

Original Article

Localization of gentamicin uptake in the acutely isolated inner ear of the rat

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Abstract: The quantitative analysis of fluorescence in frozen sections of rat inner ears exposed to Texas Red conjugated gentamicin revealed distinct gradients of gentamicin fluorescence. At 500 µg/ml gentamicin fluorescence occurred in inner and outer hair cells, the interdental cell region, the spiral limbus below the interdental cells, the nerve fiber bundle in the spiral lamina, the inner sulcus cells and the dorsal region of the spiral ligament. No gentamicin fluorescence was observed in the Hensen / Claudius cells, the ventral region of the spiral ligament, the stria vascularis and the spiral ganglion. In the vestibule only the hair cell epithelium and the transitional cells of the saccule showed gentamicin fluorescence while no gentamicin fluorescence was found in hair cell epithelia and transitional cells of utricle and ampule, nerve fibers below hair cell epithelia of saccule, utricle and ampule and in dark cells. The gentamicin fluorescence increased at higher concentrations. Gentamicin exposure led to more pronounced gentamicin fluorescence in the cochlea compared to the vestibule. Based on the predominant gentamicin fluorescence in the hair cell - limbus region of the cochlea at a low dose we propose that gentamicin may interact with the K⁺-flow from the inner hair cells back to the scala media.

Keywords: Gentamicin, Menière's disease, cochlea, vestibule, rat

Introduction

Menière's disease was first described by Prosper Menière in 1861 [1] and comprises the symptom triad of recurrent, spontaneous vertigo, sensorineural hearing loss and tinnitus. Vertigo attacks are often accompanied by nausea and vomiting. Defective hearing often fluctuates at the beginning of the disease and normalizes in the attack free intervals. During the following course of disease hearing loss persists commonly in the low frequencies. The most incriminating symptom for patients is usually vertigo [2].

The pathophysiologic mechanism for Menière's disease is an endolymphatic hydrops caused by disorders of the endolymph production or resorption. In consequence, the Reissner's membrane ruptures [3,4] and K⁺ leaks from the endolymphatic into the perilymphatic space causing a non-physiological activation of vestibular nerve fibres with the symptom vertigo [5]. Rup-

ture of Reissner's membrane in the apex of the cochlea leads to hearing loss in the lower frequency range [6].

Therapy of Menière's disease varies stepwise from conservative medical treatment to radical surgical procedures [7,8].

An innovation in treatment was the application of ototoxic aminoglycosides in 1957 by Schuknecht [9]. By inducing a pharmacological neurectomy, the symptoms of Menière's disease could be influenced beneficially. Subsequently, it was shown that a low dose intratympanic application of gentamicin was sufficient to achieve relief for the patients, without destroying the complete vestibular system [10-13]. Many studies have dealt with aminoglycosides and have suggested an effect not only on hair cells but also on the stria vascularis, marginal cells, fibrocytes [14-20] or vestibular dark cells [21]. However, in these studies high concentrations of gentamicin were applied, which

destroyed not only hair cells but also a lot of other cell types in the inner ear.

Until now, the target cells for low dose gentamicin therapy that convey the relief from Menière symptoms still remain unclear. It is the aim of the present study to identify the target cells in the inner ear of the rat by using short time exposure- and low-dose application of gentamicin.

Materials and methods

Animals

Young, 2-4 month old rats were obtained from the breeding colony of the animal facilities at the medical faculty of the University of Regensburg. Two offspring of black hooded (BDE) rats initially obtained from Boehringer Ingelheim and three offspring of the ACI strain from Harlan-Winkelmann of either sex were used in the present experiments. The use of animals in this study complies with the current „German Law on the Protection of Animals“ (Tierschutzgesetz §4 Satz 3).

Gentamicin

Texas red conjugated gentamicin has been used to analyze aminoglycoside uptake in the inner ear [e.g. 41]. The pattern of fluorescence induced by exposure to Texas Red conjugated gentamicin in the inner ear was similar to that determined by immunohistochemical analysis following exposure to native gentamicin [e.g. 46]. In the present study we used Texas Red conjugated gentamicin to analyze the pattern of fluorescence in defined cochlear and vestibular structures following a short exposure of the acutely isolated rat cochlea (for details see below). This degree of fluorescence is interpreted as an indicator of the gentamicin concentration in the respective cochlear and vestibular regions and is referred to as gentamicin fluorescence.

A stock solution of Texas Red conjugated gentamicin was prepared according to Lyford-Pike et al. [36]. First gentamicin sulfate (Sigma, Cat. # G1264) was dissolved in 0.1M potassium carbonate solution (K_2CO_3 , Sigma, Cat. # P5833) with pH 9 at a concentration of 50 mg/ml. Then Texas Red-X succinimidyl ester (Invitrogen Cat. # T6134) was dissolved in NN-

dimethylformamide (Sigma, Cat. # D4254) at a concentration of 2 mg/ml. For conjugation, 0.5 ml of the Texas Red solution and 3.67 ml of the gentamicin solution were mixed and stored under agitation on a shaker in a cold room until used (at least 24 hours). This stock solution contains 44 mg gentamicin per ml and was diluted to the desired concentrations of 500, 1250 and 2500 $\mu\text{g/ml}$ in Dulbecco's Modified Eagle Medium (DMEM with 4mM l-glutamine, 1000mg/l d-glucose and 110 mg/l sodium pyruvate; Invitrogen Cat. # 31885023). For control experiments 0.5 ml of the Texas Red solution was combined with 3.67 ml 0.1M potassium carbonate solution without gentamicin and adequate aliquots were added to DMEM for exposure in control experiments. In 3 experiments we used the lowest concentration of 500 $\mu\text{g/ml}$ and additional experiments were performed using 1250 and 2500 $\mu\text{g/ml}$, respectively.

Preparation

In order to isolate the temporal bones, the rats were killed by decapitation with a guillotine. Then the skull was opened dorsally and the brain partially removed. The tympanic bullae were exposed and the left and right side separated from the skull and placed separately into petri dishes filled with cold DMEM. Access to the inner ear was achieved by opening the bullae with blunt forceps. Excess tissue and bony structures were carefully removed. With the help of a small metal hook the stapes was removed from the oval window, the round window was ruptured and small perforations were made into the bony wall at the level of the scala tympani of the cochlear base and at the apex of the cochlea. The isolated inner ear was then transferred to fresh DMEM in a 24-well tissue culture plate and cleaned from blood by carefully flushing DMEM through the perilymphatic space with a fine-tipped pipette at the exposed oval and round windows and the apical opening. It was then transferred to the next well with the incubation solution that was again gently flushed through the perilymphatic spaces by a fine tipped pipette. The preparation of both ears until exposure to the incubation solution was typically achieved within 7-12 minutes. In each experiment one ear was exposed to gentamicin while the other served as control. In two 500 $\mu\text{g/ml}$ experiments control ears were incubated in pure DMEM without any addition while in the 3 other experiments corresponding amounts of

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Texas Red solution without gentamicin in DMEM were used for incubation.

Exposure

The inner ears were incubated for 10 minutes and then transferred to DMEM. Excess incubation solution was flushed out of the perilymphatic spaces with a fine tipped pipette and the inner ears were transferred to 4% paraformaldehyde in 0.1M phosphate buffer with pH 7.6 for fixation. Fixation in the paraformaldehyde solution was performed for 90 minutes. The solution was changed 3 times during this period and gently flushed through the perilymphatic spaces every 15 minutes to achieve a good fixation. The tissue was then transferred to 0.3% Triton X100 (Sigma, Cat. # X100) in 0.1M phosphate buffer for 90 minutes to make the cell membranes permeable for a subsequent actin label with fluorescein-conjugated phalloidin. The tissue was transferred to the fluorescein phalloidin (Invitrogen, Cat. # F432) staining solution (20 μ l stock solution prepared according to the manufacturer's instructions were diluted in 1 ml 0.1 M PBS with 0.3 % Triton). Incubation lasted 90 minutes in the phalloidin solution with a gentle flushing of the perilymphatic spaces every 15 minutes. Following the actin staining, the cochleae were washed and perilymphatic spaces flushed with decalcification solution (4 % paraformaldehyde, 0.1 M EDTA in 0.1 M phosphate buffer). Each ear was placed in a 50 ml falcon tube with decalcification solution on a shaker in the cold room at 4°C. The solution was changed every 2-3 days. After a decalcification period of 10 days the cochleae were washed in phosphate buffer and placed in 30% saccharose solution under agitation at 4°C overnight for cryoprotection of the tissue. Properly oriented 10 μ m thick frozen sections of the inner ear were collected on slides, dried and covered slipped with Vectashield with DAPI (Linaris, Cat. # H-1200) in order to stain nuclei. Using this procedure the binding or accumulation of gentamicin was indicated by the Texas Red fluorescence while the actin staining due to the green fluorescein and the nuclear staining due to the blue DAPI fluorescence provided orientation and landmarks in these sections of the inner ear.

