

Neuroprotective effects of PACAP in the retina

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) is a widespread neuropeptide that is well-known for its general cytoprotective effects in different neuronal injuries, such as traumatic brain and spinal cord injury, models of neurodegenerative diseases, and cerebral ischemia. PACAP and its receptors also occur in the retina. In this review, we summarize the retinoprotective effects of PACAP. *In vitro*, PACAP is protective against glutamate, thapsigargin, anisomycin, oxidative stress, UV light, high glucose, inflammation and anoxia. Both the neural retina and the pigment epithelial cells can be protected by PACAP in various experimental paradigms. *In vivo*, the protective effects of intravitreal PACAP treatment have been shown in the following models in rats and mice: excitotoxic injury induced by glutamate, N-methyl-D-aspartate (NMDA) or kainate, ischemic injury induced by carotid artery ligation and high intraocular pressure, degeneration caused by UV-A light, optic nerve transection, and streptozotocin-induced diabetic retinopathy as well as retinopathy of prematurity. Molecular biological methods have revealed that PACAP activates anti-apoptotic, while inhibits pro-apoptotic signaling pathways, and it also stimulates an anti-inflammatory environment in the retina. Altogether, PACAP is suggested to be a potential therapeutic retinoprotective agent in various retinal diseases.

Key words: retina, diabetic retinopathy, ischemia, excitotoxic injury, retinopathy of prematurity

Pituitary adenylate cyclase activating polypeptide (PACAP)

Pituitary adenylate cyclase activating polypeptide (PACAP), was isolated from ovine hypothalamus based on its stimulating effect on adenylate cyclase enzyme in anterior pituitary cells (Arimura, 2007; Miyata et al. 1989). PACAP has two isoforms; PACAP1-27 and PACAP1-38, containing 27 and 38 amino acids, respectively. PACAP is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon superfamily (Arimura and Shioda, 1995). Its receptors can be divided into two groups, PAC1 receptor and VPAC receptors (VPAC1 and VPAC2) (Vaudry et al. 2009). The biological effects of PACAP are diverse (Vaudry et al. 2009), but one of the most studied actions of the peptide is its potent neuroprotective effect. Earlier studies have shown that PACAP protect neurons *in vitro* against various toxic agents (glutamate, 6-OHDA, oxidative

stress etc.). Neuroprotective role of PACAP has been also demonstrated *in vivo* in different animal models, such as cerebral ischemia, traumatic brain injury, spinal cord injury, and Parkinson's disease. These neuroprotective effects have been summarized in several reviews by different authors (Ohtaki et al. 2008; Reglodi et al. 2011, 2015; Shioda et al. 2006; Shioda and Nakamachi, 2015; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009; Waschek, 2002).

PACAP is also protective in sensory organs, including the auditory and visual systems (Atlasz et al. 2010; Nemeth et al. 2014). The retina is a special tissue, an extension of the central nervous system (CNS) containing different neurons, and it also provides an excellent model to measure neuronal degenerations. PACAP has been shown to protect the different neurons of the retina in numerous *in vitro* and *in vivo* models of retinal degenerations. The purpose of the present review is to summarize the current knowledge of the retinoprotective functions of PACAP. The retinoprotective effects of PACAP provide a new, exciting area of research, and several novel data have been published since the appearance of the first two extensive reviews on the retinoprotective effects of PACAP (Atlasz et al. 2010a; Nakamachi et al. 2012). Therefore, we briefly summarize retinoprotective effects until 2010 and discuss findings in the last 5 years more in detail.

Distribution of PACAP in the retina

Several studies have described the presence of PACAP and PACAP receptors in the retina in rat. PACAP immunopositive cell bodies were found in amacrine, horizontal cells in the inner nuclear layer (INL) in the ganglion cell layer (GCL) and also in the Muller glial cells. PACAP positive nerve fibers were also present in the nerve fiber layer (NFL), and also in the inner plexiform layer (IPL), but no PACAP positivity was found in the outer nuclear layer (ONL) or the retinal pigment epithelium (RPE) layer (D'Agata and Cavallaro 1998; Hannibal and Fahrenkrug 2004; Izumi et al. 2000; Kubrusly et al. 2005; Seki et al. 2000a). The presence of PACAP was also found in the mouse (Mathis and Schaeffel, 2007), teleost (Grone et al. 2007), turtle (Reglodi et al. 2001) and chicken retina (Jozsa et al. 2001). PACAP appearance showed in a subset of retinal ganglion cells, which cells are photosensitive due to expression of melanopsin. The PACAP expression in this subset of ganglion cells is partly controlled by dopamine, while it is not affected by photoreceptor degeneration (Sakamoto et al. 2005; Wan et al. 2006). Melanopsin-containing retinal ganglion cells are known to be more resistant in various types of degeneration, such as axonal injury (Perez de Sevilla Muller et al. 2014), and it has been proven by numerous studies that these melanopsin-containing cells almost all co-store PACAP, which might contribute to the highly resistant nature of these cells (Hannibal et al. 2014; La Morgia et al. 2011; Langel et al. 2015). PACAP- and melanopsin-containing retinal ganglion cells play an important role in several light-activated non-image-forming functions of the retina and the complimentary signaling due to PACAP neurotransmission has been shown to be important in addition to the glutamatergic signaling (Delwig et al. 2013; Purrier et al. 2014).

