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Early Detection of Endolymphatic Hydrops using the Auditory Nerve Overlapped Waveform (ANOW)

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Abstract

Endolymphatic hydrops is associated with low-frequency sensorineural hearing loss, with a large body of research dedicated to examining its putative causal role in low-frequency hearing loss. Investigations have been thwarted by the fact that hearing loss is measured in intact ears, but gold standard assessments of endolymphatic hydrops are made postmortem only; and that no objective low-frequency hearing measure has existed. Yet the association of endolymphatic hydrops with low-frequency hearing loss is so strong that it has been established as one of the important defining features for Ménière's disease, rendering it critical to detect endolymphatic hydrops early, regardless of whether it serves a causal role or is the result of other disease mechanisms. We surgically induced endolymphatic hydrops in guinea pigs and employed our recently developed objective neural measure of low-frequency hearing, the Auditory Nerve Overlapped Waveform (ANOW). Hearing loss and endolymphatic hydrops were assessed at various time points after surgery. The ANOW detected low-frequency hearing loss as early as the first day after surgery, well before endolymphatic hydrops was found histologically. The ANOW detected low-frequency hearing loss with perfect sensitivity and specificity in all ears after endolymphatic hydrops developed, where there was a strong linear relationship between degree of endolymphatic hydrops and severity of low-frequency hearing loss. Further, histological data demonstrated that endolymphatic hydrops is seen first in the high-frequency cochlear base, though the ANOW demonstrated that dysfunction begins in the low-frequency apical cochlear half. The results lay the groundwork for future investigations of the causal role of endolymphatic hydrops in low-frequency hearing loss.

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Author contributions

JTL designed the research

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CL and JTL analyzed the data

CL, CVV, SSG, CAB, and JTL interpreted results

JTL wrote the manuscript

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Keywords

Ménière's disease; cochlea; cochlear action potential; compound action potential; otoacoustic emissions; low frequency hearing

Introduction

Low-frequency hearing loss can be debilitating, given that the majority of speech, music, and background sounds are of low-frequency nature. For decades, no means of objectively assessing low-frequency hearing loss < 1 kHz has existed, a vexing problem given that low-frequency sensorineural hearing loss is one of the important defining features of Ménière's disease, one of the most devastating hearing disorders. Understanding the underlying mechanisms of low-frequency hearing loss has been hampered by the absence of minimally-invasive objective measures allowing an investigation of cochlear physiology and pathophysiology in the low-frequency cochlear apex. Objective threshold estimation methods based on otoacoustic emissions (OAE), compound action potentials (CAP), auditory brainstem responses, and auditory steady-state responses perform adequately only for mid- and high-frequencies in the mid- and basal-cochlea. Most knowledge of cochlear mechanics is restricted to the extreme base mainly because of its surgical accessibility. However, new imaging techniques are shedding light on the cochlear mechanics involved in the apex (Recio-Spinoso and Oghalai 2017, 2018; Dong et al. 2018), but a non-invasive, objective measure of low-frequency hearing is necessary to assess the ear's hearing sensitivity during imaging experiments and capture the functional consequences of experimental manipulations such as surgery. We recently developed an objective measure of low-frequency hearing, the Auditory Nerve Overlapped Waveform (ANOW). The ANOW is a neural measure of responses originating in the apical half of the cochlear spiral (Lichtenhan et al. 2014, 2016) that addresses a long-standing obstacle by providing a minimally-invasive technique to measure low-frequency hearing.

Endolymphatic hydrops, an enlargement of scala media, is associated with and is a putative cause of low-frequency sensorineural hearing loss in conditions such as Ménière's disease. Yet currently the only gold standard measure of endolymphatic hydrops must be conducted with post-mortem histology. A large body of research has explored endolymphatic hydrops as a cause of low-frequency hearing loss, but the relationship remains poorly understood, and certain findings are difficult to reconcile. For example, human temporal bones with endolymphatic hydrops rarely have sensory cell loss in the apex (Lindsay and von Schulthess 1958; Lindsay et al. 1967; Schuknecht 1989).

Given that endolymphatic hydrops is a biomarker of Ménière's disease (cf. Committee on Hearing and Equilibrium, 1995; Goebel 2015), detecting endolymphatic hydropic-associated dysfunction in intact ears is critical for early identification of the disease, regardless of whether endolymphatic hydrops functions as a cause of hearing dysfunction or reflects other disease processes. Detection of low-frequency hearing loss in ears with endolymphatic hydrops is also a necessary—and previously unrealizable—step in the process of establishing whether endolymphatic hydrops is an origin of low-frequency hearing loss. This

investigation has not been possible previously since endolymphatic hydrops can be histologically assessed in humans only post-mortem, and no objective measure of low-frequency hearing with known cellular and spatial origins existed to test this relationship in animals. Therefore, in order to address this gap of knowledge in the field, we sought to determine if the recently developed ANOW can measure low-frequency hearing loss in the earliest days after a surgery to induce endolymphatic hydrops. Early detection of endolymphatic hydropic-associated dysfunction by the ANOW would allow researchers to study the disease's progression, before the ear has been severely damaged by its full development, and an *objective* means to assist with the diagnosis of Ménière's disease. Even more critically, if the ANOW can detect pathology in early development, treatments can be designed to arrest the progression of the pathology and their efficacy monitored.

To lay the groundwork for determining if endolymphatic hydrops functions as a cause of low-frequency hearing loss, we determined if all ears with surgically-induced endolymphatic hydrops (as confirmed by histological assessment) developed low-frequency hearing loss. Additionally, we evaluated if the severity of endolymphatic hydrops correlates with the degree of low-frequency hearing loss. Due to the lack of a good objective measure of low-frequency sensorineural hearing from the auditory periphery, previous studies to determine this correlation were restricted to the mid- and high-frequency cochlear regions (e.g., Klis & Smoorenburg 1988; Salt 2004). Therefore, given our recently-developed ANOW, we were able to conduct a novel investigation of this question.

Our final investigation was to determine where along the cochlear length endolymphatic hydrops begins. Ears with Ménière's disease experience only *low*-frequency hearing loss during the disease's earliest stages, and as the disease progresses, hearing loss occurs at all frequencies. Given the initial low-frequency hearing loss, a hypothesis is that the originating location of endolymphatic hydrops is in the apical cochlear regions. Locating the origin of the endolymphatic hydrops is important for precision treatment and investigations of the disease's progression.

Experimental Procedures

Overview

The endolymphatic sac was ablated in the right ears of NIH-strain pigmented guinea pigs of either sex weighing, on average, 470.4g (SD $\pm$ 58, N = 27). Animals survived up to 30 days based upon pre-defined experimental endpoints following the surgery: 1- (N = 5), 2- (N = 6), 4- (N = 5), or 30-days (N = 7). Animals then underwent a second surgery to make cochlear function measurements: stimulus-frequency otoacoustic emissions (SFOAEs), distortion-product otoacoustic emissions (DPOAEs), ANOWs, and compound action potentials (CAPs). Immediately after cochlear function measurements were made, both ears were fixed, decalcified, and embedded in plastic for histological analysis. The cross-sectional area of scala media was measured on a personal computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). All procedures were conducted under protocols 20180133 and 20150091, both of which were approved by the Washington University Institutional Animal Care and Use Committee.

