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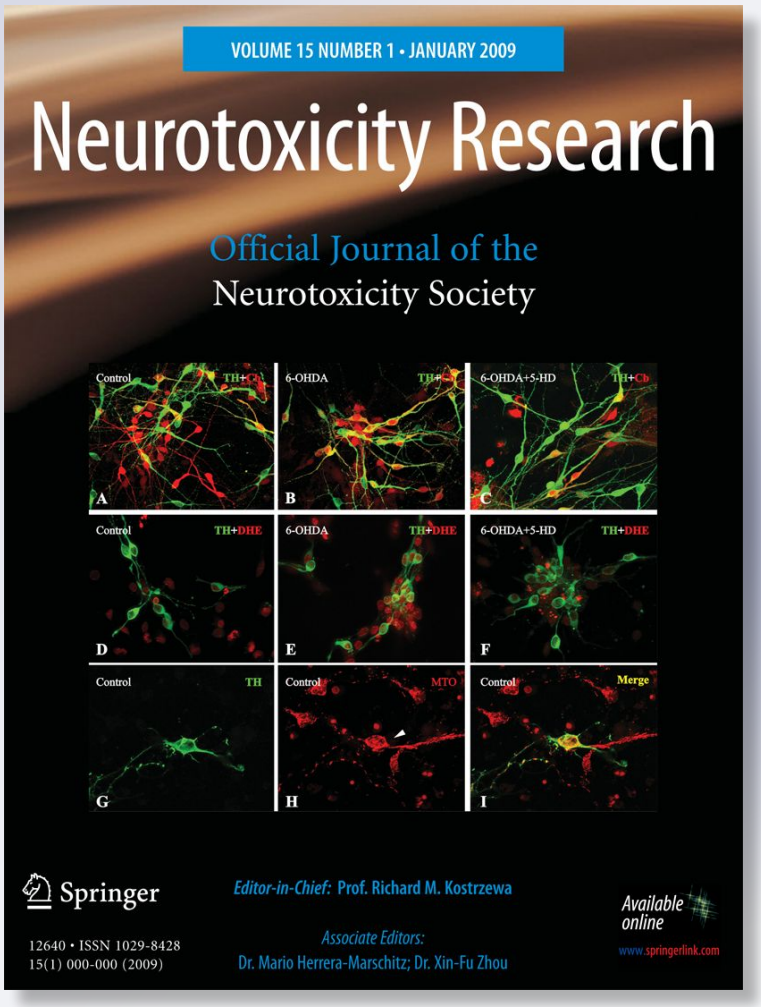

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Mice Deficient in Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) are More Susceptible to Retinal Ischemic Injury In Vivo

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Abstract Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuroprotective peptide exerting protective effects in neuronal injuries. We have provided evidence that PACAP is neuroprotective in several models of retinal degeneration in vivo. Our previous studies showed that PACAP treatment ameliorated the damaging effects of chronic hypoperfusion modeled by permanent bilateral carotid artery occlusion. 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. The aim of this study was to compare the degree of retinal damage in wild type and PACAP deficient mice in ischemic retinal insult. Mice underwent 10 min of bilateral carotid artery occlusion followed by 2-week reperfusion period. Retinas were then processed for histological analysis. It was found that PACAP deficient mice 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 retinal degeneration in PACAP deficient mice. These results clearly show that endogenous PACAP reacts as a stress-response peptide that is necessary for endogenous protection against different retinal insults.

Keywords PACAP knockout · Retina · Transient ischemia · Endogenous PACAP · Intravitreal PACAP treatment

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Introduction

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 (Bek 2009; Feigl 2009; Lelong et al. 2007; Shazly and Latina 2009; Zheng et al. 2007). The mechanism underlying ischemic retinal degeneration and potential

protective interventions are studied in a diversity of animal models (Dimitriu et al. 2008; Moren et al. 2009; Osborne et al. 2004; Vidal-Sanz et al. 2000). Similar to cerebral ischemia, the extent and degree of retinal ischemic damage largely depends on the model used and the animal strain and gender (Barnett and Osborne 1995; Davidson et al. 2000; Mester et al. 2009; Oliff et al. 1997; Yamamoto et al. 2006). Bilateral common carotid artery occlusion (BCCAO, also called 2-vessel occlusion), is a model of chronic cerebral hypoperfusion, leading also to ischemic retinal changes (Atlasz et al. 2007a, b, 2010a; Farkas et al. 2007; Osborne et al. 2004; Yamamoto et al. 2006). Previously, we have studied several candidate neuroprotective agents in rat permanent bilateral carotid artery occlusion and we have shown the protective effects of diazoxid, urocortin 2, and a PARP inhibitor (Atlasz et al. 2007a, Mester et al. 2009; Szabadfi et al. 2009, 2010).

