Investigation of the retinoprotective effects of neuropeptides

Ph.D. thesis

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INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide with widespread occurrence in various organs and diverse effects both in the nervous system and in the periphery. PACAP is strongly expressed in the central nervous system, where it exerts several effects such as it is a central regulator of circadian rhythmic activities, plays a role in memory formation and psychiatric processes, and is involved in central feeding control. It elicits these actions by binding the G-protein-coupled receptors, PAC1- and VPAC1/2, which also bind vasoactive intestinal polypeptide (VIP). PACAP is also known to be expressed in the retina, along with its receptors (PAC1, VPAC1 and VPAC2 receptors). Numerous studies have provided evidence that the neuroprotective effects are mainly mediated by the PAC1 receptor and diverging downstream pathways upon its activation.

Soon after the discovery of PACAP it became evident that the peptide has strong neuroprotective effects, in several *in vitro* and *in vivo* models. The first *in vivo* proof of its neuroprotective action came from studies of global cerebral ischemia. Subsequent studies confirmed the efficacy of PACAP in cerebral ischemic conditions, Parkinson's desease and also in ischemic lesions of other organs, including kidney, intestine and heart. In the retina, ischemic injury can be induced by ligating both carotid arteries transiently or permanently (bilateral carotid artery occlusion - BCCAO), leading to ischemia/reperfusion injury or chronic retinal hypoperfusion, respectively, methods which mimic several hallmarks of chronic retinal degeneration. PACAP is protective in both types of ischemic retinal lesions. Earlier we showed that PACAP1-38 ameliorates the reduction in thickness of different retinal layers as well as the loss of cells in the ganglion cell layer (GCL) in BCCAO-induced injury.

The bioavailability and fast degradation of PACAP limit its therapeutic use and attention has been drawn to the application of shorter fragments and/or analogs. N-terminally shorter fragments of PACAP usually have antagonistic effects, but some reports have documented agonistic behavior, depending on the cell/tissue type. In contrast, C-terminally shorter fragments usually differ in the strength of receptorial binding. Therefore, it is necessary to test whether shorter PACAP fragments have any effect, ameliorating or damaging, on retinal lesions. We have previously shown that PACAP 6-38, the most widely used antagonist of PACAP, has an aggravating effect in retinal excitotoxic lesion. However, it is not known, whether the shorter fragments of PACAP have any effect in the retina. In a recent study we

have shown that the peptide most closely related to PACAP, namely vasoactive intestinal peptide (VIP), is also protective in retinal ischemia. However, to achieve a degree of neuroprotection similar to PACAP, higher doses are required. Other members of the peptide family (secretin, glucagon) have not been tested in retinal ischemia so far.

Altogether, these results provide strong evidence that PACAP has potential therapeutic value in ischemic retinopathy. However, a major drawback of PACAP-induced retinal therapy is that in all these studies PACAP was given intravitreally. Although this route of administration is a common clinical practice in several diseases, like age-related macular degeneration and diabetic retinopathy, easier administration routes would be very important from the clinical point-of-view. Topical administration (eye drops) provides a suitable non-invasive method for treating ophthalmic diseases. PACAP eye drops has been shown to have topical effects in the cornea, but it is not known whether PACAP would penetrate the ocular barriers, reaching the retina.

AIMS OF THE STUDY

1. The first aim of our present study was to examine the effects of PACAP fragments 4-13, 4-22, 6-10, 6-15, 11-15, 20-31, and of the other members of the peptide family (secretin and glucagon) in chronic retinal hypoperfusion induced by bilateral carotid artery occlusion

2. The second aim of the present study was to investigate the retinoprotective efficacy of PACAP1-27 and 1-38 given as eye drops in ischemic retinopathy.

1. INVESTIGATING THE SECRETIN, GLUCAGON AND PACAP FRAGMENTS IN ISCHEMIC RETINOPATHY

MATERIALS AND METHODS

1. Bilateral carotis sommunis occlusion (BCCAO)

Experimental animals derived from a local colony of 3-4 month old Wistar rats (sham n=7, BCCAO n=32). Animals were housed in individual cages, fed and watered ad libitum, under light/dark cycles of 12/12 h. All animal procedures complied with the University of Pecs (No: BA02/2000- 15024/2011) for the ethical use of animals. Rats were exposed to permanent bilateral common carotid artery occlusion (BCCAO) under isoflurane anesthesia and both common carotid arteries were ligated with a 3-0 filament through a midline incision. A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid arteries. These animals served as sham-operated animals. Immediately following the operation, PACAP 1-38 or its fragments (PACAP 4-13, 4-22, 6-10, 6-15, 11-15, 20-31) or other members of the peptide family such as secretin and glucagon (100 pM/5µl) was injected intravitreally using 30 G Hamilton syringe into the right vitreous body of animals. The left eyes received the same volumes of vehicle treatment (physiological saline) and served as ischemic eyes.

