PH.D. THESIS

EXAMINATION OF THE LACK OF ENDOGENOUS PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP) IN KNOCKOUT MICE IN THE AUDITORY SYSTEM, VASCULAR SYSTEM AND DURING TOOTH DEVELOPMENT

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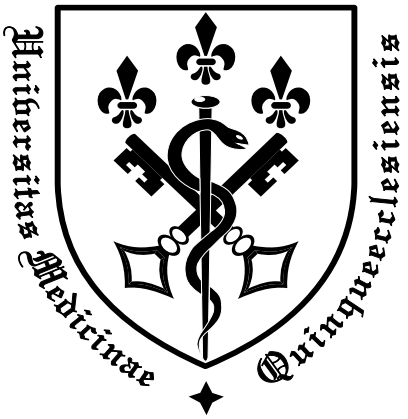

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I. INTRODUCTION

The research I was involved in investigates how the lack of the pituitary adenylate cyclase-activating polypeptide (PACAP) affects the functions of different organ systems in PACAP-knockout (KO) mice. In my thesis, the results that entail the effects on the hearing system, vascular system and teeth development are discussed.

DISTRIBUTION AND FUNCTIONS OF PACAP

PACAP is a member of the vasoactive intestinal polypeptide (VIP)/glucagon/secretin peptide family and is 68% homologous with VIP. Great quantities of PACAP are found in the nervous system and endocrine organs, and the first described functions were neurotrophic and neuroprotective. It has protective functions due to its antiapoptotic, anti-inflammatory and antioxidant effects. It has cytoprotective effects in neurodegenerative diseases, in cerebral ischemic models, in the intestine, in the kidney and in the cardiovascular system.

PACAP also has protective effects in sensory systems and influences the proliferation of sensory cells. The retinoprotective effects of PACAP were proved in retina impairments of various origins, and were documented on morphological, functional and molecular levels. These protective effects are based on the activation of antiapoptotic signalling pathways, on the restriction of apoptotic pathways and on anti-inflammatory functions. PACAP acts as a neurotransmitter and neuromodulator throughout development and it also regulates fundamental physiological processes. The peptide affects the production of different neurotransmitters and hormones, the motility of the intestines, the immune system, the circadian rhythm, the regulation of body temperature, the function of the genital organs and placenta, the development of migraine and psychiatric diseases and the function of the cardiovascular system. PACAP also regulates the development and function of the connective and supportive tissues, including bones, cartilage and teeth.

ISOFORMS AND RECEPTORS OF PACAP

PACAP naturally occurs in a 38-aminoacid (PACAP1-38) and a 27-aminoacid (PACAP1-27) forms. Its receptors are G-protein coupled receptors, from which VPAC1 and VPAC2 receptors (vasoactive intestinal peptid receptors 1 and 2) bond to PACAP and VIP with equal affinity, meanwhile PAC1 receptor (PACAP receptor type 1) is specific for PACAP. The receptors are not tissue specific, the distribution of different receptor types within the same tissue shows high variability. Due to alternative splicing, multiple variants of PAC1 receptors are formed, which could impact the intracellular signalling pathways or change the receptor affinity to different PACAP isoforms.

I. INTRODUCTION

INTRACELLULAR PATHWAYS TRANSMITTING PACAP FUNCTIONS

The canonical pathway of PACAP through PAC1 receptor activates the cyclic adenosine monophosphate - protein kinase A (cAMP-PKA) pathway. This leads to the phosphorylation of the extracellular signal regulated kinase (ERK), which aids the survival of the cell through several molecules. The PKA-ERK inhibits the c-Jun N-terminal kinase (JNK), which in turn blocks the phosphorylation of c-Jun and therewith the apoptotic pathways. Independently of cAMP, PACAP blocks the proapoptotic Bad and Bax molecules through Akt, therefore activates the anti-apoptotic B-cell lymphoma protein 2 (Bcl-2) and the B-cell lymphoma-extra large (Bcl-xL) protein which prevents the outflow of caspases and the mitochondrial apoptosis. Also independently of cAMP, PACAP also activates the phospholipase C (PLC), which also decreases the activation of caspases.

THE DISTRIBUTION AND FUNCTIONS OF PACAP IN THE AUDITORY SYSTEM

Both PACAP and PAC1 receptors have been shown in several parts of the inner ear and auditory pathway. In the inner ear, PACAP has been identified in the organ of Corti, within the spiral ganglion, and in the marginal cells of the stria vascularis. In the organ of Corti, PACAP immunopositivity and PAC1 receptor positivity has been found in the inner and outer hair cells, and PAC1 receptor was also found in supporting cells. PACAP and PAC1 receptors were identified in the stria vascularis on the basolateral surface of the marginal cells. The co-localization of PACAP and its receptor on the same cell implies its autocrine/intracrine regulation. Presumably PACAP has a crucial part in the integrity of tight junctions and K^+ transportation, which affects the composition of the endolymph and the sustainability of its proper ion concentration. From co-localization tests, it has been proved that PACAP mostly participates in the efferent innervation of the inner ear. Out of the efferent neurotransmitters choline-acetyltransferase (ChAT) and dopamine β-hydroxylase (DBH) co-localized with PACAP in the organ of Corti. Glutamate is the cochlea's primary afferent neurotransmitter, but excessive glutamate leads to hair cell damage in case if acoustic trauma or ischemia. Glutamate receptor 2/3 (GluR2/3) co-localizes with PAC1 receptor on the inner and outer hair cells and on supporting cells. PACAP reduces glutamate induced cell damage in other organs. Therefore, proposedly the co-localization of PAC1 and GluR2/3 receptors in the inner ear means that PACAP could play a protective role against excitotoxicity induced by glutamate.

In the auditory pathway, PACAP was shown in the cochlear nuclei and cochlear nerve. Thirty percent of the neurons are PACAP positive in the superior olivary complex (SOC), and in the trapezoid body 40% of the cochlear efferent neurons are also PACAP positive. Presumably these are the perikarya of the PACAP positive fibers found in the cochlea. In the olivocochlear tracts, PACAP was present in the medial olivocochlear tract which ends on the outer hair cells. That further supports the theory that the

regulatory function of PACAP in the auditory system is the efferent innervation of the cochlea. PACAP was also identified in both the inferior colliculus (IC) and medial geniculate body (CGM) in human brain.

THE EFFECT OF PACAP ON THE PROTEIN PROFILE OF THE ENDOLYMPH

The primary production site of the endolymph is the stria vascularis. The endolymph is a necessity for the appropriate function of the inner ear, and its deterioration is connected to different pathologies of the inner ear. Presumably aminoglycoside antibiotics reach the hair cells through the endolymph and therewith cause hearing impairment.

It is known that several neuropeptides such as substance P, vasopressin and somatostatin affect the composition of the endolymph. The ion concentration and electrical potential of the endolymph form a gradient from the basal to the apical turns, similarly to PACAP and PAC1 receptor in the stria vascularis. These findings propose that PACAP affects the composition of the endolymph. Our research team has examined how the proteins of the endolymph change in the cochlear duct after intraperitoneal PACAP treatment in 1-day-old chickens. The results showed multiple protein peaks between 14-80 kDa, which could correspond to the proteins found in the endolymph, such as albumin, α-chymotrypsin, α-antitrypsin, transferrin, apolipoprotein D, apolipoprotein J and fetuin. However, PACAP treatment had no effect on the composition of the proteins in this experimental setup.

