DOCTORAL (PH.D.) THESIS

Protective effect of pituitary adenylate cyclase activating polypeptide (PACAP) in the inner ear: *in vitro* and *in vivo* studies

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I. INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide first isolated from the ovine hypothalamus in 1989 based on its adenylate cyclase activating effect. It has two forms, PACAP27 and PACAP38, the latter being the dominant form in mammals. PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family, with 68% similarity to VIP. PACAP receptors are the VPAC1 and VPAC2 receptors, which bind PACAP and VIP with similar affinity, and the specific PAC1 receptor, which binds PACAP with much higher affinity than VIP. The peptide has wide distribution in the entire body. It is present in the central and peripheral nervous system, in endocrine glands, and also in the cardiovascular, gastrointestinal, urogenital and respiratory tracts. Since its discovery numerous studies have shown its neurotrophic, neuroprotective and general cytoprotective effects both *in vitro* and *in vivo*.

PACAP-deficient mice

PACAP-deficient mice are used for the examination of endogenous PACAP in different physiological functions and under pathological conditions. It is well known that PACAP-deficient mice have, among others, decreased reproductive function, behavioural abnormalities, memory disturbances, and biochemical alterations. The gross morphology of most examined organs does not show significant difference between wild type and PACAP-deficient mice. However, numerous studies have shown that PACAP-deficient mice have increased susceptibility for different nervous, renal, and intestinal injuries, supporting the protective effect of endogenous PACAP. The supposed mechanism for the increased vulnerability of PACAP-deficient mice against various harmful stimuli includes increased apoptosis, inflammatory reactions, and oxidative stress.

Organ of Corti

The organ of Corti is the receptor organ for hearing and is located in the cochlea of the inner ear. The organ of Corti is located on the basilar membrane, with the Corti tunnel between the pillar cells, surrounded by the inner and outer hair cells and phalangeal cells (Figure 1.).The lumen of the membranous labyrinth is filled with endolymph which contains more K and less sodium compared to extracellular fluid and perilymph. The endolymph is a positively polarized fluid secreted by stria vascularis, spiral ligament, and supporting cells. Evaluation of endolymph proteins indicates that they are predominantly derived from plasma. However, the profile of endolymph proteins is remarkably similar to that of perilymph and entirely different from that of plasma. The composition of the inner ear fluids, the origins of endolymph and perilymph, and the cellular mechanisms involved in the secretion of these fluids have been intensively investigated during the last years. Several neuropeptides have already been shown to influence tear and endolymph secretion and composition, such as substance P, vasopressin, and somatostatin.

