Hearing Research 350 (2017) 100–109

Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares

Research Paper

Streptococcus pneumoniae-induced ototoxicity in organ of Corti explant cultures

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ARTICLE INFO

Article history:
Received 29 November 2016
Received in revised form
11 April 2017
Accepted 23 April 2017
Available online 25 April 2017

Keywords:
Organ of Corti
Hair cells
Ototoxicity
Streptococcus pneumoniae
Pneumolysin

ABSTRACT

Hearing loss remains the most common long-term complication of pneumococcal meningitis (PM) reported in up to 30% of survivors. *Streptococcus pneumoniae* have been shown to possess different ototoxic properties. Here we present a novel *ex vivo* experimental setup to examine in detail the pattern of hair cell loss upon exposure to different *S. pneumoniae* strains, therefore recapitulating pathogen derived aspects of PM-induced hearing loss. Our results show a higher susceptibility towards *S. pneumoniae*-induced cochlear damage for outer hair cells (OHC) compared to inner hair cells (IHC), which is consistent with *in vivo* data. *S. pneumoniae*-induced hair cell loss was both time and dose-dependent. Moreover, we have found significant differences in the level of cell damage between tissue from the basal and the apical turns. This shows that the higher vulnerability of hair cells located at high frequency regions observed *in vivo* cannot be explained solely by the spatial organisation and bacterial infiltration from the basal portion of the cochlea. Using a wild type D39 strain and a mutant defective for the pneumolysin (PLY) gene, we also have shown that the toxin PLY is an important factor involved in ototoxic damages. The obtained results indicate that PLY can cause both IHC and OHC loss. Finally, we are reporting here for the first time a higher vulnerability of HC located at the basal and middle cochlear region to pneumolysin-induced damage. The detailed description of the susceptibility of hair cells to *Streptococcus pneumoniae* provided in this report can in the future determine the choice and the development of novel otoprotective therapies during pneumococcal meningitis.

1. Introduction

Sensorineural hearing loss is the most common long-term neurological deficit after bacterial meningitis (BM) with an incidence of up to 30% when caused by *Streptococcus pneumoniae* (Chandran et al., 2011; Karppinen et al., 2015; van de Beek et al., 2004). The peripheral sensory cells responsible for hearing, namely the hair cells (HCs) and the spiral ganglion neurons (SGNs), were shown to be affected in experimental studies (Coimbra et al., 2007; Klein et al., 2003; Meli et al., 2006; Perny et al., 2016) and clinical cases (Chandran et al., 2011; Karppinen et al., 2015; Merchant and Gopen, 1996; Rappaport et al., 1999; van de Beek et al., 2004) of BM. Histological damages were observed predominantly in outer HCs, inner HCs, supporting cells and SGNs. Infiltrating bacteria and inflammatory cells were mainly found in the scala tympani, having no direct contact to HCs (Klein et al., 2003, 2007; Møller et al., 2014; Osborne et al., 1995; Perny et al., 2016). A prominent inflammatory reaction is triggered during the acute phase of disease, releasing potentially ototoxic molecules, including reactive oxygen, nitrogen species and cytokines (Coutinho et al., 2013; Klein et al., 2007; Wu et al., 2015). Especially tumor necrosis factor-α (TNF- α), a pro-inflammatory cytokine, has been shown

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http://dx.doi.org/10.1016/j.heares.2017.04.012

to be directly toxic for hair cells in organ of Corti (OC) explant cultures (Wu et al., 2015). Additionally, cerebrospinal fluid concentration of TNF-α positively correlated with the severity of long-term hearing deficits after BM in an infant rat model (Perny et al., 2016). The bacterial exotoxin pneumolysin, either actively secreted or released after bacteriolytic antibiotic therapy, has been shown to be a major virulence factor responsible for postmenigitic hearing loss (Skinner et al., 2009; Winter et al., 1997). *In vitro* studies have shown that it preferentially targets inner HCs, leading to calcium-dependent apoptotic cell death (Beurg et al., 2005). Furthermore, pneumococci produce a variety of protein and non-protein toxins also implicated in disease pathologies (Barnett et al., 2015).

Our knowledge about pneumococcal ototoxicity is mostly based on clinical postmortem studies and experimental animal studies. To date, no *in vitro* system to study pathological mechanisms of BM-induced hearing loss in a controllable setup has been reported. Here we modified a previously described hippocampal slice culture system (Gianinazzi et al., 2004) on transwell inserts to culture explants of the organ of Corti. This novel *ex vivo* co-culture system allows to study the pathogenic effect of live bacteria and their secreted toxins on cochlear HCs. We analyzed the ototoxicity of different pneumococcal species co-cultivated with whole mounts of the organ of Corti, and quantified hair cell survival upon exposure to bacteria. *Ex vivo,* we could confirm the predominant vulnerability of OHCs towards pneumococci previously observed *in vivo* (Perny et al., 2016). Additionally, we characterized the contribution of PLY to the hair cell toxicity, either indirectly by using a PLY-deficient strain or directly by applying the purified toxin.

The present *ex vivo* system is a new platform to study the pathological mechanisms of BM-associated hair cell loss and to identify and develop otoprotective and/or otoregenerative compounds.

2. Materials and methods

2.1. Organ of corti organotypic cultures

All animal studies were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland (license BE 124/13 to S.L.L) and followed the Swiss national guidelines for the performance of animal experiments.

Cochlear explants were isolated from 2 to 4 days old Wistar rats. Rats were decapitated and the heads were cut sagittaly to remove the brains. The two otic capsules were isolated and transferred into ice-cold Hank's balanced salt solution (HBSS) (Invitrogen, USA) for sterile dissection under a binocular microscope (Nikon SMZ800, Japan) with forceps (World Precision Instruments, USA). After bone removal, the cochlea was transferred to a Transwell-Clear insert (6-well format, Corning, USA) with a permeable polyester membrane (0.4 μm pore size). The membranes were pre-coated with Celltak (Corning, USA) according to manufacturer's protocol. The organ of Corti (OC) was then separated from stria vascularis and the modiolus and plated on the insert, with the hair cells facing up. The explants were randomized and distributed between treatment groups. Dissection medium was carefully removed and 1.5 ml otic culture medium: (DMEM/F12 (Invitrogen, USA), 0.01% Ampicillin (Sigma, USA) and 10% fetal bovine serum (Invitrogen, USA)) was added to the lower compartment under the insert membrane. This volume generates a thin film over the OC explant and allows for a medium-air interface culture. The explants were cultured for 24 h at 37 °C and 5% CO_2 before any treatment.

2.2. Bacteria culture

A clinical isolate of *Streptococcus pneumoniae* serotype 3 (S3) from a patient with bacterial meningitis, adapted to the animal model by serial passaging, was used for a subset of the experiments (Leib et al., 2000). *S. pneumoniae* S3 were cultured overnight (16–17 h) at 37 °C and 5% CO_2 in brain heart infusion medium (BHI). On the following day, culture broth was diluted in fresh medium (1:10) and bacteria were grown for 5 h to late-logarithmic phase. Bacterial suspensions were then pelleted by centrifugation for 10 min at 3′100 rpm (4 °C), and resuspended in 10 ml of NaCl 0.85% followed by a second centrifugation for 10 min at 3′100 rpm (4 °C). Finally, bacteria were re-suspended in ampicillin- and FBS- free otic medium to the desired optical density, which resulted in a final