Analysis

The sections were examined under a Leica DM RBE microscope (Leica Mikrosysteme, Ben-

sheim, Germany) with epifluorescence. Images were digitized with a Spot RT3 Slider camera (Diagnostic instruments, Stirling Heights, Mich., USA) with the help of VisiView (Visitron Systems GmbH Puchheim, Germany). To achieve high sensitivity and resolution we used a 40x lens resulting in a nominal resolution of 0.19 μ m per pixel. For each frame, separate images were digitized with the Texas Red, the fluorescein and the DAPI fluorescence filters. The exposure time for each channel was determined in pilot experiments (2 s for Texas Red and fluorescein, 0.1 s for DAPI) and subsequently kept constant for digitizing all images with a 16 bit gray level depth. Further processing of the raw images was done using ImageJ 1.43. For each image we performed a background subtraction with the dark background option selected and a rolling ball radius of 50 pixels. The settings for optimizing brightness and contrast for each channel were also determined in pilot experiments and subsequently used for all images (Texas Red: min 80, max 350; fluorescein: min 40, max 1500; DAPI: min 100, max 1500). Following these processing steps, images were converted to 8 bit gray level depth and the red, green and blue image of each frame combined to a RGB image. To obtain overviews of a section through the cochlea including the spiral ganglion, organ of Corti, stria vascularis and the spiral ligament or covering complete sections through the vestibular organs, several overlapping frames were taken and subsequently assembled to an overview with high resolution using Photoshop CS4 (**Figure 2, 3, 4, 5**). These overviews were used for a qualitative visual evaluation of gentamicin accumulation in the inner ear.

For a quantitative analysis of Texas Red fluorescence as an indicator of gentamicin distribution in the tissue we defined 11 distinct areas in the overviews of cochlear sections and 10 distinct areas in overviews of sections through vestibular organs (**Figure 1, Table 1**). Using ImageJ 1.43 the regions of interest representing the above defined cochlear or vestibular areas were selected in the overviews using the polygon selection tool and the mean gray level of the red channel in each region of interest was calculated.

The software PASW statistics 17 (SPSS Inc.) was used for calculating the test statistics. First, data from each of the 11 cochlear and 10 vestibular areas were assigned to one of 6 groups: 1) treated with 500 μ g/ml gentamicin, 2) con-

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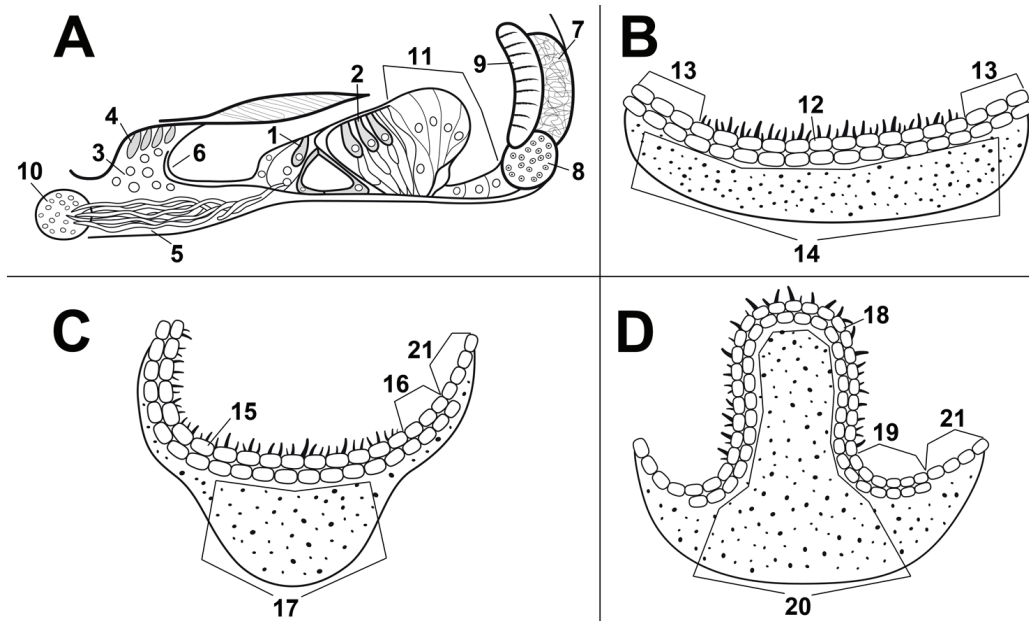


Figure 1, A-D: Schematic illustration of sections showing the cochlea (A), saccule (B), utricle (C) and ampule (D). 1 = inner hair cells, 2 = outer hair cells, 3 = lower spiral limbus, 4 = interdental cells, 5 = dendrites of spiral ganglion cells, 6 = inner sulcus cells, 7 = dorsal spiral ligament, 8 = ventral spiral ligament, 9 = stria vascularis, 10 = spiral ganglion, 11 Hensen / Claudius cells, 12 = saccule hair/supporting cells, 13 = saccule transitional cells, 14 = saccule nerve fiber region, 15 = utricle hair/supporting cells, 16 = utricle transitional cells, 17 = utricle nerve fiber region, 18 = ampule hair/supporting cells, 19 = ampule transitional cells, 20 = ampule nerve fiber region, 21 = dark cells.

Table 1. Analyzed structures

Cochlea	Vestibular organ
1 = Inner hair cell	12 = Saccule hair cells and supporting cells
2 = Outer hair cells	13 = Saccule transitional cells
3 = Spiral limbus below interdental cells	14 = Saccule nerve fiber region
4 = Interdental cells	15 = Utricle hair cells and supporting cells
5 = Nerve fiber bundle in the region of the Spiral lamina	16 = Utricle transitional cells
6 = Inner sulcus cells	17 = Utricle nerve fiber region
7 = Dorsal spiral ligament	18 = Ampule hair cells and supporting cells
8 = Ventral spiral ligament	19 = Ampule transitional cells
9 = Stria vascularis	20 = Ampule nerve fiber region
10 = Spiral ganglion	21 = Dark cells
11 = Hensen / Claudius cells	

trol in the 500 µg/ml experiment, 3) treated with 1250 µg/ml gentamicin, 4) control in the 1250 µg/ml experiment, 5) treated with 2500 µg/ml gentamicin, 6) control in the 2500 µg/ml experiment. For each area a Kruskal Wallis test was performed comparing these 6 groups to determine if gray levels differed significantly between groups. Since these tests revealed a significant effect of group for each of the 21 areas ($p \leq 0.011$) subsequent pair wise comparisons with Mann Whitney U tests were per-

formed. To identify areas where gray values were significantly elevated in the gentamicin treated ears compared to the corresponding controls comparisons were performed within each of the 3 gentamicin doses (500 µg/ml: group 1 versus group 2; 1250 µg/ml: group 3 versus group 4; 2500 µg/ml: group 5 versus group 6). To identify dose dependent effects of gentamicin, pair wise comparisons within each area were performed comparing gray levels in ears treated with 500 and 1250 µg/ml (group 1

