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Does Vestibular End-Organ Function Recover after Gentamicin-Induced Trauma in Guinea Pigs?

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Abstract

Until 1993 it was commonly accepted that regeneration of vestibular hair cells was not possible in mammals. Two histological studies then showed structural evidence for spontaneous regeneration of vestibular hair cells after gentamicin treatment. There is less evidence for functional recovery going along with this regenerative process; in other words, do regenerated hair cells function adequately? This study aims to address this question, and in general evaluates whether spontaneous functional recovery may occur, in the short or long term, in mammals after ototoxic insult. Guinea pigs were treated with gentamicin for 10 consecutive days at a daily dose of 125 mg/kg body weight. Survival times varied from 1 day to 16 weeks. Vestibular short-latency evoked potentials (VsEPs) to linear acceleration pulses were recorded longitudinally to assess otolith function. After the final functional measurements we performed immunofluorescence histology for hair cell counts. Auditory brainstem responses (ABRs) to click stimuli were recorded to assess cochlear function. As intended, gentamicin treatment resulted in significant loss of utricular hair cells and accompanying declines in VsEPs. Hair cell counts 8 or 16 weeks after treatment did not significantly differ from counts after shorter survival periods. Maximal functional loss was achieved 1–4 weeks after treatment. After this period, only 2 animals showed recovery of VsEP amplitude – all other animals did not reveal signs of regeneration or recovery. In contrast, after initial ABR threshold shifts there was a small but significant recovery. We conclude that spontaneous recovery of otolith function, in contrast to cochlear function, is very limited in guinea pigs. These results support the concept of intratympanic gentamicin treatment where gentamicin is used for chemoablation of the vestibular sensory epithelia.

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Introduction

Sensory hair cells of the inner ear are vulnerable and can be damaged by a variety of sources. These include aging, genetic defects and stresses, such as loud noises or chemotherapeutic drugs such as cisplatin or gentamicin. This causes hearing and balance disorders in millions of people each year.

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Hair cell replacement after damage to the mature inner ear was assumed to be impossible until studies in the late 1980s proved this to be false [Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988]. These studies showed that destruction of cochlear hair cells in birds by intense sound or ototoxic drugs may be followed by the generation of new hair cells. These new cells may become active and may contribute to recovery of auditory function [Hashino et al., 1988; Hashino and Sokabe, 1989; Tucci and Rubel, 1990; Ryals et al., 2013].

Vestibular hair cells undergo repair processes similar to cochlear systems, as indicated by several reports. Jorgensen and Mathiesen [1988] and Roberson et al. [1992] have provided evidence suggesting that new vestibular hair cells are produced at a low rate in the normal adult bird. Weisleder and Rubel [1992] have shown in the bird that repair of the sensory epithelium does occur following pharmacological destruction of vestibular hair cells. The production of new hair cells increases substantially during the repair process.

In 1993, two seminal studies showed histological evidence for regeneration of vestibular hair cells after gentamicin-induced damage in mature mammals [Forge et al., 1993; Warchol et al., 1993]. This is of great clinical relevance because of the balance disorders many people suffer from after treatment with ototoxic drugs. If, for example, regeneration can be stimulated pharmacologically, this may be of help for these kinds of patients. Further, knowledge on regeneration mechanisms and phenomena can be used to induce regeneration of hair cells in the cochlea. Regeneration can occur spontaneously or it can be induced with growth factors or transcription factors. Below, several aspects on spontaneous vestibular regeneration are described, after which examples of induced vestibular regeneration are given.

Since 1993, various other reports have confirmed the existence of spontaneous vestibular regeneration after ototoxic damage in mammals [Cotanche and Lee, 1994; Yamane et al., 1995; Rubel et al., 1995; Lopez et al., 1997; Stone et al., 1998; Zheng et al., 1999; Matsui and Cotanche, 2004]. For a recent review see Rubel et al. [2013]. The degree of ototoxic damage in these studies is typically severe loss of type-I hair cells and substantial loss of type-II cells. Initial signs of hair cell regeneration have been found to occur at 4 weeks post-treatment [Forge et al., 1993; Warchol et al., 1993]. After 8 weeks post-treatment, the number of type-II hair cells recovered to 55% of normal, whereas 90% had been lost after 2 weeks [Lopez et al., 1997]. Forge et al. [1998] showed that some regenerated utricular hair cells did not contain a normal

synaptic afferent bouton. In a recent study where type-I hair cell regeneration was induced with Math1 gene transfer, it was shown that the appearance of the new type-I hair cells was not normal [Xu et al., 2012]. Whether vestibular function may recover in mammals as a result of hair cell regeneration is thus an important question, and this has been addressed by only a few studies [Meza et al., 1992, 1996; Taura et al., 2006].

Meza et al. [1992] showed recovery of vestibular function in guinea pigs treated with streptomycin by assessing nystagmus responses. Meza et al. [1996] studied damage and recovery of otolith function in the rat after streptomycin treatment by swimming behaviour. Taura et al. [2006] assessed hair cell function (mechano-electrical transduction properties) by measuring Ca^{2+} responses in in vitro cultures of rat vestibular macula. Ca^{2+} responses disappeared within days after gentamicin treatment and gradually recovered to a peak 13–17 days after treatment.

The function of vestibular organs has also been studied after induced regeneration [Kopke et al., 2001; Staecker et al., 2007]. Kopke et al. [2001] assessed hair cell function using vestibulo-ocular reflexes in guinea pigs after the vestibules were lesioned with gentamicin. The hair cell renewal was enhanced with growth factor and improvement of function was seen. Staecker et al. [2007] used vestibulo-ocular reflexes and swimming performances as parameters of vestibular function in mice after aminoglycoside-induced damage of the vestibular organ. In this latter study, regeneration was induced with Math1 gene transfer. It showed recovery of the vestibular neuroepithelium within 8 weeks after Admath1.11D treatment, and improved vestibular function compared with mice treated with aminoglycoside only.

The above-mentioned studies used functional parameters which do not directly reflect end-organ function, since they do not rule out the influence of central compensation. Thus, long-term effects of ototoxic damage on the otoliths reflected by direct parameters of end-organ function are not known. To address this, we recorded vestibular short-latency evoked potentials (VsEPs) which reflect end-organ function in guinea pigs [Freeman et al., 1999; Jones and Jones 1999; Oei et al., 2001; Bremer et al., 2012; Chihara et al., 2013]. At various times before and after gentamicin treatment VsEPs were recorded in a longitudinal design. The VsEPs were evoked with linear accelerations in 3 directions in order to assess both saccular and utricular function [Bremer et al., 2012]. Histological evaluation followed after assessment of function. We used relatively long survival periods of up to 16 weeks, since functional recovery might take longer than morphological recovery.
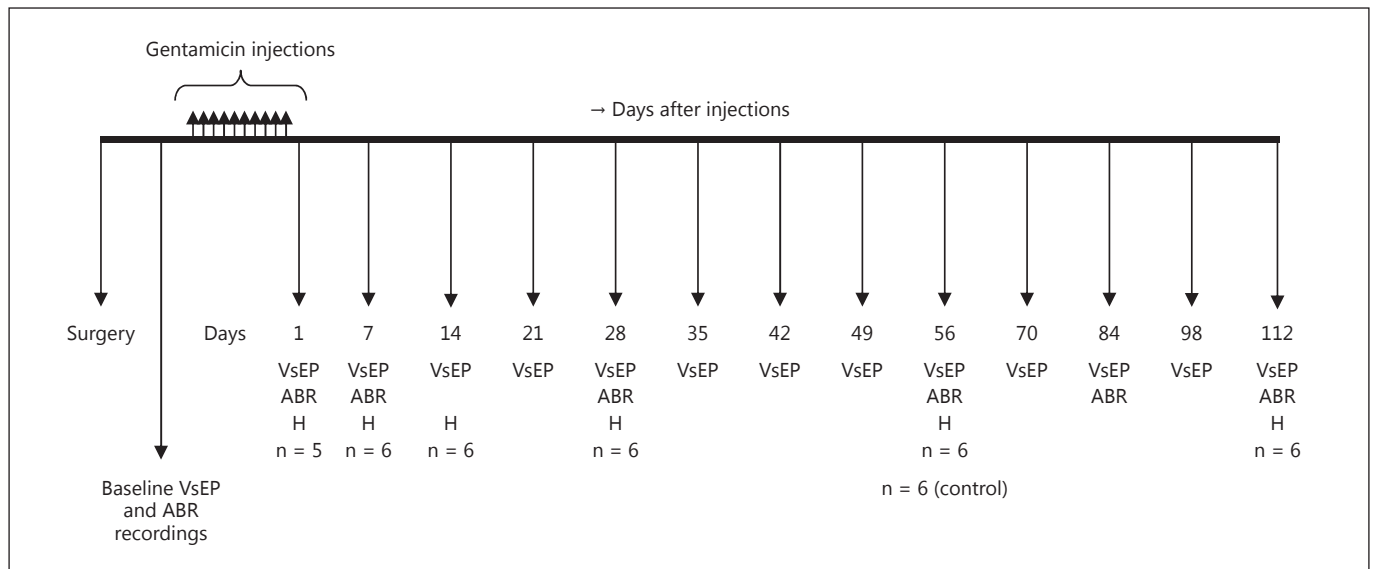
Fig. 1. Schedule of treatment and electrophysiological recordings. Guinea pigs were sacrificed at various survival times (1 day and 1, 2, 4, 8 and 16 weeks). H = Histology. Control animals were measured weekly until T = 56 days. After final recordings animals were prepared for surgery. T = 0 is defined as the 10th day of treatment.