Pituitary adenylate cyclase activating polypeptide (PACAP) is a widely distributed neuropeptide, first isolated from hypothalamic extracts (Miyata et al. 1989). PACAP belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family. PACAP occurs throughout the nervous system and in peripheral organs (Vaudry et al. 2009). 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 (Seki et al. 2000a). 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 (Laburthe et al. 2007; Vaudry et al. 2009). 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) (Seki et al. 1997; 2000b). The neurotrophic and neuroprotective effects of the peptide are now well established (Reglodi et al. 2011; Seaborn et al. 2011; Shioda et al. 2006; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009; Waschek 2002). PACAP protects neurons against different toxic agents in vitro and provides neuroprotection in several models of brain pathology (Botia et al. 2011; Shioda et al. 2006; Somogyvari-Vigh and Reglodi 2004). Among the first studies describing the in vivo neuroprotective effects of PACAP were those showing that PACAP reduces brain damage in focal and global cerebral ischemia (Dejda et al. 2011; Ohtaki et al. 2008; Reglodi et al. 2002, 2004; Shioda et al. 1998; Uchida et al. 1996). Based on the presence of PACAP and its receptors in the retina, it was suggested that PACAP would be protective also in retinal ischemia. Evidence has been provided that PACAP is protective in a rat model of chronic retinal hypoperfusion induced by permanent bilateral carotid occlusion (Atlasz et al. 2007b, 2010a, b).

Based on the important neurotrophic effects of PACAP during neuronal development, the involvement of the peptide in endogenous restorative processes was hypothesized (Waschek 2002). Indeed, several studies have demonstrated that endogenous PACAP increases upon nervous injury and PACAP deficient mice respond to insults with more severe deficits and lower level of regeneration (Armstrong et al. 2008; Larsen et al. 1997; Vaudry et al. 2005; Zhang et al. 1996). These studies support the hypothesis that PACAP plays a role in the natural defense mechanism in nervous injuries. Of special interest are studies showing that this endogenous protective effect is also present in ischemic injuries: PACAP deficient mice have larger infarct volume in a model of cerebral ischemia (Chen et al. 2006; Ohtaki et al. 2006). Based on these studies, 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.

Materials and Methods

The generation and maintenance of the knockout mice on the CD1 background have been described previously in details (Hashimoto et al. 2001, 2009), they were backcrossed for ten generations with the CD1 strain. Wild type ($PACAP^{+/+}$; $n = 11$) and homozygous PACAP deficient ($PACAP^{-/-}$; $n = 12$) mice were subjected to transient BCCAO. Animals were fed and watered ad libitum, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-20/2006). 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 (Biemer-Clip, Aesculap, Germany). Immediately following the operation, PACAP (100 pmol in 3 µl 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 (120 mg/kg pentobarbital, Nembutal, Sanofi-Phylaxia, Hungary) and the eyes were immediately dissected in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer. 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 ligation (Atlasz et al. 2007a, b). Briefly, retinal sections (2 μm) were stained with toluidine blue (Sigma, Hungary). The sections were mounted in Depex medium and examined in a Nikon Eclipse 80i microscope. Photographs were taken with a digital CCD camera using the Spot program, from central retinal areas of nearly same eccentricities. Files were then further processed with Adobe Photoshop 7.0 program. Measurements were taken from the digital photographs with the NIH Image 1.55 program. 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: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM); (ii) the width of the outer and inner nuclear (ONL, INL), outer and inner plexiform layers (OPL, IPL); (iii) the number of cells/100 μm section length in the GCL, and (iv) the number of cells in 500 μm^2 INL. Results are presented as mean ± SEM. Statistical comparisons were made using ANOVA test followed by post hoc analysis of Tukey-B test.

