The retinoprotective role of endogenous PACAP and the PAC1-receptor in retinal inflammation

Ph.D. thesis

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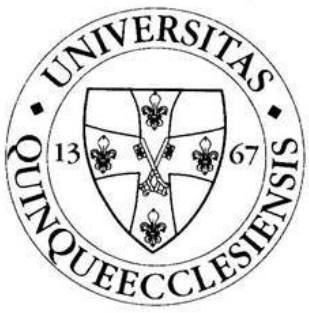

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I. Introduction

1 The anatomy of the retina

The retina is a multi-layered sensory tissue that lines the back of the eye. It contains millions of specific retinal cells that capture photons and translate light signals into electrical potential changes. The vertebrate retina has ten distinct layers. From outermost to innermost, they include:

1. Retinal pigment epithelium (PE; supporting cells for the neural portion of the retina).
2. Photoreceptor layer (PL; composed of light and colour sensitive cells called rods and cones).
3. Outer limiting membrane (OLM; a layer which imperfectly separates the inner segment portions of the photoreceptors from their cell bodies).
4. Outer nuclear layer (ONL; contains cell bodies of rods and cones).
5. Outer plexiform layer (OPL; the first layer, where connections between photoreceptors, and vertically running bipolar cells as well as horizontally oriented horizontal cells occur).
6. Inner nuclear layer (INL; somata of horizontal, bipolar and amacrine cells, main distribution of Müller cells).
7. Inner plexiform layer (IPL; in this layer bipolar cells synapse with different varieties of functionally specialized amacrine cells and dendrites of the various ganglion cells).
8. Ganglion cell layer (GCL; the layer of the retina containing primarily the cell bodies of ganglion cells, giving rise to optic nerve fibers, and some displaced amacrine cells).
9. Nerve fiber layer (NFL; fibers from ganglion cells traversing the retina to leave the eyeball at the optic disc).
10. Inner limiting membrane (ILM; inner surface of the retina bordering the vitreous body and thereby forming a barrier between neural retina and vitreous humor.).

This structure provides the neuronal background of the first steps in processing contrast, colour and motion. The retina is supplied with blood through two branches of the ophthalmic artery which is the central retinal artery and the posterior ciliary arteries.

In vivo methods for studying the retina

Electroretinography (ERG)

Electroretinography is a functional method for examining vision that measures the activity of retinal cells upon light stimulation. The method provides information on the functionality of retinal photoreceptors, bipolar and horizontal cells. The first parameter of the electrical response is the a-wave which is called late receptor potential. The a-wave indicates the function and state of photoreceptors located outside the retina, while the amplitude of the b-wave reflects the state of the inner layers of the retina, such as ON bipolar and Müller cells. The a-wave and b-wave are followed by the slow corneal-positive c-wave which originates in the pigment epithelium.

Optical coherence tomography (OCT)

Nowadays, the use of spectral domain OCT (SD-OCT) in ophthalmology has become very common in routine diagnostics. OCT is an imaging technique that uses low-coherence light to capture micrometer-resolution, two- and three-dimensional images from within optical scattering media (e.g., ocular tissue). While the scanning beam moves across tissue, the sequential longitudinal signals, or A-scans, can be reassembled into a transverse scan yielding cross-sectional images, or B-scans, of the subject. The scans can then be analyzed in a variety of ways providing both empirical measurements (e.g. retinal layer thickness/volume) and qualitative morphological information.

2. Pituitary adenylate cyclase-activating polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamus based on adenylate cyclase activating effects in pituitary cells. PACAP belongs to the secretin / glucagon / vasoactive intestinal peptide (VIP) superfamily. Two biologically active forms are present in mammals, one of which is built up of 38 amino acids (PACAP38), and the other one has a shorter structure with only 27 amino acids (PACAP27). Nearly 90% of PACAP in vertebrates is the PACAP38. The N-terminal structure of PACAP shows a very conserved amino acid sequence during evolution.

Presence of PACAP in the human body

PACAP is abundantly present in the central nervous system. The highest concentration of the polypeptide is in the hypothalamus. Its distribution has also been described in other brain areas, such as the midbrain, cerebellum, brainstem, bridge, and cerebral cortex. It has been identified in the peripheral nervous system in vegetative pre- and postganglionic neurons as well as in a number of other locations, such as the spleen, lymphoid tissue, cardiovascular, respiratory and immune systems, uterus, breast and adrenal gland.