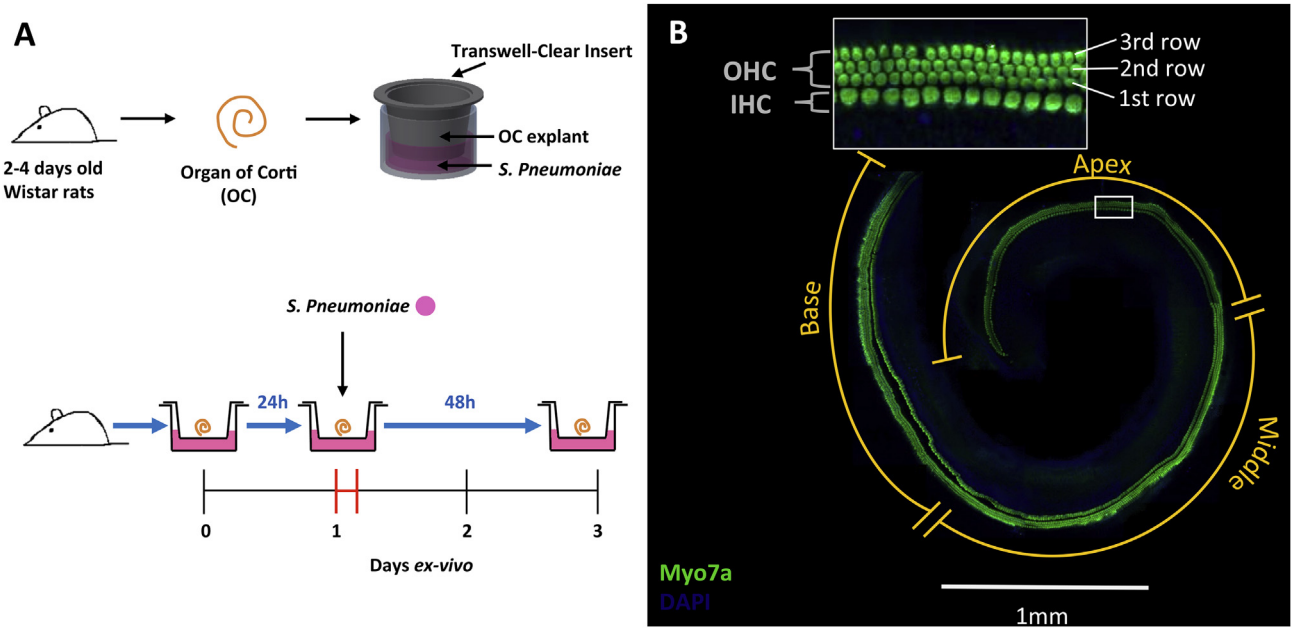


Fig. 1. Experimental setup. (A) Experimental model to assess the ototoxicity of *S. Pneumoniae* species *ex-vivo*. (B) Representative immunostaining of the organ of Corti whole-mount explant, distinguishing basal, middle and apical region. Myosin VIIa expressing inner (IHC) and outer hair cells (OHCs) are shown in green, cell nuclei in blue (DAPI). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

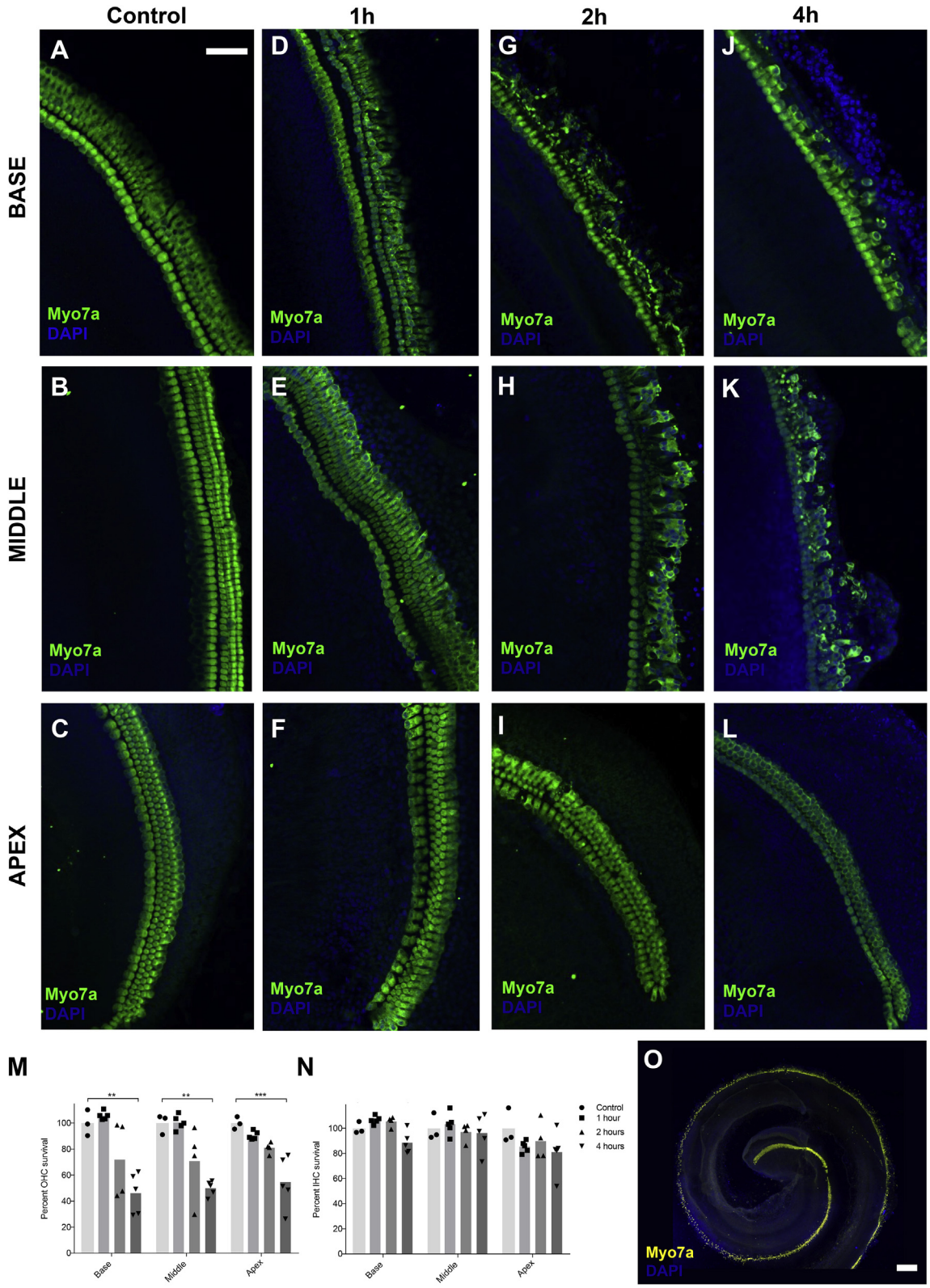


Fig. 2. *S. pneumoniae*-induced hair cell damage in cochlear explants. (A–L) Immunofluorescence stainings of whole-mount OC explants (basal, middle and apical region) of control cultures and after exposure to *S. pneumoniae* S3 for 1, 2 or 4 h, labelled with Myo7a (green) and DAPI (blue) Scale bar: 25 μm. (M, N) Quantitative analysis of inner and outer hair cells survival. The mean is indicated in the bar graph. N = 3–5 cochleas per group. (O) Immunofluorescence staining of the entire whole-mount OC explant exposed to 4×10^8 cfu/ml of *S. pneumoniae*, labelled with Myo7a (yellow) and DAPI (blue). Scale bar: 100 μm. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests to analyse differences between the controls and treated samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration of 1×10^8 colony-forming units (cfu) ml^{-1}. The accuracy was always confirmed by quantitative cultures on columbia sheep blood agar (CSBA) plates.

Bacterial preparation for *S. pneumoniae* D39 wild type (serotype 2) and a D39 strain containing a deletion affecting the pneumolysin gene and affecting both its cytolytic and complement activities (D39ΔPLY) (Berry et al., 1999) was performed analogous to the strain p21 (see above) with some modifications. Overnight cultivation was shortened to 8 h and bacteria were grown for 1.5 h to late-logarithmic phase. Re-suspension in ampicillin- and FBS- free otic medium to the desired optical density was performed to obtain the final concentrations: 1×10^7, 1×10^8, 4×10^8 or 2×10^9 cfu ml^{-1}. The accuracy was again confirmed by quantitative cultures on CSBA plates.

