NKFIH K104984 2012-2017 Grant Report

The main topic of the present closing grant was to investigate the cytoprotective effects of PACAP, mainly in models of perinatal pathologies, from basic research to possible clinical translational research and to investigate the occurrence of PACAP in biological fluids with possible clinical relevance.

The first main research area was investigating the protective effects of PACAP in models of retinopathy, especially in **retinopathy of prematurity (ROP),** the main cause of visual impairment in children. We standardized the model: oxygen-induced retinopathy (OIR) was generated by placing the animals in daily alternating 10/50 oxygen *concentrations* from postnatal day (P) 0 to P14, then returning them to room air. We could prove the well-known **retinoprotective effects of intravitreal PACAP** injection in the rat model of ROP (*Kvarik et al. J Mol Neurosci 2016*). Animals received PACAP or saline intraperitoneally or intravitreally. We found that intravitreal (but not intraperitoneal) treatment with PACAP remarkably reduced the extent of avascular area compared to the non- and saline-treated OIR groups. Alterations in cytokine profile after local PACAP injection further supported its protective role. As a continuation of this study, we have also started experiments on **PACAP knockout (KO) mice**, where, after the standardization of the model in mice, we found that the extent of retinal injury is greater in the KO mice than in the wild types, indicating the **endogenous role of the peptide** (Kvarik et al. in preparation). These are the basis of the PhD work of T. Kvarik (to be submitted in 2018). We also studied several **factors influencing the outcome of ROP**, such as **prenatal smoke exposure, prenatal stress** as well as **hyperglycaemia**, all common problems in prenatal care (presented in posters, publications in preparation, publication on the prenatal stress model: *Kvarik et al. J Neurol Neurosci 2016*). We found that smoke exposure worsens the outcome of the retinopathy, and we tested the **effects of PACAP in models of smoke and ethanol exposure, salsolinol, 6-OHDA, Tat-induced neurotoxicity and inflammation induced toxicity in vitro and in vivo**, where we found **protection by PACAP** administration (*Manavalan et al. Neurotox Res 2017* – Basis of the PhD work of Sridharan Manavalan; *Rozzi et al. J Mol Neurosci 2014; Brown et al. Neurotox Res 2014, J Mol Neurosci 2013; Maasz et al Dis Model Mech 2017*). As ROP involves several vascular components, we also studied the effects of PACAP on the **vascular responses** (*Vamos et al. J Mol Neurosci 2014* – included in the PhD thesis of I. Ivic – defense 2017 March; *Banki et al. Neuropharmacology 2014*) and on the **angiogenic capacity** of microvascular endothelial cells (*Banki et al. J Gerontol A Biol Sci Med Sci 2015*). Another aim of the proposal was to study the effects of hyperglycaemia on the retina and the possible interference with PACAP pathways. We described several pathways **beneficially altered by PACAP in hyperglycaemic retinas**, and described preservation of synaptic integrity along the vertical processing pathway (*Szabadfi et al. Neurotox Res 2016; Neurochem Int 2014, Int Rev Cell Mol Biol 2014*). Also, ROP shares several common features with **diabetic angiopathy** in general. We conducted numerous experiments with diabetic angiopathies, which can give us further insight into the mechanism of PACAP exerted protection (*Banki et al. Peptides 2013, J Mol Neurosci 2014* – included in the PhD work of E. Banki 2014).

As another aim of the project, we studied the effects of PACAP as topical application in form of **eye drops** on retinal lesions, which is a clinically more suitable route of administration. We found that both the **morphological and the functional** outcomes in **ischemic retinopathy** are **ameliorated by PACAP**, and we determined the efficacy of the eye drops treatment depending on the type of vehicle used. We established collaboration with WA Banks, a blood-brain barrier expert from the University of Washington, Seattle, USA, and with the help of this collaboration we determined the **passage of PACAP eye drops to the retina**. We found that an effective dose of PACAP can penetrate through the ocular layers to reach the retina in order to exert retinoprotective effects. PACAP1-27 and 1-38 eye drops treatment (using benzalkonium chloride vehicle) was able to **preserve the retinal structure**, to counteract the pathological Muller glial cell activation, and the elevation of several inflammatory cytokines as well as to increase antiapoptotic signaling (*Werling et al. Invest Ophthalmol Vis Sci 2016; Werling et al. Int J Mol Sci 2017*). These experiments were included in the PhD work of D. Werling (defense: 2017 March). Recent results on **LPS-induced inflammatory retinopathy** can also be decreased by PACAP eye drops treatment (Vaczy et al. in preparation - basis of PhD work of A. Vaczy).