Distribution of PACAP in the eye

The presence of PACAP has been described in several parts of the eye. Significant amount appears in the iris, ciliary body, cornea, conjunctiva, sclera choroid and retina. PACAP appears in the retina of different animal species at a very early stage of development. In zebrafish, the peptide was observed in the ganglion cell layer 72 h after fertilization. In rodents, it has been reported that from day 20 of development, the presence of the peptide appears in the ganglionic cell layer. PACAP immunopositive cell bodies were found in amacrine, horizontal cells in the INL, in the GCL and also in the Müller glial cells. PACAP positive nerve fibers were also present in the NFL, and in the IPL.

Receptors of PACAP

PACAP exerts its actions through three distinct G-protein coupled receptors: the PAC1, as well as VPAC1, and VPAC2. The PAC1 receptor is specific to PACAP, while the VPAC receptors have similar affinity to PACAP and VIP. The protective effects of PACAP are mainly mediated through activation of the PAC1 receptor which is currently considered as a potential target for the treatment of neurodegenerative diseases. The PAC1 receptor is found in many areas of the body, including the retina. Seki and co-workers described the localization and strong expression of PAC1 receptor in NFL, GCL, INL, and weaker expression in the ONL, OPL, IPL, and the outer segments of the photoreceptors in adult rodent retina. Moreover, PAC1 receptor has been described in Müller glial cells. Biding of PACAP to the PAC1 receptor elevates the intracellular cAMP concentration. The increased level of cAMP can activate protein kinase A. PACAP is also able to regulate Ca^{2+} release in various cell types. Regulation of the different pathways via the PAC1 receptor is highly dependent on PACAP concentration as well as the presence of the PAC1 receptor splice variants.

General effects of PACAP

The polypeptide is able to influence many biological processes such as nutrition, salivary secretion, circadian rhythm, thermoregulation, stress and immune processes. It is able to influence fluid uptake of the body, regulates pituitary hormone production, increasespancreatic insulin and thyroid thyroxine synthesis, and has been described as an important vasodilator and bronchodilator. The neuroprotective effects of PACAP have been shown in several different cell types in vitro against various toxic agents, such as hydrogen peroxide, glutamate or 6-hydroxydopamine. In vivo descriptions have also proven that PACAP is protective in global and focal cerebral ischemia, traumatic brain injury and neurodegenerative diseases. Another major important role of PACAP is the immune modulator effect under acute and chronic inflammatory conditions. Several studies use PACAP knock out (KO) mice to provide more insight into the endogenous protective effects of PACAP. In these studies, PACAP KO mice usually have significantly greater damage in response to harmful insults such as hypoxia, inflammation and oxidative stress compared to Wt mice.

Effects of PACAP on the eye

The neuroprotective effect of PACAP has also been demonstrated in the retina. Protective actions have been found in different pathological conditions such as diabetic retinopathy, UV light-induced degeneration, excitotoxic retinal injury, optic nerve injury and oxygen-induced retinopathy. Regarding ischemic retinopathy, our research has shown that intravitreal PACAP treatment rescued the bilateral common carotid artery occlusion (BCCAO) caused damage of several cell types in retina. PACAP was able to attenuate the reduction in thickness of all retinal layers and the loss of cells in the GCL. This morphological improvement was also accompanied by functional amelioration. In addition, our study has demonstrated that even in form of eye-drops, PACAP can lead to retinoprotective effects in ischemic retinopathy. These findings clearly shown that exogenous PACAP could be a potential therapeutic agent for neurodegenerative diseases. Furthermore it was found that endogenous PACAP has crucial retinoprotective role also in BCCAO. These results clearly show that endogenous PACAP reacts as a stress-response peptide that is necessary for endogenous protection against different retinal insults.

3. Maxadilan

Maxadilan, a 61-amino acid peptide, was initially isolated from the salivary glands of the sand fly Lutzomyia longipalpis based on its strong vasodilator action. Although maxadilan does not occur naturally in the mammalian organism and has no sequence homology with PACAP, it is the only known selective agonist of the PAC1 receptor. It has been shown to be a useful peptide to study the diverse effects mediated by the PAC1 receptor.