PACAP immunopositivity was also found in the limboretinal pathway (Vereczki et al. 2006). PACAP and its receptors were also described in the developing retina of mammals (Bagnoli et al. 2003) In developing chicken retina PACAP was expressed in

the INL from embryonic day 8 (E8) (Borba et al. 2005). In zebrafish during the pharyngula period, at 24h of post fertilization PACAP-like immunoreactivity (PACAP-LI) was detected in the superficial layer of the retina, and during the hatching period, at 72h of post fertilization in the GCL (Mathieu et al. 2005). PACAP-LI was absent from the retina in the zebrafish at day 13 of larval development, suggesting that PACAP may have a role in the control of cell differentiation and proliferation (Mathieu et al. 2004, 2005). In the rat, PACAP mRNA was expressed in the GCL in the developing retina at E20 (Skoglosa et al. 1999).

Distribution of PACAP receptors in the retina

Earlier studies showed the distribution and localization PACAP receptors in the retina. Nilsson et al. (1994) characterized the receptors of PACAP and their coupling to adenylate cyclase in albino rat eye, and D'Agata and Cavallaro also described the expression of PACAP/VIP receptor variants and their coupling to phospholipase C in the rat retina (D'Agata and Cavallaro, 1998). In chicken retina at early stage (E6) PAC1 receptor and its mRNA can be found (Borba et al. 2005). The mRNA of PACAP receptors and the protein expression have also been shown in all the layers of the neonatal rat retina (Silveira et al. 2002). Seki et coworkers described the localization and strong expression of PAC1 receptor and its mRNA in different retinal layers (NFL, INL, GCL), weaker expression in the ONL, OPL, IPL, and the outer segments of the photoreceptors in the rat retina (Seki et al. 1997, 2000b). Moreover, different PAC1 receptor mRNA variants (short, hop) were found in the cell bodies and the processes of ganglion and amacrine cells (Seki et al. 1997). Finally, PAC1 receptor has been described in Muller glial cells (Kubrusly et al. 2005; Seki et al. 2006).

PACAP also plays a role in retinal development. The expression of the specific PAC1 receptor and VPAC receptors has been confirmed in the developing mammalian retina, including the retinal progenitor cells (Njaine et al. 2010). A shift in PAC1 receptor isoform expression (null, hip, hop1, hiphop1) during development might explain the different roles exerted during differentiation of retinal neural elements (Denes et al. 2014; Lakk et al. 2012).

PACAP controls the proliferation of retinal progenitor cells through the activation of all 3 receptors, and the cAMP/PKA/CREB pathway. This activation resulted in downregulation of cyclin D1 and the transcription factor Klf4, while p27^{kip1} protein content did not change (Njaine et al. 2010, 2014). The overall effect of these changes was an anti-proliferative effect.

Protective effects of PACAP in the retina in vitro

The in vitro protective effects are summarized in Table I.

In vitro protective effects in the neural retina

The first in vitro studies presented that VIP, a related peptide to PACAP (Shoge et al. 1998), or PACAP1-27 and PACAP1-38 (Shoge et al. 1999) have retinoprotective effect against glutamate toxicity using cultured retinal neurons. PACAP1-27, PACAP1-38 and VIP increased cAMP, and the protective effects of VIP or PACAP isoforms were

antagonized by VIP6-28 (VIP antagonist) or PACAP6-38 (PACAP antagonist), respectively. In addition, H-89 (PKA inhibitor) also decreased the protective effects of VIP or PACAP1-27, PACAP1-38.

Silveira et al. (2002) found that anisomycin (1 μ g/ml) inhibits protein synthesis and inducing cell death in the neuroblastic layer of retinal explants from newborn rats. PACAP1-27 or PACAP1-38 dose-dependently prevented the anisomycin-induced cell death. 1nM PACAP1-27 or PACAP1-38 administration in parallel significantly reduced the number of dead cells with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) procedure and the number of pyknotic profiles per mm² in the neuroblast layer (NBL). A similar retinoprotective effect was observed with PAC1 receptor agonist maxadilan, but not with glucagon. Neuroprotection by PACAP1-38 was also measured in photoreceptor cell death caused by thapsigargin in the ONL (Silveira et al. 2002). In the retinal explant model, PACAP6-38 and the specific PAC1 receptor antagonist Maxd.4 antagonized the neuroprotective effect. Molecular and immunohistochemical analysis demonstrated that the retinoprotective effects are most probably mediated by the PAC1 receptor, which is expressed in all the retinal layers of the neonatal retina. Treatment of PACAP1-38 induced CREB immunoreactivity in the NBL. PACAP1-38 administration also increased cAMP, but not IP3, and a cAMP-dependent protein kinase inhibitor Rp-cAMPS could block the retinoprotective effects of PACAP1-38.

In adult turtles, horizontal cells showed light responses 18, 22, 42 and 46 h after removal of the eyes. Rabl et al. (2002) demonstrated that the amplitudes of light responses were larger in slices at all time-points in PACAP1-38-containing solution (0.165 μ M PACAP) compared to control retinal slices in anoxic condition. These results provide evidence that PACAP1-38 has neuroprotective effects in hypoxic/anoxic conditions in the adult turtle retina in vitro.

In vitro protective effects in the retinal pigment epithelial cells

Retinal pigment epithelial (RPE) cells play an essential role in photoreceptor survival and metabolism. Several diseases are associated with malfunction of the pigment cells. The first results concerning the effects of PACAP came from Zhang et al. (2005). Presence of mRNA for PAC1 and VPAC1 receptors was confirmed in unstimulated human RPE cells (ARPE19). VPAC2 mRNA was expressed in cells stimulated with IL-1 β . PACAP treatment at 0.1-1 micromolar concentrations inhibited the IL-1 β -stimulated IL-6, IL-8, and MCP-1 mRNA and protein levels. The dense immunofluorescence of NF- κ B in the nucleus after stimulation with IL-1 β was decreased by PACAP (Zhang et al. 2005). Studies following these first results in pigment cells revealed several other effects of PACAP in these retinal cells.

