NKFIK K 119759 (10.2016 – 12.2022) project final report

Pituitary adenylate cyclase activating polypeptide (PACAP) is a pleiotropic and multifunctional neuropeptide, having important roles in various physiological processes. Increasing evidence shows that plasma PACAP levels and PACAP in several other tissue samples change under different physiological and pathological conditions suggesting the potential clinical use of PACAP as a diagnostic and prognostic biomarker in certain diseases. The aim of this project was to focus on those researches and clinical collaborations where preliminary data showed significant alterations in PACAP levels such as cardiovascular, neurological, endocrinological, neoplastic disorders and different physiological conditions such as lactation and pregnancy to identify the prognostic and/or diagnostic biomarker value of PACAP. We also completed our studies with animal experiments to examine the effect of endogenous and exogenous PACAP on pathological and physiological conditions.

I., The first aim of the starting year was to compare different laboratory methods, radioimmunoassay (RIA) and ELISA, to find the most suitable technique for PACAP measurements of different clinical samples.

In our earlier experiments we used a specific and sensitive RIA method, developed in our laboratory, to measure PACAP38 and 27-like immunoreactivity (LI) in different clinical samples. Since ELISA has many advantages over RIA technique (e.g. faster and cheaper method with similar sensitivity, higher accuracy and no radioactive exposure), ***our aim was to find a reliable and cost-effective PACAP38 ELISA kit*** that is available in the market and would be applicable for routine clinical examination. Before the definitive testing period, we collected background data from eight different manufacturers (BioVendor Laboratory Medicine, Cloud Clone Corp, Creative Diagnostics, Cusabio Life Science, Elabscience, LifeSpan BioSciences Inc, MyBioSource, Peninsula Laboratories International) to find the most suitable kits for quantitative PACAP38 measurements. Finally, the sandwich ***ELISA kit #MBS109020 of MyBioSource*** was chosen, since its detection range was wider (125-4000 pg/ml vs. 0-50 pg/ml), and it was able to detect PACAP38 both in plasma and breast milk samples. Although the concentration ranges of PACAP38 determined by ELISA and RIA were not the same, they showed similar tendencies. Based on these results, the sandwich-type MBS109020 ELISA of MyBioSource was chosen as a sensitive, cost-effective, non-invasive laboratory screening method for the quantitative measurement of PACAP38 in further large-scale clinical studies.

II., Examination of PACAP in clinical researches in different physiological and pathological conditions completed with animal experiments

1., Cardiovascular disorders

We collected our clinical samples for this experiment with collaboration of the Heart Institute of the University of Pécs.

First, we examined peripheral venous blood samples of 42 patients with ***primary*** and ***ischemic dilated cardiomyopathy*** (DCM) to measure the alterations of PACAP levels in chronic heart failure caused by primary DCM or ischemic cardiomyopathy (IDCM) with RIA. We found significant correlations between PACAP and NT-proBNP levels with remarkable differences between the ischemic and non-ischemic heart failure groups suggesting that PACAP might play a significant role in the pathomechanism and progression of ischemic heart failure and might be a potential biomarker of cardiac diseases in the future *(Sárszegi et al. J Mol Neurosci 2018)*.

In another translational study we examined tissue PACAP38 in ***a translational porcine myocardial infarction (MI) model*** and plasma PACAP38 levels ***in patients with ST-segment elevation myocardial infarction (STEMI).*** In STEMI patients, plasma PACAP38 level was significantly higher before percutaneous coronary intervention (PCI) compared to controls and decreased after PCI. Significant negative correlation was found between plasma PACAP38 and troponin levels. Furthermore, a significant effect was revealed between plasma PACAP38, hypertension and HbA1c levels. This was the first study showing significant changes in cardiac tissue PACAP levels in a porcine MI model and plasma PACAP levels in STEMI patients. These results suggest that PACAP, due to its cardioprotective effects, may play a regulatory role in MI and could be a potential biomarker or drug target in MI *(Szabó et al. Int J Mol Sci 2021)*.