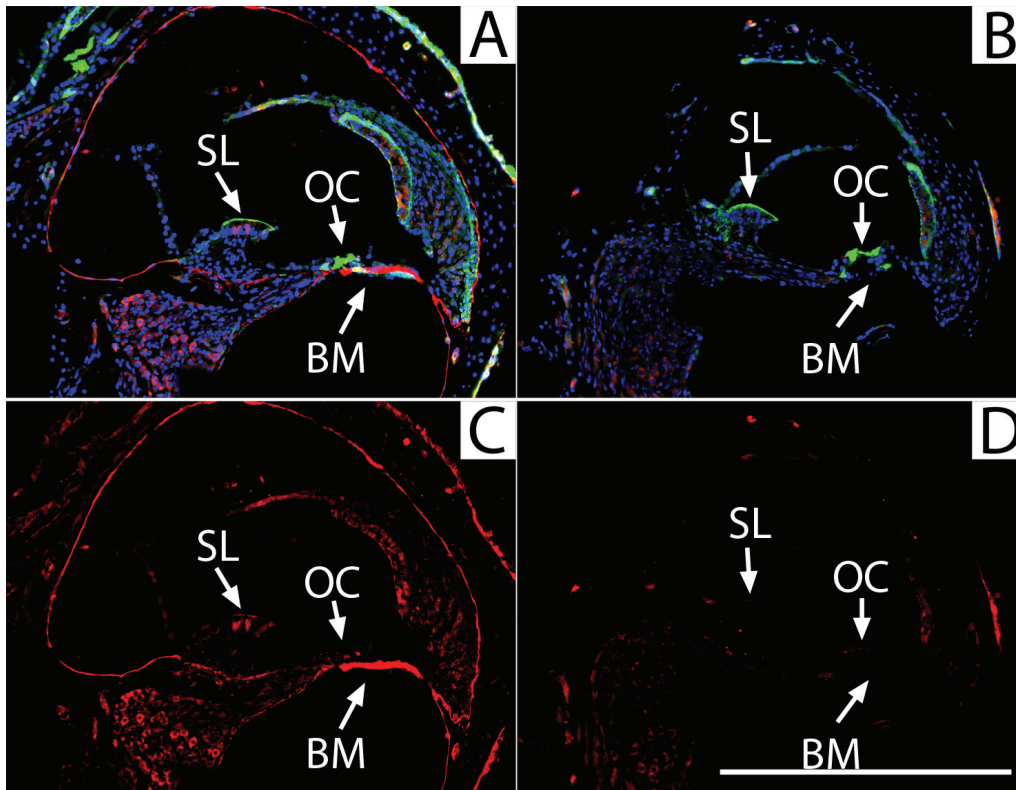


Figure 2. Overview of a section through the cochlea exposed to 500 µg/ml gentamicin (A,C) and a corresponding control exposed only to DMEM (B,D). Figures A and B show composite RGB images of the three colour channels representing Texas Red coupled gentamicin (red), FITC-phalloidin labelled actin (green) and DAPI stained nuclei (blue) and allow a clear identification of the different cochlear structures (e.g. spiral limbus, SL; basilar membrane, BM; Organ of Corti, OC). Only the corresponding images of the red colour channel are shown in Figure 2C,D and emphasize the distinct and elevated red fluorescence in the section from a cochlea exposed to 500 µg/ml gentamicin (Figure 2C) as compared to the low degree of background fluorescence in a control (Figure 2D). The labels and arrows pointing to OC, SL and BM in the images of the red channel (Figure 2C,D) resemble those from the composite images (Figure 2A,B). The scale bar in the bottom right of Fig. 2B indicates 500 µm.

versus group 3), 1250 and 2500 µg/ml (group 3 versus group 5) and 500 and 2500 µg/ml (group 1 versus group 5). A corresponding analysis was performed between the controls (group 2 versus group 4, group 4 versus group 6 and group 2 versus group 6) to identify a potential non specific binding of the Texas Red control solution without gentamicin.

Results

Qualitative analysis

We started with a qualitative analysis of Texas red conjugated gentamicin binding by visual inspection of the sections through the cochlea and the vestibular regions.

Cochlear structures

Visual inspection of the sections exposed to a concentration of 500 µg/ml gentamicin showed elevated gentamicin fluorescence in non-cellular structures such as the basilar membrane, the lining of the perilymphatic spaces and the osseous spiral lamina compared to control sections as illustrated in the overviews shown in **Figure 2**. For better orientation, the images in **Figure 2A** (exposed to 500 µg/ml gentamicin) and **Figure 2B** (control) show composite images of the three colour channels demonstrating gentamicin fluorescence in red, actin labelling by phalloidin in green and nuclear staining by DAPI in blue. In these composite RGB images different cochlear structures can

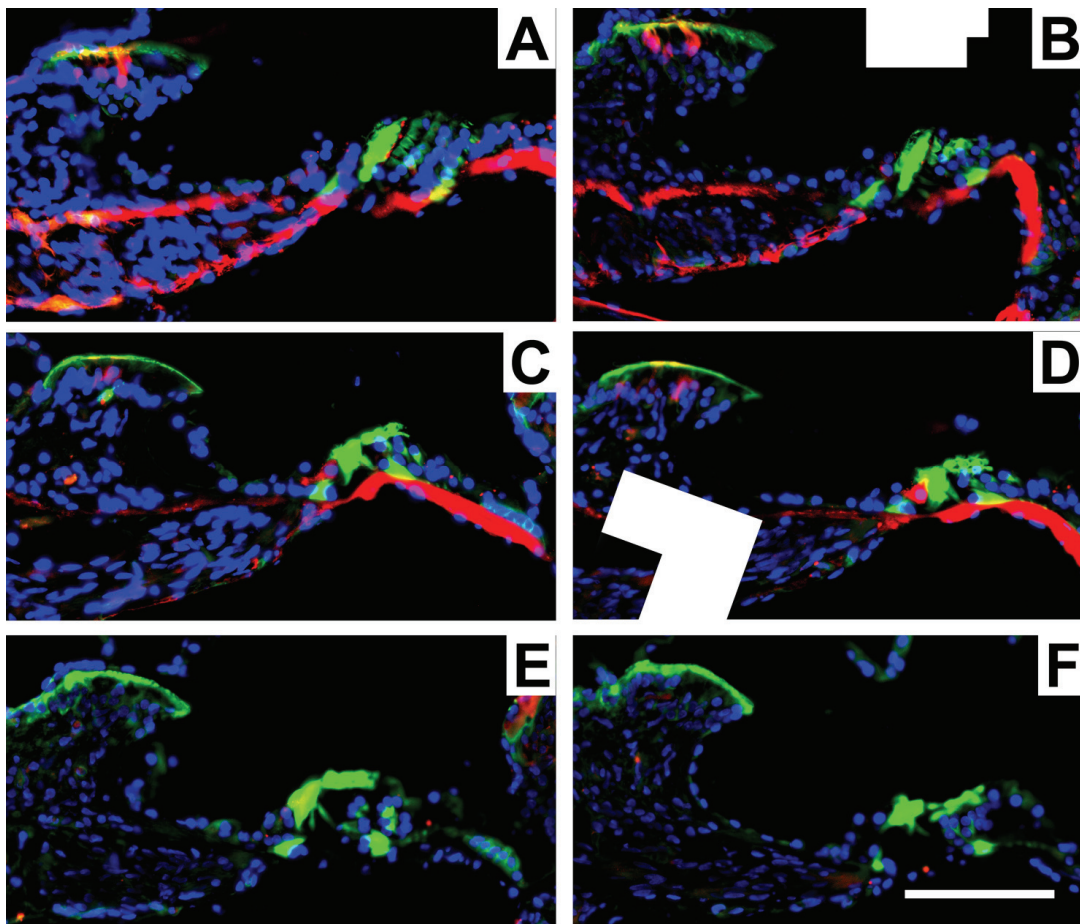


Figure 3. High magnification views of a cochlear sections exposed to 500 µg/ml gentamicin (A-D) and controls (E,F). Texas red conjugated gentamicin is visible as red fluorescence, cell nuclei are shown in blue and actin is shown by the green fluorescence. The scale bar in the bottom right of Fig. 3F indicates 100 µm.

be clearly recognized as shown by the labels and arrows pointing to the Organ of Corti (OC), the spiral limbus (SL) and the basilar membrane (BM). To emphasize the difference in the pattern of red fluorescence between gentamicin exposed and control ears only the corresponding red channel is shown in **Figure 2C** and **Figure 2D** respectively. While some unspecific red fluorescence is visible in the region of the spiral ganglion and the stria vascularis in the control (**Figure 2B,D**), most other cochlear regions were devoid of visual red fluorescence. **Figure 2D** illustrates that the Organ of Corti (OC), the spiral ligament (SL) and the basilar membrane (BM) cannot be recognized and defined in the image of the red channel despite the arrows that point to the identical positions illustrated in the composite image (**Figure 2B**). Thus, due to the low

background fluorescence of the red channel (e.g. **Figure 2D**), composite images were necessary to unequivocally identify the cochlear and vestibular regions defined in **Figure 1** for subsequent quantitative measurements.

With respect to cellular structures, red fluorescence of gentamicin was seen in some interdental cells of the spiral limbus (**Figure 2A**) and in a few inner hair cells. This is illustrated at higher magnification in **Figure 3** with red fluorescence visible in a population of interdental cells (**Figure 3 A-D**) and some inner hair cells (**Figure 3 C,D**). The examples from controls (**Figure 3 E,F**) demonstrate the specificity of gentamicin fluorescence shown in **Figure 3 A-D**.