ARPE19 cells exposed to PACAP1-38 could be rescued against oxidative stress induced by H₂O₂ in a dose-dependent manner (Mester et al. 2011). Highest efficacy was observed at 100 nM PACAP concentration, but it was effective in a range 10 pM-1 μ M, while not effective at 1 pM. Using flow cytometric and JC-1 assay, PACAP treatment was proven to reduce apoptotic cell death. PACAP1-38 and 6-38 alone had no effect on cell survival. The survival-promoting effect was inhibited by PI3K/Akt inhibitors, but not affected by MAPK inhibitors (Mester et al. 2011). A subsequent study investigated the

signaling pathways in oxidative stress-induced pigment epithelial cell damage (Fabian et al. 2012). Western blot results revealed that PACAP treatment could activate the protective ERK/CREB and Akt pathways, while it inhibited the phosphorylation of p38 MAPK and JNK. Several cytokines and other apoptotic markers were altered upon oxidative stress, as measured by cytokine and phospho-kinase arrays. PACAP administration counteracted several of these changes. 10 and 100 nM PACAP decreased the oxidative stress-induced increases of the proinflammatory and/or proapoptotic factors Bad; Bax; Trail R2 DR5; FADD; Fas TNFR; HIF-1 α ; HO-1; cIAP-1; HSP-27; SMAC/Diablo; p53; eNOS; Paxillin; PLCgamma-1; STAT4; RSK1/2. In contrast, PACAP could increase the protective Lyn; Yes; and Src.

A more recent study has reported that PACAP regulates semaphorin4A expression from RPE cells (Ko et al. 2015). The semaphorin protein family has members that influence brain and retinal development, by providing neural guidance. Semaphorin4A is expressed in the GCL, INL and RPE cell during the developmental period of contact-building between photoreceptors and RPE cells. Human ARPE cells were co-cultured with PC12 cells (prototype neuronal cells) based on a collagen vitrigel membrane. The authors found that in the presence of neural cells the protein and mRNA levels of semaphorin4A (but not other semaphorins) were increased, an effect mimicked by PACAP (but not VIP, NPY or enkephalin). Furthermore, this effect was also associated with ERK phosphorylation (but not that of JNK or p38 MAPK). All these effects of PACAP were blocked by the PACAP antagonist PACAP6-38. Furthermore, PACAP6-38 downregulated the cytokine IL-6 expression, indicating that endogenous PACAP participates in the neural cell-induced IL-6 production (other cytokines out of the tested 11 were not influenced by the neuronal co-culture). These results suggest that the PACAP secreted by neural cells regulates several factors secreted by RPE cells, important for retinal development and homeostasis (Ko et al. 2015). The local secretion of PACAP necessary for these effects is supported by the high concentration used in this study (1 μ M and 10 μ M PACAP, lower concentrations were not reported), a concentration much higher than the ones reported to exert protective effects against oxidative stress (Fabian et al. 2012, Mester et al. 2011).

Another study investigated the potential of PACAP and VIP against the disruption of tight junctions in the retina, a major factor in macular edema developing in diabetic retinopathy (Scuderi et al. 2013). ARPE19 cells were cultured either in normal glucose or in high glucose in combination with the pro-inflammatory IL-1 β . Effects on permeability were evaluated by measuring both apical-to-basolateral movements of fluorescein isothiocyanate (FITC) dextran and transepithelial electrical resistance (Scuderi et al. 2013). Results of these experiments demonstrated that normal glucose+IL-1 β and, to a greater extent, high glucose+IL-1 β significantly increased FITC-dextran diffusion, paralleled by decreased electrical resistance. PACAP or VIP reversed these effects. Furthermore, high glucose+IL-1 β -induced reduction of claudin-1 and ZO-1 expression was reversed by PACAP and VIP. Occludin expression was not affected in any of the conditions tested. Altogether, these findings show that both peptides counteract high glucose+IL-1 β -induced damage in ARPE19 cells, suggesting that they might be relevant to the maintenance of outer blood retinal barrier function in edema accompanying diabetic retinopathy (Scuderi et al. 2013). These results are in accordance with our earlier observations in an in vitro blood-brain barrier (BBB) model (Wilhelm et al. 2014). We

found that PACAP treatment improved the barrier properties of the brain endothelium. PACAP induced an increase in the transendothelial electrical resistance, which is the most important marker of the tightness of the tight junctions. Moreover, PACAP had a protective role against glucose deprivation- and oxidative stress-induced junctional damage in microvascular brain endothelial cells (Wilhelm et al. 2014).

Protective effects of PACAP in the retina in vivo

The in vivo protective effects are summarized in Table II.