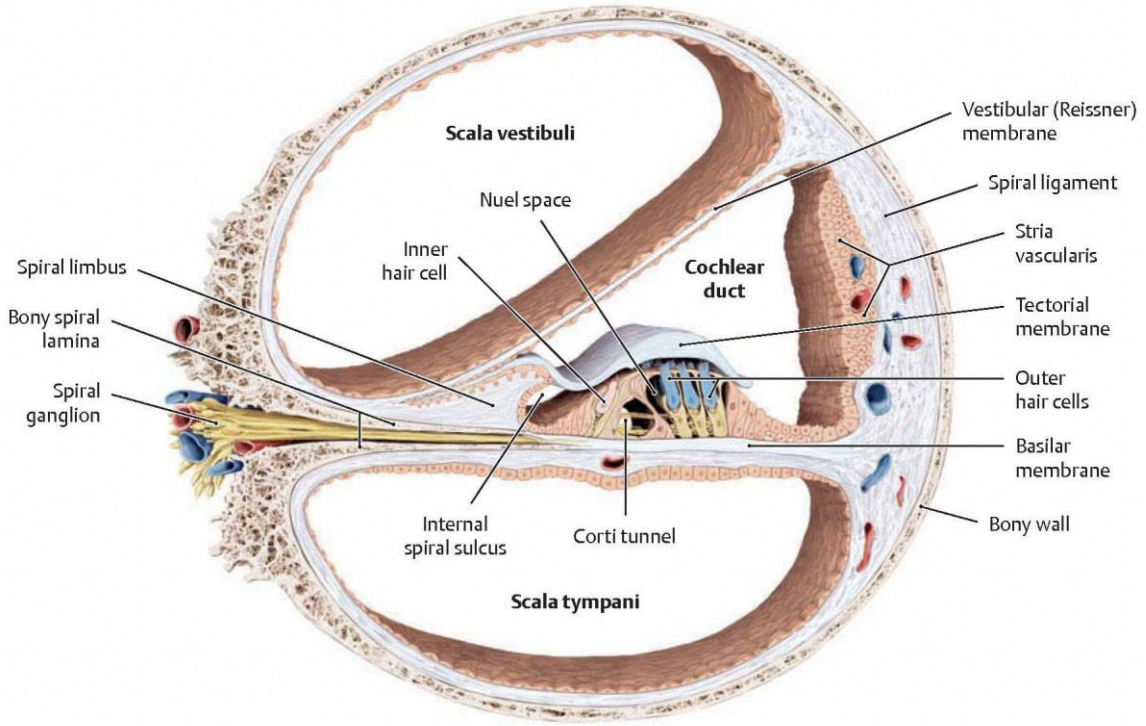

Figure 1. Organ of Corti (Thieme 2007)

PACAP in the hearing system

The involvement of PACAP in sensory processing has also been documented, mainly in the visual and olfactory systems. However, little is known about its effects in the auditory system. PACAP immunoreactive elements have been shown in the cochlear nucleus neurons of the brainstem, in the spiral ganglion and marginal cells of the stria vascularis with immunohistochemistry and in situ hybridization. PACAP precursor protein mRNA and PAC1receptor mRNA have been described by reverse transcription-polymerase chain reaction in microdissected cochlear lateral wall, organ of Corti and spiral ganglion subfractions. PACAP and PAC1 receptor play an important role in the modulation of afferent signalling of the organ of Corti and PACAP is present in the olivocochlear neurons of superior olivary complex. Localization of PACAP and PAC1 receptor in the stria vascularis close to the endolymphatic compartment suggests that PACAP might have an effect on the endolymph production, potential, and/or composition.

Apoptosis in the inner ear

Apoptosis is an important process both for normal development of the inner ear and for removal of oxidative stress-damaged sensory cells from cochlea. Increased apoptosis leads to permanent damages in the inner ear and loss of hearing. Increased cell death can be caused by lack of essential growth factors, exogenous toxins such as the ototoxic aminoglycoside antibiotics and the antineoplastic agent cisplatin, acoustic overstimulation, infections, autoimmune conditions or hereditary diseases. Several important apoptotic signaling events that regulate the death of cochlear cells have been identified. Ototoxic treatments induce the activation and redistribution of the proapoptotic cytosolic Bax and the release of cytochrome c from injured mitochondria. Activation of caspase-9, caspase-3, c-Jun N-terminal kinase and the cleavage of caspase-3 is observed within damaged hair cells. Intracellular damage caused by various ototoxic agents seems to share a final common pathway leading to caspase

activation. Inhibition of caspases protects hair cells against toxic agents. PACAP has well-known antiapoptotic and antioxidant effects; therefore, it could play an important role in the protection against aminoglycoside and oxidative stress-induced ototoxicity. Numerous studies have demonstrated that PACAP is able to inhibit proapoptotic pathways, such as JNK, caspases, cytochrome c release from the mitochondria, and to activate phosphorylation of antiapoptotic agents such as ERK, Bad, Bcl2, therefore, PACAP could be a promising otoprotective mediator during ototoxic injuries.

Ca^{2+} buffering proteins in the inner ear

In the inner ear, Ca^{2+} buffering is especially important for normal hair cell function. The endolymphatic Ca^{2+} concentration controlled by Ca^{2+} buffers of the hair cells is essential for acoustic transduction and normal hearing processes. Labyrinth destruction causes an elevation in endolymphatic Ca^{2+} concentration, which leads to disturbances in the cochlear function. Acoustic overstimulation leads to sustained increases in the Ca^{2+} concentration of the outer hair cells, which is toxic to cells. Ca^{2+} buffering proteins (for example: parvalbumin, calbindin, calretinin, calmodulin, calsequestin) have been identified in hair cells of different species, such as in frogs, turtles and mammals. Other research groups also investigated the concentrations of Ca^{2+} buffering proteins in the rat cochlear cells and presumed that high concentration of Ca^{2+} buffers may protect the cells against the toxic effect of Ca^{2+} overload after acoustic overstimulation.