Rats were sacrificed with an overdose of anesthetic after 2 weeks after BCCAO and the eyes were processed for histological analysis.The following parameters were measured: cross-section from the outer limiting membrane (OLM) to the inner limiting membrane (ILM), and the width of the outer and inner nuclear and plexiform layers (ONL, INL, OPL, IPL, respectively) and the number of cells/100 µm section length in the ganglion cell layer (GCL) the number of cells/500 μm^2 area in the INL. Results are presented as mean ± S.E.M. Statistical comparisons were made using the ANOVA test followed by Tukey-B`s *post hoc* analysis.

RESULTS

BCCAO resulted in severely reduced thickness of retinal layers as observed two weeks after ligation (compared to sham-operated controls). Morphometric analysis revealed that the most pronounced reduction in thickness in retinas with BCCAO was found in the OPL. Many cells in the ganglion cell layer also suffered degeneration, shown by necrotic cells in this layer. Intraocular PACAP 1-38 treatment following BCCAO led to nearly healthy appearance of the retinal layers. The thickness of the major retinal layers was almost identical with that of the sham-operated animals and was significantly larger than that of control ischemic ones. This was especially conspicuous in the OPL, which disappeared, and the ONL and INL layers were fused in several control animals and were preserved in all PACAP 1-38 injected animals.

Intravitreal injection of different PACAP fragments (PACAP 4-22, 6-15, 11-15, 20-31, 6-10 and 4-13) did not ameliorate the ischemic damage of the retina. In all samples, nuclear layers (ONL, INL) and plexiform layers (OPL, IPL) were reduced, and the cell number in the ganglionic layer was significantly less than in sham-operated controls. However, injection with PACAP 6-10 and 4-13 resulted in further reduction of the inner plexiform and nuclear layers. Treatment with members of the PACAP-related peptide superfamily, glucagon and secretin, led to no aggravation or amelioration of the ischemic retinal lesion. These observations were confirmed by morphometric measurements.

2. THE PACAP1-27 AND 1-38 EYE DROPS TREATEMENT IN ISCHEMIC RETINOPATHY

MATERIALS AND METHODS

1. Surgery and PACAP eye drops treatment

Experimental animals derived from a local colony of 3-4 month old Wistar rats (PACAP1-27: Σn=130: n=43 morphometric analysis, n=11 immunhistological analysis, n=20 immuno-blot analysis, n=28 citokin array, n=28 ERG analysis; PACAP1-38: n=12 morphometric analysis, n=4 immunhistological analysis). Immediately following the BCCAO operation, the rats were treated with PACAP eye drops. A group of animals underwent anesthesia and all steps of the surgical procedure except ligation of the carotid arteries. The right eye was treated with PACAP1-27 eye drops (1 μg/drops). The left eye, serving as a control, was treated only with vehicle. In the experiment for histological analysis PACAP1-27 was dissolved in following vehicles: saline, aqua ad iniectabilia, solvens viscosa pro oculoguttis cum thiomersalo, solutio ophthalmica cum benzalkonio. Solutio ophthalmica cum benzalkonio was the most effective in the histological analysis so in the other type of examinations this vehicle was used. PACAP1-38 was dissolved only solutio ophthalmica cum benzalkonio (1 μg/drops). Rats were treated for 5 consecutive days, twice a day with one drop.

2. Histological analysis of PACAP1-27 and 1-38

Rats (43 animals: PACAP1-27: n=7 sham, n=24 BCCAO; PACAP1-38: n=3 sham, n=9 BCCAO) were sacrificed with an overdose of anesthetic 2 weeks after BCCAO and eyes were processed for histological analysis. The following parameters were measured: cross-section from the outer limiting membrane to the inner limiting membrane, and width of all retinal layers: outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer. The number of cells in the ganglion cell layer was also measured. Statistical comparisons were made using the two-way ANOVA followed by Fischer's *post hoc* analysis.

