The Round Window Membrane - Gateway to the Cochlea

A Morphological and Electrophysiological study

BY

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ABSTRACT


Topical treatment of several inner ear diseases through the round window membrane (RWM) might be feasible in the near future. Bacteria toxins, ototoxic drugs and noise trauma seem to harm the inner ear by a common pathway which involves, excessive outflow of the afferent neurotransmitter glutamate and formation of nitric oxide (NO), which can severely damage cells/nerve endings and lead to cell death.

In this study we used 98 Sprague-Dawley rats and seven human temporal bones. Various substances were instilled into the middle ear of the rat, such as Pseudomonas Aeruginosa Exotoxin (PaExoA), gentamicin, NO-inhibitor N-Omega-Nitro-L-Arginine Methyl Ester (L-NAME), and glucocorticoids. The effects of the substances were studied by morphological analysis of RWM and the endolymphatic sac (ES) by light and electron microscopic. Hearing level was measured in the rats by ABR technique. The human temporal bones were studied immunomorphologically to search for glutamate.

In the human inner ear, glutamate receptors and glutamine synthetase, were identified. In the rat, we found, following PaExoA exposure, reversible and permanent hearing loss and morphological changes in the RWM. The ES showed increased numbers of macrophages and thickening of the epithelia. When L-NAME was used as an otoprotector from gentamicin ototoxicity a therapeutic effect in the high frequency area was found. Hydrocortisone (but not dexamethasone) exposure of the RWM resulted in membrane thickening, and adjacent to the membrane, inflammatory cells.

The importance of the RWM as a portal for toxic substances and topical treatment of inner ear diseases was highlighted in this study. The difficulties of applying drugs in the round window niche were exposed. The results of this study add important knowledge concerning certain mechanisms of inner ear injury and help us to understand possibilities and problems of local treatment of inner ear diseases in patients.

Key words: round window membrane, pseudomonas exotoxin, auditory brainstem response, hearing loss, endolymphatic sac, inner ear immunology, glutamate, nitric oxide inhibitor, glucocorticoid.

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To my family
Original papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:


IV. L Nordang & M Anniko. L-NAME, a potential protector from gentamicin ototoxicity [submitted].

V. L Nordang, B Linder, M Anniko. Morphological changes in round window membrane following topical hydrocortisone and dexamethasone treatment [submitted].
## Contents

**ABSTRACT** ........................................................................................................................................... 2  
**ORIGINAL PAPERS** ............................................................................................................................. 4  
**ABBREVIATIONS** .................................................................................................................................. 6  
**INTRODUCTION** .................................................................................................................................. 7  
  Otitis media and hearing loss ...................................................................................................................... 7  
  The round window membrane .................................................................................................................... 8  
  The hair cells — the target area for toxic agents? ....................................................................................... 8  
  Glutamate excitotoxicity — the final common pathway for otological disorders? ................................. 9  
  Aminoglycoside ototoxicity ...................................................................................................................... 10  
  The endolymphatic sac ............................................................................................................................... 11  
**AIMS OF THE PRESENT INVESTIGATION** ......................................................................................... 12  
**MATERIAL AND METHODS** .............................................................................................................. 13  
  **MATERIALS** ...................................................................................................................................... 13  
    Animals .................................................................................................................................................... 13  
    Temporal bones ...................................................................................................................................... 13  
  **METHODS** ....................................................................................................................................... 13  
    1. Antibodies (Paper III) ......................................................................................................................... 13  
    4. L-NAME (Paper IV) .......................................................................................................................... 14  
    5. Glucocorticoids (Paper V) .................................................................................................................. 14  
    6. ABR procedure (Papers I, II & IV) ...................................................................................................... 14  
    8. RWM thickness measurement (Papers I & V) .................................................................................... 15  
**RESULTS** ........................................................................................................................................... 17  
  **ELECTROPHYSIOLOGICAL MEASUREMENTS** .............................................................................. 17  
  **MORPHOLOGY OF THE RWM** ....................................................................................................... 17  
  **THICKNESS OF RWM** ...................................................................................................................... 18  
  **MORPHOLOGY OF ES FOLLOWING PAEXOA EXPOSURE OF MIDDLE EAR (PAPER II)** ...................... 19  
 **GLUTAMATE IN THE HUMAN INNER EAR (PAPER III)** ...................................................................... 20  
**DISCUSSION** ..................................................................................................................................... 21  
  Hearing after PaExoA exposure (Paper I) ................................................................................................. 21  
  RWM morphology after PaExoA exposure (Paper I) .............................................................................. 21  
  Endolymphatic sac morphology after PaExoA exposure (Paper II) ....................................................... 22  
  Glutamate in human cochlea (Paper III) .................................................................................................. 23  
  Hearing after L-NAME as otoprotectant from gentamicin ototoxicity (Paper IV) ................................. 23  
  RWM morphology after glucocorticoid exposure (Paper V) ................................................................. 24  
**GENERAL DISCUSSION** ..................................................................................................................... 26  
  The round window membrane – a gateway for local therapy of the inner ear ....................................... 26  
  Immunotherapy (glucocorticoids) ........................................................................................................... 27  
**GENERAL CONCLUSIONS** .................................................................................................................. 28  
**ACKNOWLEDGEMENTS** ...................................................................................................................... 29  
**REFERENCES** ................................................................................................................................. 30
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
</tr>
<tr>
<td>AG</td>
<td>aminoglycoside antibiotics</td>
</tr>
<tr>
<td>CSOM</td>
<td>chronic suppurative otitis media</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>ES</td>
<td>endolymphatic sac</td>
</tr>
<tr>
<td>GM</td>
<td>Gentamicin sulfate</td>
</tr>
<tr>
<td>HL</td>
<td>hearing loss</td>
</tr>
<tr>
<td>IHC</td>
<td>inner hair cell</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>LM</td>
<td>light microscopy</td>
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<tr>
<td>L-NAME</td>
<td>N-Omega-Nitro-L-Arginine Methyl Ester Hydrochloride</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxasolepropionate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>OHC</td>
<td>outer hair cell</td>
</tr>
<tr>
<td>OM</td>
<td>otitis media</td>
</tr>
<tr>
<td>OMA</td>
<td>otitis media acuta</td>
</tr>
<tr>
<td>PaExoA</td>
<td>Pseudomonas Aeruginosa Exotoxin A</td>
</tr>
<tr>
<td>RWM</td>
<td>round window membrane</td>
</tr>
<tr>
<td>SG</td>
<td>spiral ganglion</td>
</tr>
<tr>
<td>SNHL</td>
<td>sensorineural hearing loss</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
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Introduction

