Damage and Repair in the Inner Ear:

From Experimental Research to Clinical Aspects

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Damage and Repair in the Inner Ear:

From Experimental Research to Clinical Aspects

Schade en herstel in het binnenoor; van experimenteel onderzoek tot klinische aspecten.
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 11 december 2013 des middags te 16.15 uur

door

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geboren op 29 mei 1977
te Delft

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Voor Poem

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1.1 BACKGROUND

This thesis is about the toxic side effects of aminoglycoside drugs on the inner ear, which contains the sensory organs of the vestibular system, and the sensory organ involved with hearing. Aspects that we studied were the short- and long-term effects, following ototoxic medication induced damage, and the regenerative capacity of the vestibular system in an animal model. Finally, the ototoxicity is clinically applied as treatment modality in vestibular inner ear disease in human patient population. The emphasis of this thesis lies on the vestibular system; however, because the auditory system is so closely related to the vestibular system a portion of this thesis is dedicated to the auditory system as well.

In total, six receptor organs are located in the inner ear: the cochlea, a sensory organ with the primary purpose to aid in hearing and the utricle, saccule, the lateral, anterior, and posterior semicircular canals which together constitute the sensory organs of the vestibular system. The inner ear contains two distinct extracellular fluids, endolymph and perilymph, which are separated by a complex structure of membranes resulting early anatomists to call it the labyrinth. The vestibular system enables a person to maintain balance, visual fixation and is in part responsible for our spatial orientation. Our visus and propriocepsis are part of the vestibular system. The vestibular end organ has highly differentiated receptor organs. The three semicircular canals detect angular acceleration; the utricle and saccule (the otolith organs) are sensors for detecting linear acceleration.

The inner ear sensory organs contain hair cells, which are the sensory receptors. These hair cells convert sound vibrations and head movements into electrochemical signals that are conveyed to the brain. Damage and loss of these hair cells occur due to e.g., aging, exposure to noise, medications, disease, genetic disorders and head trauma, resulting in hearing and vestibular impairment in millions of people world wide each year.

Currently, severe vestibular dysfunction is incurable. The relief of vestibular dysfunction primarily depends on adaptation by compensation of central vestibular function; the brain learns to ignore, adjust to or compensate for the false signal caused by vestibular dysfunction and is known as central compensation [Curthoys, 2000; Dieterich and Brandt, 2008]. There is evidence for the substitution of other sensory input and responses during vestibular compensation where other sensory parts of the vestibular system, e.g. visus and propriocepsis, ‘take over’ the lost function of the affected vestibular end-organ resulting in a recovery of

sense of balance [Curthoys, 2000]. Unfortunately, this does not always occur or result in a reduction of vestibular related symptoms. Especially when there is partial or fluctuating dysfunction, the central compensation mechanisms do not manifest or seem to adapt only on the long-term [Stefanelli et al., 1978]. Such compensation mechanisms are not present to such an extent in the auditory pathway, nor in humans neither in animals; in the auditory pathway, other systems (as e.g., visus and propriocepsis in the vestibular system) cannot compensate for lost function of auditory periphery. Further, Rüttiger et al. [2013] showed that tinnitus (an annoying auditory percept that originates in the head and not from an external sound source) is linked to a failure to adapt central circuits to reduced cochlear input.

Stimulated by the success story of the effectiveness of the cochlear implant in the deaf patient, certain centers have focused on the possibility to implant a device in the vestibular system in order to restore balance. Recently, a vestibular implant has come to the attention as a possible solution for people with loss of vestibular function and it is typically applied in patients suffering from severe bilateral vestibulopathy [Rubinstein et al., 2012; van de Berg et al., 2012; Merfeld and Lewis, 2012]. However, this technique, requires extensive surgery and is, therefore, not suitable for some patients. Also, the vertigo spells may recur if the device is switched off. Furthermore, to date, this technique has no evidence in proven benefit. A strategy targeted at the main substrate of the disease, i.e. the vestibular sensory organs, seems preferable. Researchers have explored the possibility of regeneration of lost hair cells as a potential solution for recovery of vestibular function, which has yielded some promising results. Some background about regeneration of hair cells (cochlear as vestibular) is given.

In mammals, the great majority of hair cells are produced during embryogenesis. Hair cells that are lost after birth are practically irreplaceable, leading to permanent disability. Other vertebrates, such as fish and amphibians, produce hair cells throughout life. However, hair cell replacement after damage to the mature inner ear, in animal or human, was assumed to be impossible until studies in the late 1980s proved this to be incorrect. Adult birds were shown to regenerate lost hair cells in the auditory sensory epithelium after noise- and ototoxic drug-induced damage [Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988]. Since then, the field of investigators working on this topic has continued to investigate the capacity of the auditory and vestibular epithelia in vertebrates (fish, birds, reptiles, and mammals) to regenerate hair cells and to recover function.

In mammals, regeneration of cochlear or vestibular hair cells was not considered possible [Lindeman, 1969b; Hawkins and Preston 1975]. However, in 1993 there came evidence for

regeneration of vestibular hair cells in guinea pigs under very specific circumstances [Forge et al., 1993; Warchol et al., 1993]. It is important to note that to date, regeneration of the cochlear hair cells has not been found [for a review see Rubel et al., 2013],

1.1.1 Objectives of the thesis

The studies described in this thesis are part of the research line of the department of Otorhinolaryngology of the UMC Utrecht 'Experimental models for neuroprotection and neuroregeneration of the vestibular system'. The main objective of this vestibular research line is to quantify the regenerative ability of the vestibular sensory epithelia, after ototoxic treatment with aminoglycoside drugs in mammals. Fundamental insights potentially could lead to new clinical ideas that can be of help when treating people with peripheral equilibrium problems. In addition, these insights in the vestibular system may be extrapolated to the cochlea, which is another area of great interest of the department Otorhinolaryngology, to eventually treat people with sensorineural hearing loss.

1.1.2 Outline of the thesis

The thesis is globally divided in three parts. In the first part several pragmatic questions related to ototoxicity are answered. The emphasis lies on validating methods of damaging the hair cells of the inner ear (both vestibular and cochlear) efficiently and reliably to facilitate further research, such as studying the long-term effects after damage induction, or applying tissue-engineering techniques in the future. In the second part long-term effects of ototoxically induced damage are studied for signs of hair cell regeneration and functional recovery. This fundamental study is related to a randomized controlled clinical trial in the third part. In this clinical study the aminoglycoside drug gentamicin is used for chemo-ablation of the vestibular sensory epithelia in patients suffering from invalidating Ménière's disease, which severely affects the vestibular system.

1.2 Morphological aspects of the otolith organs

To understand the mechanisms of hair cell damage and possible regeneration after ototoxically induced damage fundamental research is needed. Regeneration of hair cells can be studied with several histological techniques of which a few are illustrated in this paragraph. The guinea pig was chosen for our experimental animal model for the vestibular studies because it has a vestibular organ similar to the human. Another consideration was that the guinea pig is the most frequently used animal in experimental Ménière's disease research [Kingma and Wit,

2010; Brown et al., 2013] promoted by the fact that its inner ear is fairly easy to access for electrophysiological recordings and measurements.

Of the vestibular organs the otolith organs are studied the most in this thesis and here a general introduction is given about these otolith organs. In the guinea pig, the otolith organs (together with the three semicircular channels and cochlea) are embedded in a bulla (i.e. a particular chamber of the skull), which facilitates its use in the laboratory setting and for harvesting for histology (**figure 1a**). The otolith organs are embedded in the medial wall of this bulla (**figure1b**) and they consist of the utricle and the saccule. In the normal position of the head, the utricle is positioned approximately in a horizontal plane, while the saccule is positioned more vertically and perpendicular to the utricle (**figure 2**). The utricle detects linear movements in the horizontal plane whereas the saccule detects linear movements in the vertical plane. The sensory areas, containing hair cells as sensory receptors, are located in the macula of the utricle and saccule. The macula of the utricle is triangular, the macula of the saccule hook–shaped. The striola is a ribbon-shaped zone that runs throughout much of the length of the central macula and divides it into lateral and medial extrastriolae. An otolithic membrane covers both maculae. On top of this membrane are otoconia, which in the guinea pig and other mammals consist of calcium carbonite crystals. The otoconia come in high numbers and have a large mass. They lie on a gelatin layer and the hair bundles of the sensory hair cells are connected to this gelatin meshwork, which is flexible and makes flexion and deflection of hair cell bundles possible caused by the inertia of the heavy otoconia. The sensory epithelia of the maculae contain type-I and type-II hair cells (HCs). Type I-HCs are pear-shaped and connect to an afferent neural calyx synapse. The type-II HCs are cylindrically shaped and connect to afferent bouton-like synapses. Further, the maculae consist of supporting cells and nerve fibers. About two-thirds of the striolar HCs are type-I HC's, whereas the extrastriolar regions contain type-I and type-II hair cells in approximately equal numbers (Lindeman, 1969a).

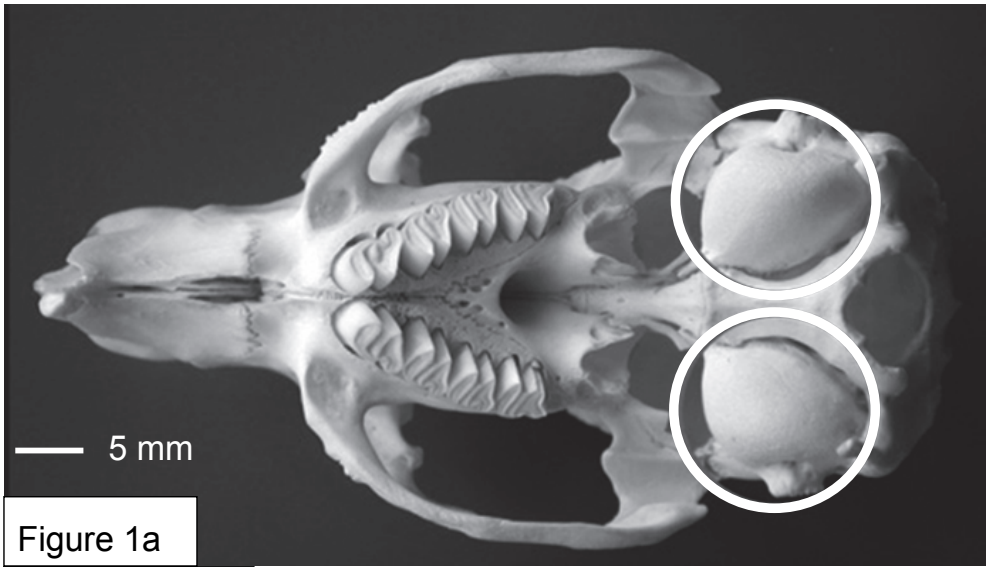

Figure 1a. Caudal view at the location of the bullae in the guinea pig (courtesy from Albuguergue et al., 2009).

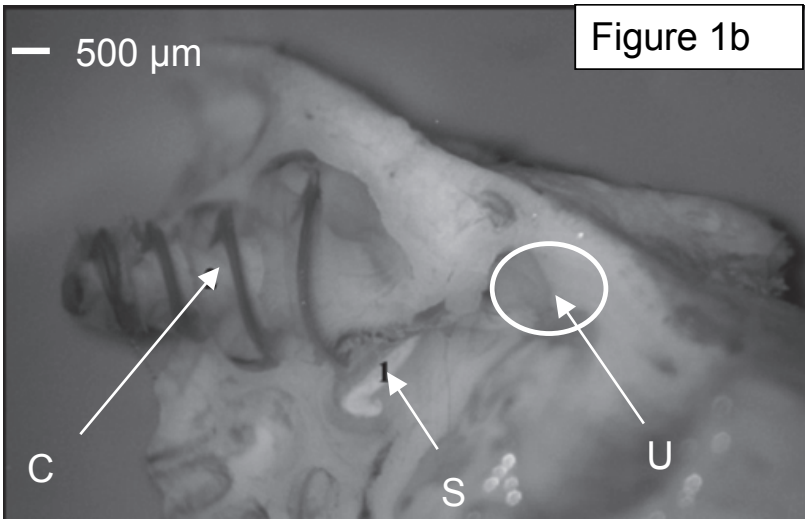

Figure 1b. U = utricle, S = saccule, C = cochlea. The circle schematically draws the location of the utricle (courtesy from Albuquerque et al., 2009).

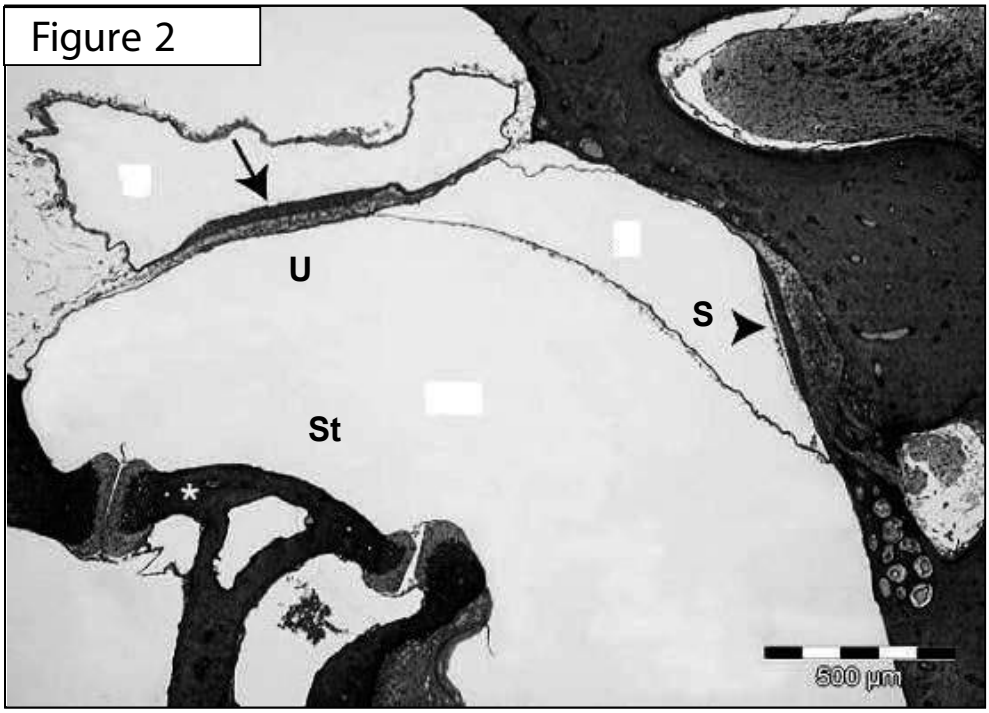

Figure 2. Light microscopic image to show the relation between utricle (U), saccule (S) and stapes (St) in the vestibulum of a guinea pig (courtesy from Oei 2003, thesis, University of Groningen).

1.2.1 Light microscopy

The inner ears of the guinea pigs were processed for microscopy according the histological methods described in chapter 2. **Figure 2** shows a light microscopic example of a section of the inner ear of the guinea pig. As can be inferred, for histological processing the utricle can be more easily harvested than the saccule which is more embedded in the bone of the cochlea. **Figure 3** shows an utricle at larger magnification (microscopic lens setting at 63x). Type-I and type-II HCs are visible where the type-I HCs can be distinguished by the calyces surrounding them. The otoconia are visible as well as the stereocilia, which are embedded in the gelatinous layer. Below the hair cells is the lamina propria. Although the emphasis in this thesis lies on the otolith organs, a significant portion is dedicated to the cochlea and we also performed light microscopic evaluation of the cochlea. **Figure 4a** shows a light micrograph of a cross-section of a guinea pig cochlea to indicate the organ of Corti. The organ of Corti contains the auditory hair cells. Of these, the inner hair cells are the receptor cells converting the mechanical sound signal into an electrical nerve signal. The outer hair cells are thought to mainly function as cochlear amplifiers. Note that the different functions of the two types of cochlear hair cells imply that

profound hearing loss only occurs when the inner hair cells are damaged. **Figure 4b** shows light micrographs of semi-thin (1 µV) midmodiolar sections of resin-embedded cochleae at a location ± 12.5 mm from the apex demonstrating 3 outer hair cells (arrowheads) and a single inner hair cell (*). **Figure 4c** shows a completely collapsed organ of Corti in the cochlea of an animal treated with kanamycin and furosemide. Both outer and inner hair cells are missing.

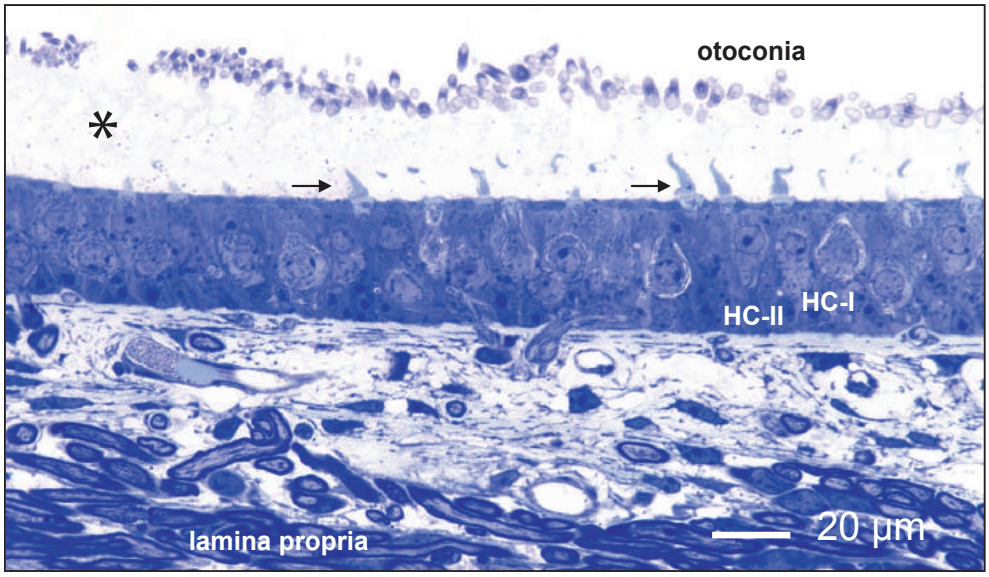

Figure 3. Light microscopic example of a section of an utricle of a guinea pig (from our laboratory). HC-I = Hair cell type I, HC-II = Hair cell type II (stained with methylene blue and azur-II). The asterisk indicates the gelatinous layer where the hair cell bundles (arrows) are embedded in.

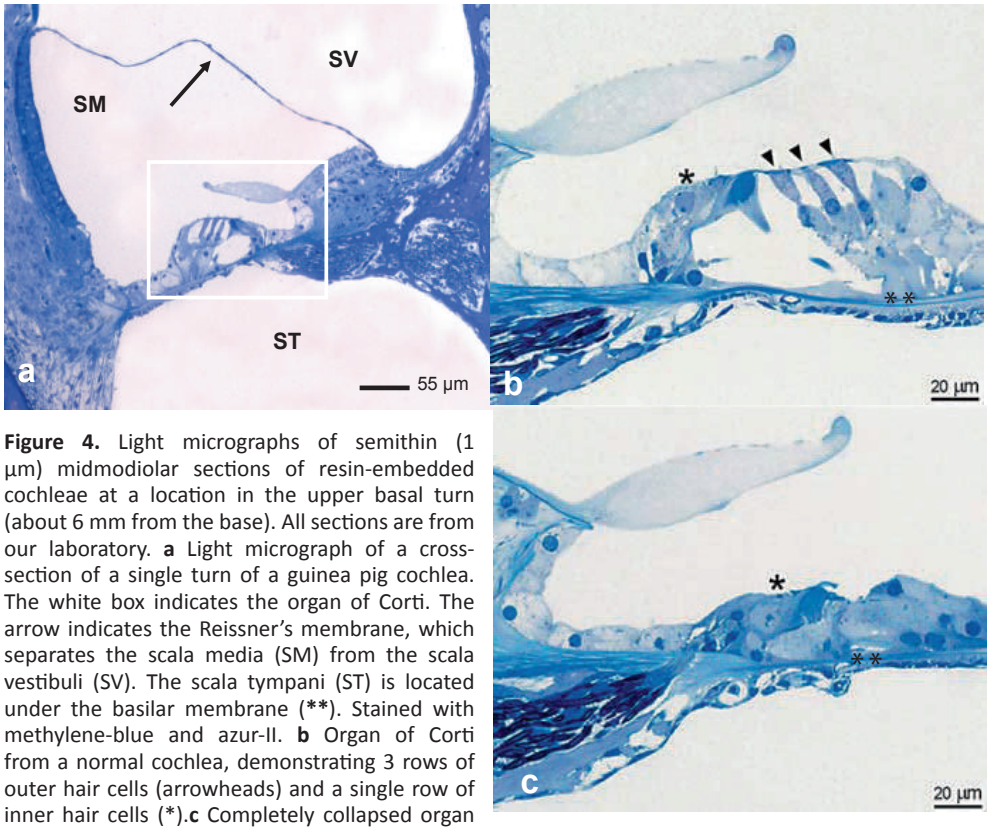

Figure 4. Light micrographs of semithin (1 µm) midmodiolar sections of resin-embedded cochleae at a location in the upper basal turn (about 6 mm from the base). All sections are from our laboratory. **a** Light micrograph of a cross-section of a single turn of a guinea pig cochlea. The white box indicates the organ of Corti. The arrow indicates the Reissner's membrane, which separates the scala media (SM) from the scala vestibuli (SV). The scala tympani (ST) is located under the basilar membrane (**). Stained with methylene-blue and azur-II. **b** Organ of Corti from a normal cochlea, demonstrating 3 rows of outer hair cells (arrowheads) and a single row of inner hair cells (*).**c** Completely collapsed organ of Corti in the cochlea of an animal treated with kanamycin and furosemide. The outer and inner hair cells are missing**.**

1.2.2 Immunofluorescent microscopy

For quantification of vestibular hair cells, immunofluorescent microscopic techniques are used in our studies. A detailed explanation of the immunofluorescent methods used is given in chapter 2 and 5. Fluorescence microscopy makes use of fluorescent dyes that illuminate when irradiated with light of a certain wavelength. We used sera of artificial antibodies (phalloidin), which are specifically bound to a protein, which is present in the stereocilia and cuticular plate of the hair cells. These antibodies contain a fluorescent dye (rhodamine), which makes them microscopically visible. **Figure 5** shows low-resolution images of a normal utricle and saccule after staining with fluorescent phalloidin. Highly stained immunofluorescent dots are seen that represent hair cell bundles. **Figure 6a** shows a view of the striola in a normal utricle. Hair cells and supporting cells can easily be distinguished. Hair cells are round and can be identified

by the hair bundles (**figure 6b**), which appear intensely stained (arrows). Supporting cells are smaller in size, polygonal in shape and contain an actin-free zone in the central portion (arrowheads). Besides hair cell counts, to quantify damage in the sensory epithelia in the following studies, we used the presence of so-called scars (circles). Scars are characterized by the cartwheel-like staining pattern of phalloidin and consist of supporting cells that fill the space left by the degenerated and extruded hair cells [Meiteles and Raphael, 1994]. Two weeks after 10 days of daily gentamicin treatment (**figure 6c**), these supporting cell scars are present, indicating loss of striolar hair cells. Remaining hair cells (arrows) still exhibit phalloidin staining of the cuticular plate and hair bundle.

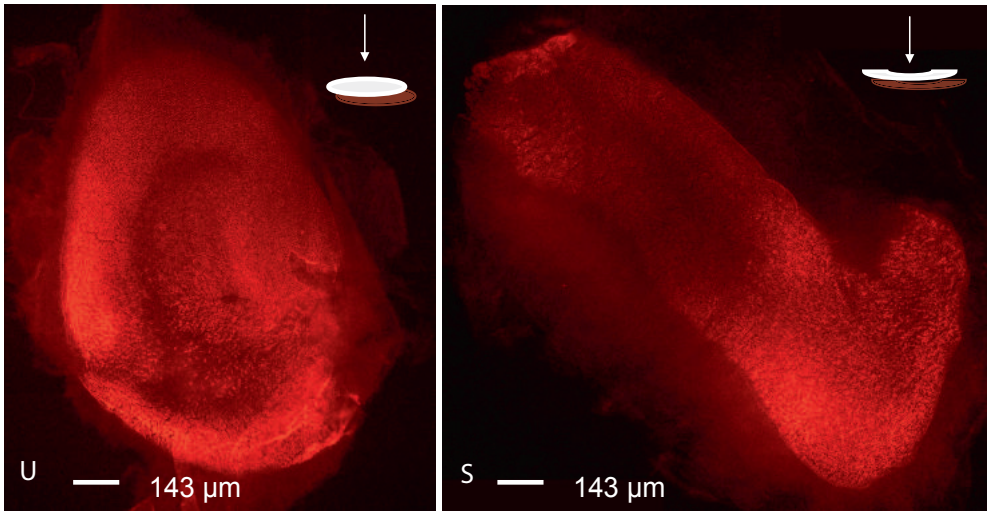

Figure 5. Low-resolution image of a normal utricle (U) and saccule (S) of a guinea pig after staining with fluorescent phalloidin as the hair cell marker. Highly stained immunoflourescent dots are seen that represent hair cell bundles.

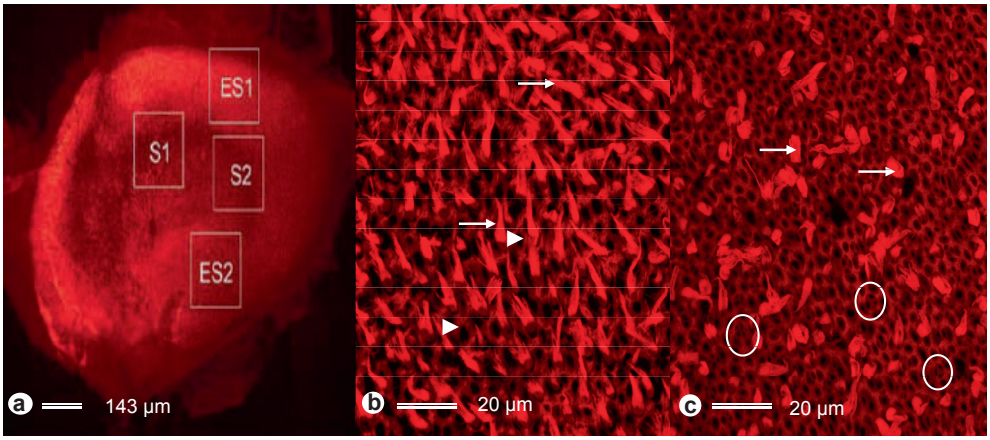

Figure 6. Confocal images of the striolar region in whole mounts of utricles of guinea pigs stained with fluorescent phalloidin as the hair cell marker. Arrows indicate hair cell bundles; arrowheads supporting cells and circles indicate scars. **a** Low-resolution image of a normal utricle. The boxes measure143 x143 µm and indicate 2 striolar (S) and 2 extrastriolar (ES) locations in which vestibular hair cells were counted. **b** High-resolution image of the striolar region in the 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** Two weeks after gentamicin treatment, remaining hair cells (arrows) still exhibit phalloidin staining of the cuticular plate and hair bundle. Several scars are visible (circles).

1.3 Functional aspects of the vestibular system

To evaluate whether potentially regenerated vestibular hair cells are functional, the combination of both morphological and functional evaluation of the vestibular system before and after damage is preferable, but few authors have performed such combination studies in animal research.

Vestibular function in human use

The vestibuloocular reflex (VOR) serves as an inertial stabilization system for vision; it controls movements of the eyes to 'compensate' for head movements. The reflex starts in the vestibular system, where semicircular canals get activated by head rotation and send their impulses via the vestibular nerve (cranial nerve VIII) through Scarpa's ganglion and end in the vestibular nuclei in the brainstem. In humans, recording of this reflex is possible by measuring the responses to a vestibular stimulus by means of a nystagmogram, which electrophysiologically records the compensatory saccadic eye movements. Most clinics use the above-described nystagmography using either a rotating chair or calorization through the ear canal, which stimulates only the lateral semicircular canal. This method relies on central processing; apart from vestibular end-organ disease, the presence of nystagmus may result from visual disease, or disorders affecting the cerebellum or brainstem.

Due to new developments a specific test for the otolith organs has become available: The vestibular evoked myogenic potential (VEMP) is an electromyographic response elicited by loud acoustic stimuli or skull vibrations. This potential measures vestibular function through the vestibulocollic reflex originating in the otolith organs and is transmitted via the ipsilateral vestibulospinal tract to the motor neurons of the sternocleidomastoid muscle and provides information about the vestibulospinal pathway [Colebatch et al., 1994; McCue et al., 1997; Kushiro et al., 1999; Rosengren et al., 2010]. This method therefore relies on vestibular function but also on central processing. Apart from the cVEMP described above, the oVEMP found its use in clinical practice, which is derived from responses from the eye muscles [Todd et al, 2003; Rosengren et al., 2005; Winters et al., 2012; 2013].

Vestibular function in animal use

The great advantage of animal studies is that we can isolate the specific end organs that we are interested in by eliminating the interference of the whole reflex chain (complex signal pathway). Another advantage of an animal model is that these measurements can be done quantitatively and longitudinally. For assessing vestibular function in guinea pigs, recording of the above mentioned VOR [Cartwright et al., 2003; King and Shanidze, 2011; King, 2013] and VEMP [Yang et al., 2005; 2010] can be used. However, these measurements rely on the presence and functioning of an intact peripheral vestibular organ, an intact oculomotor system and intact brainstem nuclei, which may interfere with the end-vestibular origin.

Therefore, a technique has been developed to elicit recordings from the vestibular end organs in animals - short latency vestibular evoked potentials (VsEPs). This technique records the compound action potential of the vestibular nerve after stimulation of the otolith organs [Böhmer et al., 1995; Jones et al., 1999; Chihara et al., 2013]. The latter study showed the VsEP to be originating from the utricle (when stimulation was done in the horizontal plane).

1.3.1 Assessing cochlear function

The auditory brainstem response (ABR) is an auditory evoked potential that records electrical activity using electrodes that are placed on the scalp. Although it is a brainstem response, the ABR reflects appropriate cochlear function in humans and animals [Kyo and Yanagihara, 1980] and has been used for several decades to investigate cochlear function [Moore and Ernest 1983; Eggermont et al., 2007; Hall and James 2007]. The ABR can be used properly for studying the effect of ototoxic effect on auditory periphery in an animal experimental design [Perez et al., 2000; Agterberg et al., 2009]: In the auditory pathway, other systems (as visus

and propriocepsis in the vestibular system) cannot compensate for lost function of auditory periphery minimizing confounding factors for the end-organ origin of the signal.

1.3.2 Stimulus

Stimulation to record ABRs is relatively simple; acoustic stimuli can easily be delivered through speakers near the ear canal. One of the main problems of vestibular function testing is the presentation of the vestibular stimulus. Since the vestibular system is located inside the skull, administering such a stimulus accurately and reproducibly requires an elaborate mechanical test set–up. A rigid coupling of the stimulator to the skull is necessary to ensure that given stimuli actually reach the vestibular organ. Elidan et al. [1987] and Plotnik et al. [1999] have used both angular and linear acceleration to elicit VsEPs recorded with skin electrodes in several species. Angular acceleration excites the hair cells in the ampullae of the semicircular canals. Hair cells in the maculae of the otolith organs respond to linear acceleration [Jones and Jones, 1999; Oei et al., 2001; Chihara et al., 2013].

In the following vestibular experimental studies, animals, when anesthetized, were coupled to a vibration exciter that was placed on a shake-proof plate (**figure 7**). In this set-up, stimulation was possible in three directions, horizontal lateral (HL), horizontal medial (HM) and vertical (V). This way we could investigate (chapter 2) whether HM and HL stimuli mainly excite the utricle and whether V stimuli excite the saccule as expected on the basis of the horizontal and vertical configurations of utricle and saccule respectively. This makes selective measurements of the utricle possible: the utricle detects mainly motions in the horizontal plane [Chihara et al., 2013]. In chapter 2 it is further investigated if the VsEP recorded after vertical stimulation reflects saccular function. An example of the used stimulus waveform is given in **figure 8**.