EFFECTS OF PACAP ON INNER EAR CELL CULTURE

Apoptosis plays an important role in the physiological processes of the inner ear, with oxidative stress being an important factor. Different ototoxic agents lead to excessive apoptosis and therefore to hair cell and neuron damage, that eventually results in hearing impairment. Based on the above, molecules with antioxidant and antiapoptotic functions, like PACAP, could play an essential role in the protection of the inner ear against damage. Our research team examined these signalling pathways in inner ear cell culture from 1-day-old chickens. MTT test showed that cell survival halved after H_2O_2 treatment, but it was significantly improved by PACAP co-treatment. Further tests showed that PACAP significantly decreased the level of apoptosis and therewith the activation of the apoptotic caspase-3 protein. Both cAMP dependent and independent pathways could participate in these processes. This could provide the molecular background how PACAP protects the inner ear from damage caused by ototoxic agents, aging, noise overstimulation or other impairing factors.

I. INTRODUCTION

THE ROLE OF PACAP IN TEETH DEVELOPMENT

The development of teeth occurs due to the continuous interaction between the mouth's ectoderm and the underlying ectomesenchyme. The indent of the ectoderm into the ectomesenchyme forms the dental lamina. The focal thickening of the dental lamina forms tooth buds which continue to develop to cup and bell stages. In the late bell stage the inner enamel cells already differentiate into enamel producing ameloblasts. They enclose the ectomesenchyme which differentiates into the following layers: dentin producing odontoblast cells, the subodontoblast layer beneath them and the dental papilla which will later transform to the pulp chamber. More than 300 factors were identified that influence the development of teeth, the most frequently analysed ones are the bone morphogenetic protein (BMP), sonic hedgehog (SHH), wingless-related integration site (WNT), fibroblast growth factor (FGF) and Notch signalling pathways.

PACAP was found in the odontoblast cells, in the subodontoblast layer and around the vessels of the pulp. Following teeth's luxation the amount of PAC1 receptor and PACAP both increased on the site of the periodontal ligament. It is also known, that PACAP affects the activation of the Runt-related transcription factor (RUN2X) through PKA and therewith it influences the expression of the SHH gene. Furthermore, in osteoblast cells, it influences the BMP and SMAD protein families. These play an integral part of the development of bone and teeth.

The Notch signalling pathway is evolutionally highly conserved and contributes to the development of multiple organs, such as teeth. Neighbouring cells express ligands and receptors and the bond between these influences the proliferation and/or apoptosis of these cells. This induces asymmetric cell division and therefore neighbouring cells of same origin differentiate in different directions. That helps the production of daughter cells with alternate functions and the formation of distinct cell layers. The Notch signalling pathway shows partial overlap with PACAP and we assume that the changes of Notch pathway elements in PACAP knockout mice are to substitute the effects of PACAP.

The Notch signalling pathway is composed of four receptor molecules (Notch1, 2, 3, 4), and five ligands (DLL1, 3, 4 and Jagged1, 2). Upon ligand binding to the receptors the TNF-α converting enzyme (TACE) cleaves the Notch intracellular domain (NICD) from the receptor and it translocates into the nucleus and binds to the CSL transcription factor. The Numb protein blocks the Notch signalling pathway (Fig. 1).

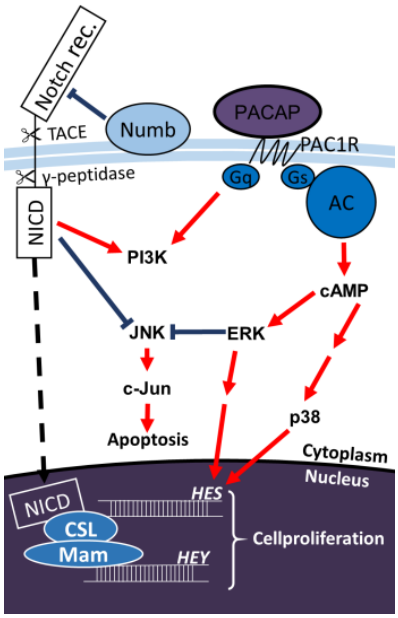

Fig. 1: The common targets of PACAP and Notch pathways. Red arrow: activation. Black blunt arrow: inhibition. ***AC:*** *adenylate cyclase;* ***cAMP:*** *cyclic adenosine monophosphate;* ***CSL:*** *CSL transcription factor;* ***ERK:*** *extracellular signal regulated kinase;* ***G_s and G_q:*** *G_s and G_q proteins;* ***JNK:*** *c-Jun N-terminal kinase;* ***Mam:*** *Mastermind;* ***NICD:*** *Notch intracellular domain;* ***Numb:*** *Numb protein;* ***p38MAPK:*** *p38 mitogen-activated protein-kinase;* ***PAC1R:*** *PACAP receptor 1 type;* ***PI3K:*** *phosphatidylinositol 3-kinase;* ***TACE:*** *TNF-α converting enzyme.*

THE FUNCTIONS OF PACAP IN THE CARDIOVASCULAR SYSTEM

PACAP has diverse effects in the cardiovascular system. Due to PACAP treatment vasodilation and drop of the systemic blood pressure occur. The vasodilation effects have been demonstrated in multiple organs in both animal models and human experiments. These functions are carried out by all PACAP receptors that are found primary in the walls of arteries and arterioles. Their presence has been shown in the aorta, coronary arteries and cerebral vessels. The distinct vasodilation effect is complicated by the fact that PACAP leads to the expression of catecholamines and therewith to the elevation of systemic blood pressure. Our research team demonstrated the angiogenic effects of PACAP on endothelial cells from microvascular vessels of the brain. PACAP decreased the apoptosis caused by oxidative stress in mouse endothelial cells through the activation of the ERK pathway, which inhibited the JNK and p38MAPK pathways. We showed the protective effect of PACAP against oxidative stress or doxorubicin induced apoptosis and against ischaemia-reperfusion induced damages in cardiomyocyte cell cultures. These protective effects partially occur through the Bad, Bcl-2, caspase-3 mitochondrial pathways. Multiple cardiovascular clinical trials were carried out that showed PAC1 receptors' mRNS in human myocardium samples, showed the changes of tissue PACAP levels in myocardial ischemia and of serum PACAP levels in dilated cardiomyopathy.

EXAMINATION OF PACAP-KNOCKOUT (KO) MICE

For the examination of the lack of endogenous PACAP, Hashimoto and colleagues have established a PACAP KO mice strain in 2001. These mice are not different from wild mice on a macroscopic level, but their functions and microscopic morphology do differ, as well as their behaviour. The gonadal function are decreased in KO mice, their mortality rates are increased and their metabolic system is damaged. They show signs of hyperactivity, depression, they have reduced sensitivity to fear, and react differently to pain and stress compared to the wild-type (WT) mice.

The microscopic structure of the cerebellum differs; abnormal neuronal arborization and aberrant myelinisation are present in the PACAP KO mice compared to the wild-type mice.

