Tutorial courses: Sunday 8th September 8h-8h50

- **A- A.F. Ryan:** "Transgenic mice for inner ear research"
- **B- T.R. Van De Water:** "Application of experimental embryology techniques to the study of inner ear development"

Cell signaling, ionic channels and receptors – Sunday 8th September 9h00-10h45

9h00-9h15 O1: Expression Patterns of Tight Junction Membrane Proteins, Occludin and Claudins, in the Inner Ear

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Tight junctions (TJs) are directly involved in maintaining the electrochemical potential gradient between the endolymph and perilymph in the inner ear. However, our knowledge on the expression and distribution of occludin and claudins, major TJ adhesion proteins, was still fragmentary. We then examined the expression and distribution pattern of claudins-1-18 (except claudin-17) in the inner ear of mice at 3 days, 6 days and 10 weeks of age by immunohistochemistry. In the organ of Corti, occludin/claudins-1/-3/-5/-9/-12/-18 and occludin/claudin-12/-14/-18 were detected in 10-week and 3-(6-)day mice, respectively. The marginal cells of the stria vascularis were positive for occludin/claudin-1/-3/-5/-8/-9/-12/-18 and for occludin/claudin-1/-3/-8/-10/-12/-14/-18 in 10-week and 3-(6-)day mice, respectively. In basal cells of the stria vascularis, occludin and claudin-11 were detectable both in 10-week and 3-(6-)day mice. Reissner's membrane was positive for occludin/claudin-1/-3/-5/-8/-9/-12/-18, for occludin/claudin-12/-18 and for occludin/claudin-3/-8/-12/-18 in 10-week, 3-day and 6-day mice, respectively. In the otolith organ, occludin/claudin-1/-5/-9/-12/-18 and occludin/claudin-1/-12/-18 were detected in 10-week and 3-(6-)day mice, respectively, and the ampullary crest of semicircular canals were positive for occludin/claudin-1/-3/-8/-9/-12/-18 in 10-week mice. These findings will be indispensable for future physiological studies of TJs in the inner ear.

9h15-9h30 O2: Expression of KCNQ1-5 voltage-gated K+ channel genes in rat and guinea pig cochleae

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Voltage-gated potassium channels are essential for regulation of membrane potential and neuronal excitability. Mutations in these ion channel genes lead to human disorders such as epilepsy, cardiac arrhythmias, and congenital deafness. Inner ear function relies mainly on two types of excitable cells, mechanosensory hair cells and primary afferent spiral ganglion neurons type I. Mechanotransduction by cochlear hair cells is further dependent on a K+ gradient across the cochlear partition. These processes involve several types of potassium channels, among them several voltage-gated subtypes. In previous work, we have identified time- and voltage dependent sub-threshold non-inactivating K+ current in cochlear outer hair cells. The aim of this study was to identify genetic correlates of these M currents in the inner ear using molecular and electrophysiological approaches. M-like potassium currents were recorded from guinea pig outer hair cells. Here we report on the expression of KCNQ subtype mRNAs as determined by reverse-transcription-polymerase chain reaction (RT-PCR) of total RNA from guinea pig and rat cochleae. PCR fragments were confirmed by sequence analysis. These results, taken together with ongoing localization of expression, will provide an extended basis for the molecular heterogeneity of M currents in cochlear tissues.

9h30-9h45 O3: Localization of Nitric Oxide Synthase I and III Isoforms in the microdissected Human Cochlea

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The role of nitric oxide(NO) in function and pathology of the inner ear is an issue of current discussion. The three isoforms of nitric oxide synthase(NOS) have been detected previously in the cochlea of mammals and recently the localization of NOS has been described in the human cochlea. Yet these previous studies have yielded conflicting results concerning the distribution of the NOS isoforms in the cochlea, depending on different methods or antibodies. In this study we examined the localization of NOS I and III in the microdissected human cochlea by immunohistochemistry. The inner ears were fixed, microdissected and prepared for FITC- immunohistochemistry without using decalcification or antigen retrieval procedures, both of them are known to alter the tissue preservation. Immunoreactivity to NOS I was detected in the region of outer(OHC) and inner hair cells(IHC), in nerve fibres and nerve endings at the sensory cells of the organ of Corti(OC), in the pillar cells and the spiral ganglion(SG) cells. Faint staining for NOS I could be observed in the supporting cells of the OC and in the lateral wall tissue. Staining for NOS III was located in endothelial cells of cochlear blood vessels, in the OHC and IHC of the OC, in nerve fibres and in the SG cells. We compare our observations with previous results in literature and discuss the role of NO as a neurotransmitter / neuromodulator at the hair cells, as regulator of cochlear blood flow and under certain conditions as mediator of excitotoxicity in the human cochlea.

9h45-10h00 O4: Biochemical evidence for the presence of serotonin transporters in the rat cochlea

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Serotonergic fibers have been recently identified innervating the cochlear receptor (Gil-Loyzaga et al, NeuroReport, 1997;8:3519-22; Gil-Loyzaga, Acta Otolaryngol, 2000;120:128-32). Besides, serotonin (5-HT) and its metabolite 5-HIAA were detected in the cochlea by HPLC-ED (Vicente-Torres et al, NeuroReport 1998;9:3699-701; Gil-Loyzaga et al, Acta Otolaryngol, 2000;120:128-32), and serotonergic receptor mRNAs by in situ hybridization (Johnson and Heinemann, Mol Cell Neurosci 1995;6:122-138) and PCR-RT (Oh et al, Mol Brain Res 1999;70:135-40). In order to clarify the cochlear serotonergic synaptic activity, the effect of 6-nitroquipazine (6-NQ), a serotonin selective reuptake inhibitor, on 5-HT and 5-HIAA concentrations was analyzed. Long-Evans rats were treated with 6-NQ or saline, exposed to silence or 90 dB SPL of white noise and perfused to remove the blood from the cochlea. Then, cochleae were removed and concentrations of monoamines and metabolites quantified by HPLC-ED, as previously described (Vicente-Torres et al, NeuroReport 1998;9:3699-701; J Neurosci Meth 2002, in press). The 5-HT cochlear concentration was higher in animals pretreated with 6-NQ than in saline treated ones, while 5-HIAA concentration was lower in 6-NQ treated animals. These changes were detected independently of the acoustic stimulation. In contrast, 6-NQ induced no modification in concentrations of dopamine, norepinephrine, DOPAC or HVA. Present results indicate that 6-NQ induced a 5-HT accumulation within the cochlea, together with a decrease in 5-HT degradation, which could be subsequent to the blockade of 5-HT transporters. Acknowledgements: This work was supported by Spanish grants BFI2001-1447 and FIS 2001/0652

10h00–10h15 O5: Thyroid hormone & ion channels

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Retardation of the expression of the fast-activating potassium channel IK,f or BK-channel was recently documented to be related with deafness in thyroid hormone receptor beta mutant mice (Ruesch et al., PNAS, 1998). In the developing cochlea various ion channels are expressed in outer and inner hair cells as well as in spiral ganglia neurons either shortly before, during or after the onset of hearing, dependent on the differentiation stage of the cell. We analysed the expression of different ion channels in hair cells and spiral ganglia neurons and studied a presumptive thyroid hormone (TH) dependancy in animals in which TH is retracted by goitrogen, TSH receptor mutations or TR mutations. Data may hint to a new principle how TH affects phenotypically important genes in the inner ear. Supported by a grant from the SFB 430 B3/Kni; IZKF A-Kni; DFG 316/3-3; Fortuene 972-0-0 Tuebingen.

10h15–10h30 O6: Reversal of vasospasms of the spiral modiolar artery (SMA): A potential new approach for

the treatment of sudden hearing loss

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Vasospasms of the SMA may cause an ischemic stroke of the inner ear that manifests itself by sudden hearing loss. Vasospasms are induced by hypercontraction of vascular smooth muscle cells; the intracellular mechanisms for these hypercontractions are still unknown, and the elucidation of these mechanisms remains an important clinical issue. We have shown previously that ET-1 induces vasospasms of the SMA via activation of ET_A-receptors. Here we tested the hypotheses that these vasospasms are a) reversible by ET_A-receptor antagonists, b) mediated by a Ca^{2+}-sensitization of the contractile apparatus via a Rho-kinase induced inhibition of myosin light chain phosphatase (MLCP), and c) reversible by the second messenger cAMP and the vasodilator CGRP. The smooth muscle cell Ca^{2+}-concentration ($[Ca^{2+}]_i$) and the vascular diameter were determined simultaneously by fluo4-microfluorometry and videomicroscopy, respectively. The Ca^{2+}-sensitivity of the contractile apparatus was evaluated by a correlation between the $[Ca^{2+}]_i$ and the vascular diameter. ET-1 induced vasospasms were prevented but not reversed by the ET_A-receptor antagonist BQ-123. The Ca^{2+}-sensitivity of the contractile apparatus was increased by ET-1 and by inhibition of MLCP with the selective inhibitor calyculin A. ET-1 induced Ca^{2+}-sensitization and vasospasms were prevented and reversed by the selective Rho-kinase antagonist Y-27632, by the CGRP-receptor agonist CGRP, and by the cAMP analog dbcAMP. We conclude that ET-1 induces vasospasms of the SMA via an ET_A-receptor mediated activation of Rho-kinase. Activation of Rho-kinase results in a Ca^{2+}-sensitization of the contractile apparatus that may be due to an inhibition of the MLCP. The observation that vasospasms were reversed by Y-27632 but not by BQ-123 suggests that Rho-kinase, rather than ET_A-receptors, are the most promising pharmacological target for the treatment of vasospasms, ischemic stroke and sudden hearing loss. Supported by NIH RO1-DC04280

10h30–10h45 O7: VEGF (Vascular endothelial growth factor) dependent activation of extracellular regulated kinase 1/2 (ERK1/2) in the cochlea of puinea pig

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Proliferation signals by growth factors such as VEGF are partialy mediated by activation of ERK1/2. Even though VEGF, its membranous receptors FLT1 and FLK1 and inactive ERK1/2, are ubiquitary expressed in the guinea pig cochlea, the diphosphorylated or active ERK1/2 can not be found under control conditions. In this in vitro experiment we aimed to clarify a VEGF dependent activation of ERK1/2. 60 guinea pigs were decapitated and the temporal bones were extracted. The cochleae were incubated for 5, 15, 30 and 60 min respectively (each n=6) in an physiological solution containing 12,5 ng/ml VEGF. The control experiment consisted of the same number of specimen immersed in a solution which did not contain VEGF. Finaly the cochleae were fixed with 4% PFA and with antibodies against ERK1/2 and diphospho-ERK1/2. A Westernblot was performed to confirm the specifity of both antibodies (N=6 each). After 5 min. of incubation with VEGF a strong staining for active ERK1/2 was found in supporting cells. In cells of Hensen and the limbus the staining became intense after 15 min. At 30 and 60 min active ERK1/2 could not be detected in the cells mentioned above anymore. Neurons and spring coil vessels showed active ERK1/2 expression after 60 min. In the animals not incuabted with VEGF no active ERK1/2 could be found at all times. No changes of inactive ERK1/2 expression could be found in any group. The VEGF incubated homogenate used for Westernblot showed mainly an increase of diphospho-ERK1. The current in vitro experiment shows a time-dependent activation of ERK1/2 in the guinea pig cochlea after incubation with VEGF. ERK1/2 is supposed to be a regulator of cell proliferation. It may also be involved in regulating nitric oxide synthase (NOS), which is plays a role in supporting cell physiology and neurotransmission in the cochlea.

Electrophysiology of cochlea and auditory pathways – Sunday 8th September 11h15-12h15

11H15–11H30 O8: Are distortion product otoacoustic emission (DPOAE) responses amplified, after carboplatin treatment ?