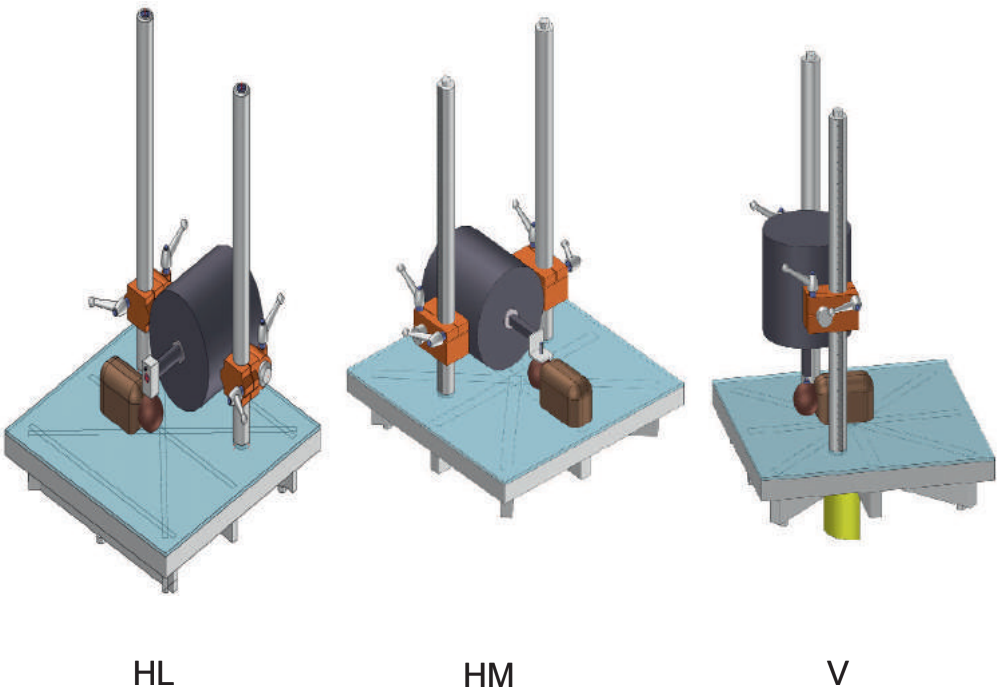

Figure 7. Schematic of the rigid set-up. The guinea pig is schematically indicated in brown, the vibration exciter in black and the shake-proof plate in blue. The animals head could be stimulated in three directions. HL = Horizontal Lateral, HM = Horizontal Medial, V = Vertical.

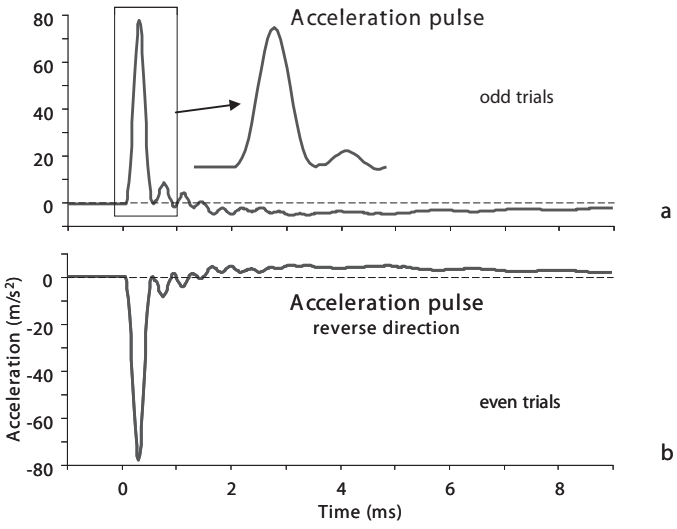

Figure 8. Example of the recorded stimulus waveform. The stimulus is also shown in detail from –0.2 to 1 ms.

1.3.3 Acquisition of the vestibular response

Near the bulla, the vestibular nerve is only separated from the facial nerve by a thin bony wall. Böhmer et al. [1995] have measured the responses to linear acceleration by inserting an electrode in the facial nerve canal. In the first ototoxic study (chapter 2) where we performed acute experiments, a platinum electrode is implanted in the bony facial nerve canal up to the first curvature according to this method. When longitudinal experiments are warranted (chapter 5) this method gives rise to morbidity because the facial nerve has to be sacrificed on one or both sides causing ingestion problems due to facial paralysis. This motivated us to investigate a method that was more suitable for longitudinal measurements. The method described by Jones and Jones [1999] makes use of an epidural electrode that is placed on top of the head through the skull resting on the dura (rats, mice, guinea pigs, and gerbils were studied). It seemed to be well reproducible in our laboratory. **Figure 9** shows examples of VsEPs acquired with both methods, with a facial nerve electrode (**9a**) and using an epidural electrode (**9b**). For a detailed explanation of the electrophysiological material and methods used see chapters 2 and 5.

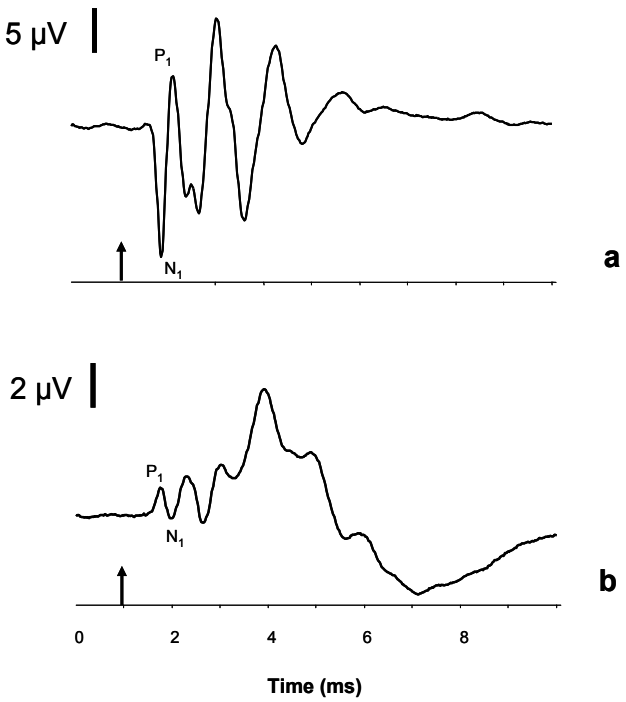

Figure 9. **a** VsEP recorded with the facial electrode (HM direction at 70 m/s²). A = Amplitude, L = Latency time, N_1 = First peak of VsEP. **b** VsEP recorded with the epidural electrode (HM direction at 70 m/s²). The P_1 and N_1 are indicated. Arrows indicate stimulus.

1.3.4 End-organ function

The first peaks of the VsEP, N_1 and P_1, occurring with a latency of about 0.8 ms (N_1) and 1.0 ms (P_1) are valid indicators of end organ vestibular function [Jones and Jones, 1999; Freeman et al., 2000; Oei et al., 2001]. Later peaks in the VsEP do not solely originate from nuclei in the vestibular pathway, but may originate from the cranial nerve nuclei as shown in the cat [Li et al., 1997] or the auditory nuclei as shown in the chinchilla [Böhmer et al., 1995] and guinea pig [Oei et al., 2001]. Three validation studies on the VsEP were done in our laboratories, which showed the first two peaks of the VsEP to be originating from the end-organ vestibular organs (**figure 10**): Loud white noise was used to stimulate the cochlear hair cells so that an added vestibular stimulus can no longer elicit a response from the cochlea. In 2 normal-hearing animals, we presented continuous acoustically broadband white noise (103 dB SPL; flat spectrum up to 25 kHz, fluctuations < 15 dB) effectively masking the acoustic component caused by the linear acceleration stimuli; the masker did not affect the N_1, but later peaks – with latencies longer than 2 ms – were substantially reduced **(figure 10b**). Secondly, we ruled out cochlear influences by mechanically destructing the cochlea, which had no influence on the first two peaks of the signal (**figure 10c**). Finally, measurements were performed using a dead animal during stimulation to check for stimulus artifacts: N_1 could not be detected anymore (**figure 10d**). In the following vestibular studies N_1 - P_1 amplitudes, N_1 - P_1 latencies and N_1 - P_1 thresholds are analyzed before and after ototoxic insult **(figure 11).**

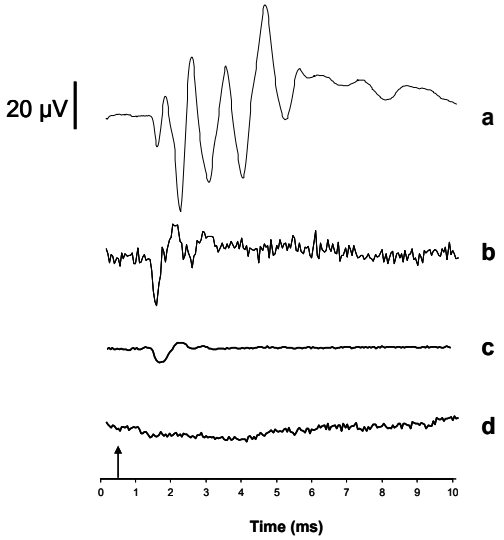

Figure 10. a Example of a normal VsEP. **b** When acoustically broadband white noise (103 dB SPL; flat spectrum up to 25 kHz, fluctuations < 15 dB) was presented. **c** Series of VsEPs after destruction of the cochlea. **d** Recording of the VsEP in a dead animal. The stimulus was in all four cases an acceleration pulse of 60 m/s^2 in the HM direction.

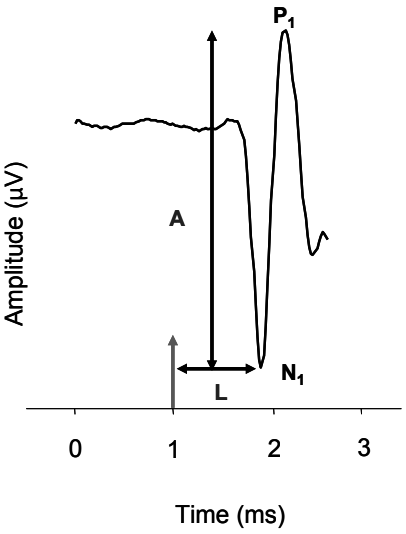

Figure 11. Detail of figure 9a to indicate the used VsEP variables. A = Amplitude, L = Latency. Arrow indicates stimulus.

1.4 Ménière's disease

In chapter 6 the ototoxic drug gentamicin is used as therapeutic agent for patients suffering from Ménière's disease, which affects both the vestibular and the cochlear system. Here, a short background is given about this disease. In 1861, Prosper Ménière was the first to describe a triad of symptoms that are characteristic for the attacks of the disease named after him: vertigo, hearing loss and tinnitus. The vertigo is accompanied by nausea and vomiting. A patient typically suffers from single-sided sensorineural hearing loss that fluctuates and especially affects the lower frequencies. During an attack there is tinnitus and/or a feeling of fullness in the ear. The attacks are invalidating and usually last several hours. After an attack and in the beginning of the disease, the hearing loss usually recovers. This recovery occurs less frequently if attacks continue and this eventually leads to a flat sensorineural hearing loss of about 50-60 dB. The course of the disease is unpredictable and may be limited to a few attacks, but also may persist for years. The frequency of attacks varies considerably between patients and within a patient in time. The condition occurs with an incidence of 10-150 per 100,000 [Schessel et al., 1998]. In about 10% of the cases, eventually both ears will be affected [Balkany

et al., 1989; Enander et al., 1967; Mizokushi et al., 1979]. Women are more often affected than men. Patients often indicate that stress is a provoking factor of an attack.

1.4.1 Pathophysiology

The pathogenesis of Ménière's disease is still not clear. Currently, it is generally assumed that the attacks are caused by an imbalance in the homeostasis of the inner ear fluid in the endolymphatic space. This creates a so-called endolymphatic hydrops [Kimura et al., 1967]. In 1938, this hydrops was first observed during autopsy in two independents patients (Hallpike and Cairns; Yamakawa). It is thought that by this endolymphatic hydrops, the Reissner's membrane ruptures (this is a two-cell layered membrane inside the cochlea that separates the scala media and scala vestibuli, see **figure 4a**), causing the endolymph and the perilymph to interflow. This then would result in a depolarization of the cochlear and vestibular hair cells. This could lead to an acute loss of hearing and vestibular function. When the defect in the membrane closes, the normal relationship between endolymph and perilymph restores resulting in normal hair cell function [Lawrence and McCabe, 1959; Coelho and Lalwani, 2008] eventually leading to recovery of hearing and balance. Also the altered mass of the increased volume of endolymph possibly accompanied by an increased pressure might play a role in the disease [Klis and Smoorenburg, 1994].

1.4.2 Diagnostics

There is no specific diagnostic test to demonstrate Ménière's disease. It is a diagnosis 'per exclusionem' of other disorders. The typical patient history is the most important in making the diagnosis. The American Academy of Otolaryngology and Head and Neck Surgery (AAO-HNS) has formulated a number of diagnostic criteria that must be met before the diagnosis should be made [Duvall et al., 1980]:

- There must have been at least two attacks of vertigo that lasted at least 20 minutes.
- Audiometric confirmation of a sensorineural hearing loss in the lower frequencies.
- Tinnitus and/or a feeling of fullness in the affected ear.
- Other disorders are excluded.

1.4.3 Therapy

Ménière's disease must be regarded as a chronic disease. Treatment is aimed at reducing the severity and frequency of attacks of vertigo, reducing the hearing loss and tinnitus associated with the attacks, relieving chronic symptoms such as tinnitus and balance problems and

minimizing disability. A good patient education is very important [Yardly and Kirby, 2006; Greenberg and Nedzelski, 2010], followed by advices on living habits and medication. In recalcitrant cases interventions as intratympanic gentamicin treatment are used. This is further studied in chapter 6.

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Chapter 2

Ototoxic study-I

Combined administration of kanamycin and furosemide does not result in loss of vestibular function in guinea pigs

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ABSTRACT

Aminoglycoside antibiotics are known to damage the vestibular and auditory sensory epithelia. Although loop diuretics enhance the cochleotoxic effect of aminoglycosides, it is not known if concomitant administration of an aminoglycoside and a loop diuretic affects the vestibular system. The aim of our study was to investigate the effect of co-administration of kanamycin and furosemide upon the otolith organs and to compare it to the known vestibulotoxic effect of gentamicin. Five guinea pigs were injected with a single dose of both kanamycin (400 mg/kg, sc) and furosemide (100 mg/kg, iv), five animals received gentamicin (100 mg/kg, ip) for ten days, and five untreated animals served as controls. After seven days, vestibular function was assessed by measuring vestibular short-latency evoked potentials (VsEPs) to linear acceleration stimuli and cochlear function by auditory brainstem responses (ABRs) to clicks. Hair cell densities were determined in phalloidin-stained whole mounts of the utricles and saccules, and in midmodiolar sections of resin-embedded cochleas. Co-administration of kanamycin and furosemide had no significant effect on VsEPs and hair cell densities in the utricles and saccules were not reduced. ABR thresholds were increased to a great extent (by ~60 dB), and histologically a severe loss of cochlear hair cells was observed. The effect of gentamicin, both on vestibular and cochlear function, was just the opposite. VsEP thresholds to horizontal stimulation were elevated and suprathreshold amplitudes showed a decrease, whereas cochlear function was not reduced. With this protocol we have a tool to selectively induce cochlear or vestibular damage, which can be of interest to researchers and clinicians alike.

1. INTRODUCTION

Aminoglycosides are ototoxic drugs that induce a severe and progressive impairment of the auditory and vestibular functions of the ear [for reviews, see Forge and Schacht, 2000; Darlington and Smith, 2003; Carey, 2005]. Aminoglycoside-induced hearing impairment is associated with an irreversible loss of the cochlear hair cells. Initially, the hair cells in the basal cochlear turn are affected resulting in loss of the high frequencies, but with continuous exposure to the drug hair cell loss gradually progresses to the more apically located cochlear turns, which eventually leads to deterioration of hearing at the middle and lower frequencies [Forge and Schacht, 2000; Ding and Salvi, 2005]. Outer hair cells are more vulnerable than inner hair cells. Similar to these intracochlear differences in susceptibility to aminoglycoside treatment, the vestibular sensory epithelia exhibit regional and cellular differences in sensitivity and also the different components of the vestibular apparatus (i.e., semicircular canals, utricle, saccule) demonstrate varying degrees of vulnerability [Lindeman, 1969; Aran et al., 1982; Twine, 1985]. In the semicircular canals, aminoglycoside-induced loss of vestibular hair cells starts in the central zone of the cristae ampullares and then progresses towards the peripheral zone. Hair cell loss in the otolith organs, however, is apparent at a later stage [Lindeman, 1969; Darlington and Smith, 2003]. In the maculae of the utricle and saccule, hair cell loss in the striolar region is more extensive and occurs earlier than in the extrastriolar region, whereas the utricle is more vulnerable than the saccule. The type-I hair cells are more sensitive to aminoglycoside exposure than the type-II hair cells [Lindeman, 1969; Lyford-Pike et al., 2007].

After systemic administration of aminoglycosides (e.g., gentamicin, kanamycin, neomycin, streptomycin), in a repetitive (i.e., daily) fashion, a substantial cochleotoxic effect is reached within several weeks. However, single high-dose application of an aminoglycoside given concurrently with a loop diuretic (e.g., ethacrynic acid, furosemide) results in massive loss of cochlear hair cells, already within days [West et al., 1973; Russell at al., 1979; Brummett, 1981; Webster and Webster, 1981; Xu et al., 1993; Nourski et al., 2004; McFadden et al., 2004; Versnel et al., 2007]. Since the first clinical observations of this potentiating effect [Mathog and Klein, 1969; Johnson and Hamilton, 1970; Meriwether et al., 1971], treatment protocols based upon concomitant administration of an aminoglycoside and a loop diuretic have been used extensively to induce rapid hair cell loss in experimental animal models of deafness.

The aim of this study was to examine if single high-dose application of kanamycin in combination with furosemide also results in impairment of vestibular function and loss of

vestibular hair cells. We have compared the functional and histological outcome parameters with those after chronic administration of gentamicin, which is known for its vestibulotoxic effect [Twine, 1985; Oei et al., 2004]. In addition, as a control, we investigated the cochleotoxic effects of both treatment protocols. Functional impairment was investigated using vestibular short-latency evoked potentials (VsEPs) and auditory brainstem responses (ABRs). The VsEPs were evoked by linear acceleration stimuli, which mainly excite the otolith organs [e.g., Freeman et al., 1999]. Hair cell loss was quantified in whole mounts of phalloidin-stained utricles and saccules as well as in midmodiolar sections of resin-embedded cochleas.

2. MATERIALS AND METHODS

2.1 Animals and Experimental Design

Fifteen 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. Animals had free access to both food and water and were kept under standard laboratory conditions. The experimental procedures were approved by the University's Committee on Animal Research (DEC #06.06.055).

Two ototoxic treatment protocols were compared with respect to vestibular and cochlear function. Five animals were treated concomitantly with kanamycin and furosemide and five animals received gentamicin during ten consecutive days. Seven days after treatment, all animals were anesthetized, underwent surgery to implant electrodes, and both vestibular and cochlear function was measured electrophysiologically. These measurements were also performed in a control group consisting of five normal animals.

After the final functional measurements, the animals were euthanized while sedated by an overdose of sodium pentobarbital (Nembutal®; Ceva Santé Animale, Maassluis, the Netherlands). Some of the left ears were selected and processed for histological evaluation of the otolith organs (utricle and saccule) and the organ of Corti, whereas the majority of utricles and saccules, both of the left and right ears, were processed for immunohistochemistry and quantitative evaluation.

2.2 Treatment with Ototoxic Drugs

The combined treatment of kanamycin and furosemide was adapted from a deafening procedure previously reported by West et al. [1973] and is routinely used in our laboratory [Versnel et al., 2007]. Animals were anesthetized with S-ketamine (Ketanest-S®, 40 mg/kg, im)

and xylazine (Sedamun®, 10 mg/kg, im). Kanamycin sulphate (Sigma-Aldrich, St. Louis, USA) in isotonic saline was administered as a single subcutaneous injection at a dose of 400 mg/kg body weight. Next, the vena jugularis externa was exposed and cannulated and a single dose of furosemide (Centrafarm Pharmaceuticals, Etten-Leur, the Netherlands) was slowly infused, at a dose of 100 mg/kg body weight.

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. 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 100 mg/kg body weight.

2.3 Measurement of Cochlear Function

Seven days after ototoxic treatment, the animals were anesthetized with Ketanest-S® (40 mg/kg, im) and Sedamun® (10 mg/kg, im) and prepared for functional measurements. Click-evoked ABRs were recorded by use of subdermal needle electrodes. The active electrode was placed at the vertex, while the reference electrode was placed on the nose and the ground electrode in the neck muscles. ABRs were evoked with monophasic clicks (40 µs) that were delivered with alternating polarity at a rate of 10/s [cf., Agterberg et al., 2009]. Stimulation was started at 77 dB nHL (~110 dB peSPL) and 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, FL, USA). The clicks were presented with an earphone (Blaupunkt PCxb352), which was held 10 cm from the animal's left ear. The responses were amplified (x5,000) using a differential amplifier (EG&G instruments, model 5113), and AD 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.

2.4 Measurement of Vestibular Function

After the ABR recordings the animals were prepared for measurement of vestibular function. The method for recording VsEPs via the facial nerve canal was based on the method originally described by Böhmer et al. [1995], but modified to allow for local circumstances and suppliers.

A retroauricular incision was made and the stylomastoid foramen was identified in both ears. The facial nerve extruding from the foramen was cut and an insulated platinum wire with exposed tip was implanted in the bony facial nerve canal up to the first curvature.

Then, a stainless steel screw was cemented with dental acrylic cement (ESPE Dental, Gorinchem, the Netherlands) upside-down on the animal's skull near bregma. The screw was attached to a Brüel and Kjær type 4809 vibration exciter. This device can be orientated in three perpendicular planes to produce linear head motions in three directions. We defined rostrocaudal movements as horizontal medial, intra-aural movements as horizontal lateral, and dorsoventral movements as vertical (see fig. 1d).

The head motions were generated in a custom-made computer program and consisted of 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 [cf., Oei et al., 2001]. At this high rate the N_1 is not reduced substantially [Jones et al., 2002], while auditory responses, which may contribute to the VsEP, are significantly reduced. Successive pulses of alternating polarity were used to reduce stimulus artifacts in the recordings; peak amplitudes varied between 1 and 78 m/s². The acceleration pulse, *a(t)*, can be described as follows:

$$a(t) = \frac{b}{2} \cdot \left\{\sin\left(2\pi f t - \pi/2\right) + 1\right\} \text{ for } t \text{ between 0 and } 1/f, \quad \text{for } t \text{ between 0 and } 1/f,$$

with f = 2 kHz, *b:* maximum acceleration. Maximum jerk, a derivative of the acceleration, equals $\pi f b$ (e.g., for b = 40 m/s², jerk is 250 km/s³). Stimuli were calibrated with a Brüel and Kjær type 4521 accelerometer and monitored during recordings. Electrical activity in the facial nerve canal was amplified (x5,000; EG&G instruments, model 5113), filtered between 0.1 and 10 kHz, and digitized at a 50-kHz sample rate. The reference electrode was placed in the neck muscles. A facial nerve electrode was placed at each side of the head to record VsEPs from the left and right ears.

An example of a recording with the pulse amplitude at 78 m/s² is presented in figure 1a. An example of the recorded stimulus waveform is shown in figure 1b (with the opposite polarity in fig. 1c). This VsEP consists of four prominent waves occurring within 5 ms after stimulus onset. The first negative peak, N_1, occurring with a latency of about 0.8 ms, has been demonstrated to be a reliable indicator for otolith organ function in various rodents [Böhmer et al., 1995: chinchilla; Jones et al., 1999: mouse; Oei et al., 2001: 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 et al., 1995: chinchilla; Oei et al., 2001: guinea pig].

We performed two tests to ensure that the N_1 we recorded reflected vestibular function. Firstly, in two normal-hearing animals we presented acoustically broadband white noise (103 dB SPL; flat spectrum up to 25 kHz, fluctuations <15 dB) simultaneously with the linear

acceleration stimuli: the masker did not affect the N_1, but later peaks – with latencies longer than 2 ms – were substantially reduced (data not shown). Secondly, measurements were done using a dead animal during stimulation to check for stimulus artefacts: N_1 could not be detected anymore. Therefore, we analysed the N_1 wave to assess vestibular function: amplitude and latencies of the N_1 were analysed as a function of acceleration. Thresholds were computed by linear interpolation between amplitude-versus-acceleration data points around an iso-response criterion of 0.5 µV.

2.5 Tissue Processing for Immunohistochemistry

The majority of the utricles and saccules from the left and right ears were processed for whole-mount fluorescent immunohistochemistry using phalloidin as a hair cell marker, and 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 phosphate-buffered saline (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 (TRITC)-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, CA, USA).

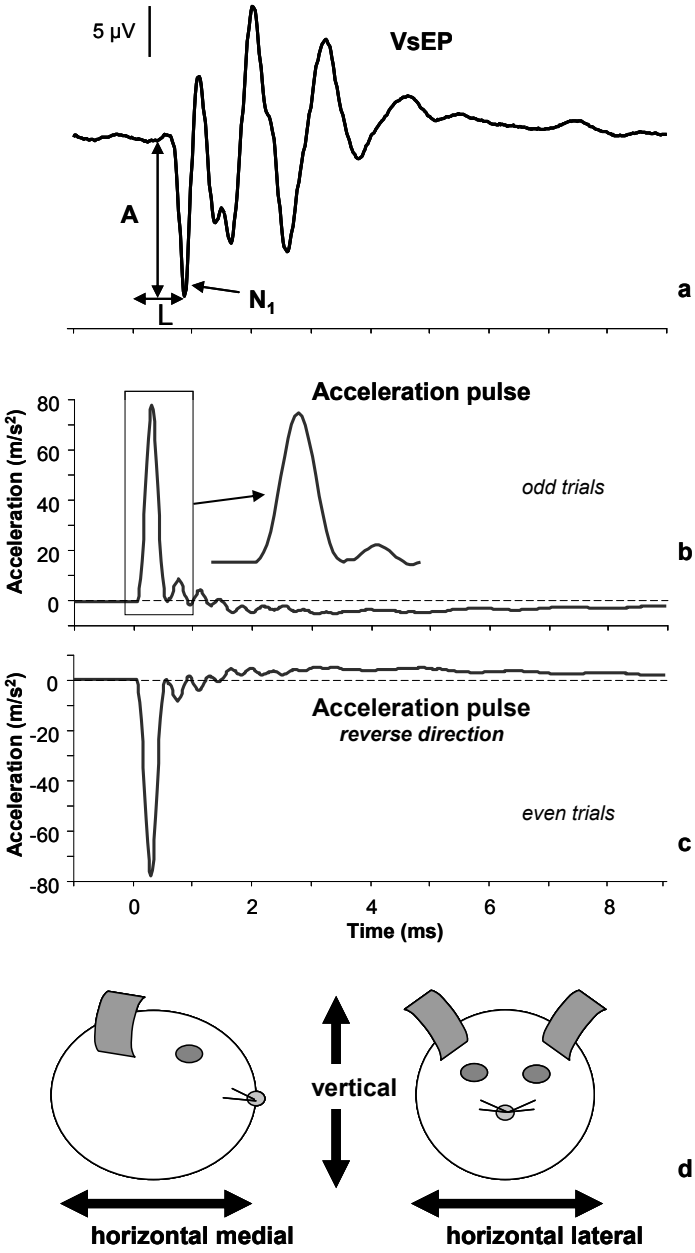

Figure 1. a Example of a VsEP waveform recorded at 78 m/s² (horizontal-medial stimulation) in a normal animal. A series of negative and positive peaks can be seen with a clear first negative peak (N_1) around 0.8 ms after stimulus onset. N_1 amplitude (A) was measured from baseline and N_1 latency (L) from stimulus onset to the minimum of N_1. **b** Example of the recorded stimulus waveform. The stimulus is also shown in detail from -0.2 to 1 ms. **c** Recorded waveform of stimulus in opposite polarity. **d** Schematic drawing to clarify the three stimulation directions.

2.6 Tissue Processing for Histology

Some of the left bullae were selected for histological evaluation of semithin transverse sections of the otolith organs (utricle and saccule) and hair cell counting in semithin midmodiolar sections of the cochlea.

After decapitation, the bullae were rapidly dissected out of the skull and opened. A hole was made in the apex of the cochlea, both the oval and round window membranes were perforated and the cochlea and vestibular labyrinth were fixed by intralabyrinthine perfusion with a tri-aldehyde fixative consisting of 3% glutaraldehyde, 2% formaldehyde, 1% acrolein, 2.5% dimethylsulfoxide in 0.08 M sodium cacodylate buffer, pH 7.4 [De Groot et al., 1987]. Perfusion was followed by immersion in the same fixative for 3 h at room temperature, after which the bulla was rinsed several times in 0.1 M sodium cacodylate buffer (pH 7.4) and stored in the same buffer at 4°C. In order to facilitate the localization of the utricle and saccule during microdissection, an aqueous solution of 1% OsO_4 containing 1% $K_4Ru(CN)_6$ [De Groot et al., 1987] was gently perfused through the hole in the apex of the cochlea, followed by immersion in the same solution for 20 min at 4°C.

After several rinses in distilled water, the bulla was immersed in physiological saline and the vestibule was opened. The utricle was removed with a set of finely pointed microhooks and a small forceps. The saccule was exposed by removing all excess bone and was then gently freed and lifted from the recessus hemisphericus taking care to leave the cochlea intact. In order to remove the otoconia and remnants of bony tissue, the utricles and saccules were decalcified for 3 days in 10% EDTA.2NA (pH 7.4) at room temperature. Cochleas were decalcified in the same decalcifying solution for 5 days. All specimens were postfixed in a 1% aqueous OsO_4 solution containing 1% $K_4Ru(CN)_6$ for 2 h at 4°C followed by several rinses in distilled water [De Groot et al., 1987]. Dehydration was performed in a graded ethanol and propylene oxide series. Specimens were embedded in Spurr's low-viscosity resin and polymerized overnight at 70°C.

Utricles and saccules were orientated in such a way that sections were obtained in a plane perpendicular to the striola. Cochleas were divided into two halves along a standardized midmodiolar plane [cf., Van Ruijven et al., 2004] and re-embedded in the same resin. Semithin (1 µm) sections were cut with a diamond knife on a Reichert-Jung 2050 SupraCut microtome, collected on glass slides, stained with 1% methylene blue and 1% azur II in 1% sodium tetraborate and examined with a Leica DMRA light microscope.

2.7 Quantitative Assessment: Vestibular Hair Cell Counts

Vestibular hair cells were counted off-line in digital images of phalloidin-stained utricles and saccules. Whole mounts were examined with a Zeiss LSM 510 META confocal laser-scanning microscope. At low magnification, the striolar and extrastriolar regions were identified and then, using a 63x objective, digital images were acquired of two randomly selected areas each in the striolar and extrastriolar regions, in each utricle and saccule (cf., fig. 8a). The field of view was 143 µm x 143 µm. Series of 3-dimensional overlapping image stacks were assembled into a composite image and digitally processed with Adobe® Photoshop® Elements, followed by a manual off-line count of the hair cells.

Hair cells were counted as present if they displayed a hair bundle and an intact cuticular plate. All hair cell counts were performed by two investigators, independently of one another, in a single-blind fashion. 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. Data of utricles and saccules were averaged for each experimental condition. Hair cell densities were expressed as the average number of hair cells per 1,000 μm^2. Using this counting method we have obtained hair cell densities for normal utricles and saccules that are comparable to the data reported by Desai et al. [2005] (see also table 2).

2.8 Quantitative Assessment: Cochlear Hair Cell Counts

The efficacy of the ototoxic treatment protocols was assessed by counting the number of outer and inner hair cells in semithin midmodiolar sections of resin-embedded cochleas at seven different locations (B1, B2, M1, M2, A1, A2, and A3) at a half-turn spacing [cf., Van Ruijven et al., 2004]. Either one of the following features was used for the identification of hair cells in case of oblique transection: (1) presence of the cuticular plate and/or stereocilia; (2) cytoplasmic staining characteristic for hair cells; and (3) presence of the basal part of the hair cell. All remaining hair cells were counted irrespective of their histological appearance. Histopathological features such as vacuolation, shrinkage, and loss of stereocilia or diminished cytoplasmic staining were not used as exclusion criteria. Hair cell counts were expressed as the mean percentage of remaining outer and inner hair cells per cochlear location.

2.9 Statistical Analysis

MATLAB 6.5 software (MathWorks, Inc.) and Microsoft Excel 2001 were used for processing data. For statistical analyses, we used STATISTICA 6.0 (StatSoft (Europe) GmbH). The effect of treatment on VsEPs (amplitude, latency and threshold), ABR threshold, and on striolar

and extrastriolar hair cell densities in the utricles and saccules was analyzed using repeated measures analysis of variance (rmANOVA) and the Student's *t* test. In case of the *t* test, VsEP data were first averaged over the two ears per animal. Differences were considered statistically significant when $p<0.05$.