Mice lacking PACAP are more vulnerable to different in vitro and in vivo insults compared to the wild mice. This was demonstrated in ischemia and ischemia-reperfusion models among others in the brain, retina, kidney and intestines. In numerous toxicity models, higher sensitivity of PACAP KO mice was detected. This includes retinal damage, autoimmune encephalomyelitis model, damage of the spinal cord and peripheral nerves, pancreas glucotoxicity model, doxorubicin heart toxicity model. The arthritis model also showed disorder in the restructuring of bones in the PACAP KO mice.

THE EXAMINATION OF THE AUDITORY SYSTEM IN PACAP KO MICE

Our research team has examined the structure of the cochlea and the presence of PAC1 receptor in heterozygote and homozygote PACAP KO mice. Both groups yielded PAC1 receptor immunopositivity in the inner and outer hair cells, in the Deiters cells and in the pillar cells, however, the intensity of the signal was significantly decreased in the PACAP KO mice compared to the wild-type mice. We also examined the immunopositivity of different Ca^{2+}-binding proteins in the absence of endogenous PACAP under control conditions, and after kanamycin-induced ototoxic treatment in both PACAP KO and wild-type mice. The results are reviewed in the discussion.

THE DEVELOPMENT OF TEETH IN PACAP KO MICE

Previously, we examined 7-day-old mice, and we found that the dentin in the molar teeth of PACAP KO mice was thinner and the disordering of the intracrystalline structure was elevated. The protein composition of the enamel was less variable in PACAP KO mice compared to wild-type ones. Examining the signalling pathways, the immunopositivity of SHH, GLI1 (glioma-associated oncogene 1) and PTCH1 (protein patched homolog 1 protein) increased in PACAP KO mice compared to the wild ones. The incisors break through 2 days earlier in PACAP KO mice than in the wild ones. In the adult PACAP KO mice, the incisors were smaller, the pulp narrower and the increased intracrystalline disordering of the dentin was also characteristic.

THE CARDIOVASCULAR SYSTEM OF PACAP KO MICE

The cardiovascular system also shows changes in PACAP KO mice compared to wild-type mice. After doxorubicin treatment, echocardiography showed impaired heart functions. Dilatation of the left chamber, increased fibrosis and extensive myocardiocyte degeneration occurred. The vasodilation capacity of the meningeal vessels were also decreased in PACAP KO mice.

PACAP AND AGING

Extensive data suggests that the antiapoptotic and anti-inflammatory effects of PACAP have an important role in slowing down and regulating aging processes. We assume that the lack of these protective functions accelerates the aging processes in PACAP KO mice. Several organ systems show more prominent signs of aging in earlier stages of their life compared to the wild-type mice. In addition, higher levels of apoptosis, oxidative stress and inflammation also support this theory. The morphology of the retina, the decrease in ganglion cells, the changes of the protein profile of the Muller glial cells all show early degeneration/aging of the retina. Our research group showed that PACAP KO mice have more severe and earlier systematic amyloidosis in almost all of their organ systems compared to the wild-type mice.

II. AIMS

During our studies our aim was to examine the alterations caused by the lack of PACAP in different organ systems of PACAP KO mice.

I. In the auditory system, we measured the hearing functions of PACAP KO animals and showed alterations of the inner ear and the auditory pathway. We compared wild-type and PACAP KO mice:
    1. We examined the hearing functions with auditory brainstem respons (ABR) tests in collaboration with the Semmelweis University. These results are not part of this thesis but they are strongly related to our morphological results.
    2. We showed the noise induced activation of neurons in the auditory pathway with c-Fos immunostaining.
    3. We performed Nissl staining to differentiate between decreased number of cells and decreased activation of cells in the cochlear nuclei.
    4. We completed PAC1 receptor immunostaining in the cochlear nuclei.
    5. We analysed the protein profile of cochlear ducts of the inner ear to elucidate the role of the inner ear in hearing impairment.

II. We examined the changes of the Notch signalling pathway in the molar teeth of 5-day-old PACAP KO and wild-type mice using immunofluorescent staining.

III. We aimed to study the function and morphology of the vascular system in PACAP KO and wild-type mice. We isolated common carotid arteries and femoral arteries from both genotype groups and induced vasorelaxation with PACAP1-38, PACAP1-27 and VIP. We also described the distribution of the different PACAP receptors in these vessels.

III. METHODS

ANIMALS

All the experiments were performed on wild-type (CD1), heterozygous and homozygous PACAP KO mice. Ethical license numbers: BA02/2000-24/2011, XIV-I-001/1028-4/2012; PE/EA/1912-7/2017.

AUDITORY PATHWAY ACTIVATION – C-FOS IMMUNOLABELLING

We performed c-Fos immunolabelling in 1.5-month-old male mice (n=9-8) (Fig. 2). Noise exposure lasted 30 min and comprised of 4 to 20 kHz white noise with 100 dB sound pressure. After noise exposure, we allowed 1 hour for c-Fos transcription, translation and for its subsequent translocation to the nucleus. Following fixation 30 µm coronal sections were prepared. We performed free-floating immunohistochemistry with anti-c-Fos antiserum followed by biotinylated goat anti-rabbit secondary antibody. For visualization diaminobenzidine (DAB) was used.

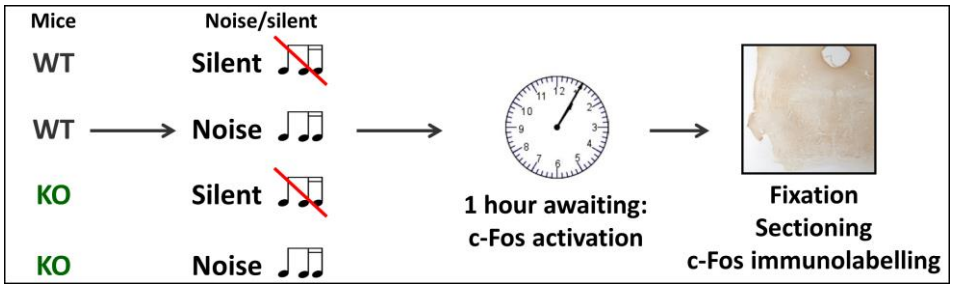

Fig. 2: Experimental setup for the measurement of the activation of the auditory pathway neurons with c-Fos immunolabelling

CELL COUNT MEASUREMENT IN THE COCHLEAR NUCLEI - NISSL STAINING

The samples of 4-month-old untreated male WT and KO animals (n = 3-3) were fixed and sectioned as described for c-Fos immunolabelling. We performed Nissl staining to count the number of neurons in the cochlear nuclei.

PAC1 RECEPTOR IMMUNOLABELLING IN THE NUCLEI OF THE AUDITORY PATHWAY

We performed PAC1 receptor immunostaining in the nuclei of the auditory pathway in 2-month-old male wild-type and PACAP KO mice (n=4-4). The samples were fixed and sectioned as described for c-Fos immunolabelling. We performed free-floating immunohistochemistry with anti-PAC1 receptor antiserum followed by biotinylated anti-rabbit secondary antibody. For visualization Cy3 conjugated streptavidin was used.

