Dear Colleagues,

We present abstracts submitted for the 59th Inner Ear Biology Workshop (IEB 2024), which will take place in Warsaw from Sunday, 15 September to Tuesday, 17 September 2024.

The Inner Ear Biology Workshop is organized annually by a group of scientists interested in and actively pursuing the research of inner ear biology. The annual meetings are held in different European academic centers with the objective of a free exchange of scientific accomplishments.

In 2024, the meeting is hosted jointly by the World Hearing Center and the Institute of Sensory Organs.

We wish you a productive and exciting meeting!

Prof. Piotr H. Skarzynski, MD, PhD, MSc
General Secretary of the 59th IEB Workshop

ISSN: 2083-389X
eISSN: 2084-3127

© J Hear Sci, 2024; 14(3): 93–143

59TH INNER EAR BIOLOGY WORKSHOP, 15–17 SEPTEMBER 2024, WARSAW, POLAND

Invited Lectures

Cellular senescence in inner ear physiology and disease

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Cellular senescence, a state of permanent cell cycle arrest, is vital in embryonic development, including inner ear formation. During this process, senescent cells aid morphogenesis by regulating tissue remodeling and ensuring proper cell differentiation. Senescent cells are characterized by distinctive morphological and physiological changes, including enlarged cell size, increased β-galactosidase activity, altered gene expression, and the secretion of various inflammatory cytokines, chemokines, and proteases, collectively known as the senescence-associated secretory phenotype (SASP). Senescence cells secrete factors that shape the development of auditory structures like the cochlea and vestibular apparatus. Deficits in embryonic senescence can lead to malformations and functional impairments in the inner ear. This highlights the importance of regulated senescence for proper inner ear development. Vestibular schwannomas, age-associated benign tumors from Schwann cells of the vestibular nerve, further illustrate senescence's role in ear health. These tumors can cause hearing loss, tinnitus, and balance issues. In tumors, senescence can both suppress and promote growth. While senescence halts uncontrolled cell proliferation, the senescence-associated secretory phenotype (SASP) can create a pro-tumorigenic environment, enhancing tumor survival and progression. Understanding senescence's dual roles in embryonic development and tumorigenesis is crucial for targeted therapies. In embryonic contexts, promoting proper senescence could prevent anomalies. In vestibular schwannomas, modulating senescence and SASP factors may improve outcomes, indicating the potential for senescence-targeted interventions in developmental and neoplastic inner ear conditions.

This work was supported by PID2020-THEARPY and BenBedPhar-COST.

Chronic electrical stimulation may slow down deafness-induced neural degeneration but does not change responsiveness of the auditory nerve

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Introduction: The auditory nerve degenerates after severe damage to the organ of Corti including loss of hair cells and/or synapses. It is often assumed, based on the use it or lose it principle, that activation of the auditory nerve by chronic electrical stimulation (CES), as delivered by a cochlear implant (CI), halts further neural degeneration. However, data in both animal and human literature (e.g., Seyyedi et al., 2013) do not convincingly support that assumption. In the present study we examined the effect of CES on the auditory nerve in deafened guinea pigs, applying both structural and functional measurements.

Material and methods: Normal-hearing guinea pigs received an intracochlear electrode array and were ototoxically deafened four weeks later by co-administration of kanamycin and furosemide. CES treatment started either one or five weeks after deafening, and it was applied for 6 days/week during 2 weeks. The CES stimuli consisted of asymmetric charge-balanced current pulses of 300 μA presented at quasi-random variable pulse rate (0.7–1.9 kHz; 1 kHz on average). Using a MED-EL PULSAR stimulator, awake eCAP recordings were weekly performed as described in Ramekers et al. (2022). Following the final eCAP recording session the animals were sacrificed, and their cochleas were processed for histological quantification of the spiral ganglion cells (SGCs).

Results: SGC survival was similar for the implanted right and the non-implanted left ears in control animals. The SGCs in the implanted ear were significantly larger than those in the non-implanted ear. Animals receiving CES showed a moderate but statistically significant increase in SGC survival in their implanted/stimulated ear compared to the contralateral ear; cell size across ears was similar in these animals. We did not observe a difference in any of the several eCAP outcome measures between CES treated animals and untreated control animals.

Conclusions: CES slows down, but does not stop SGC degeneration, which is consistent with previous studies in animal models and CI users. CES is nor beneficial neither detrimental for the nerve's responses to electrical stimuli as delivered by a CI.

Cochlear stress granules: potential regulators of stress and survival in the inner ear

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Sensory hair cell death is a primary cause of adult-onset sensorineural hearing loss. It is well established that, in mammals, cochlear hair cells are not regenerated and thus hearing loss resulting from hair cell death is permanent. Understanding the mechanisms that determine hair cell death is therefore essential to provide effective therapies for protecting those cells. Stress granules are membrane-free aggregates of mRNA and RNA-binding proteins that form during cellular stress. By controlling the fate of mRNAs, SGs play a key role in the post transcriptional regulation of gene expression during stress. Stress granules assemble rapidly when cells are exposed to stress and normally disperse when the stress is resolved. I will discuss our research on stress granules and their potential role in the homeostatic response of cochlear cells to ototoxic drugs that cause hair cell loss and deafness.

Deciphering the genetic background of autosomal dominant hearing loss

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Introduction: Autosomal dominant hearing loss (ADHL) is the second most common form of inherited HL with an onset usually after the first decade of life. It affects mainly high frequencies and progresses over time. Autosomal-dominant genes are responsible for about 20% of cases of hereditary non-syndromic deafness, with 63 different genes identified to date.

Material and methods: In this study, 105 families with a vertical inheritance pattern of hearing impairment were recruited. Genomic DNA was isolated from peripheral blood samples or buccal swabs of available family members. In all probands targeted next-generation sequencing (NGS) using a targeted multi-gene panel (237 genes) was performed. In 6 largest unsolved families linkage analysis and whole genome sequencing (WGS) were performed. Presence of the selected probably pathogenic variants and their segregation with HL within the family were confirmed by standard Sanger sequencing.

Results: Genetic cause of ADHL was identified in 43.8% (46/105) of the examined families. Among the 46 identified HL variants only 26% (12/46) have been previously reported and the remaining 74% are novel (34/46). We identified missense variants (27/46; 58.7%), splice site variant (9/46; 19.5%), stop-gain variants (5/46; 10.9%) as well as frameshift variants (5/46; 10.9%). Among the most common causative genes were *MYO6* ($n = 8$), *TBC1D24* ($n = 5$), *KCNQ4* ($n = 4$), *GSDME* ($n = 4$), *POU4F3* ($n = 4$) and *WFS1* ($n = 4$). Pathogenic variants causative of HL in the *NLRP3*, *LMX1A*, *FGFR3*, *CD164*, *GRHL2*, *TMC1*, *COCH*, *ATP2B2* and *CEACAM16* genes were detected in single families. Implementation of linkage analysis and WGS resulted in the identification of the non-coding variants in the *EYA4* and *ATP11A* genes and two novel candidate genes.

Conclusions: Our custom multigene panel has demonstrated good diagnostic performance. Considering frequent identification of novel genetic variants it is necessary to perform thorough clinical examination and variant segregation analysis with ADHL in all available family members. The use of linkage analysis and WGS increases the detection rate of causative variants, especially located in the non-coding regions, and provides the opportunity to identify novel genes.

Grant: 2016/22/E/NZ5/00470 National Science Centre, Poland.

History of the Inner Ear Biology Meeting

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The IEB's inaugural assembly convened under the name of "Arbeitstagung für Innenohrbiochemie" (Ear Biochemistry) in 1964 within the environs of Düsseldorf, Germany, orchestrated by Sigurd Rauch (1916–2003), gathering a select cohort of burgeoning otolaryngologists with a proclivity for scholarly inquiry. Spanning the period from 1964 to 1967, four symposia on Inner Ear Biochemistry were convened in Germany, marking the nascent stages of a burgeoning scholarly endeavor. However, the intellectual purview of these gatherings swiftly transcended national boundaries, metamorphosing into an international discourse. The seminal year of 1968 witnessed a pivotal milestone as the venue shifted beyond the confines of Germany, alighting upon Zürich, concurrently accompanied by an appellation modification from "Workshop on Inner Ear Biochemistry" to the more encompassing "Workshop on Inner Ear Biology". Since its seminal expansion, the convocation has traversed a series of locales and nations annually, responding to invitations from a diverse array of hosts. Attendance burgeoned precipitously, only to plateau subsequently, stabilizing at a robust cadre numbering between 100 to 200 attendees. The workshops have perennially exuded a distinctive ambiance, meticulously nurtured and preserved throughout successive iterations. Their paramount objective, both then and now, remains the fostering of an environment conducive to candid exchange, characterized by affability interwoven with rigorous intellectual debate. This ethos facilitates the cultivation of enduring professional connections, even amongst dissenting viewpoints. Notably, the workshop serves as a veritable nexus for biologists and otolaryngologists alike, fostering interdisciplinary discourse and collaborative problem-solving. Otolaryngologists, notwithstanding their clinical commitments, assume a pivotal role in fostering cohesion amidst this interdisciplinary amalgamation, thereby augmenting the workshop's enduring legacy.

Identification of feedforward/feedback contributions to age-dependent hearing loss and tinnitus using OPM-MEG

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Acquired auditory processing disorders including age dependent hearing loss, speech discrimination deficits, tinnitus or hyperacusis, require a personalized diagnosis to assign the individual cause within the auditory hierarchy to either the periphery, subcortical or distinct cortical or cortico-fugal neuronal dysfunctions. The well-functioning feedforward and feedback PV-IN network is an essential precondition for temporal intracortical network function in audition that above all senses relies on high speed of information flow (Zajac I.T. and Nettelbeck T., 2018). We hypothesize disease-specific deficits in temporal intracortical network function in auditory circuits. Therefore, the diagnostic of those should have a special significance. We used time-sensitive MEG-OPM measurements and aimed to study different auditory stimulus paradigms to detect fast auditory processing in different groups of tinnitus with and without hyperacusis or presbycusis. We expect this method to become an efficient diagnostic strategy to fathom peripheral or central contribution of the distinct auditory impairments in the future to improve individualized targeted interventional therapies. Here we will present preliminary results demonstrating the usability and function of the OPM-MEG for hearing research.

This work was supported by the Deutsche Forschungsgemeinschaft DFG KN 316/13-1, DFG RU 713/6-1, ERA-NET NEURON JTC 2020: BMBF 01EW2102 CoSySpeech and FWO G0H6420N.

Irx3/5 null deletion in mice blocks cochlea-saccule segregation

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Introduction: A gene cadre orchestrates the normal development of sensory and non-sensory cells in the inner ear, segregating the cochlea, the organ of Corti, and five vestibular endorgans. Evolution transforms the basilar papilla in sarcopterygians into the cochlea in mammals. However, the role of genes driving the ear development is largely unknown.

Material and methods: We used double null mice for Iroquois homeobox 3 (Irx3) and 5 (Irx5) transcription factors (Irx3/5 DKO). Mice can survive to about E16.5, after that they occasionally can reach at E17.5.

Results: We show that double deletion of (Irx3/5 DKO) mice leads to the fusion of the saccule with the cochlear base. The medial rows of cochlear HCs in the expanded sensory epithelium assumed vestibular-like hair cells near the modiolus while others seem like cochlear hair cells. The otoconia and tectorial membranes are needed for normal function but are absent in the Irx3/5 DKO inner ear. The mutant cochlea showed a reduced spiral ganglion neuron population, which projects fibers to both saccular- and cochlear-like HCs. The central projections from the cochlear apex-base contour are not fully segregated into a dorsal and ventral innervation in the Irx3/5 DKO cochlear nucleus. An expansion of the cochlear dorsal nuclei in the brainstem reaches vestibular fiber connections only in the Irx3/5 DKO. Additionally, the auditory and vestibular systems in Irx3/5 DKO mice are interconnected, characterized by the formation of bilateral connections between the descending vestibular and ascending apex that also demonstrates a unique interconnectedness between the cochlear apex and the vestibular neurons, a "vestibular-cochlear" nerve (VCN) in the mouse inner ear.

Conclusions: We suggest that it indicates the mammalian cochlear apex, which is a derived from the lagena. Further, a newly bilateral connection between the vestibular and apex are reminiscent of sarcopterygians based on fibers and neurons.

The cognitive ear: the emerging role in the aging

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Hearing impairment is known as a major clinical risk factor for cognitive decline, with relevant clinical implications for dementia prevention, diagnosis, and treatment. However, the complex pathophysiological relation between hearing impairment and dementia remains to be fully defined. Consistently with the concept of an "hearing health trajectory," beginning at conception/birth and continuing throughout life in which environmental factors, such as noise, medicaments, and lifestyles (e.g., alcohol, smoking, diabetes, and weight gain), contribute to affect hearing. Several studies identified the exposure noise-induced hearing loss (NIHL) as a risk factor for sensory aging and cognitive decline processes. Although the association among age related hearing loss (ARHL), NIHL, and cognitive impairment has been clinically widely documented the molecular mechanisms underlying this association are not fully understood, and it is not known how these risk factors (sensory aging and noise) can interact, affecting brain functions. We recently found that early noise exposure in an established animal model of ARHL (C57BL/6 mice) accelerates the onset of age-related cochlear dysfunctions. While an animal model of Alzheimer's disease (AD), that is the 3 × Tg-AD mice we found that NIHL before that phenotype is manifested, caused persistent synaptic and morphological

alterations in the auditory cortex and earlier hippocampal dysfunction, increased tau phosphorylation, neuroinflammation, and redox imbalance, along with anticipated memory deficits compared to the expected time-course of the neurodegenerative phenotype. Furthermore, in the WT mice also HL, can accelerate ARHL onset inducing persistent synaptic alterations in both auditory cortex and hippocampus affecting memory performance and oxidative-inflammatory injury. Collectively, our experimental data confirm the existence of "cognitive ear" that can be early affected, thus midlife HL can be responsible for a hippocampal-dependent memory dysfunction. Considering that memory dysfunction is usually the first cognitive symptom of dementia (like AD) onset, from a translational point of view, our results support the hypothesis that associating auditory and memory screenings could represent a powerful non-invasive tool to potentially identify subjects with a high risk to develop dementia, allowing early diagnosis and treatment.

The impact of OAEs on hearing science and the inner ear research

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Otoacoustic Emissions (OAEs) are vibrational patterns which are recorded in the external meatus, upon stimulation of the ear by a transient or sinusoidal signal. They were discovered by David Kemp in 1978 but their true potential in gauging the functional status of the cochlear amplifier never truly shined and thus this tool never made the impact we were hoping to make, back in the 80s. This lecture will present an excursus of some interesting developments the last 45 years, based on otoacoustic emissions in three key areas of hearing science, such as: (i) screening for hearing deficits (neonatal screening, adult screening, drug efficiency monitoring etc.); (ii) pharmacology testing models; (iii) inner ear modelling.

The resident mast cells are a component of the cochlear immune system

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The immune system is often conceptualized as the body's internal police force. Its innate components (macrophages, dendritic cells, neutrophils, eosinophils, basophils, mast cells, and the complement system), in conjunction with the acquired immunity (T cells, B cells), are responsible for the surveillance of the body, discrimination between "self" and "non-self," recognition of pathogens and toxins, and appropriate defense if needed. In addition to the classic lymphoid tissues, such as the thymus, spleen, and lymph nodes, immune system cells are found throughout the body, including the cochlea. Several publications have reported the existence of resident immune cells in the human and rodent cochlea, but their role in cochlear biology remains unclear. Our research group has concentrated on characterizing two cochlear cell types representing the innate immune system: the microglia and the mast cells. Microglia develop from yolk-derived mesodermal precursors and reside exclusively in the central nervous system. Recently, research has identified microglia in the cochlea based on the expression of the Iba1 surface molecule. To gain further insight into the nature of these cells, we employed immunofluorescence, confocal microscopy, and Western blot techniques to characterize them using another marker for microglia (TMEM119). Our findings indicate that in the murine cochlea, Iba1+ TMEM119+ double-positive cells are absent before and after the onset of hearing and after acoustic trauma in adult mice. In contrast, the second cell type, mast cells, were present in the developing and adult rodent cochleae, as evidenced by the expression of several markers, including heparin, mast cell tryptase, mast cell chymase, and IgE receptor. Their numbers were sparse, but inhibiting their degranulation reduced cisplatin-induced damage to auditory hair cells. Furthermore, adding the supernatant from degranulated mast cells resulted in the loss of inner and outer hair cells. Moreover, we identified all four receptors for the major mast cell mediator, histamine, in the murine cochlea. Furthermore, our systematic review demonstrated the association between allergies, sudden sensorineural hearing loss, Meniere disease, and acute low-tone hearing loss. We conclude that mast cells, but not microglia, are resident cells in the rodent cochlea and that their activation can contribute to cochlear pathologies.

Oral Presentations

350 families' whole genome sequencing in early onset hearing loss: the French Reference Centre's experience

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Introduction: Up to recent years, diagnosis of the genetic aetiology of early onset hearing loss (EOHL) relied on targeted gene panel analyses, with a diagnosis rate of about 50%. French clinical geneticists have now access to trio whole genome sequencing (WGS) through the Plan France Medecine Genomique 2025 sequencing platforms for most patients presenting with EOHL.

Methods: Testing criteria for WGS differ according to the subgroup of EOHL indications. The strategy is genome-first for syndromic hearing loss (HL), WGS after normal array-CGH for HL associated with malformation(s) and WGS after normal GJB2/GBJ6 and HL gene panel for patients presenting with isolated HL.

Results: From 2020 to mid-2024, 350 families assessed in the French Reference Center for genetic HL underwent whole genome sequencing on the SeqOIA platform. The diagnosis yield was 40%. We will present the main genes identified, the diagnostic rate according to subgroups of the EOHL indication, clinical vignettes, along with the advantages and limitations of our strategy.

Conclusions: WGS allows for identification of additional causes (class 4 and 5 variants) and candidate genes, but variants interpretation is more complex than exome or gene panel analysis. We show with our four-and-a-half years' experience that WGS is an enticing new tool for patient diagnosis, with its advantages and setbacks.

A mouse model of unilateral stereotactic radiosurgery-induced hearing loss

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Introduction: Stereotactic radiosurgery (SRS) is a precise, single-session irradiation technique commonly used to treat vestibular schwannomas. However, SRS can lead to irreversible hearing loss, most probably due to irradiation-induced damage to the nearby inner ear. Currently, no preventive or therapeutic options exist, highlighting the need for the development and experimental testing of novel treatments. To enable this research, we developed a protocol for inducing unilateral hearing loss in mice through targeted unilateral cochlear irradiation.

Material and methods: We used 6-week-old C57BL/6J mice and administered precise unilateral irradiation in the vicinity of the cochlea using a Leksell Gamma Knife® Icon device. The precision and reproducibility of the targeted area were ensured through radiological imaging for each mouse using the integrated cone beam CT scan and co-registering these images with MRI and CT mouse atlas images. To ensure meaningful translational data, we placed a single 4 mm isocenter lateral to the cochlea with the 80% isodose line passing through the modiolus to deliver 8 ($n = 3$), 16 ($n = 5$), 24 ($n = 8$), and 32 ($n = 8$) Gy. Auditory brainstem responses (ABR) were measured one day prior to irradiation (baseline) and at one and four weeks post-irradiation. Statistical analysis was performed using two-way repeated measures ANOVA with Bonferroni correction.

Results: In all experimental groups, the irradiation dose received by the non-irradiated cochlea was less than 15% of that received by the irradiated cochlea. In the 32 Gy group, irradiation of cochlea yielded significant threshold shifts, compared to the non-irradiated ear, at 22.6 and 32 kHz on day 7 and to a greater degree on day 28. Similar but less pronounced effects were observed in the 24 Gy group. Furthermore, we observed a unilateral decrease in the *p-p* and wave I amplitudes, following 72–78 dB SPL click stimulation, in both groups. No hearing loss was detected in the 8 and 16 Gy groups. Histopathological studies are ongoing.

Conclusions: Targeted near-cochlear irradiation in mice induces unilateral dose-dependent high-frequency hearing loss,

evidenced by increased threshold shifts and decreased ABR wave I amplitudes. This model provides a valuable tool for exploring the radiobiological mechanisms underlying SRS-induced hearing loss and for testing potential radioprotective agents.

A one-year time course of electrocochleography in cochlear implant users

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Introduction: Electrocochleography (ECochG) has become increasingly valuable in cochlear implant (CI) surgery as it allows intraoperative monitoring of effects of electrode array insertion on residual hair cell function. Additionally, ECochG has the potential to portray postoperative cochlear function. Here we examine the postoperative time course of residual hair cell function by recording ECochG in CI recipients intraoperatively and at several time points up to one year postoperatively.

Material and methods: Twenty-three patients with severe sensorineural hearing loss receiving a CI participated in a trial comparing electrode arrays (SlimJ and Mid-Scala of Advanced Bionics) and surgical approaches (Jwair et al., 2021). Here, the data are analysed by investigators blinded to the randomisation. ECochG recordings were performed at 5 time points using the active insertion monitoring (AIM) system of Advanced Bionics: intraoperatively, at 4–6 weeks, 3–4 months, 6–7 months and 12–14 months postoperatively. Responses to pure-tone stimuli with frequencies varying from 125 to 4000 Hz, at sound levels 100–115 dB HL, were recorded at each of the 16 available electrodes, in two opposite phases. The difference and sum of the recordings to the opposite phases were computed as estimates of cochlear microphonics (CM, reflecting hair cell potentials) and auditory nerve responses, respectively.

Results: Significant CM responses (>1.5 μV) were found in 12 out of 22 patients intraoperatively, and postoperatively in 10/22 at 4–6 weeks, 12/16 at 3–4 months, 11/16 at 6–7 months, and 7/11 at 12–14 months. Notably, 7 intraoperative non-responders showed significant responses in the first postoperative session. Averaged across frequencies the largest responses were observed recorded at the apical electrodes during the intraoperative session. The responses were smallest at 4–6 weeks postoperatively, then recovered after 3 to 7 months, and decreased again up to one year after implantation. Intraoperatively, the dominant frequency was 500 Hz, postoperatively it was 125 Hz.

Conclusions: Residual hair cell function gradually declines over the course of one year. Three months after cochlear implantation a short period of recovery is seen, which may be attributed to natural reduction of the acute local inflammatory response to the electrode array.

AAV-mediated precision treatment of SchABE8e in the pou4f3Q113*/+ mouse model

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Genetic mutations can cause hereditary deafness, among which DFNA15 caused by mutations in the transcription factor *POU4F3* is a clinically common autosomal dominant non-syndromic deafness, and studies have been conducted to partially restore hearing in model mice by small molecule inhibitors. However, this strategy fails to address the root cause of hearing impairment. Base editors are capable of editing mutation sites precisely and efficiently, and have the potential to completely restore hearing damage. In order to verify the effectiveness of gene editing for the treatment of this deafness disease, taking the Chinese DFNA15 (POU4F3Q113*) deaf family line as an example, we successfully constructed the Pou4f3Q113*/+ mouse model with the progressive hearing damage of this type of patients, which provides a good animal model for the study of this type of genetic deafness disease. On the basis of the three highly efficient Cas9 types discovered in the laboratory in the previous stage, a series of novel ABE toolboxes were developed through the fusion of multiple types of TadA deaminases. The test screened SchABE8e base editor in vitro can realize precise and efficient editing at the pathogenic mutation (up to 50% efficiency). Compared with ABE8e, SchABE8e has higher editing efficiency and lower off-target mutations and indels, which is safer and more precise. Subsequently, by analyzing the secondary structure of SchABE8e protein, the optimal splitting site 4 was screened to achieve similar protein expression as WT SchABE8e in the HEK293T cell. Delivery of split SchABE8e/NNGR-Target3 into the cochlea of neonatal mice by double AAV-Anc80L65 achieved long-term effective hearing recovery (hearing level close to that of WT mice) in Pou4f3Q113*/+ mice. This project provides a new strategy for the treatment of DFNA15 disease in the clinic, and the development of SchABE8e also provides a novel tool option for gene therapy of other hereditary deafness diseases.

AAV-OTOF gene therapy for autosomal recessive deafness 9: a multicenter, multiage, non-randomized controlled intervention study

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Introduction: Autosomal recessive deafness 9 (DFNB9) is a congenital auditory neuropathy with clinical features including congenital or prelingual, bilateral symmetry, severe to complete deafness, caused by OTOF mutations. We previously reported the safety and efficacy of adeno-associated virus (AAV) mediated OTOF (AAV-OTOF) delivery in children for the first time worldwide. It is believed that AAV-OTOF can restore hearing in infants and children with DFNB9, but there have been no safety and efficacy studies in elder participants. This study enrolled the adolescent and adult participants and aimed to investigate the association of age with safety and efficacy of AAV-OTOF gene therapy.

Material and methods: This study is a multicenter, open-label, single-arm and intervention trial. We recruited 9 DFNB9 participants with age diversity (1.8- to 23.9-year-old) from 4 Chinese sites. All participants carry biallelic OTOF mutations with severe to complete hearing loss, unilateral or no cochlear implantation. Participant 3 received two rounds of AAV-OTOF injection. Single injection of AAV-OTOF into the inner ear was performed in other 8 participants. The follow-up period was from July 2023 to May 2024. The primary outcomes were safety and tolerability. Secondary outcomes included auditory function assessments.

Results: We present a relevant evaluation of safety and efficacy in 9 participants 2–9 months after AAV-OTOF treatment. No serious adverse events (AEs) occurred in the dose of AAV-OTOF at 8.4×10^{11} to 1.12×10^{12} vg. No serious drug-related AEs (AEs) were observed with a total number of 8 of grade I and II AEs. Hearing recovered in 8 participants after surgery. At 1 month after surgery, the mean click ABR threshold for participants decreased from >99 dB at baseline to 56.7 dB. Notably, the thresholds of click-ABR, tone-burst ABR (TB-ABR) and pure tone audiology (PTA) thresholds for the 23.9-year-old adult participant decreased from >100 dB, >100 dB, and 93.6 dB at baseline to 70, 81, 67.9 dB at 1 month, respectively. We found that the hearing recovery effects showed a potential age correlation. Participants with hearing recovery were divided into three groups by age (1: 1–2 years; 2: 5–8 years; 3: >14 years). The thresholds of click-ABR (PTA) in the 3 groups were improved by 45 dB (7.9 dB), 51.8 dB (56.7 dB) and 20 dB (25.7 dB) at 1 month, respectively. The thresholds of TB-ABR in group 2 and 3 improved from >99 dB at baseline to 57 dB and 69 dB at 1 month, respectively. Overall, participants aged 5–8 years had a better hearing recovery.

