

Symposium

The Inner Ear in Translational Research

Closing the Gap toward Causal Treatment

29. September 2012

Abstracts

S1 Genotype-phenotype correlations in non-syndromic hearing loss

Henricus P.M. Kunst

Department of Otorhinolaryngology, Radboud University Nijmegen Medical Centre; Nijmegen, The Netherlands

More than 50% of prelingual hearing impairment is genetic, most often autosomal recessive and nonsyndromic. Postlingual hearing impairment is much more frequent than prelingual hearing impairment and has mostly a multifactorial inheritance, although monogenic forms exist with mainly autosomal dominant inheritance. The heterogeneity in nonsyndromic hearing impairment is high with multiple genes implicated in the pathogenesis.

An Age Related Typical Audiograms (ARTA) gives a comprehensive phenotype presentation and is therefore extremely useful. An ARTA can be used to compare the type of hearing impairment, the age of onset and the progression of hearing impairment in relation to the genotypes. An ARTA does not only help in selecting potentially interesting loci for linkage analysis or genes for mutation analysis, but it is also valuable for genetic and individual counseling. In addition, sensitive audiometric (psychophysical) tests can be used to determine the impact of the affected gene on the function of the inner ear. For various types of genetic hearing impairment the results of cochlear implantation can also be studied. These correlations can be helpful in predicting the outcome of cochlear implants for specific genes.

S2 Unraveling the genetics of autosomal recessive deafness

Hannie Kremer

Department of Otorhinolaryngology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Newborn hearing tests are commonly applied in many countries worldwide to provide early diagnosis of congenital hearing impairment. The underlying causes, however, often remain elusive and questions on prognosis and recurrence risk for the families unanswered. We have used a combination of strategies to unravel the genetic defects underlying autosomal recessive nonsyndromic hearing impairment (arNSHI) in a panel of 150 Dutch individuals with presumed arNSHI. Homozygosity mapping and candidate gene selection previously already revealed a number of novel deafness genes. We recently identified two novel deafness genes to be associated with moderate hearing impairment by high density SNP genotyping for homozygosity mapping and genotype sharing. Also, we found that *TMPRSS3* is an important cause of highly progressive hearing impairment with an onset in early childhood and a ski-slope audiogram. Whole exome sequencing was performed in a subset of the 'unsolved' families which revealed mutations in a number of known deafness genes and also candidate genes for arNSHI.

S3 Next-generation Sequencing: A novel screening approach in diagnosing hereditary hearing loss

Saskia Biskup

CeGaT GmbH, Tübingen, Germany

Genetic heterogeneity in hereditary hearing loss makes genetic testing time consuming and very costly when using conventional sequencing. Newly available methods like massively parallel high throughput sequencing now allows the simultaneous screening of all genes of the human genome in one sequencing run. Here, a targeted enrichment of 100 genes is compared with whole exome sequencing in a clinical setting. Targeted enrichment for all coding parts of 100 with hearing loss associated genes and SOLiD sequencing is used. In total 100 individuals with severe deafness were included. In 10 index cases with positive family history exome sequencing was performed. Potentially pathogenic variants were independently verified by Sanger sequencing. Targeted enrichment of 100 genes allows the parallel sequencing of 48 patients compared to 6 exomes in one flowcell of the SOLiD 5500xl platform. The enrichment for 100 genes is more cost efficient and reliable due to significantly higher coverage per base.

S4 Sensory hair cell death and protection: Insight into a new approach

Jonathan Gale

UCL Ear Institute, London, UK

Department of Cell & Developmental Biology, UCL, London, UK

Sensory hair cell death is the precipitating cause of adult-onset sensorineural hearing loss. It is well established that, in mammals, cochlear hair cells are not regenerated after loss 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. I will present a brief overview of the current understanding of how and why hair cells die and the approaches being taken to prevent hair cell death. Secondly I will introduce recent work from the UCL Ear Institute, a collaboration with Sally Dawson's laboratory, that has implicated a set of genes regulating the assembly of structures known as stress granules in the response of the inner ear to stress. We are currently characterising the properties of these dense cytoplasmic complexes of RNA-binding proteins and "stalled/silenced" RNA in order to increase our understanding of hair cell death and survival.

In general, the current pharmacological treatments/therapies for preventing hair cell death approach the problem by attempting to target the hair cells directly. There are many other targets within hair cell epithelia that can and do have a significant influence on hair cell death albeit indirectly. I will also present recent work, using a combination of *in-ovo* electroporation, viral transfection, organ culture and live imaging to show that supporting cells may play a more active role during hair cell death than has previously been thought. I will use this as the basis to introduce the concept of a multi-target approach to protecting hair cells. Whether such an approach can improve the efficacy of current therapeutic approaches to hair cell protection remains to be determined.

S5 Fish in a dish: Habilitation of hearing and balance disorders in the 21st Century

Edwin W. Rubel, David W. Raible, Julian Simon and Kelly Owens

University of Washington and Fred Hutchinson Cancer Research Center
Seattle Washington, USA

A variety of genetic, environment, and epigenetic factors influence our abilities to develop and maintain normal hearing and balance throughout our lives. Major causes of disorders arising after initial maturation include exposures to environmental toxins such as excessive noise and certain chemicals, ototoxic therapeutic drugs and the aging process itself. In most, but not all cases, a source of the pathology is loss of inner ear hair cells. Yet, the variability of human responses to these challenges is enormous and our understanding of this variability is minimal. Variability is opportunity! The research program I will discuss seeks to understand the cellular pathways governing the responses of mechanosensory hair cells to these challenges and discover ways to prevent the ensuing hair cell loss. We use the mechanosensitive hair cells in neuromasts of the lateral line system in free-swimming larval zebrafish as a platform to address these issues. We validate this system by showing that the integrity of neuromast hair cells are compromised by the same chemicals and can be protected in the same ways as mammalian inner ear hair cells. I will outline the strategy underlying our mutagenesis and chemical screens and present new data on two topics: a) current studies have begun to reveal intracellular Ca⁺⁺ regulatory pathways underlying aminoglycoside-induced hair cell death; and b) validation of our first lead compound, PROTO1, in mammalian hearing has led to new efforts at compound testing, and drug optimization necessary for translation to clinical use. These efforts will be discussed and evaluated.

Supported by NIH grants from NIDCD and NINDS

S6 The Role of Not for Profits in Supporting Translational Research

Ralph Holme

Head of Biomedical Research, Action on Hearing Loss, London, UK

Action on Hearing Loss want to see scientific discoveries rapidly translated into treatments to benefit people with hearing loss and tinnitus. Hearing loss is a major public health issue affecting one in six of the population. It significantly reduces quality of life, is an economic burden to society and is associated with high levels of unmet clinical need. With an ageing population, there is an urgent need for new treatments.

Rapid progress is being made towards understanding the biological causes of hearing loss and tinnitus, which in turn is leading to new opportunities for translational research. But, despite the clear clinical need and scientific progress being made there remain considerable barriers for translational research. Academic institutes tend to be driven by the need to produce high impact scientific publications and by investigator's personal intellectual curiosity. This serves fundamental research well and succeeds in generating innovative ideas, but it tends not to support the research needed to develop a potential new treatment. Many believe it is the role of industry to invest in the development of emerging new treatments, but the reality is that industry is becoming more reluctant to invest in early stage translational research requiring strong evidence that an approach is likely to be successful before investing. Industry tends also to invest in tried and tested therapeutic areas where the regulatory requirements and route to market are well understood. This presents another barrier for translating hearing research.

A new role is emerging for not for profits, such as Action on Hearing Loss, in helping to close the gap between academic and commercial research. In common with industry they are focused on seeing new treatments brought to market, but are not driven by the need to return a profit. Through our Translational Research Initiative for Hearing (TRIH) we have created a new funding scheme to support translational research that is not able to attract commercial funding and have built a consortium of 16 organisations willing to consider funding TRIH projects, making it easier for industry to identify emerging opportunities. We are also exploiting our links with people with hearing loss to better understand patient need, and links to the research, clinical and commercial communities to help forge partnerships.

By targeting funding to the most promising areas of research, breaking down barriers to translational research and being a catalyst for industry investment, we believe we can accelerate the discovery and development of treatments for hearing loss.

S7 Auditory evoked responses can be restored by human ES cell-derived otic progenitors

Marcelo N. Rivolta

Centre for Stem Cell Biology, Department of Biomedical Sciences,
University of Sheffield, Sheffield, UK

Of all the forms of deafness, auditory neuropathy is of a particular concern. This condition, defined primarily by damage to the spiral ganglion neurons with relative preservation of the hair cells, is responsible for a significant number of patients with hearing impairment. While the loss of hair cells can be circumvented partially by a cochlear implant, no routine treatment is available for sensory neuron loss since poor innervation limits the prospective performance of an implant. Using stem cells to recover the damaged sensory circuitry is a potential therapeutic strategy. Here, we present a protocol to induce differentiation from human embryonic stem cells (hESCs) using signals involved in the initial specification of the otic placode. We obtained two types of PAX8/SOX2/FOXP2 positive otic progenitors able to differentiate in vitro into hair cell-like cells and auditory neurons that display expected electrophysiological properties. Moreover, when transplanted into an auditory neuropathy model, otic neuroprogenitors engraft, differentiate and significantly improve auditory evoked response (ABR) thresholds. These results should stimulate further research into the development of a cell-based therapy for deafness. Moreover, these progenitors offer an excellent platform for screening and discovery of new drugs.

S8 Cochlear progenitor cell differentiation to hair cells

Albert Edge

Eaton Peabody Laboratory, Massachusetts Eye and Ear Infirmary, Boston, USA

Department of Otolaryngology, Harvard Medical School, Boston, USA

The vestibular and auditory organs have a limited ability to replace damaged cells but they contain cells with stem cell properties. We have identified supporting cells that express intestinal epithelial stem marker, Lgr5, as stem cells in the mammalian inner ear. FACS sorted Lgr5pos cells formed neurospheres and differentiated to hair cells at a higher rate than Sox2pos Lgr5neg cells. Upregulation of Wnt signaling and inhibition of Notch increased the percentage of hair cell markers in differentiating neurospheres. Similar treatment of explants of the organ of Corti also resulted in new hair cells. The demonstration that Wnt-responsive inner ear progenitor cells could differentiate into hair cells raises the possibility that progenitor cell-directed therapeutic agents could replace hair cells in the inner ear.

Supported by grant DC007174 from the National Institute of Health.

S9 Manipulations of the cochlea and measurement of functional status

Alec N. Salt

Washington University School of Medicine, St. Louis, USA

Many potential therapies of the ear require drugs and other compounds such as siRNAs, viruses or cells to be applied to the inner ear. The technical problems that must be overcome in delivering substances to the ear are not trivial and can result in unjustified experimental failures when the substance is not efficiently delivered. Results obtained in one species may be difficult to generalize to others, or to the human situation. Quantitative analysis of the entry of drugs and markers into the ear with computer models has allowed a theoretical basis to be established for the delivery of substances to the ear. There is typically a trade-off between efficiency of delivery and risk of the procedure to hearing. Measurement of status of the cochlea with cochlear action potentials and brainstem responses provides an indication of high and mid frequency function. New methods are becoming available to measure low frequency function which is more sensitive to fluid manipulations. Although drugs cannot yet be delivered to the ear in a well-controlled manner that does not jeopardize hearing, we are progressing towards that goal.

S10 Intracochlear drug delivery systems and new therapeutic concepts

Takayuki Nakagawa

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Sensorineural hearing loss (SNHL) is one of the most common disabilities in the world. The majority of SNHL is caused by degeneration of the cochlea. Previous studies have demonstrated the cellular or molecular targets in the cochlea for developing novel therapeutics. However, many obstacles to be overcome are present before translation of such basic findings into the clinic. Our mission is to overcome these obstacles and to provide patients with new therapeutic options for SNHL. One of obstacles for translation to the clinic is how to deliver therapeutic molecules to the targets in the cochlea. As a solution for this problem, we have developed a drug delivery system for cochleae using gelatin hydrogel, which is a biomaterial that is capable of sustained release of proteins or peptides. We chose insulin-like growth factor-1 (IGF1) as a therapeutic agent, because of both its potential for cochlear protection and clinical availability. Pharmacological assessments revealed the potential of the gelatin hydrogel for sustained delivery of IGF1 into the cochlear fluid. Topical application of IGF1 using gelatin hydrogels exhibited hair cell protection and attenuation of hearing impairment due to noise or ischemia. Based on this, we have performed a clinical trial to test the safety and efficacy of topical application of IGF1 using gelatin hydrogels in patients with sudden deafness refractory to systemic steroids. As results, no serious adverse events were observed, indicating the safety of this treatment. A half of patients exhibited hearing recovery better than 10 dB in pure tone audiometry, which is significantly better than hyperbaric oxygen therapy, a historical control. Interestingly, major hearing recovery appeared at 4 weeks after treatment, and a few patients demonstrated further recovery of hearing until 24 weeks after treatment. In this clinical trial, patients received topical IGF1 application at 3 weeks after the onset. In animal experiments, hearing recovery usually saturates at 2 weeks after therapeutic treatment. Therefore, the present findings suggest that different mechanisms for hearing recovery between humans and animals may be involved. As a possible explanation for this, regenerative processes could be involved in the process of hearing recovery observed in patients with sudden deafness. Now a randomized controlled clinical trial of topical IGF1 treatment is ongoing. In parallel, we have initiated basic research to explore mechanisms for delayed hearing recovery after topical IGF treatment.

IEB Workshop, Abstracts

Session A:

Deafness genes / Gene therapy

Chairpersons: A. Ryan
H. Bolz

O1 **Comprehensive genetic analysis of all known deafness genes by next-generation sequencing**

*H. Bolz^{1,2}, C. Neuhaus¹, C. Bergmann^{1,3}, T. Eisenberger¹

¹Bioscientia, Center for Human Genetics, Ingelheim, Germany

²University Hospital of Cologne, Institute of Human Genetics, Cologne, Germany

³RWTH Aachen University, Department of Human Genetics, Aachen, Germany

Introduction: Congenital deafness has a genetic basis in most cases, with a multitude of causative genes identified to date. Apart from *GJB2* (connexin-26), whose mutations account for 30 - 50% of cases (depending on the ethnic background), there is no other major gene locus for deafness. Therefore, genetic analysis beyond *GJB2* testing was frustrating until recently. Now, with the advent of next-generation sequencing (NGS) technologies, this genetically extremely heterogeneous condition has become accessible to comprehensive (diagnostic) analysis.

Materials and Methods: We have established NGS for genes implicated in deafness and its most frequent/important syndromes (Usher-, Pendred-, Jervell and Lange Nielsen- and SANDD syndrome). Our approach comprises the in-solution capture and massively parallel sequencing of 67 genes on a Roche 454 FLX and an Illumina MiSeq platform. NGS data were analysed using an in-house bioinformatic pipeline and JSI Medical Systems SeqNext-Software (vs 3.5). Sequence variants of interest were verified by Sanger sequencing. Different filtering strategies were applied, depending on clinical data and pedigree information (e.g. autosomal dominant versus recessive inheritance, parental consanguinity etc.).

Results: Several examples will be presented for the molecular findings. We have identified mutations in a broad range of genes, including some that have been reported as disease-related only once in the literature. We were able to genetically determine Usher syndrome type 1 before the onset of visual symptoms. NGS was particularly helpful in clinically atypical Usher syndrome and in *USH2A*-negative *USH2* patients where choosing the "right gene" for Sanger sequencing would have been difficult. Of note, some patients were found to carry mutations in several genes which required careful interpretation. Of note, we have identified causative copy number variants based on NGS data which is particularly helpful for genes where MLPA reagents are not available.

Conclusions: We have established NGS in a diagnostic routine setting for the detection of deafness-causing mutations. As a cost- and time-efficient method, it overcomes the previous bottleneck in diagnostic confirmation of presumably genetic hearing impairment, and it is an important tool to distinguish between isolated and syndromic forms. Moreover, application of the deafness gene NGS panel in a research setting can quickly identify patients who are highly likely to carry mutations in novel deafness genes.

O2 Efficacy of targeted genomic capture and massively parallel sequencing of 82 known deafness genes for molecular genetic diagnosis of hereditary deafness in Korean multiplex families

*B.J. Kim¹, G. Park², A. Kim¹, K.-H. Han¹, Y.H. Kim³, S.O. Chang¹, S.-H. Oh¹, W.-Y. Park², B.-Y. Choi^{1,4}

¹College of Medicine, Seoul National University, Otolaryngology, Seoul, Republic of Korea

²College of Medicine, Seoul National University, Biomedical sciences, Seoul, Republic of Korea

³Boramae medical center, Seoul National University – Seoul Metropolitan Government, Otolaryngology, Seoul, Republic of Korea, ⁴Seoul National University Bundang Hospital, Otolaryngology, Seongnam, Republic of Korea

Introduction: Identification of causative genes for hereditary hearing loss is important to decide treatment modalities and to counsel the patients. We intended to make molecular genetic diagnosis using next generation sequencing and to propose a decision tree upon which we could systematically find a causative mutation of genetic hearing loss patients.

Materials and Methods: Targeted exome capture of 82 known deafness genes using a NimbleGen array and massively paralleled sequencing by HiSeq2000 was performed for 19 probands from families with two or more members with variable degree of hearing loss.

To identify the causative mutations among variants found from TGS, we filtered out the variant that were not compatible with the inheritance pattern of the family, after exclusion of known common SNPs included in dbSNP132 or reported in the 1000 Genomes project. In addition, internal frequency of the variant among 19 cases was also used to narrow candidates. Then, remaining candidate variants were validated using Sanger sequencing and the resulting variants were considered responsible mutations. We further excluded the variants if they are detected frequently among Normal hearing 80 Korean controls. Finally segregation study was done for confirmation, whenever it is possible. Genotype-phenotype correlation including configurations of audiogram and the progression pattern of hearing loss was also used.

Results: About 10% of exons were uncaptured, which had below 1% of bases above 10 read depth. Critical mutations were identified in 10 of 19 probands of these multiplex families through target exome sequencing of 82 deafness genes. We identified likely deleterious mutations in WFS1, COCH, EYA4, MYO6, GJB3, MYO6, COL11A2, MYO3A and MYO7A. Most of them are private, again confirming etiologic heterogeneity of hereditary deafness in this population.

Conclusion: We suggested the diagnostic flow to identify mutation responsible for hearing loss and could make molecular genetic diagnosis in more than half of the Korean multiplex families with hearing loss. This genomic analysis procedure will enable us to evaluate causative mutation with more efficiency.

O3 Polymorphic analysis in patients with Ménière's disease and sudden sensorineural hearing loss

*M. Teranishi¹, Y. Uchida², N. Nishio¹, K. Kato¹, H. Otake¹, T. Yoshida¹, M. Sone¹, S. Sugiura³, F. Ando^{4,5}, H. Shimokata⁵, T. Nakashima¹

¹Nagoya University, Otorhinolaryngology, Nagoya, Japan

²Aichi Medical University, Otolaryngology, Aichi, Japan

³National Center for Geriatrics and Gerontology, Otorhinolaryngology, Aichi, Jordan

⁴Aichi Shukutoku University, Health and Medical Sciences, Aichi, Japan

⁵National Center for Geriatrics and Gerontology, Development of Preventive Medicine, Center for Development of Advanced Medicine for Dementia (CAMD), Aichi, Japan

Introduction: Although the etiologies of Ménière's disease (MD) and sudden sensorineural hearing loss (SSNHL) remain unclear, genetic factors could contribute, at least in part. The homeostasis of the water and blood circulation in the inner ear is essential for maintaining its hearing and equilibrium functions. On the other hand, recently, accumulating evidence has demonstrated that oxidative stress is related to the pathology of inner ear disease. We investigated the associations of polymorphisms of genes involved in the fluid or ion balance in the inner ear, or oxidative stress pathway with the susceptibility to MD and SSNHL in the present study.

Methods: Patients affected by MD or affected by SSNHL, who attended the Department of Otorhinolaryngology of the Nagoya University Hospital between November 2007 and March 2011, were enrolled in the study. The subjects of the control group were selected from the comprehensive Longitudinal Study of Aging (NILS-LSA), an ongoing population-based study with a two-year follow-up, conducted by the National Institute for Longevity Sciences. Polymorphisms in the genes *aquaporin 4* (*AQP4*) (rs2075575), *AQP5* (rs3736309), *estrogen receptor α* (*ER α*) (rs2234693), *ER α* 2 (rs9340799), *glutathione peroxidase 1* (*GPX1*) (rs1050450), *paraoxonase 1* (*PON1*) (rs662 and rs854560), *PON2* (rs7493), and *superoxide dismutase 2* (*SOD2*) (rs4880) and *NOS3* (rs1799983) were investigated for statistical analysis.

Results: Multivariable logistic regression analysis revealed *AQP5* polymorphism was significantly associated with a risk of MD. The ORs for *AQP5* polymorphism and MD risk was 0.676 (CI: 0.477-0.957) with adjustment for age and sex, which implies the variant G allele of *AQP5* (rs3736309) reduces the risk of MD. A significant higher prevalence of T allele (minor allele) of *NOS3* (rs1799983) was observed in SSNHL cases compared with controls. The remaining eight polymorphisms failed to show any associations with the risk of SSNHL and MD.

Conclusion: The variant G allele of *AQP5* (rs3736309) reduces the risk of MD and T allele of *NOS3* (rs1799983) increases the risk of SSNHL.

O4 Allelic variants of TLR10 gene and MICA-STR A.4 suggest changes in innate immune response in Ménière's disease

I. Gazquez¹, T. Requena¹, N. Perez², I. Aran³, A. Soto-Varela⁴, S. Santos⁴, H. Perez-Garrigues⁵, P. Perez^{1,6}, G. Trinidad⁷, A. Batuecas⁸, R. Teggi⁹, L. Zagato⁹, M.A. Lopez-Nevot¹⁰, *J.A. Lopez-Escamez¹

¹GENyO- Center for Genomic and Oncological Research, Otolaryngology & Neurotology Group CTS495, Granada, Spain

²University of Navarra, Otolaryngology, Pamplona, Spain

³Hospital Pontevedra, Otolaryngology, Pontevedra, Spain

⁴Hospital Clinico Universitario, Otolaryngology, Santiago de Compostela, Spain

⁵Hospital La Fe, Otolaryngology, Valencia, Spain

⁶Hospital de Cabueñes, Otolaryngology, Gijon, Spain

⁷Hospital Universitario, Otolaryngology, Badajoz, Spain

⁸Hospital Universitario, Otolaryngology, Salamanca, Spain

⁹San Raffaele Hospital, Otolaryngology, Milan, Spain

¹⁰Hospital Virgen de las Nieves, Immunology, Granada, Spain

Hypothesis: Ménière's disease (MD) is a complex trait and multiple genes and environmental factors determine its pathophysiology. Genetic and proteomics studies suggest changes in the immune response. We have studied the association of allelic variants of the genes IFNG, MIF, TNFA, MICA, TLR3, TLR7, TLR8 and TLR10 in patients with MD and their influence in hearing loss progression.

Study design. A case control study.

Methods: We genotyped four functional repeats of the genes IFNG, MIF, TNFA and MICA by a PCR-based method and seven SNP of TLR genes by a Taqman allelic discrimination assay including three cohorts of patients with MD from Spain and Italy with a total of 960 patients and 881 controls.

Results: The overall allelic frequency of the T allele at rs11096955 (TLR10 gene) was 61% in cases and 53% in controls (OR 1.36 (1.19-1.56); $P_c=6.6 \times 10^{-6}$). This association was also confirmed for the genotype TT (39% in MD and 29% in controls; OR=1.54 (1.27-1.88); $P_c=2.9 \times 10^{-5}$). Although MICA-STR were not associated with MD, the median time to develop hearing loss greater than 40 dB was 16 years (95% confidence interval, 9-23) for patients with the MICA*A.4 allele and only 10 years (95% confidence interval, 9-11) for patients with another MICA-STR allele (log-rank test, $p = 0.0038$).

Conclusions: The rs11096955 at TLR10 gene is the first risk factor identified in a large cohort of patients with MD and allelic variants of MICA and TLR10 genes may be associated with changes in the innate immune response in MD.

O5 Differential expression of apoptosis-related genes in the cochlea of conditional GJB2 gene knockout mice

*Y. Zhang¹

¹309th Hospital, Otolaryngology, Beijing, China

Introduction: Previous studies have shown that GJB2 gene mutation is the most common cause of non-syndromic hearing impairment (NSHI) in humans. GJB2 conditional knockout mice (cCx26ko) are ideal animal models for studying the mechanism of NSHI. Activated caspase 3 was detected in their cochlea and typical apoptotic morphology was found in the outer hair cells at P18, which demonstrate that apoptosis is the mechanism underlying their cochleae cells death. But to date, few apoptotic gene has been implicated in GJB2 knock out induced apoptosis. We evaluated the changes in apoptosis gene expression by quantitative real-time PCR array in cCx26ko mice at P10 and P18. Our results identified 20 genes that significantly increased or decreased expression following gene knockout. Eleven of the 20 genes have never reported expression in the cochlea.

Materials and Methods: Total RNA was purified from mice membranous cochlear canal. Total RNA was reverse-transcribed into cDNA, mixed with qPCR mastermix, and aliquoted to each well of the real-time PCR arrays. The mouse Apoptosis RT2 Profiler TM PCR Array was used. The real-time PCR cycling program was run on an ABI 7900 HT instrument. Twofold induction or repression of expression, with a P value of more than 0.05 was considered to represent significantly up- or downregulated gene expression.

Results: Sixteen genes showed a biologically relevant change ($P < 0.05$, ≥ 2 fold change) in P10. Among them 2 genes (Dapk1 and Tnfrsf10b), of which function are both induction of apoptosis, were upregulated. Other 14 genes (Bcl2l10, Naip1, Naip2, Birc3, Birc5, Casp1, Casp12, Casp14, Tsc22d3, Hells, Nol3, Pak7, Cd40 and Trp53inp1), nine (Bcl2l10, Naip1, Naip2, Birc3, Birc5, Tsc22d3, Hells, Nol3, Pak7) of which are responsible for inhibiting apoptosis, were downregulated. In P18, only 1 gene (Tnfrsf10b) was upregulated and 3 genes were downregulated (Bcl2l1, Bcl2l10 and Cd40lg). Caspase 8 and other 8 genes were detected upregulated at P18 but the changes did not show significant differences ($P > 0.05$).

Conclusions: Compared to wild type ones, obvious apoptosis gene expression changes were observed in cCx26ko mice at P10 before obvious cells loss happened. But the number of apoptosis relevant genes at P18 reduced, which may be related to the evident morphology of apoptosis already appeared in outer hair cells then. Since Dapk1, Tnfrsf10b and caspase 8 are all key elements in death signal receptor of apoptosis pathway, the action of this pathway may play an important role in the development of degeneration of cochleae cells in cCx26 ko mice.

O6 DNA vaccines and stem cell therapy restore hearing in hearing loss in human patients as well as experimental animals

B. Zhou^{1,2,3}, M. Kermany^{1,2,3}, Q. Cai^{1,2,3}, C. Cai^{1,2,3}, *T.J. Yoo¹

¹UTHSC, Dept of Medicine, Molecular Sciences, Otolaryngology, Neuroscience Institute, Memphis/TN, USA

²ImmuGen and StemGen Inc., Memphis/TN, USA

³Yoo Clinic and Institute, Seoul, Republic of Korea

Autoimmune inner ear disease, including Meniere's disease is devastating disease. The main feature of these disorders is the development and persistence of inflammatory processes in the apparent absence of pathogens, leading to destruction of target tissues. We are able to establish disease remission and re-acquire immune homeostasis by introducing immune regulatory elements by DNA vaccine and gene therapy in experimental animals as well as stem cells therapy in both human patients and experimental animals.

Mice and guinea pigs were immunized with β -tubulin and hearing loss was induced. They demonstrate low density of the spiral ganglion as well as cochlear hair cell damage. Increased ABR thresholds were shown in these animals. Serum antibody reactive to antigen was elevated. CD4 T cells as well as Th1 cytokine mediated autoimmune response were involved and CD4+CD25+Foxp3+ cells were linked to this disease.

Adoptive immunotherapy via T cell delivery of the IL-12p40 subunit was performed by iv injection of 2×10^6 T cell hybridoma transfected with IL-12p40 gene. ABR as well as DPOAE threshold for β -tubulin immunized mice at 2 weeks and 6 weeks were measured. Distortion product traces of 8, 16, 32 KHz frequency at 80 dB SPL intensity in tubulin immunized were measured. DPOAE show low response to immunized group. Therapeutic group showed restoration of hearing level at 6 weeks after therapy. Stem cell could be served as adoptive gene therapy, but the cells without gene were able to restored hearing in mice when it is given to tubulin immunized mice with hearing loss.

In mice and guinea pig, β -tubulin DNA gene constructs as well as IL10, IL4 and TGF beta gene were injected intra-muscularly. Total of 100 μ g of plasmid (mice) and 250 μ g of plasmid in guinea pigs were injected. On week in guinea pig and 2 weeks in mice after, we performed hearing test again. They have restored normal hearing. Beta tubulin gene containing DNA was also very effective vaccines to restore hearing.

Adipose derived mesenchymal stem cells also restored hearing in human with hearing loss as well as animal model of hearing loss

In summary, DNA vaccine therapy as well as stem cell therapy could restore immune based hearing loss in both human and animal experiments, however DNA vaccines study in human needs a clinical trials.

O7 Inner ear protein transduction utilizing arginine-rich cell-penetrating peptides

*R. Minoda¹, T. Miwa¹, E. Yumoto¹

¹Kumamoto University, Otolaryngology head and neck surgery, Kumamoto, Japan

Introduction: Various approaches, including viral vectors, electroporation, microinjection, and liposome encapsulation, have been used to introduce target molecules into the cells to manipulate them. In 1988, Green and Frankel independently discovered that HIV TAT proteins were able to cross cell membranes. Short peptide sequences, such as these, have been referred to as cell-penetrating peptides (CPPs). CPPs are a class of short cationic peptides that are able to traverse the cell membranes in many types of mammalian cells. Many macromolecules have been attached to these peptides and subsequently internalized. The cargo molecules delivered into cells maintain their biological activities. Among CPPs, arginine-rich CPPs have been the most widely studied. We assessed protein transducing abilities of nine-arginine-rich CPP into embryonic inner ears and adult inner ears.

Materials and Methods: Protein transduction into the developing inner ear: CD-1 pregnant mouse dams with E11.5 embryos were anesthetized. The uterus was exposed using a low midline laparotomy. EGFP conjugated to a nine-arginine peptide (EGFP-9R) was injected into the otocysts.

Protein transduction into the adult inner ear: Gelatin foam with soaked in EGFP-9R in was placed at the round window of adult CD-1 mouse cochleae.

Protein transducing abilities was assessed periodically.

Results: In the embryos that underwent EGFP-9R inoculation, EGFP-9R expression was detected in the lining cells in the otocysts and in their vicinity in a diffuse manner. Expression of EGFP-9R was maintained for 12-18 hours after otocystic inoculation. Embryos that underwent EGFP-9R inoculation developed to term normally, and P30 mice that underwent EGFP-9R inoculation showed normal auditory and vestibular functions. In adult EGFP-9R mice, EGFP-9R expression was diffusely detectable in the cochlea at 12 hours post-treatment.

Conclusions: Protein transduction may provide a useful strategy for delivering target molecules into embryonic inner ear and/or adult mice inner ear. Protein transduction via the round window membrane may be a potentially useful method for delivering therapeutically relevant molecules into the inner ear.

Session B:

Gene therapy / Delivery

Chairpersons: P. Thorne
S. Plontke

O8 Exploring novel therapeutic interventions based on gene delivery to treat hereditary deafness

*F. Galindo Ramirez¹, G. Crispino¹, G. Di Pasquale², J. Chiorini²,
F. Mammano^{1,3,4}

¹Venetian Institute of Molecular Medicine, Padua, Italy

²NIH, Bethesda/MD, USA

³University of Padua, Physic, Padua, Italy

⁴CNR Institute of Neuroscience, Padua, Italy

Introduction: Using cochlear organotypic cultures from deaf, connexin-deficient mouse models, we have recently shown that gene delivery with recombinant bovine adeno associated virus (BAAV) vectors restores protein expression and rescues gap junction coupling *in vitro* (1,2).

Methods: Canalostomy in the mouse was performed through a micro syringe controlled by a micro pump under a dissection microscope. Three weeks after injection we tested hearing by recording auditory brainstem responses (ABR) and we examined the cochlea of injected mice by confocal immunofluorescence, as previously described (2,4).

Results: We explored several routes of delivery for introduction of vectors into the inner ear *in vivo* while minimizing injury to existing structures and at the same time ensuring widespread distribution of the agent throughout the cochlea. Vector injection via a canalostomy in the posterior semicircular canal of the mouse is particularly attractive due to the simplicity of the surgical approach (3). Canalostomy is also appealing for it evolved from a selective labyrinthectomy that has been used for the treatment of benign paroxysmal positional vertigo in humans. Initially, we determined the volume of fluid that can be delivered in the mouse via canalostomy without compromising the integrity of the cochlear duct. Next we injected P4 Cx26^{loxP/loxP} mice with BAAV vectors containing a Cre-IRESGFP expression cassette (BAAVCre-IRESGFP). Cre recombinase expression resulted in a recombination of the Cx26^{loxP/loxP} gene and a decrease of Cx26 mRNA level. ABR thresholds were significantly elevated in Cx26^{loxP/loxP} mice that had received BAAVCre-IRESGFP but remained normal in siblings injected with vehicle alone. Moreover, immunofluorescence analysis revealed a drastic reduction of the Cx26 in particular in the lateral wall region of the scala media.

Conclusions: Altogether, our results confirmed the efficacy of viral transduction *in vivo* using BAAV.

Supported by MIUR PRIN grant n. 2009CCZSES and Telethon grant GGP09137 to FM.

References:

1 Ortolano et al., PNAS, 2008; 2 Crispino et al., PlosOne, 2011

3 Kawamoto et al., Mol Ther, 2001; 4 Schütz et al., Hum. Mol. Gen., 2010

O9 Mouse otocyst trans-uterine gene transfer restores hearing in connexin 30 knockdown mice

*T. Miwa¹, R. Minoda¹

¹Kumamoto University, Japan, Department of Otolaryngology-Head and Neck Surgery, Kumamoto, Japan

Objective: Mutation in gap junction beta-6 (GJB6), the gene that codes for connexin 30(Cx30), causes hereditary deafness in humans and mice¹. Short hairpin RNAs (shRNA) that are used for gene silencing by RNA interference (RNAi). In our previous study, we show that electroporation-mediated transfection of EGFP-fused Cx30-targeted shRNA (shRNA-Cx30-EGFP) into the normal mouse otocyst induced the knock-down of endogenous Cx30 in the cochlea, lack of endocochlear potential (EP) and severe hearing impairment. Now then, we investigated that co-transfection of shRNA-Cx30-EGFP and Cx30 which is resistant to shRNA-Cx30 fused EGFP (resistant-Cx30-EGFP) rescued the Cx30 knockdown mice from hearing impairment using otocystic inoculation and electroporation.

Methods: At embryonic day 11.5 (E11.5), shRNA-Cx30-EGFP and resistant-Cx30-EGFP were microinjected through the uterus into the otic vesicle of CD-1 normal mice and electroporated. Electroporated embryos were delivered at E18.5. Some fetuses were removed to prepare frozen sections, and some fetuses were raised by surrogate mothers until functional and morphological assessments on postnatal day 30 (P30). An auditory brainstem response (ABR), endocochlear potential (EP), immunohistology, surface preparation and western blot analysis were used for the assessments. As a control, a random sense shRNA expression vector fused EGFP (shRNA-scramble-EGFP) was used.

Results: Co-transfected mice showed more-expansive Cx30 expression than normal Cx30 distribution in the cochlea, except for the nascent spiral limbus. Moreover, the co-transfected mice showed significant amelioration of auditory threshold compared with shRNA-Cx30-EGFP transfected mice, and showed normal EP. The co-transfected mice showed normal surface morphology of the organ of Corti. Western blot analyses demonstrated that shRNA-Cx30-EGFP transfected mice show decrease of Cx30 expression in the cochlea, but Cx30 protein levels in the cochlea were indistinguishable between the mice who underwent co-transfection of shRNA-Cx30-EGFP and resistant-Cx30-EGFP and the normal mice.

Conclusions: Transfection of Cx30 along with shRNA-Cx30, restored down-regulated Cx30 expression in the cochlea and lack of the EP. Consequently the co-transfected mice did not show any auditory deterioration. The trans-uterine gene transfer into the otocysts utilizing shRNA appears to be an alternative strategy of ear-specific conventional conditional knock-out mice.

O10 Penetrating the inner ear with a Cell Penetrating Peptide and siRNA

*S. Dash-Wagh¹, S. Jacob¹, S. Lindberg², A. Fridberger¹, † Langel²,
M. Ulfendahl¹

¹Karolinska Institutet, Dept of Neuroscience, Stockholm, Sweden

²Stockholm University, Neurochemistry, Stockholm, Sweden

RNA interference (RNAi) using short interfering RNA (siRNA) is an attractive therapeutic approach for treatment of dominant-negative mutations. Some rare missense dominant-negative mutations lead to congenital hearing impairments. A variety of viral vectors have been tested with variable efficacy for modulating gene expression in inner ear. However, there are concerns regarding their safety for clinical use. Here, we report a novel cell penetrating peptide-based non-viral approach for delivering siRNA into inner ear tissue using organotypic cultures as model system. PepFect6 (PF6), a variant of stearyl-TP10, was specially designed for improved delivery of siRNA by facilitating endosomal release. Here we show that PF6 was internalized by all cells without inducing cytotoxicity in cochlear cultures. PF6/siRNA nanoparticles lead to knockdown of target genes, a housekeeping gene and supporting cell specific Connexin 26. Interestingly, application of PF6/Connexin 26-siRNA exhibited knockdown of both connexin 26 and 30 mRNA. Further, their absence led to impaired intercellular communication as demonstrated by reduced transfer of calcein among the PF6/Connexin 26-siRNA treated cells. Thus, we conclude that PF6 is an efficient non-viral vector for delivery of siRNA, which can be applied as a tool for the development siRNA-based therapeutic applications for hearing impairments.

O11 Transplantation of multipotent stromal cells (MSC): Is there a future role in cochlear implantation?

*A. Radeloff¹, R. Mlynski¹, K. Rak¹, K. Frölich¹, P. Schendzielorz¹, R. Hagen¹

¹Universitätsklinik, HNO, Würzburg, Germany

Autologous transplantation of multipotent stromal cells (MSC) has been successfully used in orthopedics and cardiology. Recently, we found that adipose-derived stromal cells (ADSC) survive for at least 6 month in the inner ear of guinea pigs. In these animals, spiral ganglion neurite (SGN)-outgrowth was directed towards the implanted cells. The aim of this study was to identify neuron-guiding substances secreted by MSC and, thus, to determine if cell-based therapies could help to improve the bio-electric interface in cochlea implant patients.

Human ADSC were grown under varying culture conditions. These included monolayer and spheroid condition types. Supernatants were harvested and analyzed by enzyme-linked immunosorbent assays (ELISAs). Additionally, SGN were grown in coculture with ADSC and the neurite outgrowth was determined.

ADSC produced relevant amounts of neurotrophic factors and extra cellular matrix proteins. The coculture of SGN with ADSC led to a significant increase in neurite length as compared to SGN culture without ADSC.

The transplantation of autologous MSC during cochlear implantation may provide neuroprotective and neuron-guiding factors that possibly could help to improve the bio-electric interface.

O12 Restoration of transport function and anion exchanger activity of missense pendrin mutations by salicylate

*H. Wada¹, K. Ishihara^{1,2}, S. Okuyama¹, S. Kumano¹, K. Iida¹, H. Hamana¹, M. Murakoshi¹, T. Kobayashi³, S. Usami⁴, K. Ikeda⁵, Y. Haga⁶, K. Tsumoto⁷, H. Nakamura⁸, N. Hirasawa⁹

¹Tohoku University, Department of Bioengineering and Robotics, Sendai, Japan

²Ibaraki University, Laboratory of Medical Science, Course for School Nurse Teacher, Faculty of Education, Mito, Japan

³Tohoku University Graduate School of Medicine, Department of Otolaryngology Head and Neck Surgery, Sendai, Japan

⁴Shinshu University School of Medicine, Department of Otorhinolaryngology, Matsumoto, Japan

⁵Juntendo University School of Medicine, Department of Otorhinolaryngology, Tokyo, Japan

⁶Tohoku University, Department of Biomedical Engineering, Sendai, Japan

⁷The University of Tokyo, Department of Medical Genome Sciences, Kashiwa, Japan

⁸Gakusyuin University, Department of Chemistry, Tokyo, Japan

⁹Tohoku University, Laboratory of Pharmacotherapy of Life-Style Related Diseases, Sendai, Japan

Pendrin is a membrane protein expressed in the inner ear, a member of the *solute carrier 26A (SLC26A)* family. It is known as an anion exchanger and its mutations are responsible for hereditary hearing loss. In Japanese, 10 missense mutations, i.e., P123S, M147V, K369E, A372V, N392Y, C565Y, S657N, S666F, T721M and H723R, have been found (Tsukamoto et al., 2003). We have reported that salicylate restores the localization and function of mutants of prestin (Kumano et al., 2010), which is a member of the *SLC26A* family and has about 45% similarity with the amino acid sequences of pendrin. Thus, it is hypothesized that salicylate restores pendrin mutants as in the case of prestin. In this study, the effects of salicylate on the localization and anion transporter activity of the 10 mutants were analyzed *in vitro*.