Surgical ablation of the endolymphatic sac

Guinea pigs were anesthetically induced with 50mg/kg of ketamine and 10mg/kg of xylazine, both given intraperitoneally. Guinea pigs were then placed in a neonatal warming isolette and observed closely until loss of toe-pinch reflex occurred. Once loss of toe-pinch reflexes occurred, the head of the guinea pig was shaved. A subcutaneous fluid bolus of 12mL of lactated Ringer's solution was given. Lubrication was applied to both eyes to prevent corneal abrasions. The guinea pig was then placed supine with legs raised while a 27.5 gage butterfly needle (Terumo Surflo Winged Infusion Set, Terumo Corporation) was placed intraperitoneally for administration of maintenance anesthesia (12.5mg/kg ketamine and 2.5mg/kg xylazine). After verifying that the needle was in the intraperitoneal space, the guinea pig was transferred and positioned in a custom-made stereotactic holder. A pulse oximeter was placed and secured on the foot. A rectal temperature probe was inserted to monitor the temperature. The rectal probe was part of a warming blanket in order to maintain body temperature at 38°C. Supplemental oxygen was administered to maintain oxygen saturation levels above 90%.

The operative area was prepped with iodine scrub and solution. The essence of the classic surgery to ablate the endolymphatic sac and duct was a combination of the intradural approach described by Kimura & Schuknecht (1965) and the extradural approach described by Andrews and Bohmer (1989). Briefly, guinea pigs were in a prone position held in place with a snout clamp and ear bars. A small, vertical, midline incision was made in the posterior occiput and iris scissors were used to cut the posterior cervical muscles. A small craniectomy along the right temporo-occipital suture line was made under visualization of an operating microscope with 3 mm, 2mm, 1mm, and 0.5mm diamond otologic burrs, consecutively. The sigmoid sinus was exposed and gently retracted medially. The extra-osseous portion of the endolymphatic sac was then visualized. A variety of custom angled picks were then used to destroy the intra-osseous portion of the endolymphatic sac to disarticulate it from the extra-osseous portion. Picks were also used to injure the endolymphatic duct. Once there was no indication of residual endolymphatic sac connected to the dura overlying the sigmoid sinus, bone dust was obtained from the ipsilateral temporal bone and packed into the operculum. A small piece of bone wax was then placed over the bone dust, and a large piece of bone wax was then applied to the craniectomy defect. Posterior cervical muscles were then approximated using 4-0 braided, absorbable suture. A subcuticular closure was then performed using a 4-0 braided, absorbable suture. Throughout this manuscript we will generally refer to this entire process as “endolymphatic sac ablation”.

The guinea pig was transferred immediately to the neonatal warming isolette. Atipamezole (2mg/kg) and lactated Ringer's solution (24mL) were given subcutaneously. Vital signs were obtained every 15 minutes until the guinea pig emerged from anesthesia. Additional lactated Ringer's (12mL) was given during the recovery period. Survival-day guinea pigs were then monitored two or three times daily for the first three days, and then weekly depending on assigned end point.

Physiological measurement procedure

Overview—At time points 1-, 2-, 4- and 30-days after the endolymphatic sac ablation surgery, cochlear function measurements were made. Animals were initially sedated with intraperitoneal injection of 100 mg/kg Inactin hydrate (i.e., thiobutabarbital sodium) and then the head and neck were shaved. A tracheotomy was performed to artificially ventilate and maintain anesthesia with ~1.2% of isoflurane in supplemental oxygen gas with respiration volume adjusted to maintain 5% end-tidal CO₂. A pulse oximeter was used to monitor O₂ saturation, expired CO₂ level, and heart rate. Body temperature was maintained at 38°C with a DC-powered heating blanket & rectal thermometer system. The room was heated such that the area around the animal was 25°C. The guinea pig head was secured with a bite bar, snout clamp, a hollow ear bar on the right side, and solid ear bar on the left side. While in the supine position, a canula was inserted into the left jugular vein. Lactated Ringer solution (0.5 mL / hour) was administered through the jugular vein canula to maintain hydration. The right bulla was accessed ventrally by removing soft tissue and the jaw. The right ear canal was cut so the hollow ear bar could be placed with no acoustic leaks. Measurements were made with the guinea pig in a double-walled sound treated room.

Cochlear function measurements from the right ear were made using a computer running custom MATLAB software (The MathWorks, Inc., Natick, MA) and the software utility Playrec (Humphrey 2008). Stimuli were generated in MATLAB, digitized at 96 kHz, and presented to a 24-bit sound card (RME Fireface 802) and routed to an acoustic probe system (ER-10X, Etymotic Research) that was coupled to the hollow ear bar. Ear canal pressure measurements (e.g., otoacoustic emissions) were made using the acoustic probe system connected to the 24-bit sound card. Once the ear bars were in place, and the head secured, the bulla was opened slightly and OAE measurements were made. The RW niche electrode was then positioned, Vecuronium bromide (0.1 mg / kg) was administered through the jugular vein canula to prevent middle ear muscle contractions, and ANOW and CAP were measurements made. Round-window electrode voltage measurements were band pass filtered at 0.1-3 kHz and amplified 10,000 times (GRAS CP511, Astro-Med, Inc.).

Sound calibration

A reference microphone (1/8" GRAS type 40P) and custom coupler was used for setting stimulus levels in animal ears. In these experiments, hollow ear bars (5 cm, 0.322 cm i.d.) were attached to the bony portion of the ear canals and acted as a stereotaxic device. Sound was presented through one of the hollow ear bars via an ER-10X probe. To calibrate system output, the terminal end of the hollow ear bar was coupled to a cavity having dimensions and volume similar to the bony portion of a guinea pig ear canal. The cavity was terminated by the reference microphone. Stimuli were played through the ER-10X probe loudspeakers into the ear bar and measured by the reference microphone. A transfer function was obtained and applied to all stimuli used in this experiment.

The ER-10X probe microphone was not used for setting stimulus levels in animal ears, but it was used for recording otoacoustic emissions. The ER-10X probe microphone was calibrated using a long lossy copper tube (1.83 m; 0.635 cm i.d.) closed at one end. A sound source was placed in the open end. The ER-10X probe and a reference microphone (1/8"

GRAS type 40P) were sealed in small holes located 2.5 cm from the sound source. The probe and reference microphones were located opposite of each other and perpendicular to the long axis of the tube. Care was taken to place the microphone inlet (probe) and diaphragm (reference) flush with the wall of the tube. A train of click stimuli was played through the sound source, and the incident wave was measured simultaneously by the probe and reference microphones. Clicks were temporally spaced to allow internal reflections in the tube to decay into the noise floor before the next click in the train was presented. Measurements were averaged and temporally windowed to include only the incident wave. A transfer function of the ER-10X probe microphone relative to the reference microphone was created and used to achieve a flat probe microphone response from 0.1 to 34 kHz.

Otoacoustic emission measurements

DPOAEs at $2f_1$ - f_2 were measured with pairs of one-second duration primary tones having frequencies separated $f_2/f_1 = 1.22$ and L_1 & L_2 levels being 60- and 50-dB SPL, respectively. f_2 frequencies ranged from 1 to 30 kHz in 2 kHz steps, with 12 repetitions presented at each f_2 . Noise floor measures were estimated as the standard error of the DPOAE measurement converted to dB SPL.

SFOAEs were elicited with 250 ms (10 ms rise / fall), 40 dB SPL tones using the double-evoked method with suppressor tones of 60 dB SPL that were 50 Hz above the SFOAE frequency (Keefe 1998; Keefe & Ling 1998; Schairer et al. 2003). Probe tones ranged from 1 to 30 kHz in 2 kHz steps and were presented 24 times at each probe frequency. Briefly, the double-evoked method eliminates any system distortion, leaving only a physiologic component. Noise floor measures were estimated as the standard error of the SFOAE measurement converted to dB SPL.

