The distribution of PACAP and its specific receptor, verification of the protective effect

Ph.D. thesis

Evelin Viktória Patkó

Supervisor: Tamás Atlasz, Ph.D.

Program leader: Dóra Reglődi, M.D., Ph.D., D.Sc.

Head of Doctoral School: Dóra Reglődi, M.D., Ph.D., D.Sc.

University of Pécs, Medical School

Department of Anatomy

Pécs, 2024

1. Introduction

1.1. The organ of vision

The human embryo's developing eye appears approximately in the third week of embryonic development. The retina, ciliary body, iris, and optic nerve arise from the neuroepithelium layer. The lens, corneal epithelium, and eyelid come from the surface ectoderm, while the sclera, corneal endothelium and stroma, blood vessels, muscles and vitreous arise from mesenchyme. These structures combine to form the complex sensory organ known as the organ of vision. The eyeball comprises three structural layers: the outer fibrous layer, which includes the sclera and the cornea; the vascular layer, which contains the iris, the ciliary body, and the choroid; and the innermost layer, which is called the retina. The anterior segment of the eyeball contains two chambers - the anterior and posterior chamber - separated by the iris, and connected by the aqueous humor, produced by the ciliary processes. The aqueous humor circulates from the posterior chamber to the anterior chamber, nourishing the avascular structures of the anterior segment, and drains through the trabecular meshwork. The trabecular meshwork is connected to the aqueous veins along the Schlemm's canal, which structures are connected to the venous system. Behind the posterior chamber lies the vitreous body, separated by the lens. In vertebrates, the retina, a specialized photosensitive layer of the eyeball, comprises ten histological layers, from inside to outside:

- Inner limiting membrane (ILM) – formed by the basal lamina of Müller's cells
- Nerve fiber layer (NFL) – ganglion cell processes that lead from the retina to the brain
- Ganglion cell layer (GCL) – cell bodies of the ganglion cells
- Inner plexiform layer (IPL) – horizontal-, amacrine-, bipolar-, and ganglion cell processes connected
- Inner nuclear layer (INL) cell bodies of horizontal-, amacrine-, bipolar-, and Müller's cells
- Outer plexiform layer (OPL) – processes of photoreceptors and processes of horizontal-, amacrine-, and bipolar cells connected to them
- Outer nuclear layer (ONL) – cell bodies of photoreceptors (rods and cones)
- Outer limiting membrane (OLM) – the apical boundary of Müller's cells
- Photoreceptor layer (PL) – contains the outer and inner segments of photoreceptors
- Pigment epithelium (PE) – supporting the neural part of the retina, not part of the neural retina

The structure of the eyeball and retina in rodents is similar to that of humans. The blood supply to the retina comes from branches of the ophthalmic artery, which create the central retinal artery and the posterior ciliary arteries. In the retina, there are three different vessel plexuses: the deep vascular plexus, the intermediate vascular plexus, and the superficial vascular plexus.

1.2. The *in vivo* study of the retina with optical coherence tomography and electroretinography

Optical coherence tomography (OCT) is a widely used imaging and diagnostic tool in ophthalmology. It uses interferometry and short-coherence length light to generate high-resolution images and scans in both two- and three-dimensions. By analyzing the reflected light from biological samples, OCT can provide non-invasive imaging and diagnosis of diseases related to the retina, optic nerve, and cornea.

On the other hand, Electroretinography (ERG) is another tool used for the diagnosis of retinal diseases. It provides cell-type-specific functional information of the retina by measuring electrical responses with corneal surface electrodes. ERG distinguishes different waveforms, namely a-wave and b-wave. A-wave represents the photoreceptor cell function, while b-wave gives information about bipolar-, Müller glial-, and amacrine cells. Ophthalmologists use ERG to diagnose retinal diseases such as diabetic retinopathy, retinitis pigmentosa, or retinal detachment.