Excitotoxic injury in the retina

Glutamate, a nonessential amino acid, acts as an excitatory neurotransmitter in the retina under normal conditions. However, glutamate in high concentrations can be toxic to the retinal cells by overstimulation of the glutamate receptors. Monosodium glutamate (MSG) administration given systemically passes the BBB to newborn rats, can injure the retina (Szabadfi et al. 2009) and lead to loss of the entire inner retinal layers (Babai et al. 2006). The excitotoxic damage of the retina is a main factor in retinal diseases, such as glaucoma and ischemia-induced retinopathy. We have previously shown in a number of studies that PACAP is protective against excitotoxicity induced by systemic monosodium-glutamate lesion. We showed that, three times intravitreal MSG treatment on postnatal days 1, 5 and 9 caused severe neurodegeneration in the inner retinal layers in newborn rat pups (Babai et al. 2005, 2006). Three weeks after the MSG administration the thickness of the total retina was decreased, the distance of the outer limiting membrane-inner limiting membrane (OLM-ILM) was only half of the normal retina. In addition, the IPL almost disappeared, and the fusion of the INL and GCL could be observed. The number of the cells per 100 μ m in the GCL was also decreased (Tamas et al. 2004; Babai et al. 2005, 2006; Atlasz et al. 2009a). Although PACAP1-38 is able to cross the BBB, systemic PACAP1-38 treatment caused insignificant improvement of the retinal morphology following MSG-induced excitotoxic injury (Kiss et al. 2006). In contrast, intravitreally injected PACAP1-38 (1-100 pmol) resulted in significant amelioration of this degeneration (Babai et al. 2005, 2006; Tamas et al. 2004). While 1 pmol PACAP1-38 was slightly effective, treatment with 100 pmol PACAP1-38 was able to significantly attenuate the MSG-induced toxicity and the entire retina seemed to be normal. Similar protection could be seen by PACAP1-27 treatment (Atlasz et al. 2009a). The application of the two widely used PACAP antagonists (PACAP6-38, PACAP6-27) led to a more pronounced degeneration, indicating that, endogenously present PACAP plays a key role against retinal toxicity (Atlasz et al. 2009a). A similar degree of retinoprotection was found using enriched environment, but combining these two neuroprotective strategies (PACAP and enriched environment) did not result in increased neuroprotection in excitotoxic retinal injury (Kiss et al. 2011).

Cell-type specific effects of PACAP were shown in MSG-induced retinal degeneration by means of immunohistochemistry. In the MSG-treated retinas, the cell bodies and processes in the INL, IPL and GCL layers displayed less immunoreactivity for vesicular glutamate transporter 1 (VGLUT1), tyrosine hydroxylase (TH), vesicular GABA transporter (VGAT), parvalbumin, and calretinin. After MSG treatment the

calbindin-positive horizontal cells did not appear to be affected. In MSG+PACAP1-38 co-treated retinas, the immunoreactivity patterns were similar compared to the normal eyes. These data suggest that PACAP has a general, but not subtype-specific protective mechanism against glutamate-induced excitotoxic injury in the rat retina (Atlasz et al. 2008).

We also studied potential protective signaling mechanisms in MSG treated retinas. We found that intravitreal administration of PACAP preceding the MSG treatments induced significant increases in the phosphorylation, that is, the activation of ERK1/2 and its downstream target, CREB, 12 h after the treatment compared to the contralateral untreated eye during the first two treatments, with no further elevations 24 h after treatments. These results demonstrate that the degenerative effect of MSG and the protective effect of PACAP involve complex kinase signaling pathways and are related to cAMP/ERK/CREB activation (Racz et al. 2006a). Furthermore, after MSG treatment proapoptotic signaling proteins such as caspase-3, JNK, apoptosis inducing factor (AIF) and cytochrome *c* increased, and some anti-apoptotic proteins (phospho-PKA, phospho-Bad, Bcl-xL, 14-3-3) decreased. Co-treatment with PACAP1-38 counteracted the MSG-induced apoptotic effects, the level of pro-apoptotic signals was significantly decreased, while the anti-apoptotic signals proteins were significantly increased (Racz et al. 2006a, 2006b, 2007). A follow-up study confirmed the anti-apoptotic effect of PACAP in MSG-induced retinal lesion of the newborns by reinforcing that PACAP decreased caspase-3 levels and also demonstrating the decreased level of caspase-9 (Lakk et al. 2015). In addition, we showed that the PACAP antagonist PACAP6-38 increased cytochrome-C release, caspase-3, JNK activity and decreased phospho-Bad, phospho PKA, Bcl-xL, 14-3-3 activity (Racz et al. 2006b, 2007). These *in vivo* observations, similarly to the results of Atlasz et al. (2009a), indicating that endogenously present PACAP has retinoprotective effects in glutamate-induced excitotoxicity.

We studied the short-term functional consequences of MSG treatment in the mouse retina. Spontaneous and light-evoked spikes of retinal ganglion cells (RGCs) from wild type mice were recorded using a 60-channel multielectrode array. MSG remarkably elevated the free intracellular calcium (Ca^{2+}) concentration and also increased the spontaneous spiking 4-5 minutes after drug application. During this time, spike correlations between RGC pairs were reduced. However, after 10-15 minutes of MSG application, the spontaneous activity of most RGCs was dramatically reduced or totally eliminated. Pretreatment with PACAP1-38 prevented the MSG effects as indicated by little or no change in the spontaneous spiking patterns during the course of recordings (up to 60 minutes). Moreover, the Ca^{2+} influx was noticeably decreased by PACAP1-38. In addition, MSG blocked the light-evoked responses of all recorded cells. Based on these data, application of PACAP1-38, rescued RGCs from the short-term MSG-induced insults (Atlasz et al. 2014).

Kainic acid is a glutamate receptor agonist, which leads to excitotoxic cell death in the CNS, including the retina (Seki et al. 2006). It was found that 5 nmol kainic acid caused an extensive loss of AMPA/kainate receptors expressing ganglion cells (Zhang et al. 1995). Previously we showed that microiontophoretically applied PACAP1-38 could block the excitatory effects of kainic acid in several brain areas *in vivo* (Atlasz et al. 2006). In the rat retina, pretreatment with 10 pmol intravitreal PACAP1-38, was

neuroprotective against kainic-acid induced neurotoxicity, as shown by the attenuated ganglion cell loss (Seki et al. 2006).

