PROTECTIVE EFFECT OF ANAESTHETIC PRECONDITIONING AND THE VIP/PACAP PEPTIDE FAMILY IN ISCHEMIC RETINOPATHY

Doctoral (Ph.D.) thesis

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INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (**PACAP**) is a widely distributed neuropeptide, first isolated from hypothalamic extracts. PACAP belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family. PACAP occurs throughout the nervous system and in peripheral organs. In the retina, PACAP immunoreactivity is present in the amacrine and horizontal cells, in the inner plexiform layer (IPL), in the ganglion cell layer (GCL), and in the nerve fiber layer (OFL). There are two types of PACAP receptors: PAC1 receptor, which binds PACAP with much higher affinity than VIP and VPAC1 and VPAC2 receptors, which bind VIP and PACAP with similar affinities. In the retina, the selective PAC1 receptor is predominant and its mRNA is present in the ganglion cells, amacrine cells and the inner nuclear layer (INL). The neurotrophic and neuroprotective effects of the peptide are now well established. PACAP protects neurons against different toxic agents in vitro and provides neuroprotection in several models of brain pathology. Among others, PACAP treatment results in smaller infarct size in focal cerebral ischemia, it leads to less extensive hippocampal damage in global cerebral ischemia, and it decreases postischemic endothelial dysfunction. PACAP is also protective in ischemic retinal lesions, in addition to the various other types of retinopathies in animal models, such as diabetic retinopathy, excitotoxic retinal injury, UV lightinduced degeneration, high intraocular pressure caused hypoperfusion, bilateral common carotid artery occlusion (**BCCAO**). Based on the important neurotrophic effects of PACAP during neuronal development, the involvement of the peptide in endogenous restorative processes was hypothesized. Several studies have demonstrated that endogenous PACAP increases upon nervous injury. Mice deficient in endogenous PACAP (**PACAP KO**) respond to insults with more severe deficits and lower level of regeneration, for example they have larger infarct size in models of stroke.

Vasoactive intestinal peptide **(VIP)** is a member of the secretin/glucagon/VIP superfamily. VIP is a pleiotropic neuropeptide, with various effects in the central and peripheral nervous system. VIP acts on 3 receptors, the VPAC1 and VPAC2, which bind VIP and PACAP with similar affinity, and PAC1 which binds PACAP with higher affinity. VIP is a multifunctional peptide, exerting vasoactive, immune, behavioral, and anti-inflammatory effects. VIP has also been shown to exert neuroprotective effects in

various in vitro and in vivo injury models. Less is known about the retinoprotective effects of VIP. Considering ischemic lesions, the protective effects of VIP have been shown in focal ischemia of the brain. In the retina, it has been demonstrated that VIP protects against lipid peroxidation following ligation of ophthalmic vessels.

Preconditioning is a process through which a prior exposure to certain stimuli can induce protection against the damaging effects of a subsequent insult. In the nervous system, multiple stimuli exert neuroprotection in ischemic conditions like short episodes of ischemia or hypoxia, and chemical agents including volatile anaesthetics. Isoflurane, sevoflurane and halothane have been shown to induce neuroprotection in cerebral ischemia. This phenomenon is referred to as anaesthetic preconditioning and has been demonstrated in various tissues and organs. This protection involves two phases, the early and late preconditioning. The early phase begins immediately following the stimulus and lasts for up to 3 h. The late phase develops 18–24 h after the stimulus and can last for 3 days. Retinal ischemia is a pathology involving mechanisms similar to cerebral ischemia. It is therefore hypothesized that preconditioning with sevoflurane may also lead to protection in retinal ischemia.

Retinal ischemia is a major cause of visual impairment, and is fully or partially responsible for various retinal disorders such as ischemic optic neuropathies, retinopathies following arterial occlusion, venous thrombosis, diabetic retinopathy, retinopathy of prematurity, age-related maculopathy, and even glaucoma. The mechanism underlying ischemic retinal degeneration and potential retinal ischemia/hypoperfusion is one of the leading causes of retinal degeneration and blindness, which can be modelled by permanent BCCAO (also called 2 vessel occlusion) in rats. A severe ischemic insult affects retinal ganglion cells first; additional damage to most of the inner retina develops slower and finally photoreceptors can also be affected. Blood flow in the retina is severely reduced after BCCAO. Protective interventions are studied in a diversity of animal models. Previously, we have studied several candidate neuroprotective agents in rat permanent BCCAO and we have shown the protective effects of diazoxid, urocortin 2, and a PARP inhibitor.