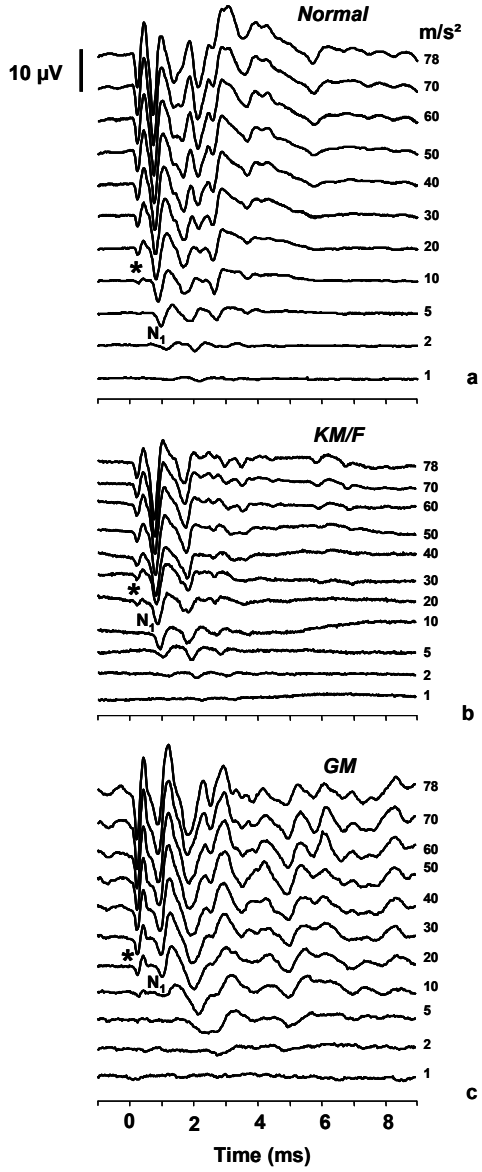

Figure 2. Examples of VsEPs with decreasing acceleration of the three groups studied. N_1 indicates the first negative response peak, appearing around 0.8 ms. An asterisk (*) near stimulus onset indicates the stimulus artefact in the waveform. **a** Normal: a clear N_1 is visible even at the lower accelerations. Later components of the VsEP waveform are also visible. **b** Combined kanamycin and furosemide treatment (KM/F): N_1 is clearly visible at the lower accelerations. **c** Gentamicin treatment (GM): N_1 is smaller than normal at the lower acceleration stimuli (<30 m/s²).

3. RESULTS

3.1 Vestibular Short-Latency Evoked Potentials: Effect of Treatment

Figure 2 shows VsEPs of a normal animal, an animal treated with kanamycin and furosemide, and an animal treated with gentamicin alone. The VsEPs in this example were evoked by pulses in the horizontal-medial direction at decreasing accelerations. The N_1 in the animal treated with kanamycin and furosemide (fig. 2b) was similar to the control N_1. The N_1 was reduced after gentamicin treatment (fig. 2c) as compared to the untreated control (fig. 2a). This was most noticeable at the lower stimulus levels, resulting in a higher threshold. The later peaks of the VsEP (latencies longer than 2 ms) were strongly reduced by combined treatment of kanamycin and furosemide (fig. 2b). The later peaks were not affected by gentamicin treatment. When stimulated with lower accelerations (2-10 m/s^2) the later peaks were visible, whereas the N_1 had disappeared (fig. 2c).

The VsEP thresholds of the three groups of animals, determined for N_1, are given in figure 3 for all directions. Treatment with kanamycin and furosemide did not cause a threshold shift in any direction. Tested over all directions of stimulation, a significant negative effect of gentamicin treatment on VsEP thresholds was found (rmANOVA with ear and direction as within factors, $F(2, 12) = 3.9$, $p<0.05$). Post-hoc analysis on the separate directions shows that the thresholds were higher in gentamicin-treated animals (5 m/s^2) than in normal animals (2.5 m/s^2) for the horizontal medial direction (*t* test, $p<0.05$). Data of the VsEP thresholds are summarized in table 1.

Table 1. Mean VsEP and ABR thresholds

Treatment	VsEP threshold (m/s^2)	ABR threshold (dB re normal)
Normal	3.1±1.0	0±3
Kanamycin/furosemide	3.2±1.3	59±13 ($p<0.001$)
Gentamicin	5.7±2.3 ($p<0.05$)	-11± 6 ($p<0.01$)

Mean and standard deviation of VsEP and ABR thresholds of the three groups of animals. Each group consists of five animals. For each animal VsEP thresholds were averaged across the three directions and the two ears. *p* values are shown for significant differences with normal.

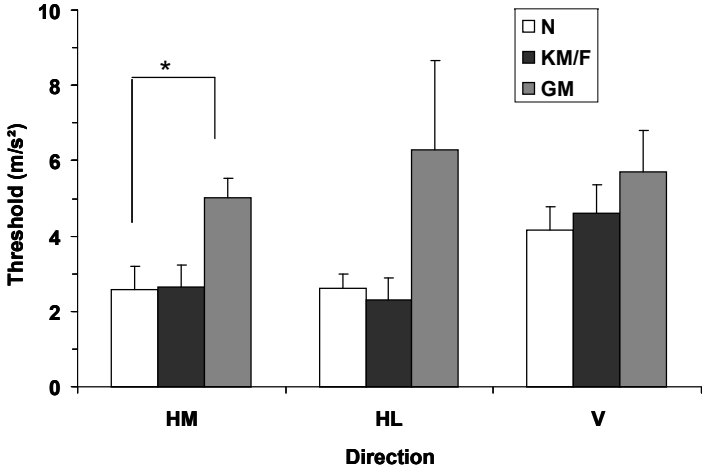

Figure 3. Average VsEP N_1 thresholds for the three directions of stimulation (HM: horizontal medial; HL: horizontal lateral; V: vertical) and different treatments (N: normal; KM/F: kanamycin and furosemide; GM: gentamicin). Thresholds are first averaged across ears and subsequently averaged across animals (n=5). Error bars represent SEM; *: $p<0.05$.

Analysis of the suprathreshold measures amplitude and latency of the N_1 of the VsEPs showed the following. The gentamicin-treated animals had significantly lower N_1 amplitudes than the normal controls and the animals treated with kanamycin and furosemide, when stimulated in the horizontal-medial direction (fig. 4a; rm ANOVA with ear and acceleration at 30-80 m/s² as within factors, $F(2, 12) = 5.7$, $p<0.05$). Also, the N_1 latencies to horizontal-medial stimuli were shorter in the gentamicin-treated animals than in the other animals (fig. 4b; rm ANOVA, $F(2, 12) = 4.0$, $p<0.05$). In the other two directions, the gentamicin-treated animals had generally smaller N_1 amplitudes and shorter latencies, but these differences were not significant. The amplitudes and latencies of the animals treated with kanamycin and furosemide were in the normal range.

3.2 Vestibular Short-Latency Evoked Potentials: Effect of Stimulus Direction

For each stimulus direction the VsEPs had similar waveforms, but the amplitudes significantly differed. Figure 5 illustrates the mean vestibular N_1 amplitudes of the control group stimulated with an acceleration stimulus of 40 m/s² in the three directions. Normal responses were significantly smaller during vertical stimulation than during stimulation in either horizontal direction ($p<0.001$, paired t test). Horizontal-medial stimulation evoked stronger responses than horizontal-lateral stimulation ($p<0.05$). This trend (HM>HL>V) was also found in animals treated with kanamycin and furosemide ($p<0.05$), but not in gentamicin-treated animals ($p>0.5$). These results were also reflected by the thresholds (fig. 3): the control animals and the animals treated with kanamycin and furosemide showed lower thresholds for stimulation in the two horizontal directions (~2.5 m/s²) than in the vertical direction (~4.5 m/s²), and gentamicin treatment annihilated these differences.

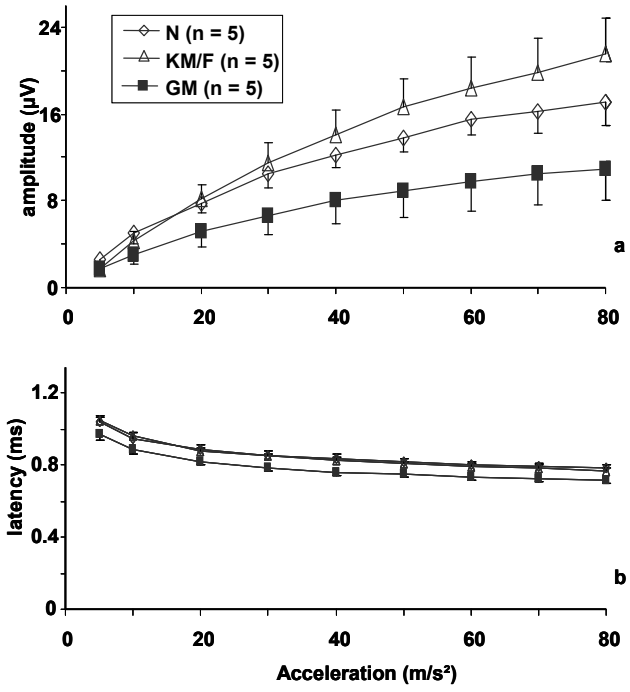

Figure 4. Averaged input/output curves of VsEP N_1 amplitudes (**a**) and N_1 latencies (**b**) for each of the three groups: normal (N), combined kanamycin and furosemide treatment (KM/F), and gentamicin alone (GM). Animals were stimulated in the horizontal-medial direction. Amplitudes and latencies are first averaged across ears and subsequently across animals (n=5). Error bars represent SEM.

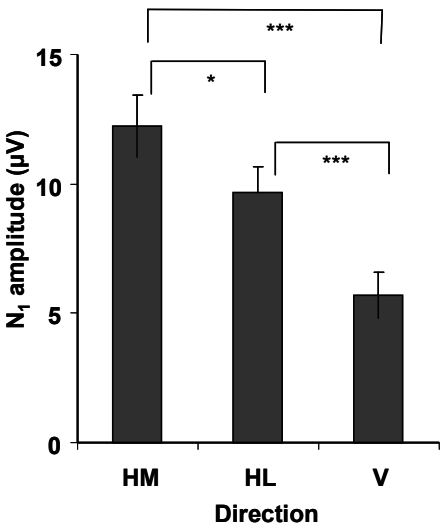

Figure 5. N_1 amplitudes of the VsEPs of the control group (at 40 m/s²) for the three stimulus directions (HM: horizontal medial; HL: horizontal lateral; V: vertical). Amplitudes are first averaged across ears and subsequently across animals (n=5). Error bars represent SEM. *: $p<0.05$; *** $p<0.001$.

3.3 Auditory Brainstem Responses

Figure 6 shows three series of ABR recordings, each series consisting of recordings at decreasing sound levels. Each series was recorded in a single animal belonging to one of the three treatment groups. The most salient result was the nearly complete absence of a discernible

waveform in the animal treated with kanamycin and furosemide (fig. 6b). Only at the highest level presented (77 dB nHL) some response could be detected. The gentamicin-treated animal showed responses down to the lower stimulus levels (-3 dB nHL, fig. 6c) like the normal control (7 dB nHL, fig. 6a). ABR thresholds determined in all animals are presented in table 1. A highly significant threshold increase of 59 dB was found in the animals treated with kanamycin and furosemide (*t* test, $p<0.001$). Remarkably, gentamicin caused a significant threshold decrease (-11 dB; $p<0.01$).

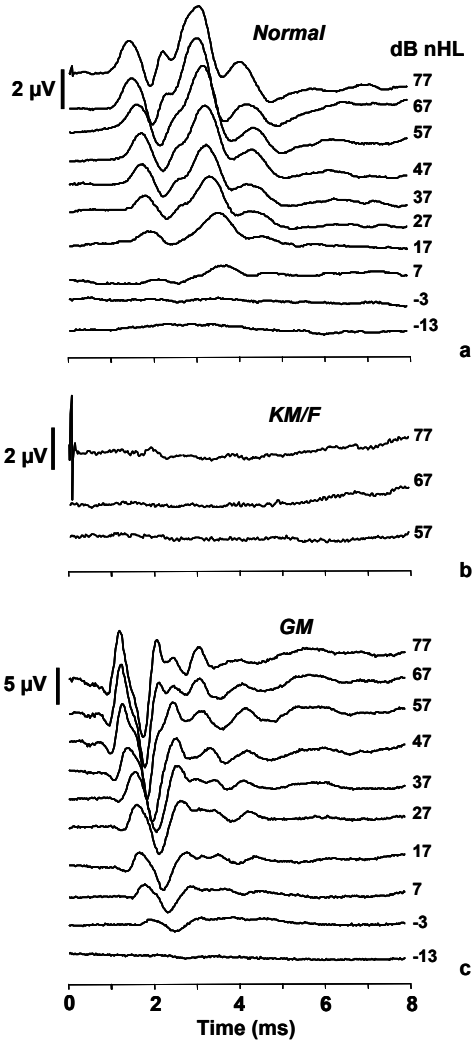

Figure 6. Examples of series of ABRs of the three groups studied. ABRs are arranged with decreasing sound level (in dB nHL). **a** Normal animal. **b** Animal treated with kanamycin and furosemide (KM/F): a discernible waveform is visible at 77 dB nHL only. **c** Animal treated with gentamicin (GM).

3.4 Morphological and Quantitative Assessment of the Utricles

Confocal images of phalloidin-stained whole mounts of utricles were analysed in striolar and extrastriolar regions (cf., fig. 7a). In animals treated with kanamycin and furosemide (fig. 7c), the striolar region demonstrates a topographical distribution of hair cells and supporting cells that is similar to that in normal utricles (fig. 7b). Quantitative analysis of utricular hair cell densities (pooled data) corroborates that treatment with kanamycin and furosemide had no effect on the number of hair cells in the striolar region as compared to untreated controls, nor in the extrastriolar region (table 2).

Table 2. Hair cell density in otolith organs

Organ	Treatment	vHC density Striolar region	vHC density Extrastriolar region
Utricle	Normal	9.6±0.3 (4)	11.5±0.3 (4)
	Kanamycin/furosemide	9.5±0.2 (8)	11.6±0.5 (9)
	Gentamicin	9.0±0.5 (4)	11.9±0.9 (5)
Saccule	Normal	11.6±1.2 (4)	11.4±0.7 (5)
	Kanamycin/furosemide	8.8±0.7 (7)	11.0±0.6 (9)
	Gentamicin	10.2±0.8 (4)	11.2±0.7 (6)

Vestibular hair cell (vHC) densities are shown for the striolar and extrastriolar regions in utricles and saccules from normal ears and ears treated with kanamycin/ furosemide and gentamicin. Hair cell densities are expressed as the average number of hair cells (± SEM) per 1,000 μm^2. Numbers between brackets denote the number of utricles and saccules used to determine vestibular hair cell densities.

In 2 out of 4 gentamicin-treated utricles numerous scars were detected in the striolar region, indicating hair cell loss (fig. 7d). Confocal images revealed the characteristic cartwheel-like staining pattern ('scars') displayed by the expanding supporting cells at the site of the missing hair cells; these scars were scattered throughout the striolar region. The hair cell densities in both specimens were lower than those in the striolar region of the other gentamicin-treated utricles and the normal utricles. Comparing the groups, the difference between gentamicin-treated and normal utricles was statistically not significant (*t*-test: $p>0.5$). The extrastriolar region of the gentamicin-treated utricles did not reveal any scars.

In semithin sections of resin-embedded utricles from animals treated with kanamycin and furosemide (fig. 8c), the macula demonstrated both areas with cellular lesions and areas that looked similar to the macula in normal utricles (fig. 8a). In the lesioned areas, many type-I hair cells showed shrinkage in a varying degree, and occasionally contained some small vacuoles

(fig. 8c). Unlike the type-I hair cells, the majority of type-II hair cells displayed a metachromatic cytoplasm and contained numerous vacuoles of varying size (fig. 8c). After gentamicin treatment, utricular hair cells demonstrated degenerative changes. Type-I hair cells especially demonstrated signs of shrinkage, whereas type-II hair cells were metachromatic in appearance and contained multiple small vacuoles (fig. 8e).

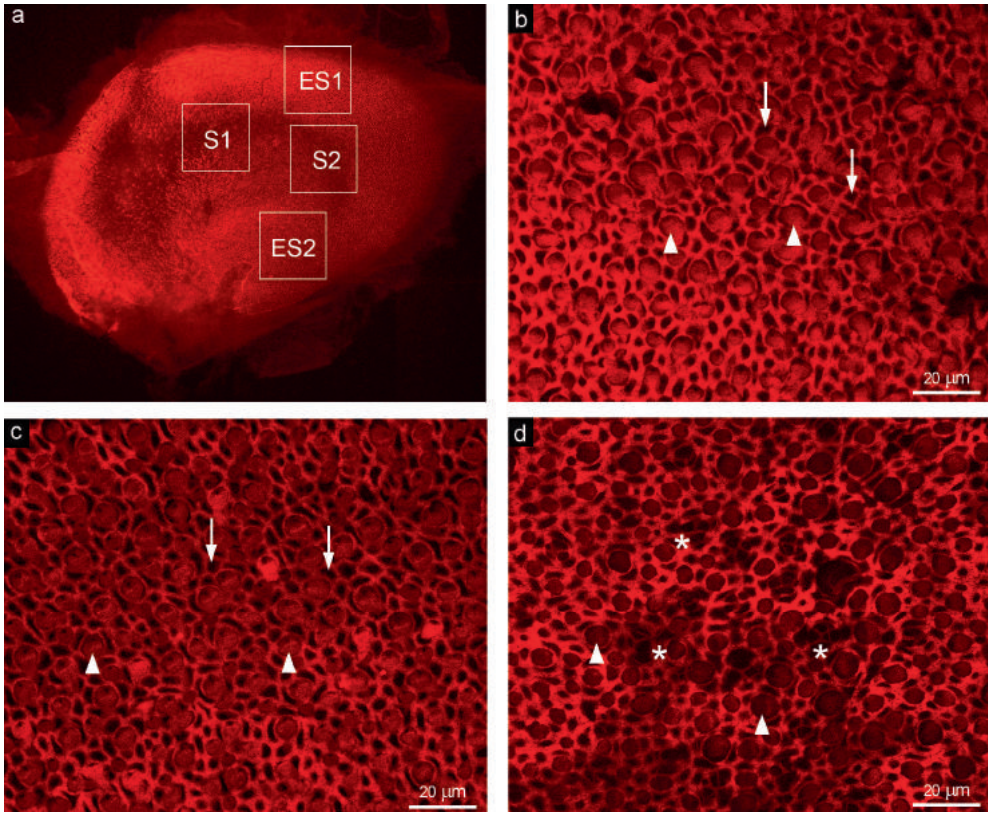

Figure 7. Confocal images of the striolar region in whole mounts of utricles stained with fluorescent phalloidin as hair cell marker. **a** Low-resolution image of a normal utricle. The boxes measure 143 µm x 143 µm and indicate two striolar (S) and two extrastriolar (ES) locations in which vestibular hair cells were counted. **b** High-resolution image of the striolar region in a normal utricle. Hair cells can be identified by their round shape and the hair bundles, which appear as intensely stained fluorescent dots (arrowheads). Supporting cells are smaller in size, polygonal in shape and contain an actin-free zone in the central portion (arrows). **c** Combined adminstration of kanamycin and furosemide does not affect the number of hair cells (arrowheads) or supporting cells (arrows). **d** After gentamicin treatment, supporting cell scars (*) are present, indicating 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. Remaining hair cells (arrowheads) still exhibit phalloidin staining of the cuticular plate and hair bundle.

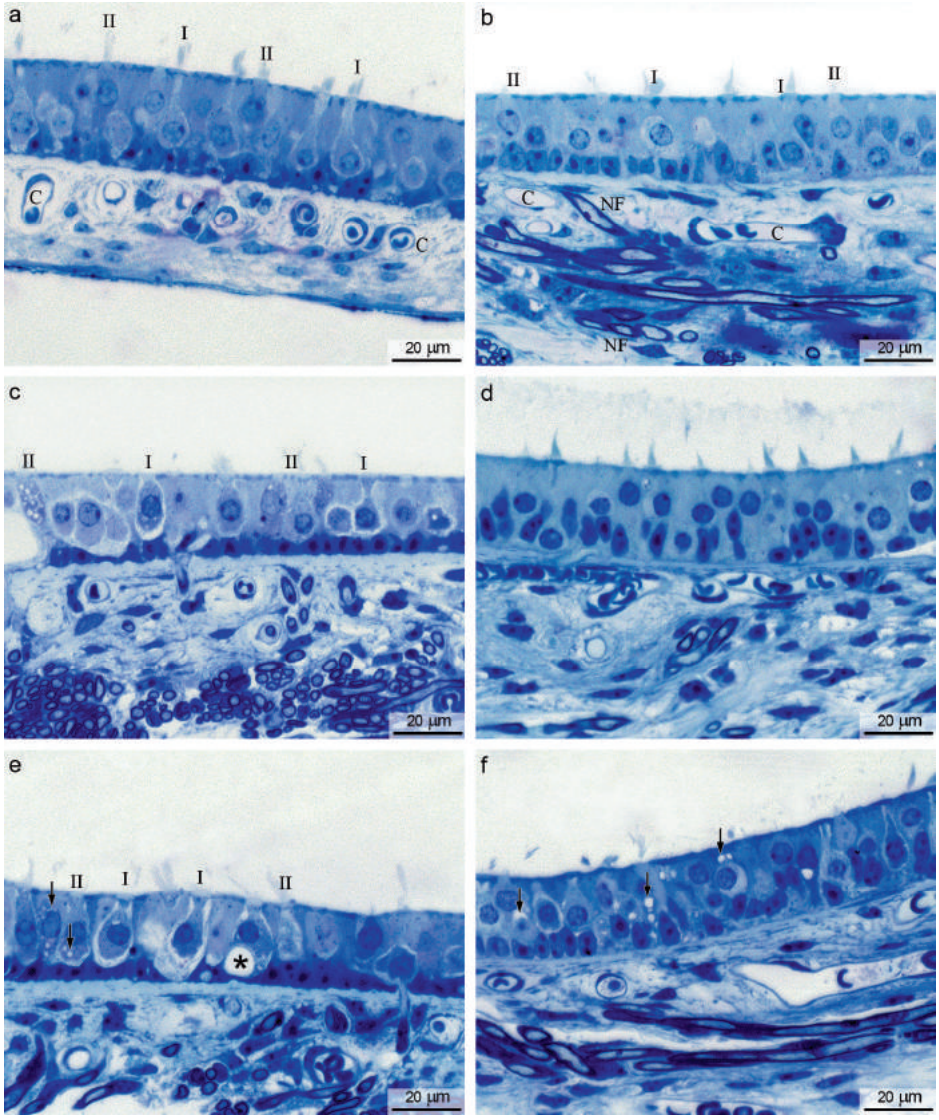

Figure 8. Light micrographs (**a**, **c**, **e**) of semithin (1 µm) transverse sections of resin-embedded utricles. **a** Normal utricule with part of the macula consisting of type-I hair cells (I) and type-II hair cells (II) surrounded by supporting cells. In the underlying stroma, numerous capillaries (C) are present. **c** After combined kanamycin and furosemide administration utricular type-I hair cells (I) demonstrate signs of shrinkage and type-II hair cells (II) contain numerous vacuoles. **e** After gentamicin treatment, both type-I hair cells (I) and type-II hair cells (II) in the utricular macula demonstrate degenerative changes, such as shrinkage (*) and vacuolation (arrows).
Light micrographs (**b**, **d**, **f**) of semithin (1 µm) transverse sections of resin-embedded saccules. **b** Normal saccular macula contains type-I hair cells (I), type-II hair cells (II) embedded in a layer of supporting cells. In the underlying stroma, nerve fibres (NF) and capillaries (C) can be seen. **d** After combined kanamycin and furosemide administration the saccular macula looks similar to that of the normal saccule. **f** After gentamicin treatment, intercellular edema is present in the saccular macula (arrows).

3.5 Morphological and Quantitative Assessment of the Saccules

Supporting cell scars were not observed in confocal images of phalloidin-stained whole mounts of saccules from animals treated with either kanamycin and furosemide or gentamicin, neither in the striolar nor in the extrastriolar region (light micrographs not shown). Additional quantitative analysis of the saccular hair cell densities (pooled data) demonstrates that combined treatment with kanamycin and furosemide and treatment with gentamicin alone had no significant effect on the number of hair cells in the striolar region as compared to untreated controls, nor in the extrastriolar region (table 2).

In semithin sections of resin-embedded saccules from animals treated with kanamycin and furosemide (fig. 8d), the macula did not look different from that in the normal saccule (fig. 8b). After gentamicin treatment, however, intercellular edema was infrequently observed in the saccular macula (fig. 8f).

3.6 Morphological and Quantitative Assessment of the Organ of Corti

Examples of light micrographs of midmodiolar sections, and the corresponding hair cell counts, from a cochlea treated with kanamycin and furosemide (figs. 9c, d) and a cochlea treated with gentamicin alone (figs. 9e, f) are compared with those from a normal cochlea (figs.9a, b). Co-administration of kanamycin and furosemide resulted in a collapse of the organ of Corti (fig. 9c). In the majority of cochleas there was extensive loss of both outer and inner hair cells, especially in the basal and middle turns (fig. 9d). After gentamicin treatment, there was no loss of the outer and inner hair cells (fig. 9f), but vacuoles can be seen in the supranuclear region of the outer hair cells (fig. 9e).

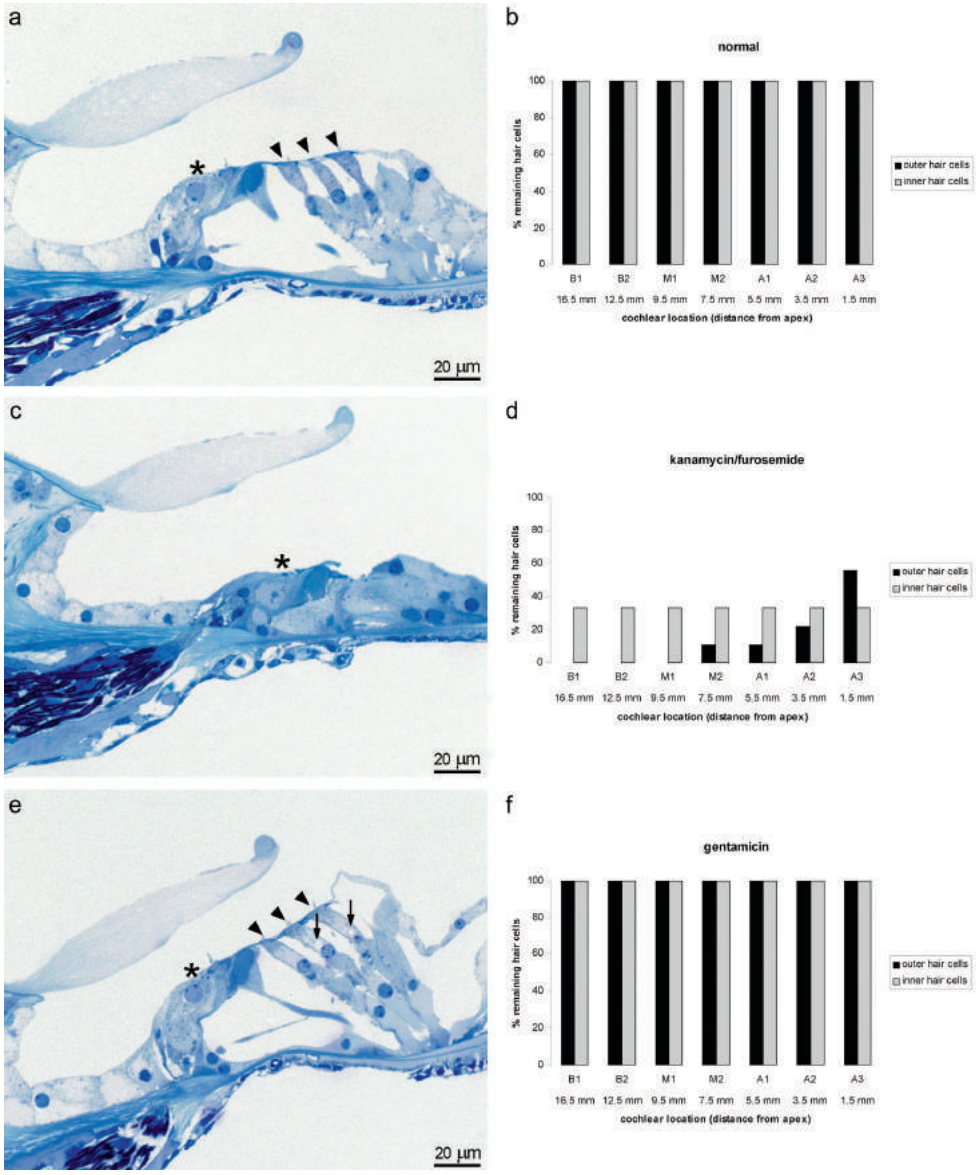

Figure 9. Light micrographs (**a**, **c**, **e**) of semithin (1 µm) midmodiolar sections of resin-embedded cochleas at location B2 (±12.5 mm from the apex). **a** Organ of Corti from a normal cochlea, demonstrating three rows of outer hair cells (arrowheads) and a single row of inner hair cells (*). **c** Completely collapsed organ of Corti in the cochlea of an animal treated with kanamycin and furosemide. The outer and inner hair cells are missing. **e** After gentamicin treatment, there is no loss of the outer hair cells (arrowheads) and inner hair cells (*), but vacuoles (arrows) are obvious in the supranuclear region of some outer hair cells. Midmodiolar hair cell counts (**b**, **d**, **f**) performed at 7 different cochlear locations (B1, B2, M1, M2, A1, A2, and A3; see Van Ruijven et al., 2004). The bars represent the percentages of remaining outer hair cells (black) and inner hair cells (gray) in normal cochleas (**b**), and cochleas treated with kanamycin and furosemide (**d**), and gentamicin alone (**f**). Loss of hair cells is observed only in the cochleas treated with kanamycin and furosemide.

4. DISCUSSION

We compared the effects of two different deafening protocols on vestibular and cochlear function and found that the protocols produced opposite effects. Combined kanamycin and furosemide treatment virtually abolished cochlear function, but had no effect on vestibular function, as assessed by the VsEP. Conversely, treatment with gentamicin alone did result in reduced vestibular function, but had no negative effect on cochlear function, at least not at the dosage regimen used in this study.

4.1 Effects of Combined Kanamycin and Furosemide Treatment

Co-administration of an aminoglycoside with a loop diuretic leads to irreversible loss of cochlear hair cells within a matter of hours (OHCs) or days (IHCs) [Russell et al., 1979; Ding and Salvi, 2005], and results in severe functional loss within 48 hours [Versnel et al., 2007]. This is in contrast to deafening protocols based upon chronic aminoglycoside administration, in which it takes several weeks for substantial hair cell loss to develop. In agreement with previous findings of other authors [Mitchell et al., 1997; Gillespie et al., 2003; Versnel et al., 2007], we observed substantially increased ABR thresholds corresponding with a severe loss of cochlear hair cells after combined treatment with aminoglycosides and loop diuretics. The pattern of hair cell loss was similar to that generally seen after aminoglycoside-alone administration. In particular, the outer hair cell loss followed a longitudinal gradient starting at the basal turn of the cochlea and decreasing towards the more apical turns [Forge and Schacht, 2000; Versnel et al., 2007, their fig. 7]. Also, we found that loss of outer hair cells was more severe than that of inner hair cells, indicating that outer hair cells are more vulnerable to this combination of ototoxic agents [Forge and Schacht, 2000].

Based upon these findings we expected to observe a damaging effect of combined kanamycin and furosemide treatment upon the otolith organs, at least functionally to some extent, but such an effect was not observed. The VsEP threshold to linear accelerations did not shift, nor did the N_1 amplitude to suprathreshold stimuli decline. Hair cell loss was not observed, although cellular changes such as cell shrinkage and vacuolation, either separate or in combination, were seen in the hair cells within the utricular macula. Note that the VsEP was sensitive to only minor otolith lesions as demonstrated in the gentamicin-treated animals, thus the absence of changes in the VsEP implies that damage induced by kanamycin and furosemide to the otolith organs was minute indeed.

The following considerations may be made to explain this striking difference in ototoxic effect between the cochlea and otolith organs. Firstly, differences in endolymphatic potential between the cochlea (~80 mV) and the vestibular system (~10 mV) as well as the different compositions of cochlear and vestibular fluids [for a review, see Wangemann and Schacht, 1996] may contribute to this difference. As furosemide temporarily decreases the endolymphatic potential [Sewell, 1984], it may thus affect cochlear function. However, furosemide alone does not affect vestibular function [Freeman et al., 1999, 2001], suggesting that the observed limited effect on the utricle may be due to kanamycin itself.