4. Bilateral carotid communis occlusion

BCCAO leads to moderate reduction in the cerebral blood flow in rats leading to subtle changes in biochemical and behavioral measures. It has been shown that BCCAO causes long-lasting white matter lesion, neuronal degeneration, microglial activation, astrocytosis, behavioral deficits and changes in several biochemical parameters. In the retina, it produces a characteristic pathologic appearance, paralleling the retinopathy of carotid artery occlusive disease in humans. Electroretinographical and morphological studies also show that BCCAO leads to ischemic damage of the retina.

5. Bacterial lipopolysaccharides (LPS)

LPS is the major molecular component of the outer membrane of Gram-negative bacteria. Bacterial infection is a powerful activator of innate immune responses and activate pro-inflammatory signaling pathways in the host. Endotoxin-induced uveitis is a classic animal model to study human uveitis. The ocular inflammation can be induced by systemic injection of LPS.

II. Aims

I.) Previous studies have shown that exogenously administered PACAP and the endogenous form have retinoprotective effects in ischemic retinopathy. The first part of our experiment was designed to demonstrate the protective effect of PAC1 receptor using maxadilan on ischemic retinopathy induced by permanent BCCAO in rats.

II.) The second half of our study investigate the protective role of endogenous PACAP and the anti-inflammatory effects of the PAC1 receptor using maxadilan in LPS-induced retinal inflammation mice model.

III. Investigation of the retinoprotective effect of PAC1 receptor in ischemic retinopathy

1. Materials and methods

Animals and treatment

Adult male Wistar rats weighing 250-300 g were subjected to permanent BCCAO (n=36) under isoflurane anesthesia. The common carotid arteries on both sides were exposed through a cervical incision in the carotid triangle of the neck. Common carotid arteries were ligated with a 3-0 filament. Following the operation, 5 µl (0.1 µM, n = 9 or 1 µM, n = 9) maxadilan solution was injected intravitreally into the right eye with Hamilton syringe. Left eyes always served as controls, receiving the same volume of phosphate buffered saline (PBS). The control group of animals (sham) underwent anesthesia, all steps of the surgical procedure, except ligation of the carotid arteries. These animals were also injected with maxadilan solution (0.1 or 1 µM) into the right eye and vehicle (PBS) into the left eye.

Histology

After two weeks the animals were sacrificed and eyes were dissected in and fixed in 4 % paraformaldehyde. Eyecups were embedded in Durcupan resin and cut at 2 µm. The samples were stained with toluidine blue and mounted in DPX medium. Retinas were measured with Nikon Eclipse 80i microscope, using the Q-Capture Pro7 program. Four tissue blocks from animals were prepared and central retinal areas within 1 mm from the optic nerve were used for

measurements. The following parameters were measured: OLM-ILM, ONL, INL, OPL, IPL, respectively, the number of cells/100 μm section length in the GCL.

Cytokine measurements

For the semiquantitative cytokine array measurement, retinas were removed twenty-four hours after the BCCAO and analyzed using Rat Cytokine Array Panel. The array was performed as described by the manufacturer. The samples were homogenized. The nitrocellulose membranes were blocked with antibody and incubated overnight with homogenates. After washing process adding streptavidin-HRP to each membrane, the plates were spread to a chemiluminescent detection reagent. The membranes were placed facing up to an X-ray film cassette. Developed films were scanned and analyzed by densitometry using the Protein Array Analyzer program for Image J software. The array was repeated three times.

Data Analysis

Data are expressed as mean ± standard error (SEM). Data of the histological and the cytokine analysis were used in the Kolmogorov-Smirnov normality test followed by two way ANOVA test and Fisher LSD's post hoc analysis with OriginPro 2016. Significant differences were considered at p values below 0.05.