ANOW Measurements

Cochlear responses were made with an Ag/AgCl ball electrode in the round window niche and platinum needle electrodes at the vertex and in the neck musculature. Tone bursts (33.3 ms duration) were presented in alternating polarity (92 repetitions) at sinusoidal frequencies, f , 300, 480, 720, and 1020 Hz. For ease, these frequencies will be respectively referred to as 300, 500, 700, and 1000 Hz for this report. In response to low- to moderate-level low-frequency tones, the cochlear microphonic is nearly sinusoidal though the neural excitation occurs mostly during one phase of the tone. Averaging responses to tone bursts presented at opposite polarities cancels the cochlear microphonic and *overlaps* the phase-locked neural firing. ANOW waveforms were made by taking 0.5 times the sum of the responses from the two tone burst phases (Fig. 1). The first 5 ms and final 8.3 ms of each waveform were removed and the remaining 20 ms ramped on and off (ramp duration = $1/f$ sec). ANOW amplitude was measured at $2f$. The mean magnitude and phase of the ANOW waveforms were computed using a digital heterodyne filter. Briefly, an analytic signal was created by adding the Hilbert transform of the original ANOW waveform multiplied by the imaginary operator to the original ANOW waveform. The analytic signal was then multiplied by a complex sinusoid of frequency $2f$. The mean of the real ($\overline{\Re}$) and imaginary ($\overline{\Im}$) parts of the resulting product were calculated. ANOW magnitude and phase were computed as $|ANOW| = \sqrt{\overline{\Re}^2 + \overline{\Im}^2}$ and $\angle ANOW = \text{atan}(\overline{\Im} / \overline{\Re})$, respectively. The heterodyne

filtering process was applied as part of a statistical resampling algorithm, applied repeatedly to the 92 measurements. The resampling was used to compute 95% tolerance regions for the mean ANOW magnitude and phase. These tolerance regions were applied as part of an algorithm that automatically determined ANOW threshold.

CAP measurements

CAPs were elicited with 128 repetitions of 13.9 ms tone bursts (1.0 ms rise/fall) that were alternated in polarity. Two tone bursts of alternating polarity were interleaved with three periods of silence of the same duration, for a total of 69.5 msec and thus a repetition rate of 14.38 / second. Tone bursts were varied from 80 to 10 dB SPL in 5 dB steps. CAP threshold was defined by a computer algorithm that included measurements of CAP latency and signal to noise ratio.

Histological preparation and analysis

At the end of the cochlear function measurements, sodium pentobarbital was administered intraperitoneally (250 mg / kg), and animals were perfused transcardially with a 0.1 M phosphate buffer (~30 mL, pH 7.4) followed by 2% paraformaldehyde and 0.5% glutaraldehyde in a 0.1 M phosphate buffer (~60 mL, pH 7.4). Fixed temporal bones were stored for at least 12 hours in the same fixative at 4°C. After fixation, cochleae were decalcified with 0.1 M EDTA in a 0.1 M phosphate buffer for 6 days at room temperature. We adjusted the pH at 7.4 in all solutions. After decalcification, cochleae were post-fixed (1% OsO₄ in dH₂O) for 1 hour, dehydrated in a graded ethanol series and propylene oxide and then embedded in Araldite resins. The plastic blocks were sectioned in a vertical plane parallel to the modiolus at 40 µm with a carbide steel knife. Each section was mounted on microscope slides in Permount, coverslipped, and visualized using an OlympusBX51 microscope and DP71 camera (Olympus America Inc.). Scala media cross-sectional area was quantified in each cochlear half turn of a mid-modiolar cut using Image J.

Control group

The control group comprised measures from two different types of ears: 1) animals that underwent a sham surgery where the endolymphatic duct was visualized but not ablated, survived 1-, 2-, 4-, or 30-days, underwent a second surgical procedure to make the cochlear function measurements, and were finally transcardially perfused to prepare the ears for histological analysis; 2) naive animals that had no procedures other than those needed to make cochlear function measurements and trans-cardiac perfusion. In summary, the control group comprised three animals surviving sham surgery and four naive animals (N = 7). Wilcoxon ranked sums test comparisons for each tone burst frequency showed that cochlear function measures for sham surgery and naive guinea pigs did not differ significantly, justifying the use of both sham surgery and naive ears as a single control group. At the time of functional measurements, average animal weight was 463g (SD = ± 85, 1-day survival group, N = 5), 437g (SD = ± 41, 2-day survival group, N = 6), 466g (SD = ± 59, 4-day survival group, N = 5), 617g (SD = ± 81, 30-day survival group, N = 7), 468g (SD = ± 80, naïve controls, N = 4), and 499g (SD = ± 41, sham surgery, N = 4). Weights of sham-surgery animals were within the range of the associated survival-day group. In other words, the weight of the 1-day sham-surgery animal was within the range of the weights of the group of

animals that had their endolymphatic duct ablated and then survived 1 day until functional measurements were made, and similarly for the 2-, 4- and 30-day surgical control ears.

Statistics

Standard descriptive statistics were used to explore distribution of different measures between groups. Shapiro-Wilk's test was used to test the normality assumption for each of the continuous level measures included in the analysis. Wilcoxon rank-sum test was used to test for differences in cochlear function measures between control group ears, sham surgery and naïve guinea pigs, for each tone burst frequency, and to compare basal-turn scala media cross sectional area measures of this control group to each of the survival groups. A generalized linear mixed model analysis with random subject effects and group and frequency as fixed effects was used to explore differences in each of the outcome measures between control and survival groups. The mixed model analysis allows for evaluating of the interaction effects between group and frequency or turn estimates. Parameters for each of the variables in the model(s) were estimated using restricted maximum likelihood estimates, and estimated marginal means and their differences were used as measures of effect size. 95% Confidence Intervals were estimated for each marginal mean difference, and if the confidence interval includes 0, the comparison is not statistically significant. Planned contrasts were used to explore comparisons with focus on comparing each survival group with the Control group. Estimated marginal means and their differences are measures of effect size for the model. Statistical analysis was performed in SAS 9.4, SAS Institute Inc., Cary, NC, USA

Results

Histological Assessment

To determine if endolymphatic sac ablation produced endolymphatic hydrops, mid-modiolar histological sections were used for evaluation of scala media cross-sectional area (Fig. 2). Figure 3 depicts mid-modiolar histological sections through cochlear turn 2.5 of a naïve control ear where endolymphatic hydrops was not measurable, and a 30-day post-surgery ear where endolymphatic hydrops was measurable. Scala media is highlighted in semi-transparent red in the bottom row. Endolymphatic hydrops was also found in the saccule and utricle (data not shown). Figure 4A shows quantification of endolymphatic hydrops as a function of cochlear half turns for the right ear. For the ears that developed endolymphatic hydrops, scala media enlargements were histologically found for all cochlear turns (Figs. 2 & 4A).

A key finding of this study is that scala media cross-sectional area for all cochlear turns of the right ear of 30-day survival animals group only was enlarged (Fig. 4A), demonstrating that all other survival-day ears would have developed histologically measurable pancochlear endolymphatic hydrops had they lived to 30 days. That is to say, survival-day groups < 30 days were in the process of developing histologically measurable hydrops throughout the cochlea when physiologic measures (below) were made. But a Wilcoxon rank sums test revealed that scala media cross-sectional area in the basal-most cochlear turn, 0.5, for all survival groups (i.e., 1-, 2- 4-, and 30-day) was significantly different from the control group

(Table 1), demonstrating that histologically measurable endolymphatic hydrops began in the cochlear base. Measures of scala media cross-sectional area were divided into cochlear base and apical halves, which are respectively cochlear turns 0.5-2 and 2.5-4. Mixed model analysis with planned comparisons focused on comparison of the control group with each of the survival groups showed that cross-sectional area for only the 30-day survival group differed from the control group's measures (Tables 2A and 2B).

