Grant Report –NKFIH FK129190

The main aims of the present proposals were to investigate the in vivo and in vitro retinoprotective effects of **PACAP or PACAP fragments/related peptides/analogs** in different retinal pathologies, such as (i) ischemia, (ii) retinopathy of prematurity (ROP), (iii) endotoxin (LPS)-induced inflammation, and (iv) glaucoma in rodents.

Firstly, we planned to ***investigate the effects of several PACAP fragments/related peptides/analogs in BCCAO-caused hypoperfusion***. One of the major disadvantages of in vivo PACAP treatments is the poor bioavailability of the peptide caused by the dipeptidyl-peptidase (DPPIV) enzyme. To increase the in vivo bioavailability of the peptide, the main goal of this project was to investigate the effects of DPPIV inhibitor (sitagliptin) and test in vivo degraded metabolites with antagonistic properties PACAP3-38 and PACAP5-38 on the retina. Our results showed oral administration of sitagliptin did not show any remarkable changes in the retina. We conclude that the inhibition of the DPPIV enzyme did not cause any effects on the degree of ischemic retinal degeneration. The goal also was, to prove that only the two biologically active isoforms (PACAP 1-27 and PACAP 1-38) of PACAP have neuroprotective effects in BCCAO given in the form of eye-drops. We investigated the role of degradation products (PACAP 3-38, 5-38) of PACAP 1-38. **Our aim was also to investigate whether the most effective PACAP fragments/related peptides/analogs can reach the retina in the form of eye-drops to provide protection in models of retinopathies and investigate PACAP solutions' stability.** We conclude that PACAP 1-27 or PACAP 1-38, delivered as an eye-drop, effectively ameliorates the effects of retinal ischemia and it is suggested to be a potential therapeutic agent in retinal diseases. For the first time, we provided evidence that topical administration of PACAP in a special form (PACAP-TAT and VIP-TAT) attenuates ischemic retinal degeneration via PAC1 receptor presumably due to a multifactorial protective mechanism. We have already published in a Q1 journal (Atlasz et al., J. Mol. Neurosci., 2019; IF: 2.454; http://real.mtak.hu/id/eprint/99151).

We have also performed measurements with PACAP in vitro. Previously, we have shown that PACAP, in the form of eye-drops, can pass through the ocular barriers and can exert retinoprotective effects. As eye-drops represent a promising form of administration of PACAP in ocular diseases, it is important to investigate the stability of PACAP in solutions used in eye-drops. The stability of PACAP1-27 and PACAP1- 38 in eye-drops was measured in four common media and a commercially available artificial tear. Mass spectrometry results show that the highest stability was gained with PACAP1-38 in water and 0.9% saline solution at +4 ºC. In summary, PACAP1-38 has higher stability than PACAP1-27, with the highest stability at +4 ºC in water solution. The present results provide a future reference for PACAP solutions to be used in the treatment of ocular diseases. We have published in a journal (Kovacs et al., Journal of Molecular Neuroscience, 2019; IF: 2.678; http://real.mtak.hu/id/eprint/114832).

The second main topic of the proposal was to investigate the effects of PACAP delivered via eye-drops in a model of the most widely occurring and devastating childhood perinatal retinopathy, the *retinopathy of prematurity (ROP)* mimicked by oxygen-induced retinopathy (OIR),

Previously, we have shown that intravitreal PACAP administration can maintain retinal structure in the animal model of ROP. Since many of our projects are based on PACAP knockout mice, we have performed an experiment using PACAP deficient mice to get closer insight into the effects of endogenous PACAP. The main purpose of this study was to examine the development of ROP in PACAP-deficient and wild-type mice to reveal the function of endogenous PACAP. The retina of PACAP-deficient OIR mice showed more central avascular area than that of the wild types. ERG revealed significantly decreased a- and b-wave amplitudes in PACAP KO compared to their wild types. Our finding was that retinopathic mice lacking PACAP showed a deteriorated vascularization, a disrupted cytokine balance, a decreased cell protective mechanism, and visual functional disturbances. These results suggest that endogenous PACAP is part of the protective machinery in this retinopathy model. We published the results in a Q1 journal (Kvarik et al., 2021 J Mol Neurosci, IF: 3.444; http://real.mtak.hu/id/eprint/129144).