Electroretinography **(ERG)** is a suitable method for determining the functional efficacy. An entire record of ERG consists of a negative wave (a wave) followed by a positive wave (b wave) with two to five oscillatory potentials on the rising slope. There is a largely variable c wave, the origin of which is attributed to the pigment epithelium or the glial (Müller) cells of the retina. The amplitude of each component of the ERG

varies from animal to animal, whereas they are very stable within the same animal. ERG is useful for the detection of early-stage retinal dysfunction. The gross physiological response of the retina to ischemia is commonly measured using the ERG, whose b wave component has attributes from the interaction between photoreceptors and ON bipolar cells. Recordings of ERG showed reduction of the b wave during the bilateral common carotid artery occlusion (BCCAO) indicating an effect at the level of the photoreceptors, bipolar cells, and Müller cells.

AIMS

1. We have previously demonstrated that BCCAO leads to degeneration of all retinal layers and causes decrease in the ganglion cell number. Several chemical agents proved to be protective in this model. In this experiment we evaluated the potential retinoprotective effect of late **sevoflurane preconditioning** in retinal ischemia in rats.

2. Our previous studies showed that PACAP treatment ameliorated the damaging effects of chronic hypoperfusion modeled by BCCAO. We have also demonstrated in earlier studies that treatment with PACAP antagonists further aggravates retinal lesions. It has been shown that **PACAP deficient mice** have larger infarct size in cerebral ischemia. Based on these results, it was hypothesized that the extent of retinal ischemic injury would also be more severe in mice lacking endogenous PACAP. Therefore, in the present study we examined the effects of mild ischemia/reperfusion in the retina of PACAP deficient mice.

3. It is not known whether **VIP** has protective effects on the retinal morphology in ischemic lesion induced by BCCAO. Therefore, the aim of the present study was to provide detailed retinal morphometric analysis following VIP treatment in a rat model of chronic retinal hypoperfusion.

4. Retinoprotective effects of PACAP are well-known and have been demonstrated in various pathological conditions, but whether this morphological improvement is also reflected in functional amelioration remains unknown. Therefore, our purpose was to investigate the protective effect of PACAP on the rat retina after BCCAO with electroretinography **(ERG)** to parallel the functional data with the previous morphological and neurochemical observations.

MATERIALS AND METHODS

1. Preconditioning with sevoflurane in ischemic retinal lesion

Rats (n=7) were subjected to BCCAO, ligating both common carotid arteries with a 3–0 filament through a midline incision. Rats were anaesthetized with 1 MAC (minimal alveolar concentration) of sevoflurane in air during the preconditioning period, while the animals were anaesthetised with 1.5 MAC of sevoflurane during the operation. The rats were breathing spontaneously during both preconditioning and operation. Concentrations of oxygen, carbon dioxide and anaesthetic vapor in the anaesthetizing box were monitored with a gas analyzer. The duration of preconditioning period was 1 hour, that was the period of exposing animals to sevoflurane and it was performed 24 hour before BCCAO. A randomized separated group of animals underwent anaesthesia and all steps of the surgical procedure, except for ligation of the carotid arteries. These subjects served as sham-operated controls. We examined four groups: sham- and BCCAO operated animals with no preconditioning and with late preconditioning. Standard histological examination was carried out 2 weeks after BCCAO. Rats were decapitated under anaesthesia and eyes were removed for standard histological examination. Retinal sections (2μm) were stained with toluidine blue, the sections were mounted and photographs were taken with a microscope combined with digital CCD camera from central retinal areas of nearly same eccentricities. Measurements were taken from the digital photographs. Samples for measurements derived from at least six tissue blocks prepared from at least three animals (n=2–5 measurements from one tissue block). The following parameters were measured in a blinded fashion: cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM), the width of the outer and inner nuclear (ONL, INL), outer and inner plexiform layers (OPL, IPL), the number of cells/100 μm section length in the GCL, and the number of cells in 500 μm^2 INL.

2. PACAP KO and wild-type mice- comparison of susceptibility to retinal ischemic injury

Wild-type (PACAP+/+; n=11) and homozygous PACAP deficient (PACAP-/-; n=12) mice were subjected to transient BCCAO. Under isoflurane anesthesia, carotid region was exposed through a midline cervical incision. In both groups, bilateral common carotid arteries were ligated for 10 min using a vascular clip for temporary use. Immediately following the operation, PACAP (100pmol in 3µl saline) was injected into the vitreous body of the right eyes and the same volume of saline was injected into the left eyes. Thus, the left eyes served as ischemic eyes (n=6 in both wild-type and PACAP KO groups) and the right eyes of the same animals served as PACAP-treated retinas (n=6 in both wild-type and PACAP KO groups). After 2 weeks of reperfusion period animals were killed with an overdose of anesthetic and standard histological examination was performed. A group of animals underwent anesthesia and all steps of the surgical procedure, except for the 10 min ischemic period, serving as sham-operated animals (n=5 in wild-type and n=6 in PACAP KO groups). Retinas were processed for histological analysis as previously described 2 weeks after carotid artery.