COCHLEAR DUCT PROTEOME PROFILE ANALYSIS

Proteome profile analysis was performed in the inner ear of 3-6-month-old male wild-type and PACAP KO mice (n=20-20). The bony cochlea was dissected and placed in a perilymph-like solution. Dissection of the cochlea occurred under operating microscope (Fig. 3). The cochlear ducts were homogenized, sonicated and centrifuged. The samples were then analysed with R&D Systems Proteome Profiler Mouse Cytokine Array Panel A and Mouse Angiogenesis Array Kit (R&D Systems, Biomedica, Hungary) and handled according to the manufacturer's description.

EXAMINATION OF NOTCH SIGNALLING PATHWAY ELEMENTS IN MOLAR TEETH

Immunostaining of Notch pathway elements was performed on molar teeth of 5-day-old WT, heterozygous (HZ) and homozygous PACAP KO mice (n=3-3-3). After Saint-Marie fixation 5 µm sagittal sections were made. Sections were incubated in anti-Notch1, 2, 3, 4, DLL1, DLL3, DLL4, Jagged 1,2, CSL, TACE and Numb antibodies then visualized by anti-rabbit Alexa555. DAPI was used to visualise the nuclei of the cells.

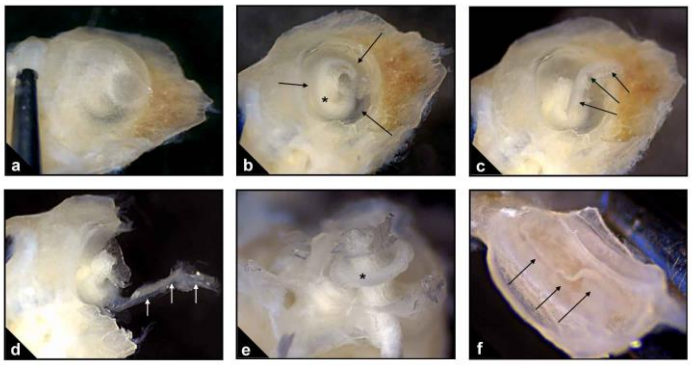

*Fig. 3. Dissection of the cochlea. (a) External aspect of the intact bony cochlea. (b) Bony wall of the cochlea partly removed. Cochlear duct: →; osseous spiral lamina: *. (c) Removal of the apical part of the cochlear duct (→). (d) Cochlea broken into two parts for gaining access to the basal part of the cochlear duct (→). (e) The main part with the modiolus after removal of the cochlear duct. Osseous spiral lamina: *. (f) The other part of the cochlea with the cochlear duct (→). Subsequently this part of the cochlear duct will also be dissected (not shown).*

VASORELAXANT EFFECTS OF PACAP1-38, PACAP1-27 AND VIP

We used 8-12-week-old WT and PACAP KO male mice (n=6-6). Under anaesthesia the common carotid artery and the proximal segments of the femoral arteries were isolated from the mice. The isometric force of 2 mm sections of the isolated vessels were examined with DMT 610 M Wire Myograph. Maximal contraction was induced by 60 mM KCl, then we measured the relaxation of the vessels to cumulative doses of PACAP1-38, PACAP1-27 and VIP in 10^{-9}-10^{-6} M concentration.

EXPRESSION OF PAC1, VPAC1 AND VPAC2 RECEPTOR MRNA

We examined the expression of PACAP receptor mRNA from common carotid and femoral arteries with RT-PCR. After reverse transcription, amplification was performed with 0.4 μM forward and reverse primers, 200 μM dNTP and 5 units of Promega GoTaq® DNA polymerase. After denaturation 35 cycles followed with final extension. PCR products were analysed by electrophoresis in agarose gel containing ethidium bromide. Actin was used as the internal control.

ANALYSIS OF PAC1, VPAC1 AND VPAC2 RECEPTORS WITH WESTERN-BLOT

Common carotid and femoral arteries were isolated (n=3-3). Mechanical grinding and sonication followed in radio immunoprecipitation assay buffer. Laemmli electrophoresis sample buffer was added to tissue lysates to set an equal protein concentration of samples, and boiled for 10 min. A total of 20 μg of protein was separated by 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Immunolabelling was performed by rabbit antibodies against PAC1, VPAC1 and VPAC2 receptors, followed by anti-rabbit seconder antibodies. Signals were detected by enhanced chemiluminescence, actin was used as internal control.

STATISTICS

Based on the type and distribution of the samples, two-way ANOVA (with Bonferroni, Fisher or Tukey post-hoc tests), one-way ANOVA and Student's t-test were used. Differences were considered significant at $p < 0.05$.

AUDITORY PATHWAY ACTIVATION – C-FOS IMMUNOLABELLING

Cochlear nuclei

There was almost no cell activation in mice, which were held in a silent environment ('silent') in either genotype groups. There was a significant elevation of activated neurons in both WT and PACAP KO animals after exposure to half an hour filtered white noise ('noise'). The elevation was significantly smaller in KO animals compared to WT ones (Fig. 4).

Central uclei of the auditory pathway

There was neuronal activation in the silent group regarding the superior olivary complex (SOC), the nuclei of lemniscus lateralis (NLL) and in the colliculus inferior (CI) in both WT and PACAP KO animals. We found significant elevation of c-Fos immunolabelling in both WT and PACAP KO animals after noise application but there was no significant difference between the WT and PACAP KO groups. In the primary auditory cortex, the high neuronal activation in the silent animal groups showed a tendency to be elevated after noise application but this elevation was not significant in either genotype groups.

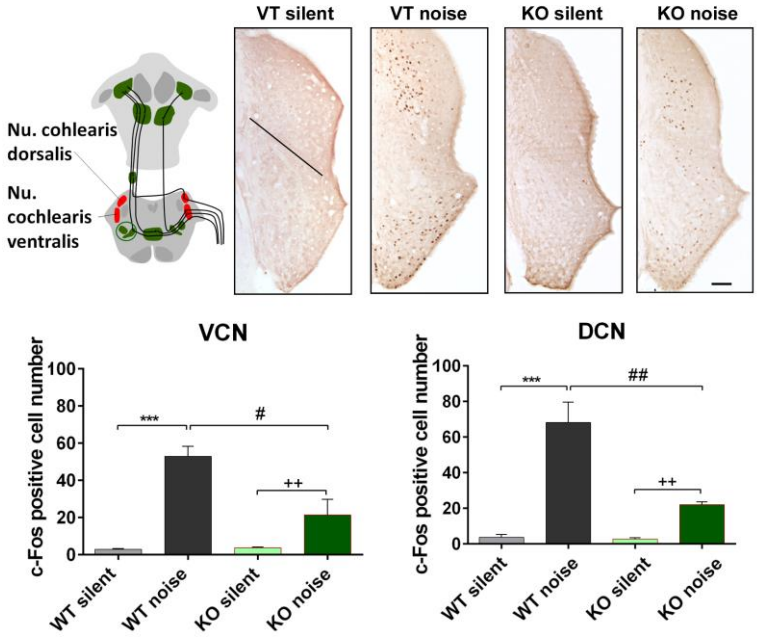

*Fig. 4: Expression of c-Fos in the dorsal and ventral cochlear nuclei (DCN, VCN) of wild-type (WT) and PACAP-deficient (KO) animals after noise exposure (noise) or without noise exposure (silent). Cochlear nuclei are labelled red on the schematic figure. Representative images of the VCN and DCN. Black line represents the border between VCN (below) and DCN (above). Scale: 100 µm. Mean±SEM; 2-way-ANOVA, Bonferroni post-hoc test, ***p < 0.001 vs. WT silent; ++p < 0.01 vs. KO silent; ##p < 0.01, #p < 0.05 vs. WT noise.*

CELL COUNT MEASUREMENT IN THE COCHLEAR NUCLEI - NISSL STAINING

We applied Nissl staining to the slides of VCN and DCN of WT and PACAP KO animals to decide whether the decreased number or the decreased activation of the cells had caused the reduced c-Fos immunopositivity in PACAP KO animals. Despite the different cell activation with c-Fos

immunostaining there was no difference in cell number in the VCN and DCN between the WT and PACAP KO animals.