2.3. Recombinant pneumolysin

Recombinant pneumolysin (PLY, a kind gift of Prof. Dr. Annette Dräger, University of Bern, Institute of Anatomy, Bern, Switzerland), was prepared as previously described (Wolfmeier et al., 2016). Toxin was kept frozen at −80 °C until use. For experiments, PLY was mixed with otic medium (DMEM/F12 (Invitrogen, USA) and 0.01% Ampicillin (Sigma, USA)) (without FBS) at final concentrations of 5–10 ng/μl, depending on the experiment.

2.4. Culture treatments

After sterile dissection and an initial incubation for 24 h, OC explants were exposed for 1, 2, 4 or 24 h to ototoxic insults. Ampicillin- and FBS-free otic medium with bacteria or FBS-free medium with pneumolysin was added under the membrane to the lower compartment of the cultivation system. After the treatment, cultures were washed 3 times with PBS and cultivated for additional 48 h in fresh full otic medium. Bacterial titers were determined with quantitative cultures on Columbia Sheep Blood Agar (CSBA) plates, by serial dilutions. Control samples exposed to ampicillin- and FBS-free otic medium were run simultaneously with the experimental samples.

2.5. Immunofluorescence microscopy

At the end of the experiment, otic medium was removed from the cultures and OC explants were washed once with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. After fixation, explants were transferred (by cutting the insert membrane) to a 24 well plate, washed three times with PBS and permeabilized with 3% Triton-X 100 for 30 min at room temperature. OC tissues were immersed in a blocking buffer containing 2% bovine serum albumin (BSA) and 0.01% Triton-X 100 for 1 h at room

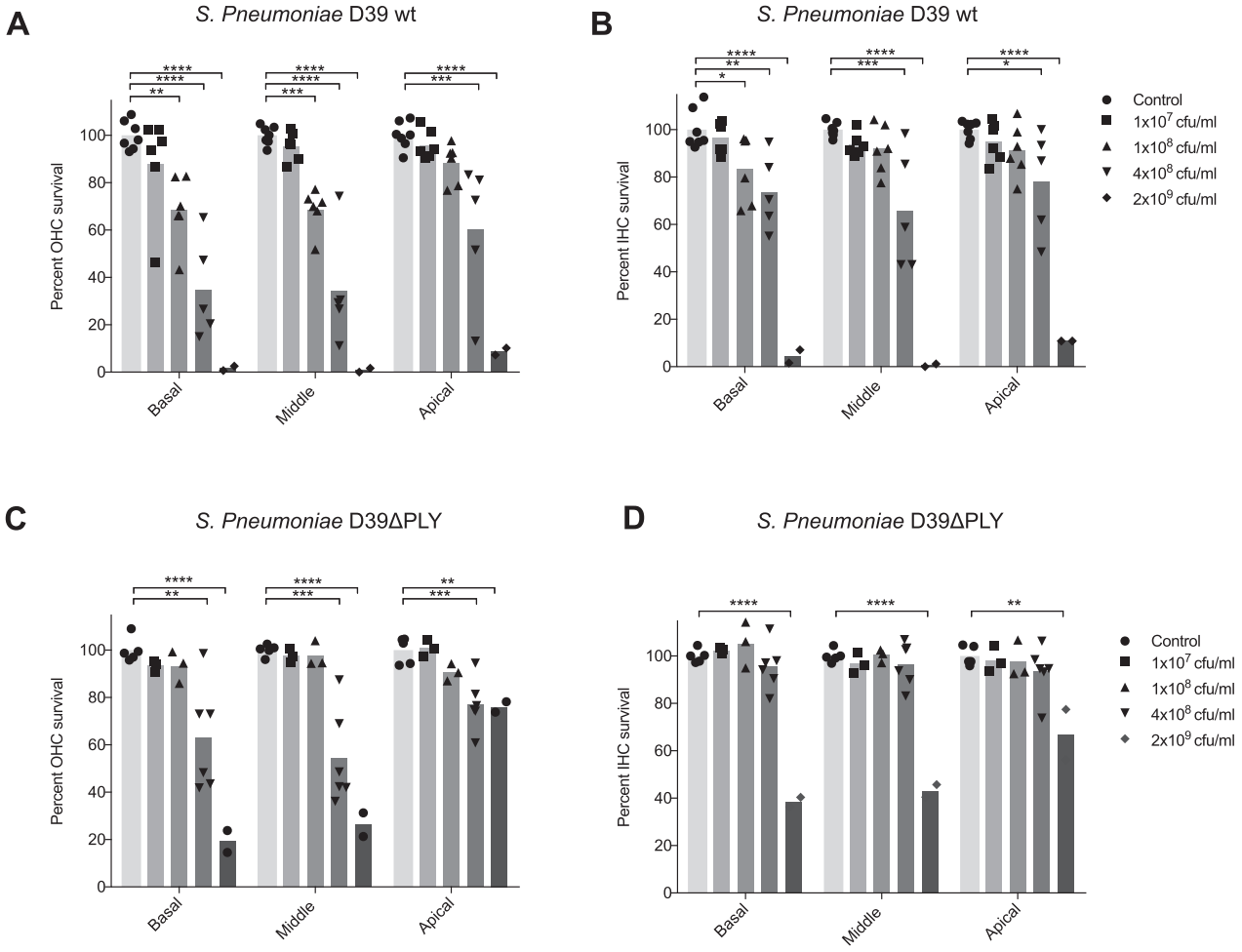
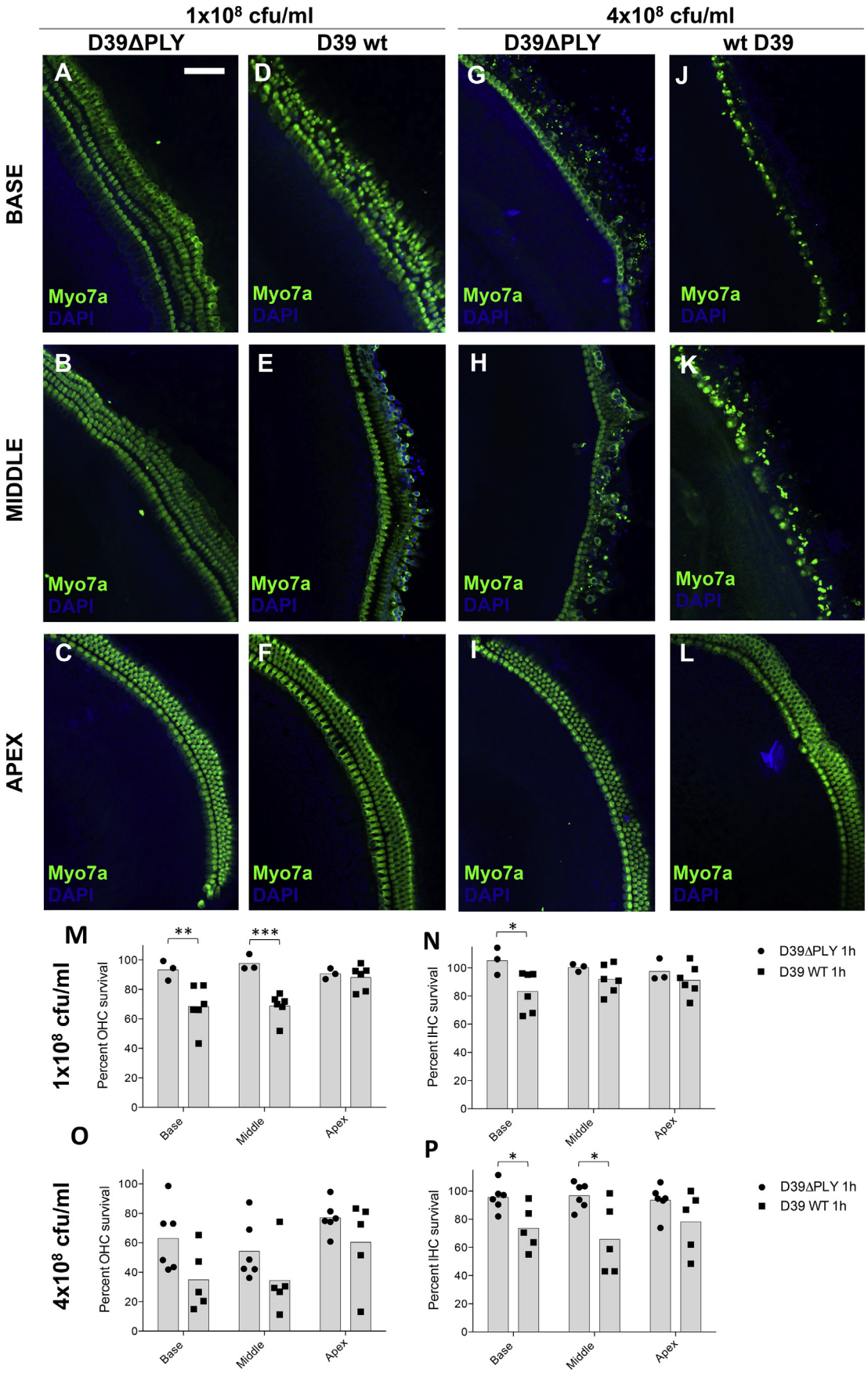