In frame of Japanese collaboration, we were able to detect **PACAP in the tear film** and described that **PACAP KO mice develop dry eye-like symptoms** such as corneal keratinization and tear reduction. PACAP immunoreactivity was co-localized with a neuronal marker, and PACAP receptor (PAC1-R) immunoreactivity was observed in mouse infraorbital lacrimal gland acinar cells. PACAP eye drops stimulateed tear secretion and increase cAMP and phosphorylated (p)-protein kinase A levels in the infraorbital lacrimal glands that could be inhibited by pre-treatment with a PAC1-R antagonist or an adenylate cyclase inhibitor. Moreover, these eye drops suppress corneal keratinization in PACAP-null mice. PACAP eye drops increase aquaporin 5 (AQP5) levels in the membrane and pAQP5 levels in the infraorbital lacrimal glands. AQP5 siRNA treatment of the infraorbital lacrimal gland attenuates PACAP-induced tear secretion. Based on these results, PACAP might be clinically useful to treat dry eye disorder (*Nakamachi et al. Nature Commun 2016*).

As far as the **protective mechanism** is conserved, we have made progress with elucidating the intracellular mechanisms through which PACAP exerts its retinoprotective effects. We showed that PACAP counteracted retinal ischemia-induced activation of apoptotic factors such as p38 MAPK and JNK, while increased the activation of the protective ERK and Akt in hypoperfused retinas. The cytokine profile was also dramatically changed after retinal ischemia, with most cytokines and chemokines showing an increase, which was attenuated by PACAP (such as CINC, CNTF, fractalkine, sICAM, IL-1, selectin, LIX, MIP-1, RANTES and TIMP-1) (*Szabo et al. Neurosci Lett 2012*). Some of these PACAP-induced effects had not been previously described. Considering excitotoxic retinal degeneration, we used perinatal monosodium-glutamate treatment. This paradigm induces severe retinal degeneration when applied in newborn rat. We described the protective effects of another stress-related peptide, urocortin, in this model (*Szabadfi et al. Acta Physiol Hung 2014 – invited manuscript for winning the Young Investigator Award of the Hungarian Physiological Society 2011*). We also standardized the ERG measurements, and described that PACAP improves

functional outcome in retinal hypoperfusion (*Danyadi et al. J Mol Neurosci 2014* – PhD work of B. Danyadi, thesis submitted).

We analyzed different **PACAP agonists and related peptides** in the well-described retinal ischemia model and found that maxadilan, a specific PAC1 receptor agonist is able to exert similar protective effects as PACAP, indicating that the protective effects of PACAP are mediated by the **PAC1 receptor** (*Vaczy et al. J Mol Neurosci 2016*). However, shorter fragments of PACAP or the related secretin or glucagon did not exert retinoprotection (Werling et al. J Ophthalmol 2014). We also studied **retinal aging**, as mechanisms that play a role in aging are commonly opposite to developmental processes and thus, they may help us understanding some developmental aspects of postnatal retinal pathologies. We described retinal aging in an octodon model, a rodent more closely resembling human retina than rats and mice (*Szabadfi et al. Front Cell Neurosci 2015*). Studying PACAP KO mice, we found that aging signs in the retina appear earlier in mice lacking endogenous PACAP, indicating the endogenous protective, "anti-aging" role of the peptide (*Kovacs-Valasek et al. Neuroscience 2017*).

Regarding the in vitro retina studies, we studied the **effects of PACAP on retinal pigment epithelial cells**. Continuing the first study, in which we described that PACAP protected human retinal pigment epithelial cells against oxidative stress-induced apoptosis, we found that PACAP influenced several angiogenetic factors and stress kinases (e.g. VEGF, endothelin, angiopoetin 2, thrombospondin, platelet factor 4, TIMP-1) in pigment epithelial cells under oxidative stress or under hyperosmotic stress. We used both array and flow cytometry methods. These works were the basis of E. Fabian's PhD work (defended in 2016). We have also performed several studies in Muller glial cells and retinoblastoma cell lines (presentations in preparation). About the retinoprotective effects, we published a review article in the handbook edited by our group (*Atlasz et al. 2016*).