2. Results

Effects of maxadilan treatment on histological changes of the retina

Maxadilan treatment did not cause any morphological alteration in sham-operated animals. Retinal layers in BCCAO animals showed signs of severe degeneration compared to sham-operated PBS-treated controls. All layers were significantly thinner than in the sham-operated group. Most marked reduction was observed in the IPL thickness but significant changes were also found in the INL, OPL, and ONL. The number of cells in the GCL/100 μm was significantly decreased compared to the controls. Intraocular treatment of 0.1 or 1 μM maxadilan caused protection in the thickness of different retinal layers, with 1 μM being more effective. This resulted in a significantly better preserved whole retinal distance between the OLM-ILM. Quantitative analysis demonstrated that the loss in the number of cells in the GCL

was also attenuated in the maxadilan-treated groups compared to the BCCAO-operated samples.

Effects of Maxadilan Treatment on Cytokine Expression

The expression level of several cytokines was increased after ischemia, including chemoattractant proteins such as CINC-1 and MIP-3α. IL-1α was also activated, while other interleukins remained unchanged, such as IL-2, IL3, IL-4, IL-6, and IL-10. The activation of fractalkine, sICAM-1, L-selectin, thymus chemokine, TIMP-1, and VEGF was increased in the retinas that underwent BCCAO compared to the control groups. Maxadilan treatment attenuated the activation of CINC-1, IL-1α, and L-selectin. The expression of other cytokines did not show any marked alterations after maxadilan treatment.

3. Discussion

In this study, we provided evidence for the involvement of the specific PAC1 receptor in the retinoprotective effects of PACAP in ischemic retinopathy using the PAC1 specific agonist, maxadilan. Cerebral ischemia often causes irreversible retinal vascular damage. We have previously studied different neuroprotective strategies in BCCAO induced retinal degeneration where we have proven that PACAP is protective in BCCAO-induced ischemic retinal lesion. PACAP protected the retinal layers and this morphological amelioration paralleled the functional improvement. Thus, it seems that PACAP and its receptors are promising therapeutic targets in various pathologies therefore, it is essential to study the involvement of the different PACAP receptors in models of neuronal degeneration. To test the involvement of PAC1 receptor in the retinoprotective effects of PACAP in hypoperfusion, we decided to use maxadilan, a selective PAC1 receptor agonist in our experiment. Maxadilan increases cyclic AMP (cAMP) formation in human neuroblastoma cells, rat PC12 pheochromocytoma cells and rabbit aortic smooth muscle cells. It has been shown to elevate MEK/phospho ERK in cardiac neurons involving both adenylate cyclase/cAMP/protein kinase A signaling pathways. Similarly to PACAP, maxadilan has been shown to exert protective effects in different tissues. Subcutaneously injected maxadilan has anti-inflammatory effects in Leishmania-infected mice by stimulating IL-6 and inhibiting TNF-alpha expressions. Maxadilan also inhibits apoptosis and promotes cell survival via caspase 3 and caspase 9 reduction as well as Bcl-2 upregulation in human adipose-derived stem cells. It prevents the

neuroblastic layer of retinal explants from anisomycin induced cell death. Maxadilan has been shown to influence cell survival and inflammatory pathways by PAC1 receptor which was confirmed in the present study in ischemic retinal lesion. In our study, ischemia could dramatically change the morphology of the retina and induced several cytokines. Decreased activation was found in the CINC-1, IL-1 alpha, and L-selectin regulatory proteins after maxadilan treatment. Members of the interleukin-1 family mainly regulate immune and inflammatory responses during infections and ischemic damages. It is primarily released in the microenvironment as an alarming factor to the cell and to activate the early mechanisms of defense. Reduced level of these IL-1 subclasses by maxadilan is similar to what we found earlier with PACAP treatment in ischemic retinal injury. Members of the selectin family play important roles in the distinct cellular cross talk between leukocytes, T cells, and endothelial cells and can cause microvascular dysfunction and reperfusion damage. Increased expression of L-selectin was observed in different model organs after ischemia, and blocking the cytokine-induced leukocyte recruitment can reduce the inflammatory process of neurological disorders. PACAP treatment could decrease these cell adhesion molecules in many different models, in accordance to our present findings with maxadilan. CINC-1 also participates in the retinal inflammatory reactions. This is stimulated by oxidative stress and ischemia/reperfusion. It is involved in several inflammatory processes, usually during the acute inflammatory response phase, and it plays a main role in the subsequent neutrophil migration. Compromise in blood flow has been shown to induce decreasing CINC levels in different tissues which are usually associated with decreased damages. Elevated CINC-1 level was significantly reduced by PACAP treatment in streptozotocin induced diabetic nephropathy. The anti-inflammatory effects of PACAP were similar in BCCAO-induced retina degeneration model where the neuroprotective peptide diminished the damages. Taken together, the maxadilan-induced cytokine alterations and the morphological amelioration are in agreement with other earlier reports, suggesting an anti-inflammatory role of the PAC1 receptor in ischemic retinal injury.