In this grant, our aim also was to analyze the possible endogenous role of PACAP, by using mice deficient to PACAP expression (PACAP -/-) in LPS-treated inflammation. The thickness of nearly all retinal layers was significantly less in LPS-injected PACAP KO mice compared to wild-type animals. Increased expression of glial fibrillary acidic protein was induced in Müller glial cells after LPS treatment, which was more intense in PACAP KO mice. LPS treatment significantly increased cytokines in both treated groups, but it was more expressed in PACAP KO animals. Furthermore, ERG responses were disturbed after LPS injection in PACAP KO mice. Our results showed the retinoprotective role of endogenous PACAP in LPS-caused retinal inflammation and we have published (Vaczy et al., Current Pharmaceutical Design, 2018; IF: 2.757; http://real.mtak.hu/id/eprint/85739). In the present year of the grant, we also started to investigate the retinoprotective role of the specific PAC-1 receptor agonist maxadilan in LPS-induced inflammation by using different methods. Expression levels of several anti-apoptotic factors and anti-apoptotic pathways were significantly increased in maxadilan-injected retinas during inflammation, whereas apoptotic pathways were decreased in LPS-injected retinas. Moreover, the retinal structure and function also were preserved from the PAC-1R agonist maxadilan during inflammation. Furthermore, maxadilan was able to reduce the inflammatory process in the corneal tissue where the endotoxin-induced central corneal edema was thinner compared to the LPS-injected ones. These experiments (LPS-caused inflammation) were the Ph.D. work of Alexandra Vaczy (member of the retina research group) and she had the Ph.D. based on these results. We have also worked on further adjustments to the mass spectrometry measurements of PACAP. We have analyzed the tear composition of PACAP KO mice and a detailed evaluation of the found differences is being done (Vaczy et al. in preparation).

Our goal was to measure PARP inhibitor olaparib in chronic hypoxia-reoxygenation (H/R) in the retina in rats. H/R induced decreases were observed in the thickness of most retinal layers and olaparib reversed these processes providing a clear amelioration of retinal morphology. Expression of chemokines and vascular endothelial growth factor (VEGF) was increased by H/R that was attenuated by olaparib. H/R caused increased levels of Akt and GSK-3β phosphorylation that was further increased by olaparib, while the H/R-induced increases of JNK and p38 MAPK phosphorylation were reduced likely via olaparib-induced MKP-1 activation. The H/R-induced HIF1α expression was decreased by olaparib contributing to reduced VEGF expression, and nuclear factor

(erythroid-derived 2)-like 2 (Nrf2) expression was increased. NFκB was activated by H/R through the phosphorylation (Ser536) and acetylation (Lys310) of p65 subunit that was significantly reduced by olaparib. These results were published in a D1/Q1 journal (Kovacs et al., Investigative Ophthalmology and Visual Science, 2019; IF: 3.388; http://real.mtak.hu/id/eprint/99406).

Our research group has also published three review papers (i) on the effects of PACAP against different neurotoxic agents (Reglodi et al., Neurotoxicology, 2018; IF: 3.076; http://real.mtak.hu/id/eprint/83645), (ii) the effects of endogenous PACAP in aging (Reglodi et al., Geroscience, 2018; IF: 6.44; http://real.mtak.hu/id/eprint/99158), and (iii) showing different alternative routes of PACAP administration (Reglodi et al., Current Pharmaceutical Design, 2018; IF: 2.757; http://real.mtak.hu/id/eprint/99159).

Our retina research group previously proved that PACAP passes through ocular barriers and so, retinoprotection can be achieved also by eye-drops). **Accordingly, in this grant, we aimed to examine the possible neuroprotective effects of topically administered (eye-drops) PACAP1-38 in glaucoma.**

Polystyrene microbeads were injected into the anterior chamber of the right eye with a Hamilton syringe. After the microbeads injections, we treated the eyes with PACAP1-38 eye-drops for 4 weeks. Intraocular pressure (IOP) was monitored, and retinal morphological changes were followed with routine histology and optical coherence tomography (OCT). In the glaucomatous, vehicle-treated group a significant elevation in the IOP was observed, however, in the PACAP1-38 eye-drops-treated group the elevation of the IOP was not significant compared to the control ones. From the IOP results, we can conclude that the PACAP1-38 eye-drops treatment stopped the intraocular pressure elevation, this way resulted in a protective effect. We have also examined the functionality of the eyes with ERG. PACAP1-38 eye-drops treated group there were not any significant changes in the a-wave amplitude compared to the control. In the case of the microbeads injected, PACAP1-38 eye-drops treated group the b wave amplitude was also close to the normal range just as in the control animals. We can conclude that in the microbeads injected, vehicle eye-drops treated group a significant loss of sign was observed. We observed a significant decrease in the number of GCs in the microbeads received, vehicle-treated group. However, in the glaucomatous PACAP1-38 eye-drops treated eyes, the GC number did not change significantly. We can confirm that PACAP1-38 eye-drops treatment had a protective effect on glaucoma, providing the basis for future therapeutic administration. We have published these data in a Q1 journal (Szabo et al, Int. J. Mol. Sci.; http://real.mtak.hu/id/eprint/129142), This work was the basis (50%) of E. Szabo's Ph.D. thesis, 2023.