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INTRODUCTION: Carboplatin is a antitumour drug which selectively alters the micromechanical function of the inner hair cells (IHCs) of the organ of Corti. Data from an earlier study (Wake et al, 1996) from a chinchilla model support the hypothesis that carboplatin administration not only disrupts the IHC function but affects the efferent feedback loop to the cochlea, causing an amplification of the otoacoustic emission responses. The present study was designed to verify the OAE amplification-issue, using distortion product emissions (DPOAEs) in a wider bandwidth range. MATERIALS AND METHODS: Carboplatin (Paraplatin,10 mg/ml, Brystol Myers) was administered by a 30 min low infusion. Pre and 72-hour post DPOAE and ABR recordings were acquired from a group of 12 Sprague -Dawley rats (mean weight 360 ± 35 gr). The animals were anesthetized with a ketamine-atropin anesthesia administered in two consecutive phases.The DPOAE responses (cubic distortion products) were recorded with 4 asymmetrical protocols P1= 60-50 ; P2 = 50-40 ; P3= 40-30 and P4= 30-20 dB SPL, in the frequency range from 5.0 to 16 kHz. ABR responses were obtained for bipolar clicks and tone-pips at the frequencies 8.0, 10.0, 20.0 and 30 kHz using stimuli in the range from 100 to 30 dB SPL . Wave-III was used to identify shifts in hearing threshold. Four animals randomly selected, underwent a SEM evaluation assessment. A repeated measures model was used for the analysis of the DPOAE responses. RESULTS: ABR threshold shifts of 20 dB were observed in the frequencies from 20 to 30 kHz and shifts of 10 dB in the frequencies 8.0 and 10.0 kHz. The comparison of pre and post treatment DPOAE responses did not revealed any significant changes for protocols P1, P2 and P3. Border line differences were observed for the P4 protocol. CONCLUSIONS: The findings from the rat model contrast the data available in the literature from a gerbil animal model and suggest that different efferent mechanisms might suppress or enhance the activity of the OHCs of the organ of Corti across various species.

11h30–11h45 O9:Auditory nerve stochasticity and deafness

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Stochastic response properties are evident in the responses of auditory neurons responding to electrical stimulation of the auditory nerve. Although the level of the stochastic behaviour is less than seen in hearing animals, the variance in spike rate that results appears to be important for the performance of perceptual tasks such as loudness discrimination (Bruce et al, IEEE Trans Biomed Eng 46:1393-1404). There is experimental evidence that the variance in spike rate from electrical pulse trains decreases with the duration of deafness (Javel and Shepherd, J.Acoust. Soc. Am., 107). These data are analysed further to determine whether the deafness-dependent reduction in variance can be attributed to changes in the neural membrane. Extracellular responses from individual auditory nerve fibres were recorded from the barbiturate anaesthetised 6 domestic cats following differing durations of deafness (Shepherd and Javel, Hear. Res. 108:112-144). In this study the deafening was achieved by kanamycin/ ethacrynic acid (5 animals), or in one case direct current. Input-output functions were recorded in response to biphasic, charge balanced electrical pulse trains, presented at 200 pulses/s (100 ms duration, repetition interval 200 ms). Data extracted were the variance in spike rate from the pulse train, and the relative spread from the I/O of the first pulse in the pulse train. The relative spread was obtained by fitting an integrated gaussian curve to the first-pulse I/O function, and defined as the standard deviation divided by the mean of the fitted curve. This reflects theoretical considerations that membrane noise has a gaussian distribution. The variance of the pulse train decreased with the duration of deafness. The relative spread decreased following short term deafness (5 animals, 2-3 months) but was relatively greater following long-term deafness (1 animal, 3 years). Conclusions: A decrease in membrane noise following short-term deafness contributes to the corresponding reduction in pulse train variance. The further reductions in variance following long-term deafness appear not to be explained by changes in membrane noise.

11h45–12h00 O10: Dependence of exocytosis at the afferent hair cell synapse on L-type Ca^{2+} channels

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Ca^{2+}-dependent exocytosis of glutamate mediates synaptic transmission at the inner hair cell (IHC) afferent synapse. Using patch-clamp membrane capacitance measurements we previously demonstrated a steep Ca^{2+}-dependence of the underlying fusion of readily releasable vesicles and showed that L-type Ca^{2+}-channels are involved in stimulus-secretion coupling. However, nifedipine (10µM), a dihydropyridine (DHP) inhibitor of L-type Ca^{2+}-channels, blocked both Ca^{2+}-current and exocytosis to the same degree (~50%), which was not expected given the high power dependence of exocytosis on Ca^{2+}. The incomplete, linear block by the DHP prompted us to further investigate the dependence of the fusion of the readily releasable vesicle pool (RRP) on Ca^{2+}-influx through L-type channels. IHCs were stimulated by short (20ms) depolarizations, which selectively recruit the RRP, in the increasing presence (up to 10µM) of nifedipine or isradipine. Thereby, we modified the Ca^{2+}-influx over a range of ~85% of control. In support of our previous result inhibition of exocytosis related linearly to the reduction of available L-type Ca^{2+}-channels. BayK8644, a DHP agonist of L-type Ca^{2+} channels, greatly augmented the IHC calcium current but only sublinearly increased the amount of RRP exocytosis. In experiments on IHCs from mice lacking the alpha1D subunit of L-type Ca^{2+}-channels (Platzer et al., 2000 Cell 102, 89-97) we observed a reduction by about 90 % of the Ca^{2+}-current and a similar inhibition of exocytosis. Therefore, the alpha1D L-type Ca^{2+}-channel is the major regulator of exocytosis in IHCs. Moreover, the linear relationship of Ca^{2+}- channel block and exocytosis suggests, that the Ca^{2+}-concentration at each release site is controlled by few L-type channels, may be a single Ca^{2+}-channel. Supported by the DFG

12h00–12h15 O11: The consequences of passive neural properties for eCAP recordings with the forward-masking paradigm

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Modern cochlear implants can record the electrically evoked compound action potential (eCAP) of the auditory nerve through the intra-cochlear electrode array. The forward masking method is most widely used to eliminate the electrical artefact, which temporally overlaps with the neural response and exceeds its amplitude by several orders of magnitude. A masker-stimulus drives the auditory nerve into the refractory state, and a probe-stimulus is used to record the response in unmasked and masked conditions. The difference between these two responses then yields the eCAP. Nevertheless, usually part of the transient waveform of the eCAP is still obscured by the stimulus artefact. With our optically insulated set-up, the amplifier (gain: 5x) is not saturated during stimulus delivery. Recordings in guinea pigs show reproducible bi- and tri-phasic eCAPs, depending on the stimulus used, without any indication of degradation by a stimulus artefact. In addition, the commonly reported decrease of eCAP amplitudes for masker-probe intervals below 300-500µs is absent if the amplitude of the masker stimulus is chosen well above that of the probe stimulus. The most probable explanatory mechanism is that a too weak masker stimulus -apart from making fibres refractory- also causes sub-threshold excitation of nerve fibres somewhat further away. During the time interval necessary to discharge their membrane capacitances these fibres exhibit a lower threshold for full depolarisation by the probe stimulus. This leads to recruitment of additional nerves by the probe, and therefore the eCAP as measured with forward-masking has lower amplitudes. We observed time constants around 400µs, which is in line with known membrane time constants. We conclude that these passive neural properties necessitate a sufficiently large masker for reliable eCAP recordings with the forward masking paradigm.

Development and regeneration – Sunday 8th September 14h00

14h00-14h15 O12: Adenovirus-mediated GDNF prevents progressive inner hair cell loss following transient cochlear ischemia in gerbils

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Sudden hearing loss, which often results from disruption of cochlear function, is devastating to patients in the prime of life and is thought to be caused by an acute interruption of the blood supply to the inner ear. However, the details of the effects of transient ischemia on the cochlea remain unclear. Using a technique called experimental hindbrain ischemia, we successfully made a chronic animal model of transient cochlear ischemia in Mongolian gerbils and demonstrated that inner ear damage was closely related to the progressive inner hair cell (IHC) loss that follows ischemia. Adenovirus has recently attracted a great deal of attention as a vector for gene therapy. In this study, we assessed the utility of an adenoviral vector expressing glial cell-derived neurotrophic factor (GDNF) in ischemia-reperfusion injury of the gerbil cochlea. The vector was injected through the round window 4 days before ischemic insult. On the seventh day of ischemia, the CAP threshold shift and inner hair cell loss were remarkably suppressed in the Ad-GDNF group compared with the control group. These results suggest that adenovirus-mediated overexpression of GDNF is useful for protection against hair cell damage, which otherwise eventually occurs after transient ischemia of the cochlea.

14h15-14h30 O13: Transplantation of hair cell precursors, hair cells and stem cells in vitro and in vivo

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The loss of hair cells is the most common cause of sensorineural hearing loss and peripheral vestibular disorders. Once lost hair cells do not regenerate in mammals. The possibility of transplantation to replace lost hair cells has received relatively little attention in the inner ear. However, successful transplantation of stem cells and fetal neurons into the brain suggests the possibility that transplantation might be successful in the inner ear, as well. We have transplanted a variety of cells into the inner ear or its sensory epithelia, including immature mouse hair cells, mature mouse hair cells, and rat neural stem cells, using genetically regulated green fluorescent protein (GFP) to identify the transplanted cells. Using *in vitro* models we have found that immature hair cells can be successfully transplanted into a previously damaged vestibular sensory epithelium. The cells can integrate into the sensory epithelium, and form new stereociliary bundles. Neither uncommitted stem cells from the developing sensory epithelium, fully differentiated sensory cells derived from the adult inner ear, or stem cells derived from the central nervous system showed similar integration into a damaged epithelium. Immature hair cells transplanted *in vivo* survived for several weeks, but appeared to remain at the site of transplantation and showed no signs of integration into sensory tissue. Neural stem cells transplanted *in vitro* or *in vivo* showed evidence of migration, and integration into sensory epithelia was sometimes observed. However, no development of hair cell characteristics was seen. Supported by NIH/NIDCD grant DC00139, the Research Service of the VA, and the NOHR. Neural stem cells were generously provided by Dr. Fred Gage of the Salk Institute.

14H30-14H45 O14: TRANSPLANTATION OF AUTOLOGOUS MESENCHYMAL STEM CELLS INTO THE SPIRAL GANGLION IN GENTAMICIN-TREATED CHINCHILLAS

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The spiral ganglion cells play an essential role in hearing as the primary neurons of the auditory tract, especially when hearing must be restored by their direct electrical stimulation in patients with inner ear deafness. There are cases, however, that spiral ganglion cells are lost secondary to degeneration of hair cells, or by direct injury to them. The aim of this study was to investigate whether autologous mesenchymal stem cells (MSC), who can differentiate into multiple cell types including neurons, harvested from the bone marrow survive in a damaged spiral ganglion in gentamicin-treated chinchillas. We used six adult chinchillas. Bone marrow was harvested by suctioning the medullary cavity of the femur of the animal, and the cell cultures were grown for 5 weeks to obtain plastic-adherent cell population including MSCs. We injected chinchillas with a single concurrent dose of gentamicin (IM) and ethacrynic acid (IV), and autologous MSCs were injected into the modiolus of the cochlea after 4 weeks of survival. The MSCs were labeled with

Di-I before injection. Animals were sacrificed at 3 weeks after injection of MSCs, and the cochlea was examined histologically. We confirmed survival of the injected cells in the modiolus near the basal turn, which became sparse at the upper turns. Some labeled cells were also observed in the peri-lymphatic space of the cochlea. Whether the cells survived in the modiolus can differentiate into neurons is still to be investigated.

14h45-15h00 O15: Proliferative generation of auditory hair cells in culture

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Hair cell (HC) and supporting cell (SC) productions are completed during early embryonic development of the mammalian cochlea. This study shows that acutely dissociated cells from the newborn rat organ of Corti, developed into so-called otospheres consisting of 98% nestin (+) cells when plated on a non-adherent substratum in the presence of either Epidermal Growth Factor (EGF) or Fibroblast Growth Factor (FGF2). Within cultured otospheres, nestin (+) cells were shown to express EGFR and FGFR2 and rapidly give rise to newly formed myosin VIIA (+) HCs and p27KIP1 (+) SCs. Myosin VIIA (+) HCs had incorporated bromodeoxyuridine (BrdU) demonstrating that they were generated by a mitotic process. Ultrastructural studies confirmed that HCs had differentiated within the otosphere, as defined by the presence of both cuticular plates and stereocilia. This work raises the hypothesis that nestin (+) cells might be a source of newly generated HCs and SCs in the injured postnatal organ of Corti.

15h00-15h15 O16: Functions of FGFs during otic vesicle induction

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The otic vesicle (otocyst) is an embryonic structure that after a complex morphogenetic process gives rise to the adult organ responsible for hearing and balance in vertebrates, the inner ear. The induction of the otocyst, adjacent to the closing neural tube in the hindbrain area, occurs during early stages of development, and starts with the formation of the otic placode. Several studies have addressed the involvement of different members of the Fibroblast Growth Factor (FGF) family in the induction and morphogenesis of the inner ear. Here, we report our findings on the implication of various FGFs during otic induction, using *gain- and loss-of-function* approaches, carried out in different animal models. FGFs were overexpressed in chicken embryos by *in ovo* electroporation, and their activity was inhibited in this species by using anti-sense morpholinos in explants. On the other hand, we have created transgenic mouse lines that overexpress FGFs in the area of the neural tube next to which the otic vesicle is induced. Our results suggest that different FGFs might play distinct roles during the induction of the otic vesicle depending on the organism and/or the signalling source.