We also studied the effects of **early postnatal enriched environment** in both ischemic and toxic retinal and other neuronal lesions (*Kiss, Szabadfi et al. Int J Mol Sci 2013; Kiss, Vadasz et al. 2013; Horvath et al. Int J Mol Sci 2013; Jungling et al. Int J Mol Sci 2017*). We determined the levels of PACAP in the brain in a model of enriched environment. We found that PACAP levels are upregulated in several brain areas after exposure to enriched environment, but this could be observed only in adulthood, not after early postnatal enrichment (*Horvath et al. Neuroendocrinol Lett 2015*). A review was written on perinatal injury models: *Horvath et al. Advances in Neurobiol 2015*. These and several earlier studies in perinatal injuries and enriched environment were the basis of Gabor Horvath's PhD thesis (2016).

Another model of prematurity complications was the **bronchopulmonary dysplasia** (BPD) model, which we aimed to standardize and test the possible effects of PACAP. The standardization of the model was very challenging and we could only complete it at the end of the grant period. Animals were operated intrauterine, on day 20 of pregnancy, by inducing inflammation 0,75 or 1 µg of LPS dissolved in 5 µl saline into their amniotic fluid through the uterine horns including the fetuses. Two days after the operation the offspring were naturally born and raised by their mothers. Animals were tested for signs of bronchopulmonary dysplasia using a wide spectrum of methods: CT scan to determine the reduction of respiratory surface in the lungs, whole body pletysmograph to test respiratory function (where breathing frequency, tidal volume,

minute volume, Penh, inspiration time, expiration time, peak inspiratory flow and peak expiratory flow were measured), and lungs were processed for histologic evaluation. A scoring system was used based on the following parameters: perivascular edema, perivascular/peribronchial acute inflammation, goblet-cell metaplasia of bronchioles, eosinophilic macrophages in alveolar spaces. Cytokine expression levels were evaluated using a rat cytokine array. Our results showed that the present model induced a low level of bronchopulmonary hyperreactivity, where the effects of PACAP will be tested in the near future.

Many of our projects involve **PACAP KO mice**, to get closer insight into the effects of and regulatory pathways influenced by endogenous PACAP (including effects during development), in addition to the above-mentioned retinal studies. Many of these experiments also have perinatal relevance, like the project on the inner ear development, where we showed altered sensitivity of newborn KO mice against kanamycin-induced toxicity (*Nemeth et al. Neurotox Res 2014* - Included in the PhD work of A. Nemeth 2015). We have also described altered sensitivity in neuropathy models (*Botz et al. Peptides 2013*). We have recently described the disturbed neurobehavioral development of PACAP KO mice (*Farkas et al. J Mol Neurosci 2017*), their altered tooth development (*Sandor et al. J Mol Neurosci 2014, 2016* - PhD to be submitted in 2017), altered spermatogenesis (manuscript under preparation), altered vascular responses (*Ivic et al. J Vasc Res 2017*), and worsening of the outcome of ischemia/reperfusion kidney injury (*Laszlo et al. Transplant Proc 2015*). We have also described altered thermogenic response (*Banki et al. J Mol Neurosci 2014*). In order to find the background for the **increased sensitivity of PACAP deficient mice** to cellular stressors, we performed proteomic analysis of PACAP deficient mice and described several differences in certain brain regions between wild type and PACAP deficient mice. We showed a disturbed balance in several metabolic pathways of PACAP deficient mice probably rendering higher sensitivity to decreased energy supply, thus increased vulnerability to insults in lack of endogenous PACAP (*Maasz et al. J Mol Neurosci 2014).* Among the altered proteins, several are involved in metabolic processes, energy homeostasis, and structural integrity. ATP-synthase and tubulin beta-2A were expressed more strongly in PACAP-knockout mice. In contrast, the expression of more peptides/proteins markedly decreased in knockout mice, like pyruvate kinase, fructose biphosphate aldolase-A, glutathione S-transferase, peptidyl propyl cis-trans isomerase-A, gamma enolase, and aspartate amino transferase. The altered expression of these enzymes might partially account for the decreased antioxidant and detoxifying capacity of PACAP-deficient mice accompanying the increased vulnerability of these animals. (This work was the basis of G. Maasz's PhD thesis 2015)