In our previous study, we described an inducible, microbeads model in Sprague Dawley (SD) rats in which we were able to prove the neuroprotective effects of PACAP1-38 eye drops treatment. Vascular factors have been suggested to play an important role in the development of glaucoma, based on numerous studies. In this period, our aim was also to examine the possible protective effects of PACAP1-38 eye drops on the retinal vasculature and the molecular patterns of hypoxia in hypertensive glaucoma models. We found several vascular parameters changed in the microbeads injected group. The examination of molecular patterns suggested hypoxic conditions in the microbeads injected rats, however after PACAP1-38 administration retinoprotective effects were observed in HIF1α and

VEGF protein levels (Patko et al., 2023 Int J Mol Sci,; http://real.mtak.hu/id/eprint/172770).

Our aim was also to develop a pharmacopeia-grade medicinal product containing natural herbal active ingredients with the potential to reduce intraocular pressure and reduce the progression of irreversible optic neuropathy through anti-inflammatory and antioxidant effects.

Microbeads injection significantly increased the intraocular pressure IOP in the vehicle-injected glaucomatous group, however, herbal treatment in eye-drops indicated a decrease in the IOP. OCT scans also showed a significant reduction in the total retinal thickness, and in several different layers of the retina in the glaucomatous vehicle-treated injected group. In the same group, ERG a-wave and b-wave were also significantly reduced. In the herbal-treated glaucomatous group, both ERG and OCT results were similar compared to the control ones.

The aim of this grant period also was to determine the influence of maternal smoking during pregnancy on the early physical and neurobehavioral development of newborn rats. Prenatal cigarette smoke exposure did not alter weight gain or motor coordination. Critical physical reflexes indicative of neurobehavioral development (eyelid reflex, ear unfolding) appeared significantly later in pups prenatally exposed to smoke as compared to the control group. Prenatal smoke exposure also resulted in a delayed appearance of reflexes indicating neural maturity, including hind limb grasping and forelimb placing reflexes. In conclusion, clinically relevant prenatal exposure to cigarette smoke results in slightly altered neurobehavioral development in rat pups. These findings suggest that chronic exposure of pregnant mothers to cigarette smoke (including passive smoking) results in persisting alterations in the developing brain. We have already published in a journal (Mammel et al., Physiology International, 2020; IF: 1.41) http://real.mtak.hu/id/eprint/114833).

We also extended the research on smoking to the retina. We aimed to examine the vulnerabilities of maternal smoking on OIR. During the pregnancy mice (C57BL/6) had to smoke two times a day for 30 minutes. To induce retinopathy pups were exposed to 75% oxygen +/- 2% from postnatal day (PD) 7-12 then returned to room air. On PD 17 the eyes were examined with OCT. Isolectin GS-IB4 was used to label the endothelial cells of the retinas. We found remarkable avascularisation areas in ROP, which were exacerbated by smoking. Furthermore, the hypoxia-caused neovascularization tufts were more in the maternal smoking+ROP group compared to the ROP group. The in vivo OCT examination and histological analysis showed alterations in the total retinal thickness in retinopathy. Based on our results we showed that maternal smoking caused a greater degree of retinal damage in ROP. However, to conclude, we need to increase the number of samples, and we are continuously working in collaboration with the Pharmacology Department of UP.

In this period we investigated the retinoprotective role of PACAP on diverse stress factors in vitro and on the retinal pigment epithelial (RPE) cell numbers in vivo.

PACAP significantly ameliorated the increased Hif1-α levels in hypoxic conditions. In H_2O_2-induced oxidative stress, PACAP had an anti-apoptotic effect, it could decrease the expression of cytochrome-c and p53, while it upregulated the concentration of three antioxidants, namely SOD2, PON2, and thioredoxin. Based on this result, we provided new information on the molecular biological background of the retinoprotective effect of PACAP. We have published the result in the following journal (Fabian et al., International

Journal of Peptide Research and Therapeutics, IF: 1.931; http://real.mtak.hu/id/eprint/129170.)

In the present year, we also tested the possible protective role of PACAP in MSG model in vitro.