Conclusions: In this trial, AAV-OTOF gene therapy was proved as a safe and effective treatment for infants to adult patients with DFNB9, which appears to be age-related therapeutics and indicated a large treatment window of AAV mediated gene therapy.

The trial has been registered on ClinicalTrials.gov, NCT 05901480, and is ongoing.

Ageing, noise, and ER stress: exploring stereocilia fusion pathology in the cochlear outer hair cells with super-resolution expansion microscopy

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Healthy hearing is fundamentally dependent on the stereocilia bundles of cochlear hair cells. Recent studies on human post-mortem cochleas have shown that ageing is associated with structural impairment of these hair bundles, specifically in outer hair cells (OHCs) (Wu and Liberman, 2022). Genetic

defects and environmental noise challenge the maintenance of the OHC hair bundle structure, contributing to age-related hearing loss. Several prior studies have described stereocilia fusion as a hair bundle pathology, yet often without a clear definition of this abnormality and its molecular mechanisms. Here, we aimed to elucidate the molecular anatomy of OHC stereocilia fusion by studying mouse models of ageing, prolonged noise-exposure, and cell-intrinsic stress caused by genetic perturbations. We utilised a novel imaging method to cochlear research, expansion microscopy, to generate super-resolution data of the OHC hair bundle structure and protein expression. Ageing in the C57BL/6J mice exhibited mild OHC stereocilia fusion, in most cases restricted to the lateral edges of hair bundles, indicating gradual progression of the fusion pathology. OHCs of young adult C57BL/6J mice exhibited elevated likelihood of stereocilia fusion following eight hours of daily, moderate-level noise-exposure (90 dB SPL) over the course of a week, with no evidence of recovery over one-month post-trauma period. Most severe phenotype was found in the genetic mouse model of perturbed endoplasmic reticulum homeostasis (ER stress), exhibiting adult-onset OHC stereocilia fusion with rapid progression to prominent fusion covering the whole hair bundle (Herranen et al., 2020; Ikäkeimo et al., 2021). This severe pathology correlated with reduced FM1-43-dye uptake through the mechanotransduction channels, loss of key stereociliary proteins (neuroplastin, PMCA2, myosin 7a, BAIAP2L2), and increased expression of the calcium buffer oncomodulin in the stereocilia, indicative of a major disturbance to mechanotransduction and to the Ca^{2+}-balance required for stereocilia maintenance (Ikäheimo et al., 2024). These hair bundle abnormalities preceded OHC death, suggesting a window of opportunity to intervene with the maintenance of hair cell survival. We conclude that understanding the molecular anatomy of the hair bundle pathology might facilitate the development of targeted therapies for maintaining bundle integrity or to promote bundle repair.

Alteration of the gut microbiome causes sensorineural hearing loss by increasing blood-labyrinth barrier permeability and cochlear inflammation through the gut-cochlear axis

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Recent advances in neuroscience have revealed a bidirectional communication between the gut microbiota and the central nervous system (CNS), known as the "gut–microbiota–brain axis." Imbalance in the gut microbiota, called dysbiosis, can increase intestinal permeability, allowing pathogens to trigger inflammation in distant organs. Despite these established connections, no research has explored the link between gut microbiota alterations and inner ear function. To address this gap, this study delved into the molecular mechanisms underlying a potential association between gut microbiota alterations and sensorineural hearing loss (SNHL). To this aim, we used a mouse model of gut dysbiosis induced by dextran sulfate sodium (DSS) treatment, supplemented with fecal microbiota transplantation (FMT) from donor patients with active (aUC) or remissive (rUC) ulcerative colitis. This enabled us to exacerbate or ameliorate the microbiome imbalance, respectively. Auditory brainstem responses (ABRs) were conducted alongside morphological, immunofluorescence, and molecular analyses. ABR results revealed a significant increase in auditory thresholds in mice subjected to DSS and FMT-aUC treatments. Conversely, FMT from rUC donors exhibited a protective effect on auditory function, highlighting the beneficial impact of microbiota restoration. Morphological evaluations revealed loss of outer hair cells (OHCs), degeneration of spiral ganglion neurons (SGNs), and atrophy of the stria vascularis in mice with gut dysbiosis. Conversely, FMT from rUC donors displayed a protective effect on cochlear structures. Immunofluorescence and Western blot analyses unveiled increased oxidative stress and inflammation in cochlear tissues of mice with gut microbiota alterations, while restoration of microbiota composition exerted otoprotective effects. These findings were associated with disruptions in the integrity of the blood-labyrinth barrier (BLB), characterized by altered expression of tight junction proteins (ZO-1 and Occludin), Na^+/K^+-ATPase levels, along with increased of pericytes damage and vascular permeability in mice with altered microbiota composition. Overall, these results suggest a mechanistic link between gut microbiota alterations and SNHL through oxidative stress and inflammation mediated by changes in BLB permeability. This study provides experimental evidence supporting the existence of a gut-cochlear axis and highlights the potential therapeutic implications of restoring gut microbiota balance in mitigating hearing impairment associated with gut dysbiosis.

Antisense oligonucleotides for dominantly inherited hearing impairment DFNA9: from cells models to humanized mice

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The c.151C>T (p.P51S) mutation in *COCH* is highly prevalent in the Dutch/Belgian population and causes DFNA9 (hearing loss and vestibular dysfunction) in > 1500 individuals. The initial symptoms manifest between the 3rd and 5th decade of life, which leaves ample time for therapeutic intervention. The clear non-haploinsufficiency disease mechanism indicates that blocking or reducing the p.P51S mutant cochlin protein levels may alleviate or prevent the DFNA9 phenotypes.

Considering the broad expression of *COCH* by the fibrocytes of the inner ear, we designed "gapmer" antisense oligonucleotides (ASO) to specifically induce RNase H1-mediated degradation of *COCH* transcripts containing the c.151C>T mutation. We established several model systems to investigate the molecular efficacy of ASOs targeting the c.151C>T mutation or low-frequency mutant allele-specific SNPs.

Using overexpression models, we identified several ASOs that efficiently induce the degradation of mutant *COCH* transcripts. By introducing chemical modification to the oligonucleotide bases, we can alter the affinity and selectivity for the mutation transcript. We identified several ASOs with a strong preference for the mutant transcript in overexpression models. To investigate allele-specificity under physiological expression levels, we exposed patient-derived otic progenitor cells (iPCS-OPCs) to different ASOs for 8 days. In parallel, we developed a genetically humanized mouse model for DFNA9 in which human sequence-specific therapeutic strategies can be evaluated. Phenotypic follow-up of mice of all genotypes indicate that the genetic humanization has no adverse effects, and removal of the Cdh23ahl allele is mandatory to observe the late-onset auditory phenotype: the first signs of high-frequency hearing loss emerged at 12 months of age.

Studies in iPSC-OPCs indicated that the ASOs identified in overexpression studies also effectively reduce mutant *COCH* transcript levels in patient-derived cells with physiological expression levels. Unfortunately, variation between replicate wells of OPC differentiation is relatively high, making it difficult to draw conclusions on allele-specificity. We selected a candidate ASO, directed against a rare mutant allele-specific intronic SNP, for subsequent studies in our humanized mouse model. First intracochlear injections will be conducted in May 2024, after which we can collect the first in vivo data on gapmer ASO uptake and efficacy in fibrocytes of the mammalian inner ear.

Assessing the cognitive decline post hearing loss

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Hearing loss is known to exacerbate cognitive decline in humans. However, the variability in the impact of cognitive function is large, making predictions and drawing causal links difficult. We tested cognitive function in mice using an 8 arm radial maze (8ARM) that has no auditory cues and found that deafened mice showed a dramatic deficit in working memory. We used a hearing loss model that placed the human diphtheria toxin receptor (HDTR) in inner hair cells (IHCs) so that upon exposure to diphtheria toxin (DT), IHCs were selectively ablated. Despite this very uniform and complete deafening, there was a large variance in cognitive function loss, implicating a biological cause to the variance. We are creating a battery of behavioral tests to better investigate this variance and to test the fundamental hypothesis that the impact of hearing loss is in part dependent on how the individual animal weights hearing in sensory integration circuits. This battery of tests has the added advantage of being utilized for assessing the impact of hearing restoration treatments and for defining thresholds of hearing loss that lead to cognitive deficits. We will include the Y-maze and Novel object recognition test (NORT) as tests for memory both short term and consolidation and with the added advantage of repeated measures. We are including visual and auditory training to assess both learning and acuity as a proxy for sensory weighting. And finally, we are measuring auditory evoked potentials in awake animals in an attempt to identify correlations that can be used as predictors of cognitive function sensitivity.

Biosafety and biodistribution study of vesicle-enriched secretome fractions in cochlear implantation trauma

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Introduction: Cochlear implantation induces trauma leading to immunological reactions compromising the performance of the implant. Vesicle-enriched secretome fractions (VSF) derived from human umbilical cord mesenchymal stromal cells is a new class of biological therapeutic targeting cochlear cells to modulate immunological processes. Previously, efficacy in spiral ganglion cell culture and attenuation of threshold shifts and protection of hair cells in vivo were shown. Safety, biodistribution and neuroprotective effects of VSF to maintain gross structural integrity of the cochlea after implantation trauma has been evaluated.

Material and methods: Hearing guinea pigs (GP) were implanted with a cochlear implant followed by administration of VSF and hearing was assessed until sacrifice after 4 weeks. A long-term group of hearing GP received VSF with a six month follow up of monthly hearing measurement. A third group of hearing GP received labelled VSF investigating distribution of VSF in the inner ear 1–2 hours post application. All groups underwent detailed histological assessment.

Results: Treatment with VSF improved hearing after cochlear implantation compared to control animals. Fibrosis 4 weeks post implantation was at similar level to control animals. Electrophysiological and histological findings of the long-term group revealed no adverse effects of VSF and ABR thresholds remained on a similar level compared to control. Positive fluorescent signal of labelled VSF was confirmed in cells of the inner ear.

Conclusions: Uptake of labelled VSF into the guinea pig cochlea has been demonstrated and treatment with VSF seems to mediate immunological processes to a healthier state and maintain gross structural integrity. Application of

VSF associated with cochlear implantation seems to be a safe and solid combination to prevent post implantation trauma and preserve residual hearing.

This work was funded by Med-El, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2177/1 – Project ID 390895286 and by the European Regional Development Fund Interreg V-A Italia–Austria 2014–2020 (Project "EXOTHERA IT AT 1036") and the Project "ExtraNeu" from the State of Salzburg.

Can you hear without FIRE: The impact of microglia loss on cochlear function

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The immune system is known to critically regulate the central nervous system during development, homeostasis, as well as during injury and repair. More recently, much work has been undertaken to characterize the role of immune cells in the cochlea. Macrophages are the resident innate immune cells of the cochlea. Several studies on mice have reported that macrophages mediate drug uptake via the stria vascularis, promote synaptic repair after noise exposure, and participate in tissue remodeling after cochlear implantation. In other organs macrophages can be heterogenous. Whether there are subpopulations of macrophages in the cochlea and the function of such subgroups are unknown. Here we report molecular heterogeneity among cochlear macrophages which include a population resembling microglia, the primary innate immune cells of brain and two molecularly distinct subpopulations of macrophages resembling two recently identified macrophage population in peripheral nerve. To begin to ascertain the function of one of these populations we examined FIRE mice (Csf1rΔFIRE/ΔFIRE), a mouse model that is entirely microglia- deficient but retains other macrophages which lacks embryonic macrophages and microglia We examined cochlear histology at postnatal days 5, 14, 6 weeks and 6 months of age. Surprisingly, FIRE mice had lower auditory brainstem response thresholds and higher wave I amplitudes when compared to aged-matched control mice at both 6 weeks and 6 months, suggesting less aged-related hearing loss. However, the FIRE mice number,organization of inner and outer hair cells and inner hair cell synapse were similar to aged-matched control C57/Bl6 mice. Taken together, these findings suggest that microglia are dispensable for cochlear development but may play a role in age-related hearing loss, a role which requires further investigation.

Causes of bilateral sensorineural hearing loss in 838 patients according to degree of progression of hearing loss

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Purpose: This study aimed to investigate the etiology of hearing loss (HL) based on the rate of its progression.

Material and methods: Pure tone audiometry was conducted on 42,744 tests (9,269 cases) at Shinshu University Hospital between 2012 and 2022. Cases with unilateral HL, conductive HL, postoperative ear issues, functional hearing loss, and those with bilateral scaling-out at initial examination or lacking detailed data were excluded. A retrospective review was performed on 838 cases followed for over 5 years. Hearing progression was categorized as "Stable" (0–1 dB HL/year), "Slow progression" (1–3 dB HL/year), and "Fast progression" (>3 dB HL/year). Causes were classified as genetic, middle ear disease, Meniere's disease-related, other known causes, and unknown etiology.

Results: Of the 838 cases, 302 were classified as "Stable", 402 as "Slow progression", and 134 as "Fast progression". Across all groups, unknown etiology was the predominant cause of HL, followed by genetic factors in Stable and Slow progression groups, and others in the Fast progression group. Regarding genetic etiology, the *GJB2* and *STRC* genes were most prevalent in the Stable group, while genes such as *SLC26A4* and *CDH23* were identified across all groups.

Conclusions: Regardless of progression rate, over half of bilateral HL cases had an unknown etiology. Genes such as *GJB2* and *STRC* were identified to exhibit stable hearing, consistent with our findings. This study suggests that genes responsible for progressive HL manifest in an intermittent, rather than continuous, manner, highlighting the clinical utility of genetic testing, particularly in cases lacking long-term follow-up data.

Cochlear health in a cohort of cochlear implant users carrying the p.Pro51Ser variant in the *COCH* gene (DFNA9): a cross-sectional study evaluating the changes in the electrically evoked compound action potential (eCAP)

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The present study focuses on DFNA9, an autosomal dominant disorder caused by pathogenic variants in the *COCH* gene. These mutations induce the formation of aggregates that are toxic to the fibrocytes in the extracellular matrix, ultimately leading to degeneration of spiral ganglion neurons (SGNs), which are crucial for transmitting auditory signals from the cochlea to the brain. An important tool for evaluating the function of the SGNs, which are the target cells of a cochlear implant (CI), is the electrically evoked compound action potential (eCAP). Therefore, the main objective is to evaluate the eCAP to describe the function of the SGNs and study cochlear health in CI patients with DFNA9.

For this reason, we included 15 carriers of the p.Pro51Ser variant in the *COCH* gene who received a Med-El CI (DFNA9 group) and 15 matched control CI subjects without DFNA9 to compare the impedances and subsequently the threshold, amplitude and slope of the eCAP amplitude growth function (AGF). These parameters were evaluated from intraoperative autoART recordings (Med-El) during CI surgery. Matching of the two groups was based on sex, age at implantation, duration of deafness, and type of implant. The first results, regarding the difference in impedance between DFNA9 and non-DFNA9 patients, show a significant interaction between time and group in the middle and basal electrodes, indicating that electrode impedances were similar in the early phase after implantation between the two groups, but increased significantly more for the DFNA9 group up to one year after implantation. Secondly, the results show that the success rate (present or absent) to record eCAP responses is lower in the DNFA9 group: eCAPs were detectable in 75.5% of the intraoperative measurements (145/192) in comparison to 96.9% (186/192) in the group without DFNA9. ECAP absence in the DFNA9 group was observed across the whole electrode array, but more pronounced in the basal region (channels 11 and 12). Additionally, comparing the parameters of the AGF, the maximum eCAP amplitude was consistently smaller and the AGF slope consistently shallower for the DFNA9 group compared to the control group throughout the entirety of the electrode array. Finally, the eCAP thresholds in patients with DFNA9 were higher compared to those in the control patients for all cochlear locations. To our knowledge, this is the first study to investigate the eCAP measurements in patients with DFNA9. As proven in the literature, eCAP measures correlate well with the health and survival of SGC. This means that the results of our study predominantly suggest that DFNA9 leads to an even stronger reduction in excitability and neuronal health than seen in other causes of deafness.

Development of a ouabain-induced hearing loss guinea pig model for preclinical efficacy assessment

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Ouabain is a cardiac glycoside acting as a Na^+/K^+-ATPase inhibitor. It is widely used in the treatment of congestive heart failure and arrythmia, but currently less used due to its narrow therapeutic index. Several animal studies demonstrated ouabain's ototoxicity, its contribution to spiral ganglion neuron (SGN) loss and subsequent hearing loss. The objective of this study was to compare various methods of ouabain administration in the middle and inner ear to characterize its ototoxic effects in guinea pigs. Different routes of administration and doses of ouabain were tested: 1, 3 and 10 mM administered via round window injection (RWI), a round window membrane deposit (RWM) or transtympanic administration (TT). Several parameters were assessed, from the injection volumes (5, 10, 20 µL), to the injected ear (L/R) and the repetition of administration (up to 3 times). Specific care was provided to the treated guinea pigs with consideration to hydration and food supplementation. ABR thresholds and DPOAE amplitudes were measured between baseline (BL) and T+7DAYS or T+14DAYS to evaluate hearing impairments. These functional measures were correlated with histological analyses, SGN counts, inner hair cell (IHC) and outer hair cell (OHC) counts. Both RWI and RWM, at 3 and 10 mM, induced important hearing loss demonstrated by a significant increase of ABR thresholds followed by considerable SGN loss. Moreover, a decrease of DPOAE amplitudes and some inflammatory processes were observed, even at the lowest dose of all the ouabain administrations. Interestingly, IHC and OHC numbers were similar after a 1 mM ouabain injection whereas DPOAE amplitudes dramatically decreased. However, a 1 mM dose of ouabain administered via TT generated a decrease of DPOAE amplitudes, without affecting ABR thresholds and neither the SGN nor the HC count. Apart from the TT ouabain administration, all the tested approaches generated various health issues, such as major weight loss, ear necrosis, rectal prolapse and high mortality. Ultimately, ouabain administration in the middle or inner ear of guinea pigs is ototoxic with heterogenous results, not limited to SGN loss. Concomitantly, most of the tested routes of administration generated variable health issues.

Discovery of NOX3 inhibitors for the prevention of acquired hearing loss

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The accumulation of reactive oxygen species (ROS) in cells and tissues contributes to the development and the progression of numerous diseases such as cancers, metabolic syndromes, or sensorineural disorders. NAPDH oxidases, a family of enzymes which sole function is to produce reactive oxygen species (ROS), appeared as relevant therapeutic targets in the treatment of oxidant-mediated pathologies. More specifically, the NOX3 isoform is only expressed in the inner ear and, although its physiological role in the cochlea is not known, there is increasing evidence that NOX3 is involved in different forms of acquired hearing loss. Thus, the inhibition of NOX3 would provide an efficient otoprotective strategy, notably by preventing ROS-induced damages to the auditory synapse. This project aims at discovering NOX3 small molecule inhibitors for the prevention of acquired sensorineural hearing loss. We developed a cell-based high-throughput screen using an inducible system allowing the expression of NOX3 upon 24 h treatment with tetracycline. NOX3 activity was assessed through the detection of generated extracellular superoxide radical anion ($O2^{\bullet-}$) using the colorimetric assay WST-1. The non-specific NOX inhibitor diphenyleneiodonium chloride (DPI) was used as a reference compound for maximal inhibition. Among the 15,511 compounds screened, 115 showed an inhibitory activity on NOX3 equal to or higher than 50% and were considered as hits. These hits were further tested in dose-response using WST-1 and validated using orthogonal assays detecting hydrogen peroxide (Amplex Red/HRP and CBA fluorometric assays) and cytotoxicity. The specificity of the validated hits for NOX3 over the 6 other isoforms was also assessed. This critical early drug discovery step paves the way to the development of new small molecule therapeutics for the prevention of sensorineural hearing loss.

EDNRB2 is a novel marker for hair cell precursors in chick auditory epithelia

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Although mammalian cochleae have no capacity for hair cell (HC) regeneration, the avian auditory epithelium, namely basilar papilla (BP), can regenerate HCs through direct conversion of supporting cells (SCs) or mitotic proliferation of SCs. To explore molecular mechanisms for HC regeneration in chick BP, we previously established an explant culture model of chick BP, in which HC regeneration occurs predominantly via direct conversion of SCs (Matsunaga et al., 2020) and performed single-cell RNA sequencing (Matsunaga et al., 2023) by using our model. A pseudotime trajectory analysis for the process of SC-to-HC conversion revealed that SCs were once reprogrammed to the precursor state, followed by differentiation into HCs (Matsunaga et al., 2023). In addition, we observed temporal upregulation of EDNRB2 encoded endothelin receptor b2 in reprogrammed SCs as differentially expressed genes during SC-to-HC conversion. In the current study, we examined the expression patterns of EDNRB2 in the developing chick BP to determine which stage progenitor or precursor populations express EDNRB2. The expression of EDNRB2 in BP was found only in the time points when HC differentiation was initiated, not in common progenitors for HCs and SCs or immature HCs and SCs after fate determination. The result supports our hypothesis that EDNRB2 is a marker for HC precursors and indicates that SCs are reprogrammed to the precursor state just before fate determination, not to the common progenitor state. We also assessed the roles of EDNRB signaling during SC-to-HC conversion in regenerating chick BP explants using a selective inhibitor for EDNRB signaling. The pharmacological inhibition of EDNRB signaling significantly reduced the number of regenerated HCs, not reduced the number of SCs, which indicates that EDNRB signaling may play a role in the differentiation of precursors into HCs. Further, RNA sequencing is underway to identify the critical molecules downstream of EDNRB signaling. In conclusion, EDNRB2 is a novel marker for HC precursors in chick auditory epithelia and may be involved in HC differentiation.

Efficacy of cochlear implantation in patients with severe to profound hearing loss without prior hearing aid use

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Introduction: Previous studies have suggested a negative association between the duration of hearing loss and post-cochlear implantation (CI) audiometry performance. Consequently, it has been assumed that cochlear implantation may be less effective in ears without prior hearing aid (HA) use. However, some cases of CI recipients with no history of HA use (non-aided) have demonstrated favorable hearing outcomes. This study aimed to explore the relationship between the duration of hearing loss in non-aided ears and postoperative auditory performance, as well as to compare CI outcomes between non-aided and aided ears.

Material and methods: This retrospective study included 153 ears (127 cases) with postlingual hearing loss that underwent CI for bilateral severe to profound hearing loss between April 2011 and March 2023. Among them, 28 patients (29 ears) received CI in non-aided ears, while 100 patients (125 ears) received CI in aided ears. One patient (2 ears) underwent CI in both non-aided and aided ears, and six patients (12 ears) received CI in non-aided ears initially, followed by

CI in aided ears. Audiometry performance was assessed using the Japanese monosyllable test (CI2004).

Results: No significant correlation was found between audiometry performance at 1 year postoperatively in non-aided ears and the duration of hearing loss without HA use. Additionally, there was no significant difference in audiometry performance between non-aided and aided ears at 1 year after surgery.

Conclusions: This study revealed that irrespective of the duration of hearing loss, postoperative CI outcomes in non-aided ears were comparable to those in aided ears. Patients with severe-to-profound hearing loss often exhibit asymmetric hearing loss, leading to requests for CI on the worse hearing ear. While surgeons may hesitate to provide CI to ears without prior HA use, our findings suggest that such hesitancy may not be warranted, assisting the decision-making process for CI in patients with postlingual hearing loss.

Epigenetic landscape of supporting cell reprogramming toward hair cell regeneration in chick auditory epithelia

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Sensorineural hearing loss is usually intractable because of virtually no capacity for hair cell (HC) regeneration in mammals. In contrast to mammals, lower vertebrates including birds have the capability for HC regeneration. Elucidation of precise mechanisms for HC regeneration in avian auditory epithelia will contribute to exploring key molecules for inducing HC regeneration in mammals. We previously established an explant culture model of the chick auditory epithelium, namely basilar papilla (BP), showing total HC loss and consecutive HC regeneration through direct conversion of supporting cells (SCs) to HCs (Matsunaga, 2020). Single-cell RNA sequencing using this model illustrated dynamic changes in expressed genes in BP SCs during direct conversion, which indicates that reprogramming of SCs to the precursor state occurs before differentiation into HCs (Matsunaga, 2023). To explore mechanisms of SC reprogramming toward hair cell regeneration in chick BP, we performed an integrated analysis of RNA- and ATAC-seq of chick BP explant cultures during the early phase (three time points) of HC regeneration. ATAC sequencing detected 80,366 peaks. Based on the distance from the transcription start site, and correlations between read counts and peak depths and between their alterations in a time course, we determined enhancer candidate loci for differentially expressed genes. We focused on a set of temporally upregulated genes during SC reprogramming in our previous single-cell RNA sequencing data (Matsunaga, 2023). In temporally upregulated genes during SC reprogramming, enhancer candidate loci were identified in nine genes. Motif enrichment analysis of these loci indicated transcription factors that may control chromatin remodeling in SC reprogramming. In the near future, we will examine spaciotemporal expression patterns of these transcription factors in regenerating chick BP.

This work is supported by KAKENHI (Grants-in-Aid for Scientific Research, 23K08983) from the Japan Society for the Promotion of Science.

Essential role of *ISL1* in the development and survival of spiral ganglion neurons in the inner ear

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ISL1, a LIM-homeodomain transcription factor, is expressed during embryonic development of the inner ear in both hair cells and spiral ganglion neurons (SGNs), but its function is still unknown. For the first time, we created a mouse model with Isl1 conditional deletion in the SGNs, and in our previous study of adult Isl1CKO mice, we identified the significant role of Isl1 in the phenotype and function of the SGNs. Here, we present the results of our cellular and molecular assessment of the SGNs' embryonic development in the Isl1CKO. Using immunolabeling, we studied the 3D structure of the cochlea by confocal and light-sheet microscopy through different stages of development. Exploiting the reporter protein, we inspected the axon projection characteristics in the Isl1CKO by time-lapse imaging of the cochlear explant. At the molecular level, we performed RNA sequencing to assess the transcriptional regulation impact of *ISL1*. In addition, we investigated the involvement of *ISL1* in epigenetics regulation by performing the CUT&Tag assay for important histone markers. We observed that from early developmental stages, the SGNs of the Isl1CKO manifested a problem in migration and axonogenesis. And while the proliferation of SGNs was not affected a gradual increase in apoptosis was detected. In Isl1CKO, despite the wrong location, SGNs projected axon fibers towards the target cells, however, axonogenesis started earlier and advanced faster than the control, resulting in disorganized and overshooting axons. Similarly, our RNA sequencing of the Isl1CKO's SGNs showed massive changes in the expression of the genes essential for neuronal communication, migration, survival, and axon projection including *DCC*, *Robo*, *Unc-5*, *Nrp*, members of the *EphA* family, *Ntrk2*, *Ntrk3*. Interestingly, we identified a sizeable overlap between the downregulated genes and the genes with altered histone modification, such as *EphA5*, *Unc5b*, and *Grin3a*, indicating that *ISL1* not only directly interacts with downstream genes to regulate their expressions, but also employs the epigenetics machinery to demethylase H3K27me3, and promote the expression of genes essential in neuronal development, axonogenesis, and synaptogenesis. Here, we unfold the prominent

regulatory role of *ISL1* in developing SGNs and provide insight into its direct and indirect targets.