Otitis media acuta (OMA) is extremely common as more than 80% of all children suffer at least one episode of OMA [1]. Sequelae of OMA include chronic suppurative otitis media (CSOM), conductive hearing loss and sensorineural hearing loss (SNHL). Health care concerning otitis media (OM) stands for great costs and lot of people suffer from this disease, especially in the developing countries. Hitherto we have not succeeded in preventing or treat inner ear impairment following OM. However, some interesting attempts have recently been made to tackle our greatest challenge: to help patients suffering from such inner ear disturbances as SNHL and tinnitus.

Otitis media and hearing loss

OMA and CSOM are common all over the world and the risk of inner ear disturbances consequent on OM has been investigated in several studies [2, 3], [4]. Both temporary and permanent threshold shifts were found, most commonly in the high frequency corresponding to the basal turn of the cochlea. Several factors are involved in the pathogenesis of OMA, such as abnormalities of the Eustachian tube (cleft palate, Downs syndrome) impaired immunity (low IgG2), viral infections preceding bacterial infection (mostly RSV) and defective immune response. In OMA, the most common bacteria are: *Streptococcus pneumonia*, *Haemophilus influenza* and *Moraxella catharralis*. In CSOM the bacterial panorama differs: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and other enterobacteria are often found [5].

Several of these bacteria produce toxins – either exotoxins (peptides) or endotoxins (lipopolysaccharides). The highly toxic *Pseudomonas aeruginosa* Exotoxin A (PaExoA) from *Pseudomonas aeruginosa* was used in our model for CSOM. Knowledge of PaExoA regarding pathophysiology, and the activation and mechanism of the immune response are important matters requiring research to elucidate the way CSOM leads to inner ear impairment.

Pseudomonas Aeruginosa Exotoxin A

PaExoA can damage the inner ear, either reversibly or irreversibly, as has been proven morphologically [6] and electrophysiologically [7]. PaExoA, produced by most strains of *Pseudomonas aeruginosa* as a 'protoxin', binds to the α-2-macroglobulin receptor [8] and
enters the cell by endocytosis. The toxin is cleaved into a larger 45 kD receptor binding domain and a smaller 27 kD enzymatically active domain which inhibits the mammalian protein synthesis by blocking elongation factor 2 [9]. The target area in the inner ear is not yet known, though for *Haemophilus influenzae* endotoxin [10] the stria vascularis seems to be affected very early.

**The round window membrane**

Only a three-cell layer membrane separates the middle ear lumen from the inner ear (scala tympani). The thickness of the normal human RWM is 60 to 70 µm [11], but increases in different stages of OM, being thickest in individuals suffering from CSOM. The thickness of the membrane differs between species [11, 12] and is generally thinner in rodents than in Man. The ultrastructure of the RWM consists of an outer epithelium of low cuboidal cells lining the middle ear, an inner epithelium of squamous cells bordering the inner ear and a layer of connective tissue between the epithelial layers. The connective tissue layer consists of fibroblasts, collagen and elastic fibres. The membrane has several functions [13]: it releases mechanical energy to the labyrinthine fluids, can serve as an alternative route for sound energy to enter the cochlea, participates in absorption and secretion from the perilymph and is involved in the defence system of the inner ear.

Passage through the membrane is possible for small molecules by passive diffusion and for larger molecules probably by endocytosis [12, 14]. Passage of various medium-sized molecules such as bacteria toxins has been used as a models for the study of inner ear impairment caused by OM [6, 15, 16]. Some toxins even ease the passage by creating pores in the RWM [17]. The importance of the RWM in inner ear impairment led us to investigate the RWM morphology in CSOM (paper I).