The second main goal of the project was to analyze **PACAP levels in human biological fluids** and tissues. We have performed measurements in several groups of patients and have started to collect samples also from neonates and their mothers. We have standardized the RIA and ELISA measurements with a novel antiserum. We determined PACAP levels in the cerebrospinal fluid and serum in patients with head trauma (*Bukovics et al. Peptides 2014*). We collected tissue samples from various human cancer types and described alterations in PACAP and/or receptor expressions (*Bardosi et al. J Mol Neurosci 2016, Tamas et al. J Mol Neurosci 2016*). Considering measuring PACAP in human milk samples, we showed that PACAP is also present in different

infant formula samples and commercially available milk products (*Csanaky et al. Acta Physiol Hung 2013 - invited manuscript for winning the Young Investigator Award of the Hungarian Physiological Society 2012 – Andrea Tamas*) and showed that the PACAP present in the milk might influence differentiation and growth factor secretion of the mammary cells (*Csanaky et al. J Mol Neurosci 2014 – PhD thesis of K. Csanaky 2015*). We have also worked on further adjustments of the mass spectrometry measurements of PACAP. As far as tear composition is concerned, we are currently analyzing tear composition of PACAP KO mice and the detailed evaluation of the found differences is currently being done (Vaczy et al. in preparation). We have also obtained amniotic fluid samples from pregnant women and the pool of data obtained so far indicates that in some pathological conditions the PACAP levels are extremely low. However, in order to draw final conclusions, we need to increase the number of samples, and we are continuously working in collaboration with the Obstetrics Department.

Several further developmental and other aspects of PACAP-signaling have been elucidated. We have described that PACAP is protective in mechanical load-induced chondrogenic cell culture (*Juhasz et al. Int J Mol Sci 2015*). We have also studied the effects of endogenous PACAP in bone fracture healing process (manuscript in preparation – PhD work of G. Jozsa). We found significant delay and altered bone structure in mice lacking endogenous PACAP. We have done some further studies on the protective effects of PACAP, topics that are related to the OTKA project and could help us understanding the actions of PACAP. Among others, we studied the expression and effects of PACAP in the skin (*Helyes et al. J Invest Dermatol 2015*). We described that PACAP influences bone and cartilage development using in vitro and in vivo systems (*Botz et al. Arth Rheumat 2014, Juhasz et al. PLOS One 2014, J Mol Neurosci 2014*). We found protective effects in experimental ileitis in mice (*Heimesaat et al. PLOS One 2014*), blood brain barrier integrity (*Wilhelm et al. J Mol Neurosci 2014*) and in zebrafish hair cells against oxidative stress (*Kasica et al. Neurotox Res 2016*). We could show reversal of age-related learning deficit by PACAP in an invertebrate model of aging (*Pirger et al. J Gerontol 2014*), the evolutionarily conserved effects on neuromuscular junctions (*Krajcs et al. J Mol Neurosci 2015*) and the effects in trigeminal sensory neurons (*Saghy et al. Neuroscience 2015*). Regarding the developmental effects of PACAP in placental growth and trophoblast signaling, we published the results of a long series of experiments using human and mouse cell lines and migration, invasion, differentiation and angiogenic assays (*Horvath et al. J Mol Neurosci 2014*). We found that PACAP mainly influences angiogenic pathways and trophoblast invasion/proliferation depending on the cell line and environmental circumstances. We also described involvement of PACAP signaling in the hypothalamo-pituitary-thyroid axis (*Egri et al. Endocrinology 2016*).

We published review papers on the effects of PACAP in the reproductive system, with special regards to female fertility and reproduction (*Reglodi et al. Front Endocrinol 2013*), on the protective effects of PACAP in Parkinson's disease (*Reglodi et al. Progr Neurobiol 2015*), on the protective effects of ischemic kidney injuries (*Laszlo et al. Acta Biol Hung 2014*) and on bone and cartilage physiology and repair (*Juhasz et al. Peptides 2015*). We edited a handbook on PACAP (Springer, New York, 2016, eds. Dora Reglodi and Andrea Tamas), which contains 49 review chapters on PACAP, from which was prepared by our research group: on retinoprotective effects: Atlasz et al; on intestinal

protective effects: Horvath et al.; on PACAP in the milk: Tamas et al.; on PACAP in the auditory pathway: Fulop et al.; on PACAP in the placenta: Horvath et al.; on PACAP in chongdro and osteogenesis: Juhasz et al.; and on PACAP in biological fluids and as a potential biomarker: Reglodi et al. 2016.