HEK 293 cells were transfected with each expression vector with the 10 mutants. After the cells were incubated with salicylate for 12 h, for the analysis of localization, they were stained with TRITC and its fluorescence was observed using a confocal laser scanning microscope. For the analysis of anion transporter activity, they were incubated in high I⁻ buffer containing 200 kBq/ml ¹²⁵I, promoting uptake of ¹²⁵I, and then incubated with high Cl⁻ buffer, leading to the export of ¹²⁵I from the cells to extracellular media by transfected pendrin. Radiolabeled iodide in the cell lysates was measured using a scintillation counter.

Immunofluorescent staining revealed that K369E and C565Y, as well as

wild-type pendrin, were transported to the plasma membrane while 8 other mutants were retained in the cytoplasm. Incubation with salicylate induced the transport of 4 pendrin mutants (P123S, M147V, S657Y and H723R) from the cytoplasm to the plasma membrane. The remaining radioactivity in cells transfected with such 4 mutants was significantly decreased by salicylate treatment as in the case of WT pendrin, indicating that their anion exchanger activity was restored. These findings suggest that salicylate possibly contribute to development of a new method of medical treatment for sensorineural hearing loss caused by pendrin mutations.

O13 Leakage control after intracochlear drug delivery by injections through the round window membrane

*S. Plontke¹, J. Hartsock², A. Salt²

¹University of Halle, Dept. of Otorhinolaryngology (HNO), Halle (Saale), Germany

²Washington University, Dept. of Otolaryngology, St. Louis/MO, USA

Intratympanic (extracochlear) drug delivery to the round window membrane (RWM) provides only limited control of perilymph drug levels. Direct injections of drugs into the perilymphatic space, e.g. through the RWM, are more efficient and can result in more consistent drug levels. Perforation of the ear in guinea pigs, however, results in a flow of CSF between the cochlear aqueduct and the site of perforation. For injections through the RWM, this can result in a rapid washout of the applied drug. In the present study, fluorescein was used as a marker to assess the effectiveness of a number of procedures for leakage control (hydrostasis). Injections through the RWM or through the bony wall of the cochlea were performed with sharply beveled glass pipettes. The ability of combinations of gels and biologically compatible cyanoacrylate tissue sealants to prevent leakage at the injection site was investigated. Gels were intended to plug the perforation site from within scala tympani (ST), while tissue sealants applied to the RW membrane were intended to exteriorly seal the perforation site. Injection of fluorescein dissolved in gels resulted in the marker being distributed more apically. This suggests the gel may be influencing the rate of longitudinal perilymph flow along the scala. Covering the RWM with cyanoacrylate adhesive allowed the highest retention of fluorescein in perilymph but, surprisingly, the same result was obtained when injection occurred through the bony wall of the cochlea, without perforation of the RWM. The systematically lower perilymph levels found when the RWM was not occluded may be the result of transport of fluorescein out of ST at this site. Fluorescein levels were found to be higher in fluid present in the RW niche than were found in perilymph from the basal turn of ST, suggesting that efflux at the RW membrane could be active. This process is currently being investigated further. The study shows that gels and adhesives can control fluid leakages from the ear after injection through the RWM, although the procedures may also alter other physiologic processes, such as flow and elimination.

Studies supported by NIH/NIDCD grant RO1 DC01368

Session C:
Development I

Chairpersons: I. Varela-Nieto
A. Forge

O14 Early otic development depends on autophagy for apoptotic cell clearance and neural differentiation

*M. Magarinos^{1,2,3}, M.R. Aburto^{1,3}, H. Sanchez-Calderón¹, J.M. Hurlé⁴, I. Varela-Nieto^{1,3,5}

¹Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain

²Universidad Autónoma de Madrid, Biology, Madrid, Spain

³Instituto de Salud Carlos III, Unit 761, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER, Madrid, Spain

⁴Universidad de Cantabria, Departamentos de Anatomía y Biología Celular and Fisiología y Farmacología, Santander, Spain

⁵IdiPAZ, Madrid, Spain

Introduction: Early inner ear development requires a strict regulation of the dynamics of cellular proliferation, survival, migration and differentiation in order to generate the different cell types of the adult inner ear, including the neurons for the acoustic-vestibular ganglion. Autophagy is a program of self-degradation of the cytosolic constituents crucial for balancing sources of energy in response to different extracellular stimuli. In vertebrates, autophagy is a highly regulated process that plays key roles during early development and in adult cell growth and homeostasis.

Material and Methods: To investigate the role of autophagy in the developing inner ear we have studied the expression of autophagy genes in early stages of otic development and the consequences of inhibiting pharmacologically and genetically the autophagic pathway in organotypic cultures of explanted chicken otic vesicles.

Results: Here we show the expression of Beclin-1, Atg5 and LC3B in the otocyst and the presence of autophagic vesicles by using transmission electron microscopy in the otic neurogenic zone. The inhibition of autophagy causes accumulation of apoptotic cells and missregulation of the cell cycle at the otic epithelia. Moreover, neurogenesis is severely impaired, thus the number of neural cells decreases and axonal outgrowth is reduced. Finally, our results indicate that autophagy provides the energy required for the clearing of neuroepithelial dying cells and suggest that it is required for the migration of otic neuronal precursors.

Conclusions: Taken together, our results show for the first time that autophagy is an active and essential process during early inner ear development.

O15 The incoherent feed-forward loop regulation of *Atoh1* by *Sox2* provides a mechanism for sensory commitment and deferred hair cell differentiation in the developing inner ear

J. Neves¹, *F. Giraldez¹

¹Universitat Pompeu Fabra-PRBB, CEXS, Barcelona, Spain

The proneural gene *Atoh1* is crucial for the development of inner ear hair cells and it requires the function of the transcription factor *Sox2* through yet unknown mechanisms. In the present work, we used the chicken embryo and HEK293T cells to explore the regulation of *Atoh1* by *Sox2*.

The results show that hair cells derive from *Sox2*-positive otic progenitors and that *Sox2* directly activates *Atoh1* through a transcriptional activator function that requires the integrity of *Sox2* DNA binding domain. *Atoh1* activation depends on *Sox* transcription factor binding to specific sites (*Sox*TFBS) present in the *Atoh1* 3' enhancer, as shown by site directed mutagenesis and chromatin immunoprecipitation (ChIP). In the inner ear, early *Atoh1* enhancer activity in the neurosensory competent domain depends on *Sox2*, and dominant-negative competition (*Sox2*HMG-Engrailed) or mutation of the *Sox*TFBS abolish reporter activity *in vivo*. ChIP assay in isolated otic vesicles also shows that *Sox2* is bound to the *Atoh1* enhancer *in vivo*. However, besides activating *Atoh1*, *Sox2* also promotes the expression of *Atoh1* negative regulators that results in an incoherent feed-forward loop that prevents *Atoh1* expression. This is illustrated by the transient nature of the activation of *Atoh1* by *Sox2*, which is turned into a steady activation after the mutation of HLH binding sites in the *Atoh1* regulatory region.

We suggest that as a consequence of this dual interaction, otic progenitors are committed to sensory fate early in development, but hair cell differentiation is procrastinated until later stages of ear development.

We thank MICINN BFU2011-24057 and PLE-2009-0098 (Spain), and the fellowship SFRH/BPD/70691/2010 to Joana Neves from FCT, Portugal

O16 Septin protein expression in the embryonic and neonatal mouse cochlea

*N. Yamamoto¹, A. Yoshida¹, T. Nakagawa¹, J. Ito¹

¹Graduate School of Medicine, Kyoto University, Otolaryngology, Head and Neck Surgery, Kyoto, Japan

Introduction: Septin proteins are evolutionally well-conserved proteins that constitute non-canonical cytoskeleton. Their functions include the lateral compartmentalization of membranes and the regulation of membrane trafficking. The former function contributes to the formation of polarity of cells and the latter function includes the formation of presynaptic vesicles in neurons. Considering that the cochlea contains many cells that have a strong polarity and have many synapses, we assumed that Septin proteins had important roles in the cochlea.

To elucidate the functions of Septin proteins in the cochlea, we decided to determine the distribution of Septin proteins in embryonic and neonatal mouse cochleae using immunohistochemistry. We chose three Septin proteins, Septin 4 (Sept4), Septin 5 (Sept 5), and Septin 7 (Sept7) that are abundantly expressed in brain tissues. Especially, Sept7 is a core component of most multimeric Septin complexes, indicating that the expression pattern of Sept7 can cover the whole localization of Septin proteins.

Materials and Methods:

RT-PCR

cDNA was prepared from the brains and cochleae of postnatal day 7 (P7) ICR mice to amplify the transcripts of Sept1-12 and 14.

Immunohistochemistry

Frozen sections were prepared from the inner ear tissues of embryonic day 13, 15 (E13, 15) and P1, 2, 3, and 7 mice and were immunostained with rabbit polyclonal anti-Sept4, -Sept5, and -Sept7 antibodies.

Results: Sept4 and Sept5 were undetectable in the cochlea from E13 to P3. Sept4 started its expression in hair cells, outer pillar cells, and Deiters' cells at P7. Sept5 started its expression in outer pillar cells and presynaptic vesicles of efferent nerve terminals also at P7. In contrast, Sept7 started its expression in most cochlear epithelial cells at E13. The expression of Sept7 in cochlear epithelial cells became limited to pillar cells, Deiters' cells, Hensen's cells, and Claudius cells at P1. Sept7 became strongly expressed in pillar cells and Deiters' cells by P7.

Conclusion: These results suggested that Sept7 contributed to the development of whole cochlear epithelia and the maturation of supporting cells was dependent on Sept4, 5, and 7.

O17 Ras/p38 and PI3K/Akt but not Mek/Erk signaling mediate BDNF-induced neurite formation on neonatal cochlear spiral ganglion explants

*Y. Brand^{1,2}, L.M. Mullen¹, K. Pak³, E. Chavez¹, K. Kondo¹, A.F. Ryan^{1,3}

¹UCSD School of Medicine, Departments of Surgery/Otolaryngology, San Diego/CA, USA

²University Hospital Basel, Department of Biomedicine and Clinic of Otolaryngology, Head and Neck Surgery, Basel, Switzerland

³VA Medical Center, San Diego/CA, USA

Introduction: Neurotrophins participate in regulating the survival, differentiation, and target innervation of many neurons. In the cochlea, spiral ganglion neuron survival is strongly dependent upon neurotrophic input, including brain-derived neurotrophic factor (BDNF). Less is known about signal transduction pathways linking the activation of neurotrophin receptors to spiral ganglion (SG) neuron nuclei.

Material and Methods: To explore BDNF signal transduction in SG neurons, SG explants of p3-p5 rats were treated with BDNF in the presence of specific inhibitors of intracellular signaling pathways involved in TrkB signaling in the inner ear and other neuronal systems, and activation of signaling proteins was assessed by Western blotting.

Results: We found that inhibition of Ras, p38, PI3K or Akt signaling reduced or eliminated BDNF mediated increase in number of neurite outgrowth, while inhibition of Mek/Erk had no influence. Inhibition of Rac/cdc42, which lies upstream of JNK, modestly enhanced BDNF induced formation of neurites. Western blotting implicated p38 and Akt signaling, but not Mek/Erk.

Conclusions: The results suggest that the Ras/p38 and PI3K/Akt are the primary pathways by which BDNF promotes its effects. Activation of Rac/cdc42/JNK signaling by BDNF also appears to reduce the formation of neurites. Neurotrophins may thus exert opposing effects on spiral ganglion neurons, the balance of competing signals influencing the generation of neurites. This competition could provide a potential mechanism for the control of neurite number during development.

O18 Neural cell adhesion molecule (NCAM) mediates glial cell line-derived neurotrophic factor (GDNF) induced neuritogenesis in the neonatal spiral ganglion

*S. Euteneuer^{1,2,3}, K.H. Yang², E. Chavez², A. Leichtle^{2,4}, G. Loers³,
A. Olshansky², K. Pak², M. Schachner³, A.F. Ryan²

¹University Hospital Heidelberg, Otorhinolaryngology, Heidelberg, Germany

²University of California San Diego, Surgery/ Otolaryngology, La Jolla/CA, USA

³Zentrum fuer Molekulare Neurobiologie Hamburg, ZMNH, Hamburg, Germany

⁴Klinikum Bielefeld, Otorhinolaryngology, Bielefeld, Germany

Question: Glial cell line-derived neurotrophic factor (GDNF) increases neurite extension and survival of spiral ganglion neurons (SGNs) via yet unknown signaling mechanisms. In other cell types, signaling is achieved by the GPI-linked GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) via recruitment of transmembrane receptors: Ret (re-arranged during transformation) and/or NCAM (neural cell adhesion molecule).

Methods: Cell culture, western blotting, rtPCR, immunohistochemistry

Results: Here we show that GDNF enhances neuritogenesis in organotypic cultures of neonatal spiral ganglia (P5) in mice and rats. GDNF/GFR $\alpha 1$ -Fc stimulation activates intracellular PI3K/Akt and MEK/Erk signaling cascades in P5 and P20 rats. Both cascades mediate GDNF stimulation of neuritogenesis, since application of the Akt inhibitor Wortmannin or the Erk inhibitor U0126 abolished GDNF/GFR $\alpha 1$ -Fc stimulated neuritogenesis in P5 rats. Cultures of P5 NCAM-deficient mice fail to respond with increased neuritogenesis to GDNF/GFR $\alpha 1$ -Fc stimulation.

Conclusion: NCAM mediates neuritogenesis induced by GDNF in the early postnatal spiral ganglion.

O19 Reduction of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] synthesis causes graded alteration of Ca²⁺ signaling along the postnatal cochlea

*E. Simeonato^{1,2}, L. Rodriguez^{1,3}, P. De Camilli⁴, T. Pozzan^{1,5,6},
F. Mammano^{1,5,3}

¹Vimm, Padua, Italy

²University of Padua, Departement of Molecular Medicine, Padua, Italy

³University of Padua, Departement of Physics, Padua, Italy

⁴Yale University School of Medicine, Departements of Cell Biology and Neurobiology, New Haven/CT, USA

⁵Istituto CNR di Neuroscienze, Padua, Italy

⁶University of Padua, Departement of Biomedical Sciences, Padua, Italy

Introduction: PI(4)P 5-kinases type I (PIPKI) generates most plasma membrane PI(4,5)P₂, a minor glycerophospholipid that is hydrolysed by phospholipase C (PLC) into the second messengers DAG and IP₃; the latter binds to IP₃R to activate Ca²⁺ efflux from ER, raising the cytosolic free [Ca²⁺]_i. Knockout mice for PIPKI γ die within 24h while PIPKI γ (+/-) mice breed normally and have no overt phenotype [1].

Materials and Methods: We analyzed ATP evoked intracellular [Ca²⁺]_i oscillations in the lesser epithelial ridge (LER) as well as spontaneous Ca²⁺ transients in the greater epithelial ridge (GER) of cochlear organotypic cultures obtained from P5 WT and PIPKI γ (+/-) mice. Ca²⁺ signals were measured as fura-2 emission ratio changes (ΔR).

Results: In cochlear organotypic cultures obtained from P5 WT mice, [Ca²⁺]_i oscillations evoked by 200 nM ATP persisted for more than 10 min, as previously reported [2, 3]. In contrast, [Ca²⁺]_i oscillations in cultures from PIPKI γ (+/-) mice ceased abruptly and rarely exceeded 5 min. By plotting the maximal fura-2 ΔR vs. [ATP] in cultures from P5 mice, we created ATP dose-response functions. In the middle turn, the EC₅₀ of the dose-response function shifted significantly from 96 nM [ATP] in WT cultures to 353 nM in PIPKI γ (+/-) cultures. In the basal turn, the shift was more dramatic: from 72 nM in WT cultures to 448 nM in PIPKI γ (+/-) cultures. Also the amplitude of the [Ca²⁺]_i oscillations was affected in PIPKI γ (+/-) middle and basal turn cultures, with a reduction of 24% and 22%, respectively, compared to WT controls. In addition, we also examined the mean frequency of occurrence of spontaneous [Ca²⁺]_i transient in the GER of WT and PIPKI γ (+/-) cultures: differences were discernible in the middle turn while they became evident (5-fold reduction) in the basal turn. Also [Ca²⁺]_i transient amplitudes in the middle and basal GER of PIPKI γ (+/-) mice were reduced, by 21% and 33% respectively, compared to WT controls.

Conclusions: The results presented here demonstrate that PLC-mediated PI(4,5)P₂ hydrolysis more rapidly depletes the specific pool of PI(4,5)P₂ necessary for normal Ca²⁺ signaling both in the GER and in the LER in PIPKIγ(+/-) mice, suggesting that this enzyme is critical for producing the PI(4,5)P₂ pool responsible for IP₃ generation along the developing cochlea.

1. Di Paolo, G., et al., Nature, 2004.
2. Piazza, V., et al., Cell Calcium, 2007.
3. Majumder, P., et al., Pur. Signaling, 2010.

Session D:
Development II

Chairpersons: J. Ashmore
M. Göpfert

O20 A Hopf bifurcation controls intracellular calcium oscillations in the developing mouse cochlea

*F. Ceriani^{1,2}, F. Mammano^{1,3,2}

¹Università degli Studi di Padova, Dipartimento di Fisica e Astronomia 'G. Galilei', Padova, Italy

²Istituto Veneto di Medicina Molecolare, Padova, Italy

³Istituto CNR di Neuroscienze, Padova, Italy

Introduction: The non-sensory epithelium that lines the developing cochlear duct, which before the onset of hearing comprises the greater epithelial ridge (GER) and the lesser epithelial ridge (LER), is the site of intense ATP-mediated Ca^{2+} signals, i.e. changes in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), that propagate in a coordinated fashion across non-sensory cells (1,2,3,4). We investigated the properties of Ca^{2+} signals in mice cochlear non-sensory cells of the LER using a combined experimental and computational approach.

Materials and Methods: Ca^{2+} signals were elicited in the LER of cochlear apical turns dissected from P5 wild type mice by direct applications of ATP (from 2 nM to 5 μM) using glass micropipettes, or by photolytic release of caged IP_3 . Fluorescent Ca^{2+} indicators were used to measure the time course of Ca^{2+} signals.

Data analysis and computational simulations were performed using custom-built MATLAB routines.

Results: We found that: (i) the peak of ATP-induced Ca^{2+} responses exhibits a sigmoid-shaped dependence on extracellular ATP concentration; (ii) ATP levels above 20 nM promote the insurgence of $[\text{Ca}^{2+}]_i$ oscillations. Our mathematical analysis indicates that the increase in extracellular ATP concentration promotes self-sustained $[\text{Ca}^{2+}]_i$ oscillations through a supercritical Hopf bifurcation. Next, we extended our model to simulate an entire network of cochlear non-sensory cells coupled by gap junction channels aiming to study the spreading of Ca^{2+} signals elicited by photolytic release of IP_3 . The extended model reproduced the experimental data assuming a junctional transfer rate of 0.05 s^{-1} for IP_3 , which is compatible with the value previously measured (5).

Conclusions: The investigation of Ca^{2+} signaling in the context of the inner ear is a rapidly growing field of research. Our computational approach could help dissecting the role of individual molecular players involved in these intriguing phenomena.

References:

- (1) Gale et al., Curr. Biol, 2004.
- (2) Majumder et al., Purinergic Signal., 2010.
- (3) Piazza et al., Cell Calcium, 2007.
- (4) Anselmi et al., Proc. Natl. Acad. Sci. U S A, 2008.
- (5) Hernandez et al., Nat. Methods, 2007.

O21 Spatio-temporal pattern of action potential firing in mouse inner hair cells

*G. Sendin¹, J. Bourien¹, J.-L. Puel¹, R. Nouvian¹

¹Institute for Neurosciences, Inserm U1051, Montpellier, France

Introduction: Inner hair cells (IHCs) are the primary transducer for sound encoding in the cochlea. In contrast to the graded receptor potential of adult IHCs, immature hair cells fire spontaneous calcium action potentials during the first postnatal week. Here, we investigate the pattern of spontaneous action potential firing in mammalian IHCs during development.

Materials and Methods: We recorded action potentials from neonatal mouse IHCs (P1 to P7) of apical or basal coils from freshly dissected organs of Corti using the perforated patch-clamp technique in the current clamp mode ($I_{inj}=0pA$).

Results: IHCs fire bursts of action potentials and this pattern is indistinguishable between basal and apical hair cells. However, the bursting behavior became more salient during development, i.e, from a broad and stochastic structure right after birth to a sharp and stereotyped motif at the end of the first postnatal week.

Conclusions: The IHCs spiking activity has been proposed to shape the tonotopic map along the ascending auditory pathway. We suggest that in addition to carrying place information to the ascending auditory nuclei, the firing pattern conveys temporal information of the cochlear development.

O22 Loss of stria vascularis integrity and endocochlear potential in E-cadherin KO mice

*H. Maier^{1,2}, M. Schweizer³, M.-O. Trowe^{1,2,4}, A. Kispert⁴

¹MHH, HNO, Hannover, Germany

²Medizinische Hochschule Hannover, HNO, Hannover, Germany

³Universitätsklinikum Hamburg-Eppendorf, ZNMH, Hamburg, Germany

⁴Medizinische Hochschule Hannover, Institut fuer Molekularbiologie, Hannover, Germany

Introduction: The endocochlear potential (EP) is required for the sensitivity of the cochlea. It crucially depends on the insulation of the intrastrial compartment by epithelial basal cells. For the formation of the basal cell layer E-cadherin is a key factor in mesenchymal to epithelial transitions. Here we demonstrate that E-cadherin is required for late differentiation but not for formation of basal cells. Its deletion results in reduction of the EP and severe hearing deficits.

Methods: Experiments were performed in conditional E-cadherin mice $Tbx18^{cre/+}$; $Ecadherin^{flox/flox}$ (EcadKO), while $Tbx18^{cre/+}$; $Ecadherin^{flox/+}$ and $Tbx18^{+/+}$; $Ecadherin^{flox/+}$ littermates served as controls. Hearing thresholds were determined by auditory evoked brainstem responses (ABR) to alternating clicks and the EP was recorded in the 1st turn of anesthetized animals.

Results: In adult wild type mice E-cadherin is found in the spiral ligament except in type III and IV fibrocytes. In the stria vascularis it is located in basal and marginal cells, but not in intermediate cells. In EcadKO mice gross morphological changes were absent and otic fibrocyte differentiation, condensation and initiation of basal cell differentiation were normal. Beside strial hypoplasia the dense infoldings of basal cells were affected in EcadKO mice and the expression of Kir4.1 in intermediate cells was mainly reduced. ABRs thresholds were significantly increased in EcadKO mice from 58.6 ± 5.2 dB peSPL to 86.6 ± 3.7 dB peSPL (MV \pm SD; n=13/10) at 4 weeks and 59.6 ± 3.8 dB peSPL to 125.9 ± 6.4 dB peSPL (n=8/8) at 12 weeks of age. The EP was significantly decreased from 119.0 ± 6.2 mV to 65.0 ± 9.4 mV (n=7/6, 4w) and 114.9 ± 11.1 mV to 31.0 ± 7.5 mV (n=6/5, 12w).

Discussion: We suggest that loss of E-cadherin interferes with the establishment of the EP at two levels: first, by the failure to establish a tight, functional basal cell layer, and second, by the loss of Kir4.1-positive functional intermediate cells.

O23 Transmembrane channel-like proteins Tmc1 and Tmc2 influence the tonotopic variation in the mechanotransducer channels of mouse cochlear hair cells

*R. Fettiplace¹, K.X. Kim¹

¹University of Wisconsin-Madison, Neuroscience, Madison/WI, USA

The first step in sound detection is vibration of the stereociliary bundles of cochlear hair cells, which activates mechanotransducer (MT) channels, large conductance calcium selective cation channels located at the tips of the stereocilia. The molecular identity of the MT channels has not yet been firmly established but recent evidence indicates that Tmc1 and Tmc2, isoforms of the transmembrane-channel-like family, play a central role in transduction (Kawashima et al., 2011).

We have characterized the properties of MT channels in wild type, Tmc1 (dn) knockout and Tmc2 knockout mice as a function of age and cochlear location.

Peak MT amplitudes measured in outer hair cells of neonatal (post-natal days, P2 to P10) mice were 0.9, 1.2 and 1.5 nA at apex, middle and base respectively. Similar values were obtained in wild type and mutants in the first post-natal week, but after P7 the MT current in the Tmc1 knockouts declined to zero, which may account for the deafness phenotype (Steel & Bock 1980). The relative calcium permeability of the MT channel, P_{Ca}/P_{Cs} , deduced from reversal potentials, showed a small but significant decrease from apex (6.1 ± 0.3 ; $n=7$) to base (4.4 ± 0.1 ; $n=21$) in wild type mice (P2 - P6). In Tmc1 homozygotes over the same age range, the gradient disappeared with the permeability at the base (5.9 ± 0.2 ; $n=3$) being not significantly different from that at the apex (6.3 ± 0.1 ; $n=4$). In contrast, the calcium permeability in the Tmc2 knockout at the apex (4.3 ± 0.1 ; $n=5$) became comparable to the base.

Our results suggest that in the first neonatal week, there are tonotopic differences in the MT channel calcium permeability that are partly determined by expression of Tmc1 at the base and Tmc2 at the apex.

Supported by NIDCD grant RO1 DC01362 to RF

Session E:

Hair cells

Chairpersons: J. Santos-Sacchi
J. Engel

O24 Mechanotransduction current adaptation differs between lower vertebrate and mammalian hair cells

A.W. Peng¹, *T. Effertz¹, A.J. Ricci^{1,2}

¹Stanford, School of medicine, Otolaryngology-Head & Neck surgery, Palo Alto/CA, USA

²Stanford University, Molecular and Cellular Physiology, Palo Alto/CA, USA

To hear, animals rely on specialized mechanosensory cells, the hair cells. All hair cells feature a sensory organelle called hair bundle that is comprised of actin-filled microvillae arranged in a staircase pattern. While the staircase of inner hair cells (IHCs) and outer hair cells (OHCs) consist of three rows, the hair bundles of lower vertebrate (and vestibular hair cells) harbor up to 10 rows. At the tip of these stereocilia reside mechanoelectrical transduction channels (MET-channels). The MET-channel's open probability (P_o) increases with stereociliary deflections towards the tallest row and decreases with stimulation in the opposite direction. Channel dynamic range is enhanced by adaptation, a process (es) that attempts to compensate for static hair bundle deflections and thus restore P_o to its resting value. Adaptation may also be important for establishing the hair cell's resting potential, providing tonotopic mechanical filtering to the hair bundle and driving an amplification process. Though significant controversy exists over the mechanisms of adaptation, it has been universally found that calcium drives adaptation. This data largely comes from frog saccule, turtle auditory papilla and chick cochlea while some supporting data comes from mammalian vestibular and cochlea cells. In general lowering external calcium or increasing intracellular calcium buffering results in a slowing of adaptation kinetics and a leftward shift in the steady-state activation curve resulting in a larger P_o . Similarly depolarizations eliminate adaptation and also result in a leftward shift in the activation curve. To our surprise, we find that rat IHCs show little effect on adaptation to lowering external calcium or to depolarizations that reduce calcium entry. Activation curves show little shift, kinetics are unaffected or slowed slightly and the extent of adaptation remains constant. OHCs are more sensitive to these manipulations than IHCs but much less sensitive than the more established models.

Funding: NIH-NIDCD RO1 DC003896 to AR; NIH-NIDCD F32 DC10975 to AWP; NIH-NIDCD core grant P30-44992; DAAD postdoc fellowship to TE

O25 Microdomains shift and rotate in the lateral wall of cochlear outer hair cells

R. Kitani¹, C. Park¹, *F. Kalinec¹

¹House Research Institute, Cell Biology & Genetics, Los Angeles/CA, USA

There is strong consensus that OHC electromotility, a response is associated with the concerted conformational change of hundreds of thousands of plasma membrane-embedded prestin molecules, confers to the mammalian hearing its remarkable sensitivity and frequency resolution.

Looking for a better understanding of the structural basis of this motile mechanism we labeled isolated guinea pig OHCs with polystyrene microspheres and, using high-speed video recording, investigated their movements at the apical, middle and basal regions of osmotically and electrically stimulated cells.

Following hypo-osmotic challenge, OHCs shortened and their diameter increased, with microspheres moving always toward the central plane; iso-osmolarity returned OHCs to their original shape and microspheres to their original positions. Under stimulation with an external alternating electrical field microspheres exhibited robust back and forth displacements, but their trajectories changed in direction from random to parallel to the electrical field with angular frequencies of up to 6 rad/s, and returned to random following 5 min without stimulation. Alterations in plasma membrane cholesterol levels as well as cytoskeleton integrity affected microspheres responses.

We concluded that microspheres attach to different molecular microdomains, and these microdomains are able to shift and rotate in the plane of the OHCs' lateral wall with a dynamics tightly regulated by membrane lipid composition and the cortical cytoskeleton.

O26 Does DDR1 contribute to the cytoarchitecture and stability of motile cells?

*A.-M. Meyer zum Gottesberge¹, S. Hansen¹

¹University of Düsseldorf, ORL, Düsseldorf, Germany

Discoidin domain receptor 1 (DDR1) is a non-integrin tyrosine kinase receptor that is activated by native collagen. DDRs regulate cell adhesion, epithelial integrity, but also cell motility, which is linked to actomyosin organization (Hidalgo et al. 2011). In the inner ear a deletion of the DDR1 gene causes ultrastructural alterations including a heterogeneous group of cells which contain contractile elements of the actomyosin complex (Meyer zum Gottesberge et al. 2008), especially it causes deformation of outer hair cells and separation of their lateral wall.

Using immunohistochemical staining, we identified a co-localization of DDR1 and NMHC-IIA, a non-muscle myosin heavy chain isoform encoded by MYH9, especially in the spiral ligament in particular region adjacent to bony wall, which is populated by myofibrocytic cells (fibrocyte type III), and in the outer hair cells. Electron microscopy revealed an alteration of the coupling of lateral wall to the cytoskeleton of the body in the outer hair cells in DDR1^{-/-} mice.

Conclusion: We propose that DDR1 together with proteins of the actomyosin complex are involved in the tension of the spiral ligament as well as in the control of the cellular shape of the outer hair cells. The co-localization of NMHC-IIA and DDR1 confirms the idea that hearing loss in mice lacking DDR1 may be due to alterations of the micromechanics of the basilar membrane and of the outer hair cells.

O27 Slow down prestin – a very simple model says you're moving too fast!

*J. Santos-Sacchi¹, L. Song¹

¹Yale University School of Medicine, Surgery, New Haven/CT, USA

Outer hair cells (OHC) drive cochlear amplification, which enhances auditory detection and discrimination. The motor protein, prestin, which evolved from the SLC26 anion transporter family, underlies the OHC's electromotility. Here we report on simultaneous measures of prestin's voltage sensor charge movement (NLC) and electromotility that evidence disparities in their voltage dependence and magnitude as a function of intracellular chloride. A simple kinetic model, possessing fast anion binding transitions and fast voltage dependent transitions, coupled together by a much slower transition recapitulates these disparities and a host of other biophysical observations on the OHC. The intermediary slow transition probably relates to the transporter legacy of prestin, and this intermediary gateway which shuttles anion bound molecules into the voltage-enabled pool of motors, provides molecular delays that present as phase lags between membrane voltage and electromotility. Cochlear modelers find that such phase lags are required to effectively inject energy at the appropriate moment to enhance basilar membrane motion. Thus, slowing down prestin has its benefits.

Supported by NIDCD R01 DC000273 to JSS.

O28 The striated organelle in inner ear hair cells

*A. Lysakowski¹

¹Univ. of Illinois at Chicago, Anatomy and Cell Biology, Chicago/IL, USA

We have recently described the striated organelle (SO) in vestibular hair cells, where it is particularly well developed (Vranceanu et al., *PNAS*, 2012). The SO is located in the subcuticular region of hair cells and consists of alternating thick and thin bands (Friedman et al., 1965; Ross and Bourne, *Science*, 1983). In type I hair cells, the SO is shaped like an inverted open-ended cone that contacts the cell membrane along its entire circumference and is separated from the cuticular plate by a layer of large mitochondria. Stereociliar rootlets have been observed bending at an angle of 110 degrees, traversing the cuticular plate and inserting in the plasma membrane in the vicinity of the SO, opposite the kinocilium. The SO is also present in cochlear inner hair cells. In hair cells other than vestibular type I hair cells, it has seemed to be a much smaller structure and it appeared to be free-floating. We have now studied it in more detail with EM tomography and confocal microscopy and find it much more extensive (in height, breadth and radial depth) in type II hair cells than in type I hair cells. In a 3-D tomographic reconstruction, we have not yet found a connection to actin rootlets in type II hair cells, as there was with type I cells, but it is closely associated with mitochondria, although these are smaller in volume and surface area than in type I cells. EM immunogold experiments have demonstrated that antibodies to the actin-binding proteins α -2 and β -2 spectrin (also called fodrin or non-erythroid spectrin) and γ -actin label the SO. We are performing co-immunoprecipitation experiments to determine other protein-protein interactions. Confocal immunolabeling shows that the SO in type II hair cells extends down from the cuticular plate as two large sheets. Unlike the SO in type I hair cells, those in type II cells are not associated with a constriction in the neck of the hair cell, although thick filaments do form cross-links and occasionally, “morph” into thin filaments. We continue to study the structure, protein composition, and function of this intriguing organelle.

Supported by NIH DC-02521, the 2008 Tallu Rosen Grant in Auditory Science (from the National Organization for Hearing Research Foundation), the American Foundation for Hearing Research and a grant from The Knowles Hearing Center at Northwestern University.

Session F:

Hair cells / Non-sensory cells

Chairpersons: A. Nuttall
S. Klis

O29 How are inner hair cells stimulated? Evidence for multiple mechanical drives

*J. Guinan¹

¹Mass. Eye & Ear Infirmary; Harvard Medical School, Eaton Peabody Lab, Boston/MA, USA

Introduction: Deflections of inner-hair-cell (IHC) stereocilia bring about auditory nerve (AN) output, but the mechanisms involved are poorly understood. Recent studies indicate that the gap between the reticular lamina (RL) and the tectorial membrane (TM) varies cyclically during low-frequency sound. Variation in the RL-TM gap produces radial fluid flow in the gap that can drive IHC stereocilia.

Methods: We form hypotheses of how fluid flows drive IHC stereocilia, and how they may explain formerly anomalous AN data.

Results: We distinguish three IHC drives in addition to classic SHEAR: (1) OHC-MOTILITY: Upward basilar-membrane (BM) motion causes OHC somatic contraction which tilts the RL, compresses the RL-TM gap over IHCs and expands the RL-TM gap over OHCs, thereby producing an outward (away from the IHCs) radial fluid flow. (2) TM-PUSH: For upward BM motion, the force that moves the TM compresses the RL-TM gap over OHCs causing inward radial flow past IHCs. (3) CILIA-SLANT: Motions that produce large tilting of OHC stereocilia squeeze the supra-OHC RL-TM gap and cause inward radial flow past IHCs. For upward BM motion, IHC stereocilia are deflected in the *excitatory* direction by SHEAR and OHC-MOTILITY drives, but in the *inhibitory* direction by TM-PUSH and CILIA-SLANT drives. Combinations of these drives explain: (1) the reversal at high sound levels of AN initial peak (ANIP) responses to clicks, and medial olivocochlear (MOC) inhibition of ANIP responses below, but not above, the ANIP reversal, (2) dips and phase reversals in AN responses to tones in cats and chinchillas, (3) hypersensitivity and phase reversals in tuning-curve tails after OHC ablation, and (4) MOC inhibition of tail-frequency AN responses.

Conclusions: The ability of these IHC drives to explain previously anomalous AN data provides strong, although indirect, evidence that these drives are significant. The OHC-MOTILITY drive provides another mechanism, along with BM motion amplification, that uses active processes to enhance cochlear output. Overall, the success of the hypotheses presented here argues for a new view of how the cochlea works at frequencies below 3 kHz.

Supported by NIDCD RO1DC00235, P30DC005209

O30 Testing the cochlear amplifier with otoacoustic emissions

*R. Sisto¹, A. Moleti², T. Botti²

¹INAIL Research, Occupational Hygiene, Monteporzio Catone (Rome), Italy

²University of Rome Tor Vergata, Department of Physics, Rome, Italy

Introduction: Otoacoustic emissions (OAE) provide acoustic imaging of cochlear functionality. Their response is related to the physical properties of the active feedback mechanism inside the organ of Corti, which is associated with the outer hair cells electromotility and with the dynamical properties of the “hair bundle”.

Two main generation mechanisms must be considered, nonlinear distortion and coherent linear reflection.

In this study it is shown how time-frequency domain filtering of the SFOAE, TEOAE and DPOAE responses to stimuli varying in a wide level range yields a coherent picture showing universal behavior of each mechanism, as a function of the stimulus level, across different acquisition paradigms.

Materials and methods: High-resolution SFOAE and DPOAE have been measured at different stimulus levels using a chirp technique. TEOAE were measured with a double evoked paradigm. Time-frequency wavelet analysis has been applied to separate OAE components of different phase-gradient delay.

In the TEOAE and SFOAE cases, time-frequency analysis permitted to separate the main reflection component from shorter latency components, and from multiple-reflection components.

Results: The correlation between the responses associated with the same generation mechanism is very high. The separated components corresponding to the same mechanism also show similar growth rate as a function of stimulus level, demonstrating that the OAE response is strictly related to the nonlinear physics of the underlying cochlear mechanisms.

Conclusions: The separated OAE components show universal behavior across different experimental acquisition paradigms and stimulus levels. Universality of the OAE response, when it is suitably time-frequency filtered to isolate different generators, permits to detect the signature of the behavior theoretically predicted for a wide class of nonlinear dynamical systems operating close to a Hopf bifurcation. This study confirms the importance of cochlear mechanical insight and advanced signal analysis methods to fully exploit the potentially high OAE diagnostic power.

O31 The effect of aging on cochlear performance: A simulation approach using a physiologically-based electromechanical model of the cochlea

*A. Saremi¹, S. Stenfelt¹

¹Technical Audiology, IKE, Linköping, Sweden

Our aim is to explicitly integrate the electrical, acoustic, and mechanical elements of the cochlea. The elements of the model are chosen to have a physiological interpretation of the human cochlea in so far as is known. As a result, the model enables simulation of specific cochlear lesions such as metabolic presbycusis whereby this finite element model is used to study the effect of age-related degenerations on cochlear performance. Besides, the longitudinal coupling between cochlear structures is included here which is often neglected in auditory models.

The model incorporates passive vibrations; nonlinear mechano-electrical transduction of outer hair cells (OHCs) and inner hair cells (IHCs), active somatic motility, as well as tectorial membrane (TM) shearing and vibration to neural conversion at the inner hair cells (IHCs). The input to the model is the sound-induced acoustical vibration of stapes and its output is the neural activity on the auditory nerve. A linearized version of the model is created here, according to small-signal analysis, to study the cochlear performance in frequency domain.

The prevailing hypothesis is that high-frequency audiometric threshold elevations in older adults are due to metabolic presbycusis whereby there is a reduction in the endocochlear potential (EP) as a result of age-related cellular degeneration in the lateral wall of the cochlea. Our simulations quantitatively confirm this hypothesis. Specifically, in our model, as the EP decreases, the OHC's transduction mechanism produces less receptor current such that there is a reduction in the battery of the somatic motor (OHC motility) which leads to a drastic decrease in cochlear amplification and frequency sensitivity. Furthermore, the model successfully predicts how the position-frequency map of cochlea changes as the somatic motor declines.

Besides of lowering the cochlear amplification, degeneration of EP can also affect the sensory mechanism of the inner hair cells by lowering the firing rate of the auditory nerve; thereby increasing the time interval between firings which may, at least partially, explain declines of temporal resolution in an aging auditory system.

O32 Acoustic signal transduction in a simple ear - the *crista acustica* of bushcrickets

*M. Nowotny¹, A. Palghat Udayashankar¹, J. Hummel¹, M. Kössl¹

¹Institute for Cell Biology and Neuroscience, Frankfurt, Germany

Sound-induced force transduction is essential in the hearing process. We study sound transduction in bushcrickets (Tettigoniidae), which offer the unique opportunity of easily accessing the hearing organ in their forelegs *in-vivo*. The high-frequency hearing organ, called *crista acustica* (CA), is sensitive to airborne sound up to about 80 kHz and the mechanical motion of the CA was investigated by laser-Doppler-vibrometer measurements. Using pure tone stimuli, we found traveling waves along the CA. These traveling waves form the basis of tonotopy in bushcrickets similar to mammals. Very little data is available on the radial structure of the traveling waves in the mammalian cochlea. The ease of access to the CA allowed us to study the radial structure of traveling waves in detail. Traveling waves along the hearing organ were reflected off the lateral walls during propagation. Further, a clear lateralization of energy in the magnitude response of the CA towards the anterior part of the leg was found. Propagation by reflection and lateralization of energy is predicted by mathematical models of the mammalian cochlea and now we are able to show these effects experimentally in bushcrickets. In addition, propagation by reflection induces a rotation of the CA and the resulting force presumably stretches the sensory dendrite of the receptor cell resulting in transduction. Further investigations of the receptor cells using intracellular recordings will help clarify the characteristics of the transduction process in the bushcricket hearing organ.

O33 Drastic impairment of calcium signaling in cochlear non-sensory cells of postnatal Cx30 KO mice

*L. Rodriguez Hernandez¹, E. Simeonato¹, F. Mammano¹

¹Venetian Institute of Molecular Medicine, Padua University, Padua, Italy

Introduction: Connexin 30 (Cx30) and connexin 26 (Cx26) are the only connexins expressed in organ of Corti non-sensory cells and they are essential for the cell-to-cell propagation of Ca²⁺ signals [1-3].