In the outer hair cells no gentamicin fluores-

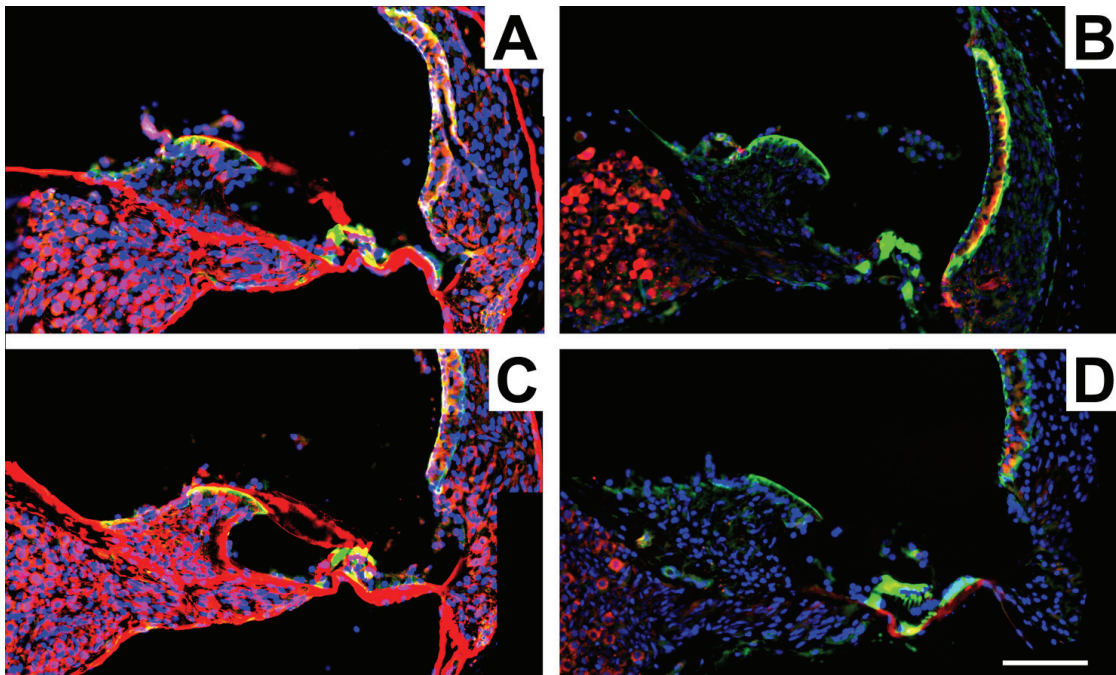


Figure 4. High magnification views of cochlear sections exposed to 1250 µg/ml gentamicin (**A**) and 2500 µg/ml gentamicin (**C**) and corresponding controls (**B,D**). Texas red conjugated gentamicin is visible as red fluorescence, cell nuclei are shown in blue and actin is shown by the green fluorescence. The scale bar in the bottom right of Fig. 3F indicates 100 µm.

cence could be detected visually (**Figure 3A-D**) at a gentamicin concentration of 500 µg/ml. The visual interpretation of the red fluorescence in the cells of the spiral ganglion, between the basal and the marginal cells of the stria vascularis and the ventral spiral ligament was hampered by background fluorescence in controls (e.g. **Figure 2B**).

At higher drug concentrations of 1250 (**Figure 4A**) and 2500 µg/ml (**Figure 4C**), gentamicin fluorescence appeared much more widespread in the cochlea, especially, inner as well as outer hair cells, inner sulcus cells, interdental cells, fibrocytes of the spiral limbus, the spiral ganglion, stria vascularis, the ventral spiral ligament and also non-cellular structures like the basilar membrane, osseous spiral lamina, the lining of the perilymphatic spaces and the tectorial membrane showed a clear gentamicin fluorescence (**Figure 4A,C**). The specificity of gentamicin fluorescence is illustrated by the corresponding controls shown in the right column of **Figure 4 (B,D)**. The images in **Figure 4 A, C** demonstrate a more intense red gentamicin fluorescence signal as compared to the corresponding controls shown in **Figure 4 B, D**.

Vestibular structures

Following an exposure with 500 µg/ml gentamicin no specific gentamicin fluorescence was seen in vestibular structures while at higher concentrations gentamicin fluorescence became visible. This is exemplified in **Figure 5** for the saccule. The left column shows sections from inner ears exposed to 500 (**Figure 5A**), 1250 (**Figure 5C**) and 2500 µg/ml (**Figure 5E**) while corresponding controls are shown in the right column. Over all, gentamicin fluorescence in vestibular structures appeared lower compared to the cochlea (compare **Figure 3-5**).

Over all, the qualitative visual analysis of the sections indicated only for cochlear interdental and inner hair cells specific gentamicin fluorescence at the lowest gentamicin concentration tested (500 µg/ml). At the higher concentrations visual assessment suggested more pronounced gentamicin fluorescence in cochlear compared to vestibular structures (**Figure 3-5**).

In addition to this qualitative visual evaluation of gentamicin fluorescence we performed a quantitative gray level analysis of the red fluo-

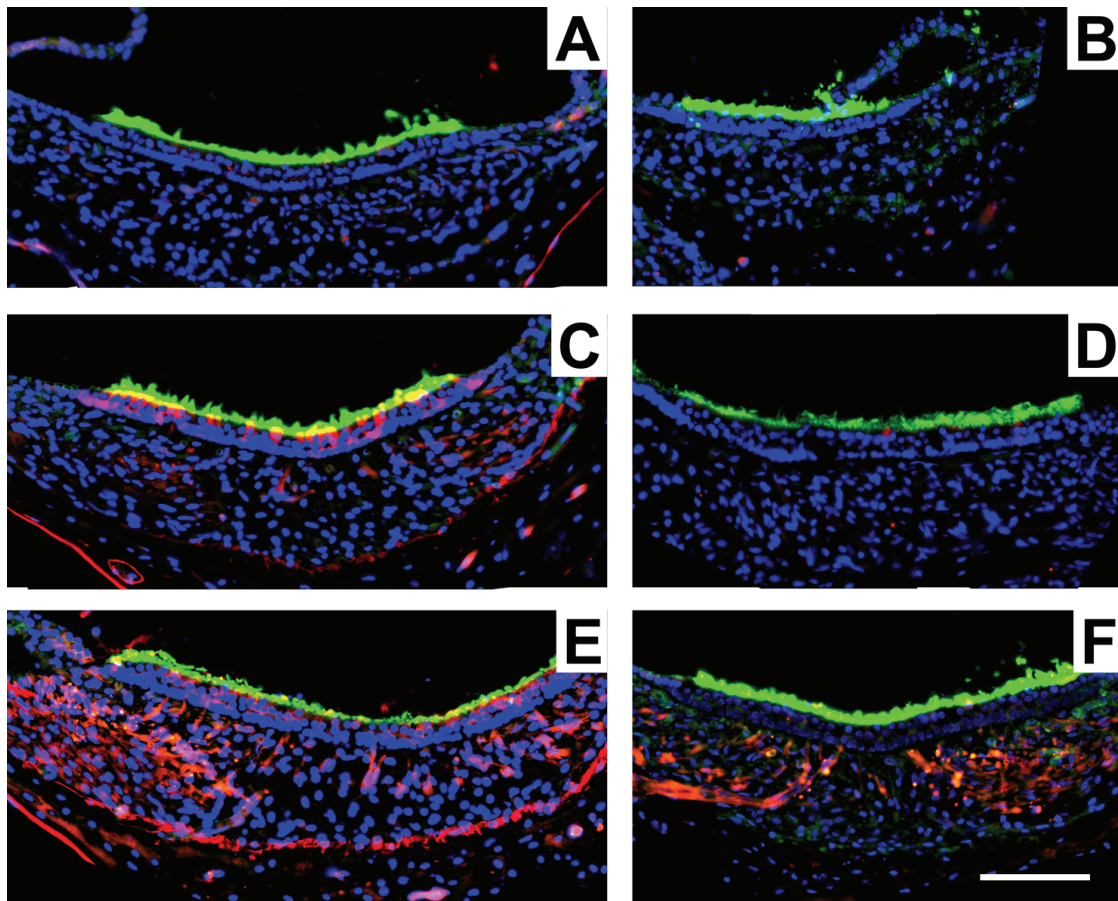


Figure 5. Sections through the saccule exposed to 500 $\mu\text{g/ml}$ gentamicin (A), 1250 $\mu\text{g/ml}$ gentamicin (C) and 2500 $\mu\text{g/ml}$ gentamicin (E) and corresponding controls (B,D,F). Texas red conjugated gentamicin is shown as red fluorescence, cell nuclei are shown in blue and actin is shown by the green fluorescence. The scale bar in the bottom right of Figure 5F indicates 100 μm .

rescence for the cochlear and vestibular regions defined in **Figure 1** and listed in **Table 1**.