Finally, we investigated the alterations of PACAP38 level in blood and heart muscle samples in **acute** and ***chronic heart failure (HF) patients***. We found significantly higher plasma PACAP38 in patients with acute HF, while a lower PACAP38 level was observed in chronic HF patients compared to healthy controls with ELISA method. Strong negative correlation was identified between plasma PACAP38 and NT-proBNP levels in chronic HF, as opposed to the positive connection seen in the acute HF group. We also measured the level of different cytokines in plasma samples with LUMINEX method. Plasma IL-1 β, IL-2 and IL-4 levels were significantly lower in chronic HF, and IL-10 was significantly higher in patients with acute HF. Human heart samples were collected in the Department of Heart Failure and Transplantology, Cardinal Stefan Wyszynski National Institute of Cardiology, Warszawa, Poland. PACAP38 levels of myocardial tissues were lower in all end-stage HF patients and lower PAC1 receptor levels were detected in the primary dilated cardiomyopathy group compared to the controls. We conclude that PACAP38 and PAC1 expression correlates with some biomarkers of acute and chronic HF; therefore, further studies are necessary to explore whether PACAP could be a suitable prognostic biomarker in HF patient *(Szabó et al. Int J Mol Sci 2022)*.

We also summarized the effects of PACAP on cardiovascular system in a review paper *(Szabó et al. Cardiologica Hungarica 2018).*

2., Parkinson's disease

The aim of this study was to examine the alterations of PACAP level in ***parkinsonian patients.*** We collected clinical samples with the collaboration of the Neurology and Neurosurgery Clinics of the University of Pécs. PACAP levels were measured with ELISA and correlated with clinical parameters, age, stage of the disorder based on the Hoehn and Yahr (HY) scale, subtype of the disease, treatment, and specific scores measuring motor and non-motor symptoms, such as movement disorder society-unified Parkinson's disease rating scale (MDS-UPDRS), Epworth sleepiness scale (ESS), Parkinson's disease sleep scale (PDSS-2), and Beck depression inventory (BDI). Our results showed significantly decreased PACAP levels in PD patients without deep brain stimulation (DBS) therapy and in akinetic-rigid subtype; additionally, we also observed a further decrease in the HY stage 3 and 4. Elevated PACAP levels were found in patients with DBS. Based on these results, we suggest that following the alterations of PACAP with other frequently used clinical biomarkers in PD patients might improve strategic planning of further therapeutic interventions and help to provide a clearer prognosis regarding the future perspective of the disease *(Pham et al. Geroscience 2022).*

The neuroprotective effects of environmental enrichment and PACAP are already well-known. We also described the protective effect of postnatal ***enriched environment in a rat model of Parkinson's disease*** *(Jüngling et al. Int J Mol Sci 2017).*

The aim of our study with aging parkinsonian rats was to investigate the possible beneficial effects of enriched environment and PACAP treatment on a human-relevant model of Parkinson's disease, by inducing the dopaminergic cell loss in ***aging (14-18-months-old) rats*** by a unilateral nigral lesion. In healthy, unoperated animals we have found an age-related decrease of dopamine (DA) levels. Aging parkinsonian rats raised under enriched conditions were more protected against the toxin, and PACAP treatment could counteract the toxin-induced lesion. All injured PACAP-treated rats showed remarkably higher protective PARK7 levels. The slightly higher DA and PARK7 levels after 6-OHDA treatment could suggest a better ability of compensation in enriched animals. Our novel observation in aging parkinsonian rats is that the protective effect of PACAP correlates well with the increase of DA and PARK7 protein levels. However postnatal environmental enrichment could not strengthen the effect of PACAP therapy *(Jüngling et al. Life Basel 2021).*

We investigated the molecular background of the neuroprotective effect of PACAP in DA-based neurodegeneration using ***rotenone-induced snail and 6-OHDA-induced rat models of Parkinson's disease*** *(Maász et al. Des Model Mech 2017).* Behavioral activity, monoamine (DA and serotonin), metabolic enzyme (S-COMT, MB-COMT and MAO-B) and PARK7 protein concentrations were measured before and after PACAP treatment in both models. The neuroprotective effect of PACAP in these animal models of Parkinson's disease is well correlated with the neurotransmitter, enzyme, and protein levels. In another Parkinson's model our research group showed decreased PAC1-receptor expression in the basal ganglia of ***MPTP-induced parkinsonian monkeys*** *(Fehér et al. Neurotox Res*

2018).