3. Protective effects of VIP in ischemic retinal degeneration

Adult male Wistar rats (n=17) weighing 250–300g were subjected to permanent BCCAO under isoflurane anesthesia. Immediately following the BCCAO operation, VIP (100 pmol, n=5 or 1,000 pmol, n=6 /5 µl saline) was injected into the vitreous body of the right eye with a Hamilton syringe. The left eye received the same volume of vehicle treatment, serving as the control bilateral carotid-occluded eyes. A group of animals underwent anesthesia and all steps of the surgical procedure, except ligation of the carotid arteries, with saline or VIP treatment (100 or 1000 pmol). These animals served as sham-operated animals (n=6). Two weeks after the carotid occlusion, rats were sacrificed under isoflurane anesthesia and eyes were removed for standard histological examination.

4. Effect of intravitreal PACAP treatment in ischemia induced retinal degeneration- functional analysis with ERG

In order to induce hypoperfusion of the retina, BCCAO was carried out on Wistar rats (n=8; 3 months of age, weighing 350g). Carotid arteries on both sides were permanently ligated under isoflurane anesthesia. The operation was immediately followed by intravitreal PACAP treatment (100pmol in 5µl vehicle/eye) into the right eye, while saline was injected into the other eye as a self-control. ERG measurements were performed to assess retinal function in each group, after an overnight dark adaption. The animals were anesthetized by intraperitoneal injection of ketamine 5% and xylazine 20% during the electrophysiological measurements. The pupils were dilated with 0.5% cyclopentolate. Oxybuprocaine 0.4% eye drops were used for the topical anesthesia. Flash ERG was recorded before BCCAO as nontreated controls and on postoperative days 2, 6, 10, and 14 in each group. ERG potentials arise in the retina after light stimulation and they are detectable all around the eye, being the largest in the center of the cornea. ERGs were recorded by surface electrodes from the center of the cornea with the negative electrode placed under the skin of the cheek and the ground electrode stuck under the skin of the neck. All procedures were performed under a dim red LED light. The responses to light flashes were pre-amplified, amplified, and recorded with an A/D converter. Responses (n=150) were averaged with the software of the A/D converter to draw the graph of each measurement. Histological examination was carried out to confirm the severity of the retinal lesions and correlate functional changes with morphological alterations induced by ischemia and PACAP treatment.

RESULTS

1. Preconditioning with sevoflurane in ischemic retinal lesion

All characteristic retinal layers of the mammalian retina were well visible in sham preparations with or without sevoflurane-preconditioning. The photoreceptor layer (PL) was followed by several rows of photoreceptor cell bodies, forming the outer nuclear layer (ONL). The first synaptic layer, a thin outer plexiform layer (OPL) was followed by the rows from the cell bodies of bipolar, horizontal and amacrine cells form the inner nuclear layer (INL). The inner plexiform layer (IPL) was followed by ganglion and displaced amacrine cells in the ganglion cell layer (GCL). The outer limiting membrane

(OLM) marks the border of the PL and ONL layers and the inner limiting membrane (ILM) was the innermost layer of the retina. No apparent morphological differences could be observed between sevoflurane-preconditioned and nonpreconditioned sham-operated animals. Differences between absolute control and sham-operated rats could not be detected in morphological or morphometrical parameters. BCCAO in non-preconditioned animals resulted in severe retinal degeneration and reduced thickness of the whole retina and all retinal layers as observed 2 weeks after ligation compared to sham-operated animals. Cell body-shaped holes in the nuclear layers and intermingled retina structure were detected. All retinal layers suffered marked reduction compared to the sham-operated animals: 66% in the whole retina thickness (OLM-ILM); 40% in the OPL; 53.5% in the INL; 42.5% in the IPL and 40.5% in the cell number of 100 μm/GCL.

In BCCAO-induced ischemic injury sevoflurane preconditioning ameliorated retinal damage as evidenced by morphological and morphometrical analysis. Sevoflurane preconditioning led to a nearly intact appearance of the distinct retinal layers. This resulted in the clear separation of the nuclear layers in contrast to the non-preconditioned BCCAO rats, where the ONL and INL fused in most animals. This is reflected in the data of the morphometrical analysis: the retinal thickness and the cell number in the ganglion cell layer were more retained in preconditioned animals after BCCAO compared to the non-preconditioned group. In all retinal layers significant amelioration of the damage is seen as reflected in the percentage of differences, which was significant between sevoflurane nonpreconditioned and preconditioned BCCAO animals: the degree of amelioration after preconditioning was 14% in the whole retina thickness (OLM-ILM); 10% in the ONL; 23% in the OPL; 20% in the INL; 16% in the IPL and 18% in the cell number of 100μm/GCL. We noted that the OPL and INL layers are retained better than average, indicative of the importance of the first synaptic layer in this preconditioning phenomenon.