Secondly, it has been demonstrated that loop diuretics facilitate aminoglycoside entry into the endolymph as well as accelerate aminoglycoside uptake into cochlear hair cells [Tran Ba Huy et al., 1983; Yamane et al., 1988; Hayashida et al., 1989]. This facilitating process might differ in the otolith organs due to regional differences in the blood-labyrinth barrier, different uptake and saturation levels, or delayed entry from the vestibular endolymph. On the other hand, this phenomenon may be absent altogether in the otolith organs.

Thirdly, the differences in ototoxic effect may be explained in terms of the microenvironment of the hair cells. In particular outer hair cells differ greatly from vestibular cells. Outer hair cells in the organ of Corti are more vulnerable to ototoxic drugs than inner hair cells and immunohistochemical studies have demonstrated that aminoglycosides initially accumulate within the outer hair cells [De Groot et al., 1990; Ding and Salvi, 2005]. One explanation would be that since inner hair cells are almost entirely surrounded by supporting cells – except for their apical membranes, which are in direct contact with endolymph – they are exposed to lower drug levels and/or delayed exposure. This is because ototoxic drugs (i.e., aminoglycosides) from the endolymph can enter the inner hair cells only via their apical membranes, unlike the outer hair cells, which are directly exposed to aminoglycosides that are present in the cortilymph (i.e., perilymph) as well as in the endolymph. As vestibular hair cells, like inner hair cells, are surrounded by supporting cells (see figs. 8a, b), and contact the endolymph only by means of their apical membranes, this may explain why we observed hardly any hair cell loss in the utricular and saccular maculae. While differing in microenvironment, OHCs also differ from vestibular hair cells with respect to their motility. Furosemide reduces this motility [Ruggero and Rich, 1991], and this motility reduction might facilitate kanamycin uptake. However, it should be noted that furosemide-induced reduction of OHC motility might also have a protective effect as shown by Adelman et al. [2010] after noise exposure.

Finally, it has been suggested that a plethora of subcellular mechanisms – such as depletion of intracellular glutathione, increased intracellular levels of free radicals, interference with

intracellular signaling pathways, and decreased membrane permeability [Oesterle et al., 2008; Warchol, 2010; Ding et al., 2010] – are involved in drug-induced degeneration of cochlear hair cells. Differences in intracellular expression of key molecules in the various signalling pathways or the intracellular levels of free radical scavengers may explain the differences in ototoxic effect.

To the best of our knowledge there are no other reports on the toxic effect(s) upon vestibular function after combined kanamycin and furosemide adminstration, neither in clinical nor in experimental studies. Recently, the effect of concomitant administration of an aminoglycoside and a loop diuretic upon vestibular hair cell distribution in young mice has been studied by means of immunofluorescent techniques [Taylor et al., 2008; Oesterle et al., 2008]. Using several specific markers to identify vestibular hair cells, these authors could not find any evidence for hair cell loss in the utricular maculae of drug-exposed mice, not even within the striolar region. This agrees with the findings in the present study. None of the clinical studies reporting on the effect upon hearing mentions any disturbances on equilibrium, but it should be kept in mind that the incidence of vestibular ototoxicity in humans may be severely underestimated [cf., Rotstein and Mandell, 2004; Kisilevsky et al., 2004].

Our data confirm observations made in several rodent species [Böhmer et al., 1995: chinchilla; Jones et al., 1999: mouse; Oei et al., 2001: guinea pig] that the N_1 of the VsEP is of mainly vestibular origin, especially since it is not affected in animals deafened with kanamycin and furosemide, thus excluding the possibility of a cochlear contribution to the N_1.

4.2 Effects of Gentamicin Treatment

Gentamicin treatment has a damaging effect on vestibular hair cells [Forge and Schacht, 2000; Nakashima et al., 2000; Darlington and Smith, 2003; Lyford-Pike et al., 2007]. In the present study, this treatment caused a significant reduction in N_1 amplitude of the VsEP and an elevation in N_1 threshold. A statistically significant decline in the number of vestibular hair cells was not seen, but intracellular alterations (shrinkage and vacuolation) were apparent in the utricular macula. Thus, the VsEP proved to be sensitive for these minor histological alterations. A decline in vestibular function was also found in other studies [Elidan et al., 1987; Perez et al., 2000; Oei et al., 2004]. The effects found in these studies were larger than in our study, which might be related to the longer survival time applied in those studies (e.g., Oei et al. [2004], 3 weeks vs. 1 week) and mode of application (Perez et al. [2000] applied gentamicin topically).

N_1 amplitudes of the VsEPs recorded after vertical stimuli were significantly smaller and thresholds were higher than after horizontal stimuli. Similar findings for vestibular function

have been described before [Freeman et al., 1999, 2001; Plotnik et al., 1999; Jones et al., 2001]. Gentamicin treatment had more effect on VsEP outcomes when stimulated in the two horizontal planes than in the vertical plane (fig. 3). This concurs with our histological findings (figs. 8, 9) and those of other authors [Lindeman, 1969; Aran et al., 1982; Twine, 1985] that the utricle, which primarily detects horizontal accelerations, is more vulnerable to gentamicin than the saccule.

When aminoglycosides are administered without loop diuretics, hearing loss develops over a period of several weeks. This delay is due to the time needed for the drug to accumulate within the hair cells in sufficient amounts to provoke hair cell degeneration [De Groot et al., 1990; Hashino et al., 2000]. Although gentamicin causes subcellular alterations in cochlear hair cells already at an early stage of intoxication, it will take at least ten days for a substantial hair cell loss to occur [Aran et al., 1982; De Groot et al., 1991; Song et al., 1997; Aran et al., 1999; De Groot et al., 2005; Wang et al., 2007]. Functional damage of the cochlea in these studies was seen only after 20 days. In early stages of treatment the uptake of gentamicin by the cochlear hair cells occurs without affecting cochlear function [Hayashida et al., 1989]. Therefore, in our study substantial loss of hearing after seven days was not expected, but it came as a surprise that gentamicin caused an increase in auditory sensitivity. ABRs were recorded in free field. Hence both ears were stimulated. Since the most sensitive ear will determine the threshold, it cannot be excluded that the threshold decrease is due to changes in only one of both ears. However, because the drugs were systemically administered, it is plausible to assume that both ears will be affected similarly, and it is likely that this threshold decrease was caused by changes in both ears. Possibly, the biological availability of gentamicin to the cochlea at the time of measurements was very low. A self-protecting effect of low-dose gentamicin before high-dose gentamicin was recently described using guinea pigs and DPOAEs as indicators of cochlear function [Maudonnet et al., 2008]. Similar results have been found in guinea pigs after administration of amikacin [De Oliveira et al., 2004] and kanamycin [Fernandez et al., 2010]. In the latter study, it was found that low doses of kanamycin protect against acoustic noise trauma. Interestingly, similar to our findings, kanamycin-treated animals had less-than-normal thresholds (1-6 dB; their fig. 2), but this was statistically not significant.

5. CONCLUSION

The main question of this study was if combined administration of kanamycin and furosemide, which is known for its cochleotoxic effect, would also induce vestibular damage. Our results suggest that the answer to this question is negative. In general, aminoglycosides are both cochleo- and vestibulotoxic, but factors such as survival time and dosage determine whether effects are first seen in the cochlea or the vestibular organs. Under the conditions used in this study, there was a clear cross-effect of both treatment protocols. With this protocol we have a tool to selectively induce cochlear or vestibular damage, which can be of interest to researchers and clinicians alike.

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Chapter 3

Ototoxic study-II

Deafness induction in mice

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ABSTRACT

Hypothesis: How to induce most efficiently severe sensorineural hearing loss in mice using a single coadministration of an aminoglycoside antibiotic and a loop diuretic? **Background**: The coadministration of aminoglycosides and a loop diuretic has been widely used to induce hair cell and spiral ganglion cell loss in guinea pigs. However, the development of new treatment strategies against sensorineural hearing loss, such as tissue engineering techniques, requires the use of mouse models. Previous attempts to induce hearing loss in mice have rendered inconsistent results because of resistance to aminoglycoside-induced ototoxicity. Especially inner hair cells seem to be resistant to aminoglycoside-induced ototoxicity. **Methods**: In the present study, we aim to optimize hearing loss in mice, using a single high-dose kanamycin (700 and 1,000 mg/ kg) injection followed by a furosemide (100 mg/kg) administration. Although previous studies used intraperitoneal furosemide injections 30 minutes after kanamycin administration, we used intravenous furosemide injections administered within 5 minutes after kanamycin treatment. **Results**: Auditory brain stem responses illustrated severe threshold shifts, and histologic analysis showed marked outer hair cell destruction as well as spiral ganglion cell loss. The present protocol results in more severe inner hair cell loss when compared with the results of previous researches. **Conclusion**: We conclude that severe sensorineural hearing loss can be induced in mice. Moreover, we found that this mouse model can be augmented via the use of rapid intravenous furosemide administrations to maximize inner hair cell loss.

1. INTRODUCTION

The ototoxic effects of a single dose of combined administration of an aminoglycoside and a loop diuretic have long been studied to create animal models with sensorineural hearing loss (SNHL) [West et al., 1973; Webster and Webster, 1981]. The addition of a loop diuretic to an aminoglycoside administration has a more than additive ototoxic effect because diuretics facilitate aminoglycosides to pass the blood-cochlea barrier [Ding et al., 2003; Ding et al., 2002; Li et al., 2011]; they allow aminoglycosides to more effectively spread through the cochlea by altering the endolymphatic potential [Asakuma and Snow, 1980; Emst et al., 1997] and they decrease renal kanamycin clearance [Ohtani et al., 1978].

The combined administration is particularly effective in guinea pigs and has been frequently used in our department [Versnel et al., 2007; Agterberg et al., 2008; 2009; 2010; Bremer et al., 2012]. Unfortunately, lesioning the adult mouse cochlea using aminoglycosides has proven to be considerably more difficult than lesioning that of other rodents [Henry et al., 1981; Wu et al., 2001; Poirrier et al., 2010]. In guinea pigs 100mg/kg furosemide combined with 400mg/kg kanamycin was routinely used for effective hair cell (HC) and spiral ganglion cell (SGC) destruction. In mice however, for a similar procedure to generate comparable effects, much higher aminoglycoside concentrations are required [Wu et al., 2001]. For example, Taylor et al. [2008] and Oesterle et al. [2008] combined a high dose loop diuretic intraperitoneally with 1000 mg/kg kanamycin subcutaneously. Both regimens successfully induced elimination of almost all outer hair cells (OHCs), yet inner hair cell (IHC) loss was less evident and the percentages of animals having marked IHC loss were not optimal. Although these results are promising, for future experiments with mouse models, it is desirable to assure reliable reproducibility and severity. Furthermore, the use of larger studies with many experimental animals would benefit from rapid hearing loss induction as realised by a single administration of both an aminoglycoside and a loop diuretic [Oesterle et al., 2008], instead of more complex regimens using several administrations per day for several consecutive days [Wu et al., 2001; Hirose and Sato, 2011].

We studied a single dose deafening procedure in mice similar to the procedure for deafness induction in guinea pigs. Two high dose kanamycin regimens of 700 and 1000 mg/kg were compared. These doses were chosen because they are midway between the concentrations used for guinea pigs (400 mg/kg) and the LD50 value of kanamycin for mice (1300 mg/kg; *http://www.duchefa.com*). A rapid subsequent intravenous furosemide injection in the tail vein was used to enhance the ototoxic effects. Hearing was evaluated by recording acoustically

evoked auditory brainstem responses (ABR) using click stimulation and histological analysis in terms of HC and SGC counts.

2 MATERIALS AND METHODS

2.1 Study population and experimental design

Twenty-seven wild type C57Bl6 male mice of 6-8 weeks old were used (Purchased from Harlan Laboratories, Horst, The Netherlands). Weights from all animals were recorded before the deafening procedure. All animals had free access to food and water and were kept under standard laboratory conditions. Qualified personnel performed all procedures and the protocol was approved by the institutions animal experimentation committee (DEC) under number 2010.1.12.258.

There were 2 experimental groups. Group 1 consisted of 11 mice treated with 700 mg/kg kanamycin; group 2 consisted of 12 mice treated with 1000 mg/kg. Four mice served as control. All mice were tested for hearing loss by recording ABRs and sacrificed for histological preparation after 4 weeks. The researcher evaluating the histological preparations microscopically was blinded until data was statistically analyzed.

2.2 Deafening procedure

Mice were treated using kanamycin sulphate 700 mg/kg or 1000 mg/kg (stock solution 100 mg/ml in saline) subcutaneously. Within 5 minutes after kanamycin administration a 10 mg/ml furosemide (Centrafarm®; Etten-Leur; the Netherlands) was infused in the tail vein at 100 mg/kg. Control animals were treated with similar amounts of saline, subcutaneously and intravenously. Animals were weighed on a weekly basis.

2.3 Auditory brainstem responses

Methods for recording ABRs were described extensively for guinea pigs before [Versnel et al., 2007; Agterberg et al., 2008] and were essentially the same for the animals used in this study. The ABRs were recorded in a sound attenuated chamber under general anaesthesia using three subcutaneously positioned scalp electrodes. The ground electrode was inserted over the bregma, the recording electrodes were positioned lateral from the bregma just behind the pinna. Stimulus generation and data acquisition were controlled by custom-written software involving a personal computer and a Tucker-Davis Technology TDT3 system (modules RP2, PA5 (2x) and SA1). Acoustic stimuli consisted of click trains with an inter-stimulus interval of 99 ms.

99 Hz. Click duration was 40 µs. They were presented in an open-field configuration with a Blaupunkt speaker (PCxb352; 4 Ohm; 30 W) positioned at 10 cm from the pinna. Using a pair of attenuators (TDT3 system; module PA5), we varied sound levels from about 100 dB peak equivalent SPL down to below threshold in 10 dB steps. Calibration was performed with Bruel and Kjaer equipment. The ABR threshold was determined visually.

2.4 Histological tissue processing

After ABR measurements the animals were decapitated and the cochlea's quickly harvested. First, midmodiolar sections were prepared. Histological workup was performed as described for guinea pigs by de Groot et al. [1987]. Cochleae were treated with a tri-aldehyde fixative followed by decalcification, dehydration, embedding *in toto* in Spurr's low-viscosity resin and subsequent sectioning in semi thin midmodiolar sections (1 µm). Cochleae were examined with a Zeiss axiophot light microscope. An example of a midmodiolar section is shown in Fig. 1. Midmodiolar sections were used to study histological alterations qualitatively.

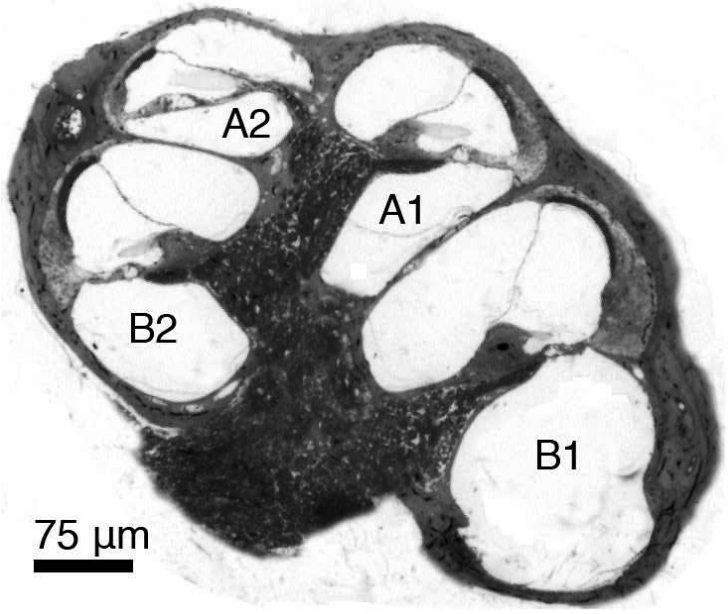

Figure 1 Light micrograph of a midmodiolar section of an entire normal mouse cochlea showing the different cochlear locations where we investigated spiral ganglion cells (staining: methylene blue and azure B).

We studied HC presence and absence quantitatively in a detailed way in surface preparations. The half-cochleae, which remained after taking a maximum of 16 midmodiolar sections, as described above, were cut in the transverse plane, just above the organ of Corti and below the basilar membrane. This was done for each half turn separately. These transverse surface preparations were then fixed to glass plates to allow for evaluation of hair cell presence over the entire length of the basilar membrane. The half turns were studied using Nomarski microscopy with a 40x objective lens. Microscopic pictures were taken with a video camera and projected on an LCD-monitor to enable counting. The width of the screen corresponded to 150 µm in the preparation; the height corresponded to 110 µm. For each cochlea, total length of the BM was calculated by adding the length of all the different monitor projections. To acquire an estimation of the cutting loss per cochlea, the total length of each individual BM was subtracted from the average mouse BM length (6.15 mm) calculated from BM length reports of Spongr et al. [1997]. On average, we lost a total of 1.5 mm per mouse, whereas Ehret and Frankenreiter [1977] reported an average loss of 0.8 mm per mouse. The higher cutting loss noted in our study was attributable to prior midmodiolar sectioning of the cochleas. HC counts were performed as follows: HCs were classified as absent when no stereocilia, cuticular plate or cell nucleus could be detected, or in case a phalangeal scar had replaced the HC. Percentages of HCs remaining were plotted against the distance from the helicotrema, expressed in percentage of total BM length.

For each animal, one fourth of the average total 1.5mm was added at each sawing place to calculate the total BM length. In five animals a single half turn was missing due to technical failures. In those cases the HCs could obviously not be counted. The length of missing half turns was set at the average of all other corresponding half turns in the other animals.

2.5 Data analysis

The statistical analysis was performed using SPSS® 15.0 for windows. All graphs were made using GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, California).

3. RESULTS

3.1 Survival and weight post treatment

One animal in the 700 and 1 animal in the 1,000 mg/kg group immediately died after kanamycin administration. The 25 remaining animals all survived the 4 weeks after treatment period and kept gaining weight (Fig. 2). Using a 2-way analysis of variance, we found that there is a significant increase in weight over time for all groups ($F_{2, 100} = 13.3$, $p < 0.001$). However, we

found no significant difference in weight gain over time between the 3 groups (as illustrated by the nonsignificant interaction effect between time and group; $F2, 100 = 0.2$, $p > 0.05$). Also, we used an independent samples t test with post hoc Bonferroni correction to evaluate the difference in weight gain at 2 different time points, Weeks 1 and 4. At Week 1, we found that the 1,000 mg/kg condition differed significantly from the control condition ($t15 = 5.4$, $p < 0.05$). The control condition did not differ significantly from the 700 mg/kg group ($t13 = 1.1$, $p > 0.05$). There was also no difference between the 700 and 1,000 mg/kg condition at Week 1 ($t23 = 2.4$, $p > 0.05$). At Week 4, the t test with post hoc Bonferroni correction illustrated that there was no significant difference between the control and experimental conditions (control versus 700 mg/kg: $t13 = 0.8$, $p > 0.05$; control versus 1,000 mg/kg: $t13 = 0.8$, $p > 0.05$). Thus, the differences between the control and 1,000 mg/kg group were not significant anymore at 4 weeks, indicating that the treated animals had caught up with their weight, and therefore, there are no indications for long-lasting systemic effects, which might compromise this model.

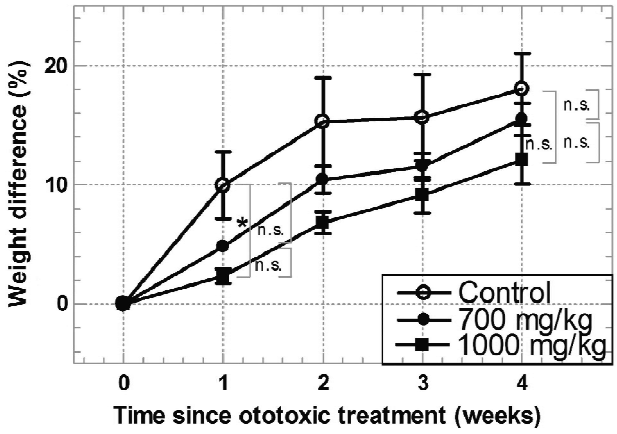

Figure 2 Average percentage of weight gain relative to the weight just prior to treatment of mice with saline, 700 and 1,000 mg/kg (*significant difference between groups, $p < 0.05$; n.s.: nonsignificant).

3.2 Auditory brainstem responses

Figure 3 depicts the ABR threshold shift for each individual animal, relative to the average threshold in the control animals. These results show a clear dichotomy, illustrating a significant difference between the case and control group using an independent samples t test ($t26 = -2.98$, $p = 0.006$). Sixteen of the 21 treated animals showed a threshold shift of more than 50 dB (76%). Five of the 21 animals have thresholds close to control value, (3 in the 700 mg/kg condition and 2 in the 1,000 mg/kg condition) representing a lack of effect, which Taylor et al. [2008] and Oesterle et al. [2008] observed in 20% of mice. Furthermore, among these nonresponders, 1 animal in each experimental group showed a partial response (14 and 18 dB

shift, respectively). If we restrict the analysis to the 16 animals, which showed a clear effect, we found no significant difference between the 2 dose groups of 700 and 1,000 mg/kg kanamycin (independent samples t test: t15 = 0.71, p = 0.709). Because we used ABR measurements as an exclusion criterion, just the 16 above-mentioned animals, which had a threshold shift of more than 50 dB, were considered for histologic analysis.

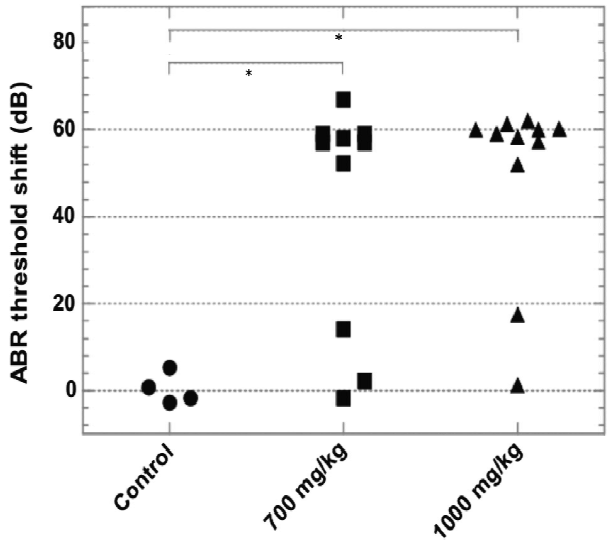

Figure 3 ABR threshold shift for each individual animal in each of the three groups (*significant difference between groups, $p < 0.05$).

3.3 Hair cell elimination

Figure 4 illustrates a representative surface preparation of the organ of Corti at cochlear location B1 (Fig. 1), from a control (A) and a deafened mouse treated with 700 mg/ kg kanamycin (B). All hair cells were destroyed in the treated mouse, and flat epithelium remained. Further quantitative analysis showed that all 16 animals, which did show marked ABR threshold shift illustrated severe HC loss in surface preparations (OHC loss: overall, 96%; range, 90%-100%; IHC loss: overall, 47%; range, 20%-80%). Using a 2-way analysis of variance, we found no significant difference between both drug concentrations (IHC survival: F1, 19 = 1.2, p = 0.28; OHC survival: F1, 19 = 0.1, p = 0.76). IHC survival was, however, significantly related to the location of HC counting (F19, 1 = 4.8, $p < 0.001$), following an apex to base gradient. Because there was not much difference between the 700 and 1,000 mg/kg group, we pooled them into a single group in Figure 5. This figure shows the percentages of average IHC and OHC loss plotted against the distance from the helicotrema, expressed in 5% intervals of total BM length. It shows that OHCs were severely damaged over the entire BM length. Mean IHC counts for both the 700 and 1,000 mg/kg group pooled decrease from 90% present at the apex to 0% at the base.

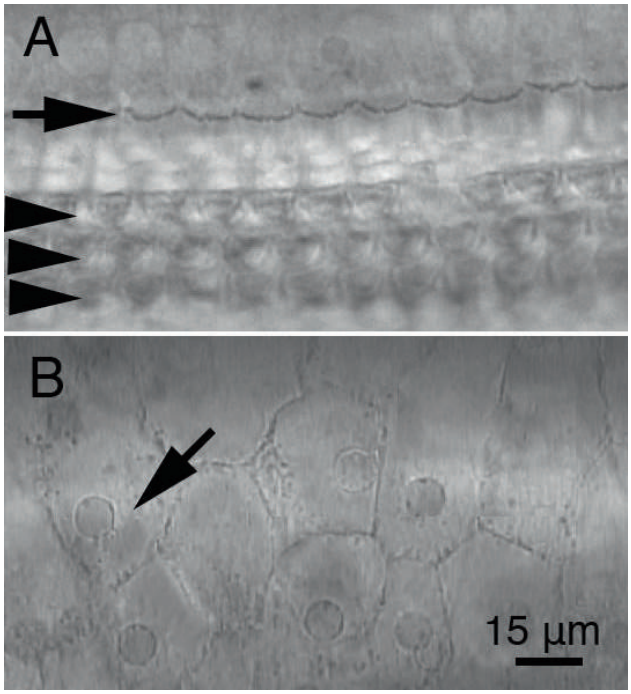

Figure 4 Surface preparation of the basilar membrane at cochlear location B1 of a mouse from the control (A) and a mouse treated with 700 mg/kg kanamycin (B). A, The arrows indicate IHCs, and the arrowheads illustrate OHCs. B, The arrow indicates a squamous cell.

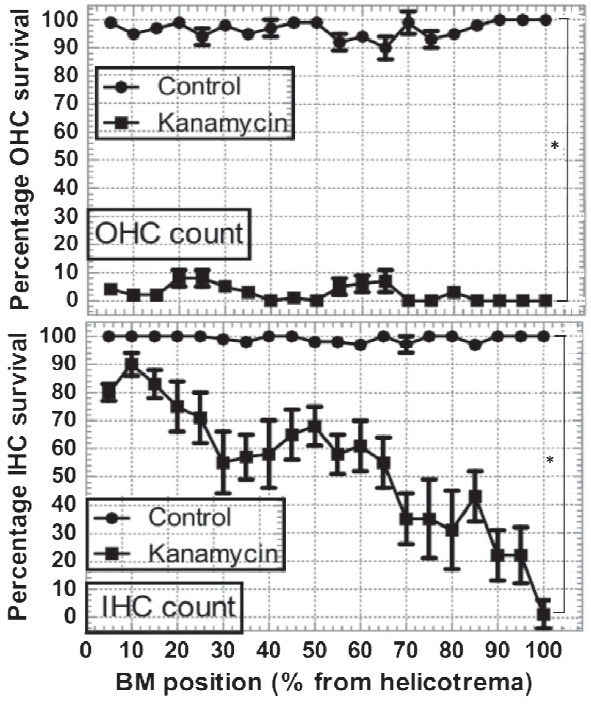

Figure 5 Mean percentages of missing OHCs and IHCs in 5% intervals as a function of percent total distance from the apex of the cochlea. Error bars are SEM (*significant difference between groups, $p < 0.05$).

3.4 Spiral ganglion cell degeneration

Figure 6 presents representative light micrographs of Rosenthal's canal at cochlear locations B1 and A1 of a control and deafened animal. In the control group and at cochlear location A1 of the kanamycin-treated group (Figs. A, B, and C), Rosenthal's canal contained the full complement of SGCs, whereas marked SGC destruction was found after kanamycin treatment (D) in the more basal turns.

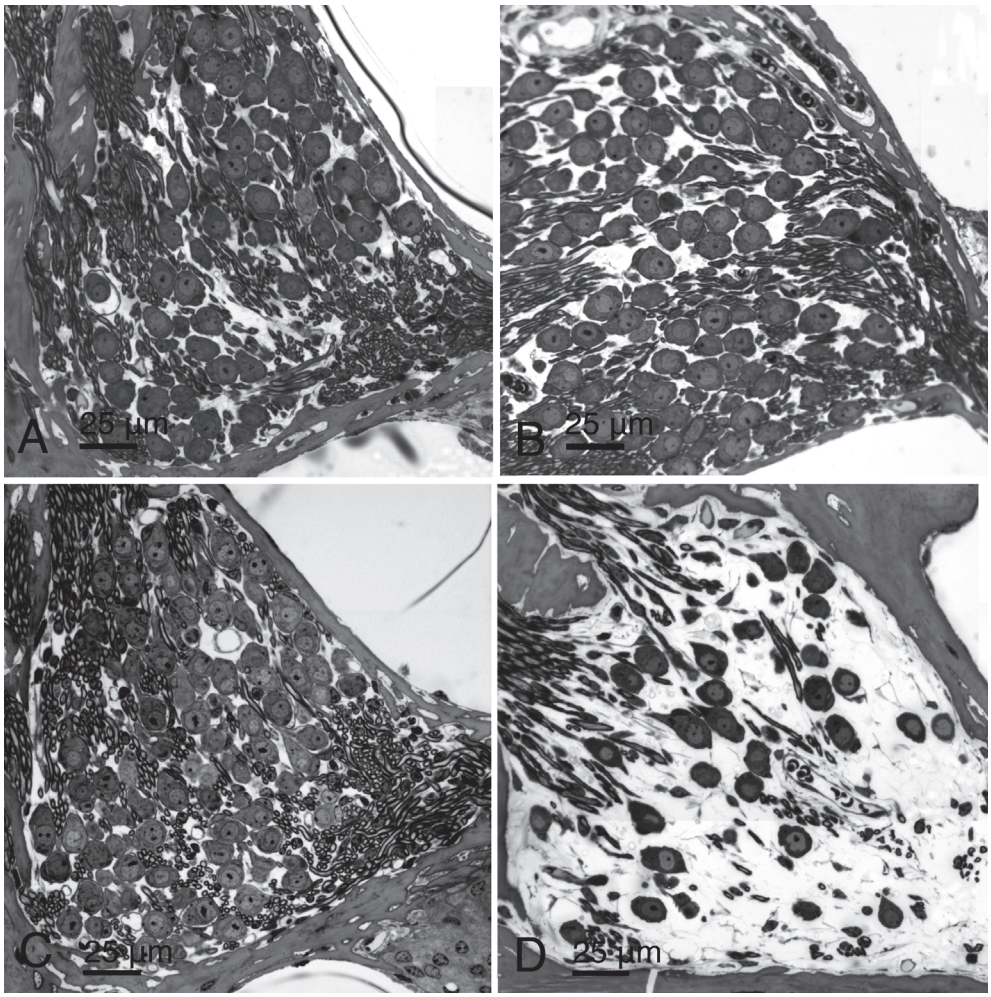

Figure 6 SGCs in a normal cochlea at level A1 (A) and level B1 (C) and in a deafened mouse (after treatment with kanamycin, 700 mg/kg s.c.) at level A1 (B) and B1 (D). Staining: methylene blue and azure B.

4. DISCUSSION

Our results show that induction of severe SNHL is possible in 6- to 8-week-old C57Bl6 mice using a singledose administration of 700 or 1,000 mg/kg kanamycin (s.c.) combined with 100 mg/kg furosemide (i.v.). Both concentrations induced comparable threshold shifts and severe HC and SGC loss without observable side effects.

4.1 Auditory brainstem responses

To evaluate the actual extent of SNHL, we used ABR measurements, which illustrated that almost 76% of the treated animals showed a threshold shift of more than 50 dB. There is no difference in the response rate and the size of the threshold shift between both experimental groups. This probably makes the 700 mg/kg condition the most preferable because it might induce less systemic toxicity. Moreover, our results have illustrated that ABRs evoked with click stimulation are suitable for selection of animals with severe SNHL with marked HC and SGC degeneration. Until now, no experiments using a single procedure for deafness induction used functional measurements to a control (A) and a deafened mouse treated with 700 mg/kg kanamycin (B). All hair cells were destroyed in the treated mouse, and flat epithelium remained. Further quantitative analysis showed that all 16 animals, which did show marked ABR threshold shift illustrated severe HC loss in surface preparations (OHC loss: overall, 96%; range, 90%-100%; IHC loss: overall, 47%; range, 20%-80%). Using a 2-way analysis of variance, we found no significant difference between both drug concentrations (IHC survival: $F1, 19 = 1.2$, $p = 0.28$; OHC survival: $F1, 19 = 0.1$, $p = 0.76$). IHC survival was, however, significantly related to the location of HC counting ($F19, 1 = 4.8$, $p < 0.001$), following an apex to base gradient. Because there was not much difference between the 700 and 1,000 mg/kg group, we pooled them into a single group in Figure 5. This figure shows the percentages of average IHC and OHC loss plotted against the distance from the helicotrema, expressed in 5% intervals of total BM length. It shows that OHCs were severely damaged over the entire BM length. Mean IHC counts for both the 700 and 1,000 mg/kg group, showed a decrease from 90% present at the apex to 0% at the base.

