Final report of grant K-128875

entitled

'Hearing and hearing loss: purinergic signaling and noise-induced hearing loss'

Hearing impairment is the most common sensory deficit among the human populations. Over 5 % of the world's population – or 430 million people – require rehabilitation to address their disabling hearing loss (including 34 million children) and this prevalence is estimated to be 10 % by 2050 (https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss). Deafness may occur at any age with any degree of severity. Hearing loss halts speech acquisition and threatens personal autonomy resulting in major difficulties in daily life and, ultimately, social isolation and depression.

Contrary to the conductive hearing losses (HLs), there are no specific drug therapy for the sensorineural forms (SNHLs; e.g., ototoxicant drug- and noise-induced (NIHL) or age-related hearing loss (ARHL)). The majority of the causes of SNHLs resides in the cochlea, the sensory organ of hearing. A promising way of inventing pharmaceuticals to treat SNHLs, including ARHL could be the positive modulation of endogenous protective mechanisms.

Purinergic signaling – modulation of hearing and protection in NIHL and other SNHLs

Both hearing and its protection is regulated by purinergic signaling in the supporting cells of the organ of Corti (OoC). Extracellular ATP controls the intracellular Ca^{2+} concentration in the supporting cells of the OoC and the intercellular Ca^{2+} waves that travel through these cells, and has been suggested to play an important role in the protection against noise trauma and the repair mechanism in NIHL. Inadequate knowledge of the basic molecular mechanisms of normal and impaired adult hearing and of endogenous protective factors, including purinergic signaling, is one of the main reasons for the lack of specific tools to prevent and cure SNHLs, including NIHL. Our work has advanced knowledge in this area at the molecular, cellular and hearing system levels.

Development, auditory neurotransmission, control of sensitivity, mechanism of pathological impairments in the hearing organ and protection against NIHL or other SNHL forms all involve extracellular purines as transmitters and paracrine regulators in the cochlea. We reviewed the functions of purinergic signaling in the cochlea focusing primarily on the possible therapeutic targets for SNHLs, including NIHL. (*Brain Res Bull, 2019*)

A quick, straightforward and reliable Ca^{2+} imaging method with high spatial and temporal resolution in the mature organ of Corti was missing. We have established a way of targeted single-cell electroporation loading of Ca^{2+} indicators in the mature hemicochlea preparation for the first time in the literature. This simple, rapid and reliable method of Ca^{2+} indicator dye loading into individual supporting cells of the organ of Corti prepared from hearing mice is a proper tool for performing our study. We demonstrated that the loading is selective to the target cell and causes little dye spill-over in the extracellular space. By using this technique we were able to investigate the purinergic P2, TRPA1, TRPV1 and acetylcholine receptor (AChR) agonist-evoked cellular and subcellular dynamics of intracellular Ca^{2+} concentration

in Deiters', Hensen's and Claudius' cells in different turns of the cochlea. The functional role of AChRs in Hensen's cells and the lack of functional role of TRPA1 and TRPV1 channels in Ca^{2+} signaling in the three supporting cell types have not been described before. (*Hear Res, 2019*)

Exploring the development of the hearing organ helps in the understanding of hearing and hearing impairments and it promotes the development of the regenerative approaches-based therapeutic efforts. The use of targeted electroporation loading of single Deiters' cells allowed us to perform intracellular Ca^{2+} measurements in different subcellular compartments (soma, phalangeal process), in two different cochlear turns (apical, middle) and in the critical P5-25 developmental period of the cochlea. Soma and process of Deiters' cells elongated, and the process became slimmer by maturation without tonotopic preference. The tonotopically heterogeneous spontaneous Ca^{2+} activity less frequently occurred by maturation and implied subcellular difference. The exogenous ATP- and UTP-evoked Ca^{2+} responses were maturation-dependent and showed P2Y receptor dominance in the apical turn. By monitoring the basic structural dimensions of this supporting cell type as well as its spontaneous and evoked purinergic Ca^{2+} signaling in the hemicochlea preparation in different stages in the critical postnatal P5-25 developmental period for the first time, we showed that the soma and the phalangeal process of the Deiters' cells go through age- and tonotopy-dependent changes in the morphometric parameters and purinergic signaling. (*Cells, 2019*)