PAC1 RECEPTOR IMMUNOLABELLING IN THE NUCLEI OF THE AUDITORY PATHWAY

We performed PAC1 receptor immunolabelling in the cochlear nuclei. The stratum granulosum, which is found between the VCN and DCN, showed decreased number of PAC1 immunopositive cells compared to the wild-type mice. Neither other parts of the cochlear nuclei nor other nuclei of the auditory pathway (SOC, NLL, CI) showed any differences between the wild-type and PACAP KO mice. The density of the PAC1 receptor within the cells did not show significant difference between the genotype groups.

COCHLEAR DUCT PROTEOME PROFILE ANALYSIS

To elucidate the molecular mechanisms resulting in the functional and morphological changes, we used Proteome Profilers from the R&D Systems to elucidate the protein composition of the cochlear ducts of WT and PACAP KO mice. From the lysates of cochlear ducts endostatin, acidic FGF, osteopontin, BLC, CD54, PF4, TF, DPPIV, IGFBP-2, Serpin F1 and CXCL12 were in detectable quantity with Mouse Cytokine Array Panel A and the Mouse Angiogenesis Array Kits. There were no significant differences between the WT and PACAP KO groups.

CHANGES OF NOTCH SIGNALLING PATHWAY ELEMENTS IN MOLAR TEETH

Notch receptors

Notch receptor examinations (Notch1, 2, 3, 4) showed a significantly elevated level of Notch2 receptor in the cytoplasm of ameloblast cells in PACAP HZ and KO animals compared to WT mice (Fig. 5). There was no significant difference in the odontoblast cells. The other receptors also showed a similar tendency but the difference was not significant under the examined circumstances.

Notch ligands

The ligands (DLL1, 3, and 4, Jagged1 and 2) of Notch signalling pathway also displayed alterations in PACAP deficient mice. DLL1 positivity was high in the WT mice which increased significantly further in the ameloblast cells of PACAP HZ animals, and PACAP KO animals displayed an additional elevation. Jagged1 showed significant elevation in the odontoblast cells of PACAP KO animals. Other ligands were unchanged in PACAP-deficient mice compared to the wild-type mice.

Notch intracellular elements

CSL, the target of the canonical Notch pathway, showed significantly elevated expression levels in the ameloblast and odontoblast cells in the PACAP HZ and KO mice compared to the WT mice. The lack

of PACAP had no effect in our experimental setup in the examined TACE or NUMB protein expression in the odontoblast or ameloblast cells.

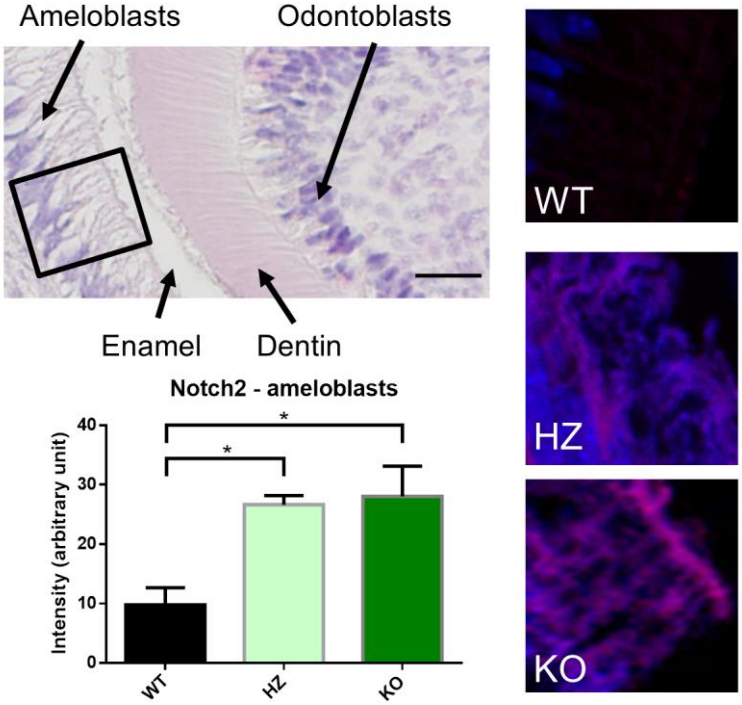

*Fig. 5: Notch2 receptor immunolabelling in ameloblast cells with Alexa Fluor555 (red) in molars of 5-day-old wild-type (WT), PACAP HZ and PACAP KO mice. Nuclei labelled with DAPI (blue). We labelled the examined area with black rectangle on the HE stained figure. Scale: 20 µm. Notch2 receptor immunopositivity elevated significantly in the ameloblasts of PACAP HZ and KO mice compared to wild-type mice. Mean ± SEM. One-way ANOVA, Fisher post-hoc analysis, *p<0,05 vs. WT.*

VASORELAXANT EFFECTS OF PACAP1-38, PACAP1-27 AND VIP

The maximal contraction induced by KCl was affected differently by PACAP1-38, PACAP1-27 and VIP in the vessels of wild-type and KO animals. PACAP1-38 induced greater relaxation in the wild-type mice than in the KO animals in both the common carotid and femoral arteries. However, PACAP1-27 and VIP provoked larger relaxation in the KO than in the wild-type animals in both vessels. In wild-type mice, PACAP1-38 induced larger relaxation than PACAP1-27 or VIP in the common carotid artery. In PACAP KO mice, PACAP1-27 and VIP provoked greater relaxation than PACAP1-38 in both arteries.

EXPRESSION OF PAC1, VPAC1 AND VPAC2 RECEPTORS IN THE VASCULAR SYSTEM

We measured the mRNA and protein expression of PAC1, VPAC1 and VPAC2 receptors from common carotid and femoral arteries of wild-type and PACAP KO animals. RT-PCR and Western-blot analysis showed lower PAC1 receptor mRNA and protein level in the KO animals compared to the wild-type ones. VPAC1 receptor mRNA and protein was present in both arteries in both genotypes, without significant differences between the genotype groups. However, neither the mRNA nor the protein of VPAC2 were detectable in the vessels of KO or wild-type mice.

V. DISCUSSION

In our research, we examined the consequences of PACAP deficiency in the auditory system, the vascular system and during tooth development in PACAP KO mice.