1.3. Glaucoma

Glaucoma is a group of optic disorders that cause gradual loss of peripheral vision and can eventually lead to permanent blindness. The condition progresses slowly as the retinal ganglion cells (RGCs) and their axons, which include the optic nerve, degenerate over time. Although there are various types of glaucoma, they all share this common characteristic. In primary open-angle glaucoma, blockage of the trabecular meshwork and the aqueous humor drainage system leads to ocular hypertension. The increased intraocular pressure (IOP) eventually causes the degeneration of the sensitive ganglion cells. Although agents such as alpha adrenergic receptor blockers and beta-blockers can help facilitate the aqueous humor outflow or reduce its production, they can only slow down the progression of blindness. This highlights the strong need for a long-term neuroprotective treatment for glaucoma.

To better understand the development of the disease, reproducible and reliable animal models are crucial for investigating pathophysiology and testing treatments.

1.4. PACAP

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide found throughout the body that performs a variety of biological functions. PACAP belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily and is the most conserved member. PACAP has two biologically active forms: the shorter PACAP27 and the longer PACAP38, with the latter being the dominant form. PACAP activates its effects via the G-protein coupled PAC1 receptor (PAC1-R) or VPAC1 and VPAC2 receptors, which can also bind VIP. PACAP has a variety of physiological functions, including the modulation of neuronal excitability, immunomodulation, reproduction, and embryonic growth. Since its discovery, it has become clear that PACAP has a robust neuroprotective effect in various *in vivo* and *in vitro* studies. In several models of retinopathy, PACAP has been shown to have a protective effect. There is a large body of evidence confirming its widespread presence in human organs, including the organ of vision.

Previous studies have found that PACAP and its receptor are present in several structures of the eye, such as the cornea, sclera, iris, ciliary body, choroid, conjunctiva, and lacrimal glands. In animal models, PACAP has been found in various layers and cells of the retina, including retinal ganglion cells, bipolar cells, amacrine cells, horizontal cells, and the inner plexiform layer (IPL) and nerve fiber layer (NFL) of the retina.

2. Aims

Our research group has previously conducted studies on the distribution of PACAP and its receptor (PAC1-R) in the eyes of various animal species.

I, In the first part of our experiments, we aim to investigate the distribution of PACAP and its specific PAC1-R in human eyes. This way emphasizes the translational significance of the results from previous animal model studies.

These studies have shown that PACAP has a retinoprotective function in various ophthalmic diseases. In particular, we have confirmed the retinoprotective and IOP-lowering effects of PACAP eye drops in a rat glaucoma model. Since glaucoma is a common ophthalmic disease with a multifactorial pathomechanism, we believe it is important to investigate the effect of PACAP eye drops on other factors underlying the disease.

II, In the second part of our studies, we aim to investigate the effect of PACAP1-38 eye drops on the retinal vasculature and the presence of hypoxic molecules in a rat glaucoma model.

3. Research I. Distribution of PACAP and PAC1 receptor in the human eye

3.1. Materials and methods

Samples

Human eyes (n = 7 patients; 6 boys, 1 girl) were used in the experiment (ethical permission No: 6383-PTE 2018). The age of patients, undergoing enucleation surgery because of retinoblastoma, was 16 ± 10 months. Only the tumor-free, normal parts were used for histological analysis.

Immunohistological analysis

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 3-µm-thick sections with a rotational microtome. After epitope retrieval in 1 mM (pH = 6.0) citrate buffer, samples were incubated in anti-PAC1-R antibody and anti-PACAP38. After sections were incubated with HISTOLS-AP-R anti-rabbit alkaline phosphatase labeled detection system. The reaction was developed with HISTOLS Resistant AP-Red Chromogen/substrate System in the dark, for the chromogen substance magenta color was chosen, so positive immunoreaction would also be visible in the pigmented cells. Sections were counterstained with hematoxylin solution. Negative control was obtained when the primary antibody was replaced with TBS. The slides were digitalized and sections were analyzed using a semiquantitative approach. Immunoreactivity was scored by 3 researchers, between 0- +-+ +-+ + + depending on the staining intensity.

3.2. Results

The outer layer of the eyeball, known as the tunica fibrosa, is composed of the sclera and cornea. There was no PACAP or PAC1-R immunosignal detected in the sclera. However, the cornea's epithelium and endothelium showed moderate immunopositivity. Surprisingly, the middle stroma layer had no signal. The middle layer of the eyeball, known as the vascular layer, is made up of the iris, ciliary body, and choroid. The magenta-stained positive immunoreaction was visible in all layers of the iris. However, the sphincter pupillary muscle showed very weak immunoreactivity for PAC1-R. The ciliary body's pigmented and non-pigmented epithelium exhibited strong immunopositivity for both PACAP and PAC1-R. The stroma had weaker PACAP reactivity. The choroid was negative for the antibodies.