Purinergic Ca^{2+} signaling in the supporting cells of the organ of Corti sets the sensitivity of hearing and also provides protection against NIHLs in the mature cochlea. We have continued to work on the characterization of ATP-induced calcium signaling in the supporting cells of the OoC from hearing mice (P15-25). ATP evoked Ca^{2+} transients in supporting cells of the OoC (Deiters', Hensen's and pillar cells) along the whole tonotopic axis (in apical, middle and basal cochlear turns). The ATP response was concentration dependent and present in supporting cells of three different mouse strains (BALB/c, C57Bl/6 and CD1 mice). The role of adenosine receptors was ruled out by showing the lack of effect of adenosine and the adenosine receptor antagonist 8-SPT. We performed a more detailed analysis of the ATP-evoked Ca^{2+} response in one of the most interesting supporting cells, the Deiters' cells. The columnar body of these polar cells are located under the outer hair cells (OHCs), while the head of their long process is incorporated into the cuticular plate. This special anatomical structure, the dense actin content in the process and the innervation of these cells supposed their role - besides the well known metabolic support of the OHCs - also in the mechanics of the OoC, thus their role in setting hearing sensitivity and protection against noise trauma. Previously we have shown that the ATP and UTP-induced Ca^{2+} transients in these cells involve both extracellular Ca^{2+} dependent P2X and intracellular Ca^{2+} store dependent P2Y receptors. Using our new method of Ca^{2+} dye loading and imaging supporting cells on the subcellular level, we could measure the ATP responses separately in the soma and process of the Deiters' cells. We have discovered that the response in the phalangeal process preceded the soma's one and the delay was more pronounced in the middle turn, vs. the apical one. This result suggests the tonotopic nature of purinergic Ca^{2+} signaling in the mature organ of Corti. Inhibition of extracellular Ca^{2+} influx (either by Ca^{2+} withdrawal or voltage-gated Ca^{2+} channel inhibition) reduced the delay, while inhibition of the release of Ca^{2+} from intracellular stores (by sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibition) enhanced it. Inhibition of both the influx and intracellular release of Ca^{2+} reduced the amplitude of the ATP-evoked Ca^{2+} response. The simultaneous inhibition of Ca^{2+} influx and store release of

Ca^{2+} suppressed the ATP response at significantly higher magnitude than the sum effect of the separate inhibitions. This supra additive effect suggests the involvement of calcium-induced calcium release in the ATP response.

Based on the effect of different purinoceptor ligands (PPADS, suramin, TNP-ATP, UTP and ADP) on the ATP-evoked Ca^{2+} signal, we have assumed the role of P2X2 and P2Y4 receptors in the response. Performing immunofluorescence staining on these receptors indicated that the higher expression of P2X2 in the processes of Deiters' cells is more pronounced in the middle turn. The presence of P2Y4 purinoceptors in the two cellular compartments did not show tonotopical difference. This may support our working hypothesis that the delay is caused by the predominant role of the "faster" ionotropic P2X receptors in the process vs. the prominence of the "slower" metabotropic P2Y purinergic receptors-induced Ca^{2+} signaling in the soma and that this expression difference also has a tonotopicity. In order to investigate the involvement of P2X7 and P2Y12 receptors in the intracellular Ca^{2+} response, we tested the effect of ATP on cochleae from P2X7 and P2Y12 KO mice. The absence of neither receptors did alter the response significantly.

Deciphering the details of purinergic Ca^{2+} signaling at cellular and subcellular level in the light of tonotopicity - which aspect has been studied very poorly because of its technical difficulty - is a fundamental step to understand its role in hearing (mal)function and reveals possible targets for therapeutic interventions in NIHL and in SNHLs in general. It is worth noting that lesions of the OoC in SNHLs also typically show tonotopicity. (*Finishing the pharmacological characterization and immunostainings are under way to complete the results for publication in a research paper*.)

