP and the reaction visualized using HRP-DAB. Fig. 6b1-6b3 shows higher magnification view from Fig. 6b. Fig. 6b1 shows GCR-IR in the spiral ligament, organ of Corti and Reisner's membrane, Fig. 6b2 shows GCR-IR in the stria vascularis Fig. 6b3 shows GCR-IR in the basal portion of the spiral ganglia.

3.4. Quantitative analysis

Comparison of GCR-IF between the normal and Meniere's and normal and NIHL specimens showed that there were statistically significant differences between the different structures (Table 2).

Normal vs. MD: In the organ of Corti there were significant differences in GCR-IF at the apical, middle and the base of the cochlea. In the stria vascularis no significant changes were found in GCR-IF at the apical and middle portion but there were significant differences at the base portion. In the spiral ligament there were significant differences in GCR-IF at the apical, middle, and base. In the spiral ganglia there were not differences in GCR-IF at the apical portion but there were significant differences at the middle and basal portion (Table 2).

Normal vs NIHL: In the organ of Corti there were not significant differences in GCR-IF at the apical, middle, and basal portion. In the stria vascularis there were significant differences in GCR-IF at the middle portion but not at the apical and base. In the spiral ligament there were not significant differences in GCR-IF at the apical and base portion but there were significant differences at the middle portion. In the spiral ganglia there were not significant differences in GCR-IF at the apical middle and the basal portion (Table 2).

4. Discussion

GCRs-IF was ubiquitous localized in cell nuclei of the human cochlea, this IF distribution was almost similar the mouse cochlea models (Shimazaki et al., 2002;Zuo et al., 1995). In the normal cochlea, sensory hair cells nuclei showed less GCR-IF signal, than supporting cells and mesenchymal cells. Cells of the stria vascularis and spiral ligament showed also strong GCR-IF. Spiral ganglia neurons cell nuclei showed no GCR-IF. There was a differential distribution of GCR-IF in the normal, Meniere's disease and NIHL cochlea.

4.1. Organ of Corti

GCRs were previously found to be expressed in the organ of Corti in several animal models (Zuo et al., 1995). GCRs-IF has been previously detected in outer hair cells, inner hair cells, and supporting cells nuclei in the rat cochlea, and supporting cells nuclei in the mouse cochlea (Shimazaki et al., 2002). In guinea pigs, GCRs are localized in hair cell cytoplasm and nuclei, and dexamethasone induces GCRs and MCRs translocation from cytoplasm to nuclei in these cells (Kil and Kalinec, 2013). Direct infusion of dexamethasone into the perilymphatic space has protective effects against noise-induced trauma in the guinea pig outer hair cells (Takemura et al., 2004). Shen et al., (2011) showed inner hair cell protection in old mouse after acoustic trauma after the activation of glucocorticoid signaling pathways. GCRs expression in the human organ of Corti support a functional role depending on different pathologies and treatments. AT-IF allowed to identify pillar cells and Deiter's cells that showed GCR-IF. Additional markers need to be tested together with GCR to identify sensory inner and outer hair cells as well as other supporting cell types and ascertain a specific role of GCR receptors in the human cochlea.

4.2. Reissner's membrane

Reissner's membrane epithelium forms the barrier that produces and sustains the large ionic differences between cochlear endolymph and perilymph. In mice, Reissner's membrane is mediated by apical Epithelial Na^+ channels and/or other amiloride-sensitive channels, basolateral Na^+-K^+-ATPase, and K^+-permeable channels, which is under the control of glucocorticoids (Kim et al., 2009). Our findings of GCR-IF in the Reissner's membrane suggested that GCR receptors may participate in ion transport homeostasis.

4.3. Stria vascularis and spiral ligament

GCRs-IF has been detected in the mouse (Erichsen et al., 1996) and rat stria vascularis (ten Cate et al., 1993). Terakado et al., (2011) showed the translocation of GCRs from the cytoplasm to the nuclei in cultured stria vascularis. In a guinea pig model glucocorticoids decrease TNF-α activity and protect the stria vascularis from atrophy (Waissbluth et al., 2013). In the rat cochlea, the ototoxic effects of cisplatin are prevented when dexamethasone is administered with cisplatin (Capelo et al., 2017). GCRs in cell nuclei of the human stria vascularis suggest an involvement in ion transport to maintain cochlea homeostasis, and that they may have a protective effect on NIHL or other pathologies.

GCRs-IF has been reported in cell nuclei of the spiral ligament of mice and rats (Erichsen et al., 1996; Kil and Kalinec, 2013; ten Cate et al., 1993). Fibrocytes in the spiral

ligament release proinflammatory cytokines, such as interleukin-1beta and interleukin-6 immunoreactivity after in noise-exposed rat cochlea (Fujioka et al., 2006). Spiral ligament fibrocytes seems to be a target cell population of glucocorticoids in the inner ear (Kil and Kalinec, 2013). The expression of GCRs in cell nuclei in the human spiral ligament may be involved in inflammatory response.