Quantitative analysis

Over all conditions, 1126 gray level measurements were obtained from cochlear areas and 396 from vestibular areas. The majority of measurements was collected for the 500 $\mu\text{g/ml}$ concentration of gentamicin (cochlear 705; vestibular 207) since we were primarily interested in the uptake following exposure to a low dose of gentamicin.

The gray level is a quantitative measure of the fluorescence signal. Low gray levels are associated with a weak fluorescence and high numbers are associated with a high fluorescence.

Cochlear structures

Figure 6 compares mean gray level and standard deviation obtained in eleven cochlear regions in controls (white bars) and following gentamicin exposure at a concentration of 500 $\mu\text{g/ml}$ (filled black bars). Because in controls and in samples exposed to 500 $\mu\text{g/ml}$ the red fluorescence signal was typically low (**Figure 2**) the gray levels are shown on a logarithmic scale to emphasize potential small differences. For most cochlear structures the background fluorescence in controls was low (below 10), only the ventral spiral ligament (mean 13), stria vascularis (mean 42), the spiral ganglion (mean 32) and the Hensen/Claudius cells (mean 14) showed a higher degree of background fluorescence (see also background fluorescence in Fig.

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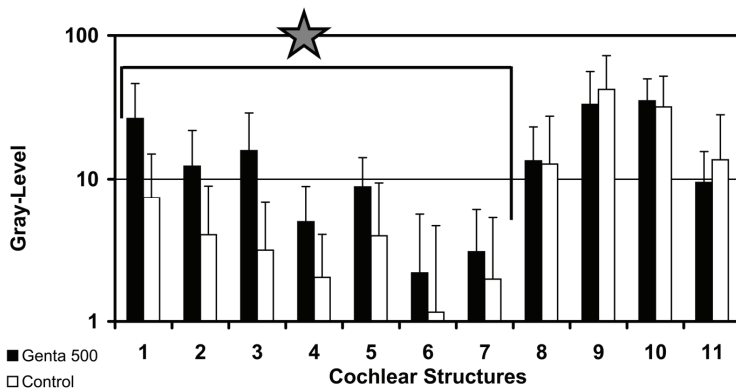


Figure 6. Comparison of the gray levels in controls (white bars) and gentamicin exposed (black bars) cochlear regions for the 500 $\mu\text{g}/\text{ml}$ condition. A significant difference in gray level between exposed cochleae and controls is indicated by a star. Note the logarithmic gray level scale to visualize small differences. This quantitative analysis revealed that exposure to 500 $\mu\text{g}/\text{ml}$ gentamicin was associated with a significantly higher fluorescence in seven of the eleven analyzed cochlear structures as compared to background fluorescence in controls. 1 = inner hair cells, 2 = outer hair cells, 3 = lower spiral limbus, 4 = interdental cells, 5 = dendrites of spiral ganglion cells, 6 = inner sulcus cells, 7 = dorsal spiral ligament, 8 = ventral spiral ligament, 9 = stria vascularis, 10 = spiral ganglion, 11 = Hensen / Claudius cells.

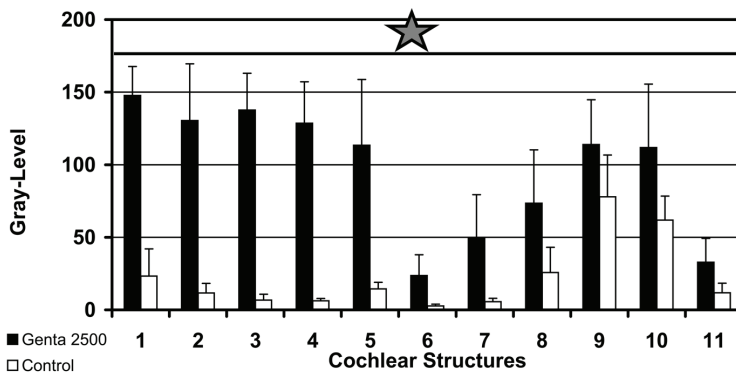


Figure 7. Comparison of controls (white bars) and gentamicin exposed (black bars) cochlear regions for the 2500 $\mu\text{g}/\text{ml}$ condition. Significantly elevated gentamicin fluorescence in comparison to controls is indicated by a star. At this high gentamicin concentration all examined cochlear structures showed a significant elevation of gentamicin fluorescence above background in controls. 1 = inner hair cells, 2 = outer hair cells, 3 = lower spiral limbus, 4 = interdental cells, 5 = dendrites of spiral ganglion cells, 6 = inner sulcus cells, 7 = dorsal spiral ligament, 8 = ventral spiral ligament, 9 = stria vascularis, 10 = spiral ganglion, 11 = Hensen / Claudius cells.

some inner hair cells that appeared red following gentamicin treatment, the quantitative analysis presented in Fig. 6 indicates that red fluorescence following 500 $\mu\text{g}/\text{ml}$ gentamicin treatment significantly exceeded background fluorescence of controls in inner hair cells, outer hair cells, lower spiral limbus, interdental cells, dendrites of the spiral ganglion, inner sulcus cells and the dorsal spiral ligament. For the ventral spiral ligament, the stria vascularis, the spiral ganglion and the Hensen / Claudius cells there was no significant difference between controls and sections from inner ears exposed to 500 $\mu\text{g}/\text{ml}$ gentamicin.

At a gentamicin concentration of 1250 $\mu\text{g}/\text{ml}$ fluorescence in exposed inner ears was generally higher compared to 500 $\mu\text{g}/\text{ml}$. In inner ears exposed to 1250 $\mu\text{g}/\text{ml}$ gentamicin gray levels exceeding a value of 100 were found in outer hair cells, the stria vascularis and the spiral ganglion. Gray levels in the range 50-100 were found for inner hair cells, interdental cells and for the dendrites of the spiral ganglion. The lowest mean gray level of 9 was found for inner sulcus cells but this was considerably higher than the mean gray level of 2 in inner sulcus cells of controls. Compared to controls, all cochlear structures except for the Hensen / Claudius cells showed a significantly elevated fluorescence signal following 1250 $\mu\text{g}/\text{ml}$ gentamicin exposure.

At a gentamicin concentration of 2500 $\mu\text{g}/\text{ml}$ the fluorescence signal was significantly elevated compared to background fluorescence in controls for all 11 cochlear regions analyzed (Figure 7). With the exception of inner sulcus cells, the dorsal and

2B,D, 4B,D). Although visual inspection of the images revealed only some interdental and

cochlear regions analyzed (Figure 7). With the exception of inner sulcus cells, the dorsal and

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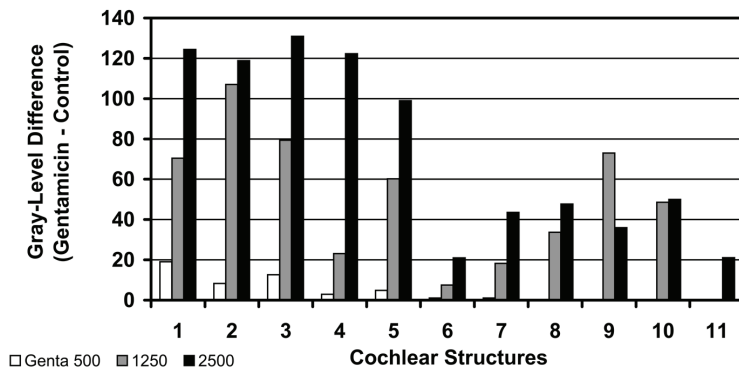


Figure 8. Comparison of gray levels exceeding significantly background fluorescence in corresponding controls for the three gentamicin concentrations (500, 1250, 2500 µg/ml) tested in eleven cochlear structures. Shown is the difference of the gray level in gentamicin exposed regions and corresponding controls. White bars represent exposure to 500 mg/ml gentamicin, grey bars represent 1250 µg/ml gentamicin and black bars represent 2500 µg/ml gentamicin. 1 = inner hair cells, 2 = outer hair cells, 3 = lower spiral limbus, 4 = interdental cells, 5 = dendrites of spiral ganglion cells, 6 = inner sulcus cells, 7 = dorsal spiral ligament, 8 = ventral spiral ligament, 9 = stria vascularis, 10 = spiral ganglion, 11 = Hensen / Claudius cells.

ventral spiral ligament and the Hensen / Claudius cells mean gray level exceeded a value of 100, representing a bright fluorescence. The data in **Figure 7** show that the fluorescence in controls was low.