**The hair cells — the target area for toxic agents?**

One row of inner hair cells (IHC) in the organ of Corti has a central function in hearing. The IHC are connected to the majority of afferent neurons (95%) in the spiral ganglion. The movement of the basilar membrane and the stereocilia of the IHC initiates the conversion of peri- and endolymphatic waves to electrical impulses in the cochlear nerve. Influx of calcium triggers a depolarization of the IHC, resulting in glutamate release in the synaptic cleft to activate the glutamate receptors on the postsynaptic membrane. This cascade (Fig. 2) is central to the process of hearing, to the pathophysiology of SNHL, and a basis for future treatment of inner ear diseases [18].
The three rows of outer hair cells (OHC) are probably responsible for the tuning of the hearing and are connected mainly to efferent nerve fibres. In pathophysiology the OHCs are often shown to be the more vulnerable of the hair cells, especially the first row.

**Glutamate and its receptors**

Glutamate is a common amino acid in all cells as a component of proteins. Mammalian cells use different amino-acids as neurotransmitters in communication and glutamate is the most important afferent neurotransmitter between the hair cells and the afferent neurons in the mammalian cochlea [19]. Glutamate stimulates two main types of receptor, the ionotropic and the metabotropic. Ionotropic receptors can be subdivided into the N-methyl-D-aspartate (NMDA) and the non-NMDA (α-amino-3-hydroxy-5-methyl-4-isoxasolepropionate {AMPA} and kainate) receptors which can be further divided into several subtypes [20]. The purpose of this differentiation in relation to the hearing process is still unclear, however. NMDA receptors require strong and repeated depolarization in order to be activated, possibly corresponding to sound stimuli of high intensity [21]. In Paper III we studied the distribution of glutamate in the human inner ear.

**Glutamate excitotoxicity — the final common pathway for otological disorders?**

Many neurological disorders may in part be caused by receptor hyperstimulation by excitatory amino acids. Glutamate is the principal excitatory neurotransmitter and excess of glutamate has proved to be toxic to the postsynaptic neuron [18]. The toxic effect of glutamate in the organ of Corti is prevented by an enzymatic re-uptake, inactivation and recycling of glutamate (Paper III, fig. 1). Release of glutamate in the synaptic cleft activates ionotropic glutamate receptors, thus opening Ca channels. An increase in intracellular Ca\(^{2+}\) concentration initiates the formation of NO and free radicals (see below), a cascade of events probably involved in several inner ear disorders.

**Nitric oxide**

Nitric oxide (NO) is an uncharged small molecule that easily passes all membranes and has a half-life of 2–30 seconds. NO-synthase (NOS) is essential for the production of NO, a byproduct when L-arginine is converted to citrulline. Subtypes of NOS have been identified as constitutive (cNOS) and inducible (iNOS). cNOS (endothelial and neuronal) which helps regulate vascular tone and neuronal transmission, is produced in small amounts and is activated by Ca\(^{2+}\) that binds to calmodulin. iNOS can be produced in large amounts by
macrophages leading to NO production followed by formation of reactive oxygen species (ROS), which are toxic to proteins and lipids leading to apoptosis and cell death [22, 23]. Endotoxin, γ-interferon (TNF-γ), interleukin (IL) 1, IL-2 and tumour necrosis factor (TNF) can all induce iNOS synthesis. Glucocorticoids, IL-4 and IL-10 inhibit transcription for iNOS.

Cells have an internal defence against ROS, including glutathione (GSH), various antioxidant enzymes and vitamins, but are unable to prevent large-scale iNOS production and subsequent cell death. GSH concentration is particularly high in stria vascularis, but is also present in IHC, OHC and the supporting cells of the organ of Corti [24].

The toxic sequence of events resulting in free radical production is central the mechanism of both glutamate excitotoxicity and aminoglycoside ototoxicity.

**Aminoglycoside ototoxicity**

Streptomycin was the first effective antibiotic against tuberculosis. From streptomycin several substances have been developed in this group called aminoglycoside antibiotics (AG), all effective against Gram-negative bacteria. AGs bind to the 30S subunit of the bacterial ribosome, causing an alteration of the codon: anticodon, resulting in misreading of the messenger RNA and consequently in the production of defective bacterial proteins. The bactericidal effect of AG is achieved by a forming pore-effect on the cell membrane [25], in addition to inhibiting protein synthesis. The half-life of AG in plasma is 2-3 hours and eliminated by glomerular filtration. In certain tissues such as endolymph and perilymph, AG is less effectively cleared and its half-life in inner ear tissues and fluids may exceed 30 days [26]. Its most serious side effects are the kidney and inner ear impairment, the renal damage being mostly reversible. All AGs can damage both the labyrinth and the cochlea, gentamicin and streptomycin being more likely to interfere with vestibular function, while kanamycin and neomycin are more likely to produce SNHL. AG destroys both the hair cells and the supporting cells of the organ of Corti, chiefly the OHC and later the IHC as an effect of increasing dosage and/or longer treatment. The damage begins in the basal part of the coil, spreading apically causing primary SNHL in the high frequency [26]. Gentamycin, an AG, is used worldwide in spite of its side effects, probably because of its low price and broad spectrum. The mechanism behind the cochlear damage of topically instilled gentamicin seems to follow a multi-step sequence as the positively charged agent is attracted to the negatively charged outer plasma membrane [26, 27, 28]. Then follows chelating to iron, forming a redox-active compound that can promote the formation of NO and ROS [29] (Fig. 2). This
mechanism prompted us to study the effect of a NO-inhibitor as a therapeutic drug against gentamicin ototoxicity (Paper IV).