Specific characterization of fibrocytes in the human cochlea and colocalization with GCR remains to be characterized to ascertain a specific role of the GCR receptor in this region.

4.4. Spiral ganglia

GCRs-IF as been detected in cell nuclei and cytoplasm in the rat's spiral ganglia (ten Cate et al., 1993). Nuclear and cytoplasmic staining has been observed by immunohistochemistry in the mouse spiral ganglia (Erichsen et al., 1996; Shimazaki et al., 2002). Loss of spiral ganglion cells in older mice is observed in mice with chronically high systemic levels of glucocorticoids (Shen et al., 2011).

In the normal, Meniere's and NIHL cochlea, GCR-IF was present in cell nuclei of the spiral ganglia. Using AT-IF SGNs were identified and showed no GCR-IF in the cell nuclei. SGNs are surrounded by satellite cells and there are also Schwann cells and macrophages (Noonan et al., 2020), it is likely that GCR-IF cells in the SG are among these cellular population. Specific double labeling of this cells and GCR-IF needs to be done to very their identity. GCR expression in these cells suggest that they may have a protective role towards the SGN. The lack of GCR-IF in the human SGNs needs to be further investigated with molecular biological techniques like *in situ* hybridization, it is also necessary to investigate the presence of other steroid receptors (like mineralocorticoid receptor) in the SGN.

In the present study, we also detected GCRs-IF in cell nuclei around the blood vessels of the spiral ganglia. In this respect vascular endothelial cells are the primary targets of oxidative stress, and pericytes play a role in the loss of the blood labyrinthine barrier and triggering of inflammatory pathways thus GCRs in the pericytes and endothelial cells may respond upon oxidative stress (Ishiyama et al., 2018).

4.5. Limitations and advantages

There are several limitations in the interpretation of the cellular localization of GCRs by immunohistochemical techniques using celloidin embedded human inner ear sections. First, tissue integrity is dependent on the agonal and postmortem time before the temporal bones are harvested. Second the processing of embedding celloidin-embedded sections which includes long fixation (temporal bones are immersed in 10% formalin for 2–4 weeks) and decalcification (6–9 months), dehydration until celloidin is immerse (2–3 weeks). These steps undoubtedly affect the stability of antigens. This may explain the absence of GCR-IF in the cytoplasm, the source of antibodies and species differences may also contribute to different results. It is important to mention that GCR-IF were detected in cell nuclei but not in cell cytoplasm. GCR immunoreactivity have been detected using other tissue embedding methods (paraffin embedded tissue, or formalin frozen tissue), however other antigens have not been observed on celloidin-embedded specimens (Lopez et al., 2016). 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 include the use of GCR-IF together with antibodies against myosin VIIa to identify hair cells in the organ of Corti, fibrocytes markers for the spiral ligament, GFAP or SOX-2 to identify supporting cells and satellite, Schwann, and macrophages in the spiral ganglia.

Some advantages to the use of celloidin embedded sections for immunohistochemistry include: First, we can refer to the clinical history of the bone donors and consider the result of immunostaining. Second, only one in every tenth section is stained with hematoxylin & eosin, so we can evaluate, identify, and classify the specimens with the underlying pathology.

5. Conclusion

GCRs were detected in the nuclei of the human cochlea. GCR-IF the distribution of was different in each compartment. The localization of glucocorticoids in the human inner ear remains to be investigated, given the evidence of their presence in animal models (Yao and Rarey, 1996). The differential expression of GCR receptors found in the human cochlea may help to understand the site of action of glucocorticoids administration in different ear diseases.

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Data availability

Data will be made available on request.