Materials and Methods

Animals and Experimental Design

A total of 41 healthy, female albino guinea pigs (strain: Dunkin-Hartley, weighing 250–350 g) were obtained from Harlan Laboratories (Horst, The Netherlands) and housed in the Central Laboratory Animal Institute of Utrecht University. The animals had free access to both food and water, and they were kept under standard laboratory conditions. The experimental procedures were approved by the University's Committee on Animal Research (DEC 2007.I.07.093). All animals underwent surgery for implantation of a recording electrode. Control electrophysiological measurements (VsEPs and auditory brainstem responses, ABRs) were performed prior to gentamicin treatment. The animals were clustered in groups of 6, and were selected for different survival times, varying from 1 day to 16 weeks (see fig. 1). Electrophysiological recordings were performed 1 week after gentamicin treatment; in animals with a survival time of 1 day, electrophysiological recordings were performed on that final day. Longitudinal measurements were also performed in a control group, consisting of 6 animals, with a survival time of 8 weeks – 3 were given NaCl 0.9% solution instead of gentamicin and the other 3 received no treatment.

After the final functional measurements, the animals were euthanized while sedated by an overdose of sodium pentobarbital (Nembutal®; Ceva Santé Animale, Maassluis, The Netherlands). Utricles and saccules were dissected for immunohistochemistry and quantitative histology.

Surgery

Surgery was performed to enable longitudinal VsEP recordings. The animals were anesthetized with Ketanest-S® (40 mg/kg, i.m.) and Sedamun® (10 mg/kg, i.m.). The skin on the skull was

locally infiltrated with lidocaine 2%. An incision was made from the vertex until 2 cm rostral of the bregma [Bremer et al., 2012]. The skin including periost was incised and lateralized for exposition of the skull. A hole was drilled for a screw that was placed into the epidural space and that served as active electrode for VsEP recordings [Jones and Jones, 1999]. Small holes were drilled in the skull for anchor screws. Dental acrylic cement (ESPE Dental, Gorinchem, The Netherlands) was poured to encase these anchor screws and a coupling screw was placed upside down on the midline. The head of the coupling screw was embedded in the cement, and the exposed threaded post was used to secure the skull via a metal bar to a vibration exciter.

Gentamicin Treatment

Gentamicin was applied according to the protocol used by De Groot et al. [1991] in a cochlear study and by Twine [1985] in a vestibular study. The animals received daily intraperitoneal injections of gentamicin sulphate (Centrafarm Pharmaceuticals, Etten-Leur, The Netherlands) for a period of 10 consecutive days, at a daily dose of 125 mg/kg body weight. Treatment was stopped earlier than the intended period if weight loss was more than 10% of the initial weight.

Measurement of Vestibular Function

VsEPs were recorded weekly or, in case survival lasted beyond 8 weeks, biweekly. The method for recording VsEPs was based on the method originally described by Jones and Jones [1999], but modified to allow for local circumstances and suppliers.

The animals were sedated with Ketanest-S (13.3 mg/kg, i.m.) and Sedamun (3.3 mg/kg, i.m.); these sedation dosages were one third of surgical dosages. The epidural screw served as active electrode, and subdermal needles placed in the neck and dorsum

served as reference and ground electrodes, respectively. For delivering linear acceleration stimuli we used a Brüel and Kjær vibration exciter, type 4809. This device could be orientated in 3 perpendicular planes to inflict head motions in 3 directions. We defined rostrocaudal movements as horizontal medial (HM), intra-aural movements as horizontal lateral (HL), and dorsoventral movements as vertical (see fig. 2d).

The head motions were generated in a custom-made computer programme and consisted of acceleration pulses amplified by a Brüel and Kjær type 2718 power amplifier. The pulses were single haversine waveforms of 0.5 ms and were applied at a rate of 51/s [see Oei et al., 2001]. At this high rate the negative peak, N_1, is not reduced substantially [Jones et al., 2002], while auditory responses, which may contribute to the VsEP, are significantly reduced. Peak amplitudes varied between 1 and 78 m/s². Successive pulses of alternating polarity were used to reduce stimulus artefacts in the recordings. The acceleration pulse, $a(t)$, can be described as follows:

$$a(t) = \frac{b}{2} \cdot \left\{ \sin\left(2\pi ft - \pi/2\right) + 1 \right\}$$

for t between 0 and $1/f$, with f = 2 kHz and b = maximum acceleration. Maximum jerk, a derivative of the acceleration, equals πfb (e.g. for b = 40 m/s², jerk is 250 km/s³). The accelerometer (Brüel and Kjær type 4521), which was attached to the metal bar that connected the animal's head to the vibration exciter, monitored the stimuli during recordings. Electrical activity was amplified (×5,000; EG&G instruments, model 5113), filtered between 0.1 and 10 kHz, and digitized at a 50-kHz sample rate. An example of a recording with the pulse amplitude at 78 m/s² is presented in figure 2a. The VsEP typically consists of 3 or 4 prominent waves occurring within 5 ms after stimulus onset. The positive and negative peaks, P_1 and N_1, occurring with latencies of about 0.75 and 1.0 ms, respectively, have been demonstrated to be reliable indicators for otolith organ function in various rodents [Böhmer, 1995: chinchilla; Jones and Jones, 1999: mouse; Oei et al., 2001; Bremer et al., 2012; Chihara et al., 2013: guinea pig]. Later peaks in the VsEP do not solely originate from nuclei in the vestibular pathway, but may originate from the cranial nerve nuclei [Li et al., 1997: cat] or the auditory nuclei [Böhmer, 1995: chinchilla; Oei et al., 2001; Bremer et al., 2012: guinea pig]. Three control experiments on the VsEP in our laboratory showed that the N_1 peak of the VsEP originates from the end-organ vestibular organs [Bremer et al., 2012]. Furthermore, destruction of the cochlear hair cells with a single co-treatment of kanamycin and furosemide, which leaves the vestibular hair cells intact, did not reduce the N_1 [Bremer et al., 2012], which confirmed that the N_1 is mainly vestibular.

The P_1-N_1 amplitudes and P_1 latencies were analysed as a function of acceleration. Thresholds were determined by visual inspection of the series of P_1-N_1 waveforms recorded at various accelerations (see fig. 3). When a threshold could not be determined because no response was found at the highest possible acceleration of 78 m/s², the threshold was assigned a value of 78 m/s².

Measurement of Cochlear Function
Click-evoked ABRs were recorded immediately before gentamicin treatment, 1 week after treatment and on the final day. Dependent on the survival time, additional recordings were performed (typically with a 4-week interval).

After vestibular recordings, click-evoked ABRs were recorded in sedated condition by use of subdermal needle electrodes. The active electrode was placed at the vertex, the reference electrode in the neck and the ground electrode in the dorsum. ABRs were evoked with monophasic clicks (electric input of speaker: 20 μs). The polarity of each click alternated in polarity from that of the previous click at a rate of 10/s [see Agterberg et al., 2009]. Stimulation was started at 77 dB nHL (approx. 110 dB peSPL) and the level was decreased down to threshold in 10-dB steps. The stimuli were synthesized and attenuated using Tucker-Davis Technologies system 3 (modules RP2, PA5 and HB7; Tucker-Davies Technologies Inc., Alachua, Fla., USA). The clicks were presented with a speaker (Blaupunkt PCxb352), which was held 10 cm from the animal's left ear. The responses were amplified (×5,000) using a differential amplifier (EG&G instruments, model 5113), and A/D converted with a sample rate of 50 kHz (RP2, TDT-3). Response thresholds, more appropriately called 'iso-response levels', were defined as the sound level required to evoke an ABR with an amplitude of 0.3 μV for the most prominent peak. We defined the mean threshold of the normal group as 0 dB nHL.

Tissue Processing for Immunohistochemistry
Utricles and saccules from one side (randomly chosen left or right) were processed for whole-mount fluorescent immunohistochemistry, using phalloidin-rhodamine as a hair cell marker, followed by quantitative evaluation.