Fig. 3. Ototoxic damages in cochlear explants exposed to *S. pneumoniae* D39 and its pneumolysin-deficient strain (D39ΔPLY). Quantification of hair cell loss at different cochlear regions of control cultures and after exposure to 4 different concentrations of *S. Pneumoniae* D39 (A and B) and D39ΔPLY (C and D) (1×10^7, 1×10^8, 4×10^8, 2×10^9 cfu/ml). The mean is indicated in the bar graph. N = 3–6 cochleas per group. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests to analyse differences between the controls and treated samples.


1x10⁸ cfu/ml
4x10⁸ cfu/ml
D39ΔPLY
D39 wt
D39ΔPLY
wt D39
BASE
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Myo7a
DAPI
M
N
O
P
1x10⁸ cfu/ml
4x10⁸ cfu/ml
Percent OHC survival
Percent IHC survival
Base
Middle
Apex
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*
D39ΔPLY 1h
D39 WT 1h

temperature. Explants were incubated with the anti-MyoVIIa (1:500, rabbit; Proteus, USA) antibody in blocking buffer overnight at 4 °C. On the following day, tissues were rinsed three times with PBS and incubated with the secondary antibody anti-rabbit Alexa Fluor 488 or 555 (1:500; Invitrogen, USA) in blocking buffer for 2 h at room temperature. Explants were again washed 3 times with PBS and stained for 30 min with phalloidin (1:50; Sigma, USA) diluted in blocking buffer solution. Finally, samples were rinsed three times with PBS and mounted on a glass slide with Fluoroshield containing DAPI (Sigma, USA).

The labelled cells were visualized with a confocal laser-scanning microscope (Zeiss LSM710) equipped with a CCD camera (Leica Microsystems) with a Plan-apochromat 20×/0.8 NA objective. Several z-planes were imaged to ensure visualization of all hair cells in each picture.

2.6. Data analysis/hair cells quantification

Confocal images were processed and analyzed using ImageJ. The entire OC whole mount was imaged with 16–20 confocal pictures to cover the entire length of the basilar membrane. This was divided into 3 groups (base, middle and apex) (5–6 pictures each). The number of surviving hair cells was assessed for each region of the explants. A semi-automatic ImageJ quantification plugin was developed, to facilitate and automate the evaluation of our ototoxicity screens. The number of surviving hair cells (IHCs and OHCs) was divided by the length of the basilar membrane. The control samples were cultured for 4 h (longest time point) for the S. pneumoniae serotype 3 experiments and for 1 h for *S. pneumonia* D39 and D39ΔPLY under exactly the same conditions but without the corresponding bacteria. Hair cell survival rates were calculated relative to the mean value of the respective control cultures.

2.7. Statistical analysis

All obtained data were processed and analyzed using GraphPad Prism software version 5.0 (GraphPad Software, USA). HC survival rates are presented in the figures as individual data points for each cochlea (standard deviations are mentioned in the text) and the mean value (bar graph). The number of experimental replicates (N = 2–6 cochleas, depending on the experiments) is described in the corresponding figure legend. Nonparametric Spearman test was used for the correlation analysis. One-way analysis of variance (ANOVA) was conducted to test for differences in inner and/or outer hair cells survival across time of bacterial exposure (Fig. 2), bacterial concentrations (Fig. 3), cochlear regions (Base, Middle, Apex; Fig. 5) and pneumolysin concentrations (Fig. 5). Following significant main effects, *post hoc* Dunnett's multiple comparison tests were performed to look for differences in inner and outer hair cells survival between treated and control cultures or Tukey's multiple comparison tests to assess statistical differences across cochlear regions. Where appropriate, two experimental groups were compared using an unpaired *t*-test with Welch's correction (Fig. 4).

3. Results

3.1. S.pneumoniae serotype 3 induced hair cell loss

In order to study the toxic effect of *S. pneumoniae* on sensory hair cells, we analyzed hair cell density and morphological integrity after exposing organ of Corti explants indirectly to bacteria as schematized in Fig. 1. The variables assessed were time of exposure and bacteria concentration. The damage was quantified for basal, middle and apical turns (as illustrated in Fig. 1 B), and the effect on OHC or IHC was assessed by immunostaining the whole mounts at the end of the experiment for the hair cell marker Myo7a. Representative pictures of OC explants exposed to *S. pneumoniae* for different durations are shown in Fig. 2 A–L. The bacterial concentration (1×10^8 cfu/ml) was chosen based on our *in vivo* studies, representing the peak concentration during the acute phase of disease when antibiotic therapy was initiated (Perny et al., 2016). A one-way ANOVA revealed a significant effect of the time of bacterial exposure on survival of outer ($p < 0.001$) but not inner hair cells (Fig. 2 M and N). Dunnett's multiple comparison *post hoc* test showed that there was a significantly lower survival of OHCs after 4 h in the basal (46.1% ± 13.9 of survival, $p < 0.01$), middle (49.9% ± 5.1 $p < 0.01$) and apical turn (54.61% ± 17.7, $p < 0.001$) compared to the corresponding cochlear regions in control cultures. Hair cell damage was already observed after 2 h of bacterial exposure (72% ± 26.0 of OHC survival at the base; in the middle turn 70.8% ± 24.9 and at the apex 80.9% ± 3.8), but without reaching statistical significance. A significant inverse correlation was found between the time of exposure and the number of OHCs, which survived the treatment (Spearman: basal turn: $r = -0.8177$, $p = 0.001$; middle turn: $r = -0.8177$, $p = 0.0001$, apical turn: $r = -0.9225$, $p < 0.0001$). Hair cells located at the most apical region were never damaged, even after 4 h of treatment (Fig. 2 C, F, I, L, O). However, no significant difference was found between the three different cochlear regions (Fig. 2 M and N) because only the most apical cells showed increased survival (≈20% of hair cells in the apical turn).

In contrast IHCs were not significantly damaged at all time points (2 h: base: 105.4% ± 3.5 of survival; middle: 97.1% ± 6.4 of survival; apex: 89.8% ± 13.2 of survival; 4 h: base: 88.6% ± 7.9 of survival; middle: 96.3% ± 13.3 of survival; apex: 81.1% ± 15.6 of survival) (Fig. 1 G–L, M and N).

Our data reveal a higher susceptibility of OHCs to *S. pneumoniae,* that was time-dependent, compared to the highly resistant IHCs.