In the pigmented epithelial layer of the retina positivity for PACAP and a strong signal for PAC1-R was observed. However, the photoreceptor layer (rods and cones), and the outer nuclear and plexiform layers displayed weak or no immunostaining. Only in some samples, a weak signal for PAC1-R was shown. In the inner nuclear layer the cell bodies of the bipolar cells, amacrine cells, horizontal cells, and Müller glial cells, samples were positive in most cases for both the peptide and the receptor. In the ganglion cell layer, an interesting staining pattern was observed, some of the ganglion cells showed very strong immunosignal, while others were negative. The optic nerve had moderate immunoreactivity for PACAP and PAC1-R in the neuropil, while the glial cells only displayed immunopositivity for the receptor.

3.3. Discussion

In the present study, we describe the distribution of PACAP and PAC1-R-like immunoreactivity in the human eye. Our findings indicate that there is immunopositivity in the corneal epithelium and endothelium, as well as in the stroma and muscles of the iris and ciliary body. The retina shows strong immunoreactivity in several layers, including the pigment epithelial cells, while the optic nerve has weaker immunoreactivity. Previous studies have shown that PACAP occurs in the retinas and other ocular tissues of various species, including rats, mice, turtles, and fish. However, other parts of the eye have been less investigated. For example, PACAP immunoreactivity has been described in the cat choroid, and a study of the rabbit eye revealed PACAP immunoreactivity in homogenates of the iris, ciliary body, cornea, retina, and choroid. Our study is the first to provide a detailed description of the distribution of PACAP and PAC1-R-like immunoreactivity in the human eye.

The protective effects of PACAP on the retina have been widely researched and proven by numerous animal models and *in vitro* studies. PACAP has also been found to play a role in preventing retinal aging, as early signs of aging have been observed in animals lacking PACAP. Additionally, it has been shown that the melanopsin-containing retinal ganglion cells, which also express PACAP, are more resistant to degeneration, suggesting that PACAP is involved in endogenous protective mechanisms. The retinal pigment epithelial cells, which are the first layer of the neural retina, are crucial for the photoprotection, metabolism, membrane renewal, vitamin A storage, and growth factor supply of the photoreceptors. Studies have suggested their involvement in various retinal diseases such as diabetic retinopathy and age-related degeneration, and *in vitro* studies have shown that PACAP can protect human retinal pigment epithelial cells against various harmful effects.

We found that both layers of the blind part of the retina express strong immunoreactivity for PACAP and its receptor. The presence of PACAP in the aqueous humor has been investigated in rabbit and human fluid samples, which showed that PACAP could not be detected under normal conditions, only after stimulation, when PACAP levels increased in the aqueous humor. Although the direct involvement of PACAP in aqueous humor production is not yet established, several lines of evidence support this hypothesis. cAMP is known to trigger transepithelial fluid transport across the ciliary epithelium in mammals. As PACAP is a cAMP-stimulating peptide, it can be assumed that the neuropeptide plays a role endogenously in the aqueous humor production. The role of PACAP has been implied not only in the production but also in the absorption of the aqueous humor, as our most recent data have provided evidence that PACAP treatment leads to reduced intraocular pressure in a rat model of glaucoma.

In addition to the inner and middle layers of the eye, we found strong immunoreactivity in the cornea part of the outermost, fibrous layer of the eye, where the outer epithelial and inner endothelial layers were positive for both the peptide and its receptor. PACAP treatment on the corneal surface has been shown to induce recovery of the epithelial cells and also of the sensory innervation. PACAP KO mice present dry eye symptoms with corneal hyperkeratinization, also pointing to the importance of endogenous PACAP. Our finding that the endothelial cells display strong immunoreactivity for both PACAP and PAC1 receptors is in agreement with previous studies, which showed the presence of PACAP and PAC1-R in corneal endothelial cells isolated from human corneal cells. Among others, PACAP showed protective effects against growth factor deprivation and induced epidermal growth factor receptor phosphorylation. These results show that PACAP may be an important factor in corneal integrity.