The results show a dose dependent variation of gentamicin fluorescence in cochlear structures, higher concentrations of gentamicin were associated with a higher fluorescence signal. This was confirmed by a statistical analysis. Pair wise comparisons (Mann Whitney U tests) revealed that rising gentamicin concentration from 500 µg/ml to 1250 µg/ml led to a significant increase of fluorescence (and consequently gentamicin binding) in all 11 cochlear structures analyzed ($p < 0.001$).

Increasing the gentamicin dose further from 1250 to 2500 µg/ml was associated with a significantly increasing fluorescence in inner hair cells, spiral limbus, interdental cells, the dendrites of the cochlear ganglion cells, inner sulcus and dorsal spiral ligament ($p \leq 0.022$), demonstrating a systematic dose dependent increase of gentamicin fluorescence in these cochlear structures for gentamicin concentrations varying between 500 and 2500 µg/ml. In outer hair cells, the ventral spiral ligament, the stria vascularis, the spiral ganglion and the Hensen / Claudius cells no significant increase of fluores-

cence was found for increasing gentamicin concentration from 1250 to 2500 µg/ml ($p > 0.05$) suggesting that gentamicin concentration in the tissue may be saturated by doses below 2500 µg/ml in these cochlear structures.

The variation of background fluorescence between the different cochlear regions in controls (**Figure 6, 7**) complicates the direct comparison of gentamicin fluorescence.

To minimize the contribution of background fluorescence in controls for the evaluation of gentamicin fluorescence, we subtracted the gray level obtained in controls from the value obtained in gentamicin treated inner ears.

Figure 8 compares the difference obtained at the three gentamicin

concentrations (500 µg/ml: white bars; 1250 µg/ml: grey bars; 2500 µg/ml: black bars) for the cochlea. Data are only shown when the difference between control and exposed tissue was significant. This analysis confirms that the highest dose dependent increase of gentamicin fluorescence preferentially occurs in cochlear regions closer to the modiolus (inner and outer hair cells, spiral limbus including interdental cells and the dendrites of the spiral ganglion) while gentamicin fluorescence appeared less pronounced in the dorsal and ventral spiral ligament, the stria vascularis and the cochlear ganglion. Although the inner sulcus cells showed over all a very limited gentamicin fluorescence, fluorescence was significantly higher in the exposed as compared to the control at 500 µg/ml and fluorescence increased systematically by increasing the gentamicin dose to 1250 and 2500 µg/ml. The data in Fig. 8 demonstrate a differential, region specific and dose dependent degree of gentamicin fluorescence in the cochlea.

Vestibular structures

Mean gray levels obtained with exposure to 500 µg/ml gentamicin (black bars) and corresponding controls (white bars) are shown in **Figure 9** for vestibular structures. This comparison demonstrates only for the hair cell / supporting cell

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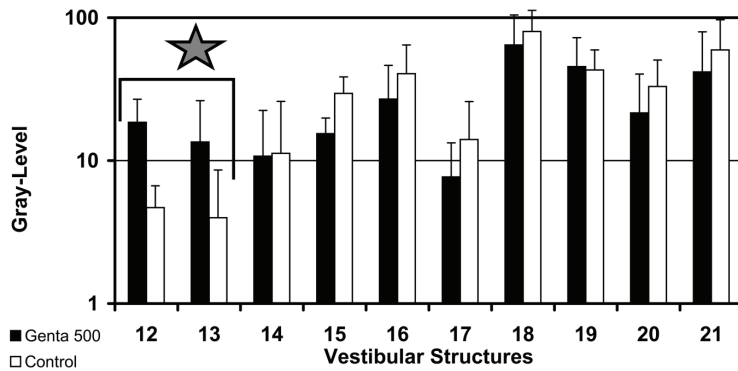


Figure 9. Comparison of controls (white bars) and gentamicin exposed (black bars) vestibular regions for the 500 $\mu\text{g}/\text{ml}$ condition. A significant difference of gentamicin fluorescence between exposed and control conditions is indicated by the star. Note the logarithmic gray level scale to visualize small differences. Only the hair cells / supporting cells and the transitional cell region of the saccule showed a specific gentamicin fluorescence above background fluorescence in controls. 12 = saccule hair/supporting cells, 13 = saccule transitional cells, 14 = saccule nerve fiber region, 15 = utricle hair/supporting cells, 16 = utricle transitional cells, 17 = utricle nerve fiber region, 18 = ampule hair/supporting cells, 19 = ampule transitional cells, 20 = ampule nerve fiber region, 21 = dark cells.

and the transitional cell region of the saccule an obvious increase of fluorescence above background. The statistical analysis confirmed that this increased fluorescence was significant ($p \leq 0.004$; indicated by the asterisk in **Figure 9**). All other vestibular structures (utricle, ampule and dark cells) did not show a significant increase of gentamicin fluorescence above background.

At a gentamicin concentration of 1250 $\mu\text{g}/\text{ml}$ all vestibular regions except the regions of saccular afferent dendrites and the utricular transitional cells showed significantly elevated fluorescence compared to controls ($p \leq 0.025$).

The mean gray levels obtained in the vestibular regions at a gentamicin dose of 2500 $\mu\text{g}/\text{ml}$ are shown in Fig. 10. Compared to the cochlea (**Figure 7**) the gentamicin fluorescence at a dose of 2500 $\mu\text{g}/\text{ml}$ appeared less pronounced in the vestibular regions. For each vestibular region, the mean gray value in the presence of gentamicin was elevated compared to controls. However, most likely due to the limited sample size, this difference was not significant for the transitional cells and the region of afferent dendrites of the utricle (3 exposed versus 5 controls) and the ampullar transitional cells (6 exposed versus 2 controls).

Comparing the mean gray levels following 500 $\mu\text{g}/\text{ml}$ with those following 2500 $\mu\text{g}/\text{ml}$ revealed a significant increasing fluorescence in all vestibular structures ($p \leq 0.024$) except for the region of saccular afferent dendrites. Increasing the gentamicin dose from 1250 to 2500 $\mu\text{g}/\text{ml}$ was only associated with a significant increase of the gray level ($p = 0.025$) for the region of utricular afferent dendrites. In most vestibular regions, significant gentamicin fluorescence compared to controls was found at a concentration of 1250 $\mu\text{g}/\text{ml}$ and further increasing the dose of gentamicin was not associated with increased fluorescence.

The difference of gray levels in the presence of gentamicin and the corresponding controls is shown in **Figure 11** for the vestibular region. Compared to the cochlea (**Figure 8**) the increase of fluorescence above control levels is on average lower in the vestibular regions. In contrast to a number of cochlear regions a systematic dose dependent increase of gentamicin from 500 to 2500 $\mu\text{g}/\text{ml}$ was not seen in the vestibular system. Significant gentamicin fluorescence above background in controls at 500 $\mu\text{g}/\text{ml}$ was only seen in the hair cells /supporting cells and the transitional cells of the saccule.