3., Polytraumatic injury

Our other aim was to determine the changes of PACAP38 levels **in polytrauma patients** in the early post-traumatic period with collaboration of the Department of Anaesthesiology and Intensive Therapy of the University of Pécs. We observed significant correlation between PACAP38 and CRP levels on day 4 and 5 as well as between PACAP38 and LAR levels all days. This could be due to the anti-inflammatory and cytoprotective functions of PACAP38 as part of an endogenous response to the trauma induced systemic inflammatory response syndrome. These significant correlations could have clinical importance to monitor the dynamic balance of pro- and anti-inflammatory processes in case of polytraumatic patients *(Tamás et al. Peptides 2021).*

4. Lactation and pregnancy

Earlier we investigated the presence of PACAP in different milk samples and we described the changes during different periods of lactation. One of the aims of this project was to complete the examination of ***human milk samples*** with other ***bioactive factors.*** At the beginning of the project, we started to collect milk samples with collaboration of Special Nurse Network and the Obstetrics and Gynaecology Clinic of the University of Pécs. We investigated the concentrations of several cytokines (CD40, Flt-3L), chemokines (MCP-1, RANTES, GRO, MIP-1ß, MDC, eotaxin, fractalkine), and epidermal growth factor (EGF) with Luminex technology. We published our results about the presence of each bioactive factor in every layer of the milk samples during the first 6 months of breastfeeding *(Vass et al. Int Breastfeed J 2019)*.

We also collected ***milk samples of lactating women with mature and premature babies.*** In our preliminary examination we separated the milk samples to water phase and lipid phase to measure the PACAP level in different fraction of the mature milk. In both fractions we could detect PACAP which level was significantly higher in the water phase of the human milk (not published).

With the collaboration of Department of Medical Microbiology and Immunology and the Department of Obstetrics and Gynecology of the University of Pécs we examined blood samples from ***healthy pregnant women and in toxaemia***. In this preliminary experiment we measured significantly elevated PACAP38 levels both in early and late toxaemia compared to healthy samples (not published).

It is well known, that PACAP plays an important role in reproductive physiology and development, it was of interest to examine whether this neuropeptide occurs in the ***human amniotic fluid.*** Amniotic fluid samples were collected between the 15-19th weeks of gestation. PACAP was measured by radioimmunoassay and could be detected in all samples *(Tóth et al. Reprod Biol 2020).*

We completed our human experiments with ***animal research.*** The aim of this part of the study was to determine PACAP in ***female suckling lambs compared to ewe plasma and mammary gland,*** as well as their age-dependent alterations. PACAP38-LI was 5, 6 times higher in the milk compared to the plasma of lactating sheep. It significantly increased in the lamb plasma 1 h, but returned to basal level 2 h after suckling. PACAP accumulated in the milk might be synthesized in the mammary gland or secreted from the plasma of the mothers. We suggest that PACAP has differentiation/proliferation promoting and immunomodulatory effects in the newborns and/or a local function in the mammary gland *(Pohóczky et al. Physiol Int 2020).*

In an animal experiment with the collaboration of University of Veterinary Medicine we completed our studies in reproductive processes. We analysed the ***mRNA expression of PACAP in embryos and pregnant uterus in BDF1 mice.*** Our results showed significantly higher PACAP levels in females growing embryos compared to non-mated ones and robust elevation of PACAP was detected in females with blastocyst embryos compared with non-blastocysts. However, we did not find detectable PACAP mRNA in embryos suggesting that PACAP might play a role during the peri-implantation period of early mouse embryo development *(Somoskői et al. Reprod Biol. 2020).*

5., Neoplastic disorders

Earlier we showed altered PACAP level in different tumor samples, therefore, we continued our research with neoplastic samples and different tumor cells. PACAP and PAC1 receptor expressions were investigated from thyroid gland samples of patients with ***papillary carcinomas.*** Our results reveal that both PACAP and PAC1 receptor LI are altered in papillary carcinoma *(Bárdosi et al. J Mol Neurosci 2016).*

In another in vitro preliminary experiment, we applied ***breast cancer adenocarcinoma cell lines***, MCF-7 and MDA MB231 to describe the effect of PACAP1-38 and PACAP6-38 (PAC1 receptor antagonist) in different concentrations (not published) *(Vass et al. FENS 2017 abstract)*.