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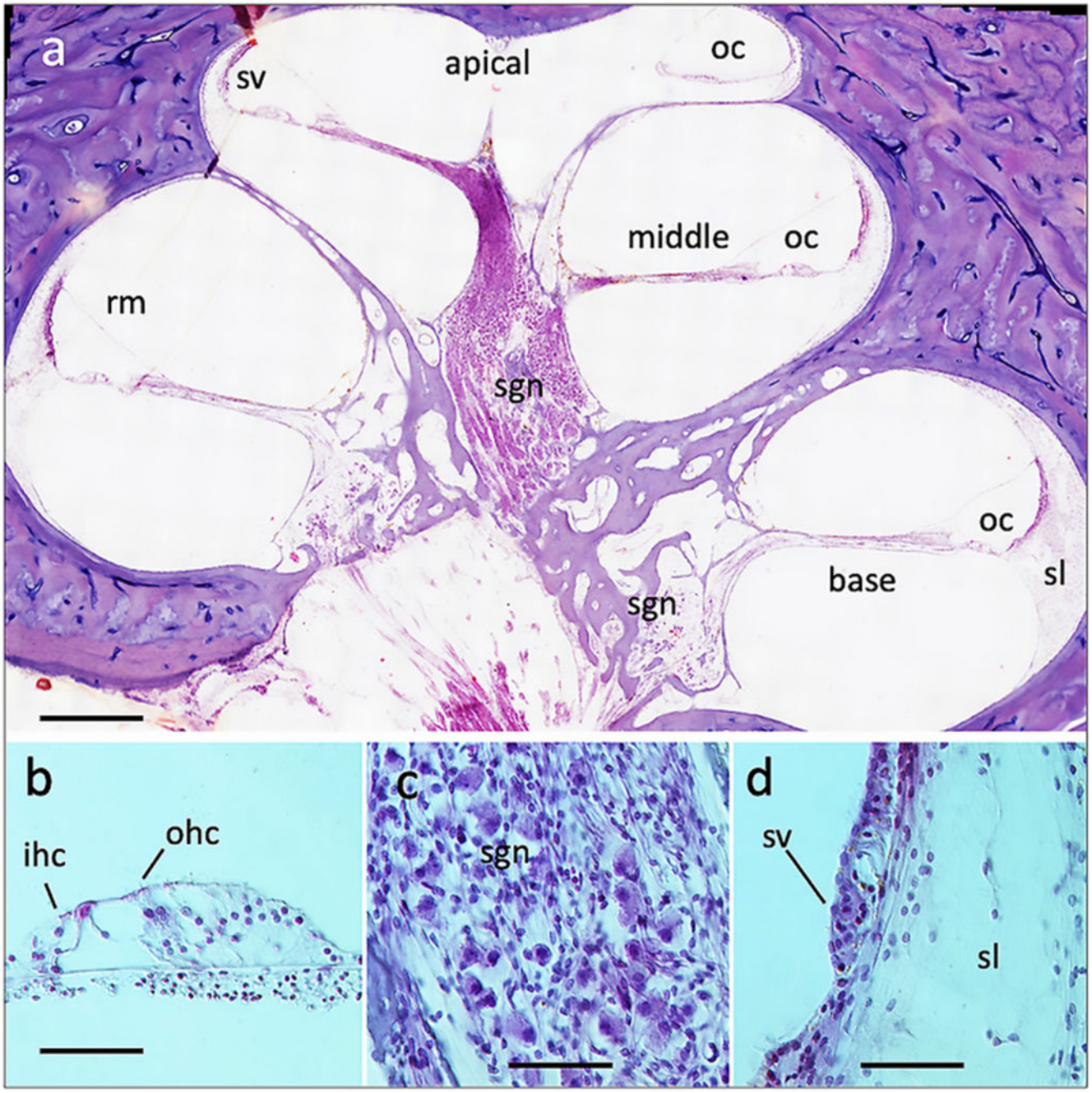

Fig. 1.

A. the normal human cochlea showing the apical, middle, and base region (hematoxylin and eosin stained celloidin section, 65-years-old male); b, c, d high magnification views from a; b. the organ of corti (oc), c. spiral ganglia neurons (sgn), d. the stria vascularis (sv), and spiral ligament (sl), reissner's membrane (rm). magnification bar in a = 250 μm, b = 50 μm, c = 100 μm, d = 100 μm.