Results

All layers were visible in sham-operated control preparations (Fig. 1a, b). 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 each present. No marked differences were observed between the control retinas of wild type (Fig. 1a) and PACAP deficient mice (Fig. 1b) 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 (Fig. 1c, d). In the wild-type ischemic retinas empty cell body shapes and tissue gaps were the only changes that could be noticed in the INL (Fig. 1c). Packing of cells in the INL was particularly loose and the large cells almost completely disappeared from the GCL in ischemic PACAP KO retinas (Fig. 1d). Intravitreal PACAP treatment significantly ameliorated the retinal degeneration induced by transient BCCAO (Fig. 1e, f). 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 (Fig. 2a, b). 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%; OPL: 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% (Fig. 2c). The observed degree of retinal degeneration corresponds to our previous results with the BCCAO model (Atlasz et al. 2007b). The cell number of the GCL was also reduced in ischemic conditions (in wild type: 65%; in PACAP KO: 48% of sham-operated control retinas; Fig. 2d). 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 (Fig. 2). This could be particularly well demonstrated in the case of the IPL thickness and the number of cells in the GCL.

Discussion

These 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 (Gray et al. 2002), have decreased fertility and reproductive functions (Isaac and Sherwood 2008), display early neonatal death (Cummings et al. 2004), and react to hormonal and metabolic changes in an altered manner (Hatanaka et al. 2008; Nakata et al. 2004; Tomimoto et al. 2008). Furthermore, PACAP deficient mice have memory disturbances, abnormal nonvisual photoreception, behavioral abnormalities, and altered pain and inflammatory reactions (Hashimoto et al. 2001, 2009; Kawaguchi et al. 2010; Kemeny et al. 2010; Matsuyama et al. 2003; Sandor et al. 2010).

It has been reported that the gross cerebral and cerebellar morphology is not altered in PACAP deficient mice (Vaudry et al. 2005). 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 (Allais et al. 2007;