IV. Investigation of the protective effect of endogenous PACAP and PAC1 receptor in LPS-induced retinitis

1. Materials and methods

Animals and treatment

Adult three-month-old male (CD1 strain) Wt and PACAP KO mice (n=124) were used in the experiments. Mice (n=37; Wt+LPS; n=25 PACAP KO+LPS group) received a single intraperitoneal injection of 6.0 mg/kg body weight of LPS from Escherichia coli in phosphate buffered saline (PBS). Control groups (n=37; Wt+LPS; n=25 PACAP KO+LPS csoport) were injected PBS intraperitoneally. To investigate the role of the PAC1 receptor in inflammatory processes (n= 24), 1µM maxadilane was applied intravitreally (ivi) to the right eye (Wt + ivi 1µM maxadilane; Wt + LPS + ivi 1µM maxadilane) and their left eye was treated with vehicle (PBS) (Wt + ivi PBS; Wt + LPS + ivi PBS).

Histology

To prove the protective role of endogenous PACAP in retinitis, the animals (n=6/group: Wt; PACAP KO; Wt+LPS; PACAP KO+LPS) were sacrificed 7 days after the LPS injection. To analyze the protective role of PAC1 receptor, we killed the mice 5 weeks after the endotoxin treatment (n=24 animal, in these group: Wt+ ivi 1µM maxadilán; Wt+LPS+ivi1µM maxadilán, Wt+ ivi PBS; Wt+LPS+ ivi PBS). Eyes were fixed in 4 % paraformaldehyde and embadded Durcupan resin. We used the same protocol as already described in the first part of my experiment.

Optical coherence tomography examination

Animals (n = 24) were monitored after LPS treatment week by week with SD-OCT in the following groups: Wt + ivi 1µM maxadilane, Wt + LPS + ivi 1µM maxadilane, Wt + ivi PBS, Wt + LPS + ivi PBS. 5 weeks after inflammation was proven to be a sufficient time interval for the development of permanent retinal damage. Mice were anesthetized and placed on the animal holding pad. For the measurement pupils were dilated with one drop of 1% homatropine and we applied artificial tears. OCT scanning and the funduscopy examination were recorded by the retina lens.

Glial fibrillary acidic protein (GFAP) immunohistochemistry

Animals were sacrificed 24 h after LPS or vehicle (PBS) injections (n=5 animals/each condition). For measurement of GFAP activity in the Müller glial cells, eyes were postfixed, washed and dehydrated. The eyecups were vertically sectioned in tissue freezing medium at 16 µm thickness on a freezing microtome. Sections were permeabilized and incubated with polyclonal antibodies against anti-GFAP (1:1000) antibody. On the following day, the second fluorescent anti-rabbit antibody Alexa Fluor 488 (1:200) was added. After washing, propidium iodide (1:500) was used to detect the nuclear components. Preparations were mounted with Fluoroshield and detected by a fluorescent microscope. Central retinal areas were used for immunohistochemical analysis. The percentage of GFAP labeled area was measured in each picture using an ImageJ macro (NIH).

Cytokine Array Analysis

One day after the administration of LPS (n=7 animals/ each condition), retinas were dissected. Proteome Profiler Mouse Cytokine Array Kit, Panel A from R&D System was used for the analysis. The array is based on antibodies binding with nitrocellulose membranes and it was performed as described by the manufacturer. We used the same step of the protocol the details of which were described for the first experiment.