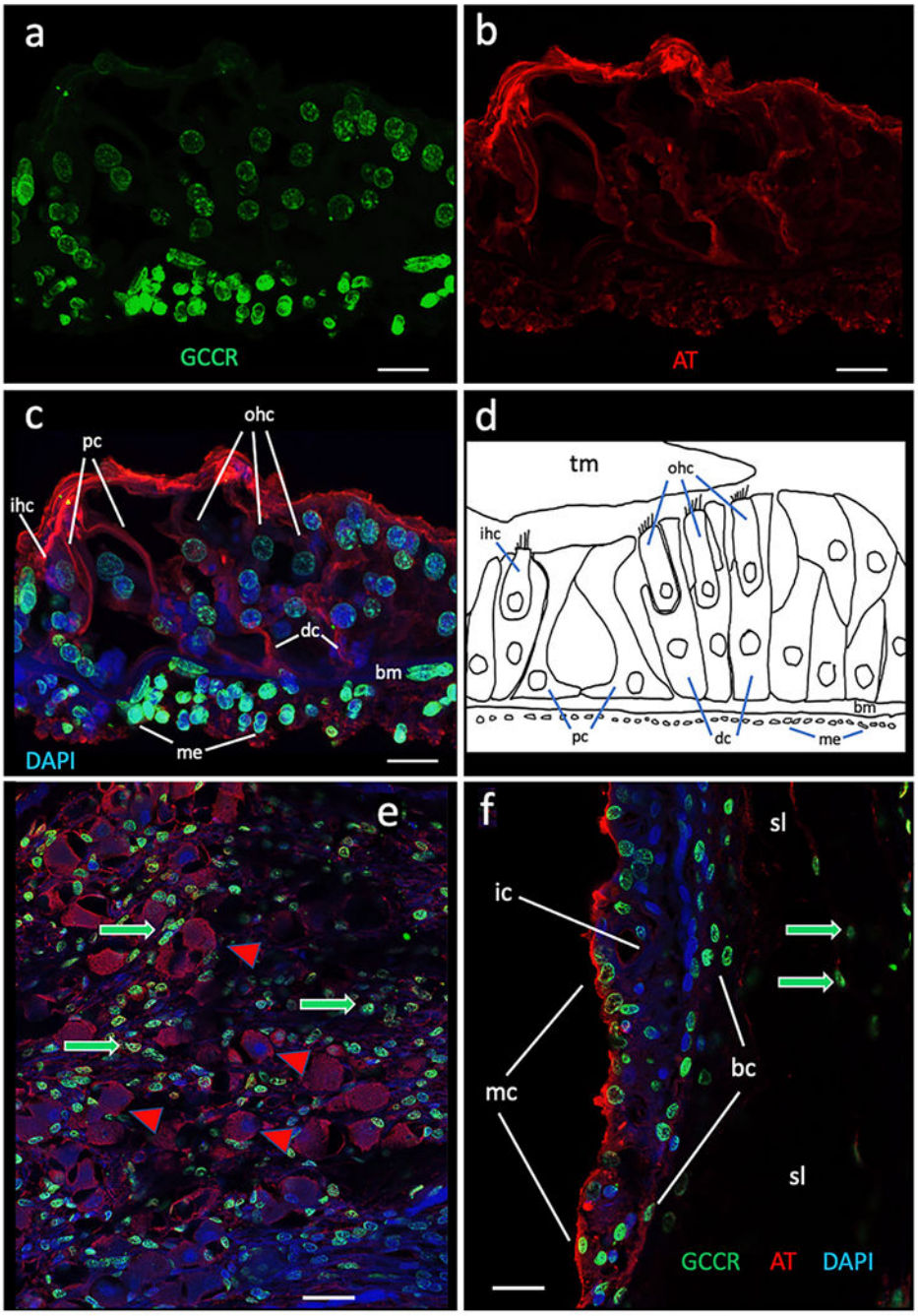

Fig. 2.

GCRs-IF in the apical portion of the normal human cochlea (65-years-old male); a. GCR-IF (green) in the organ of Corti cell nuclei; b. Acetylated tubulin-IF (AT) (red) allows the identification of Dieters' cells (dc) and pillar cells (pc); c. Merged image from Fig a. and b. GCR-IF was detected outer hair cells nuclei (ohc), inner hair cells nuclei (ihc), pillar (pc) and Deiter's cells (dc) of the organ of Corti; GCR-IF was also present in mesenchymal cell (me) nuclei under the basilar membrane (bm). DAPI identified cell nuclei; d: diagram to illustrate the location of the different cell types in the organ of Corti; (e) In the spiral

ganglia, GCR-IF was seen cell nuclei (green arrows) intermingle with sgn (red arrowheads) that shows no GCR-IF; (e) GCR-IF was detected in marginal (mc), intermediate (ic) and basal (b) cell nuclei of the stria vascularis (sv, green arrows). Magnification bar in a, b, and c = 25 μm in e and f = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