The endolymphatic sac
The resting cochlea does not contain immunocompetent cells, but the endolymphatic sac (ES) is known to contain T-cells, macrophages, and B-cells bearing immunoglobulins such as IgM, IgG and IgA. Antigen in the scala tympani gives rise to macrophages and granulocytes, observed in the ES 6 hours post-challenge. As PaExoA instilled into the middle ear can pass the RWM [6] it might even initiate a reaction in the ES (Paper II).
Aims of the present investigation

1. To perform a qualitative and quantitative morphological analysis of the RWM during experimental CSOM.
2. To study the response in ES after exposure of the middle ear to PaExoA.
3. To investigate the presence of glutamate in the human inner ear.
4. To investigate the ability of a possible otoprotectant (a NO-inhibitor) to reduce damage from gentamicin ototoxicity.
5. To investigate the morphological effect of steroids on the round window membrane.
Material and Methods

Materials

Animals
98 male albino Sprague-Dawley rats (body weight of 250 g) were used for the experiments in Papers I, II, IV and V. They were purchased from a local breeder (B & K Universal AB). The animals were housed in Plexiglas cages (45x23x20 cm) on wood chips bedding, in temperature controlled rooms (20 – 22°C), humidity 40 – 50%, ambient noise level < 40 dBA and a 12/12 hour lighting cycle. The rats were fed ad lib on Beekay Feeds standard diet. The experimental series was approved by The Swedish National Board for Laboratory Animals (C276/98).

Anaesthetics
During middle ear instillation and ABR measurement, the animals were anaesthetized with 90 mg/kg bw i.p. ketamine (Ketalar, Parke-Davis) and 10 mg/kg bw i.p. xylazine (Rompun vet., Bayer) initial dose and, when needed, supplementary doses of ketamine 30 mg/kg.

Temporal bones
Seven human temporal bones from subjects in the age range 14 – 71 years were used for the investigation in Paper III.

Methods

1. Antibodies (Paper III)
The ionotropic NMDA receptor subunit NR2B was identified with monoclonal antibodies (mAbs) of mouse IgG2B from Transduction Laboratories (Lexington, USA); glutamine synthetase was visualized with mAbs of mouse IgG2A from Transduction Laboratories; and L-glutamate with mAbs of mouse IgG from Sigma BioSciences (St. Louis, Mo, USA).

2. Pseudomonas aeruginosa Exotoxin (Paper I)
PaExoA was obtained from Sigma-Aldrich Corp., Milwaukee, Wisc., USA. The rats were given varying numbers of doses of PaExoA. In Papers I and II each dose consisted of 1 µg PaExoA diluted in 20 µl saline, instilled into the right middle ear cavity through a minute puncture in the posterior-superior quadrant of the tympanic membrane. Left ears served as
unexposed controls. One control series had vehicle (saline) instilled in the same manner as in toxin-exposed animals (see below).

3. Gentamicin (Paper IV)
Gentamicin sulfate (GM) was obtained from Sigma-Aldrich Sweden AB. GM was dissolved in bicarbonate buffer to a concentration of 30-480 mg/ml. Doses of 20 µl were instilled through a puncture in the posterior part of the tympanic membrane (Paper IV). One dose of GM was given on day 0, one on day 1 and one on day 3.

4. L-NAME (Paper IV)
N-Omega-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) was obtained from Sigma-Aldrich Sweden AB. 1 mg of L-NAME was instilled into the middle ear after GM (Paper IV).

5. Glucocorticoids (Paper V)
Hydrocortisone as Solu-cortef ® (Pharmacia) was purchased from a local pharmacy and dexamethasone sodium 21-phosphate was purchased from Sigma Chemical Corp., St Louis, Mo., USA. Rats in Group 1 (n=10) received dexamethasone in the right middle ear as five or ten doses of 1 µg (20 µl) every second day. The left ears served as unexposed controls. Group 2 (n=10) received five or ten doses of 2% hydrocortisone solution (20 µl). The left ears of Group 2 rats served as control and received 20 µl saline doses according to the procedure used for the glucocorticoid exposed ears (Paper V).

6. ABR procedure (Papers I, II & IV)
A frequency-specific ABR technique was used, consisting of tone bursts of 5 ms overall duration. The stimuli were presented at a rate of 16/s (Tektronix SG 505 oscillator). The attenuation spanned a range of 0 to 88 dB (Somedic TMS 110 attenuator). Tone bursts at 10 frequencies (2, 4, 6, 8, 10, 12, 14, 16, 20 and 31.5 kHz) were presented in an electrically shielded, soundproof box. To distinguish between conductive and SNHL, latency/intensity curves at 4, 12 and 20 kHz were constructed [7].
Control thresholds (Paper IV)
Because of a technical failure and recalibration of the ABR equipment before commencing the Gentamicin/L-NAME experiment (Paper IV), the control thresholds in Paper IV differed from previous measurements in Paper I. The thresholds in Paper I are therefore not comparable to those in Paper IV.

7. Histological preparation, light and electron microscopy (Papers I, II & V)
After the final instillation (described in Papers I, II, IV & V), the animals were decapitated after a supplementary dose of general anaesthetic. The temporal bones were removed and immersed in a fixative containing 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M Na-phosphate buffer, pH 7.2 – 7.4, for 24 h and subsequently decalcified in Na-EDTA, postfixed in 1.5% osmium tetroxide and embedded in Epon 812. The specimens were sectioned for light microscopy (LM) and/or transmission electron microscopy (TEM) in a JEOL 100 SX (Papers II & V).