2. PACAP KO and wild-type mice- comparison of susceptibility to retinal ischemic injury

All layers were visible in sham-operated control preparations. Under the pigment epithelium, several rows of photoreceptors with a thin OPL as well as the cell rows of

the INL followed by the thick IPL, were all present. No marked differences were observed between the control retinas of wild-type and PACAP deficient mice by standard histological methods. Ten minutes BCCAO led to a mild reduction in the thickness of the retinal layers after 2 weeks reperfusion period as compared to sham-operated control mice. The degree of retinal degeneration was markedly worse in PACAP deficient mice: all layers suffered more severe damage than in wild-type mice. In the wild-type ischemic retinas empty cell body shapes and tissue gaps were the only changes that could be noticed in the INL. Packing of cells in the INL was particularly loose and the large cells almost completely disappeared from the GCL in ischemic PACAP KO retinas. Intravitreal PACAP treatment significantly ameliorated the retinal degeneration induced by transient BCCAO. Retinas of both wild-type and PACAP deficient mice had nearly normal appearance, except that neurons in the PACAP KO retina seemed swollen compared to the wild-type animals. Morphometric analyses also demonstrated that PACAP deficient mice had a more severe ischemic retinal damage, supported by the measurements of the whole retina and individual retinal layers. Each retinal layer showed width reductions suggestive of severe degeneration. Reductions in percentage of respective sham-operated animals were the following: ONL: 93%; OPL: 77%; INL: 69%, and IPL: 81% in wild-type animals; and ONL: 84%; PL: 66%; INL: 56%, and IPL: 56% in PACAP deficient mice. As a consequence, the distance between the OLM and the ILM was significantly less than in sham preparations (wild-type retina: 89%; KO retina: 69%). The reduction in cell numbers was also detected after transient BCCAO. In the INL (500 μm^2), the cell number was significantly decreased in wild-type mice by 33% and in PACAP KO mice by 39%. The observed degree of retinal degeneration corresponds to our previous results with the BCCAO model. The cell number of the GCL was also reduced in ischemic conditions (in wild-type: 65%; in PACAP KO: 48% of shamoperated control retinas). The protective effect of intravitreal PACAP administration was proven by quantitative analysis: the thickness of the retinal layers as well as the cell number in the INL and GCL were partially or totally compensated after PACAP treatment. This could be particularly well demonstrated in the case of the IPL thickness and the number of cells in the GCL.

3. Protective effects of VIP in ischemic retinal degeneration

In sham-operated control preparations, all rat retinal layers were visible. Under the pigment epithelium, several rows of photoreceptors with a thin OPL as well as the cell rows of the INL followed by the thick IPL were all present. VIP (100 or 1000 pmol) treatment in sham-operated animals did not cause any morphological alteration in retinal structure. BCCAO resulted in severely reduced thickness of retinal layers compared to sham-operated controls. All retinal layers bore the marks of severe degeneration and were significantly thinner than sham-operated preparations. The distance between OLM and ILM was significantly decreased. Most marked reduction in thickness was found in the OPL and IPL, and a subtle but significant change was observed in the cellular layers ONL and INL. Several empty cell body-shaped spaces were seen in the ONL and INL which layers intermingled with the OPL. Numerous cells in the GCL displayed severe degeneration, which was well reflected in the reduced number of cells in the GCL. Intravitreal treatment with 100pmol VIP following BCCAO caused no visible improvement in the degenerated retina structure. Significant differences could not be observed between the BCCAO and BCCAO+100pmol VIP groups by morphometrical analysis. Quantitative analysis demonstrated that 100pmol VIP administration could not protect the cells in the GCL. However, 1000pmol VIP treatment after BCCAO led to a nearly intact appearance of the retinal layers. Intravitreal administration of VIP led to the preservation of the retinal structure, with wellvisible OPL and INL with three cell rows. However, the differences between BCCAO- and BCCAO+1000pmol VIP-treated retinas were statistically significant in almost all retinal layers, except for the OPL. The number of cells in the GCL was higher in the BCCAO+1000pmol VIP-treated group compared to the BCCAO group. Based on our previous data on the protective potential of PACAP in BCCAO-induced injury, we compared the protective potential of PACAP and VIP. PACAP was more effective, already protective at 100pmol doses. VIP, in contrast, was only effective in higher doses. Differences could not be observed in the thickness of the whole retina and the individual layers between 1000pmol VIP and 100pmol PACAP treatment. However, PACAP was more effective in preserving cells in the GCL where significant differences could be observed between the protective potential of 1000 pmol VIP and 100pmol PACAP.