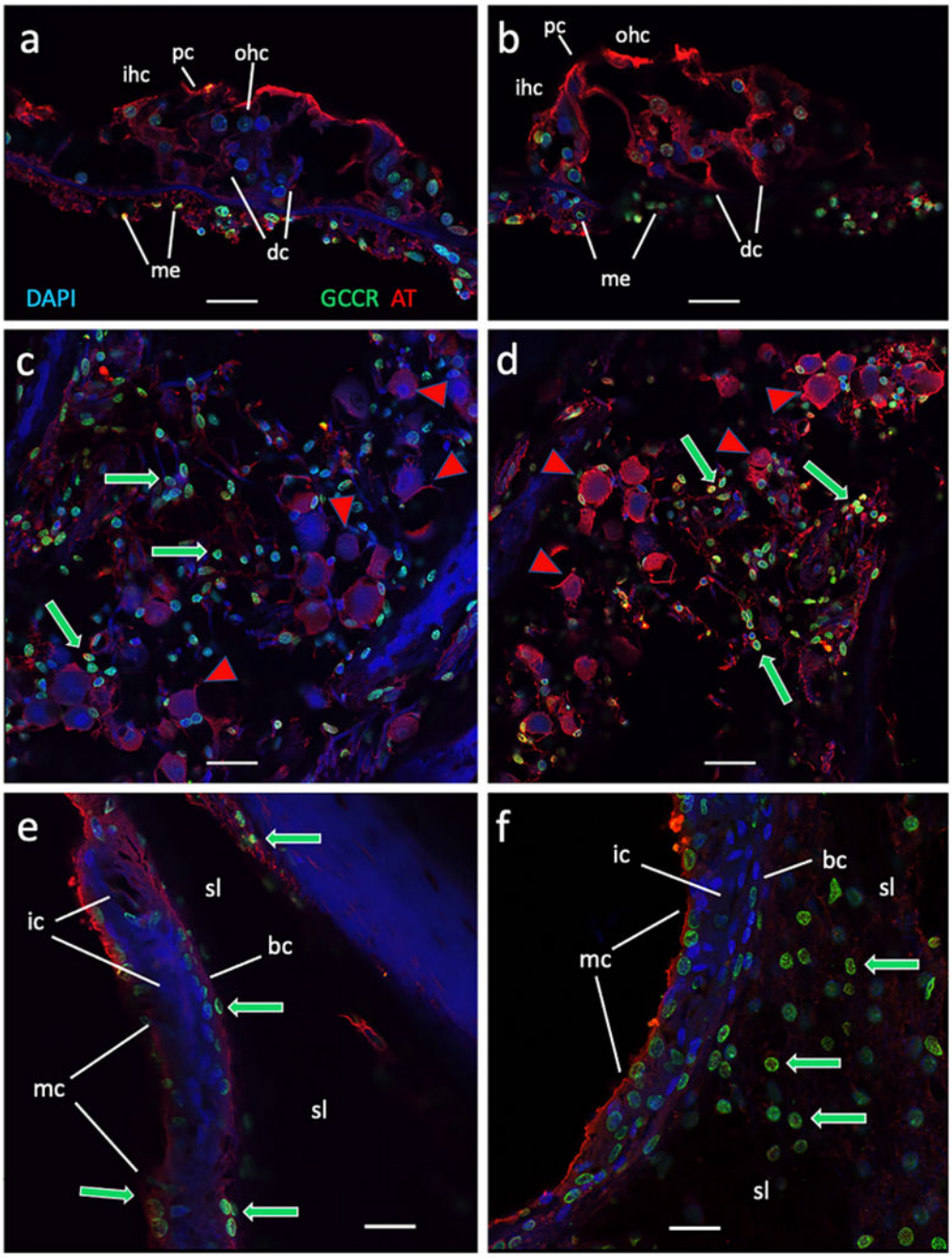

Fig. 3.

GCRs-IF in the mid and basal portion of the normal human cochlea (65-years-old male) (continuation) from Fig. 2. GCR-IF (green) and AT-IF (red). Fig a, c and e shows GCR-IF and AT-IF in the organ of Corti, spiral ganglia and stria vascularis at the middle region of the cochlea; Fig I), d, and f shows GCR-IF and AT-IF in the Organ of Corti, spiral ganglia and stria vascularis at the basal region of the cochlea. Abbreviations: ohc: outer hair cells; dc: Deiters' cells; pc: pillar cells; dc: Deiters' cells, me: mesenchymal cells; sl: spiral ligament; m: marginal cells; i: intermediate cells; basal: basal cells. Magnification bar in a and b = 25