Neural Measurement of Hearing Dysfunction

In order to ascertain how early objective measurements could detect low- and mid-frequency hearing loss in ears which would develop endolymphatic hydrops, ANOW measurements were employed to quantify low-frequency (0.3 – 1 kHz) auditory thresholds and CAPs for mid- (2 – 4 kHz) and high-frequency (> 4 kHz; Fig. 4B) thresholds in 1-, 2-, and 4-day survival ears. Mixed model analysis showed that ANOW thresholds of control and all survival groups did not differ by probe tone frequency (i.e., there was no interaction effect). There was a significant main effect showing that, averaged across probe tone frequencies, there was a significant group difference between ANOW thresholds. Specifically, ANOW thresholds of the control group differed significantly from each survival group (Table 3A), i.e. the low-frequency hearing loss was permanent. Average high-frequency auditory thresholds were elevated in the 1-day survival group, and sequentially recovered to thresholds that were within the range of control-ear thresholds by 30 days. The configuration of thresholds as a function of frequency for 30-day survival animals was consistent with that which was found previously using an intradural approach to endolymphatic sac ablation (Horner 1991), demonstrating good agreement of results from our less aggressive extradural approach. There was a significant main effect showing that, averaged across probe tone frequencies, there was a significant group difference between CAP thresholds. Specifically, CAP thresholds for 1-day and 2-day survival groups differed significantly from the control group (Table 3B). In summary, *a central finding of this report is that average low- and mid-frequency thresholds were permanently elevated after endolymphatic sac surgery in 1-, 2-, and 4-day survival ears, but before endolymphatic hydrops was able to be measured histologically. These results demonstrate that objective measures of cochlear function detected dysfunction before histologically measurable endolymphatic hydrops developed (cf. Fig. 4A and Fig. 4B).*

Having found neural-based measurements of low- and mid-frequency hearing loss, we also evaluated outer hair cell physiology to further assess hearing function in the mid- and high-frequencies. DPOAE and SFOAE amplitudes were measured in all survival-day groups (Fig. 5). High-frequency (>5 kHz) DPOAE amplitudes were reduced in the 1-day survival group, and measures from 2-day, and 4-day survival groups sequentially recovered toward control-ear measures (Fig. 5A). Mid-frequency (i.e., $f_2 = 1\text{--}5$ kHz) DPOAE amplitudes were reduced in all survival-day groups and did not recover. There was a significant main effect showing that, averaged across probe tone frequencies, DPOAE amplitudes of the control group differed significantly from each survival group (Table 4). The 1- and 2-day high-frequency DPOAE amplitudes differed from controls; and 1-, 2-, and 4-day mid-frequency DPOAE amplitudes differed from control (Table 4A and 4B). DPOAE amplitudes for control and survival groups did not differ by frequency (i.e., there was no interaction effect).

SFOAE amplitudes from 1-day survival ears were lower than control-ear measures at all probe frequencies (Fig. 5B). Mixed model analysis showed that SFOAE amplitudes for control and survival groups did not differ across probe tone frequency (i.e., there was no interaction effect). However, there was a significant main effect showing that, averaged across probe tone frequencies, control group SFOAE amplitudes significantly differed from survival group. Planned comparisons showed that SFOAE amplitudes for 1-day survival ears differed from controls (Table 4C).

Degree of low- and mid- frequency hearing loss correlated with the severity of histologically measurable endolymphatic hydrops—

Only the 30-day survival animals could be used to assess the relationship between the severity of endolymphatic hydrops and the degree of low- (0.3 – 1 kHz) and mid- (2 – 4 kHz) frequency hearing loss, as other survival-day animals did not yet develop histologically measurable endolymphatic hydrops in the apical half. ANOW measurements were used to quantify low-frequency auditory thresholds and CAPs for mid- and high-frequency (> 4 kHz; Fig. 4B) thresholds. The low- and mid-frequency threshold differences from the respective measures of the control group were calculated to yield a measure of “threshold shift.” Thirty-day survival group data revealed no significant high-frequency hearing loss (Fig. 4B), so thus there was no statistically significant relationship between basal endolymphatic hydrops and high-frequency hearing loss to assess. Scala media cross-sectional area measures of cochlear turns 2.5-4 were summed for individual 30-day ears, the regions of which ANOW and mid-frequency CAPs originate (Lichtenhan et al. 2014, 2016; Lee et al. 2019). ANOW and mid-frequency CAP threshold shifts correlated with scala media cross-sectional area ($y = 44.29x - 14.023$, $r^2 = 0.6842$ and $y = 73.524x - 31.13$, $r^2 = 0.8693$, respectively; Fig. 6). These results demonstrate that low-frequency (0.3 – 1 kHz) and mid-frequency (2 – 4 kHz) thresholds correlated with the severity of histologically measured endolymphatic hydrops (Fig. 6), suggesting that endolymphatic hydrops may function as an origin of the associated low-frequency hearing loss. Further reinforcing these conclusions, one animal that underwent endolymphatic sac ablation surgery did not develop endolymphatic hydrops, presumably due to human error during surgery, yet critically, did not develop low- or mid-frequency hearing loss (animal number SAC61). As such, this animal was excluded from further analyses. *These data show, for the first time, that the severity of histologically measurable endolymphatic hydrops correlated with the degree of low- (0.3 – 1 kHz) or mid-frequency (2 – 4 kHz) hearing loss.*

Sequential recovery of hearing function measures: Hearing loss was not caused from acoustic trauma during the surgery—

Low-frequency ANOW and mid-frequency CAP thresholds were permanently shifted, in that they did not recover toward control values with increasing survival day. In contrast, high-frequency CAP thresholds and DPOAE amplitudes deviated from the control group measures in the 1-day survival group, but sequentially recovered to control-group values by 30- days (Figs. 4B & 5A). Unlike high-frequency CAP thresholds and DPOAE amplitudes, SFOAE amplitudes showed permanent alteration relative to control. Sequential recovery of temporary hearing thresholds shifts and DPOAE amplitudes in humans has been attributed to acoustic trauma associated with skull drilling (Karatas et al. 2007; Migirov & Wolf 2009; Goyal et al. 2013;

Baradaranfar et al. 2015; Shenoy et al. 2015). The attributes of acoustic sound from drilling on the human skull are complicated, particularly when the type of drill and possible trauma from both air- and bone-conduction sounds are considered, but a few generalizations are possible. Air-conduction sounds during drilling can exceed 100 dBA with a spectrum ranging from 2.5 – 6.3 kHz (e.g., Hilmi et al. 2011; Hetzler et al. 2012; Vaisbuch et al. 2018). The previous studies on human skull drilling involved multiple hours of drilling, far longer than the ~5-10 minutes of drilling needed in the present study to create the necessary amount of space to visualize the operculum prior to expunging the endolymphatic sac with a pick. The characteristics of bone-conducted skull drilling sound entering the guinea pig cochlea are unknown, as is the extent to which such sound could potentially be traumatizing. An exposed and open skull with brain retraction would likely influence what is known about bone conduction sound from guinea pig skull drilling, further complicating an understanding of the possible influence of skull drilling on guinea pig hearing. While the 1-day sham surgery ear was removed from the control group because high-frequency (> 8 kHz) CAP thresholds and DPOAE amplitudes were consistent with temporary acoustic trauma from skull drilling, thresholds for 2-day survival sham surgery ears were within normal limits suggesting that temporary acoustic trauma had resolved after 1 day. Thus, acoustic trauma from skull drilling is not the sole origin of the sequential recovery of high-frequency CAP thresholds and DPOAE amplitudes.