N-methyl-D-aspartate (NMDA) treatment also leads to similar lesions. A radioimmunoassay analysis showed that in contrast to VIP, retinal PACAP levels do not change after intravitreal NMDA injection, which might be caused by a compensatory upregulation (Teuchner et al. 2011). A recent study has confirmed that PACAP-mediated pathways are protective in NMDA-induced retinal lesion. Cheng et al. (2014) showed that intravitreal NMDA injection led to a decrease in light aversion, and decreases in the a and b-waves in the electroretinogram accompanied by a photopic negative response. These alterations were reversed by cyclic PACAP1-5 treatment. This treatment also resulted in decreased ganglion cell loss and apoptosis assessed by TUNEL-staining (Cheng et al. 2014).

A similar protective effect was described in mice with PACAP1-38, at a concentration of 100 pmol. The PACAP-attenuated NMDA-induced retinal damage in mice was associated with modulation of the microglia/macrophage status into an acquired deactivation subtype (Wada et al. 2013). NMDA injection led to an increase in microglia/macrophage number, while PACAP co-administration attenuated this effect in addition to the attenuated ganglion cell loss. The co-administration of the antagonist PACAP6-38 counteracted these protective effects and the beneficial effects were not observed in IL-10 knockout mice. PACAP administration also led to elevated expression of the mRNA for the protective cytokines IL-10 and TGF- β (Wada et al. 2013). In summary, these results show that acquired deactivation type of the microglia/macrophage cells favors neuroprotection in the retina and this is stimulated by PACAP.

The question was raised whether these apparent morphological improvements by PACAP administration also lead to functional amelioration in MSG-induced retinal damage (Varga et al. 2011). Electroretinographic recordings revealed a marked decrease in both the b- and a-wave values after MSG treatment, reflecting the function of the inner retinal layers and the photoreceptors, respectively. In retinas receiving intravitreal PACAP treatment, these values were significantly increased. Thus, the functional outcome, although not parallel with the morphology, was significantly improved after PACAP treatment. These observations are important from the clinical point of view, showing, that PACAP treatment is able to improve the functional properties of the retina in excitotoxic damage (Varga et al. 2011).

Ischemic injury in the retina

Retina is one of the most sensitive tissues to hypoxia in the human body. Several diseases such as artery occlusion, diabetic retinopathy and high intraocular pressure cause hypoperfusion in the retina. Depending on the rat strain, permanent bilateral common carotid artery occlusion (BCCAO) leads to reduction in the cerebral blood flow, causes hypoperfusion-induced biochemical and behavioral changing and also produces retinal degeneration (Atlasz et al. 2007; Mester et al. 2009). Our research group showed in a series of experiments that intravitreal PACAP is highly protective in ischemic retinal lesion. We found that BCCAO caused severe damage in all layers of the retinas. Two weeks after permanent carotid artery ligation, the thickness of the plexiform layers were

markedly decreased, and as a consequence, the distance between the OLM-ILM was significantly less compared to the control retina. In several treated animals, in the IPL some dots of about 1 μm in diameter were seen. Based on the relatively even distribution, these dots seemed to be degenerating bipolar cell terminals. In addition, the outer segments of the photoreceptors became shorter, and their structure was also damaged after the insult (Atlasz et al. 2007). Intravitreal PACAP1-38 treatment following BCCAO led to a nearly intact appearance of the retinal layers, which is well reflected by the morphometric analysis. The thickness of the all retinal layers, but not the number of cell in GCL, was preserved in PACAP1-38 treated animals. PACAP antagonist PACAP6-38 significantly attenuated the protective effects of PACAP1-38.

In 2010, we provided immunohistochemical description of several retinal cell types that were damaged by BCCAO and could be partially or fully rescued by intravitreal PACAP1-38 treatment. Using the following cell type specific markers (VGLUT1, TH, VGAT, parvalbumin, calbindin, calretinin, PKC α , and GFAP) marked changes were detected after BCCAO, and the alterations observed in the immunostaining for all examined antisera were counteracted by intravitreal PACAP1-38 treatment (Atlasz et al. 2010b). These studies provided the basis for the promising therapeutic potential of PACAP in ischemic retinal diseases (Szabadfi et al. 2010).

Subsequent studies confirmed these original observations. Seki et al. (2011) showed that intravitreal PACAP treatment protected ganglion cells against a different kind of ischemic lesion induced by high intraocular pressure. In this model, PACAP exerted a bimodal protective effect with peaks at 10 fM and 10-1000 pM. The protective effect was blocked by cAMP antagonist at both high and low protective doses, while a MAP kinase inhibitor only blocked the effects of PACAP at a low dose, suggesting that the protective effects of PACAP are mediated by different signaling at low and high doses (Seki et al. 2011).

In a series of studies, we confirmed these findings and compared the efficacy of several analogs and related peptides to PACAP1-38 (Szabadfi et al. 2012a; Werling et al. 2014). We investigated the potential retinoprotective effects of the following PACAP fragments: PACAP 4-13, 4-22, 6-10, 6-15, 11-15, and 20-31 and three related peptides (VIP, secretin, glucagon) in BCCAO-induced ischemic retinopathy. We confirmed that the most effective molecule is PACAP1-38, while the other fragments or related peptides did not lead to retinoprotection (Werling et al. 2014). The only exception was VIP, which showed retinoprotection in a dose 10 times higher than that of PACAP (Szabadfi et al. 2012a). In addition, our preliminary results demonstrate that even in form of eye-drops, PACAP can lead to retinoprotective effects in ischemic retinopathy, a route of application with major possible clinical relevance (Werling et al. 2015).