μm in c, d, e, and f = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

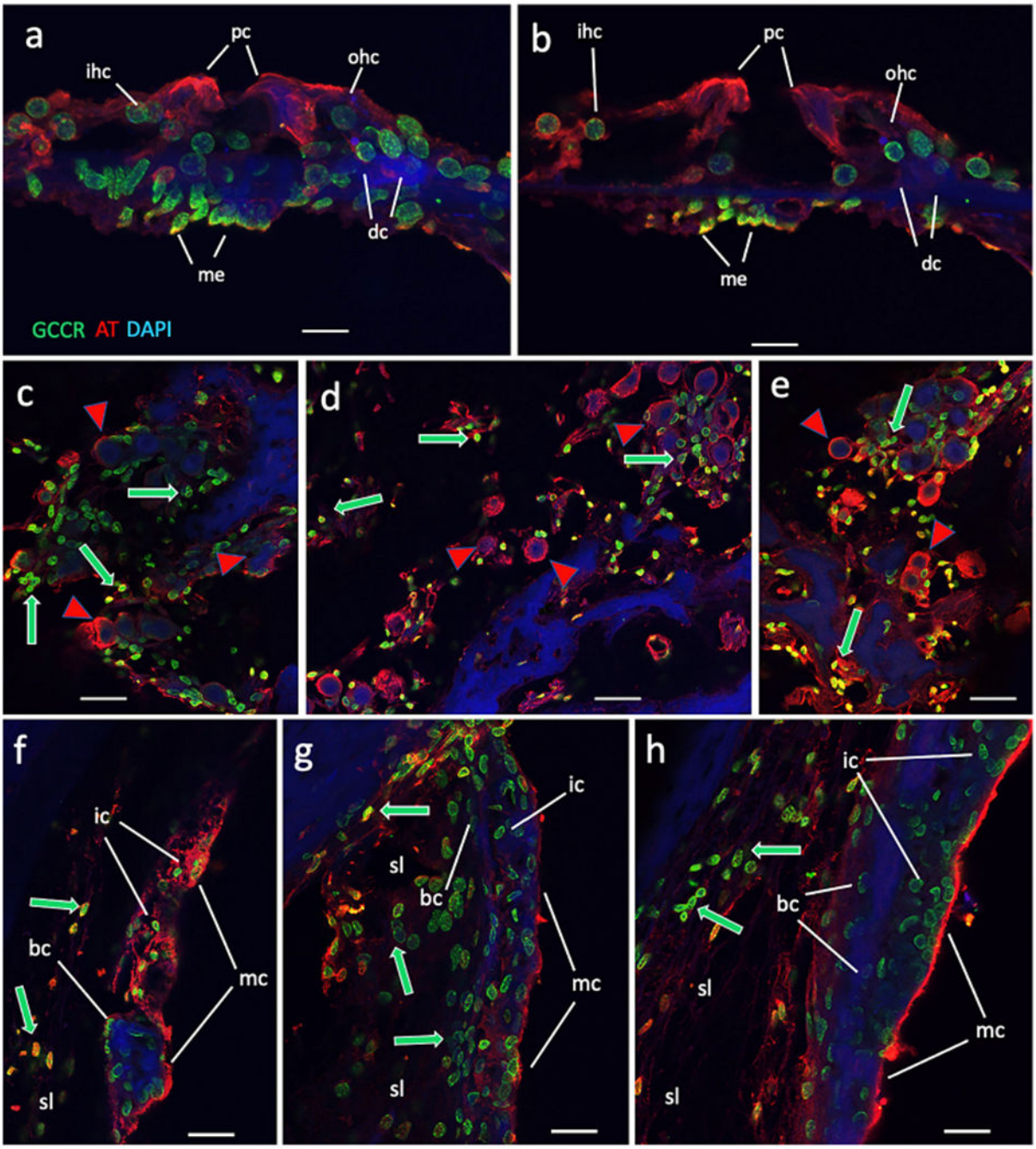

Fig. 4.

GCR-IF in a Meniere's disease cochlea section (65 years old-male). GCR-IF (green) and AT-IF (red). Fig a, and b shows the organ of Corti at the mid-apical, and mid-basal portion of the cochlea respectively, GCRs-IF was detected in the nuclei of outer hair cells, inner hair cells, and supporting cells, AT-IF allowed the identification of pilar and Deiter's cells. Fig c, d, and e shows the spiral ganglion at the mid-apical, middle, and base of cochlea. GCRs-IF was present in cell nuclei (arrows), however sgn (red) showed not GCR-IF. Fig f, g, and h shows the stria vascularis (sv) at the mid-apical, middle, and base of the cochlea. GCRs-IF

was seen in mc, ic and bc of the stria vascularis, and cell nuclei of spiral ligament (sl). Abbreviations: ohc: outer hair cells; dc: Deiters' cells; pc: pillar cells; dc: Deiters' cells, me: mesenchymal cells; m: marginal cells; i: intermediate cells; basal: basal cells.Magnification bar in a and b = 25 μm in c to h = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