Extended high frequency thresholds and their association with otoacoustic emissions and demographic factors

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Objectives: Hearing assessments typically cover frequencies up to 8 kHz, although testing can extend to 16 or 20 kHz. The range beyond 8 kHz is commonly referred to as the extended high frequency (EHF) range. This study aimed to investigate the connection between EHF hearing thresholds (HTs) and distortion product otoacoustic emissions (DPOAEs) in adult subjects. Factors such as the presence of spontaneous otoacoustic emissions (SOAEs), gender, ear side, and aging were of interest.

Material and methods: Participants consisted of 95 adults. The age ranged from 21 to 77 years, with an average of 42 ± 14. There were 55 women, comprising 58% of the whole group. All subjects had normal middle ear function verified by 226 Hz tympanometry. None had any known history of otologic disease. DPOAEs were measured using the HearID system (Mimosa Acoustics Inc., Champaign, IL, USA) with an ER-10C probe (Etymotic Research, Elk Grove Village, IL, USA). DPOAEs were measured at 9 selected frequencies for F2 of 1, 1.5, 2, 4, 6, 8, 10, 12, and 16 kHz. Only ears which gave a signal-to-noise ratio (SNR) greater than 6 dB at 3 of the 4 frequencies from 2, 3, 4, and 6 kHz were analyzed. SOAEs were acquired using the in-built routine (SOAE50) provided by the HearID system, resulting in a measurement of so-called synchronized SOAEs (SSOAEs).

Results: The key findings indicate that DPOAEs, both within the standard frequency (SF) range (0.125–8 kHz) and the EHF range (10–16 kHz), decrease as thresholds deteriorate. Age significantly influences DPOAEs and HTs in both ranges, with EHFs being particularly affected. The presence of SOAEs was the only other significant factor influencing DPOAE level. Gender and ear side had minor and non-significant effects on both DPOAEs and HTs.

Conclusions: In conclusion, DPOAEs in the EHF range emerge as reliable predictors of EHF HTs, and given their correlation with age, they may serve as suitable markers for early signs of presbycusis.

From cells to cures: hiPSC-derived inner organoids and RNA therapy to resolve genetic hearing loss

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Genetic hearing loss impacts millions worldwide, yet effective treatments remain unavailable, leaving patients reliant on technological aids such as hearing aids or cochlear implants. One major obstacle in therapy development is the lack of representative in vitro models of the human inner ear capable of mimicking genetic inner ear diseases and facilitating treatment validation. In this study, we present a novel approach to address this challenge. We differentiated human induced pluripotent stem cells (hiPSCs) derived from patients with genetic hearing diseases into 3D self-organizing inner ear organoids. Specifically, we focused on two genes associated with significant auditory impairments: *USH2A*, hereditary deafblindness, and *COCH*, implicated in late-onset genetic hearing loss, the latter presenting a window for intervention. We successfully generated disease-specific inner ear organoids by growing patient hiPSCs through precise modulation with small molecules and growth factors at distinct intervals. With immunohistochemistry we showed the presence of organ-specific cell structures within both *USH2A*- and *COCH*-inner ear organoids, including otic vesicles, hair cells and periotic mesenchymal cells. We compared the disease-specific inner ear organoids with healthy inner ear organoids through molecular and structural analyses and confirmed the presence of mutant transcripts in the patient-derived inner ear organoids. Moving beyond characterization, we demonstrate the clinical relevance of the model by countering the disease phenotype with antisense oligonucleotides (ASOs) in vitro. ASOs can specifically target and modify RNA transcripts and slow down or halt genetic disease progression. We applied ASOs to late-stage disease-specific inner ear organoids via gymnotic delivery and observed its effect on mutant transcript expression through PCR analysis following ASO therapy. This study underscores the potential of human inner ear organoids as a platform for modelling genetic inner ear diseases and evaluating potential therapeutic interventions. Our findings offer promising avenues for increasing treatment options for individuals affected by genetic hearing loss, offering hope for improved outcomes and quality of life.

HMGA2 mediates tonotopic identity in the developing mouse cochlea

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HMGA2 belongs to the non-histone chromosomal high--mobility group (HMG) protein family and binds to DNA at promoter and enhancer regions. The chromatin modifier plays a role in recruiting other factors such as histone acetylases, which together with *HMGA2* form a structure called the enhanceosome that has impact on local chromatin structure and therefore gene expression. In the cochlea, *HMGA2* is tonotopically expressed. However, its relevance for frequency specific hearing remains to be determined. Sonic hedgehog, a tonotopic morphogen, forms an apex-to-base decreasing gradient in the cochlea. To study the impact of hedgehog signaling on *HMGA2* expression, the hedgehog pathway was activated in vivo using a gain-of-function mouse model. On the other hand, retinoic acid forms a base-to-apex decreasing morphogen gradient, thereby opposing sonic hedgehog. To modulate retinoic acid levels in vivo, gain- and loss-of-function mouse models were used. Furthermore, conditional knockout of *Hmga2* from the otocyst stage was established to study the relevance of *HMGA2* using histological staining and hearing measurements. Constitutive activation of the HH pathway resulted in ectopic expression of *HMGA2*. Retinoic acid in turn was found to limit the extension of the *HMGA2* gradient towards the base. This finding indicates that positional information mediated by tonotopic morphogen gradients establish the *Hmga2* gradient in vivo. Conditional knockout of *Hmga2* did not affect the cellular composition of the organ of Corti. However, adult mice fail to develop normal low-frequency hearing as determined by ABR and DPOAE measurements. In this work, we used different transgenic mouse lines to establish that embryonic patterning via retinoic acid and sonic hedgehog establish the *HMGA2* gradient along the tonotopic axis. Also, loss of *Hmga2* demonstrated that the chromatin modifier is necessary for the development of low-frequency hearing in the adult animal. Together, these results indicate that *HMGA2* is an integral part in mediating tonotopic identity in the mouse cochlea.

Human pluripotent derived auditory neuron progenitors (LCTANP1) for the treatment of auditory neuropathy spectrum disorder

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Introduction: Loss of auditory nerve cells can lead to auditory neuropathy, even when the hair cells and the cochlear nucleus remain intact. Cell-based therapy for replacing lost or dysfunctional auditory neurons may restore hearing in these cases and enhance the degree of success of a cochlear implant procedure by repopulating the cochlea with transplanted, functional auditory neurons. We developed a novel proprietary differentiation process to manufacture LCTANP1 composed of Auditory Neuron Progenitors from clinical grade line of pluripotent human stem cells.

Material and methods: The manufacturing process starts with a series of differentiation cues, in specific time frames, and ends by harvesting the auditory neuron progenitors cells, and cryopreserving them as LCTANP1 drug product in a ready to administer format. LCTANP1 cells were characterized by biological and functionally relevant sets of markers, using different quantitative methods that we newly developed and customized such as analysis of specific protein marker expression by flow cytometry, and immunofluorescence and expression profiles, including RNA sequencing. Functional in-vitro assays were developed to measure neuronal properties of LCTANP1, such as the ability to elicit calcium influx. Fluorescent labeled LCTANP1 cells were delivered to the base of the cochleae of eight ouabain-treated guinea pigs by a cochleostomy and via the scala tympani, or the modiolus. Seven days later, animals were euthanized and labeled LCTANP1 cells were visualized within the cochlea using a fluorescence stereoscope and by human specific immunofluorescent staining.

Results: LCTANP1 cells were successfully manufactured at scale, met pre-set release criteria, and demonstrated relevant activity in in-vitro functional tests. LCTANP1 cells were cryopreserved in a ready-to-administer, thaw and inject format and were successfully thawed, successfully transplanted and survived a 7-days study in an in-vivo Guinea pig model. LCTANP1 cells are currently being evaluated in a functional model of hearing simultaneous with additional manufacturing enhancements.

Conclusions: LCTANP1 is a novel cell-based product composed of Auditory Neuron Progenitors derived from clinical grade pluripotent stem cells. LCTANP1 completed initial CMC and Preclinical POC.

In situ 3D fluorescence microscopy mapping in the Prphp-mCherry mouse line differentiates inner ear afferent populations

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The inner ear facilitates hearing and balance sensory encoding through its complex 3D structure and heterogeneity of primary afferent neurons. In the cochlea, spiral ganglion neurons (SGN) directly encode sound via the inner hair cell-Type I SGN circuit and regulate cochlear amplification feedback via the outer hair cell-Type II SGN circuit. Vestibular ganglion neurons (VGN) are divided into calyx afferents (Type I hair

cells), dimorphic (both Type I and Type II hair cells), and bouton afferents (Type II hair cells), that map to regions of the cristae and otolith organs to encode features of head position and acceleration. Both Type II SGN and vestibular bouton afferents are marked by the Type III intermediate filament protein Peripherin (Prph). We developed a transgenic mouse model using Prph promotor elements which demonstrated mCherry reporter expression (Prphp-mCherry) in SGN and VGN throughout postnatal development, characterised using CUBIC1/PEGASOS clearing and Lightsheet fluorescence microscopy. We found overlap of the Prphp-mCherry and Prph immunopositive populations in the hook and basal regions of the cochlea, but significant mismatch in mid-apical regions. In the vestibular ganglion, mCherry immunolabelling was confined to small diameter afferent somata by adulthood, colocalising with Prph positive bouton afferent fibres, although mismatch in fibre staining suggests a subpopulation has been identified. Using nanopore sequencing, the integration site of the Prphp-mCherry transgene cassette was located within the *Grm8* gene encoding metabotropic GluR8, where exon reshuffling was evident. Intriguingly, *Grm8* is a marker of Type Ic SGN, which synapse on the modiolar face of the inner hair cells and are particularly vulnerable to noise and aged-related hearing loss. Significant overlap of Type Ic SGN markers and Prphp-mCherry neurons has been quantified, indicating the integration site of the transgenic construct may have influenced transgene expression. Mapping the distribution of type and subtype markers in the cochlea SGN and under-resolved landscape of the VGN in 3D has revealed new protein marker compartmentalization, uncovering broader afferent heterogeneity in the inner ear.

Influence of impeded biomechanics after implantation

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The hearing preservation has become a standard goal of cochlear implantation. A new method of monitoring cochlear function has become available using the intracochlear electrocochleography or intracochlear ECochG. This measurement allows via intracochlear electrodes of the cochlear implant itself to record the response to acoustic stimuli. The feasibility of such recordings was presented at the XXXII World Congress of Audiology in 2014. Intracochlear ECochG is a sensitive measurement of cochlear function that allows for detection of basilar membrane contact in Flex arrays. Our previous study (Lorens et al., 2019) showed that the measurements of acoustically evoked intracochlear potentials via the location dependent intracochlear electrodes are systematically recordable in a wide range of postoperative hearing abilities of cochlear implantees. The most sensitive location within the cochlea to record CM potentials depends on the frequency tone used. The deeper in the cochlea the mean maximum CM peak-to-peak amplitude is, the lower the stimulating tone frequency will be. Multiple recordings along the cochlea provide a method for assessing cochlear mechanics. In this study, we use this approach to test for electrophysiological evidence of basilar membrane fixation, demonstrated by a substantial basal or apical shift of maximum ECochG response away from the characteristic frequency of the stimulus. The hearing preservation rates and speech outcomes will be presented and compared in 16 cochlear implantees.

Input ear impedance and eardrum energy reflectance variations related to increase in intracochlear and intracranial pressure

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Variations of the intracranial pressure (ICP) are expected in particular pathologies, in microgravity conditions, and associated with postural changes. Lumbar puncture is a direct but highly invasive ICP diagnostic method. Mechanical models of the inner and middle ear suggest an indirect non-invasive method based on acoustic measurements. Since the cerebro-spinal fluid is in contact with the intracochlear fluid, a change of the pressure in the lymphatic fluid of the peripheral hearing system gives information about variations of the ICP. In particular, the increase in the cochlear fluid pressure is measurable as increase of the middle ear reflectance and, consequently, the otoacoustic emission (OAE) phase (Buki et al., 1996; Avan et al., 2018; Voss et al., 2010). Avan et al. (2000) theoretically characterized the enhancement of the middle ear stiffness in terms of stapedius reflex activity as a reduction of the stapes compliance and observed the relative increase of the distortion product OAEs (DPOAEs) phase. As ICP changes can be induced by postural changes, we use theoretical models of middle ear transmission to discuss how and why the reactance of input ear impedance Z changes with the body posture, showing correlation with the increase and the frequency shift of the eardrum energy reflectance, and the DPOAE phase changes. Specifically, in the low-frequency range, the reactance of Z is stiffness-dominated, thus it is a negative function of (and inversely proportional to) frequency. When a tested subject changes position from orthostatism to clinostatism, the negative term of reactance decreases and the frequency of null reactance increases. This evidence is in accordance with the reduction of the stapes compliance which is most relevant at very low frequency. These results help providing a theoretical basis to (and a connection among) the empirical methods for the indirect estimate of ICP variations based on the monitor of different observable consequences of the variation of the middle ear transmission, which can be applied in microgravity conditions or also in cerebrospinal diseases.

Intracochlear administration methods across species

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Introduction: The delivery of therapeutic molecules to the inner ear represents a challenge due to the blood-labyrinth barrier, necessitating local delivery approaches, particularly for expensive medications or drugs with limited therapeutic range or susceptibility to systemic side effects. This is particularly relevant for viral gene transfer and cell-based therapies, but could be meaningful for small molecules, peptides or antibodies. Adeno-associated virus (AAV) vector emerges as a prominent choice for gene therapy due to its infection efficiency, low toxicity, sustained gene expression, and cost-effectiveness.

Purpose: This study aimed to compare different intracochlear (IC) injection techniques in mice and guinea pigs.

Material and methods: Mice were administered via posterior semicircular canal (PSCC) and round window (RW) injections, while guinea pigs received cochleostomy infusion, RW injection (RWI) or deposit (RWD) due to anatomical differences. Auditory function effects of IC administration were evaluated in both species via auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) measurements. Following AAV delivery, GFP expression from AAV-mediated cell transfection enabled the identification of transfected cochlear regions. Both PSCC and RW injections successfully targeted hair cells, albeit with variations in transfection patterns and intensity depending on the injection method and AAV subtypes. Regarding molecules delivery, the continual renewal of inner ear fluid may necessitate chronic infusion for maintaining active concentrations, assessable through pharmacokinetics evaluation. Finally, impacts on auditory function varied among injection methods, with PSCC and IC routes showing less effect on DPOAE amplitudes and ABR thresholds compared to the RW route.

Conclusions: In conclusion, our study highlights the importance of refined intracochlear delivery method in animals accordingly to the delivered product and targeted inner ear disorders. While all methods effectively enabled to reach cochlear hair cells, variations in transfection patterns or in product concentration at targeted cells, and effects on auditory function highlight the need for careful consideration of injection strategies. It is worth mentioning that the posterior semicircular canal (PSCC) and cochleostomy route, though effective in animals, lacks translational relevance to humans. Moving forward, further research into optimizing delivery methods and understanding their specific effects on cochlear function will be essential for advancing therapeutic interventions in auditory disorders.

Investigating the genetic bases of age-related hearing loss – human GWAS to mouse models

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The most common form of sensory disability is age-related hearing loss (ARHL), a complex disorder involving genetic and environmental factors. While not life-threatening, ARHL causes communication difficulties and is associated with social isolation, depression and reduced physical and cognitive function. Moreover, there is a growing literature suggesting causal links between ARHL and dementia. Presently, there are no biological therapies for the condition, and our limited knowledge of the underlying genetic mechanisms of ARHL is a severe impediment to the design of interventions.

As with other complex disorders, genome-wide association studies (GWAS) have had limited success in identifying susceptibility genes. However, the advent of the UK Biobank, with ~500k participants, is a game-changer having the statistical power to identify genome-wide significant loci. Indeed, a recent study reported 44 significant loci associated with self-reported hearing difficulty or hearing aid use. These data represent an opportunity to increase our understanding of ARHL, but first the gene responsible for the association at each locus needs to be confirmed.

Mice are the predominant model organism for hearing research. Similarities in auditory structure and physiology with humans, close evolutionary relationship of genomes and the available genetic toolkit, make mice an ideal system for studying the functional genomics of hearing. To elaborate upon the genetics of ARHL, we are generating knockout mice for genes located in close proximity to the strongest associations and assessing their hearing; we are utilising the IMPC programme, importing knockout lines and undertaking recurrent auditory phenotyping up to 15-months of age.

In addition to *Clrn2*, using this approach we have validated *Baiap2l2* and *Klhdc7b* as important for mammalian hearing. However, no overt hearing phenotype was evident in *Arhgef28*, *Nid2* or *Fto* mutant mice.

These findings highlight the potential of the UKBB data to elaborate upon the genetics of mammalian hearing, but also the difficulty of translating results from human GWAS to animal models. Importantly, here we have only investigated gene loss-of-function, which may not be appropriate in all cases. Validated mouse models will provide essential information regarding the pathobiology of ARHL, and this knowledge will lay the foundation necessary for developing preventive strategies.

Isolated early onset hearing impairment? Diagnosis of syndromic forms by whole genome sequencing

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Introduction: Clinical geneticists involved in the diagnosis of early onset hearing loss can to encounter young patients with apparently isolated hearing loss or cochleovestibular pathology and a diagnosis of a syndromic form. For example, diagnosis in a young child of Usher syndrome, that associates congenital hearing loss (and vestibular areflexia in the type 1) and later onset retinitis pigmentosa, is not rare in our practice.

Material and methods: In France, patients presenting with early onset isolated or syndromic hearing loss have access to diagnostic trio whole genome sequencing through the Plan France Medecine Genomique 2025 sequencing platforms. From 2020 to 2023, almost 500 families have undergone whole genome sequencing for this indication on the SeqOIA platform only.

Results: Among patients assessed in the Paris Reference Center for Genetic Deafness and presenting with apparently isolated early onset hearing loss or cochleovestibular pathology, whole genome sequencing identified one patient with Usher syndrome (*USH2A*) and four patients with very rare syndromic forms. Syndroms identified are Heimler syndrome (*PEX6*), a Perrault syndrome phenocopy (*NARS2*), Dystonia Deafness Cerebral Hypomyelination syndrome (*BCAP31*) and Hypoparathyroidism Deafness Renal syndrome (*GATA3*).

Conclusions: Diagnostic whole genome sequencing enables the diagnosis of ultra rare conditions, which would not have been possible with usual panel sequencing testing strategies. For the patients, the diagnosis of a syndromic form in an apparently isolated presentation allows for better care and genetic counselling but can also be distressing. Indeed, the delay between the result and the manifestation of additional symptoms and the severity of these symptoms can be uncertain.

Lef1 and Tcf7l2 are Wnt signalling effectors with contrasting functions during inner ear sensory organ formation

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The inner ear is composed of several sensory organs responsible for the detection of sound, head position and acceleration. During embryonic development, these organs originate from neurosensory-competent domains within the otocyst, but the molecular signals controlling sensory organ formation remain unclear. The transcription factor *Sox2* is required for neurosensory specification since its deletion abolishes the differentiation of sensory organs and their associated neurons. *Sox2* is initially present throughout the otocyst, but it becomes restricted to the ventro medial aspect. Our recent work suggests that this restriction is regulated in a dose-dependent manner by a dorso ventral gradient (from high to low) of canonical Wnt activity. Dorsally, high levels of Wnt activity inhibit sensory organ formation whereas ventrally, low levels are needed to maintain prosensory specification. To find out how Wnt signalling can exert these two contrasting functions, we analysed the expression and function of four members of the Tcf/Lef family of transcription factors (*Lef1*, *Tcf7*, *Tcf7l1* and *Tcf7l2*) in the chicken otocyst. We found that all members of the family are expressed in the otocyst but that each factor has a unique expression pattern. Our functional studies suggest that only *Lef1* and *Tcf7l2* contribute to prosensory specification. The expression pattern of *Lef1* and its gain-of-function effect reflect high levels of Wnt activity, while the distribution of *Tcf7l2* and the effect of its overexpression are consistent with low levels of Wnt activity. In summary, our results suggest that *Lef1* and *Tcf7l2* transcription factors are key effectors of the Wnt activity gradient during inner ear sensory organ formation.

Lgr5+ endogenous progenitor cells in the adult (deafened) cochlea

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Introduction: *LGR5* positive supporting cells (SCs) in the cochlea give rise to hair cells (HCs) during embryonic development. Neonatal SCs have increased progenitor potential compared to adult and only a few studies (including ours) have shown survival of SCs with progenitor cell markers after severe HC loss in adult mice. In mammals, there is no evidence for spontaneous HC regeneration in adulthood. However, three-dimensional cultures have allowed the expansion and experimentation of human (and mouse) inner ear organoids. Here, we evaluated HC differentiation from human cochlear organoids and from adult normal-hearing and deafened mice.

Material and methods: Adult patients undergoing surgery for skull base tumors were included. Sensory epithelium of the cochlea and vestibular organ was collected in medium and tissue was digested to single cell suspension. Adult Lgr5-eGFP-IRES-creERT2 heterozygous mice were used. Mice were deafened with a single dose of furosemide in combination with kanamycin and deafening was confirmed by auditory brainstem responses (ABRs). Cochleas were harvested and digested to single cell suspension and after filtering, 3D drops were made with Matrigel. Cells were grown on expansion medium (EM) for 10 days and differentiation medium (DM) for 3–10 days after. Organoids were fixed, permeabilized and processed for immunofluorescence and whole-mounted for imaging in a confocal microscope.

Results: Vestibular-organ-derived organoids were generated in EM from all seven patients so far included. Cochlea--derived organoids were generated in five out of seven patients. After exposure to DM, vestibular organ-derived and cochlea-derived organoids produced MYO7A+ HC-like cells. Cochlear organoids from normal-hearing mice expressed *LGR5* and Ki67 in EM and *MYO7A* after differentiation. Significantly less cochlear organoids were produced from deafened mice; however the organoids reached similar size as NH-cochlear organoids, expressed *LGR5* and *Ki67* in EM and *MYO7A* after differentiation.

Conclusions: Cochlear and vestibular tissue from adult patients (and adult normal-hearing and deafened mice) possess progenitor potential and the capacity to generate inner ear organoids in vitro. After differentiation, HCs were visible in tissue derived from human cochlea, human vestibular organ, and adult mouse cochlea. The adult inner ear has (limited) regenerative capacity and can produce new MYO7A+ HCs.

Loud low frequency sound-induced pathophysiology of cochlear sound transmission and sound transduction

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Introduction: Prolonged exposure to loud sounds can result in noise-induced hearing loss. Among sounds of different frequencies, loud high frequencies are damaging to base of the cochlea while loud low frequencies induce damage in the larger range of cochlea. Research has predominantly focused on the effects of high-frequency noise, though the consequences of loud low-frequency sounds (LFS) on cochlear function have received almost no attention.

Objective: The primary objective of this study was to investigate adverse effects of loud-LFS on cochlear sound transduction and sound transmission in guinea pigs.

Material and methods: Under anesthesia, guinea pigs were exposed to 200 Hz at 120 dB SPL for 40 mins, while controls received similar exposure at 55 dB SPL. The distortion product otoacoustic emissions (DPOAEs) and compound actional potential (CAP) thresholds were measured to evaluate outer hair cell (OHC) function and sound transmission to brain, respectively. In a different cohort of surgical guinea pigs, organ of corti (OoC) vibrations were recorded at the apex of cochlea, using optical coherence tomography. All measurements were taken before and after the sound exposure.

Results: We present that loud-LFS knocks out CAP for all tested frequencies, from 0.5 to 32 kHz. However, DPOAEs amplitudes remain unchanged to similar exposure. These findings suggest that loud-LFS selectively impairs sound transmission to brain without altering the hearing threshold, particularly in the cochlear region specific to middle to high frequencies. However, subsequent investigations of loud-LFS impact on apical cochlear mechanics revealed that loud-LFS alters apical frequency resolution by causing an upward shift in OoC best frequency. Our results also show a decrease in OoC vibrations at all frequencies, particularly more pronounced at frequencies below and above the best frequency. Vibration magnitude reductions were largest at low stimulus levels while alterations at intense stimulus levels were insignificant, reflecting a loud-LFS mediated impairment of OHC driven amplification at the apex of the cochlea. These response-magnitude changes were accompanied by morphological alterations within the cochlea.

Conclusions: These findings suggest that loud-LFS causes selective damage, impairing cochlear sound transmission across a wide range while preserving OHC transduction at cochlear base and damaging it at the apex.

Mapping human inner ear development: insights from single-nucleus transcriptomics

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The inner ear, essential for auditory perception and balance, relies on specialized cell types whose developmental mechanisms are incompletely understood. While animal models provide valuable insights, human inner ear development remains predominantly characterized by descriptive approaches. The advent of single-cell transcriptomics offers a promising approach to decode the complexities of human inner ear development. In this study, we introduce the Human Inner Ear Developmental snRNAseq Atlas (HIEDRA), a comprehensive single-nucleus RNA transcriptomic dataset that covers the entire membranous human inner ear at nine developmental stages from fetal weeks 7 to 15. Our analysis of over 55,000 cells reveals the molecular dynamics that drive the differentiation of epithelial, neuronal, and mesenchymal cell populations in both vestibular and cochlear regions. We provide new insights into specific gene markers that differentiate cochlear from vestibular cell types. We additionally find the involvement of canonical signaling pathways such as Notch, Wnt, and Hippo in sensory cell type development – a correlation previously established in non-human models. Moreover, our data reveal the contribution of additional signaling pathways, including TNF, to hair cell formation in silico. We also identify the role of ErbB, Notch and Hippo signaling pathways in the specification of key nonsensory epithelial cell types: vestibular dark cells and cochlear marginal cells, which are crucial for inner ear function. Our findings not only clarify the complex landscape of human inner ear development but also highlight the diverse roles of otic mesenchymal cells,

previously underappreciated in this context. This characterization deepens our understanding of human inner ear development and offers potential to explore pathophysiological mechanisms that lead to hearing loss and balance disorders. Furthermore, leveraging these insights to improve culture models, such as human pluripotent stem cell-derived inner ear organoids, could significantly boost their applicability for in vitro studies of developmental processes and their efficacy as disease models.