II. AIMS OF THE THESIS

1. The aim of the first study was to examine H_2O_2-induced cell death in an inner ear in vitro model, chicken cochlear cell culture, and the effect of PACAP on cochlear cell survival under oxidative stress.

2. In the second study the aim was to investigate whether systemic injection of PACAP has any modulatory effect on the protein composition of the endolymph, using chip electrophoresis analysis.

3. Given the known neuroprotective effects of PACAP and its involvement in sensory processing and the importance of Ca^{2+} buffering in the inner ear, the aim of the third study was to investigate whether there is any difference in the localization and expression of PAC1-receptor and Ca^{2+} binding proteins (parvalbumin, calretinin, calbindin) in cochlear cells between wild type and PACAP-deficient mice.

4. The aim of the fourth study was to examine the effect of kanamycin treatment on the expression of Ca^{2+}-binding proteins (parvalbumin, calretinin) in hair cells lacking endogenous PACAP compared with wild-type animals.

III. PACAP AMELIORATES OXIDATIVE STRESS IN THE CHICKEN INNER EAR: AN IN VITRO STUDY

Materials and methods

Cochlear cell cultures were isolated from newly hatched domestic chickens (in 6 series, 40 chickens/series). Animals were sacrificed by decapitation following anaesthesia with halothane. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (No: BA02/2000-20/2006). The middle ear space was exposed, and the bone overlying the proximal (basal) end of the cochlea was broken away. Cochleae were removed by grasping their very proximal tips with fine forceps. Afterwards cochlear cells were isolated under steril circumstances. PACAP1-38 was synthesized by Prof. Tóth Gábor (SZTE OVI / University of Szeged, Faculty of Medicine, Department of Medical Chemistry).

Cultured cochlear cells were randomly assigned to one of 4 experimental groups: (1) control group of cells, incubated in DMEM/F12 (normal medium) without treatment; (2) cells incubated in medium containing 100 nM PACAP1-38; (3) cells treated with 1 mM H_2O_2; (4) cells treated with 1 mM H_2O_2 together with 100 nM PACAP1-38. Cells were exposed to the mentioned concentrations of chemicals for 2 hours. Evaluation of cell survival was performed immediately after termination of treatments.

Viability of cochlear cells was determined by colorimetric MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). The assay is based on the reduction of MTT into a blue formazan dye by functional mitochondria of viable cells. Optical densities were determined by an ELISA reader at the wavelength of 550 nm representing the values in arbitrary unit. All experiments were run at least four parallels and repeated six times. Results are expressed as percentage of control values. Data are presented as mean±SEM from six independent experiments, analyzed with one-way ANOVA followed by Neuman–Keul's post hoc analysis and two-way ANOVA.

Results

Immunohistochemical identification of the cells revealed that the cochlear cell culture used in the present experiment contained both sensory hair cells and supporting cells. H_2O_2 treatment alone led to a manifest decrease of cell viability compared to control values. Treatment with PACAP alone did not alter cell viability. Cell viability significantly increased when H_2O_2-treated cells were co-incubated with PACAP.

IV. INVESTIGATION OF PACAP EFFECT ON THE COMPOSITION OF ENDOLYMPH PROTEINS

Materials and methods

Chicken was used as a model animal for evaluating the composition of endolymph because of the simpler organization of its membranous labyrinth. Endolymph was collected from newly hatched, 1-day-old chickens. PACAP1-38 (20 μg intraperitoneal) was given, and endolymph was collected 1, 6, and 24 h after PACAP injection (n=5 in each group). The control group received saline injection. The whole membranous labyrinth was removed, and the endolymph was squeezed onto a sterile paper strip with sterile forceps. Protein composition of the chicken endolymph and effect of PACAP on the protein expression were analyzed by the commercially available Agilent 2100 Bioanalyzer System. Laser induced fluorescence detection was applied in the measurements. Evaluation of the results was performed by the Protein 230 assay software.