Methods: We prepared cochlear organotypic cultures from P5 WT, Cx30(+/-) and Cx30(-/-) mice, and examined the frequency of occurrence of spontaneous [Ca²⁺]_i transients in non-sensory cells by counting all events in the field view while performing ratiometric imaging with the membrane permeable AM ester derivatives of fura-2. In addition we quantified the level of mRNA for both connexins using quantitative real time polymerase chain reaction (qPCR).

Results: Spontaneous [Ca²⁺]_i transients showed no sign of fatigue in recordings lasting 15 min or more in the greater epithelial ridge (GER) of WT cultures. The frequency of occurrence of spontaneous [Ca²⁺]_i transients in Cx30(+/-) cultures was comparable to that of WT cultures in the apical turn (36±14 events/min in Cx30(+/-) vs. 38±11 events/min in WT), in the middle turn (34±6 events/min in Cx30(+/-) vs. 32±7 events/min in WT), as well as in the basal turn (22±6 events/min in Cx30(+/-) vs. 21±2 events/min in WT; P = 0.97). In contrast, in Cx30(-/-) cultures the frequency of spontaneous [Ca²⁺]_i transients was drastically and uniformly reduced in all turns (10±2 events/min, in the apical turn; 7±5 events/min, in the middle turn and 5±2 events/min, in the basal turn; P<0.001). Amplitudes of these spontaneous [Ca²⁺]_i transients were significantly reduced in the middle and basal turn of Cx30(+/-) cultures (P<0.005) and in all turns of Cx30(-/-) cultures (P<0.001). In addition, our prior work showed that the whole cochlea samples from postnatal Cx30(-/-) mice, which have no detectable levels of Cx30, have only ~8% residual Cx26 mRNA [4]. Here we found that the levels of Cx26 and Cx30 in the cochlea of Cx30(+/-) mice were reduced by 50%±10% compared with age-matched WT controls.

Conclusions: The amplitude and frequency of spontaneous [Ca²⁺]_i transients were drastically reduced in each cochlear turn of Cx30(-/-) mice, but preserved in cultures of Cx30(+/-). The present work highlights the essential role of inner ear connexins in the generation of spontaneous [Ca²⁺]_i transients in cochlear non-sensory cells.

1. Schutz M., et al., Hum Mol Genet, 2010.
2. Anselmi et al., Proc. Natl. Acad. Sci. U S A, 2008.
3. Majumder P., et al., Purinergic Signaling, 2010
4. Ortolano S., et al., Proc Natl Acad Sci. 2008

O34 A molecular dynamics investigation of Connexin 26 and Connexin 30 channels

*F. Zonta^{1,2}, G. Polles³, G. Zanotti^{4,5}, F. Mammano^{1,2,5}

¹Padua University, Department of Physics and Astronomy "G. Galilei", Padua PD (Italy), Italy

²Consorzio Nazionale Universitario per le Scienze Fisiche della Materia (CNISM), Padua PD (Italy), Italy

³International School for Advanced Studies (SISSA), Statistical and Biological Physics, Trieste TS (Italy), Italy

⁴Padua University, Department of Biological Chemistry, Padua PD (Italy), Italy

⁵Venetian Institute of Molecular Medicine, Foundation for Advanced Biomedical Research, Padua PD (Italy), Italy

Introduction: Mutations in the genes GJB2 and GJB6 encoding human connexin26 (hCx26) and connexin30 (hCx30), respectively, are the leading cause of non-syndromic prelingual deafness in several human populations.

Methods: In this work, we exploited the high degree (77%) of sequence similarity shared by hCx26 and hCx30 to create atomistic models of homomeric hCx26 and hCx30 connexons starting from the X-ray crystallographic structure of an intercellular channel formed by hCx26 protomers at 3.5-Å resolution (1). The equilibrium dynamics of the two protein complexes was followed for 40 ns each by Molecular Dynamics (MD) simulations.

Results: Our results indicate that in hCx26, positively charged Lys41 residues establish a potential barrier within the fully open channel, hindering ion diffusion in the absence of an electrochemical gradient. A similar role is played, in hCx30, by negatively charged Glu49 residues. The different position and charge of these two ion sieves account for the differences in unitary conductance observed experimentally. Moreover, our study suggests that Ca²⁺ ions can bind with millimolar affinity to acidic side chains in the extracellular mouth of the connexon, producing an occlusion of the pore, which could be related to the phenomenon known as "loop gating".

Conclusions: By use of Molecular Dynamics simulations we provided insight on connexin channels behavior and their permeation and gating properties.

For a full account of this work, see (2).

Supported by MIUR PRIN grant no. 2009CCZSES and Telethon grant GGP09137 to FM, and from a University of Padova grant to FZ (prot. GRIC101108). Computer simulations were performed at the CINECA and CASPUR supercomputer centers. 1. Maeda, S., Nakagawa, S., Suga, M., Yamashita, E., Oshima, A., Fujiyoshi, Y. & Tsukihara, T. (2009) *Nature* 458, 597-602. 2. Zonta, F., Polles, G., Zanotti, G. & Mammano, F. (2012) *Journal of Biomolecular Structure and Dynamics* 29, 985-998.

Session G:
Neurotransmission

Chairpersons: R. Fettiplace
J. Guinan

O35 The presynaptic scaffold Bassoon and the synaptic ribbon control synaptic strength at the mouse inner hair cell afferent synapse

*H. Takago^{1,2,3}, N. Strenzke⁴, T. Moser^{1,2,5}

¹University Medical Center Göttingen, InnerEarLab, Department of Otolaryngology and Collaborative Research Center 889, Göttingen, Germany

²University of Göttingen, Bernstein Focus for Neurotechnology, Göttingen, Germany

³Research Institute of National Rehabilitation Center for Persons with Disabilities, Department of Rehabilitation for Sensory Functions, Tokorozawa, Japan

⁴University of Göttingen, Auditory Systems Physiology Group, InnerEarLab & Department of Otolaryngology and Collaborative Research Center 889, Göttingen, Germany

⁵University of Göttingen, Center for Molecular Physiology of the Brain, Göttingen, Germany

Faithful sound encoding at the inner hair cell (IHC) ribbon synapse is a crucial step for hearing. To ensure action potential generation in the spiral ganglion neurons (SGNs) in response to ultrafast sound stimuli, this synapse takes advantage of multiquantal (MQR) release of neurotransmitter glutamate. Although a set of previous studies using presynaptic patch clamp recording from IHCs demonstrated that disruption of presynaptic scaffolding protein Bassoon and the synaptic ribbon reduces Ca^{2+} influx and exocytic rate in the IHCs (Khimich et al., 2005; Frank et al., 2010), it remains unclear whether these might be accompanied by a reduction in EPSC amplitude.

To address this question, we performed whole-cell patch clamp recording from afferent dendrites of type I SGNs of postnatal day 9- 11 mutant mice with deletion of exon 4 and 5 of the Bassoon gene ($BSN^{\Delta Ex4/5}$) and their wild-type littermates (BSN^{wt}).

We found reductions in EPSC amplitude and charge transfer in $BSN^{\Delta Ex4/5}$ afferents compared with controls. We also found EPSC frequency upon depolarizing high K^+ stimulation was lower in $BSN^{\Delta Ex4/5}$ than that in BSN^{wt} , compatible with previous data obtained by the presynaptic membrane capacitance measurement.

In conclusion, Bassoon and the synaptic ribbon are essential to MQR and replenishment of synaptic vesicles at the IHC afferent synapse.

O36 Voltage-gated ionic conductances required for action potential firing in auditory nerve fibers

*C. Michel^{1,2}, R. Nouvian^{1,2}, J. Santos-Sacchi³, J.-L. Puel^{1,2}, J. Bourien^{1,2}

¹Inserm UMR 1051, Institute for Neurosciences of Montpellier, Montpellier, France

²University Montpellier 1, Montpellier, France

³Yale University School of Medicine, Dept. of Surgery (Otolaryngology), Neurobiology and Cellular and Molecular Physiology, New Haven/CT, USA

Introduction: Depolarization of the inner hair cell triggers glutamate release onto the dendrite-like processes of spiral ganglion neurons (SGNs) and drives action potentials, which are conveyed to the brain. Whereas knowledge of the transfer function at the ribbon synapse has considerably progressed, little is known about the voltage-gated ionic channels which shape the action potential firing. Here, we provide a comprehensive computational model bridging the gap between the voltage-dependent currents measured *in vitro* on fresh isolated SGNs and spikes (extracellular action potentials) recorded *in vivo* from guinea pig auditory nerve fibers.

Materials and Methods: Voltage-dependent currents (Na⁺ and K⁺) of SGNs somata patch-clamp recordings were fitted by a Hodgkin-Huxley model with a full-trace fitting Willms algorithm. Node of Ranvier model was designed from the hypothesis that channels expressed on soma were identical, but differ in density. Simulated spikes were adjusted in order to match *in vivo* single-unit recordings with gradient-descent algorithm.

Results: Computation of the data allows for the identification of: i) one fast inward Na⁺ current (GNa, activation: $V_{1/2} = -33$ mV, $\tau < 0.5$ ms; inactivation: $V_{1/2} = -61$ mV, $\tau < 2$ ms); and ii) two K⁺ delayed-rectifier conductances, a low voltage-activated component (GKL, activation: $V_{1/2} = -56$ mV; $\tau < 5$ ms) and a high voltage-activated component (GKH, activation: $V_{1/2} = -41$ mV; $\tau < 2.5$ ms). Node of Ranvier model generates spikes that fit with *in vivo* recordings. Interestingly, the different spike durations along the tonotopic axis measured *in vivo* (i.e. 450 ms peak-to-peak duration versus 250 ms for 1 to 20 kHz, respectively) was explained by a gradual change in Na and K channel densities along the cochlea (GNa ~78 nS, GKL ~9 nS, GKH ~3 nS at 1 kHz versus GNa ~90 nS, GKL ~12 nS, GKH ~6 nS at 20 kHz).

Conclusion: This study identifies the ionic conductances and densities, which shape the action potential waveform of auditory nerve fibers and suggests that the interplay of fast inward Na⁺ current and the two K⁺ delayed-rectifier enables the auditory nerve fibers to sustain high firing rates.

O37 Logic of sound level encoding in the auditory nerve

*J. Bourien^{1,2}, Y. Tang^{1,2}, M. Lenoir^{1,2}, S. Ladrech^{1,2}, C. Batrel^{1,2},
R. Nouvian^{1,2}, J.-L. Puel^{1,2}, J. Wang^{1,2}

¹Inserm UMR 1051, Institute for Neurosciences of Montpellier, Montpellier, France

²University Montpellier 1, Montpellier, France

Introduction: In mammal auditory system, sound encoding over vast level range (~120 dB), relies on auditory nerve fibers (ANFs) with different threshold and dynamic range. High- and medium-spontaneous rate (SR) fibers detect low-sound level, but rapidly saturate (i.e. ~30 dB). In contrast, low-SR fibers translate high-levels with little or no saturation. However, this general view has been challenged because of the large number of high- and medium-SR fibers versus small number of low-SR fibers in the auditory nerve, leading to a limited dynamic range for sound level encoding.

Materials and Methods: Using a combination of pharmacological (application of ouabain on the round window membrane), anatomical (light microscopy and 3D-confocal imaging) and computational approaches (cochlea biophysical model), we investigate the weight of each of the auditory fibers sub-populations on the threshold and amplitude of sound-evoked compound action potential (CAP) of the auditory nerve.

Results: Infusion of ouabain into the gerbil cochlea leads to the selective loss of auditory afferent fibers. Strikingly, the threshold shift and CAP amplitude reduction induced by the ANFs loss enable to distinguish 3 populations of fibers, which match near perfectly to the ANFs SR-based distribution. CAP threshold is only governed by high-SR fibers, and CAP amplitude strongly depends of high- and medium-SR fibers. In all cases, CAP threshold and amplitude are independent of the low-SR fibers. Computational modeling further suggested that large dynamic range encoding might be achieved through the recruitment of adjacent ANFs (spread of excitation hypothesis) and through the intrinsic properties of high-SR fibers.

Conclusion: Our result argue against a continuum recruitment of all the ANFs to encode vast dynamic range sound level but rather rely on the spread of excitation toward the basal region and the intrinsic properties of high-SR fibers.

O38 A new physiologic technique for assessing apical cochlear function: The Auditory Nerve Overlapped Waveform (ANOW)

*J. Lichtenhan¹, A. Salt¹, J. Guinan, Jr.²

¹Washington University School of Medicine, Department of Otolaryngology, St. Louis/MO, USA

²Harvard Medical School, Department of Otolaryngology, Boston/MA, USA

Introduction: Objective measures of low-frequency auditory function could be important for managing some forms of human hearing loss and for determining the health of the cochlear apex during *in vivo* animal experiments. Recently, Lichtenhan et al. (Ear & Hearing 2012 In Press) described a new technique - the auditory-nerve overlapped waveform (ANOW) - that used round window potentials to quantify low-frequency auditory thresholds. ANOW thresholds from 0.3-1 kHz tones were ~10-15 dB less sensitive than single-auditory-nerve-fiber thresholds in cats. Thus, ANOW and typical compound action potential (CAP) thresholds from tone-bursts >1 kHz relate similarly to single-fiber thresholds. Here we address the question of what cochlear regions contribute to the ANOW response.

Materials and Methods: ANOW and cochlear microphonic (CM) from 500 Hz tones, as well as CAP thresholds from 1.4-16 kHz tone-bursts, were monitored through time while KCl was injected at a low flow rate from a pipette sealed into the cochlear apex of guinea pigs. The goal of KCl injections was to abolish cochlear function progressively from apex to base. Flow rate was incrementally increased from 50 nl/min to 200 nl/min to compensate for varying cross sectional scala tympani area. An established model of the cochlear fluids was used to estimate how KCl concentration varied with time and location along scala tympani.

Results: ANOW responses from low- and moderate-level stimuli (35-65 dB SPL) were quickly abolished when KCl was injected. Then, as expected, CAP thresholds were slowly and systematically raised, confirming their generation in the basal half of the cochlea. ANOW responses to high-level (80 dB SPL) stimuli had different characteristics, consistent with being influenced by CM asymmetry related to the transducer operating point in the basal turn.

Conclusions: These findings confirm that ANOW from low- to moderate-level stimuli is generated in the apical half of the cochlea. In contrast, ANOW from high-level stimuli becomes contaminated by un-cancelled CM components generated in the cochlear basal region.

Supported by NIDCD grants F32 DC010112, RO1 DC00235, RO1 DC03687, R01DC001368, and P30 DC005209

O39 Functional and histological characterization of the degenerating auditory nerve in the deafened guinea pig

*D. Ramekers¹, H. Versnel¹, E.M. Smeets¹, S.B. Strahl², W. Grolman¹, S.F.L. Klis¹

¹University Medical Center Utrecht, Otorhinolaryngology, Utrecht, Netherlands

²MED-EL GmbH, R&D, Innsbruck, Austria

Question: After severe hair cell loss, secondary degeneration of spiral ganglion cells (SGCs) is observed - a gradual process that takes years in humans but only weeks in guinea pigs. Being the target for cochlear implants, both the number and the physiological state of the SGCs are important determinants for the effectiveness of a cochlear implant. Therefore, our goal is to provide a comprehensive characterization of both functional and histological properties of the SGCs after deafening.

Methods: Guinea pigs were deafened by co-administration of kanamycin and furosemide two or six weeks before acute experiments. We used a MED-EL PULSAR_{CI}¹⁰⁰ cochlear implant to electrically evoke and record compound action potentials (eCAPs). Amplitude growth, recovery and pulse train sequences were measured varying the following stimulus parameters: phase duration, inter-phase gap, current level, inter-pulse interval and pulse train duration. The eCAP was evaluated with respect to amplitude, threshold, dynamic range and refractoriness. Immediately thereafter, the animals were sacrificed for histological analysis of the SGCs and both their central and peripheral processes.

Results: With increasing duration of deafness eCAP amplitude, slope and latency decreased, while the dynamic range increased. The number and average size of SGCs and their central and peripheral processes decreased after deafening. In all animals' eCAP amplitude, slope, dynamic range and latency increased with inter-phase gap, while threshold decreased. The positive effect of inter-phase gap diminished significantly after deafening, and correlated with the number of surviving SGCs. Whole-nerve absolute refractoriness did not change, while the relative refractory period was increased in deafened animals.

Conclusions: We have shown that a variety of electrophysiological measures correlate with the histological state of the auditory nerve. The potential of these objective measures towards assessment of the condition of the auditory nerve can be of great benefit to clinical diagnostics.

O40 Effects of inner ear biology on cochlear implant function

*B.E. Pfingst¹, Y. Raphael¹, D.J. Colesa¹, C.L. Budenz¹, M.M. Watts¹,
G.L. Su¹, L.L. Kabara¹, S.B. Strahl^{1,2}, S.N. Garadat^{1,3}, N. Zhou¹,
K.M. Masterson¹

¹Kresge Hearing Research Institute, Otolaryngology, University of Michigan, Ann Arbor/MI, USA

²MED-EL GmbH, R&D Worldwide Headquarters, Innsbruck, Austria

³University of Jordan, Hearing and Speech Sciences, Amman, Jordan

Studies in guinea pigs and humans are used to test the hypothesis that the health of the implanted cochlea affects the functional responses to cochlear implant stimulation. "Cochlear health" includes presence or absence of hair cells, densities of surviving spiral ganglion cell bodies and peripheral processes, and amount of ensemble spontaneous activity in the auditory nerve recorded from the implanted electrodes. To determine the effects of these variables on cochlear implant function we conducted psychophysical and electrophysiological studies across a population of guinea pigs that had a broad range of cochlear health.

Some animals were implanted in a hearing ear, resulting in surviving hair cells, excellent spiral ganglion cell survival, and ensemble spontaneous activity. Other animals were deafened by antibiotic perfusion to destroy the hair cells and then gene-therapy procedures were used to upregulate the secretion of neurotrophins, which resulted in good preservation of spiral ganglion cells over periods of a year or more. For comparison, deafened animals without the neurotrophin therapy showed no hair cells, only sparse spiral ganglion cell survival, and no detectable spontaneous activity. We found several functional measures reflected cochlear health. These included slopes of psychophysical multipulse integration and temporal integration functions and slopes of ECAP and EABR amplitude-growth functions.

Studies using these psychophysical and electrophysiological measures in humans with multisite cochlear implant electrode arrays found significant variation in functional responses from one stimulation site to the next, consistent with the idea that conditions near each individual site have significant effects on the functional responses. We use site-specific functional data to select the best stimulation sites for the subject's processor and achieve improvements in speech recognition over that obtained with the everyday processor map that uses all available stimulation sites.

These results suggest that cochlear health is important for implant function, that it can be monitored *in vivo* and that the data can be used to improve implant function.

Session H:
Homeostasis / Diagnostics

Chairpersons: A. Schrott-Fischer
H. Wada

O41 Anti-diuretic hormone can regulate water homeostasis of the inner ear

*C. Gleiser¹, A. Eckhard², D. Taguchi³, M. Müller⁴, H. Löwenheim⁴, B. Hirt^{4,1}

¹University of Tübingen, Institute of Anatomy - Clinical Macroscopic Anatomy, Tübingen, Germany

²University of Tübingen Medical Centre, Hearing Research Centre, Department of Otorhinolaryngology – Head & Neck Surgery, Tübingen, Germany

³Kochi Medical School, Department of Otolaryngology, Kochi, Japan

⁴University of Tübingen Medical Center, Hearing Research Centre, Department of Otorhinolaryngology – Head & Neck Surgery, Tübingen, Germany

Hearing and balance functions of the inner ear rely on homeostasis of the inner ear fluid spaces. Disturbance of this equilibrium leads to pathologic conditions, for example the endolymphatic hydrops (EH) often observed in Ménière's disease (MD). While several specific transmembrane proteins have been identified that facilitate endolymphatic ion homeostasis, the knowledge about the molecular determinants of endolymphatic water homeostasis and its volume regulation is rather limited. A positive correlation between the presence of EH and blood concentrations of the anti-diuretic hormone (ADH) was reported from clinical observations and animal experiments. Therefore, the inner ear has been suggested as a direct target of ADH.

Using a confocal laser scanning technique the transepithelial and transcellular water flow in the endolymphatic sac epithelium and the epithelial supporting cells of the cochlea were measured in response to osmotic challenge under different pharmacological treatment conditions.

Our data show that the inner ear is a direct target of ADH and that the endolymphatic water homeostasis may be influenced by pharmacological substances which are involved in an ADH-mediated signal transduction cascade. Aquaporins (AQPs) facilitate water transport across cell membranes along osmotic gradients and are shown to be regulated by ADH. As knowledge of the expression patterns of various AQP subtypes in the cells of the endolymphatic sac is restricted to the cellular level, we analyzed the subcellular localization of AQP2 and AQP3 in the cells of the endolymphatic sac using pre- and post-embedding techniques. Confocal microscopy and immuno-electron microscopy revealed the expression of AQP2 in the ribosomal rich cells (RRCs) and AQP3 in the mitochondria rich cells (MRC).

These results add to the understanding of the mechanisms of water related volume regulation of the inner ear and have direct implications for potential treatment options in MD.

O42 Raised static pressure in scala media of gerbils is consistent with endolymphatic hydrops

*E. LePage^{1,2}, P. Avan¹, Y. Pavier¹, T. Mom¹

¹School of Medicine, Laboratory of Sensory Biophysics, Clermont-Ferrand, France

²PO Box 2564, Mount Claremont, Australia

Question: It has often been supposed, but never demonstrated - presumably due to experimental difficulty - that endolymphatic hydrops (EH) is associated with raised static pressure in the endolymph. The existing literature suggests that such measured pressures rarely differ much from perilymphatic pressure. Alternately, perhaps the swelling evident in post-mortem histology from Ménière's patients occurs primarily because Reissner's membrane has lost its stiffness? Maybe higher pressures are not registered, 1) if there exist leaks in the vessel e.g. due to multiple penetrations - which may still show an endocochlear potential (EP), or 2) if the exact status of the preparation needed to see such pressures is unknown.

Materials and Methods: We report experiments on 50 Mongolian gerbils using a WPI-900A micropressure measurement system. Extensive care has been given to calibration of the device. Each animal is anaesthetised with Ketamine. Its head is immobilised in a head-holder with earbar for sound delivery. A glass micropipette is inserted through the round window for perilymph recordings and later advanced into scala media (SM) through the basilar membrane. The pipette gives independent measures of EP and pressure in SM recorded continually. On two extra channels we record distortion products in the ear canal and the round window potential. The results presented are for manipulations due to sound, applied cerebrospinal fluid (CSF) pressure, furosemide injection and hypoxia.

RESULTS: Applied pressures to the CSF through a cranial canula produce expected behaviors in perilymph pressure. Raised pressures have not been found in fresh preparations with or without sound. However, in 16 gerbils pressures exceeding 60 mmHg have been registered under circumstances in which cochlear homeostasis is absent, i.e. loss of normal EP due to furosemide injection and hypoxia. With improvements in technique, higher pressures have been recorded. Termination tends to result in sustained pressures. Pipette withdrawal releases any pressure.

Conclusions: The results suggest the clinical effects of EH are consistent with raised static pressure in scala media when homeostasis is disabled.

O43 Visualization of endolymphatic hydrops in living animals using optical coherence tomography (OCT)

*Y. Tona¹, T. Sakamoto¹, M. Taniguchi¹, H. Torii¹, T. Nakagawa¹, J. Ito¹

¹Kyoto University, Otolaryngology, Kyoto, Japan

Introduction: Endolymphatic hydrops has been believed to be the most important feature for the pathogenesis of Meniere's disease. However, the mechanisms of its dynamic pathology are not well understood due to the lack of *in vivo* diagnostic tools in animals and humans.

Optical Coherence Tomography (OCT) is a relatively new imaging modality that utilizes near infra-red light to obtain cross sectional images of opaque biological tissues. OCT has already been clinically applied in ophthalmology and coronary vessels.

Targeted disruption of 2 alleles of *Slc26a4* (*Pds*, a causative gene of Pendred syndrome) results in dilated endolymphatic sac and enlarged scala media. (Everett, 2001; Wangemann, 2009), and is regarded as one of model mice of Meniere's disease. In this study, we tried to visualize endolymphatic hydrops in living *Pds*-knockout mice using OCT.

Materials and methods: Littermates of *Pds*-knockout mice at the age of 11 weeks were used in this study. All animals were subjected to the auditory and vestibular evaluations. Auditory brainstem responses (ABR) were recorded at 8, 16, and 32 kHz. Reaching responses on suspension by their tails were also studied.

Under general anesthesia, bulla was removed to expose the bony wall of cochlea, and OCT images were acquired. Animals were placed to obtain the optical section that includes the apex and the round window niche in the same image.

Then, animals were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA) before excision of temporal bones. Inner ears were re-fixed by cochlear perfusion with 4% PFA and postfixed at 4 °C for more than 4 h. Hematoxylin and eosins staining of 10- μ m-thick frozen sections were used for the morphological study.

Results: Homo mice showed profound inner ear dysfunction, and hetero and wt mice were normal.

In hetero and wt, Reissner's membranes were visible at normal position in OCT images. While, in homo mice, OCT images showed extreme enlargement of scala media with thinned basal membrane, which is consistent with the histology.

Conclusions: OCTs have the potential to visualize endolymphatic hydrops *in vivo*.

OCT should facilitate understanding dynamic mechanisms of Meniere's disease.

O44 Inner ear MRI using a multi-functional nanoprobe for MRI contrast enhancement and radical scavenging

*J. Zou¹, M. Zhen², J. Zheng², I. Pyykko¹, C. Shu², C. Wang²

¹University of Tampere Medical School, Inner Ear Research Unit, Tampere, Finland

²Institute of Chemistry, Chinese Academy of Sciences, Beijing National Laboratory for Molecular Sciences, Key Laboratory of Molecular Nanostructure and Nanotechnology, Beijing, China

Gadolinium is among the most convenient substances in developing targetable MRI contrast agent, but its relaxivities are significantly compromised after being encapsulated inside the certain nano-composites. In this work one kind of multi-functional magnetic resonance imaging (MRI) contrast agent is designed by hydroxyl and amino-bearing C₆₀ derivatives (C₆₀O₋₁₀(NH₂)₋₇(OH)₋₁₂) covalently binding with Gd-DTPA, which exhibited superior contrast enhancement in comparison to any reported nano-composites as well as intriguing radical scavenge capability. The relaxivity of synthesized nano-composite C₆₀O₋₁₀(OH)₋₁₂(NH₂)₋₇/(Gd-DTPA)₃ (abbreviated as DF₁Gd₃) represents more than fourfold improvement over the commercial MRI contrast agent [Gd-DTPA (H₂O)]²⁺ (MagnevistTM) (20.4 mM⁻¹s⁻¹ vs 4.5 mM⁻¹s⁻¹, at 0.5 T, 37°C). Simultaneously, it shows excellent quenching effects on hydroxyl radical studied by the DMPO-spin trap/ESR method. To test MRI contrast enhancement of DF₁Gd₃ in the inner ear, the nano-composites were directly delivered into rat cochlea through cochleostomy. Perilymphatic compartments of both cochlea and vestibule were efficiently highlighted on MR images acquired immediately after intracochlear injection, but endolymphatic compartment of neither cochlea nor vestibule was contrasted at the concentration of either 500 mM or 100 mM. This indicates that DF₁Gd₃ did not pass the endolymph-perilymph barrier. The nano-composites also appeared in the modiolus which contains that auditory nerve. This suggests that DF₁Gd₃ might be developed as a multifunctional agent to diagnose and treat auditory neuropathy, which is a challenge for the otologists. The efficient contrast enhancement on *in vivo* middle and inner ear MR imaging along with the radical scavenge capability of DF₁Gd₃ will potentiate the novel nano-composite a diagnostic and therapeutic agent in combating human diseases including sensorineural hearing loss.

Session I:
Ototoxicity / Prevention

Chairpersons: J. Syka
R. Nouvian

O45 Cell-specific accumulation of gentamicin and doxycyclin within the guinea pig cochlea after intratympanic application

*U.-R. Heinrich¹, I. Schmidtman², I. Fischer¹, W.J. Mann¹, K. Helling¹

¹University Medical Center of the Johannes Gutenberg - University Mainz, Otorhinolaryngology, Head and Neck Surgery, Mainz, Germany

²University Medical Center of the Johannes Gutenberg - University Mainz, Germany, Institute for Medical Statistics, Epidemiology and Informatics, Mainz, Germany

Introduction: Intratympanic medication might alter cochlear NO-production resulting in damage or protection, respectively. In this study, the quantitative distribution patterns of gentamicin and doxycyclin were determined after intratympanic application in the guinea pig animal model.

Materials and Methods: Gentamicin or doxycyclin were injected into both middle ears of male animals (n = 24 and n = 3, respectively). The cochleae were removed 1, 2 and 7 days after gentamicin injection or 1, 6 and 24 h after doxycyclin application, transferred into fixative and embedded in paraffin. The cellular staining reactions by specific antibodies against gentamicin and doxycyclin were quantified computer-assisted on semi-thin sections for seven different cochlear regions.

Results: Gentamicin was identified in all experimental groups in numerous regions of the cochlea but with quantitative cell-specific differences. An intense accumulation was observed within the spiral ligament, organ of Corti, nerve fibers, interdental cells and fibrocytes in the limbus-area. A low gentamicin accumulation was seen in the stria vascularis and spiral ganglion cells. Statistical analysis revealed fixed effects of cell type and an interaction between treatment and cell type, but no effects of cochlea turn and of treatment. Analysis over time identified a reduction of gentamicin within the spiral ligament and nerve fibers. Doxycyclin was preferentially located in the stria vascularis and in hair cells.

Conclusion: In respect to the route of infiltration, a contribution of the vessel systems is discussed. The identified accumulation of gentamicin in those cochlea regions which are responsible for potassium recycling might result in a local NO-increase leading finally to cochlear damage. The specific accumulation of doxycyclin in the stria vascularis and hair cells might prevent an NO up-regulation in stress situations in these areas and offers a promising approach for human medication.

O46 Variability of ototoxicity but not antimicrobial potency of gentamicin in-vitro

*M. Huth^{1,2}, A. Vu², A. Cheng², A. Ricci^{2,3}

¹Inselspital, Otorhinolaryngology, Head & Neck Surgery, Bern, Switzerland

²Stanford University, Otolaryngology, Head & Neck Surgery, Stanford/CA, USA

³Stanford University, Molecular and Cellular Physiology, Stanford/CA, USA

Aminoglycosides (AGs) are potent antibiotics. Their inherent ototoxic side effects, however, restrict the use of AGs to few and otherwise fatal indications. But the incidence of ototoxicity is inconsistent. Ototoxic damage is reported in 0-58% after systemic administration of gentamicin (GM). In 1/3 of patients, the ototoxic damage can be explained by a genetic susceptibility. The difference of the remaining 2/3 in comparison to AG-treated patients without ototoxic damage remains unknown. Interestingly, gentamicin as used in patients is not a pure substance but a complex of gentamicin derivatives with different organic rests. This study investigates the ototoxic variability of gentamicin in-vitro to dissociate toxicity from antimicrobial potency (AP).

Cochleae from rats were cultured on postnatal day 4, treated for 1h after 24h in culture and kept in culture for additional 48h. Treatments were performed with different bottles of injectable gentamicin sulfate solution (gentamicin complex) from the same lot as used in patients, isolated and purified Gentamicin compounds A, C1, C1a, C2, C2a, and X2 as well as the synthetic gentamicin-derivative sisomicin for comparison. Hair cell damage was quantified by immunostaining and fluorescence microscopy. Inhibiting and bactericidal AP was tested by serial dilution of decreasing AG doses against *E. coli* bacteria.

Ototoxic hair cell loss varies between hospital bottles (GC) of the same lot number. With repeated use of the same bottle, ototoxicity decreases over time while AP remains intact. The purified isolates gentamicin A, C1, C1a, C2, C2a and X2 demonstrate different degrees of ototoxicity and not all compounds are antimicrobial. However, the variability over time is decreased compared to the GC. Sisomicin demonstrates a constant ototoxicity with persistent AP.

Different compounds of the GC demonstrate different degrees of ototoxicity. Reduced ototoxic variability in the purified isolates suggests that impurities of the GC might be causal. Further quantitative and qualitative research is needed based on the most extreme findings in this study to further reduce the risk of permanent cochlear damage from AGs.

O47 Dissociation of antibacterial activity and aminoglycoside ototoxicity: Prevention is better than protection

*J. Schacht¹, T. Matt², N.C. Leong³, K. Lang³, S.-H. Sha^{1,4}, R. Akbergenov², D. Scherbakov², M. Meyer², S. Duscha², J. Xie^{1,5}, S. Dubbaka⁶, D. Perez-Fernandez⁶, A. Vasella⁶, V. Ramakrishnan³, E. Böttger²

¹Kresge Hearing Research Institute, Otolaryngology Head & Neck Surgery, Ann Arbor/MI, USA

²Institut für Medizinische Mikrobiologie, Zürich, Switzerland

³MRC Laboratory of Molecular Biology, Structural Studies Division, Cambridge, United Kingdom

⁴Medical University of South Carolina, Department of Pathology and Laboratory Medicine, Charleston/SC, USA

⁵Beijing Capital University, Tongren Hospital, Beijing, China

⁶ETH Zürich, Laboratorium für Organische Chemie, Zürich, Switzerland

We have shown clinically successful pharmacological protection against drug-induced hearing loss (New Engl. J. Med. 354:1856-1857, 2006). Such approaches do not, however, address today's major problem in infectious diseases, namely increasing microbial resistance. In order to impact chemotherapy of the future, new antibiotics are needed that are effective against multi-drug resistant bacteria but have no or low ototoxic potential.

Our mechanistic concept postulates a key role for the mitochondrial ribosome (mitoribosome) in aminoglycoside ototoxicity. We now report that apramycin, a structurally unique aminoglycoside in veterinary use, shows little activity towards eukaryotic ribosomes, including hybrid ribosomes carrying the aminoglycoside-susceptibility A1555G allele.

In cochlear explants, apramycin caused only little hair cell damage. *In vivo*, and in contrast to gentamicin, ototoxicity developed more gradually and only at higher concentrations. In accordance with its lesser toxicity, apramycin did not elicit free-radicals (ROS) at low concentrations. However, for both gentamicin and apramycin, the ROS marker nitrotyrosine appeared at antibiotic levels that caused loss of hair cells. This correlation with ototoxicity - rather than with drug concentration - supports the notion that generation of ROS is an integral part of the mechanisms that lead to cell death.

Apramycin exhibits high antimycobacterial activity, including against *M. tuberculosis*, and rapidly growing non-tuberculous mycobacteria, such as *M. abscessus*, *M. massiliense* and *M. boletii*. The latter are difficult-to-treat lung pathogens in patients with cystic fibrosis or *bronchiectasis*. Apramycin also inhibits the growth of multidrug-resistant clinical strains of *M. tuberculosis*.

These data are proof-of-concept that antibacterial activity can be dissected from aminoglycoside ototoxicity. Together with three-dimensional structures of apramycin-ribosome complexes at 3.5 Å resolutions our results provide a conceptual framework to develop less toxic aminoglycosides by hypothesis-driven chemical synthesis. For the present, apramycin's high efficacy combined with low ototoxic potential makes it a drug of immediate clinical interest.

Support: Grants from the University of Zurich and the European Community (PAR, FP-7 HEALTH-2009-241476) to ECB and grant DC-003685 from the National Institutes on Deafness and Other Communication Disorders, NIH, to JS. VR was supported by the Medical Research Council (UK) and the Wellcome Trust.

O48 Sepsis otopathy: Proof of concept

*J. Schmutzhard¹, R. Glueckert¹, C. Pritz¹, M. Blumer¹, M. Bitsche¹,
H. Riechelmann¹, A. Schrott-Fischer¹

¹Medical University Innsbruck, ENT Department, Innsbruck, Austria

Introduction: Intensive care treatment is known to result in hearing loss. Several medications used in intensive care treatment have been identified as ototoxic. Till today sepsis, a frequent cause of intensive care hospitalization has not been suspected to cause inner ear damage. The goal of the present study is to examine the otologic influence of severe sepsis in the Cecal Ligation Puncture mouse model, an established murine sepsis model.

Material and Methods: Twenty five two month old C57BL/6 mice have been included into the study. Seven animals served as control and 18 as sepsis group (belly incision, with perforation and ligation of the cecum). Prior to inclusion hearing levels were measured for the first time with auditory evoked brainstem responses and at the peak of the disease for the second time. Afterwards the animals were sacrificed and the inner ears were harvested for further light microscopic, electron microscopic and immunohistochemical evaluation. The severity of disease was objectified with blood cultures and measurement of blood lactate levels and blood ph levels.

Results: The statistical evaluation comparing the hearing results at the first and the second measurement did show a significant hearing loss ($p=0.012$) in the Sepsis group and no hearing loss in the control group. The light microscopy, electron microscopy and immunohistochemistry for cleaved Caspase 3 did reveal an induction of apoptosis in the Deiter cells, Claudius cells and Hensen cells.

Conclusion:

Sepsis leads to a hearing loss in the Cecal ligation puncture mouse model. The functional impairment can be linked to an induction of apoptosis in the supporting cells.

O49 Upstream JNK signaling and JNK isoforms in hair cell damage

*A. Ryan¹, K. Pak², J. Savas³

¹UCSD School of Medicine, ENT & Neurosciences, La Jolla/CA, USA

²UCSD School of Medicine, ENT, La Jolla/CA, USA

³The Scripps Research Institute, Chemical Physiology, La Jolla/CA, USA

Hair cell (HC) damage is a complex process involving multiple molecular substrates leading to the death of the cell. Within these substrates, signaling via the c-Jun N-terminal kinase (JNK) pathway has been proposed to play a central role in HC damage due to several causes.

To expand our knowledge of this pathway, we evaluated signaling molecules upstream of JNK using proteomics and specific inhibitors, in a gentamicin-induced *in vitro* model of HC loss. We also used proteomics in an *in vivo* model of noise-induced hearing loss, by comparing cochlear proteins from mice exposed to a brief noise at an intensity that induced only temporary threshold shift (TTS), versus exposure to an intensity that induced permanent threshold shift (PTS). To assess the role of JNK isoforms, we evaluated *in vitro*, gentamicin-induced HC loss in organs of Corti from mice deficient in JNK1 or JNK2.

Inhibitor and proteomic data indicated that gentamicin activated small GTPase proteins including kRas, Rac and cdc42. Inhibitors further implicated mixed lineage kinases and JNK itself. Comparison of proteins from cochleae with TTS versus those with PTS identified rapid, noise-induced reductions in several small GTPases of the Ras and Rho families in PTS cochleae, compared to their levels in TTS cochleae, consistent with high levels of activation. In addition, a cdc42/Rho activating protein and a protein downstream from RhoA were significantly reduced. Preliminary data from mice deficient in JNK1 and JNK2 indicate that gentamicin induces the expected level of HC loss.

These results suggest that JNK signaling, which includes the activation of upstream small Ras and Rho family GTPases, is involved in HC damage due to both drugs and noise. In addition, we have identified new molecular candidates for roles in regulating this pathway within the HC, providing additional targets for potential pharmacological manipulation. Our data to date suggest that JNK1 and JNK2 play redundant roles in HC damage.

(Supported by the US NIH/NIDCD and the Veterans Administration.)

O50 AMP-activated protein kinase in BK-channel regulation and protection against hearing loss following acoustic overstimulation

*M. Jaumann¹, M. Föller², J. Dettling¹, A. Saxena², T. Pakladok², C. Munoz², W. Singer¹, P. Ruth³, M. Sopjani², G. Seebohm⁴, L. Rüttiger¹, M. Knipper¹, F. Lang²

¹THRC, Molecular Physiology of Hearing, Tübingen, Germany

²University of Tübingen, Department of Physiology, Tübingen, Germany

³Institute of Pharmacy, Department of Pharmacology and Toxicology, Tübingen, Germany

⁴Faculty of Medicine, Prishtinë, Kosovo

The energy-sensing AMP-activated serine/threonine protein kinase (AMPK) confers cell survival in part by stimulation of cellular energy production and limitation of cellular energy utilization. AMPK-sensitive functions further include activities of epithelial Na⁺-channel ENaC and voltage-gated K⁺-channel KCNE1/KCNQ1. AMPK is activated by increased cytosolic Ca²⁺ concentration. The present study explored, whether AMPK regulates the Ca²⁺-sensitive large conductance and voltage-gated potassium (BK) channel.

BK-channel encoding cRNA was injected into *Xenopus* oocytes with and without additional injection of wild type AMPK (AMPK α 1+AMPK β 1+AMPK γ 1), constitutively active AMPK^{VR70Q}, or inactive AMPK^{aK45R}. BK-channel activity was determined utilizing two-electrode voltage-clamp. Moreover, BK-channel-protein abundance in the cell membrane was determined by chemiluminescence and confocal immunomicroscopy. As BK-channels are expressed in outer hair cells (OHC) of the inner ear and lack of BK-channels increases noise vulnerability, OHC BK-channel expression was examined by immunohistochemistry and hearing function analysed by auditory brain stem response measurements in AMPK α 1-deficient mice (ampk^{-/-}) and in wild type mice (ampk^{+/+}).

As a result, coexpression of AMPK or AMPK^{VR70Q} but not of AMPK^{aK45R} significantly enhanced BK-channel-mediated currents and BK-channel-protein abundance in the oocyte cell membrane. BK-channel-expression in the inner ear was lower in ampk^{-/-} mice than in ampk^{+/+} mice. The hearing thresholds prior to and immediately after an acoustic overexposure were similar in ampk^{-/-} and ampk^{+/+} mice. However, the recovery from the acoustic trauma was significantly impaired in ampk^{-/-} mice compared to ampk^{+/+} mice.