Mechanotransduction molecules regulate stereocilia membrane mechanics

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Hair cells are the mechanoreceptors of the auditory and vestibular sensory systems. The sensory hair bundle is the organelle that senses movement and converts this movement to an electrical signal using mechanosensory ion channels. These channels can operate at high frequencies and with sensitivities at molecular dimensions. How is this achieved? A growing body of data supports the hypothesis that the stereocilia membrane plays a role in modulating mechanotransduction. Biochemical modulations happens with PIP2, for example, regulating permeation and conductance. Mechanical modulation is implicated by fluorescent recovery after photobleaching which demonstrated that the MET open probability co-varied with membrane diffusivity; lower diffusivity correlated with more open channels. We now demonstrate that the MET channel complex directly regulates membrane viscosity using a newly developed viscosity sensor, BODIPY 1c. This sensor shows a strong correlation between MET channel activity and membrane viscosity both during development and in mutant mice that disrupt mechanotransduction. Biophysically dissecting current, voltage and calcium demonstrates that scramblase activity associated with the MET channel is responsible for the lower membrane viscosity. Conventional MET channel blockers block the scramblase activity resulting in an elevation in viscosity. We suspect an as yet undefined membrane flippase/floppase system creating an asymmetric membrane that is then regulated by the MET channel's scramblase activity. The reduced viscosity will directly impact MET channel kinetics and sensitivity.

This work was supported by R21DC021027-01 to SSG and RO1DC021448 to AJR from NIDCD.

NEUROD1 orchestrates cell fate changes and neurogenesis

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NEUROD1, a basic helix-loop-helix (bHLH) transcription factor, plays an essential role in neurogenesis within both the central and peripheral nervous systems. Recent studies demonstrate its capability to directly reprogram various cell types into neurons. However, these cell-reprogramming experiments indicate that NEUROD1 possesses the ability to instigate cell-fate changes but only under specific conditions that remain incompletely characterized. To gain further insights into the ability of NEUROD1 to change cell-fate and promote neurogenesis in vivo, we used three distinct gain-of-function of Neurod1 mouse models. Each model was designed to explore different temporal and spatial overexpression of NEUROD1. Overexpression of NEUROD1 in non-neuronal progenitors resulted in the induction of neurogenesis and neuron generation, while in neuronal progenitors it yielded no discernible changes. Our study provides new evidence that NEUROD1 is an efficient reprogramming factor of non-neuronal progenitors into neurons and confirms its potential for facilitation of reprogramming therapeutic strategy.

Novel antisense therapy for USH2A patients

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Perilymph proteome in prelingual deafness

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Introduction: Inner ear liquid biopsy would help in understanding the molecular environment of the diseased organ. In cases of prelingual deafness it is postulated that cochlea' insufficiency, both congenital and acquired in early postnatal period is connected with remarkable changes in the perilymph proteome and the protein pathways. In cases in which deafness results from genetic change of the cochlea structures, like *GJB2*-related deafness, it is probably expected that proteins encoded by the defected gene should not be detected in the perilymph or should be detected at much different levels. Analogically, in cases of deafness acquired due to ototoxic agents exposure one could expect that proteome characteristics would be altered, among others towards inflammatory products and proteins involved in oxidative stress reactions. Having known the proteome status of the perilymph we could approach to more precise prognosis of the disease, especially in cases of residual hearing.

Material and methods: We have collected perilymph samples using glass capillaries from preliminary group of prelingually deaf children during the procedure of cochlear implantation in Institute of Physiology and Pathology of Hearing, Warsaw. Each sample was no less than 2 µl and was harvested via round window approach. Method: using mass spectrometry (MS) protein quality analysis was performed in each sample.

Results: Number of detected proteins across samples varied from 1324 to 2103. Further samples taken from patients with different kinds of deafness are needed to continue the analyses.

PHOENIX – an animal free platform to accelerate the development of new therapeutics against sensorineural hearing loss

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Cochlear hair cells and their associated auditory neurons do not regenerate after injury, leading to irreversible sensorineural hearing loss. This lack of regenerative potential of cochlear progenitors is a major obstacle to developing efficient in vitro models, delaying new therapeutic advancements for hearing loss treatment. Consequently, from early preclinical stages, testing new treatments relies on animal-based models, resulting in low throughput, significant variability, and limited predictive value. We have recently identified the signaling pathways that can reprogram stemness in senescent auditory neuroprogenitors. By synergistically targeting the WNT and TGFβ/Smad pathways using small molecules or genetic means, we achieved virtually unlimited expansion of auditory neuroprogenitors in vitro. This reprogramming does not compromise their ability to differentiate into mature and functional auditory neurons, even after 40 passages and a thousand-fold amplification. The so-called phoenix auditory neuroprogenitors can be frozen and thawed, leading to the creation of a cell bank and offering an efficient alternative to animal-based models. The phoenix platform provides numerous advantages, including suitability for high-throughput technologies, low experimental variability, single-cell resolution, and a significant reduction in the number of animals used. Furthermore, it maintains the phenotype of auditory neurons in a primary culture-like setup. Initially developed using mouse neural cells, we are currently implementing this model with human fetal otic neural stem cells. This reprogramming method represents a significant breakthrough in overcoming a major bottleneck in auditory research. The phoenix platform offers an efficient, high-throughput, cost-effective, and 3R-compatible approach for in vitro screening of potential otoprotective and otoregenerative drug candidates. In addition, the precise investigation of the mechanisms leading to phoenix proliferation opens new avenues in the field of inner ear regeneration.

Quantitative RNA-scope study of the expression patterns of Tcf/Lef transcription factors in the otocyst

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Canonical Wnt signalling is a critical morphogenic pathway regulating cell fate choices and tissue patterning during embryogenesis. In the developing inner ear, Wnt signalling is implicated in both otic induction and hair cell differentiation. Our recent work revealed that it also regulates prosensory specification, which is the formation of inner ear sensory organ precursor cells. We observed a dorso-ventral gradient of Wnt signalling activity throughout the early otocyst and showed that it has two opposing functions: inducing and repressing prosensory specification in a manner that is dose-dependent. Wnt signalling operates through the activation of different sets of target genes, expression of which is regulated by four transcription factors from the Lef/Tcf family. Thus, to better understand the process and mechanisms of Wnt gradient activity during prosensory specification, we analysed the expression patterns of *Lef1*, *Tcf7*, *Tcf7l1*, and *Tcf7l2* in the early chicken otocyst using RNA-scope fluorescent in situ hybridisation and developed a quantification pipeline to better determine their activity levels. Our results show that *Lef1*, *Tcf7*, *Tcf7l1* and *Tcf7l2* exhibit distinct expression patterns across the dorsal and ventral axes of the otocyst. The different levels and patterns of expression quantified using our pipeline suggested that *Lef1* and *Tcf7* are candidates for effectors of

high Wnt signalling activity and *Tcf7l2* is an effector of low activity. Further functional validation confirmed that *Lef1* and *Tcf7l2* act downstream of Wnt signalling to regulate its two contrasting functions during otic prosensory specification and thereby substantiate our RNA-scope pipeline as a valuable tool for quantitative spatial analysis.

Sensory transduction plays an essential role in the maturation of inner hair cells, afferent ribbon synapses and auditory nerve fibers

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Inner hair cells (IHCs) convert sound stimuli into electric signals through activation of mechanosensory transduction channels (*TMC1* and *TMC2*) localized at the tip of the stereocilia (Pan et al., 2013, 2018; Kurima et al., 2015). Hair cells acquire transduction, progressively, from the basal end of the cochlea to its apical end, during the first postnatal week in mice (Lelli et al., 2009). Before the onset of hearing, IHCs also fire spontaneous action potentials which are believed to play a role in the development and maturation of the auditory system (Kros et al., 1998; Trish et al., 2007; Johnson et al., 2011, 2017). Alteration in sensory transduction has been shown to affect hair cell physiology (Marcotti et al., 2006, Corn et al., 2018) and maturation of IHC synapse morphology (Lee et al., 2021). Here we further investigate how sensory transduction affects IHC physiology, afferent ribbon synapses, as well as downstream type-I auditory nerve fibers (ANF) properties. To tackle this question, we took advantage of several mouse models with altered sensory transduction: mice lacking or carrying dominant mutation in TMC proteins. We performed single cell electrophysiological recordings to assess voltage-dependent calcium currents and exocytosis and examined ribbon synapse with immunostaining and transmission electron microscopy at 2 weeks and 3 weeks. We assessed the spontaneous and evoked firing properties of ANF, in vivo, using single fiber recording in anesthetized mice (2–4 months). Finally, we assessed RNA expression of the ANF fibers by RNA single cell sequencing in P24–P28 mice. Our work demonstrates preservation of synaptic properties and features in *Tmc2* KO mice and alterations in fast and sustained exocytosis along with impairment of voltage-dependent calcium currents *Tmc1* KO and double *Tmc1*/*Tmc2* KO mice. These changes are also associated with alteration in the morphology of the synapse as we demonstrated previously (Lee et al., 2021) and further validated in this study. Our work demonstrates that sensory transduction plays an important role in the development and maturation of hair cells, their afferent synaptic machinery as well as maturation of the ANF.

Single-cell transcriptomic atlas reveals increased regeneration in diseased human inner ear balance organs

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Mammalian inner ear hair cell loss leads to permanent hearing and balance dysfunction. In contrast to the cochlea, vestibular hair cells of the murine utricle have some regenerative capacity. Whether human utricular hair cells regenerate in vivo remains unknown. Here we procured live, mature utricles from organ donors (9 ears from 6 organ donors) and vestibular schwannoma patients (24 ears from 24 patients), and presented a single-cell transcriptomic atlas at unprecedented resolution. We validated marker genes using immunostaining and RNAscope in situ hybridization and described previously unknown markers of 13 sensory and non-sensory cell types. In addition, we compared and found partial overlap and correlation between transcriptomes of human and mouse hair cells and supporting cells. We further uncovered transcriptomes unique to hair cell precursors, which are validated in both organ donor and vestibular schwannoma utricles. Unexpectedly we found 14-fold more hair cell precursors in vestibular schwannoma utricles, demonstrating the existence of ongoing regeneration in humans. Lastly, supporting cell-to-hair cell trajectory analysis revealed 5 distinct patterns of dynamic gene expression and associated pathways. Our dataset constitutes a foundational resource, accessible via a web-based interface, serving to advance knowledge of the normal and diseased human inner ear.

Styrene ototoxicity is associated with memory impairment and hippocampal dysfunctions

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Styrene is an organic solvent commonly used in industries with well-documented ototoxicity. Although styrene toxicity on cochlear structures has been extensively documented, its detrimental effect on the central nervous system and on brain structures involved in the auditory (i.e. auditory cortex) and extra-auditory (i.e. hippocampus) pathway has not been established yet. Our recent study reveals that styrene exposure increases oxidative stress in both the cochlea and auditory cortex activating macrophages and glial cells (Paciello et al., 2024). Considering that alterations in the auditory cortex induced by peripheral damage can be linked with cognitive impairment and altered hippocampal functions (Paciello et al., 2021; Paciello et al., 2023), we wondered if the ototoxic effect of styrene could also be associated with cognitive dysfunctions. Therefore, the aim of our study was to investigate the relationship between styrene ototoxicity and cognitive impairment. To this aim, adult male Wistar rats were exposed to styrene for 5 days a week during 3 weeks at a dose of 400 mg/kg. Hearing loss and damage to neural transmission were assessed by recording auditory brainstem responses (ABR) and by performing wave II latency and amplitude analysis. At the end of treatment (day 21) animals underwent behavioural test (Novel object recognition test-NOR) to evaluate recognition memory. Then, we performed morphological analyses and western blot assays in hippocampal samples to evaluate the level of oxidative stress, macrophage infiltration, glial cell activation and inflammation in the hippocampus. Results revealed a decrease in auditory threshold in styrene-exposed animals compared to control animals. Hearing loss was associated with memory deficits and hippocampal dysfunction with increased oxidative stress, lipid peroxidation, inflammation, and *Iba-1* and *CD68* expression suggesting microglia-induced inflammation. Overall, the present study suggests that styrene can exert an oto/neurotoxic effect not only in the cochlea but also in brain structures involved in auditory and extra-auditory pathways, leading to altered hippocampal functions and memory impairment.

Supporting cell responses to sensory cell damage: novel insights from a quantitative analysis of cyclodextrin--induced ototoxicity in mice

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Introduction: The cochlea is vulnerable to various pathological conditions, with sensory cells typically being the primary target of damage. However, supporting cells also experience significant impacts. Despite their critical role in maintaining the structural and functional integrity of the sensory epithelium, the responses of supporting cells to cochlear damage are not well understood. This study aims to characterize the reactions of supporting cells to sensory cell damage in mouse cochleae.

Material and methods: The study utilized a mouse model of cochlear damage induced by cyclodextrin to simulate the ototoxicity of the cochlea. A single dose of cyclodextrin treatment caused cochlear damage with a damaging pattern similar to that caused by aminoglycoside antibiotics. The cochleae were examined at various time points after the treatment to evaluate supporting cell survival patterns and the roles of various types of supporting cells in the repair process of the organ of Corti. We also examined the vulnerability of different supporting cell populations and cochlear responses to supporting cell pathogenesis.

Results: Cyclodextrin exposure caused considerable sensory cell loss, particularly outer hair cell loss. Despite significant sensory cell damage, most supporting cells survived. These surviving cells not only helped maintain the structure of the organ of Corti but also expressed immune molecules. However, the basal end of the cochlea exhibited noticeable supporting cell death, with quantitative analysis indicating that pillar cells were the most vulnerable, followed by Deiters' cells. This supporting cell death triggered the local expression of immune molecules in the surrounding supporting cells. Additionally, macrophages were observed in areas where supporting cells were absent at the chronic phase but not in regions with sensory cell loss at the acute stage of cochlear damage.

Conclusions: This study elucidates the complex dynamics of supporting cell responses in the cochlea following damage, demonstrating that while most of these cells retain their structural integrity and initiate immune responses, they exhibit varied vulnerability to cochlear insults. The findings emphasize the importance of supporting cells in cochlear recovery processes and the potential of targeting these cells for therapeutic strategies aimed at restoring auditory function.

The border and inner-phalangeal cells are required to synchronize the calcium action potentials in developing inner hair cells

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Introduction: Developing cochlear inner hair cells (IHCs) elicit sensory-independent Ca^{2+} action potentials (APs) that propagate along the auditory pathway and is required for the maturation of the IHCs and for the refinement of the neural circuitry. The AP activity in IHCs is believe to the synchronized by spontaneous ATP-induced Ca^{2+} waves originating in the non-sensory supporting cells (SCs). This ATP signalling triggers fluid secretion from the SCs by activating Ca^{2+}-activated Cl^- channel (TMEM16A), which has been reported having bipolar influence on the IHC excitability.

Whether TMEM16A channels are involved in the functional maturation of IHCs is still unclear.

Material and methods: We used conditional Tmem16afl/flPlp1-cre mice in which the expression of TMEM16a was downregulated specifically in the inner phalangeal and inner border cells (IPhC and IBC), which are the SCs adjacent to the IHCs. Cell-attached path-clamp electrophysiology was used to monitor the SAP activity from ex-vivo cochlear tissue, while whole-cell patch-clamp was used to record current and voltage responses in pre- and post-hearing IHCs.

Results: We showed that the absence of TMEM16A in IPhC and IBC significantly prolonged the inter-spike intervals (ISIs) of spontaneous APs in the IHCs. High-frequency burst of APs (≥10 Hz) in IHCs were almost completely eliminated in the absence of TMEM16a channels. Calcium imaging also revealed a significantly reduced correlation in APs between nearby IHCs from Tmem16afl/flPlp1-cre mice. Although the IHCs from Tmem16afl/flPlp1-cre mice appeared to experience an initial delay in the maturation of their basolateral membrane currents, they were indistinguishable from control IHCs.

Conclusions: We showed that IPhCs and IBCs mediate the synchronization of APs in nearby IHCs and that the activation of TMEM16A channels is critical to elicit high-frequency burst (>10 Hz) in developing IHCs. We also found that IHCs from Tmem16afl/flPlp1-cre mice show a delay in their maturation, highlighting the possible role of ATP signalling from the SCs is driving the normal development of IHCs.

The clinical effect of steroids on hearing preservation in PDT patients in cochlear implantation

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Introduction: Recent advances in cochlear implantation which consist of: the design of the electrodes, atraumatic surgical techniques and monitoring of cochlear function during implantation, draw attention also to administration of steroids and other anti-inflammatory drugs for preserving residual hearing.

Aim: The main aim of this study was to assess the clinical effect of steroids (dexamethasone and prednisone) on hearing preservation in patients who underwent cochlear implantation with different cochlear implant systems (Oticon®, Advanced Bionics®, Med-El®).

Material and methods: 147 adult patients met the inclusion criteria and were enrolled to the study and divided into three groups depending on the brand of cochlear implant they received and participated in all follow-up visits regularly. They were also randomly divided into three subgroups depending on the steroid administration regime: (1) intravenous dexamethasone (0.1 mg/kg body weight twice a day for three days); (2) combined intravenous and oral steroids (dexamethasone 0.1 mg/kg body weight twice a day plus prednisone 1 mg/kg weight once a day); and (3) no steroids (control group).

Results: The results were measured by pure tone audiometry (PTA) at three time points: (i) before implantation, (ii) at processor activation, and (iii) 12 months after activation. A hearing preservation (HP) figure was also calculated by comparing the preoperative results and the results after 12 months. Further measures collected were electrode impedance and hearing threshold in the non-operated ear. The highest HP measures were obtained in the subgroups who were given steroids. Of the 102 patients given steroids, HP was partial or complete in 63 of them (62%). In comparison, partial or complete HP was achieved in only 15 patients out of 45 (33%) who were not given steroids. There were differences between the three cochlear implant groups, with the Med-El and Advanced Bionics groups performing better than the Oticon group (45% and 43% of the former two groups achieved partial or complete HP compared to 20% in the latter). Hearing thresholds in the non-operated ear were stable over 12 months.

Conclusions: Pharmacological treatment with steroids in patients undergoing cochlear implantation helps to preserve residual hearing.

The effect of brain-derived neurotrophic factor and neurotrophin-3 on the auditory nerve response to cochlear implant stimulation

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Cochlear implants rely on the integrity and correct functioning of auditory nerves. However, after sensorineural hearing loss there is a well-characterised degeneration of auditory nerves. One proposed mechanism of this degeneration is a reduction in the levels of neurotrophins, which are naturally occurring proteins that aid the growth and maintenance of neurons. Neurotrophin application to the cochlea of animals prevent morphological degeneration of the auditory nerves after deafness, making it a promising treatment for improving cochlear implant outcomes. The current study examined the effects of two major neurotrophins present in the cochlea, brain-derived neurotrophic factor and neurotrophic factor-3, on the function of individual auditory nerve fibres to cochlear implant stimulation.

Guinea pigs were ototoxically deafened and then divided into groups that received treatment for four weeks with either

brain derived neurotrophic factor, neurotrophin factor-3 or Ringer's solution as a control. Treatments were administered one week after deafening and delivered to the left cochlea through a combined cannula-electrode array that was attached to a mini-osmotic pump. Additional control groups included guinea pigs deafened for five weeks and an acutely deafened group. At the end of the treatment period responses of individual auditory nerve fibres to acute electrical stimulation of the cochlea at rates from 200–5000 pulses per second were recorded.

Both brain derived neurotrophic factor and neurotrophin-3 treatment generally normalized the reduction in spike latency of auditory nerve fibres observed after deafness. BDNF significantly reduced thresholds compared to all control groups, while NT-3 did not. Both BDNF and NT-3 increased the first-spike dynamic range compared to untreated groups. These results were largely similar regardless of the stimulus rate used.

These results suggest that neurotrophin treatment of the cochlea after deafness appears to preserve the latency of auditory nerve fibres, but may alter the response threshold and dynamic range. Further investigations are required to determine if neurotrophins are likely to preserve or improve auditory nerve function when used with a cochlear implant.

The expression and functional role of histamine receptor 3 in the mammalian inner ear c57BL/6 mice

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Introduction: Histamine receptor 3 (H3R) is known for its regulatory functions in the central nervous system, but its role in the mammalian inner ear is poorly understood. This study investigates the expression and functional implications of H3R in the inner ear of postnatal c57BL/6 mice.

Material and methods: Immunofluorescent staining was employed to determine the localization of H3R in the cochlea of postnatal day 3–5 (P3-5) mice. Cochlear explants were cultured for 24 hours in the presence of one of two H3R agonist/antagonists, Ciproxifan or Pitolisant, at various concentrations (10 μM, 50 μM, 100 μM). The effect of Ciproxifan and Pitolisant on hair cells (HCs) and spiral ganglion neurons (SGNs) morphology was assessed using fluorescent microscopy.

Results: H3R expression was detected in HCs and SGNs. Exposure to Ciproxifan induced significant damage and loss of inner and outer HCs in a concentration-dependent manner. Moreover, the typical apex-to-base directional growth of some type II SGN fibers appeared to be reversed (base-to-apex). Exposure to Pitolisant did not reduce the number of HCs. Still, it caused morphological changes in HCs cilia and a reversed directional growth of type II SGNs but to a lesser extent than the equivalent concentrations of Ciproxifan.

Conclusions: This study validates the expression of H3R in the inner ear of P3-5 c57BL/6 mice and suggests its potential role in the development and maintenance of hair cells. The differential effects of H3R antagonists underscore the necessity of pharmacovigilance for this class of drugs, particularly in the fields of otology and audiology. Further research is necessary to elucidate the mechanisms underlying the role of H3R in auditory development and to explore its potential as a therapeutic target for hearing and balance disorders.

The functional integrity of the mechanoelectrical transduction complex in the hair cells of the mature cochlea requires MYO7A

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Introduction: The transduction of acoustic information into electrical signals depends on the mechanically induced displacement of stereociliary bundles projecting from the apical surface of the sensory hair cells. Hair bundle deflection opens mechanoelectrical transducer (MET) channels located at the tips of the shorter rows of adjacent stereocilia. The gating of the MET channels requires force supplied by the tensioning of tip links during sound-induced bundle displacement. The motor protein MYO7A, which is an unconventional myosin responsible for syndromic (Usher 1B) or non-syndromic recessive deafness in humans when mutated, has long been associated with tip-link tensioning, but conclusive evidence is still lacking. In this study, we investigated the role of MYO7A in mature hair cells using conditional knockout mice.

Material and methods: The role of MYO7A in mature hair cells was investigated using conditional Myo7afl/flMyo15-cre mice in which the delayed downregulation of the protein allowed normal cochlear development and hearing function up to about postnatal day 20. Patch clamp electrophysiology was used to record the MET current, which was elicited by displacing the hair bundles of the IHCs and OHCs with a piezo-driven fluid jet. The morphology of the stereociliary bundles and their molecular composition was investigated using immunofluorescence microscopy and scanning electron microscopy. Hearing function was measured using auditory brainstem responses.

Results: We found that mature hair cells from MYO7A-deficient mice progressively lose their MET current while still having normal hair bundle morphology (up to at least 1 month of age), albeit with a considerably reduced stiffness. Surprisingly, the resting open probability of the MET channel and its sensitivity to intracellular and extracellular Ca^{2+} were not affected in the absence of MYO7A. By 2 months of age, the hair bundles of the hair cells started to become disorganised and by 7 months the organ of Corti was almost completely devoid of hair cells. We also found that the

progression of hearing loss and deterioration of the stereociliary hair bundles in Myo7a-deficient mice was accelerated by noise insults. Finally, transcriptomic analysis showed that the absence of MYO7A in 1 month-old mice caused the downregulation of a number of genes known to be essential for mechanoelectrical transduction.

Conclusions: We found that MYO7A is required for maintaining the functional integrity of the stereociliary hair bundles, but it is not essential for setting the resting tension on the mechanoelectrical transduction complex in mature cochlear hair cells.

Funding: RNID (G94) to WM and CJK; BBSRC (BB/T004991/1 and BB/S006257/1) to WM.

The hidden truth of hereditary hearing loss: gaining insight into the genetic basis of non-syndromic mimics

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Introduction: The definition of a molecular diagnosis for patients affected by hereditary hearing loss (HHL) is significantly hampered by the extreme clinical and genetic heterogeneity that characterise the condition. In particular, peculiar and understudied cases are those of non-syndromic mimics (NSM), meaning patients with particularly mild forms of syndromic HHL or initially presenting isolated deafness and delayed onset of other clinical signs.

Material and methods: In the last 18 months, a cohort of 73 apparently non-syndromic Italian HHL patients has been enrolled in the study. All the individuals were negative at *GJB2* and *STRC* genetic tests and underwent whole-exome sequencing, aiming to define a molecular diagnosis and eventually identify NSMs.

Results: A molecular diagnosis was provided for 36/73 patients (49.3%), and 12 of them could be classified as NSMs. In detail, two groups of patients could be highlighted: (1) patients presenting subtle additional signs that were missed during the first clinical evaluation and (2) patients whose molecular diagnosis suggests the future development of additional clinical features. In Group 1, two patients were identified, and they carried pathogenic variants within the *MITF* and *GATA3* genes, which are associated with Waardenburg and Barakat syndromes, respectively. As regards Group 2, ten patients were detected, and the involved genes were *CDH23* (one patient), *USH2A* (six patients) and *ADGRV1* (three patients). Thus, these results suggest that Usher syndrome type 2 accounts for the vast majority of NSMs (75%). Moreover, these considerations further confirm our previous findings regarding the high prevalence of Usher syndrome type 2 carriers in the Italian population (1: 70).

Conclusions: Identifying patients within Group 1 of NSMs highlights the importance of a critical re-evaluation of the diagnostic criteria of each condition and provides crucial insight into the clinical characteristics of very mild forms of syndromic deafness. On the other hand, the clinical condition of Group 2 NSM patients will be evaluated by a multidisciplinary team in order to provide personalised follow-up and specific preventive strategies.

The pharmacological action of Pimozide on vestibular Type-I and Type-II hair cells

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Pimozide is a conventional antipsychotic of the diphenylbutylpiperidine class widely used for treating schizophrenia, delusional disorders, and managing motor and phonic tics in Tourette's syndrome. Its primary mechanism of action in the central nervous system is as a dopaminergic D2 receptor antagonist. Additionally, Pimozide is known for blocking various types of voltage-gated calcium and potassium channels. Among its side effects, dizziness and balance disorders are the most observed.

This study delved into the effects of Pimozide on ionic currents in vestibular hair cells. Using the patch-clamp whole-cell technique, we studied the effect of Pimozide at a concentration of 3 μM on the ionic currents expressed by chicken embryo vestibular Type-I and Type-II hair cells, as well as on mammalian Type-II hair cells. Consistent with a previous report on chicken embryo, Pimozide significantly increased the delayed outward rectifying K+ current of Type-II hair cells on mouse. In chicken embryo, the drug also notably reduced the inward (anomalous) rectifying K+ current and the mixed Na+/K+ (Ih) current.