8. RWM thickness measurement (Papers I & V)
The thickness of the RWM was measured using a Zeiss Axiophot light microscope connected to a TV camera (PCO Computer Optics) and analysed with KS 300 image analysis software. Three measurements were made at the mid-point of each membrane and the mean value was calculated.

9. Immunomorphological procedure (Paper III)
The human temporal bones were perfused with sublimate–formaldehyde within 6 h after death and immersion fixed for a further 24 h. Blocks containing the inner ear were cut out with a band saw and immersed in EDTA solution until complete decalcification, dehydrated and embedded in Paraplast® (at 54°C). The preparations were then serially sectioned in the horizontal plane, deparaffinized in xylol and trypsinized. 30 deparaffinized sections were used for the immunomorphological analysis. For control puposes, the primary antibody was replaced by PBS or rabbit serum.

The sections were immunostained using the FITC fluorescence technique and viewed in a Zeiss Axiophot photomicroscope. Each section was also analysed in phase contrast.
Saline control series (Papers I & II)

To ensure that the morphological findings in the RWM (Paper I) and the ES (Paper II) were not caused by the instillation technique, we added a control group of animals ($n=8$) in which the middle ear was exposed to saline by the same procedure as in Paper I. The temporal bones were subjected to the same preparation technique, the RWM’s examined by LM and the thickness was measured. The ES was sectioned and examined for the same morphological parameters as we used in Paper II. Two animals, given one dose of saline (20 µl) in the right ear and one in the left, survived 7 days; another 2 animals, given one dose of saline (20 µl) in the right and one in the left ear, survived 14 days. Another 2 received two saline doses with an interval of 7 days and survived 7 days after the final instillation. The final 2 animals received four doses of saline following the same schedule.
Results

Electrophysiological measurements

1. ABR results following PaExoA exposure (Paper I)
   All animals but one suffered a hearing loss (HL). In Group 1 (single application) we found both progressive and, more commonly, reversible HL. In some of the animals the threshold shift was of the flat-loss type, but more often the higher frequencies were affected. The results are presented Paper I, Fig. 1. In Group 2 (several applications), the most common effect was high frequency impairment. Group results are presented in Paper I, Fig. 2. Latency/intensity curves were constructed (Paper I, Fig. 3), the sensorineural component of the HL being indicated as a steeper slope of the curve.

2. ABR results following gentamicin/L-NAME exposure (Paper IV)
   Temporary threshold shifts in the high frequency area (16 – 31.5 kHz) were found in some of the animals exposed to a low dose of gentamicin. The maximum HL was found at the 7 or 14-day measurement. The severity of the HL increased with increasing concentration of gentamicin. At doses of 480 mg/ml (9.6 mg/20 µl), we more often found a permanent HL and impairment even at the lower frequencies (Paper IV, Fig. 1, Table 2). In animals given gentamicin doses of 480 mg/ml and L-NAME, the HL ran a course similar to that described for the gentamicin group, but the recovery appeared earlier, at 20 and 31.5 kHz (Paper IV, Fig. 3). The difference resulted in a p-value of 0.0655 at 20 kHz. Latency/intensity curves verified the cochlear involvement of the HL (Paper IV, Fig. 2). In animals given L-NAME only, we found a slight HL in the high frequency area during the first days, sloping to normal hearing after 2–3 weeks (Paper IV, Fig. 1).

Morphology of the RWM

1. RWM morphology following PaExoA exposure (Paper I)
   Generally we found an increasing height and change from the flat to cubic shape of the RWM epithelia facing the middle ear when the middle ear was exposed to PaExoA. We also found increasing edema of the middle layer and increasing layers of fibroblasts. In animals exposed to one or several doses of PaExoA we found similar changes in the different layers of the RWM. The differences between membranes exposed to two, three, or four doses of toxin were
slight as regards both the epithelial layer and the middle layer. In membranes exposed to five doses a return toward normal morphology was seen.

2. RWM morphology following glucocorticoid exposure (Paper V)
In the group of dexamethasone-exposed ears, nine cochleas were prepared for LM. The membranes had a normal appearance of the epithelia and two exposed ears showed an accumulation of inflammatory cells adjacent to the RWM (Paper V, Table 1). In the group of hydrocortisone-exposed ears, 10 membranes were examined and in eight an accumulation of inflammatory cells was found (Paper V, Fig. 1). In three membranes we found a thickening of the epithelia facing the middle ear side of the RWM.

Among the unexposed membranes (n=9) we found neither accumulation of inflammatory cells nor thickening of the epithelia (Paper V, Fig. 2). Among the saline exposed ears (n=10) we found an accumulation of inflammatory cells adjacent to one RWM and epithelial thickening in another (Paper V, Table 1).

The inflammatory cells adjacent to the rim of the RWM were identified by TEM as polymorphonuclear leukocytes and macrophages. The latter contained large numbers of vesicles and some were filled with filament-like structures. Polymorphonuclear leukocytes were even found subepithelially in the RWM (Paper V, Fig. 3).