PACAP and/or its receptors have been shown in most human tissues, with the eye being an exception. In the present study, we provided evidence for the widespread occurrence of PACAP and its PAC1-R in the human eye. In human tissues, expression levels of PACAP and/or its receptors show alterations in various diseases. Very limited data had been available on the occurrence and almost no data on the distribution of PACAP and its receptors in the human eye. As dozens of studies have described different effects of PACAP in the eye, our study indicating the widespread occurrence of PACAP and its specific receptor in the human eye implies that the *in vitro* cellular effects and *in vivo* results from animal studies have translational value and most probably are also present in the human eye.

4. Research II. Protective effects of PACAP on retinal vasculature and molecular responses in a rat model of glaucoma

4.1. Materials and methods

Animals, microbeads injection, and treatment

Adult male Sprague–Dawley (SD) rats (n = 30) weighing 300–500 g were maintained under a 12 h light/dark cycle and fed and watered ad libitum. All procedures were undertaken following the Animal Research Review Committee of the University of Pecs, Hungary (No. BA02/2000-50/2022). Rats were divided randomly into four experimental groups: (i) PBS + vehicle (Systane (S)) n = 5; (ii) PBS + PACAP1-38 (P) n = 5; (iii) microbeads + vehicle (S) n = 10; and (iv) microbeads + PACAP1-38 n = 10, referred to as PBS+S; PBS+P; Beads+S; and Beads+P, respectively. Polystyrene microbeads were injected (10 µl) into the anterior chamber of the eye, control animals received the same amount of PBS. The procedure was repeated two weeks later. The eye drops treatment was started one day after the microbeads injection, eyes were treated with Systane or PACAP1-38 eye drops (1 µg/drop) for four weeks. Intraocular pressure changes were monitored with the help of a rebound tonometer. OCT imaging was performed one day before and 8 weeks after the first microbeads injections. Radial volumetric images, centered on the optic nerve, were acquired from both eyes with SD-OCT and were analyzed by the Bioptigen Diver program.

Immunohistochemistry, retinal whole mount, vessel analysis

Rats were killed and eyes (n = 20) were fixed in 4% paraformaldehyde 8 weeks after the microbeads injection. Eyecups were immersed into a 10–20–30% sucrose solution and embedded in O.C.T. compound-mounting media, later thin sections were made (15–17 µm) on gelatin-coated slides with cryostat. Sections were blocked and incubated overnight at 4 °C with rabbit anti-HIF1-α diluted in 1:200 or mouse anti-VEGF-A diluted in 1:200. Immunoreactivity was detected with Alexa Fluor-488 diluted 1:800 in PBST. Cell nuclei were stained with propidium iodide, and were mounted with Fluoroshield. Microphotographs were made with a Nikon Eclipse Ti2-E microscope with a Nikon C2 confocal detector.

For retinal whole mounts (n = 24), eyecups were fixed in 4% paraformaldehyde for 2 h at room temperature, after washing the retina was removed from the eyecup, and made four small cuts. The vasculature staining was obtained with fluoresceinated isolectin solution. The labeled, isolated retinas were placed and unfolded on a glass slide and mounted with Fluoroshield

mounting medium. Images were made of the retinal whole mounts with a Nikon Eclipse 80i epifluorescence microscope. The lectin-stained retina images were thresholded and corrected with Adobe Photoshop CS6, and whole retinas were analyzed with the use of AngioTool software. The following vessel morphological parameters were measured: total blood vessel length, total number of junctions, total number of endpoints, and lacunarity (the distribution of gap area surrounding the vessels). The following parameters were used in the program: blood vessel diameter (2–30 μm) and pixel intensity (0–255). Vascular density was measured using the ImageJ Vessel Analysis plugin.

Western blot analysis

For western blot analysis, retinas (n = 16) were removed 8 weeks after the first injections. Samples were homogenized and protein concentrations were determined. Membranes were probed with rabbit anti-HIF1-α (1:2000) and mouse anti-VEGF-A (1:100; ó) for 1 h. Non-phosphorylated anti-GAPDH (1:20000) was used as an internal control. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h at room temperature. The antibody–antigen complexes were visualized through enhanced chemiluminescence. For the quantification of blots, band intensities were quantified by the NIH ImageJ program.