In Type-I hair cells, Pimozide showed no significant effect on IK,L, a large low-voltage activated outward rectifying K+ current absent in Type-II cells, nor on the small delayed outward rectifying K+ current. The latter result suggests that the delayed rectifying K+ current involves different channel subunits in the two hair cell types. Additionally, Pimozide did not alter the inward Na+ current expressed by Type-I hair cells.

In conclusion, these findings highlight that Pimozide selectively impacts potassium channels in Type-II, but not Type-I, hair cells. The drug acts as a delayed outward rectifying potassium channel opener in Type-II cells, potentially leading to a decrease in afferent signal transmission from these cells to primary sensory neurons. While providing a possible explanation for the vestibular side effects of Pimozide, the above results also open up possibilities for its use in reducing altered vestibular input in various vestibular disorders.

Unraveling age-related cellular and molecular mechanisms associated with vestibular sensory epithelium and its prolonged resilience compared to cochlear aging

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Aging of the inner ear contributes to age-related hearing loss (ARHL) and vestibular dysfunction (ARVL). Many studies have examined the underlying mechanisms involved in ARHL, while ARVL remains poorly understood. ARVL is the gradual loss of bilateral vestibular function accompanied by interruptions to visual and proprioceptive inputs, increasing the risk of imbalance, geriatric dizziness, and injurious falls. According to the National Institute of Health, age-related falls account for 50% of all accidental deaths, and it is the 6th leading cause of death in the elderly, highlighting the urgency of understanding the molecular basis to develop targeted therapeutics. Evidence from human and animal studies indicates age-related functional and morphological alterations in the vestibular sensory epithelia with a slow pace of aging in contrast to the cochlea, suggesting distinctive age-related cellular and molecular mechanisms between the two systems. Thus, in the current study, we investigated the age-related cellular and molecular alterations in the vestibular system particularly focusing on how it differs from cochlear aging.

Our vestibular sensory evoked potential, auditory brainstem response, and distortion product otoacoustic emissions and the endolymphatic potential measurements revealed age-related vestibular and auditory functional decline and the different paces of aging of the two sensory systems in the same mice. Morphological analysis using histology, super-resolution confocal-microscopy, and scanning electron microscopy revealed degeneration of stereocilia and alterations in hair and supporting cell soma. Using single-cell RNA sequencing of hair cells and supporting cells from the auditory and vestibular sensory epithelia from adult and aging CBA/J mice, we were able to identify shared and unique genes and molecular processes associated with vestibular aging and the disparity of aging trajectories of the two systems contributing to differential onset of ARVL and ARHL.

Our findings delineated the relationship between the onset of age-induced vestibular dysfunction and cellular and molecular degeneration of the vestibular sensory epithelium, uncovering novel insights into mechanisms governing vestibular aging leading to ARVL. Moreover, the comparative analysis between vestibular and cochlear aging revealed mechanism(s) contributing to the delayed onset of vestibular aging, in contrast to cochlea. Our findings pave the way to developing selective therapeutic interventions to prevent ARVL.

Validation of a newly developed SPL Chirp for intracochlear ECochG measurement

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Introduction: Intracochlear electrocochleography (ECochG) records electrical potentials generated in the inner ear in response to acoustic stimuli. Previous studies have demonstrated that ECochG recordings are related to the remaining inner ear function. Recently intracochlear ECochG measurement tool was applied during CI surgery to gain a better understanding of the impact of the implant on the inner ear function. For the stimulation, a newly developed SPL chirp will be applied.

Aim: The aims of this study were to validate SPL chirp and secondly, to perform real time intracochlear EcochG recordings during the electrode advancement and maneuvering during the cochlear implantation

Material and methods: Ten patients implanted with the Flex electrodes, with various degree of hearing preservation were postoperatively tested for SPL chirps and tone bursts of 250, 500, 1000, 2000 and 4000 Hz. The recordings was performed for each active electrode in alternating mode. The frequency specific response amplitudes of tone bursts were compared with those of SPL chirp1 and SPL chirp2.

Results: In every subject we obtained response to tone bursts and SPL chirp responses. Generally, SPL chirp frequency specific amplitudes were equal or lower than those for tone bursts obtained at the same stimuli level. The frequency specific amplitudes varied from more than1μV (noise flor) to about 100uV.

Conclusions: SPL chirps are useful stimuli to be used during the intraoperative monitoring of hearing preservation cochlear implant surgery as a time-reduction paradigm comparing to burst stimulation. Such stimuli may provide additional information of cochlea specific information related to hearing preservation.

Whole organ imaging of the mature and aged mammalian vestibular system

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Aging is associated with an increased risk of falling, which is contributed to by diminished balance. However, the precise anatomical changes occurring within the vestibular system as it ages are debated. In this study, we used

immunofluorescence, tissue clearing, and 2-photon microscopy to visualize the anatomical changes that occur as the vestibular system ages. The entire temporal bone was dissected and decalcified, instead of dissecting individual vestibular organs, to minimize tissue damage and distortions during dissection. We compared tested multiple clearing methods, both aqueous and solvent based methods to find which works best for the vestibular organs and settled on using the ethyl cinnamate method. These methods allow imaging of the entire vestibular system sensory cells in their native orientations. Using a combination of antibodies, we can label all hair cells as well as the type II hair cells. Using automated analyses, we delineate the region of the sensory epithelia and automate the counting and mapping of the locations of each cell. Using this protocol, we quantify the number of hair cells in both mature (1–2 months) and aged (36–40 months) epithelia to determine how hair cell numbers change with age. We also correlate these changes with vestibular function using vestibular evoked potential recordings. This study lays the groundwork for determining changes in the vestibular system with age to determine the pathophysiological changes.

Zebrafish in vivo functional investigation of *TBC1D24* linked with autosomal dominant hearing loss reveals structural and functional defects of the inner ear

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TBC1D24 genetic variants are causally involved in the development of both autosomal recessive hearing loss and epilepsy syndromes, and autosomal dominant hearing loss (ADHL). So far, our group published four novel ADHL-causative *TBC1D24* probably pathogenic variants by performing high-throughput genetic testing in families with ADHL, and more variants are yet to be revealed. In the light of current discoveries, variants in *TBC1D24* emerge as a more significant cause of ADHL.

The molecular mechanism behind the *TBC1D24*-associated ADHL is unknown. Using a zebrafish model, we investigated involvement of *TBC1D24* in hearing and the functional effects of the associated ADHL-causing genetic variants. Different methodological approaches were used in the study, including (i) expression studies by whole mount in situ hybridization (WISH), qPCR on different developmental stages and cryosections, (ii) assessment of the zebrafish ear and neuromast hair cell morphology by high-resolution imaging and (iii) behavioral studies in a developed tbc1d24-deficient zebrafish models (by knock-down or knock-out of tbc1d24) and in overexpression and rescue tbc1d24 models.

We show that the morpholino-mediated knockdown of Tbc1d24 resulted in defective ear kinocilia structure and reduced locomotor activity of the embryos. The observed phenotypes were rescued by a wild-type *TBC1D24* mRNA but not by a mutant mRNA carrying the ADHL-causing variant c.553G>A (p.Asp185Asn), supporting its pathogenic potential. CRISPR-Cas9-mediated knockout of tbc1d24 led to mechanosensory deficiency of lateral line neuromasts. Overexpression of *TBC1D24* mRNA resulted in developmental abnormalities associated with ciliary dysfunction and mesendodermal mispatterning. We observed that the ADHL-causing *TBC1D24* variants: c.553G>A (p.Asp185Asn); c.1460A>T (p.His487Leu), c.1461C>G (p.His487Gln) or a novel variant c.905T>G (p.Leu302Arg) alleviated the effect of overexpression, indicating that these variants disrupt the *TBC1D24* function. Furthermore, the zebrafish phenotypes correspond to the severity of ADHL. Specific changes in ear structures upon *TBC1D24* overexpression further highlighted its tissue-specific role in ciliary function and inner ear development.

Our findings provide functional evidence for the pathogenic potential of the ADHL-causing *TBC1D24* variants and lead to new insights into the function of *TBC1D24* in cilia morphogenesis.

Grant: 2016/22/E/NZ5/00470 National Science Centre, Poland.

Posters

AAV-regulated Serpine2 overexpression promotes hair cell regeneration

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Inner ear hair cell (HC) damage is irreversible in mammals, but is has been shown that supporting cells (SCs) have the potential to differentiate into HCs. Serpine2, a serine protease inhibitor, encodes protease nexin 1, and this has been suggested to be a factor that promotes HC regeneration. In this study, we overexpressed Serpine2 in inner ear SCs cultured in two- and three-dimensional(2D and 3D) systems using the Adeno-associated virus-inner ear (AAV-ie) vector, which promoted organoid expansion and HC differentiation. Overexpression of Serpine2 in the mouse cochlea through the round window membrane (RWM) injection promoted SC proliferation and HC regeneration, and the regenerated HCs were found to be derived from Lgr5+ SCs. In conclusion, our findings indicate that Serpine2 overexpression promotes HC regeneration and suggest that the utilization of inner ear progenitor cells in combination with AAVs might be a promising therapeutic target for hearing restoration.

Accuracy and consistency of ChatGPT responses to questions related to physiology of hearing

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Introduction: ChatGPT has been tested in many disciplines, but only a few studies have involved hearing diagnosis, and none have focused on hearing physiology. The consistency of the chatbot's responses to the same questions posed multiple times has not been well investigated either. This study aimed to assess the accuracy and repeatability of ChatGPT 3.5 and 4 on test questions related to otoacoustic emissions and auditory brainstem responses. Of particular interest was the short-term repeatability of responses, which was tested over four separate days within one week.

Material and methods: The questions which focused on hearing physiology were posed five times to both ChatGPT 3.5 and ChatGPT 4 on each of four days (two days in one week and two days in the following week). The accuracy and the repeatability of the responses over time were evaluated.

Results: The overall accuracy of ChatGPT 3.5 was 48–49%, while that of ChatGPT 4 was 65–69%. ChatGPT 3.5 consistently failed to pass the threshold of 50% correct responses. Within a single day, the percent agreement was 76–79% for ChatGPT 3.5 and 87–88% for ChatGPT 4. The percent agreement between responses from different days was 75–79% for ChatGPT 3.5 and 85–88% for ChatGPT 4.

Conclusions: ChatGPT 4 outperforms ChatGPT 3.5 both in accuracy and repeatability over time. However, the significant variability in responses raises doubts about the potential professional applications of both versions.

Characterizing hair bundle maturation in the mouse utricle during embryonic and postnatal development

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Sensory hair cells are mechanoreceptors required for hearing and balance functions. Stereociliary bundles play a critical role in mechanoelectrical transduction (MET), many prior studies examining developing and regenerated hair cells have assessed bundle morphology to determine cell maturity. However, only few studies have systematically assessed hair bundle dimensions during embryonic and postnatal development. In this study, utricles were collected from embryonic (E) 13.5, 15.5, *E*18.5, postnatal day (P) 0, P37 and P180 wild type mice, and immunostained with the kinocilia marker α-Tubulin and stereocilia marker phalloidin. From 3D reconstructed images of hair cells, kinocilia height, tallest and shortest cilia, and volume were measured. At E13.5, height of kinocilia, tallest and shortest cilia were relatively uniform. Starting at E15.5, wide distributions of hair bundle and kinocilia heights were observed, possibly because of maturing kinocilia and bundles in older hair cells and the emergence of hair bundles and kinocilia in new hair cells in late embryonic and early postnatal periods. Some longer kinocilia appeared curled or bent. Short bundles were still observed in adult mouse utricle. Expression of the actin-crosslinking protein FSCN2 was also examined to characterize the maturity of hair bundles. FSCN2 was absent at E13.5 and E15.5, became detectable in most hair bundles by E18.5 and *P*0, but

some short bundles still lacked FSCN2 even at 6 months. To mark newly born hair cells, we fate-mapped supporting cells in Plp1CreERT/+; Rosa26RtdTomato/+ mice by treating them with tamoxifen at P3. At 1, 2 and 6 months, most bundles of traced hair cells still appeared relatively shorter than untraced ones, and continue to display short, curled or bent kinocilia, resembling those in the early embryonic stages. By 6 months, bundles of only 46% of traced hair cells expressed FSCN2, compared to 97% of the untraced hair cells. Together, our data indicate that hair cells in the embryonic utricle display short kinocilia and stereocilia that elongate over time. Newly added hair cells display short bundle and kinocilia that resemble those during embryonic periods, with some remain detectable in the adult utricle.

Comparing the protective effect of antioxidant and anti-inflammatory drugs, anakinra and rosmarinic acid, against styrene-induced ototoxicity

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Among various solvents used in industries, the aromatic hydrocarbon styrene is strongly associated with ototoxic effects in workers. Our research group previously demonstrated that the primary ototoxic effect of styrene is mediated by the interplay between oxidative stress and inflammation. Indeed, as reactive oxygen species accumulate, the cochlea's natural antioxidant defenses become inadequate, leading to oxidative status imbalance and enhanced inflammatory markers, responsible for hair cell death and hearing loss. Nowadays effective therapeutic interventions for styrene-induced ototoxicity are still lacking. In this study, we compared the protective effects of an antioxidant molecule, rosmarinic acid (RA), and an anti-inflammatory agent, anakinra (Ana), an antagonist of the IL-1β receptor, to evaluate their potential as novel pharmacological treatments against styrene-induced ototoxicity. To this aim, adult male Wistar rats were exposed to styrene (400 mg/kg) by gavage for 3 weeks, 5 consecutive days/week. Before each styrene administration, a subgroup of animals was treated with RA at a dosage of 10 mg/kg intraperitoneally injected, whereas a second subgroup received a dosage of 40 mg/kg of Ana by intramuscular injection. To assess the efficacy of the two different treatments, we performed the auditory brainstem responses (ABRs) at 7, 14, and 21 days after styrene and antioxidant or anti-inflammatory treatment onset. Our results showed a protective effect of both RA and Ana against styrene-induced cochlear damage, with a significant decrease in hearing thresholds in treated animals, compared to styrene-exposed animals. At the end of treatment, we conducted immunofluorescence and molecular biology analyses on cochlear specimens to evaluate changes in molecular markers linked to oxidative stress and inflammation, thus assessing the treatment efficacy. We observed a decrease of oxidative stress markers, as well as of inflammatory agents, indicating that both the antioxidant and the anti-inflammatory treatment can potentiate endogenous responses counteracting oxidative stress and inflammation, thus reducing hearing loss. Collectively, our data show that the treatment with an antioxidant or an anti-inflammatory drug can be effective against styrene-induced ototoxicity, with potential clinical applications to prevent worker health and reduce hearing loss.

Computational model of the peripheral auditory system: ion channel distribution in inner hair cell synapses

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This poster presents a comprehensive computational model of the mammalian peripheral auditory system, encompassing the outer and middle ears, cochlea, and auditory nerve. While the model integrates various physiological aspects across the entire auditory system, this study primarily examines the distribution of CaV1.3 channels in inner hair cell ribbon synapses and their impact on synaptic behavior. The model's holistic approach allows for the examination of individual hearing components and their interactions, providing valuable insights into normal hearing processes and the impacts of various defects. These findings have potential applications in studying hearing impairments and developing auditory prosthetics. Our simulations show that different spatial distributions of CaV1.3 channels result in varying spontaneous rates, thresholds, and sensitivities of the ribbon synapse, thereby affecting auditory signal processing. At low stimulus levels, single CaV1.3 channel openings significantly contribute to vesicle release events, highlighting nanodomain control. As stimulus levels increase, vesicle releases are predominantly influenced by multiple channels, indicating a shift towards microdomain control. This dual mechanism ensures high sensitivity and a wide dynamic range of the ribbon synapse.

Diagnostic genome sequencing improves diagnostic yield in a single center study of 100 patients with non-syndromic and syndromic hearing impairment

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Introduction: Hearing impairment (HI) is a common sensory disorder, which is genetically heterogeneous. Identification of causative variants underlying HI is challenging, since >100 genes for non-syndromic HI and >450 genes for syndromic HI have been reported.

Material and methods: In this study, 100 index patients with HI and variable additional clinical features underwent whole genome sequencing (WGS) in clinical settings. The samples were analyzed using virtual gene-panels of 174 (76% of patients) or 500 genes, respectively, for patients suspected of

having non-syndromic and syndromic HI. Nine patients had prior to WGS been prescreened for *DFNB1*, *SLC26A4*- and/or *STRC*-related HI and six using gene-panels for HI.

Results: A definite genetic diagnosis was made in 42/100 patients, distributed in 25 different genes. In total, 45 different likely pathogenic/pathogenic variants were detected, and 14 variants were novel. In addition, six patients had variants of uncertain significance (VUS) identified, where further work-up was recommended, which might change the classification to likely pathogenic. Finally, in an additional ten patients only one pathogenic variant was identified, so far. Variants in rare/recently identified genes causative of HI included *PLS1* and *ATOH1*.

Conclusions: WGS allowed detection of a definite or possible genetic diagnosis in ~48% of 100 cases (~53% excluding patients with unilateral HI and patients prescreened with previous NGS HI panel). Causative variants were found in >25 different genes, including both common and rare/recently identified HI genes, emphasizing the genetic heterogeneity of the condition. In non-solved selected families, the data will be re-analyzed with improved methods.

Differences in petrosal bone marrow distribution between rat and mouse

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Using tissue clearing and immunofluorescence, we have recently characterized the adult rat temporal bone marrow distribution, and in particular the distribution and connections to the inner ear of petrosal bone marrow (doi: 10.3389/fneur.2024.1386654). Bone marrow was identified by its high cellular content and by the presence of cell populations belonging to the hemopoietic niche (e.g. megakaryocytes, see companion abstract from our group). In the cleared rat petrosal bone, autofluorescence allowed delineation of the otic capsule. The largest marrow island was found outside of the otic capsule, surrounding semicircular canal arms, and connecting to the dura through bone channels similar to those of calvarial bone, with only a few channels directed towards the bony labyrinth. Unexpectedly, bone marrow was also observed within the otic capsule endochondral layer, forming small clusters associated to the vestibule (VEM) and cochlear apex (CAEM). Endochondral bone marrow was connected through vascular loops to the labyrinth, and through straight channels to dural sinuses. The latter also received vascular connection from marrow located in surrounding bones, suggesting a role as immune barrier restricting pathogen spread from ear to brain. In mouse, petrosal bone marrow distribution was overall similar to the rat but displayed a few differences. The most evident difference was the volume ratio of CAEM over total petrosal bone marrow (23 ± 1% in mouse, $n = 2$; 2 ± 1% in rat, $n = 8$; $p < 10E\text{-}8$). Moreover, CAEM and VEM were connected by vascular bridges in mouse but not in rat. Given the importance of local bone marrow in the immune reactions of brain (doi: 10.1111/imr.13120) and middle ear (doi: 10.3389/fgene.2022.985214), this difference in CAEM volume and connectivity calls for attention in choosing a model for human inner ear immune reactions.

Disrupted *GRHL2* transcriptional activity as a mechanism of autosomal dominant hearing loss development (DFNA28)

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Introduction: *GRHL2* is one from over 50 genes causative of autosomal dominant hearing loss (ADHL); it is also implicated in other disorders, including cancers. *GRHL2* encodes a transcription factor but up to now only a handful of ADHL-related *GRHL2* pathogenic variants have been reported. Their mode of action leading to ADHL development remains unknown. The aim of the study was to identify the genetic basis of ADHL in a multigeneration family with postlingual, progressive HL and to gain insight into the molecular mechanism of the ADHL-related (DFNA28) *GRHL2* mutations.

Material and methods: Genomic DNA was isolated from the peripheral blood samples of the proband and other family members ($n = 8$). Next-generation sequencing was performed using a multi-gene panel with 237 HL-related genes. Segregation analysis of the selected *GRHL2* variant with HL in the family was performed by Sanger sequencing. For four different ADHL-related *GRHL2* variants expression vectors were prepared and luciferase reporter gene assay was conducted in HEK293T cells.

Results: In the family a novel heterozygous *GRHL2* variant (NM_024915.4: c.1061C>T; NP_079191.2: p.(Ala354Val)) segregating with HL was identified. It localizes in the region corresponding to the DNA binding domain. The functional effect of the variant as well as of the other two *GRHL2* variants located in the DNA-binding domain (i.e. c.1258-1G>A, p.(Gly420Glufs*111) and c.1276C>T, p.(Arg426*)) was a reduction in *GRHL2* transcriptional activity. In contrast, the c.1609–1610insC (p.(Arg537Profs*11)) variant affecting the DNA dimerization domain of the *GRHL2* protein acted in a different way leading to a strong activation of the GRHL-responsive promoter.

Conclusions: Our data show that only truncating *GRHL2* mutations can cause ADHL. The pathogenicity of the novel missense ADHL-related *GRHL2* variant was strengthened by the results of functional assays. *GRHL2* mutations causing ADHL demonstrated both suppression and activation of *GRHL2* transcriptional activity and the effect seems to depend on where the variant is located. While the variants located in the DNA-binding domain showed haploinsufficiency, the variant located in the DNA dimerization domain presented a gain of function effect. Our study sheds new light on the mechanism of *GRHL2* mutations leading to hearing loss.

Grant: 2016/22/E/NZ5/00470 National Science Centre, Poland.

Early transtympanic administration of rhBDNF exerts a multifaceted neuroprotective effect against cisplatin--induced hearing loss

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Cisplatin-induced sensorineural hearing loss is a significant clinical challenge and, currently, only one drug has been approved by Food and Drug Administration as an effective treatment. Thus, several efforts are needed to better understand cisplatin mechanism of damage and to explore new therapeutic strategies. Although the potential effects of brain-derived neurotrophic factor (BDNF) have previously been investigated in some ototoxicity models, its efficacy in cisplatin-induced hearing loss remains uncertain. This study aimed to investigate the therapeutic potential of recombinant human BDNF (rhBDNF) local delivery in counteracting cochlear damage in an in in vivo model of cisplatin-induced ototoxicity. Thus, adult Wistar rats were treated with cisplatin (12 mg/kg, intraperitoneally injected) and after one hour, they received 5 mg/kg of rhBDNF suspended in a thermogel by a transtympanic injection. Auditory brainstem responses were recorded to evaluate hearing function at 1, 3 and 7 days after treatment. Seven days after cisplatin treatment, we collected cochlear samples to perform, morphological, immunofluorescence, and molecular analyses to investigate the molecular mechanisms underlying the beneficial effects of our rhBDNF formulation. Our data showed that rhBDNF mitigates hearing loss in cisplatin-exposed rats by preserving synaptic connections in the cochlear epithelium and reducing hair cell and spiral ganglion neuron death. rhBDNF maintains the balance of its receptor levels (pTrkB and p75), boosting TrkB-CREB pro-survival signalling and reducing caspase 3-dependent apoptosis in the cochlea. Additionally, it activates antioxidant mechanisms while inhibiting inflammation and promoting vascular repair. Overall, our study demonstrates that the early transtympanic treatment with rhBDNF plays a multifaceted protective role against cisplatin-induced ototoxicity, thus holding promise as a novel potential approach to preserve hearing in adult and pediatric patients undergoing cisplatin-based chemotherapy.

Electrical and cytotoxical examination of electrospun PVDF-TrFE fiber mats

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Implantation of cochlear implants (CI) triggers various biological reactions in the patient's cochlea. Being unable to remove the foreign structure by itself, the body insulates the implant by surrounding it with connective tissue. Modification of the CI electrodes surfaces is one way to reduce the adhesion of connective tissue. Electrospinning is an electrohydrodynamic process used to generate thin mats. These fine micro networks can be used to alter the surface of CI electrodes. In this study, fiber meshes consisting of the hydrophobic poly(vinylidene fluoride-trifluoroethylene) (PVDF-TrFE) were used to investigate their contribution to electric conductivity and their cytotoxic potential for fibroblasts. The fiber mesh was cut into Ø 14 mm circular samples. Conventional SEM-holders were used as sample holders for the electrical measurements. Using a self-constructed chamber with four identical measuring cells, the increase in impedance due to the additional layer of fibers was investigated. In addition, cytotoxicity assays with rectangular samples as described in ISO 10993-12 were performed. For further investigations, model electrodes out of platinum-iridium wire were manufactured such that their surface area resembles that of CI electrodes. These models were embedded in silicone (Sylgard 184) and electrically analysed to test different wettability methods. Measured impedances for the fiber mesh samples show a mean increase in impedances of 225.99 ± 107.09 Ω compared to the reference samples. The only constraint in biocompatibility was found with 100% extraction solution (69 ± 4.88% cell viability). The mean surface area of the model electrodes was 0.386 ± 0.024 mm2 with impedances of about 1 kΩ (1024.19 ± 107.61 Ω). Cytotoxic and electric characterisation of PVDF-TrFE electrospun fiber meshes was successfully performed. Reliable model electrodes were manufactured and can be used in further investigations regarding the influence of the fiber mats on the impedance of CI electrodes. Additionally, cell proliferation behaviour on the fiber mats will be investigated to refine the cytotoxic influence.

Elucidating the molecular diversity of the non-human primate cochlea

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Unraveling the intricate composition and function of the cochlea is paramount to comprehending the mechanisms underlying sound perception and the pathogenesis of auditory disorders. The mammalian cochlea displays a highly organized structure, which contributes to the diversity and complexity of auditory processing. However, the cellular

intricacies in non-human primates remain largely unexplored. In the present study, we employed high throughput transcriptomic sequencing to profile over 11,280 nuclei across virtually all cochlear cell types in both juvenile and adult *Macaca fascicularis* at single-cell resolution. Our analysis unveiled remarkable heterogeneity both across and within cell types. Despite a largely conserved cellular composition of the cochlea, glial cells exhibited substantial species-specific diversity, while hair cells and spiral ganglion neurons with specialized transcriptional programs were well mapped onto their murine counterparts, underscoring the similarities that persist despite evolutionary divergence. Furthermore, we constructed a disease map associated with hearing loss, establishing this transcriptomic atlas of the macaque cochlea as an indispensable resource for future investigations in both human and non-human primates.

Enhanced spiral ganglion neuron transduction for neurotrophin gene therapy with novel capsid-engineered AAV vector

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Hearing loss (HL) affects over 460 million people worldwide, significantly affecting quality of life. Genetic analysis has identified more than 150 causative monogenic genes for non-syndromic sensorineural hearing loss, presenting attractive targets for gene therapy interventions. These approaches also have the potential to enhance conventional treatment strategies. Particularly interesting in this regard are cochlear implants (CI), used for severe to profound HL. However, their efficacy is believed to depend on spiral ganglion neuron (SGN) survival. Recognizing the potential of neurotrophins to enhance SGN survival and improve CI outcomes, we here report on the development of a novel adeno-associated virus (AAV) vector optimized for transducing SGN, even in adult mice and at low vector doses. For this purpose, the capsid was engineered to display a heptamer peptide, which was previously derived from a phage library screen.