3.2. Reduced virulence of the D39 pneumolysin-deficient mutant of Streptococcus pneumoniae

To evaluate the contribution of the bacterial toxin pneumolysin, released by the bacteria, to the ototoxic damages observed, we compared HC loss in OC explants exposed for 1 h to four different concentrations (1×10^7, 1×10^8, 4×10^8, 2×10^9 cfu/ml) of *S. pneumoniae* D39 and its pneumolysin-deficient mutant (D39ΔPLY). All data collected from experiments performed with the PLY-producing D39 wt strain and the D39ΔPLY is summarised in Fig. 3 (A–D) and supplementary Table 1.

A one-way ANOVA revealed a significant effect of the S. pneumoniae D39 wt concentration on the survival of inner ($p < 0.001$ in basal, middle and apical region) and outer ($p < 0.001$ in basal, middle and apical region) hair cells in all cochlear regions. Dunnett's multiple comparison *post hoc* tests showed significant differences in inner and outer hair cell survival between explants treated with 1×10^8, 4×10^8, 2×10^9 cfu/ml *S. Pneumoniae* D39 wt compared to control cultures, which was dependent on the

Fig. 4. Side-by-Side comparison of hair cell damage upon exposure to *S. pneumoniae* D39 and its pneumolysin-deficient strain (D39ΔPLY). (A–L) Representative immunofluorescence stainings of the whole-mount OC explants (basal, middle and apical region) after exposure to 2 different concentrations (1×10^8 cfu/ml and 4×10^8 cfu/ml) of S. pneumoniae D39 and D39ΔPLY, labelled with Myo7a (green) and DAPI (blue). Scale bar: 25 μm. (M–P) Quantitative comparison of HC survival rates in cochlear explants exposed to 1×10^8 cfu/ml and 4×10^8 cfu/ml of D39 and D39ΔPLY. The mean is indicated in the bar graph. N = 3–6 cochleas per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ unpaired *t*-test with Welch's correction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

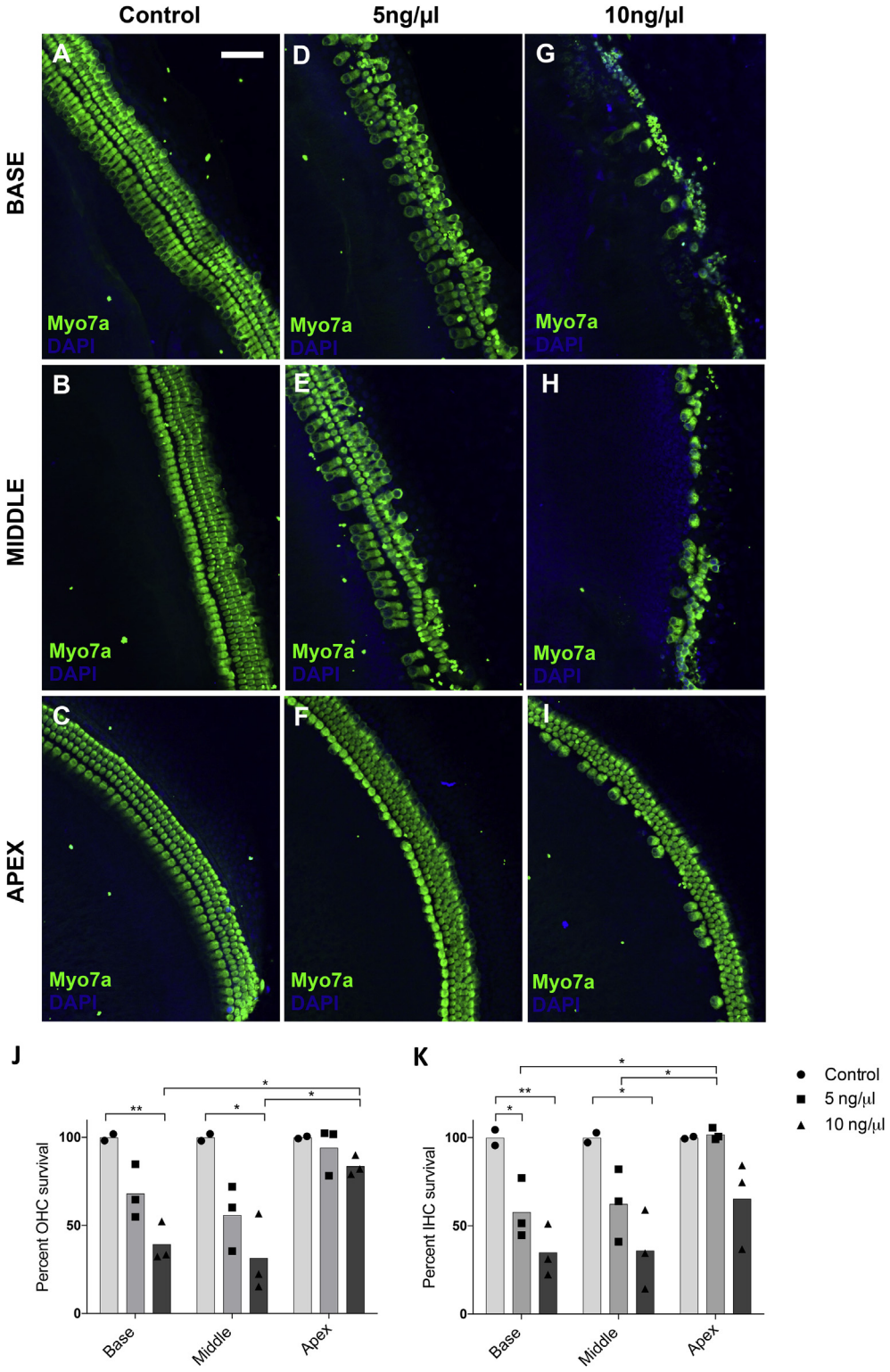

Fig. 5. Pneumolysin-induced damage in cochlear explants. (A–I) Representative Immunofluorescence stainings of the whole-mount OC explants (basal, middle and apical region) of control cultures and after exposure to two different concentrations of pneumolysin (5 ng/µl and 10 ng/µl), labelled with Myo7a (green) and DAPI (blue). (J–K) Quantification of HC survival rates in cochlear explants challenged with PLY (5 ng/µl, 10 ng/µl). The mean is indicated in the bar graph. N = 2–3 cochleas per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0. one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests to analyse differences between the controls and treated samples. Tukey's multiple comparison *post hoc* tests were performed to assess differences across the cochlear regions (Base, Middle, Apex). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cochlear region. The lowest concentration (1×10^7 cfu/ml) didn't induce statistically significant hair cell damage. A significant inverse correlation was found between hair cell survival and bacteria concentration for both outer (Spearman: basal turn: $r = -0.8422$, $p < 0.0001$; middle turn: $r = -0.9090$, $p < 0.0001$, apical turn: $r = -0.8203$, $p < 0.0001$) and inner hair cells (Spearman: basal turn: $r = -0.7169$, $p < 0.001$; middle turn: $r = -0.6072$, $p < 0.01$; apical turn: $r = -0.5267$, $p = 0.0205$).

The same experiments were also performed with *S. pneumoniae* D39ΔPLY in order to analyse the contribution of secreted pneumolysin on hair cell survival (Fig. 3 C, D and supplementary Table 1). Again, a one-way ANOVA revealed a significant effect of the bacterial concentration on the survival of inner (basal: $p < 0.0001$, middle: $p < 0.0001$, apical: $p < 0.01$) and outer (basal: $p < 0.0001$, middle: $p < 0.0001$, apical: $p < 0.001$) hair cells in all cochlear regions. Pairwise comparisons with the Dunnett's multiple comparison tests showed significant loss of outer hair cells in all cochlear regions only for explants treated with 4×10^8 and 2×10^9 cfu/ml compared to corresponding controls (Fig. 3 C). Loss of inner hair cells reached statistical significance only with the highest bacterial concentration in the basal, middle and apical turn (Fig. 3. D). A significant inverse correlation was found between bacteria concentration and OHCs survival (Spearman: basal turn: $r = -0.7354$, $p < 0.05$; middle turn: $r = -0.8949$, $p < 0.0001$; apical turn: $r = -0.7955$, $p < 0.001$).