4.2 Hair cell elimination

We found a general destruction of almost all OHCs over the entire BM length after 4 weeks. Overall, 47% of all IHCs died (range, 20%-80% in different animals) with survival decreasing from apex to base. In line with the ABR measurements, no difference between the 2 dosage

regimens could be found. Similar OHC counts were found by other studies using single procedure regimens. Taylor et al. [2008] who used 1,000 mg/kg kanamycin (s.c.) combined with 400 mg/kg bumetamide intraperitoneally - instead of intravenously as we applied - found severe OHC loss within 48 hours posttreatment as well. Moreover, Oesterle et al. [2008] and Hartman et al. [2009], both using a protocol consisting of 1,000 mg/kg kanamycin (s.c.) combined with 400 mg/kg furosemide, again intraperitoneally, described overall OHC degeneration. IHC vulnerability, however, was proven to be more variable in these previous studies: Taylor et al. [2008] illustrated a marked IHC loss in 50% of treated mice after 3 months, whereas Oesterle et al. [2008] found IHC destruction in no more than 5% of treated animals after 11 weeks. Hartman et al. [2009] found no IHC destruction at all after 48 hours. Interestingly, when compared with Taylor et al. [2008], Oesterle et al. [2008], and Hartman et al. [2009], we managed to induce relatively high rates of IHC destruction, despite the fact that the mouse strain we used (C57Bl6) is more resistant to kanamycin-induced IHC destruction than the CBA strain used by most other studies [Wu et al., 2001]. With respect to Hartman et al. [2009], the difference in effect is easily explained, as their posttreatment time window of 48 hours is probably too short to allow for marked IHC loss. Regarding the study by Oesterle et al. [2008], it could be argued that the age of their mice partially accounts for their decreased IHC destruction rates because some of their mice were older (entire sample, 4-20 wk) compared with ours (6-8 wk) and IHC susceptibility decreases with age [Wu et al., 2001]. However, part of the animals of Oesterle et al. [2008] and all the animals of Taylor et al. [2008] were younger (2-4 wk) than ours. Therefore, the posttreatment window and mouse strain and age cannot account entirely for the discrepancy found in IHC destruction rates. An alternative explanation might be that the difference in both the access route and the timing of the loop diuretic administration influenced the degree of IHC destruction. We administered the furosemide intravenously in the tail vein, which is a more effective route than the intraperitoneal furosemide or bumetamide infusions used by the others. In addition, the time interval between kanamycin treatment and the subsequent loop diuretic administration differed between studies. In our study, this interval was 2 to 8 minutes, whereas in the other studies, this interval was 30 to 40 minutes. It is likely that their larger time window combined with a less effective access route would have resulted in less IHC destruction. In line with this supposition, Li et al. [2011], who treated Sprague- Dawley rats with 75 mg/kg of ethacrynic acid (i.v.) combined with 500 mg/kg kanamycin (i.m.), concluded that a short time interval between the loop diuretic and the aminoglycoside antibiotic administration is critical for inducing permanent hearing loss and cochlear degeneration. Taylor et al. [2008] and Oesterle et al. [2008] used single procedure

protocols comparable to ours except for the access route for furosemide. We also managed to induce more IHC loss when compared with the more complex multidose regimens of Wu et al. [2001] and Hirose and Sato [2011]. Wu et al. [2001] described severe OHC damage with exception of the apex. Similarly, Hirose and Sato [2011] described marked OHC destruction as well, with survival at the apex in 40% of cases. With respect to IHC destruction: Wu et al. [2001] did not describe this, whereas Hirose and Sato [2011], who used a tenfold lower dose of furosemide, describe IHC survival rates of 20% yet confined to the base.

It should be noted that no left and right comparisons were performed in our study. In our experiment, both drug administrations, reach their target - the cochlea - via the systemic blood circulation. Therefore, there is no reason to assume that the 2 cochleas would have responded differently to the treatment. This rationale has been confirmed by several authors who used similar methods for deafness induction in mice and guinea pigs. For example, Oesterle et al. [2008] performed left and right comparisons in mice, using s.c. kanamycin administrations and i.p. bumetamide treatments and found no differences within subjects. Furthermore, Agterberg et al. [2010] and Ding et al. [2002] also described high concordance between left and right cochleas.

4.3 Spiral ganglion cell degeneration

The second histologic parameter we considered was SGC survival. We found marked SGC packing density decreases at the basal sites. Oesterle and Campbell (24), who used a single procedure regimen of 1,000 mg/kg kanamycin (s.c.) and 400 mg/kg furosemide (i.p.) in 3- to 4-month-old Swiss Webster mice, considered SGC counts in mice before. In line with our results, they found marked SGC loss confined to basal sites at 30 days posttreatment.

5. GENERAL CONSIDERATIONS AND CONCLUSION

The use of systemic drug administration could have caused additional organ failure, such as kidney and liver failure, which might interfere with experimental outcomes. However, it is unlikely that the animals used in the current study have experienced permanent liver or kidney damage because there were no clinical signs for these side effects as observed by an independent veterinarian study monitoring board. Supporting evidence for this conclusion is that all the animals in the 2 experimental conditions did show a catch-up in weight gain. Nonetheless, additional research might be required to evaluate the potential hepatotoxic and nephrotoxic effects of our cotreatment.

Conclusively, we state that induction of severe hearing loss is possible in male mice belonging to the C57Bl6 strain, as measured in ABR threshold shifts and decreased HC and SGC survival. Moreover, we found that there is no significant difference between the 700 and 1,000 mg/ kg kanamycin dose, which allows us to use doses away from the LD50 value. Our mouse model of severe SNHL will be relevant for hearing impairment research in mice as it allows approaches using tissue-engineering techniques.

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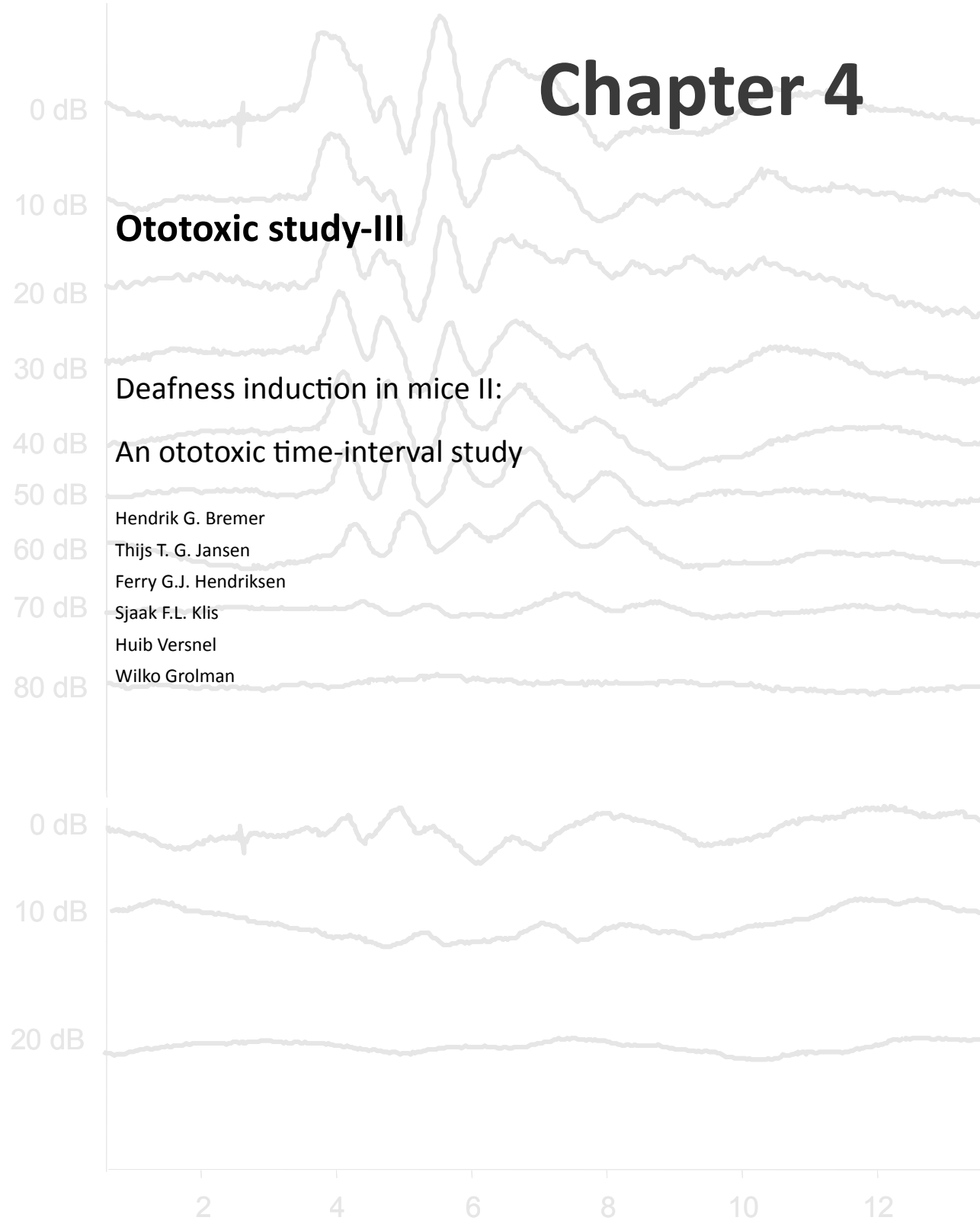

Chapter 4

Ototoxic study-III

Deafness induction in mice II:

An ototoxic time-interval study

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Thijs T. G. Jansen
Ferry G.J. Hendriksen
Sjaak F.L. Klis
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Wilko Grolman

ABSTRACT

Background: Due to ample availability of antibodies and probes, mice have long served as the model for research in tissue engineering techniques. Therefore, for the development of new treatment strategies in the domain of sensorineural hearing loss, a reliable and repeatable mouse model for deafness research is required.

Recently, our laboratory has improved the protocol introduced by Taylor et al. [2008] and Oesterle et al. [2008] which uses a single co-treatment of a loop diuretic combined with a high dose of aminoglycosides, to induce rapid hair cell loss [Jansen et al., 2013]. In the latter study, we noticed that the earlier the furosemide would be administered relative to the kanamycin infusion, hearing threshold shifts, assessed with auditory brainstem response (ABR) recordings to click stimuli, were the largest. **Methods:** In the present study, we aim to optimize hearing loss in mice. Twenty-four animals were divided in 3 experimental groups and 1 control group. We compared the effect of furosemide administered 10 minutes prior to -, simultaneous with - and 15-20 minutes after kanamycin administration. Hearing was evaluated by recording click-evoked ABRs. **Results:** Overall, more animals in the simultaneous group (five out of six) showed significant threshold shifts than the animals in the other groups (two and three out of six). This difference, however, was not significant (P>0.2). **Conclusion:** We could not find an efficacy difference of deafness induction between administering the furosemide with a 10 minute or 15 minute interval following the kanamycin, which we expected to find based on our previous work.

1. INTRODUCTION

Sensorineural hearing loss is one of the most common disabilities in our society, and it is usually associated with the loss of cochlear hair cells. The current treatment for sensorineural hearing loss, depending on its severity, is sound amplification or electrical stimulation using a cochlear implant. Cochlear implantation requires extensive surgery and this is not possible in all patients. Moreover, not all patients reach satisfactory auditory perception with either treatment options. More research towards unravelling the pathogenesis of sensorineural hearing loss is needed and new more cause-directed treatment strategies need to be developed. Such strategies may include tissue-engineering techniques aiming to protect and regenerate sensorineural epithelium. Due to ample availability of antibodies and probes, mice have long served as the model for research in tissue engineering techniques. Therefore, for the development of new treatment strategies in the domain of sensorineural hearing loss, a reliable and repeatable mouse model for deafness research is required.

Recently, our laboratory has improved the protocol introduced by Taylor et al. [2008] and Oesterle et al. [2008] which uses a single co-treatment of a loop diuretic combined with a high dose of aminoglycosides, to induce rapid hair cell loss [Jansen et al., 2013]. Since the inner hair cells transfer acoustic information to 90-95% of the afferent auditory neurons, their loss is more relevant than outer hair cell loss. The outer hair cells are thought to function as cochlear amplifiers. Earlier reports [Taylor et al. 2008; Oesterle et al. 2008; Hirose and Sato, 2011] found massive outer hair cell loss but only limited inner hair cell loss. We found that more radical inner hair cell loss can be obtained upon intravenous administration of furosemide in a time window of several minutes after kanamycin administration than a high dose loop diuretic intraperitoneally with 1000 mg/kg kanamycin subcutaneously [Taylor et al., 2008; Oesterle et al., 2008]. Mice were treated using kanamycin sulphate 700 mg/kg or 1000 mg/kg (stock solution 100 mg/ml in saline) subcutaneously. After kanamycin administration a 10 mg/ml furosemide (Centrafarm®; Etten-Leur; the Netherlands) solution was infused in the tail vein at 100 mg/kg. Herewith, we established a reliable method for reproducible IHC loss induction in an apex to base gradient. Kanamycin sulphate 700 mg/kg appeared as effective as 1000 mg/kg. We noticed that the earlier the furosemide would be administered relative to the kanamycin infusion, hearing threshold shifts, assessed with auditory brainstem response (ABR) recordings to click stimuli, were the largest (figure 1). The notion that the timing of furosemide potentially affects aminoglycoside induced ototoxicity is not a novel observation. For example

Brummet et al. [1979] found in the guinea pig that loop diuretic administrations will be solely effective when treated within hours. The maximal time interval between treatments in this study was three hours. In guinea pigs both a virtually simultaneous administration [Gillespie et al. 2003] and an interval of 2 hours are applied to obtain profound deafening [Mitchell et al. 1997; Miller et al. 2007]. Moreover, Ding et al. [2003] showed in the guinea pig that a relatively short time interval (2.5 hours) between injections of the loop diuretic (ethacrynic acid) and gentamicin compared with longer time intervals (5-12 hours interval) is the key point in causing permanent hearing loss and cochlear degeneration. The relevance of proper furosemide timing with respect to the kanamycin administration could be explained as both ototoxic drugs have a relatively small time window at which they maximally affect the cochlea. The time interval in mice may be more critical than in guinea pigs. Wu et al. [2001] found that after subcutaneous kanamycin injections in mice the maximal serum peak is reached within 15 to 30 minutes. Therefore one could argue that the diffusion capacity of kanamycin from the serum to the cochlear fluids is the largest in this window as well. The addition of a loop diuretic facilitates aminoglycoside entry into the cochlea by reversibly breaking the blood cochlea barrier and altering the endolymphatic potential. Both these effects are reached maximally within 15 and 20 minutes respectively. Therefore, we suggest that proper timing of furosemide administrations relative to the kanamycin administration results in more effective aminoglycoside entry into the cochlea, and hence more inner hair cell degeneration and corresponding hearing loss. However, in some animals in the study by Jansen et al. [2013] the intervals between administrations were larger than in others because the intravenous administration of the furosemide took more time. This may have caused spilling of furosemide leading to a less effective administration and therefore less cochlear availability and damage, as illustrated in Fig. 1 or it may be a real physiological effect. Thus, in the present study we systematically varied the interval. We compared the effect of furosemide administered 10 minutes prior to -, simultaneous with - and 15-20 minutes after kanamycin administration. Hearing was evaluated by recording click-evoked ABRs. Because of the previous observations (Figure 1; Jansen et al. 2013) we hypothesised that simultaneous administration of kanamycin and furosemide is a more efficient method of deafening mice compared to the larger interval method.

2. MATERIALS AND METHODS

2.1 Study population and experimental design

Twenty-four wild type C57Bl6 male mice of 6-8 weeks old were used (Harlan Laboratories, Horst, the Netherlands). Weights from all animals were recorded before the deafening procedure. All animals had free access to food and water and were kept under standard laboratory conditions. Qualified personnel performed all procedures and the protocol was approved by the institution's animal experimentation committee (DEC) under number 2012.I.02.025. Animals were divided in 3 experimental groups and 1 control group. Group 1 consisted of 6 mice treated first with furosemide (Centrafarm®; Etten-Leur; the Netherlands) at 100 mg/kg, infused in the tail vein at 100 mg/kg and after 10 minutes with 700 mg/kg kanamycin (stock solution 100 mg/ml in saline; subcutaneously) group 2 consisted of 6 mice treated with furosemide 100mg/kg directly followed with 700 mg/kg kanamycin (we considered a maximum of a three minute interval as simultaneous administration); group 3 consisted of 6 mice that were treated first with kanamycin 700 mg/kg and after 15 minutes with furosemide 100 mg/kg. Six mice served as control. Control animals were treated with similar amounts of saline, subcutaneously and intravenously, respectively. Weight was recorded on a weekly basis

2.2 Auditory brainstem responses

Methods for recording ABRs were described extensively for guinea pigs before [Bremer et al., 2012] and were essentially the same for the animals used in this study. The ABRs were recorded in a sound attenuated chamber under general anesthesia using 3 subcutaneously positioned scalp electrodes. The active electrode was positioned behind the pinna, the reference electrode on the bregma and the ground electrode was inserted at the dorsum. Stimulus generation and data acquisition were controlled by custom-written software involving a personal computer and a Tucker-Davis Technology TDT3 system (modules RP2, PA5(2x) and SA1). ABRs were evoked with monophasic clicks that were delivered with alternating polarity with an interstimulus interval of 99 ms. Click duration was 20 μs. Sounds were presented in an open-field configuration with a Blaupunkt speaker (PCxb352; 4 Ohm; 30 W) positioned at 10 cm from the pinna. Using a pair of attenuators (TDT3 system; module PA5), we varied sound levels from about 100 dB peak equivalent SPL down to below threshold in 10 dB steps. Calibration was performed with Bruel and Kjaer equipment. 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.

2.3 Data analysis

The statistical analysis was performed using the non-parametrical Kruskal-Wallis test (ABRs) and the one way-ANOVA test (weight) with SPSS® 20.0 for windows.

3. RESULTS

3.1 Weight, health and quality of injections.

Weight was recorded on a weekly basis because furosemide and aminoglycosides can cause loss of weight what could compromise the animal's health. This was not of interest in the current study (P>0.7, figure 4). Further, there were also no other signs of compromised health during the experiments. All deafening procedures went smoothly without spilling of furosemide.

3.2 Auditory brainstem responses

A series of click-evoked ABRs of a control animal is illustrated in figure 2a where ABR waveforms are seen up to 70 dB attenuation. When an animal is treated with kanamycin and furosemide (in this particular animal kanamycine was applied 15 minutes after furosemide) waveforms are only seen until 10 dB attenuation (figure 2b). Thus, in this example, the deafening treatment resulted in a threshold shift of 60 dB relative to normal. Figure 1 illustrates data from our first ototoxic study in mice [Jansen et al., 2013]. ABR threshold shifts are plotted against the time interval in which furosemide was given after kanamycin. There appears to be a bimodal distribution of threshold shifts, i.e., threshold shifts > 50 dB or a shift < 20 dB after treatment. When the time interval between treatments is more than 6 minutes all four animals showed only a small threshold shift. As mentioned before, this result triggered the present study, in which we specifically investigated the relative timing of the two injections. In Fig. 3 the ABR threshold shifts are plotted for each individual animal from the current study in relation to the average ABR threshold in the control animals. Again the distribution of threshold shifts after deafening is bimodal, comparable to the situation in Figure 1. Two animals, which received kanamycin 10 minutes after furosemide showed no ABR threshold shift while the other four showed a threshold shift of 50 dB or more. Of the six animals, which received furosemide 15 minutes after kanamycin three showed only a mild ABR threshold shift (0-20 dB). In the group where kanamycin and furosemide was administered simultaneously only one animal showed a low threshold shift of 5 dB. The other five showed ABR threshold shifts of 45 dB or more. Overall, more animals in the simultaneous group (five out of six) showed significant threshold

shifts than the animals in the other groups (two and three out of six). This difference, however, was not significant (P>0.2). We performed a meta-analysis over the results of this study together with the results of the first deafening study [Jansen et al. 2013] excluding the animals that did not receive the full dosage of the furosemide and categorising the intervals between treatments in groups. The group where the administration was performed simultaneously did show the highest number of large ABR threshold shifts. However, this was also not significant (P>0.6).

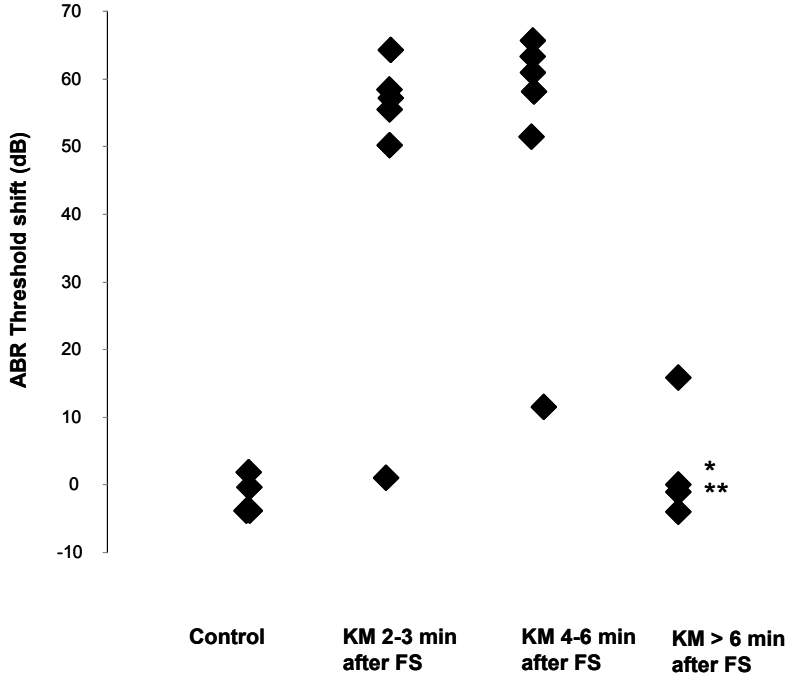

Figure 1. Click-evoked ABR threshold shifts of the animals from the first ototoxic study on deafening mice [Janssen et al., 2013]. The ABR threshold shifts of the experimental groups were plotted against the time intervals between administration of kanamycin and furosemide. The asterisks indicate two animals were there was observed spilling of furosemide.

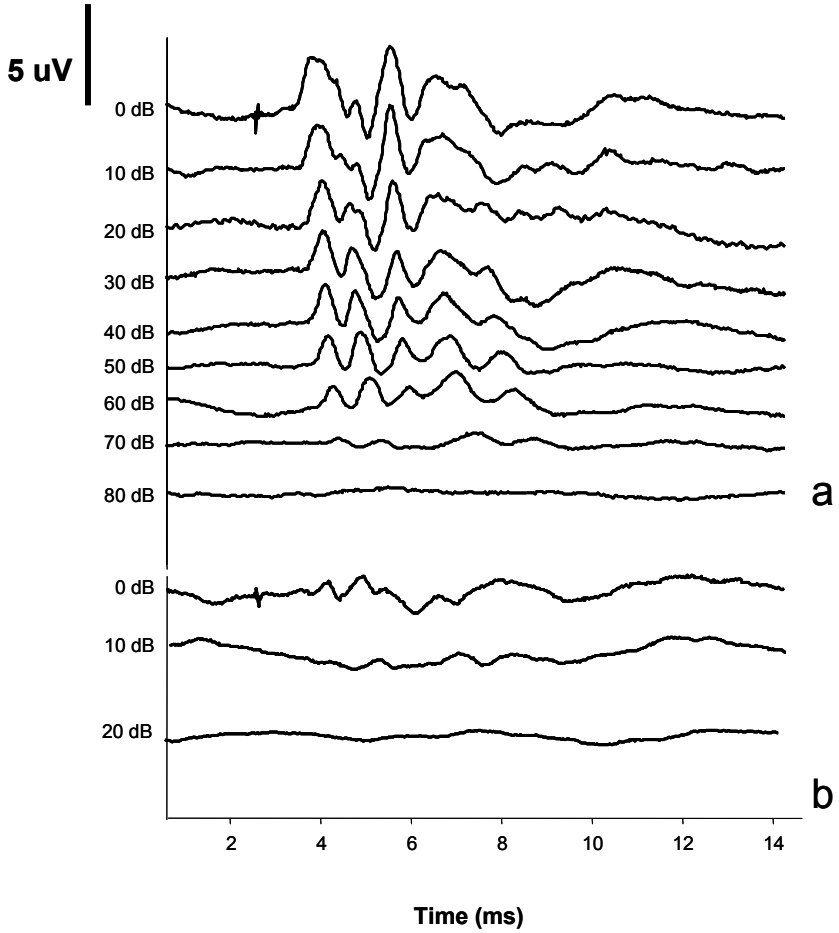

Figure 2. A series of click-evoked ABRs of an animal 28 days after saline injections. b Series of ABRs of an animal 28 days after co-treatment of kanamycin and furosemide. The furosemide was given 15 minutes after kanamycin.

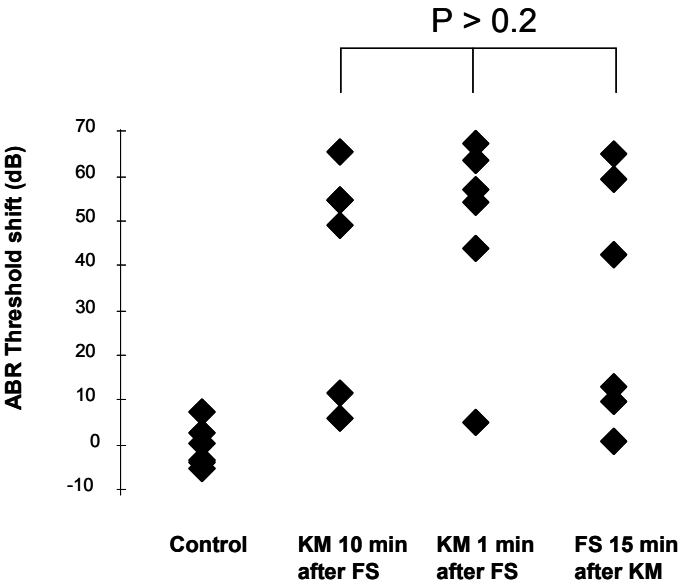

Figure 3. ABR threshold shifts of the animals of the experimental groups and of the control group. The ABR threshold shifts between the experimental groups did not significantly differ as evaluated by the non-parametrical Kruskal Wallis test.

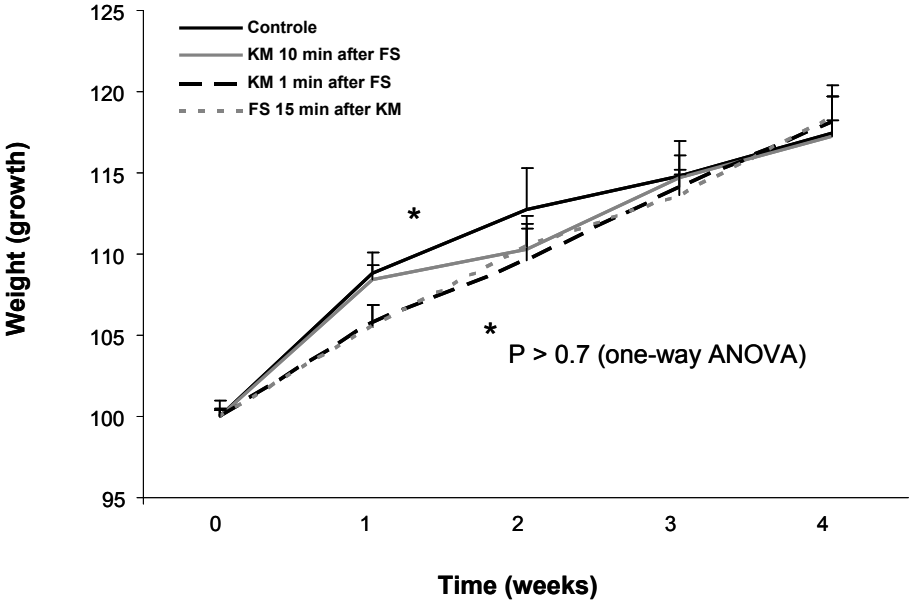

Figure 4. Time courses of the weight growth of the 4 groups. Error bars indicate S.E.M.
The weight growths between the groups did not significantly differ as evaluated by one-way ANOVA test.

4. DISCUSSION

We compared interval times between administration of furosemide and kanamycin regarding its effect on ABR threshold shifts as a measure for the induced deafness. There was no significant difference found on the ABR threshold shifts between the experimental groups ($P>0.2$). Overall, six of the eighteen animals (33%) showed a lack of effect after treatment as demonstrated by shifts in ABR thresholds of 0-20 dB. This is comparable to the response rates acquired by Jansen et al. [2013]. Hirose et al. [2011] used a dose of 900 mg/kg body weight kanamycin that was given every 12 h for 15 consecutive days by intraperitoneal injection. Intraperitoneal injections of furosemide at 50 mg/kg were given every morning, 20 min after the injection of kanamycin, and were done for 15 consecutive days. They found a profound ABR threshold shift in all mice using the co-administration. Hair cell loss was limited to the outer hair cells. This method is unpractical since it requires several injections per day for 15 consecutive days. Furthermore, this method gave rise to mortality. Also, a method that implies several injections per day is a burden for the animals, which is for obvious reasons undesirable. Taylor et al. [2008] and Oesterle et al. [2008] found a lack of effect in ~ 20-25% of their mice (both used a co-administration of kanamycin and furosemide) regarding the amount of outer hair cell loss. These studies suggested that the lack of effect was due to complications arising during kanamycin or furosemide infusion such as fluid leakage from the injection site. In this study the chance of complication bias caused by leakage was diminished by deliberately making several interval groups. Further, all the infusion procedures went smoothly. So the reasons for non-responding to treatment for the other animals in this study are probably due to individual resistance, as was also encountered in studies in guinea pigs using kanamycin and a loop diuretic [Nourski et al. 2004; Versnel et al. 2007]. It is known weight loss has a positive effect on the degree of hearing loss after administration of ototoxic drugs [Forge and Schacht, 2000], but this was not of interest in the current study ($P>0.7$, figure 4). The fact that the weight of the animal was not a significant factor determining the threshold shifts was seen also in Versnel et al. [2007] for guinea pigs.

5. CONCLUSION

The results of this study show that in individual animals either substantial hearing loss was induced or hardly any hearing loss was induced at all when using the co-administration of kanamycin and furosemide in mice. We could not find an efficacy difference of deafness

induction between administering the furosemide with a 10 minute or 15 minute interval following the kanamycin, which we expected to find based on our previous work. This ototoxic technique is helpful for future research on hearing impairment using a mouse model. However, we need to take note of poor reproducibility since not all mice are equally affected.

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Chapter 5

Applied study-I

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 were varied from 1 day to 16 weeks. Vestibular short latency evoked potentials (VsEPs) to linear acceleration pulses were recorded longitudinally to assess end-organ vestibular function. After 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 to 4 weeks after treatment. After this period, only two 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 vestibular end-organ 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 chemo-ablation of the vestibular sensory epithelia.

1. 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.

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] as well as Roberson et al. [1992] have provided evidence suggesting that new vestibular hair cells are produced at a low rate in the normal adult bird. Furthermore, Weisleder and Rubel [1992] have shown in the bird that repair of the sensory epithelium does occur following pharmacological destruction of vestibular hair cells and that 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.

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., 1994; 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% was 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 were 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 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 they gradually recovered to a peak 13-17 days after treatment.

Function of vestibular organs has been studied after *induced* regeneration as well [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 aminoglycoside-only-treated mice.

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 otolith organs 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 [Freeman et al., 1999; Jones and Jones 1999; Oei et al., 2001; Bremer et al., 2012; Chihara et al., 2013], in a longitudinal study in guinea pigs. At various times before and after gentamicin treatment VsEPs were recorded. The VsEPs were evoked with linear accelerations in three 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.

2. MATERIALS AND METHODS

2.1 Animals and Experimental Design

Forty-one 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. 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, and control electrophysiological measurements (VsEPs and auditory brainstem responses, ABRs) were performed prior to gentamicin treatment. Animals were clustered in groups of six and were selected for different survival times, varying from 1 day to 16 weeks (see Figure 1). One week after gentamicin treatment, electrophysiological recordings continued. 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 with a survival time of 8 weeks consisting of six animals. Three were given NaCl 0.9% solution instead of gentamicin and the other three 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.