Structure-focused modeling of our novel vector Var9 indicated a clear change in cell attachment receptor binding due to peptide insertion compared to its parental serotype AAV2. Predictions were confirmed using affinity chromatography and competition assays. Interestingly, Var9 demonstrated faster transgene expression in HEI-OC1 cells, a murine otic progenitor cell line, despite significantly lower entry efficiency, indicating enhanced intracellular processing of the vector. Subsequent IF-FISH analysis at single-cell level as well as our indirect uncoating assay revealed that Var9 vectors clearly outperform AAV2 vectors regarding kinetics and level of uncoating (3-fold), i.e. release of their genome from the capsid, a prerequisite for transgene transcription. Finally, in a neurotrophic gene therapy approach, Var9 effectively prevented SGN degeneration by overexpressing BDNF in SGN of deafened mice. Histological analysis of the cochlea revealed remarkable protective effects of SGN comparable to untreated control levels in all cochlear turns of mice treated with Var9-BDNF.

In conclusion, our novel AAV vector demonstrated superior properties crucial for efficient SGN transduction and will be further refined for clinical applications, aiming to enhance neural survival and improve outcomes for cochlear gene therapy in CI recipients.

Exploring the link between noise-induced trauma and peripheral inflammation

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Traditionally, the cochlea has been considered as an immunological privileged site, as the blood-labyrinth barrier provides isolation from the systemic immune system. However, the presence of immune cells in the cochlea has been recently reported, suggesting that immune-mediated processes can play a crucial role within the auditory system. In this context, both protective and detrimental T-cell functions have been linked to normal hearing and hearing loss, respectively, thereby emphasizing the dual role of T cells in cochlear health. Current knowledge also suggests that a balanced presence and activity of T cells is crucial for tissue homeostasis in the cochlea, avoiding pathologies such as age-related hearing loss and autoimmune inner ear diseases. Although cochlea-resident T cells are scarce under physiological conditions, the number of T cells appear to rise in response to acoustic trauma revealing the active recruitment of peripheral immune cells through cochlear blood vessels. In order to evaluate the induction of a peripheral inflammatory response upon noise trauma, we exposed mice to excessive noise (115 dB) for two hours. Hearing levels were determined 72 h and 1 week after noise-exposure, which revealed a substantial increase in hearing thresholds in all mice. To evaluate if the auditory stimulation would trigger a peripheral immune response we isolated splenocytes and analyzed cytokine production in different immune subsets

by high parametric flow cytometry. The analyses revealed increased activation of both CD4+ and CD8+ T cells, illustrated by significant increases in the expression of INF-γ, TNF and CD44. These data suggest a peripheral component in the elevated inflammatory response following acoustic trauma. Future research will be performed to explore the link between noise-induced hearing loss, peripheral inflammation and more specifically antigen-specificity of the observed increase in activated CD4+ and CD8+ T-cell populations.

Expression of P2X2, P2X4, and adenosine A1 receptors in sheep and human cochlea: a translational study

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Purinergic receptors have been identified in virtually all mammalian tissues to regulate fundamental cellular processes, leading to increased focus on its therapeutic potential. In rodent cochlea, purinergic receptors (P2X1,2,3,4,6,7, P2Y1,2,4,6,12, adenosine A1, A2A, A3) have been identified to play roles in cochlear (patho)physiology. However, previous findings from rodent models have yet to be translated into other mammalian species. Our earlier screening showed that several purinergic receptor subtypes are also expressed in sheep and human cochlea. The aim of this study is to characterize in detail the immunolabelling of P2X2, P2X4, and adenosine A1 receptors (P2X2R, P2X4R, adenosine A1R) in the sheep and human cochlea to contribute to understanding the potential functional roles played cochlear (patho)physiology.

Fixed, decalcified adult New Zealand Romney sheep temporal bones and celloidin-embedded adult human temporal bone sections were used for immunohistochemistry. Sub-type specific rabbit polyclonal antibodies raised against P2X2, P2X4, and adenosine A1 receptors were used with cellular markers. Super-resolution confocal imaging (Zeiss LSM800 Airyscan) was used for data acquisition.

P2X2R immunolabelling was present in outer hair cells (OHCs), inner hair cells (IHC), Deiters' cells, outer sulcus cells, basal cells of the stria vascularis, and the Reissner's membrane in sheep cochleae. At high resolution, strong immunolabelling was observed along the reticular laminar. Strong immunolabelling was also observed in the stereocilia and within the cuticular plates. P2X4R immunolabelling was present in OHCs and IHC, and the Reissner's membrane sheep cochleae. However, P2X4R immunolabelling was predominantly localized to the cytoplasm of OHCs. Adenosine A1R immunolabelling was predominantly localized to the IHC and Deiter's cells in sheep cochleae. Comparative data for P2X2R, P2X4R, and adenosine A1R immunolabelling in adult human cochleae with no known history of hearing impairment will also be presented.

Our results show that the expression patterns of P2X2R, P2X4R, and adenosine A1R appear comparatively conserved across mammalian species. A future study will be conducted to test the inferred functional role of P2X2R, P2X4R, and adenosine A1R in adult sheep models. Furthermore, our results support the potential for purinergic signaling as a target for future pharmacological-based interventions aimed at mitigating hearing loss.

GelMA promotes inner ear organoidogenesis by regulating Mmp-mediated extracellular matrix remodeling

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Permanent damage and loss of inner ear hair cells due to genetic mutations or external factors such as drugs and noise are the main causes of irreversible hearing loss. Inner ear organoids can provide an in vitro model for studying the underlying mechanisms of injury and for developing new therapeutic approaches. However, there is lack of protocols for the rapid and efficient establishment of inner ear organoids. In this study, we established a novel method for creating inner ear organoids using a synthetic GelMA hydrogel culture system to promote the spontaneous aggregation and assembly of inner ear stem cells in order to rapidly form organoids, and we found that the extracellular matrix undergoes extensive and rapid remodeling during self-assembly. The expression and activity of the Mmp family of extracellular matrix degradation enzymes, especially matrix metalloproteinase 9 (Mmp9), were increased in inner ear organoids in the hydrogel culture system, and inhibition of Mmps significantly inhibited the formation of inner ear organoids. Our study is the first to combine analysis of the extracellular matrix with inner ear organogenesis and provides a rapid and efficient method to model inner ear organoids.

Genetic analysis reveals novel variants in a cohort of patients affected by sensorineural hearing loss and enlarged vestibular aqueduct (EVA)

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Biallelic pathogenic variants in the *SLC26A4* gene, coding for the anion exchanger pendrin, are responsible for Pendred syndrome and nonsyndromic recessive hearing loss DFNB4. Both are associated with an enlarged vestibular aqueduct (EVA), the most common malformation of the inner ear. We recruited the first Austrian cohort of patients with hearing loss and EVA to define the prevalence of pathogenic variants in *SLC26A4* and discover novel EVA-associated genes.

The coding region and intron-exon boundaries of known EVA genes were amplified by PCR and Sanger sequenced. The presence of the Caucasian EVA (CEVA) haplotype was determined with the rhAmp® SNP Assays (IDT). Copy number variation (CNV) in the *SLC26A4* and *STRC* genes was assessed using a TaqMan™ Assay on QuantStudio3D. For undiagnosed patients, whole exome sequencing (WES) was performed. The pathogenicity of novel *SLC26A4* and TJP2 variants was evaluated by functional and molecular assays.

Biallelic pathogenic variants in *SLC26A4* were detected in 5/33 patients. Based on the perchlorate discharge test, one had Pendred syndrome. Monoallelic variants in *SLC26A4* were detected in 5/33 patients. Two were benign based on functional and molecular tests. The CEVA haplotype was found in 6 patients, 3 carried monoallelic pathogenic *SLC26A4* variants, 2 carried biallelic pathogenic variants, and one carried a monoallelic benign variant. Pathogenic variants in *FOXI1* (1/33), *POU3F4* (2/33) and *GJB2* (2/33) were also identified. No CNV of *SLC26A4* and *STRC* was found. WES of patients negative for known causative genes (15/33) detected variants in 6 EVA-unrelated genes (*SCD5*, *REST*, *EDNRB*, *TJP2*, *TMC1*, and *CDH23*) in 5 patients. Cell-based assays showed that the novel TJP2 variant leads to an aberrantly localized protein product, supporting its pathogenicity.

Sequence alterations in *SLC26A4* and/or the CEVA haplotype, *FOXI1*, *POU3F4*, and *GJB2* genes are responsible for hearing loss and EVA in 14/33 patients of this cohort. WES led to the identification of 6 genes previously not associated with EVA and allowed for the diagnosis of additional 5 patients. The genetic causes remain unidentified in 42% (14/33) of patients. Functional and molecular studies are needed to define the pathogenicity of novel variants and establish a causal link with disease.

Genetic diversity of hearing loss and its connection to auditory development of cochlear-implanted children

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Introduction: Each year, approximately 1–6 out of every 1000 children are born with severe to profound hearing loss (HL). In the majority of them HL is genetically determined and usually two pathogenic variants are detected in the DFNB1 locus. The aim of the study was to dissect the genetic background of non-DFNB1 HL in CI patients and to analyze their auditory development.

Material and methods: The study group ($n = 51$) was recruited from patients with isolated profound prelingual deafness who received CI before the age of 24 months. All patients were negative for DFNB1 locus pathogenic variants. Genomic DNA was isolated from blood samples. In probands whole exome sequencing (WES) was performed. Validation of selected variants and family segregation analysis were performed using standard Sanger sequencing. Identified copy number variants were examined with aCGH and qPCR. Evaluation of patients auditory development was performed with the LittlEARS questionnaire (LEAQ) in three subsequent intervals – at the time of cochlear implant activation as well as in 5th and 9th month after CI.

Results: Causative variants were identified in 74.5% of patients (38/51). The majority of them are localized in the *MYO15A* ($n = 7$) and *PAX3* ($n = 5$) genes. Among the detected genetic variants, 28% (15/54) were inherited in an autosomal dominant manner and eight of them occurred de novo. A syndromic form of HL was diagnosed in 27% (14/51) of patients. The auditory development of the studied children was the most dynamic in the first 5 months after CI and slowed down between the 5 and 9 months of using the device. No differences were observed between the auditory development of patients with an identified and unknown genetic causes of HL.

Conclusions: Obtained results show a high heterogeneity of genetic HL causes in the population of Polish DFNB1-negative cochlear-implanted patients. All tested children were good candidates for CI as their HL causative genetic variants are localized in genes preferentially expressed in the cochlea. In a group of patients without an identified genetic cause, the tested area should be expanded and more advanced technologies enabling full genome analysis (WGS) should be used.

Grant: 2017/27/N/NZ5/02369, National Science Centre, Poland.

Harnessing AI for enhanced analysis of cochlear imaging data

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The sensory epithelium of the mammalian cochlea exhibits a tightly organized pattern of sensory hair cells along the so-called tonotopic axis. High-resolution imaging now commonly generates large datasets from light and electron microscopy, but analyzing these massive datasets has become a bottleneck, exacerbated by the lack of efficient tools that can mitigate user biases and manual labor.

Recent advances in Artificial Intelligence and Machine Learning (AI/ML) are transforming our ability to analyze extensive datasets and accelerate scientific discovery, particularly in tasks related to bio-image analysis. We will present examples of AI/ML-based applications we have developed for analyzing large inner ear imaging datasets, demonstrating how these technologies can expedite traditional time-consuming analyses and help overcome barriers in the field. These tools serve as a blueprint for developing novel applications in the field of auditory neuroscience.

To develop one such tool, we first assembled a diverse, carefully annotated dataset comprising 2D images of auditory hair cells captured using fluorescence microscopy, contributed by the global auditory research community. We then developed an AI/ML-based application trained on this dataset that automates the detection, classification, and quantification of hair cells along the tonotopic axis. The tool leverages advanced deep learning libraries and architectures, resulting in robust, generalizable models. Next, we extended AI/ML models to a more complex challenge: analyzing serial 3D electron microscopy datasets. We developed a novel tool for volumetric instance segmentation of mitochondria, which significantly enhances the structural analysis of subcellular organelles in electron microscopy volumes.

Our results illustrate significant time savings and increased reproducibility, utilizing open-source technologies and free software to build tools that can be shared as standalone tools or ImageJ plugins. These developments streamline data processing across various imaging modalities commonly used in the field of auditory neuroscience and enable detailed, quantitative analysis of large datasets to aid in discoveries that may have been overlooked otherwise.

While not exhaustive, these case studies underscore the essential steps for developing and employing AI/ML-based tools to address complex biological questions, highlighting the potential of these technologies to advance studies that rely heavily on detailed imaging data analysis.

Hearing loss as the main clinical presentation in NLRP3-associated autoinflammatory disease

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The NLRP3 gene mutations are the cause of autosomal dominant autoinflammatory disorders (*NLRP3*-AID). Recently, hearing loss (HL) has been found to be the sole or major manifestation of *NLRP3*-AID. Here, we tested 110 autosomal dominant HL families with a custom panel of 237 HL genes and found one family carrying the *NLRP3* c.1872C>G, p.Ser624Arg mutation. Functional studies revealed that this novel variant is a gain of function mutation, leading to increased activity of caspase-1 and subsequent oversecretion of proinflammatory interleukin-1b. Clinical reanalysis of the affected individuals, together with serological evidence of inflammation and pathological cochlear enhancement on FLAIR-MRI images, guided our diagnosis to atypical *NLRP3*-AID. The study highlights the role of genetic analysis in patients with progressive postlingual HL. This can help to identify individuals with hereditary HL as a consequence of *NLRP3*-AID and allow timely and effective treatment with interleukin-1-receptor antagonist.

How do you define bone marrow in the petrosal bone?

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Bone marrow is a highly cellular connective tissue, containing both fixed elements (blood vessels, adipocytes, stromal and staminal cells) and mobile cells (blood cellular elements and their precursors). Since mature immune cells are also found in other connective tissues, especially during inflammation, the only way to identify bone marrow with certainty is by labelling its unique components, i.e. blood cell precursors and stromal components. This however creates two problems: 1-mobile cells are lost, in variable measure, during tissue sectioning, and 2-most bone marrow cells do not display unique epitopes, just unique combinations of them.

Within most bones, these problems do not significantly affect marrow identification, since the latter is contained in large, well-defined bone cavities, and even after some cell loss, most of its elements are still present in sufficient quantity to be visualized by immunofluorescence. The temporal bone,

however, displays unique structural complexity, and in particular the petrosal bone surrounding the inner ear is made of extremely dense bone formed by endochondral ossification (different from calvaria, which are formed by membranous ossification) and even displays cartilage remnants in the adult. Although immune cells have been observed throughout the petrosal bone, only at the petrosal apex bone marrow has been identified as such.

Recently, however, in the human temporal bone, cavities compatible with bone marrow were found by synchrotron imaging to be located between the cochlear base and endolymphatic sac and connected with the latter through bone channels (Liu et al., 2024, doi: 10.3389/fneur.2024.1355785). In a similar position, by using tissue clearing, we observed in the rat temporal bone highly cellular cavities with a similar connection pattern (Perin et al., 2024, doi: 10.3389/fneur.2024.1386654). These cavities could be as small as only containing a few hundred cells only, and in previous work they had been addressed as perivascular connective. However, even the smallest cavities contained megakaryocytes, which are platelet precursor and are confined to marrow in other bones. Therefore, the small cavities within the petrosal bone can be identified as bone marrow, and may be involved in local inner ear immune responses.

Identification of novel components of the lower tip-link complex: a proteomic and AI-based approach

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Understanding the complex workings of auditory mechano-electrical transduction (MET) in the auditory hair cells is essential for comprehending the mechanisms underlying hearing. We present a novel approach integrating single-cell proteomics, affinity-purification mass spectrometry (AP-MS), and AI modeling to identify new components of the lower auditory MET complex, crucial for auditory function. Specifically, we seek to identify new components of the lower tip-link complex via AP-MS with specialized antibodies against the known tip-link component protocadherin 15 (Pcdh15). Protein-protein interactions between potential new and known MET components will be validated using Alphafold2. Simultaneously, we construct an exhaustive proteomic profile of inner hair cells (IHCs) and outer hair cells (OHCs) isolated from the murine cochlea, employing a suction pipette technique for single-cell isolation. Our sample preparation method yields promising results, with over 500 protein groups per single OHC. While proteomic analyses of vestibular hair bundles exist, none focus specifically on IHCs and OHCs. Ultimately, the aim is to conduct a comparative analysis between OHCs and the more difficult-to-isolate IHCs. Furthermore, we intend to correlate this dataset with established molecular structures in auditory mechanisms and have them serve as complimentary validation for AP-MS experiments with known components of the hearing machinery. These findings will offer critical insights into cochlear mechanosensation, advancing our understanding of auditory biology and providing a framework for innovative therapeutic strategies to address hearing impairments.

Implementing swept-tone and level distortion-product and stimulus-frequency otoacoustic emission recording protocols in rats

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Age-related hearing loss (ARHL) progression and outer hair cell (OHC) functional decline have previously been reported in Wistar rats but limited to non-linear distortion-product otoacoustic emissions (DPOAEs). Linear reflection-type stimulus-frequency otoacoustic emissions (SFOAEs) have not yet been established for this species and may provide additional information about cochlear amplification, tuning and onset of hearing loss. In human studies, inclusion of both OAE types in cochlear assessment may provide complimentary information. Here we report initial efforts to broaden the toolbox for time-efficient and objective assessment of cochlear function in rats with swept-stimulus DPOAE and SFOAE measurements.

SFOAE measurement feasibility and repeatability were first confirmed in male Wistar rats. Then, for fixed level OAEs measured across range of frequencies, we implemented a "swept-tone" paradigm, testing various frequency sweep rates. To further speed up data collection, the frequency range (~4–40 kHz) was divided into 2 or 3 subranges played simultaneously. Results of single vs. multiple frequency sweep SFOAE and DPOAE at varying rates were compared to OAEs measured with steady-state discrete tones. We also studied the effects of sweeping the stimulus level, at fixed frequency for measurements of DPOAE input-output functions at six f2 frequencies. Stimulus levels were swept at variable rates with up to three pairs of stimulus frequencies were played simultaneously. Results were compared to DPOAE input-output functions measured with discrete tones.

For the SFOAE repeatability test, a conventional discrete-tone suppression paradigm was used. The maximum average within-subject differences were 5.0 ± 7.1 dB within-day and 6.2 ± 13.5 dB between days.

Swept-tone SFOAE and DPOAE recordings showed amplitudes similar to discrete-tone with rates up to 2 octaves/sec. Whereas DPOAEs could be recorded with 3 simultaneous sweeps (<3 dB difference), SFOAE results from multiple sweeps were not as consistent. Swept-level DPOAEs produced results identical to discrete-tone up to a 80 dB/sec rate and allowed simultaneous recording of 3 frequencies without interference. These data confirm that SFOAEs can be measured in a consistent and time-efficient manner in rats, with levels comparable to other laboratory species. Further work aims

to demonstrate the evolution of SFOAEs with aging, in comparison to ABRs and DPOAEs.

In vitro biocompatibility study of Polyvinyl difluoride piezoelectric nanofibers for cochlear implants

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The cochlear implant (CI) is currently the gold standard in treating sensorineural hearing loss (SNHL) as it activates the cochlear nerve bypassing the hair cells, allowing the brain to hear sound waves. The goal of ongoing research is to create self-powered cochlear stimulation devices based on piezoelectric nanomaterials that can improve the quality of life for patients with SNHL and reduce the side effects of traditional CIs.

Polyvinyldifluoride (PVDF) piezoelectric nanofibers are a new type of piezoelectric nanostructure developed for biological purposes. The fiber can be coated with barium titanate (BaTiO3) nanoparticles or graphene nanosheets (GN) to enhance the piezoelectric coefficients. The purpose of the study is to evaluate the in vitro biocompatibility of PVDF nanofibers, which appear promising as alternatives for producing next-generation CIs.

Pure PVDF, BaTiO3-coated PVDF, and GN-coated PVDF were evaluated on three cell lines: HaCaT, OC-k3, and PC12. Viability, morphological changes, and neuritic outgrowth were evaluated in vitro using these cell lines as a model for the cochlear tissues.

The morphology study indicated that HaCaT, OC-k3, and PC12 cells were healthy, well-preserved, and had normal structural characteristics at all times tested. According to cell viability results, the fibers caused an increase in cell metabolism after 72 hours of incubation, especially on OC-k3 and PC12. On the HaCaT cell line, the fibers exhibited a slight but not significant reduction of cell metabolic activity starting from 48 hours of exposure. In addition, BaTiO3-coated PVDF have the most favorable results when it comes to the number of branch points and average length of neurites in PC12 cells, leading to a conclusion that BaTiO3 nanoparticles enhance the complex processes of PC12 cells.

To summarize, the investigation revealed that the tested nanofibers exhibited high biocompatibility in vitro, particularly with cochlear and neuronal cells, and the piezoelectric nanofibers with barium titanate particles be used to develop the next generation of self-powered cochlear implants. To conclude, these piezoelectric nanofibers have the ability to stimulate the cochlea, even though additional research is needed to achieve adequate mechanical and electrical performance.

In vivo calcium imaging in the developing mouse cochlea

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Introduction: Sensory-independent calcium activity regulates the development of mammalian sensory systems. Our current understanding of the origin and modulation of these calcium signals comes from ex vivo experimental work, which cannot replicate the sophisticated anatomy, innervation and physiology of the intact mammalian cochlea. We have developed surgical and microscopy approaches that, combined with transgenic animals expressing fluorescent indicators, allow us to study how mammalian sensory hair cells operate in vivo. Using this approach, we investigated the dynamics of spontaneous calcium activity in the prehearing cochlea of live mice at the cellular level.

Material and methods: Mice (P3–P10) expressing the genetically encoded calcium indicator GCaMP6f in either the hair cells or the supporting cells were anaesthetised using isoflurane and their body temperature maintained with a heat mat. The surgical procedure only led to a very small opening in the apical coil of the cochlear bone, leaving the cochlear canals intact and unopened. The mouse was then transferred on the stage of a two-photon microscope equipped with long working distance water immersion objectives for imaging.

Results: This approach allowed us to record from the same cochlear region spanning 15–30 IHCs. We found that IHCs and supporting cells displayed spontaneous calcium activity in vivo throughout the age-range investigated. IHC activity mostly appeared in bursts and some IHCs appeared to transition between quiescent periods and periods of prolonged spontaneous activity. Nearby IHCs displayed both independent and coordinated activity, which was compatible with the modulation on IHC excitability by calcium waves from the supporting cells.

Conclusions: Our approach provides significant insights into the nature of spontaneous cochlear activity in prehearing mice. These findings provide the first in vivo physiological recordings of spontaneous calcium activity occurring in the mouse pre-hearing cochlea. The application of two-photon imaging to study cochlear activity in vivo offers a promising avenue for future research.

Funding: The Wellcome Trust (224326/Z/21/Z) to WM.

Inner ear malformations caused by mutations in *Slc26a4* gene and it's regulative elements presented in a zebrafish model

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Congenital hearing loss can be caused by genetic abnormalities leading to inner ear malformations (IEM). SLC26A4 (pendrin) is an anion exchanger expressed in the inner ear, thyroid and β-intercalated cells in the kidney. Mutations in *SLC26A4* cause a common form of IEMS: enlarged vestibular aqueduct (EVA), often accompanied by incomplete partition type 2 (EVA/IP2). However, only 25% of patients have confirmed mutations in *SLC26A4*. It has been reported that a group of polymorphisms upstream of the *SLC26A4* gene, the CEVA haplotype, is frequently found in patients with monoallelic *SLC26A4* mutations. Since the publication of the zebrafish genome in 2001, the zebrafish has become an increasingly popular animal model for studying human diseases. The transparency of the larvae, the large number of offspring and the accessibility of various methods of genetic, chemical and physical manipulation make it a very useful model for studying inner ear development. During early development, the inner ear undergoes dynamic changes from an otic placode and otic vesicle to a labyrinth of semicircular canals. Since data on the expression of genes associated with hearing are incomplete, we present here a detailed account of expression during early development. Secondly, we plan to create a zebrafish model for human *SLC26A4* gene expression by introducing human upstream regulatory elements into the zebrafish genome.

Investigation of cochlear implant impedances over time

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Introduction: Structure and hearing preservation are important factors in cochlear implant (CI) surgery. To improve outcomes, impedance measurements of the CI electrode can be used to monitor these parameters after electrode insertion to detect delayed hearing losses: electrode impedance is thought to be a biomarker for inner ear inflammation after insertion of a CI electrode into the cochlea. Within the present study, impedance changes over time were analyzed using an app-based solution for daily in-vivo impedance measurements of CI recipients.

Material and methods: $N = 22$ participants were asked to use a research software, the Telemetry Study App, to measure the impedance of all 12 electrode contacts morning and evening for a period of at least 4 months after implantation. Depending on the start of electrical stimulation, the cohort was divided into (1) direct activation (DA) and (2) non-DA group.

Results: In both groups, lower impedances were observed in the evening compared to the morning with the onset of electrical stimulation. An increase in mean impedances was shown up to 10 days after surgery. Mean impedances of the DA group reached a plateau after about 30 days after surgery while the non-DA group continued to show a slight increase. Impedance values of the DA group remained mostly unaffected from the start of the first fitting week (~day 40 postoperatively) but decreased in the non-DA group. Mean impedances of the non-DA group were slightly lower than those of the DA group at this time but converged at the end of the investigation period.

Conclusions: The Telemetry App allowed for daily impedance measurements and hence for monitoring of the inner ear condition. With increased measurement frequency, the impedance development over time after CI surgery could be shown. In addition, daily impedance fluctuations were observed with the onset of electrical stimulation.

Is there a relationship between voice and hearing?

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Introduction: Nowadays, hearing impairment is one of the key challenges at the forefront of paediatric otorhinolaryngology. In the absence of auditory feedback, children with impaired hearing suffer from voice and speech disturbances due to inability to control their own voices. Thus, the purpose of this study was to describe the nature of voice abnormalities in children with hearing loss.

Material and methods: The study involved 100 aged 4–12 years with a diagnosis of bilateral chronic sensorineural hearing loss. The children underwent subjective and objective voice research methods.

Results: The most prevalent complaint in children (72.94%) was a change in voice quality. Endoscopy of the larynx showed no pathological findings in 87.0%. Acoustic analysis of the voice in children with hearing loss showed the following voice disorders: in Grades 3 to 4 hearing loss, a shift in F0 towards low frequencies of 239.78 Hz is seen (95% CI 228.6–250.95) ($p < 0.05$). Children with hearing loss had high Jitter of 1.82 (95% CI 1.22–2.43) that was nearly three times over the normal limits ($p < 0.05$). MPT in children with Grades 3 to 4 hearing loss was lower than that in children with normal voice and hearing and measured 5.41 s (95% CI 1.22–2.43) ($p < 0.05$).