After decapitation, the bullae were rapidly dissected out of the skull and immersed in physiological saline. Next, the cochlea was partly obliterated, leaving intact the bony wall separating the vestibule from the basal turn of the cochlea, and the vestibule was opened. The utricle was removed with a set of finely pointed microhooks and a small forceps. The saccule was then gently freed and lifted out from the recessus hemisphericus. The otoconia were removed by a stream of physiological saline applied via a syringe with a 25-gauge needle [Cunningham, 2006]. Next, the utricles and saccules were fixed overnight in 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C. After several rinses in PBS, the utricles and saccules were immersed overnight in 10% EDTA 2Na (pH 7.4) at 4°C to remove remnants of bony tissue. After thorough rinsing in PBS, the utricles and saccules were incubated with rhodamine-conjugated phalloidin (1 μg/ml; Molecular Probes, Leiden, The Netherlands) for 1 h at ambient temperature, rinsed in distilled water, and whole-mounted in Vectashield® mounting medium (Vector Labs, Burlingame, Calif., USA).

Quantitative Assessment: Vestibular Hair Cell Counts
Vestibular hair cells were counted offline in digital images of phalloidin-stained utricles and saccules. Phalloidin is a hair cell marker for both type-I and type-II hair cells. Whole mounts were examined with a Zeiss LSM 510 META confocal laser scanning microscope. At low magnification, the striolar and extrastriolar regions were identified. Then, using a 63× objective, digital images were acquired of two randomly selected areas each in the striolar and extrastriolar regions, in each utricle and saccule. The field of view was 143 × 143 μm. Series of 3-dimensional overlapping image stacks were assembled into a composite image and digitally processed with Adobe® Photoshop Elements. This was followed by a manual offline count of the hair cells.
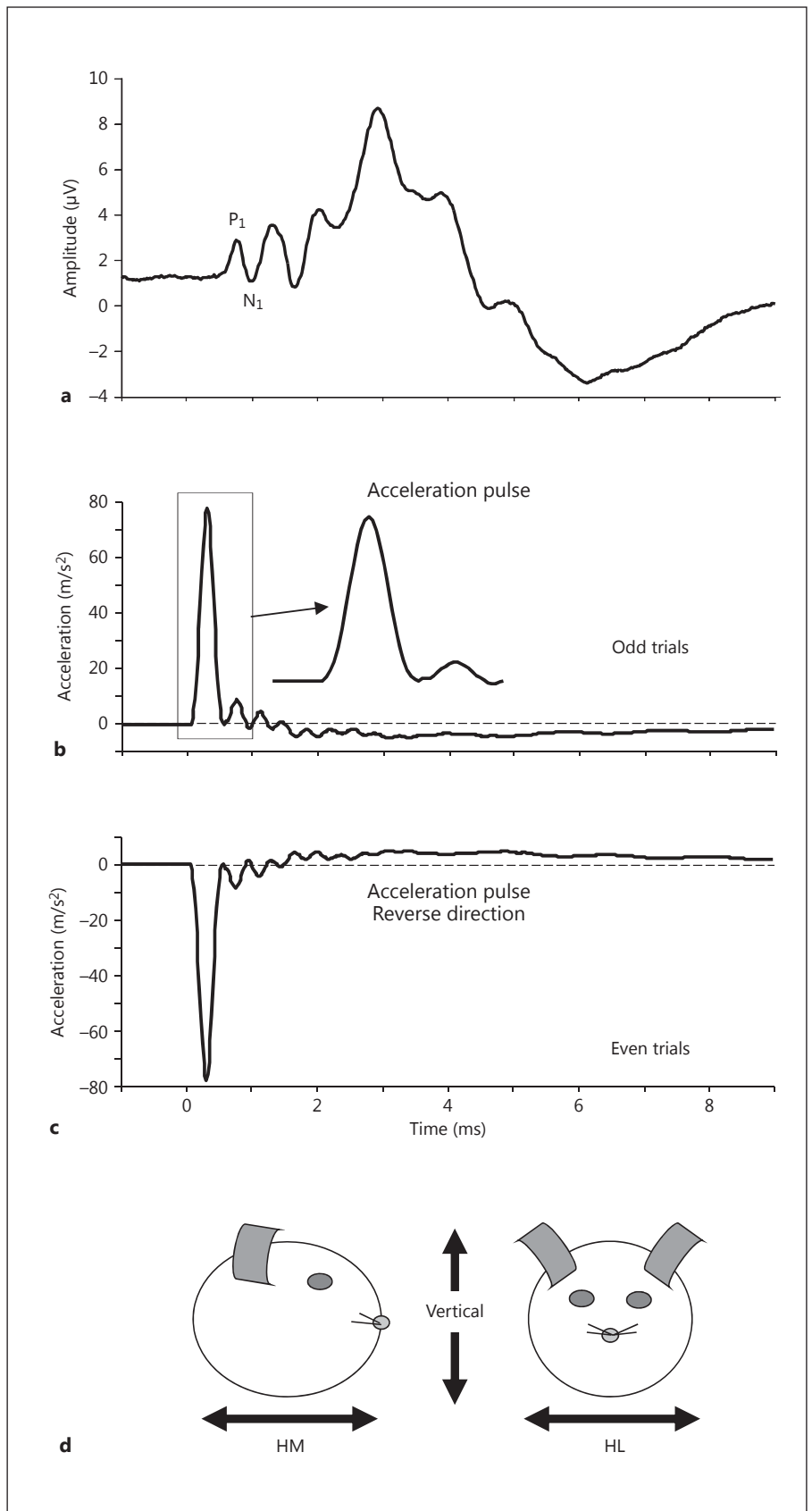
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Fig. 2. a Example of a VsEP recorded at a stimulus level of 70 m/s² in the HM direction in an untreated animal. P_1 and N_1 are indicated. **b** Example of the recorded stimulus waveform. The stimulus is also shown in detail from –0.2 to 1 ms. **c** Recorded waveform of the stimulus in opposite polarity. **d** Schematic drawing to clarify the 3 stimulus directions.
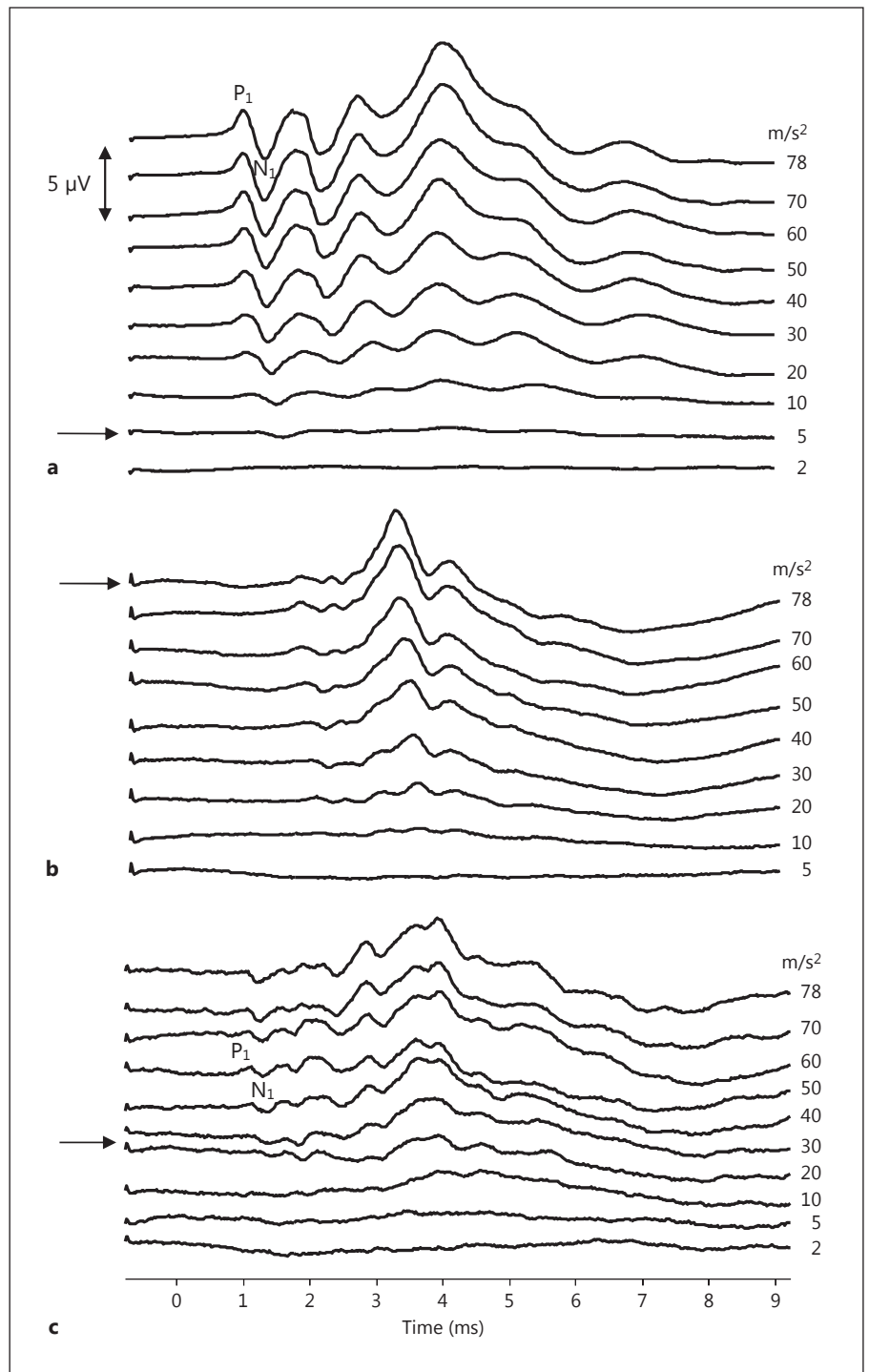
Fig. 3. a A series of VsEPs of a control animal stimulated in the HM direction. **b** A series of VsEPs of another animal treated with gentamicin 3 weeks after treatment stimulated in the HM direction. P_1 and N_1 can no longer be detected. Later peaks are still visible. **c** A series of VsEPs of an animal treated with gentamicin after 16 weeks stimulated in the HM direction. Here, P_1 and N_1 can be detected from stimulus level of 20 m/s². Arrows indicate threshold.