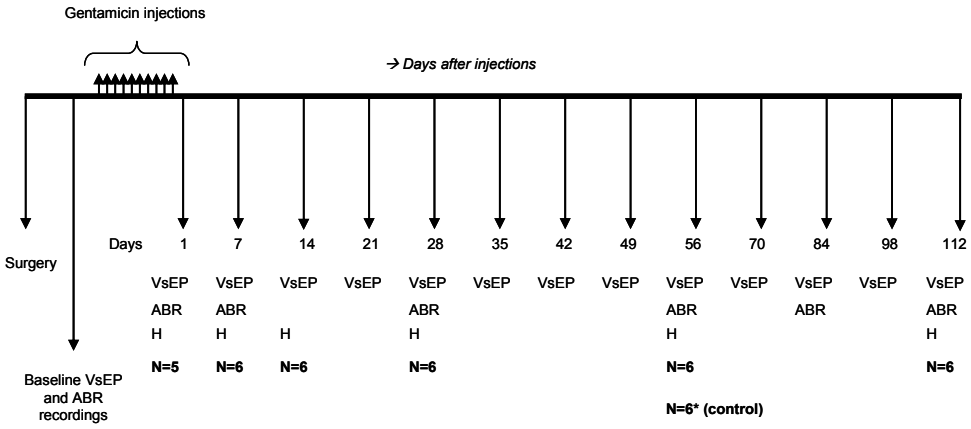


Figure 1. Schedule of treatment and electrophysiological recordings. Guinea pigs were sacrificed at various survival times (1 day, 1, 2, 4, 8 and 16 weeks). VsEP: Vestibular short-latency Evoked Potentials; ABR: Auditory Brainstem Responses; H: Histology. * Control animals were measured weekly until T=56 days. After final recordings animals were prepared for surgery. T=0 is defined as the tenth day of treatment.

2.2 Surgery

Surgery was performed to enable longitudinal VsEP recordings. Animals were anesthetized with Ketanest-S® (40 mg/kg, im) and Sedamun® (10 mg/kg, im). The skin on the skull was locally infiltrated with lidocaine 2%. An incision was made from the vertex till 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 screw was embedded in the cement and the exposed threaded post was used during VsEP recordings to secure the skull to a vibration exciter, which delivered linear acceleration stimuli.

2.3 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. 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.

2.4 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.

Animals were sedated with Ketanest-S® (13.3 mg/kg, im) and Sedamun® (3.3 mg/kg, im); 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. The threaded post of the screw attached to the skull was connected to a Brüel and Kjær vibration exciter, type 4809. This device could be orientated in three perpendicular planes to inflict head motions in three directions. We defined rostrocaudal movements as horizontal medial (HM), intra-aural movements as horizontal lateral (HL), and dorsoventral movements as vertical (V, see figure 2d).

The head motions were generated in a custom-made computer program 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 [cf., Oei et al., 2001]. Peak amplitudes varied between 1 and 78 m/s². Successive pulses of alternating polarity were used to reduce stimulus artifacts in the recordings. The acceleration pulse, *a(t)*, can be described as follows:

$$a(t) = \frac{b}{2} \cdot \left\{\sin\left(2\pi f t - \pi/2\right) + 1\right\} \text{ for } t \text{ between 0 and } 1/f, \quad \text{for } t \text{ between 0 and } 1/f,$$

with f = 2 kHz, b: maximum acceleration. Maximum jerk, a derivative of the acceleration, equals $\pi f b$ (e.g., for b = 40 m/s², jerk is 250 km/s³). Stimuli were calibrated with a Brüel and Kjær type 4521 accelerometer and monitored during recordings. Electrical activity was amplified (x5,000; 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 three or four 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 et al., 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 et al., 1995: chinchilla; Oei et al., 2001: guinea pig, Bremer et al., 2012: guinea pig].

The P_1-N_1 amplitudes and P_1 latencies were analyzed as a function of acceleration. Thresholds were determined by processing using Matlab software and by visual inspection of the series of P_1-N_1 waveforms recorded at various accelerations (see Fig. 3). When a threshold was 78 m/s² or higher, it was quantified as 78 m/s².

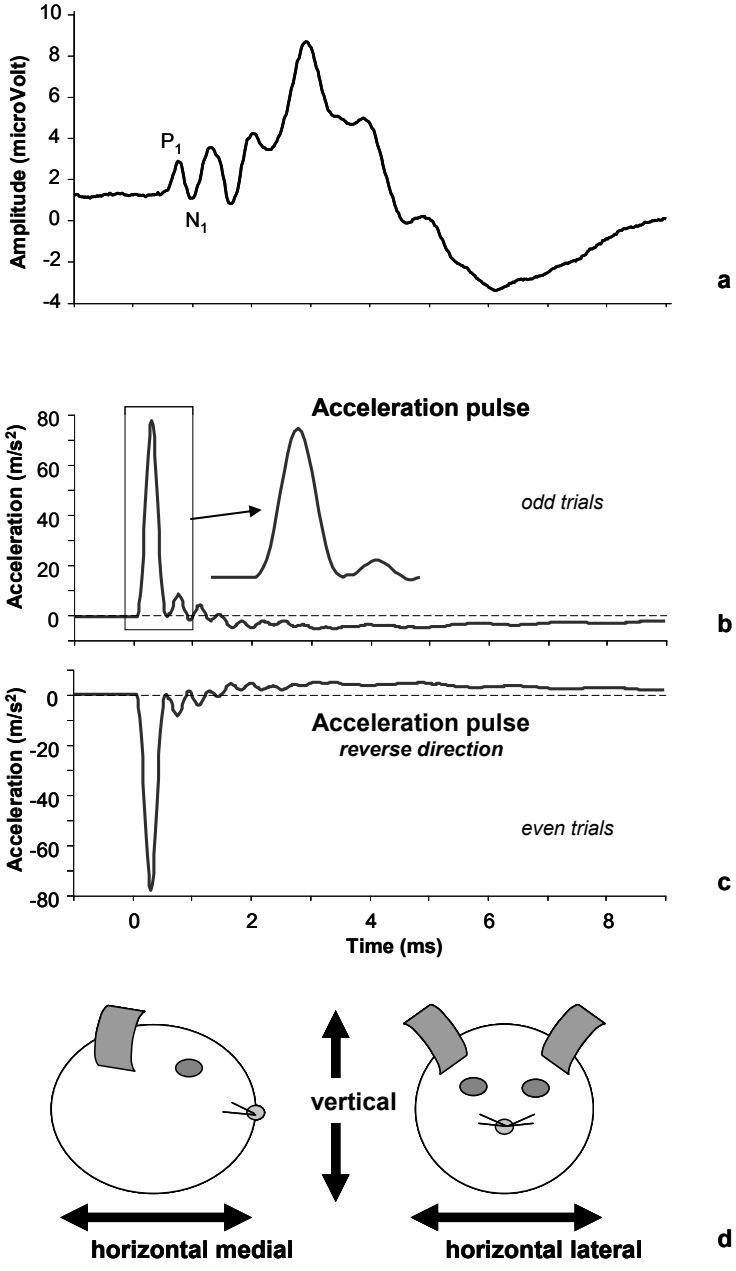


Figure 2. a. Example of a VsEP recorded at a stimulus level of 70 m/s^2 in the horizontal-medial (HM) direction in an untreated animal. The P_1 and N_1 are indicated. **b**. Example of the recorded stimulus waveform. The stimulus is also shown in detail from –0.2 to 1 ms. **c** Recorded waveform of the stimulus in opposite polarity. **d**. Schematic drawing to clarify the three stimulus directions.

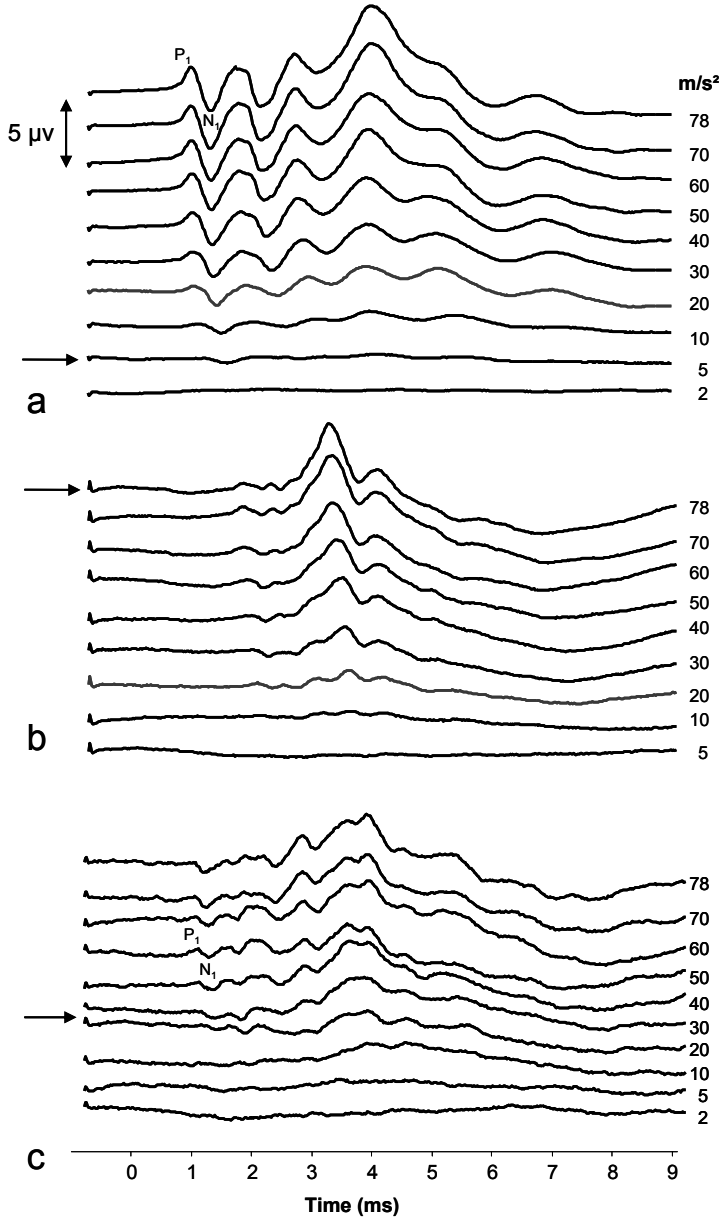


Figure 3. a . A series of VsEPs of a control animal stimulated in the HM direction. **b**. A series of VsEPs of another animal treated with gentamicin 3 weeks after treatment stimulated in the HM direction. The P_1 and N_1 can no longer be detected. Later peaks are still visible. **c**. A series of VsEPs of an animal treated with gentamicin after 16 weeks stimulated in the HM direction. Here, a P_1 and N_1 can be detected from stimulus level of 20 m/s². Arrows indicate threshold.

2.5 Measurement of Cochlear Function

Click-evoked ABRs were recorded immediately before gentamicin treatment, one 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 was placed in the neck and the ground electrode in the dorsum. ABRs were evoked with monophasic clicks (20 μs) with alternating polarity that were delivered at a rate of 10/s. Stimulation was started at 77 dB nHL (~110 dB peSPL) and 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, FL, USA). The clicks were presented with an earphone (Blaupunkt PCxb352), which was held 10 cm from the animal's left ear. The responses were amplified (x5,000) using a differential amplifier (EG&G instruments, model 5113), and AD 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.

2.6 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 micro hooks 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 phosphate-buffered saline (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, CA, USA).

2.7 Quantitative Assessment: Vestibular Hair Cell Counts

Vestibular hair cells were counted off-line 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 and then, using a 63x 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 µm x 143 µm. Series of 3-dimensional overlapping image stacks were assembled into a composite image and digitally processed with Adobe® Photoshop Elements, followed by a manual off-line count of the hair cells.

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 μm^2.

2.8 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 on striolar and extrastriolar hair cell counts in the utricles and saccules was analyzed using repeated measures analysis of variance (rmANOVA) and the Student's *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 $p<0.05$.

3. RESULTS

3.1 Normal VsEPs

Figure 2a shows an example of a VsEP after stimulation in the horizontal medial 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, later peaks originate from mixed auditory and cranial nerve nuclei (see also Materials and Methods section). 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).

3.2 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 and a clear P_1 and N_1 were visible down to a low stimulus level (5/ms²). Figure 3b shows a series of VsEPs (of another animal) at different stimulus levels three weeks after gentamicin treatment. No P_1 or N_1 can 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 VsEPs recorded 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 figure 3a) at higher stimulation levels down to 20 m/s². Amplitudes were much smaller than in control animals (figure 3a).

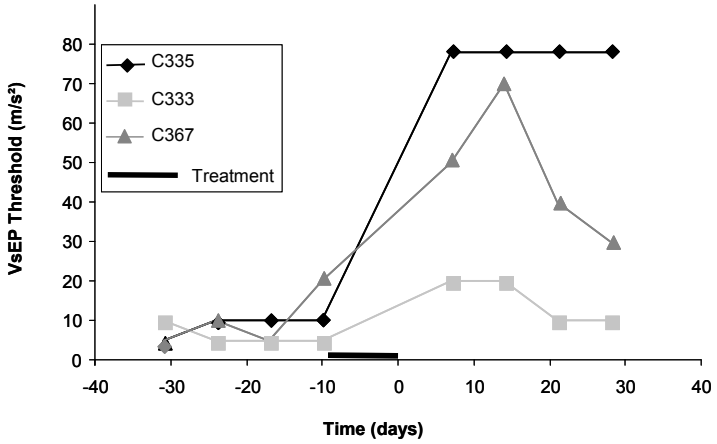


Figure 4. Threshold time courses of three different animals before and in the first four weeks after gentamicin treatment. Baseline thresholds before treatment were stable. After ten days of treatment the thresholds vary widely among the three animals from 20 m/s² to ceiling levels (78 m/s²).

The maximal degenerative effect of gentamicin treatment on electrophysiological function appeared in the first four 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 three directions in the first four weeks after treatment. When HL stimulated (Fig. 5b) threshold shifts were significantly larger than in the other two 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/s². 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 V stimuli (paired t test, p<0.001).

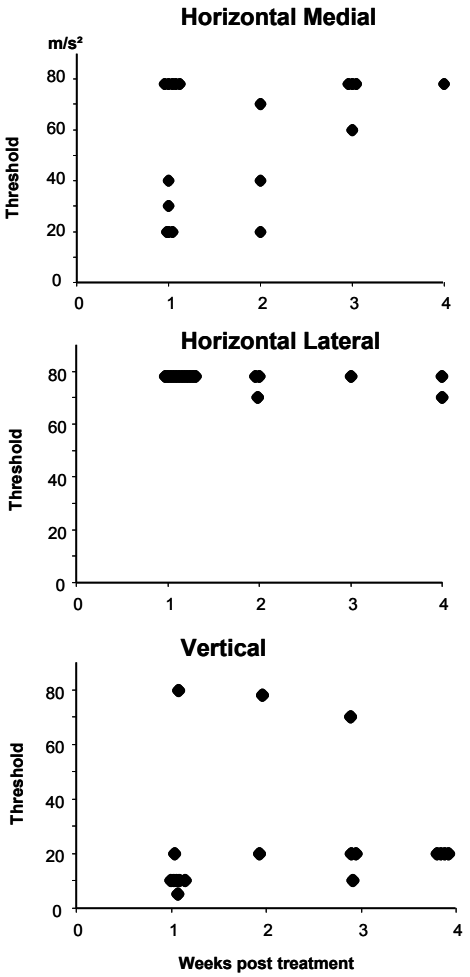


Figure 5. Maximum VsEP P_1-N_1 thresholds of each animal with survival times of 4 weeks or longer (n=18) plotted at the first moment the maximum threshold was recorded.. **a.** Stimulation in the horizontal medial (HM) direction. **b**. Horizontal lateral (HL) stimulation. **c**. Vertical (V) stimulation.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

3.3 VsEP: Long-term Effects

Amplitudes

Figure 6 shows individual time courses of the P_1-N_1 amplitudes of the 16-week survival group at 4 weeks (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 1.0 µV until 70 days.

After this time point there was a gradual increase in amplitude in two animals from about 0.5 to 1 µV. Two other animals showed a moderate increase and two showed no sign of increase or further decrease. Considered over all six animals, from 3 weeks after ototoxic insult, when effects were maximal (see also Figs. 4, 5), to final recordings after 16 weeks, recovery of VsEPs to HM stimuli appeared to be nearly significant (rm ANOVA: $F(8, 40)=2.13$, $p = 0.056$).

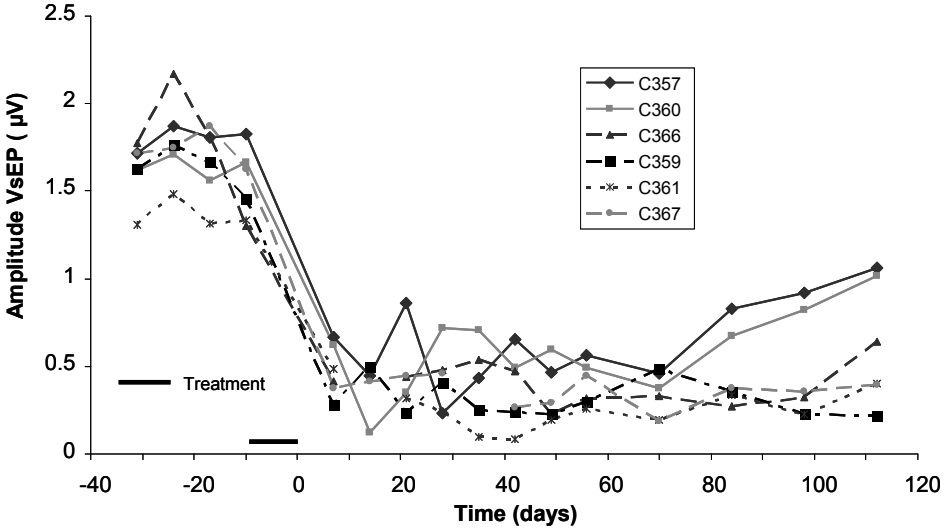


Figure 6. The individual time courses of the VsEP P_1-N_1 amplitudes of the animals in the 16-week survival group (n=6, stimulation at 70 m/s² in the HM direction).

Figure 7 shows the mean P_1-N_1 amplitude time course of the control group (a), the 8-week survival group (b), and the 16-week survival group (c). During control measurements there was amplitude variability between 1 µV and 2 µV. After treatment there was a rapid decline of amplitude in the experimental groups as described above. Considering both 8-week and 16-week groups from about three to eight weeks survival time there was no systematic trend. As already shown in Fig. 6, after 60 days there appeared to be a gradual increase in amplitude for the HM direction. Such trend was present to some extent for the V direction, but it was absent for the HL direction. Statistical analyses over the three directions confirmed the trend ($F(8, 40)=1.85$, $p= 0.096$).

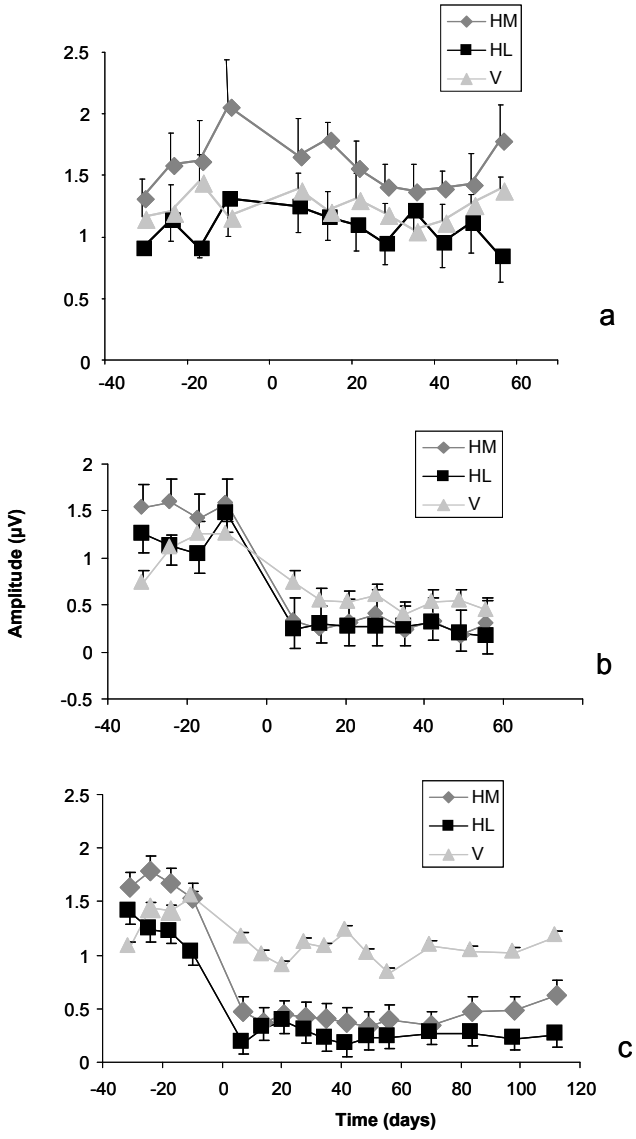


Figure 7. Averaged P_1-N_1 VsEP amplitudes for the three directions (horizontal medial, horizontal lateral, vertical). **a**. Control group (n=6). **b**. 8-week group (n=6). **c**.16-week group (n=6). Error bars represent standard error of means. Day 0 corresponds to the tenth day of treatment.

Thresholds

Figure 8 shows the mean threshold time course of the control group (a), the 8-week group (b) and the 16-week group (c). The control group showed stable VsEP-thresholds over 16 weeks between 5 and 10 m/s^2. In the 8-week group there was a tendency of a threshold decrease after 30 days for all three stimulus directions (i.e., recovery). This was not statistically significant considering 5 time points post-treatment (3 - 8 weeks; rm ANOVA, 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 or increase (rm ANOVA, F(8, 40) = 0.43, p = 0.89).

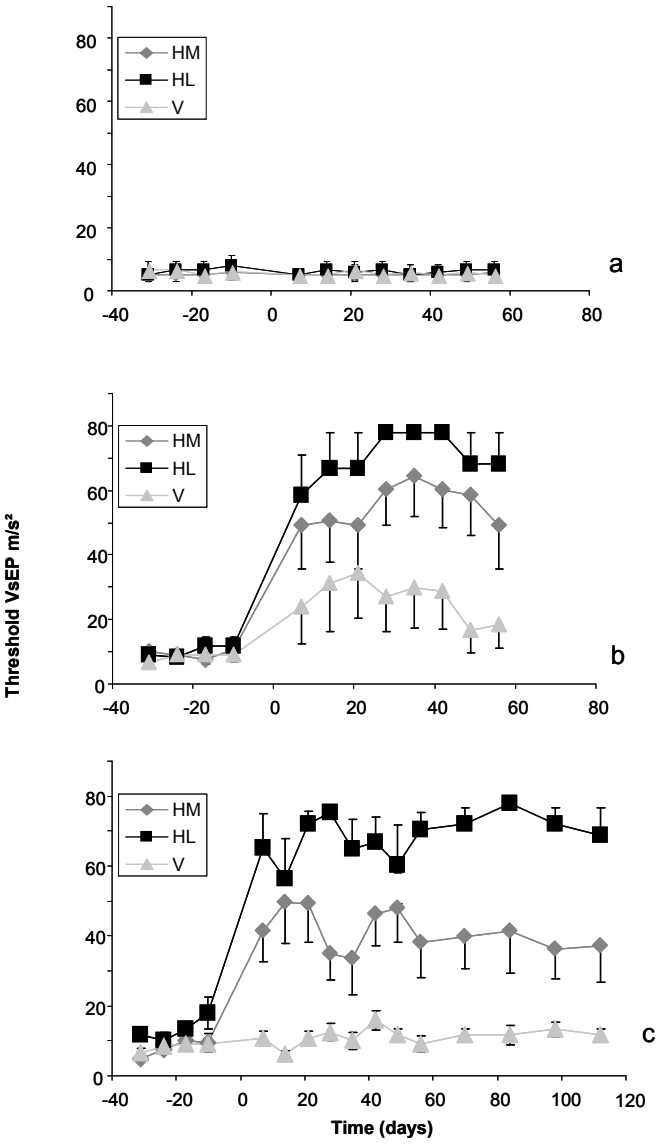


Figure 8. VsEP P_1-N_1 thresholds for the three directions. **a**. Control group (n=6). **b**. 8-week group (n=6). **c**.16-week group (n=6). Error bars represent standard error of means. Day 0 corresponds to the tenth day of treatment.

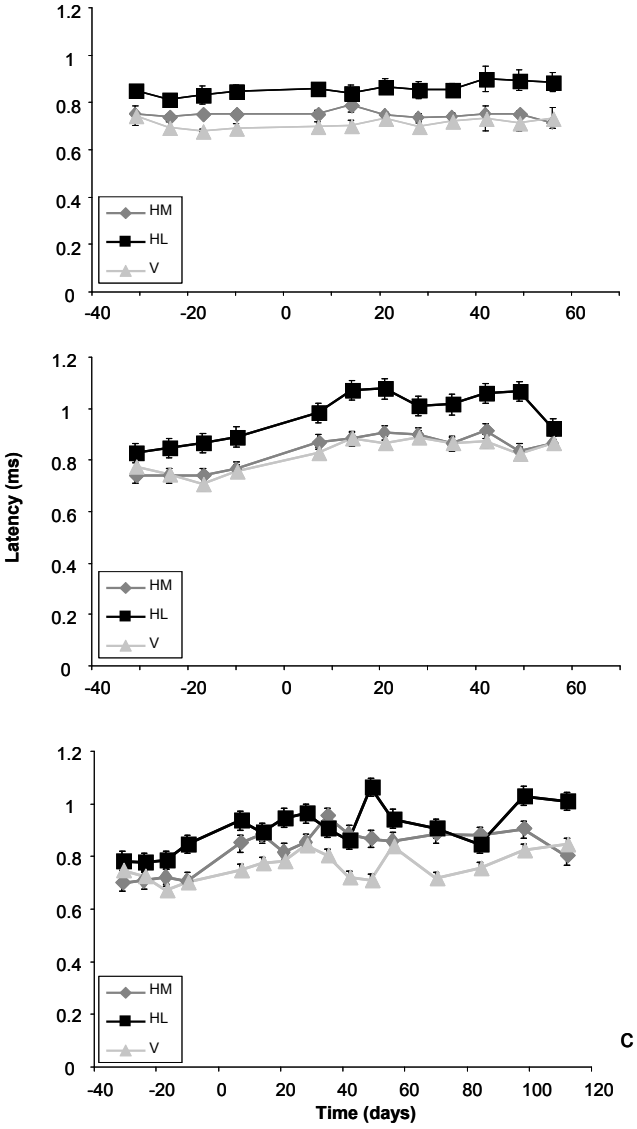
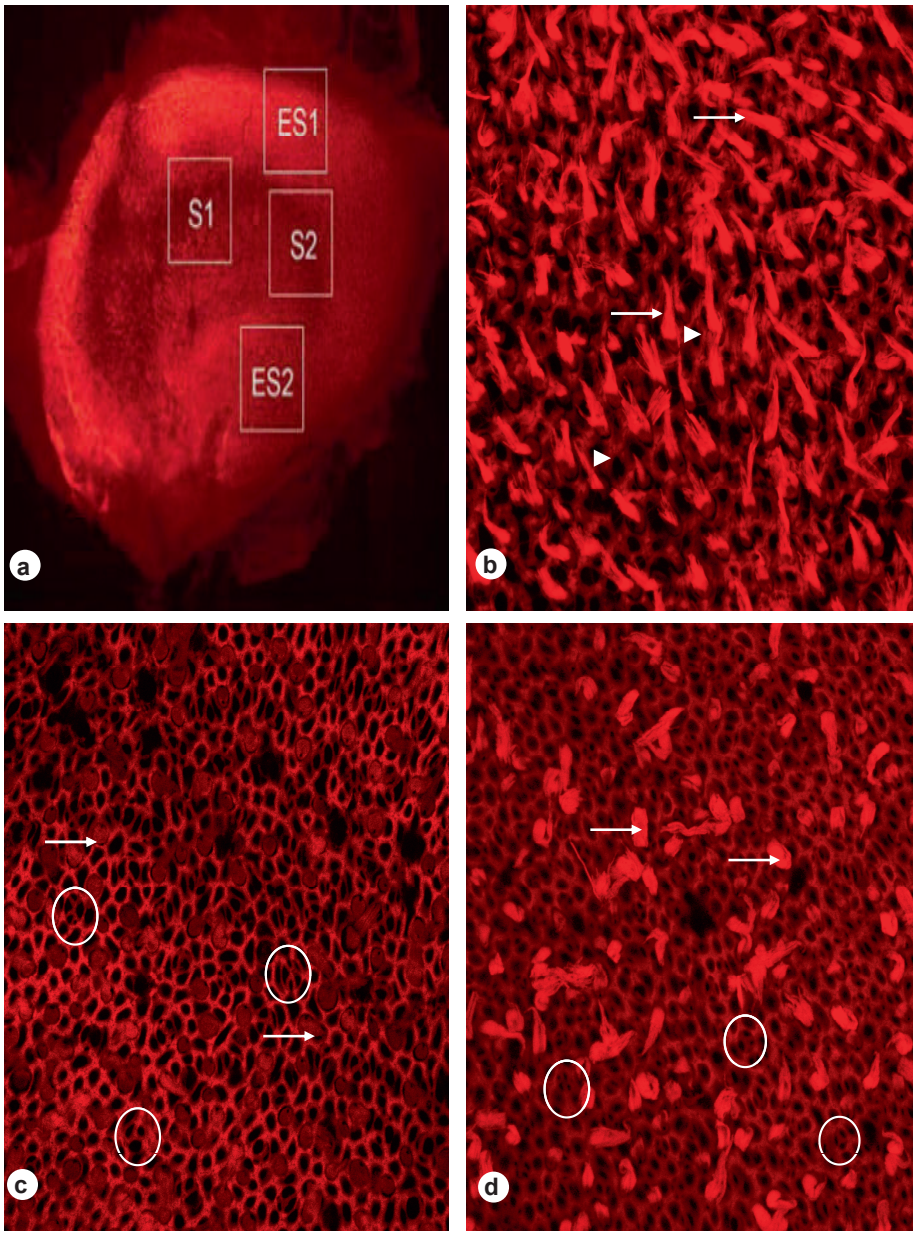


Figure 9. VsEP P_1 latencies for the three directions. **a**. Control group (n=6). **b**. 8-week group (n=6). **c**.16-week group (n=6). Error bars represent standard error of means. Day 0 corresponds to the tenth day of treatment.

Latencies

In figure 9 the latencies are shown for the control group (a), the 8-week group (b) and the 16-week group (c). In the control group latencies were stable over time (Fig. 9a). Across all groups, HL stimulation resulted in longer latencies than HM and V stimulation (before treatment: 0.83 ms versus 0.73 ms). Treatment resulted in significant latency increases of about 0.1 ms for each stimulation direction (rm ANOVA, $F(5,85) = 30.8$, $p < 0.0001$), as illustrated in Fig. 9b,c.

In neither the 8- nor the 16-week group a decrease (or increase) in latency was observed over time (rm ANOVA, $F(4, 44) = 0.73$, $p = 0.58$).