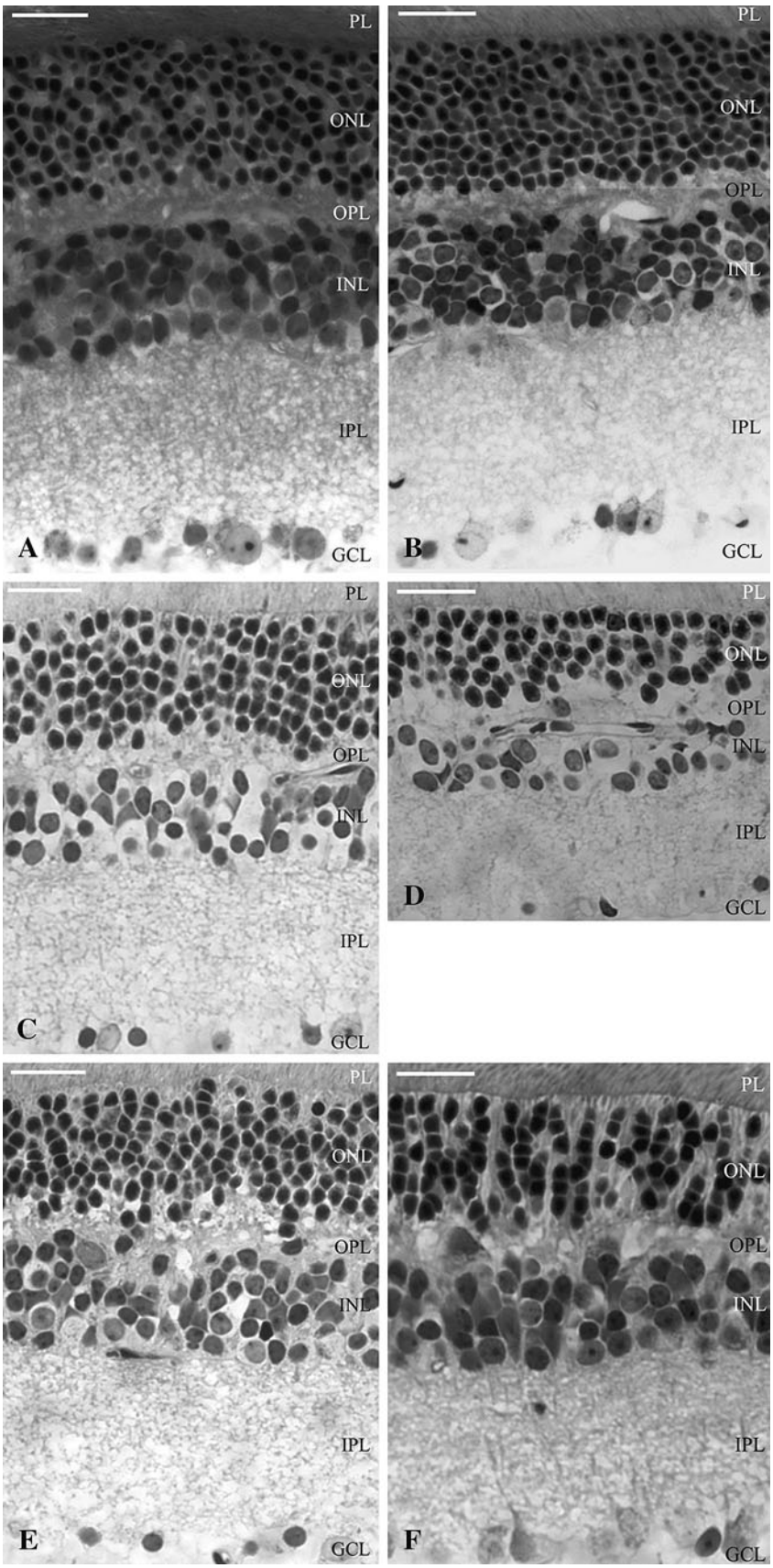

Fig. 1 Light microphotographs of toluidine blue-stained retinas from sham-operated wild type (**a**) and homozygous PACAP deficient mice (**b**); wild type after 10 min BCCAO (**c**); PACAP deficient mice after 10 min BCCAO (**d**); BCCAO + PACAP-treated wild type (**e**), and PACAP deficient mice (**f**). No marked differences could be detected between the retinas of wild type (**a**) and PACAP deficient mice (**b**). The wild-type retina of BCCAO-operated animals did not show significant morphological differences compared to **a**, **b** (**c**). Severe degeneration of the homozygous PACAP KO retinas was observed in histological preparations after transient BCCAO (**d**). The protective effects of intravitreal PACAP treatment could be observed in both groups (**e**, **f**). *PL* photoreceptor layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer. *Scale bar* 20 μm