Supporting cells in the OoC form a network in which they modulate each other's function. This synchronized network activity involves intercellular Ca^{2+} waves triggered by paracrin purinergic signaling. ATP evokes both Ca^{2+} increase and ATP release from adjacent cells through pannexin channels. The intercellular Ca^{2+} waves underpins endogenous noise-protection. Blocking pannexin channels (carbenoxolon), inhibited significantly the ATP response. This is indicative of the involvement of surrounding supporting cells in the ATP-induced Ca^{2+} response in our experiments. In order to understand and describe tonotopic and subcellular differences in ATP-evoked intra- and intercellular Ca^{2+} dynamics in and between Deiters' cells at a more complex level, we have set up a closed cell, minimal model, using the parameters of our experimental results. The mathematical model supports the hypothesis that Deiters' cells are glia-like cells and suggests the tonotopical distribution of IP_3 degrading enzymes and the similarity of the calcium-induced calcium release rate to the one in the astrocytes. (*Int J Mol Sci, 2023*)

Modulation of other endogenous cochleo-protective mechanisms

During the implementation of the project plan, it became evident that, in addition to the purinergic system, other endogenous signaling systems play a pivotal role in hearing and NIHL or ARHL, in interaction with the former. Consequently, these systems inevitably influence the project's objectives and are intrinsically linked to the project's experiments. Modulation of the release of dopamine (DA) from lateral olivocochlear (LOC) efferents, the

protective neuropeptide pituitary adenylate-cyclase-activating polypeptide (PACAP) and of the immune system are promising ways to treat NIHLs and ARHLs.

DA release from LOC efferents – NIHL, ARHL

Repeated noise exposure during lifespan is one of the major cause of ARHL. Loud noise, ageing or insults in SNHLs evoke an excessive release of glutamate (Glu) from the inner hair cells (IHCs) resulting in the excitotoxic damage of the primary auditory neurons and their synapse with the IHCs. The excitotoxic overactivation of neurons is inhibited by DA released from the LOC efferents forming axodendritic synapses on the auditory neurons, thereby protecting the IHC-afferent nerve synapse and the auditory neurons. Based on our previous results, we hypothesized that selegiline, an antiparkinsonian drug could be a promising candidate for the treatment of SNHLs due to its complex neuroprotective, antioxidant, immune modulatory and DAergic neurotransmission enhancing effects. We monitored by repeated auditory brainstem response (ABR) measurements the effect of chronic p.o. selegiline administration on the hearing function in BALB/c and DBA/2J mice, which strains shows different sensitivity against noise exposure and exhibit - as life advances - moderate and rapid progressive high frequency hearing loss, respectively. The treatments were started at 1 month of age and lasted till almost a year and 5 months of age, respectively. In BALB/c mice, 4 mg/kg selegiline significantly mitigated the progression of ARHL at higher frequencies. Used in a wide dose range (0.15 – 45 mg/kg), selegiline had no effect in DBA/2J mice. Our results suggest that selegiline can partially preserve the hearing in certain forms of ARHL by alleviating its development. It is presumably also otoprotective in other mammals or humans who have a similar DAergic LOC system and it may also be effective in other SNHL forms. (*Int J Mol Sci, 2021*)

PACAP – NIHL, ARHL

Many genetic defects, including genes of the purinergic system, contribute and form the basis of sensitivity to NIHL and ARHL, the two most prevalent forms of SNHLs. We investigated the effect of the deficiency of the regulatory and cytoprotective neuropeptide PACAP, present in the auditory system, on hearing and showed its protective effect against NIHL and ARHL. ABR tests found higher hearing thresholds in PACAP-KO mice at click and low frequency burst stimuli. Hearing impairment at higher frequencies showed as reduced ABR wave amplitudes and latencies in KO animals. Increase in neuronal activity, demonstrated by c-Fos immunolabeling, was lower in KO mice after noise exposure in the ventral and dorsal cochlear nuclei. Our results support that certain transmitters, mediators and signaling pathways in the auditory system can efficiently provide protection on the hearing function. (*Sci Rep, 2019*)

Immune system – NIHL, ARHL

The immune system is also playing a fundamental role in cochlear homeostasis and pathology, as it was recognized in the very recent years. Virtually all immune cells express P2 and P1 receptors and purinergic signaling plays a pivotal role in the regulation of the immune system. Supporting cells in the OoC, like the Deiters' cells can contribute to the homeostatic function of the immune system, among others by removing debris of the damaged hair cells and promoting scar formation, thus maintaining endocochlear potential after harmful noise insults.