EXAMINATION OF THE AUDITORY SYSTEM IN PACAP KO MICE

Examining the auditory system of PACAP KO mice, we found decreased neuron activity and altered PAC1 receptor immunoreactivity in the auditory pathway and unchanged protein profile in the cochlear duct compared to wild-type animals.

Around 500 million people are affected by hearing loss world-wide. Therefore, it is important to explore the molecules which could have protective functions in the auditory system. As PACAP is known for its neuroprotective and cytoprotective effects, our aim was to examine its effects in the auditory pathway and in the inner ear. The presence of PACAP and the mRNA of the PAC1 receptor was shown earlier in the inner ear and in the auditory pathway nuclei; supposedly it has a major role in the efferent innervation of the cochlea.

In collaboration, we first examined the hearing functions of the mice with ABR tests. In this collaboration, the tests were carried out by Viktoria Humli in auditory laboratory of dr. Tibor Zelles, thereby the detailed analysis of the results are not part of this dissertation. However, the results are crucial to interpret the morphological experiments, therefore find hereby a short description. Auditory thresholds of PACAP KO mice were significantly higher using low frequency stimulus in 1.5, 4 and 8 months old mice compared to their wild-type counterparts. For the CD1 mouse strain, age-related hearing loss is known; we have detected hearing impairment with aging in both PACAP KO and wild-type mice. This could explain that the difference at 8.2 kHz disappeared in the 8-month-old mice. Fine analysis at higher frequencies showed differences in the amplitude and latency results between the two genotype groups. Decreased amplitude and latency values are probably the subtle indications of hearing impairment not revealed by hearing threshold measurements.

Parallel to the functional examinations we conducted morphological studies; these results serve as part of my thesis. We evaluated the activation of neurons in the auditory pathway with c-Fos immunohistochemistry following sound stimulus. We found only a small number of c-Fos positive cells in the VCN and DCN in both genotype groups kept in silent environment. The number of activated cells was elevated after noise exposure in both groups; however, the increase in PACAP KO mice was significantly smaller. These result correlate with the results of the functional ABR examinations: the hearing loss of PACAP KO animals is shown by the increase of the hearing threshold as well as by the decreased number of activated neurons in the auditory pathway. Nissl staining showed that there is no

decrease in the number of nerve cells in the area of VCN-DCN of the KO mice. Therefore, the lower c-Fos positivity was rather due to the reduced activation of the nerve cells than to the reduced number of cells. In the cochlear nuclei, we also examined the immunoreactivity of the PAC1 receptor. In accordance with previous studies, we detected the presence of PAC1 receptor in the cochlear nuclei of wild-type mice in our experiment, whereas we found fewer PAC1 receptor positive cells in the stratum granulosum of the cochlear nuclei of the KO mice. That correlates to our previous findings in the inner ear and to the present findings regarding the vessels namely the decrease of PAC1 receptor in PACAP KO mice compared to wild-type mice.

In the central nuclei of the auditory pathway, we have found that there is a baseline neuron activation in the silent group in all examined areas (SOC, NLL, IC, AU1). In both genotypes, the number of activated cells increased significantly after noise exposure in the areas of SOC, NLL, and IC; however, there was no difference between the two genotypes. These nuclei are not only relay stations of the auditory pathway but they have complex functions in information processing. They receive innervation from various other parts of the brain, thereby many different neurotransmitters of different nerve cells are excreted here. We hypothesize that in a more elaborate system with complex afferentation there is a higher chance of compensating the lack of PACAP. That justifies the elevated c-Fos levels in the silent groups in these nuclei and also explains why the difference between the wild-type and the PACAP KO mice disappeared. It still remains a question exactly which signalling pathway replaces the role of PACAP; however, it seems that not one sole system is responsible for the substitution of the lack of PACAP.

To discover the exact cause of hearing loss, we continued our experiments in the inner ear, where we performed protein profile analysis from the lysates of cochlear ducts of wild-type and PACAP KO mice inner ears. The literature implies that PACAP plays a role in the production of the endolymph and the efferent innervation of the cochlea. It has been shown, that PACAP increases dopamine secretion. That could have protective effects against the excitotoxicity on type I afferent cells of the spiral ganglion. In the hippocampus, PACAP protects against glutamate induced deleterious Ca^{2+} concentration increase; thereby we assume that in the inner ear PACAP could also have a protective role against glutamate excitotoxicity.

The Ca^{2+} level of the endolymph and in the hair cells elevates in case of inner ear insults. This is damaging for the hair cells and is necessary for the execution of apoptosis. Ca^{2+}-binding proteins play an important role in binding large quantities of Ca^{2+}-ions, therewith protecting the hair cells from the damage caused by high Ca^{2+} concentration. However, their Ca^{2+} binding capability is limited. Our research team has previously examined the immunopositivity of Ca^{2+}-binding proteins in the inner ear.

We have found that under normal circumstances the quantity of the proteins is low in wild-type mice, however ototoxic kanamycin treatment increases the Ca^{2+}-binding protein immunopositivity. In PACAP KO mice, Ca^{2+} binding proteins showed already high immunopositivity without treatment, this however did not increase further with kanamycin treatment. We hypothesize, that the protective effects of PACAP are missing in PACAP KO mice Therefore, impacts which cause no cell damage in the inner ear of wild-type mice will lead to different processes in the KO mice. It will result in the activation of the apoptotic pathways, therewith elevation of the intercellular Ca^{2+} concentration and hence elevation in the level of Ca^{2+}-binding proteins. The protective effects of Ca^{2+}-binding proteins seem to be however limited, which is indicated by the fact that the quantity of these proteins do not elevate further after kanamycin treatment in the PACAP KO mice.

Lesions involving inflammation and the lesions of the vascular system play an important role in the different types of sensorineural hearing loss. Therefore, in our research we examined cytokines and angiogenic proteins in the cochlear ducts of both genotype groups. Cytokines are markers for lesions caused by inflammation and angiogenic proteins are involved in the deterioration of the vasculature. The protein profile analysis identified several molecules in both wild-type and PACAP KO mice, which take part in angiogenic processes (FGF, CCXCL12), antiangiogenic processes (endostatin, Serpin F1), have chemotactic effect (BLC, PF4, CXCL12) or take part in coagulation (PF4, TF). Furthermore, we showed the ubiquiter cell surface protein DPPIV, the antiapoptotic osteopontin and the intercellular adhesion molecule CD54. However, there was no significant difference between the PACAP KO and the wild-type mice in regards of these proteins. According to our results we can state that the hearing loss in PACAP KO mice is probably not caused by inflammation or angiogenic lesions of the inner ear, because the quantity of these proteins in the examined conditions did not differ significantly compared to the wild-type mice.