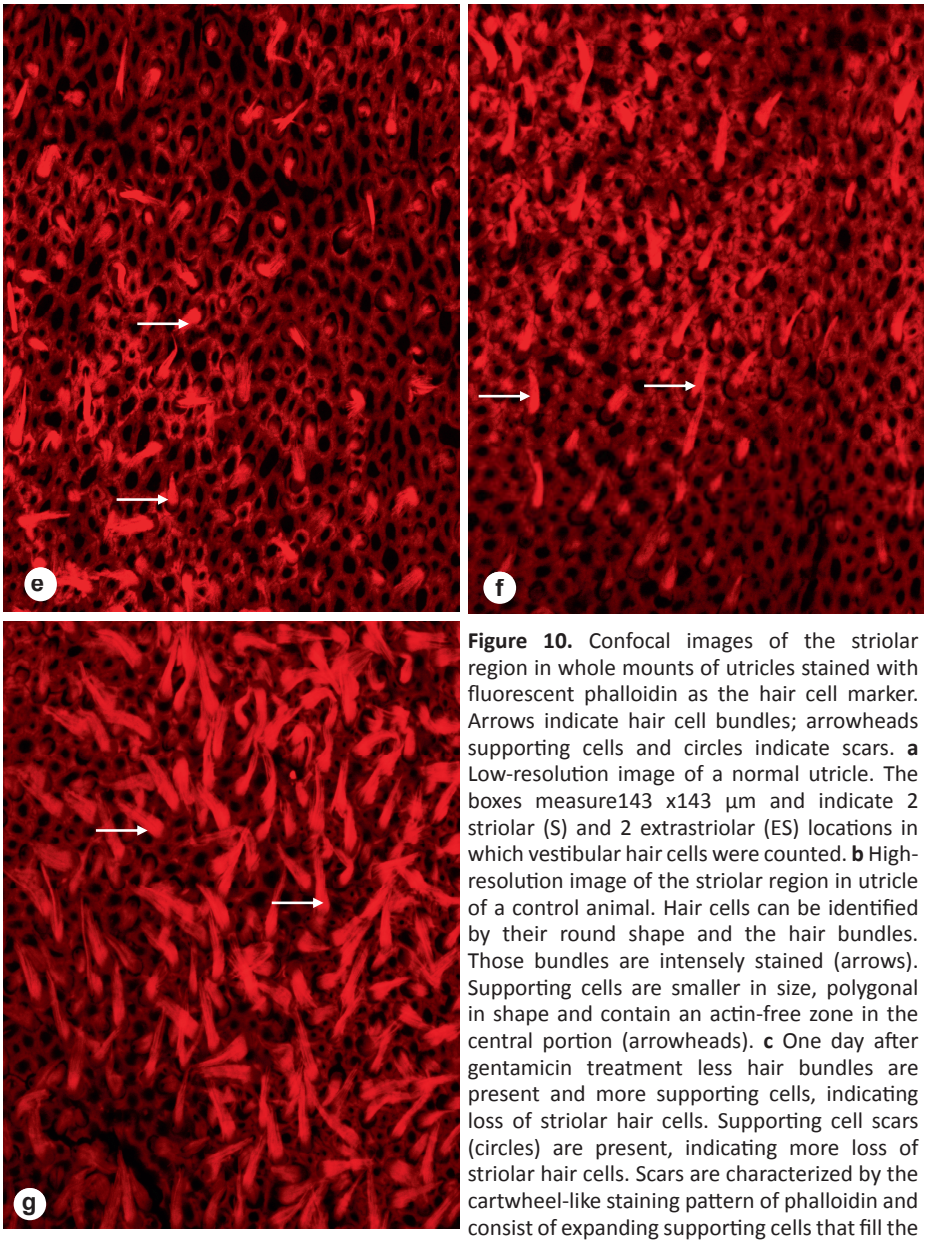


Figure 10. Confocal images of the striolar region in whole mounts of utricles stained with fluorescent phalloidin as the hair cell marker. Arrows indicate hair cell bundles; arrowheads supporting cells and circles indicate scars. **a** Low-resolution image of a normal utricle. The boxes measure143 x143 µm and indicate 2 striolar (S) and 2 extrastriolar (ES) 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** One day after gentamicin treatment less hair bundles 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** Two weeks after gentamicin treatment, remaining hair cells (arrows) still exhibit phalloidin staining of the cuticular plate and hair bundle. Scars are also visible. **e** Four weeks after gentamicin treatment. Areas of hair cell loss are seen. Hair cell bundles are organized in a chaotic manner. **f** Eight weeks after gentamicin treatment. Here, hair cell bundles are organized more alike. **g** Sixteen weeks after gentamicin treatment. There is an increase of hair cell density.

3.4 Histology

Figure 10 shows immunohistological examples of areas in the striolar region of a normal utricle (b) and of utricles at one day (c), 2 weeks (d), 4 weeks (e), 8 weeks (f) and 16 weeks (g) after gentamicin treatment. In the gentamicin-treated utricles, from one day after treatment, numerous scars were detected in the striola, indicating hair cell loss (figure 10 c,d). In the 4- (e) and 8-week (f) group there was stable loss in hair cell density. 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 (g), there was a larger hair cell density than after shorter survival times (c-f). Besides this indication of hair cell regeneration, this specific animal showed functional recovery as shown by the stable increase in VsEP amplitude (animal C360, see figure 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 V direction (one-way ANOVA: $p = 0.85$). In contrast, hair cells in the utricle diminished substantially after treatment, as well in the striolar as in the extra-striolar region (one-way ANOVA: $p<0.001$). Maximum loss was at two and four weeks after treatment. After four weeks the hair cell counts slightly increased in both zones of the utricle, more apparent in the striolar regions than the extra-striolar regions as shown in the upper left histogram. This was not statistically significant (linear regression, $p = 0.23$).

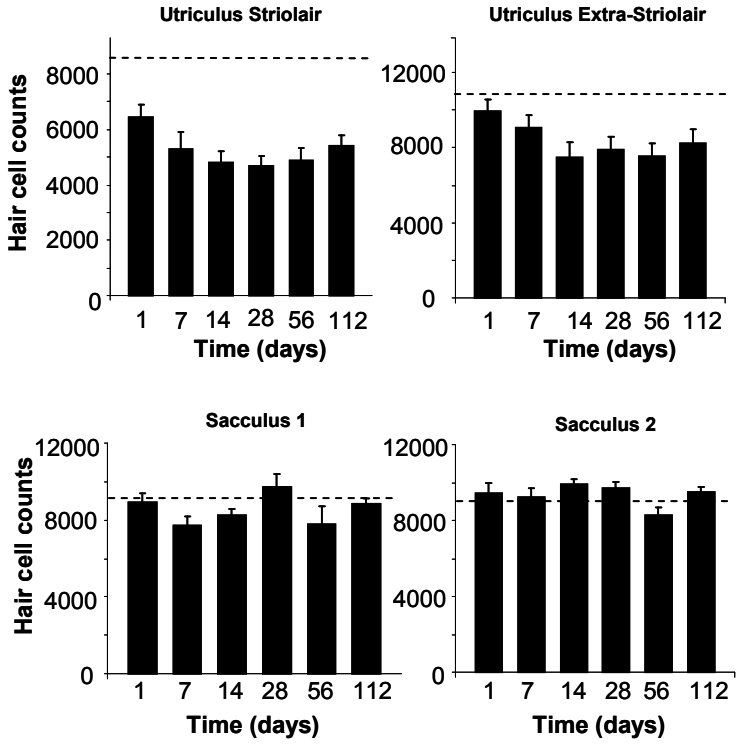


Figure 11. Histograms on hair cell counts of the different survival groups (averaged data) for the striolar and extra-striolar regions in the utricle and saccule. The dotted lines in the figures represent hair cell counts of the control animals. Error bars represent SEM.

Figure 12 shows the correlation between the VsEP P_1-N_1 amplitudes found to HM stimuli (at 70 m/s²) 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 two animals in the 16-week group that showed functional recovery (Fig. 6), one (same animal as in figure 10g) showed relatively high hair cell counts (6400) compared to the average number of hair cells in that group (5400), and in other experimental groups (~4800 in the 2-, 4- and 8-week groups). The other animal that showed functional recovery had a hair cell count (5120) comparable to the average number of hair cells in that group. There is some correlation within the 8-week and 16-week group between amplitudes and hair cell count ($R^2 = 0.24$, $p=0.11$).

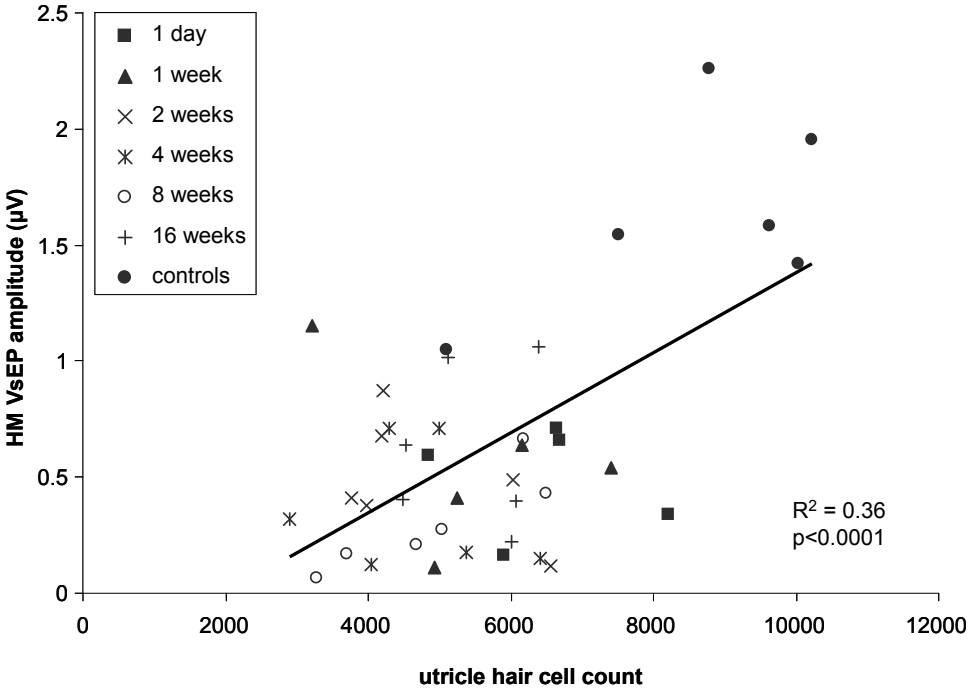


Figure 12. Correlation between VsEP amplitudes and utricular hair cell counts. Stimulation for the VsEPs was in the HM direction at 70 m/s². Hair cell counts were done in the striolar regions of the utricles.

3.5 ABRs

Figure 13 shows the mean click-ABR thresholds over time for the 4-week, 8-week and the 16-week group. One week after ototoxic insult, there was a significant threshold shift, 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).

3.6 VsEP vs ABR Thresholds

We examined whether VsEP threshold shifts correlated to ABR threshold shifts. We considered VsEPs in HM direction since those showed the largest variance. It appeared that ABR en VsEP threshold shifts one 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 where 2 did show VsEP recovery after 4 weeks. Of the two animals showing VsEP recovery after 16 weeks (Fig. 6), one had shown ABR recovery but the other not.

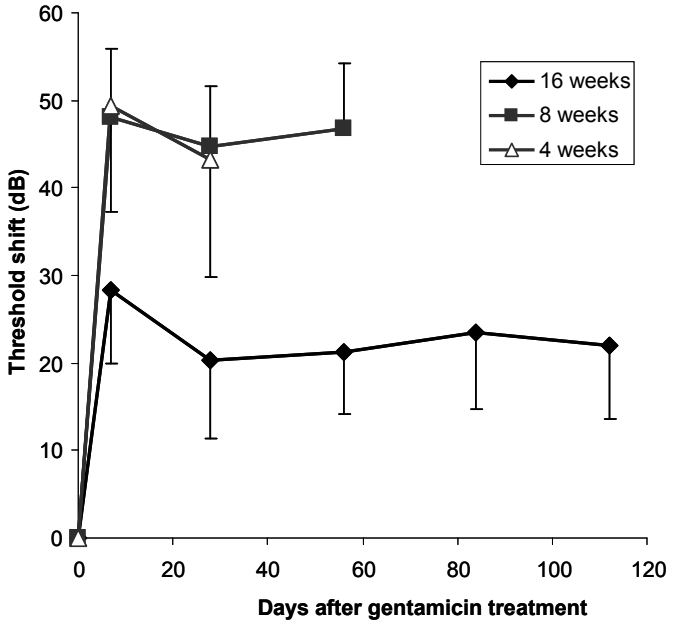


Figure 13. Mean click-ABR thresholds for the 4,-, 8- and 16-week groups. Error bars represent SEM.

4. DISCUSSION

This study directly assessed otolith function after significant gentamicin-induced utricular hair cell loss (~55%) at several survival times up to 16 weeks. Functional assessment by recording VsEPs and 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.

4.1 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. Further, 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].

4.2 Long-term Effect of Gentamicin Treatment

After ototoxic insult, we explored the longer-term histological aspects and functional phenomena of the otolith organs using direct end-organ parameters (i.e., VsEP amplitude, threshold and latency). Overall, the end-organ parameters were stable between 2 and 16 weeks (Figs. 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 on the longer term (16 weeks). In mammals, although morphological evidence is available pointing to regeneration of hair cells [Forge et al., 1993; Sobkowicz et al., 1996; Warchol et al., 1993; Lopez et al., 1997] functional recovery was not confirmed using direct end-organ parameters. Studies that had shown functional recovery after ototoxic treatment used functional parameters that (partially) were 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 longer-term the effects of central compensation did not bias 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 8 months survival. In a recent study were 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 (the shorter survival times, i.e. the 1 week, 2 week and 4 week group), 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 two 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 mentioned studies the recovery was mostly due to re-organizing of the hair bundles back to the normal pre-lesional situation, which include repair of ruptured tip links confirmed by restoration of the mechano-electrical transduction responses [Jia et al., 2009], 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 on the longer term.

We applied the same type of strains (Dunkin Hartley) and ototoxic model (10 days 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 (55%) as compared to those studies (90%).

4.3 Saccule versus Utricule

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 (Figs. 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 (Figs. 7, 8). This corresponded to significant hair cell loss in the utricle (Fig. 11). This is congruent with findings in 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.

4.4 Clinical Implications

Because of the shown limited regenerative capacities of the vestibular end organs, 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 chemo-ablation 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 with as result 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 favor of this treatment. On the other hand, limited functional recovery may be the cause that in some cases several treatments with intratympanic gentamicin are necessary.

4.5 Future Considerations

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

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Chapter 6

Applied study-II

Intratympanic gentamicin treatment for Ménière's disease: A randomized, double-blind, placebo-controlled trial on dose efficacy. Results of a prematurely ended study

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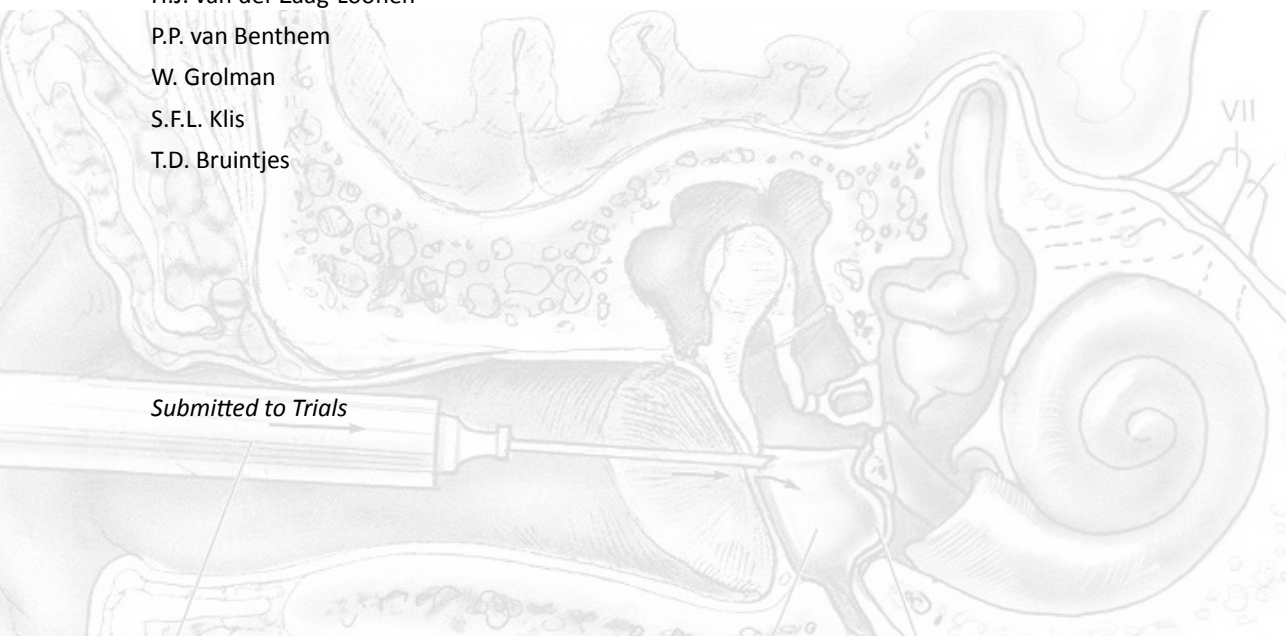

Submitted to Trials

ABSTRACT

Background: Gentamicin is used as a therapeutic agent for Ménière's disease because of its vestibulotoxicity causing chemo-ablation of the vestibular sensory epithelia. Its use has increased in recent years. However, there is still no consensus about the dose regimen of gentamicin in the treatment of Ménière's disease. In this study two different dose regimen treatment protocols are compared in a placebo controlled study design. The primary objective is effect of treatment on dizziness. Secondary objective is hearing evaluation.

Methods: We performed a randomized, double blind, placebo-controlled study in adults with unilateral Ménière's disease according to the AAO-HNS guidelines resistant to conservative medication. Three groups received four injections, administered weekly (four intratympanic injections with 40 mg/ml gentamicin solution, two injections gentamicin solution and two injections of placebo in random order, or four injections with placebo). Outcome parameters were the score on the Dizziness Handicap Inventory and pure tone audiometry (PTA). Intended follow-up was two years.

Results: During follow up one patient exceeded the accepted amount of hearing loss. Further, enrollment was very slow (until 12 months between two patients) and new insights showed an apparent benefit of ITG. Therefore we performed an unscheduled interim analysis, which showed that PTA threshold shifts had reached the stopping criteria in two more patients. Because of this, this study was ended. Of the three patients with the significant PTA threshold shift two were enrolled in the gentamicin-group.

Conclusion: No conclusions can be drawn concerning doses regimens. Now that new publications have shown that ITG treatment can be an effective and safe treatment, a placebo controlled RCT may not pass the ethical committee because these recent reports in literature. Still, a dose regimen study (without placebo) on ITG treatment needs to be performed.

1. INTRODUCTION

Ménière's disease (MD) is an invalidating illness characterized by attacks of vertigo with hearing loss, tinnitus and or aural fullness of the affected ear. The exact origin of this disease is not known, but it is generally accepted that endolymphatic hydrops is the pathophysiological substrate. Treatment, besides advises on living habits and diet, exists of medication. This treatment gives unsatisfying results in many cases. The natural course of this disease often results in a decline of the sensitivity of the vestibular peripheral system and progressive hearing loss [Minor et al., 2004]. Gentamicin is an aminoglycoside antibiotic that is applied systemically for serious infections. Some of the adverse events of gentamicin treatment are vestibulotoxicity and cochleotoxicity. For several reasons gentamicin is more vestibulotoxic than cochleotoxic [Bremer et al., 2012]. Intratympanic gentamicin treatment (ITG) for control of vertigo has become popular worldwide. In this treatment gentamicin solution is applied in the middle ear with the aim of damaging the vestibular hair cells with an intended result of a decrease (or disappearing) of vertigo complaints while preserving hearing [Minor et al., 2004; Blakley 1997; Pender, 2003; Lii et al., 2004; Pullens and van Benthem, 2011].

Several studies have been published about this topic. These are mainly descriptive studies that used different gentamicin concentrations and administration protocols. Unambiguous in these publications is the high success rate in reducing the number of vertigo attacks. However, still obscure is the best treatment scheme and optimal concentration of gentamicin. Two dose regimen protocols are mostly used. One model uses a fixed number of administrations (fixed dose regimen) and the other model uses a titration regimen where gentamicin is administered until attacks disappeared or other terms are reached [Atlas and Parnes, 1999; Atlas and Parnes, 2003; Blakley, 1997; Carey, 2004; Chia et al., 2004; Cohen-Kerem et al., 2004; Diamond et al., 2003; Nedzelski et al., 1993]. There are to date a few placebo-controlled studies on this topic available. E.g., the study by Stokroos et al. [2004] used a titration model and the study by Postema et al. [2008] used a fixed dose regimen. Although the studies used a different treatment protocol, both studies demonstrated the efficacy of gentamicin treatment for Ménière's disease when compared to placebo treatment. A weakness of these studies is the limited follow-up time. Stokroos et al. used a follow-up period of 6 months and Postema et al. of 12 months. The advice of the Committee on Hearing and Equilibrium of the American Association of Otolaryngology- Head and Neck Surgery (1995) is to use a follow-up of at least 24 months, taking into consideration the expected placebo effects (Committee on Hearing and Disequilibrium, 1995). In this study we compare the efficacy of two different administration

protocols. One uses 4 injections with gentamicin solution (40 mg/ml) versus 2 injections with gentamicin solution (40 mg/ml), both compared to placebo treatment. The main question is whether ablation of the peripheral vestibular system (4 injections) gives a better clinical result than more subtle damage to the peripheral vestibular system (2 injections). Secondary outcome is the effect of treatment on hearing. Numbers of injections are based on the studies by Stokroos et al., (1.5 +/- 0.5 injections) and Postema et al. (4 weekly injections). The fact that to date no placebo controlled dose efficacy study is available is an extra argument for a placebo group. We expect a better clinical result after 4 injections with gentamicin solution.

2.SUBJECTS AND METHODS

The study was started in two Hospitals, the University Medical Center in Utrecht (UMC Utrecht) and Gelre Hospital Apeldoorn. Before entering the study all patients gave written informed consent for participation in the study. Patients were eligible for the study if they had been diagnosed as having unilateral Ménière's disease according to the 1995 AAO-HNS criteria: two or more spontaneous episodes of vertigo each lasting 20 minutes or longer, sensorineural hearing loss documented audiometrically in the diseased ear and the presence of tinnitus and/or aural fullness in this ear. Causes other than Ménière's disease were excluded by a diagnostic protocol, including vestibular tests, MRI of the cerebellopontine angle, clinical history and physical examination. Other inclusion criteria were: Ménière's disease resistant to conservative medical treatment executed longer than six months, (i.e. Dizziness Handicap Score of at least 30 points) and ability to provide written informed consent. Patients had to have compromised hearing on the affected side without fluctuations. Exclusion criteria were ipsilateral middle ear pathology, contralateral ear pathology or contralateral hearing loss, allergy for aminoglycosides or earlier treatment with intratympanic gentamicin. Approval of the study was granted by the medical ethics committee (on 30th December 2008, Protocol ID: 07/343, EudraCT number 2006-005913-37).

For assignment of the participants a computer-generated list of random numbers was used. Patients were randomized to one of three treatment groups. The three groups all received four weekly intratympanic injections. Group 1 received placebo injections (sterile NaCl 0.9 % solution), group 2 received two injections gentamicin 40 mg/ml and two injections placebo in random order, and group 3 received four injections with gentamicin 40 mg/ml. After treatment the intended follow-up period was two years. Three monthly questionnaires were taken by telephone. If there was any problem the patients were free to contact one of

the researchers and all the time they were free to withdraw from the study. At the end of the follow-up a last audiogram, ENG and the DHI questionnaire were taken.

Primary outcome parameter was the total score on the Dizziness Handicap Inventory (DHI). The DHI is a standardized and validated questionnaire assessing impairments due to dizziness [Jacobson and Newman, 1990; Vereeck et al., 2006; 2007]. It comprises of 25 items, leading to a score range from 0 to 100, with higher scores indicating more perceived impairments. The secondary outcome parameter was hearing loss. Intended follow-up was two years. Stopping criteria were the occurrence of SUSARs (Suspected Unexpected Serious Adverse Reactions), one of which was an average hearing deterioration of 30 dB or more over the frequencies of 500, 1000, 2000 and 4000 Hz of the treated ear, or an average deterioration of 15 dB or more over the frequencies of 500, 1000, 2000 and 4000 Hz at the contralateral ear. During follow-up at the outpatient clinic, the audiograms of the patients were visually compared with the former audiograms.

3. STATISTICAL ANALYSIS

Power analysis (alfa at 5% and power at 80%) showed that 16 patients per group were needed to show a clinically relevant difference of 12 points on the DHI scores between the three groups [Albera et al., 2003]. The first interim analysis was planned to be performed when 18 patients were included.

Baseline characteristics (sex, age and MD duration), DHI scores and hearing outcomes were compared between the three groups with non-parametric statistics, due to the small numbers (Kruskal Wallis test for continuous variables).

4. RESULTS

Between June 2009 and January 2012 we included 15 patients. One patient was included in the UMC Utrecht and 14 in Gelre Hospital. The time between inclusion of the 14th and the 15th patient was 12 months. Mean age was 64.8 years (SD 12.5 years), eight (57%) were male. Five (one male) were randomized in group 1, five (four males) in group 2, and five (three males) in group 3 (table 1). However, one patient (group 2) withdrew from the study after randomization. Although informed consent was obtained before inclusion, she changed her mind and did not want the chance to be treated with placebo and was not included in the further follow up and analyses. Another patient withdrew from the study after two injections

because he suffered from Tumarkins crises. One patient deceased during the study (as a result of co-morbidity). This patient was enrolled in the gentamicin-placebo group (group 2). The median duration in years of MD was 2.5 (range 0.1-18.2) for the placebo-group, 3.3 (0.7-7.5) for the placebo-gentamicin group and 3.1 (1.1-19.6) or the gentamicin group. Because of the slow enrollment, apparent hearing loss in one patient and new insights with respect to the benefit of ITG treatment the randomization code was broken and we performed an unscheduled interim analysis on which the following results are based.

Table 1. Demographic, clinical and treatment characteristics of included patients

Characteristics	Total n = 14	Placebo n = 5	Gentamicin/ Placebo n = 4	Gentamicin n = 5	P value
Sex					
male	8 (53%)	1 (20%)	4 (80%)	3 (60%)	0.16
Age (mean, (SD))	64.8 (12.5)	57.3 (16.7)	72.6 (5)	64.5 (8)	0.16
Duration of MD in years (median (range))	3 (0.1-19.6)	2.5 (0.1-18.2)	3.3 (0.7-7.5)	3.1 (1.1-19.6)	0.42
DHI	49 (20)	54 (18)	41 (16)	51 (25)	0.61
Averaged (0.5, 1, 2, 4kHz) pure-tone-hearing loss (dB HL)					
Right	42 (20)	35 (25)	30 (7)	59 (9)	0.046
Left	50 (24)	44 (22)	61 (31)	47 (21)	0.53

Effect of treatment: Vertigo

The median DHI score before treatment was 54 points (range 32-76), 44 points (range 18-56) and 46 points (range 20-88) for the placebo group, the gentamicin-placebo group and the gentamicin group respectively. At the time of the interim analysis the median DHI score for the placebo group was 24 points (range 4-34, decrease of 20 points), for the gentamicin-placebo group 34 points (range 4-58, decrease of 10 points) and for the gentamicin group 5 points (range 0-76, decrease of 41 points); the latter group showed the largest decrease in DHI score but this was not statistical significant ($P > 0.5$).

Side effect of treatment: Hearing outcome

During follow-up, one patient had a hearing loss of 50 dB of the treated side averaged over the four frequencies which was more than the criterion of 30 dB. The interim analysis showed two more patients with a hearing loss of more than 30 dB. Both had an exceeding of the accepted threshold shift (30 dB) of ~ 5 dB. After breaking the randomization code, the patient with

the PTA shift of 50 dB was enrolled in the gentamicin group. Of the patients with the minor exceeding, one was also enrolled in the gentamicin group and the other patient was enrolled in the placebo group. The mean increase in PTA threshold shift for the placebo group was 10 dB, for the gentamicin-placebo group 0.9 dB and for the gentamicin group 27.4 dB ($P > 0.5$).

5. DISCUSSION

The present study was performed to assess a good dose regimen protocol for ITG therapy in patients suffering from Ménière's disease. Unfortunately, no conclusive statements can be made about this due to the fact that the study was ended prematurely and at that time too few patients have been included to make significant conclusions. However, our results indicate that there does seem to be efficacy of gentamicin treatment in comparison to placebo but the significant risk for hearing loss with four injections enervates these results.

The time between inclusion of the 14th patient and 15th patient was 12 months indicating a very slow enrollment. During that time a Cochrane Review was published which stated that ITG treatment was an effective and safe therapy for patients with Ménière Disease [Pullens and van Benthem, 2011]. Also, a meta-analysis showed an effective and safe treatment with intratympanic steroids [Philips and Westerberg, 2011]. Data monitoring committees have an ethical obligation to ensure that patients are offered effective treatment as soon as it is clear that an effective treatment is indeed available [Bassler et al., 2010]. Based on the slow enrollment, the suspicion of more hearing loss besides the noticed exceeding in PTA shift of one patient and together with the new scientific insights we decided to perform the unscheduled interim analysis over the 15 patients that had been included in our study until that time. The results of this interim analysis showed two other (minor) exceeding in PTA shift. This meant that the stopping criterion was reached and the study was ended. Because of this unexpected stop of the study full inclusion was not reached resulting in too small a sample size to support a strong external validity of our study. Clinicians should consider these results with caution [Montori et al., 2005].

Retrospectively, visual determination of the audiograms was not sufficient because two of the three exceedings of the criterion for hearing loss were only noticed after the unscheduled interim analysis. If, e.g., the time courses of hearing loss are calculated during outpatient clinic visits, also a small exceeding in accepted hearing loss is noticed. Two of the three patients were enrolled in the gentamicin group so the PTA threshold shifts were probably the result of the cochleotoxicity of gentamicin. The third patient was enrolled in the placebo group so in this

case the MD itself instead of the ototoxicity of gentamicin may have caused the PTA threshold shift; the natural course of Ménière's disease eventually leads to a sensorineural hearing loss of ~ 50 dB [Minor et al., 2004]. The two patients of the gentamicin group are content with their treatment as indicated by a strong reduced DHI score. One other patient suffered from residual Ménière's attacks with Tumarkins crises after four injections and withdrew from the study. This patient was enrolled in the placebo-group, so the cause of the crises was MD itself and not gentamicin treatment. Subsequently, this patient received one injection with gentamicin in the outpatient clinic (outside the scope of this study) with satisfying results. We assessed the stopping criteria for hearing loss for the PTA threshold shift at 30 dB averaged over the four frequencies. Maybe some MD patients will accept the chance of hearing loss, as they are eager to be treated for their main problem, i.e., disabling vertigo attacks. So maybe the determined stopping criterion for hearing loss can be augmented in future studies.

The difficulty of recruiting enough Ménière subjects for a 2-year follow-up period with adherence to the 1995 AAO-HNS guidelines for reporting is well known. There is also a certain placebo effect when treating patients with MD. This was the reason that this RCT contained a placebo-group. Frequently, this discouraged patients to participate in the trial since they did not want to receive placebo instead of gentamicin. These patients suffered from recalcitrant MD, with a great morbidity, and they demanded a 'real' treatment and did not accept the risk to get treated with placebo. For this reason, studies have been performed were 2 treatment modalities (e.g., intratympanic gentamicin vs. dexamethason or prednison) are being compared [Casani et al., 2012; Gabra and Saliba, 2013]. The latter study implicated less efficacy of prednison compared to ITG injections without a difference in hearing level. A low-dose protocol can be used [Longridge and Mallinson, 2000; Harner et al., 2001; Quaranta et al., 2001], administrating just 1 or 2 gentamicin injections with a similar effectiveness for vertigo control and with a lower risk for major side effect (i.e. hearing loss and a prolonged period of imbalance after treatment) as compared with high-dose protocol [Chia et al., 2004]. But there are limitations on the latter study regarding the variability in the total dosage of gentamicin delivered (2.4–720 mg). Wasson et al. [2013] indicated efficacy of ITG also on the longer-term (mean 17 years and 3 months).

6. CONCLUSION

The known inherent risk of gentamicin treatment induced hearing loss was also present in our study. Due to a slow enrollment of patients into our study and recent new clinical insights into the role and treatment benefits of ITG in MD patients we terminated our study prematurely. We performed an interim analysis that revealed a potential SUSAR that we reported to our Medical Ethical Committee. This interim analysis did show efficacy of four injections of gentamicin compared with placebo. Important consideration beside its potential benefit is the risk to perceptive hearing loss. To adapt a placebo controlled study design, now that the reports in literature show effectiveness for ITG seems unethical. [Pullens and van Benthem, 2011; Philips and Westenberg, 2011; Wasson et al., 2013]. However, a randomized controlled trial on dose regimens still has to be performed because of the lack of consensus on the intratympanic dose of gentamicin that needs to be used to have the best balanced result between vertigo reduction and hearing preservation.

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Chapter 7

Conclusions and General Discussion

1. Abstract thesis
   1.1. Main conclusions thesis

2. Drug induced damage to the inner ear
   2.1. Site specific actions of the used ototoxic drugs in the guinea pig inner ear
   2.2. Use of the co-treatment in mice

3. Effects after damage
   3.1. Histological vestibular regeneration in guinea pigs?
   3.2. Functional recovery of the VsEP and ABR
   3.3. Recommendations on future fundamental research

4. Clinical aspects of the ototoxic studies
   4.1. General clinical considerations on aminoglycosides
   4.2. Translational aspects of the ototoxic studies on ITG treatment

5. Methodological aspects on assessing end-organ function
   5.1. Aspects on acquisition of the vestibular response
   5.2 Alternatives for assessing end-organ vestibular function

6. Methodological aspects of the ITG study
   6.1. Main outcomes of the ITG study
   6.2 Inclusion of patients
   6.3 Aspects on the adverse event hearing loss
   6.4 Alternative study on ITG treatment

1. ABSTRACT THESIS

In this thesis, fundamental research is performed on the sensory epithelia of the inner ear in guinea pigs and mice. The outcomes of these fundamental studies are discussed in relation to clinical vestibular research. Methods to efficiently, repeatedly and reproducibly damage the sensory epithelia of both the otolith organs and cochlea have been described in chapters 2 (guinea pigs; cochlear and vestibular damage), 3 and 4 (mice; cochlear damage). These methods are crucial for studying regeneration and/or recovery phenomena in the otolith organs or cochlea in the future. Furthermore, these animal models of vestibular disease or deafness may facilitate in studies of regeneration induction or application of tissue-engineering techniques. Using one of these methods the regenerative capacities of the vestibular sensory epithelia were quantified in the guinea pig, which provided only limited evidence for recovery (chapter 5). These animal data speak in favor of intratympanic gentamicin treatment in patients with Ménière's disease when gentamicin is used as a treatment modality. This modality was studied in a randomized placebo controlled design in chapter 6. Gentamicin had a positive effect on incapacitating dizziness complaints in patients but one of the dosages of gentamicin used inflicted more hearing loss than was accepted in the protocol. Therefore, this study was ended prematurely. In the following paragraphs these topics will be further discussed and elaborated upon in relation to the results that were obtained in the previous chapters.