Western Blot Measurements

For western blot experiments, retinas were removed 24 h after LPS injection (n=7 animals/ each condition). Tissues were homogenized and protein concentration was determined. Membranes were probed overnight with the primary antibodies: phosphospecific anti-Akt-1 Ser473 (pAkt; 1:1000), phospho-specific glycogen synthase kinase-3β Ser9 (pGSK; 1:1000). Nonphosphorylated total-Akt (tAkt; 1:1000) antibody was used as an internal control. Horseradish peroxidase-conjugated secondary antibody (1:3000) were added to the membranes. The antibody-antigen complexes were visualized by means of enhanced chemiluminescence. For quantification of blots, band intensities were quantified by NIH ImageJ program.

Electroretinography

Scotopic ERGs were performed to assess retinal function in Wt and PACAP KO groups. ERG flashes were recorded before LPS treatment and 24 h after LPS-induced inflammation (n=6 animals/ each condition). Mice were dark adapted for at least 12 h and prepared under dim red illumination (632 nm), anesthetized with intraperitoneal injection of ketamine. The pupils were dilated with one drop of 1% homatropine. ERGs were recorded by surface electrodes from the center of the cornea. The reference electrode and the ground electrode were placed subcutaneously under the skin. Responses (n=50/eye) were averaged with Ratsoft software. The graphs were analyzed with OriginPro 2016. The following parameters were measured: amplitude of the a- and b-wave.

Data analysis

Data are expressed as mean ± SEM. Data were analyzed using Kolmogorov-Smirnov normality test followed by ANOVA test and Fisher LSD's post hoc analysis. Significant differences were considered at p values below 0.05.

2. Results

Effects of LPS treatment on histological changes of the retina

No differences were observed between the control retinas of Wt and PACAP KO mice. Wt retinas in the LPS treated group did not show remarkable differences (except in INL layer) compared to control groups. In LPS-treated KO animals, all retinal layers were significantly thinner than in the control and LPS-treated Wt groups. The marked reduction was observed in the ONL, but significant changes were also found in the INL, OPL and IPL as well as in the OLM-ILM distant.

Optical coherence tomography investigation on histological changes of the retina

Endotoxin caused chronic inflammation in the eye and it resulted permanent retinal degeneration. Maxadilan treatment was able to ameloriate these damages and protect the thickness of different retinal layers which resulted a better preserved entire retinal thickness

compared to the LPS+PBS treated ones. Fundus photograph from control mice did not show any vascular dysfunction. However, signs of ocular inflammation were observed in the LPS+PBS treated animals. This inflammation was manifested by perivascular exudates leading to infiltration of mononuclear cells in LPS+PBS treated group. Maxadilan was able to prevent this proecess and the endotoxin induced vascular leakage had no sign in LPS+maxadilan treated group.

Analysis of glial fibrillary acidic protein in Müller glial cells

GFAP was markedly upregulated following LPS treatment in the retinas of Wt and PACAP KO mice. Expression was more intense in the entire cell from the OLM to ILM in LPS-treated PACAP KO animals compared to the LPS-injected Wt mice.

Effects of LPS treatment on cytokine expression profile of the retina

The activation of sICAM-1 (soluble intercellular adhesion molecule-1), TIMP-1 (tissue inhibitor of metalloproteinase-1) and JE (monocyte chemoattractant protein-1) was increased in the retinas that underwent LPS inflammation compared to control groups. The expression level of these three cytokines was significantly stronger in the LPS-treated PACAP KO group compared to the LPS-injected Wt group.

3. Discussion

We showed, for the first time, that endogenous PACAP is protective in LPS-induced ocular inflammation in the retina using PACAP KO mice. In our present study, we detected dramatic changes of the retinal layers after LPS-induced inflammation in the PACAP KO groups compared to the Wt ones. These findings correlated with results of other research groups, where PACAP KO mice showed increased severe retinal abnormalities in aging or ischemia. We showed an irregularity in Müller glial cells in LPS-induced inflammation, which was more intense in the PACAP KO group. The decreased uptake of GABA and glutamate results in accumulations of these proteins and causes abnormalities in the retinal neurons. PACAP is retinoprotective in Müller glial cells and stimulates the release of interleukin-6, which has been confirmed in ischemic and excitotoxic brain lesions. In our experiment, TIMP-1 level showed a strong activation 24h after the LPS treatment in both treated groups but was more severe in