Hair cells were counted as present if they displayed a hair bundle and an intact cuticular plate. The two striolar and two extrastriolar hair cell counts were each averaged to produce one striolar and one extrastriolar density for each utricle and saccule. Hair cell counts were expressed as the average number of hair cells per 1,000 μm².

Statistical Analysis

MATLAB 6.5 software (MathWorks, Inc.) and Microsoft Excel 2001 were used for processing data. For statistical analyses, we used SPSS for Windows (version 20.0). The effect of gentamicin treatment on VsEPs (amplitude, latency and threshold), ABR threshold, and striolar and extrastriolar hair cell counts in the utri-
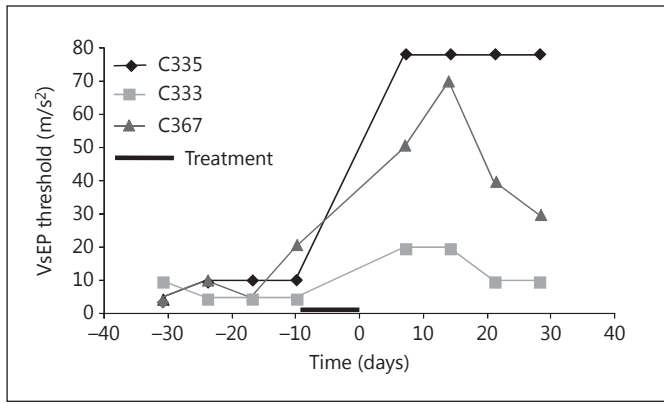
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Fig. 4. Threshold time courses of 3 different animals before and in the first 4 weeks after gentamicin treatment. Baseline thresholds before treatment were stable. After 10 days of treatment the thresholds varied widely among the 3 animals from 20 m/s^2 to ceiling levels (78 m/s^2).

cles and saccules was analysed using repeated measures analysis of variance (rmANOVA) and the Student t test. Linear regression analysis was applied to correlate hair cell counts with survival time, VsEP amplitudes with hair cell counts, and VsEP threshold shifts with ABR threshold shifts. Effects were considered statistically significant when p < 0.05.

Results

Normal VsEPs

Figure 2a shows an example of a VsEP after stimulation in the HM direction. This VsEP consists of 4 prominent waves occurring within 5 ms of stimulus onset. The first two peaks, P_1 and N_1, are of end-organ vestibular function, and later peaks originate from mixed auditory and cranial nerve nuclei (see also Materials and Methods). P_1 and N_1 had latencies varying between 0.7 and 0.8 ms and between 0.95 and 1.05 ms, respectively (at accelerations of 78 m/s^2). The P_1-N_1 amplitude varied between 1.5 and 2.5 μV. An example of the recorded stimulus waveform is shown in figure 2b and c (with the opposite polarity).

VsEP: Short-Term Effects

Figure 3a shows a series of VsEPs of an untreated animal at different stimulus levels. Amplitudes decreased and latencies increased with decreasing stimulus level. A clear P_1 and N_1 were visible down to a low stimulus level (5 m/s^2). Figure 3b shows a series of VsEPs (of another animal) at different stimulus levels 3 weeks after gentami-
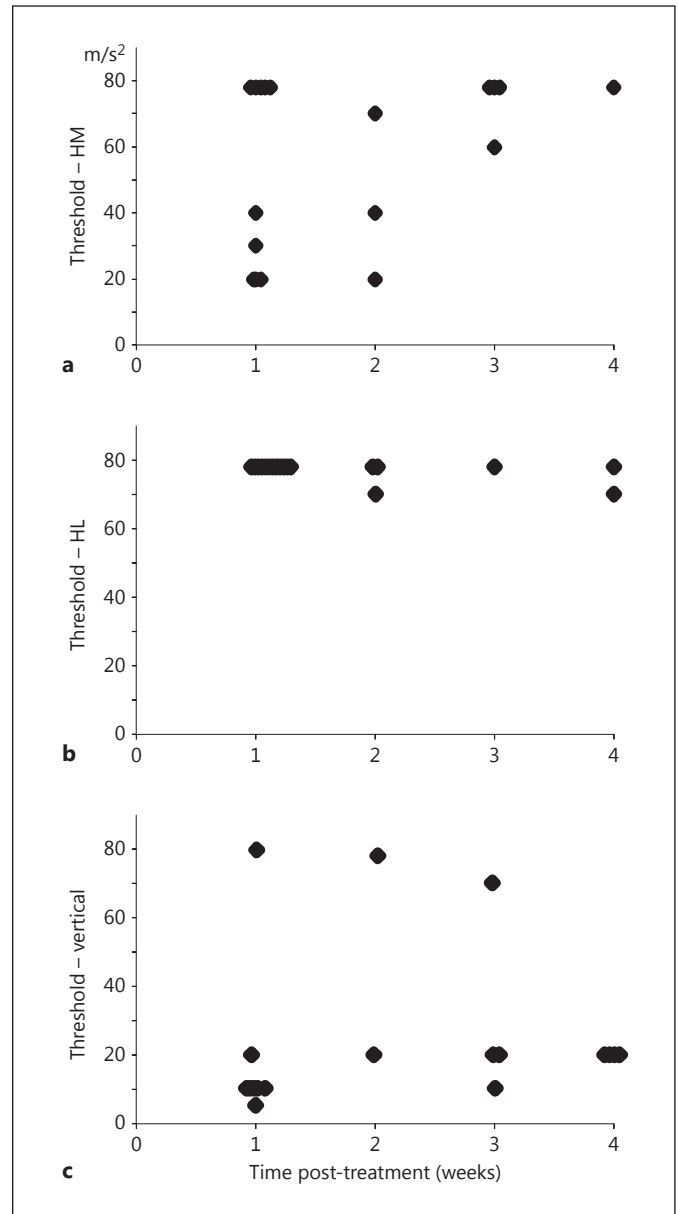

Fig. 5. Maximum VsEP P_1-N_1 thresholds of each animal with survival times of 4 weeks or longer (n = 18) plotted at the first moment the maximum threshold was recorded. **a** Stimulation in the HM direction. **b** HL stimulation. **c** Vertical stimulation. When HL-stimulated, threshold shifts were largest and fastest, and when vertically stimulated, threshold shifts were minimal and relatively slow.

cin treatment. Neither P_1 or N_1 could be detected anymore even at the higher stimulus levels, indicating an abolishment of vestibular end-organ function. Later peaks were still visible, meaning that auditory function was still present. Figure 3c shows a series of VsEPs re-
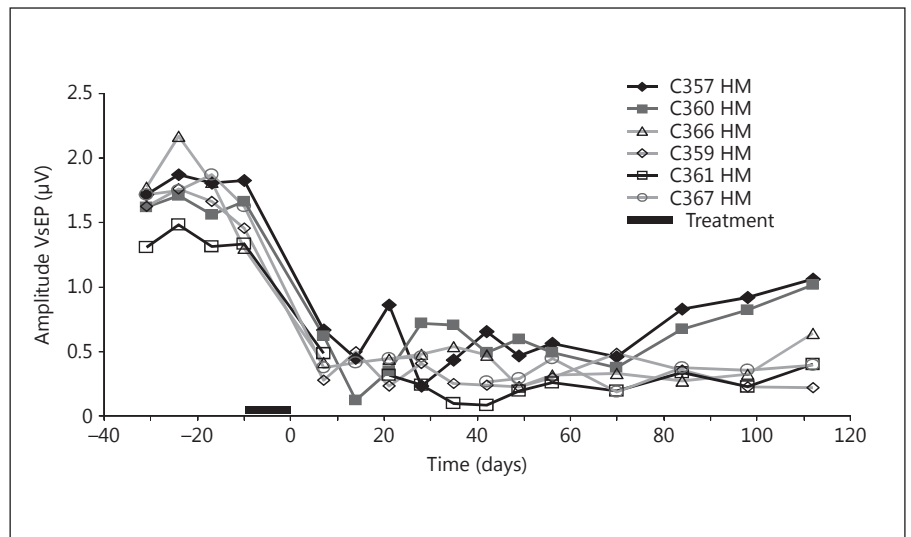
Fig. 6. The individual time courses of the VsEP P_1-N_1 amplitudes of the animals in the 16-week survival group (n = 6, stimulation at 70 m/s^2 in the HM direction).

corded in an animal at 16 weeks after treatment. Now, P_1 and N_1 peaks could be detected again (with similar latencies as in the control animal in fig. 3a) at higher stimulation levels down to 20 m/s^2. Amplitudes were much smaller than in control animals (fig. 3a).