Thickness of RWM
1. Thickness of RWM following PaExoA exposure (Paper I)
In general, membranes were thicker in the exposed ears, than in the controls.
In the group of two to five applications, membranes were initially thick but thinned gradually over time in spite of adding a weekly dose of PaExoA (Paper I, Figs. 4 & 5).

2. Thickness of RWM following glucocorticoid exposure (Paper V)
Membranes exposed to ten doses of hydrocortisone had the highest mean value. The mean values of all groups, with standard deviations are presented in Fig. 4 (Paper V).

3. Thickness of RWM following saline exposure
These membranes showed a slight thickening (Fig. 1, S1 7d and S4 7d), when compared with unexposed RWM (Fig. 1, U1 7d and U4 7d).
Morphology of ES following PaExoA exposure of middle ear (Paper II)

Serial sections of ES were prepared from 32 of 40 rats. In 18 animals (56%) the ES showed LM changes, compared with the control ear suggesting an increased activity. The morphological results are presented in Tables 1 & 2, (Paper II) together with the ABR difference means. For a description of morphological changes, we studied: number of free-floating cells (macrophages), thickness of epithelia and proliferation, densification of the homogenous substance (HS), and numbers of perisaccular leukocytes.

Morphology of ES following PaExoA exposure of middle ear (Paper II)

Serial sections of ES were prepared from 32 of 40 rats. In 18 animals (56%) the ES showed LM changes, compared with the control ear suggesting an increased activity. The morphological results are presented in Tables 1 & 2, (Paper II) together with the ABR difference means. For a description of morphological changes, we studied: number of free-floating cells (macrophages), thickness of epithelia and proliferation, densification of the homogenous substance (HS), and numbers of perisaccular leukocytes.
TEM of the epithelium showed the dark cells veiling over the light cell. The dark cells displayed abundant rough endoplasmatic reticulum. Macrophages in the ES lumen sometimes formed cell clusters containing abundant vesicles.

Glutamate in the human inner ear (Paper III)

Immunoreactivity to L-glutamate, the receptor subunit NR2B and glutamine synthetase, varied considerably in different parts of organ of Corti. Staining for L-glutamate was intense close to the IHC base and OHCs (Paper III, Fig. 2). NR2B was visualized in the basal part of the organ of Corti, especially adjacent to OHCs. Glutamine synthetase was found primarily in the basal part the OHC area, possibly in the cytoplasm.

In the spiral ganglion (SG). L-glutamate immunofluorescence appeared as individual dots within the ganglion cells (Paper III, Fig. 3). Staining for NR2B occurred preferentially at one end of the ganglion cells (Paper III, Fig. 4). Glutamine synthetase was identified in the SG cells as numerous intensely stained dots. All control specimens proved negative.
Discussion

PaExoA exposure in the middle ear
In our animal model of CSOM we studied the effect of PaExoA on the RWM and the ES. As the hearing level of the animals was determined at different times, we could compare the morphology of the RWM and ES with that of the HL, thus increasing our knowledge of CSOM as a source of inner ear impairment.

Hearing after PaExoA exposure (Paper I)
The profound effect on hearing of a single dose of PaExoA and the lack of additive effect of multiple toxin applications were the most unexpected findings. Some animals suffered an irreversible HL, others only a partially reversible HL. In contrast to Stenqvist et al. [7] we found a greater variation in the threshold shift levels after exposure to only one PaExoA dose (1µg/20µl). Anatomical variations in the round window niche or varying inner ear sensitivity may explain these variations [30].

The sensorineural component of HL was identified by calculating the mean of the latency/intensity values [7]. The most severe HL was seen after 7 days. Even if this was partly attributable to OM, it was also an effect of inner ear impairment as the latency/intensity curve showed a steeper slope. Hearing improved at 14 and 21 days, probably by healing of the OM and the reversibility of the cochlear impairment. The change in threshold shifts was more often significant at high frequencies. The short distance from the point of application of toxin to the high frequency area in the basal turn of the cochlea may explain this finding. These results are consistent with those of Lundman et al. [6].

In Group 2 (multiple exposures) the HL changes over time showed, surprisingly, a tendency to diminish in spite of repeated toxin applications. This diminishing effect of the instilled doses of PaExoA can perhaps be attributed to the increasing impermeability of the RWM, or to an increasing inner ear resistance to the toxin.

RWM morphology after PaExoA exposure (Paper I)
The epithelium facing the middle ear side of the RWM was the first layer to react and the last to normalize, whereas the onset of reaction in the middle layer lagged somewhat and normalized more rapidly. Inflammatory changes were about the same following both single and multiple exposures, except for membranes exposed to one dose followed by 2 weeks of
survival, and in the membranes exposed to five PaExoA doses. The milder inflammatory reaction might be explained by an anatomical hinder preventing the toxin from reaching the RWM [30]. In our study we did not identify many inflammatory cells, as polymorphonucleated leukocytes and macrophages, in contrast to Goycoolea’s description [12]. The disparity might be due to differences in determining the borders of the RWM, or to different species and procedures.