4. Effect of intravitreal PACAP treatment in ischemy induced retinal degeneration- functional analisys with ERG

We investigated the possible effects of PACAP treatment in BCCAO-induced ischemic retinal degeneration on neuronal cell activity by recording ERGs at various time points before (control measurements) and 2, 6, 10, and 14 days after the induction of ischemia. Control average ERG waveforms were similar in both left and right eyes. Our results show that BCCAO caused a severe functional damage reflecting the previously described alteration of the histological structure. BCCAO-induced functional retinal degeneration was already observed on postoperative days 2 and lasted throughout the entire observation period. Intravitreal injection of PACAP immediately after BCCAO resulted in a more retained retinal function as assessed by average ERG waveforms compared to the BCCAO-operated groups on both postoperative 2nd and 14th days. The same tendencies could be observed on postoperative day 6 and 10. Average amplitudes of waves decreased with the duration of ischemia in BCCAO eyes. Differences were found in the average amplitudes of the a waves and b waves between control and vehicle-treated BCCAO eyes, but no differences could be observed in the case of c wave on postoperative day 14. ERGs of PACAP-treated ischemic eyes were similar to the intact controls in contrast to the ERGs of saline-treated BCCAO retinas. PACAP treatment significantly counteracted the ischemia induced alterations in the amplitudes of both the a and b aves of the ERG on postoperative day 14. Amplitudes of the b wave decreased in BCCAO (33% of controls). Significant difference could be detected between BCCAO and PACAP-treated BCCAO groups on postoperative day 14 (53% of control). The same tendency could be observed on postoperative day 2 in BCCAO-treated and BCCAO+PACAP-treated groups in the b wave amplitudes. A slight but not significant decrease in the b wave/a wave amplitude ratio (b/a) was observed in the BCCAO and PACAP-treated BCCAO retinas. The latency of b/a waves was significantly decreased in both BCCAO- and PACAP-treated BCCAO groups compared to their controls, but no differences could be observed between the treated and untreated ischemic groups. The elapse time of the five major oscillatory potentials (OPs) was reduced in the BCCAO ischemic group, but PACAP treatment led to significant protection. The morphological parameters are in accordance with our previous

observation that PACAP treatment ameliorated the BCCAO-induced retinal degeneration.

DISCUSSION

1. Preconditioning with sevoflurane in ischemic retinal lesion

We showed that late-preconditioning with the volatile anaesthetic sevoflurane induced retinal protection in carotid artery occlusion-induced ischemic lesion. Sevoflurane, along with other volatile anaesthetics, has already been shown to provide protection in conditions of cerebral ischemia. This is the first time to demonstrate similar protective potential of sevoflurane in retinal ischemia. The protective effect of this preconditioning was long-lasting, observable also when the morphological changes induced by different injuries reach final degree in the retina (2 weeks). Postoperative visual loss is a rare but serious consequence of both ophthalmic and non-ophthalmic surgery. Regarding non-ophthalmic procedures it affects most frequently patients after cardiac and spine operations. The prevalence following cardiac surgery may be as high as 0.08 %. In most of the cases ischemic retinal damage is responsible for the visual loss. Sevoflurane preconditioning has been shown to provide neuroprotection in the brain, the exact mechanism of which is not known, however. Recently, several pieces of the puzzle have been elucidated and a complex neuroprotective mechanism is suggested. For example, sevoflurane preconditioning has been demonstrated to upregulate antioxidant enzyme activity before ischemic injury in a model of focal cerebral ischemia. Anti-inflammatory effects have also been shown: sevoflurane preconditioned animals have displayed suppressed expression of inflammatory cytokines, NK-kappa B and p38 MAPK in a stroke model. The involvement of other MAP kinases has also been suggested. It has been reported that sevoflurane pre- and postconditioning protect the brain via the mitochondrial K ATP channel. A further possible mechanism could be the hyperpolarizing effect of sevoflurane anesthesia, which has been described in hippocampal slices after hypoxia. Even anti-apoptotic pathways have been shown to be activated upon sevoflurane exposure. Although the mechanism of retinal protection can only be hypothesized at the moment, based on the similar pathways involved in retinal and cerebral ischemic degeneration, similar mechanisms can be proposed in the sevoflurane-induced retinal protection.

2. PACAP KO and wild-type mice- comparison of susceptibility to retinal ischemic injury

Our results showed that the extent of retinal ischemic injury was more severe in mice lacking endogenous PACAP in a mouse model of transient retinal ischemia. Exogenous PACAP administration partially compensated the severe degeneration in PACAP deficient mice. PACAP deficient mice display several abnormalities under both physiological and pathological conditions. Mice lacking PACAP are temperature-sensitive, have decreased fertility and reproductive functions, display early neonatal death, and react to hormonal and metabolic changes in an altered manner. Furthermore, PACAP deficient mice have memory disturbances, abnormal nonvisual photoreception, behavioral abnormalities, and altered pain and inflammatory reactions. It has been reported that the gross cerebral and cerebellar morphology is not altered in PACAP deficient mice. However, subtle morphological differences and alterations in neurochemical markers could be identified in the cerebellum and altered axonal arborization has been found in the dentate gyrus. Of great importance are the findings showing that PACAP deficient mice respond to stressors in a more sensitive manner: mice lacking endogenous PACAP have increased cellular death in cerebellar granule cells upon exposure to ethanol or oxidative stress. In vivo, PACAP deficient mice are more vulnerable to experimental autoimmune encephalomyelitis and display slower recovery after peripheral nerve crush injury. It has also been shown that these mice develop larger infarct volume and increased edema in focal cerebral ischemia. This endogenous protective effect of PACAP in ischemic lesions seems to be not restricted to the brain, but similar findings have been described in peripheral tissues. For example, recent results have found that PACAP deficient mice show increased susceptibility to in vivo renal and intestinal ischemia/reperfusion. These results are in accordance with our observations that mice lacking PACAP have no gross morphological deficiencies in the retina, but they are more susceptible to retinal ischemia. Previous studies have shown the protective effects of exogenously applied PACAP in several different kinds of retinal injuries, like excitotoxity, optic nerve transection, anisomycin-induced lesion and UV light-induced degeneration. We have provided evidence that PACAP also protects against ischemic injury caused by carotid occlusion in rats, which has subsequently been confirmed by others in a different model. Studying the potential protective effect