The maximal degenerative effect of gentamicin treatment on electrophysiological function appeared in the first 4 weeks after treatment in all animals. In figure 4, three representative examples of time courses of VsEP thresholds for HM stimulation are given to demonstrate the variability of outcomes. Some animals were recalcitrant against treatment, showing only a small increase in threshold, while others showed maximal threshold shift (i.e. no responses at highest stimulation level). Figure 5 shows the maximum threshold for each animal plotted versus the first moment this threshold has been reached; these data are shown for the 3 directions in the first 4 weeks after treatment. When HL stimulated (fig. 5b), threshold shifts were significantly larger than in the other 2 stimulus directions (paired t tests, p < 0.01). VsEPs measured in the vertical direction (fig. 5c) were modestly affected – for 15 out of 18 animals threshold shifts did not exceed 20 m/s^2. When stimulated in the HM direction (fig. 5a), an increase in threshold was apparent in the first weeks after treatment but not as extensive as in the HL direction – in half of the animals the thresholds reached plateau level (78 m/s^2), while in most of the other animals thresholds were moderately increased (20–40 m/s^2). Maximum threshold shifts to HM stimuli were significantly higher than for vertical stimuli (paired t test, p < 0.001).

VsEP: Long-Term Effects
Amplitudes
Figure 6 shows individual time courses of the P_1-N_1 amplitudes of the 16-week survival group (stimulation in the HM direction at 70 m/s^2). After a steep decline of amplitudes due to ototoxic insult, there was amplitude variability between 0.2 and 0.8 µV until 70 days. After this time point there was a gradual increase in amplitude in 2 animals from about 0.5 to 1 µV; 2 other animals showed a moderate increase and 2 showed no sign of increase or further decrease. Considered over all 6 animals, from 3 weeks after ototoxic insult (when effects were maximal; see also fig. 4, 5) to final recordings after 16 weeks, recovery of VsEPs to HM stimuli appeared to be almost significant (rmANOVA: $F_{8, 40}$ = 2.13, p = 0.056).

Figure 7a–c shows the mean P_1-N_1 amplitude time course of the control group, and the 8- and 16-week survival groups. During control measurements amplitude variability was between 1 and 2 µV. After treatment, there was a rapid decline of amplitude in the experimental groups as described above. Considering both 8- and 16-week groups from a survival time of about 3–8 weeks, there was no systematic trend. As already shown in figure 6, after 60 days there appeared to be a gradual increase in amplitude for the HM direction. Such a trend was present to some extent for the vertical direction but was absent for the HL direction. Statistical analyses over the 3 directions confirmed the trend ($F_{8, 40}$ = 1.85, p = 0.096).
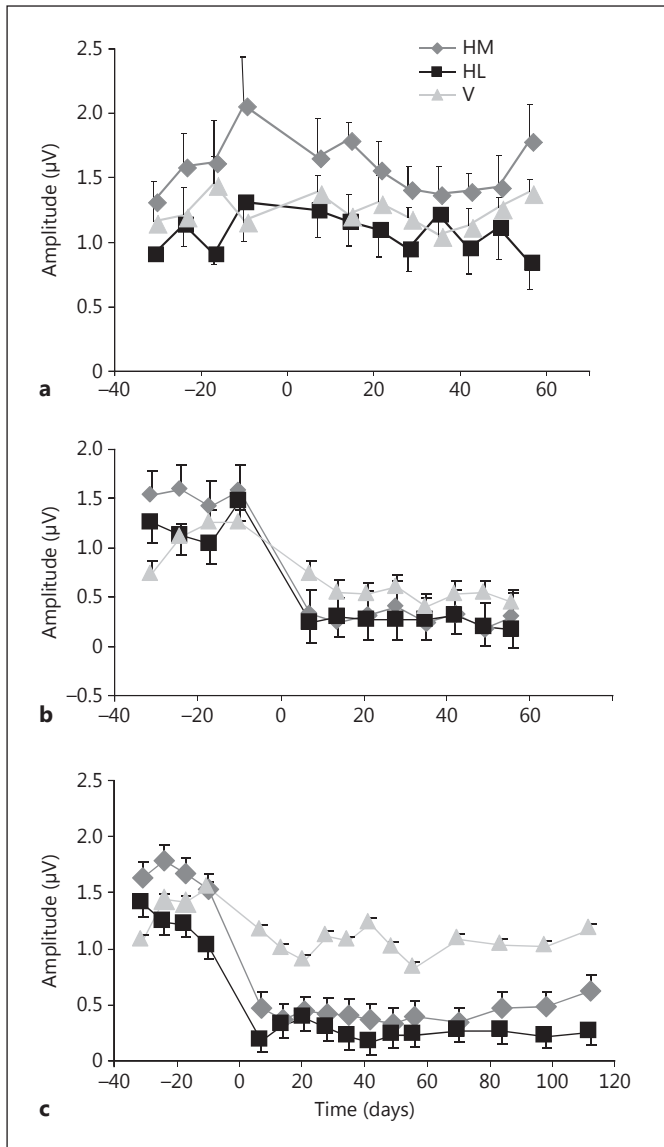
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Fig. 7. Averaged P_1-N_1 VsEP amplitudes for the 3 directions. V = Vertical. **a** Control group (n = 6). **b** 8-week group (n = 6). **c** 16-week group (n = 6). Error bars represent standard error of mean (SEM). Day 0 corresponds to the 10th day of treatment.
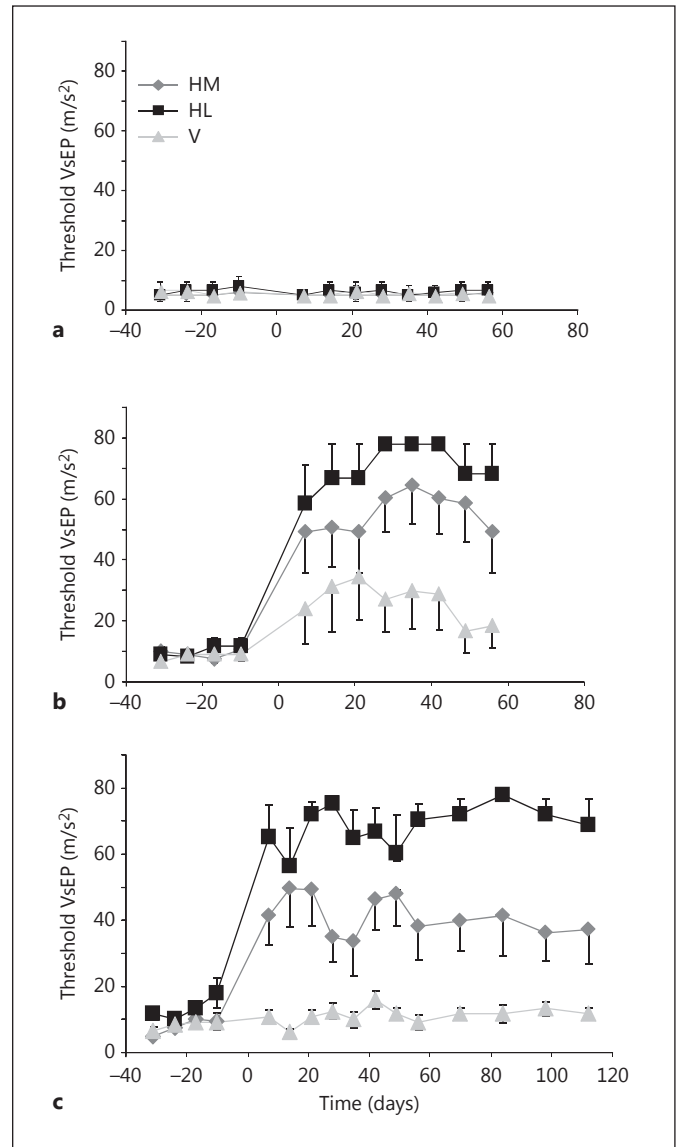
Fig. 8. VsEP P_1-N_1 thresholds for the 3 directions. V = Vertical. **a** Control group (n = 6). **b** 8-week group (n = 6). **c** 16-week group (n = 6). Error bars represent SEM. Day 0 corresponds to the 10th day of treatment.