15h15-15h30 O17: FGF Signaling Regulates Pillar Cell Development in the Organ of Corti

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One of the most striking aspects of the cellular pattern within the sensory epithelium of the mammalian cochlea is the presence of two rows of pillar cells in the region between the single row of inner hair cells and the first row of outer hair cells. The factors that regulate pillar cell development have not been determined, however, the results of previous studies suggested a key role for fibroblast growth factor receptor 3 (FGFR3). To examine the specific effects of FGFR3 on pillar cell development, receptor activation was inhibited in explant cultures established from embryonic cochlea. Results indicated that differentiation of pillar cells is dependent on continuous activation of FGFR3. Moreover, transient inhibition of FGFR3 did not permanently inhibit the pillar cell fate, since reactivation of FGFR3 resulted in the

resumption of pillar cell differentiation. The effects of increased FGFR3 activation were determined by exposing cochlear explants to FGF2, a strong ligand for all FGF receptors. Treatment with FGF2 lead to a significant increase in the number of pillar cells and to a small increase in the number of inner hair cells. These effects were dependent on the dose of FGF2 as well as on the timing of growth factor addition, but were not dependent on cellular proliferation. Therefore, it seems likely that additional pillar cells and inner hair cells were a result of increased recruitment into the prosensory domain. These results indicate that FGF signaling plays a critical role in the commitment and differentiation of pillar cells. Moreover, the position of the pillar cells appears to be determined by the activation of FGFR3 in a subset of the progenitor cells that initially express this receptor.

15h30-15h45 018: Activity-dependent Expression of different BDNF splice variants in the cochlea

Justin Tan, I. Köpschall, K. Rohbock, U. Zimmermann, M. Knipper
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Activity-dependent rearrangement and maintenance of fibers is a general property of the developing and adult nervous system and the brain-derived neurotrophic factor BDNF is one of the activity-dependent genes shown in several studies to be involved in plasticity phenomena. It has been shown that four promoters direct the expression of rat brain-derived neurotrophic factor (BDNF) gene (Timmusk et al. 1993). To examine a possible role of the different BDNF splice variants in activity-dependent paradigms in the cochleae, we generated riboprobes of the different BDNF exons I-IV. Using *in situ* hybridization and northern blot approach. our data indicated that adult rat spiral ganglion neurons do not express BDNF exon I and II transcripts, but exon III and IV. BDNF transcripts are expressed in a tonotopic manner with the highest intensity observed in the basal spiral ganglion neurons and lowest in the apical. Interestingly, this is in contrast to NT3, which shows the lowest expression in basal turns. Different paradigms of activity modulation (eg. kainate and salicylate treatment) are currently used to analyse an alteration of BDNF mRNA level in the cochlea, as well as the central auditory structures such as the auditory cortex. This expression pattern was compared with other activity-dependent genes, namely c-fos and Arg 3.1. Our data will be discussed in the context that BDNF may influence activity patterns in the auditory system. Supported by Deutsche Forschungsgemeinschaft Kni 316/3-1 and Fortüne 817-0-0.

15h45-16h00 019: Signalling networks controlling apoptosis during inner ear development

Isabel Varela-Nieto, Itziar Gorospe, Yolanda Leon

Instituto de Investigaciones Biomédicas Alberto Sols. CSIC-UAM. Madrid. Spain.

Programmed cell death is a critical process for normal development and tissue homeostasis. While the basic program of apoptosis execution remains conserved, distinct regulatory signals have been described depending on the cell type and developmental stage. Particularly interesting are the opposite actions displayed by nerve growth factor (NGF) that acts either as a survival factor or as a death-inducing factor. A coherent understanding of the regulation of programmed cell death during development requires a coordinated study of some of the multiple signals acting on the cells. We are interested in the molecular mechanisms by which these signals initiate and pattern the vertebrate inner ear. IGF-1 is a member of a family of structurally related genes that have pleiotropic actions on embryonic cells. In vitro culturing and knock out mice analysis have determined that IGF-1 is critical for the proper development and maturation of the inner ear. IGF-1 stimulates the generation of lipidic second messengers, activates the Raf/mitogen-activated protein kinases cascade and increases AP-1 and PCNA levels leading to cell growth and survival. On the contrary, NGF, after binding to p75 low affinity receptors, activates Jun N-terminal kinase and increases ceramide levels, in a process that regulates apoptotic cell death. In this context, we have explored the interactions between the pathways activated by IGF-1 to prevent apoptosis and those activated by NGF to induce cell death. We propose that the dynamic balance between levels of ceramide metabolites and the consequent regulation of Akt phosphorylation are important factors that determine whether a cell survives or dies.

Central and peripheral auditory morphology – Sunday 8th September 16h30-17h30

16h30-16h45 020: Induction of MHC class II antigens of the inner ear

Bertrand Gloddek[1], Daniel Bodmer[2], Dominik Brors[2], Allen Ryan[2]

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Growing evidence supports the concept that immune reactions occur in the cochlea where they can function either in protection or as a source of inflammation. Since immunity regularly starts with antigen presentation of foreign substances to T-cells, antigen presenting cells expressing major histocompatibility complex (MHC) class II molecules are required. Under resting conditions, cochlear cells usually express no MHC class II. However we show that exposure to interferon-y (IFN-y) induces an increase in MHC expression in neonatal cochlear cells of mice, in vitro. In addition, MHC class II molecules were localized in the inner ear of adult mice after induction of sterile labyrinthitis. The induction of MHC class II molecules either by IFN-y or inflammation may render cochlear cells competent to initiate and participate in immune reactions and may therefore contribute to both immunoprotective and immunopathological responses of the inner ear.

16h45-17h00 021: Cochlear aqueduct flow resistance in the guinea pig, measured with small middle ear pressure changes

Hero P. Wit, Robert A. Feijen, Hans M. Segenhout, Frans. W. J. Albers

Department of ORL, University Hospital Groningen, The Netherlands

Inner ear fluid pressure was measured in the guinea pig with a micropipette and a WPI micropressure system. The pipette was introduced through a hole in the bulla and the rim of the round window membrane. After this the hole in the bulla was closed with dental cement. Through a hole in the tympanic membrane middle ear air pressure could be varied and measured. The middle ear pressure stimulus was a combination of a constant level (-2.5, 0, 2.5 and 5 cm water with respect to ambient pressure) and a sudden upward pressure step of 0.5 cm water , followed after 60 seconds by a downward step of the same magnitude. The time course of inner ear pressure recovery after a sudden change of middle ear pressure was recorded. From these recordings the flow resistance of the cochlear aqueduct was calculated, assumimg a constant compliance for the round window membrane. Results are that this flow resistance depends on the above mentioned constant level of middle ear pressure and does not depend on the direction of fluid flow through the aqueduct. (A dependence on flow direction was proposed as an outcome of earlier experiments: Feijen et al., Acta Otolaryngologica 122 (2002) 138-145). The dependence of resistance on middle ear pressure can be explained by assuming a relation between round window position and aqueduct permeability.

17h00-17h15 022: Cochlear aqueduct flow resistance in the guinea pig depends on (quasi-)static middle ear pressure

Robert A. Feijen, Hans M. Segenhout, Frans A. Albers, Hero P. Wit

Department of ORL, University Hospital Groningen, The Netherlands

Inner ear pressure was measured in the guinea pig with a micropipette connected to a WPI micropressure system. The pipette was introduced through a hole in the bulla and the rim of the round window membrane. After this the hole was closed with dental cement. Through a hole in the tympanic membrane middle ear pressure could be varied by applying different pressures to the ear canal. Through a small tube glued into a hole in the skull CSF pressure was sinusoidally modulated with constant amplitude. These sinusoidal pressure variations could be clearly measured in the inner ear. Simultaneously middle ear pressure was changed very slowly from negative (-5 cm water) to positive pressure (5 cm water) with respect to ambient pressure. As a consequence the amplitude of the inner ear pressure variations consequently changed gradually between a maximum and a minimum value. This led to the conclusion that cochlear aqueduct flow resistance can be changed by changing middle ear pressure. The dependence of resistance on middle ear pressure can be explained by assuming a relation between round window position and aqueduct permeability.

17h15-17h30 023:Deafness and degeneration of the auditory receptor in two experimental mouse models.

Mª Visitación Bartolomé, Francisco Carricondo, Eduardo del Castillo, Mª Angeles Vicente-Torres, Pablo Gil-Loyzaga
Dept. Surgery II (ORL), Fac Medicina Univ Complutense, Madrid, Spain
The TGK8-4 transgenic mice containing 12 Kb of K8 human cytokeratin (HK8) (Casanova et al, Cell Science 1995 108:811-820) showed a highly abnormal and immature auditory receptor (Bartolomé et al, Histol Histopathol 2002 17:827-836). Electrophysiological recordings of transgenic mouse cochleae showed a significant latency increase and a significant amplitude decrease of the N1 wave of the compound action potential (CAP), with respect to the controls. Morphological studies of TGK8-4 transgenic mouse cochleae showed the degeneration of Corti's organ (absence or immaturity of sensory hair cells, supporting cells, and the tunnel of Corti) and distortion of the tectorial membrane. The remaining cells could be epithelial ones, neither sensory nor supporting cells. All these results indicate a relevant auditory injury, which starts at the basal coil reaching, progressively, the lower middle cochlear coil. Similar alterations were observed in other degenerative cochlear pathologies. In particular, the presbycusis process, analyzed in C57BL/6J mice cochleae (Bartolomé et al, NeuroReport 2001, 12:3107-3110; Adv Otorhinolaryngol 2002 59:106-111), or the rat cochlear degeneration after aminoglycoside administration (Bartolomé et al, Brain Res 1999 822, 43-51). All these situations exhibited Corti's organ totally substituted by an epithelial scare, composed by strongly anti-galectine-1 immunolabeled cells. The high similarity with TGK-8 transgenic mouse auditory receptor degeneration could also point out the existence of a common scare epithelization process after several types of inner ear degeneration.
Acknowledgments: The authors wish to thank to Dr JL Jorcano and Dra ML Casanova. This work was supported by Spanish grants BFI 2001-1447 and FIS 2001/0652.

17h30-17h45 024:Dose-dependant viral vector mediated gene transfer in the cochlea of the mouse *in vivo*

Maoli Duan[1], Thierry Bordet[2], Zhiqiang Chen[1], Mauro Mezzina[3], Axel Kahn[2], Mats Ulfendahl[1].
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Gene therapy has attracted widely interest as a molecular tool for basically research in biomedicine. In addition, it has also potential treatment for human being diseases that range from monogenic disorders to complex diseases such as cancer, AIDS and neurodegenerative disorders. Clinical trials of gene therapy have already been begun in different diseases. Gene therapy in the inner ear research is just at beginning. However, the inner ear is a rather isolated organ, and fluid via perilymph and endolymph filled the cochlea. Thus, the gene therapy will have more chance to succeed in the inner ear than other organ. Adeno viral (Ad) vectors can infect both dividing and nondividing cells. Thus Ad vector can transfer gene into cochlea cells. Ad vectors are very attractive in that they may carry large foreign DNA fragments (8kb) and can be generated at extremely high titers. At present study, we investigate roles of Ad vector in cochlea *in vivo*. We choose mouse as subject to investigate the dose-dependant gene expression in the mouse *in vivo* and found that there was no detective gene expression in the mouse cochlea when the mouse was injected 0.5 µl-1 µl Ad-lacZ vector through the round window membrane but there was strong gene expression when the volume of the Ad-lacZ was increased to 5µl in the cochlea. There is even more strong gene expression when the volume was increased to 10µl. The present findings demonstrated that Ad mediated gene expression in the cochlea of the mouse but in dose-dependant way.

Tutorial courses: Monday 9th September 8h-8h50

A. **M.W Kelley:** "Cell fate determination in the sensory epithelium of the inner ear"
B. **B. Lonsbury-Martin:** "Of mice, rabbits, guinea pigs, and monkeys: Otoacoustic emission measures of cochlear function."