Fixative osmolarity can modulate endolymphatic volume—Histologically quantifying endolymphatic hydrops critically depends on the position of Reissner's membrane that has tight junctions and mediates a boundary between scala media and scala vestibuli. The membrane itself is permeable only to water, and the stabilization of local scala media fluid volume should likely be regulated by aquaporins (Eckhard et al. 2014). Histology has long been considered a gold-standard method to quantify endolymphatic hydrops, though histological fixation processes are not standardized and can vary across laboratories. It is possible that the hit rate of successfully imaging induced endolymphatic hydrops in animal models is influenced by fixative osmolarity. Most reports on studies of endolymphatic hydrops do not specify osmolality or osmolarity of fixative solutions. Moreover, perfusing the perilymphatic space with hypotonic artificial perilymph has been shown to treat volume increases of the endolymphatic space (Kim et al. 2018). We thus used a subset of animals to evaluate the effect of hypertonic fixative solutions on Reissner's membrane to optimize our histological data.

A subset of animals that did not undergo endolymphatic sac surgery were perfused with ~2003 (N = 4 animals) or ~500 mM/kg (N = 6 animals) fixative solution (Fig. 7). Fixative solution from transcardiac perfusion likely enters the scala media through stria vascularis capillaries, evidenced by small empty vacuoles in the stria vascularis (e.g., Figs. 2 and Fig. 3). Guinea pig endolymph osmolarity is approximately 316 mOsm/L (Sziklai et al., 1992). Hypertonic fixative solution modulated endolymphatic volume, in that the scala media enlarged in response to ~2003 mM/kg fixative solution. But scala media cross-sectional area values with ~500 mM/kg fixative were within the normal range of those made from intact, non-fixed guinea pig ears (cf. Fig. 2; Salt et al. 1995b). Hypertonic fixative solution could have mediated water entry into the scala media through osmosis and caused the bowing

effect on Reissner's membrane into scala vestibuli. These results demonstrate that osmolarity of perfused solutions ought to be comparable to that of scala media when volume measurements are of interest. Otherwise, a hypertonic fixative solution could cause a false-positive interpretation of an enlarged endolymphatic space. All survival-day guinea pigs in the current study were perfused with ~500 mM/kg fixative solution for histological analysis.

Discussion

Our investigation of the functional consequences of surgically-induced endolymphatic hydrops revealed the following: 1) the ANOW can detect low-frequency hearing loss in ears with surgically-induced endolymphatic hydrops well before gold standard histological assessment can detect the presence of endolymphatic hydrops; 2) surgically inducing chronic endolymphatic hydrops reliably results in low-frequency hearing loss, where there is a strong linear relationship between the degree of endolymphatic hydrops and severity of low-frequency hearing loss once endolymphatic hydrops was detected by histological assessment; and 3) permanent hearing loss begins in the low-frequency apical cochlear half, though histologically-ascertained endolymphatic hydrops is seen first in the high-frequency cochlear base without associated high-frequency hearing loss. Jointly, these results provide evidence that the ANOW can detect endolymphatic hydropic-associated low-frequency hearing loss as early as one day post-endolymphatic hydrops induction procedures, and suggest a causal role for endolymphatic hydrops in low-frequency hearing loss.

Low-frequency hearing loss as an early detector of endolymphatic hydrops

Previously we found that the ANOW can detect low-frequency hearing loss upon induction of *acute* endolymphatic hydrops in animals—critically, when conventional measurements of hearing are normal (Lichtenhan et al. 2017). Establishing the ANOW's ability to detect low-frequency hearing loss upon induction of *chronic* endolymphatic hydrops was an essential next step, because the endocochlear potential can differ in acute vs. chronic endolymphatic hydrops (cf. Meyer zum Gottesberge 1988, Salt 2004) and, moreover, patients present to the clinic with chronic conditions, not acute. Low-frequency (0.3 – 1 kHz) ANOW and mid-frequency (2 – 4 kHz) CAP thresholds were elevated for all 1-, 2-, 4-, and 30-day survival groups that underwent an endolymphatic sac ablation (Fig. 4A). This hearing loss was found before histological assessment revealed endolymphatic hydrops. The elevated low- and mid-frequency thresholds, as well as decreased DPOAE amplitudes in survival ears 4-days post endolymphatic sac ablation, demonstrated that objective measures of hearing detected hearing dysfunction before the gold standard histology-based measures of endolymphatic hydrops. Critically, the ANOW did not detect low-frequency hearing loss in control ears (i.e., it did not produce any false positives), and it detected low-frequency hearing loss in all 30-day ears that developed histologically measurable endolymphatic hydrops (i.e., it did not produce any false negatives), meaning that early survival day ears with hearing loss during the early stages would have developed hydrops had they lived to 30 days.

This study's CAP findings accorded with that of Horner and Cazals (1987), who found CAP-based measures of mid-frequency hearing loss at ~5-to-10-days post endolymphatic sac ablation, though they did not make physiological measurements in the earliest days as

we did here. However, exercising caution in the interpretation of CAP findings is warranted. Lee et al. (2019) demonstrated that the spatial origin of mid-frequency CAPs amplitude measures can shift dramatically with the increases in stimulus level necessary for measuring hearing loss. Further, mid-frequency CAP thresholds are elevated relative to single fiber thresholds (e.g., Fig. 6B of Lichtenhan et al. 2013). Additionally, response averaging can also influence the signal-to-noise of CAP measures. Together, these findings suggest that CAP thresholds evoked from mid-frequency tone bursts are not good estimates of auditory threshold, leaving interpretation of these measures somewhat unclear depending on the research or clinical question being addressed. Because ANOW measurements are not derived from onset responses and are more consistent with low-frequency spatial origins, the ANOW is a safer choice to assess dysfunction associated with endolymphatic hydrops.

Degree of low- and mid-frequency hearing loss correlated with the severity of histologically measurable endolymphatic hydrops

The scientific and clinical value for the sought-after relationship between hearing loss and endolymphatic hydrops is high because the cause of the hearing loss is not known (e.g., Klis & Smoorenburg 1988; Salt 2004; Merchant et al. 2005; Nadol 2010). Prior studies have been restricted to examining the relationship of hearing loss and the severity of enlarged scala media area in mid- and high-frequency cochlear regions (e.g., Klis & Smoorenburg 1988; Salt 2004), and have found no correlation. Yet critically, it is *low-frequency* hearing loss that is the hallmark clinical symptom of endolymphatic hydrops. Until recently, a good objective measure of low-frequency hearing from the auditory periphery has not been available, rendering a challenge to the exploration of the relationship of the severity of endolymphatic hydrops with low-frequency hearing loss. Therefore, given our recently-developed objective measure of low-frequency hearing (ANOW), we were able to appropriately investigate the sought-after relationship. Animals in the 30-day survival group did not have significant high-frequency hearing loss, demonstrating that endolymphatic hydrops in the cochlear base did not have functional consequences (cf. Figs. 4A and 4B). However, we established that low- (0.3 – 1 kHz) and mid-frequency (2 – 4 kHz) hearing loss relates to the severity of enlarged scala media area in the apical cochlear half (Fig. 6). Presumably, permanent endolymphatic hydrops was developing in earlier-day survival ears that had low-frequency hearing loss, but was not measurable with classic histological techniques. Our findings from 30-day survival ears are a basis for understanding that endolymphatic hydrops may function as an origin of low-frequency hearing loss, but more work is needed to unequivocally establish a causal relationship between endolymphatic hydrops and low-frequency sensorineural hearing loss.

Endolymphatic hydrops as a cause of low-frequency hearing loss: Alternative considerations

Previous attempts to elucidate whether endolymphatic hydrops functions as a cause of low-frequency hearing loss have been unable to probe the question appropriately because of a lack of an objective measure of low-frequency hearing. Here, we were able to employ our recently-developed neural measure, the ANOW, to study the endolymphatic hydrops as a putative cause of low-frequency hearing loss. Specifically, endolymphatic hydrops was reliably created in most ears and, in those ears, produced low-frequency hearing loss in all

ears. However, careful consideration of alternative contributors to the low-frequency hearing loss found here are in order.