Thresholds

Figure 8a–c shows the mean threshold time course of the control group and the 8- and 16-week groups. The control group showed stable VsEP thresholds over 16 weeks of between 5 and 10 m/s². In the 8-week group there was a tendency of a threshold decrease after 30 days for all 3 stimulus directions (i.e. recovery). This was not statistically significant considering 5 time points post-treatment (3–8 weeks; rmANOVA: $F_{4,44}$ = 2.0, p = 0.12).

In the 16-week group such recovery did not appear to manifest beyond 8 weeks – there was a more or less stable threshold value showing neither decrease nor increase (rmANOVA: $F_{8,40}$ = 0.43, p = 0.89).

Latencies

In figure 9a–c the latencies are shown for the control group and the 8- and 16-week groups. In the control group latencies were stable over time (fig. 9a). Across all
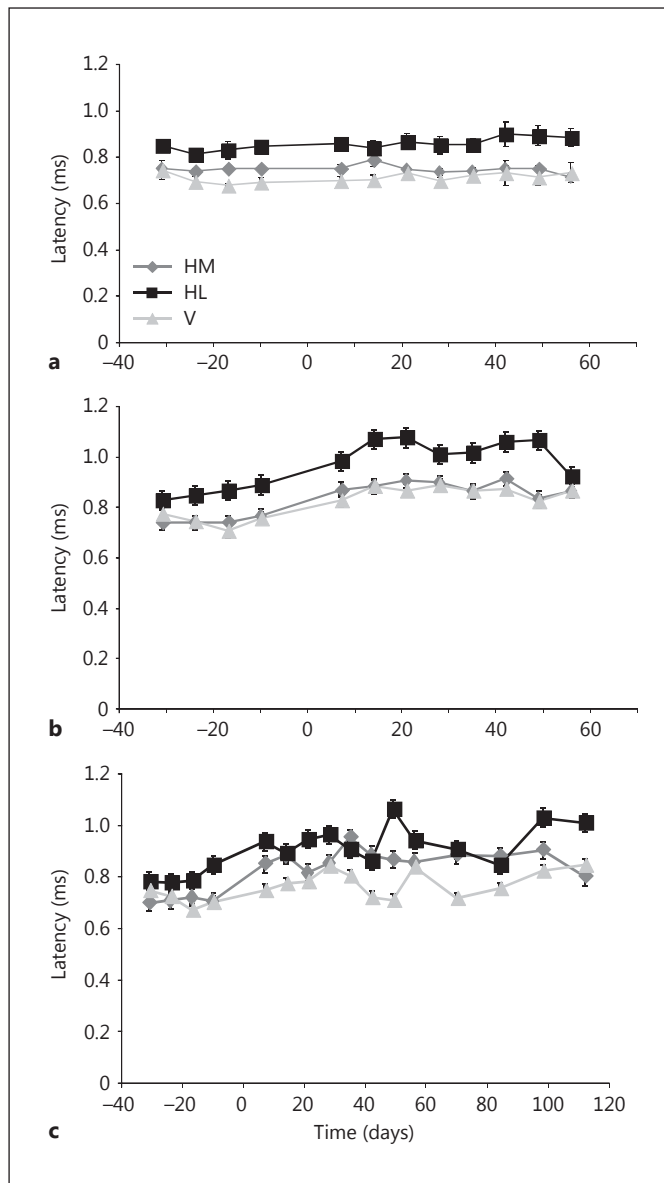
Fig. 9. VsEP P_1 latencies for the 3 directions. V = Vertical. **a** Control group (n = 6). **b** 8-week group (n = 6). **c** 16-week group (n = 6). Error bars represent SEM. Day 0 corresponds to the 10th day of treatment.

groups, HL stimulation resulted in longer latencies than HM and vertical stimulation (before treatment: 0.83 vs. 0.73 ms). Treatment resulted in significant latency increases of about 0.1 ms for each stimulation direction (rmANOVA: $F_{5,85} = 30.8$, $p < 0.0001$), as illustrated in fig. 9b and c. A decrease (or increase) in latency was observed in neither the 8- nor the 16-week group over time (rmANOVA: $F_{4, 44} = 0.73$, $p = 0.58$).

Histology

Figure 10 shows immunohistological examples of areas in the striolar region of a normal utricle and of utricles at 1 day and at 2, 4, 8 and 16 weeks after gentamicin treatment. In the gentamicin-treated utricles, from 1 day after treatment, numerous scars were detected in the striola, indicating hair cell loss (fig. 10c, d). In the 4- and 8-week group there was stable loss in hair cell density (fig. 10e, f). High-resolution imaging revealed the characteristic cartwheel-like staining pattern ('scars') displayed by the supporting cells at the site of the missing hair cells [Meiteles and Raphael, 1994]. The extrastriolar region of the gentamicin-treated utricles also showed scars (not shown). No scars were observed in the zones of the saccule. In the example of 16 weeks after treatment shown here (fig. 10g), there was a larger hair cell density than after shorter survival times (fig. 10c–f). Besides this indication of hair cell regeneration, this specific animal showed functional recovery as seen by the stable increase in VsEP amplitude (animal C360, see fig. 6).

Figure 11 shows mean hair cell counts for the different survival times. The saccule was not affected by the treatment protocol used in this study, comparable to the functional measurements recorded in the vertical direction (one-way ANOVA: $p = 0.85$). In contrast, hair cells in the utricle diminished substantially after treatment, as well as in the striolar and extrastriolar region (one-way ANOVA: $p < 0.001$). Maximum loss was at 2 and 4 weeks after treatment. After 4 weeks the hair cell counts slightly increased in both zones of the utricle – more apparent in the striolar than the extrastriolar regions, as shown in the upper left histogram. This was not statistically significant (linear regression: $p = 0.23$).