3. Radioactive labeling of PACAP1-27 and 1-38

This part of the experiment was done in collaboration with William A. Banks, és Therese S. Salameh (Education, and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, WA, USA; Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA). PACAP1-27 was labeled with ^{125}I using the lactoperoxidase method. Male CD-1 mice were anesthetized with urethane and administered PACAP1-27 and 1-38 as an eye drop ($1x10^6$ cpm/eye in benzalkonium-chloride). At various time points, blood was collected from the carotid artery, and the eyes and whole brain removed. The time points used in this study were 5, 30, 60, and 120 minutes. Mice (n=3-6) were used at each time point.

4. Measurement of glial fibrillary acidic protein (GFAP) activity in the Müller glial cells after PACAP1-27 and 1-38 eye drops treatments

For immunohistochemical analysis, 2 weeks after the induction of ischemia, animals (PACAP1-27: n=2 sham, n=5 BCCAO; PACAP1-38: n=2 sham, n=2 BCCAO) were sacrificed with an overdose of anesthetic, the eyes were immediately dissected in ice-cold PBS and fixed in 4% paraformaldehyde dissolved in 0.1M PB (pH 7.4) for 4 hours at room temperature, similarly to earlier descriptions. Briefly, tissues were washed in 0.1M PB (6x10 minutes) and cryoprotected in 10% and 20% sucrose for 1 hour, followed by 30% sucrose in PBS overnight at 4 °C. For cryostat sectioning, retinas were embedded in tissue freezing medium, and cut in a cryostat at 10–12μm. Central retinal areas within 2mm from the optic nerve head were used for immunocytochemical analysis.

5. Immuno-blot analysis after PACAP1-27 eye drops

For western blot experiments, a separate group of retinas was removed 2 weeks (n=3 sham and n=9 BCCAO animals) and 24 hours (n=4 sham and n=4 BCCAO animals) after BCCAO. Samples were processed for western blot analysis as described earlier. Membranes were probed overnight at 4°C with anti-total Akt, phospho-specific anti Akt, phospho-specific anti-ERK1/2 and anti-actin.Total-Akt (tAkt) antibody was used as internal control for phospho-Akt (pAkt), actin antibody was used as control for phospho-ERK (pERK) 1/2. The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After the scanning step, results were quantified using the NIH ImageJ program. Pixel volumes of the spot were normalized to the internal controls. Data are represented by pixel density in arbitrary units (two-way ANOVA test followed by Fischer's post hoc analysis).

6. Cytokine array after PACAP1-27 eye drops treatments

For semiquantitative cytokine array, retinas (n=11 sham and n=17 BCCAO) were removed after 24 hours of BCCAO operation and homogenized in PBS with protease inhibitors. Samples were pooled in three replicates (n=3 per replicate). Samples were stored at −80 °C prior to use. Cytokine array from tissue homogenates was performed using Rat Cytokine Array Panel A Array kit from R&D Systems. After washing with buffer 3 times and addition of horseradish peroxidase-conjugated streptavidin to each membrane we exposed them to a chemiluminescent detection reagent then to an X-ray film cassette. The developed films were scanned, and the pixel volumes of the spot were determined by using ImageJ Protein Array Analyzer. Pixel volumes of the spot of interest were normalized to the control (sham retinas).The array was repeated four times.

7. Electroretinography (ERG) measurement

ERG measurements were performed to assess retinal function in each group (n=11 sham and, n=17 BCCAO animals), after an overnight dark adaptation. Animals were anesthetized by 0.7ml intraperitoneal injection of ketamine and xylazine cocktail during the measurements. The pupils were dilated with 0.5% cyclopentolate and oxybuprocaine 0.4% eye drop was used for the topical anesthesia. The responses to light flashes (5.0cd/m2, 0.25Hz, 503nm green LED light) were pre-amplified, amplified and recorded with an A/D converter. Responses were averaged with the software of the A/D converter to draw the graph of each measurement. The values of the selected parameters were analyzed by two-way ANOVA test followed by Fischer's *post hoc* analysis.