In a review paper we summarized the purine-immune interactions in the cochlea. The role of harmful immune mechanisms, like chronic inflammation in SNHLs has been emerging in the horizon of cochlear pathologies. Influencing the immune system can be an additional avenue for pharmacological targeting of purinergic signaling in the cochlea. Elucidating the complexity of purinergic effects on cochlear functions and immune responses are necessary and it can result in the development of new therapeutic approaches in hearing disabilities, especially in the NIHL and AHRL ones. (*Int J M Sci, 2019*)

Contrary to previous notions, the inner ear is not an immune privileged organ. It contains bone-marrow derived resident macrophages and insults of the inner ear induces the invasion of the cochlea by monocytes. This cochlear immune system is involved in both the homeostatic or injury repair processes and the pathogenesis of all SNHL forms. In order to check its role in NIHL and ARHL, we inhibited the negative immune regulation to activate the immune system by PD-1 receptor inhibitor monoclonal antibody (mab) treatment (disinhibition) and investigated its consequences on the hearing function and cochlear morphology. This immune checkpoint inhibitor (ICI) treatment, a new and efficient class of human antitumor therapy, may result in immune-related adverse events in different organs like the heart, GI tract, lung or the skin. But it's side effect is largely unexplored in the hearing organ. In our experiments the 4-week long mouse specific anti-PD-1 mab treatment did not influence the hearing thresholds in click or tone burst stimuli at 4-32 kHz frequencies measured by ABR. Number and morphology of spiral ganglion neurons were unaltered in all cochlear turns. The apical-middle turns (< 32 kHz) showed preservation of the IHCs and OHCs, whilst ICI treatment mitigated the age-related loss of OHCs in the basal turn (> 32 kHz; in mice, frequency range of hearing: 2 – 90 kHz, frequency of max sensitivity: 16 kHz). The number of Iba1-positive macrophages has also increased moderately in this high frequency region. We concluded that a 4-week long anti-PD-1 mab treatment did not affect functional and morphological integrity of the inner ear in the most relevant hearing range (4-32 kHz; apical-middle turns), but a noticeable preservation of OHCs and an increase in macrophage activity appeared in the >32 kHz basal part of the cochlea, where the high frequency sound is detected. This part of our results also draws attention to the possibility of using immune modulation by the purinergic system for otoprotective purposes. (*Int J Mol Sci, 2020*)

We continued to investigate our interesting recent observation that certain types of activation of the immune system may result in the preservation of hearing, not exclusively in inducing inflammation in the cochlea with consequent SNHLs, described in the literature. After targeting a single immune checkpoint (PD-1, see above), now we used a 4-week long combined inhibition of PD-1 and CTLA4 (combination used also in the clinical practice) in C57BL/6J mice. This resulted in the mitigation of the age-dependent progressive impairment of hearing. Over the repeated objective audiometry measurements (ABR and Distortion Product Otoacoustic Emissions, DPOAE) before and at the end of the treatment period, we also investigated the morphological and RNA expression changes in the cochlea in all three treatment groups (combined ICI, isotype monoclonal antibody control and vehicle control). The number of the primary auditory neurons, the spiral ganglion cells was higher in the combined ICI treated group (vs. controls) in the apical and middle turns of the cochlea, but we did not see significant differences in the number of hair cells. Expression of mRNA of the

measured immune function markers (IL1β, Iba1, CD3e, IL17) did not change either significantly in the whole cochlea extracts.