We also collected samples from haematological disorders in collaboration with the Division of Haematology of the University of Pécs. Based on our preliminary results, we showed that the level of endogenous PACAP38 was significantly lower in patients with treated multiple myeloma compared to the healthy control group. We found significantly lower PACAP levels in patients in remission phase, and numerous significant correlations with laboratory parameters (not published).

Plasma samples were collected from ***patients with lung tumors*** from radial artery and cubital vein before (preop) and after (postop) the operation, and patients with kidney transplantation. Our preliminary results need further analysis (not published) *(Tamás et al. FENS 2018 abstract).*

III. Examination of allelic variants of PACAP receptors

Examination of allelic variants of PACAP receptors collection of Roma samples has been continued in the Department of Public Health Medicine (214 Roma and 198 non-Roma samples). The database containing the required data for the epidemiological-statistical evaluation from the recorded epidemiologic/demographic variables has been constructed, to describe and analyse the distribution of different allelic variants of PACAP receptor in Roma and non-Roma general populations. Analysis for the representativity of the group of participants to the given settlements and regions has been made. Isolation of DNA has been performed for all the samples. Genotyping of PAC1 (ADCYAP1R1) receptor gene rs2267735 and rs2302475 polymorphisms has been performed on 120-120 samples, in order to test the methodology, and to get the expected approximate allelic distributions. We are collecting the clinical samples form patients, but this group still did not reach the required and designed size needed for statistical analysis.

IV. Animal research with PACAP-deficient animals

The original project did not contain animal research; therefore, we have submitted an amendment request in the second year of the project to complete the work plan with animal experiments. In addition to clinical examinations our further aim was to continue our previous investigations in PACAP-deficient (KO) mice to study the consequences of the absence of endogenous PACAP in different physiological and pathological conditions such as tooth development, hearing, neurological development, bone formation, gastrointestinal system, cardiovascular system and age-related degenerative processes.

The aim of our work was to evaluate the possible effects of ***PACAP on Notch signaling pathway during tooth development.*** Immunohistochemical staining was performed of Notch receptors (Notch1, 2, 3, 4), their ligands (DLL 1, 3, 4, Jagged1, 2], and intracellular target molecules (CSL, TACE, NUMB) in molar teeth of 5-day-old wild type (WT), homozygous and heterozygous PACAP-deficient mice. We measured immunopositivity in the enamel-producing ameloblasts and dentin-producing odontoblasts. We found that the lack of PACAP leads to upregulation of Notch pathway elements in the odontoblast and ameloblast cells. We hypothesize that this compensatory upregulation of Notch signaling by the lack of PACAP could represent a salvage pathway in PACAP-deficient animals *(Fülöp et al. J Mol Neurosci 2018).*

We also performed ***functional and morphological examinations of the auditory pathway, and proteome profile analysis*** of the inner ear in PACAP KO and WT mice. Auditory brainstem response (ABR) tests found higher hearing thresholds in KO mice. Increase in neuronal activity, demonstrated by c-Fos immunolabeling, was lower in KO mice after noise exposure in the ventral and dorsal cochlear nuclei. Similar inflammatory and angiogenic protein profile data were measured from cochlear duct lysates *(Fülöp et al. Sci Rep 2019).*

Our research group also showed altered signalling in ***spermatogenesis and in bone of PACAP-deficient mice*** *(Józsa et al. Int J Mol Sci 2018, Reglődi et al. Reproduction 2018).*