Figure 12 shows the correlation between the VsEP P_1-N_1 amplitudes found to HM stimuli (at 70 m/s^2) and the number of utricular hair cells across all experimental groups (the different gentamicin-treated animals and controls). There was a clear overall positive correlation between function and hair cell counts (linear regression: $R^2 = 0.36$, $p < 0.001$). In contrast, there was no correlation ($R^2 = 0.00$) between function and hair cell counts over only the gentamicin-treated animals (without controls). Indeed, in several of those animals, in spite of only modest hair cell loss, VsEP amplitudes were relatively small. Considering the 2 animals in the 16-week group that showed functional recovery (fig. 6), 1 (same animal as in fig. 10g) showed relatively high hair cell counts (6,400) compared to the average number of hair cells in that group (5,400) and in other experimental groups (approx. 4,800 in the 2-, 4- and 8-week groups). The other animal
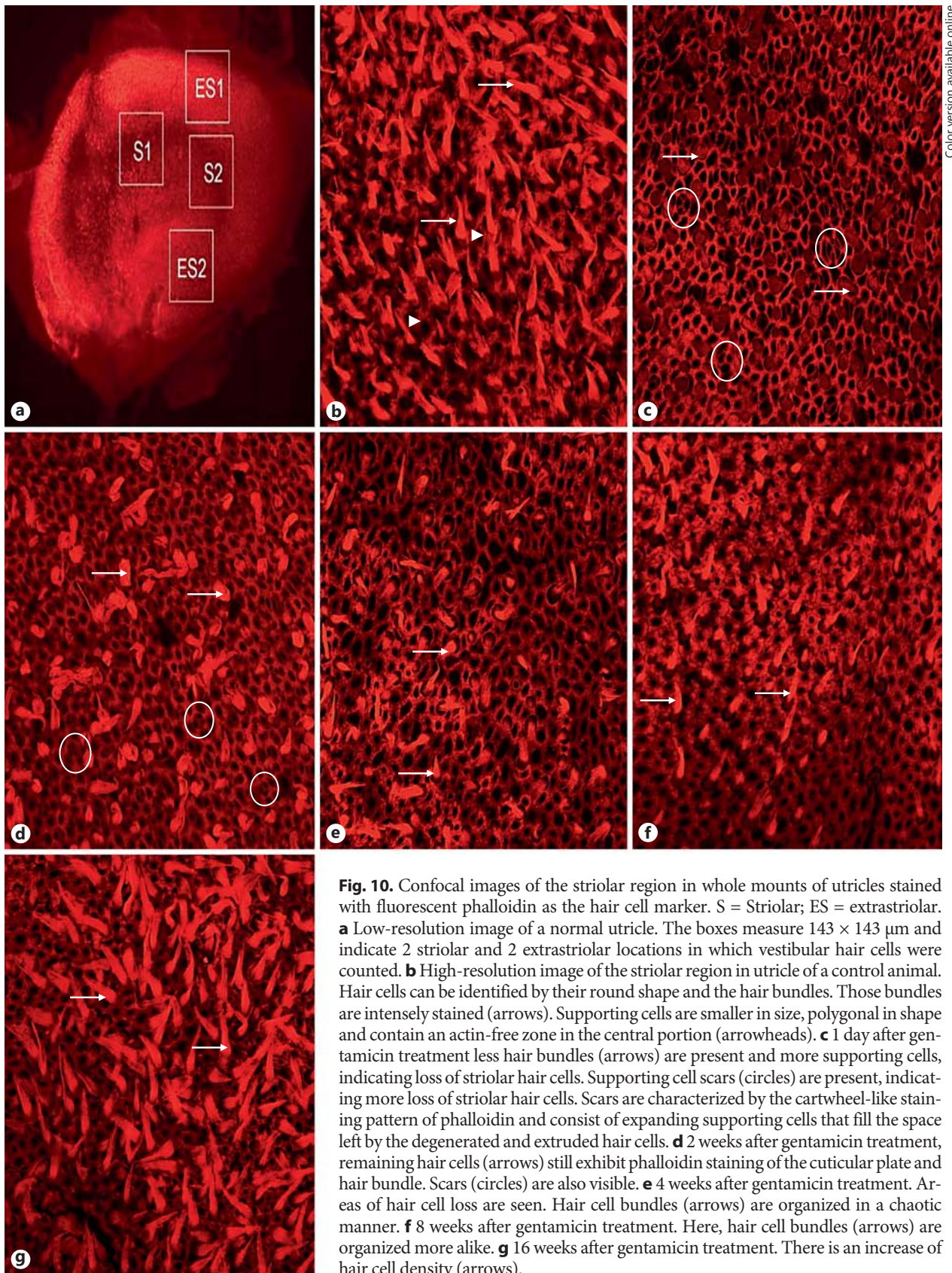
Fig. 10. Confocal images of the striolar region in whole mounts of utricles stained with fluorescent phalloidin as the hair cell marker. S = Striolar; ES = extrastriolar. **a** Low-resolution image of a normal utricle. The boxes measure 143×143 μm and indicate 2 striolar and 2 extrastriolar locations in which vestibular hair cells were counted. **b** High-resolution image of the striolar region in utricle of a control animal. Hair cells can be identified by their round shape and the hair bundles. Those bundles are intensely stained (arrows). Supporting cells are smaller in size, polygonal in shape and contain an actin-free zone in the central portion (arrowheads). **c** 1 day after gentamicin treatment less hair bundles (arrows) are present and more supporting cells, indicating loss of striolar hair cells. Supporting cell scars (circles) are present, indicating more loss of striolar hair cells. Scars are characterized by the cartwheel-like staining pattern of phalloidin and consist of expanding supporting cells that fill the space left by the degenerated and extruded hair cells. **d** 2 weeks after gentamicin treatment, remaining hair cells (arrows) still exhibit phalloidin staining of the cuticular plate and hair bundle. Scars (circles) are also visible. **e** 4 weeks after gentamicin treatment. Areas of hair cell loss are seen. Hair cell bundles (arrows) are organized in a chaotic manner. **f** 8 weeks after gentamicin treatment. Here, hair cell bundles (arrows) are organized more alike. **g** 16 weeks after gentamicin treatment. There is an increase of hair cell density (arrows).
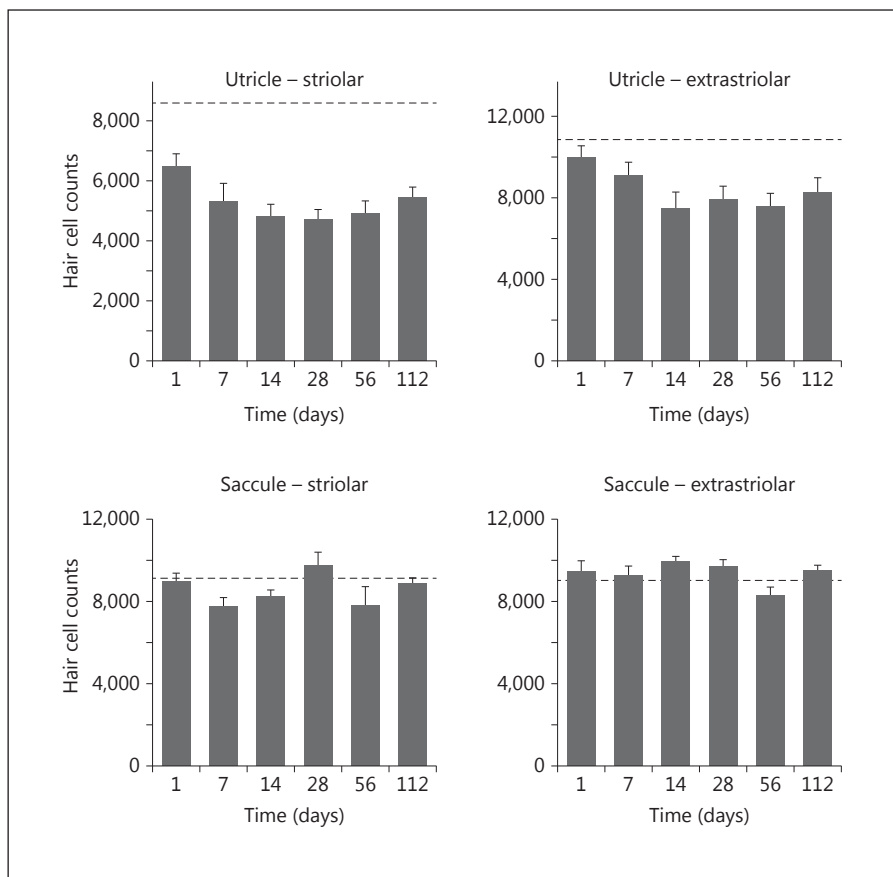
Regeneration of Vestibular Hair Cells
after Gentamicin Treatment

Fig. 11. Histograms on hair cell counts of the different survival groups (averaged data) for the striolar and extrastriolar regions in the utricle and saccule. The dotted lines in the figures represent hair cell counts of the control animals. Error bars represent SEM.
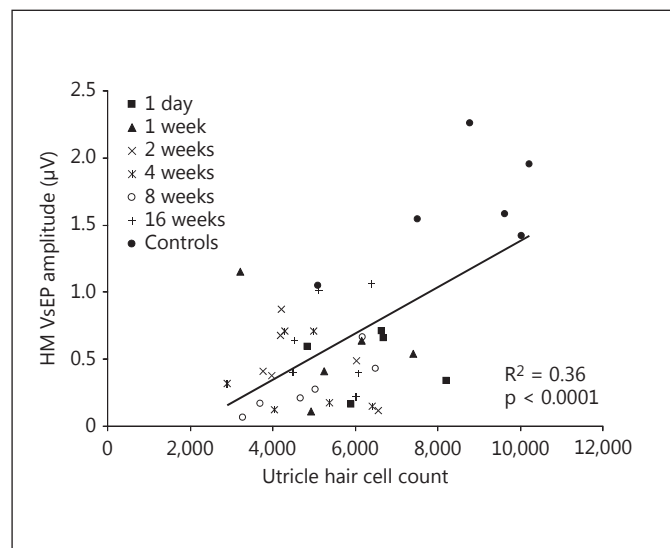

Fig. 12. Correlation between VsEP amplitudes and utricular hair cell counts. Stimulation for the VsEPs was in the HM direction at 70 m/s^2. Hair cell counts were done in the striolar regions of the utricles.
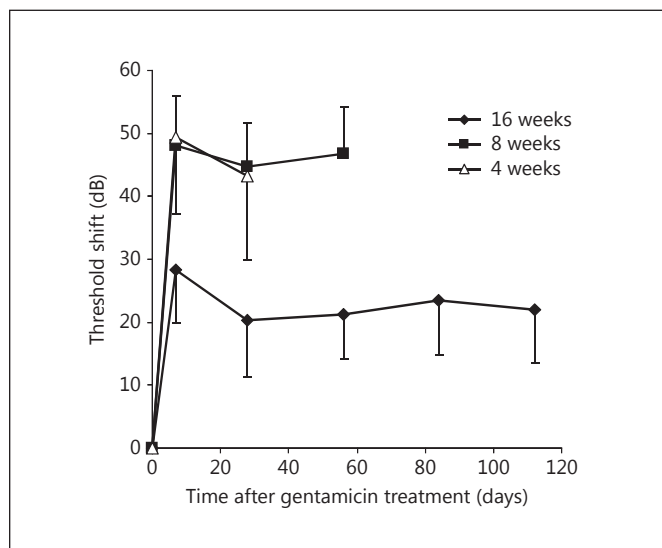

Fig. 13. Mean click-ABR thresholds for the 4-, 8- and 16-week groups. Error bars represent SEM.