Statistics

Statistical comparisons were made using two-way ANOVA followed by Fischer's post hoc analysis (OCT results; AngioTool vessel analysis; vessel density; Western blot). Differences $p < 0.05$ were considered significant.

4.2. Results

The effect of PACAP1-38 eye drops on changes in intraocular pressure

In absolute control, the average intraocular pressure (IOP) value was 11.93±0.22 mmHg. The intraocular pressure of the control groups (PBS+S, PBS+P) showed no significant changes apart from the normal daily rhythm-dependent fluctuation. In the case of the glaucoma groups, a significant 40% increase in IOP was observed in the 2nd week after microbeads injection. From the 4th week onwards, a drop in IOP was observed in the Beads+P group. In the 8th week, we measured significantly increased IOP in the Beads+S group compared to the control groups, while the values of the microbeads-injected group receiving PACAP1-38 treatment did not show a significant difference compared to the controls.

Morphological changes of the retina

In the control groups 8 weeks after the microbeads injections there was no difference even in case of PACAP1-38 administration. In the case of the microbeads-injected vehicle-treated (Beads+S) animals severe retinal degeneration was observed compared to the PBS controls. Significant decreases in the following layer thicknesses were obtained: in the whole retinal thickness, in the RNFL, OPL, and the IS, OS of the photoreceptor layer. PACAP1-38 treatment led to a significant amelioration in the total retinal thickness. In summary, the microbeads-injected vehicle-treated group showed typical signs of glaucoma with structural degeneration, while PACAP1-38 treatment could counteract the deteriorating effects of high IOP.

Vessel analysis

The PBS-injected control groups had no difference in vessel morphology. The analysis of the Beads+S groups indicated a significant, 20% reduction in the total vessel length, and vessel density compared to the control groups. Also, a major difference was found in the total number of junctions, endpoints, and in the lacunarity. In case of the PACAP1-38-treated (Beads+P) group, the vessel morphology was similar to the control groups.

Immunohistochemical and protein level changes

The PBS-injected groups (PBS+S; PBS+P) did not show notable immunopositivity either in HIF1-α or VEGF-A expressions. In the Beads+S group, an intense HIF1-α immunoreactivity was detected in the retinal sections (IPL, GCL) and western blot panels. In the Beads+P group a slightly higher HIF1-α positivity was observed within the retina (GCL) and the western blot panels than the controls. The VEGF-A expression was high in the Beads+S group and also in the retinal sections (particularly within the GCL and RNFL) and western blot analysis. The PACAP1-38-treated group (Beads+P) had lower level of VEGF-A signal compared to the Beads+S group.

4.3. Discussion

Our study aimed to investigate the potential protective effects of PACAP on a rat model of glaucoma, focusing on the vascular theory of glaucoma pathogenesis. Recent research has shown that vascular disruption is a critical factor in the development of glaucoma. Our findings demonstrate that PACAP can reduce hypoxia and maintain the retinal vasculature in a hypertensive glaucoma model.

Based on our findings, we concluded that PACAP has the ability to pass through the ocular barriers when administered as eye drops. Furthermore, the presence of the specific receptor in the ciliary body and iris indicates that PACAP can bind to these receptors when provided in the form of eye drops.

In vivo imaging of retinal structures has been increasingly recognized as a valuable tool in the investigation of retinal degeneration in animal models. Also, recent studies on glaucoma patients have confirmed that OCT enables the detection of structural damage in the RNFL. A previous study has suggested that the thickness between the retinal pigment epithelium (RPE) and OS is associated with visual sensitivity in glaucoma. In our microbeads model, moderate hypertension induced morphological changes in several retinal layers (total retinal thickness, RNFL, OPL, IS, OS). These changes were similar to another SD glaucoma model induced by episcleral vein occlusion. The segments of the photoreceptor layer showed a significant decrease, which is in accordance with our previous study where we suggested there to be functional damage of the photoreceptors. As was also described earlier, we could demonstrate that the outer retina is affected along with the expected thinning of the RNFL. Glaucoma also affected the whole retinal thickness. A decrease of 10% was observed in the RNFL layer of the Beads+S group compared to the PBS-treated groups. These findings in the rat model are comparable to those in human glaucoma in terms of reduced total retinal thickness in the early stage of the disease. The application of PACAP eye drops could protect the whole retinal morphology in glaucoma and could also preserve the inner and outer layers of the retina.