Conclusions: The study found that hearing impairment is associated with the development of voice abnormalities. Medical care for children with Grades 3 to 4 hearing loss should include measures to identify and treat those voice abnormalities.

KDM5B controls sensory neuron subtype diversity

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Introduction: The transformation of initial auditory stimuli is fundamentally upon the synaptic connections originating from hair cells and projecting to the spiral ganglion neurons (SGNs) within the cochlea. During stages of neuronal development, epigenetic modulations coordinated synergistically with transcription regulators play a pivotal role in the determination and preservation of neuron subtypes. Mature murine cochlear SGNs comprise four distinct subtypes, specifically designated as Type Ia, Ib, Ic, and II; however, the definitive regulatory factors conferring specification and maintenance of these subtypes are yet to be comprehensively elucidated.

Methods: 1. Single-cell transcriptomic analysis: Seurat, Harmony, SCENIC, Metascape. 2. Adono-associated virus (AAV)-based delivery system to murine cochlea. 3. Single-molecular in situ hybridization (RNASCOPE). 4. Electrophysiological patch-clamp recording. 5. Immunofluorescence staining.

Results: In this study, we initially constructed a single-cell transcriptomic landscape of SGNs from embryonic, neonatal, and adult developmental stages. Utilizing the SCENIC algorithm designed to predict transcription factor (TF) activity, we were able to identify a sequence of TFs exhibiting time-specific expression patterns. Interestingly, our data revealed a substantial association between the epigenetic cofactor KDM5B (Lysine-specific demethylase 5B) and the timing of initial subtype specification which typically occurs at birth or just prior. Altering the gene expression of Kdm5b in the mouse cochlea at birth through an AAV-based round window injection revealed bidirectional effects on subtype markers (Calb1, Calb2, and Lypd1). Specifically, a significant downregulation or even complete ablation was observed subsequent to shRNA--mediated knockdown of *Kdm5b*. Conversely, overexpression of Kdm5b resulted in a reinforcement of mixed SGN identity. Notably, both genetic manipulations consequently induced auditory impairments in the mice, as determined via the auditory brainstem response (ABR) test. Additionally, bulk RNA sequencing data derived from profiling cochlear tissue echoed these findings, reinforcing the importance of Kdm5b in the specification process of SGN subtypes. In sum, our results indicate that the epigenetic regulator *Kdm5b* is indispensable in effectively determining SGN subtype specification.

Conclusions: 1. *Kdm5b* expression is restricted to early SGN development. 2. *Kdm5b* specifies and maintains SGN subtype identity.

Long term expansion of *Lgr5* positive supporting cells and differentiation into a hair cell-like phenotype from adult mouse derived cochlear organoids

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Introduction: Over 400 million people worldwide suffer from hearing loss, which requires medical intervention. The current treatments available to these patients are the use of hearing aids or cochlear implants. Although they improve the patients quality of life, 'normal' hearing is not completely restored. A primary cause of hearing loss results from damage to hair cells (HCs) due to excessive noise exposure, ageing, or the exposure to ototoxic drugs. These damaged HCs are not regenerated in humans and can continue to deteriorate with time. In non-mammalian vertebrates, supporting cells (SCs) in the cochlea have regenerative capacities and give rise to new HCs after damage, even in adulthood. Research with neonatal mice has also shown that SCs can give rise to new HCs after damage in vivo. Furthermore, neonatal SCs can be expanded and differentiated into hair cells in vitro. However, for translational purposes, it is important to evaluate if this is also possible with adult-derived tissue.

Material and methods: Young adult and mature adult Lgr5GFP transgenic and C57CB/Bl6 mice were used for isolating cochleas. Cochleas were harvested and, after digestion with thermolysin and accumax, single cells were mixed with Matrigel, plated in 3D and grown in a high growth factor medium containing Wnt, R-spondin, and Noggin conditioned mediums. After passaging with trypsin and mechanical trituration, we evaluated proliferation and differentiation by immunofluorescence microscopy and qPCR or bulk sequencing.

Results: Lgr5-expressing SCs derived from adult (p30-p200) Lgr5GFP transgenic and C57CB/Bl6 mice cochleas can be expanded to at least 9 passages without losing differentiation capacity. *Lgr5* is highly expressed in expansion medium and lost after differentiation. *Atoh1* expression increases during expansion and it is lost after differentiation. Myosin7 is not expressed in expansion medium and it is highly enhanced after differentiation. In parallel, we aim to setup a co-culture system where we will culture differentiated cochlear organoids with spiral ganglion neurons (SGN), to further mimic the in vivo physiology. This research will be the first step to building a functional primary cochlear organoid model to get a better understanding of the regenerative capacities of adult derived tissue.

Loud noise exposure is unlikely to cause DNA damage within the organ of Corti

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Introduction: Noise-induced hearing loss (NIHL) affects a large proportion (Daniel, 2007) of the estimated 1.5 billion people currently living with hearing loss (WHO, 2021). NIHL, just like all other forms of hearing loss, can lead to depression and anxiety (Shukla et al., 2020), and it increases the risk of dementia (Livingston et al., 2020). However, we know little about the pathogenesis of NIHL, which is important for helping us identify prevention methods and therapies. Based on previous work showing that oxidative stress is liekly to be part of the pathogenesis of NIHL (Kishimoto-Urata et al., 2022), we hypothesise that cells within the cochlea experience DNA damage following loud noise exposure, leading to cell death seen in NIHL.

Material and methods: We exposed 1 month-old C57Bl/6N mice to 120dB SPL of sound (1–16 kHz) for 2 hours, before sacrificing them and fixing their cochleae. Following fixation and dissection, organs of Corti were immunolabelled for the DNA damage markers γH2AX and 53BP1. To test how the different cell types within the cochlea respond to oxidative stress, we treated the organs of Corti explanted from 1 month-old mice with the oxidising agent hydrogen peroxide, before fixing and labelling them for γH2AX and 53BP1.

Results: One hour post-noise exposure, γH2AX and 53BP1 immunofluorescence labelling in the cochlear cells appeared comparable to that observed in non-exposed mice. We also found that following peroxide treatment, adult hair cells show relatively little change in γH2AX labelling when compared to the supporting cells.

Conclusions: Our preliminary study shows that DNA damage is unlikely to mediate the cell death seen in NIHL, and that there is a difference in how adult hair cells and adult supporting cells respond to oxidative stress. One explanation for the different cellular responses is that adult hair cells are better protected against DNA damage than supporting cells. The other explanation is that adult hair cells are less able to recognise and repair DNA damage. Identifying which explanation is correct will help us understand why adult hair cells degenerate and die following an increase in oxidative stress.

Mammalian TMC1 or 2 are necessary for scramblase activity in cochlear hair cells

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Auditory sensory transduction converts sound information into electrical signals through opening of mechanosensitive ion channels located at the tips of hair cell stereocilia (reviewed by Zheng and Holt, 2021). Among the proteins of the ion channel complex, we find the transmembrane channel-like (TMC) proteins 1 and 2 form the pore of hair cell transduction channels (Pan et al., 2013, 2018). The structure of TMC proteins in C. elegans worms (Jeong et al., 2022; Clark et al., 2024) and predicted mammalian TMC structures (Hahn et al. 2009; Ballesteros et al., 2018; Pan et al., 2018) are reminiscent of TMEM16 proteins, which function as Ca^{2+}-activated ion channels and lipid scramblases. For the current study, we confirmed lipid scramblase activity in auditory hair cells with genetic or pharmacologic disruption of *TMC1*, consistent with a previous report (Ballesteros and Swartz, 2022). We used the Annexin-V marker coupled with a fluorophore emitting at 647nm to label the phosphatidyl serine (PS) localized in the membrane at the tips of hair cell stereocilia. PS externalization was triggered by disruption of sensory transduction using the established non-ototoxic blocker, benzamil, or by genetic mutations that affect permeation properties of *TMC1*. Following application of 5 μM FM1-43 to label hair cells, we compared externalization of PS before and after benzamil treatment. We found that expression of either *TMC1* or *TMC2*, were essential for PS externalization. Tmc1/Tmc2 double knockout mice lacked PS externalization completely. We also determined that expression of exogenous human TMCs (hTMC1 or hTMC2) can induce PS externalization. Finally, we demonstrated that hair cells expressing two different human mutations in Tmc1 can constitutively evoke PS externalization. In conclusion, here we show that not only *TMC1* is essential for the lipid scramblase function in hair cells but *TMC2* can also promote scramblase activity in the absence of *TMC1*. The PS externalization can be triggered by human TMC proteins. Our data suggest that human TMC dysfunction, like mouse, may lead to dysregulation of membrane homeostasis at the tips of hair cells stereocilia and thus may contribute to auditory dysfunction due to *TMC1* mutations.

Minigene assay as important tool in determining the pathogenicity of genetic variants in hereditary hearing loss

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Introduction: Next generation sequencing (NGS) is a method which is becoming increasingly available in clinical setting, especially in genetically heterogenous diseases as hereditary hearing loss. For each patient there are thousands of variants, including benign polymorphisms, pathogenic variants and variants of unknown clinical significance. The last ones are difficult to interpret, especially in case of silent variants and variants in non-coding parts of the gene – their mode of pathogenicity might be more elusive, i.e. alteration of splicing. Such variants require functional studies to properly assess their impact and pathogenic potential.

Aim: To assess the pathogenicity of 11 novel variants with possible effect on splicing using minigene assay.

Material and methods: Selected variants were: *ATP2B2* c.941-7C>G, EYA1 c.1475+1G>T, *EYA4* c.1282-12T>A, *GSDME* c.991delT, *GSDME* c.1127A>G, *MYO6* c.816+1G>A, *MYO6* c.1984-1G>A, *MYO6* c.3281-13A>G, *MYO7A* c.2829G>A, *MYO15A* c.9230-4 A>T, *SLC26A4* c.1001+1G>A. Each variant was detected in the custom HL gene panel performed for patient of Department of Genetics, Institute of Physiology and Pathology of Hearing. Fragments of genes of interest encompassing closest introns and exons were introduced into expression vector pDEST pCI-Neo RHO using Gateway cloning system. Cell cultures of HEK293T line were transfected with expression vectors for each gene, containing either wild type sequence or sequence with studied variant. After 48 h of incubation cell lysis and RNA isolation were performed. Transcripts were analyzed by subsequent RT-PCR, gel electrophoresis and Sanger sequencing.

Results: The majority of studied variants displayed their effect on splicing (9 out of 11, 82%). The most common aberrations were exon skipping and incorporation of intron fragment to the transcript, which usually resulted in frameshift and introduction of premature stop codon. In case of variants *ATP2B2* c.941-7C>G and *MYO15A* c.9230-4 A>T there were no observable signs of splicing alteration.

Conclusions: Genetic variants affecting splicing emerge as an important contributor to HL. The performed minigene assays allowed for better variant interpretation, which in turn allowed for the correct genetic diagnosis. The study demonstrates the significance of functional testing especially when it comes to the silent variants and intronic variants.

Grants: 2016/22/E/NZ5/00470 and 2021/41/B/NZ5/04390 National Science Centre, Poland.

Modeling genetic inner ear hearing loss: development of hiPSC-derived inner ear organoids harboring *GJB2* mutations

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Hearing loss is the foremost common sensory disorder globally, impacting approximately 5% of the population. The current treatment options are limited to hearing aids or cochlear implants, which mitigate symptoms but are not a permanent solution. In the context of genetic hearing loss, gene therapy could be the ultimate solution.

In Europe, the most common mutations occur in the *GJB2* gene, which encodes Connexin 26 (Cx26), a component of gap junctions, which facilitate ion transport between cells. The exchange of ions is an essential process for hearing as it functions as a message between sound detection by the hair cells and transmission of this message to the brain. Disease-causing mutations in *GJB2* lead to aberrant or non-functional protein, probably leading to improper exchange of ions between cells and thereby reduced hearing.

The only available human model for inner ear research is the inner ear organoid model, generated from human induced pluripotent stem cells (hiPSCs). This model recapitulates the diverse cell types found within the inner ear, providing insights into hearing-related mechanisms. We are employing this model system to study how *GJB2* mutations result in hearing loss.

To select the most relevant *GJB2* mutations, we performed a literature search to identify mutations associated with hearing disorders. Multiple sequence alignment and AlphaFold 2 predictions were performed to assess mutations at conserved protein sequence positions. Two selected mutations, c.35delG and c.269T>C/p.L90P, commonly found in European patients, induce a shortened Cx26 protein and a missense mutation with a structural change, respectively. Guide RNAs for these mutations are undergoing efficiency testing.

We outline selection criteria for mutations that can be studied in hiPSC-derived inner ear organoid models. This will facilitate the study of *GJB2* mutations on essential hearing cells, enhancing understanding of the disease and guiding future therapy development.

Monitoring the negative effects of music listening on otoacoustic emissions: a preliminary report

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Introduction: Many people consider music an essential aspect of daily life, often listening to it for extended periods, surpassing safe sound levels. Prolonged exposure to loud sounds (>85 dBA) can harm the hearing organ, leading to irreversible changes in the inner ear and noise-induced hearing loss. Regular hearing tests, such as otoacoustic emission testing, are crucial for early detection of cochlear changes, particularly in cases of noise-induced hearing loss. Fortunately, advancements in technology grant us nearly limitless access to tools for monitoring hearing health.

Purpose: The purpose of this study was to see how exceeding recommended sound doses affects the magnitude of different types of otoacoustic emissions (OAEs).

Material and methods: Measurements were made on 1 person with normal hearing listening to music on an Android mobile device. Three types of OAEs were measured: click-evoked OAEs (CEOAE), distortion product OAEs (DPOAE) and spontaneous emissions (SOAE). The application's (HearAngel)

which monitors musical multimedia internal measure, the daily sound allowance (DSA), was used to estimate music exposure time. OAE measurements were made for: 100% DSA, 100% DSA + 10 minutes, 100% DSA + 30 minutes, 7 hours.

Results: Slightly exceeding the daily limit calculated with the app does not cause significant changes in OAE response levels. Some differences are noticeable only after several hours of musical exposure. The biggest changes occurred in the SOAEs. The amplitude of all SOAEs decreased for DSA + 30 minutes and for 7 h.

Conclusions: SOAEs appear to be the most sensitive type of OAE to changes in the auditory system. Unfortunately, SOAEs are currently recorded in about 60–70% of the population with normal hearing, which is the biggest limitation in the use of these measurements. However, the results obtained are promising and encourage further research.

Multiplexed TMT-based quantitative proteomics identified essential players involved in the mechanism of action of SENS-401 observed under normal or ototoxic conditions in intact cochlear organ cultures

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Introduction: SENS-401, known as R-azasetron besylate, is a first-in-class drug candidate to treat Sudden Sensorineural Hearing Loss (SSNHL) and in clinical development for inner ear protection against Cisplatin-Induced Ototoxicity (CIO) and hearing preservation after cochlear implantation.In all three indications SENS-401 demonstrates a promising potential to improve hearing (https: //www.sensorion.com/en/). The goal of this study is to conduct a systematic analysis of proteins and pathways involved in the protective action of SENS-401.

Material and methods: P3-P5 Wistar rat organotypic explant cultures, including both spiral ganglion and organ of Corti intact tissue, were exposed or not to 20 h-cisplatin (Cis) and co-treated or not with SENS-401. We used unbiased tandem mass tag multiplexed quantitative proteomics coupled with high performance liquid chromatography and mass spectrometry (FPP Montpellier). Bioinformatics data processing was done with MaxQuant & Perseus software. Differentially expressed proteins (DEPs) obtained by comparing conditions were analysed using the Panther Classification System, the Database for Annotation, Visualization and Integrated Discovery (DAVID), and KEGG/Reactome/Wikipathways database resources. Differential analysis with corrected t-test (FDR 5% and s0 = 1) was considered significant when the p-value was < 0.05.

Results: We provide for the first time proteomics data that revealed an overview of proteins/pathways involved in the SENS-401 effects, identifying 186 DEPs associated with 16 pathways, by comparing the SENS-401 to the Control condition. While 38% of the DEPs were significantly upregulated by SENS-401, 62% of DEPs were downregulated, highlighting the involvement of several combined protective pathways. In the CIO context, our proteomics data revealed that SENS-401 treatment affects up to 417 DEPs (with 52% DEPs upregulated and 48% downregulated) involving 38 pathways (SENS-401+Cis versus Cis) over the 799 DEPs and 62 pathways characterizing the ototoxic effects of Cis (Cis versus CTL). Finally, the co-treatment of SENS-401+Cis showed only 84 DEPs with 5 associated pathways when compared to Control, suggesting that SENS-401 is strongly shifting protein profiles and biological pathways towards normal condition.

Conclusions: We present for the first time a systematic analysis of proteins/pathways involved in SENS-401 effects under normal or ototoxic conditions. Replication and extension of these exploratory studies, including in vivo, will provide valuable insights into potential molecular targets.

***Net1* overexpression promotes the trans-differentiation of Lgr5-positive progenitor cells into hair cells**

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Introduction: Sensorineural hearing loss is mainly caused by loss of sensory hair cells (HCs) due to ototoxic drugs, aging, and environmental noise. Unfortunately, hair cells are non-regenerative in the cochlea of adult mammals. Recent studies have shown that Lgr5-positive progenitors have the properties to regenerate hair cells. Net1 is a guanine nucleotide exchange factor of RhoA GTPase, which is involved in a variety of biological processes in cancer cells, including cell proliferation and differentiation. However, its roles in the cochlea have not been widely reported in vivo.

Material and methods: We inserted a *Net1* gene expression sequence at Hipp11 (H11) to construct Net1loxp/+ mice using CRISPR/Cas9 technology. After injecting Tamoxifen at P0-P1 to activate Cre recombinase, *Net1* was specifically overexpressed in Lgr5+ progenitor cells. We observed ectopic HCs by immunofluorescence, and determined whether the HCs were proliferated from Lgr5+ progenitor cells by EdU assay and lineage tracing, respectively, and the mechanism of Net1 enhancing HC regeneration was explored by real-time fluorescence quantitative PCR.

Results: By immunofluorescence, we found a large numbers of ectopic HCs in cochlea of Net1 conditionally overexpress(cOE) mice, and the EdU assay failed to detect any EdU+/Sox2+ cells. The lineage tracing results showed that more tdTomato+HCs significantly derived from Lgr5+ progenitors of *Net1* cOE mice than control. More importantly, real-time qPCR results showed that the hair cell-associated transcription factor Atoh1, was significantly increased, Wnt/β-catenin pathways was activated and TGFβ pathway was up-regulated in cochlear basilar membranes (BMs) of Net1 cOE mice. All results indicated that the Net1 cOE promoted

HCs regeneration, and these regenerated ectopic HCs may be directly transdifferentiated from Lgr5+ progenitors.

Conclusions: In summary, we specifically overexpressed *Net1* in neonatal mouse cochlear Lgr5+ progenitor cells and found a remarkably increased in the number of ectopic HCs compared to control mice. This study provides new evidence for the regulation of *Net1* on the regeneration of neonatal mouse cochlear HCs.

Neural health assessments in cochlear implant recipients using electrically evoked compound action potentials

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Introduction: Hearing performance of cochlear implant (CI) users relies on the condition of the auditory nerve. Animal studies have shown that electrically evoked compound action potentials (eCAPs; responses of the nerve to current pulses) can be applied to assess this condition. In particular, relative eCAP measures, obtained by comparing eCAPs to different stimuli, are strongly correlated with neural survival (Ramekers et al., 2014). Studies in humans demonstrated the value of such relative measures in their correlations with hearing performances (Zamaninezhad et al., 2023). Here, we recorded eCAPs to various stimuli in CI recipients to examine the predictive value for hearing performance using both linguistic and non-linguistic perception tasks.

Material and methods: Ten subjects with severe sensorineural hearing loss, aged 60 to 80 years, received a CI (Flex28 arrays of Med-El GmbH). Intraoperatively and approximately 4 months postoperatively, eCAPs were recorded to biphasic current pulses with varying interphase gaps (IPGs; 2.1 to 30 μs) and varying current levels up to saturation level. Outcome measures include amplitude and latency at maximum current levels, and the current level halfway the amplitude growth function, level 50%. Relative eCAP measures were obtained by the difference between measures at IPG of 30 and 2.1 μs. Hearing performance was assessed by CVC in noise (+5 and +10 dB signal-to-noise ratio) perception and spectral ripple discrimination using the spectral-temporally modulated ripple test (SMRT; Aronoff and Landsberger, 2013).

Results: In all patients eCAPs could be recorded with amplitudes between 200 and 1000 μV. The absolute eCAP measures for IPG of 30 μs were similar for postoperative and intraoperative recordings. The IPG-difference measures substantially differed between postoperative and intraoperative recordings with postoperative measures aligning more with outcomes from animal studies. One notable correlation between hearing performance and eCAP outcome was observed: the ripple discrimination score increased with decreasing eCAP latency ($R2 = 0.5$, $p < 0.05$). Speech perception scores did not significantly vary with absolute or relative eCAP measures.

Conclusions: The eCAP latency appeared to have predictive value for a non-linguistic hearing outcome. We argue that speech perception is harder to predict from eCAP measures because of cognitive factors involved.

Non-invasive monitoring of intracranial pressure changes: utilizing otoacoustic emissions

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Introduction: Invasive techniques for objectively measuring intracranial pressure (ICP) pose significant risks including infection, intracerebral hemorrhage, and brain injury (Maniker et al., 2006; Wolfe and Torbey, 2009; Scheithauer et al., 2009). Otoacoustic emissions (OAEs) provide non-invasive indicators of ICP changes, as the OAE phase reflects middle ear transmission, which is influenced by ICP via its connection to intracochlear pressure. A calibration of the technique is based on the ICP and OAE phase data collected by Buki et al. (1996) in patients undergoing controlled ICP changes during neurosurgery. Forward pressure level (FPL) calibration provides additional insight into middle ear transmission, complementing and validating OAE phase measurements.

Material and methods: Five International Space Station (ISS) normal-hearing astronauts were tested for distortion product otoacoustic emissions (DPOAEs) pre-flight, in microgravity in-flight conditions, and post-flight. Ground experiments were conducted involving 20 young volunteers, who were DPOAE tested in different body postures. The DPOAE response was time-frequency filtered (Moleti et al., 2012) to unmix the distortion and reflection components. The FPL calibration data provided, as a byproduct, a direct estimate of the load impedance measured in the ear canal, which is related to the middle ear reflectance.

Results: Systematic changes associated with microgravity and postural changes were observed for all considered physical quantities, with the DPOAE phase generally yielding the best results. Although physical quantities such as reflectance and load impedance are more directly related to ICP changes than the OAE phase, their numerical evaluation may be more difficult, because it involves ratios between complex quantities that are very sensitive to phase uncertainties.

Conclusions: DPOAE measurements with FPL calibration and component unmixing provide effective non-invasive indicators of ICP changes. Future research directions include applications in Glaucoma patients and absolute calibration in neural surgery environments for several types of clinical population such as hydrocephalus and intracranial tumor.

Overexpression of Serpine2 promotes trans differentiation of Lgr5+ progenitors into hair cells in the neonatal mouse cochlea

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Lgr5+ progenitors in neonatal mouse cochlea have the ability to regenerate hair cells (HCs) by directly transdifferentiation or mitotic regeneration, which could be induced by several genes and pathways. However, the regeneration ability of Lgr5+ progenitors is still limited in neonatal mice and is almost lost in adults. Considering HC regeneration is a complicated process involving lots of genes and pathways, it is necessary to find more key genes which could induce the proliferation and differentiation of Lgr5+ progenitors to promote HC regeneration. Here, we conjoint analysis our three previous RNA-seq data: Lgr5+ progenitors in the apical (ALPs) and basal of mouse cochlea (BLPs), neomycin-treated Lgr5+ progenitors (NLPs) and untreated Lgr5+ progenitors (ULPs), Lgr5+ progenitors and Lgr5-supporting cells (SCs), screened novel genes which we further explored their effects on the proliferation ability of Lgr5+ progenitors by sphere assay in vitro. We found that knockdown of Serpine2 inhibit only the proliferation of Lgr5+ progenitors. Serpine2, a member of the Serpins family, is involved in proliferation of various tumor cells in breast, pancreas and other organs. Our in vitro experiment data showed that Serpine2 may be also involved in the regulation of proliferation and differentiation of Lgr5+ progenitors. Here we studied the roles of Serpine2 in HC regeneration in vivo. We found that Serpine2 conditional overexpression (cOE) in Lgr5+ progenitors induced the number of ectopic HCs, especially inner HCs (IHCs) at postnatal day (P)7. Lineage tracing assay showed that these ectopic HCs are probably originated from Lgr5+ progenitors through direct trans-differentiation. Together, our data suggest that Serpine2 exerts a functional effect during the development of mouse cochlea, and may participate in the regulation of HC regeneration from supporting cells (SCs) and Lgr5+ progenitors in the neonatal mouse cochlea.

Refining rodent cochlear explant models for screening therapeutic drugs against ototoxicity

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Ototoxicity is defined as damage to the inner ear, targeting cochlear and vestibular structures and sensory function, due to exposure to certain pharmaceuticals or chemicals. Drug classes most associated with ototoxicity include antibiotics, such as aminoglycosides and platinum-based chemotherapeutic agents (cisplatin). Although ototoxicity mechanisms of action are not fully elucidated, much progress has been made in identifying otoprotective solutions and/or drug replacement with reduced or no ototoxicity. There are ongoing efforts to get alternative tests and techniques to the in vivo tests, to predict early in the development the ototoxicity risk, in a rapid manner and reduce the number of animals used for in vivo tests.

Cochlear explants in neonatal rodents are an organotypic culture of the immature cochlea, facilitating the presentation of organized cellular structures within the inner ear, which are otherwise hard-to-access.The objective of this study was to develop the most accurate ototoxic ex vivo model, using rat and mice cochlear explants. The technical challenges are presented and discussed.

We worked on the different components of the explant to provide the most relevant and reliable method to analyze drug ototoxic effects on the rodent cochlear explants: (1) the parameters characterizing the explant model: age of the pups, composition of culture medium, dissection method, ototoxic reference drug dose, duration of drug exposure and culture period. (2) the markers to visualize the various structures of the cochlea, such as hair cells, supporting cells, fibers, and neurons. (3) the method of image acquisition using a laser scanning confocal microscope and the histological analysis methods based on a qualitative and quantitative assessment of hair cells (scoring of hair cell organization and counting of hair cell numbers).