Results

Protein content of the chicken endolymph showed a complex pattern; several protein peaks could be detected within the 14 to 80 kDa molecular mass region. Most of the proteins were presented in this molecular mass range. Because of the complexity of the pattern, baseline separation of the protein peaks could not be achieved. There was no significant difference between control and PACAP-treated endolymph samples at any examined time point as determined by chip electrophoresis.

V. COMPARATIVE EXAMINATION OF INNER EAR IN WILD TYPE AND PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP)-DEFICIENT MICE

Materials and methods

Wild type ($PACAP^{+/+}$, n = 9) and homozygous PACAP-deficient mice ($PACAP^{-/-}$, n = 9) were used. Animals were fed and watered ad libitum, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-20/2006). 5-day-old pups were sacrificed with an overdose of anaesthetic. We made approximately 10–12 sections from the cochlea, and we analyzed only those slides in which the modiolus was visibly surrounded by bony spiral canal. We compared only the middle turns of the cochlea. Cryosections were stained with haematoxilin-eosin to identify the different cell types in the organ of Corti and to make a comparison of the inner ear between the wild type and PACAP-deficient mice. The sections were also processed for immunohistochemical examination. Sections were incubated with antibodies directed against PAC1-receptor (PAC1-R) (anti-rabbit; 1:100; Sigma, Hungary); Ca^{2+}-binding proteins: calretinin (anti-mouse; 1:1000; Swant, Switzerland), parvalbumin (anti-mouse; 1:500; Sigma, Hungary), and calbindin (anti-mouse; 1:500; Sigma, Hungary) for overnight at room temperature. After several washes in PBS, sections were incubated for 2 h at 37ºC in the dark with Alexa Fluor ‘‘568’’ and ‘‘488’’ secondary antibody (1:1000; Southern Biotech, Hungary). For control experiments, primary antisera were omitted and no specific cellular staining was found. Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were taken with the Spot software package. Photographs were further processed with the Adobe Photoshop 7.0 program. We compared

the localization of the different markers stained with immunofluorescence in the cochlear hair cells between wild type and homozygous PACAP-deficient mice. Immunofluorescence intensity was measured by ImageJ 1.440 software, expression levels were corrected with the tissue background. The statistical analysis was made by GraphPadPrism 5.01. Results are presented as mean ± SEM, statistical comparisons were made using Student-t test with Welch's correction and Mann–Whitney test.

Results

There was no difference in the gross microscopical structure of the inner ear between wild type and PACAP-deficient mice: the different cell types of the organ of Corti could be observed in both groups.

PAC1-R expression was found in inner hair cells, outer hair cells, outer phalangeal cells (Deiters' cells), and pillar cells. The distribution of PAC1-R immunopositivity was not different between the two groups, but we observed differences in the intensity of the immunolabeling of inner and outer hair cells of wild type and PACAP-deficient mice. In wild-type mice the hair cells and outer phalangeal cells showed more intense immunolabeling compared to PACAP-deficient mice.

Examining Ca^{2+}-binding proteins, the hair cells showed weak parvalbumin, calretinin and calbindin-immunopositivity in wild-type mice. In contrast, both inner and outer hair cells showed significantly stronger immunopositivity to parvalbumin in the PACAP-deficient mice. The immunostaining was more intense in the entire cell bodies compared to wild-type cochleas, but especially strong in their hair bundles (stereocilia) (Figure 2.).. Inner and outer hair cells of PACAP-deficient mice also showed more pronounced calretinin immunopositivity compared to wild-type animals. In PACAP-deficient mice calretinin was present, accumulated in the hair bundles of the hair cells and less intensely stained in their cell bodies. A weaker calbindin labeling was found in wild-type mice compared with parvalbumin or calbindin. In contrast, a more pronounced calbindin expression was observed in PACAP-deficient mice. All of the observed differences between the wild type and PACAP-deficient animals were significant according to the immunofluorescence intensity measurements.