In conclusion, AMPK is a potent regulator of BK-channels. It may thus participate in the signaling cascades that protect the inner ear from damage following acoustic overstimulation.

O51 Oxidative stress in acoustic trauma: Cochlear and cortical responses following the increase of antioxidant defense

A.R. Fetoni¹, S.L. Eramo², R. Rolesi¹, F. Paciello¹, P. de Bartolo³, C. Bergamini⁴, D. Troiani², *G. Paludetti¹

¹Catholic University of Rome, Institute of Otolaryngology, Rome, Italy

²Catholic University of Rome, Institute of Physiology, Rome, Italy

³University "La Sapienza" of Rome, Institute of Psychology, Rome, Italy

⁴University of Bologna, Dept. of Biochemistry, Rome, Italy

Introduction: Several studies have recently identified the oxidative stress, induced by acoustic trauma, as the pivotal pathway of cochlear damage.

The present study addresses the effect of noise exposure on inner ear function, the morphological changes on cortical auditory regions and the potential protective effect of an antioxidant agent as water soluble Coenzyme Q₁₀ (Q-ter), in a chronic model of hearing loss.

Material and Methods: Acoustic trauma was induced in adult Wistar rats by the exposure to a continuous pure tone of 10 kHz. All animals were exposed for 60 minutes to 100 dB SPL for 10 days. Of these, a group was simultaneously treated with the antioxidant Q-ter (100mg/Kg) over the same period. Intact animals were used as control group. Auditory function was evaluated by recording auditory brainstem responses (ABR) at 6-32 kHz (before and 1, 3, 7, 11, 30 and 60 days after trauma). The animals were then sacrificed with a lethal dose of anesthesia; the cochleae and brains were quickly removed and processed for morphological analyses. Golgi-Cox staining was used to evaluate the morphological changes in cortical auditory regions comparing spine density and dendritic branching of II-III and V-VI layer pyramidal neurons of primary and secondary auditory cortices. The oxidative stress was determined by ROS assay and 4HNE (4-hydroxynonenal, a marker of lipid peroxidation) staining in the organ of Corti, spiral ganglion and acoustic cortex.

Results: Hearing loss and damage of hair cells and spiral ganglion neurons (SGNs) as well as changes in the acoustic cortices were accompanied by a marked noise-induced oxidative stress as evidenced by the enhanced reactive oxygen species (ROS) production and lipid peroxidation in hair cells and SGNs and by the decreased levels of CoQ₉ and CoQ₁₀ in the cortex. The acoustic trauma increased dendritic length and decreased spine density in pyramidal neurons of II-III and V-VI layers of auditory cortical areas. The systemic administration of the water-soluble CoQ₁₀ formulation (Q_{ter}[®]) reduced both hearing loss and damage in the organ of Corti and reversed auditory cortex modifications by decreasing the oxidative stress.

Conclusion: The present findings indicate that protection of noise induced peripheral damage and prevention of cortical changes can be obtained by re-establishing the redox status.

Session J:

Protection

Chairpersons: A. Szczepek
J. Schacht

O52 Lack of brain-derived neurotrophic factor hampers inner hair cell synapse physiology, but protects against noise-induced hearing loss

*W. Singer¹, A. Zuccotti¹, S. Kuhn², S.L. Johnson², C. Franz¹, D. Hecker³, H.-S. Geisler¹, I. Köpfschall¹, K. Rohbock¹, K. Gutsche⁴, J. Dlugaiczyk³, B. Schick³, W. Marcotti², L. Rüttiger¹, T. Schimmang⁴, M. Knipper¹

¹ENT Clinic, THRC, Molecular Physiology of Hearing, Tübingen, Germany

²University of Sheffield, Department of Biomedical Science, Sheffield, United Kingdom

³Saarland University Hospital, Department of Otorhinolaryngology, Homburg/Saar, Germany

⁴Universidad de Valladolid y Consejo Superior de Investigaciones Científicas, Instituto de Biología y Genética Molecular, Valladolid, Spain

Brain derived neurotrophic factor (BDNF) has been suggested by analogy to the known functions of neurotrophins, to be a critical survival and growth factor for the central nervous system. Recent studies, however suggest that BDNF is not essential for postnatal survival of neurons in the brain but rather defines dendritic complexity and spine density (Rauskolb et al. 2011). As constitutive BDNF KO mice die prematurely the role of BDNF for mature sensory systems is still elusive.

We generated viable conditional BDNF mouse mutants (BDNF^{Pax2} KO) that lead to deletion of BDNF in the peripheral auditory organ. We found that BDNF in the adult mammalian cochlea is essential for maintaining a complete set of maximal transmitter release sites at the inner hair cells (IHCs) and number of afferents in high frequency cochlear turns. This minor auditory deprivation can however perfectly be compensated through altered central brain responsiveness as detected through elevated ABR wave amplitudes and tracing brain centers with activity related genes (*Arc/Arg3.1*) in the ascending pathway. After acoustically overstimulation BDNF^{Pax2} WT developed severe afferent neurodegeneration at the inner hair cell level with a failure to elevate responsiveness in central auditory circuitries. Strikingly BDNF deletion in the cochlea in BDNF^{Pax2} KO did prevent this harmful event.

Data describe a crucial beneficial and harmful role of BDNF at the first synapse within the auditory pathway with dramatic consequences for the adaptive responsiveness.

Acknowledgements: This work was supported by the Marie Curie Research Training Network CavNET MRTN-CT-2006-035367, the Deutsche Forschungsgemeinschaft DFG-Kni-316-4-1 and Hahn Stiftung (Index AG) and by the Wellcome Trust (088719 and 091895) to WM. WM and SLJ are Royal Society University Research Fellows. Present address of A.Z.: Dept. Clinical Neurobiology of University Hospital and DKFZ Heidelberg, In Neuenheimer Feld 280, D-69120, Heidelberg, Germany.

O53 The hearing function of the deletion of L-type CaV1.2 in the peripheral and central auditory system

*S.C. Lee¹, A. Zuccotti^{1,2}, S.V. Satheesh³, T. Schimmang⁴, L. Rüttiger¹, H.G. Nothwang³, M. Knipper¹

¹Tübingen Hearing Research Centre, Department of Otolaryngology, Head and Neck Surgery, Tübingen, Germany

²Current address: Dept. Clinical Neurobiology of University Hospital and DKFZ Heidelberg, Heidelberg, Germany

³Department of Neurogenetics, Carl von Ossietzky University, Oldenburg, Germany

⁴Instituto de Biología y Genética Molecular, Universidad de Valladolid y Consejo Superior de Investigaciones Científicas, Valladolid, Spain, Spain

In a previous study we could show that BDNF in the cochlea is required for normal hearing function. However, BDNF reduction was found out to be protective for IHC and IHC synapse following noise induced acoustic trauma. (Zuccotti et al. 2012). In the brain, BDNF expression is controlled by L-type voltage-gated Ca²⁺ channel 1.2 (CaV1.2) activity at the transcriptional level (West et al., 2001, McDowel et al., 2010). CaV1.2 channel is expressed in spiral ganglion neurons in the auditory system but its regulatory function for BDNF in the cochlea and its effect on hearing is yet unknown.

Constitutive CaV1.2 knockout mice die in utero before embryonal day 15 (Seisenberger et al., 2000). We therefore inactivated CaV1.2 in a conditional mouse line (CaV1.2^{Pax2/Cre}) tissue specifically in the cochlea, dorsal cochlear nucleus and inferior colliculus. Complementarily, we conditionally inactivated CaV1.2 in the olivary complex (MSO/LSO) but not in the cochlea by crossing the CaV1.2^{fl/fl} mouse line with a mouse line expressing Cre under a promotor specifically expressed in the central auditory system (CaV1.2^{Egr2/Cre} Satheesh et al., 2012). For both mouse lines, we compared the hearing thresholds and the waveform fine structure (wave amplitudes, growth function and latencies) of auditory brainstem responses (ABR) before and after noise exposure.

After noise, CaV1.2^{Pax2/Cre} mice had less ABR threshold loss and less reduction in ABR wave I amplitude, similar to what was found for the BDNF^{Pax2} KO mice described before (Zuccotti et al., 2012). However, deletion of CaV1.2 in the auditory brainstem in the CaV1.2^{Egr2/Cre} mouse line did not affect, or protect, hearing function after noise. This suggests that CaV1.2 channel activity and the increase of BDNF may exacerbate damage in the cochlea after noise.

Supported by the Marie Curie Research Training Network CavNET MRTN-CT-2006-035367, Deutsche Forschungsgemeinschaft, grant DFG Kni316/4-1

O54 Cisplatin- and ROS-induced ototoxicity and otoprotection in a whole organ culture model of the postnatal and functionally mature mouse inner ear

*H. Arnold¹, A. Tropitzsch¹, M. Müller¹, H. Löwenheim¹

¹Univ.-HNO Klinik Tübingen, Otolaryngology, Head & Neck Surgery, Tübingen, Germany

Introduction: Ototoxicity is a major dose-limiting side-effect of the chemotherapeutic drug Cisplatin. Hair cell damage is essentially mediated by reactive oxygen species (ROS). *In vitro*, ototoxicity of Cisplatin as well as otoprotective effects have been assessed in cell and tissue cultures requiring fragmentation and isolation of the sensory epithelia from its natural environment. We developed a bioreactor system, facilitating the organ culture of the entire and intact inner ear within the bony labyrinth for the use in drug screening assays.

Material and Methods: In a horizontally rotated bioreactor applying simulated microgravity conditions, inner ears of postnatal day 7 (P7) mice could be maintained in culture for up to seven days. Using a modification of this technique, also the functionally mature (P21) inner ear could be cultivated for 24h. Assessment of hair cell loss and protection was performed by Myosin VIIa and phalloidin stainings in whole mount preparations.

Results: Cisplatin and ROS induced hair cell loss occurred in a dose and time dependent manner. For Cisplatin EC50 was 3.20 µg/ml for the outer hair cells (OHC) at 24 h exposure time, 1.52 µg/ml at 48 h and 0.97 µg/ml at 96 h exposure. Thus, for the OHC doubling the exposure time approximately halved the EC50. For the inner hair cells EC50 values were calculated as 8.34 µg/ml, 1.77 µg/ml and 0.80 µg/ml respectively. Hair cell loss followed a base to apex gradient. In the functionally mature stage 50% OHC loss was found after application of 0.25 µg/ml at 24 h exposure. In both stages a statistically significant protection resulted by co-administration of 1.0 mM L-NAC to the culture. Using H₂O₂ to simulate ROS, a dose dependent OHC loss with a base to apex gradient was seen as well. After 24 h of exposure application of 1.0 mM, 5.0 mM 10.0 mM and 100 mM H₂O₂ resulted in 17%, 42%, 75% and 100% outer hair cell loss. IHC were almost not affected.

Conclusions: The applicability of the whole organ culture technique for both the postnatal and functionally mature inner ear in a Cisplatin-based model of hair cell loss and otoprotection is suggested. The system remarkably resembles the *in vivo* situation in terms of organ integrity in tissue interaction and includes the common standardization advantages of *in vitro* approaches. It is validated as a new tool for inner ear toxicology and the identification of otoprotective agents.

O55 Activation of gp130-STAT3 signaling pathway protects the inner and outer hair cells from the cisplatin-mediated toxicity

*A. Szczepek¹, E. Gerschner¹, O. Hegend¹, H. Olze^{1,2}, B. Mazurek³

¹Charité University Hospital, ORL CCM, Berlin, Germany

²Charité University Hospital, ORL CVK, Berlin, Germany

³Charité University Hospital, Tinnitus Center, Berlin, Germany

Explanting and *in vitro* culture of the membranous parts of cochlea isolated from postnatal Wistar rats (p3 - p5) is an established method to study ototoxicity and its prevention. Using this method, we have previously shown that simultaneous addition of interleukin-6 (IL6) and cisplatin to cochlear explant cultures induces modest but significant otoprotection of the inner hair cells. This was demonstrated by scoring of phalloidin-TRITC stained hair cells using the epifluorescent and confocal microscopy. Significant protection was noted only in the apical portion of explants. Here, we show that a 24h pre-incubation of cochlear explants with [30 ng/ml] of IL6, enhances survival not only of the inner but also of the outer hair cells in the cisplatin [15µM] exposed explants. The protection was evident in the apical, medial and basal portion of the organ of Corti. In addition, we have visualized spiral ganglion neurons with antibodies against neurofilament 200 and observed that the pre-incubation of explants with IL6 improves survival and morphology of neurons after the exposure of explants to cisplatin, as compared to control. Upon binding to the IL6-alpha receptor and the constant chain gp130, IL6 induces phosphorylation and nuclear translocation of the signal transducer and activator of transcription 3 (STAT3). Using Western blotting, we have confirmed rapid phosphorylation of STAT3 on serine and tyrosine residues, as well as its translocation into the nucleus in the IL6-treated cochlear explants. We then hypothesized that the otoprotection is likely due to the immediate rapid events induced by IL6 *via* gp130 and STAT3. Thus, other cytokines from IL6 family (e.g., oncostatin M) should also be otoprotective, since they too signal *via* gp130 and STAT3 and display degree of functional redundancy in other biological systems. In fact, we have demonstrated that oncostatin M induces protective effect against cisplatin similar to these induced by IL6. Taken together, we are tempted to speculate that activation of gp130 or STAT3 pathway in the inner ear prior to exposure to cisplatin could prevent cisplatin ototoxicity.

O56 Identification of novel downstream effectors of IGF-1 signal pathways using a comprehensive gene expression analysis

*Y. Hayashi¹, N. Yamamoto¹, T. Nakagawa¹, J. Ito¹

¹Kyoto University, Otolaryngology, Kyoto, Japan

Introduction: We have revealed that insulin-like growth factor1 (IGF-1) protects cochlear hair cells of neonatal mice against aminoglycoside via both the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the mitogen-activated protein kinase kinase /extracellular signal-regulated kinase (MEK/ERK) pathway. However the effector genes of PI3K/Akt and MEK/ERK which work directly on cochlear hair cell protection have not been unveiled so far. To identify the effectors of IGF-1, we screened whole sets of expressed genes in the cochlear explant cultures using microarray and confirmed the results using quantitative RT-PCR (qRT-PCR).

Materials and Methods:

- Total RNA was extracted from the cochlear explant cultures of neonatal mice that were treated with only neomycin or both neomycin and IGF-1 for various durations. cDNAs were prepared from the total RNA and the expression levels of whole genes were compared between the control and IGF-1-treated groups using microarray provided by Affymetrix. We picked up the genes whose expression levels increased twofold in the experimental groups compared with those in the control groups. The differences in the expression levels were confirmed by qRT-PCR.
- To confirm the identified genes were really at the downstream of the IGF-1 pathways, the changes of their expression levels after treatment with AKT or MEK inhibitor were tested using qRT-PCR.

Results: The expression levels of Gap43 and Ntn1 were up-regulated in the IGF-1-treated cochlear explant cultures compared with the control samples. The expression levels of Gap43 and Ntn1 were significantly reduced by the addition of Akt inhibitor or MEK inhibitor.

Conclusions: As effectors of IGF-1 signaling in the context of hair cell protection from aminoglycoside, we identified two kinds of genes, Gap43 and Ntn1 whose expression level increase was canceled by the addition of the PI3K or MEK inhibitor. These findings indicated that Gap43 and Ntn1 are the downstream effectors of IGF-1 in the cochlear sensory epithelium that is under the regulation of both the PI3K/Akt and MEK/ERK pathways.

O57 Neuronal erythropoietin overexpression protects mice against presbycusis

*A. Monge Naldi¹, C. Belfrage^{1,2}, M. Gassmann², J. Vogel²

¹University Hospital Zürich, University of Zürich, Clinic for Otolaryngology, Head and Neck Surgery, Zürich, Switzerland

²University of Zürich and Zürich Center for Integrative Human Physiology (ZIHP), Institute of Veterinary Physiology, Vetsuisse Faculty, Zürich, Switzerland

Introduction: Erythropoietin (Epo) a hematopoietic growth factor has been shown to act also in other cells and tissues, amongst others by protecting cells from apoptosis. Since a few years neuroprotective properties are known. Also in the inner ear a protective effect by ischemia- and gentamicin induced *in vitro* auditory hair cells damage could be shown. In contrast, the first *in vivo* study using an animal model of noise-induced hearing loss showed a negative effect of Epo. The aim of this study was to investigate the effect of a constant Epo-expression on the auditory system and on age related hearing loss (presbycusis) in mice.

Methods: We used transgenic modified mice with constant Epo-overexpression in neurons (tg21). The strain of these mice has a known age related hearing loss. Hearing tests were performed by using a “plug-and-play” system for behavioral audiometry and by auditory brainstem response. Hearing threshold was recorded at the age of 5 months and 11 months. At the end of the experiments cochlear histology was performed.

Results: At 5 months there was nearly no difference in threshold between tg21 and wt mice, so a negative effect of Epo could be excluded. The same animals were tested at the age of 11 months. The hearing loss in the wt animals was more pronounced at higher frequencies; over 32kHz threshold could not be recorded. High frequency hearing loss is typical for presbycusis. At that age tg21 mice showed a significant improvement of threshold at all frequencies compared to their wild type littermate. The protective effect of Epo overexpression was very impressive, as in tg21 mice all frequencies could be measured, also high frequencies. In cochlear histology of aged mice a dramatically reduced number of spiral ganglion neurons mainly in the basal turn, the location of high frequencies was found in wt mice. This finding correlates with the previous described high frequency hearing loss in this group. Tg21 mice had significantly higher density of spiral ganglion neurons, as a morphological correlation of the hearing improvement in aged animals.

Conclusion: Epo promotes survival of spiral ganglion neurons and has a protective effect against presbycusis. This is the first *in vivo* study demonstrating a protective effect of Epo on the auditory system. This finding can help to better understand the mechanisms of presbycusis and may help to find protective compounds against presbycusis in a future.

O58 NOS inhibition enhances myogenic tone by increasing rho-kinase mediated Ca²⁺ sensitivity in the male but not the female gerbil spiral modiolar artery

*K. Reimann^{1,2}, G. Krishnamoorthy², P. Wangemann²

¹Eberhards Karls Universität, HNO-Klinik, Tübingen, Germany

²Kansas State University, Anatomy and Physiology, Manhattan/KS, USA

Blood flow regulation is critical for hearing due to the extreme vulnerability of the cochlea to ischemia and oxidative stress. The main blood supply of the cochlea is provided by the spiral modiolar artery (SMA), which generates a myogenic tone that is enhanced in males by L-N^G-Nitro-Arginine (LNNA), a competitive inhibitor of nitric oxide synthase (NOS). Here, we investigated the mechanism of LNNA-induced myogenic tone in the SMA of male and female gerbils. Vascular diameter, myogenic tone, global cytosolic Ca²⁺ in smooth muscle cells and Ca²⁺ sensitivity were evaluated based on laser-scanning and microfluorometric measurements in pressurized vessel segments. LNNA-induced tone in the SMA was observed in response to intramural pressure changes in males but not in females. No gender differences were observed in the tone induced by 150 mM K⁺ and endothelin-1. LNNA did not increase the global Ca²⁺ in the smooth muscle cells but increased the Ca²⁺ sensitivity, which was abolished by the rho-kinase blocker Y27632. Neither LNNA nor Y27632 changed the Ca²⁺ sensitivity in female SMA. The data suggest that the gender difference in LNNA-induced tone is not a manifestation of a difference in the contractility but a difference in the LNNA-induced increase in the Ca²⁺ sensitivity that is mediated by rho-kinase. Rho-kinase and NO thus emerge as critical factors in the regulation of cochlear blood flow. The larger role of NO-dependent mechanisms in male SMA predicts greater restrictions on cochlear blood flow under conditions of impaired endothelial cell function.

Poster Presentations

Posters should remain posted for the entire length of the meeting.
Authors should be at their posters during poster presentation time.

There will be organized on-site poster discussions.

Poster board dimensions: 140 cm wide, 95 cm high.

Deafness genes / Gene therapy / Delivery

P1 Expression of HERG and ERG channels in the rat cochlea

M. Sato¹, M. Miyashita¹, K. Saito¹, K. Terao¹, K. Doi¹

¹Depart. Otolaryngology, Kinki University School of Medicine, Osaka, Japan

Introduction: The human ether-a-go-go-related gene (*erg*), HERG, was originally cloned by homology to the *Drosophila* K⁺ channel gene *erg*. It has six putative membrane-spanning domains, a conserved putative pore domain, and a putative CNB domain. HERG channels produce an inwardly rectifying K⁺-selective current that is blocked by methanesulfonanilides, such as E-4031 or dofetilide, and is enhanced by extracellular K⁺. Mutations of HERG on human chromosome 7q35-q36 cause the autosomal dominant Long QT syndrome, which gives rise to arrhythmias, an increased incidence of sudden death, and sensorineural deafness.

Three transcripts from the *erg* gene (HERG/*erg1*, ERG2/*erg2*, and ERG3/*erg3*) are expressed abundantly in the rat and human nervous systems. In the present study, we examined the expressions of these three transcripts in the rat cochlea to determine their possible roles in cochlear functions.

Materials and Methods: Total RNA was extracted from the rat cochlea and cDNA was prepared for PCR-analysis. Three sets of degenerated oligonucleotides directed against the specific sequences for HERG1, ERG2, and ERG3 were designed. Set 1 for HERG was consisted of forward primer (tccagcggctgtactcgggc) and reverse primer (tagacaaaagtgtctgagaactc). Set 2 for ERG2 was consisted of forward primer (tcacgggcccgaacactccaagca) and reverse primer (acaggtaccgcgctgtgattcgtc). Set 3 for ERG3 was consisted of forward primer (atacgaaggcctgatacagccta) and reverse primer (tctgatgtggatcccaacaggctt). The frozen sections of the rat cochlea were prepared and incubated with anti-human HGF antibodies.

Results: RT-PCR analyses identified the intense expression of HERG channel in the rat cochlea. The faint but consistent expressions of ERG2 and ERG3 channels in the rat cochlea were confirmed, too.

Immunohistochemistry demonstrated that HERG was expressed in the fibrocytes of the spiral ligament and the epithelia lining the inner surface of Scala tympani. Cochlear ganglion cells were lacking in HERG expression.

Discussion: The previous studies reported the ERG2 and ERG3 are expressed exclusively in the nervous system, in marked contrast to HERG, which is expressed in both neural and non-neural tissue. The ERG3 channel produces a current that has a large transient component at positive potentials, whereas the other two channels (HERG and ERG2) are slowly activating delayed rectifiers. The present study indicated HERG channel was mainly expressed in non-neural tissue involved in the K⁺-recycling system in the rat cochlea, suggesting a possible role in generation of the EP (endocochlear potential).

P2 Loss of mammal-specific tectorial membrane component carcinoembryonic antigen cell adhesion molecule 16 (CEACAM16) leads to hearing impairment at low and high frequencies

*L. Rüttiger¹, R. Kammerer², R. Riesenberger³, C. Schäuble³, R. Krupar³, A. Kamp³, K. Sunami⁴, A. Eisenried³, M. Hennenberg⁵, F. Grunert⁶, A. Bressl¹, S. Battaglia⁷, H. Schrewe⁷, M. Knipper¹, M.R. Schneider⁸, W. Zimmermann³

¹Tübingen Hearing Research Centre, Molecular Physiology of Hearing, Tübingen, Germany

²Friedrich-Loeffler Institute, Institute of Immunology, Tübingen, Germany

³University Clinic, Tumor Immunology Laboratory, Life Center, München, Germany

⁴University Medical School, Department of Otolaryngology, Osaka, Japan

⁵University Clinic, Department of Urology, München, Germany

⁶Aldevron Freiburg, Freiburg, Germany

⁷Charité University Medicine, Institute of Genetics, Berlin, Germany

⁸University of München, Institute of Molecular Animal Breeding and Biotechnology, Gene Center, München, Germany

The mammal-specific, secreted *CEACAM16* is a member of the carcinoembryonic antigen gene family. *CEACAM16* is exceptionally well conserved among species.

We studied the hearing function and the cochlear expression of *CEACAM16* to elucidate its importance for auditory function in young and aged *Ceacam16^{-/-}* mice. *Ceacam16^{-/-}* mice were analyzed for age related and trauma induced hearing deficits by ABR and DPOAE measurements and exposure to noise. In cochlear sections of *Ceacam16^{-/-}* mice the tectorial membrane was inspected and gene expression was studied by in situ hybridization and immunohistochemistry. Protein expression was furthermore studied by Western blot analyses.

CEACAM16 is specifically expressed in the inner ear. The hearing phenotype of *Ceacam16^{-/-}* mice was similar to the observed hearing-impairment in members of human Family 1070 with non-syndromic autosomal dominant hearing loss (DFNA4) who carry a missense mutation in *CEACAM16*.

CEACAM16 can probably form higher order structures with other tectorial membrane proteins and influences the physical properties of the tectorial membrane. Evolution of *CEACAM16* might have been an important step for the specialization of the mammalian cochlea, allowing hearing over an extended frequency range.

P3 *Grxcr1* mutation affects development and function of cochlear hair cells in Tasmanian devil mice

*S. Kuhn¹, S.L. Johnson¹, B. Lorente², K.P. Steel², W. Marcotti¹

¹University of Sheffield, Biomedical Science, Sheffield, United Kingdom

²Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom

Introduction: Mutations of *Grxcr1* (glutaredoxin cysteine-rich 1) lead to nonsyndromic autosomal recessive deafness DFNB25 in humans (Schraders et al. 2010 *Am J Hum Gen* 86:138-47). Several mouse lines exist with mutations of *Grxcr1*, which exhibit vestibular defects and profound deafness (Erven et al. 2002 *Eur J Neurosci* 16:1433-41; Odeh et al. 2010 *Am J Hum Gen* 86:148-60). *Grxcr1* is expressed in the hair bundles of vestibular and cochlear hair cells and has been suggested to play a role in the development of the inner ear (Odeh et al. 2010).

We here investigated the physiology of inner and outer hair cells of Tasmanian devil mice bearing a mutation in *Grxcr1* (Erven et al. 2002).

Methods: We performed whole cell patch-clamp recordings on inner and outer hair cells of Tasmanian devil mice in order to investigate the effect of a *Grxcr1* mutation on their transducer currents, basolateral ion channels and efferent innervation.

Results: Mechanotransducer currents were strongly reduced in outer hair cells of Tasmanian devil mice. Furthermore, basolateral currents of adult inner and outer hair cells showed an immature-like profile in terms of their potassium conductances and responsiveness to efferent input.

Conclusions: Together our results indicate that the stereocilia protein *Grxcr1* is essential for the normal physiological development of cochlear inner and outer hair cells. The exact mechanism by which *Grxcr1* controls the maturation of the biophysical properties of hair cells is still under investigation.

This work is supported by the Wellcome Trust to KPS and WM.

P4 Effects of salicylate derivatives on changes in localization of H723R pendrin mutant expressed in cultured cells

*S. Koyama¹, H. Sato², M. Murakoshi¹, T. Kobayashi³, S. Usami⁴, K. Ikeda⁵, Y. Haga⁶, K. Tsumoto⁷, H. Nakamura⁸, N. Hirasawa⁹, K. Ishihara¹⁰, H. Wada¹

¹Graduate School of Engineering, Tohoku University, Department of Bioengineering and Robotics, Sendai, Japan

²Graduate School of Engineering, Tohoku University, Department of Nanomechanics, Sendai, Japan

³Graduate School of Medicine, Tohoku University, Department of Otolaryngology Head and Neck Surgery, Sendai, Japan

⁴Shinshu University School of Medicine, Department of Otorhinolaryngology, Matsumoto, Japan

⁵Juntendo University School of Medicine, Department of Otorhinolaryngology, Tokyo, Japan

⁶Graduate School of Biomedical Engineering, Tohoku University, Department of Biomedical Engineering, Sendai, Japan

⁷Graduate School of Frontier Sciences, University of Tokyo, Department of Medical Genome Sciences, Tokyo, Japan

⁸Faculty of Science, Gakusyuin University, Department of Chemistry, Tokyo, Japan

⁹Laboratory of Pharmacotherapy of Life-Style Related Diseases, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan

¹⁰Laboratory of Medical Science, Course for School Nurse Teacher, Faculty of Education, Ibaraki University, Mito, Japan

Pendrin is a transmembrane protein which is encoded by the *solute carrier 26A (SLC26A)* gene and thought to maintain the ion concentration of endolymph in the inner ear. Mutations in the *SLC26A* gene are responsible for hereditary hearing loss. Pendrin must be located on the plasma membrane to function. In our previous study, we found that 8 missense pendrin mutants were retained in the cytoplasm and that they lost their anion exchanger activity. Of these 8 mutants, 4 pendrin mutants (P123S, M147V, S657Y and H723R) restored the localization from the cytoplasm to the plasma membrane and their anion exchanger activity by the administration of 10 mM-salicylate (Ishihara et al., 2010). However, a high concentration of salicylate is thought to be ototoxic.

In this study, a low concentration of two salicylate derivatives, namely, 2-hydroxybenzyl alcohol and 2,3-dihydroxybenzoic acid, chemical structures of which are very similar to that of salicylate, was used to confirm the possibility of transportation activity from the cytoplasm to the plasma membrane.

The H723R pendrin mutant protein, which is the most frequently observed *SLC26A* mutant in Japanese patients, was investigated. Firstly, to confirm the non-toxicity, the growth of HEK293 cells with 1 mM of these two

derivatives was examined. HEK293 cells were then transfected with the expression vectors of human pendrin wild-type (WT) and H723R mutant. After transfection, salicylate derivatives were administered. At 1, 3, 6 and 12 h after incubation with these derivatives, cells were immunostained and the fluorescence of the cells was observed by using a confocal laser scanning microscope.

One millimolar of these two derivatives did not retard cell growth. The immunofluorescent experiment indicated that the localization of H723R pendrin mutant from the cytoplasm to the plasma membrane by the administration of 1 mM-2-hydroxybenzyl alcohol was restored and completed within 3 hours. However, 1 mM-2,3-dihydroxybenzoic acid did not restore the localization. As 1 mM-2-hydroxybenzyl alcohol was not toxic for cell growth, it might be effective to administer it in order to cure hearing loss.

P5 The controversial p.M34T mutation in GJB2: Report on three Portuguese patients with NSSHL

*A.C. Gonçalves^{1,2}, J. Chora^{1,2}, R. Santos³, P. Cavilhas³, M. da Silva³, A. O'Neill³, G. Fialho^{1,2}, H. Caria^{1,4}

¹BioFIG - Centre for Biodiversity, Functional and Integrative Genomics, Lisbon, Portugal

²Faculty of Sciences, University of Lisbon, BioFIG - Centre for Biodiversity, Functional and Integrative Genomics, Lisbon, Portugal

³Centro Hospital de Lisboa Ocidental EPE, Hospital Egas Moniz, ENT Service, Lisbon, Portugal

⁴Polytechnic Institute of Setúbal, Portugal, School of Health, Setúbal, Portugal

Introduction: The pathogenicity of some *GJB2* mutations, such as p.M34T, is still controversial, being this one reported as a recessive allele by some authors and as a benign polymorphism by others. Insofar, the study of more cases is thus important and may provide a new insight on the role of this variant. Here we report on three Portuguese patients, two sisters and one unrelated individual, presenting moderate to severe nonsyndromic sensorineural HL and harbouring p.M34T.

Methods: All the individuals were evaluated in both ears by pure tone audiometry and blood samples were collected after written informed consent was signed. DNA extraction and PCR amplification of *GJB2* coding exon followed standard methodologies. PCR products were automatically sequenced and deletions in *GJB6* gene were assessed by multiplex PCR.

Results: The two sisters presented compound heterozygosity for two different *GJB2* mutations, p.M34T and p.R184P. Their hearing mother is a p.M34T carrier and their hearing father is a p.R184P carrier. The other unrelated patient is a compound heterozygous for two mutations in *GJB2* gene, p.M34T and p.I140S. All the three patients carried no *GJB6* deletion.

Conclusions: Mutations p.R184P and p.I140S, which are not very common and are reported as recessive ones, don't explaining by themselves the HL of the individuals here reported. In this way, the compound heterozygosity observed in these three Portuguese patients leads us to consider that their HL might be due to the *GJB2* genotype [p.Met34Thr]+[p.Arg184Pro] and [p.Met34Thr]+[p.Iso140Ser], thus pointing to the pathogenic role of p.M34T, either as a recessive allele or as a polymorphism increasing the severity of the recessive monoallelic mutation identified.

P6 A novel wolframin mutation, p.D171N, in a nonsyndromic sensorineural low-frequency hearing loss case

*A.C. Gonçalves^{1,2}, T.D. Matos^{1,2}, R. Santos³, P. Cavilhas³, M. da Silva³, A. O'Neill³, G. Fialho^{1,2}, H. Caria^{1,4}

¹BioFIG - Centre for Biodiversity, Functional and Integrative Genomics, Lisbon, Portugal

²Faculty of Sciences, University of Lisbon, BioFIG - Centre for Biodiversity, Functional and Integrative Genomics, Lisbon, Portugal

³Centro Hospital de Lisboa, Ocidental EPE, Hospital Egas Moniz, ENT Service, Lisbon, Portugal

⁴Polytechnic Institute of Setúbal, Portugal, School of Health, Setúbal, Portugal

Introduction: Nonsyndromic sensorineural hearing loss affecting predominantly the high frequencies is a genetically heterogeneous common disorder. On the contrary, low frequency sensorineural hearing loss (LFSNHL) is an unusual type in which frequencies at 2000 Hz and below is predominantly affected. Mutations in the WFS1 gene, coding for wolframin, a membrane glycoprotein localized in the endoplasmic reticulum (ER), are responsible for Wolfram Syndrome type 1 (WFS1), WFS-like syndrome, and are a common cause of nonsyndromic LFSNHL. The expression pattern of wolframin in the human cochlea remains unknown but its localization in the ER suggests a role in ion homeostasis maintained by the canalicular reticulum, a specialized form of ER.

Methods: A Portuguese individual presenting with bilateral nonsyndromic progressive LFSNHL was audiologicaly evaluated by pure tone audiometry and blood sample was collected after written informed consent was signed. Testing for mutations in the GJB2 coding exon and for the common GJB6 deletions was first performed by automated sequencing and by multiplex PCR, respectively. Automated sequencing of exons 5 and 8 of WFS1 gene was later performed.

Results: A novel mutation, p.D171N, was found in exon 5 of WFS1 gene, in heterozygosity. One intronic variant previously reported, IVS4 - 9 A>G, was also identified in homozygosity in this patient. No mutations were found in GJB2 and GJB6 genes. The novel mutation p.D171N, occurring at the codon 511, changes an aspartic acid residue to an asparagine residue at position 171 in the extracellular N-terminus domain of the protein.

Conclusions: The auditory phenotype observed in this patient might probably be due to the novel mutation p.D171N. Its functional characterization should be further performed in order to assess its effect on the mutated protein, and to investigate in which way the change introduced in the aminoacid residue 171 leads to low frequency hearing loss.

P7 Lack of fatty acid binding protein 7 slows the progression of age-related hearing loss in mice

*J. Suzuki^{1,2}, T. Oshima¹, R. Kimura², K. Yoshizaki², Y. Takata¹, Y. Owada³, T. Kobayashi¹, N. Osumi²

¹Tohoku University Graduate School of Medicine, Department of Otorhinolaryngology-Head and Neck Surgery, Sendai, Japan

²Tohoku University Graduate School of Medicine, Division of Developmental Neuroscience, Sendai, Japan

³Yamaguchi University Graduate School of Medicine, Department of Organ Anatomy, Ube, Japan

Objective: Fatty acid binding protein 7 (*Fabp7*) is a brain-specific member of intracellular lipid trafficking protein family that bind to long chain fatty acids and mediate their cellular uptake and transport, thereby regulating metabolic pathways and gene expressions. Our group has reported that *Fabp7* is required for the maintenance of embryonic and adult neural stem cells in the brain. In the cochlea, *Fabp7* is reported to localize in satellite cells in the spiral ganglion, supporting cells in the organ of Corti, and fibrocytes in the spiral limbus. However, the role of *Fabp7* in these tissues is still unknown. Here we analyzed the role of *Fabp7* in age-related hearing loss (ARHL).

Methods: *Fabp7*(+/+), *Fabp7*(+/-), and *Fabp7*(-/-) mice on the C57BL/6 background were used. An auditory function was examined by auditory brainstem responses (ABR: 4, 8, 12, 16, 32 kHz) at 2, 7, 12, 15-20 months. Cochlear morphology was assessed with hematoxylin and eosin (HE) staining and immunostaining. The number of spiral ganglion neurons and spiral limbus fibrocytes was counted in HE staining, and the rate of outer hair cell (OHC) loss was measured by a surface preparation method.

Results: ABR thresholds of *Fabp7* (-/-) mice were significantly reduced in every frequency compared to *Fabp7* (+/+) mice at 12 months. *Fabp7* (+/-) showed the phenotype at milder levels. High-frequency (32 kHz) ABR threshold of *Fabp7* (-/-) mice was significantly preserved compared to *Fabp7* (+/+) mice at 7 and 15-20 months. Additionally, the numbers of spiral limbus fibrocytes and spiral ganglion neurons were remarkably preserved in *Fabp7* (-/-) mice at 12 months. It also seemed that the number of OHCs was retained in *Fabp7* (-/-) mice at 7 and 12 months.

Conclusions: Contrary to our expectation, ARHL in *Fabp7* (-/-) mice was delayed in onset. In addition, *Fabp7* (-/-) mice exhibited delay in morphological changes related to aging. These results suggest that *Fabp7* deeply contributes to aggravation of ARHL. In the future, we plan to analyze the mechanism of hearing preservation in *Fabp7* (-/-) mice.

P8 Expression of pejvakin in human cochlea-an immunohistochemical study

*W. Liu^{1,2}, A. Kinnefors¹, F. Edin¹, H. Rask-Andersen¹

¹Uppsala University Hospital, Otolaryngology, Uppsala, Sweden

²Sweden

Auditory neuropathy is characterized by a preservation of the outer hair cell integrity and an impaired neuronal transmission evident in an absent ABR. DFNB59 gene encodes protein pejvakin (PJVK) and its mutation causes autosomal recessive auditory neuropathy. Since Delmaghani and colleagues identified the gene DFNB59 and its product in 2006, observation of the distribution of pejvakin protein in human auditory system has not been reported. Our research on the expression of PJVK in human cochlea was based on adult human specimens.

Materials and Methods: Study on human materials was approved by the local ethics committee and patient consent was obtained. Three cochleae belonging to three adult patients (1 male, 2 females; age 40-56 years) were dissected out, during petro-clival meningioma surgery. The cochleae were fixed, decalcified and sectioned, and the sections subject to immunohistochemistry (IHC). IHC was performed using primary antibodies against pejvakin and Tuj1. Laser confocal microscopy was used for analyzing and imaging the immunostaining.

Results: Pejvakin labeling was seen in the neuron cell bodies rather than the nerve fibers (both dendrites and axons) that were labeled with Tuj1 antibody. As Tuj1 antibody stained the cytoplasm of type1 cell, pejvakin antibody labeled both type1 and type2 cells. The nuclei of the neurons were also PJVK-positive. No labeling was seen in the structures within the organ of Corti, the stria vascularis, etc.

Discussion: The mutation of PJVK was found to be linked to auditory neuropathy in both patients from affected families and DFNB59 knock in mice. This protein was detected in the spiral ganglion, cochlear nuclei, superior olivary nucleus and the inferior colliculus in mouse by Delmaghani and coworkers. The mutation in the DFNB59 gene has been identified in many consanguineous families from different ethnic groups. Our study demonstrated for the first time the expression of PJVK in human spiral ganglion. Its role for neural synchrony and the implication of its nuclear localization needs further elucidation although a functional role in the propagation of action potentials has been proposed (Delmaghani et al., 2006).

P9 Histone acetylation and methylation indicates epigenetic change in the aged cochlea of mice

*K.-I. Watanabe¹, W. Bloch²

¹Nippon Medical School, ENT, Tama, Japan

²Deutsch Sporthochschule Koeln, Kreislaufforschung und Sportmedizin, Koeln, Germany

Introduction: It is currently accepted that epigenetics plays an important role in normal genetics and differentiation and its failure triggers various diseases, such as cancer, aging, metabolic diseases, and abnormal differentiations. The typical mechanism involves the modification of histones and the methylation of DNA. In this study, we investigated the modification of histones in the aged cochlea of mice using immunohistochemistry.

Methods: Eight mice (C57BL/6(B6)) at the age of 8 weeks (young group) and 132 weeks (aged group) were used. Cochleas were fixed with paraformaldehyde and then decalcified. Hematoxylin-eosin staining was performed for morphological study using a light microscope. After removing paraffin, the sections were incubated with the primary antibody to acetyl-histone H3 Lys9 or dimethyl-histone H3 Lys9. Confocal scanning microscopy was performed for observation.

Results: The degeneration was severest in the spiral ganglion cells and the organ of Corti of the basal turn as determined by light microscopy. Acetylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the young group but not in those of the aged group. Methylated histone H3 was detected in the spiral ganglion cells and the organ of Corti of the aged group but not in those of the young group. Acetylation was switched to methylation during ageing.

Conclusions: Histone modification is known to have a critical role in neuro-degeneration. Our findings suggest that epigenetic change participates in the process of presbycusis.