RESULTS

1. Retina morphology and morphometry

BCCAO resulted in severely reduced thickness of retinal layers two weeks after ligation, compared to sham animals. PACAP1-27 alone in sham animals did not result in alteration of any retinal layer. PACAP dissolved in saline, aqua ad iniectabilia or solvens viscosa pro oculoguttis cum thiomersalo did not result in any protective effect. However, PACAP1-27 dissolved in solutio ophthalmica cum benzalkonio led to significant protection in the retina. The BCCAO retinas treated with PACAP1-27 dissolved in solutio ophthalmica cum benzalkonio had a more preserved structure compared to vehicle-treated retinas. OLM-ILM distance was reduced by 50.7% in ischemic retinas compared to sham controls, but was reduced only by 36.1% in the eyes treated with PACAP1-27. Similar protection could be observed in the OPL (BCCAO: 54.2%, PACAP1-27: 47.9%), INL (BCCAO: 46.5%, PACAP1-27: 30.7%), and IPL (BCCAO: 61.4%, PACAP1-27: 38.3%), while no protection could be seen in the ONL (BCCAO: 35.3%, PACAP1-27: 36.3%).

BCCAO resulted in severely reduced thickness of retinal layers two weeks after ligation, compared to sham retinas. PACAP1-38 alone in sham animals did not result in changes in any of the retinal layers. PACAP1-38 dissolved in solutio ophthalmica cum benzalkonio led to significant protection in the retina in BCCAO-lesioned retinas, retinas treated with PACAP1-38 eye drops had a more preserved structure compared to control retinas. OLM-ILM distance was reduced by 49.7 % in BCCAO retinas compared to sham controls, but it was only 40.6 % in the eyes treated with PACAP1-38 eye drops. A protection to a similar degree was found in the INL (BCCAO: 38.5 %, PACAP1-38: 30.5 %), and IPL (BCCAO: 64.8 %, PACAP1-38: 38.2 %) while no statistically significant attenuation of the damage was observed in the ONL (BCCAO: 36.5 %, PACAP1-38: 37.7 %) and OPL (BCCAO: 53.0 %, PACAP1-38: 48.2 %). The number of cells in the GCL were significantly decreased in ischemic retinas (left eyes) and was significantly ameliorated by PACAP1-27 and 1-38 dissolved in solutio ophthalmica cum benzalkonio (right eyes). Based on the findings, solutio ophthalmica cum benzalkonio was the most effective vehicle for PACAP1-27 and 1-38 to exert neuroprotective effect in the retina; therefore, we used this solution for further experiments.

2. PACAP1-27 and 1-38 uptake after ocular administration

Radioactively labeled PACAP (I-PACAP) 1-27 and 1-38 was administered ocularly in solution ophtalmica cum benzalkonio to male CD-1 mice for a period ranging from 5 - 120 minutes. I-PACAP1-27 showed transport across the cornea, peaking 60 minutes, and I-PACAP1-38 peaking 120 minutes after application. The vitreous humor showed a similar profile to the cornea (I-PACAP1-27 and 1-38 peaking 60 minutes after application). In the retina, I-PACAP1-27 uptake increased with time, plateauing at 60 minutes, and I-PACAP1-38 plateauing 30 minutes. I-PACAP1-27 was transported rapidly to the whole brain before starting to decline after 30 minutes. By 120 minutes post application, the I-PACAP1-27 signal was barely detectable in the brain. Appearance of I-PACAP1-27 and 1-38 in the blood stream after ocular administration was delayed.

3. Analysis of Muller glial cells

GFAP filaments are intermediate filaments expressed mainly by astrocytes and ependymal cells in the central nervous system. In the retina, they are normally present in the inner part of the Muller glial cells and their endfeet forming the ILM. GFAP was markedly upregulated following BCCAO with an altered distribution pattern: immunopositive signal was observed in the entire cell from the OLM to ILM. It is that the PACAP1-27 and 1-38 eye drops appeared to ameliorate GFAP upregulation to some extent.

4. Phosphorylation of Akt and ERK1/2 after PACAP1-27 treatment

No changes were detected between groups in retinas removed two weeks after BCCAO, however, marked alterations were seen in retinas 24 hours after the induction of ischemia. Ischemia itself caused a decrease in the expression of phospho Akt (pAkt), which was not only reversed by PACAP1-27 dissolved in solution ophthalmica cum benzalkonio, but a robust increase could be observed. PACAP1-27 eye drops treatment alone led to a slight decrease of phospho ERK1/2 (pERK1/2) compared to the sham group. Ischemia induced a strong phosphorylation of ERK1/2 compared to sham retinas. This was further increased by PACAP1-27 eye drops treatement.