Elevated Endolymphatic Ca²⁺—Chronic endolymphatic hydrops is associated with high endolymph Ca²⁺ levels, which likely mediates closure of mechanoelectric transducer channels and thus endolymph accumulation in the distensible scala media of the cochlear apex (Ninoyu & Meyer zum Gottesberge 1986; Meyer zum Gottesberge & Ninoyu 1987; Fettiplace & Ricci 2006; Salt & DeMott 1997). One possible factor driving hearing loss could be elevated endolymphatic Ca²⁺ (Salt & DeMott 1994). Indeed, we previously iontophoresed Ca²⁺ into the scala media to create an acute model of endolymphatic hydrops (Lichtenhan et al. 2017). But Salt & DeMott (1994) found that Ca²⁺ did not increase until ~8-16 weeks after endolymphatic sac ablation, a time point that far exceeds our maximum 30-day survival. Therefore, we do not suspect that increased Ca²⁺ is a cause of the low- (0.3 – 1 kHz) and mid-frequency (2 – 4 kHz) hearing loss we found in our ears after endolymphatic sac ablation.

Sensory Cell Loss—The low- and mid-frequency hearing loss found in our study is unlikely to originate from sensory cell loss following endolymphatic sac ablation. First, existing literature does not support the likelihood of this hypothesis. Kimura (1967) found sensory cell loss to occur in the cochlear apex of a mere 20% of guinea pigs surviving 30–60 days after endolymphatic sac ablation, and no sensory cell loss in ears surviving 4-days. Given the boundaries of Kimura's 30-60-day survival group, it is unclear if sensory cells were lost in any of his animals surviving 30 days, which is the longest survival-day group in our study. Others have found both neuronal loss in guinea pig ears with histologically measurable endolymphatic hydrops, but at survival-day times that far exceeded our 30-day survival group (e.g., Megerian 2005; Bixenstine et al. 2008; Momin et al. 2011). Further, our histological examination did not yield any evidence of obvious sensory cell loss. Therefore, sensory cell loss is an unlikely origin of the low- and mid-frequency hearing loss found in our study.

Cochlear Synaptopathy—A recent study found cochlear synaptopathy to be associated with acute endolymphatic hydrops, though it was unclear if synapses of low- or high-threshold auditory nerve fibers were affected, or if the synaptic loss was enough to affect threshold- or suprathreshold-based measures of hearing (Fig. 6J-Q of Kim et al. 2018). It is not yet clear how acute endolymphatic space enlargements in mice can inform the current study of chronic endolymphatic sac ablation in guinea pigs, as mice hear solely at mid- to high-frequencies (1-3 kHz, Taberner and Liberman 2005) and guinea pigs can hear in the low-frequency region that is clinically associated with endolymphatic hydrops. Moreover, acute and chronic models of endolymphatic hydrops can have opposing effects on the endocochlear potential (cf. Meyer zum Gottesberge 1988; Salt 2004). Be that as it may, mid-frequency hearing loss found in all survival-day ears (Fig. 4B) was corroborated with reduced mid-frequency DPOAE amplitudes (Fig. 5A), which are not reduced in synaptopic ears (Lin et al. 2011; Furman et al. 2013), arguing against synaptopathy associated with low-threshold fibers being a contributor to the reduced auditory sensitivities found in our study. High-threshold fibers – not low-threshold fibers that apparently govern hearing thresholds –

appear to be the most vulnerable to synaptopic events (Kujawa & Liberman 2009). The ANOW amplitude measures at higher stimulus levels (i.e., 60 dB SPL) were not reduced, reinforcing the lack of functional significance of synaptopathy of high-threshold fibers in our study (Fig. 8).

It is well known that synaptopathy of high-threshold fibers can result from temporary acoustic trauma. Skull-drilling noise ranging from 2.5 – 6.3 kHz was a possible source of temporary high-frequency acoustic trauma, as mentioned above. Yet critically, acoustic trauma generally does not affect low-frequency hearing (Royster et al. 2000). Measures of hearing below the acoustic exposure frequency are affected little, if at all (Lin et al. 2011; Furman et al. 2013), and the majority of acoustic trauma occurs approximately one octave *above* the frequency of the acoustic over-exposure. Therefore, acoustic trauma is an unlikely origin of the low- and mid-frequency hearing dysfunction we found, as even the most sensitive events – synaptic loss of high-threshold afferent auditory nerve fibers – requires an acoustic overexposure causing a temporary threshold shift lasting longer than the single-day found in our 1-day sham surgery control ear (Kujawa & Liberman 2009). Cochlear synaptopathy has been studied in guinea pigs (e.g., Lin et al. 2011; Furman et al. 2013), though not in the apical cochlear half of ears with endolymphatic hydrops, leaving it unclear how to extrapolate all that has been learned about synaptopathy in the cochlear base to understand the cochlear apex.

Endocochlear Potential—It is unlikely that changes in endocochlear potential following endolymphatic sac ablation could function as a mechanism of the low-frequency hearing loss found in our study. Salt & DeMott (1994) found that 4 days after guinea pig endolymphatic sac ablation the endocochlear potential in the third and fourth cochlear turns decreased by approximately 6 mV and 8 mV, respectively, although these decreases were statistically insignificant. By 4 weeks the endocochlear potential in the third and fourth turns respectively decreased by approximately 16 mV and 19 mV and these decreases were statistically significant. Considering measures made across all Salt & DeMott's survival days (i.e., 4 days to 16 weeks), hearing threshold measures at 3.36 kHz increased by 1 dB for every 1 mV decline of endocochlear potential in the second cochlear turn (Table 1 of Salt & DeMott 1994). Others have used the non-endolymphatic hydropic model of furosemide administration and found a 1 dB / mV relationship with higher-frequency regions (Sewell 1984; Schmiedt et al. 2002; Wang et al. 2019). The average $12.45 \text{ dB} \pm 8.73 \text{ SD}$ low-frequency ANOW threshold shift found in our 30-day animals is generally consistent with the ~15-20 mV endocochlear potential decrease that Salt & DeMott found in the apical cochlear half at 4 weeks. However, a ~15-20 mV decrease in the basal cochlear half that Salt & DeMott found at 4 weeks is puzzling given the normal high-frequency CAP thresholds found in our study at 30 days. Therefore, it does not seem to be the case that the 1 dB / mV relationship between hearing threshold and endocochlear potential is the sole driver of hearing loss after endolymphatic sac ablation, as the endocochlear potential seemingly does not change by 4 days when we found low- and mid-frequency hearing loss; but does indeed decrease by ~15-20 mV by 4 weeks in the basal turns where we found normal high frequency thresholds at 30 days. Future research could investigate the relationship between ANOW measurements and endocochlear potential in individual ears.