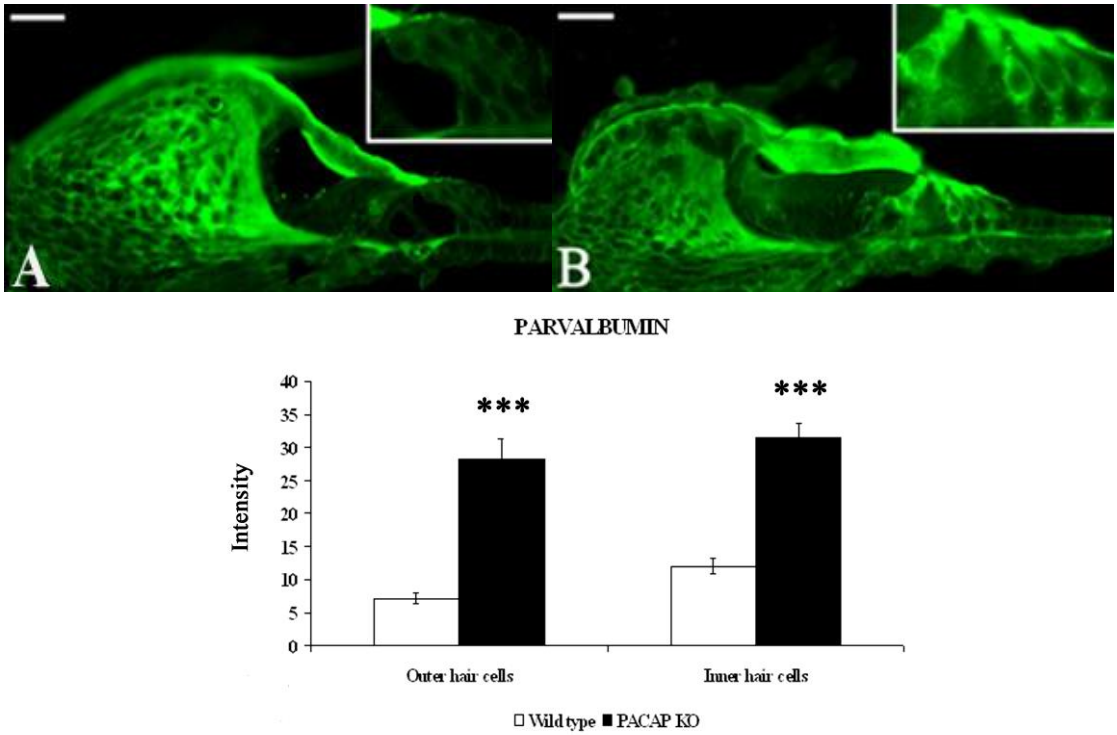

Figure 2. Representative parvalbumin immunohistochemical sections from the organ of Corti of wild type (A) and homozygous PACAP-deficient mice (B). Scale bar: 20 μm.
Bar diagram: Statistical analysis of parvalbumin immunofluorescence intensity between the outer and inner hair cells of wild type and homozygous PACAP-deficient mice (PACAP KO)
**** p <0,0001 vs. wild-type animals*

VI. EXAMINATION OF CALCIUM-BINDING PROTEIN EXPRESSION IN THE INNER EAR OF WILD TYPE, HETEROZYGOUS AND HOMOZYGOUS PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)-DEFICIENT MICE IN KANAMYCIN-INDUCED OTOTOXICITY

Materials and methods

Wild type ($PACAP^{+/+}$, n=6), heterozygous ($PACAP^{+/-}$, n=6) and homozygous PACAP-deficient mice ($PACAP^{-/-}$, n=6) were used in the experiment. Animals were fed and watered ad libitum, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-15024/2011). Five-day-old pups were treated with kanamycin solution (50 mg/ml, Sigma, Hungary) in 1 mg/g body weight concentration and control animals received 50 µl physiological saline subcutaneously. Two days later, the animals were killed with an overdose of anaesthetic. Heads were cut in a cryostat at 10-µm midsagittal sections. We made approximately 10–12 sections from the cochlea, and analyzed only those slides in which the modiolus was visibly surrounded by bony spiral canal. We compared only the middle turns of the cochlea as we described above.

Cryosections were stained with haematoxilin-eosin to identify the different cell types in the organ of Corti and the sections were also processed for parvalbumin and calretinin immunohistochemical examination as previously described. To compare the intensity of expression in the different groups we standardized the parameters by analyzing every slide from every group at the same time, in the same environment and the same settings of the program as we described earlier. Results are presented as mean ± SEM. The statistical analysis was made by GraphPadPrism 5.01 program and statistical comparisons were made using two-way ANOVA and Bonferroni post-tests.