Our experiments so far do not determine whether the loss of hearing functions is caused by sole inner ear lesions, sole auditory pathway damage or both parts of the auditory system are involved. It is known, that isolated lesions of the inner ear can cause complex changes in the auditory pathway. Therefore, it is possible, that the changes we have found were caused by sole damage of the inner ear. This is also confirmed by our Nissl staining, where only the activation and not the number of neurons changed in the cochlear nuclei of PACAP KO animals. The proposed development of hearing loss in PACAP KO mice is as follows. It is known that CD1 mice endure age related hearing loss. This process involves the loss of inner and outer hair cells in the inner ear. It is also known that aging processes are accelerated in PACAP KO mice affecting several organ systems. An acceptable hypothesis could be that the changes we found in the auditory system of the PACAP KO mice are the result of the accelerated

aging of the inner ear and auditory pathway. The decay of hair cells is accelerated and the hearing loss develops faster than in wild-type animals. This theory is confirmed by the results of the functional experiments. The actual loss of hair cells was already shown in CD1 wild-type animals; we are currently running experiments to examine the hair cell loss in PACAP KO animals. We have similar processes, however, to a different extent in the PACAP KO and wild-type animals. This could be the reason why we did not detect significant differences in the proteome profile of WT and PACAP KO mice. As PACAP already showed protective effects in other sensory systems, the long term goal of our experiments are to determine whether exogenous PACAP or PACAP agonists could have protecting functions in the auditory system in the case of aging or other ototoxic noise/drug induced hearing loss.

TOOTH DEVELOPMENT OF PACAP KO MICE

In addition to examining the auditory system, our team investigated the role of PACAP in tooth development. We showed elevated immunopositivity of the Notch2 receptor, of DLL1 and Jagged1 ligands and of the intracellular signalling molecule CSL in the ameloblast and odontoblast cells of the developing molar tooth of PACAP KO mice compared to wild-type ones.

Our previous experiments showed several morphological differences between the teeth of PACAP KO and wild-type mice. The dentin layer of 5-day-old mice is thinner than in the wild-type mice. The hydroxyapatite crystals have a higher disordering in dentin, the incisors appear 2 days earlier and are smaller in PACAP KO mice. SHH, its PTCH1 receptor and GLI1 intracellular target show increased immunopositivity in the secretory ameloblasts of PACAP KO mice compared to wild-type ones.

Based on our previous experiments our goal was to elucidate the changes of Notch signalling pathway molecules in PACAP KO mice. Cells expressing Notch receptors stay in the phase of cell division, whereas the blockage (e.g. by the Numb protein) or downregulation of Notch pathway shift the cells toward differentiation. Notch is crucial for the proper communication between the mouth ectoderm and ectomesenchyme, which is essential for normal tooth development. In our experimental setup, we examined the changes of Notch pathway molecules in ameloblast and odontoblast cells from sections of molar teeth of 5-day-old PACAP HZ and PACAP KO mice.

During the examination of Notch receptors in wild-type mice, we found that the immunopositivity in the odontoblast and ameloblast cells is low. However, Notch2 receptor significantly increases in the ameloblast cells of PACAP HZ and PACAP KO mice. The level of other Notch receptors is also tendentiously but not significantly higher in the PACAP KO mice. Physiological downregulation of the Notch receptors pushes the ameloblast and odontoblast cells in the direction of differentiation, and it helps converting the cells to dentin and enamel producing cells.

V. DISCUSSION

The elevated expression of Notch ligands (DLL1, Jagged1, 2) in ameloblast and odontoblast cells is necessary for the lateral inhibition on the adjacent cells of stratum intermedium and subodontoblast layers keeping these cells in a proliferative phase. For the first time, we described a high level of Jagged1 expression in the odontoblast cells of WT animals, which was further elevated in PACAP KO animals. The low baseline expression of DLL1 in the ameloblast cells in WT animals was also elevated in PACAP KO animals. During our experiment we examined the intracellular elements of the Notch signalling pathway. Upon ligand binding, TACE cleaves the receptor, which results in the dissociation of the NICD. NICD subsequently translocates to the nucleus and binds to CSL, the downstream target of the canonical pathway of Notch. That results in the transcription of the members of the Notch target *Hes* and *Hey* gene families. These genes are involved in cell proliferation; Numb directs the cells toward cell differentiation through the inhibition of these genes. In our experiment, we did not find differences in the TACE and Numb protein levels between PACAP KO and wild-type mice, however the CSL was increased in both ameloblast and odontoblast cells in both PACAP HZ and KO mice compared to their wild-type counterparts. We hypothesize, that the amount of CSL increased upon the elevation of Notch receptors.

The PACAP KO mice are viable without PACAP, thereby we assume that compensatory signalling pathways exist, which in case of the lack of PACAP at least partially take over its functions. Despite several promising theories, it is for now known that neither the monoaminergic pathways nor alone VIP serve as a salvage pathway in the lack of PACAP. We showed in our experiment, that Notch signalling pathways are upregulated in PACAP KO mice, which can be an alternative to counterbalance the lack of PACAP. However, the direct connection between PACAP and Notch signalling pathways is unknown. Both signalling pathways have antiapoptotic and cell proliferative effects and identical targets (Fig. 1). Both PACAP and Notch activates the PI3K/Akt pathway and inhibit the JNK and therefore c-Jun. The *Hes* gene family is a common target of both pathways. With these common targets arises the possibility that Notch signalling pathway can take over the functions of PACAP in PACAP KO mice. However, the precise upregulation of the Notch signalling pathway is unknown in the PACAP KO mice. According to our hypothesis, complex regulation processes provide the molecular background, explaining the detected changes. The increase of the SHH pathway described above can indirectly cause the increase of Jagged1. In PACAP knockout mice, the PACAP independent dimerization of PAC1 receptor can be observed, which leads to the activation of WNT/β-catenin pathway. That increases directly the gene transcription of *Jagged1* and could elevate the Notch2 receptor level through the FGF10-lunatic fringe (LF) pathway.

To summarize, we found that the immunoreactivity of different elements of the Notch signalling pathway increased in PACAP KO animals and we suggested some hypotheses for the possible underlying molecular mechanisms. We presume that the elevation of Notch pathway elements in ameloblasts and

odontoblasts of PACAP KO mice leads to disturbed proliferation and differentiation of these cells. However, based on our results, it is not yet possible to address exactly how Notch pathway contributes to the morphological changes found in the teeth of PACAP KO animals. The elevation of Notch pathway is either caused by the lack of the regulatory, probably suppressing, effects of PACAP on Notch elements or it could serve as a salvage pathway in the tooth development of PACAP KO animals. These processes need further examination to elucidate the precise connection between PACAP and Notch in tooth development and in other organs.

VASCULAR SYSTEM OF PACAP KO MICE

Examinations of the vascular system of PACAP KO animals revealed increased vasorelaxation to PACAP1-27 and VIP, and less responsivity to PACAP1-38. Regarding the receptors downregulation of PAC1 was found.

Many cardiovascular effects of PACAP are known. It causes vasodilation and, accordingly, systemic blood pressure drop, and also influences angiogenic processes. It improves capillary forming ability and reduces apoptosis in endothel cell cultures and protects against oxidative stress in cardiomyocyte cell cultures. Our research team also studied PACAP in human blood samples as a potential biomarker for cardiac failure.

The vasorelaxation effects of PACAP1-38, PACAP1-27 and VIP have been shown in several vessel types; in arteria carotis communis and arteria mesenterica superior of rats, in coronary arteries of pigs and in the middle cerebral artery among others. The effects of PACAP are highly species and region specific: in the superior mesenteric artery of rats PACAP1-38, while in coronaries of pigs PACAP1-27 and VIP induced larger relaxations. The relaxation effects of PACAP may vary even in the same organ; it has different effects on the macroscopic and microscopic vessels of the brain. A number of studies examine the role of PACAP in migraine that may be connected, among others, with its vasodilation effect. The presence of different receptor splice variants in different regions may be the cause of the various effects, which might be explained by the fact that different organs shall give different vasomotor responses to the same stimuli. Knowing this, the effects that we observed in wild-type animals can be regarded also as new results, because the effects of PACAP have not been described yet in common carotid and femoral arteries of mice.