**RWM thickness after PaExoA exposure (Paper I)**

As RWM from animals with 7 and 21 days of survival had a mean thickness similar that in animals exposed to two and three doses, it is conceivable that several toxin doses in animals with 14 days of survival failed to penetrate the RWM. In animals given two exposures we found a membrane thickness of 22 µm (mean) or twice the control thickness, i.e. very similar to the thickness in animals given one exposure and 21 days of survival. This finding might indicate the actual reaction time for the membrane to reach the maximum thickness. In groups given 3-5 doses, the membranes thinned in spite of the repeated exposures. The most likely explanation for this sloping thickness is resorption of the acute inflammatory edema in the membrane. The cause in the groups with 4 – 5 applications of PaExoA is not known but could be a ‘vaccination’ rendering the animal susceptible to subsequent instillations or infections. In the human CSOM, the RWM is known to remain persistently thickened, with little inner ear disturbance.

**RWM morphology and thickness after saline exposure**

The saline exposed membranes showed a slight thickening, compared with the unexposed control membranes in Paper I. Saline exposure resulted in a remarkable thickening in Paper V. Morphological changes were not found more often in the saline exposed membranes than in unexposed ears. Vehicle reaction in the RWM was also reported by Fauser et al. [31] and the question arises: does the RWM react to any vehicle? We have discussed the choice of control group and concluded that it might be necessary in future studies to use both unexposed and vehicle controls, as the middle ear is normally an air-filled cavity and exposure to saline or any other vehicle is unnatural.

**Endolymphatic sac morphology after PaExoA exposure (Paper II)**

This is the first study that showed signs of inflammatory reaction in the ES after toxin instillation in the middle ear. Our findings, such as increased numbers of macrophages and the
epithelial reaction, might be an end-result of an immune reaction to toxin application in the middle ear. The swift of ES response [32] and the fact that ES can react to a challenge in scala tympani within hours indicate that the ES may react within hours or days to middle ear exposure to toxin. This study therefore needs to be followed up by others with shorter observation period to reveal the course of the ES reaction.

This new observation lends further support concerning the immunological role of the ES in the inner ear. As animals with ES response after toxin exposure more often suffered from HL than animals without such signs, a destructive effect of the response was suspected. These results should lead us to consider a more active immuno-suppressive therapy when the inner ear is threatened.

Glutamate in human cochlea (Paper III)
The study on glutamate in the human cochlea identified L-glutamate, an NMDA receptor, and glutamine synthetase in synaptic regions in rather the same way as described in many other animal species [19, 33]. It can be assumed that glutamate acts as an afferent neurotransmitter in the human cochlea. The less intense staining of glutamine synthetase in relation to the IHC than OHC can be attributed to the small quantity of the enzyme. However, a distribution similar to that in previously tested species seems the most likely explanation, because the enzyme is closely involved in the recycling of glutamate. Species differences have been described, for instance concerning glutamine synthetase [34]. The distribution of glutamate in other mammals may result in a more intense staining for glutamate at the base of the IHCs [18], though this has not been found in the gerbil cochlea (16), or in our human material.

An understanding of glutamate receptors in the human cochlea may be a prerequisite for the treatment of disorders related to excitotoxicity [35, 36]. Clinically tolerated and effective glutamate release blockers acting at the hair cells, may radically modify therapeutic concepts in otology in the near future [37]. As glutamate has now been found in the human cochlea, it is conceivable that the treatment for conditions resulting from glutamate dysfunction in the inner ear could be made available.

Hearing after L-NAME as otoprotectant from gentamicin ototoxicity (Paper IV)
In the study on the role of the NO-inhibitor L-NAME as an otoprotectant from gentamicin induced HL, we found indications of a beneficial effect in the higher frequencies. L-NAME did not prevent HL, but an increasing difference in ABR threshold shifts between animals
given gentamicin only and those treated with L-NAME indicated that HL healed more quickly in the treated animals. The causes of the differing effects in lower vs. higher frequencies could be the differing distance from the RWM of the frequency-specific areas in the cochlea, a higher rate of metabolism [24], or richer efferent innervation of the OHC [26] in certain parts of the cochlea.

In a previous study [38] L-NAME was shown to be effective against PaExoA-induced HL. The reason why L-NAME was less effective as a protector from gentamicin ototoxicity might be differences in the ototoxic mechanism. L-NAME was even shown to protect the cochlea from gentamicin ototoxicity in an ‘in-vitro’ model [39]. In our ‘in-vivo’ model, the pharmacokinetics of L-NAME and the aminoglycoside are considerably more complex. The timing of the L-NAME instillation and the ototoxic effect of gentamicin are problematical as L-NAME must pass both the RWM and several inner ear structures before entering ‘the target area’.

The limited otoprotective effect of L-NAME, found in this study, might be improved by combining the agent with other drugs [40]. Access of the drug to the RWM could even be ensured by using a microcatheter [41].

**RWM morphology after glucocorticoid exposure (Paper V)**

The most conspicuous finding in the study on exposure of the RWM to glucocorticoid was an accumulation of inflammatory cells close to membranes exposed to hydrocortisone, whereas there were no such signs of inflammation among the dexamethasone exposed membranes. The inflammatory cells, identified as macrophages and polymorphonuclear leukocytes, were the most common finding. The microfilament-like structures found in many of the vesicles of the macrophages need to be identified in greater detail in order to increase our understanding of the RWM’s reaction to hydrocortisone. It is possible that the accumulation of inflammatory cells is merely a sign of raised immunological alertness, as no inflammatory signs were seen in the rest of the middle ear. Local application of glucocorticoids has previously been found to cause morphological changes in the RWM [31, 42]. We therefore suggest care when selecting hydrocortisone for treatment of the middle or inner ear.