In order to elucidate some of the molecular mechanisms, we studied MAP kinases and Akt expression as well as cytokines in BCCAO-induced retinal ischemia in rats (Szabo et al. 2012). We revealed that PACAP treatment alone did not influence the phosphorylation of Akt or the MAPKs, but decreased the hypoperfusion-induced activation of both p38MAPK and JNK and increased the activation of the protective Akt and ERK1/2 in hypoperfused retinas. The cytokine profile dramatically changed after BCCAO, with most cytokines and chemokines (such as CINC, CNTF, fractalkine, sICAM, IL-1, LIX, Selectin, MIP-1, RANTES and TIMP-1) increased, a result attenuated by PACAP. In addition, PACAP increased the expression of vascular endothelial growth

factor (VEGF) and thymus chemokine. These results provided further insight into the neuroprotective mechanism induced by PACAP in ischemic retinal injuries, showing that PACAP ameliorated hypoperfusion injury involving Akt, MAPK pathways and anti-inflammatory actions (Szabo et al. 2012).

Whether the morphological amelioration is accompanied by functional improvement was tested using electroretinography (ERG) in another set of experiments (Danyadi et al. 2014). Retinal damage and protective effects of PACAP were quantified by the changes in the waveforms and amplitudes. 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. 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' waves of the ERG on postoperative day 14. 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 results confirm that the previously described morphological protection induced by PACAP treatment is reflected in functional improvement in ischemic retinal lesions (Danyadi et al. 2014).

A recent study has revealed several metabolomics changes induced by PACAP in ex vivo retinal ischemia induced by sodium azide treatment for various periods of time (D'Alessandro et al. 2014). PACAP induced several changes in addition to the reduction of ischemia-induced cell death, VEGF overexpression, and glutamate release. Apoptosis, as evaluated by TUNEL staining, was reduced in the ONL, INL and GCL. Elevated levels of caspase-3 mRNA were also reversed by PACAP treatment. Glutamine, a precursor of glutamate, was significantly elevated in ischemic conditions, and reversed by PACAP administration. Ischemia is associated with oxidative injury and so the authors evaluated the oxidative status of the retinal explants. It was found that PACAP had a positive effect on glutathione while it reduced the oxidized glutathione levels, and counteracted the increased gamma-glutamyl cysteins levels with accompanying increase of cysteine. The accumulation of pro-inflammatory factors and peroxidized lipids was prevented by PACAP. Three nitric oxide metabolites were found to be upregulated in ischemic retinas: arginine, ornithine and citrulline. Treatment with PACAP reversed these changes. The significantly decreased level of cAMP was restored by PACAP treatment and glycolytic flues were normalized preventing the over-accumulation of lactate or promoting the down-regulation of the glyoxalate antioxidant system. Upregulated inositol levels were also abolished. PACAP seemed to promote a shift towards pentose-phosphate pathway while maintaining higher levels of NADPH compared to ischemic controls. It also led to a down-regulation of purine metabolism. This study concluded that peptidergic systems, such as PACAP, deserve attention in treating retinal ischemia (D'Alessandro et al. 2014).

Thanks to all these results, PACAP is now on the list of potential neuroprotective molecules in combatting ischemic retinopathy (Cervia and Casini, 2013; Szabadfi et al. 2010).

Optic nerve injury in the retina

Optic nerve transection is a model of apoptotic neuronal cell death in the adult CNS (Quigley et al. 1995). Seki et al. (2008) reported that optic nerve trauma resulted approximately 55% loss of cells in the GCL. In this model, the number of cells in the GCL in animals treated before the operation with PACAP1-38 at 10 and 100 pmol in 3 μ l saline (dose-dependent) intravitreally, was significantly increased compared with the control group on the 14th day after surgery (Seki et al. 2008), providing evidence that PACAP is protective in this injury model.

UV radiation injury in the retina

We showed, for the first time, that PACAP protected against in two different kinds (diffused and focused) of ultraviolet (UV)-A induced degenerations. Thickness of all layers as well as the number of cells in the ONL and INL and in the GCL was evaluated by standard morphological and morphometrical analyses. After diffuse UV-A irradiation, significant decrease of the number of the cells in the GCL was observed. In addition, severe degeneration in the ONL and INL was seen, and the IPL was swollen. PACAP1-38 treatment after the UV-A irradiation significantly ameliorated the light-induced degeneration, the ONL and INL thickness seemed to be normal, and the number of cells in INL, ONL and GCL was significantly increased (Atlasz et al. 2011). In a model of focused UV-A irradiation, the shape of the laser focus was observed on the retina, tissue gaps were found in the ONL and in the photoreceptor layer, and the IPL was reduced. The total thickness of the retina was significantly less than in unaffected areas. We observed protective effects of PACAP in all of the different retinal layers (Atlasz et al. 2009b).

Succeeding studies confirmed this effect in several different models. In an in vitro model of UV-B irradiation, PACAP was shown to protect RGC-5 retinal ganglion cells expressing PAC1 receptors (Cheng et al. 2014; Ding et al. 2012). This effect was mimicked also by a cyclic PACAP derivative, cyclic PACAP1-5 (Cheng et al. 2014). Furthermore, pretreatment with this short derivative effectively blocked apoptosis, maintained structural integrity, decreased production of reactive oxygen species (ROS). Both PACAP and cyclic PACAP1-5 counteracted the UV-B-induced decrease in the protective bcl-2 and the increase in the deleterious bax protein levels (Cheng et al. 2014).