The involvement of cochlear amplification alterations in low-frequency hearing loss caused by endolymphatic hydrops

Sensitive (i.e., low) hearing thresholds are associated with normal cochlear amplification. Cochlear amplification was recently found to exist in the cochlear apex of normal guinea pigs (Recio-Spinoso & Oghalai 2017, 2018), albeit the location and spatial extent of amplification is still not understood. Measures of low- (0.3 – 1 kHz) and mid-frequency (2 – 4 kHz) hearing thresholds were elevated in all survival-day ears of the current study (Fig. 4), suggesting that low- and mid-frequency cochlear amplification was abnormal after the surgery to induce endolymphatic hydrops. Indeed, DPOAE amplitudes were decreased in the mid-frequency (2 – 4 kHz) regions where CAP thresholds from all survival-day groups were elevated (cf. Figs. 5A and 4B). While normal cochlear amplification has been found in mice ears with acute enlargements of scala media from lowering perilymph osmolarity, the hearing sensitivity was not known (Fig. 6G-I of Kim et al. 2018). As described above, it is not clear how results from mice that hear solely at mid and high frequencies can help with interpreting results from guinea pigs that hear in the low-frequency region classically associated with endolymphatic hydrops. Additionally, acute and chronic models of endolymphatic hydrops can differ. Given the current knowledge, the low- (0.3 – 1 kHz) and mid-frequency (2 – 4 kHz) hearing loss we found after endolymphatic sac ablation originated from abnormal cochlear amplification in the apical cochlear half.

All ANOW thresholds from 0.3 – 1 kHz were affected by endolymphatic sac ablation. Mechanical filtering in the apical cochlear half is low-pass in nature, not band-pass as in the cochlear base (Dong et al. 2018; Spinoso & Oghalai 2017, 2018). Because single auditory nerve fibers encode signals in a band-pass fashion, a high-pass filter must be present between the low-pass mechanical filter and the band-pass filter of single auditory nerve fibers. While the origin of the high-pass filter is unknown (Guinan 2017), it may be affected by endolymphatic dysfunction that follows endolymphatic sac ablation because thresholds at all ANOW frequencies were affected; therefore suggesting that responses were driven by the broad low-pass nature of the mechanical motion in the cochlear apex.

Endolymphatic sac surgery differentially affected DPOAEs and SFOAEs evoked from mid-frequency probe tones (i.e., 1-5 kHz, Fig. 5). OAEs can be affected by hydropic-based shifts in the operating-point of the sigmoidal saturating nonlinearity that is involved with the *in vivo* transfer of acoustic sound into neural excitation (Sirjani et al. 2004; Brown & Gibson 2011), but this consideration has yet to be studied in ears during the early stages before endolymphatic hydrops can be histologically identified. Reduced DPOAE amplitudes in the face of normal SFOAE amplitudes may suggest that endolymphatic hydrops affected the mid-frequency sigmoidal saturating nonlinearity that mediate DPOAE production, but not the micromechanical irregularities that mediate SFOAE production (Zweig & Shera 1995). However, the aging process occurring over the duration of a lifespan can be associated with decreased cochlear amplification, inferred by loss of hearing sensitivity, and thus reduce DPOAE amplitudes more than SFOAE amplitudes. Subsequent OAE modeling showed that the consequences of decreased cochlear amplifier gain can be at least partially compensated for by an increase in micromechanical irregularities (Abdala et al. 2018). Elevated thresholds at low- and mid-frequencies after endolymphatic sac ablation is consistent with

decreased cochlear amplification, suggesting that endolymphatic hydrops may have increased the micromechanical irregularities that facilitate SFOAE amplitudes that were within normal limits by 30-days of survival. Another possibility is that endolymphatic hydrops did not increase micromechanical irregularities and the SFOAEs from mid-frequency probe tones originated in part from more basal cochlear regions that were associated with measures of normal hearing threshold and DPOAE amplitudes, an issue that is being addressed in our ongoing research (Goodman et al. 2019). Additional research is needed to understand how OAEs can be used for the early detection of dysfunction associated with endolymphatic hydrops.

Where does endolymphatic hydrops begin?

Previous studies found endolymphatic hydrops to be more prevalent in either the cochlear base or apex, though such findings do not identify where endolymphatic hydrops formation *begins* (e.g., Kimura & Schuknecht 1965; Salt et al. 1995a,b; Nadol 2016; Li et al. 2018; Bier et al. 2018). Gold standard histological data from the current study showed that endolymphatic hydrops was detected first in turn 0.5 in the base of the cochlear spiral, as scala media cross-sectional area of 1-, 2-, and 4-day survival ears was significantly greater than that of control ears (Fig. 4A). However, the lack of high-frequency hearing loss in 30-days survival animals suggests that the histologically measured endolymphatic hydrops in the basal-most turn of our study was not functionally significant. We do not suspect that the post-operative high-frequency temporary threshold shifts were related to endolymphatic hydrops being detected first in the extreme cochlear base, as all high-frequency thresholds were temporarily affected though endolymphatic hydrops was not seen in throughout the cochlear base in the earliest survival days.

Clinical hearing loss associated with endolymphatic hydrops classically begins in the low frequencies, suggesting that endolymphatic hydrops formation begins in apical cochlear regions. The current study's permanently elevated low- and mid-frequency auditory thresholds in 1-, 2-, and 4-day survival ears identified dysfunction in the apical cochlear half during the early stages of when endolymphatic hydrops was developing but not yet histologically measurable. Scala media area and Reissner's membrane length are essentially constant along the cochlear length of normal ears, though the basilar membrane is narrower and stiffer in the cochlear base and wider and more distensible in the apex. In ears with endolymphatic hydrops, attributes such as these may mediate the lack of dysfunction in the basal cochlear half, and the presence of hydropic-associated dysfunction in the apical half.

In summary, by ablating the endolymphatic sac in guinea pigs, we induced endolymphatic hydrops and examined the functional consequences using the ANOW to objectively assess low-frequency hearing. We found that the ANOW detected low-frequency hearing loss in 1-, 2-, and 4-day survival ears during endolymphatic hydrops development before the gold standard histological assessment was able to identify the presence of hydrops. The histologically measured endolymphatic hydrops in 30-day survival ears reliably produced low-frequency hearing loss, where the degree of hearing loss strongly correlated with the severity of endolymphatic hydrops. Histological data showed that endolymphatic hydrops is detectable first in the high-frequency cochlear base, though the ANOW demonstrated that

dysfunction begins in the low-frequency apical cochlear half. These findings suggest that endolymphatic hydrops, or the process of endolymphatic hydrops development, causes low-frequency hearing loss but not high-frequency hearing loss.

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Highlights

- Endolymphatic hydrops (EH) is associated with low-frequency hearing loss (LFSNHL).
- The cause of the LFSNHL is still unknown.
- In the early days after inducing EH, ears had LFSNHL but no measurable EH.
- These results suggest that LFSNHL is an early detector of developing EH.
- Once EH developed in the later days, data suggests that EH is a cause of LFSNHL.

Figure 1.

Measurements used to obtain the Auditory Nerve Overlapped Waveform (ANOW).

Responses to tone bursts of alternating polarity (Panels A and B) can be added to *overlap* the neural contribution and cancel the hair cell contribution (Panel C). See text for ANOW signal processing details. Tone burst level and frequency were respectively 60 dB SPL and 480 Hz for this example.

Figure 2. Mid-modiolar sections of the right (R) and left (L) cochlea of guinea pigs from the 1-, 2-, 4-, and 30-day survival group after a surgery to ablate the endolymphatic sac of the right ear. A black line was placed on Reissner's membrane for display purposes. Scala media cross-sectional area was enlarged in the right ear of the 30-day ear only, demonstrating endolymphatic hydrops.

Figure 3. Representative histological images of the second cochlear turn of the right ear from a naïve control and 30-day survival ear that underwent a surgery to ablate the endolymphatic sac. Scala media is highlighted in the bottom row.

Figure 4.