Genetic: human hereditary deafness – Monday 9th September 9h00-10h45

9h00-9h15 O25: Expression of a dominant-negative connexin26 in mice causes disorganization of organ of Corti and non-syndromic deafness

Katsuhisa Ikeda[1],Takayuki Kudo[1,2], Shigeo Kure[2], , An-Ping Xia[1], Yukio Katori[1], Masaaki Suzuki[1], Toshimitsu Kobayashi[1], Kanako Kojima[2], Yoichi Suzuki[2], Yoko Aoki[2] & Yoichi Matsubara[2].
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Hereditary deafness affects about 1 in 2,000 children and mutations in the *GJB2* gene, which encodes gap junction protein connexin26, are the major cause in various ethnic groups. However, the pathogenesis of deafness due to *GJB2* mutations remains obscure. Mice with targeted disruption of the gene were embryonic lethal in a previous study. To elucidate the pathological role of connexin26 in the inner ear, we produced transgenic mice carrying a R75W mutation in the *GJB2* gene, which was identified in a hereditary deafness pedigree and showed a deleterious dominant-negative effect.

The R75W+mice showed severe hearing loss from an early stage of development. Histological analysis of the mutants revealed hyperplasia of supporting cells, failure in the formation of the tunnel of Corti, and degeneration of sensory hair cells. Despite robust expression of the transgene, no obvious structural change was observed in the stria vascularis and spiral ligament that are rich in connexin26 and generate the endolymph. The high resting potential in cochlear endolymph essential for hair cell excitation was normally sustained. These results indicate that the *GJB2* mutation associated with sensorineural deafness affects the differentiation of supporting cells resulting in disorganization of the organ of Corti, rather than affecting endolymph homeostasis, in mice and probably in human.

9h15-9h30 O26: Degeneration pattern of the organ of Corti of Mpv17-negative mice.

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Loss of function of the peroxisomal Mpv17-protein leads to kidney failure and to early sensorineural deafness. The onset of the structural changes, as lamination of the glomerular basal membrane and of the capillary of the stria vascularis, occurs simultaneously in both organs. However, concomitant ultrastructural alterations were also observed in the outer hair cells. Whereas the apoptotic processes (apoptotic bodies, TUNEL) cause degeneration of the stria vascularis, the degeneration of the organ of Corti is not yet clarified. In order to understand the degeneration pattern ultrastructural studies have been performed on Mpv17-negative and wild type mice at an age from 14 days up to 2 months. The outer hair cell lateral membrane appeared almost floppy and wrinkly. Furthermore, a focal disruption of Hansen's body and endoplasmic reticule followed by vacuolization of the cytoplasm and lyses of the cells occurred. Simultaneously the supporting cells were affected; however, the degeneration pattern differed clearly from the outer hair cells. The structural degeneration pattern of the outer hair cells appears to be similar to the recently described parapoptotic processes (alternative form of programmed cell death) discussed as a cause of some type of neurodegeneration (Sperandino et al. Proc Natl Acad Sci USA 97:14376:2000; Oppenheim et al. J Neurosci 21:4752:2001). (Supported by DFG Me 890/4).

9h30-9h45 O27: Otosclerosis: a review

Kris Van Den Bogaert, Carole Faghel, Guy Van Camp
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Otosclerosis is caused by an abnormal bone homeostasis of the otic capsule and represents a frequent cause of hearing impairment among white adults. The conductive component of this hearing impairment is due to bony fixation of the stapedial footplate in the oval window, disturbing the conduction of sound vibrations from the middle to the inner ear. In some cases, a sensorineural component develops when the disease extends to the cochlea, leading to a mixed hearing impairment. The etiology of otosclerosis is controversial and both a viral and genetic cause have been proposed. However, large autosomal dominant families segregating otosclerosis are very rare and in the majority of cases there are only a few, if any, other affected family members. While this presentation has been interpreted as reduced penetrance

only, it is more consistent with a complex interaction between genes and environment. Otosclerosis can then be considered as a complex disease with rare monogenic autosomal dominant families. At the moment, three large families segregating autosomal dominant otosclerosis have been used to identify three otosclerosis loci: *OTSC1* on chromosome 15q25-26, *OTSC2* on chromosome 7q34-36 and *OTSC3* on chromosome 6p21-22. Much can be learned from the analysis of monogenic otosclerosis that may be applicable to otosclerosis as a complex disease, but in general, the analysis of these two types of otosclerosis will require different research strategies.

9h45-10h00 O28: A combination of mouse and human studies reveals new genes and mutations associated with auditory and vestibular dysfunction

Karen B. Avraham[1], Nadav Ahituv[1], Zippora Brownstein[1], Ronna Hertzano[1], Sarah Vreugde[1], Tom Walsh[2], Vanessa Walsh[2], Mary-Claire King[2], Martin Hrabe de Angelis[3], Alexandra Erven[4], Charlotte Rhodes[4], Karen P. Steel[4]

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The past years have provided an explosion in our understanding of how the ear functions. This dramatic increase is due in large part to the genes found to be associated with inherited hearing loss. Since 1995, mutations in 27 genes have been found, providing clues about auditory transduction, ion homeostasis, and inner ear development. In particular, mouse models for human deafness, with mutations in orthologous genes, have revealed essential information about the pathophysiology caused by these mutations. The combination of mouse and human studies in hereditary deafness has allowed the genetic and phenotypic heterogeneity of deafness to be analysed in the most optimal fashion, covering cochlear, vestibular, or middle ear defects; early-onset or late-onset hearing loss; and dominant or recessive deafness. A summary of our latest work in these areas will be presented, including: myosin IIIA mutations in a family with late-onset hearing loss inherited in a recessive manner DFNB30); *AP-2_* mutation in Doarad mice with middle ear defects; *Tmc1* mutation in Beethoven mice with cochlear defects (Kurima *et al*., Vreugde *et al. Nat Genet* 2002); and *Myo7a* mutation in Headbanger mice with vestibular defects. In the context of our responsibility in educating the hearing impaired community about our work, a brochure explaining the genetics of hearing loss is being distributed and will be presented.

10h00-10h15 O29: Beethoven, a mouse model for dominant, progressive hearing loss DFNA36.

Sarah Vreugde[1], Alexandra Erven[2], Corné J. Kros[3], Walter Marcotti[3], Helmut Fuchs[4], Kiyoto Kurima[5], Edward R. Wilcox[5], Thomas B. Friedman[5], Andrew J. Griffith[5], Rudi Balling[4], Martin Hrabé de Angelis[4], Karen P. Steel[2] & Karen B. Avraham[1].

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Despite the rapid progress in the past few years in localizing and identifying genes underlying deafness, there are still only a few mouse models for specific forms of human deafness. The Beethoven mouse mutation (*Bth*) arose in a large-scale ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis programme. Both homozygote and heterozygote mutants show a progressive loss of response to sound associated with progressive degeneration of cochlear hair cells. The mutation has been mapped to chromosome 19, in a region showing conserved synteny with human chromosome 9q21, the location of the DFNA36 and DFNB7/B11 nonsyndromic deafness loci. The gene involved in these human forms of deafness has recently been identified as *TMC1*, which encodes a protein with several predicted transmembrane domains and may act as a channel or transporter. We show here that the Beethoven mutation is a nonconservative missense mutation in the mouse *Tmc1* gene. Using whole mount *in situ* hybridization, *Tmc1* expression was shown in cochlear hair cells at P3, and expression was seen from P5 to P90. As the *Bth* mutation may affect the function of a channel expressed in cochlear hair cells, we assessed hair cell function. We used patch clamp electrodes to record from individual IHCs and OHCs from *Bth*/+ and wild type mice before the onset of hair cell degeneration. Both transducer and basolateral currents appeared to develop normally in *Bth*/+ hair cells between P6 and P15. Scanning electron microscopy of *Bth*/+ cochleas

revealed progressive hair cell degeneration from P20 onwards. Studying the Beethoven mouse may increase our understanding of the hair-cell degeneration assumed to be associated with progressive hearing loss with ageing (presbyacusis) in a large proportion of the human population.

10h15-10h30 O30: Mutation and expression analysis of the WFS1-gene which is a frequent cause of autosomal dominant low frequency hearing loss

Kim Cryns[1], Sofie Thys[1], Lut Van Laer[1], Markus Pfister[2], Kris Flothmann[1], Hannie Kremer[3], William Reardon[4], Richard J.H. Smith[5], Guy Van Camp[1]

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Hereditary hearing impairment is an extremely heterogeneous trait, with more than 70 identified loci. Only two of these loci are associated with an auditory phenotype that predominantly affects the low frequencies (DFNA1 and DFNA6/14). We have completed mutation screening of WFS1 in 8 autosomal dominant families and 10 sporadic cases in which affected persons had low frequency sensorineural hearing impairment (LFSNHI). Mutations in this gene were already known to be responsible for the autosomal recessive Wolfram syndrome or DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness). We identified 7 missense mutations and a single amino acid deletion affecting conserved amino acids in 6 families and one sporadic case. Among the 10 WFS1-mutations reported in LFSNHI, none is expected to lead to premature protein truncation and 9 cluster in the C-terminal protein domain. In contrast, 64% of the Wolfram syndrome mutations are inactivating. Our results indicate that only non-inactivating mutations in WFS1 are responsible for non-syndromic LFSNHI. In addition, immunohistochemistry and in situ hybridization was performed in order to study the expression pattern of WFS1 in mouse inner ear. Both techniques showed compatible results and indicated a clear expression in different cell types of the cochlea.

10h30-10h45 O31 : The DFNA5 mouse: the first analysis of the phenotype

Lut Van Laer[1], Markus Pfister[2], Sofie Thys[1], Lieve Umans[3], Lutgarde Serneels[3], Frank Kooy[1], Jean-Pierre Timmermans[4], Fred Van Leuven[3], Guy Van Camp[1]

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An autosomal dominant form of hearing impairment, starting in the high frequencies at an age between 5 and 15 years and progressively affecting all frequencies, segregates in an extended Dutch family with more than 100 patients. A complex intronic mutation resulting in skipping of exon 8 of a new gene that is expressed in the cochlea was demonstrated to be responsible for the hearing loss in the Dutch family. As no physiological function could be deduced despite of extensive computational analysis, the gene was designated *DFNA5* in correspondence with the locus name. To study the function of DFNA5 as well as the pathological processes leading to the hearing impairment in the Dutch family, a mouse model was generated. For this purpose, the mutation that segregates in the Dutch family was mimicked in the mouse through deletion of exon 8 by targeted recombination in ES cells derived from the 129 mouse strain. As the latter exhibits early onset hearing loss itself, the genetic background of the DFNA5 mouse was replaced by backcrossing during 6 generations with two other inbred mouse strains: C57Bl, which exhibits hearing loss only at an older age, and the good-hearing reference strain CBA/Ca. The complete absence of DFNA5 protein was demonstrated by Western blotting on brain tissue derived from homozygous animals, indicating that the DFNA5 mouse is a knockout. The hearing impairment was tested at different ages using frequency-specific ABR, and the inner ear morphology was studied using both light- and electron microscopy.

Otoprotection – Monday 9th September 11h15-12h15

11h15-11h30 O32: Pharmacokinetics of Caroverine in the Inner Ear and Its Effects on Cochlear Function after Systemic and Local Administrations in Guinea Pigs

Zhiqiang Chen[1, 2], Maoli Duan[2], Howsung Lee[3], Runsheng Ruan[1], Mats Ulfendahl[2]

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Caroverine, an N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, has been shown to protect the inner ear from excitotoxicity and to be effective in the treatment of cochlear synaptic tinnitus. Local administration of caroverine on the round window membrane (RWM) could be a more effective means of administration to avoid systemic side/adverse effects. The present study investigates the pharmacokinetics of caroverine in the perilymph, cerebrospinal fluid (CSF) and plasma following intravenous and local applications at different dosages. High performance liquid chromatography (HPLC) was used to determine the drug concentrations. Our results show much higher caroverine concentrations in the perilymph with lower concentrations in CSF and plasma following local applications compared with systemic administration. Auditory brainstem responses (ABR) were measured to evaluate the changes in auditory function. The effects on hearing were transient and fully reversible 24 h after local caroverine applications. The findings suggest that local application of caroverine on RWM for the treatment of excitotoxicity-related inner ear diseases, such as tinnitus and noise induced hearing loss, might be both safe and more efficacious while avoiding high blood and CSF caroverine levels seen with systemic administration.

11h30-11h45 O33: Systemic α-MSH treatment can delay ototoxicity caused by local administration of cisplatin.

Francisca L.C. Wolters[1], Sjaak F.L. Klis[1], Frank P.T. Hamers[1], John C.M.J. de Groot[1], Diane M. Prieskorn[2], Josef M. Miller[2] and Guido F. Smoorenburg[1].