A side-by-side comparison of ototoxic damages caused by *S. pneumoniae* D39 and its pneumolysin deficient strain D39ΔPLY is presented in Fig. 4. Survival rates of OHCs located at basal and middle region were significantly lower after exposure to D39 (base: 63.98% ± 16.8 of survival, $p < 0.01$; middle: 71.78% ± 3.91 of survival, $p < 0.001$) in comparison to OHCs survival rate after exposure to the same concentration (1×10^8 cfu/ml) of D39ΔPLY (base: 101.49% ± 6 of survival; middle: 103.02% ± 4.69 of survival) (Fig. 4 M). The same was true for IHC survival in the basal region of the cochlea (Fig. 4 N, $p < 0.05$).

When samples were exposed to 4 times higher bacteria concentration (4×10^8 cfu/ml) the ototoxic effect was strongly potentiated for both strains (Fig. 4 G, H, J and K) and substantial damage has also been observed for the explants exposed to D39ΔPLY. However, no statistically significant difference between the two strains was found based on the analysis of outer hair cell survival rates (Fig.4 O) due to the high variability. On the contrary, comparison of IHCs survival at basal and middle region (Fig.4 P), revealed significant ($p < 0.05$) differences (D39: base: 77.95% ± 14.51 of survival; middle: 66.73% ± 7.87 of survival; D39ΔPLY: base: 98.68% ± 8.61 of survival; middle: 95.71% ± 4.8 of survival).

3.3. Hair cell ototoxicity of recombinant pneumolysin

To investigate the ototoxic properties of pneumolysin, OC *ex-vivo* cultures were exposed for 24 h to two different toxin concentrations (5 ng/μl, 10 ng/μl). Representative pictures of OC explants incubated with recombinant PLY are shown in Fig. 5 A–I. A one-way ANOVA revealed a significant effect of the pneumolysin concentration on the survival of inner (basal: $p < 0.05$, middle: $p < 0.05$) and outer (basal: $p < 0.01$, middle: $p < 0.05$) hair cells in the basal and middle region. Dunnett's multiple comparison tests showed a statistically significant loss of inner hair cells in the basal turn (57.8% ± 17.1 of survival, $p < 0.05$) after challenging the cultures with 5 ng/μl of pneumolysin. Increasing the toxin concentration (10 ng/μl) resulted in more excessive loss of both hair cell populations compared to control cultures (OHC: base: 39.3% ± 11.2 of survival, $p < 0.01$, middle: 33.1% ± 23.2 of survival, $p < 0.05$; IHC: base: 34.9% ± 14.7 of survival, $p < 0.01$, middle: 36% ± 22.4 of survival, $p < 0.05$). Performing a one-way ANOVA revealed a statistically significant effect of the cochlear region on inner hair cell survival upon treatment with 5 ng/μl ($p < 0.05$) and on outer hair cell survival upon treatment with 10 ng/μl pneumolysin ($p < 0.01$). Tukey's multiple comparison *post hoc* tests showed a reduced survival of inner hair cells in the base (57.8% ± 17.1 of survival, $p < 0.05$) and middle turn (62.2% ± 20.6 of survival, $p < 0.05$) compared to the apex after exposure to 5 ng/μl. Furthermore, survival of outer hair cells in the basal (39.3% ± 11.2 of survival, $p < 0.05$) and middle turn (33.1% ± 23.2 of survival, $p < 0.05$) were significantly lower compared to the apex after exposure to 10 ng/μl. A significant inverse correlation was found between hair cell survival and PLY concentration for both, IHCs (Spearman: basal turn: $r = -0.8693$, $p < 0.01$; middle turn: $r = -0.8693$, $p < 0.01$; apical turn: $r = -0.7559$, $p < 0.05$) and OHCs located at the basal and middle region (Spearman: basal turn: $r = -0.9449$, $p < 0.0001$; middle turn: $r = -0.8693$, $p < 0.01$).

4. Discussion

4.1. Ex vivo model of meningitis-induced deafness

Post-meningitis hearing loss caused by pneumococcal infection is believed to be the result of the combined action of inflammatory cells, inflammatory cytokines and reactive oxygen species, bacterial products and toxins that infiltrate the inner ear and damage the resident sensory cells. In order to develop targeted therapies protecting the inner ear from these insults it is of importance to be able to investigate the respective contribution of these different elements separately. In this study we investigated in detail the pattern of *Streptococcus pneumoniae* induced hair cell loss using an *ex vivo* co-culture system. Using a Transwell-clear insert system with a permeable membrane, the sensory epithelium and the bacteria were co-cultivated in separate compartments. This mimics to a certain extent the *in vivo* situation in which bacteria are not in direct contact with the sensory hair cells (Møller et al., 2014; Osborne et al., 1995) and allows for gaining mechanistic and dynamic insight into the consequences of bacterial infiltration into the cochlea. A similar *ex vivo* approach has already been tested and used for ototoxicity studies (Dalian et al., 2012; Huth et al., 2011) and to study the effect of bacteria on the hippocampus (Gianinazzi et al., 2004). With this approach, we can discriminate between the direct contribution of the invading bacteria and their toxins from that of the recruited neutrophils, absent in our system.

After *ex vivo* exposure of OC explants to different strains of *Streptococcus pneumoniae* we found similar patterns of hair cell loss. OC cultures showed a tendency for more pronounced OHC loss, while IHCs exhibited higher resistance to bacterial exposure. These findings are in accordance with our recently published data on meningitis-induced HC loss obtained with an *in vivo* animal model, where we have found preferential OHC damage in animals that developed pneumococcal meningitis (Perny et al., 2016). OHCs were primarily damaged over IHCs in a dose- and time-dependent manner. Differences in survival capacity of OHCs and IHCs were previously reported during studies on ototoxic properties of pneumolysin (Beurg et al., 2005) and aminoglycosides (Zholudeva et al., 2015). Distinct cellular characteristic, calcium-buffering systems and specific mitochondrial distribution were mainly proposed as a possible explanation of different sensitivity of these two HC population to ototoxic insults. Furthermore, we observed (qualitatively) a higher susceptibility of the third row of OHCs. Unequivocal quantification of the three individual rows of OHCs was not possible in our system due the high disorganization upon bacterial exposure.

In addition we observed that exposure of OC explants to

different strains of *S. pneumoniae* or recombinant PLY tended to result in a damage that was graded along the base-to apex axis. HC located at the very apical region often showed higher level of survival in comparison to HC located at the basal and middle turn. These findings are in agreement with the *in vivo* characterization we have recently carried out (Perny et al., 2016). However, we had previously speculated that these effects could be explained by the site-dependent infiltration of bacteria at the cochlear base via the cochlear aqueduct, resulting in higher bacteria concentration in this region than the apex (Merchant and Gopen, 1996; Møller et al., 2014). However, in our *ex vivo* system with a "flattened" OC, we show that the more specific susceptibility to *S. pneumoniae* insults in the cells from the cochlear base is also an intrinsic HC feature. Different levels of glutathione in apical and basal HCs could account for differential antioxidant effects that lead to more/less resistant cells (Sha et al., 2001).

Post meningitis hearing loss is closely related to pneumococcal serotypes (Heckenberg et al., 2012; Worsøe et al., 2010). Despite the fact, that the same tendency towards more pronounced OHC destruction was found for all tested *Streptococcus* strains, OC explants exposed for 1 h to 1×10^8 cfu/ml of *S. Pneumoniae* S3 and D39 exhibited clearly different levels of hair cell survival (Figs. 2 A, B and 3. A, B). These differences may be explained by different hypotheses. First, the strains may express different amount of toxins, including PLY. Second, they may also differ in how effectively they release the toxins. Indeed, PLY has been hypothesized to be released upon autolysis or, alternatively, via a nonautolytic mechanism (Price et al., 2012) It is therefore conceivable that the S3 and D39 strains differ in this nonautolytic mechanism or that they have a different propensity to undergo autolysis. Our S3 strain is characterized by a thick capsule, which has been acquired by serial passage in the animal model (personal observation). This, or difference in capsule types, may influence autolysis, in accordance to a study reporting that capsular polysaccharides negatively regulate the lytic process in pneumococci (Fernebro et al., 2004).