Results

Our immmunohistochemical staining revealed significant differences in Ca^{2+}-binding protein expression in the organs of Corti from the different animal groups, in spite of no basic structural differences observed in the haematoxilin-eosin stained slides. We observed marked differences in the expression of parvalbumin and calretinin between the inner and outer hair cells from wild type, heterozygous and homozygous PACAP-deficient animals both in control conditions and after kanamycin treatment (Figure 3.).

In control homozygous PACAP-deficient mice treated with physiological saline only, stronger expression of parvalbumin was observed in the inner and outer hair cells than in their wild-type control mates. The outer hair cells of heterozygous PACAP-knockout animals also showed stronger parvalbumin expression compared to wild types. In contrast, no significant differences in parvalbumin expression was observed in the inner hair cells of wild type and heterozygous PACAP-deficient mice under control conditions. After kanamycin treatment parvalbumin expression was significantly increased in wild type and heterozygous PACAP-deficient animals, but the stronger baseline parvalbumin expression in hair cells of homozygous PACAP-deficient mice did not show further changes. We showed significantly stronger parvalbumin expression after kanamycin treatment in heterozygous PACAP-deficient mice compared to kanamycin-treated wild-type animals (Figure 3.).

Calretinin immunohistochemistry yielded similar results in wild type and homozygous PACAP-deficient animals in control and kanamycin-treated groups. We observed stronger calretinin expression in the hair cells of both homozygous and heterozygous PACAP-deficient mice compared to wild-type animals after physiological saline treatment. After kanamycin

treatment the calretinin expression was increased in the hair cells of wild-type mice but there was no further increase in Ca^{2+}-binding protein expression of the cochlea of either homozygous or heterozygous PACAP-deficient animals. The inner hair cells of heterozygous mice showed significantly higher calretinin expression after kanamycin treatment compared to wild-type animals.