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Cisplatin is a widely used anticancer drug with severe side effects including ototoxicity. Previously, our group has demonstrated that ototoxicity induced by systemic administration of cisplatin is reduced by concomitant administration of melanocortins, like α-MSH. However, these experiments were hampered by large interanimal variability. Therefore, we have developed a model that involves intracochlear administration of cisplatin into the scala tympani via a microcannulation-osmotic pump system in combination with chronic, daily recordings of the compound action potential (CAP) from a permanent round-window electrode. The present study was designed to investigate the protective effects of systemic α-MSH treatment on ototoxicity caused by local administration of cisplatin. Guinea pigs, with an implanted electrode and an osmotic pump, pumping 0.5 µl saline or cisplatin (15 µg/ml) per hour, were treated daily with a systemic bolus injection of either α-MSH (75 µg/kg/day) or saline for one week or until the electrocochleogram showed a persistent decrease in CAP amplitude (40 dB threshold shift at 8 kHz). Next, the animals were sacrificed and the cochleas were processed for histological examination and OHC counts. After 2 to 3 days of cochlear application, cisplatin alone caused a threshold shift at all frequencies (2-16 kHz), in all animals. α-MSH co-administration consistently delayed the threshold shift by 1 to 2 days. At the moment the 40 dB criterion had been reached, similar hair cell loss in both α-MSH and saline co-treatment groups was observed. These data show that α-MSH delays cisplatin ototoxicity, but does not prevent OHC loss. Nevertheless, a delay of 1 to 2 days is of potential clinical significance. Moreover, since the cisplatin was delivered directly to the cochlea, the ameliorating effect must involve a cochlear target. Supported by the Dutch Cancer Institute and Foundation "De Drie Lichten", the Netherlands

11h45-12h00 O34: Are perilymphatically administered anti-oxidants effective against cisplatin ototoxicity?

Sjaak F.L. Klis, Natalie L.M. Cappaert, Jeroen Wijbenga, Guido F. Smoorenburg
Hearing Research Laboratories, University Medical Center, Utrecht, The Netherlands

Cisplatin (cisPt), a widely used antitumor agent, has dose-limiting side effects such as ototoxicity. Several studies have reported that systemically administered anti-oxidants like 4-methyl-thio-benzoic-acid (4-MTBA) and sodium-thiosulphate (STS) provide protection against cisPt ototoxicity. In the present study we attempted to suppress cisplatin-induced ototoxicity in guinea pigs by administering STS or 4-MTBA intraperilymphatically. Guinea pig cochleas were perfused for 10 minutes with artificial perilymph (ArtP) containing cisPt at 0.3 mg/ml either alone, or in combination with 4-MTBA (0.1 or 1.0 mg/ml) or STS (0.75 or 3.0 mg/ml). In addition, ArtP and anti-oxidant alone control perfusions were performed. The compound action potential (CAP) and the summating potential (SP), evoked by 8 kHz tone bursts, were measured just before and 1, 2, 3 and 4 hours after the perfusion. After perfusion of ArtP alone, all the measured potentials remained constant for 4 hours. After perfusion of cisPt alone, the CAP amplitude decreased gradually over time. STS, in both concentrations, delayed, but did not prevent, the effects of cisPt on cochlear potentials. After cisPt in combination with 4-MTBA (both concentrations), the CAP amplitude decreased faster than after cisPt alone, thus, 4-MTBA aggravates the cisPt effect. In some animals the endocochlear potential (EP) was measured after perfusion with cisPt with or without 1.0 mg/ml 4-MTBA. These measurements revealed that the EP starts to collapse very early after application of cisPt and that 4-MTBA has no effect on this collapse. Thus, the enhanced effect of cisPt when combined with 4-MTBA is not related to strial activity. Our experiments show that the extrapolation from systemic treatment to local treatment is not straightforward.

12h00-12h15 O35:Cell therapy for application of neurotrophins into mouse inner ear

Takayuki Nakagawa, Fukuichiro Iguchi, Ichiro Tateya, Tae Soo Kim, Tsuyoshi Endo, Yasushi Naito, Juichi Ito
Department of Otolaryngology-Head and Neck Surgery, Kyoto University, Japan

In the auditory system, efforts to reduce degeneration of auditory neurons have an immediate objective of improving clinical benefits of cochlear implants, which are small devices to stimulate auditory neurons electronically. Recent studies have indicated several neurotrophins can enhance survival of auditory neurons. However, the strategy for application of neurotrophins into inner ear is still a discussed problem. Although implantable pomps and gene transfection have been used for this purpose, application periods are limited. In this study, we examined potential of transplantation of neural stem cells into the inner ear for application of nurotrophins, because a major part of transplanted neural stem cells into retina or brain differentiate into glial cells. Glial cells are well known to produce neurotrophins. Neural stem cells obtained from GFP mice were used as donor cells. Donor cells were injected from the lateral semicircular canal of mice. Histological analysis 4 weeks later showed that transplant-derived cells survived in the inner ear. In cochleae, most of transplant-derived cells were seen in the perilymphatic space. Immunohistochemical analyses revealed that most of transplant-derived cells differentiated into glial cells. In addition, transplant-derived cells frequently exhibited expression of glial-derived neurotrophic factor and brain-derived neurotrophic factor. The findings indicate that transplantation of neural stem cells will be a useful strategy for application of neurotrophins into inner ear. This work is supported by The Naito Foundation.

Ototoxicity: apoptosis and cell death (I) – Monday 9th September 14h00 -16h00

14h00-14h15 O36: Expression of hypoxia inducible factor-1 (HIF-1) in rat cochlea

Johann Gross[1], Cornelia Rheinländer[1], Julia Fuchs[1], Brigitte Mazurek[1], Thomas Kietzmann[2]
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Hypoxia/Ischemia is a major pathogenetic factor in the development of hearing loss. Hypoxia inducible factor–1 (HIF-1) is the key transcription factor involved in the signalling and adaptation to hypoxia/Ischemia. Because of differential vulnerability of cochlea structures we sought to study the HIF-1 response to hypoxia of stria vascularis (SV), organ of Corti/limbus (OC/LI) and of modiolus (MO). We used an *in vitro* hypoxia model of the cochlea of 3-5-day-old Wistar rats. HIF-1 activity was measured using a reporter gene assay. A cell monolayer culture each of SV, OC/LI and MO was prepared and transfected using CalPhos™ Mammalian Transfection Kit (Clontech). The transfected specimens were exposed to 6 h, 13 h, 24 h and 36 h hypoxia in a Billups-Rothenburg-chamber (pO_2 levels 5-10 mm Hg). Hypoxia

increased HIF–1 activity in a dose dependent manner: 6 h hypoxia increased HIF-1 activity 2-fold, 36 h hypoxia 7-15-fold. 24 h hypoxia increased the HIF-1 activity in MO 14.1 ± 3.5-fold, in OC/LI 9.4 ± 3.0-fold and in SV 6.4 ± 1.5-fold (mean ± SEM, p< 0,02, n=6). Parallel experiments using an ischemic model resulted in a similar pattern of HIF–1 activation. The data indicate a differential expression of HIF-1 activity in MO, OC/LI and SV using reporter gene assay resulting from region- and cell-specific posttranscriptional and posttranslational regulation. The differential activation could play an important role in the region specific adaptation to hypoxia and in the induction of HIF-1 target genes.

14h15-14h30 O37: Time sequence of degeneration pattern in the cochlea during cisplatin treatment: A quantitative study.

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Systemic treatment with cisplatin (CPT) can result in sensorineural hearing loss. There is still some debate with regard to its primary target in the cochlea, especially since evidence is accumulating that CPT may act at 3 major levels, i.e. the organ of Corti, the stria vascularis, and the spiral ganglion. The aim of this study was to establish if any causal relationship exists between these interactions and to determine to what extent CPT ototoxicity involves a single or multiple target(s). Albino guinea pigs were treated with CPT (2.0 mg/kg/day) by daily i.p. injections for 4 days (n=10), 6 days (n=10), and 8 days (n=10). Following ECoG, the cochleas were processed for histologic examination and morphometry. In midmodiolar sections the following parameters were determined: OHC counts, strial cross-sectional areas, endolymphatic hydrops, spiral ganglion cell shrinkage and packing densities of the spiral ganglia. Also, OHC counts were determined in surface preparations of resin-embedded cochleas (8-day group only). OHC loss was observed first in the 6-day group, but it was not statistically significant. In the 8-day group, significant ($p<0.0001$) OHC loss was present in the basal and middle turns. OHC counts in surface preparations did not differ from those in midmodiolar sections. Strial cross-sectional area was neither enlarged or diminished immediately after CPT treatment. In none of the experimental groups, an endolymphatic hydrops was found. Loss of spiral ganglion cells was not observed, but significant ($p<0.05$) cell shrinkage was present in the 6-day and 8-day groups. These data demonstrate that CPT acts simultaneously at the organ of Corti and the spiral ganglion. The absence of strial changes may be due to the limited time-window used in this study, especially since previous long-term studies have implicated the stria vascularis as one of the sites of CPT action. A causal relationship between the strial effect and the other targets remains to be investigated. This study was supported by the Heinsius-Houbolt Foundation.

14h30-14h45 O38: Cisplatin cytotoxicity in Organ of Corti-derived immortalized cells

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Cisplatin is an anticancer drug currently used the treatment of a wide range of tumors. This drug shows two major undesired side effects, nephro and ototoxicity, which mechanisms of action are only partially understood. We have studied cisplatin cytotoxicity in a cell line (OC-k3) derived from the Organ of Corti of transgenic mice, which, expressing constitutively the large T antigen of SV40, is able to proliferate indefinitely. In our cell model, cytotoxicity occurred after24- 48 hour by cisplatin incubation at concentration from 13 to 200 μM, with concomitant MDA increase and intracellular GSH diminution. Adherent cells showed, by DAPI staining and electron microscope examination, some morphological hallmarks of apoptosis, as nuclear fragmentation in presence of membrane and mitochondrial integrity. No DNA ladder was detected, indicating absence of relevant nucleasic activity.

Several substances reduced cisplatin-induced apoptosis, as suramin and PD98059, indicating the involvement of specific transduction pathways in cisplatin toxicity, and in particular of ERK cascade; actually, a time dependent and suramin-inhibitable ERK phosphorylation can be assessed after cisplatin treatment

As a whole, these data shows that cisplatin can trigger apoptosis in a time- and concentration-dependent manner, while excess of these factors lead to cell necrosis. Complex pathways could be involved in inducing apoptosis, including ERK recruiting and production of ROS.

14h45-15h00 O39: Pathomechanisms of cisplatin and gentamicin ototoxicity

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Gentamicin and cisplatin are clinically widely used pharmacological agents which may induce irreversible hearing loss as a side effect. In this study we investigated pathomechanisms of cochlear toxicity for these pharmacological agents. In an in vitro-model of the unfixed cochlear neurosensory epithelium the protective effect of iron- and calcium-chelators against cisplatin- and gentamicin-toxicity was investigated using vitality tests. Cellular, redox-reactive iron, the production of superoxide-anions as well as the role of mitochondria were visualized using dyes. The iron-chelators deferoxamine and 2,2'-dipyridyl protected against cisplatin- and gentamicin-induced hair cell loss. Both iron-chelators decreased redox-reactive iron in the cochlear neurosensory epithelium. The calcium-chelator Quin-2 AM protected against cisplatin- but not gentamicin-induced cell-damage. An increased cellular formation of superoxide-anions is an early event in cisplatin-induced cell death. The mitochondrial membrane potential was lost before gentamicin-induced cell death. Despite a similar damage to the inner ear there are differences in the pathomechanisms of cisplatin- and gentamicin-toxicity which may have an impact on clinical prevention of ototoxicity.

15h00-15h15 O40: NF-κB is a rescue pathway in aminoglycoside-induced ototoxicity

Hongyan Jiang, Suhua Sha, Jochen Schacht
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Cellular responses to a traumatic insult include the activation of multiple pathways that lead either to cell death or survival. The control of these pathways requires the concerted efforts of second messengers and transcription factors that will ultimately change the patterns of gene expression in the cell. Ototoxicity of aminoglycoside antibiotics is primarily manifested in outer hair cells and can be prevented by antioxidants such as dihydroxybenzoate or salicylate. Neither the reason for survival of supporting cells nor the precise mechanism of protection is known. We report here on the activation of the transcription factor NF-κB in surviving cells.