A rich body of literature supports the hypothesis that hearing defects, as a consequence of bacterial meningitis, are developed during the early stage of infection and that they progress quickly within the time when subject is not treated (Bhatt et al., 1993; Kesser et al., 1999; Richardson et al., 1997). This is in agreement with our data, as we have shown that ototoxic damage after exposure to *S. pneumonaie* is highly time-dependent and already occurs a few hours after exposure (Fig. 2 M, N). Thus, we confirmed that the severity of *S. pneumoniae*-induced hearing loss is closely related to the total duration of bacterial exposure.

Experimental data about the ototoxic properties of PLY showed that a PLY-deficient strain causes significantly less HC damage (Winter et al., 1997). *Ex vivo* cultures presented in this paper revealed clearly a reduced level of hair cell damage after exposure of the OC to the PLY deficient strain, underlining the importance of PLY secreted by the bacteria. Our results with the recombinant PLY contrast with the finding that PLY causes preferential IHC loss at low concentrations (Beurg et al., 2005; Comis et al., 1993; Skinner et al., 2009). In fact, after challenging the OC explants with recombinant PLY, we observed loss of both HC populations. Only with high PLY concentration (10 ng/μl) and in the less affected apical region, we found a selective loss of IHCs. Differential susceptibilities of basal/apical inner/outer hair cells might create the specific pattern of hair cell damage, which clearly depends on the PLY concentration. Similar to *S. pneumoniae*, HC loss progressed in a dose-dependent manner (Beurg et al., 2005; Comis et al., 1993; Skinner et al., 2009).

Reactive nitrogen and oxygen molecules are besides PLY and other potential toxins important bacteria-derived factors involved in the pathogenesis of cellular brain damages, which could be responsible for the observed ototoxic effect of the pneumolysin-deficient strain (Braun et al., 2002; Klein et al., 2007).

Assessing hair cell ototoxicity on postnatal (2–4 days old) organ of corti explant cultures creates some limitations regarding the extrapolation of the results because functional hair cell maturity is reached more than two weeks after birth in rodents (Eatock and Hurley, 2003). Many aspects important for their normal function are not yet fully developed in postnatal hair cells, i.e. hair bundle morphology and transduction, OHC electromotility, basolateral channels, synapses and cochlear potentials. Therefor, bacterial ototoxicity could be different if assessed on mature hair cells due to altered cell permeability or metabolic activity. Nevertheless, the mechanotransduction channels (METs), required for aminoglycoside ototoxicity, are already present at birth in the basal turn with a tonotopic gradient already five days after birth (Alharazneh et al., 2011; Waguespack et al., 2007). Hair cell maturity might be less important in the case of the pore-forming toxin pneumolysin because of its distinct cytotoxic mechanism (Beurg et al., 2005).

One limitation of our study is the chosen outbred rat strain (Wistar) for the organotypic cultures together with the rather small number of experimental replicates. The variability of the observed hair cell toxicity could be reduced by using an inbred strain, thereby allowing to compare the response from cultures derived from genetically identical backgrounds.

In conclusion, our results reveal a predominant susceptibility of OHCs to ototoxic insults exerted by pneumococci, and a bigger vulnerability of HCs located at the basal cochlear region. This is consistent with our previous observations *in vivo* (Perny et al., 2016), suggesting an intrinsically different robustness of apically/basally located hair cells. With our setup we have confirmed that pneumolysin is an important factor involved in the development of ototoxic damages. We assume that the detailed pattern of *S. pneumoniae*-induced HC loss, described in this report, will support the development of novel otoprotective therapies.

Funding

This work was supported by the European Commission (OTOSTEM Grant FP7-Health-603029, to P.S., S.L.L.) and the Swiss National Science Foundation (Grant 310030 162583 to S.L.L).

Acknowledgments

The authors thank Prof. Dr. Annette Dräger (University of Bern, Institute of Anatomy, Bern, Switzerland) for providing the recombinant purified PLY and Dr Lucy J. Hathaway (University of Bern, Institute for Infectious Diseases, Bern Switzerland) for providing the D39ΔPLY strain.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.heares.2017.04.012.

References

Alharazneh, A., Luk, L., Huth, M., Monfared, A., Steyger, P.S., Cheng, A.G., Ricci, A.J., 2011. Functional hair cell mechanotransducer channels are required for aminoglycoside ototoxicity. PLoS One 6, e22347. http://dx.doi.org/10.1371/journal.pone.0022347.

Barnett, T.C., Cole, J.N., Rivera Hernandez, T., Henningham, A., Paton, J.C., Nizet, V., Walker, M.J., 2015. Streptococcal toxins: role in pathogenesis and disease. Cell Microbiol. 17, 1721–1741. http://dx.doi.org/10.1111/cmi.12531.

Berry, A.M., Ogunniyi, A.D., Miller, D.C., Paton, J.C., 1999. Comparative virulence of streptococcus pneumoniaestrains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. Infect. Immun. 67, 981–985.

Beurg, M., Hafidi, A., Skinner, L., Cowan, G., Hondarrague, Y., Mitchell, T.J., Dulon, D.,