Mice in the above experimental run were kept in individually ventilated cages (IVCs) with ~76 dB ambient noise. We repeated the experiment on mice kept in regular animal housing where the ambient noise was significantly lower (~60 dB). Under this circumstances the threshold shifts (differences of hearing thresholds at the end of the experiment and before the onset of treatment) at different frequencies (4 – 64 kHz) in the vehicle control group were in the 0 – 10 dB range contrary to the 3 – 22 dB control shift range of mice kept in IVCs. The lack of ICI protection in the second set of experiments was due to the non-existing or minimal progression of hearing impairment that was not enough robust to be mitigate by the treatment at statistically significant level. Then we repeated the experiment for a second time in IVCs with mid-range ambient noise (~70 dB). The frequency-dependent threshold shifts in the control mice, i.e., the deterioration of hearing function, showed also mid-range values and the combined ICI treatment produced a moderate protection. We hypothesize that the continuous, 4-week long ambient noise accelerates the ARHL, that this mouse strain has. The activation of the immune system with combined ICI treatment promoted the homeostatic function of the immune system that results in a mitigated progression of the hearing function.

Morphological analysis of the cohleae dissected out from mice of all three treatment groups and sets of experiments is currently in progress. We count the inner and outer hair cells and spiral ganglion cells along the tonotopic axis and also work on the determination of M1 and M2 macrophages in the fixed cochlear samples. (*A manuscript is in preparation and we are going to submit it after the completion of these morphological experiments.*)

Project prolongation:

We requested the no-additional-cost prolongation of the project. The measures taken to control the epidemic have significantly impeded the conduct of both the in vitro and the in vivo experiments. This is due to the delays in the procurement of compounds and mice for the experiments, as well as the repair of our calcium imaging setup. Furthermore, the illness and quarantine of research personnel, along with restrictions on workplace availability, have also resulted in delays.

List of publications with indicated K-128875 support in the Acknowledgements:

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2. Berekméri E, Fekete Á, Köles L, **Zelles T**. Postnatal development of the subcellular Structures and purinergic signaling of Deiters' cells along the tonotopic axis of the cochlea. *Cells*. 2019 Oct 17;8(10). pii: E1266. doi: 10.3390/cells8101266.
3. Fulop DB, Humli V, Szepesy J, Ott V, Reglodi D, Gaszner B, Nemeth A, Szirmai A, Tamas L, Hashimoto H, **Zelles T***, Tamas A*. Hearing impairment and associated morphological changes in pituitary adenylate cyclase activating polypeptide (PACAP)-deficient mice. *Sci Rep*. 2019 Oct 10;9(1):14598. doi: 10.1038/s41598-019-50775-z. **Co-correspondent authors*

4. Köles L, Szepesy J, Berekméri E, **Zelles T**. Purinergic signaling and cochlear injury – targeting the immune system? *Int J Mol Sci.* 2019 Jun 18;20(12). pii: E2979. doi: 10.3390/ijms20122979.
5. Berekméri E, Szepesy J, Köles L, **Zelles T**. Purinergic signaling in the organ of Corti: potential therapeutic targets of sensorineural hearing losses. *Brain Res Bull.* 2019 Sep;151:109-118. doi: 10.1016/j.brainresbull.2019.01.029.
6. Szepesy J, Miklós G, Farkas J, Kucsera D, Giricz Z, Gáborján A, Polony G, Szirmai Á, Tamás L, Köles L, Varga ZV, **Zelles T.** Anti-PD-1 therapy does not influence hearing ability in the most sensitive frequency range, but mitigates outer hair cell loss in the basal cochlear region. *Int J Mol Sci.* 2020 Sep 13;21(18):E6701. doi: 10.3390/ijms21186701.
7. Szepesy J, Humli V, Farkas J, Miklya I, Tímár J, Tábi T, Gáborján A, Polony G, Szirmai Á, Tamás L, Köles L, Vizi ES, **Zelles T.** Chronic oral selegiline treatment mitigates age-related hearing loss in BALB/c mice. *Int J Mol Sci.* 2021 Mar 11;22(6):2853. doi: 10.3390/ijms22062853.
8. Moysan L, Fazekas F, Fekete A, Köles L, **Zelles T***, Berekméri E*. Ca^{2+} dynamics of gap junction coupled and uncoupled Deiters' cells in the organ of Corti in hearing BALB/c mice. *Int J Mol Sci.* 2023 Jul 4;24(13):11095. doi: 10.3390/ijms241311095. **Shared last authorship*