In a recently established model of chronic aminoglycoside ototoxicity, adult CBA/J mice received kanamycin (700 mg/kg bid) for two weeks, a regimen producing threshold shifts of up to 50 dB and corresponding loss of outer hair cells. Co-administration of salicylate (150 mg/kg bid) significantly reduced the ototoxic effects. Cochlear tissues were analyzed for the presence of the NF-κB and AP-1 complexes during kanamycin treatment. Gel shift and supershift assays showed that the level of nuclear NF-κB increased after 3, 5 and 7 days with c-Rel, p50 and p65 as the major subunits. However, only the nuclei of inner hair cells and supporting cells stained for p50 and c-Rel immunoreactivity while labeling was absent from the nuclei of outer hair cells. Following co-administration of salicylate, immunostaining for c-Rel and p50 was evident in the nuclei of outer hair cells. These results suggest the involvement of NF-κB as a protective pathway in aminoglycoside-induced ototoxicity.

(Supported by grant DC-03685 from the National Institute for Deafness and Communication Disorders, National Institutes of Health).

15h15-15h30 O41: Cochlear effects of Hexachlorobenzene, a dioxin-type compound, in male adult rats.

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EPI 9902 INSERM – MARSEILLE - FRANCE

The distribution of hexachlorobenzene (HCB) as a by-product of several industrial processes has resulted in its widespread occurrence in the environment. Because of its lipophile character, HCB accumulates in food-chains. Whereas many studies have examined its influence on numerous physiologic functions, nothing was known about auditory effects.

The present study investigated the effects of HCB on the cochlea. Conscious free-moving male rats were randomly assigned to either treatment (HCB) or control (C) groups. Treated rats were dosed daily *per os* with HCB, in concentration of 0; 0.16; 4; 16 mg/kg body weight, diluted in olive oil, for 25 days, whereas the control group (C) received the vehicle only. The effects of HCB were evaluated during the 4 weeks of treatment and the following 4 weeks. Auditory nerve acoustic sensitivity was measured through compound action potential audiograms recorded from the auditory nerve. HCB dosing at 4 and 16 mg/kg/day caused permanent cochlear sensitivity deficits (20-30 dB threshold shift) for all frequencies tested (from 1 to 64 kHz). Exposures at 0.16 mg/kg/day had no effect. However in this last group, after acoustic trauma (8 kHz; 105 dB, 5 min) nerve threshold did not recover over a period of 24 hours, whereas complete recovery was observed in control animals. At fluorescence microscopy, no hair cell loss was observed after HCB treatment (16 mg/kg/day). These data indicate that exposure of rats to HCB could produce a permanent and non-reversing cochlear dysfunction without hear cells death in the organ of Corti.

15h30-15h45 O42: Effects of oxidative stress on mitochondrial function in vitro

Hinrich Staecker [1] and Daqing Li [1]

1) Division of Otolaryngology, University of Maryland, USA

Oxidative stress resulting in induction of apoptosis has been demonstrated in several different types of cochlear pathology. Ototoxic drugs such as aminoglycosides or cis platin produce free radicals resulting in apoptosis. Noise trauma also appears to partially mediated through oxidative damage. Some studies now suggest that the aging in the nervous system as well as in the organ of Corti may be the result of prolonged recurrent oxidative stress resulting in eventual apoptosis of the damaged cells. Although the stria vascularis, auditory neurons and inner hair cells are all susceptible to oxidative damage, the outer hair cells appear particularly vulnerable to oxidative damage and loss of outer hair cells is seen in all of the disease processes outlined above. The mitochondrion has been shown to play a pivotal role in apoptosis in a variety of experimental system and auditory diseases. Using P3 mouse oragnotypic cultures we demonstrate that low dose oxidative stress results in damage to mitochondrial DNA, changes in mitochondrial gene expression and ultimately collapse in of the mitochondrial membrane potential in OHCs leading to apoptosis.

15h45-16h00 O43: Aspirin "Miracle Drug": It can stop your headache but can it prevent cisplatin-induced hearing loss?

Tom Van De Water[1], Geming Li[2], Su-Hau Sha[3], Jochen Schacht[3]; 1) Ear Institute, ENT, University of Miami School of Medicine, 2) ENT, Albert Einstein College of Medicine, 3) Kresge Hearing Research Institute, University of Michigan School of Medicine

Cisplatin and aminoglycosides are potent generators of oxidative stress within auditory sensory cells both *in vitro* and *in vivo*. Salicylate has been shown to provide near complete protection against aminoglycoside induced-damage to auditory sensory cells and hearing loss in both laboratory animal experiments and in ongoing clinical trials. The similarities between the ototoxic mechanism(s) of damage for aminoglycoside antibiotics and cisplatin suggests that treatment with salicylate may also be effective in protecting the auditory receptor from the ototoxic damage caused by treatment of neoplasms with cisplatin. To test the ability of salicylate to protect hearing during cisplatin chemotherapy we used a rat cancer model, scanning electron microscopic evaluation of hair cell integrity and ABR responses to pure tone stimuli. Fisher 344 strain of rats were implanted with a highly metastatic form of breast cancer and then treated with cisplatin to control and eradicate the cancer. As an otoprotectant, salicylate was given for two days prior to the initiation of cisplatin chemotherapy and then continuously throughout the administration of cisplatin. The hearing was monitored prior to and during the administration of cisplatin, while the integrity of the outer hair cells and the functional ability of the kidneys were monitored at the end of the period of cisplatin treatment. The efficacy of cisplatin control of the breast cancer was monitored throughout the period of treatment. Both cisplatin and cisplatin + salicylate rats had effective control of their breast cancer, while the untreated and salicylate control rats had uncontrolled growth of their cancer with metastasis. Cisplatin treated animals had outer hair cell losses, hearing deficits and kidney damage, while the untreated control and the cisplatin treated, salicylate protected rats had almost no outer hair cell losses or hearing loss and had a

partial protection of their kidney function. The results of this study suggest that salicylate administration prior to and during cisplatin chemotherapy may an effective otoprotective and nephroprotective treatment strategy in the clinic.

Ototoxicity: apoptosis and cell death (II) – Monday 9th September 16h30 -17h30

16h30-16h45 O44: Multiple Cochlear Hair Cell Death Responses to Noise

Thomas M. Nicotera[1], BoHua Hu[2] and Donald Henderson[2]
1) Roswell Park Cancer Institute, USA 2) State University of NY at Buffalo, USA

We have previously reported that intense noise exposure causes outer hair cell (OHC) death primarily through apoptosis. Here we investigated the intracellular signal pathways associated with apoptotic OHC death. Chinchillas were exposed to a 4 kHz narrow band noise at 110 dB SPL for 1 hour. After the noise exposure, the cochleas were examined for the activity of each of three caspases, including caspases-3, -8 or -9 with carboxyfluorescein labeled fluoromethyl ketone (FMK)-peptide inhibitors. The cochleas were further examined for cytochrome c release from mitochondria by immunohistology and for DNA degradation by the TUNEL method. The results showed that the noise exposure triggered activation of caspase-3, an important mediator of apoptosis. The noise exposure also caused the activation of caspase-8 and -9, each of which is associated with a distinct signaling pathway that leads to activation of caspase-3. Caspase activation occurred only in the apoptotic OHCs and not in the necrotic OHCs. These results indicate that multiple signaling pathways leading to caspase-3 activation take place simultaneously in the apoptotic OHCs. In addition to caspase activation, noise exposure caused the release of cytochrome c from mitochondria, resulting in a punctate fluorescence in the cytosol. In contrast to activation of caspases, the release of cytochrome c took place in both apoptotic and necrotic OHCs. Moreover, the release of cytochrome c in a subpopulation of OHCs took place early in the cell death process, prior to any outward signs of necrosis or apoptosis. These data suggests that, in this subpopulation, there exists a common step that is shared by cell death pathways before entering either necrosis or apoptosis. Research supported by NIH/NIDCD grant to T. Nicotera (1R21DC04984-01)

16h45-17h00 O45: ROS as a Factor in TTS and PTS

Donald Henderson[1], BoHua Hu[1], Eric Bielefeld[1] and Thomas M. Nicotera[2]
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There is an increasing body of evidence implicating reactive oxygen species (ROS) in hair cell pathology. However, most of this evidence is indirect and not specific for any single species of ROS. We have attempted to develop approaches that provide a more direct assessment of ROS and to determine the role of ROS in apoptosis. In order to address this question we utilized the herbicide paraquat, delivered directly to the round window of the chinchilla. Paraquat (PQ) is reduced by endogenous NADPH to generate the PQ^+ radical, which in turn, reacts with molecular oxygen to generate the superoxide radical. After the PQ treatment, hearing loss can be documented with evoked potentials or cochlear peroxide content by staining the cochlea with dichlorofluorescein. Apoptosis was assessed by the combination of propidium iodide incorporation and caspase-3 activation. Our results demonstrate that both continuous and impulse noise results in the dramatic increase in DCF fluorescence that continues over for at least four days. Secondarily, PQ was shown to mimic the effects of noise in that both treatments resulted in the induction of apoptosis. The maximum hearing loss occurred at about 8 hours post-treatment and partially recovered to a PTS. The addition of glutathione monoethyl ester prevented the DCF fluorescence and dramatically inhibited the apoptotic responses of hair cells. We can conclude that superoxide may be the biologically relevant species generated which initiates the oxidative cascade of events leading to the formation of highly reactive forms of ROS and lipid peroxidation. Lipid peroxidation products such as 4-hydroxy-nonenal are known inducers of apoptosis and may provide the long-term basis for hair cell loss. Supported by NIH/NIDCD grant to Donald Henderson (DC03600-04)

17h00-17h30 O46: Extremely rapid induction of hair cell apoptosis by impulse noise exposure in the chinchilla cochlea

Bo Hua Hu[1], Wei Ping Yang[1], Thomas M. Nicotera[2] and Donald Henderson[1]

Center for Hearing and Deafness, State University of New York at Buffalo, Buffalo, NY, USA 2) Roswell Park Cancer Institute, Buffalo, NY USA

Death of hair cells (HCs) in the inner ear following exposure to an intense noise is believed to be associated with the combined effect of two distinct mechanisms, the direct mechanical trauma and subsequent metabolic impairments. This study was designed to investigate the effect of the mechanical factor on the pathways of HC death. Chinchillas were exposed to 75 pairs of impulse noise (1 second interval between each pair) at 155 dB pSPL. Immediately or 1 hour after the noise exposure, the organ of Corti was processed for examination of morphological and biological indices for apoptosis and necrosis of outer hair cells (OHCs). Propidium iodine labeling revealed an extremely rapid nuclear condensation of OHCs which was evident in less than 10 min after the noise exposure. The plasma membrane staining with 3,3'-dioctadecyloxacarbocyanine perchlorate revealed cytoplasmic shrinkage in these OHCs having condensed nuclei. Further detection with two biological markers for apoptosis, TUNEL assay and caspase-3 staining, showed the spatial correlation between the condensed nuclei and TUNEL positive as well as caspase-3 positive staining. One hour after the exposure, there were several clear differences. The size of the lesion had increased and, interestingly, there was evidence of necrotic OHCs. Two themes will be discussed: 1) the initial OHC death caused by mechanical trauma is apoptotic. The necrotic death is secondary to apoptosis; 2) the induction of OHC apoptosis after the mechanical injury is an extremely rapid process. Research supported by NIH/NIDCD grant to Donald Henderson (DC03600-04).

17h30-17h45 O47: The role of the MAPK pathway in oxidative stress-induced apoptosis of auditory sensory cells

Tom Van De Water[1,4], Ulysses Scarpidis[2] , DilipMmadnani[2], Philippe Lefebvre[3,4], Brigitte Malgrange[4], Hinrich Staecker[5]

1) Ear Institute, ENT, Univ. Miami School of Medicine; 2) ENT, Albert Einstein Coll. Med.; 3) ENT Univ. Liege; 4) Cellular & Molecular Neurosci., Cntr. Univ. Liege; 5) ENT, Univ. Maryland School Medicine

Oxidative stress can be generated within the sensory cells of the auditory receptor by a wide variety of insults which include: 1) loss of trophic support; 2) aminoglycoside expodsure; 3) cisplatin toxicity; 4) sound trauma; 5) presbycusis; and 5) exposure to other toxic substance in the environment, i.e. carbon monoxide. Oxidative stress can generate many toxic molecules within a sensory cell such as reactive oxygen species, 4-hydroxy-2-nonenal, and free radicals (e.g. hydroxy radicals). The reaction of these toxic compounds within the auditory sensory cells can initiate damage to the sensory cells which can then trigger the initiation of an apoptotic cascade and the loss of the damaged sensory cell via programmed cell death. To formulate effective therapies to counteract damage initiated loss of auditory sensory cells, an important step is to more fully understand the sequence of events that results in the cell death process activated within a damaged auditory sensory cell. To accomplish this we have utilized an *in vitro* model of oxidative stress-induced auditory sensory cell apoptosis using a series of three insult paradigms that vary in the extent of oxidative stress generated within cell cultures of dissociated auditory neurons, i.e. 1) neurotrophin withdrawl; 2) exposure to 4-hydroxy-2-nonenal; and 3) cisplatin exposure which are listed in an assending order of their ability to generate oxidative stress. To define one of the cell death pathways that participates in the apoptosis of auditory sensory cells we used immunolabeling of c-Jun complexes (i.e. anti-ASP and anti-AP1), several inhibitors (i.e. curcumin and PD 098059) of the MAPK (stress activated) cell death pathway and an antisense oligonucleotide that is dicted against the mRNA of *c-jun.* The results of this study have identified the MAPK cell death pathway as a major contributor to the programmed cell death of the auditory sensory cells in response to all three of the insults paradigms tested in our *in vitro* system. These results correspond with and support other studies that have utilized inhibitors of the MAPK cell death pathway (e.g. CEP1347 and D-JNKI-1) to stop oxidative stress damage-induced cell death of auditory sensory cells both *in vitro* and *in vivo*. The results of this study and those off other investigators support a therapeutic approach that targets the MAPK cell death pathway for the prevention of loss of oxidative stress-damaged auditory sensory cells.