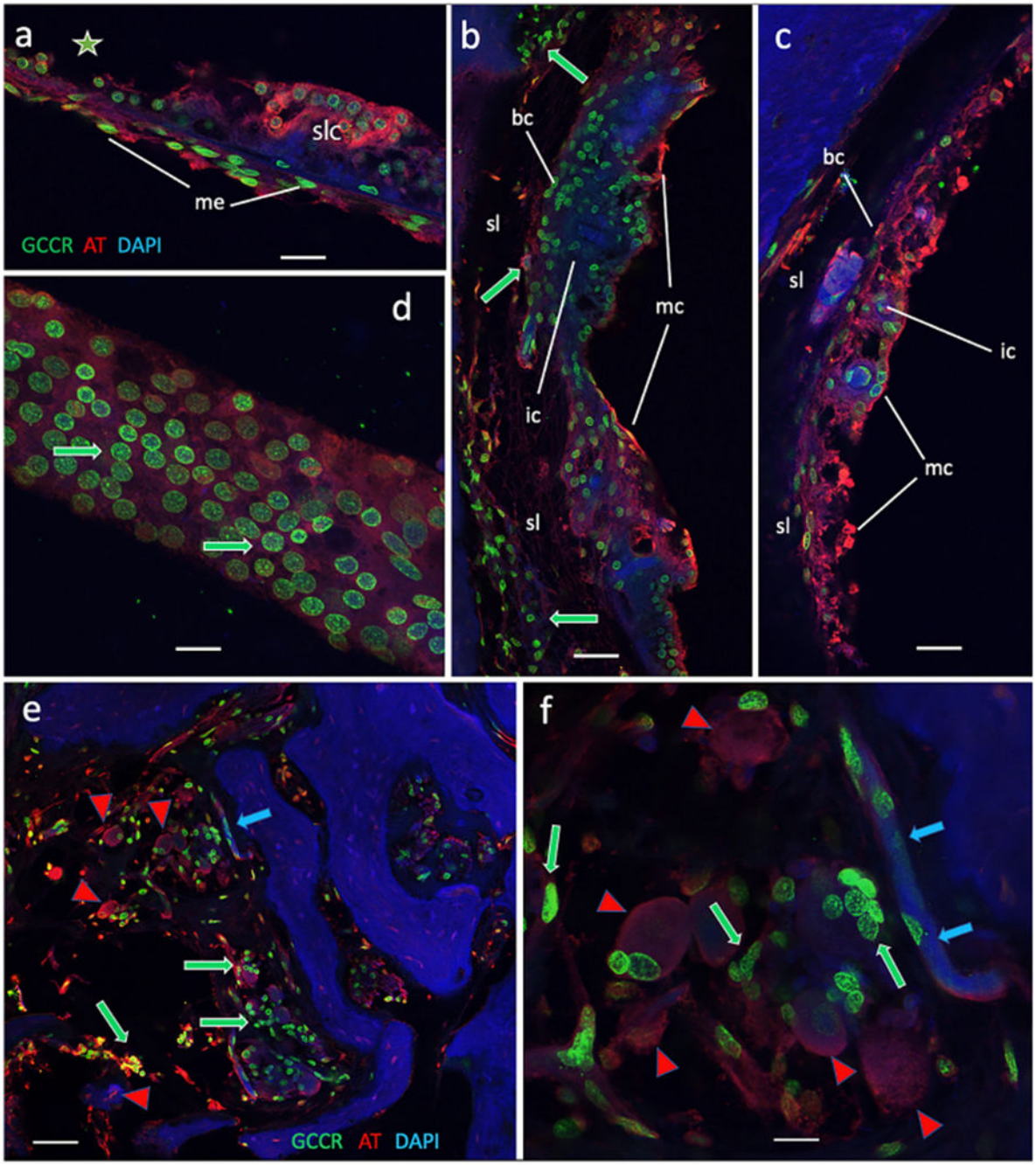

Fig. 5.

GCR-IF in another Meniere's disease cochlea (75-year-old-male). GCR-IF (green) and AT-IF (red). a. The apical organ of Corti, most supporting and hair cells are missing (green star), spiral limbus cells (slc) showed GCR-IF; b and c, GCR-IF in the stria vascularis and spiral ligament at the mid-apical and mid-base cochlea; d. GCR-IF in cell nuclei of the Reisner's (oblique section) at the mid-base of the cochlea; e, GCR-IF in the spiral ganglia at the mid-basal, fig f shows a high magnification view from e, GCR-IF was seen in cells around the sgn (non-GCR-IF). Pericytes and vascular endothelial cells delineate a blood vessel (bv).

Abbreviations; mc: marginal cells; i: intermediate cells; basal: basal cells. Magnification bar in a, d, f = 25 μm, b, c, e = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