of endogenous PACAP, we have demonstrated earlier that intravitreal injection of the PACAP antagonist PACAP6-38 aggravates retinal injury caused by glutamate excitotoxicity and activates pro-apoptotic signaling pathways. A recent study has shown that even the partial lack of PACAP aggravates the death of retinal ganglion cells induced by NMDA toxicity using heterozygous PACAP deficient mice. These studies along with our present observations show that PACAP is able to exert long-term neuroprotection, since its protective effects could be observed even 2 weeks after the injury. Most studies investigating the neuroprotective effects of PACAP perform measurements a few hours or a few days following insults. It is important to note that in case of retinal protection, these effects are present 2 weeks after the insult, so PACAP is a long-term neuroprotective agent. The exact molecular mechanism of the endogenous protective action of PACAP is not known at the moment, but studies done in the retina and other parts of the nervous system have revealed that PACAP influences numerous protective pathways. PACAP is a strong anti-apoptotic agent, which has been proven also in the retina. In glutamate-induced retinal degeneration, PACAP activates anti-apoptotic signaling pathways (like ERK, CREB, Bcl-2, Bcl-xL, Akt, 14-3-3 protein), while inhibits pro-apoptotic proteins, such as caspases and Bad. Furthermore, PACAP also acts indirectly, through glial cells, by increasing the release of neuroprotective factors. PACAP acts on Müller glial cells in the retina, where it stimulates release of interleukin-6, the protective effects of which have been confirmed in ischemic and excitotoxic brain lesions. Other mechanisms might also play a role in retinal protection, for example the antiinflammatory effects of PACAP and the action against free radicals. In summary, our present findings further support the endogenous protective effect of PACAP in the retina and indicate that PACAP is part of the natural defense mechanism against retinal injuries.

3. Protective Effects of VIP in Ischemic Retinal Degeneration

In the present study, we showed that intravitreal VIP exerted neuroprotective effects in the retina in ischemic retinal lesion, given at 1000pmol (1nmol) dose. However, it was not effective at lower doses. The mechanism of the neuroprotective effects of VIP is not fully understood. It is suggested that VIP has a complex action, including antiapoptotic, anti-inflammatory, and antioxidant effects. VIP shares receptors with PACAP, namely the VPAC1 and VPAC2 receptors, to which the two peptides

show similar affinity and PAC1, which bind PACAP with higher affinity than VIP. Not surprisingly, VIP and PACAP also share common actions in various systems, while they have different actions in others. The neuroprotective effects of both peptides are widely accepted. A novel neuroprotection target has been described by VIP acting through specific splice variant of the PACAP receptor providing cellular protection. The main mechanisms involved in their neuroprotective effects are antiapoptotic, anti-inflammatory, and antioxidant properties. PACAP is shown to have stronger antiapoptotic effects in most studies, while VIP has better known anti-inflammatory actions. A recent study showing that VIP protected the ischemic brain in focal cerebral ischemia found decreased number of apoptotic cells and attenuated S100B (a glial derived calcium binding protein) immunoreactivity after VIP treatment. VIP also acts indirectly, by inducing the synthesis and secretion of neuroprotective proteins from astrocytes. Activity-dependent neuroprotective protein (ADNP) and its smallest active element NAP have been discovered as a glial mediator of VIP-induced neuroprotection. Both ADNP and NAP have been shown to have strong neuroprotective effects in various systems, including retinal cells. In the retina, both PACAP and VIP have neuroprotective effects. It has been shown in several studies using similar models. For example, PACAP is highly effective against glutamate-induced excitotoxicity in vitro and in vivo, and the same has been shown for VIP in vitro. Similarly, both PACAP and VIP have been documented to be effective in light-induced damage: we have shown that PACAP protects against UV light-induced retinal lesion, while the VIP-mediator NAP protects against laser-induced retinal damage as reported by others. The putative protective effects of VIP have been proposed also in streptozotocin-induced diabetic retinopathy, due to the significant reduction in the endogenous VIP levels in the retina. Recently, we have shown that PACAP effectively prevents several morphological changes in the same model. Regarding hypoxic/ischemic retinal lesions, it has been shown that PACAP protects against permanent carotid occlusion-induced retinal degeneration and injury induced by high intraocular pressure. Similarly, intraperitoneal injection of NAP protects retinal ganglion cells in high intraocular pressure-induced retinal ischemia. Another study has demonstrated that NAP in retinal Müller glial cells prevents hypoxia-induced injury and promotes neuron growth. An earlier study reported that VIP protected the retina against ischemia/reperfusion injury induced by ligation of ophthalmic vessels. The authors showed that both systemic and intravitreal VIP significantly decreased malondialdehyde levels, indicating decreased oxidative stress.