Tutorial courses: Tuesday 10th September 8h-8h50

C. **E.W. Rubel:** "Cellular and molecular approaches towards understanding hair cell death"
D. **H.Staecker:** "Gene therapy"

Cochlear fluids – Tuesday 10th September 9h00-9h45

9h00-9h15 O48:Endocytosis in the epithelial cells of the endolymphatic sac

Akinobu Kakigi[1], Teruhiko Okada[2], Taizo Takeda[1], Sawada Shoichi[1], Shunji Takeuchi[1]
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The endolymphatic sac (ES) is thought to play an important role in regulation of the inner ear fluid. In this study, we investigated the early phase of endocytosis in the endolymphatic sac using transmission electron microscopy (TEM). As tracers of the endocytosis, horseradish peroxidase (HRP), microperoxidase (MPO) and cationized ferritin (CF) were infused into the endolymphatic space of the endolymphatic sac. At 30 min after the infusion, HRP, MPO and CF particles bound the apical plasma membrane of epithelial cells of the sac. Concerning to the endocytosis, HRP were observed in the early to sorting endosomes. The diameter of these early endosomes showed two types. One has smaller diameter (less than 200 nm). This type of endosome was given rise from non-coated pit. The other has larger diameter (over 200 nm). This type of endosome was given rise from macropinocytosis. These results suggest that non-coated vesicles and macropinocytosis may play an important role of the endocytosis in the endolymphatic sac. On the contrary, MPO and CF were much less observed in the early endosomes. As CF is one of the tracers for coated vesicles, this result suggests that coated vesicles may not play an important role of the endocytosis in the endolymphatic sac.

9h15-9h30 O49: Otoacoustic emissions reveal intracochlear pressure involvement in patients with fluctuating hearing loss and vertigo

Paul Avan[1], Béla Büki[1,2], Laurent Gilain[1], Thierry Mom[1]
1) School of Medicine, Clermont-Ferrand, France 2) Krankenhaus Krems, Austria

Theory predicts, and animal experiments show, that changes in intracranial and intracochlear pressure (ICoP) influence stapes stiffness in such a way that otoacoustic emissions exhibit a characteristic pattern of phase shift around the resonance frequency of the stapes system (i.e., 1 kHz in man), and almost no level shift. Accordingly, body tilt from upright to supine posture normally induces a phase lead of otoacoustic emissions that peaks between 0 and 50° (these limits corresponding to mean effect +/- 2 s.d.) around 1 kHz. Otoacoustic emissions were tested in supine vs. upright position in 49 consecutive patients referred for attacks of fluctuating (mainly, low-frequency) hearing loss and vertigo, possibly indicative of Menière disease. The rationale was that posture might help to reveal or enhance any disruption of ICoP regulation. Menière disease was later confirmed in 30 patients, 14 of whom had no emission in the impaired ear. In the 16 Menière ears with residual emissions, 13 (81%) exhibited a posture-induced shift of 60 to 190°, i.e., up to 9 s.d.'s outside the normative range. In contrast, in the 19 patients whose Menière diagnosis was not confirmed, 17 (89%) had normal posture shifts while 2 showed an excessive shift in the symptomatic ear. Regardless of the size of the effect, its frequency dependence was always in good accordance with the model assuming a simple change in stapes stiffness with ICoP. These findings suggest that a non invasive objective test of ICoP could be designed at early stages of pressure-induced cochlear dysfunction. *Work supported by grant # ET1-304, Fondation de l'Avenir, France.*

9h30-9h45 O50: The effects of V2-antagonist (OPC-31260) on experimental endolymphatic hydrops in guinea pigs

Taizo Takeda(1), Shoichi Sawada(1), Setsuko Takeda(1), Hiroya Kitano(2), Mikio Suzuki(3), Akinobu Kakigi(1), Shunji Takeuchi (1)
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There is considerable evidence for the belief that water homeostasis in the inner ear is regulated via

vasopressin-aquaporin2 (VP-AQP2) system in part and that endolymphatic hydrops, the morphological characteristics of Meniere's disease, reflects the mal-regulation of VP-AQP2 system in inner ear fluid. If so, V2-antagonist (OPC-31260) also seems to act on the VP-AQP2 system in the inner ear in the same fashion as in the kidney, resulting in the reduction of the endolymphatic hydrops. In the present study two experiments were performed to investigate the influence of OPC on experimentally-induced endolymphatic hydrops and on a regulation of AQP2 mRNA expression in the rat inner ear. In the morphological studies, the increase ratios of the cross-sectional area of the scala media (IR-S) were quantitatively assessed among 3 groups of hydrops guinea pigs: no treatment (Ge), infusion of physiological saline into the scala tympani (Gp) and infusion of 3% OPC into the scala tympani (Gopc). Perilymphatic infusion was carried out using Alzet osmotic mini pump. IR-Ss of Ge, Gp group (48.8 - 49.3 %) were not statistically different ($P< 0.01$). But, IR-S of Gopc-group (-14.8%) was significantly smaller compared with those of Ge- and Gp-groups ($p<0.01$). In the PCR study, quantitative analyses of AQP2 mRNA expression were performed with the LightCycler analysis software. To assess the effects of OPC on the expression of AQP2 mRNA in the cochlea and the endolymphatic sac, a comparison of the ratio of AQP2 and -actin mRNA was made among non-treatment rats, sham-injection rats and OPC treated rats. An intravenous injection of OPC resulted a significant decrease in the ratio of AQP2 and ?-actin mRNA both in the cochlea and in the endolymphatic sac (t-test). These results indicated that water homeostasis in the inner ear is regulated via vasopressin-aquaporin2 (VP-AQP2) system, and that V2-antagonist is the promising drug for the therapy of Meniere's disease.

9h45-10h00 O51: Different types of rat endolymphatic sac mitochondria-rich cells

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Ultrastructural characteristics of endolymphatic sac mitochondria-rich (light) cells indicative for cellular function were studied concisely at distinct developmental stages and compared with renal mitochondria-rich cells (i.e. the intercalated cells). In addition, expression of cytokeratins 7 and 19 was determined.

Until birth, only one type of mitochondria-rich cell is observed in the ES. In young adult (P60) animals, distinct differences in mitochondria-rich cell ultrastructure enables classification into subtypes or configurations. Comparison of ES mitochondria-rich cells with renal intercalated cells reveals striking similarities and provides additional information on their specific function in endolymph homeostasis. Furthermore, differences in cytokeratin 19 expression are determined in ES mitochondria-rich cells. Differences in morphology of ES mitochondria-rich cells develop after birth and may reflect a distinct functional or physiological state of the cell. In analogy to renal intercalated cells, the distribution patterns of H^+-adenosine triphosphatase and Cl^-/HCO_3^- exchanger may differ between subtypes. We propose that subtype A mitochondria-rich cells, from which protruding A mitochondria-rich cells are the activated state, are involved in proton secretion (apical H^+-adenosine triphosphatase) and thus are potential candidates for hearing loss observed in renal tubular acidosis (dRTA). Subtype B mitochondria-rich cells are the most likely candidates to be affected in Pendred syndrome because of the assumed function of pendrin as apical Cl^-/HCO_3^- exchanger.

Clinical otorhinolaryngology (I) – Tuesday 10th September 10h00-10h45

10h00-10h15 O52: 3D-reconstruction of the vestibular endorgans in pediatric temporal bones

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Because of its bony protection inside the temporal bone, direct examination of the peripheral vestibular end organs is not yet feasible without permanent damage. Therefore the final diagnosis can mostly be made by post-mortal histological examination. The temporal bones of a newborn child (age: 17 hours, 42 weeks of gestation) without known peripheral vestibular pathology and a child with Goldenhar syndrome (age: 155 days, 42 weeks of gestation) were prepared by the celloidin technique and sectioned in 20 µm. Every 5th to 10th section was digitized. The imaging data were layered anatomically correct in Adobe Photoshop 5.5 and exported for rendering into AVS-Express 5.1. The obtained 3D models were used for measurements. With this technique the vestibular end organs were reconstructed. The angle between the

utricular and saccular macula of the normal vestibular endorgans ranged between 52.1-127.0°. The saccular macula had a length of 2.26 mm and a width of 1.63 mm. The utricular macula was slightly bigger with a length of 2.59 mm and a width of 2.09 mm. Both maculae were curved in longitudinal and transverse axis. In both maculae the transverse axis described a curve of approximately 35°. In the saccular macula the longitudinal axis was 82.7° opposite to the utricular macula with an elevation of 37.7°. The pathological model differed significantly from all measured distances and angles of the normal model. We found that the angles between the semicircular canals were not right angled as Curthoys et al. had suggested [*Curthoys I, Oman M (1987). Acta Otolaryngol (Stockh) 103: 254-261*]. Our findings indicate that each macula is able to analyze more than one direction of linear accelerations in 3D space. The findings of the abnormal peripheral vestibular endorgans in the child with Goldenhar syndrome suggest vestibular deficits which, however, could not be evaluated because of the patient's young age.

10h15-10h30 O53: A NEW CONCEPT OF TINNITUS AND ITS TREATMENT

George Offutt Ph.D.and Burkhard Franz MD.

The electromodel is a revised concept of auditory function (Offutt, 1984). Although all aspects of this model have not been proven, it is based upon and is compatible with experimental results. By rejecting dubious assumptions, this model has led to a successful treatment of peripheral tinnitus.

There are three postulates that underlie this concept of auditory function and tinnitus. First, the inner hair cells (IHC) are electroreceptors that are sensitive to negative potentials. Second, positive potentials in the cochlear fluids suppress the excitability of the IHC. Third, natural suppression is provided by the outer hair cells (OHC) that generate certain positive potentials such as the summating potential (+SP). Thus, when there is a loss of the OHC, as following an injury, there is decreased suppression of the remaining IHC. The IHC then become hypersensitive and the result is tinnitus.

By using these concepts, procedures are proposed to treat peripheral tinnitus. Techniques will be suggested that either decrease the stimulation of the IHC or decrease the IHC sensitivity. One procedure (TST) that has proven to be successful apparently increases the positive suppressive potentials. This has been achieved by sonically stimulating residual OHC that generate +SP and probably suppress the IHC resulting in a decrease in tinnitus.

10h30-10h45 O54: TINITUS SUPPRESSION THERAPY (TST) WITH SUBTHRESHOLD SOUND STIMULI

Burkhard Franz1 and George Offutt2

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It has long been suspected that an important relationship exists between inner hair cells and outer hair cells, and that a break down of this relationship could lead to the phenomenon of tinnitus. The electromodel of the auditory system describes one mechanism of this inner-outer hair cell relationship. Following this model mechanosensitive outer hair cells influence inner hair cell sensitivity through suppression. Loss of functional outer hair cells can lead to reduced suppression and tinnitus. In tinnitus suppression therapy (TST) mechanosensitive outer hair cells are recruited by specific sound stimuli that remain at subthreshold level. We present a preliminary report of 34 tinnitus sufferers that were investigated for the suitability of tinnitus suppression therapy. 64.7% experienced suppression, 14.7% had partial suppression, and 20.6% were non-responders

Clinical otorhinolaryngology (II) – Tuesday 10th September 11h00-12h15

11H00-11H15 O55: The totally implantable micro drug delivery system – future implications

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The cochlea is a target for local pharmaceutical treatment options in Otolaryngology. The totally implantable micro pump device has been developed as a possible device for this task. It is composed of biocompatible titanium and silicone surface to stay in a body for a lifetime, it can be refilled easily and its delivery is on demand at the users finger tip. We show here a series of experiments with the pump system implanted into rats. They were subcutaneously implanted on the

back and left for four weeks to six months. The catheter was placed underneath the skin leading to the bulla and its tip facing the round window membrane. The device stayed in place over the time of the experiment. No substantial adverse effects as rejection or severe inflammation were observed. Possible future applications are discussed regarding therapeutical approaches in inner ear diseases as vertigo, sudden hearing loss or tinnitus, when local therapy seems to be an appropriate option.