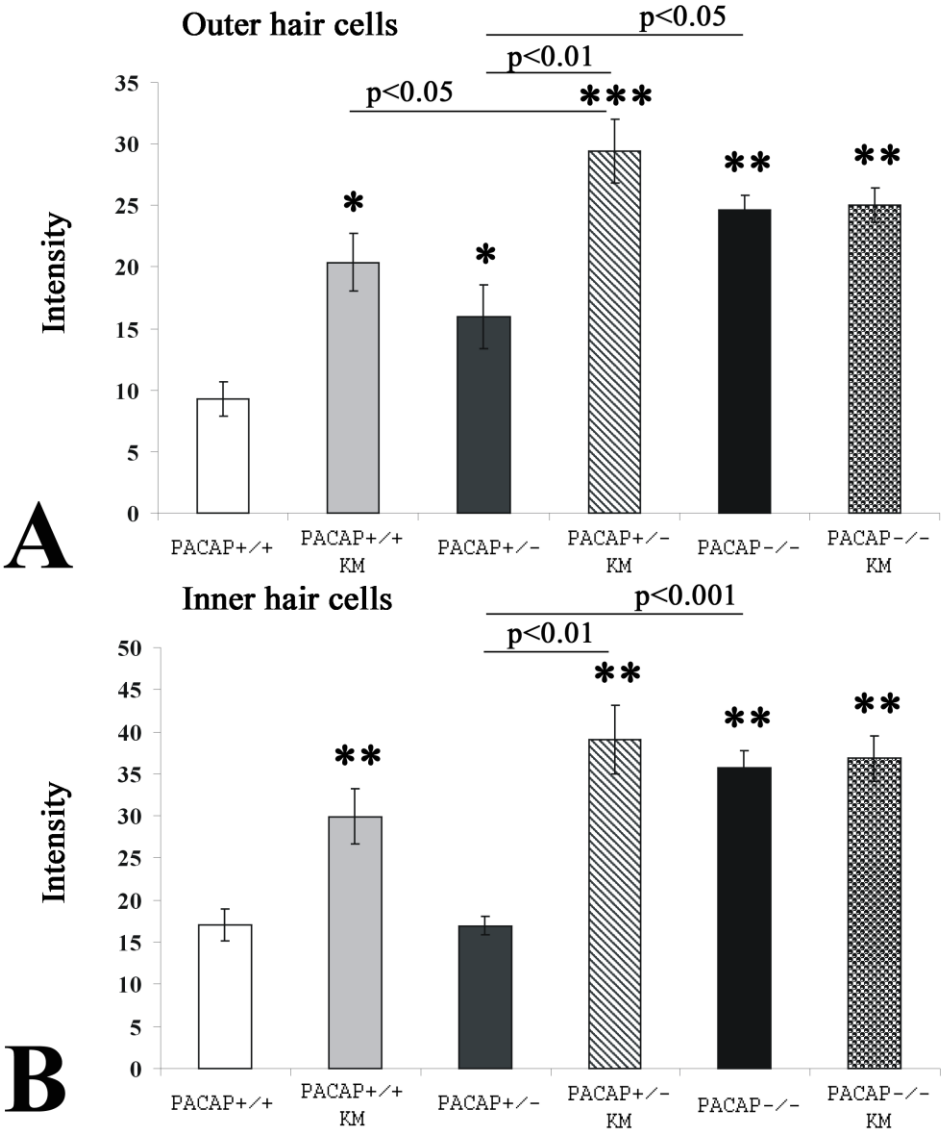

*Figure 3. Statistical analysis of parvalbumin immunofluorescence intensity between the outer (A) and inner (B) hair cells of wild type ($PACAP^{+/+}$), heterozygous ($PACAP^{+/-}$) and homozygous PACAP-deficient mice ($PACAP^{-/-}$) under normal conditions ($PACAP^{+/+}$; $PACAP^{+/-}$; $PACAP^{-/-}$) and after kanamycin treatment ($PACAP^{+/+}$ KM; $PACAP^{+/-}$ KM; $PACAP^{-/-}$ KM), respectively. **p<0.05; **p<0.01; ***p<0.001 vs. control wild-type animals ($PACAP^{+/+}$)*

VII. DISCUSSION

In the first study we showed that PACAP had antiapoptotic effects in a cochlear cell culture and thus, it increased cell survival against H_2O_2-induced oxidative stress. In this study we also showed that oxidative stress caused a marked increase in caspase-3 activation, while PACAP counteracted this effect of H_2O_2. These results in accordance with numerous other studies provided evidence for the strong antiapoptotic effects of PACAP in neuronal and non-neuronal cells against oxidative stress-induced apoptosis.

Examination of the endolymph proteome is important in understanding inner ear physiology, and hormonal influence on the function and certain pathological conditions of the labyrinth. Localization of PACAP and PAC1 receptor in the stria vascularis close to the endolymphatic compartment suggests that PACAP might also have an effect on the endolymph production, potential, and/or composition. In the second study there was no significant difference between control and PACAP-treated endolymph samples at any examined time point as determined by chip electrophoresis.

In the third study, we provided evidence for the altered expression of PAC1-R and Ca^{2+}-binding proteins in the inner ear of PACAP-deficient mice. Although the gross microscopical structure of the inner ear did not show alteration in PACAP-deficient mice, comparative immunohistological examination revealed significant differences in the expression of PAC1-R and Ca^{2+} binding proteins (parvalbumin, calretinin, and calbindin) of the inner and outer hair cells between wild type and PACAP-deficient animals. In our study we demonstrated that both hair cell types and Deiters' cells of PACAP-deficient mice expressed PAC1-R, but this expression was significantly lower in PACAP-deficient mice compared to their wild-type mates. The regulation of the PACAP receptor subtypes in the central and peripheral nervous system is not well understood, but other researchers have also found a developmental delay in the expression of all three receptor subtypes in the brains of PACAP- and VIP-deficient animals compared to their wild-type counterparts. Next we investigated the expression of Ca^{2+} binding proteins in wild type and PACAP-deficient mice. We found that inner and outer hair cells showed parvalbumin, calretinin, and calbindin-immunopositivity in the cochlea of the wild-type mice and PACAP-deficient mice on postnatal day 5. Although PACAP itself is able to increase intracellular Ca^{2+} through phospholipase C signalling, it has also been described that the glutamate-induced pathological increase of intracellular Ca^{2+} concentration can be inhibited by PACAP and thereby neuroprotection can be achieved in hippocampal neurons. Our third study presents data, for the first time, on the inner ear structure of PACAP-deficient mice and suggests that PACAP deficiency may mean higher vulnerability of cochlear cells against toxic agents. As an endogenous compensatory mechanism, a higher Ca^{2+} binding protein concentration can be observed in the cells of the inner ear.