P10 Differentiation of human induced pluripotent stem cells into otic sensory neural fate

*A. Dos Santos¹, N. Abboud¹, E. Savary¹, I. Watabe¹, J. De Vos², F. Feron³, A. Zine¹

¹CNRS UMR 7260, Integrative and Adaptative Neurosciences, Marseille, France

²INSERM U 1040, Biotherapy Institute, Montpellier, France

³CNRS UMR 7259, NICN, Marseille, France

Induced pluripotent stem cells, like embryonic stem cells, are known to be able to generate any cell type of differentiated cells of the adult organism. Due to this quality, stem cells can be used for the purposes of developmental modeling, drug screening and possibly as novel cell-based treatments for some diseases.

While the ability of human embryonic and induced pluripotent stem cells (hiPSC) to generate differentiated cell types from other sensory placodes, such as the retina, has been well characterized (Lamba et al., 2006; 2010), the capacity for human embryonic pluripotent stem cells to form otic neural placode derivatives has not been clearly determined.

In the quest to develop the tools for a possible cell-based therapy to generate lost sensori-neural elements of the human inner ear, a critical step is to establish a reliable *in vitro* model for the expansion of hiPSC, either on human fibroblast feeder or Matrigel, and their differentiation on a basement membrane matrix.

We have developed an *in vitro* method to expand and differentiate hiPSC to an otic neural progenitor cell fate. The hiPSC, through the stepwise supplementation with selected developmental cues i.e., growth factors and signaling molecules, are able to generate a population of cells with neurite-like outgrowths emerging from the embryoid bodies and otic sensory neural immuno-phenotypes (i.e., Brn3a, GATA3, NeuroD, peripherin).

We will present preliminary findings of our ongoing studies aimed to optimize the method of maintaining and directing the differentiation of human induced pluripotent stem cell types towards an inner ear sensory neural fate.

Supported by "Les Gueulles Cassées" & "Danet Mazet- Fondation de France"

P11 Pilot study of stem-cell based therapy for auditory spiral ganglion loss after experimental bacterial meningitis

*P. Senn¹, J. Zimmermann², D. Grandgirard², S. Hofer², A. Mina^{1,2}, S. di Santo³, H.R. Widmer³, S. Leib²

¹Univ. Dept. for Otorhinolaryngology, Head & Neck Surgery, Bern, Switzerland

²University of Bern, Neuroinfection Laboratory, Institut for Infectious Diseases, Bern, Switzerland

³University of Bern, Laboratory of Neural Repair, Department of Neurosurgery, Inselspital, Bern, Switzerland

Question: Bacterial meningitis leads to long-term neurological sequelae in up to 30% of survivors, including seizure and hearing loss. Hearing loss is caused by the loss of auditory afferent spiral ganglion neurons (SGN) and the loss of hair cells. The ability to regenerate lost sensory hair cells and auditory neurons in the mammalian inner ear is very limited. The question to answer was, whether transplantation of neural stem cells into the postmeningitic inner ear is feasible and whether the transplanted cells are surviving and differentiating into neuronal cell types.

Methods: 11 day old male Wistar rats were infected intracisternally by injection of 5×10^5 live *Streptococcus pneumoniae*. 18h after infection, antibiotic treatment was started, using ceftriaxone (100mg/kg, twice daily by subcutaneous injection) and continued for a total of 3 days. Two weeks after the infection, spiral ganglion stem cells, isolated from infant (P2-P4) GFP rats were injected into the modiolus of the postmeningitic deaf animals via a retroauricular, transbullar approach. Transplanted animals received the painkiller rimadyl (50mg/kg) and the antibiotic ceftriaxone (100mg/kg) subcutaneously twice a day for the following 3 days. Rats were sacrificed 1 and 4 weeks after transplantation to dissect the cochleas for histopathological analysis following staining of neurons and hair cells with the respective markers β -III-tubulin and MyoVIIa.

Results: The surgical access and the stem-cell transplantation have been successfully completed in n=6 animals. The injected animals survived for one and four weeks, respectively, with minimal trauma to the middle and inner ear structures. The transplanted spiral ganglion-derived stem cells integrated into the modiolus, survived and formed long axons in the injured cochlea.

Conclusions: A protocol for a safe and effective surgical transplantation of stem cells into the postmeningitic modiolus could be established in this preliminary study. In the future, intramodiolar transplantation of stem cells may increase the effectiveness of cochlear implants. In the long-term, other stem cell types (hair cell progenitors) may be simultaneously transplanted, which may help to advance cellular therapies for hearing loss in the future.

P12 Creatine supplementation promotes propagation and differentiation of rat spiral ganglion-derived stem cells

*A. Mina¹, S. di Santo², A. Ducray², H.R. Widmer², P. Senn¹

¹Univ. Dept. for Otorhinolaryngology, Head & Neck Surgery, Bern, Switzerland

²University of Bern, Laboratory of Neural Repair, Department of Neurosurgery, Inselspital, Bern, Switzerland

Question: Creatine administration has been reported previously to exert neurotrophic properties on neuronal progenitor cells isolated from different brain regions. In the present study we investigated whether Creatine might enhance the long term propagation and differentiation of inner ear stem cells.

Methods: Stem cells were isolated from the neonatal rat spiral ganglion and propagated in basal medium containing mitogens. Creatine supplementation was started from the third passage. The number of spheres was assessed at each passage. To address the effects of Creatine on differentiation of expanded spiral ganglia cells, number of neuronal cells was analyzed by beta-III-tubulin immunohistochemistry after two weeks culture in absence or presence of Creatine. Moreover, the differentiation properties of primary versus *in vitro* expanded spiral ganglion stem cells were compared.

Results: We observed that Creatine administration promoted number of spiral ganglia derived spheres as compared to controls. Moreover, creatine significantly increased the yield of neuronal cells of differentiated progenitors by 40%, while the total cell number remained steady in both experimental conditions. Notably, both the total cell count and the number of beta-III-tubulin positive cells were more than two fold higher in the primary cultures compared to passaged cultures.

Conclusion: Our study shows that inner ear stem cells can be expanded over considerable time periods. Creatine supplementation promoted both the propagation and neural differentiation of rat spiral ganglia stem cells.

P13 Topical application of dexamethasone in a thermoreversible hydrogel for hearing preservation in an animal model of cochlear implantation

*C. Honeder¹, W. Gstoettner¹, E. Engleder², F. Gabor², R. Plasenzotti³, E. Vyskocil¹, C. Arnoldner¹

¹Medical University of Vienna, Department of Otorhinolaryngology, Vienna, Austria

²University of Vienna, Department of Pharmaceutical Technology and Biopharmaceutics, Vienna, Austria

³Medical University of Vienna, Department of Biomedical Research, Vienna, Austria

Introduction: Limited effectiveness and severe side effects of systemic glucocorticoid (GC)-treatment make topical GC delivery to the inner ear an interesting alternative. The application of thermoreversible hydrogels has emerged as a promising approach to improve local drug administration. Dexamethasone (dexa) has been shown to possess otoprotective effects in guinea pig models of cochlear implantation, if applied preoperatively. Nevertheless, the intraoperative application, using a thermoreversible hydrogel in an attempt to achieve a prolonged delivery, has not been evaluated up to date. We therefore established a guinea-pig model for cochlear implantation, which was used to evaluate the effects of such a hydrogel.

Materials and Methods: After implantation of a cochlear implant electrode, 50 µl of 6% dexa-hydrogel, hydrogel only or physiological saline were applied to the round window niche of the experimental guinea pigs. Hearing was evaluated pre- and postoperatively as well as after 3, 7, 14, 21 and 28 days by the measurement of compound action potentials and auditory brainstem responses.

Results: A preliminary statistical evaluation of the click-thresholds showed no statistical difference between the treatment group and the control groups on day 28. Analysis of the frequency specific measurements showed only minor frequency shifts in the low frequencies (0.5-2kHz), which did not differ relevantly between the groups. In the middle frequencies (2-8 kHz) threshold shifts were significantly higher in the physiological saline-treated group than in the hydrogel treated groups (both control-gel and dexa-gel). In the high frequencies (8-32kHz), threshold shifts were significantly higher in the physiological saline and dexa treated groups as compared to the poloxamer-gel treated group.

Conclusions: It seems that the application of a poloxamer hydrogel in the area of the round window membrane can reduce postoperative hearing threshold-shifts. To verify these preliminary findings, the animal numbers in the groups need to be increased and the trauma caused by cochlear implantation needs to be further standardized.

P14 Thermoreversible hydrogels: Alternative-therapy to systemic glucocorticoid therapy for inner ear diseases

*E. Engleder¹, C. Honeder², J. Klobasa¹, C. Arnoldner², F. Gabor¹

¹University Vienna, Department of Pharmaceutical Technology and Biopharmaceutics, Vienna, Austria

²Medical University of Vienna, Department of Otorhinolaryngology, Vienna, Austria

Introduction: Thermoreversible hydrogels, applied liquid on the round window membrane and then forms a gel at body temperature, possess a promising potential for local intratympanic therapy for inner ear diseases, instead of common systemic glucocorticoid therapy. As a matter of fact, through the gelling and hence the extended contact with the round window membrane, a drug delivery system with higher drug release is expected.

Materials and Methods: The triamcinolone acetonide containing hydrogel with optimal Sol/Gel transition is developed. Further on, the release profile will be evaluated by using in-vitro models with semipermeable membranes to mimic the round window membrane. Physiological conditions are adjusted with an artificial perilymph fluid as acceptor medium. To examine the albumin-binding capacity of triamcinolone acetonide, albumin modified artificial perilymph fluid is used.

Additionally, the release in-vivo will be investigated in animal trials on a guinea pig model. Samples of perilymph fluid, liquor and plasma will be analysed after 24, 72 and 240 hours through HPLC-mass spectrometry with a limit of detection of 5ng/ml.

Results: As a result of Sol/Gel transition studies, the optimized hydrogel is a liquid at room temperature that forms a gel after ten minutes at body temperature. In-vitro release investigations by use of a semipermeable membrane, showed a release for triamcinolone acetonide of 1% after 24 hours, 1.3% after 72 hours and 2.16% after 240 hours. No significant influence of the release rate through addition of different amounts of albumin in the acceptor medium was observed. Yet, in-vivo studies have resulted in a release rate of 0.3% in perilymph fluid.

Conclusion: The optimization of the gel-recipe results in a hydrogel that is applicable in clinic, due to the observed delayed release in therapeutic amounts in-vitro as well as in-vivo. A new approach to treat inner ear diseases and postoperative traumata such as after cochlear implantation is therefore developed.

P15 Effects of TrkB antibody functionalized silica nanoparticles on the survival of spiral ganglion neurons

C. Pritz¹, *A. Schrott-Fischer¹, M. Bitsche², W. Salvenmoser³, J. Dudas¹, R. Glueckert¹

¹Medical University Innsbruck, Department of Otolaryngology, Innsbruck, Austria

²Division of Clinical and Functional Anatomy, Medical University, Innsbruck, Austria

³Institute of Zoology, Innsbruck, Austria

In the recent years the use of nanoparticles in medicine and biology has become widespread. Third generation nanoparticles bear surface modifications to fulfill tasks such as targeting of certain cell types or penetration of epithelia and are able to deliver chemically diverse cargo. Recently, this approach of gene and drug delivery has also been implemented for spiral ganglion neurons. After acoustic trauma, inner and outer hair cells can be lost. In this case, spiral ganglion cells lose their neurotrophic support by the hair cells. Apoptosis of spiral ganglion neurons is consequential. Most conventional approaches to prevent spiral ganglion neurons from cell death involve the application of brain derived neurotrophic factor and neurotrophin 3, which induce survival signaling in the remaining spiral ganglion neuron population. In our strategy we use nanoparticles, which carry molecules which are acting agonistic on the TrkB receptor. Recently we succeeded in activating TrkB signaling in TrkB over expressing SH-SY5 cells by the use of surface modified polymericosomes. These nanoparticles were carrying hNGF-EE peptide. Future efforts will involve the agonistic TrkB antibodies (anti-TrkB) grafted to silica nanoparticles. These antibodies fulfill two fundamental roles. On the one hand they are homing the nanoparticles specifically to the TrkB positive spiral ganglion neurons and on the other hand the antibody surface modification is needed to induce TrkB mediated survival signaling in these neurons. Subsequently nanoparticles bound to TrkB receptors will be internalized into signaling endosomes. We have already investigated the mode of internalization of silica nanoparticles in a cell line derived from the organ of Corti and identified macropinocytosis as the principle mode of internalization. Further we gained insight into the subcellular localization and endocytic trafficking of silica nanoparticles in these cells. So we laid the cornerstone for future investigations of the biological effect of the anti-TrkB surface modification on spiral ganglion cells. These results will open new therapeutic applications for nanoparticles against acoustic trauma.

P16 Surgical approach for local treatment strategies of noise induced hearing loss

*J.A. Sistiaga^{1,2}, L. Sanz^{1,2}, S. Murillo Cuesta^{3,2,4}, R. Martinez Vega³, C. Avendaño⁵, I. Varela Nieto^{3,2,4}, T. Rivera^{1,2}

¹Hospital Universitario Príncipe de Asturias, ENT, Madrid, Spain

²Center for Biomedical Network Research on Rare Diseases (CIBERER), ISCIII, Valencia, Spain

³Institute of Biomedical Research Alberto Sols (CSIC-UAM), Madrid, Spain

⁴Research Institute Hospital La Paz (IdiPAZ), Madrid, Spain

⁵Department of Anatomy, Histology & Neuroscience, Medical School, Autónoma University of Madrid, Madrid, Spain

Introduction and Objective: Local delivery of new drugs into the middle ear represents a valid therapeutic alternative for cochlear damage. However, this strategy requires an innocuous surgical approach and efficient drug delivery systems. The objective of this study is to determine the feasibility and safety of a model of local treatment for noise induced hearing loss in mice, based on the ventral surgical approach to the middle ear and the use of a soaked gelfoam.

Materials and Methods: 34 two month-old male CBA/CaOlaHsd mice were evaluated with auditory brainstem responses (ABR) before surgery and divided in 2 groups: SHAM (n=20) and noise-exposed (violet swept sine noise, 105 dB SPL for 30 minutes 24h before surgery, n=14). Surgical process consisted in a transcervical approach to the middle ear and a bullostomy, in order to reach the round window niche, where a saline-soaked gelfoam was placed. ABR measurements were repeated 1, 14 and 30 days after surgery. Morphological changes were evaluated with histology and stereological hair cell quantification.

Results: Baseline ABR thresholds were in the normal hearing range, as expected for this strain. No statistically significant threshold shifts were observed in the SHAM group after surgery. Accordingly, mice from control group presented a normal cytoarchitecture and a homogeneous distribution of hair cells along the cochlea. Mice exposed to noise showed an evident threshold shift (40-50 dB SPL) and altered cochlear morphology, with disruption of stereocilia and loss hair cells, mainly OHC, especially in the basal turn of the cochlea.

Conclusion: Here we present an easy-to-perform local treatment model with no evidence of functional or morphological damage to the cochlea. This strategy could be used to deliver hydrophilic drugs to the inner ear, although further studies are needed to optimize it.

Acknowledgements: This research was funded by grants from the Spanish Ministry of Science and Innovation FIS (PI10/00394) to TR and SAF2011-24391 to IVN. RMV and SMC were supported by CSIC and CIBERER, respectively. We appreciate the help of R. Cediél (UCM) and L. Barrios (CTI CSIC).

Development

P17 Expressions of HGF and its high-affinity receptor C-MET in the early developing rat cochlea

*K. Doi¹, M. Sato¹, M. Miyashita¹, K. Matsumoto¹, T. Nakamura¹, K. Saito¹, K. Terao¹

¹Department of Otolaryngology, Kinki University School of Medicine, Osaka, Japan

Introduction: Hepatocyte growth factor (HGF) has mitogenic, motogenic and morphogenic activities for various cell types and function as an organotrophic factor for regeneration of the several organs. Various biological activities of HGF are mediated by a membrane-spanning tyrosine kinase receptor C-MET encoded by the c-met proto-oncogene. We had identified the expressions of both HGF and C-MET receptor in the adult rat cochlea. In the present study, we examined the expressions of these two molecules in the developing rat cochlea to determine their possible roles in organogenesis and maturation of the rat cochlea.

Materials and Methods: The rat cochlea sections were obtained at embryonic day 10 (E10), E12, E14, E16, and E18. The sections were fixed in 4% paraformaldehyde (PFA) in PBS, treated with protease K, then slides were post-fixed in 4% PFA acetylated and dehydrated. EcoRI-HindIII fragment of rat HGF cDNA (625 bp) corresponding to position 1115-1787, and 825 bp rat c-met cDNA were used as templates for synthesization of antisense and sense RNA probes. After in-situ hybridization (ISH), the sections were immersed and washed in SCC containing PFA several times. The recovered and dehydrated sections were coated with Kodak NTB-2 emulsion and exposed for 2 weeks. The frozen sections of the rat cochlea at E10, E12, E14, E16, and E18 were prepared and incubated with anti-rat and anti-human HGF antibodies, and anti-mouse and anti-human C-MET antibodies.

Results: ISH indicated that the expressions of HGF and C-MET mRNAs were moderate by E12, while those dramatically increased after E14 in the developing rat cochlea. HGF and C-MET mRNAs were mainly localized in the developing epithelia, cochlear and vestibular ganglion cells, and surrounding mesenchymal cells. Both molecules were co-expressed in each cell at almost the same time.

Immunohistochemistry demonstrated that HGF and C-MET proteins were expressed in the developing epithelia, cochlear and vestibular ganglion cells, and surrounding mesenchymal cells, indicating the locations and the timings of the expressions were identical to their mRNAs expressions.

Discussion: The present study indicated HGF and its high-affinity receptor C-MET should play important roles in organogenesis and maturation of the rat cochlea. HGF and C-MET were co-localized in the developing epithelia, cochlear and vestibular ganglion cells, and surrounding mesenchymal cell, suggesting the functional coupling of these two molecules in both autocrine and paracrine manner.

P18 C-RAF deficiency causes cochlear abnormalities and profound sensorineural deafness in mice

R. de Iriarte¹, *M. Magarinos^{1,2,3}, U.R. Rapp⁴, I. Varela-Nieto^{1,2,5}

¹Instituto de Investigaciones Biomédicas "Alberto Sols", Madrid, Spain

²Instituto de Salud Carlos III, Unit 761, Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain

³Universidad Autónoma de Madrid, Biology, Madrid, Spain

⁴Max Planck Institute, Munich, Germany

⁵diPAZ, Madrid, Spain

Introduction: Insulin-like growth factor I (IGF-I) is fundamental for neurogenesis and neuronal differentiation during inner ear development. IGF- I deficiency is associated with deafness in man and mice. IGF-I binds to its high affinity receptor and activates downstream signaling as the RAF-MEK-ERK pathway. RAF kinases are essential for cell proliferation, survival and differentiation during development and in the adult tissues homeostasis. RAF proteins have redundant but also specific cellular and tissular functions. In the developing chicken inner ear the activation of C-RAF and B-RAF are critical for otic neurogenesis.

Material and Methods: To further study the role of RAF kinases in the auditory receptor, we have analysed C-RAF mRNA and protein expression patterns in the mouse inner ear along development. To explore its functional relevance we have preformed ABR to study the auditory phenotype of the C-Raf^{-/-} null mouse.

Results: Our results show that C-RAF is differentially expressed and that the protein is active and able to phosphorylate downstream substrates. Mutants present an all-frequency profound sensorineural hearing loss with a mean auditory threshold of 90 dB SPL. The study of the general cochlear cytoarchitecture indicates that the main structures and cell types have been formed, although the expression of proteins essential for hearing is altered. Thus the levels of the Kir4.1 potassium channel in the stria vascularis are reduced in the C-Raf^{-/-} null when compared to the wild type littermates.

Conclusions: In summary, these results show that C-RAF is expressed in the developing cochlea and that its activity is essential for the onset of hearing.

P19 Role of p75NTR-positive Schwann cells on spiral ganglion neurite outgrowth

*S. Hansen¹, K. Schaumann¹, T. Klenzner¹, J. Schipper¹

¹University Hospital Düsseldorf, ENT, Düsseldorf, Germany

Introduction: Degeneration of spiral ganglion fibers and spiral ganglion neurons (SGN) after hair cell loss may be a problem in adequate hearing rehabilitation of patients with a Cochlea Implant (CI). Additional impact for preservation of spiral ganglion fibers comes from regenerative therapeutic strategies e.g. hair cell regeneration or stem cell therapy. The intact connections from the hair cell to the secondary neuron of the auditory pathway are required for a successful approach to these future techniques. The Schwann cells of the spiral ganglion and its secreted factors may be another resource to influence SGN degeneration or regeneration. It is meanwhile known that nonmyelinating Schwann cells facilitate nerve regeneration after nerve injury or axotomy in the peripheral nervous system, whereas some myelin components like Myelin-Associated Glycoprotein (MAG) and its receptor p75NTR are axonal growth inhibitors.

Materials and Methods: Here, we demonstrated the expression of MAG and p75NTR during hearing development in cryosections of the mouse cochlea. Additionally, we cultured SGN of newborn mice and analyzed the Schwann cell-Neuron-interaction with and without neurotrophic support.

Results: Although MAG expression could be seen around SGN fibers (except the SGN soma) *in vitro*, MAG expression was absent *in vivo* during 2 to 10 days of culture. However, strong p75NTR expression was present in neurite accompanying Schwann cells. The density of p75NTR-positive Schwann cells correlated negatively with the neurite length of SGN and was influenced by supplemented factors like NT-3, BDNF, and FGFs.

Conclusion: We suppose an inhibitory role of p75NTR-positive Schwann cells of the SGN, which may be helpful for plasticity during hearing development, but could also be a handicap for cochlear nerve regeneration.

P20 Connexin 26 (Cx26) is crucial for the development and the preservation of the organ of Corti

*G. Crispino¹, F. Galindo Ramirez¹, G. Di Pasquale², J. Chiorini²,
F. Mammano¹

¹Vimm, Padua, Italy

²NIH, Bethesda/MD, USA

Introduction: In prior work (1) we characterized Cx26 expression from postnatal day 5 (P5) to adult age in double transgenic Cx26^{Sox10Cre} mice obtained by crossing Cx26 floxed mice with a deleter Sox10-Cre line. Cx26^{Sox10Cre} mice presented with complete Cx26 ablation in the epithelial gap junction network of the cochlea. Functional experiments performed in postnatal cochlear organotypic cultures showed impaired gap junction coupling in Cx26^{Sox10Cre} mice and auditory brainstem responses (ABR) revealed profound hearing loss. Furthermore, using cochlear organotypic cultures, we showed that recombinant bovine adeno associated virus (BAAV) vectors efficiently transduce non-sensory epithelial cells *in vitro* (1,2).

Methods: We injected P4 or P20 Cx26^{loxP/loxP} mice *in vivo* by canalostomy with BAAV vectors containing a Cre-IRESGFP expression cassette (BAAVCre-IRESGFP). Three weeks later, the injected cochleae were analyzed by confocal immunofluorescence, Cx26 mRNA levels were quantified by RT-qPCR and hearing function was tested by ABR as previously described (1,2).

Results: Cre recombinase expression after delivery of BAAVCre-IRESGFP, locally catalyzed recombination of Cx26 *in vivo*. RT-qPCR highlighted a 50% reduction of Cx26 mRNA level in the injected cochleae. Subsequent immunofluorescence analysis revealed a more prominent Cx26 ablation in the lateral wall region of the scala media and a drastic alteration of the anatomy of the organ of Corti, compared to contralateral non injected ear. In particular, a massive degeneration of outer hair cells was observed, increasing from the base to the apex of the cochlea. Moreover, Cx26^{loxP/loxP} mice that had received BAAVCre-IRESGFP presented with significantly elevated ABR thresholds.

Conclusions: Targeted ablation of Cx26 by BAAVCre-IRESGFP injection *in vivo* both in P4 and P20 Cx26^{loxP/loxP} mouse cochlea revealed that Cx26 expression is crucial not only for the development of the inner ear but also for the preservation of the mature organ of Corti and the consequent maintenance of hearing function.

Supported by MIUR PRIN grant n. 2009CCZSES and Telethon grant GGP09137 to FM.

References:

1 Crispino et al., PlosOne, 2011

2 Ortolano et al., PNAS, 2008

P21 Study of the Boettcher cells along their development: Junctions and expression of the urea-transporter B (UT-B)

*M. Cloes¹, T. Renson¹, N. Johnen¹, M.-E. Francart¹, N. Thelen¹, M. Thiry¹

¹Université de Liège, GIGA-Neurosciences, Liège, Belgium

The Boettcher cells (BC) lie on the sensory epithelium of the cochlea. Their function has never been clearly defined. However it has been suggested that they may influence the ionic composition of the fluids of the inner ear, which play a central role in the conduction of the sensory information. In this context the compartmentating function of the BC around and after the onset of hearing may influence the subsequent refining of hearing.

We collected ultrastructural and immunohistological data during the final maturation stage of the sensory epithelium. In particular the cell junctions were investigated to clarify the compartmentating function of the BC at early stages. As a potential actor in the ion flow in the sensory epithelium, the urea transporter-B (UT-B) was also immunolocalised during the development of the BC.

At the mature stage (P25) the BC are linked to the adjacent cells by numerous adherens and non-adherens junctions. They rest on a basilar membrane to which they are attached by hemidesmosomes. They typically exhibit large basolateral interdigitations. We found that, at the 8th postnatal day, the BC are separated from the neighbouring cells by wide spaces entered by scarce cytoplasmic extensions. These spaces are interrupted by areas of close contact, where adherens and non-adherens junctions may be found. Thus, although there seems to be fewer interdigitations at P8, gap junctions probably still allow easy cell-to-cell exchanges. Moreover non-adherens junctions can systematically be identified apically. Although it was impossible to differentiate tight and gap junctions without specific labeling, we postulate that these non-adherens junctions correspond to tight junctions and seal the apex of the BC. This feature is necessary to enable the control of the ion concentrations surrounding the sensory epithelium. We also found that UT-B, known for water and urea transport in red blood cells, is present in the membranes of the BC from P12 (the earliest stage tested) to P25. Thus UT-B may play a role in the regulation of the ionic concentrations of the inner ear fluids.

P22 Evidence for a partial epithelial-mesenchymal transition in rat auditory organ development

*N. Johnen¹, M.-E. Francart¹, N. Thelen¹, M. Cloes¹, M. Thiry¹

¹University of Liege, Life Sciences, GIGA Neurosciences, Cell and Tissue biology Unit, Liege, Belgium

An epithelial-mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. During this process, epithelial cells loosen cell-cell adhesion, modulate their polarity and rearrange their cytoskeleton. They also enhance their motility capacity. The EMT plays key roles in the formation of the body plan and in the differentiation of multiple tissues and organs but it is also involved in tissue repair, tissue homeostasis, fibrosis, and carcinoma progression. Until now, EMT has been rarely mentioned in the inner ear organogenesis and it has never been observed during auditory organ morphogenesis. The auditory organ, the organ of Corti, is a highly specialized structure composed by specific cellular types. The sensory cells are characterized by stereocilia at their apex and are necessary for the sound perception. These cells are supported by supporting cells. Based on their morphology and physiology, at least four types of supporting cells can be identified in the organ of Corti: inner and outer pillar cells, phalangeal cell and Deiter's cells. The inner pillar cells and outer pillar cells combine to form the tunnel of Corti, a fluid filled triangular space that separates the single row of inner hair cells from the first row of outer hair cells. The Nuel spaces are another interval in the organ of Corti that is situated between the outer pillar cells and the different rows of outer hair cells and Deiters cells. To determine whether an EMT may play a role in the morphogenesis of the auditory organ, we studied the spatial localization of several EMT markers, the cell-cell adhesion molecules and intermediate filament cytoskeletal proteins, in epithelium of the dorsal cochlea during development of the rat organ of Corti from 18th embryonic day until 25th postnatal day. We examined by confocal microscopy immunolabelings on cryosections of whole cochleae with antibodies anti-cytokeratins as well as with antibodies anti-vimentin, anti-E-cadherin and anti-beta-catenin. Our results showed a partial loss of E-cadherin and beta-catenin between supporting cells at P8 and P12, respectively, and a temporary appearance of vimentin in pillar cells and Deiters between P8 and P10. Our results show a local loss of adhesion between supporting cells of the OC from P8, an increase expression of cytokeratins in supporting cells around P10 and a temporary appearance of vimentin in supporting cells at P8-10. These observations suggest that a partial EMT might be involved in the remodeling of the Corti organ during the postnatal stages of development in rat.

P23 Absence of Plastin1 does not impair stereocilia formation but results in a moderate hearing loss

*R. Taylor¹, S.L. Johnson², E.-M. Grimm-Günter³, F. Rivero³, W. Marcotti², A. Forge¹, N. Daudet¹

¹The Ear Institute, University College London, London, United Kingdom

²Dept of Biomedical Science, University of Sheffield, Sheffield, United Kingdom

³The Hull York Medical School and Department of Biological Sciences, University of Hull, Hull, United Kingdom

The stereocilia of hair cells contain a central core of F-actin filaments, organized into tightly packed parallel bundles. Proteins that bind to F-actin and promote such assembly are known as actin-bundling proteins, some of which such as espin, harmonin and eps8 are critical for stereocilia formation. However the function of the most abundant actin-bundling protein expressed in stereocilia, plastin1 (pls1, also known as fimbrin), has not been tested so far.

In this study, we investigated hair cell morphology, physiology, and auditory function in pls1 knock-out (ko) mice. Unexpectedly, hair cells developed normally in the vestibular and auditory epithelia of pls1 ko mice. Immunocytochemistry and ultrastructural studies did not show any apparent defect in the actin content, height, width, and general organization of hair cell stereociliary bundles. Despite this, auditory brainstem responses recordings in 1 to 8 month-old animals revealed that pls1 ko mice have a moderate hearing loss across all frequencies when compared to wild-type and heterozygous littermates. Furthermore, we found that the adaptation properties, but not the size, of the mechanotransducer current were altered in pls1-deficient auditory hair cells.

Altogether, these data show that pls1 is not essential for the formation of stereocilia and their central core of parallel actin bundles. However the moderate hearing loss together with the changes in adaptation properties suggest that some components of the mechano-electrical transduction apparatus may be affected by the loss of pls1 in stereocilia.

P24 The resting mechanotransducer current drives spontaneous action potentials in pre-hearing mammalian cochlear inner hair cells

*S.L. Johnson¹, H. Kennedy², M. Holley¹, R. Fettiplace³, W. Marcotti¹

¹University of Sheffield, Department of Biomedical Science, Sheffield, UK, United Kingdom

²University of Bristol, School of Physiology and Pharmacology, Bristol, UK, United Kingdom

³University of Wisconsin Medical School, Department of Neuroscience, Madison/WI, USA

Introduction: Spontaneous electrical activity that occurs during critical periods of immature development drives the refinement of neuronal circuits in sensory organs before the onset of sensory experience (Blankenship & Feller, 2010 Nat. Rev. Neurosci 11:18-29). In the mammalian auditory system, action potentials occur spontaneously in inner hair cells (IHCs) during the first postnatal week (Johnson et al. 2011 Nat. Neurosci. 14:711-17.). During the second postnatal week, which is before the onset of hearing in most rodents, the resting membrane potential for IHCs is apparently more hyperpolarized (about -75 mV: Johnson et al. 2011) and it remains unclear whether spontaneous action potentials continue to occur. In this study we determined the IHC resting membrane potential using near-physiological recording conditions.

Methods: We performed patch-clamp recordings from P7-P10 mouse IHCs at body temperature and with an *in vivo*-like endolymphatic Ca²⁺ concentration of 0.3 mM estimated to be present in the immature cochlea.

Results: We found that when mouse IHC hair bundles were exposed to the *in vivo* endolymph-like 0.3 mM Ca²⁺ concentration, the increased open probability of the mechanotransducer channels provided enough inward current to depolarize the cells to around the action potential threshold (about -55 mV) and elicit repetitive action potential firing.

Conclusions: We propose that, *in vivo*, spontaneous Ca²⁺ action potentials are intrinsically generated by IHCs up to the onset of hearing and that they are likely to influence the final sensory-independent refinement of the developing cochlea.

This work is supported by the Wellcome Trust to WM and HK; RO1 DC013672 from the NIDCD to RF.

P25 Regulation of vestibular organs epithelial morphogenesis by the vertebrate planar cell polarity pathway is dependent on P120-catenin

*D. Ren¹, R. Ma¹, K. Jin¹, F. Chi¹, P. Chen²

¹EENT Hospital, Fudan University, Otolaryngology, Shanghai, China

²Emory University, Cell Biology, Atlanta/GA, USA

Objective: The mammalian vestibular sensory organs consist of alternating arrangement of sensory hair cells and supporting cells. Each hair cell shows distinctively an intrinsic polarity and rows of microvilli-derived stereocilia with graded heights. All of the hair cells are coordinately oriented within each vestibular organ, displaying a tissue polarity that is parallel to the plane of the sensory epithelium and known as planar cell polarity (PCP). In this study, we established the timeline to explore the sensory epithelium morphogenesis. We compared the PCP mutants and conditional knockout p120-catenin mutants to explore the role of the cell adhesion in the PCP pathway during the development of vestibular organs.

Methods: We established the timeline and used scanning electron microscope(SEM) to observe the morphological development and hair-bundles formations of the vestibular organs. We further analyzed the basal body polarity and subcellular localization of membrane associated PCP proteins, Vang-like 2 (Vangl2) and Frizzled3 (Fz3). We also compared the epithelial morphogenesis and PCP establishments in vestibular organs using Vangl2^{L^{pl}L^p} mutants -homozygous mutations in the PCP gene Vangl2 and p120^{CKO/CKO} mutants- conditional knockout mutants of p120-catenin.

Results: The hair cells of the vestibular organs initiate differentiation at embryonic day (E) 11.5. The hair- bundles first appear at embryonic day (E) 12.5. The first sign of hair cell polarity is observed around the line of polarity reversal and expands from the striolar region of the saccule and utricle. Membrane-associated PCP proteins show polar distribution along the PCP axis. The polar distribution of PCP proteins precedes the formation of hair cell polarity. PCP mutants show normal morphology of maculae but randomly oriented hair cells and the lost of polar distribution of PCP proteins. In contrast, p120-catenin mutants lead to morphogenesis defects, including significantly reduced size, and an undulated appearance of the epithelium. These morphologic defects, however, are not accompanied by loss of asymmetric partition of PCP protein Fzd3 or PCP defects in the vestibular organs.

Conclusions: During development, the different vestibular organs show a distinct gradient of cell proliferation, differentiation, and polarity establishment in a continuous epithelium. Together, these data suggest that p120-dependent cell adhesion is not essential for hair cell polarity while it is required for integrity and morphogenesis of the vestibular epithelia.

P26 The molecular development of the mouse dorsal cochlear nucleus

*M. Mao¹, P. Thorne¹, F. Kubke¹, J. Montgomery¹

¹University of Auckland, Physiology, Auckland, New Zealand

The cochlear nucleus (CN), including the dorsal (DCN) and ventral (VCN) parts, is the first structure in the CNS auditory pathway. Development of the VCN has been well studied but less is known about the DCN. It has a layered structure and receives both auditory and multimodal non-auditory inputs.

Question: This study was undertaken to investigate the molecular development of the DCN.

Methods: Expression of excitatory (GluR1, 2, 2/3 and 4) and inhibitory (Glycine α 1 and GABA_A α 1) neurotransmitter receptor subunits was studied by immunohistochemistry in CD1 mouse DCN (P0- P21). Sections were double labeled with fluorescent Nissl to aid in cell type identification. Results: Excitatory receptors were present at birth and labeling was intense between P0 and P6 after which expression reduced and became localized to the soma and proximal dendrites of the pyramidal (Py) and cartwheel cells between P9 and P21, except for GluR2, which primarily labeled neuronal processes. Inhibitory receptors were not specifically expressed until after P6. Both Glycine and GABA_A α 1 subunits were first detected at P9. The GABA_A α 1 subunit was mainly expressed in granule cells and labeling increased between P9 and P12 and then underwent substantial refinement. In contrast, the expression of Glycine α 1 did not change significantly during the same period and labeling was seen mostly in the soma and processes of the Py cells.

Conclusions: Thus, the expression of excitatory receptors occurs before the inhibitory ones in the developing DCN. Both excitatory and inhibitory receptors were present before hearing onset (P12) and were subsequently refined, implying initial expression does not require sound stimulation, although auditory input would probably significantly influence further maturation of the expression pattern. Functional studies are required to correlate the expression pattern of these receptors with the onset of function.

P27 Evaluation of the neurogenic potential in the postnatal cochlear nucleus

*K. Rak¹, J. Völker¹, S. Frenz¹, A. Radeloff¹, R. Hagen¹, R. Mlynski¹

¹Universität Würzburg, Klinik und Poliklinik für Hals-Nasen und Ohrenkrankheiten, plastische und ästhetische Operationen, Würzburg, Germany

Neuronal stem cells (NSC) have been identified in the neonatal rat cochlear nucleus (CN) recently. The identified cells display all features of NSC, particularly the capacity to perform mitosis and differentiation in neuronal progenitor cells as well as all cells of the neuronal lineage. The aim of the present study was to determine the postnatal neurogenic potential until adulthood. Furthermore the capability of these NSCs to differentiate after allogeneic transplantation should be assessed.

The CN was prepared from the brain of Sprague-Dawley rat until P40. Neurosphere formation, BrdU-incorporation in whole-mount assay and immunohistological analyses of stem cell markers were performed. In addition expression profile of NSC-related genes was studied by quantitative Real Time PCR. For transplantation experiments P6 NSCs were marked by Dil, co-cultured with CN of P6 animals and analysed by immunohistochemistry.

Neurosphere formation could be detected until adulthood. BrdU incorporation was evident over the span of life, although the numbers of positive cells were reduced at later ages. Immunohistological and gene expression analysis of stem cell markers displayed the existence of NSC until adulthood in an age dependent matter. After allogeneic transplantation immigrated cells showed markers positive for NSCs and co-localized with neuronal structures.

The present results reveal the potential of neurogenesis in the CN right up to adulthood in rat and the capability of the NSCs to immigrate and differentiate in brain tissue. The potential role of this postnatal neurogenesis in the CN is still unclear. On one hand, NSC might be involved in the development of the cochlear nucleus and the auditory function at younger ages. On the other hand, a present neurogenic niche could be present for later activation induced by definite damages in the auditory pathway.

P28 A transient, afferent input-dependent, postnatal niche for neural progenitors in the cochlear nucleus

*S. Volkenstein^{1,2}, S. Sinkkonen², T. Jan³, S. Most³, A. Cheng³, K. Oshima², S. Dazert¹, S. Heller²

¹Ruhr-University of Bochum, St. Elisabeth-Hospital, Department of Otorhinolaryngology, Head & Neck Surgery, Bochum, Germany

²Stanford University School of Medicine, Departments of Otolaryngology - Head & Neck Surgery and Molecular & Cellular Physiology, Stanford/CA, USA

³Stanford University School of Medicine, Department of Otolaryngology - Head & Neck Surgery, Stanford/CA, USA

Many sensory systems undergo a critical period of postnatal plasticity in which input from the periphery affects the development of neurons and connections in the connected central pathways. In the cochlear nucleus, the first central relay of the auditory pathway, the survival of neurons during the critical period depends on afferent innervation from the cochlea. Although input-dependent neuron survival has been extensively studied, neurogenesis in the cochlear nucleus has not been evaluated as a possible mechanism of postnatal plasticity. Here we show that NeuN-expressing neurons are being generated in the neonatal cochlear nucleus. We further demonstrate that the cochlear nucleus harbors neurosphere-forming neural progenitor cells that are maintained during the critical period of postnatal plasticity. Our results suggest that neurosphere formation is controlled by a complex interplay of Wnt, Notch, and TGF β /BMP signaling, in which absence of TGF β /BMP signaling is permissive for progenitor proliferation that is promoted by Wnt and Notch activation. Using Axin2-lacZ reporter mice, we show that cochlear nucleus-derived neurospheres display active Wnt signaling and further, that cells with activated Wnt signaling reside in the cochlear nucleus. Finally, when we eliminated afferent innervation by cochlea ablation, we found a significant reduction of neurosphere-forming cells. Our results suggest that the neonatal cochlear nucleus maintains an afferent innervation-dependent niche for progenitor cells. These progenitors display active Wnt signaling, and we hypothesize that they are candidates for mediating neuro- and gliogenesis during the postnatal plasticity period of the auditory system.

Hair cells / Non-sensory cells

P29 The chloride-channel blocker anthracene-9-carboxylic acid reduces the nonlinear capacitance of outer hair cells by acting directly on prestin

*C. Harasztosi¹, A.W. Gummer¹

¹Eberhard Karls University Tübingen, Section of Physiological Acoustics and Communication, Faculty of Medicine, Tübingen, Germany

The chloride-channel blocker anthracene-9-carboxylic acid (9AC) has been shown to influence the effective stiffness of outer hair cells (OHCs) and reduce the electrically induced motion of the organ of Corti. The aim of this study is to identify the target of 9AC on OHCs, namely if it acts on the cytoskeleton or alters the function of the motor protein by blocking chloride channels or by directly interacting with prestin.

We measured the nonlinear capacitance (NLC) as a signature of prestin function in OHCs isolated from guinea pig and in prestin-transfected human embryonic kidney 293 (HEK293) cells. Measurements were made using the patch-clamp technique in whole-cell and inside-out configurations. The absolute electrical admittance parameters of OHCs were determined using the Lindau-Neher algorithm.

500-mM 9AC applied to the extracellular fluid significantly and reversibly reduced the NLC of both OHCs and prestin-transfected HEK293 cells. Experiments on excised patches, formed from the plasma membrane of OHCs, revealed that 9AC can reduce the NLC not only from the extracellular side, but also from the intracellular side of the plasma membrane. Reduction of the extracellular and intracellular chloride concentration to 5 mM by replacing chloride with gluconate caused a significant negative shift of the dose-response curve of the extracellular applied drug.