The development of reliable and consistent rodent explant cultures provides significant advantages for investigating drug mechanisms of ototoxicity and developing novel therapies. Preclinical testing is a critical phase in new drug development, making it essential to continually refine and expand tools, including in vivo and in vitro models, to advance the progress of new treatments. Ultimately, these efforts contribute to reducing the burden of drug-induced hearing loss in clinical practice.

Sex-dependent expression of glutamate receptors in the developing murine organ of Corti

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Introduction: In rodents, synaptic connections between inner hair cells (IHC) and type I spiral ganglion neurons (SGN) undergo extensive functional and structural changes before the onset of hearing (around postnatal day P12). Each synapse comprises the presynaptic ribbons containing the structural cytomatrix protein Ribeye and the postsynaptic glutamate receptors on the peripheral afferent fibers. The major excitatory neurotransmitter glutamate signals primarily via AMPA-type receptors. AMPARs are heterotetramers made up of GluA1–4 subunits. It has been shown that in the developing cochlea, the GluR3 subunit is essential for the right assembly of AMPAR GluR2 and GluR2 subunits on cochlear afferent synapses and for presynaptic ribbon morphology. Interestingly, only adult female GluR3-KO mice present early-onset hearing loss (1,2). Also, studies in rats showed that changes in AMPA receptor subunits due to neonatal handling differ for males and females (3). Based on that, the objective was to determine whether sex influences the expression or

localization of AMPA receptor subunits (GluR1-4) in cochlear synapses in young animals.

Material and methods: Cochlear explants were prepared from young (P4-5) C57BL/6 mice of both sexes. An enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of glutamate receptors (GluR1-GluR4) in tissue lysates. Semiquantitative RT-PCR was used to compare GluR2 gene expression. Immunofluorescence and fluorescence or confocal microscopy were used to determine GluR2 protein localization and morphology. Statistical analyses were performed using IBM SPSS.

Results: Glutamate receptor 2 (GluR2) protein levels in the organ of Corti lysates differed between male and female mice, with males having higher GluR2 protein levels than females ($p < 0.05$) and at the gene expression level ($p < 0.01$). Interestingly, other AMPA subunits (GluR1, GluR3, and GluR4) did not differ between the sexes. In addition, sex-specific differences in synapse morphology were detected by immunofluorescence.

Conclusions: Our study demonstrates that sex-related differences in GluR2 exist in the developing cochlea. It remains to be established whether sex-dependent differences in AMPA composition can also be detected in adult animals and how they affect hearing. The results obtained confirm that sex should be considered a biological variable in ex vivo studies.

Success of targeted sequencing in the search for genetic causes of Usher syndrome type 2

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Introduction: Usher syndrome is one of the most common rare diseases in which both hearing impairment and retinitis pigmentosa coexist. Currently, four types of Usher syndrome are known. They are genetically heterogenous and clinically characterized based on the age of hearing loss and retinitis pigmentosa diagnosis, the degree of hearing loss and the presence of vestibular dysfunction. The aim of the study was to characterize the genetic background of Usher syndrome type 2 (USH2) in a group of Polish patients.

Material and methods: A total of 55 patients with a clinical diagnosis of USH2 were recruited to the study. The DNA was isolated from peripheral blood and genetic testing was performed using three different methods: real-time genotyping with TaqMan probes, high-throughput sequencing of the *USH2A* gene, and a panel of 237 hearing-related genes. Bioinformatic and expert analysis focused on the search for single nucleotide variants (SNVs) and copy number variants (CNVs). Segregation analysis was performed using Sanger sequencing and quantitative real-time PCR. Selected novel variants probably affecting splicing were tested using minigene assay.

Results: The cause of *USH2* was identified in all patients. In 98% (54/55) of the individuals, causative variants were located in the *USH2A* gene. In one patient (2%; 1/55), a new homozygous terminating variant in the *ADGRV1* gene was identified. In the *USH2A* gene, 42 different genetic variants were identified (28 known and 14 novel). A total of 74% (31/42) of the variants were deleterious. The most frequently identified genetic cause of USH2 was c.11864G>A (p.Trp3955Ter), present in 29 of the studied alleles. Deletions of exons 22–24 (17 alleles) and 10–11 (8 alleles) of the *USH2A* gene also played a significant role in USH2 development.

Conclusions: The obtained results characterize the mutation profile responsible for USH2 development in Polish patients. Genetic testing of USH2 patients should be based on high-throughput tests that enable simultaneous identification of SNVs and CNVs. The gathered data can serve as a starting point for further genotype-phenotype association analyses and may, in the future, identify patient groups that could benefit from developing molecular and cellular therapies.

Grant: 2020/37/N/NZ5/02800 National Science Centre, Poland.

Tailoring AAV vectors for gene therapy of inner ear disorders by directed evolution

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Hearing loss (HL) affects approximately 20% of the global population and the treatments are currently limited to hearing aids and cochlea implants. Gene therapy offers a possibility to prevent or even cure HL. With the aim to optimize the adeno-associated virus (AAV) vector system for inner ear directed gene therapy, we generated AAV peptide display libraries based on the AAV1, AAV2 and AAV6 capsid backbones. All libraries present random unique 7-mer peptide inserts at variable region VIII of the capsid protein with diversities ranging from 80,000–622,000 (maximum likelihood estimate, MLE). We conducted high-throughput in vivo selection screens in the inner ear of adult mice, testing alternative administration routes that demand overcoming robust biological barriers. The target tissue is the organ of Corti with its crucial mechanosensory hair cells (HCs) of the inner ear, the supporting cells (SCs) and the underlying spiral ganglion neurones (SGNs). Distinct variants were found to be accumulated to up to 5% for AAV2-based variants and up to

2.5% for AAV1-derived capsids after two rounds of in vivo selection. Interestingly, some top variants were already accumulated for the AAV6 KO library after only one selection round. A total of 20 top candidates from the AAV1 and AAV2 libraries were produced as vectors. They outperform the parental serotypes and show diverse expression patterns in the adult mouse cochlea. Three promising variants had the ability to transduce outer HCs, a challenging cell type to infect, and many also targeted inner HCs. Almost half of the variants also strongly transduced all layers of the stria vascularis – a viable target tissue for the treatment of age-related HL – and the SGNs were targeted, with 4 variants being highly specific for SGNs. In addition, different intensities of fluorescent transgene expression suggest differential efficacy in delivery or vector uncoating within the cells of the inner ear. Thus, we report on a set of promising new AAV variants with distinct features developed by in vivo high throughput selection screens for improving inner ear directed gene therapy.

The cargos and potential roles of small extracellular vesicles derived from mouse cochlear explants in a model of cisplatin-induced ototoxicity

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Introduction: Cisplatin, an effective chemotherapeutic agent, is clinically limited by its side effects, such as high incidence rate of ototoxicity and renal toxicity. Small extracellular vesicles (sEVs) are derived from almost all cells, and they can reflect the physiological and pathological information of parent cells and participate in intercellular communication in the progression of pathologies. However, the cargos and roles of sEVs in the progression of cisplatin-induced ototoxicity are unclear until now.

Material and methods: Here, we established an ex vivo model of neonatal mouse cochlea to scrutinize cisplatin-induced ototoxicity. And then, we isolated sEV from the conditional culture medium of cisplatin-induced cochlea explants and characterized it by TEM, NTA, and western blotting. Next, we used small RNA sequencing and label free LC-MS/MS to profile the miRNAs and proteins cargos of sEV, respectively.

Results: The small RNA sequencing of sEVs indicated that 74 microRNAs (miRNAs) were significantly upregulated and 9 miRNAs were downregulated in cisplatin-treated group (referred to as Cis-sEV) compared with control group (referred to as Ctrl-sEV). Furthermore, the targets of these differential expressed miRNAs were mainly enriched in apoptosis, inflammation, and other cell damage-associated signaling pathways, which suggested that the miRNAs of sEVs probably participate in signal communication in cisplatin-induced damage. On the other hand, LC-MS/MS analysis of sEV suggested that there is an obviously differentiation between Cis-sEV and Ctrl-sEV, with 90 proteins being upregulated and 150 proteins being downregulated, including numerous proteins that could regulate the damage response. Furthermore, we found 3 proteins (Cltc, Cct2, and Hspa8), which are potentially involved in protein homeostasis and autophagy, are verified to be up-regulated in Cis-sEV compared to Ctrl-sEV rather than in cisplatin-damaged cochlear tissue lysis, indicating that they are likely involved in important and specific intercellular communication mechanisms underlying cisplatin-induced ototoxicity via sEVs, rather than involving in intracellular roles.

Conclusions: Overall, this investigation offers an innovative and promising perspective on the molecular changes that occur in cochlear cells in response to cisplatin, which could lead to a better understanding of ototoxicity and potential targets for therapeutic intervention.

The cross-rode between oxidative stress and inflammation in the auditory system damage: role of via glial cell and macrophages activation in ototoxicity

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Redox imbalance and inflammation have been proposed as the principal mechanisms of damage in the auditory system, resulting in functional alterations and hearing loss. Microglia and astrocytes play a crucial role in mediating oxidative/inflammatory injury in the central nervous system; however, the role of glial cells in the auditory damage is still elusive. In this study, we investigated glial-mediated responses to toxic injury in peripheral and central structures of the auditory pathway, i.e., the cochlea and the auditory cortex (ACx), in rats exposed to styrene, a volatile compound with well-known oto/neurotoxic properties. To this aim, male adult Wistar rats were treated with styrene (400 mg/kg daily for 3 weeks, 5/days a week). At the end of treatment (day 21) electrophysiological, morphological, immunofluorescence and molecular analyses were performed in both the cochlea and in ACx samples to evaluate the mechanisms underlying styrene-induced oto/neurotoxicity in the auditory system. We showed that the oto/neurotoxic insult induced by styrene increases oxidative stress in both cochlea and ACx. This was associated with macrophages and glial cell activation, increased expression of inflammatory markers (i.e., pro-inflammatory cytokines and chemokine receptors) and alterations in connexin (Cxs) and pannexin (Panx) expression, likely responsible for dysregulation of the microglia/astrocyte network.

Specifically, we found downregulation of Cx26 and Cx30 in the cochlea, and high level of Cx43 and Panx1 in the ACx. Collectively, our results provide novel evidence on the role of immune and glial cell activation in the oxidative/inflammatory damage induced by styrene in the auditory system at both peripheral and central levels, also involving alterations of gap junction networks. Our data suggest that targeting glial cells and connexin/pannexin expression might be useful to attenuate oxidative/inflammatory damage in the auditory system.

The current knowledge of spiral ligament fibrocytes in cell culture: a systematic review

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The spiral ligament in the cochlea has been suggested to play a significant role in the pathophysiology of sensorineural hearing loss (SNHL). Positioned between the stria vascularis and the bony otic capsule, this structure contains spiral ligament fibrocytes (SLFs), categorized into five main types based on their structural characteristics, immunostaining patterns and location within the spiral ligament. Together with the stria vascularis, the SLFs maintain a positive endocochlear potential (EP) in the scala media via K+ recycling, which is an essential component in the transduction mechanism of the auditory pathway. It has also been suggested that the SLFs contribute to the cochlear immune response, glutamate homeostasis and cochlear blood flow regulation. Spiral ligament damage disrupts K+ recycling, reducing the EP and subsequently causing SNHL. Despite their pivotal roles, a lot remains unknown about the SLFs. Therefore, this systematic review about SLFs in cell culture could give an overview of the current state of the art, providing a basis for future studies trying to investigate those cells in vitro.

A literature search was performed using PubMed, Web of Science and Scopus taking into account the PRISMA guidelines. Twenty-five studies were included in this review that report on SLFs in cell culture. The differences in species, sex, age, method of culturing and used antibodies for immunohistochemistry are discussed. The majority of these studies cultured spiral ligament fragments onto type I collagen-coated petri dishes; a protocol described by Gratton et al. in 1996, while some recent studies are focussing more on growing these cells in a 3D environment.

A better understanding of the currently published methods will help us to optimize fibrocyte culture techniques, thereby allowing to investigate a variety of possibilities that can significantly increase our knowledge about SLFs. Among others, these possibilities include to discover their functional characteristics, such as growth patterns, protein expression profiles and ion channel physiology, to expose the cells to hypoxia or other toxic conditions and to assess the resulting effects, to genetically modify SLFs, to explore drug repurposing possibilities, to evaluate potential therapies for SNHL and to investigate their capability as a source for transplantable cells.

The efficacy of the chemical chaperone TUDCA in the preservation of cochlear ribbon synapses

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The ageing process, gene mutations and noise exposure cause two types of hearing problem arising from pathology in the cochlea; the hair cell damage causing elevation of hearing thresholds and the damage of the ribbon synapses between inner hair cells (IHCs) and spiral ganglion neurons causing an auditory neuropathy syndrome, called hidden hearing loss. Tauroursodeoxycholic acid (TUDCA), a derivative of naturally occurring bile acids, can antagonize protein misfolding and endoplasmic reticulum stress as well as mitochondrial oxidative stress. As both these stress response mechanisms are thought to be involved in cochlear pathology, our aim here was to find out if TUDCA protects against progressive hearing loss and acute noise-induced hearing loss. We used the ICR (CD-1) mouse strain as a model of early-onset progressive hearing loss. We injected these mice with TUDCA (250 mg/kg sc) or PBS, twice per week, from 3 to 9 weeks of age, and then assessed the outcome. We exposed CBA/Ca mice to noise; 98 dB SPL, 8–16 kHz frequency band for 2 h. These mice received TUDCA injections on two consecutive days before the exposure and thereafter daily for 7 days, after which the outcome was analyzed. We recorded ABRs and quantified hair cell and ribbon synapse (presynaptic ribbons) numbers within the 8-to-32 kHz cochlear frequency region. CtBP2 immunostaining marked presynaptic ribbons and CtBP2/Homer 1 double-staining pairing of pre- and postsynaptic components. Compared to PBS-treated mice, systemic TUDCA administration did not prevent ABR threshold elevations or OHC loss in either trauma model. However, TUDCA conferred statistically significant protection against IHC synaptopathy in the ICR mouse model of progressive hearing loss. The concomitant robust OHC loss in these mice prevented us from assessing the physiological relevance of synapse preservation (ABR wave I amplitude). Quantification revealed a preservation of ~20% of synapses per IHC across the frequency region studied. A recent publication showed using physiological and behavioral measures that this extent of synapse preservation improves auditory temporal processing in mice (Ji et al., 2024). Together, our results suggest that TUDCA pharmacotherapy can slow down progressive ribbon synapse loss, but not synaptopathy following acute noise trauma.

The human iPSC-derived inner ear organoid as a model for ototoxicity studies

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Drug treatment with platinum-based chemotherapeutics or aminoglycoside antibiotics can lead to inner ear damage and subsequent hearing loss and balance disorders. Studies on ototoxicity are limited to investigations in animal models or rare and difficult to acquire human inner ear tissues. In this pioneering study, we use human induced pluripotent stem cell (hiPSC)-derived inner ear organoids (IEOs) as a model system to investigate ototoxic effects of these drugs on human inner ear cells. Here, we aim to validate the IEOs as an effective model for assessing the ototoxic effects of cisplatin and gentamicin in cultured human inner ear cells. IEOs were generated from hiPSCs and cut into 200 µm thick vibratome sections at day 75 to access the hair-cell-containing inner ear vesicles within the cultured aggregates. The ototoxic compounds were applied for 24 hours (cisplatin doses 0–100 µM; gentamicin doses 0–1000 µM) and sections were kept in culture for up to one week. Evaluation techniques included H&E and immunofluorescent staining for assessing cell morphology, protein expression and apoptosis, along with cytotoxicity assays and qPCR to analyse stress signalling and cell death pathways.

Cisplatin-treated samples showed loss of hair cells, neurons, and structural integrity of the otic vesicle in the first few days after treatment, with apoptotic nuclei in the otic epithelium and its direct surroundings. Hereafter, recovery of architecture and hair cells was observed, potentially indicating that intrinsic regenerative capacities are present in the current model. Gentamicin affected the structural integrity of the vesicle with loss of cell polarity and collapse. Also, neuronal damage and extruded cells in the lumen were observed. Together, these results validate the human inner ear organoid as a model for assessing ototoxicity. Ongoing work focusses on the time course and the ototoxic effects of both compounds by RNA expression analysis of cell stress and cell death mechanisms. This study underscores the potential translational impact of the human inner ear organoid model for ototoxicity.

The influence of operating point position in nonlinear undamping feedback force on amplitude minima in simulated distortion-product otoacoustic emissions

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Furosemide has been demonstrated to reduce the endocochlear potential and influence distortion-product otoacoustic emissions (DPOAEs). Lukashkin et al. (2002) administered furosemide intraperitoneally in guinea pigs and presented cubic (2f1-f2) and quadratic (f2-f1) DPOAE input/output (I/O) functions recorded both before injecting furosemide and within the first half-hour after the injection. Over time, the amplitude of the cubic DPOAE I/O at L1 intensities up to about 50 dB SPL decreased. The cubic I/O function exhibited an amplitude minimum (notch) at L1 near 60 dB SPL, and the position of this notch shifted towards higher intensities, becoming shallower with increasing time. At the highest intensities, the cubic DPOAEs seemed to be almost unaffected by furosemide. Quadrature DPOAEs appeared to be less affected at intensities up to about 60 dB SPL, but the notch position shifted from L1 of about 50 dB SPL to about 70 dB SPL. We adjusted the operating point (OP) in the sigmoidal nonlinearity used to transform undamping feedback force in a cochlear model. The sigmoidal nonlinearity is proportional to the 2nd-order Boltzmann function, which is asymmetrical with the default position of the operating point at the inflection point. We observed that the OP position affected notches in simulated cubic DPOAE I/O functions only when shifted into the center of the sigmoidal function. In this case, the DPOAE amplitude was most reduced at the lowest intensities, and the effect of OP position was diminished at the highest intensities. Additionally, the notch shifted towards higher intensities. Simulated quadrature DPOAEs also contained a notch, and the OP point affected its position more than for the cubic DPOAEs. Direct changes to the undamping feedback force did not cause any change in the notch position. All these results indicate some agreement with the effects observed by Lukashkin et al. (2002) after the application of furosemide. However, the results should be interpreted cautiously due to several reasons. Furosemide affects the endocochlear potential, which cannot be directly simulated in our cochlear model. We can only adjust the operating point in the nonlinear function or the gain in the undamping feedback force. Additionally, the used cochlear model was designed to simulate the human cochlea, and the notch in DPOAE amplitude is located differently in L1, L2 space than in the case of the guinea pig. Lukashkin et al. measured DPOAE I/O for L1 = L2 + 10 dB SPL. We would miss the notch in simulated cubic DPOAEs for this level condition. Despite that, it is interesting that the notch position is affected only if the OP position is changed towards the center of the sigmoid. These simulations would provide a different explanation for the change in the OP position after furosemide application than that provided in recent studies applying furosemide intravenously (e.g., Strimbu et al., 2020).

Supported by the project 23-07621J of the Czech Science Foundation (GAČR).

The research of development of a miniature DNA base editor for *DFNB9* gene therapy in hereditary deafness

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OTOFERLIN (*OTOF*) mutation can lead to autosomal recessive deafness 9 (*DFNB9*), which is the main cause of auditory neuropathy. Delivering the exogenous *OTOF* gene through double AAV can restore the hearing of genetically deaf *DFNB9* mice, but the efficacy of the gene replacement therapy may diminish over time, and it cannot really solve the deafness caused by the gene mutation. In contrast, gene editing can correct mutant genes at the DNA level and fundamentally treat hereditary deafness. We screened and developed a mini-base editor and optimised AAV expression elements for constructing a single AAV delivery base editing system to cure gene therapy of genetic deafness caused by *OTOF* point mutations. Specifically, we constructed mini-ABE and sgRNA (SchABE8e-sgRNA4) on a single AAV vector and screened out small promoter and short-polyA elements for the expression of the base editing system. At the same time, we found that targeting the promoter for expression in the reverse direction achieving a more efficient cleavage efficiency. We successfully restored the hearing of *OTOF* point mutant mice to WT level and maintained it for a long time by delivering the single-base editing system to the inner ear using AAV serotype-Anc80, which is capable of efficiently transducing inner ear hair cells. Subsequently, we evaluated the safety of the SchABE8e-sgRNA4 single-base editing system in WT mice and found that both vestibular and hearing levels were maintained at the WT level, indicating the safety of our delivery system. Taken together, these findings provide new strategies for treating *DFNB9* in the clinic and lay the theoretical and experimental foundation for the clinical translation of gene editing therapy for deafness.

The role of post-translational modifications of microtubules in the inner ear: insights from knockout mice studies

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Microtubules, an essential component of the eukaryotic cytoskeleton, perform a variety of essential functions within cells. One mechanism that regulates these diverse functions is the post-translational modification of tubulin. Although these modifications have been known for decades, research into them has only really taken off in the last few years. In particular, the impact and importance of these modifications in the sensory epithelia of the inner ear have only been marginally explored. With the discovery of tubulin-modifying enzymes and the availability of knockout mice, it is now possible to investigate the biological functions and molecular mechanisms underlying these modifications. Here, we have initiated an immunohistochemical study of the effects of post-translational modification knockout mice in the inner ear. Our results indicate that the absence or accumulation of polyglutamylated tubulin leads to different morphological changes in the mouse cochlea, whereas deacetylation results in a defective epithelium at an early age. A deeper understanding of the specific post-translational modification and the cochlea may provide new insights into the mechanisms of hearing and potential therapeutic approaches for the treatment of hearing disorders.

Tmprss3 expression in the mouse cochlea

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Cochlear implants (CIs) have shown variable performance outcomes in DFNB8/10 patients carrying pathogenic mutations in *TMPRSS3*. A study by Shearer et al. (2018) which involved electrical stimulation of spiral ganglion neurons (SGNs) in CI patients revealed on average smaller electrical responses in DFNB8/10 patients compared to ones with other forms of deafness, indicating a loss of SGN function. Another study by Fasquelle et al. (2011) had previously shown a loss of more than half of SGN cell bodies in Rosenthal's canal, observed between days 90 and 180, in a mouse model with a premature stop codon in *Tmprss3* (Y206X). These observations indicate a loss of *TMPRSS3* function-mediated damage to the SGN health which could plausibly explain such variability in CI performance. However, the cell-type specific expression and function of *Tmprss3* in the cellular mosaic of Rosenthal's canal remains unclear. Combining an RNAscope assay for mRNA localization with immunohistochemistry, we semi-quantitatively assessed the cell-type specific expression levels of *Tmprss3* mRNA in the murine cochlea, with a focus on SGN subtypes. We also performed an RT-qPCR assay

with TaqMan probes on RNA isolated from mouse brainstem, and a direct few-cell RT-qPCR on the cells of the organ of Corti and SGNs. We report a strong expression of *Tmprss3* mRNA and protein in the cells of the organ of Corti with RNAscope, immunohistochemistry, and few-cell RT-qPCR. In addition, *Tmprss3* expression was detected in specific cells of the stria vascularis. Interestingly, in Rosenthal's canal, we observed *Tmprss3* mRNA enrichment in the type-II SGNs with RNAscope and immunohistochemistry in the mature cochlea. Hardly any *Tmprss3* transcripts were detected in the brainstem. Few-cell RT-qPCR revealed no abundance of *Tmprss3* mRNA transcripts in type-I SGNs. In conclusion, in contrast to our expectation, type-I SGNs do not or hardly express *Tmprss3*. Thus, the data suggest an indirect role of *TMPRSS3* for the health and function of SGNs, which will need to be studied further to understand varying CI performance in DFNB8/10 patients.

Treatment following triple-AAV delivery in mature murine model of human *CDH23*-associated hearing loss

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Gene therapy holds promise as a curative therapeutic method with the potential to suppress HL progression or restore hearing function, something unattainable with hearing aids or cochlear implants. To expand the scope of target genes for cochlear gene therapy, patients with progressive genetic hearing loss (HL) are considered suitable candidates in terms of the therapeutic time window for gene therapy intervention. *CDH23* is a common deafness gene that can cause either Usher syndrome type 1D or non-syndromic HL (DFNB12). The phenotype range of DFNB12 is variable from congenital to adult-onset HL. Adult-onset, *CDH23*-related HL is progressive, beginning as high-frequency HL that gradually affects low frequencies, ultimately resulting in HL across all frequencies. While this gene is an ideal target for cochlear gene therapy, the size of the *CDH23* coding sequence is 10.1 kb; therefore, the development of gene therapy using triple adeno-associated virus (AAV) vectors is necessary. In this study we aimed to investigate the transduction efficiency of triple-AAV. This study aimed to investigate the transduction efficiency of triple AAV vectors in the cochleae of adult mice, focusing on large-gene-associated HL. Additionally, we sought to evaluate the feasibility of cochlear gene therapy in a mouse model of human *CDH23*-mediated HL using the triple AAV approach. To create a reporter protein, we fused EGFP to mCherry, which was then divided into three parts, each packaged in a separate AAV2/2 vector. Four weeks after co-injecting the triple AAV vectors into 4–5-week-old mice, we assessed transduction efficiency. We found that up to 5.9% of inner hair cells were positive for both EGFP and mCherry. Subsequently, we developed triple *CDH23*-AAV vectors for therapeutic purposes. After administering these vectors to 4- to 5-week-old C57/BL6 mice, we conducted auditory tests and immunohistochemistry studies over a period of 60 weeks. Co-injecting triple *CDH23*-AAVs did not alter auditory function or lead to hair cell degeneration. In conclusion, this study confirms the feasibility of the triple-AAV approach for cochlear gene delivery. While this strategy did not produce any treatment effects, our findings suggest that large deafness genes could be potential future targets for cochlear gene therapy.

Trichostatin A suppresses hearing loss by activating HO-1 in an Alport syndrome model

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Alport syndrome (AS) is a genetic disorder, which is characterized by mutations in type IV collagen, leading to kidney and cochlea dysfunction and late-onset progressive hearing loss. We investigated the effect of Trichostatin A (TSA), an HDAC inhibitor, in an AS mouse model to assess its potential to inhibit hearing deterioration. Col4α3 knockout (KO) mice were treated with TSA at 3 weeks of age and hearing levels were measured using auditory brainstem response (ABR). The results demonstrate that TSA treatment significantly protects the hearing of KO mice compared to the untreated group. The TSA-treated group exhibited a reduction in the levels of oxidative stress markers 4-HNE and 3NT, along with a decrease in inflammatory cytokines, in both the mouse cochlea and in vitro cell studies. TSA treatment induced HO-1 signaling, which increased HO-1 levels and contributed to the inhibition of oxidative stress and inflammatory cytokines. These findings suggest that TSA represents a promising candidate molecule for mitigating the progression of hearing loss in AS.