Lipid peroxidation is characteristic for the reperfusion period of this type of injury. VIP administration also prevented histological alterations of the retina analyzed after a 1.5 hour ischemia and 3 hour reperfusion. Our present results are in accordance with these previous observations. However, we showed that the protection by VIP is not only observed shortly after the injury, but it is long lasting: VIP-treated retinas which were analyzed 2 weeks after ischemia were well preserved in contrast to control retinas. Also, the dose was much lower in our present study than the dose used in the above-mentioned earlier study. One nanomole VIP preserved the retinal structure in our present hypoperfusion model. However, similar to other studies, we found that VIP must be given at least in 10 times higher dose than PACAP to achieve a similar degree of neuroprotection in the retina. This 10- to 100-fold difference in the neuroprotective efficacy between the two related peptides has been reported in several other systems. However, opposite effects have also been reported: while neonatal white matter lesion is reduced by VIP, PACAP was not found to be effective. Based on currently available information, higher efficacy of PACAP can be observed in systems where apoptosis is the main reason for cellular loss. In models, where inflammation is responsible for damage, VIP seems to be as effective as PACAP or even more effective. In the present study, we hypothesize that the higher efficacy of PACAP could be due to apoptotic processes as principal causes of cell death in retinal ischemia and that the PACAP-specific PAC1 receptor plays a major role in retinal protection. However, other reasons could also be responsible for this difference in potency between the two peptides. In summary, the present results provide detailed morphometric analysis for VIP-induced retinoprotective effects in chronic hypoperfusion injury of the retina.

4. Effect of intravitreal PACAP treatment in ischemia induced retinal degeneration- functional analysis with ERG

Our results show that the morphological protection exerted by PACAP in ischemic retinal lesion is accompanied by functional amelioration, as reflected by electrical activity. We have previously described that PACAP exerts morphological and neurochemical protection in BCCAO-induced retinal degeneration. However, functional outcome measures, such as ERG, were missing. Electroretinogram displays a composite retinal electric activity which depends upon different cells, extending from the pigment

epithelium to the innermost retinal layers, allowing differential participation of cells within different layers. This method is attractive in assessing retinal neurodegeneration because it is not invasive and can thus be used in longitudinal assessments. The ERGs for all control eyes in our present study were well within the range of previously published normative data. The results of this study demonstrate that the b wave is the most sensitive detectable ERG parameter of functional abnormalities in this rat model of BCCAO-induced retinal ischemia. Our observations suggest that BCCAO and PACAP treatment also led to changes in neuronal cell activity of the retina. The main functional protective effects of PACAP were showed by the alteration of the b wave amplitudes. Although retinal ganglion cells are the most vulnerable to severe ischemia of the retina and their damage was found most prominent after BCCAO, it has been shown that 2 weeks after the operation, other cellular layers are also seriously degenerated. Therefore, deterioration of the ERG can be expected. Former studies have identified the OPs of the flash-elicited ERG b wave as sensitive indices of abnormalities within the retinal circulation. The OPs are quantitative and objective index for dysfunction and manifested earliest not only in ischemic conditions but also in diabetic retinopathy, which also involves worsened circulation and production of reactive oxygen species. Compared to nonischemic (control) values, the ERG a and b wave amplitudes were strongly reduced after the BCCAO operation. PACAP treatment alleviated the functional consequences of BCCAO-induced ischemia. In our previous studies, the beneficial effects of PACAP were observed at histological and neurochemical levels. The present results indicate the PACAP treatment induced a significant recovery of the waveform and amplitudes of a wave and b wave as seen in ERG. PACAP treatment did not completely prevent retinal dysfunction; however, the ERG recovery was a sustained effect noted as early as 2 days after BCCAO. There are two further interesting results in the ERG recordings. One is that after BCCAO, the a wave amplitudes increase slightly, possibly reflecting a relatively late and low level of photoreceptor damage and the lack of intraretinal negative feedback. This is supported by the fact that the thickness of the outer nuclear layer did not change as much as the inner retinal layers and the photoreceptors look relatively healthy in the histological preparations. The second observation is that the b/a wave latency ratio did not improve significantly after PACAP treatment. A possible explanation for that is that the time after BCCAO was not sufficient for the degenerations to reach the photoreceptors retrogradely. Our present results confirm our earlier findings in excitotoxic retinal damage, where PACAP

treatment led to functional amelioration parallel with protection reflected in morphological structure. In conclusion, the results reported in the present study demonstrate a functional protective effect of PACAP against retinal ischemic injury caused by BCCAO. Since this is the second metabolically induced retinal degeneration model where we can demonstrate such protection, data are convincing that morphological and neurochemical protection go parallel with functional protection.