11h15-11h30 O56: Idiopathic Sudden Sensorineural Hearing Loss-Will a national database solve the mystery?

Elisabeth Hultcrantz, Stig Arlinger and Ramesh Zarenoe , Dept of Otorhinolaryngology Linköping university Sweden

Idiopathic SuddenSensoneural Hearing Loss (ISSHL) has an incidence of 5-10/100.000/year. Different treatment policies have been developed according to different hypotheses about the etiology. The number of treated cases at any given clinic in Sweden is too low to provide a basis for studies to demonstrate an effect on outcome due to treatment. A national database for ISSHL is planned and 90% of the ENT-clinics are positive to participate. Demographic data, type of audiogram, time before treatment, treatment, hospitalization, sick leave and final outcome (audiogram) after three months would be requested. The data would help to clarify the disease both with respect to whether any of the present forms of treatment help, and which variables (audiogram type etc) can be used to predict the outcome. Data from about 400 patients/ year will be available. The clinics will be clustered according to their preferred treatment policy and controlled double blind studies will be planned. Knowing the local incidence in advance would make it possible to estimate how long it will take to perform the studies withsufficient statistical power. To prepare for the start of the database the questionnaire to be used has been tested retrospectively on all patients who has got the diagnosis ISSHL between 1997-2002 at their initial visit to the ENTclinic at the university hospital in Linköping. Out of the 153 patients with an initial diagnosis of ISSHL 15 developed Menieres disease, six were diagnosed with acustic neurinoma. Nine were caused by trauma (acoustic and baro-). 93 of the remaining patients were possible to evaluate: 40 completely recovered or were markedly improved (>30dB), 18 partly recovered (10-30dB) and 35 had no restitution. 20 were treated with steroids, surgery in 3 cases and different degrees of rest for the remaining. Upsloping och midfrequency loss had 5 times chance of recovery compared to others. Most patients who did not improve at all had an initial audiogram of flat loss. A database, which after four years should encompass 1600-2000 patients will be extremely valuable for the present and future research.

11h30-11h45 O57: Effect of intratympanic gentamicin in vestibular function

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The use of intratympanic gentamicin is an accepted method of treatment for patients with Ménière's disease when dizzy spells are no longer prevented by the use of diuretics and restricted-salt diet. We have used gentamicin (27mg/ml) in weekly injections until the appearance of signs of vetibular hypofunction. With this method 83% control of vertigo (according to AAO-HNS 1995 guidelines) was obtained.

As during the treatment the tympanic membrane is perforated for the installment of gentamicin the only way to assess vestibular function otherwise than clinical, is with the roatory cahir test and dynamic postrography. We have analysed the results in 20 patients treated with gentamicin. Before treatment a complete otological and oto-neurological assessment was done with caloric test, rotatory test (step or impulsive test, sinusoidal harmonic acceleration and sinusoidal high velocity tests) and the sensory organization test of the dynamic posturography. These last tests, with the corresponding ocular-motor examination and audiometric studies, were repeated every week the patient returned for treatment until signs of hypofunction develop. There is a marked reduction in the time constant and gain of the VOR immediately at the time of ending the treatment and signs precluding this were observed in the majority of the patients.

11h45-12h00 O58: Fluid Dynamics of Ground-based and Microgravity Caloric Tests

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This paper is concerned with the fluid and structural dynamics of the lateral semicircular canal system during both 1g and microgravity caloric stimulations. Robert Barany received the 1914 Nobel prize in medicine for describing the cupular deflections caused by the ampullopetal and ampullofugal flows of the hot and cold irrigations in terms of a buoyancy-driven natural convective mechanism. Microgravity caloric tests aboard the 1983 SpaceLab1 mission produced nystagmus with an intensity comparable to those elicited during post- and pre- flight tests, thus contradicting the basic premise of Barany's convection hypothesis. In this work, we present a finite element numerical model for the caloric stimulation of the lateral semicircular canal based on two simultaneous driving forces for the endolymphatic flow: *natural convection* driven by the temperature-dependent density variation in the bulk fluid and *expansive convection* caused by direct volumetric displacement of the endolymph during the thermal irrigation. The two-dimensional fluid-structural model includes a rigorous two way coupling between the endolymph and the cupula. Direct numerical simulations indicate that on earth, the natural convection mechanism is dominant. But in the microgravity environment of orbiting spacecraft, where buoyancy effects are mitigated, the expansive convection becomes the sole mechanism for producing cupular displacement. A series of transient numerical simulations are presented to delineate the different dynamics of the 1g and of the microgravity endolymph flows. The associated fluid-structural interactions are analyzed parametrically based on the time evolution of cupular displacements and cupular velocities during microgravity and 1g cold and hot caloric stimulations.

12h00-12h15 O59: Saccular function testing with VEMP (vestibular evoked myogenic potentials): added value in a clinical setting ?

Floris Wuyts, Mieke Hoppenbrouwers, Griet Pauwels, Wendy Moeyersons, Paul Van de Heyning
University of Antwerp, Edegem, Belgium

The recent developed vestibular evoked myogenic potentials (VEMP) test is beginning to take it's place in the clinical test battery for vestibular evaluation. Contrary to other vestibular tests that are mainly based on the vestibulo-ocular reflex, the VEMP is based on the vestibulo-collic reflex, where auditory stimulation of the saccule induces a myogenic response in the sternocleidomastoid muscle. This test is achieved with standard EMG equipment, combining 'Auditory Brainstem Evoked Responses' stimuli with EMG response measurements. We here present normative data, obtained in 39 healthy subjects, of latencies and amplitudes of the EMG responses. The subjects' age ranges between 19 to 83 years of age. A new method is proposed to obtain the desired responses also in the elderly group of subjects. Indeed, to measure a VEMP response, continuous contraction of the sternocleidomastoid muscle is request during the test, which is not trivial.
The 95% Prediction Interval ($\mu \pm 2s$) for the first response, called the p13 yields [12.15 ms – 17.8 ms] and for the second response, the n23 equals [19.31 ms – 26.51 ms]. These are well within accordance with the literature. Additionally, we present several clinical cases with vestibular pathology, where the data obtained from VEMP are compaired with other vestibular test results, such as caloric examination, rotary chair evaluation and utricular function testing. In several cases the VEMP test appears to be normal where other investigations are aberrant or vice versa. This is mainly due to the fact that the caloric and the rotation tests, focus on the function of the horizontal semi-circular canal, innervated by the superior branch of the vestibular nerve, whereas the saccule is innervated by the inferior branch. This study illustrates the complementary character of the VEMP to the classical vestibular tests, and shows a new method for obtaining the desired responses in elderly subject.

12h15-12h30 O60: Pharmaceutical countermeasures for space motion sickness and their effect on the otoliths and canals

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A mismatch between utricular and semi-circular canal output is believed to provoke space motion sickness. To assess the utricular and the horizontal canal functions, we apply the unilateral otolith test (UOT) and the standard electronystgmograpic (ENG) test battery. We use a modified paradigm of the unilateral otolith test during which the

subject is rotated about an earth vertical axis at a velocity of 400 degrees per second and 4 cm translated along an interaural axis to the right and to the left. When the axis of rotation is positioned through one utricular system, only the contralateral utricle is stimulated. Consequently, the centrifuged utricle feels an outward pulling force equal to 0.4g, corresponding to a gravito-inertial acceleration (GIA) tilt of 21 degrees. This utricular stimulation induces an ocular counter rolling (OCR), that is measured on-line using validated three dimensional video-oculography. For analysis of the experimental data, we use a theoretical model proposing a linear relationship between the OCR and the GIA tilt felt by a transducer placed at the centre of the head, behind the subject: (OCR = intercept + slope x GIA tilt). The function of each utricle is assumed to be additive. The slope of the linear regression is a measure of the responsiveness of both utricles whereas the intercept is a measure of lateralisation. These results are presented in the framework of a study Pharmacological countermeasures for space motion sickness (NSBRI / NASA grant: #NCC9-58), the aim of which is to assess the effect of promethazine, scopolamine, lorazepam and meclozine in healthy subjects. We present here preliminary results of the effect of these medications. Material and methods: Nine healthy volunteers (five female, four male) with an average age of 27.1 year (21-47 year) were recruited. All subjects had three sessions: one control session (UOT and ENG) one week prior to the intake of the drug, one session with UOT and ENG at the maximum response of the drug and one control session with UOT one week later. Results: Wilcoxon Matched pairs signed rank test indicates a significant decline in utricular responsiveness after intake of promethazine ($p = 0.044$) in reference to the control measurements. The effect on the horizontal canal responsiveness is even stronger ($p = 0.012$ for the caloric sum and $p=0.012$ for the gain on rotation). Scopolamine however, has no significant effect on the utricular responsiveness ($p = 0.16$). The effect on caloric sum and gain are however significant ($p=0.012$ each). Conclusion: Our results indicate that intake of promethazine leads to a suppression of the utricular function, a declined responsiveness of the horizontal semicircular canals and also to central inhibition. On the other hand, scopolamine does not reduce the utricular sensitivity, although it induces central inhibition as well as a significant decline in horizontal semicircular canal function

12h30-12h45 O61: Unilateral otolith function testing - Is the utricular function additive ?

F.L.Wuyts, M.Hoppenbrouwers, G.Pauwels, P.Van de Heyning, University of Antwerp, Edegem, Belgium

The utricle plays a crucial role in the detection of gravity. Knowledge about its function is usually obtained using lateroflexion tests or centrifuge tests. The newly developed unilateral otolith function test, based on work by Von Baumgarten in the early 90ies, and further elaborated by A. Clarke in Berlin is a method to evaluate each utricular system separately. Material and methods: We present a modified paradigm of this test during which the subject is rotated about an earth vertical axis at a velocity of 400 degrees per second and consecutively translated (2 mm/s) along an interaural axis for 4 cm to the right and to the left. In each eccentric position along this path, both utricles are centrifuged at a different magnitude. When the axis of rotation is positioned through one utricular system, only the contralateral utricle is stimulated. Consequently, the centrifuged utricle feels an outward pulling force equal to 0.4g, corresponding to a gravito-inertial acceleration (GIA) tilt of 21 degrees. This utricular stimulation induces an ocular counter rolling (OCR), that is measured on-line using three dimensional video-oculography, a method validated by a special calibration device.

Model for utricular function We present a theoretical model and experimental data describing a linear relationship between the OCR and the GIA tilt, felt by a transducer placed at the centre of the head behind the subject: (OCR = intercept + slope x GIA tilt). The function of right and left utricle is assumed to be additive during the unilateral otolith function test, as long as the axis of rotation is situated between both utricular systems. The slope of the linear regression is a measure of the responsiveness of both utricles (similar to the gain obtained from rotary chair testing with electronystagmography), whereas the intercept is a measure of lateralisation of the utricular response (similar to labyrinth asymmetry obtained in caloric testing). Results Healthy subjects (N=34) In a group of 34 healthy subjects, this linear relationship (OCR as a function of tilt) was measured for both eyes. The average (± se) responsiveness yields -0.222 (± 0.010) deg_OCR/deg_tilt and the lateralisation = -0.49 (± 0.19) deg_OCR. Acoustic Neuroma subjects (N=14). The responsiveness of the utricular system in patients with acoustic neuroma (UVD), after surgery, is only half of the value in healthy subjects. Therefore, according to the additive model should the slope of the OCR tilt relationship only be half of the slope in healthy subjects. Data obtained from 14 UVD patients yield 0.107 (± 0.007) deg_OCR/deg_tilt, as predicted. The intercept for right UVD patients is 0.73 (± 0.22) deg_OCR, where the intercept for left UVD patients is 1.22 (± 0.23)

deg_OCR, as predicted by the theoretical model. Conclusion The data are well in accordance with the theoretical model proposing an additive function of the utricle. This model and the data provide a quantitative tool for evaluating utricular function in subjects, especially when no proper knowledge of the vestibular diagnosis is available.