2005. The mechanism of pneumolysin-induced cochlear hair cell death in the rat. J. Physiol. 568, 211–227. http://dx.doi.org/10.1113/jphysiol.2005.092478.
Bhatt, S.M., Lauretano, A., Cabellos, C., Halpin, C., Levine, R.A., Xu, W.Z., Nadol, J.B., Tuomanen, E., 1993. Progression of hearing-loss in experimental pneumococcal meningitis - correlation with cerebrospinal-fluid cytochemistry. J. Infect. Dis. 167, 675–683.
Braun, J.S., Sublett, J.E., Freyer, D., Mitchell, T.J., Cleveland, J.L., Tuomanen, E.I., Weber, J.R., 2002. Pneumococcal pneumolysin and H_2O_2 mediate brain cell apoptosis during meningitis. J. Clin. Investig. 109, 19–27. http://dx.doi.org/10.1172/JCI12035.
Chandran, A., Herbert, H., Misurski, D., Santosham, M., 2011. Long-term sequelae of childhood bacterial meningitis: an underappreciated problem. Pediatr. Infect. Dis. J. 30, 3–6. http://dx.doi.org/10.1097/INF.0b013e3181ef25f7.
Coimbra, R.S., Loquet, G., Leib, S.L., 2007. Limited efficacy of adjuvant therapy with dexamethasone in preventing hearing loss due to experimental pneumococcal meningitis in the infant rat. Pediatr. Res. 62, 291–294. http://dx.doi.org/10.1203/PDR.0b013e318123fb7c.
Comis, S.D., Osborne, M.P., Stephen, J., Tarlow, M.J., Hayward, T.L., Mitchell, T.J., Andrew, P.W., Boulnois, G.J., 1993. Cytotoxic effects on hair cells of Guinea pig cochlea produced by pneumolysin, the thiol activated toxin of *Streptococcus pneumoniae*. Acta Otolaryngol. 113, 152–159. http://dx.doi.org/10.3109/00016489309135784.
Coutinho, L.G., Grandgirard, D., Leib, S.L., Agnez-Lima, L.F., 2013. Cerebrospinal-fluid cytokine and chemokine profile in patients with pneumococcal and meningococcal meningitis. BMC Infect. Dis. 13 http://dx.doi.org/10.1186/1471-2334-13-326, 1–1.
Dalian, D., Haiyan, J., Yong, F., Salvi, R., Someya, S., Tanokura, M, 2012. Ototoxic effects of carboplatin in organotypic cultures in chinchillas and rats. J. Otol. 7, 92–101. http://dx.doi.org/10.1016/S1672-2930(12)50023-1.
Eatock, R.A., Hurley, K.M., 2003. Functional development of hair cells. Curr. Top. Dev. Biol. 57, 389–448.
Fernebro, J., Andersson, I., Sublett, J., Morfeldt, E., Novak, R., Tuomanen, E., Normark, S., Normark, B.H., 2004. Capsular expression in *streptococcus pneumoniae* negatively affects spontaneous and antibiotic-induced lysis and contributes to antibiotic tolerance. J. Infect. Dis. 189, 328–338. http://dx.doi.org/10.1086/380564.
Gianinazzi, C., Grandgirard, D., Simon, F., Imboden, H., Joss, P., Täuber, M.G., Leib, S.L., 2004. Apoptosis of hippocampal neurons in organotypic slice culture models: direct effect of bacteria revisited. J. Neuropathol. Exp. Neurol. 63, 610–617. http://dx.doi.org/10.1093/jnen/63.6.610.
Heckenberg, S.G.B., Brouwer, M.C., van der Ende, A., Hensen, E.F., van de Beek, D., 2012. Hearing loss in adults surviving pneumococcal meningitis is associated with otitis and pneumococcal serotype. Clin. Microbiol. Infect. 18, 849–855. http://dx.doi.org/10.1111/j.1469-0691.2011.03668.x.
Huth, M.E., Ricci, A.J., Cheng, A.G., 2011. Mechanisms of aminoglycoside ototoxicity and targets of hair cell protection. Int. J. Otolaryngol. 2011, 19. http://dx.doi.org/10.1155/2011/937861.
Karppinen, M., Pelkonen, T., Roine, I., Cruzeiro, M.L., 2015. Hearing impairment after childhood bacterial meningitis dependent on etiology in Luanda, Angola. Int. J. Pediatr. Otorhinolaryngol. http://dx.doi.org/10.1016/j.ijporl.2015.08.015.
Kesser, B.W., Hashisaki, G.T., Spindel, J.H., Ruth, R.A., Scheld, W.M., 1999. Time course of hearing loss in an animal model of pneumococcal meningitis. Otolaryngol. Head Neck Surg. 120, 628–637.
Klein, M., Koedel, U., Kastenbauer, S., Pfister, H.W., 2007. Nitrogen and oxygen molecules in meningitis-associated labyrinthitis and hearing impairment. Infection 36, 2–14. http://dx.doi.org/10.1007/s15010-007-7153-1.
Klein, M., Koedel, U., Pfister, H.-W., Kastenbauer, S., 2003. Morphological correlates of acute and permanent hearing loss during experimental pneumococcal meningitis. Brain Pathol. 13, 123–132. http://dx.doi.org/10.1111/j.1750-3639.2003.tb00012.x.
Leib, S.L., Leppert, D., Clements, J., Täuber, M.G., 2000. Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. Infect. Immun. 68, 615–620. http://dx.doi.org/10.1128/IAI.68.2.615-620.2000.
Meli, D.N., Coimbra, R.S., Erhart, D.G., Loquet, G., Bellac, C.L., Tauber, M.G., Neumann, U., Leib, S.L., 2006. Doxycycline reduces mortality and injury to the brain and cochlea in experimental pneumococcal meningitis. Infect. Immun. 74, 3890–3896. http://dx.doi.org/10.1128/IAI.01949-05.
Merchant, S.N., Gopen, Q., 1996. A human temporal bone study of acute bacterial meningogenic labyrinthitis. Otol. Neurotol. 1–11.
Møller, M.N., Brandt, C., Østergaard, C., Cayé Thomasen, P., 2014. Bacterial invasion of the inner ear in association with pneumococcal meningitis. Otol. Neurotol. 35, 1–9.
Osborne, M.P., Comis, S.D., Tarlow, M.J., Stephen, J., 1995. The cochlear lesion in experimental bacterial-meningitis of the rabbit. Int. J. Exp. Pathol. 76, 317–330.
Perny, M., Roccio, M., Grandgirard, D., Solyga, M., Senn, P., Leib, S.L., 2016. The severity of infection determines the localization of damage and extent of sensorineural hearing loss in experimental pneumococcal meningitis. J. Neurosci. 36, 7740–7749. http://dx.doi.org/10.1523/JNEUROSCI.0554-16.2016.
Price, K.E., Greene, N.G., Camilli, A., 2012. Export requirements of pneumolysin in *Streptococcus pneumoniae*. J. Bacteriol. 194, 3651–3660. http://dx.doi.org/10.1128/JB.00114-12.
Rappaport, J.M., Bhatt, S.M., Lauretano, A.M., Kimura, R.S., Levine, R.A., 1999. Electron microscopic temporal bone histopathology in experimental pneumococcal meningitis. Ann. Otol. Rhinol. Laryngol. 108, 537–547. http://dx.doi.org/10.1177/000348949910800603.
Richardson, M.P., Reid, A., Tarlow, M.J., Rudd, P.T., 1997. Hearing loss during bacterial meningitis. Arch. Dis. Child. 76, 134–138.
Sha, S.-H., Taylor, R., Forge, A., Schacht, J., 2001. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Annu. Rev. 2011 (155), 1–8.
Skinner, L.J., Beurg, M., Mitchell, T.J., Darrouzet, V., Aran, J.-M., Dulon, D., 2009. Intracochlear perfusion of pneumolysin, a pneumococcal protein, rapidly abolishes auditory potentials in the Guinea pig cochlea. Acta Otolaryngol. 124, 1000–1007. http://dx.doi.org/10.1080/00016480410017125.
van de Beek, D., de Gans, J., Spanjaard, L., Weisfelt, M., Reitsma, J.B., Vermeulen, M., 2004. Clinical features and prognostic factors in adults with bacterial meningitis. N. Engl. J. Med. 351, 1849–1859. http://dx.doi.org/10.1056/NEJMoa040845.
Waguespack, J., Salles, F.T., Kachar, B., Ricci, A.J., 2007. Stepwise morphological and functional maturation of mechanotransduction in rat outer hair cells. J. Neurosci. 27, 13890–13902. http://dx.doi.org/10.1523/JNEUROSCI.2159-07.2007.
Winter, A.J., Comis, S.D., Osborne, M.P., Tarlow, M.J., Stephen, J., Andrew, P.W., Hill, J., Mitchell, T.J., 1997. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in Guinea pigs. Infect. Immun. 65, 4411–4418.
Wolfmeier, H., Radecke, J., Schoenauer, R., Koeffel, R., Babiychuk, V.S., Drücker, P., Hathaway, L.J., Mitchell, T.J., Zuber, B., Draeger, A., Babiychuk, E.B., 2016. Active release of pneumolysin prepores and pores by mammalian cells undergoing a *Streptococcus pneumoniae* attack. Biochim. Biophys. Acta (BBA) General Subj. 1860, 2498–2509. http://dx.doi.org/10.1016/j.bbagen.2016.07.022.
Worsøe, L., Cayé Thomasen, P., Brandt, C.T., Thomsen, J., Østergaard, C., 2010. Factors associated with the occurrence of hearing loss after pneumococcal meningitis. Clin. Infect. Dis. 51, 917–924. http://dx.doi.org/10.1086/656409.
Wu, Q., Wang, G.-P., Xie, J., Guo, J.-Y., Gong, S.-S., 2015. Tumor necrosis factor-α-induced ototoxicity in mouse cochlear organotypic culture. PLoS One 10, e0127703. http://dx.doi.org/10.1371/journal.pone.0127703. EP.
Zholudeva, L.V., Ward, K.G., Nichols, M.G., Smith, H.J., 2015. Gentamicin differentially alters cellular metabolism of cochlear hair cells as revealed by NAD(P)H fluorescence lifetime imaging. J. Biomed. Opt. 20 http://dx.doi.org/10.1117/1.JBO.20.5.051032, 051032–051032.