SUMMARY

- We demonstrated with our experiments the retinoprotective effect of late sevoflurane preconditioning in retinal ischemia in rats.
- We verified the reduced tolerance of PACAP knock out mice in ischemia-reperfusion retinal lesion with morphological and functional results.
- Our results provide detailed morphometric analysis for VIP-induced retinoprotective effects in chronic hypoperfusion injury of the retina. Based on our previous results regarding the effective PACAP concentration we established that the effective PACAP dosis is ten times lower than the effective VIP dosis.
- We demonstrated that the morphological protection exerted by PACAP in ischemic retinal lesion is accompanied by functional amelioration.

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List of publications used for the theses

Danyadi B., Szabadfi K., Reglodi D., Mihalik A., Danyadi T., Kovacs Zs., Batai I., Tamas A., Kiss P., Toth G., Gabriel R. (2014) PACAP application improves functional outcome of chronic retinal ischemic injury in rats – evidence from electroretinographic measurements. J. Mol. Neurosci.; 54: 293-299 (IF: 2.343)

Szabadfi K., **Danyadi B.***, Kiss P., Manavalan S., Gabriel R., Reglodi D., Tamas A., Trasy D., Batai I. (2012) Preconditioning with volatile anaesthetic sevoflurane in ischemic retinal lesion in rats. J. Mol. Histol.; 43: 565-569 (IF: 1.551). (*Equal contribution)

Szabadfi K., Atlasz T., Kiss P., **Danyadi B.**, Tamas A., Helyes Zs., Hashimoto H., Shintani N., Baba A., Toth G., Gabriel R., Reglodi D. (2012) Mice deficient in pituitary adenylate cyclase activating polypeptide (PACAP) are more susceptible to retinal ischemic injury in vivo. Neurotox. Res.; 21: 41-48. (IF: 2.865)

Szabadfi K., **Danyadi B.**, Kiss P., Tamas A., Fabian E., Gabriel R., Reglodi D. (2012) Protective effects of vasoactive intestinal peptide (VIP) in ischemic retinal degeneration. J. Mol. Neurosci.; 48: 501-507 (IF: 2.891)

List of publications not used for the theses

Werling D, Reglodi D, Banks WA, Salameh TS, Kovacs K, Kvarik T, Vaczy A, Kovacs L, Mayer F, **Danyadi B**, Lokos E, Tamas A, Toth G, Biro Zs, Tamas A, Atlasz T (2016). Ocular delivery of PACAP1-27 protects the retina from ischemic damage in rodents. Invest Ophthalmol Vis Sci; 57:6683-6691 (IF: 3.303)

Atlasz T., Vaczy A., Werling D., Kiss P., Tamas A., Kovacs K., Fabian E., Kvarik T., Mammel B., **Danyadi B.**, Lokos E., Reglodi D. (2016) Neuroprotective effects of PACAP in the retina. Pituitary Adenylate Cyclase Activating Polypeptide – PACAP, edited by Dóra Reglődi and Andrea Tamás, Springer Nature, New York. pp. 501-527.

Dányádi B. (2015) Ebola vérzéses láz: növekvő kockázat, nem csak Nyugat-Afrikában. Honvédségi szemle; 143., 97-106

Szabadfi K., Kiss P., Reglodi D., Fekete E.M., Tamas A., **Danyadi B.**, Atlasz T., Gabriel R. (2014) Urocortin 2 treatment is protective in excitotoxic retinal degeneration. Acta. Physiol. Hung.; 101: 67-76 (IF: 0.734)

Szabo A., **Danyadi B.**, Bognar E., Szabadfi K., Fabian E., Kiss P., Mester L., Manavalan S., Atlasz T., Gabriel R., Toth G., Tamas A., Reglodi D., Kovacs K.(2012) Effect of PACAP on MAP kinases, Akt and cytokine expressions in rat retinal hypoperfusion. Neurosci. Lett.; 523: 91-98. (IF: 2.026)

Szabadfi K., Atlasz T., Reglodi D., Kiss P., **Danyadi B.**, Fekete E.M., Zorilla E.P., Tamas A., Szabo K., Gabriel R.. (2009) Urocortin 2 protects against retinal degeneration following bilateral common carotid artery occlusion in the rat. Neurosci. Lett.; 455: 42-45. (IF:1.925)