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that showed functional recovery had a hair cell count (5,120) comparable to the average number of hair cells in that group. There is some correlation within the 8- and 16-week group between amplitudes and hair cell count ($R^2 = 0.24$, $p = 0.11$).

ABRs

Figure 13 shows the mean click-ABR thresholds over time for the 4-, 8- and 16-week groups. There was a significant threshold shift 1 week after ototoxic insult, which was about 40 dB on average and which varied quite substantially from 7 to 80 dB. After these initial threshold shifts there was a small but significant recovery between 1 and 4 weeks (6 dB; $p < 0.01$, $n = 17$).

VsEP versus ABR Thresholds

We examined whether VsEP threshold shifts correlated with ABR threshold shifts. We considered VsEPs in HM direction since those showed the largest variance. It appeared that ABR and VsEP threshold shifts 1 week after gentamicin treatment were not correlated ($R^2 = 0.049$, $p > 0.3$) – there were small ABR threshold shifts with both small and large VsEP threshold shifts and vice versa. Changes in thresholds from 1 to 4 weeks were not correlated either ($R^2 = 0.009$, $p > 0.7$). Out of 4 animals with a large ABR threshold recovery (>15 dB), 2 did not show recovery in the VsEP whereas 2 did show VsEP recovery after 4 weeks. Of the 2 animals showing VsEP recovery after 16 weeks (fig. 6), 1 had shown ABR recovery but the other had not.

Discussion

This study directly assessed otolith function after significant gentamicin-induced utricular hair cell loss (approx. 45%) at several survival times up to 16 weeks. Functional assessment by recording VsEPs and using survival times of such long periods (16 weeks) has not been applied in previous studies. We found no significant recovery of end-organ function, while the number of hair cells was stable with survival time. This contrasts with the observed significant recovery of cochlear function in the same animals.

Short-Term Effect of Gentamicin Treatment

Gentamicin treatment has a damaging effect on utricular hair cells [Wersäll et al., 1969; Forge et al., 1993; Perez et al., 2000; Gale et al., 2002; Lyford-Pike et al., 2007; Lue et al., 2009], which is also clearly demonstrated in this study. In our study discrimination between the two types of hair cells was not possible but based on above-mentioned studies we assume mostly type-I hair cells were damaged. Furthermore, along with hair cell loss, gentamicin treatment caused a significant reduction in VsEP amplitude and an elevation in VsEP threshold as found in other studies [Elidan et al., 1987; Perez et al., 2000].

Long-Term Effect of Gentamicin Treatment

After ototoxic insult, we explored the longer-term histological aspects and functional phenomena of the otoliths using direct end-organ parameters (i.e. VsEP amplitude, threshold and latency). Overall, the end-organ parameters were stable between 2 and 16 weeks (fig. 7–9); there were some tendencies of hair cell regeneration (fig. 11) and recovery in the utricle (fig. 6) but these were not significant. Thus, the capacity for spontaneous regeneration of hair cells in the mammalian inner ear and corresponding functional recovery is limited, even when studied in the longer term (16 weeks). In mammals, although morphological evidence is available pointing to regeneration of hair cells [Forge et al., 1993; Warchol et al., 1993; Sobkowicz et al., 1996; Lopez et al., 1997; Zheng et al., 1999] functional recovery was not confirmed using direct end-organ parameters. Studies that had shown functional recovery after ototoxic treatment used functional parameters that were (partially) based on central compensation [Meza et al., 1992, 1996; Taura et al., 2006; Staecker et al., 2007]. In this study we used parameters of direct end-organ function, so the effects of central compensation did not bias the longer-term results. There was a wide spread of functional outcomes for animals with relatively high hair cell counts (fig. 12), and thus a low correlation between function and hair cell number in the long-survival groups (16 weeks). Forge et al. [1998] showed that regenerated utricular hair cells did not contain a normal synaptic afferent bouton even at a survival time of 8 months. In a recent study where type-I hair cell regeneration was induced with Math1 gene transfer it was shown that the appearance of the new type-I hair cells was not normal [Xu et al., 2012]. Thus, even if hair cells were regenerated in some of the animals, it would not automatically imply that function improved. In animals with poor VsEPs and relatively high hair cell counts (shorter survival times, i.e. the 1-, 2- and 4-week groups), a subpopulation of hair cells may not function well because of intracellular damage that was not detected with immunofluorescent microscopy. The gradual recovery of VsEPs after 16 weeks in 2 animals (fig. 6) may be related to hair cell proliferation indeed or it may be caused by other mechanisms like self-repair [Zheng et al., 1999; Gale et al.,

2002; Taura et al., 2006; Jia et al., 2009]. Self-repair refers to repair of damaged cellular structures. In the aforementioned studies the recovery was mostly due to reorganizing of the hair bundles back to the normal prelesional situation, which included repair of ruptured tip links confirmed by restoration of the mechano-electrical transduction responses [Jia et al., 2009], and restoration of extruded stereocilia bundles and part of the cell's apical cytoplasm.

In the cochlear system there was a significant but mild recovery of the ABR threshold shifts after 7 days. This recovery is often seen in the cochlea for a wide range of hearing losses after various types of insults such as noise [Miller et al., 1963], cisplatin [Klis et al., 2002] and combined treatments of kanamycin and furosemide [Versnel et al., 2007; Havenith et al., 2013]. In the mammalian cochlea self-repair mechanisms contribute to recovery of hearing [Jia et al., 2009]. Assuming self-repair underlies functional recovery, our data suggest that self-repair does not occur in the mammalian vestibular system as much as in the cochlea. This is consistent with the lack of correlations between the ABR threshold shifts and the VsEP threshold shifts over the longer term.

We applied the same type of strains (Dunkin-Hartley) and ototoxic model (10 days of intraperitoneal gentamicin injections) as in studies that demonstrated vestibular hair cell regeneration [Forge et al., 1993; Warchol et al., 1993; Lopez et al., 1997]. One reason why our study did not show evidence for hair cell regeneration may be that the initial gentamicin-induced loss of hair cells was not as severe (45%) as in those studies (90%).

Saccule versus Utricle

There was a significant difference in outcomes between horizontal measurements, which reflect mainly utricular function, and vertical measurements, which reflect mainly saccular function [Plotnik et al., 1999; Bremer et al., 2012]. When stimulated in the vertical plane there was a limited loss of function (fig. 7, 8), which corresponded to a near-normal hair cell count in the saccule (fig. 11). Apparently the saccular hair cells also did not suffer from functional disturbances. In both the horizontal planes there was a salient loss of function (fig. 7, 8). This corresponded to significant hair cell loss in the utricle (fig. 11), which is congruent with findings in the literature. For instance, Lindeman [1969] and Li et al. [1995] showed more hair cell loss in the utricle than in the saccule under the same ototoxic conditions. Bremer et al. [2012] showed an effect of a 100 mg/kg gentamicin (lower dose than the current study) on utricular function (VsEPs to horizontal stimuli) but not on saccular function (VsEPs to vertical stimulation).

This study assessed function of the otolith organs only and not that of the semicircular canals. The latter are stimulated by rotational rather than linear acceleration stimuli used in this study. The results of this study can therefore not be extrapolated to the semicircular canals.

Clinical Implications

Because of the limited regenerative capacities of the vestibular end-organs shown, finding a good treatment modality based on repopulation of hair cells remains challenging, even when induction is applied.

Ménière's disease is an invalidating illness characterized by attacks of vertigo with hearing loss, tinnitus and/or aural fullness of the affected ear. Intratympanic gentamicin treatment is often used for chemoablation of the vestibular hair cells in these patients. In this treatment, gentamicin solution is applied in the ear with the aim of damaging the vestibular hair cells, resulting in a decrease (or disappearing) of vertigo attacks [Blakley, 1997; Minor et al., 2004]. The treatment makes use of the selective vestibulotoxicity of gentamicin, causing vestibular damage. In these cases, it is undesirable that eliminated vestibular hair cells regenerate and function recovers, possibly leading to recurrence of vertigo complaints. So the main results of this study (i.e. mostly stable functional loss after gentamicin-induced trauma) are in favour of this treatment. On the other hand, limited functional recovery may be the reason that, in some cases, several treatments with intratympanic gentamicin are necessary.

Future Considerations

Spontaneous vestibular regeneration is limited in mammals, as confirmed in the current study. Therefore, histological regeneration and functional recovery must be induced, for example with growth factors, neurotrophic treatment and gene expression profiling, as has already been shown in several studies [Kopke et al., 2001; Xu et al., 2012; Brigande and Heller, 2013; Jung et al., 2013]. The question remains whether end-organ function recovers after (induced) regeneration of hair cells.

Acknowledgements

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