Since 9AC reduced the NLC not only of OHCs, but also of prestin transfected HEK293 cells, we suppose that 9AC directly interacts with the motor complex. The chloride sensitivity of the blocking effect of 9AC indicates that 9AC influences the chloride-binding capability of prestin. Since the drug reduces the NLC applied to either side of the plasma membrane, we conclude that 9AC could change the conformation of prestin by binding from either side of the plasma membrane.

P30 The RC time constant problem of outer hair cells: A re-examination

*K. Iwasa¹, M. Ospeck²

¹Max-Planck-Institut für Physik Komplexer Systeme, Dresden, Germany

²Independent, Boulder/CO, USA

The mammalian ear is a highly sensitive and frequency specific organ, owing to a built-in amplifier, where outer hair cells play a key role in providing motile feedback. This amplifier works in a wide frequency range, up to 40 kHz for guinea pigs, for example, to cover the auditory range. For the effectiveness of the motile mechanism based on prestin, a piezoelectric membrane protein specific to those cells, a bottleneck is the low-pass nature of the intrinsic cellular electric circuit, arising from resistance-capacitance (RC) coupling. This is called the RC time constant problem. It has been argued that the RC time constant should match the operation frequency for the effectiveness of the cochlear amplifier. Here this issue is examined based on a set of new experimental data [1,2].

The RC time constant issue has been discussed for a considerable time using various models [6, 8-14]. The present analysis using a simple model leads to the following conclusions:

1. Near isometric condition is required for the effectiveness of prestin-based motility of outer hair cells.
2. The limiting frequency based on this motility is much higher than previously estimated, likely covering the auditory frequency range for most mammals.
3. The RC-roll off frequency can be about 10% of the characteristic frequency.
4. Echo locating mammals, including dolphins and bats, have auditory ranges, which exceed 100 kHz, and share significant mutations in their prestin. In those animals, a larger value for the piezo coefficient could be also a factor.

[1] He, D. Z. Z., S. Jia, and P. Dallos, 2004. *Nature* 429:766-770. [2] Johnson, S. L., M. Beurg, W. Marcotti, and R. Fettiplace, 2011. *Neuron* 70:1143-1154.

P31 Membrane recycling at the basal pole of outer hair cells

E. Harasztosi¹, *C. Harasztosi¹, A.W. Gummer¹

¹Eberhard Karls University Tübingen, Section of Physiological Acoustics and Communication, Faculty of Medicine, Tübingen, Germany

Rapid endocytic activity of outer hair cells (OHCs) in the guinea-pig cochlea has been studied using the fluorescent membrane marker FM1-43 (Griesinger et al., 2004; Kaneko et al., 2006; Meyer et al., 2001). It was demonstrated that membrane particles were intensively endocytosed at the apical pole of OHCs and trancytosed to different locations, namely into the basolateral membrane and to the subnuclear region. In this study we are investigating local membrane recycling located to the subnuclear region of OHCs.

We used confocal laser scanning microscopy to visualize membrane recycling at the basal pole of isolated OHCs from the guinea-pig cochlea with the application of styrylpyridinium fluorescent dye FM1-43. In our study perfusion system was used that maintains a constant laminar flow around the entire OHC. This perfusion system guarantees that upon dye application the FM1-43 arrives at the same time and concentration to both the apical and basal membranes.

We demonstrate that after dye application the fluorescence intensity immediately starts increasing in both the infracuticular and the subnuclear regions. However, the signal in the subnuclear region increased slower, up to 62 % relative to the fluorescence intensity measured in the infracuticular zone after 50-s long dye application. In comparison, the signal in the supranuclear region was significantly slower than the signal in the subnuclear region and it reached the maximum value of 80 % relative to the subnuclear-pole fluorescence intensity after the 50-s long dye application. The dynamics of this signal showed similarity to the fluorescence signal recorded 15 - 20 μm below the infracuticular zone.

These data indicate that vesicles accumulating in the subnuclear pole of the OHCs are not exclusively trancytosed from the apical pole of the cell, but also locally endocytosed, therefore recycled at the basal pole of OHCs.

P32 Voltage gated sodium channels in immature cochlear hair cells

*T. Eckrich¹, K. Varakina², S.L. Johnson¹, C. Franz², W. Singer², S. Kuhn¹, M. Knipper², M.C. Holley¹, W. Marcotti¹

¹University of Sheffield, Department of Biomedical Science, Sheffield, United Kingdom

²University of Tübingen, Department of Otolaryngology, Tübingen, Germany

Introduction: Inner hair cells (IHCs), the genuine sensory cells for sound in mammals, are able to generate spontaneous calcium-dependent action potentials prior to the onset of hearing (Marcotti et al. 2003 J Physiol 552,743-61). Action potentials are thought to instruct developmental changes in the immature cochlea such as those involved in gene expression and/or synaptic refinement of the auditory fibres (Kros et al. 1998 Nature 394,281-284; Kandler et al. 2009 Nat Neurosci 12,711-717). Several membrane currents are involved in the generation and modulation of action potential activity (Johnson et al. 2011 Nat Neurosci 14,711-717), including the transiently expressed voltage gated Na⁺ current. To date, the biophysical properties and functional role of the Na⁺ current are poorly understood. We investigated the Na⁺ current in rat IHCs as a function of development and cochlear region.

Methods: We performed whole cell patch-clamp recordings from IHCs maintained in acutely dissected organs of Corti from pre-hearing rats. Both apical and basal IHCs were investigated at body temperature. The molecular composition of the pore forming α subunit was tested using immunocytochemistry.

Results: All immature IHCs tested showed a Na⁺ current with very rapid activation and inactivation kinetics, which were highly temperature dependent. The size and kinetics of the Na⁺ current changed as a function of IHC age and position along the cochlea. Antibodies against NaV1.2 and 1.6, but not NaV1.1 and 1.7, labeled IHCs.

Conclusions: Our results reveal that the Na⁺ current is active at physiological membrane potentials and involved in action potential generation.

This work is supported by the Wellcome Trust to WM

P33 BK channel ablation in hair cells is critical for coding the temporal structure and dynamic range of auditory information for central auditory processing

*S. Kurt¹, M. Sausbier², L. Rüttiger³, N. Brandt^{4,5}, C. Möller^{6,7}, J. Kindler⁶, U. Sausbier², U. Zimmermann^{3,5}, H. von Straaten^{3,5}, W. Neuhuber⁸, J. Engel^{4,5,9}, M. Knipper^{3,5}, P. Ruth², H. Schulze^{9,7}

¹University Ulm, Institute of Neurobiology, Ulm, Germany, ²Institute of Pharmacy, Department of Pharmacology and Toxicology, Tübingen, Germany, ³Molecular Physiology of Hearing, Department of Otorhinolaryngology, Tübingen, Germany, ⁴Institute of Physiology II, Tübingen, Germany, ⁵Tübingen Hearing Research Centre, Tübingen, Germany, ⁶Department of Experimental Otolaryngology, Erlangen, Germany, ⁷Leibniz Institute for Neurobiology, Magdeburg, Germany, ⁸Institute for Anatomy, Erlangen, Germany, ⁹Saarland University, Department of Biophysics, Homburg, Germany

BK channels in inner hair cells (IHCs) of the cochlea are essential for hearing. However, germline deletion of BK α , the pore-forming subunit KCNMA1 of the BK channel, did not affect hearing thresholds in the first postnatal weeks even though altered IHC membrane time constants, decreased IHC receptor potential AC/DC ratio, and impaired spike timing of auditory fibers were reported in these mice. To investigate the role of IHC BK channels for central auditory processing, we generated a conditional mouse model with hair-cell specific deletion of BK α from day 10 onwards. Whole-cell voltage-activated K⁺ currents were recorded in IHCs. Also we tested the acuity of central temporal coding of conditional BK mice by recording responses to temporally modulated sounds from neurons in the central nucleus of the inferior colliculus (ICC). Additionally a behavioral sound discrimination task was performed.

A number of significant differences between the hair cell-specific BK mutants and their littermate controls could be identified, such as the absence of fast BK currents in IHCs. The mutants had an unexpected impact on temporal coding in the central auditory system: neuronal single and multi-unit responses in the inferior colliculus showed higher excitability and greater precision of temporal coding that may be linked to the improved learning performance in a behavioral auditory discrimination task of temporally modulated sounds. The higher precision of temporal coding, however, was restricted to slower modulations of sound and reduced stimulus-driven activity. This suggests a diminished dynamic range of stimulus coding that is expected to impair signal detection in noise. These differences shed light on the functional role of the BK channel in hair cells for normal hearing function, as they point to a crucial role of BK in IHCs for defining the temporal fine structure of sound and the dynamic range of auditory information, e.g. for detection of signals in a noisy environment in central auditory system.

P34 The properties of short hair cells in the avian basilar papilla

*R. Fettiplace¹, M. Beurg¹, X. Tan¹, S. Mahendrasingam¹, C. Hackney¹

¹University of Wisconsin-Madison, Neuroscience, Madison, United Kingdom

The origin of frequency selectivity in the avian basilar papilla is incompletely understood. The tall hair cells (THCs) are thought to be electrically tuned over part of the frequency range but little is known about the short hair cells (SHCs). To address this problem, we made patch clamp recordings from SHCs in an isolated chicken basilar papilla preparation (E18 to P5; T = 33 °C; at fractional distances along the papilla from the apex, $d = 0.1-0.6$). SHCs are analogous to mammalian outer hair cells in having a large efferent but sparse afferent innervation and are distinguished by possessing an A-type inactivating K⁺ current (Murrow 1994). We found that SHCs are also electrically tuned by a Ca-activated K⁺ conductance, but such tuning is observed only at resting potentials positive to -40 mV where the A-current is inactivated. The depolarized resting potential stems from a standing inward current through the mechanotransducer (MT) channels, whose resting open probability, P_r , is about 0.35. Two factors produce the high P_r : a low endolymph calcium (0.24 mM) and high intracellular calcium buffering. With perforated patch recordings using the MT current P_r as an assay, we estimated the intracellular calcium buffer as equivalent to 0.5 mM BAPTA ($d = 0.4$). To confirm the high levels of buffer, the distribution of calcium binding proteins in SHCs was examined with post-embedding immunogold labeling in transmission electron micrographs. Heavy stereociliary labeling was seen with antibodies to both calbindin-28K and parvalbumin-3 and displayed an apex to base gradient matching the tonotopic gradient in the MT current amplitude. The stereociliary concentrations of both calcium-binding proteins were estimated by calibrating immunogold counts against gels containing defined amounts of the proteins (Hackney et al 2003). We conclude that avian SHCs resemble mammalian outer hair cells in their large calcium buffer content, high resting open probability for MT channels and depolarized resting potential. However, SHCs do not contain prestin and the source of any electromechanical amplification remains to be established.

Funded by RO1 DC01362 from NIDCD to RF

P35 Evidence for K⁺ accumulation around mammalian vestibular Type I hair cells: An electrophysiological study

*E. Tavazzani¹, D. Contini¹, G. Russo¹, S. Masetto¹, I. Prigioni¹

¹University of Pavia, Dept. of Public Health and Neuroscience, Pavia, Italy

Type I vestibular hair cells are contacted by an unusual calyx nerve terminal that envelops the entire basolateral membrane. In addition, they express a signature low-voltage activated outward K⁺ current (IK,L) that dominates the membrane conductance. The functional meaning of IK,L and of the afferent calyx is still enigmatic. It has been speculated that the calyx is responsible for K⁺ accumulation in the synaptic cleft and that this accumulation cooperates with vesicular synaptic release in sustaining afferent transmission. By combining the patch-clamp whole-cell configuration with the whole crista preparation, we have recorded the current and voltage responses of mouse semicircular canal Type I and Type II hair cells in situ. Depolarizing voltage steps elicited in many Type I hair cells the conventional large and sustained outward K⁺ current. However, in a notable percentage (51%) of Type I hair cells, the outward K⁺ current showed an evident time-dependent relaxation. In these cells, moreover, upon repolarization to -40 mV, the instantaneous current was inward, reversing to outward slowly with time. A reasonable explanation for the above results is that during large outward K⁺ currents, K⁺ accumulates around Type I hair cells, thus shifting the K⁺ reversal potential (VrevK⁺) toward more positive values. Since we never observed such effects when recording from Type II hair cells, we hypothesized that the presence of a residual nerve calyx was responsible for K⁺ accumulation. We also found that by using voltage protocols that increased extracellular K⁺ accumulation, IK,L deactivation was slowed down. Similar results, i.e. VrevK⁺ rightward shift and IK,L deactivation slowdown, were obtained by local perfusion of the preparation with an extracellular solution enriched in K⁺. In conclusion, our results provide electrophysiological evidence for an increased K⁺ concentration in the synaptic cleft between Type I hair cell and its calyx ending during outward K⁺ current activation. The resulting depolarization might be aimed at reinforcing and prolonging Ca²⁺ channels activation and thus afferent transmission during slow head movements detected by vestibular organs.

P36 Sodium-activated potassium-current expression in the vestibular afferent neurons of the vestibular ganglion of the rat

*E. Soto¹, R. Vega¹, B. Cervantez¹

¹Instituto de Fisiología - BUAP, Puebla, Pue., Mexico

Vestibular afferent neurons transmit information from the vestibular end organs to central nuclei. That information is coded within the firing discharge of these cells and is heavily influenced by the specific set of K^+ conductances located throughout the vestibular neuron. In this study we describe the presence of a Na^+ -activated K^+ conductance (K_{Na}) previously not identified in vestibular ganglion neurons isolated from the Long-Evans rats. We found that the blocking of Na^+ channels by 100 nM TTX, or the substitution of choline for Na^+ in the extracellular solution during voltage clamp pulses, revealed a sustained outward current that depended on extracellular Na^+ . Furthermore, increments in the intracellular concentration of Na^+ by blocking the Na^+/K^+ ATPase with 1 m Mouabain decreased the amplitude of the inward Na^+ current and increased the amplitude of the outward current. Substitution of Li^+ for Na^+ in the extracellular solution significantly reduced the amplitude of the outward current in voltage clamp pulses and decreased the after hyperpolarization of action potentials in current clamp experiments. The electrophysiological results are consistent with the presence of mRNA transcripts for the K_{Na} subunits Slick and Slack in the vestibular ganglia and in the sensory epithelium determined by RT-PCR, and with the immunolabeling of Slick and Slack in isolated vestibular neurons, in the vestibular ganglion and in the vestibular sensory epithelium. These results indicate that K_{Na} channels are expressed in vestibular afferent neurons and their terminals, and may contribute to the firing regularity and precision of spike timing of vestibular information.

This work was supported by PIFI-2011 and BUAP-VIEP grants to ES and RV.

P37 Ca²⁺ channel $\alpha_2\delta_3$ subunits in spiral ganglion neurons – establishing a cell culture system for functional analysis

*V. Scheuer¹, G. J. Obermair², T. Eckrich¹, B. Schick³, J. Engel¹

¹Saarland University, Department of Biophysics, Homburg/Saar, Germany

²Innsbruck Medical University, Dept. of Physiology and Medical Physics, Innsbruck, Austria

³Saarland University, Dept. of Otolaryngology, Homburg/Saar, Germany

Voltage-gated calcium channels (VGCCs) are protein complexes composed of one pore-forming subunit α_1 and auxiliary subunits $\alpha_2\delta$ and β . The $\alpha_2\delta$ subunits (SU) 1-4 are known to assist in Ca²⁺ channel trafficking and to modulate I_{Ca} gating properties, however, their role in the hearing process remains elusive. Mice deficient for the $\alpha_2\delta_3$ SU ($\alpha_2\delta_3^{-/-}$) have mildly elevated hearing thresholds, distorted ABR waveforms and less Ca_v2.1 immunostaining in spiral ganglion (SG) neurons suggesting reduced expression of presynaptic voltage-activated Ca²⁺ channels at the auditory nerve - bushy cell synapse (Pirone et al., Engel, submitted).

We want to find out which $\alpha_2\delta$ SUs (mRNA and protein) are expressed in wildtype mouse SG neurons and how deletion of $\alpha_2\delta_3$ changes the normal expression pattern of VGCCs. We are further interested in the types of voltage-gated Ca²⁺ currents in SG neurons, in tonotopic gradients and possible effects of $\alpha_2\delta_3$ deletion. Analysis of SG neurons is complicated by the fact that the ganglia are encapsulated in bone. Therefore, we use SG explants and primary cultures at ages \leq postnatal day 6 (P6) before ossification is complete.

Quantitative RT PCR of cDNA synthesized from mRNA isolated from whole-mount SG stripes of wild type mice (WT) at P4-P6 revealed expression of $\alpha_2\delta_1$ -3 but not $\alpha_2\delta_4$ in the apical and basal half of the cochlea, with $\alpha_2\delta_3$ being significantly stronger expressed over $\alpha_2\delta_1$ and $\alpha_2\delta_2$ in the basal half. In $\alpha_2\delta_3^{-/-}$ mice, transcript numbers of $\alpha_2\delta_1$ and $\alpha_2\delta_2$ were significantly elevated as compared with WT.

Currently we are establishing primary cell culture systems of SG explants as well as enzymatically isolated SG neurons for electrophysiological and Ca²⁺ imaging analyses. First immunohistochemical characterization of explant cultures revealed co-staining of an $\alpha_2\delta_3$ antibody with the neuronal marker beta-tubulin (TUJ1) and staining of about 10% of SG neurons with anti-peripherin, a marker specific for SG neurons type II. Specificity tests for $\alpha_2\delta_3$ and Ca_v2.1 antibodies as well as functional characterization of isolated cultured SG neurons are in progress.

P38 Role of nitric oxide on the P2Y₁ purinoceptor-mediated Ca²⁺ waves in Hensen's cells

*N. Harada¹, K. Tomoda²

¹Harada ENT Clinic, Osaka, Japan

²Kansai Medical University, Otolaryngology, Osaka, Japan

ATP is known to act as an extracellular messenger mediating the propagation of Ca²⁺ waves in Hensen's cells. It has been shown that metabotropic P2Y₂ and P2Y₄ receptors predominantly act via the PLC-IP₃ signal transduction pathway for Ca²⁺ waves in Hensen's cells. Here we investigated whether the activation of P2Y₁ purinoceptor can induce the Ca²⁺ waves in Hensen's cells. We also investigated the role of nitric oxide (NO) on the P2Y₁ purinoceptor-mediated Ca²⁺ waves in Hensen's cells. 2-MeSATP, a selective P2Y₁ receptor agonist mediated the propagation of Ca²⁺ waves in Hensen's cells in the Ca²⁺-free medium. Simultaneous measurements of intracellular Ca²⁺ changes and NO production revealed that 2-MeSATP-mediated Ca²⁺ waves were accompanied with NO production in Hensen's cells. 2-MeSATP-induced NO production was blocked by the selective P2Y₁ antagonists, MRS2179 and A3P5P in Hensen's cells. L-N^G-nitroarginine methyl ester, a non-specific NO synthase inhibitor inhibited the 2-MeSATP-mediated Ca²⁺ waves in Hensen's cells while the 2-MeSATP-induced Ca²⁺ increase was seen in the stimulated cell . Therefore, functional role of P2Y₁ receptors on ATP-mediated Ca²⁺ waves may exist in Hensen's cells. NO may play a role in the P2Y₁-mediated Ca²⁺ waves in Hensen's cells.

Cochlear mechanics

P39 Frequency dependent material properties of wild-type and mutant tectorial membranes

*G. Jones¹, V. Lukashkina¹, I. Russell¹, S. Elliott², A. Lukashkin¹

¹University of Brighton, School of Pharmacy and Biomolecular Sciences, Brighton, United Kingdom

²University of Southampton, Institute of Sound and Vibration Research, Southampton, United Kingdom

Introduction: The refined sensitivity, frequency selectivity, and impressive dynamic range of the mammalian cochlea depend on its intrinsic graded properties and unique active feedback mechanism. Timing and spatial distribution of the active feedback are determined, in particular, by the mechanical properties of the tectorial membrane (TM). These properties are frequency dependent and vary within the physiologically realistic experimental frequency range (2-20 kHz) used in this study; this frequency dependency has not yet been considered in mathematical models of the cochlea.

Methods: Segments of TM (~400-100 μm) were isolated from wild-type and mutant mice (*Tecta*^{Y1870C/+}, *Tectb*^{-/-} and *Otoa*^{EGFP/EGFP}). Using laser interferometry and a model accounting for both the frequency dependent nature of the viscoelastic properties of the TM, and the *in vitro* fluid environment, the longitudinal propagation of radially shearing travelling waves was tracked in an isolated segment of TM. This segment of TM was positioned between two supports, one of which was vibrated radially at frequencies 2-20 kHz. The laser was focused on the marginal edge of the isolated TM segment, and stepped longitudinally. Phase and amplitude data collected at each point allowed the calculation of the wave's propagation velocity and spatial decay. Using the model, the shear storage modulus (G') and shear viscosity (η) of the TM were calculated across the experimental frequency range.

Results: Wave propagation varied as a function of frequency in wild-type mice, but less so in mutant mice. In the wild-types the stiffness of the TM increased and viscosity decreased as stimulation frequency increased, in segments of TM isolated from both the basal and apical regions of the cochlea. The TM segments from the mutant groups were affected to differing degrees; in general they were less stiff and did not increase in stiffness with stimulation frequency.

Conclusion: The resulting stiffening of the TM and decrease in its viscosity at high stimulus frequencies facilitates active feedback and energy transmission, especially in the basal, high-frequency, region of the cochlea. An important outcome of our analysis of the mechanical properties of the TM is the prediction that TM resonance becomes sharper in the basal, high frequency region of the cochlea, as is indeed confirmed by *in vivo* neural recordings. From a comparison between the mechanical properties of the TM and the neural responses of the cochlea, we conclude that a substantial part of energy in the cochlea is dissipated due to internal friction within the TM.

P40 About general properties of cochlear nonlinearity

*H. Duifhuis¹

¹University of Groningen, emeritus Fac Math & Natural Sc, Paterwolde, The Netherlands

Question: Are auditory nonlinear (NL) phenomena independent or do they originate from a common source?

Method: Analysis of NL cochlear models

Results: Using the tool of time domain analysis of the NL cochlea, basically because the frequency domain analysis, which is generally used, may be unreliable for NL systems, we analyzed several classical NL phenomena, such as:

- Tartini tones or combination tones ($2f_1 - f_2$ and difference tone $f_2 - f_1$, around 1710)
- NL level effects in pure tone masking (Wegel and Lane, 1924)
- NL level effects in loudness growth (ISO: approximately the dB-scale)
- 2-tone suppression (Nomoto et al, Sachs & Kiang, sixties)
- Auditory emissions (Kemp and others, since end seventies)

Conclusions: We propose that these phenomena are all related to NL properties of the intact cochlea. Cochlear hearing loss will affect the integrity of the NL properties, which are relevant for normal hearing.

P41 Comparison of low-frequency acoustical biasing of DPOAEs in humans and guinea pigs

*T. Weddell¹, A. Lukashkin¹, E. Krause², R. Gürkov², M. Drexel²

¹University of Brighton, Brighton, United Kingdom

²Ludwig-Maximilians University Munich, German Dizziness Centre (IFB), Munich, Germany

Introduction: Distortion product otoacoustic emission (DPOAE) input/output functions in guinea pigs and many rodents show differences in growth as the levels of the primaries are increased compared to humans. In rodents this growth is non-monotonic and contains a consistent and reproducible 'notch' at primaries between 60-70 dB SPL and an associated phase shift of 180 degrees, whereas human DPOAE input/output functions rarely contain notches and when they do they tend to be inconsistent between individuals. Different models have been hypothesized to account for these DPOAE input/output functions and caution has been called for when extrapolating from animal investigations to humans.

Methods: DPOAEs were recorded from humans over a range of primary tone levels in the presence of a low frequency biasing tone of 30 Hz at 120 dB SPL. The resultant modulated 2f1-f2 patterns observed were compared with a similar study in guinea pigs and both sets of results used to derive, through non-linear fitting based on the Boltzmann model, the underlying transfer function and resting OP for both species.

Results: The results obtained suggest that for the parameters used in this study DPOAE generation in both species can be predicted by a single saturating non-linearity that is spatially localized.

Conclusions: Any differences found in input/output growth functions between species can be predicted by the same model whereby guinea pig and humans have different cochlear operating points. Therefore the study supports the transferability of DPOAE findings between common laboratory species such as rodents and humans.

P42 Age-related changes in the frequency of sound perception: The biophysical basis

*E.L. Ovchinnikov¹

¹Samara State Medical University, Samara, Russian Federation

Introduction: Auditory effects with age are accompanied by changes (and sometimes by distortions) of frequency perception. Usually there is a decrease of the upper and increase of the lower frequency, although a cochlea, as bone formation, does not develop after birth. Biophysical study of the effect is not present.

The purpose and methods: Changing the perception of sound frequency is an important theoretical and practical problem. The etiology and mechanisms of the phenomenon were not identified. The aim of this study is to establish the biophysical mechanisms of age-related (time) perception of sound frequency for the prediction and reconstruction of the dynamics of the process in time. The problem is solved on the basis of biophysical and mathematical modeling.

Results: We can assume that the sound waves act on the cochlear duct throughout life, creating a continuous destruction of loose membranes in apex and reducing the length of the duct. This process is due to the cyclic action of sound on the cochlear duct and with a decrease in the number of auditory receptors. This phenomenon we identified as an effect of the age evolution of the cochlear canal and its structural elements.

The quantitative result, which characterizes the process, was marked only G. von Békésy (1960), which believes that every six months of life, is a reduction in the upper limit of audible range of 80 Hz (or 1% of the audible range during the year).

The experimental fact can be represented by the statement that for every year of life, a person loses an equal share of their perceived sound diapason: $-\Delta f/f=r\Delta t$.

Turning to the infinitely small, we have the differential equation $-df/f=rdt$.

Integrating, we obtain the general solution of the $-\ln(f/f_{mo})=rt$, and $f(t)=f_{mo}\exp(-rt)$.

The coefficient r is interpreted as the rate of loss of high frequency range (with the unit $[r]=1 \text{ year}^{-1}$). Using the condition G.von Békésy: for $t=1 \text{ year}$, $f_{mo}=20$ and $f_{1 \text{ year}}=0.99 f_{mo}=19.8 \text{ kHz}$, we have $r = 0.0100 \text{ year}^{-1}$.

The solution is defined as the standard frequency-time law of age evolution of the cochlear duct of the human inner ear.

Discussion: The calculation shows that for the hearing organ a safety margin rate up is to 700 years, a lot higher ($8 \div 10$ times) than human life. But this is not the case. The membranes cut off with the same speed low frequencies, reducing the lifetime of the inner ear in half that it is already in compliance with technical devices.

Conclusions: Knowledge of the actual boundaries of the perceived range of sounds is diagnostic, therapeutic, preventive and environmental basis of human life for the solution of practical problems.

Neurotransmission

P43 Spike encoding of neurotransmitter release timing by spiral ganglion neurons of the cochlea

*M. Rutherford¹, N. Chapochnikov¹, T. Moser¹

¹University of Goettingen, InnerEarLab, Goettingen, Germany

Mammalian cochlear spiral ganglion neurons (SGNs) encode sound with microsecond precision. Spike triggering relies upon input from a single ribbon-type active zone of a presynaptic inner hair cell (IHC). Using patch-clamp recordings of rat SGN postsynaptic boutons innervating the modiolar face of IHCs from the cochlear apex, at room temperature, we studied how spike generation contributes to spike timing relative to synaptic input. SGNs were phasic, firing a single short-latency spike for sustained currents of sufficient onset slope. Almost every EPSP elicited a spike, but latency (300-1500 μ s) varied with EPSP size and kinetics. When current-clamp stimuli approximated the mean physiological EPSC (\sim 300 pA), several times larger than threshold current (rheobase, \sim 50 pA), spikes were triggered rapidly (latency, $<$ 500 μ s) and precisely (SD, $<$ 50 μ s). This demonstrated the significance of strong synaptic input. However, increasing EPSC size beyond the physiological mean resulted in less-potent reduction of latency and jitter. Differences in EPSC charge and SGN baseline potential influenced spike timing less as EPSC onset slope and peak amplitude increased. Moreover, the effect of baseline potential on relative threshold was small due to compensatory shift of absolute threshold potential. Experimental first-spike latencies in response to a broad range of stimuli were predicted by a two-compartment exponential integrate-and-fire model, with latency prediction error of $<$ 100 μ s. In conclusion, the close anatomical coupling between a strong synapse and spike generator along with the phasic firing property lock SGN spikes to IHC exocytosis timing to generate the auditory temporal code with high fidelity.

P44 Multiple actions of GABA at murine LSO neurons

*A. Fischer¹, M.A. Xu-Friedman², E. Friauf¹, D. Griesemer¹

¹TU Kaiserslautern, Biology, Kaiserslautern, Germany

²University at Buffalo, Biological Sciences, Buffalo/NY, USA

Bilateral information from the auditory periphery converges in the superior olivary complex of the mammalian brainstem, a group of nuclei processing sound location. Here, we investigate the inhibitory projection from the medial nucleus of the trapezoid body (MNTB) to the lateral superior olive (LSO) where inhibition is mediated by Gamma-aminobutyric acid (GABA) together with glycine. In gerbils and rats GABA is described to be of great importance during early postnatal development. GABA is released by exocytosis from synaptic terminals or through channels and transporters. The subcellular localization, and the intrinsic properties of GABA_A and GABA_B receptors, together with variances of GABA concentration in the synaptic cleft, as well as of the "ambient" GABA levels always present in the extracellular space, constitutes major sources of diversity in GABA-mediated signaling. GABA transporters are thought to influence GABA concentration and thereby GABA action, but the details of this regulation are unclear. We use the inhibitory projection from the MNTB to the LSO to analyze the contribution of the different receptor- and transporter types to the GABA signaling. To do so, whole-cell voltage-clamp recordings were obtained from LSO principal neurons in acute mouse brain slices at postnatal day 11 ± 1 . GABA (100 μ M) was focally applied onto cell somata to evoke currents. While repetitive application of GABA (24 pulses at 0.2 Hz) displayed constant amplitudes of 1.07 ± 0.17 (1st pulse) to 0.93 ± 0.16 nA (24th pulse), pharmacological treatment with specific GAT blockers (GAT1: NO-711/GAT3: SNAP5114) resulted in a strong depression of 55.1 ± 4.4 % (1st vs. 24th pulse; $p < 0.001$). Concomitantly, the average current decay time significantly increased 3.4-fold from 264.8 ± 0.6 to 903.5 ± 10.7 ms ($p < 0.001$). Application of GAT3 blocker alone led to a mild 16.0 ± 5.1 % depression ($p < 0.05$) and an intermediate 1.8-fold increase of the average current decay time ($p < 0.001$). To further elucidate presynaptic actions of GABA we established an exclusively presynaptic calcium imaging assay by which we could show a GABA_BR-mediated 20.1 ± 3.3 % decrease of calcium influx due to application of baclofen (100 μ M).

Given the functional presence of postsynaptic GABA_A- and presynaptic GABA_B- receptors, together with GAT1 and GAT3 dependent modulation of GABAergic currents during focal transmitter application in the LSO, we will now focus on GAT-dependent modulation of synaptic transmission at the MNTB-LSO synapse.

P45 Auditory-task-evoked efferent inhibition in the cochlea enhances hearing and increases with task difficulty

N. Francis^{1,2}, *J. Guinan^{3,1}

¹Harvard-MIT Division of Health Sciences and Technology, Program in Speech and Hearing Bioscience and Technology, Cambridge/MA, USA

²Mass Eye & Ear Infirmary, Eaton Peabody Lab, Boston/MA, USA

³Mass. Eye & Ear Infirmary; Harvard Medical School, Eaton Peabody Lab, Boston/MA, USA

Introduction: Natural acoustic environments often contain background noise that interferes with the perception of sounds in the auditory foreground. It has been hypothesized that auditory-task-evoked activity in the brainstem's medial olivocochlear (MOC) efferent pathway inhibits cochlear responses to the background noise, thereby enhancing the perception of brief sounds in the auditory foreground. However, conclusive evidence for this hypothesis has been elusive.

Methods: Here, we used a novel, non-invasive measurement paradigm in which human subjects did an auditory discrimination of brief tones in acoustic background noise, while efferent inhibition was measured by the change in otoacoustic emission magnitudes in the task ear. Efferent inhibition was also measured while presenting the same sounds, but without task performance.

Results: The results show that attending to brief tones in noise caused, in each trial, a transient increase in efferent inhibition that was significantly larger than the efferent inhibition measured without a task. When the results from the task were divided into trials with correct and incorrect auditory discriminations, significantly more efferent inhibition was found on the correct trials, which is strong evidence for a perceptual benefit of MOC efferent activity. In addition, we found that efferent inhibition increased with auditory task difficulty, suggesting that efferent inhibition may indicate listening effort.

Conclusions: Our results provide compelling evidence that there exists a dynamic and task-dependent interaction between the central and peripheral auditory systems that changes the operation of the cochlea to enhance hearing in noisy acoustic environments.

Supported by NIDCD RO1DC005977, P30DC005209

P46 The inhibitory effects of ANP in rat auditory system using ABR

*Y.J. Yoon¹, S. Hellstrom²

¹Chonbuk National University Hospital, Otolaryngology, Jeonju, Republic of Korea

²Karolinska University Hospital, Audiology, Stockholm, Sweden

ANP (atrial natriuretic peptide), which is the inhibitory neurotransmitter in the CNS, is thought to be the transmitter in the hair cells' efferent nerve synapse in the cochlea. Under the suggestion that ANP may be caused inhibitory action in the auditory system, the effect induced by ANP on ABR was examined in anesthetized rats. Twenty-five healthy Sprague-Dawley rats were deeply anesthetized with an intravenous injection of Brietal through a tail vein follow by Mebumal intraperitoneally. In the first study, we examined the effects of intra-arterial bolus injection of ANP (0.1mg/Kg, 4mg/Kg and 8mg/Kg) dissolved in 0.2ml physiologic saline. After injection of ANP, ABR was continuously monitored for about 30 min. The frequencies tested were done at three different frequencies (1, 2, and 20kHz). Intra-arterial infusion of ANP (0.1mg/Kg, 4mg/Kg and 8mg/Kg; bolus injection) resulted in a marked and significant increase in ABR thresholds for both 1kHz and 2kHz. Increased amount of ANP significantly altered the ABR response to ANP in rats. Significant shift in the ABR wave II latency were observed in lower frequency, which was appeared dose dependent. The significant increase in ABR threshold produced by infusion of ANP may create on biological effects in the regulation of cochlear fluid or in the neuromodulation of auditory nervous system.

P47 Modulation of the vestibular afferent neuron discharge rate by opioid peptides in the isolated inner ear of the rat

*R. Vega¹, A. Ortega¹, E. Seseña¹, E. Soto¹

¹Instituto de Fisiología - BUAP, Puebla, Pue., Mexico

We have previously reported the inhibitory action of Kappa opioid receptor activation on the calcium current in hair cells and the modulatory action of the Mu opioid receptor activation on the response of amphibian vestibular afferent neurons to excitatory amino acid agonists (Vega and Soto, 2003). In this work we studied the action of the Mu opioid receptor agonists and antagonists in the electrical discharge of the vestibular afferent neurons in the isolated vestibule preparation of the rat and also in the current clamp response in isolated vestibular ganglion neurons of the rat. The multiunit vestibular nerve discharge was registered using a suction electrode in C2 Long-Evans rats of age P14-18. The isolated inner ear was continuously perfused with Tyrode saline solution at 37 C and oxygenated using 95% O₂ and 5 CO₂. The nerve discharge of the isolated vestibular preparation showed a stable discharge during a period of about two hours. Experiments using microperfusion (40 µl) of the Mu opioid selective agonists 1 µM endomorphin-1 produced a huge 10-times increase in the discharge rate (n = 9), and 100 nM endomorphin-1 produced a 2.8-times increase of the discharge rate (n = 5). The co-perfusion of 1 µM endomorphin-1 and the Mu receptor selective antagonist 10 µM H-D-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) produced no effect on the discharge of vestibular afferent neurons. Indicating that the excitatory effect produces by endomorphin-1 is specific. In current clamp recordings using isolated afferent neurons from the rat inner ear shown that the use of the Mu selective agonist [D-Ala, N-Me-Phe, Gly-ol]-enkephalin (DAMGO, 1 µM) potentiates the response produced by 100 nM Kainic acid perfusion 2-times (n = 9), thus corroborating in isolated cells the modulatory action of Mu opioid receptor activation on the excitatory amino acid input to vestibular afferent neurons. These results further support the evidence indicating that Mu opioid receptors exert a significant modulatory action in the vestibular afferent neuron electrical activity, and that the mechanism of action is most probably mediated by a postsynaptic action upon the vestibular afferent neurons potentiating the cell response to the afferent neurotransmitter. Also the use of the rat inner ear extends these results to mammals, showing that opioid modulation of vestibular input is a conserved mechanism during evolution.

This work was supported by PIFI-2011 and BUAP-VIEP grants to ES and RV.

Homeostasis

P48 Diffusive water permeability of the cochlear duct is in the range of other aquaporin-expressing epithelia

*A. Eckhard¹, M. Müller¹, H. Löwenheim¹

¹University of Tübingen, Department of Otolaryngology, Head and Neck Surgery, Tübingen, Germany

Introduction: Endolymph and perilymph are separated by the epithelial boundary of the cochlear duct and its intercellular tight-junctions, the "perilymph-endolymph barrier" (PEB). Despite such strict separation, the PEB allows for a rapid exchange of ions and water between endolymph and perilymph. Molecular substrates of ion exchange across the PEB (e.g. ion channels, ion pumps) have been exceedingly investigated, while the remarkably high water permeability of the PEB (130-times greater than ions) remains unexplained at the molecular level. The discovery of aquaporins (AQPs) in various water transporting epithelia including the cochlear duct opens up new avenues to explain the high water permeability of the PEB.

Materials and Methods: We determined the diffusive water permeability coefficient (P_D) of the cochlear PEB. Calculations of P_D were performed for a 'closed' and an 'open' compartment model of the cochlear endolymphatic fluid space. Therefore, literature data about transepithelial water dynamics across the cochlear duct, morphological data about cochlear fluid spaces, as well as software-based water dynamics simulations between cochlear endolymph and perilymph using the Cochlear Fluids Simulator were applied.

Results: For the 'open' compartment model P_D of the whole cochlear PEB was calculated with $11.17 \times 10^{-5} \text{ cm} \times \text{s}^{-1}$, PEB of Reissner's membrane was $18.83 \times 10^{-5} \text{ cm} \times \text{s}^{-1}$ and that of the neurosensory epithelium on the basilar membrane $9.33 \times 10^{-5} \text{ cm} \times \text{s}^{-1}$. The P_D -values calculated for the 'open' compartment model were in accordance with the data determined in *in vivo* experiments on guinea pig cochleae.

Conclusions: P_D -values of the cochlear PEB are comparable to those of various other AQP-expressing epithelia. These range from $0.036 \times 10^{-5} \text{ cm} \times \text{s}^{-1}$ (distal airway epithelium) to $640 \times 10^{-5} \text{ cm} \times \text{s}^{-1}$ (kidney collecting duct epithelium). To definitively determine the role of aquaporins for the transepithelial water permeability in the cochlea, the osmotic water permeability coefficient (P_f) of the cochlear PEB has to be determined experimentally. However, our results serve as a basis to develop hypotheses about the function of AQPs in transepithelial cochlear water homeostasis.

P49 Expression of aquaporins (AQPs)-10/11/12 in the lateral wall of the rat cochlea

*R. Nishioka¹, T. Takeda², A. Kakigi³, M. Komori⁴, T. Kobayashi⁴, M. Hyodo⁴

¹Tosa Municipal Hospital, Otolaryngology, Tosa, Japan

²Nishinomiya Municipal Cent. Hospital, Otolaryngology, Nishinomiya, Japan

³Tokyo University, Otolaryngology, Bunkyo, Japan

⁴Kochi Medical School, Otolaryngology, Nankoku, Japan

Introduction: To maintain water homeostasis in the inner ear, both of the absorption and production of inner ear fluid is well balanced. Generally the stria vascularis (SV) is thought to be the site of endolymph production. For the purpose to evaluate the role of water channel system in the SV we have examined the localization of aquaporins (AQPs). And we reported AQP1/2/3/6/7/9 in the stria vascularis. In this study the local expression of new AQPs (AQP10/11/12) was molecular biologically and immunohistochemically examined.

Materials and Methods: Wistar rats (body weight100~150g) were used in this study. Total RNA was extracted from inner ear tissues (cochlea), using the RNAeasy Mini kit (Qiagen, Valencia, CA, USA). The RNA was reverse transcribed to cDNA, using SuperscriptII (Invitrogen Corp., Carlsbad, CA, USA). PCR for AQP11 and 12 detection was carried out using HotStar Taq (Qiagen, Hilden, Germany).

Temporal bones were immersed in fixative overnight, decalcified in 0.12 M EDTA for 7 days, and finally soaked in sucrose. They were then dissected into cochlea under a stereomicroscope. Frozen sections prepared by Cryostat (10µm thick) were used for immunocytochemistry. The primary reagent used to detect AQP10/11/12 was a rabbit anti-AQP10/11/12 polyclonal antibody. The sections were examined using an Axiovert 200M controlled by Axiovision LE software.

Result: Only AQP11 mRNA was expressed in the rat cochlea. The nucleotide sequences of the band agreed completely with the known sequence of the rat AQP11.