**RWM thickness after glucocorticoid exposure (Paper V)**

The hydrocortisone exposed membranes were thicker than both dexamethasone exposed membranes and unexposed controls. This thickening of the RWM may reflect an injurious effect of hydrocortisone. It was also remarkable to find the saline exposed membranes thicker
than the dexamethasone exposed membranes and scarcely any difference between
dexamethasone and unexposed membranes. The difference between five or ten exposures to
hydrocortisone might indicate some effect of repeated hydrocortisone application.

Our explanation for the finding that even saline in the middle ear induces RWM
thickening is that any topical application probably initiates a certain thickening and that
hydrocortisone, being a less potent glucocorticoid, is unable to reduce the thickness of the
membrane. Another possible mechanism of potent glucocorticoids may be a disturbed
collagen synthesis. Osmosis might also have some effect but it is difficult to understand how
such an effect can be sustained 2 weeks after the last exposure. The frequent finding of
inflammatory cells in the hydrocortisone exposed membranes still suggests hydrocortisone to
be a less favourable drug than dexamethasone.

The most important question is whether treatment of the RWM with
hydrocortisone increases permeability. Some studies have found decreased permeability of the
membrane when it is thickened by fibrosis. The increased thickness might instead express
edema and membrane leakage [13]. The fact that hydrocortisone was previously found to
affect the cochlea electrophysiologically [43] indicates that RWM permeability might be
increased by hydrocortisone exposure.
General discussion

The round window membrane – a gateway for local therapy of the inner ear

The importance of the RWM in local inner ear therapy is highlighted in this study. The RWM is accessible in mammals and has been used for several years as a gateway for the treatment of vertigo in Ménière’s disease. Intratympanic steroid treatment has also been tested both experimentally [44] and in Man [45]. This topical technique for inner ear treatment will probably develop for other drugs in the coming years and with higher accuracy of application as devices for local application are developed [41]. Various drug groups may prove efficacious for topical inner ear therapy.

Glutamate antagonists

The identification of glutamate in the human inner ear opens up possibilities for further attempts to use glutamate antagonists such as Memantine [37] for the treatment of inner ear disorders caused by glutamate hyperstimulation.

Aminoglycosides and NO-inhibitors

Aminoglycosides are widely used to treat the vertigo in Meniere’s disease. When instilled locally into the middle ear the patient benefits from the ototoxic effect. Aminoglycosides
induce free radical formation, using the common pathway for inner ear disorders [24] leading to apoptosis and cell death.

Several substances have the capacity to block the formation of free radicals [46, 47]. L-NAME is an interesting substance believed to be an irreversible inhibitor of cNOS and a reversible inhibitor of iNOS. Such NO-inhibitors may hopefully prove useful in forthcoming attempts to treat inner ear disorders.

**Immunotherapy (glucocorticoids)**

Immune reaction has been proposed as the mechanism for certain inner ear diseases [48], leading to several attempts to treat instant sudden deafness with immunotherapy. Steroid hormones in particular, both systemically and locally, have been tried [43, 44, 49, 50, 51]. The identification of glucocorticoid receptors in the cochlea [52, 53], has raised hopes of successfully using glucocorticoids to treat inner ear diseases.

Various Glucocorticoids may have varying effects on the RWM. In Paper V we investigated and compared the morphological effect on the RWM of two glucocorticoids already in use as components of ear-drops. Our findings indicated that dexamethasone would be a safer drug for inner ear treatment than hydrocortisone.

**Clinical implications**

The importance of the RWM as the main gateway for local therapy of inner ear diseases seems obvious. The permeability and morphological characteristics of the RWM can be changed under the influence of toxins and drugs; even saline can induce thickening. The effect of glucocorticoids on the membrane illustrates some of the problems of local treatment of the inner ear. We have found signs of response in the ES to bacterial toxin instilled into the middle ear, indicating the importance of the organ even in middle ear inflammation. This fact indicates that the cascade of events in inner ear impairment probably includes ES response—and the possibility of using immunotherapy to diminish the damage caused by this response. Blocking the cascade of inner ear impairment at its different stages has become possible since glutamate was found in the human inner ear and opens up possibilities for further studies on inner ear therapy. The ultimate goal must be to offer effective therapy to patients suffering from inner ear diseases.
General conclusions

Paper I: Exotoxin induces inflammatory changes in the round window membrane, including thickening, and leading to either reversible or permanent hearing loss. Repeated exposure to exotoxin increases the thickness of the membrane and the profoundness of the hearing loss only to a limited extent.

Paper II: The endolymphatic sac reacts to middle ear exotoxin instillation by displaying signs of inflammatory activity.

Paper III: As L-glutamate, a glutamate receptor, and glutamine synthetase is found in the human inner ear: Glutamate is the afferent neurotransmitter in the human cochlea.

Paper IV: L-NAME offers a potential protection from high-frequency hearing loss caused by gentamicin ototoxicity.

Paper V: Hydrocortisone (but not dexamethasone) initiates inflammation in the round window membrane and disfavour hydrocortisone as a drug for inner ear therapy.
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