In conclusion, a 10 minute exposure of the freshly isolated rat cochlea to gentamicin is associated with more pronounced gentamicin fluorescence in the cochlea as compared to vestibular organs (see **Figure 3-5**). At the lowest dose tested (500 $\mu\text{g}/\text{ml}$) significantly elevated gentamicin fluorescence above background in controls was observed in 7 of the eleven analyzed cochlear structures (**Figure 6**), but only in 2 of the 10 vestibular structures (**Figure 9**). A systematically increasing gentamicin fluorescence for doses from 500 to 2500 $\mu\text{g}/\text{ml}$ was found in 7 cochlear structures (**Figure 8**) but not in the vestibule (**Figure 11**). Thus, under these experimental conditions, gentamicin fluorescence is

Gentamicin uptake in the acutely isolated rat inner ear

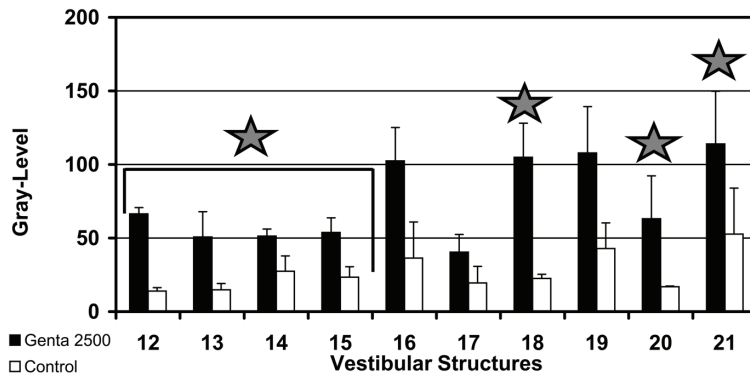


Figure 10. Comparison of controls (white bars) and gentamicin exposed (black bars) vestibular regions for the 2500 µg/ml condition. Significantly elevated gentamicin fluorescence in comparison to controls is indicated by a star. Significant elevation of gentamicin fluorescence above background in corresponding controls was found in seven of the ten examined vestibular structures. Compared to the cochlea (Figure 7) gentamicin fluorescence appeared less pronounced in vestibular structures. 12 = saccule hair/supporting cells, 13 = saccule transitional cells, 14 = saccule nerve fiber region, 15 = utricle hair/supporting cells, 16 = utricle transitional cells, 17 = utricle nerve fiber region, 18 = ampule hair/supporting cells, 19 = ampule transitional cells, 20 = ampule nerve fiber region, 21 = dark cells.

higher in the cochlea and lower in vestibular organs.

Discussion

The isolated cochlea preparation

In the present study we analyzed gentamicin fluorescence in a freshly isolated cochlea preparation following a 10 minute gentamicin exposure. The physiological state of the isolated cochlea differs from the situation in vivo. The disruption of blood and oxygen supply is associated with a very fast decline of the endocochlear potential [22,23] leading to a decreased gain of the cochlear amplifier [24] that will affect hair cell transduction [25,26]. In addition immersing the isolated cochlea in the cold incubation solution will reduce over all metabolic activity.

Although the physiological state of the isolated cochlea differs from the in vivo situation, isolated cochlea preparations have been useful for studying different aspects of cochlear function [e.g. 27,28,29,30,31,32]. The advantage of the isolated preparation with perfusion of gentamicin solution through the scalae is that cochlear and vestibular structures are rapidly exposed to a well defined concentration of gen-

tamicin. Over all, the pattern of gentamicin fluorescence that we found in the cochlear and vestibular structures analyzed shows many parallels and similarities with other published data (for details see discussion below) suggesting that the isolated cochlea is in general useful for analyzing the distribution of gentamicin in the inner ear. For the stria vascularis Wang and Steyger [46] reported prominent gentamicin fluorescence following intraperitoneal application in the mouse that differs from the moderate gentamicin fluorescence that we observed in the stria vascularis following gentamicin exposure of the isolated rat cochlea. While gentamicin applied systemically by intraperitoneal injection enters the cochlea through the vasculature of the stria vascularis [46], gen-

tamicin reaches cochlear structures without passage through the stria vascularis by perfusion of the cochlear scalae (like in the present experiments) or by intratympanic application (that is used clinically). Thus different forms of gentamicin application could explain the different pattern of gentamicin fluorescence in the stria vascularis. An additional potential difference to other published data may be related to the uptake in hair cells. Most other studies report the most prominent presence of gentamicin in inner and outer hair cells [e.g. 19] while our qualitative observations found visible gentamicin fluorescence only in interdental cells and some inner but not in outer hair cells (Fig. 3) at the lowest 500 µg/ml concentration. We suggest that the loss of EP in the isolated cochlea affects gentamicin accumulation in hair cells compared to the in vivo situation.

In summary, interpretation of the present findings in comparison with other studies needs to consider the physiological status in the isolated preparation as well as other methodological aspects outlined above. Bearing this in mind our analysis provides a quantitative description of the distribution of gentamicin fluorescence that is in most aspects consistent with previous qualitative descriptions of the location of gentamicin in the inner ear. Our data allow direct

Gentamicin uptake in the acutely isolated rat inner ear

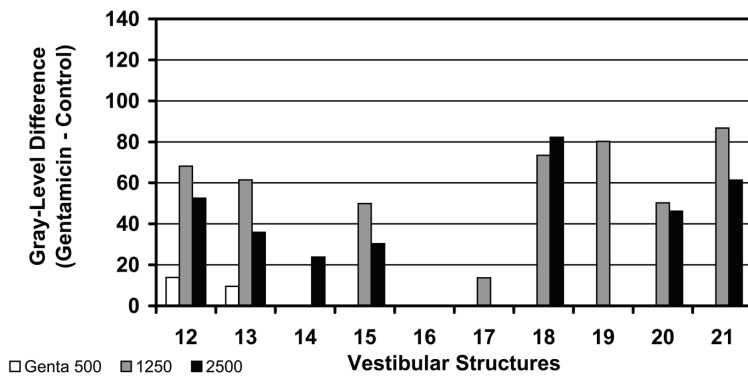


Figure 11. Comparison of gray levels exceeding background fluorescence in corresponding controls for the three gentamicin concentrations (500, 1250, 2500 µg/ml) tested in 10 vestibular structures. Shown is the difference of the gray level in gentamicin exposed regions and corresponding controls. White bars represent exposure to 500 mg/ml gentamicin, grey bars represent 1250 µg/ml gentamicin and black bars represent 2500 µg/ml gentamicin. 12 = saccule hair/supporting cells, 13 = saccule transitional cells, 14 = saccule nerve fiber region, 15 = utricle hair/supporting cells, 16 = utricle transitional cells, 17 = utricle nerve fiber region, 18 = ampule hair/supporting cells, 19 = ampule transitional cells, 20 = ampule nerve fiber region, 21 = dark cells.

comparisons of gentamicin fluorescence for distinct cochlear and vestibular regions (see **Figure 6-11**).

Concentration of gentamicin

An innovation in the therapy of Menière's disease was the local intratympanic application of ototoxic streptomycin in 1957 [9]. The rationale behind this strategy was the aim of a chemical destruction of vestibular hair cells. A severe and undesired side effect was the associated cochleotoxicity. Based on experience with patients treated between 1967 – 1977 with intratympanic aminoglycosides [33] the focus of therapy shifted from destroying vestibular hair cells to interfering with the secretory epithelium involved in inner ear ion homeostasis, and consequently avoiding hair cell loss and cochleotoxicity. This work formed the basis for the empirical establishment of low dose gentamicin therapy that allows sufficient control of Menière attacks without destroying the complete vestibular system and preserving hearing [10,34,11,12,13]. Typically 10-40 mg/ml gentamicin were applied intratympanally [35,13]. Despite successful clinical use of low dose gentamicin therapy the detailed mechanisms of action and the targets of gentamicin within the

inner ear are not fully understood. The present study tried to attempt to identify the cells of the inner ear that accumulate gentamicin within a short period following exposure.

Aminoglycosides have a variety of adverse effects on the inner ear, such as inducing apoptosis in cochlear and vestibular hair cells [36, 37, 38, 9]. High concentrations of 40 mg/ml gentamicin applied via an osmotic pump into the perilymphatic space resulted in a reduction of function of the cochlear and vestibular organs in guinea pigs, whereas lower concentrations (4 mg/ml gentamicin) did not [40].

Most studies have examined the changes of cochlear and vestibular structures following longer time periods (up to months) and higher gentamicin concentrations in comparison to our experiments [compare 41, 42, 43, 19, 44, 40]. We were interested in identifying those cells and structures within the cochlea and the vestibular organs that show elevated concentrations of gentamicin (as indicated by a higher degree of fluorescence) following acute exposure to the lower range of concentrations reached in the inner ear following intratympanic administration of therapeutic doses.

Plontke et al. developed models for the prediction of gentamicin gradients in the inner ear of chinchilla and guinea pig following intratympanic application [45,43]. Their model predicts a high concentration at the base of the cochlea (in the range of 16% of the intratympanic concentration) that rapidly declines towards the apex reaching 1-3 % of the applied intratympanic concentration in the apical half of the cochlea (see **Figure 7** in Plontke et al., 2007 [43]). The concentration of gentamicin solution applied intratympanally for the therapy of Menière's disease is typically 40 mg/ml [e.g. 13]. Based on the Plontke model, we chose to expose the freshly isolated inner ears in the present experiments to gentamicin concentrations of 500, 1250 and 2500 µg/ml because

these concentrations correspond to 1-6% of the intratympanally applied 40 mg/ml solution used for the clinical treatment of Menière's disease [e.g. 13].