We also examined the physical signs of ***postnatal development and neurological reflexes in mice*** partially or completely lacking PACAP. Our results show that mice lacking endogenous PACAP have slower weight gain during the first weeks of development and slower neurobehavioral development regarding a few developmental hallmarks, but the incisor teeth erupted earlier in mice lacking PACAP compared to wild animals *(Farkas et al. J Mol Neurosci 2017).*

In PACAP-deficient animals we found distinct changes in ***faecal microbiota.*** Faecal enterobacteria and enterococci were lower, mouse intestinal bacteroides were slightly higher in PACAP-deficient mice compared to wild-type animals. Furthermore, health-beneficial bifidobacteria were virtually absent in the intestines of PACAP-deficient animals *(Heimesaat et al. Eur J Microbiol Immun 2017).*

In another experiment we measured the ***vasomotor responses of carotid and femoral arteries*** isolated from male wild-type and PACAP-deficient mice. In PACAP-deficient mice, PACAP1-38 did not elicit relaxation, whereas in WT animals it induced significant relaxation *(Ivic et al. J Vasc Res 2017).* We found that in female WT mice, VPAC1 receptors (R) appear to play a dominant role in PACAP-induced vasorelaxation both in carotid and in femoral arteries. On the other hand, in the PACAP KO group PAC1R activation exerts vasorelaxation in the femoral arteries, however, in carotid arteries there is no significant effect of the activation of this receptor. In the background of this regional difference, we showed decreased PAC1R and increased VPAC1R availability in the carotid arteries *(Ivic et al. PLoS One 2019).* In another experiment we proved that PACAP administration reduced endothelial dysfunction after a 1-h hyperglycaemic episode and PACAP was able to restore acetylcholine-induced relaxation of the vessels and improved sodium nitroprusside-induced relaxation *(Solymár et al. Diab Vasc Dis Res 2019).* These data also proved the important function of endogenous PACAP in the regulation of vascular tone.

Finally, we described ***accelerated systemic senile amyloidosis*** in PACAP-deficient mice, which might indicate an early aging phenomenon in this mouse strain *(Reglődi et al. J Pathol 2018).*

V., Book, book chapters review publications

In the first year of the project, we have edited ***a book*** "Pituitary adenylate cyclase activating polypeptide-PACAP" with 49 chapters about the effects of PACAP in different fields *(Reglődi and Tamás 2016)*. Four review chapters have been published about PACAP experiments from different clinical research included our earlier clinical data *(Tamás et al. 2016, Reglődi et al. 2016)*. Although the book mainly contains ***review publications***, some original data have been described in relation to changes in PACAP levels in inflammatory bowel disease and in placental samples from pathologic pregnancies *(Horváth et al. 2016a,b).* We also published review papers about the novel neuroprotective strategies in Parkinson's disease *(Reglődi et al. Prog. Neurobiol 2017)*, the neuroprotective effect of PACAP against different neurotoxic agents *(Reglődi et al. Neurotoxicology 2018)*, the alternative routes of PACAP treatments *(Reglődi et al. Curr Pharm Design 2018)*, the presence and effect of PACAP in the stomach *(Reglődi et al. Front Endocrinol 2018)*, the proteomic studies focusing on the neuroprotective role of PACAP *(Rivnyák et al. Int J Mol Sci 2019)* and about PACAP-deficiency as a good model of premature aging *(Reglődi et al. Geroscience 2018).*

In the second part of the 4th year because of the COVID crisis we were not able to continue the collection of clinical samples, therefore, we published two other review papers. In the first review paper we summarized the protective effect of PACAP in peripheral organs *(Tóth et al. Front Endocrinol 2020),* and we also published a review about the neuroprotective and biomarker potential of PACAP in human traumatic brain injury *(Tóth et al. Int J Mol Sci 2020).*

In summary, we published 34 publications (cumulative impact factor: 122.667) and one book with 4 original book chapters. We attended more than 50 national and international conferences and we published more than 110 poster and oral presentations. Our students presented more than 30 oral presentations in national and international student research conferences and won 18 awards (Ist price: 7, IInd price: 10, IIIrd: 1). Four Ph.D. students defended their thesis: Sándor Balázs (2018), Fülöp Balázs Dániel (2020), Szabó Dóra (2022), Józsa Gergő (2019).