Panel A. Scala media cross-sectional area measures for each cochlear half turn made from mid-modiolar histological cuts from control ears and survival group ears after an endolymphatic sac ablation surgery to create endolymphatic hydrops. Cochlear turn 0.5 corresponds to the base, and 4 corresponds to the apex. Measures in both Panel A and B are estimated marginal means from the mixed model analysis and error bars are standard error of the mean estimates. **Panel B.** Auditory Nerve Overlapped Waveform (ANOW) and compound action potential (CAP) thresholds. *Endolymphatic sac ablation elevated ANOW (< 1 kHz) and 2-4 kHz CAP thresholds in all survival groups, though only 30-day ears had developed histologically measurable endolymphatic hydrops, demonstrating that low- and mid-frequency electrophysiological thresholds identified endolymphatic dysfunction before the development of histologically measurable endolymphatic hydrops.*

Figure 5.

Otoacoustic emission amplitudes (OAEs) from control and survival-day after an endolymphatic sac ablation surgery to create endolymphatic hydrops. Amplitudes are estimated marginal means from the mixed model analysis and error bars represent SEM estimates. Dotted lines are average noise floor measures for each group. **Panel A:** Distortion product otoacoustic emission (DPOAE) amplitudes. Mid-frequency f_2 (5 kHz) DPOAE amplitudes were reduced in all survival-day groups. **Panel B:** Stimulus frequency otoacoustic emission (SFOAE) amplitudes. SFOAE amplitudes were reduced compared to the control group at all probe frequencies for the 1-day survival group only.

Figure 6.

Low- and mid-frequency thresholds shifts related to the severity of endolymphatic hydrops measured histologically in the apical cochlear half of the six out of seven 30-day survival group animals that had histologically measurable endolymphatic hydrops in the cochlear apex. All low-frequency ANOW (0.3 – 1 kHz) and mid-frequency CAP (2 – 4 kHz) thresholds were averaged, and the apical half (i.e., cochlear turns 2.5-4) scala media cross-sectional area measures were summed for individual ears. *Objective measures of low- and mid-frequency hearing loss correlated with the severity of histologically measurable endolymphatic hydrops in the cochlear apex.*

Figure 7.

Hypertonic fixative solution (~2003 mM/kg) enlarged scala media area, likely through osmosis of water. Guinea pig endolymph osmolarity is ~316 mOsm/L. The ~500 mM/kg fixative solution yielded scala media cross-sectional area measures within the nonnal limits. Osmolarity of perfused solutions ought to be comparable to that of scala media when volume measurements are of interest.

Figure 8.

Auditory Nerve Overlapped Waveform (ANOW) amplitudes as a function of tone burst level. ANOW amplitude decreased relative to controls in response to low- and mid-level tone bursts, consistent with statistically significant threshold elevation (Fig. 3B). Measurement made above ~70 dB SPL are not ANOW and have unknown cellular or spatial origin (Fig. 9 of Lichtenhan et al. 2014), so we thus restricted our measurements to ~70 dB SPL for the current study.

Table 1.

Cochlear Turn 0.5 Scala Media Cross-Sectional Area: Control Group vs. Survival Groups

Survival Group	Wilcoxon <i>p</i> -value	S* (n1, n2)
1-day	0.0087	44 (6, 5)
2-day	0.0087	44 (5, 6)
4-day	0.0519	41 (5, 6)
30-day	0.0047	23 (7, 6)

Statistic for Wilcoxon rank-sum test

Table 2A.

Scala Media Cross-Sectional Area, Cochlear Basal Half: Control Group vs. Survival Groups

Survival Group	Mean (SD) (mm ²)	Estimated Marginal Mean Difference Control – Survival Group (mm ²)	Confidence Interval (mm ²)	
			<i>Upper</i>	<i>Lower</i>
Control	0.077 (0.019)			
1-day	0.100 (0.033)	–0.023	–0.071	0.024
2-day	0.118 (0.034)	–0.041	–0.089	0.007
4-day	0.112 (0.044)	–0.035	–0.083	0.012
30-day	0.192 (0.068)	–0.111	–0.155	–0.066

Estimated marginal mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 2B.

Scala Media Cross-Sectional Area, Cochlear Apical Half: Control Group vs. Survival Groups

Survival Group	Mean (SD) (mm ²)	Estimated Marginal Mean Difference Control – Survival Group (mm ²)	Confidence Interval (mm ²)	
			<i>Upper</i>	<i>Lower</i>
Control	0.084 (0.021)			
1-day	0.071 (0.022)	0.013	–0.020	0.046
2-day	0.076 (0.024)	0.007	–0.026	0.040
4-day	0.089 (0.033)	–0.006	–0.039	0.027
30-day	0.149 (0.049)	–0.066	–0.097	–0.034

Estimated Marginal mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 3A.

ANOW Thresholds: Control Group vs. Survival Groups

Survival Group	Mean (SD) (dB SPL)	Estimated Marginal Mean Difference Control – Survival Group (dB)	Confidence Interval (dB)	
			<i>Upper</i>	<i>Lower</i>
Control	24.643 (6.929)			
1-day	39.000 (8.046)	–14.357	–22.494	–6.220
2-day	39.375 (7.418)	–14.732	–22.464	–7.001
4-day	35.750 (11.951)	–11.107	–19.244	–2.970
30-day	36.964 (10.830)	–12.321	–19.750	–4.893

Estimated marginal mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 3B.

CAP Thresholds: Control Group vs. Survival Groups

Survival Group	Mean (SD) (dB SPL)	Estimated Marginal Mean Difference Control – Survival Group (dB)	Confidence Interval (dB)	
			<i>Upper</i>	<i>Lower</i>
Control	29.683 (8.907)			
1-day	45.778 (11.267)	–16.095	–24.421	–7.770
2-day	44.537 (10.257)	–14.855	–22.765	–6.944
4-day	38.000 (12.416)	–8.318	–16.643	0.008
30-day	35.437 (15.467)	–5.754	–13.354	1.846

Estimated marginal mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 4A. High-frequency ($f_2 = 7\text{-}29$ kHz) DPOAE Amplitudes: Control Groups vs. Survival Groups

Survival Group	Mean (SD) (dB SPL)	Estimated Marginal Mean Difference Control – Survival Group (dB)	Confidence Interval (dB)	
			<i>Upper</i>	<i>Lower</i>
Control	13.973 (12.303)			
1-day	3.541 (15.467)	10.432	2.980	17.885
2-day	5.962 (13.681)	8.011	0.931	15.092
4-day	7.780 (15.178)	6.194	−1.259	13.646
30-day	13.377 (12.864)	0.596	−6.207	7.399

Estimated mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 4B. Mid-frequency ($f_2 = 1\text{--}5$ kHz) DPOAE Amplitudes: Control Groups vs. Survival Groups

Survival Group	Mean (SD) (dB SPL)	Estimated Marginal Mean Difference Control – Survival Group (dB)	Confidence Interval (dB)	
			<i>Upper</i>	<i>Lower</i>
Control	8.506 (5.952)			
1-day	–12.353 (11.543)	20.858	10.357	31.360
2-day	–3.689 (9.047)	12.195	2.218	22.173
4-day	–7.445 (14.206)	15.951	5.450	26.452
30-day	–5.346 (14.863)	13.852	4.266	23.438

Estimated mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.

Table 4C.SFOAE ($f=1\text{--}29\text{ kHz}$) amplitudes: Control Group vs. Survival Groups

Survival Group	Mean (SD) (dB SPL)	Estimated Marginal Mean Difference Control – Survival Group (dB)	Confidence Interval (dB)	
			<i>Upper</i>	<i>Lower</i>
Control	–2.137 (10.595)			
1-day	–12.376 (10.200)	10.238	2.957	17.520
2-day	–9.020 (9.534)	6.883	–0.035	13.801
4-day	–5.463 (13.283)	3.326	–3.956	10.607
30-day	–0.432 (13.182)	–1.706	–8.353	4.941

Estimated mean differences of each survival group from the control group. Confidence intervals that include zero identify statistically insignificant results.