the PACAP KO mice. Our present findings are in accordance with earlier studies, where increased expression of TIMP-1 was associated with many pathological conditions, such as diabetic nephropathy, glaucoma or ischemic retinopathy. sICAM-1 activation was detectable in PACAP KO mice in LPS-induced inflammation. Upregulation of sICAM-1 is enhanced by inflammatory cytokines, including tumor necrosis factor alpha and it produces pro-inflammatory effects such as recruitment of leukocytes into the site of the inflammation. Increased expression of sICAM-1 was also observed in ischemia/reperfusion induced injury in several organs, and PACAP treatment partially or totally blocked this cytokine. MCP-1 is identical to JE in mice, where the upregulation of this cytokine has been implicated in a number of acute and chronic inflammatory diseases, such as ischemic retinopathy or LPS-induced uveitis. An elevated level of this cytokine was observed in our study also where endogenous PACAP could preventd this. Inhibition of Akt activation by harmful stimuli, such as LPS-induced inflammation, prevents the inhibitory phosphorylation of GSK-3, promotes its kinase activity and increases the degree of organic injury. Consistent with results from other studies, our observation showed a decreased level of phosphorylated Akt and GSK during LPS-induced inflammation. The reduction of pAKT and pGSK was more severe in the LPS-treated PACAP KO group. This study tested the hypothesis that endogenous PACAP plays an anti-inflammatory role in LPS-induced retinal damage through preservation of PI3K/Akt functional activity. Previous studies have shown the functional protective effects of exogenously applied PACAP in different retinal injuries. Similarly to earlier studies, we demonstrated severe disturbance of visual function in the inflamed retinas by ERG. Endogenous PACAP successfully prevented pathologic changes, prevented the a-wave amplitude of ERG, thus protecting the photoreceptor cell function in LPS-induced retinal inflammation. The malfunction of Müller glial cells is involved in the decreased responses of b-wave in ERG, which was also preserved in the presence of the endogenous peptide. Our findings further suggest that endogenous PACAP represents an important part of the natural defense mechanism against retinal inflammation.

V. Summary of novel findings

I) In the first part of my Ph.D. work, we demonstrated the retinoprotective role of the PAC1 receptor in BCCAO-induced retinal damage using maxadilan.

In our histological analysis, we showed that intravitreal administration of maxadilan significantly inhibited the retinal damage caused by hypoperfusion. Our cytokine array results further confirmed that the PAC1 receptor plays an important anti-inflammatory role in ischemic retinal degeneration.

II) In the second half of the thesis, we confirmed the protective role of endogenous PACAP and we provide evidence that these anti-inflammatory effects are mostly mediated by PAC1 receptor in LPS-induced retinal inflammation.

The morphological investigation and the functional analysis demonstrated the protective role of endogenous PACAP in endotoxin-induced retinal degeneration. Intravitreal maxadilan treatment was able to reduce the tissue damage induced by LPS. From these findings we can conclude that regulation of inflammation by PACAP is mediated via PAC1 receptor in the retina.

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Publications that related to the thesis (cumulative impact faktor (IF: 4,986):

- **Váczy A,** Kővári P, Kovács K, Farkas K, Szabó E, Kvárik T, Kocsis B, Fülöp B, Atlasz T, Reglődi D. (2018) Protective role of endogenous PACAP in inflammation-induced retinal degeneration. *Curr Pharm Des* 24(30):3534-3542. /**IF: 2,757/**
- **Váczy A**, Reglődi D, Somoskeöy T, Kovács K, Lőkös E, Szabó E, Tamás A, Atlasz T. (2016) The protective role of PAC1-receptor agonist maxadilan in BCCAO-induced retinal degeneration. *J Mol Neurosci* 60(2):186-94. **/IF: 2,229/**
- Reglődi D, **Váczy A**, Rubio-Beltran E, MaassenVanDenBrink A. (2018) Protective effects of PACAP in ischemia. *J Headache Pain* 19(1):19. (Review) /**IF:** 3,403/

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- Kovács K, **Váczy A**, Fekete K, Kővári P, Atlasz T, Reglődi D, Gábriel R, Gallyas F (2019) PARP Inhibitor Protects Against Chronic Hypoxia/Reoxygenation-Induced Retinal Injury by Regulation of MAPKs, HIF1α, NRF2 and NFκB. *Ophthalmol Vis Sci* 19(60). /**IF**: 3,388/
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