5. Effect of PACAP1-27 eye drops on ischemia-induced changes in cytokine expression

Cytokine expression was tested using a cytokine array. Expression of several cytokines increased after ischemia, including chemoattractant proteins, chemokines of the CINC (cytokine-induced neutrophil chemoattractant) and MIP (macrophage inflammatory protein) families: CINC-1 and MIP-3α. The activation of the thymus chemokine, TIMP-1 (tissue inhibitor of metalloproteinase) and VEGF (vascular endothelial growth factor) was also increased in the retinas that underwent BCCAO. PACAP1-27 eye drops treatment attenuated activation of all the above-mentioned cytokines. Expression of other cytokines analyzed by the array did not show any marked changes. Although several other cytokines showed slight alterations, only those are displayed in graphs and discussed, where we detected more than 20% change in the normalized data compared to sham group.

6. Protective effect of PACAP1-27 eye drops on visual responses after retinal ischemia

We investigated the possible effects of PACAP1-27 eye drops treatment in BCCAO-induced ischemic retinal degeneration on neuronal cell activity by recording ERGs 14 days after the induction of ischemia. ERG wave forms in sham animals were similar in both left and right eyes. Differences were found in the average amplitudes of the b waves between sham and BCCAO eyes (decrease by 25.1% compared to the sham groups). There was no statistical difference in the ERGs of BCCAO + PACAP1-27 and BCCAO eyes, however PACAP1-27 treatment seemed to slightly increase b wave amplitude comparing to BCCAO (decrease by 19.9% compared to the sham + PACAP1-27 groups). Analysis of other wave features (amplitude of a wave, oscillatory potential, implicit times) of ERG recording did not show any differences.

DISCUSSION, CONCLUSIONS

1. Summary of the experiments with secretin and glucagon, and PACAP fragments in ischemic retinopathy

Besides its supposed physiological actions in the retina, PACAP is a well-established retinoprotective peptide. Endogenous protection is proven by the increased vulnerability of the retina in mice lacking endogenous PACAP in ischemic retinal lesion, and in NMDA-induced excitotoxic injury. These observations are further supported by the increased apoptotic activity in retinas injected by the PACAP antagonist PACAP 6-38. Given the very potent actions of PACAP in retinal and other pathologies, there is an urging demand for developing novel analogues and or fragments to increase the bioavailability and stability of the peptide for future potential therapeutic use. Binding PACAP to other carrier molecules is a possibility to increase its capacity to traverse through membranes and to exert cytoprotective effects. It has also been shown that PACAP is able to pass through cell membranes and convey non-receptor mediated effects. This has raised the possibility of using PACAP fragments also for supporting transport of other non-penetrable molecules. Several analogues have also been developed for these purposes. Although a lot of pharmacological and receptor binding studies have been performed with fragments/analogues of PACAP, only few studies have tested the biological efficacy of these forms. Our present results show that the shorter fragments of PACAP, as expected, do not provide any neuroprotection. Relatively little is known about other members of the peptide family in the retina. Glucagonergic amacrine cells represent a small subpopulation of the amacrine cells possibly playing a role in the visual processing. An early study has shown that glucagon increases cAMP in chick retinal Muller cells, similarly to the actions of VIP. In cerebral ischemia, glucagon has been shown to exert neuroprotective effects in vivo. It has not been investigated so far whether secretin or glucagon are protective in retinal lesions. Based on our present study, neither glucagon nor secretin has protective action in retinal ischemia.

Our present results confirm that the natural forms of the peptide, PACAP1-38 and 1-27, are the most effective peptide forms in retinal ischemia, and the 38 and 27 amino acid form of the peptide cannot be replaced by another fragment or another member of the peptide family we know of. Our results further support the potent retinoprotective effects of PACAP and call for further studies to establish the future possible clinical introduction of PACAP-related retinoprotective therapeutic approach.

2. Summary of the experiments with PACAP1-27 and 1-38 eye drops in BCCAO induced ischemic retinopathy

One major disadvantage of *in vivo* PACAP treatments is the poor bioavailability of the natural peptide due to its fast cleavage by the dipeptidyl-peptidase (DPPIV) enzyme. PACAP has systemic effects when given intravenously or intraperitoneally, such as a decrease in blood pressure, facial flushing, migraine-like attacks in migraineurs and hormonal changes. Given these side effects and the fact that as far as ocular diseases are concerned, the first choice of treatment is topical application of drops or oinments, we aimed at investigating whether topical PACAP administration would be beneficial in a model of chronic retinal hypoperfusion.