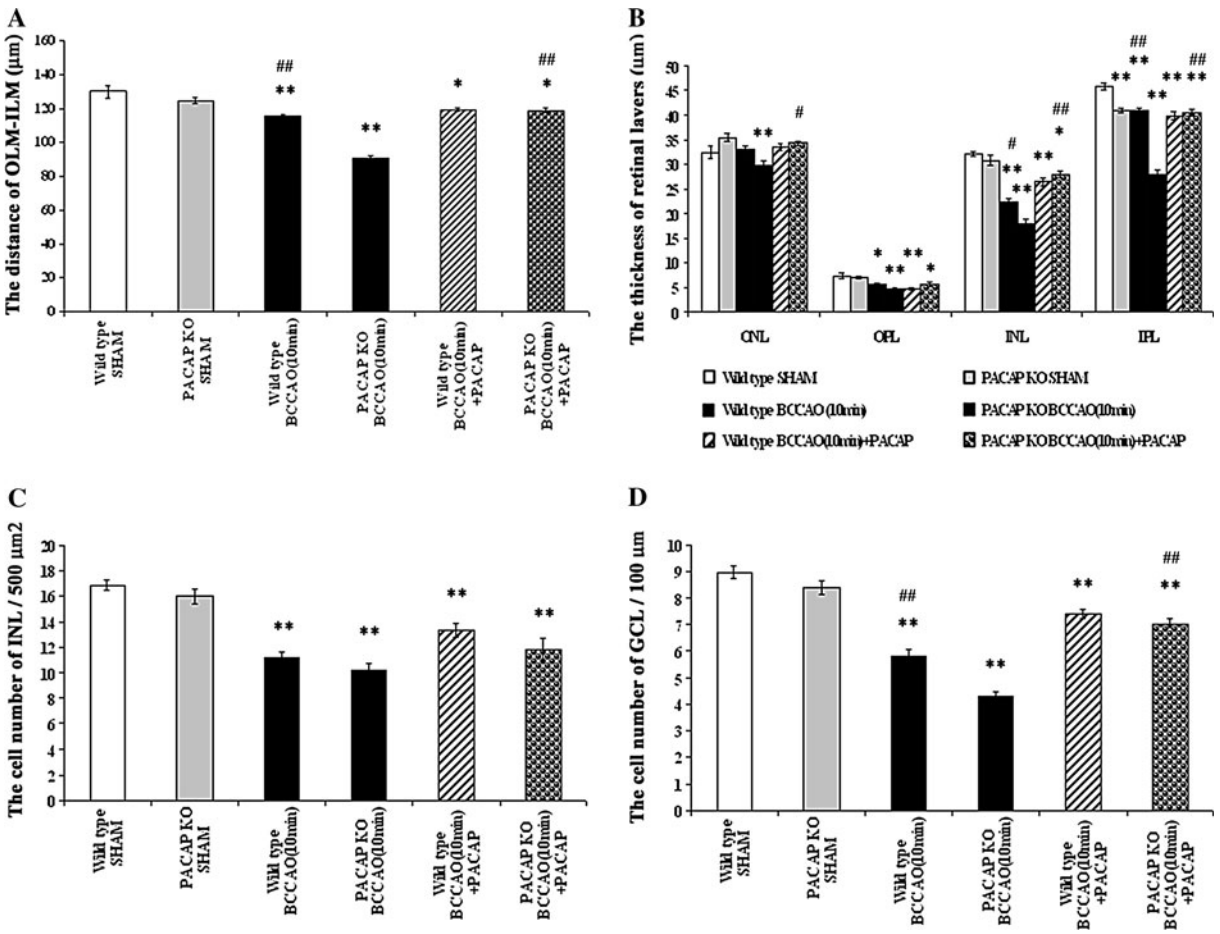

Fig. 2 Quantification of the whole retina thickness (**a**), distinct retinal layers (**b**), the cell numbers in 500 μm^2 INL (**c**), and in 100 µm length of GCL (**d**) in sham-operated, ischemic, and ischemic + PACAP-treated animals. No significant differences could be detected between the sham-operated retinas of wild type and PACAP deficient mice. The degree of BCCAO-induced retinal degeneration and also the neuroprotective effects of PACAP were quantified by the thickness of the whole retina (**a**) and of different retinal layers (**b**). In addition, cell numbers were counted in the INL and GCL (**c**, **d**). A significant amelioration of the retinal structure could be observed after PACAP treatment in both wild type and PACAP deficient mice, as shown by the thickness of the retinal layers and cell number in INL and GCL (**a**, **b**, **c**, **d**). $^{*}P < 0.01$ and $^{**}P < 0.001$ compared to sham-operated wild-type retinas; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ compared to retinas of $PACAP^{-/-}$ mice after 10 min BCCAO

Yamada et al. 2010). 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 (Vaudry et al. 2005). In vivo, PACAP deficient mice are more vulnerable to experimental autoimmune encephalomyelitis (Tan et al. 2009) and display slower recovery after peripheral nerve crush injury (Armstrong et al. 2008). It has also been shown that these mice develop larger infarct volume and increased edema in focal cerebral ischemia (Chen et al. 2006; Nakamachi et al. 2010; Ohtaki et al. 2006). 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 (Ferencz et al. 2010; Szakaly et al. 2011).

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 (Endo et al., 2011; Racz et al. 2006, 2007; Seki et al. 2006; Tamas et al. 2004; Varga et al. 2011), optic nerve transection (Seki et al. 2008), anisomycin-induced lesion (Silveira et al. 2002) and UV light-induced degeneration (Atlasz et al. 2011). We have provided evidence that PACAP also protects against ischemic injury caused by carotid occlusion in rats (Atlasz et al. 2007a, b), which has subsequently been

confirmed by others in a different model (Seki et al. 2011). 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 (Atlasz et al. 2009) and activates pro-apoptotic signaling pathways (Racz et al. 2006). 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 (Endo et al. 2011). 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 (Reglodi et al. 2011; Seaborn et al. 2011). 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 (Racz et al. 2006, 2007). Furthermore, PACAP also acts indirectly, through glial cells, by increasing the release of neuroprotective factors (Nakamachi et al. 2011). PACAP acts on Muller 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 (Liu et al. 2011; Nakatani et al. 2006; Ohtaki et al. 2008). Other mechanisms might also play a role in retinal protection, for example the anti-inflammatory effects of PACAP and the action against free radicals (Abad and Waschek 2011; Ohtaki et al. 2010; Vaudry et al. 2009).

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.

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