Spontaneous and light-evoked spikes of RGCs from wild-type mice were recorded using a 60-channel multielectrode array (MEA). We found that MSG had clear short-term effects on the spontaneous and light-evoked spiking of mouse retinal ganglion cells (RGCs). The application of PACAP, well-known for its long-term neuroprotective effects, also rescued RGCs from short-term MSG-induced insults. We propose that PACAP exerts its protective effects either through the desensitization of postsynaptic glutamate receptors and/or the extrusion of excess glutamate from the synaptic gap.

The neuroprotective function of PACAP was mostly studied on the retina, and only a few research groups focused on the cornea, so in this period, **we also investigated the retinoprotective role of PACAP on corneal epithelial wound healing in rats.**

PACAP application enhanced corneal wound healing, as the area of injury was significantly less in PACAP-treated groups. Furthermore, both ERK1/2 and Akt signaling were induced upon PACAP administration in both injured and intact corneas. In summary, the present results show that PACAP enhances corneal wound healing in a rat model of corneal abrasion. (Kiss et al, 2022 International Journal of Peptide Research and Therapeutics, IF: 1.931) http://real.mtak.hu/id/eprint/149175.

Several studies show that PACAP has cytoprotective effects mediated by its specific PAC1 receptor and plays a key role in several diseases. There is no data on the distribution and expression of PACAP and its receptors in the human eye bulb. Our research group aimed to describe the distribution of PACAP-like immunoreactivity in the human eye, and the presence of PAC1 receptors in different parts of the eye. We found positive immunoreactivity in the corneal epithelium and also in the endothelium, the stroma, the muscles of the iris, and also in the ciliary body. (Patko et al, 2022 J Mol Neurosci, IF: 3.1) http://real.mtak.hu/id/eprint/149174.

In our retina research group has been published the protective role of PACAP1-38 injected intravitreally. During this grant period, **we also examined the role of PACAP administration (eye-drops) in type 2 diabetes**. We found that both morphological and functional outcomes in type 2 diabetic retinopathy (T2DR) are ameliorated by PACAP1-38, and we determined the efficacy of the eye drops treatment in this model. In conclusion, based on the functional, structural, IHC staining, and microvascular analysis, our study suggested that PACAP 1-38 had a potent protective effect against developing diabetic retinopathy, and proved its power as a therapeutic approach to treat T2DR in the form of eye-drops. (Li et al. in preparation - basis of Ph.D. work of L. Li).

The first year (2019) of the work supported by the present NKFIH FK129190 grant was dedicated to the standardization of the novel methods described in the proposal. The impact factor of the publications was 20.872 in the first year. This year, new medical students and several BSc, and MSc biologist students joined our retina research group: Dorottya Molitor, Balazs, Meresz, Anne Schmidt, Hatem Abo Obaid, Rafaella Riszt, Diana Denes, Refiloe Malebo, Balint Becsi. In this year Evelin Patko and Lina Li started their Ph.D. in our research lab.

In the second year (2020) the impact factor of the publications was 6.828 in the second year in this NKFIH grant. We have also presented our results in 4 conference presentations (the PACAP conference at UCLA; the Janos Szentagothai Multidisciplinary Conference at UP; and the Medical Conference for Ph.D. Students at UP). Diana Denes joined our research team as a Ph.D. student.
In the third year (2021) the impact factor of the publications was 11.298. We have also presented our results in 14 conference presentations (Vision Science and Eye 2020; Medical Conference for Ph.D. Students and Experts of Clinical Sciences 2020; Janos Szentagothai Multidisciplinary Conference 2020, 2021). In this year Alexandra Vaczy received her Ph.D. degree. Dorottya Molitor and Balazs Meresz started their Ph.D. works in our lab.
In the fourth year of the work (2022) we have published our results in 2 papers (impact factor is 5.6) and presented in 12 conferences (Istico-Huphar-Iuphar Conference; Retina Workshop 2021; NEPSY 2021; HuNDoc 2022; HMAA Conference; Janos Szentagothai Multidisciplinary Conference 2022). Inez Bosnyak has joined to our research group as a MD/Ph.D. student.
In the last year (2023) we have published our result in a Q1 paper (Int. J. Mol. Sci., IF: 5.6) and also presented 21 conferences (VPAC ISBAP, TDK, OTDK, Koranyi Frigyes, ISCOMS PTE Neuroscience Centrum Ph.D. and TDK Conference, Cholnoky Symposium, Romhanyi Symposium, Students Symposium “Medicine and Science 2022”; Janos Szentagothai Multidisciplinary Conference 2023, MedPecs Medical Conference, MAT2023). In this year Edina Szabo defended her Ph.D. thesis.