The morphological results of the present study showed that PACAP1-27 and 1-38, dissolved in solution ophthalmica cum benzalkonio, significantly ameliorated the BCCAO-induced retinal damage, indicating that applying the appropriate vehicle, PACAP can reach the retina from the ocular surface. Indeed, using radioactive labeling, we provided evidence that PACAP passes through the cornea, vitreous body to the retina. In light of the above-mentioned side effects, an important finding of our study is that very little of the PACAP1-27 and 1-38 administered in solution ophthalmica cum benzalkonio as eye drops reached the systemic circulation or the brain. Topical application to skin was used to achieve dermal vasodilation while buccal delivery for treatment of type 2 diabetes. Topical ocular application of PACAP has already been used to test local, corneal effects of the peptide. It has been found that PACAP, given in eye drops, enhances corneal wound recovery, stimulates lacrimal secretion and accelerates nerve regeneration. However, the effects of topical PACAP on the retina have never been investigated. Some other growth factors have already been used as potential topical treatments, such as nerve growth factor (NGF). It has anti-apoptotic/anti-oxidant effects and promotes an anti-inflammatory status. We have previously shown that PACAP, binding to its receptors present in the retina, stimulates anti-apoptotic, whereas inhibits pro-apoptotic pathways, including the pathways studied here: ERK1/2 and Akt. PACAP not only has strong anti-apoptotic properties, but also has anti-inflammatory and anti-oxidant actions. In the retina, we have previously described that PACAP shifts the injury-induced inflammatory cytokine/chemokine profile towards an anti-inflammatory profile in a very similar manner to our present findings, where the BCCAO-induced increase of the cytokines CINC-1, MIP-3α, thymus chemokine, TIMP-1 and VEGF was attenuated by PACAP treatment. Glial cell activation is a pathological sign typically present in lesions, including retinal ischemia. Here we showed that Muller glial cell activation was markedly increased after induction of ischemia, whereas ocular PACAP1-27 and 1-38 dissolved in solution ophthalmica cum benzalkonio

attenuated it, similarly to our earlier descriptions in ischemic and diabetic retinal lesions. Functional amelioration with PACAP treatment in ocular injuries has also been shown earlier by ERG. We could not find such a marked functional protection in the present study, but a slight ameliorating effect was found.

In summary, we found that PACAP dissolved in solution ophthalmica cum benzalkonio and delivered as eye drops exerted retinoprotective effects and deserves further investigation in order to exploit its therapeutic potential in retinal degenerations in an easy-to-use form of application.

PUBLICATIONS

1. Publications related to the thesis

Werling D, Reglődi D, William AB, Theresa SS, Kovács K, Kvárik T, Váczy A, Kovács L, Lőkös E, Tamás A, Tóth G, Biró Zs, Atlasz T (2016) Ocular delivery of PACAP1-27 protects the retina from ischemic damage in rats. Invest Ophthalmol Vis Sci 57:6683-6691. (IF: 3,427)

Werling D, Reglődi D, Kiss P, Tóth G, Szabadfi K, Tamás A, Biró Z, Atlasz T (2014) Investigation of PACAP fragments and related peptides in chronic retinal hypoperfusion. J Ophthalmol 2014:563812. (IF: 1,425)

Atlasz T, Váczy A, **Werling D**, Kiss P, Tamás A, Kovács K, Fábián E, Kvárik T, Mammel B, Dányádi B, Lőkös E, Reglődi D (2016) Neuroprotective effects of PACAP in the retina. in Pituitary Adenylate Cyclase Activating Polypeptide – PACAP, edited by Dóra Reglődi and Andrea Tamás. New York: Springer Nature 501-527.

2. Publications not related to the thesis

Fehér A, Pusch G, Harang G, Gasztonyi B, Papp E, **Werling D**, Menyhárt M, Komáromy H, Szapáry L, Feher G (2011) Aspirin resistance in cerebrovascular patients. Int J Cardiol 152:111-112. (IF: 3,47)

Fehér A, Pusch G, Harang G, Gasztonyi B, Papp E, **Werling D**, Menyhárt M, Komáromy H, Szapáry L, Feher G (2011) Az acetilszalicilsav-rezisztencia klinikai jelentősége a cerebrovascularis betegek esetében. LAM 21:719-724.

Kvárik T, Mammel B, Reglődi D, Kovács K, **Werling D**, Bede B, Váczy A, Fábián E, Tóth G, Kiss P, Tamás A, Ertl T, Gyarmati J, Atlasz T (2016) PACAP is protective in a rat model of retinopathy of prematurity. J Mol Neurosci 60:179-85. (IF: 2,352)

Impact factor of publications related to the thesis: **4,852**
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