Our research team detected vasorelaxation in the presence of each molecules. In wild-type animals, PACAP1-38 induced significantly higher relaxation in the common carotid arteries than PACAP1-27 or VIP. It matches with the results in rats published by Huang and co-workers. As for the KO mice, we found that PACAP1-27 and VIP induced higher vasorelaxation than PACAP1-38. Accordingly, it can be stated that the responsivity to the members of the secretin/glucagon/VIP peptide family is changed in

PACAP KO mice, and rather VIP than PACAP induces higher vasorelaxation. We assume that this change increases the sensitivity of the vessel in PACAP KO animals to VIP, probably in order to compensate for the lost functions of PACAP.

PAC1, VPAC1 and VPAC2 receptors are expressed in the vessels to a great extent, as shown in rat and human samples; however, their distribution is highly dependent on the very organ, in accordance with the vasodilation effects. Several research demonstrated that the walls of the vessels have high amounts of PACAP containing fibres but the distribution of PACAP receptors in the vessels of the wild-type and KO mice is unknown. Therefore, our research team examined the amounts of mRNA and proteins of these receptors in the common carotid and femoral arteries. In KO animals, the PAC1 receptor mRNA and protein level decreased. These results match with our former results in the inner ear where the decrease of PAC1 receptor was detected in the hair and supporting cells of PACAP KO animals. The change in the receptor amount can also explain our other results, namely, that PACAP1-27 and VIP induced a stronger response in KO animals than in the wild-type ones.

CONCLUSION

PACAP deficiency contributes to the acceleration of aging processes, which has been shown by our research team in isolated endothelial cell cultures and in different organs of the PACAP KO animals. We had the same assumption for the auditory system namely that the aging processes accelerate in PACAP KO mice: age-related hearing loss occurs earlier and to a greater extent compared to the wild-type mice. Our present examinations confirm our former results that also the auditory system of PACAP KO animals shows the signs of early aging, and my thesis also discusses the possible compensatory mechanisms. As for the examinations of tooth development of PACAP KO mice, the components of the Notch signalling pathway – the target genes of which are partially overlapping with PACAP – showed increased immunopositivity. The examination of the vascular system showed that the expression of PAC1 receptor is reduced in the vessels of PACAP KO mice. The relaxation responsivity of the vessels to PACAP1-38 is decreased, and increased to PACAP1-27 and VIP. These changes supposedly aim to counterbalance PACAP deficiency partly through the suppression of the accelerated aging processes, however, they cannot fully compensate it. We intend to continue to study the exact role of PACAP in aging processes by modelling various physiological and pathological conditions.

V. DISCUSSION

SUMMARY OF NEW RESULTS

The following changes were detected in our studies by comparing different organ systems of PACAP KO mice to wild-type mice.

I. Examination of the auditory system:

1. Following sound stimulus, decreased activation of neurons was found in the cochlear nuclei (VCN, DCN) of PACAP KO mice compared to the wild-type ones using c-Fos immunostaining. In the central nuclei of the auditory pathway there was no difference between the two genotypes.
2. Using Nissl staining in the cochlear nuclei, the same number of neurons was found in both wild type and PACAP KO mice. The lower c-Fos positivity was rather due to the reduced activation of the nerve cells than to the reduced number of cells.
3. We used PAC1 receptor immunostaining in the cochlear nuclei. In the area of the stratum granulosum between VCN and DCN, less cells were PAC1 receptor positive in PACAP KO mice than in the wild-type mice.
4. We carried out a protein profile analysis from the cochlear duct of the inner ear and it showed several proteins both in wild-type and KO mice. No difference was found between the two genotypes.

II. Examination of tooth development showed higher Notch2, Jagged1, DLL1 and CSL immunopositivity of the Notch pathway molecules in KO mice compared to the wild-type ones. Notch either may take part in compensating PACAP deficiency, or the Notch pathway could be normally under PACAP regulation and may get released from PACAP suppression in PACAP deficient mice. Low DLL1 and high Jagged1 immunoreactivity was found in the wild-type mice.

III. When examining the common carotid and femoral arteries, we found that the mRNS and protein level of PAC1 receptor decreased in PACAP KO mice compared to the wild-type ones. VPAC1 receptor was detected in both genotypes, while VPAC2 receptor in none of them. PACAP1-38 induced greater relaxation in wild-type mice than in KO ones, while PACAP1-27 and VIP led to a higher vasorelaxation in KO animals compared to the wild-type ones. These results show a change of the receptor profile in the vascular system due to PACAP deficiency, and indicate different vasorelaxation mechanisms in KO mice.

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VII. PUBLICATIONS BY THE AUTHOR

The thesis is based on the following publications:

1. **Fulop DB**, Humli V, Szepesy J, Ott V, Reglodi D, Gaszner B, Nemeth A, Szirmai A, Tamas L, Hashimoto H, Zelles T, Tamas A (2019) Hearing impairment and associated morphological changes in pituitary adenylate cyclase activating polypeptide (PACAP)-deficient mice. Sci Rep 9:14598. (shared first author article. IF: 4,116/2=2,058; Q1 in Multidisciplinary)
2. **Fulop BD**, Sandor B, Szentleleky E, Karanyicz E, Reglodi D, Gaszner B, Zakany R, Hashimoto H, Juhasz T, Tamas A (2019) Altered Notch signalling in developing molar teeth of pituitary adenylate cyclase-activating polypeptide (PACAP)-deficient mice. J Mol Neurosci 68(3):377-388
   (IF: 2,544; Q1 in Medicine miscellaneous)
3. Ivic I, **Fulop BD**, Juhasz T, Reglodi D, Toth G, Hashimoto H, Tamas A, Koller A (2017) Backup mechanisms maintain PACAP/VIP-induced arterial relaxations in pituitary adenylate cyclase-activating polypeptide-deficient mice. J Vasc Res 54(3):180-192.
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4. **Fulop BD**, Reglodi D, Nemeth A, Tamas A (2016) Pituitary adenylate cyclase-activating polypeptide in the auditory system. In: Pituitary Adenylate Cyclase Activating Polypeptide – PACAP (Reglodi D, Tamas A, eds), pp 529–549. New York: Springer, Cham. (book chapter)

Impact factors of these publications are 9,064,
After division of the first co-author publication: 7,006.

Other co-author publications by the author

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Cumulative impact factor of the author: 71,24

Co-author book chapter by the author

1. Horvath G, Illes A, Heimesaat M, Bardosi A, Bardosi S, Tamas A, **Fulop BD**, Opper B, Nemeth J, Ferencz A, Reglodi D (2016) Protective intestinal effects of pituitary adenylate cyclase activating polypeptide. In: Pituitary Adenylate Cyclase Activating Polypeptide — PACAP, Springer International Publishing, pp. 271–288. ISBN: 978-3-319-35133-9.