Diabetic retinopathy in the retina

Our recent studies have shown that PACAP is also protective in diabetic retinopathy, a major microangiopathy affecting the vision of diabetic patients. Our first results demonstrated that in the early stages of diabetic retinopathy, amacrine cells underwent characteristic degeneration, especially the dopaminergic amacrine cells. PACAP prevented the typical alterations induced in 1-month diabetes: the degeneration in arborization and synapses of tyrosin-hydroxylase positive dopaminergic amacrine cells (Atlasz et al. 2010b). In a rat model of streptozotocin-induced diabetic retinopathy (DR), early signs do not include overall changes in thickness of retinal layers, but the decrease

in the number of ganglion cells and the upregulation of GFAP as a sign of Müller glial cell activation, can be observed. These changes were counteracted by 3 times 100 pmol/5µl intravitreal PACAP administration (Szabadfi et al. 2012b). Furthermore, the degeneration of the photoreceptor outer segments and terminals was attenuated in PACAP-treated rats. PACAP increased the levels of PAC1-receptor and TH determined with molecular biological methods. These early results suggested a therapeutic potential in DR.

Subsequently, our research group and others confirmed these early findings (Szabadfi et al. 2014a). Intraocular PACAP injection markedly attenuated diabetic retinal injury: increased levels of the anti-apoptotic p-Akt, p-ERK1, p-ERK2, PKC, Bcl-2, while decreased levels of the pro-apoptotic p-p38MAPK and activated caspases (8, 3, 12) were detected. The number of apoptotic cells increased in all nuclear layers of diabetic retinas, but significantly decreased after PACAP treatment. Our results clearly demonstrated that the protective effects of PACAP are mediated, at least partly, by attenuating apoptosis, including also that of the dopaminergic amacrine cells (Szabadfi et al. 2014a). Our most recent study shed further light on this PACAP-induced retinoprotection. Electron microscopic analysis revealed that retinal pigment epithelium, the ribbon synapses and other synaptic profiles suffered alterations in diabetes. However, in PACAP-treated diabetic retinas more bipolar ribbon synapses were found intact in the inner plexiform layer than in DR animals. The ribbon synapse was marked with C-terminal binding protein 2 (CtBP2)/Bassoon and formed horseshoe-shape ribbons, which were more retained in PACAP-treated diabetic retinas than in DR rats. These results are supported by molecular biological data. The selective degeneration of related structures such as bipolar and ganglion cells could be ameliorated by PACAP treatment. In summary, intravitreal administration of PACAP may have therapeutic potential in streptozotocin-induced DR through maintaining synapse integrity in the vertical pathway (Szabadfi et al. 2016).

D'Amico and coworkers (2015) analyzed the expression of hypoxia-induced factors (HIF) in DR 3 weeks after the induction of diabetes with streptozotocin. They found that the expression of HIF-1 α and HIF-2 α significantly increased in diabetic rats, but not after 1 intravitreal PACAP treatment. Conversely, the expression of HIF-3 α was significantly downregulated in retinas of diabetic rats, and increased after PACAP treatment. These results suggest that the retinoprotective effects of PACAP are partially mediated by interfering with the expression of HIFs that play an important role in pathological vasculogenesis occurring in DR (D'Amico et al. 2015).

These studies, together with the results demonstrating the effects of PACAP on the blood-retinal barrier properties in vitro (see above), show that PACAP has therapeutic potential in diabetic retinopathy (Gabriel 2013; Marzagalli et al. 2015; Szabadfi et al. 2014b). Several experimental evidence support the efficacy of PACAP treatment in animal model of diabetes-related diseases, such as diabetic nephropathy, another major microangiopathic complication of diabetes (Banki et al. 2013, 2014). Thus, PACAP is among the emerging molecules to fight diabetic complications, similarly to VEGF antagonists, antioxidants, anti-inflammatoxy agenst and other neuropeptides (Gabriel 2013; Ola et al. 2015; Scuderi et al. 2015).

Retinopathy of prematurity (ROP)

ROP is a major complication in premature newborns. It is a vasoproliferative disorder, the main features of which are well depicted in a rat model of oxygen-induced retinopathy. Our preliminary studies have shown that PACAP is able to reverse some of the deleterious effects of this retinopathy. Intravitreal PACAP1-38 treatment ameliorated the vascular extent of the retinopathic eyes without alterations in vessel morphology. Moreover, cytokine expression profiles indicate that PACAP may block the new vessel formation and have a positive influence on the remodeling of the tissue (Kvarik et al. 2015).

Studies in PACAP and receptor gene modified animals

We have described that although PACAP deficient mice have normal retinas at young age, aging signs appear earlier than in their wild-type mates (Szabadfi et al. 2012c). As it has been shown in numerous models, PACAP knockout (KO) mice are more vulnerable to different types of tissue injuries from ischemia to oxidative stress and even trauma (Reglodi et al. 2012). In our experiments, PACAP KO mice that underwent 10 min of BCCAO followed by 2-week reperfusion period had significantly greater retinal damage, as shown by the thickness of the whole retina, the morphometric analysis of the individual retinal layers, and the cell numbers in the inner nuclear and ganglion cell layers. Exogenous PACAP administration could partially protect against this retinal degeneration in the KO mice. These results clearly show that endogenous PACAP reacts as a stress-response peptide that is necessary for endogenous protection against different retinal insults (Szabadfi et al. 2012d).

The higher vulnerability of PACAP deficient mice against lesions was confirmed by another study by Endo et al. (2011). The authors found, in accordance with our results, that under normal circumstances, there was no difference in the number of retinal ganglion cells in heterozygous PACAP deficient mice and wild-type counterparts (Endo et al. 2011). However, when these mice were subjected to NMDA-induced toxicity, PACAP heterozygous mice reacted with increased loss of ganglion cells (Endo et al. 2011). The number of apoptotic cells, as measured by TUNEL staining, peaked on day 1 in heterozygous mice, while the peak was on day 3 in wild-types, suggesting that apoptotic cell death began earlier in mice deficient in PACAP. These effects were reversed by simultaneous injection of PACAP in heterozygous mice. These results confirm that endogenous PACAP is protective in the retina and prove that even partial loss of PACAP-driven neuroprotection decreases resistance against harmful effects (Endo et al. 2011). Our recent, preliminary results also confirm that endogenous PACAP is protective in lipopolysaccharide (LPS)-induced retinal inflammation, as shown by the increased inflammatory status of PACAP KO. Twenty-four hours after LPS treatment several cytokines and chemokines showed significant increase in the PACAP knockout mice compared to the normal, wild types (Atlasz et al. 2015).