Accumulation of gentamicin in the inner ear

A number of studies have analyzed the accumulation of gentamicin in the cochlea in some detail [19,42,46]. Comparable data with respect to the vestibular system are not very detailed [19,42] or restricted to specific end organs [47,48]. A direct comparison of previously published data with our present findings is complicated by methodological differences; however, a number of similarities as well as discrepancies can be identified.

A comparison of **Figure 6 - 8** representing the cochlea with **Figure 9 -11** representing the vestibular organs illustrates a more pronounced gentamicin fluorescence in some cochlear as compared to the vestibular regions analyzed in the present study.

Within the cochlea gentamicin fluorescence at the lowest dose analyzed (500 µg/ml, **Figure 6**) was significantly above background fluorescence predominantly in regions closer to the medial portion of the cochlea (both hair cells types, the spiral limbus including interdental cells, the dendrites of the spiral ganglion cells, and the inner sulcus cells). On the lateral side of the hair cells only the region of the dorsal spiral ligament showed significantly elevated gentamicin fluorescence above background. The stria vascularis, the ventral spiral ligament, the Hensen / Claudius cell region and the spiral ganglion did not show significant elevation of gentamicin fluorescence at this low dose. At the highest gentamicin dose tested (2500µg/ml) all cochlear structures showed elevated gentamicin fluorescence (**Figure 7**). The most pronounced systematic dose dependent increase of gentamicin fluorescence was present in both types of hair cells, the spiral limbus including interdental cells and the dendrites of the auditory nerve fibers (**Figure 8**).

In the vestibular system we found a significant elevation of gentamicin fluorescence only for the saccular hair cells and transitional cells at 500 µg/ml (**Figure 9**). Gentamicin fluorescence increased with increasing dose (**Figure 10**), however, compared to the cochlea the degree of fluorescence appeared more limited

(compare **Figure 11** with **Figure 8**).

Our results are in many aspects consistent with previous reports. A comparison of our data collected after a 10 minute exposure of the inner ear to the gentamicin solution with short survival times (up to 24 hours) reported by Imamura and Adams [19] for the guinea pig following systemic or intratympanal gentamicin application show many parallels. Consistent with our data is the pattern of gentamicin distribution in the spiral limbus including interdental cells, in the dendrites of auditory nerve fibers and a more pronounced fluorescence in the dorsal as compared to the ventral spiral ligament. Our qualitative observation of predominant gentamicin fluorescence at 500 µg/ml in inner but not outer hair cells (**Figure 3**) contrasts with the predominant gentamicin uptake in outer hair cells of the guinea pig. The observation by Imamura and Adams [19] that gentamicin was observed in saccular hair cells at a shorter time following exposure compared to those of utricle and ampule parallels our finding that at the lowest concentration of gentamicin significant increase of fluorescence above background was only seen in saccule.

Roehm et al. report the results of intratympanal gentamicin application in the chinchilla inner ear [42]. They found gentamicin located throughout the infused ear for survival times between 4 hours and 2 weeks following exposure and mention the presence of gentamicin in cochlear and vestibular hair cells, in the spiral ligament, in cochlear and vestibular nerve fibers, in the stria vascularis, in vestibular dark cells and in the neurons of the spiral and Scarpa ganglion. These observations appear at least qualitatively comparable to our findings of the distribution of gentamicin fluorescence.

Wang and Steyger compared the distribution of native gentamicin (demonstrated by immunohistochemistry) with the distribution of Texas-Red-conjugated gentamicin following intraperitoneal application in the mouse cochlea [46]. In contrast to our data in the rat, they describe the most prominent presence of gentamicin for the stria vascularis. In addition, and consistent with our data, they report the distribution of gentamicin in hair cells, the spiral limbus including interdental cells, dendrites of auditory nerve fibers, the spiral ligament and the basilar membrane. The difference in the pattern of gentamicin in the stria vascularis between the

mouse [46] and our data in the rat could be related to the different mode of gentamicin application. We perfused the gentamicin solution directly through small holes in the bony wall of the cochlea at the apex and the basal turn near the round window and the round and oval window, most likely providing direct access of gentamicin to the cochlea and the vestibular organs. In contrast, as suggested by Wang and Steyger the systemically applied gentamicin will enter the cochlea via the capillary network of the stria vascularis.

Steyger et al. [48] report that Texas red conjugated gentamicin was found in hair cells of the isolated bullfrog saccule while Lyford-Pike et al. [47] found Texas-Red-conjugated gentamicin preferentially in Type I hair cells of the chinchilla crista ampullaris. Our observation of gentamicin fluorescence in the hair cell epithelia at 500 µg/ml and above for saccule and at 1250 and 2500 µg/ml for utricle and the ampule (**Figure 9-11**) is consistent with these reports. However, since we included the cochlea as well as the vestibular organs in our quantitative analysis simultaneously (e.g. **Figure 1**) our data suggest, that gentamicin fluorescence is higher in the cochlear compared to the vestibular regions analyzed (**Figure 8,11**).

Considering the hypothesis that the therapeutic effect of gentamicin for treating Menière's disease is associated with a regulation of ion homeostasis [33], the most plausible targets would be those cells primarily involved in regulating the ionic composition of endolymph, especially the cycling of K⁺ in the inner ear.

K⁺ is taken up from the endolymph and released at the basolateral membrane of hair cells in the course of the mechano-electrical transduction process. To maintain the high K⁺ concentration in the endolymph, K⁺ released by the hair cells needs to be returned to the endolymph. For the vestibular system K⁺ is returned to the endolymph by the dark cells [49,50]. In the cochlea K⁺ recycling involves a lateral and a medial pathway [51]. The well characterized lateral pathway involves a shift of the K⁺ released by the hair cells via fibrocytes of the spiral ligament to stria vascularis and secretion by the marginal cells back to endolymph [52,53]. The less well characterized medial pathway involves inner sulcus cells, fibrocytes of the spiral limbus and interdental cells for the transport of K⁺ released by inner hair cells back to the endo-

lymph [54,55,56,57].

At the lowest gentamicin concentration tested (500µg/ml) cochlear structures that showed a stronger gentamicin fluorescence and that are regarded as relevant for K⁺ regulation were spiral limbus including interdental cells and to a lower degree inner sulcus cells and the dorsal spiral ligament (**Figure 6**) while vestibular dark cells showed no significant gentamicin fluorescence (**Figure 9**). These data suggest that at a low dose gentamicin will mainly affect the recycling of K⁺ released by inner hair cells via the medial pathway in the cochlea. The lateral pathway of K⁺ recycling via the spiral ligament and the stria vascularis in the cochlea and vestibular K⁺ recycling via dark cells appear more resistant to low concentrations of gentamicin. At higher doses, gentamicin fluorescence appears more generalized affecting most cell types (**Figure 8, 11**).

Our data on the pattern of gentamicin fluorescence are also consistent with previous studies describing the damage and loss of cells potentially involved in the regulation of K⁺ following aminoglycoside treatment. Imamura and Adams [19] found degeneration in the spiral limbus, the spiral ligament and the stria vascularis following intratympanic application of gentamicin resembling previous reports showing pathological changes following gentamicin treatment in the spiral limbus and interdental cells [58], in the stria vascularis, mainly affecting the marginal cells [16,17] and in vestibular dark cells [21].

Conclusions

Following a 10 minute exposure of the acutely isolated rat cochlea to Texas-Red-conjugated gentamicin at concentrations of 500, 1250 and 2500 µg/ml we found distinct and dose dependent patterns of gentamicin fluorescence in the cochlear and vestibular regions analyzed (**Figure 8, 11**).

Over all gentamicin fluorescence appeared more pronounced in the cochlea compared to vestibular organs, suggesting that the target of a low dose gentamicin therapy may be the cochlea rather than the vestibular organs.

Within the cochlea evaluation of the cells and regions most likely involved in K⁺ recycling, our finding of significant gentamicin fluorescence

predominantly in the spiral limbus including the interdental cells and to a lesser degree in inner sulcus cells and the dorsal spiral ligament (**Figure 6**) suggest that gentamicin at a low dose may rather affect the recycling of K⁺ released by inner hair cells via the medial pathway (inner sulcus, spiral limbus, interdental cells) and to a lesser degree the lateral pathway (fibrocytes of the spiral ligament and the marginal cells of stria vascularis).

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