1.1 Main conclusions thesis

Chapter 2:

1. Using a moderate dose, gentamicin treatment is effective for inducing vestibular hair cell damage in the guinea pig without having a negative effect on the cochlea.
2. Co-treatment with kanamycin and furosemide is effective for inducing severe cochlear damage in the guinea pig without affecting the vestibular system.
   Value: Now there are selective tools to induce damage to either the cochlea or the otolith organs in guinea pigs. These methods are crucial for studying regeneration and/or recovery phenomena in the otolith organs or cochlea in the future, and can de used to induce regeneration, or to apply tissue engineering.

Chapter 3 and 4:

1. Co-treatment with kanamycin and furosemide (the method applied in guinea pigs in chapter 2 but with a higher kanamycin dose) also induces deafness in mice.
2. The time window between administrations of kanamycin and furosemide is not crucial for the deafening effect in mice.
   Value: This model will be appropriate for hearing impairment research in mice. A murine model has more advantages than the guinea pig model since there are specific mouse strains available for stem cell research.

Chapter 5:

1. There is no evidence for spontaneous regeneration of vestibular hair cells and only very limited evidence for vestibular recovery of end-organ function in guinea pigs after ototoxic induced damage.
   Value: These results pose severe challenges in the field of research on (induction of) re-population of lost vestibular hair cells in the future in guinea pigs.

Chapter 6:

1. In this dose-efficacy study, intratympanic gentamicin (ITG) treatment in Ménière's disease patients had a decreasing effect on dizziness.
2. A suitable dose regimen of ITG treatment has not been found because this study was prematurely ended since the endpoint for the predefined acceptable hearing loss was reached.

3. Lack of consensus remains on the intratympanic dose of gentamicin that needs to be used to have the best-balanced result between reduction of incapacitating vertigo and hearing preservation.

2. DRUG INDUCED DAMAGE TO THE INNER EAR

2.1 Site specific actions of the used ototoxic drugs in the guinea pig inner ear

Treatment with gentamicin and kanamycin/furosemide showed opposite effects on vestibular and auditory sensory organs assessed both histologically and electrophysiologically (chapter 2). In particular kanamycin/furosemide treatment, which virtually annihilated cochlear function, had no effect on vestibular end-organ function. In contrast, a toxic effect of gentamicin was seen only on vestibular function, but not on cochlear function. The results of chapter 2 provide tools to selectively induce damage to the cochlea or the vestibular end organ. There is an effect difference of gentamicin dosage on ABR threshold shifts, which is illustrated clearly by the different outcomes in ABR thresholds between chapter 2 and 5 (both studies used a guinea pig model). In chapter 5, gentamicin was used in a higher concentration (125 mg/kg body weight vs. 100 mg/kg in chapter 2) resulting in a negative effect on hearing.

Besides dosage, also the animal model used is of importance for the ototoxic potential found. The inner hair cells of the mouse cochlea are more resistant to aminoglycosides than the inner hair cells of the guinea pig [Wu et al., 2001; Poirrier et al., 2010]. It has to be noted however, that at high dose all aminoglycoside drugs are cochleo- and vestibulotoxic. The role of furosemide is an enormous enhancement of the ototoxic effect. As discussed in chapter 2, this role seems to be restricted to the cochlea when applying the specific kanamycin dosage. Other factors than dosage and species as time, route of administration, frequency and duration of treatment may determine where the damage is observed first [Forge and Schacht, 2000]. The toxic potential and organ preference, however, vary among the different aminoglycosides. For example kanamycin and amikacin are more cochleotoxic where streptomycin and gentamicin are more vestibulotoxic [Schacht et al., 2008]. The reason for this predilection is not well understood [Nakashima et al., 2000]. Site-specific drug level for example is not a determining factor for the amount of toxicity, and cochleolotoxic drugs do not reach a higher concentration in the cochlea than in the vestibular system and vice versa [Dulon et al. 1986; Nakashima et al., 2000].

2.2 Use of the co-treatment in mice

The results of chapters 3 and chapter 4 render a pragmatic advantage for auditory research in the mouse model in particular. In chapter 2 the co-administration of kanamycin and furosemide is used for inducing hearing loss in the guinea pig and is a widely used model which causes substantial hair cell loss and therefore loss of cochlear function (Versnel et al., 2007; Agterberg et al., 2008, 2009; Havenith et al., 2013]. In contrast to the guinea pig, previous attempts to induce hearing loss in mice have shown inconsistent results due to their resistance to aminoglycoside-induced ototoxicity [Henry et al., 1981; Wu et al., 2001; Poirrier et al., 2010]. Especially inner hair cells of mice seemed resistant to aminoglycoside induced ototoxicity. We created a model using the combined administration of kanamycin and furosemide (adapted from chapter 2, guinea pig) that efficiently induced deafness in mice. The optimal time interval at which the co-administration must be applied was further studied in chapter 4. It seemed that in a time window of 15 minutes between administrations of both drugs there is no difference in the extent of induced hearing loss. Our controlled ototoxic drug induced mouse model for sensorineural hearing loss can potentially help in our quest to find future treatment modalities such as stem cell therapy.

3. EFFECTS AFTER DAMAGE

3.1 Histological vestibular regeneration in guinea pigs?

It is widely accepted that spontaneous regeneration is not possible in the mammalian cochlea [Rubel et al., 2013]. In contrast to the mammalian cochlea, transdifferentiation mechanisms were found to be present to some extent in mammalian vestibular hair cells [Warchol et al., 1993; Forge et al., 1993, 1998 (all used guinea pigs)]. However, other studies showed no evidence [Meiteles and Raphael 1994] or only very limited evidence for mitotic activity in the vestibular sensory epithelia after ototoxic damage [Rubel et al., 1995; Li and Forge, 1997; Kuntz and Oesterle, 1998; Ogata et al., 1999; Oesterle et al., 2003]. Most survival times in these studies did not exceed 4 weeks after treatment.

In chapter 5, we addressed this question of possible regeneration of mammalian hair cells in otolith organs and especially studied this question on the longer term. We described a significant loss of vestibular hair cells after gentamicin treatment, and up to 16 weeks later, we found no evidence for histological regeneration. Our histological results agree with most of the earlier mentioned studies in this paragraph.

3.2 Functional vestibular end-organ recovery

Recovery of vestibular function in mammals as a result of possible hair cell regeneration is an important question addressed by only a few studies [Meza et al., 1992, 1996; Kopke et al., 2001; Taura et al., 2006; Staecker et al., 2007]. These studies based their findings on methods (e.g. improved swimming behavior and a decrease of nystagmus as indicators for functional recovery), which do not exclude central compensation mechanisms. When the influences of central compensation are unwanted, as when measuring vestibular end-organ function, the vestibular short-latency evoked potential (VsEP) is a better method of assessment. This technique we applied in chapter 2 and 5. In chapter 5 long-term functional and histological effects after gentamicin-induced damage on the otolith organs were studied. Direct vestibular end-organ functional assessment by VsEPs and the 16-week survival time have not been applied in previous studies. Congruent with the findings on the vestibular hair cells, after gentamicin treatment there was a significant loss of vestibular end-organ function after which we found limited evidence for functional end-organ recovery (two of the six animals in the 16-week survival group showed a modest growth of the VsEP amplitude).

The limited VsEP recovery was in contrast to the findings in the cochlea where significant recovery of hearing thresholds was seen after gentamicin induced hearing loss in the same animals that were tested with VsEPs. This recovery of the hearing thresholds in mammals after ototoxic damage was also seen in other studies [Klis et al., 2002; Versnel et al., 2007; Havenith et al., 2013]. In the mammalian cochlea this is mainly caused by damage to and recovery of the endocochlear potential [Klis et al., 2002] or the hair cell's self-repair mechanisms [Jia et al., 2009]. Self-repair refers to repair of damaged cellular structures without mitotic activity or transdifferentiation [Zheng et al., 1999; Gale et al., 2002; Taura et al., 2006; Jia et al., 2009]. Our results suggest that this mechanism was not present in our vestibular regeneration study.

In general, according to our data, spontaneous vestibular hair cell regeneration and end-organ functional recovery are very limited in guinea pigs.

3.3 Recommendations on future fundamental research

Histological regeneration and functional recovery can be induced, e.g., with growth factors, including neurotrophins, and gene expression profiling as shown in several studies already [Kopke, 2001; Albu and Muresanu, 2012; Xu et al., 2012; Jung et al., 2013; Brigande et al., 2013]. Especially, with gene expressing profiling techniques the notch signaling pathway is being studied as a potential for inducing vestibular hair cell regeneration in mammals [Wang

et al., 2010; Jung et al., 2013]. However, the very limited capacity of the vestibular sensory epithelia to regenerate limits this field of research. A few suggestions for other fields of research, especially on drugs that can prevent the effects of ototoxicity, are now mentioned below.

Inner ear exposure to aminoglycoside drugs eventually results in apoptotic destruction of auditory and vestibular hair cells. This ototoxic hair cell death follows the caspase activation pathway. This pathway is interrupted by inhibition of especially caspase-8 and caspase-9. This implies a target for prevention of aminoglycoside induced hearing loss [Forge and Li, 2000; Cunningham et al., 2002; Cheng et al., 2005]. Further, one of the key processes in hair cell death is the regulation of Ca^{2+}. Recently it was shown that alterations in intracellular Ca^{2+} homeostasis play an essential role in aminoglycoside-induced hair cell death in vivo [Esterberg et al., 2013]. Improvements on clinical therapy may lie in designing drugs that can prevent the aminoglycoside-induced ototoxicity. Especially drugs that can interfere with the caspase pathway in apoptotic hair cell death and drugs that interact with the Ca^{2+} dynamics.

4. CLINICAL ASPECTS OF THE OTOTOXIC STUDIES

4.1 General clinical considerations on aminoglycosides

It has to be emphasized that all aminoglycoside drugs are cochleo- and vestibulotoxic but factors as dosage and duration of exposure determine where damage first initiates (see also 2.1). Renal and liver impairment (systemic administration), round window permeability (local administration), stress and disease are known predisposing factors for aminoglycoside induced toxicity, and patients receiving these drugs are thus the most sensitive population [Becvarovski, 2004; Forge and Schacht, 2000]. When disease is combined with a poor nutritional status, as found in many developing countries, a higher incidence of aminoglycoside ototoxicity is encountered [Forge and Schacht, 2000].

Further, the notion that furosemide enhances the cochleotoxic effects of aminoglycoside drugs should be recognized by the clinician working with patients receiving these drugs. So, if opportune, alternatives for these drugs should be used. A suggested alternative for furosemide, bumetanide, does not enhance the ototoxic potential of aminoglycosides in the way furosemide does [Brummett et al., 1981].

4.2 Translational aspects of the ototoxic studies on ITG treatment

The results of our first ototoxic study, described in chapter 2, showing that gentamicin was more or less exclusively vestibulotoxic, speak in favor of intratympanic gentamicin (ITG) treatment. The target of this chemo-ablation therapy is the vestibular sensory epithelium and as the cochlear hair cells are expected not to be damaged, unwanted sensorineural hearing loss should not occur. Applying a higher dosage of gentamicin did induce hearing loss as was shown in chapter 5 (125 mg vs. 100 mg) meaning that the therapeutic spectrum is relatively small.

Also, the results of the longitudinal vestibular regeneration study described in chapter 5, where there was no evidence for histological regeneration and only limited evidence for functional recovery after gentamicin induced damage, are advantageous for ITG treatment. Using gentamicin as chemo-ablation of the vestibular hair cells, regeneration and recovery is undesirable leading to recurrence of complaints. On the other hand, considering the recovery of the VsEP $N_1 - P_1$ amplitudes in a few guinea pigs, such functional recovery could be the reason that in some cases more ITG injections has to be given than in other cases as is known to clinicians applying this therapy [Stokroos and Kingma., 2004].

5. METHODOLOGICAL ASPECTS ON ASSESSING END-ORGAN FUNCTION

In this thesis the VsEP was used for studying end-organ vestibular function. Recording of the VsEP was a stable and reproducible method to assess function electrophysiologically. Some important methodological aspects about this evoked potential are discussed.

5.1. Aspects on acquisition of the vestibular response

In chapter 2 an electrode was used that was placed in the canal of the facial nerve whereas in chapter 5 acquisition of the vestibular response was done with an electrode placed on top of the head, resting on the dura. The $N_1 - P_1$ amplitudes were larger using the facial electrode because the electrode placed in the canal of the facial nerve is in closer proximity to the electrical activity of the vestibular nerve than the epidural electrode. Recording with a facial nerve electrode has an advantage over recording VsEPs using an epidural electrode because with large amplitudes there is a clear discrimination of the actual response from the physiological noise (a larger signal-to-noise ratio) requiring less averaging. However, the technique used in chapter 2 (where the active electrode is placed in the facial canal) caused problems with ingestion and can be a source for infection and is therefore not suitable for

longitudinal studies and can only be applied in acute studies. For longitudinal studies, recording VsEPs with an epidural electrode as in chapter 5 appeared suitable because by this technique there are no problems arising from damage to the facial nerve. An epidural electrode can also be a source of infection but fortunately; this was not present in our study. Further, in the longitudinal study of chapter 5 there was one disconnection of the cement cap (that is used in the preparation for recording with an epidural electrode, see the methods described in chapter 5) from the head of the animal. This seemed to be caused by the physical growth of the animal, which had a survival time at that moment of 16 weeks. The fact that there was only one dropout in the 16-week group because of methodological setbacks proved that the epidural electrode method was very suitable for longitudinal studies. Adapting longer survival times in future studies implicate more growth and then the problem of disconnection of the caps can be more prominent.

5.2 Alternatives for assessing end-organ vestibular function

Because of the above-mentioned issues some alternatives for assessing vestibular end-organ function are now discussed. Besides recording VsEPs, end-organ function can also be reflected by in vitro techniques as assessing hair cell function with use of mechano-electrical transduction properties. Here, Ca^{2+} responses are measured in *in vitro* cultures of the vestibular macula [Taura et al., 2006]. There is a disadvantage when using in vitro techniques as this technique reflects a non-physiological situation.

The vestibular evoked myogenic potential (VEMP) may be suitable for assessing end-organ function in mammals. As mentioned in the general introduction, it is being applied both in clinical settings and in an animal experimental design. Evidence is available for the saccular origin of the collicular VEMP and the utricular origin of the ocular VEMP in humans [Curthoys, 2010; Winters et al., 2012, 2013] and in guinea pigs [Yang et al., 2005, 2010]. The latter study showed successfully recorded cVEMPs in alert guinea pigs using air-conducted sound and recorded oVEMPs after bone-conduction vibration (see the general introduction for background information on the VEMP). Therefore, these techniques may also be used for studying end-organ otolith function in guinea pigs. However, although rare, central disorders could influence the end-organ outcomes. Maybe outweighing this, VEMP recordings require only minimally invasive techniques, reducing risks for infection and may thus also be suitable for longitudinal studies. Conclusively, because of the new insights concerning the utricular and saccular origin of the ocular and cervical VEMP respectively, besides the VsEP recorded with an epidural electrode, we also recommend using this technique that is also minimally invasive, in studying long-term vestibular end-organ function in experimental animal designs in the future.

6. METHODOLOGICAL ASPECTS OF THE INTRATYMPANIC GENTAMICIN (ITG) STUDY

6.1. Main outcomes of the ITG study

The ITG study was designed to assess an appropriate dose regimen on ITG treatment of Ménière's disease patients in a randomized placebo controlled design. For obvious reasons the study design had termination criteria in the case the amount of hearing loss exceeded a shift of 30 dB pure tone average (PTA). As was shown in chapter 5 in guinea pigs, gentamicin can have a clear cochleotoxic effect. This effect unfortunately occurred in the ITG study in three patients when 15 of the 48 patients were included resulting in termination of the trial. In addition we found it difficult to recruit the intended 48 patients. Recently, ITG treatment was reported to be beneficial in the treatment of Meniere's disease related complaints [Pullens et al., 2011]. We reported the exceeding of the accepted hearing loss of 30 dB PTA with 20 dB PTA of ITG treatment as a suspected unsuspected serious adverse reaction (SUSAR) to our Medical Ethical Committee. The interim analysis (over 15 patients) did show a higher efficacy of four injections of gentamicin over two injections gentamicin alternated with placebo or four injections with placebo. Important consideration of ITG treatment besides its potential benefits remains its risk of induction of perceptive hearing loss.

6.2 Inclusion of patients

The main reason for refusing participation was that patients did not want to receive placebo treatment. The difficulty of recruiting enough Ménière subjects for a 2-year follow-up period with adherence to the 1995 AAO-HNS guidelines for reporting is well known [Stokroos and Kingma, 2004; Postema et al., 2008]. Advices on how to efficiently include Ménière's disease patients are difficult to give.

6.3 Aspects on the adverse event hearing loss

Exceeding of the accepted hearing loss (30 dB PTA, averaged over 0.5, 1, 2 and 4 kHz of the treated ear) with 20 dB PTA in one patient and of 2-5 dB PTA in two more patients were the reason to end the ITG study and these were reported as a SUSAR to the medical ethical committee. However, the disability of the hearing loss is outweighed by the profits regarding the vertigo spells. Furthermore, the hearing loss that appeared in these patients could also be the result of the natural course of Ménière's disease which eventually leads to a sensorineural hearing loss of ~ 50 dB [Minor et al., 2004].

6.4 Alternative study on ITG treatment

Intratympanic dexamethason treatment has been described to be an alternative for ITG for controlling vertigo spells in Ménière's disease patients [Longridge and Mallinson, 2000; Barrs, 2004]. An important question remains which of the treatment options give the best relief of complaints with the least side effects. This may be answered by a RCT with both treatment modalities. Currently a RCT is being designed where the two treatment modalities (ITG vs. intratympanic dexamethason) are being compared considering their effect on vertigo spells and hearing. However which gentamicin dose regime should be used remains a subject of debate to achieve the best-balanced result between vertigo reduction and hearing preservation. As was shown in chapter 2 and 5, the therapeutic spectrum of gentamicin in this application is small especially in relation to the risk of inducing hearing loss.

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Chapter 8

Dutch and English Summary

Hoofdstuk 1 geeft de generale introductie van het proefschrift. Er wordt een achtergrond van het onderzoek gegeven, en de lijn van het proefschrift wordt besproken. Deze lijn betreft bestudering van het effect van verschillende ototoxische medicijnen op het binnenoor, kwantificering van functionele en histologische regeneratie van de otolietorganen na ototoxische schade, en de toepassing van de ototoxische eigenschap van gentamicine in de behandeling van patiënten met de ziekte van Ménière. In deze introductie wordt tevens de normale anatomie en functie van het binnenoor beschreven en in het bijzonder van het vestibulair systeem. Verscheidene lichtmicroscopische en immuunfluorescente voorbeelden worden gegeven. Voorbeelden worden gegeven van metingen die de functie van de otolietorganen weergeven. Met name de in dit proefschrift gebruikte term, de '*Vestibular short-latency Evoked Potential*' (VsEP) wordt toegelicht.

In **hoofdstuk 2** wordt een ototoxische studie beschreven waarin gekeken wordt welk middel het best gebruikt kan worden om trauma in de otolietorganen op te wekken met het oog op een latere studie naar regeneratieverschijnselen. Twee veel gebruikte methoden, enerzijds tien opeenvolgende dagen gentamicine en anderzijds eenmalig kanamycine/furosemide, werden met elkaar vergeleken wat betreft hun uitwerking op het gehoor- en het evenwichtsysteem. Gentamicine bleek het meest geschikt omdat het in dit model een selectief vestibulotoxisch effect had. Kanamycine/furosemide had op de otolietorganen geen effect.

In **hoofdstuk 3** wordt een tweede ototoxische studie beschreven. Hier wordt gekeken hoe nu het auditieve zintuigepitheel in de cochlea van de muis, waarvan bekend is dat de (met name binnenste) haarcellen recalcitrant zijn tegen schade, het meest efficiënt beschadigd kan worden voordat bijvoorbeeld regeneratieverschijnselen kunnen worden bestudeerd of om in de toekomst stamcel-therapie toe te passen in het auditieve systeem. De gecombineerde toediening van kanamycine en furosemide welke in hoofdstuk 2 in de cavia is gebruikt, kan ook goed in de muis worden gebruikt om doofheid te induceren. Een hogere dosering van kanamycine was wel nodig.

Hoofdstuk 4 is een vervolgstudie op de studie in hoofdstuk 3. In hoofdstuk 3 waren er aanwijzingen dat het interval tussen het toedienen van kanamycine en furosemide van belang kan zijn op de mate van gehoorsverlies in de muis. In deze studie vergeleken we het verloop van de gehoorfunctie middels het meten van '*auditory brainstem responses*' (ABR's) bij drie groepen muizen waarbij er verschillende tijdsintervallen werden vastgesteld tussen het

toedienen van kanamycine en furosemide. Tussen de onderzochte tijdsintervallen bleek er geen verschil in ototoxisch effect te zijn. De uitkomsten van hoofdstuk 3 en 4 dragen bij aan fundamenteel onderzoek naar gehoorbeperking als bijvoorbeeld gebruik gemaakt wordt van stamcel-therapie waarvoor eerst het binnenoorepitheel beschadigd dient te worden.

In **hoofdstuk 5** werd de uitkomst van de studie in hoofdstuk 2 toegepast bij de kwantificering van herstel en/of regeneratie in de otolietorganen. In deze longitudinale studie werd gentamicine gebruikt als ototoxisch agens om haarcelverlies te induceren waarna de langere termijn effecten (tot en met 16 weken na behandeling) op de otolietorganen werden bestudeerd. De effecten werden zowel functioneel, weergegeven door de VsEP, als histologisch aan de hand van haarceltellingen met behulp van immunofluorescentie-microscopie bekeken. Na behandeling met gentamicine bleek er een forse degeneratie van functie en haarcellen waarna spontane regeneratie of herstel nauwelijks optrad. Dit in tegenstelling tot een significant herstel van de ABR drempel wat duidde op herstel (maar geen regeneratie) in de cochlea.

In **hoofdstuk 6** werd klinisch gebruik gemaakt van de relatief selectieve schade die gentamicine toebrengt aan de vestibulaire haarcellen zoals bleek uit hoofdstuk 2. Het beschrijft een prospectieve, dubbelblinde, placebo-gecontroleerde en gerandomiseerde studie naar de beste dosering van de wereldwijd veelgebruikte methode van intratympanale gentamicine (ITG) toediening bij patiënten met de ziekte van Ménière. Hier werden de langere termijn effecten van twee injecties vergeleken met vier injecties. Uitkomstparameters waren onder andere het effect op de duizeligheid als de mate van gehoorsverlies. Deze studie is na een interim-analyse voortijdig stopgezet gezien er meer gehoorsverlies optrad dan van te voren voorzien. Daarnaast bleek er uit de literatuur een duidelijk voordeel van ITG behandeling bij patiënten met de ziekte van Ménière wat doorgang van een studie met een placebo groep uit ethisch standpunt onwenselijk zou maken.

Hoofdstuk 7 beschrijft de generale discussie over de translationele waarde van de fundamentele studies waarbij ook aanbevelingen worden gedaan voor toekomstig onderzoek. Daarnaast worden methodologische aspecten van de VsEP besproken als methodologische aspecten van de intratympanale gentamicine studie.

Chapter 1 describes the general introduction. A background of the study is given, and the outline of the thesis is discussed: Studying the effects of various ototoxic drugs on the inner ear, quantification of functional and histological regeneration in the otolith organs after ototoxic damage, and the application of the ototoxic property of gentamicin in the treatment of patients with Ménière's disease. Also the normal anatomy and function of the inner ear is described, in particular of the vestibular system. Several light microscopic and immune-fluorescent histological examples are given and measurements will be discussed that represent the function of the otolith organs. Especially the parameter used in this thesis, the 'Vestibular short latency Evoked Potential' (VsEP) is explained.

In **chapter 2** a fundamental ototoxic study is performed that addresses the most suitable method for assessing damage in the otolith organs before we study the longer-term effects after damage in chapter 5. Here, two methods, often used in basic inner ear research, gentamicin and co-administration of kanamycin and furosemide, were compared to each other with regard to their effects on the vestibular and cochlear system. Gentamicin appeared to be the most suitable where kanamycin/furosemide had no effect on the otolith organs.

An increasing amount of research is being performed on regeneration and stem cell phenomena in the auditory system of the mouse**.** Its auditory system is known to be recalcitrant against aminoglycoside ototoxic damage. **Chapter 3** describes a second ototoxic study that investigates the best method for assessing damage in the mouse inner ear (cochlea). The method for assessing damage in the guinea pig cochlea (co-administration of kanamycin and furosemide, which is routinely used in our laboratories and also used in chapter 2) appeared very suitable for assessing damage in the mouse auditory sensory epithelia. This model is crucial for further research on hearing impairment.

Chapter 4 is a sequel on the study in chapter 3. In the study of chapter 3 there were indications that the interval between the administration of kanamycin and furosemide was of interest on the degree of hearing loss. In this study, we compared the amount of hearing loss of three groups of mice that received the administered drugs at different time intervals. The time window between administrations of kanamycin and furosemide is not crucial for the deafening effect in mice.

The model described in chapter 3 and 4 is very suitable for hearing impairment research in mice in the future. A mice model has advantages over the guinea pig model since there are specific murine strains available for stem cell research.

The study in **chapter 5** makes use of the results of chapter 2. In this longitudinal study gentamicin was used as ototoxic agens. After damage was assessed, the long-term effects (up to 16 weeks after treatment) in the otolith organs were examined. These long-term effects were studied functional, reflected by recording VsEPs, as histological with hair cell counts using immunofluorescent techniques. After gentamicin treatment there appeared to be a rapid degeneration of function and hair cell counts. After damage, there was no evidence for spontaneous regeneration of vestibular hair cells and only very limited evidence for vestibular recovery of end-organ function in guinea pigs after ototoxic induced damage. These results pose severe challenges in the field of research on (induction of) re-population of lost vestibular hair cells in the future in the guinea pig.

Chapter 6 makes use of the more or less selective vestibulotoxicity of gentamicin (chapter 2) when gentamicin is used as treatment modality. Intratympanic gentamicin treatment induces chemo-ablation of the vestibular organs in patients suffering from Meniere's disease. The study was designed as a double-blind, prospective, randomized, placebo-controlled study on dose efficacy of intratympanic gentamicin treatment. It compared two gentamicin injections with four injections on the effects on dizziness and degree of hearing loss of. A suitable dose regimen of ITG treatment has not been found because this study was prematurely ended (the endpoint for the predefined acceptable hearing loss was reached). Lack of consensus remains on the intratympanic dose of gentamicin that needs to be used to have the best-balanced result between reduction of incapacitating vertigo and hearing preservation.

The general discussion is described in **chapter 7** where the main results and the translational values of the fundamental studies are discussed and recommendations are given for future research. Further, methodological aspects about the VsEP are given. Finally, methodological aspects of the RCT are discussed.

Dankwoord

Curriculum Vitae

Lijst met afkortingen

DANKWOORD

Ik dank iedereen die heeft bijgedragen aan de totstandkoming van dit proefschrift maar in het bijzonder wil ik de volgende personen bedanken.

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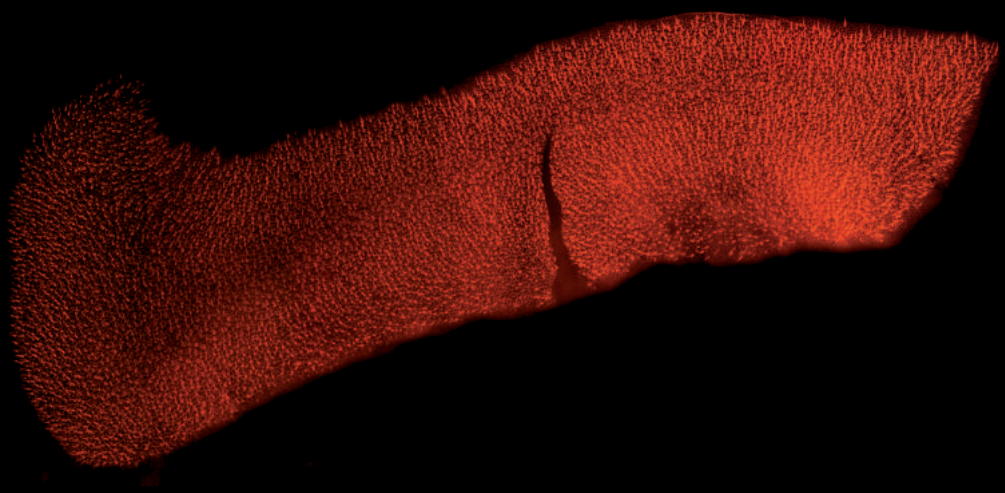
CURRICULUM VITAE

Hendrik Bremer werd geboren op 29 mei 1977 in Delft. In 1996 haalde hij het gymnasiumdiploma aan het VCL in Den Haag. Na een jaar uitgeloot te zijn begon hij in 1998 met de studie Geneeskunde in Leiden. Om ervaring op te doen in het buitenland volgde hij enkele co-schappen in Suriname. In 2005 haalde hij zijn artsenbul waarna hij tijdelijk werkte als arts docent aan het maritiem trainingscentrum Nutec. In 2006 begon hij als arts onderzoeker bij de afdeling KNO van het UMC Utrecht onder leiding van prof. dr. F. Albers. De opleiding tot KNO-arts begon in 2008 onder leiding van dr. A. van Olphen en prof. dr. W. Grolman c.s. De B-opleiding werd in het Ziekenhuis Gelderse vallei Ede gevolgd bij dr. M. Majoor c.s. en in het Gelre ziekenhuis Apeldoorn bij dr. PP. van Benthem c.s. Tijdens de opleiding is hij twee keer voor de stichting Eardrop naar Ethiopië gegaan om KNO-onderwijs te verzorgen voor lokale medici. Na het voltooien van de opleiding heeft hij enkele maanden als staflid gewerkt in het UMC Utrecht waar hij dit proefschrift heeft voltooid. Hierna heeft hij als waarnemer KNO-arts gewerkt in het Gelre ziekenhuis Apeldoorn. Sinds december 2013 werkt hij als KNO-arts in het Elkerliek Ziekenhuis Helmond in maatschapverband met dr. P. Schuil c.s. De auteur is getrouwd met Fieke Friedeman en woont in Utrecht.

LIJST MET AFKORTINGEN

AAO-HNS	American Academy of Otolaryngology- Head and Neck Surgery
ABR	Auditory Brainstem Response
BM	Basilar Membrane
CoV	Control of Vertigo
dB	Decibel
DHI	Dizziness Handicap Inventory
ENG	Electronystogram
HC	Hair Cell
HL	Horizontal Lateral
HM	Horizontal Medial
IHC	Inner Hair Cell
ITG	Intratympanic Gentamicin Treatment
kHz	kilo Hertz
MD	Ménière's disease
NaCl	Natrium Chloride
OHC	Outer Hair Cell
PTA	Pure Tone Audiometry
RCT	Randomized Controlled Trial
SD	Standard Deviation
SEM	Standard Error of Mean
SGC	Spiral Ganglion Cell
SNHL	Sensory Neural Hearing Loss
SUSAR	Suspected Unexpected Serious Adverse Reaction
UMCU	University Medical Center Utrecht
V	Vertical
VsEP	Vestibular short-latency Evoked Potential

''This thesis is about the toxic side effects of aminoglycoside drugs on the inner ear, which contains the sensory organs of the vestibular system, and the sensory organ involved with hearing. Aspects that we studied were the short- and long-term effects, following ototoxic medication induced damage, and the regenerative capacity of the vestibular system in an animal model. Finally, the ototoxicity is clinically applied as treatment modality in vestibular inner ear disease in human patient population. The emphasis of this thesis lies on the vestibular system; however, because the auditory system is so closely related to the vestibular system, a portion of this thesis is dedicated to the auditory system as well.''



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