The “vascular theory” of glaucoma pathogenesis hypothesizes the association of optic nerve damage and glaucoma with the changes in retinal vasculature. Microcirculatory changes have also been observed in glaucoma patients, and disrupted ocular blood flow leads to retinal injury. We observed compromised vascularization in the glaucomatous group, but PACAP could prevent these changes. Animal studies found similar changes in the retinal microvasculature in a rat magnetic bead model of ocular hypertensive glaucoma. In a previous study, it was described that the retinal structure changes appeared after the decrease in the retinal blood flow in glaucoma patients. Our present results suggested the disruption of the retinal vasculature in the glaucomatous group. In the case of the PACAP eye drops, the vasculature was similar to the control groups.

The dysregulation of blood flow with subsequent hypoxia in glaucoma has been suggested to have a connection to retinal ganglion cell death. Immunohistochemical studies described that HIF1-α levels were elevated in human post-mortem glaucomatous retinal tissue, which

indicates hypoxic conditions. Accordingly, our results demonstrated a similar change in the HIF1-α levels in retinal section. Also, this difference was supported by Western blot analysis. In the present study, we obtained results similar to previous findings that indicate HIF1-α was increased in the retinal tissues after IOP elevation. Elevated IOP is one of the most critical risk factors of glaucoma which can result in retinal ischemia. In the affected tissues, HIFs upregulate the production of some growth factors, mainly VEGF-A, which is produced in the eye, not only by RPE but also by ganglion cells, Müller glia, pericytes, and endothelial, glial, neural, and smooth muscle cells. VEGF-A acts on small blood vessels, inducing leakage of fluid in the retina and obliteration of capillaries, causing extra hypoxia and a further increase in VEGF-A production. VEGF-A levels were shown to be increased in the plasma of glaucoma patients when compared to healthy controls and in the aqueous humor of glaucoma patients compared to their plasma VEGF-A levels. Despite these findings, neovascularization is not impacted in glaucoma and the exact role of VEGF-A has not been examined in the glaucomatous retina. We showed the localization of VEGF-A within the retina, which was similar to that previously found, primarily localized to the GCL and the inner nuclear layer. Previously, evidence demonstrated that PACAP led to a decrease in HIF1-α and VEGF-A expression in a model of diabetic macular edema. Our present results confirmed elevated expression of HIF1-α and VEGF-A in glaucoma, and our findings suggest that PACAP is able to reduce the hypoxia-induced retinal and microvascular damage by decreasing HIF1-α and VEGF-A expression in glaucoma.

The increased intraocular pressure (IOP) can lead to damage to the inner lining of blood vessels (endothelium) and the expression of a molecule called NOX-2, which in turn causes the formation of reactive oxygen species (ROS). ROS formation can cause a state of reduced blood flow (ischemia) and subsequent lack of oxygen (hypoxia). In a hypoxic state, significant expression of a protein called VEGF-A can lead to the deterioration of the vascular network, as well as glial activation, neuroinflammation, and ultimately apoptosis. However, the protective peptide called PACAP can increase the production of a molecule called cAMP via the PAC1-R receptor, through the ERK-CREB pathway, thereby protecting cells from apoptosis and inhibiting the expression of inflammatory proteins.

It is well known that PACAP has anti-apoptotic, anti-inflammatory, and anti-oxidant effects, leading to neuroprotection. It has been stated that PACAP eye drops can suppress the symptoms of dry eye syndrome. It has also been described that PACAP has a protective effect in hypoxic conditions in BCCAO-induced retinopathy, in diabetic macular edema, and retinopathy of

prematurity. This list of retinopathies is now extended to glaucoma. In summary, our study provided evidence that PACAP, in a model of glaucoma, can preserve retinal structure, decrease vascular damage, and decrease hypoxia markers. These results suggest that PACAP eye drops could be a potential future therapeutic agent in glaucoma treatment. However, further study is needed to understand the exact underlying mechanism behind the protective effect.