AQP11 was immunohistochemically expressed in the basal cells and marginal cells of the stria vascularis. However AQP10 and 12 were not expressed.

Conclusions: AQP10 have accepted not to express in rat. So in the present study we did not examine AQP10 molecular biologically. Endolymphatic hydrops is generated when homeostasis of inner ear fluid is broken. We have suggested that the increase of vasopressin might cause the development of the endolymphatic hydrops experimentally and clinically. Focused on the stria vascularis, VP regulates water flux at the basal cell, and AQP11 might work the role of water flux.

P50 Expression of aquaporin-11 in the rat endolymphatic sac

*D. Taguchi¹, H. Löwenheim², A. Kakigi³, T. Takeda⁴, H. Kitano¹

¹Tottori University Faculty of Medicine, Division of Otolaryngology, Head and Neck Surgery, Department of Medicine of Sensory and Motor Organs, School of Medicine, Yonago, Japan

²University of Tübingen School of Medicine, Department of Otolaryngology, Head and Neck Surgery, Tübingen, Germany

³University of Tokyo Faculty of Medicine, Department of Otolaryngology, Tokyo, Japan

⁴Kochi Medical School, Department of Otolaryngology, Kochi, Japan

In maintaining inner ear function, the homeostasis of inner ear fluid is essential. Water channels and ion channels play an important role in the fluid homeostasis of the living body. Indeed, multiple subtypes of aquaporins (AQPs) are expressed in the inner ear. Among AQPs, aquaporin-11 (AQP11) is expressed in the kidney, brain, liver and testis. Microscopically, AQP11 is distributed in the proximal tubule of kidney, and localized in the intracellular area as well as AQP6 and AQP8. Immunofluorescence-microscopic studies made AQP11 expression confirmed in the rat cochlea and vestibulum, but that in the endolymphatic sac has not yet been evaluated. In the present study, we investigated AQP11 expression in the rat endolymphatic sac by immunofluorescence microscopy. We newly observed AQP11 expression in the intracellular area of the rat endolymphatic sac. Since AQP11 expression is localized in the sites of the secretion and absorption of endolymph, AQP11 might play some role in the homeostasis of endolymph in the inner ear. However, its lack of expression on the plasma membrane indicates that AQP11 does not take direct part in water flux via the plasma membrane.

P51 Perivascular resident macrophage-like melanocytes in the inner ear are essential for intrastrial fluid-blood barrier integrity

W. Zhang^{1,2}, M. Dai¹, A. Fridberger³, A. Hassan⁴, J.M. DeGagne¹,
L. Neng^{1,2}, F. Zhang¹, W. He¹, T. Ren¹, D. Trune¹, M. Auer⁴, *X. Shi¹

¹Oregon Health & Science University, Oregon Hearing Research Center, Portland/OR, USA

²Zhengzhou University, Department of Otolaryngology/Head & Neck Surgery, Zhengzhou City, China

³Karolinska Institute, Department of Clinical Science, Intervention and Technology, Stockholm, Sweden

⁴Lawrence Berkeley National Laboratory, Life Sciences Division, Berkeley/CA, USA

The microenvironment of the cochlea is maintained by the barrier between the systemic circulation and the fluids inside the stria vascularis. However, the mechanisms that control intrastrial fluid-blood barrier permeability remain largely unknown. The barrier comprises endothelial cells connected to each other by tight junctions and an underlying basement membrane. In a recent study, we discovered that the intrastrial fluid-blood barrier also includes a large number of perivascular cells with both macrophage and melanocyte characteristics. The perivascular resident macrophage-like melanocytes (PVM/Ms) are in close contact with vessels through cytoplasmic processes. Here we demonstrate that PVM/Ms have an important role in maintaining intrastrial fluid-blood barrier integrity and hearing function. Using a new cell culture-based *in vitro* model and a genetically-induced PVM/M-depleted animal model, we show that absence of PVM/Ms increases the permeability of the intrastrial fluid-blood barrier to both low- and high- molecular-weight tracers. The increased permeability is caused by decreased expression of pigment epithelial-derived factor (PEDF), which regulates expression of several tight junction-associated proteins instrumental to barrier integrity. When tested for endocochlear potential (EP) and auditory brainstem response, PVM/M-depleted animals show substantial drop in EP with accompanying hearing loss. Our results demonstrate a novel and critical role for PVM/Ms in the regulation of intrastrial fluid-blood barrier permeability for establishing a normal hearing threshold.

Ototoxicity

P52 Transport mechanism for cisplatin to the cochlea - an *in vivo* and *in vitro* study in the guinea pig

*V. Hellberg¹, C. Gahm¹, H. Ehrsson², G. Laurell³

¹Karolinska Institutet, CLINTEC, Stockholm, Sweden

²Karolinska University Hospital, Karolinska Pharmacy, Stockholm, Sweden

³Umeå University, Department of Clinical Sciences, Umeå, Sweden

Introduction: Cisplatin is a platinum anticancer drug associated with ototoxicity. It is not known how cisplatin is transported from the systemic circulation to the target cells in the inner ear, but an active transport mechanism has been proposed. Organic cation transporter 2 (OCT2) has been identified to be involved in cisplatin-induced oto- and nephrotoxicity. The aim of the study was to investigate if immunoreactivity of OCT2 could be localized in the guinea pig cochlea *in vitro* and also to evaluate if a potential blocker of OCT2 (phenformin) could reduce the ototoxic and nephrotoxic side-effects of cisplatin *in vivo*.

Materials and methods: Immunoreactivity for OCT2 was analysed in paraffin embedded cochlear slides from untreated guinea pigs. Slides from the whole cochlea were analyzed in a fluorescence microscope. Guinea pigs given an i.v. injection of an ototoxic dose of cisplatin (8 mg/kg) were divided into two groups. Group I was given only cisplatin and group II was given phenformin i.v. 30 minutes before cisplatin. Four days later hearing thresholds were determined, thereafter the animals were sacrificed and loss of hair cells were assessed. Total amount of platinum in cochlear and renal tissue was analysed with mass spectrometry. Blood creatinine and urea were measured to evaluate the renal function.

Results: OCT2 was expressed in outer and inner hair cells, supporting cells and spiral ganglion cells. In the *in vivo* study there was no evidence of reduction of the nephrotoxic side-effect induced by cisplatin when guinea pigs were treated with phenformin. The level of platinum in cochlear and renal tissue did not differ between the groups. Hearing data will be presented at the meeting.

Conclusions: OCT2 might play a role in an active transport mechanism for the transport of cisplatin to the inner ear and also for the uptake of cisplatin to the hair cells. Systemic administration of an OCT2-blocker could be beneficial in reducing the ototoxic effect of cisplatin. However, phenformin did not reduce the uptake of platinum to the inner ear.

P53 Ototoxic effect of daptomycin applied to the guinea pig middle ear

M. Yamazaki¹, H. Oshima¹, *K. Nomura¹, T. Oshima¹, T. Kawase¹,
T. Kobayashi¹

¹Tohoku University, Otolaryngology, Head and Neck Surgery, Sendai,
Japan

Introduction: Ototoxic antibiotic eardrops are frequently used for external and middle ear infections. Fluoroquinolones are commonly used and provide adequate coverage for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas aureginosa* and *Moraxella catarrhalis*. However, MRSA is not responding to those antibiotics. Recently, ototoxic effect of topically used vancomycin, which is anti-MRSA antibiotics, has reported. Daptomycin is new anti-MRSA drug and its ototoxicity has not been known. The current study was designed to examine the ototoxic effect of daptomycin.

Materials and Methods: Male Hartley guinea pigs (weight, 500-640 g) were used. Twelve animals were divided into 3 groups. The first group received daptomycin (25mg/ml), the second received gentamicin (50mg/ml, positive control), and the third group received saline solution (negative control). After insertion of a pressure-equalizing tube, pretreatment auditory brainstem responses (ABRs) were obtained. Topical solutions of 0.1 ml were applied through the tube into the middle ear twice a day in 7 days. Post-treatment ABRs were obtained 7 days after the last treatment.

Results: Saline applied groups (negative control) and daptomycin treated groups had no deterioration of hearing threshold on ABR. Active control groups treated with gentamicin showed significant change in the threshold.

Conclusions: Daptomycin at the concentration of 25mg/ml was considered to be safe when topically applied. Although further investigation, such as the appropriate concentration, is needed and species difference should be considered, daptomycin might be a candidate for an ototopical drop effective for MRSA.

P54 Investigating free radical production in the cochlea during ototoxicity

P. Majumder¹, M. Duchen², J. Gale^{1,2}

¹UCL Ear Institute, London, United Kingdom

²UCL, Cell & Developmental Biology, London, United Kingdom

Research over the last decades has implicated reactive oxygen species (ROS) as a major causative factor for acquired hearing loss. Despite much research in this area the sites, sources and dynamics of ROS production have evaded a thorough examination. We are using live imaging to investigate ROS production during exposure of the cochlea to the ototoxic aminoglycoside neomycin. We use explant cultures from the basal and middle cochlear turns of 4-7 day old mice. ROS is measured using dihydroethidium (HET), and in we also measure intracellular calcium (Ca^{2+}) using Oregon Green BAPTA (OGB). When HET is oxidized by ROS it forms ethidium which intercalates within DNA and fluoresces in the red/far-red. Explants are loaded and maintained in 5 μ M HET, and in some experiments also loaded with 10 μ M OGB for 30 minutes. Confocal images stacks containing the inner sulcus (IS), inner hair cells (IHC), outer hair cells (OHC), Hensen's cells, outer sulcus (OS) and stria vascularis (SV) are recorded every 1 to 5 min over ~4 hour experiment using a Zeiss 510 NLO META.

HET fluorescence shows a steady increase indicating a basal rate of ROS production in different cells/regions. After an initial 30 minutes of pre-exposure imaging, 1mM neomycin was applied. In the first hour of neomycin exposure the rates of ROS generation were increased by the following (fold values) in IS (3.5 ± 2.2 n=5), IHC (1.1 ± 0.4 n=5), OHC (1.3 ± 0.4 n=15), Hensen's (1.3 ± 0.5 n=5), OS (1.5 ± 0.6 n=5) and SV (0.9 ± 0.2 n=5). By the second and third hours of neomycin exposure rates of ROS production were reduced down to values close to baseline. In order to investigate the role of mitochondria in neomycin-induced ROS production, explants were treated with an inhibitor of complex I (rotenone 20 μ M), prior to neomycin exposure. Rotenone pre-treatment reduced the neomycin-induced increase in ROS in almost all of the cell types suggesting that mitochondria are a significant source of the ROS produced in cochlear cells in response to aminoglycosides. In additional experiments we measured the dynamics of intracellular Ca^{2+} and ROS by multi-wavelength imaging. A number of OHCs in a field of view die during the 4 hours. Preliminary analysis of those cells indicates that a sample of 17 such OHCs (6 experiments) revealed an intracellular Ca^{2+} increase 42 \pm 10 minutes before cell death. In 8/17 of the cells there was second Ca^{2+} increase just prior to cell death (2 to 4 minutes) coinciding with a large increase in ROS production.

Experiments to further characterize the sites and mechanisms of ROS production in the cochlea are ongoing.

Protection / Prevention / Regeneration

P55 Protective effect of Curcumin in cisplatin-induced ototoxicity in the guinea pig: A functional and morphological study

*S.L. Eramo¹, F. Paciello², R. Rolesi², D. Troiani¹, A.R. Fetoni², G. Paludetti²

¹Catholic University of Rome, Institute of Physiology, Rome, Italy

²Catholic University of Rome, Institute Of Otolaryngology, Rome, Italy

Introduction: Cisplatin (cis-diamine-dichloroplatinum II [N₂Cl₂PtH₆]) is a widely used chemotherapeutic agent for the treatment of various tissue malignant neoplasms; however, its usefulness is limited by nephrotoxicity, neurotoxicity and ototoxicity. Although the mechanisms of the antineoplastic effects of cisplatin are well known, the cellular and molecular mechanisms of cisplatin-induced ototoxicity are not well described, even if it seems that the production and/or release of Reactive Oxygen Species (ROS) may play a key role. Because both the therapeutic effects and the ototoxicity of cisplatin are dose-dependent, there is great interest in developing effective strategies to protect or rescue the auditory function from cisplatin ototoxicity without affecting the antitumoral activity of the drug. Many agents have been tested to ameliorate cisplatin-induced hearing loss and in this study we focused on curcumin (*Curcuma longa*), a dietary pigment with antioxidant, anti-inflammatory and anti-tumoral properties.

Material and Methods: The guinea pigs were used as model of cisplatin-induced cochlear damage (16 mg/kg IP) and the effectiveness of curcumin at dose 200mg/kg IP, 4 days consecutively of was studied by measuring Auditory Brainstem Response (ABR) thresholds. The extent of damage has been evaluated with cochleogram and the magnitude of lipid peroxidation by the expression of 4-hydroxynonenal (4HNE). Finally, the role of heme-oxygenase 1 (HO-1) were studied.

Results: Our results demonstrated that the protective effect of curcumin can be mediated by both the direct free radical scavenging activity and the activation of HO-1: (a) decreased ABR threshold shifts at 3 and 5 days after cisplatin administration, (b) decreased oxidative stress biomarkers as shown by decreased expression of 4HNE, (c) increased survival of hair cells as evidenced by rhodamine-phalloidin staining and (d) increased expression of HO-1 at 3 and 5 days from the onset of treatment.

Conclusion: These results demonstrate the antioxidant properties of curcumin as free-radical scavenger and suggest that activation of HO-1 can represent an additional mediator against ototoxicity.

P56 Oncostatin M protects against cisplatin ototoxicity

*E. Gerschner¹, O. Hegend¹, H. Olze^{1,2}, B. Mazurek³, A. Szczepek¹

¹Charité University Hospital, ORL CCM, Berlin, Germany

²Charité University Hospital, ORL CVK, Berlin, Germany

³Charité University Hospital, Tinnitus Center, Berlin, Germany

In our previous report, we have demonstrated that interleukin-6 (IL6) protects the apical part of post-natal rats' inner hair cells from cisplatin-induced toxicity. Encouraged by these results, we wanted to investigate otoprotective properties of other IL6 family members. We have chosen to focus our attention on Oncostatin M (OCM), because of high expression level of OCM receptor's in the inner ear. OCM signals *via* Oncostatin M receptor (OCMR) and gp130, downstream *via* signal transducer and activator of transcription 3 (STAT3). STAT3-induced signaling has recently been shown to be neuroprotective.

For our experiments, we have isolated and next explanted and cultured organs of Corti from the postnatal Wistar rats (p3-p5). In contrast to our previous experiments, explants were preincubated with OCM [30ng/ml] for one day and then simultaneously incubated with cisplatin [15µM] and OCM [30ng/ml] for another day. Explants were stained with phalloidin-TRITC to visualize and score the hair cells under epifluorescent microscope. OCM-mediated protection from cisplatin was visible for inner as well as outer hair cells in the apical and medial section of the explants.

Additionally, we have incubated the explants with antibodies against alpha neurofilament 200 and secondary antibody labeled with Alexa633. Using confocal microscopy, we have assessed the morphology and survival of spiral ganglion neurons. Preliminary investigations confirmed protective role of OCM for this cell type as well.

Based on our *in vitro* results, we hypothesize that activation of STAT3 signaling pathways *via* IL6-family cytokine OCM may counteract ototoxicity by protecting different cell types from cisplatin's deleterious side effects.

P57 Antioxidants against Cisplatin ototoxicity in the rat cochlea *in vitro*

*A. Maniu¹, M. Schrepler¹, M. Cosgarea¹

¹University of Medicine, Otorhinolaryngology, Cluj Napoca, Romania

Introduction: Neurosensorial hearing loss involves an irreversible damage of sensory cells (hair cells), supporting cells and neurons in the cochlea caused by ototoxic drugs, acoustic overstimulation, etc. Cisplatin is a platinum- based chemotherapeutic agent intensely used in the treatment of several solid tumors. Its clinical use is limited by the severe side effects like ototoxicity and nephrotoxicity. Sensorial cell death after Cisplatin exposure is caused by the release of large quantities of reactive oxygen species, that's why supplementation with antioxidants appears to a rational approach to prevent and improve hearing disorders in combination with standard therapy.

Purpose. The aim of this study was to test if L-N-acetylcysteine (L-NAC) can protect hair cells against cisplatin-induced damage *in vitro*.

Material and methods: Cochleae were dissected from new-born CD1 rats, and placed on Millicell membrane inserts in DMEM high glucose, N1 supplement. The explants were exposed to Cisplatin for 24h, three explants for each concentration (0;5; 10;15; and 20 μ M/L); three cochleae were left as control explants. Half of the explants were pretreated for 24h with antioxidant before exposure to Cisplatin: 20 μ g/ml grape seed extract (GSE) and 10mM L-N-acetylcystein (L-NAC), respectively. After treatment, the explants were stained with TRITC- phalloidin and examined at a Zeiss AxioObserver D1 inverted microscope. Apoptosis was assessed using AlexaFluor 488- Annexin V staining, combined with the specific staining of hair cells with phalloidin.

Results: Control explants had an average number of 43 OHC/ 0.1 mm, while IHC were 13.5 cells/ 0.1 mm. Increasing concentrations of Cisplatin resulted in a dose dependent reduction of the number of intact hair cells, outer hair cells (OHC) being more susceptible than inner hair cells (IHC). The pretreatment with 10 mM L-NAC offered significant protection.

Conclusions: Antioxidants, both GSE and L-NAC protected inner ear hair cells against Cisplatin toxicity in cochlear explants, significantly reducing the number of apoptotic cells, compared with the explants exposed only to Cisplatin. The use of antioxidants, before and during treatment with Cisplatin could represent a logical strategy in preventing neurosensorial hearing loss which occurs frequently as a serious limiting side-effect of chemotherapy with Cisplatin.

P58 Distribution of glucocorticoid receptors and 11 β -hydroxysteroid dehydrogenase isoforms in the human inner ear

*H. Kumagami¹, M. Terakado¹, H. Takahashi¹

¹Nagasaki University, Department of Otolaryngology Head and Neck Surgery, Nagasaki, Japan

Objectives/Hypothesis: Glucocorticoids (GCs) are widely used as a therapeutic modality for inner ear disorders including Ménière's disease (MD). The concentration of GCs in the target cells is known to be regulated by 11 β -hydroxysteroid dehydrogenase (11 β -HSD), an enzyme complex responsible for the conversion of hormonally active cortisol into inactive cortisone. There is no morphological indication of glucocorticoid receptors (GRs) and 11 β -HSD isoforms (11 β -HSD1 and 2) in human inner ear. Thus, we conducted a study to determine whether GRs and the isoforms of 11 β -HSD were present in human inner ear tissues and to establish their precise distribution. In addition, we performed a new trial using dexamethasone to prevent hearing disturbance during endolymphatic sac surgery in 10 patients with MD. In the present study, the results of the surgery are also presented.

Results: Immunoreactivity of GRs was detected in the stria vascularis (SV), the outer hair cells (OHCs), the inner hair cell (IHC), the spiral ligament (SLig), Reissner's membrane (RM), the vestibular hair cells (VHCs) the vestibular nerve (VN), the transitional cells (TCs) and the dark cells (DCs) of the crista ampullaris. 11 β -HSD1 was observed in the apical area of the VHCs, the TCs, and the DCs. However, no immunoreactivity of 11 β -HSD2 was observed. After surgery, hearing improved in 9 of the 10 patients.

Conclusions: Our data regarding GRs and 11 β -HSD isoforms indicates that different local steroid regulation by GRs and the isoforms of 11 β -HSD is present in various parts of human inner ear tissues, and that the tissues are a direct therapeutic target of glucocorticoids in the inner ear diseases. Regarding the results of hearing preservation in patients with MD, topical use of dexamethasone seems to be an effective method for preventing hearing disturbance over long periods as well as during endolymphatic surgery.

P59 Distribution of glucocorticoid receptors and 11 β -hydroxysteroid dehydrogenase isoforms in the stria vascularis

*M. Terakado¹, H. Kumagami¹, H. Takahashi¹

¹Nagasaki University, Department of Otolaryngology Head and Neck Surgery, Nagasaki, Japan

Glucocorticoids (GCs) and glucocorticoid receptors (GRs) play a role in sensory transduction and homeostasis in the inner ear. In humans, GCs are widely used as a therapeutic modality to treat many pathologic conditions of the inner ear or to prevent inner ear damage by various manners of administration. Protective effects of GCs against stress to hair cells have already been known, however, in the stria vascularis (SV), cellular distribution of GRs, a factor controlling GCs concentration in the target cells, and actions of GCs are not fully demonstrated. In the present study, PCR (polymerase chain reaction) and immunohistochemistry were conducted to investigate the distribution of GRs and isoforms of 11 β -hydroxysteroid dehydrogenase (11 β -HSD1 and 2) that regulate concentration of GCs in the target cells in the rat and human. In addition, translocation of GRs by stimulation of dexamethasone (DXM) and protective effect against hypoxia were investigated in rat SV tissue explants. Although GRs and 11 β -HSD1 were found to be in the plasma membrane and cytoplasm of all cell types of the SV, GRs were translocated into the nucleus by the stimulation of DXM. Only a few cells died in the medium containing DXM, however, the number of dead cells significantly increased in the medium without DXM. GRs and GCs regulate the SV function and play a role in protection of the SV against stress in cells.

P60 Changes in expression levels of NADPH oxidases in the cochlea in response to noise

*S. Vljakovic¹, C.-Y.S. Lin¹, P. Thorne¹

¹The University of Auckland, Physiology, Auckland, New Zealand

NADPH oxidases are membrane bound enzymes catalyzing the production of superoxide radical from molecular oxygen. The current study investigated the expression and distribution of six members of the NADPH oxidase family (Nox1-4 and Duox1-2) in the rat cochlea and their regulation in response to noise.

Methods: Gene and protein expression levels were studied using quantitative RT-PCR and semi-quantitative immunohistochemistry. Wistar rats (8-10 weeks) were exposed for 24 hours to band noise (8-12 kHz) at a moderate (100dB) or traumatic (110dB) sound pressure level. Animals exposed to ambient noise in the animal facility served as controls.

Results: At the transcript level, significant up-regulation was observed for Duox2 (2.5 fold) in the cochlea exposed to moderate noise, whereas Nox3 was down-regulated (7 fold). Immunohistochemistry demonstrated predominant expression of NADPH oxidases in the sensory and supporting cells of the organ of Corti, with very limited expression in the lateral wall tissues and spiral ganglion neurons. A significant reduction in the intensity of Nox1 and Nox3 immunostaining was observed in the organ of Corti after noise exposure, whilst Nox4 immunolocalisation shifted from the blood vessels to the marginal cells of the stria vascularis.

Conclusion: The down-regulation of Nox1 and Nox3 at a protein level may be a protective mechanism to reduce oxidative stress in the noise-exposed cochlea. In contrast, noise-induced changes in Nox4 immunolocalisation could be linked to cochlear injury. Further studies, however, are required to determine the role of NADPH oxidases in noise-induced oxidative stress.

This study was approved by the University of Auckland Animal Ethics Committee.

P61 STAT3 and the noise-induced stress response in the inner ear

T.M. Wilson¹, I. Omelchenko¹, Y. Yang¹, M. Dai¹, X. Shi¹, *A.L. Nuttall¹

¹Oregon Health & Science University, Oregon Hearing Research Center, Portland/OR, USA

Introduction: Signal transducers and activators of transcription 3 (STAT3) mediates a complex range of cellular responses to stress. Many of its target genes such as MnSOD, HIF-1 α and Survivin are involved in the regulation of pro-survival and cellular proliferation functions, and STAT3 activity is protective against both cerebral and myocardial ischemia/reperfusion injury. However, through transcriptional regulation of VEGF, a key proangiogenic factor, STAT3 also plays a major role in inflammation and vascular paracellular permeability under both normal and pathological conditions. Here, we examined the role of STAT3 in the cochlea to gain a better understanding of factors involved in the noise-induced stress response in the inner ear.

Materials and Methods: Male (8 to 10 weeks old) CBA/CAJ mice were used. The effect of noise exposure (NE) on STAT3 levels, localization and phosphorylation status in the cochlea was examined by proteomics, immunohistochemistry and Western blot analysis. Inhibition of STAT3 activity was accomplished using the JAK/STAT3 pathway inhibitor JSI-124.

Results: Our proteome of noise exposed stria vascularis (SV) capillaries revealed that acoustic trauma increased STAT3 protein levels. Further, NE induced the phosphorylation and nuclear translocation of STAT3 in many cell types in the inner ear including the marginal cells of the stria vascularis, type II fibrocytes of the spiral ligament, inner hair cells in the supporting cells in the organ of Corti. JSI-124 attenuated the noise-induced increase of STAT3 phosphorylation as well as VEGF transcript levels in the cochlea. Super resolution-structured illumination microscopy demonstrated that VEGF treatment increased STAT3 phosphorylation in isolated SV capillary endothelial cells. The contribution of noise-induced STAT3 activation to cochlear physiology and function is currently being examined through the use of JSI-124.

Conclusion: NE activated STAT3 in many different cell types of the cochlea. STAT3 is likely involved in both protective and injurious activities in response to NE depending upon the cell type and location.

Supported by: 5R01DC000105 (ALN), 1R01DC010844 (XS) and P30DC005983.

P62 Local corticosteroid application reduces permanent threshold shift after impact noise trauma

*M. Müller¹, H. Löwenheim¹

¹University Tübingen, Otolaryngology, Tübingen, Germany

Exposure to loud noise leads to an acute threshold shift which disappears within days. However, quite frequently permanent threshold shift (PTS) remains. Treatments to limit the PTS show that corticosteroids may provide an effective medication. Here we investigated the use of corticosteroids in a preclinical guinea pig model of acute noise trauma.

Exposure to impact noise led to a permanent hearing loss in the entire frequency range. Hair cell loss was observed in the middle and apical region of the cochlea. A catheter was used to apply Dexamethasone, Prednisolone or Methylprednisolone to the round window of the cochlea. Osmotic pumps connected to the catheter were subcutaneously implanted immediately, 1, 3, or 7 days after the noise exposure. The pumps (0.5 µl/h) lasted for weeks, then PTS and hair cell loss was determined.

The treatment with corticosteroids resulted in a dose-dependent rescue of hair cells and reduction in PTS. Also a dependence on the onset of treatment was found, onset immediate or one day after noise exposure resulted in lowest PTS. When treatment started 3 days after the exposure a reduced effectiveness of rescue in the high frequency range was observed. When treatment started 7 days after noise exposure, effectiveness was reduced further, at most frequencies in the range of untreated controls.

Permanent hearing loss and hair cell loss was reduced using corticosteroids applied at the round window of the cochlea. The results show a rescue effect for an onset of treatment of at least up to 3 days after the noise insult.

P63 IGF-I deficit predisposes to noise-induced hearing loss in mice

A. Celaya¹, S. Murillo-Cuesta^{1,2,3}, L. Rodríguez-de la Rosa^{1,2,3}, R. Cediel^{1,2,4}, J. Contreras^{1,2,4}, C. Avendaño⁵, *I. Varela-Nieto^{1,2,3}

¹Instituto de Investigaciones Biomédicas Alberto Sols CSIC-UAM, Madrid, Spain

²Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Valencia, Spain

³Instituto de Investigación Sanitaria Hospital La Paz (IdiPAZ), Madrid, Spain

⁴Facultad de Veterinaria UCM, Madrid, Spain

⁵Facultad de Medicina UAM, Madrid, Spain

Human IGF-I deficiency (ORPHA73272, OMIM608747) is a rare disease associated with poor growth rates, mental retardation and syndromic hearing loss. Equally, *Igf1*^{-/-} mice are dwarfs with poor survival rates and congenital profound deafness¹. IGF-I is a neuroprotective agent, and accordingly, low circulating IGF-I levels have been related to cognitive and brain alterations. *Igf1*^{-/-} mice present undetectable serum levels of IGF-I throughout their life, whereas *Igf1*^{+/-} and *Igf1*^{+/+} littermates show an age-dependent decrease in IGF-I serum levels, especially from 6 months of age on^{2,3}, which correlates with the increase in ABR thresholds. There is little information on the potential protective actions of IGF-I against noise-induced hearing loss (NIHL).

We have studied the susceptibility of *Igf1*^{+/-} and *Igf1*^{+/+} mice to NIHL at different ages, with functional (auditory brainstem responses, ABR), morphological (cochlear histology and stereological hair-cell quantification) and molecular (Western Blotting) studies. Noise-exposure experiments with 3 months-old mice did not reveal differences between genotypes. However, 6 month-old *Igf1*^{+/-} mice presented a greater susceptibility to noise damage, with higher threshold shifts and a poorer recovery compared to control mice. *Igf1*^{+/-} mice showed severe morphological changes, as well as an altered intracellular signaling response to IGF-I. These changes correlated with low IGF-I serum levels in the heterozygous mice, compared to wild type.

These results support the idea that IGF-I-based therapies could contribute to prevent or ameliorate age-related and noise-induced hearing loss.

Acknowledgements: This research was funded by grants from the Spanish Ministry of Science and Innovation SAF2011-24391 and Intra-CIBERER programs to IV-N. SMC and LRR hold contracts from CIBERER.

P64 Otoprotection by stimulation of cGMP cascade in a gerbil and rat animal model

*K. Varakina¹, B. Müller¹, M. Jaumann¹, M. Knipper¹, L. Rüttiger¹

¹Tübingen Hearing Research Centre, Tübingen, Germany

Recent findings indicate that also reversible hearing loss, when characterized by temporary threshold shifts, can be connected with slow degeneration of auditory nerve fibers (AN) and progressive hearing loss (Kujawa and Liberman, 2009), even if the damage initially would not become apparent in conventional clinical threshold testing. For acute persistent hearing loss after temporary noise exposure, we could recently show that the deterioration of inner hair cell (IHC) synapses following noise exposure can be overcome by the modulation of the cGMP signaling cascade (Jaumann et al., 2012). Whether the stimulation of cGMP cascade is also protective for slowly progression hearing loss with age is unclear.

Current project aims to highlight molecular and physiological basis of presbycusis and to find the molecular basis of deterioration of auditory fiber and IHC synaptic function.

We investigate whether stimulation of cGMP signaling cascade hearing loss progression and auditory fiber degeneration following mild noise exposure. In a rat and gerbil animal model (Rüttiger et al., 2007) functional hearing measurements were performed before and up to 8 weeks after exposure to TTS-inducing noise. Aged and young animals were treated with cGMP cascade modulating drug or vehicle as control. Additionally, a group of animals was held in enriched environment conditions (with physical and sensory stimulation). Morphology of auditory tissues was analyzed by the presence of pre- and postsynaptic molecular markers. The results will help to identify compounds with otoprotective effect for presbycusis that could be a promising candidate for preventive therapy of age-related hearing loss in humans.

This work is supported by RNID. The cGMP cascade modulating drug was kindly provided by Bayer Pharma AG.

P65 Characterizing the proliferation and differentiation potential of different supporting cell populations within the organ of Corti using fluorescence- (FACS) and magnetic-activated cell separation (MACS) techniques

*A. Eckhard¹, N.-N. Luu¹, J. Waldhaus², M. Müller¹, H. Löwenheim¹

¹University of Tübingen, Department of Otolaryngology, Head and Neck Surgery, Tübingen, Germany; ²Stanford University, School of Medicine, Departments of Otolaryngology, Head and Neck Surgery and Molecular & Cellular Physiology, Palo Alto/CA, USA

Introduction: The vulnerability of sensory hair cells in organ of Corti to ototoxic insults combined with the lack of hair cell regeneration is the leading cause of incurable hearing loss. Towards a rational therapy for the cure of hearing loss, the use of *in vitro* cultures of sensory hair cells for pharmacological screenings is indispensable. Currently, *in vitro* cultures of organ of Corti stem cells are capable to generate approximately 2% new hair cells *in vitro*. Substantially higher numbers of *in vitro* generated hair cells are required for high-throughput pharmacological screenings of otoprotective/-regenerative drugs. Here we developed a method to enrich hair cells *in vitro* by using FACS and MACS.

Materials and Methods: Supporting cells near the inner hair cell, that express the glutamate aspartate transporter GLAST (GLAST⁺ cells) and those near the outer hair cells that express the p75-neurotrophine receptor (p75-NTR⁺ cells) were purified from the murine neonatal (P4) organ of Corti by FACS and MACS. The *in vitro* proliferative capacity as well as the potential to re-differentiate into hair cells was investigated by immunohistochemical and qRT-PCR analyses of their *in vitro* stem cell-, supporting cell- and hair cell-marker expression.

Results: Purified GLAST⁺, p75-NTR⁺ and GLAST⁻/p75-NTR⁻ cells proliferate *in vitro* as determined by EdU-incorporation and express stem cell markers. Determined from their hair cell marker expression patterns, GLAST⁺ and p75-NTR⁺ cells differentiated into more hair cells *in vitro* than GLAST⁻/p75-NTR⁻ cells. Addition of a notch-signal inhibitor (gamma-secretase) to the *in vitro* culture of purified cells further increased the number of newly generated hair cells.

Conclusions: Purified GLAST⁺, p75-NTR⁺ and GLAST⁻/p75-NTR⁻ cells from the murine neonatal organ of Corti proliferate *in vitro*. GLAST⁺ and p75-NTR⁺ serve as hair cell progenitors *in vitro*. The notch-signal inhibitor pushed the cell fate of these cells toward the expression of hair cell markers. The method developed combines FACS/MACS purification of cells derived from the postnatal organ of Corti with chemical stimulation to robustly generate hair cell like cells *in vitro*.

P66 Defining the role of integrins in the repair and regeneration of hair cells in the human vestibular system

*K. Hussain¹, A. Forge¹, S. Saeed¹, R. Taylor¹

¹The Royal College of Surgeons of England, London, United Kingdom

Integrins are cell adhesion receptors that play important roles in physiological and pathological processes throughout the body. Integrins comprise alpha and beta subunits and the 24 $\alpha\beta$ heterodimeric members mediate the interaction of cell - cell signaling as well as communication between the cell and the extracellular matrix.

The lesion created by the death of a hair cell is closed by supporting cells in a manner that maintains the permeability barrier at the luminal surface of the epithelium. This controlled process relies on cell shape changes and spreading that likely involves integrins. Furthermore, the supporting cells are thought to remove these dead hair cells by a phagocytic process. Certain integrin receptors recognize apoptotic cells mediating phagocytosis of the apoptotic body.

Following hair cell loss in the vestibular system of mammals there is limited regeneration which may be due to the direct phenotypic conversion of supporting cell into hair cells without an intervening mitotic event. We hypothesize that integrins play an integral role in the repair and recovery process. We are able to test this hypothesis with human vestibular tissue obtained from patients undergoing trans labyrinthine procedures for acoustic neuromas and provided by a National consortium of surgeons.

Real Time PCR has been used to identify specific integrins in human vestibular explants. A number of integrins have been identified and on-going work will examine integrin protein location and further elucidate their role in the repair and regeneration of hair cells.

Pathology / Therapy

P67 Optogenetic stimulation of the auditory nerve

*V.H. Hernandez^{1,2}, G. Hoch^{1,2}, M. Bartels³, G. Vogt⁴, C.W. Garnham⁴, T. Salditt³, N. Strenze¹, T. Moser^{1,2}

¹University of Göttingen Medical Center, InnerEarLab, Dept. of Otolaryngology, Göttingen, Germany

²University of Göttingen, Bernstein Focus for Neurotechnology, Göttingen, Germany

³University of Göttingen, Department of Physics, Göttingen, Germany

⁴MED-EL Company, Innsbruck, Austria

Cochlear implants are among the most advanced neuroprostheses, enabling open speech comprehension in the majority of the implanted deaf subjects. However, employing only a dozen of electrodes in order to avoid electrode cross-talk, sound encoding driven by the current cochlear implants is limited making limited use of the tonotopically ordered projections to the brain. Frequency resolution might be improved by optical stimulation. Here, we explored the use of channelrhodopsin-2 expression in spiral ganglion neurons for optical stimulation of the auditory pathway. Optical stimulation of ChR2-expressing SGNs by a light emitting diode (LED) activated the auditory pathway in hearing mice and in mouse models of acute and chronic human deafness. The dependence of response amplitude on stimulus duration, rate and light power was systematically explored. Auditory pathway activation has also been tested by inferior colliculus recordings after light cochlear stimulation. Frequency specificity of optical stimulation has been measured using tone on tone and tone on light methods. Resulting tuning curves show best frequencies around 12 kHz. In summary, ChR2-mediated optical stimulation of cochlea is feasible.

P68 Neurotrophin mediated stimulation of neurite outgrowth in a mouse spiral ganglion model

*B. Kramer^{1,2}, M. Müller^{1,2}, M. Knipper^{1,2}, H. Löwenheim^{1,2}, A. Tropitzsch^{1,2}

¹University Tübingen, Otolaryngology, Tübingen, Germany

²University Tübingen, Hearing Research Centre, Department of Otorhinolaryngology, Tübingen, Germany

Cochlea implant (CI) technology is a milestone for deaf people. Yet electric stimulation by a CI is always inferior to normal hearing, because of the limited number of information channels by which the auditory nerve is electrically stimulated. To achieve a higher performance the nerve-electrode interaction needs to be improved. One way to achieve this is stimulating the outgrowth of the neurites towards and onto the electrode. This may be achieved by neurotrophins such as brain derived neurotrophic factor (BDNF). Via TrkB receptors neurite outgrowth is stimulated, however as a paradox, this effect is inhibited by simultaneous BDNF binding to the low-affinity p75^{NTR} receptor. We thus set out to investigate the TrkB and P75 mediated pathways in more detail.

An organotypic culture model of postnatal (P4-6) mouse spiral ganglion was used. Neurite outgrowth was analyzed using micrographs quantified with a custom adapted Sholl analysis. Stimulation of neurite outgrowth was quantified after application of BDNF, synthetic, selective TrkB ligands and Rho-associated kinase (ROCK) inhibitors. Inhibition of neurite outgrowth was assessed for application of MAG-Fc (myelin-associated glycoprotein), TrkB-inhibitors, PI3K (phosphoinositide-3-kinase) inhibitors and PKA (protein kinase A) inhibitors.

BDNF application resulted in a dose- and a time-dependent response of neurite outgrowth. Outgrowth was also stimulated by a synthetic TrkB ligand. Inhibitors were capable to inhibit these effects in a dose dependent manner. Using MAG-Fc an inhibitory environment was created which suppressed BDNF activated outgrowth. The artificial inhibitory environment could be avoided by a selective TrkB ligand, also compensated by a Rho-associated kinase inhibitor.

The organotypic culture model of the spiral ganglion is suitable for the evaluation of compounds stimulating or inhibiting neurite outgrowth. A synthetic, selective TrkB ligand proves to be a potential candidate for selective stimulation of neurite outgrowth. To overcome inhibitory environment, more potent TrkB-agonists are required. Selective inhibition of the p75^{NTR} pathway improves neurite outgrowth in an inhibitory environment.

P69 The influence of different sealing techniques on ABR thresholds and fibrous tissue development after CI implantation

*A. Burghard¹, A. Kral¹, T. Lenarz¹, G. Paasche¹

¹Hannover Medical School, Otolaryngology, Hannover, Germany

For patients suffering from severe to profound hearing loss a cochlear implant (CI) is the treatment of choice. Since more and more patients with some residual hearing get implanted, a preservation of this residual hearing is desired. Additionally, reduction of fibrous tissue reaction after cochlear implantation is also in the focus of research. In this study we wanted to investigate the influence of different sealing techniques on the development of auditory brainstem response (ABR) thresholds and fibrous tissue formation after implantation.

Normal hearing guinea pigs were used as animal model. The animals were implanted through the round window with a CI model electrode on day 0. Prior to surgery their hearing threshold was determined by ABR measurements. After implantation the round window was sealed by different sealing techniques: 1) no additional seal, 2) muscle, 3) bone cement. On day 28 the hearing threshold was tested again before the animals were sacrificed and the cochleae harvested. The cochleae were fixed and embedded in epoxy. Due to the platinum wire in the CI model electrodes a grinding technique was performed for histological evaluation (40 µm steps). The amount of fibrous tissue and/ or new bone formation was determined at the insertion site, the tip of the model electrode as well as at several steps in between.

The hearing threshold increased in almost all animals with the lowest increase in the group without any seal. First histological evaluations revealed a decrease of fibrous tissue growth from the insertion side towards the tip of the model electrode in all groups. The least fibrous tissue formation was found in the group without any seal followed closely by the group with a muscle graft as seal. The group with a bone cement seal showed the highest amount of fibrous tissue development. In this group also strong new bone formation was observed.

In conclusion the sealing technique not only has an influence on the formation of fibrous tissue and new bone formation after implantation of a CI but also on the threshold shift induced by cochlear implantation.

P70 Dexamethasone eluting cochlear implant for fibrous tissue and impedance reduction

M.E. Wilk^{1,2}, R. Hessler³, M. Fehr², T. Lenarz¹, *V. Scheper¹

¹Hannover Medical School, ENT, Hannover, Germany

²University of Veterinary Medicine Hannover, Clinic for exotic pets, reptiles, pet and feral birds, Hannover, Germany

³MedEl Hearing Implants, Innsbruck, Austria

Introduction: Shortly after cochlear implantation an increase of impedances is observed which is typically explained by fibrous tissue growth around the electrode carrier. Dexamethasone (DEX) eluting cochlear implants (CI) may affect impedances, fibrous tissue growth around the CI and residual hearing.