In the fourth study, we provided evidence for the altered expression of Ca^{2+}-binding proteins (parvalbumin and calretinin) in the inner ear of wild-type, heterozygous and homozygous PACAP-deficient mice under normal conditions and after kanamycin treatment. Kanamycin is one of the most frequently used aminoglycoside antibiotics in mouse hearing loss models causing apoptotic hair cell damage mainly through generation of reactive oxygen species. Several promising therapeutic agents have been studied in ototoxic damage such as antioxidant drugs and antiapoptotic strategies to protect hair cells against aminoglycoside induced ototoxicity. PACAP has well-known antioxidant and antiapoptotic effects, but there are no data about its otoprotective effect in kanamycin-induced ototoxicity. After physiological saline treatment we found significant differences between wild type and PACAP-deficient mice in Ca^{2+}-binding protein expression, which was significantly lower in

wild-type animals. Control heterozygous PACAP-deficient animals showed significantly lower expression compared to homozygous PACAP-deficient mice. After kanamycin treatment, Ca^{2+}-binding protein expression significantly increased in wild-type and heterozygous PACAP-deficient animals, but the baseline stronger expression in homozygous PACAP-deficient mice did not change significantly.

The exact cause of elevated Ca^{2+}-binding protein expression in the hair cells of PACAP-deficient mice is unknown. Numerous studies provided evidence for the protective role of Ca^{2+}-binding proteins against different toxic agents. We suggest that the lack of endogenous PACAP is a pathological condition for the hair cells, leading to a compensatory baseline increase in Ca^{2+}-binding protein expression. However, this can reach a maximum level in PACAP-deficient mice, where the expression of Ca^{2+}-buffers can no longer be upregulated upon ototoxic insults. This study presents data, for the first time, on the otoprotective effect of endogenous PACAP in kanamycin induced inner ear toxicity. These results suggest that exogenous PACAP could have promising protective effect in different ototoxic models, but further investigations are necessary to describe the exact mechanism of PACAP in aminoglycoside-induced ototoxicity.

VIII. NEW RESULTS

1., Our results of the first experiment demonstrated that PACAP effectively protected cochlear cells against oxidative stress-induced apoptotic cell death.

2., In the second study there was no significant difference between control and PACAP-treated chicken endolymph samples at any examined time point as determined by chip electrophoresis.

3., We did not find differences in the distribution pattern of PAC1 receptors between wild type and PACAP-deficient mice, but wild-type animals showed significantly higher PAC1 receptor expression. In contrast, inner and outer hair cells of PACAP-deficient mice showed more pronounced parvalbumin, calbindin, and calretinin immunopositivity compared with wild-type mice.

4. We found stronger expression of Ca^{2+}-binding proteins in the hair cells of control heterozygous and homozygous PACAP-deficient mice compared with wild-type animals. Kanamycin induced a significant increase in Ca^{2+}-binding protein expression in wild-type and heterozygous PACAP-deficient mice, but the baseline higher expression in homozygous PACAP-deficient mice did not show further changes after the treatment.

IX. PUBLICATIONS RELATED TO THE THESIS

Németh A, Szabadfi K, Fülöp B, Reglődi D, Kiss P, Farkas J, Szalontai B, Gábriel R, Hashimoto H, Tamás A (2014) Examination of calcium-binding protein expression in the inner ear of wild-type, heterozygous and homozygous pituitary adenylate cyclase activating polypeptide (PACAP)-knockout mice in kanamycin-induced ototoxicity. Neurotox Res 25:57-67 (IF: 3.151)

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Impact factor of publications related to the thesis: 8,077
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Cited by others: 34
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