5. Summary of novel findings

I, In the first part of my Ph.D. thesis, we investigated the occurrence of PACAP and its specific receptor (PAC1-R) in the human eye. Our study involved a semiquantitative analysis following immunohistochemical labeling. Based on the results, we concluded that the distribution of PACAP and PAC1-R in the human eye is similar to that previously described in mammalian models. We were the first to report on the occurrence of PACAP and its specific receptor in the human eye. Our findings confirm that previous *in vitro* and *in vivo* research on animals has translational value.

II, In the second half of my Ph.D. thesis, we examined the role of topically applied PACAP eye drops in protecting the retinal vasculature in a microbeads-induced glaucoma model under hypoxia. We conducted OCT, immunohistochemistry, vascular network, and western blot analyses to evaluate the effect of PACAP eye drop treatment. Our results confirmed that the treatment had a protective effect. Our results can form the basis of a future therapeutic option, which can be included in the supplementary treatment of glaucoma in an easily applied, non-invasive way by developing an eye drops therapy.

In summary, our research concludes that PACAP and its specific receptor are widely present in the human eye, which confirms the translational value of previous *in vitro* and *in vivo* results. Based on our study and previous research, we believe that the results of PACAP research can have significant therapeutic potential in the treatment of glaucoma.

6. Acknowledgements

I am thankful for my supervisor, **Tamás Atlasz**, Ph.D., who has always been supportive of my scientific career. I am also grateful to **Dóra Reglődi**, M.D., Ph.D., D.Sc., for providing me with many opportunities to learn and improve. Their guidance has been invaluable in my journey.

I would like to express my gratitude to **Edina Szabó**, Ph.D. You have been my biggest supporter, my friend, and I will never forget it.

I am also thankful for **Alexandra Váczy**, Ph.D. supported my work, and provided guidance.

I am also thankful for **Dorottya Molitor**, who has supported me both professionally and as a great friend. We were always there for each other in good and bad times.

I would like to thank **Inez Bosnyák** and **Dénes Tóth** for their help with my work. I am also grateful to **Adrienne Csutak**, M.D., Ph.D., D.Sc., for sharing her clinical experiences with me.

I am thankful to every member of the **Retina Research Group** and all my colleagues at the **Department of Anatomy** who have helped me with my work.

Finally, I would like to express my gratitude to **my family** and **my friends** who have always been supportive of me.

Supports: NKFIH FK129190, NKFIH K135457, ÚNKP-21-3-I-PTE-1299, ÚNKP-22-3-II-PTE-1402, ÚNKP-23-3-II-PTE-2034.

7. Publications

The thesis is based on the following publications (cumulative impact factor (IF): **9,652**)

Patko E., Szabo E., Toth D., Tornoczki T., Bosnyak I., Vaczy A., Atlasz T., Reglodi D. (2022). Distribution of PACAP and PAC1 receptor in the human eye. J Mol Neurosci 72(11):2176-2187, https://doi.org/10.1007/s12031-022-01985-0

(IF=3,444)

Patko E., Szabo E., Vaczy A., Molitor D., Tari E., Li L., Csutak A., Toth G., Reglodi D., Atlasz T. (2023). Protective effects of pituitary adenylate-cyclase-activating polypeptide on retinal vasculature and molecular responses in a rat model of moderate glaucoma. Int J Mol Sci 24(17):13256, https://doi.org/10.3390/ijms241713256

(IF= 6,208)

Other publications (cumulative IF: **9,123**)

Szabo E., **Patko E**., Vaczy A., Molitor D., Csutak A., Toth G., Reglodi D., Atlasz T. (2021). Retinoprotective effects of PACAP eye drops in microbead-induced glaucoma model in rats. Int J Mol Sci 22, 8825. http://doi.org/10.3390/ijms22168825

(IF=5,923)

Tóth D., Fabian E., Szabo E., **Patko E.**, Vicena V., Vaczy V., Atlasz T., Tornoczky T., Reglodi D. (2024). Investigation of PACAP38 and PAC1 receptor expression in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells. Life 14(2), 185; https://doi.org/10.3390/life14020185

(IF=3,2)

Total cumulative impact factor of all publications: **18,775**