Materials and Methods: The electrode arrays' silicone of experimental CIs was provided with DEX. Normal hearing guinea pigs were implanted with those electrodes containing three different concentrations of DEX: A) 1% DEX (n=10), B) 10% DEX (n=10), C) 0% DEX (n=9). The devices were inserted via round window approach into the scala tympani for a depth of 3mm and left in situ for a period of 91 days. Hearing thresholds were measured via acoustically evoked auditory brainstem response before implantation and on experimental days 7, 28 and 91. Impedances were measured before and after an electrical stimulation of 60 minutes to investigate stimulus effects. Impedance measurements were performed on day 0 and then weekly for 3 months. On experimental day 91 cochleae were harvested, embedded in epoxy resin, grinded, and the fibrous tissue growth was examined histologically.

Results: DEX treatment had no effect on residual hearing and after 28 days of implantation the impedances of DEX treated animals did not differ significantly compared to the control. But after 91 days the impedance levels in both DEX-groups were significantly lower than in the control group - before and after electrical stimulation. DEX concentrations of 1% and 10% significantly reduced the formation of fibrous tissue sheaths around the electrode part of the CI. The highest amount of fibrous tissue growth was detected in the basal region of the cochlea of all experimental groups. CIs containing 10% DEX led to better functional and histological results than 1% DEX CIs.

Conclusions: DEX significantly reduced impedances and fibrous tissue growth after 3 months observation. These findings were concentration dependent since electrodes containing 10% DEX showed better results than those containing 1% DEX. DEX eluting CIs are a promising device for impedance and fibrosis reduction in cochlear implant patients.

P71 Real-time impedance measurement feedback for intracochlear electrode insertion

*C.-T. Tan¹, M. Svirsky¹, A. Anwar¹, S. Kumar², B. Caessens², C. Treaba², P. Carter², J.T. Roland Jr¹

¹New York University, School of Medicine, Otolaryngology, New York/NY, USA

²Cochlear Ltd, Sydney, Australia

Objectives: This pilot study details the use of a software tool that measures electrode impedance continuously during intracochlear electrode insertion, with the eventual potential to assess and optimize electrode placement and reduce insertional trauma.

Methods: A prototype program to measure electrode impedance and display it graphically in real time has been developed. The software was evaluated in human cadaveric temporal bones and in two live surgeries during intracochlear electrode insertion. Electrode position was cross-evaluated with real time fluoroscopic analysis.

Results: Several electrode designs were evaluated with the new software system and data were obtained during electrode insertion with fluoroscopic guidance. Impedance changes were observed with various scalar positions. Using Contour Advance™ electrodes, impedance values increased after stylet removal, particularly when the electrodes are stimulated in the monopolar mode.

Conclusions: Impedance values seem systematically affected by electrode position, with higher values being associated with proximity to the cochlear wall. The new software is capable of acquiring impedance measurements during electrode insertion and this data may be useful to guide surgeons to achieve optimal and atraumatic electrode insertion, to guide robotic electrode insertion, and to provide insights about electrode position in the cochlea.

This study was supported by Cochlear Ltd. (PI: Roland). Dr. Tan's participation in the study was supported by NIH grant K25-DC010834 (PI: Tan), and Dr. Svirsky's participation was supported by NIH grant R01-DC003937(PI: Svirsky).

P72 The 'bounce' phenomenon in humans – a transient model for Ménière's disease?

M. Überfuhr¹, T.D. Weddell², E. Krause³, R. Gürkov³, L. Wiegrebe¹,
*M. Drexl³

¹University of Munich, Department Biology II, Munich, Germany

²University of Brighton, School of Pharmacy and Biomolecular Sciences, Brighton, United Kingdom

³University Hospital Munich, German Dizziness Centre, Munich, Germany

Introduction: Loud, non-traumatic low-frequency tones can induce transient, oscillatory changes in mammalian cochlear sensitivity and gain, for which the term 'bounce' phenomenon (BP) has been coined. Manifestations of the BP are (amongst others) oscillatory changes of hearing thresholds, of Distortion Product Otoacoustic Emissions (DPOAE) amplitudes and of transient (putatively cochleogenic) tinnitus. The BP is considered to originate in the cochlea due to low-frequency induced changes of ionic homeostasis. A complete description of the BP in humans is relevant to our understanding of control systems (and pathologies thereof) enabling the cochlea to maintain its function during periods of mechanical and ionic disturbances.

Methods: Three non-invasive measures of the BP were studied in each of 22 normal-hearing human subjects. The amplitude and phase of quadratic and cubic DPOAEs, the auditory threshold, and the amplitude of the perceived tinnitus were followed over time after low-frequency stimulation (30 Hz, 120 dB SPL, 90 s).

Results: The majority (70%) of subjects showed a change in the phase and a biphasic modulation in the amplitude of quadratic (but not cubic) DPOAEs. This modulation consisted of an increase (mean 3.8 dB, N=14) followed by a decrease (mean 3.1 dB, N=14) of the DPOAE amplitude. Auditory threshold changes, ranging from desensitisation or sensitisation only to biphasic oscillation, occurred more often and lasted longer at lower probe tone frequencies. All subjects reported a tinnitus lasting for about two minutes that decreased in amplitude in a monotonic manner.

Conclusions: The relative ease of evoking the three measures of the BP in humans indicates that they share at least a common origin caused by the low-frequency stimulation, which are possibly, as has been suggested before, oscillatory changes of calcium levels in the outer hair cells. However, the different time courses of the three measures indicate that increased calcium levels trigger different mechanisms causing the manifestations of the BP. If the BP was an epiphenomenon of a tight cochlear control mechanism ensuring cochlear function, we can speculate that pathologies leading to a failure of these mechanisms could cause more pronounced symptoms resembling those we observe in Ménière's disease patients.

P73 Intraoperative observation of the endolymphatic sac by narrow band imaging

*H. Kumagami¹, M. Terakado¹, Y. Sainoo¹, M. Hara¹, H. Takahashi¹

¹Nagasaki University, Department of Otolaryngology Head and Neck Surgery, Nagasaki, Japan

A decrease or occlusion of vessels in the endolymphatic sac (ES) is suggested to be related to the pathogenesis of Ménière's disease (MD) by observing the temporal bone obtained from a few cases of MD. However, whether the findings are an essential causative factor is controversial. Thus, in the present study, the ESs were observed by a narrow band imaging endoscopy during surgeries in patients with MD (n=5) and vestibular schwannoma (VS, n=6) to evaluate characteristics of vessels in the ES. According to the guidelines for MD defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) 1995, all the patients of MD were definite cases and underwent endolymphatic surgery. In the patients with VS, the ES was observed during translabyrinthine removal of the tumor. The ESs were sampled and observed electron-microscopically. Comparing with the ES of the VS, the ES of MD was less abundant in vessels and showed degeneration of the epithelium. In some cases with MD, a decrease of vessels is indeed likely to play a role in the genesis of the disease.

P74 Molecular basis of tinnitus

*M. Knipper¹, L. Rüttiger¹, W. Singer¹, A. Zuccotti¹, R. Panford-Walsh¹, M. Matsumoto¹, U. Zimmermann¹, M. Jaumann¹, C. Franz¹, H.-S. Geisler¹, I. Köpschall¹, K. Rohbock¹, H. Xiong¹, K. Varakina¹, S.C. Lee¹, S. Verpoorten¹, T. Schimmang²

¹Tübingen Hearing Research Centre, Molecular Physiology of Hearing, Tübingen, Germany

²Universidad de Valladolid y Consejo Superior de Investigaciones Científicas, Instituto de Biología y Genética Molecular, Valladolid, Spain

Tinnitus is a non-curable stress-related brain disorder, that is mostly noise-induced and whose origin is unknown. We have addressed the molecular and physiological basis of this disease using a combined approach that included behaviorally tested tinnitus (Rüttiger et al., Knipper, *Hear Res* 2003), hearing measurements (including DPOAEs, ABRs and ABR wave analysis) and markers that trace network activity (Arc/Arg3.1). Data analyzed the first time equally hearing impaired animals that were behaviorally distinguished in hearing impaired animals with and without tinnitus. We compared animals between the periphery of the cochlea and the auditory cortex, including the hippocampus and amygdala. We also included an analysis of altered responsiveness after stress priming. We unraveled a tinnitus specific trait that may explain some of the existing controversies about the molecular basis of tinnitus.

Acknowledgements: This work was supported by the Marie Curie Research Training Network CavNET MRTN-CT-2006-035367, the Deutsche Forschungsgemeinschaft DFG-Kni-316-4-1 and Hahn Stiftung (Index AG).

P75 Alteration of inhibitory feedback mechanisms in the cochlea and dorsal root ganglion by KCC2 and NKCC1 after injury

*D. Campanelli¹, J. Tsung-Chieh Chen², W. Singer¹, A. Zuccotti¹, L. Rüttiger¹, J. Hu², M. Knipper¹

¹Tübingen Hearing Research Centre, Molecular Physiology of Hearing, Tübingen, Germany

²CIN, Centre for Integrative Neuroscience, Tübingen, Germany

The symptoms and signs of phantom sensations like tinnitus and neuropathic pain have many similarities (Moller AR., 2007). Similar hypothesis have been presented regarding how the symptoms are generated. The changes in the auditory system that cause tinnitus and the changes in the somatosensory system that cause neuropathic pain may be initiated from the periphery, i.e. the inner ear or the auditory nerve for tinnitus and peripheral nerve endings for neuropathic pain (Moller AR., 2007).

There is considerable evidence that expression of neuronal plasticity plays a central role in the development of the abnormalities that cause tinnitus and neuropathic pain. Changes in neuronal plasticity can affect the balance between excitation and inhibition, promote hyperactivity, and cause re-organization of specific parts of the nervous system e.g. redirection of information to parts of the nervous system normally not involved in processing information (Moller AR., 2007).

Since much more is known about the generation of neuropathic pain than about tinnitus, it is valuable to take advantage of the knowledge about neuropathic pain in efforts to understand the pathophysiology of tinnitus.

It is known that the chloride transporters NKCC1 (Sodium-Potassium-Chloride Co-transporter 1) and KCC2 (Potassium-Chloride Co-transporter 2) as well as the neurotrophin BDNF (Brain-derived neurotrophic factor), play a pivotal role in the generation of neuropathic pain (Price et al., 2009; Coull et al., 2005). Although most of the studies are focused the central nervous system (Blaesse et al., 2009), there are some experimental evidences of an altered expression of NKCC1, KCC2 and BDNF, after nerve injury, in the dorsal root ganglia (DRG) (Kanaka et al., 2001; Funk et al., 2008).

The aim of this study is to investigate if expression of NKCC1, KCC2 and BDNF is altered following tinnitus-inducing trauma in the cochlea and also nerve injury in DRGs

This work was supported by the Deutsche Forschungsgemeinschaft (EXC 307).

P76 Hyperhomocysteinemia causes premature hearing loss in C57BL/6J mice

*R. Martínez-Vega¹, G. Varela-Moreiras², T. Partearroyo², M. Pajares³, I. Varela-Nieto^{1,4,5}

¹Instituto de Investigaciones Biomédicas Alberto Sols, Fisiopatología Endocrina y del Sistema Nervioso, Madrid, Spain

²Universidad San Pablo CEU, Ciencias Farmacéuticas y de la Alimentación, Boadilla del Monte, Madrid, Spain

³Instituto de Investigaciones Biomédicas Alberto Sols, Metabolismo y señalización celular, Madrid, Spain

⁴Centro de Investigación Biomédica Red de Enfermedades Raras (CIBERER), Unit 761, Valencia, Spain

⁵Instituto de Investigación Hospital Universitario La Paz (IdiPAZ), Madrid, Spain

Introduction: Alterations in plasma homocysteine (pHcy) levels and betaine homocysteine methyltransferase (BHMT) expression have been reported in genetic mouse models of human deafness (1). BHMT is one of the enzymes responsible for Hcy remethylation leading to methionine synthesis, its changes being one of the causes of increased pHcy levels and indirectly altering production of glutathione (2). Nutritional factors modulate Hcy metabolism, and hence we have studied the impact of a dietary-induced folic acid deficiency on the cochlear methionine metabolism and in hearing.

Material and Methods: Hearing capacity from the control and folate deficient diet groups was assessed by ABR threshold analyses after 8 weeks of treatment. RT-PCR and western blot were used to determine the cochlear levels of methionine metabolism enzymes, whereas pHcy was determined by HPLC. Cochlear morphology was evaluated by hematoxylin-eosin staining and immunohistochemistry techniques.

Results: The control group showed normal ABR thresholds (8 to 28 KHz, 27- 48 dB SPL) whereas the folate-deficient group presented moderate to severe hearing loss (8 to 28 KHz, 52-85 dB SPL). Folate deficiency caused hyperhomocysteinemia together with significant changes in protein and mRNA levels of enzymes involved in methionine metabolism and oxidative stress biomarkers. Control and folate-deficient mice showed normal cytoarchitecture and signs of severe sensorineural hearing loss, respectively.

Conclusions: Folate deficiency causes alterations in the cochlear methionine cycle leading to an increase in pHcy levels, which is concomitant with molecular and cellular alterations in this organ and premature hearing loss in the C57BL/6J mouse.

1. - Cohen-Salmon, M. et al. (2007).Proc Natl Acad SciUSA104:6229.
2. - Pajares, M.A. and Pérez-Sala D. (2006).Cell Mol Life Sci63:2792.

Acknowledgments: RMV holds a CSIC predoctoral JAE fellowship. This work was supported by grants from Ministerio de Economía y Competitividad (SAF2011-24391, BFU2009-08977) and PULEVA. We appreciate very much the collaboration of Dr. C. Martínez-Alvarez (UCM, Madrid, Spain).

P77 A discussion of the relationship between the inner ear, bone and kidney in traditional Chinese medicine

*M. Long¹

¹University of Hawaii, Complametary Medicine, Honolulu/HI, USA

The theory behind the practice of Traditional Chinese Medicine (TCM) states that “the kidney controls the bone and ear”. Physiologically, this means that bone and ear development are related to kidney function. Pathologically, this means that that chronic bone disease and hearing loss are same caused by kidney deficiency. Therapeutically, this means that the promotion of bone and hearing repair may first entail a decision to improve kidney function. Finally, pharmacologically speaking, bone diseases, including fracture and osteoporosis, and hearing function deficiency can be treated with herbs that are understood to be kidney tonics. It may be conjectured that kidney function is essential to maintaining bone and ear health.

A kidney tonic herb, *Drynaria fortunei*, with a Chinese name, Gu-Sui-Bu (GSB), was classified to be a kidney affective herb in TCM. This herb has been used for promoting bone reformation after fracture or osteoporosis and is also used in the treatment of back pain, tinnitus and deafness, and tooth loss. An active flavanoid fraction (FF) from GSB was isolated by screening with multiple experiments using a gentamicine ototoxicity guinea pig model. Our animal experiments show that FF protect against hearing loss and promotes the repair of inner ear hair cell damage in gentamicine-induced ototoxicity animal model. FF also promoted the repair of kidney damage in guinea pig gentamicinel and mercury chloride -induced renal toxicity animal models. FF promoted renal proximal tubule regeneration and prolonged survival time in 5/6-nephrectomy mice model. FF also promoted bone reform in Retired rat model.

A component, kaempferol, was isolated from FF that shows a stimulatory effect on renal tubule cell proliferation, but no significantly effect on osteoblast and fiblast proliferation. This *in vitro* experiment indicates that the effect of kaempferol on renal tubular cell proliferation is consistent with the effect of FF *in vivo* experiments. However, this was inconsistent with the effect of FF on bone formation in vivo and clinical.

Consistent with the theory of TCM that “kidney dominates the bone”, the experiments showed tubular cell-conditioned culture medium increased osteoblasts proliferation and kaempferol -conditioned tubular cell culture medium significantly increased osteoblast proliferation above that of tubular cell-conditioned culture medium. This indicates that the kidney tubular cell-produce osteoblast growth factor(s) (OGF), kaempferol, increase the production of OGF and lends confirmation to the theory of TCM in which “the kidney dominates the bone”.

Questions that remain to be answered include: Does inner ear function depend on kidney function? Does FF or kaempferol promote inner ear hair cell proliferation through kidney metabolism?

P78 Anatomical preparation and characterization of human vestibular ganglia in the context of vestibular neuritis

*S. Himmelein¹, I. Sinicina², A. Horn-Bochtler³, K. Hübner¹

¹Klinikum Grosshadern, Neurology, German Dizziness Center, Munich, Germany

²Institute of Legal Medicine, Munich, Germany

³Ludwig-Maximilians-University, Institute of Anatomy & Cell Biology, Dept. I, German Dizziness Center, Munich, Germany

Introduction: Herpes-simplex-virus type 1 (HSV-1) is a neurotropic DNA-virus. Following primary infection (stomatitis aphthosa) the virus may reach the geniculate ganglion via the lingual nerve and subsequently infect cells in the vestibular ganglion (VG). After establishing latency in sensory neurons, reactivation can result in different diseases including Herpes labialis or vestibular neuritis (VN). VN is known to mostly affect the superior portion of the vestibular nerve, whilst sparing the inferior part. The reason for this clinical phenomenon remains uncertain.

Methods: We established and validated a method for the preparation of human VG from the temporal bone, involving rapid and gentle processing of post-mortem specimens. Fifty-six VG samples were used to perform various micro- and macro-anatomical studies.

Results: First we performed macro-anatomical studies of the VG and its position within the temporal bone. We evaluated the anastomoses between the facial and vestibular nerves, which are proposed as a route of infection. These were found more frequently to the superior part of the VG nerve. We also noted that in 65% of the temporal bones the posterior canal is innervated by 2 separate portions of the inferior vestibular nerve running in 2 separate canals, and that the superior vestibular nerve is on average 2.5mm longer than the inferior vestibular nerve. These anatomical differences could contribute to the clinical sparing of the inferior vestibular nerve in VN. We also evaluated the localization of the neuron fields within 8 VG and found neurons predominantly within individual branches, rather than the assumed ganglion stem. On a micro-structural level we studied the distribution of calcium-binding proteins in VG neurons and found about 20% of VG neurons had strong expression of Calbindin or Calretinin.

Conclusion: HSV-1 infection and reactivation in the VG is thought to cause VN, which mainly affects the superior vestibular nerve. By evaluating the micro- and macro-anatomy of human VG, new aspects of the tissue that could be implicated in Herpes virus infections were revealed.

P79 Expression of insulin signaling components in the sensory epithelium of the human inner ear

*E. Degerman¹, U. Rauch², S. Lindberg³, A. Hultgårdh², M. Magnusson⁴

¹Lund University, Diabetes, Metabolism and Endocrinology, Lund, Sweden

²Lund University, Vascular Wall Biology, Lund, Sweden

³Skåne University Hospital, OtoRhinoLaryngology, Lund, Sweden

⁴Lund University/Skåne University Hospital, OtoRhinoLaryngology, Lund, Sweden

Introduction: A number of studies have demonstrated an association between diabetes and inner ear morphological as well as functional alterations including degeneration of inner ear structures, sensorineural hearing loss, vestibular manifestations, presbycusis etc. However, the expression of the insulin receptor, downstream signaling components and interacting signaling networks, have not been demonstrated in the vestibular system.

Materials and Methods: Human saccule was obtained during the removal of vestibular schwannoma via the translabyrinthine approach. The sample was immediately fixed and embedded. Sections were cut with a cryostat and collected on slides and were stored at -80°C until use. Sections were immunohistochemically stained and observed using a camera-equipped fluorescence microscope.

Results: Key insulin signaling components, the insulin receptor, the insulin receptor substrate 1 (IRS1), protein kinase B (PKB) and two PKB targets, the insulin sensitive glucose transporter (GLUT4) and the insulin activated cAMP degrading enzyme, phosphodiesterase (PDE) 3A, were shown to be expressed in the human saccule sensory epithelium. In many cell-types insulin signaling networks cross-talk with cAMP signaling systems. Two calcium sensitive PDEs, PDE1B and PDE1C, the vasopressin receptor 2, and the transcriptional factor TORC2, components of relevance in cAMP signaling contexts, were shown to be expressed in human saccule. IRS1 and PDE1C were selectively expressed in hair cells whereas the other components were expressed in supporting cells or in both cell types as judged from co-expression or not with glial fibrillary acidic protein, a marker for supporting cells. In addition to being expressed in the sensory epithelium, IRS1 and PDE1C appeared to be localized in connection to sensory nerves whereas GLUT4 was expressed in the perinuclear area of stromal cells.

Conclusion: Insulin as well as some selected cAMP signaling components were shown to be expressed in human saccule which could have a role in the observed link between diabetes and balance/hearing disabilities and these components provide a new platform for drug development.

P80 Microarray analysis of the mouse cochlea in induced sepsis by lipopolysaccharide induced sepsis model

*J.-W. Choi¹, A.-N. Choi¹, B.-Y. Choi², J.-W. Koo^{2,3}

¹Otorhinolaryngology-Head and Neck Surgery, School of Medicine, Chungnam National University, Daejeon, Republic of Korea

²Seoul National University Bundang Hospital, Otorhinolaryngology, Seongnam, Republic of Korea

³Seoul National University Medical Research Center, Research Center for Sensory Organs, Seoul, Republic of Korea

Introduction: Several risk factors predispose the patients to increase ototoxicity during aminoglycoside treatment. Bacterial sepsis had been shown to potentiate inner ear uptake of aminoglycoside not only by increased serum level of aminoglycoside secondary to poor renal function, but also by increased permeation of aminoglycoside across the blood-labyrinthine barrier (BLB) into the stria. In this study, we investigated the modulation of cochlear gene expression in lipopolysaccharide (LPS) induced mouse sepsis model, thus hoped to find the potential mechanism related to increase BLB permeability during sepsis.

Materials and methods: Three groups of mice (C57BL/6, 4~5 weeks) received were injected intraperitoneally one of the followings via intraperitoneal injection: saline (control), 1 mg/kg of LPS (group A) and 10 mg/kg of LPS (group B). Differential gene expression profiles at 24 h after injection were analyzed with DNA microarray that covers 28,853 genes.

Results: When compared with control group, 103 genes and 327 genes were up-regulated in group A and group B, respectively. 12 genes and 131 genes were down-regulated in group A and group B, respectively. When compared between Group A and B, 44 up-regulated genes and 2 down-regulated genes were identified. Among of those genes, positive linear increase was observed *innrf125*, *gbp2*, *5*, and *cxcl10*.

Conclusions: Microarray analysis identified 44 up-regulated and 2 down-regulated genes in LPS induced sepsis model. Sepsis results in numerous gene expression of mouse inner ear. Interferon related genes such as *gbp2*, *gbp5* and *cxcl 10* were are significantly up-regulated with dose dependent manner of LPS. Investigating the role of modulated genes in sepsis is necessary to unveil the mechanism facilitating stria permeability

P81 Quantitative MRI assessment of the inner ear blood labyrinthine barrier: A translational effort towards clinical applications

J. Le Floc'h¹, S. Vljakovic¹, A. Nuttall², W.D. Rooney³, *P. Thorne⁴

¹The University of Auckland, Physiology, Auckland, New Zealand

²Oregon Hearing Research Center, Department of Otolaryngology, Oregon Health and Science University, Portland/OR, USA

³Oregon Health and Science University, Advanced Imaging Research Center, Portland/OR, USA

⁴The University of Auckland, Section of Audiology, Auckland, New Zealand

Introduction: Hearing loss is a major health problem affecting 1 in 6 of the New Zealand population and is mostly caused by cochlear diseases. To date, there have been no reports which quantify changes in vascular permeability of the inner ear in animals or in humans, which may underpin diseases.

Purpose: The primary purpose of this work is to develop and apply dynamic contrast enhanced (DCE) MRI techniques to quantify changes in vascular permeability of the inner ear in normal and inflamed guinea pig cochleae. A secondary purpose is to investigate the feasibility to translate these DCE-MRI techniques to study the vascular permeability of the human inner ear.

Methods: Anaesthetized guinea pigs (GPs, n=8) were inoculated intratympanically by bacterial lipopolysaccharide (LPS, 0.8mg/kg) and DCE-MRI was performed at 4 days (7), 7 days (4) and 10 days (3) after LPS sensitization. Using a 4.7T MR system, control saline-treated guinea pigs (n=2) were each scanned at 4, 7 and 14 days after injection of saline. A two-compartment pharmacokinetic (PK) model was used to determine the rate constant (K^{trans}) characterizing gadolinium based contrast agent (GBCA) leakage from the vascular space into the extravascular, extracellular space of the cochlear tissues. Human participants with normal hearing (n=4) underwent a DCE-MRI study using a 3T clinical MRI system (GBCA administered as a bolus at a dose of 0.1mmol/kg).

Results: PK modelling showed that the vascular permeability K^{trans} of the GP cochlea 4 days after LPS injection ($0.0799 \pm 0.0110 \text{ min}^{-1}$) was 2.6-fold greater than K^{trans} in GPs 7 days after LPS injection and 3-fold greater than K^{trans} in control and in LPS-treated GPs 10 days after LPS injection. No significant GBCA uptake was observed in the normal human inner ear 70 minutes after GBCA injection, suggesting a very small K^{trans} .

Conclusion: It is possible to quantitatively assess inner ear vascular permeability in normal and inflamed cochleae in animals. The results establish DCE-MRI as a promising diagnostic tool for human hearing conditions such as labyrinthitis, autoimmune inner ear disease or sudden deafness. This study was approved by the University of Auckland Animal Ethics Committee and the Northern X Regional Ethics Committee.

P82 Magnetic resonance imaging of the inner ear after intratympanic and intravenous gadolinium injections

*T. Nakashima¹, T. Iida¹, M. Teranishi¹, T. Yoshida¹, M. Kato¹, M. Sone¹, M. Yamazaki¹, S. Naganawa¹

¹Nagoya University, Department of Otorhinolaryngology, Nagoya, Japan

Objectives: Endolymphatic hydrops can be visualized by magnetic resonance imaging (MRI) after intratympanic (IT) or intravenous (IV) injection of gadolinium-based contrast material (Gd). In general, the IT method has higher enhancement of the perilymph and better ability to predict IT drug transition to the inner ear. The IV method is less invasive and enables stable bilateral observation of the condition of the cochlea. We performed MRI of the inner ear after IT and IV Gd injections (IT + IV) to obtain merits of both methods simultaneously.

Methods: Twenty-four hours before the MRI, Gd diluted eightfold with saline (v/v 1:7) was injected intratympanically in the ear with unilateral Meniere's disease. Four hours before the MRI, standard-dose Gd (0.1 mmol/kg or 0.2 ml/kg) was injected intravenously. Three-dimensional fluid-attenuated inversion recovery (3D-FLAIR), three-dimensional real inversion recovery (3D-real IR), heavily T2-weighted 3D-FLAIR, positive endolymph image (PEI), HYbriD of Reversed image Of Positive endolymph signal and native image of positive perilymph Signal (HYDROPS) MRIs were taken as described in the literature published recently (Naganawa et al., *Magn Reson Med Sci*, 2010-2012, in press).

Results: The IT + IV method revealed advantages of IT and IV methods simultaneously. In some patients who were clinically diagnosed as having unilateral Meniere's disease, asymptomatic endolymphatic hydrops was observed in the contralateral ear.

Conclusions: Degree of endolymphatic hydrops and disruption of the blood-labyrinthine barrier can be evaluated by MRI with advanced techniques. Each IT and IV Gd administration method has its advantages and disadvantages. IT + IV method is useful especially in patients with unilateral Meniere's disease before intratympanic gentamicin therapy.

P83 The early onset of oxidative stress processes in the organ of Corti after intense noise exposure

*A.R. Fetoni¹, G. Maulucci², S.L. Eramo³, F. Paciello¹, M. De Spirito², G. Paludetti¹, D. Troiani³

¹Catholic University of Rome, Institute of Otolaryngology, Rome, Italy

²Catholic University of Rome, Institute of Physic, Rome, Italy

³Catholic University of Rome, Institute of Physiology, Rome, Italy

Introduction: It is well known that outer hair cells (OHCs) are more vulnerable to insult than inner hair cells (IHCs) to the acoustic trauma, drug ototoxicity or presbycusis. The common basis for OHC loss appears to be apoptosis triggered by the generation of reactive oxygen species (ROS) and the main source for ROS is the mitochondrial respiratory chain. The current study was designed to investigate the onset of oxidative mechanisms and the initial pattern of OHC death induced by exposure to intense noise.

Material and Methods: The guinea pigs were used as model of acute acoustic trauma. Functional, morphological and immunohistochemical changes in OHC during the first 48 hours after noise exposure were studied. Two-photon microscopy was applied to study the NAD(P)H fluorescence as a measure of superoxide production and membrane stiffness in OHCs in a living explanted organ of Corti preparations.

Results: One hour after noise exposure the threshold shift was elevated to about 40-50 dB and the hearing loss level and its recovery was paralleled by a rapid ROS production in OHCs. NADPH oxidation showed a different distribution related to the different vulnerability of noise induced damage of OHCs and activation of endogenous mechanisms of protection. Furthermore, the acoustic trauma induced both a rigidification and a loss of fluidity polarization of the plasma membrane indicating that the modification of active processes of OHCs is involved in NHIL.

Conclusion: The results of the study demonstrated that induction of oxidative stress in OHC after the noise exposure is an extremely rapid process and perturbation of OHC electromotility is involved in NIHL.

P84 Comparison of effect of impulse and continuous noise exposure on mouse cochlea

*Y.H. Kim¹, *J.H. Park¹, B.J. Kim¹

¹Seoul National University Boramae Medical Center, Seoul, Republic of Korea

Various types of noises can lead to hearing threshold shift. Noise-induced hearing loss (NIHL) by impulse noise has been made with gunshot noise before. The present study was performed to investigate the effect of noise of titanium head golf driver on mouse cochlea and we also compared the effect of impulse noise and continuous noise. A total thirty-one balb/C mice (20 - 22 g) with normal hearing were used in this study. Among these animals, 26 mice were exposed to noise and remaining 5 animals without exposure to noise served as normal control. For the noise exposure, impulse noise (titanium head golf driver's hitting noise) centered around 4.5 kHz with 120.5 dB SPL for 2 hrs (1440 repetitions; 1/5 sec) was used. And continuous noise at the same Hz with same dB SPL was exposed for 288 seconds. Auditory brainstem response (ABR) was measured before noise exposure, day 7, and day 14 after noise exposure. And, histopathological examinations were done on day 14 after noise exposure. ABR in impulse noise group showed PTS at 4, 8, 16, 32 kHz, and click after noise exposure and threshold shifts at immediate, 1 week, 2 weeks after noise exposure were statistically significant compared to baseline. And, the threshold shifts in impulse noise group were greater than those in continuous noise group. Two weeks after noise exposure, histopathologic findings were well correlated with auditory functions. These findings indicate that repeated exposure of impulse noise may cause greater damage to the cochlea than continuous noise with same exposure time.

P85 About electromyographic signals and the automatic detection of the stapedius reflex

*R. Warmuth¹

¹University of Rostock, Rostock, Germany

Cochlear implants help regain the auditory sense in cases where the cochlea is not able to transform the sound signals into electric stimulation for the auditory cortex. By their very nature, cochlear implants require a calibration process, which is often based on the electrically evoked stapedius reflex threshold (ESRT), since it very well correlates with a person's most comfortable loudness level. During the ESRT-based calibration process, the implant stimulates the cochlea with increasing currents until the stapedius muscle contraction can be observed visually. The calibration process described above yields a first, rough implant adjustment, which requires an additional, extensive calibration procedure a few weeks after the implantation.

The goal of this project is the development of an autonomous calibration scheme that is constantly monitoring and occasionally re-calibrating the implant. To this end, the system utilizes the electromyographic (EMG) signals that it directly derives at the stapedius muscle by means of specific electrodes.

The system basically correlates the external (objective) loudness and the observable stapedius muscle activity, and correlates them with the expected, pre-defined stapedius activity. Too large a difference between the expected and observable stapedius activity, the system triggers an adaptation cycle of the implant's gain factors. The processing of the EMG signals of the stapedius muscle employs a series of three filters: (1) a 4th-order band-pass filter with corner frequencies of 30 and 500 Hz for removing steady components and high-frequency noise, (2) a rectifier, and (3) a 4th-order low-pass filter with a corner frequency of 2 Hz for extracting the EMG signal's envelope. In its subsequent processing stages, the system considers this envelope as the physical activity of the stapedius muscle. This system has undergone a series of different tests.

Since no solid EMG signal data base of the stapedius muscle exists nor can it be derived for obvious medical reasons, the first evaluation was done with labeled data (external forces) that was derived at the quadriceps femoris muscle as well as the soleus muscle, which are also skeletal muscles as the stapedius is. The results show a qualitative correlation between both the calculated and the true external force. Afterwards, the available EMG data of the stapedius muscle were fed into the same system. Similarly, the system's output indicates an increasing muscle force as the external stimulation increases.

In future research, this continuous, non-linear dependency between EMG data and the muscle force will be used as the core principle of the autonomous adaptation process.

P86 Otosclerosis as a neoplastic replica of the outer layer of the otic capsule

L. Michaels¹, V. Chrobok², *S. Soucek³

¹University College London, Histopathology, London, United Kingdom

²Charles University, Otolaryngology, Hradec Kralove, Czech Republic, Czech Republic

³Imperial College London, Surgery and Cancer, London, United Kingdom

Introduction: The object of this work was to determine the pathological basis of otosclerosis, by histologic study of the developing otic capsule and of temporal bones with otosclerosis.

Materials and Methods: Step sections of 60 celloidin-embedded temporal bones from fetuses and 24 from patients aged between 1 and 52 years, were examined to determine the development of the otic capsule outer layer. Step sections of 63 temporal bones, each with 2 or more deposits of otosclerosis, were surveyed to correlate pathological appearance of otosclerosis with normal development.

Results: The normal otic capsule outer layer is formed of lamellar bone accompanied by very numerous intercommunicating canals, "Volkmann's canals" (like Haversian canals, but multidirectional). All of the temporal bones with otosclerosis, showed prominent plaques of the disease apparently derived from periosteum. The presumed invasive front of all the plaques on the edge opposite to their origin (i.e. labyrinthine side) showed a variable level and a darkly-stained appearance with large numbers of osteoblasts, poorly differentiated Volkmann's canals and poorly-formed osteoid. The otosclerotic bone became progressively better differentiated away from the darkly-stained zone, often with dilated Volkmann's canals (otospongiosis) and definite lamellar bone. Maturation was most marked in the suggested origin region of the plaque near the periosteum.

Conclusions: Otosclerotic plaques present a histologic replica of external layer otic capsule. They seem to arise from similar cells in the periosteum superiorly, and form tumour-like masses invading inferiorly and laterally towards the labyrinthine spaces.

Morphological techniques

P87 Establishment of a glial cell free culture of dissociated spiral ganglion neurons

*S. Frenz¹, K. John¹, K. Rak¹, S. Jablonka², R. Hagen¹, R. Mlynski¹

¹Klinik und Poliklinik für Hals-, Nasen- und Ohrenkrankheiten, plastische und ästhetische Operationen, Würzburg, Germany

²Institut für Klinische Neurobiologie, Würzburg, Germany

Auditory neuropathy is a sensorineural hearing disorder wherein the outer hair cells are functional but the transmission of the acoustic information to the brainstem is lacking. Since spiral ganglion neurons are crucial for sending the generated nerve impulses from the hair cells to the brainstem, a reliable method to culture them *in vitro* might open new possibilities to study auditory neuropathy. Cultured neuronal cells are highly dependent on neurotrophic factors, which are secreted by non-neuronal cells like glial cells. Since the secretion of growth factors by these cells is not controllable *in vitro*, the aim of this study was to develop a glial cell free culture for spiral ganglion neurons.

Cells were isolated from the spiral ganglion of C57Bl/6 mice at postnatal day 5 and cultured on coverslips with different coatings. Various cell culture media and factors were tested. After 4 days cultured cells were fixed and stained by DAPI, β -Tubulin and β -III Tubulin. Number of glial cells and neurons were evaluated as well as neurite length.

No significant difference in glial and neuronal cell number between Polyornithin and Poly-D Lysine as coating material could be evaluated. However, additional laminin coating increased significantly axon length and raised the ratio between neurons and glial cells. Furthermore the use of DMEM medium increased the percentage of neurons compared to Neurobasal medium. Arabinosylcytosine as additive decreased the glial cell number, but increased the numbers of neurons whereas neurite length was not affected. Addition of leukemia inhibitory factor, a pleiotropic cytokine which plays a role in survival of sensory and motoneurons, increased the percentage of neurons in culture, too.

In conclusion laminin coating, the use of DMEM medium and addition of Arabinosylcytosine and leukemia inhibitory factor results in nearly glial cell free culture conditions for *in vitro* experiments of spiral ganglion neurons. These optimized conditions will prove to be useful to further investigate the molecular properties of the cultured auditory neurons without uncontrollable endogenous stimulation. This could possibly lead to new insights regarding the pathophysiological findings in auditory neuropathy.

P88 Spiral ganglion neuron quantification in the guinea pig cochlea using CLSM

A. Wrzeszcz¹, V. Scheper¹, M. Hütten¹, H. Meyer¹, I. Nolte², T. Lenarz¹, *G. Reuter¹

¹Hannover Medical School, Department of Otolaryngology, Hannover, Germany

²University of Veterinary Medicine Hannover, Small Animal Clinic, Hannover, Germany

Neuron counting in the cochlea is a crucial but time-consuming operation for which various methods have been developed. One of them is embedding the cochlea into paraffin with subsequent generation of histological sections; another is embedding into epoxy resin and gradual grinding with documentation of every slice. To improve simplicity and efficiency, we tested a semi-automatic method based on Confocal Laser Scanning Microscopy (CLSM) and the free software ImageJ to visualize and quantify Rosenthal's Canal and the neurons within.

8 Cochleae from approx. 3 months old guinea pigs were fixated in paraformaldehyde (PFA) and decalcified. After dehydration with ethanol the cochleae were cleared in Spalteholz solution and transferred into glass chambers filled with it. CLSM was performed using the tissue's autofluorescence generating about 20 slices of the modiolus in 50µm steps. With a 10x objective, 3 z-series stacks were obtained and stitched using the freeware XuvTools. In ImageJ, in 7 locations in 5 slices per cochlea the perimeters of the Rosenthal's Canal were surveyed and the neurons first counted manually and then automatically by a plugin. Representative neuron diameters were measured randomly.

In contrast to the embedding methods the CLSM method has the advantage that the cochleae remain intact as an organ and keep their geometrical structure. Generation of three dimensional figures by digital imaging provides good orientation support. The tissue shows minimal or no shrinking artifacts and damages typical for embedding and sectioning. In comparison to the cell diameters of about 9µm in embedded sections, the cell diameters in the cleared cochleae reach an average of 20µm. Using the PFA-induced autofluorescence instead of stains saves time and costs. Z-stack creation is nearly fully-automatic and is frequently repeatable with various objectives, step sizes and without visible bleaching. First results show that the difference between the manual and the automatic counts is negligible.

We expect the CLSM method to be simpler and more effective compared to the other two methods. It provides high decrease of tissue damage and the automatic stack-generation as well as the count plugin reduces the effort considerably.

P89 Counting method of hair cells in Corti's organ

*L. Sanz^{1,2,3}, C. Avendaño⁴, S. Murillo-Cuesta^{2,5,6}, R. Martínez-Vega⁶,
J.A. Sistiaga^{1,2,3}, F. García-Alcántara^{1,2,3}, I. Varela-Nieto^{2,5,6}, T. Rivera^{1,2,3}

¹Hospital Universitario Príncipe de Asturias, ENT, Madrid, Spain

²Center for Biomedical Network Research on Rare Diseases (CIBERER), ISCIII., Valencia, Spain

³University of Alcalá, Alcalá de Henares, Spain

⁴Autónoma University of Madrid, Department of Anatomy, Histology & Neuroscience, M, Madrid, Spain

⁵Research Institute Hospital La Paz (IdiPAZ), Madrid, Spain

⁶Institute of Biomedical Research Alberto Sols (CSIC-UAM), Madrid, Spain

Introduction and Objective: The cochleogram is a graphic record which represents hair cells along the length of the basilar membrane and relates cell damage with frequency specific values in hearing thresholds. The purpose of this study is to design a simple and robust method to quantitatively determine the distribution of the inner and outer hair cells at the organ of Corti in the mouse cochlea.

Materials and Methods: Six male CBA/CaOlaHsd mice with normal auditory brainstem responses were sacrificed at 2 months of age. The cochleae from both ears (n=12) were extracted, fixed and decalcified, and then divided in two parts (apical-middle and basal), obtaining around 80% of the whole extent of the basilar membrane. The organ of Corti (OC) was isolated and phalloidin-stained in multiwell glass slides. Using a fluorescence microscope and stereological software, the total length of the OC was divided into equidistant 5% sectors¹. The number of inner (IHC) and outer (OHC) hair cells in randomly distributed areas were determined, and cell density (cells/mm²) was estimated for each sector.

Results: The distribution of hair cells along the apical, middle and available basal turns of the cochlea was fairly homogeneous. The overall mean density for IHC and OHC was similar in both sides (Left IHC: 4.10, OHC: 12.12; Right IHC: 4.20, OHC: 12.22 (p>0.05), with a ratio IHC/OHC of 2.95 and 2.91 (right and left sides, respectively), without significant differences between them, and similar to physiological 3:1 ratio.

Conclusion: This method allows us to quantify the hair cell populations throughout the cochlear turns in a precise and reproducible manner and can be used to assess hair cell damage or regeneration in experimental mice models.

Acknowledgements: This research was funded by grants from the Spanish Ministry of Science and Innovation FIS (PI10/00394) to TR and SAF2011-24391 to IVN. RMV and SMC were supported by CSIC and CIBERER, respectively. We appreciate the help of R. Cediél (UCM) and L. Barrios (CTI CSIC).

¹Viberg et al. *Hear Res.* 2004 Nov;197(1-2):1-10.