Little is known about retinal morphology or function in mice with PAC1 receptor abnormalities. Engelund and coworkers (2012) described that pupillary light reflex was altered in PAC1 receptor knockout mice. The finding that PACAPergic signaling is essential for normal retinal development is also confirmed by studies with transgenic

mice. It was described by Lang et al. (2010) during development that overexpression of the PAC1 receptor led to an early exit from retinal proliferation, reduced signaling of GABAergic neurons. Furthermore, this led to decreased visual function (Lang et al. 2010). These data show that a proper balance of PACAP signaling is required for appropriate retinogenesis and visual acuity.

Studies in tumor cells

PACAP can exert different effects in cells derived from various sources and especially from tumors. While in most cell types PACAP is protective, in some tumors it exerts toxic effects (Brubel et al. 2010). Furthermore, PACAP6-38 is in most cell types an antagonist, it can also behave as an agonist in some, tumorous and even non-tumorous, cells (Juhász et al. 2015, Saghy et al. 2015). Retinoblastoma is a malignant tumor of retinal progenitor cells. Human retinoblastoma cells are known to express PACAP receptors (Olianas et al. 1996). In Y79 retinoblastoma cells, PACAP1-38 did not influence cell survival at nanomolar concentrations, but at 1-5 micromolar concentrations, it reduced survival (Wojcieszak and Zawilska, 2014). Interestingly, PACAP1-27 and maxadilan had negligible effects, while two membrane-penetrating analogs of PACAP also decreased cell viability. The authors also revealed that this cytotoxic effect might be via an interaction of p38, MEK1/2 and JNK signaling, because inhibitors of these pathways led to an even more expressed cytotoxicity (Wojcieszak and Zawilska, 2014). These results suggest that high concentrations of PACAP1-38 might lead to a transmembrane penetration and lead to direct cytotoxicity at sites other than the conventional PACAP receptor-mediated signaling.

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Abbreviations:

6-OHDA: 6-hydroxydopamine
AIF: apoptosis inducing factor
AMPA receptor: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Bad: Bcl-2-associated death promoter
Bax: Bcl-2-associated X protein
BBB: blood-brain barrier
BCCAO: bilateral common carotid artery occlusion
Bcl-2: B-cell lymphoma 2
Bcl-xl: B-cell lymphoma-extra large
cAMP/PKA/CREB: ciklikus AMP/protein-kina A/ cAMP response element-binding protein
CIAP-1: cellular inhibitor of apoptosis protein-1
CINC: cytokine-induced neutrophil chemoattractant
CNS: central nervous system
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CtBP2: C-terminal binding protein 2
DR: diabetic retinopathy
eNOS: endothelial nitric oxide synthase
ERG: electroretinography
ERK: extracellular-signal-regulated kinase
Fas TNFR: Fas tumor necrosis factor receptor
FITC: fluorescein isothiocyanate
GCL: ganglion cell layer.
GFAP: glial fibrillary acidic protein
HIF1 α ,2 α ,3 α : hypoxia-inducible factor 1-alpha, 2-alpha, 3-alpha
HO-1: heme oxygenase 1
HSP-27: heat shock protein 27
IL-1 β : interleukin-1 beta
INL: inner nuclear layer
IP3: inositol trisphosphate
IPL: inner plexiform layer
JNK: c-Jun N-terminal kinase
LPS: lipopolysaccharide
MAPK: mitogen-activated protein kinase
MIP-1: macrophage inflammatory protein 1
MSG: monosodium glutamate
NBL: neuroblast layer
NF- κ B: nuclear factor- κ B
NFL: nerve fiber layer
NMDA: N-methyl-D-aspartate
NPY: neuropeptide Y
OLM-ILM: outer limiting membrane-inner limiting membrane

ONL: outer nuclear layer
OPL: outer plexiform layer
Ops: oscillatory potentials
PAC1: PACAP receptor 1
PACAP KO: PACAP knockout
PACAP: adenylyl cyclase activating polypeptide
PKC α : protein kinase C alpha
PLCgamma-1: phospholipase C gamma-1
RANTES: regulated on activation, normal T cell expressed and secreted
RGCs: retinal ganglion cells
ROP: retinopathy of prematurity
ROS: reactive oxygen species
RPE: retinal pigment epithelial
RPE: retinal pigment epithelium
RSK1/2: ribosomal s6 kinase 1,2
sICAM: soluble intercellular adhesion molecule
STAT4: signal transducer and activator of transcription 4
TH: tyrosine hydroxylase
TIMP-1: tissue inhibitor metalloproteinase inhibitor 1
Trail R2 DR5: TRAIL receptor 2 - death receptor 5
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
TGF- β : transforming growth factor beta
UV-A, B: ultraviolet A, ultraviolet B
VEGF: vascular endothelial growth factor
VGAT: vesicular GABA transporter
VGLUT1: vesicular glutamate transporter 1
VIP: vasoactive intestinal peptide
VPAC1: vasoactive intestinal peptide (VIP) receptor type 1
VPAC2 (vasoactive intestinal peptide (VIP) receptor type 2
ZO-1: zona occludens protein 1