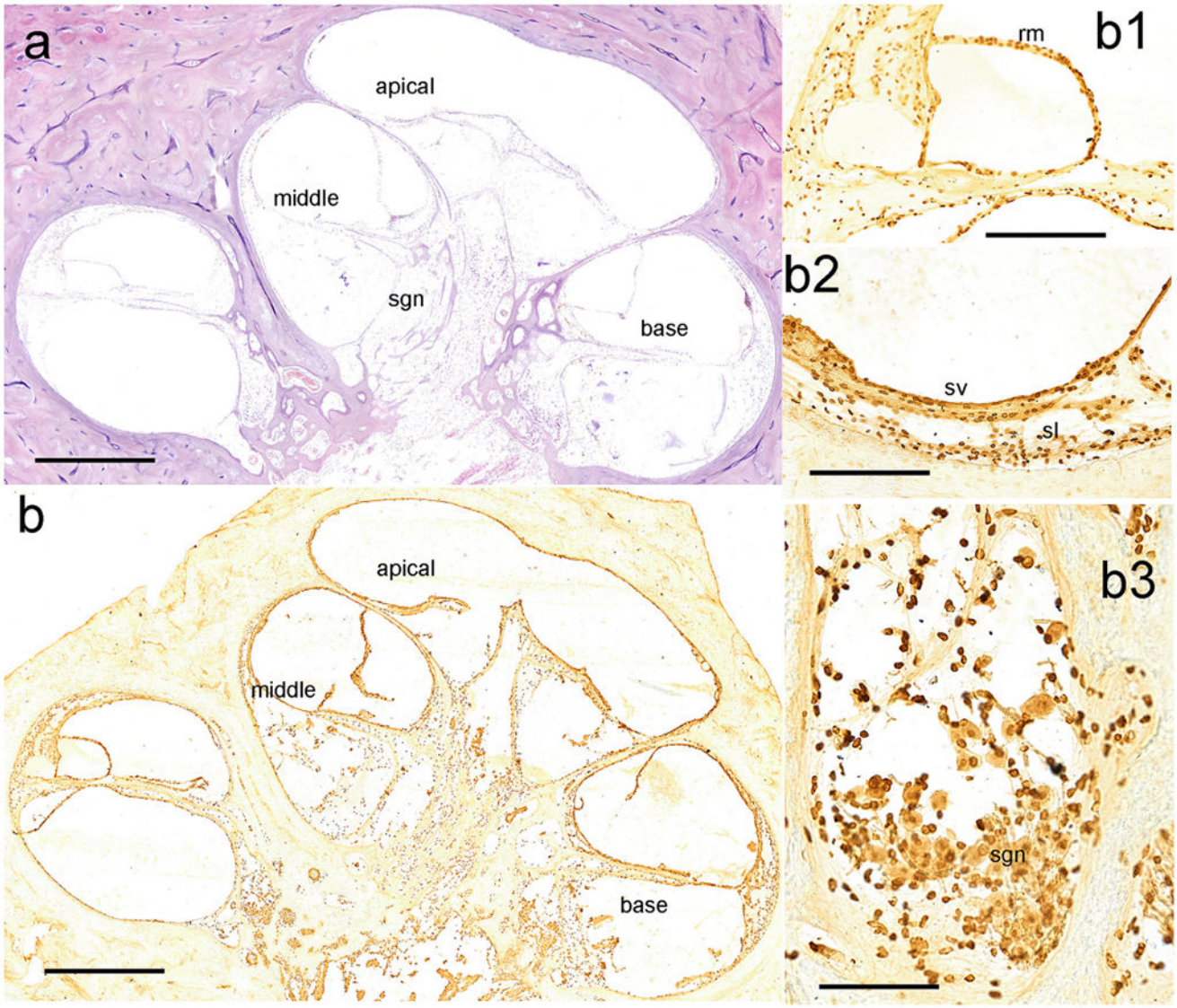

Fig. 6.

GCR-IR in a cochlea from 80 years old male, diagnosed with NIHL. a. Low magnification view of the cochlea (H&E staining), b) GCR-IR adjacent section (same specimen), GCR-IR was widely present in cell nuclei of the cochlea., b2. Base of the cochlea (high magnification view from b) GCR-IR was present in the lateral wall, Reisner's membrane, inner and other cells as well as supporting cells of the organ of Corti were loss., b2. Stria vascularis at the mid-apical zone, few GCR-IR cell nuclei were present in the atrophic stria, GCR-IR was present in cell nuclei of the spiral ligament., b3. GCR-IR was seen in cells of the spiral ganglia. Magnification bar in a and b = 500 μm; b2 and b3 = 150 μm.

Table 1

Temporal bones used in this study.

Specimen	Age	Gender	Diagnosis	Duration of disease in years
1	65	M	Normal	–
2	34	M	Normal	–
3	72	F	Normal	–
4	67	F	Normal	–
5	55	M	Normal	–
6	65	M	MD*	8
7	52	M	MD*	5
8	55	F	MD*	7
9	82	M	MD*	15
10	80	F	MD*	10
11	75	M	NIHL	5
12	65	M	NIHL	6
13	80	M	NIHL	7
14	84	F	NIHL	21
15	73	F	NIHL	10

Abbreviation. F: female, M: male. Age in years. MD: Meniere's disease, NIHL: noise induced hearing loss.

*= Meniere's disease was active at the time of dead i.e., patients suffer hearing loss, tinnitus, and reports of dizziness.

Table 2

GCR immunoreactive area in the different regions of the cochlea in the normal, MD and NIHL.

Location	N (n = 5)	MD (n = 5)	NIHL(n = 5)
OC Apical	20.5 ± 0.94	26.31 ± 1.7 *	20.9 ± 0.82 **
OC Middle	8.71 ± 0.99	14.02 ± 0.94 *	12.6 ± 0.82 **
OC Base	7.67 ± 0.4	12.79 ± 1.41 *	9.96 ± 0.65 **
SV Apical	12.50 ± 0.8	10.16 ± 1.5 **	9.65 ± 0.73 **
SV Middle	13.76 ± 0.955	12.65 ± 1.63 **	9.66 ± 0.95 *
SV Base	6.48 ± 0.58	14.46 ± 0.83 *	10.64 ± 1.71 **
SL Apical	13.44 ± 1.25	15.07 ± 0.41 *	11.15 ± 1.21 **
SL Middle	5.36 ± 0.0.62	13.58 ± 0.91 *	8.58 ± 0.59 *
SL Base	8.27 ± 0.89	15.13 ± 2.66 *	9.47 ± 0.85 **
SG Apical	4.84 ± 1.2	6.34 ± 0.63 **	5.40 ± 0.74 **
SG Middle	5.34 ± 0.45	10.17 ± 1.01 *	5.34 ± 0.44 **
SG Base	4.89 ± 0.42	9.08 ± 0.64 *	4.02 ± 0.42 **

Abbreviations: OC: organ of Corti; SV: stria vascularis; SL: spiral ligament; SG: spiral ganglia. N = normal; MD: Meniere's disease; NIHL: noise induce hearing loss; Statistical comparisons of normal values vs. MD and normal vs NIHL: $p < 0.05$ value statistically significant;

* S: significant

** NS: not significant. ± = standard error of the mean.