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64 Abstract

Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide, acting as a neuromodulator and neuroprotective peptide in the CNS after injuries. We have previously described that pituitary adenylate cyclase-activating polypeptide, another member of the same peptide family, is retinoprotective in ischemic lesions. The aim of this study was to investigate the protective potential of VIP in bilateral common carotid artery occlusion (BCCAO)-induced ischemic retinal lesion. Two-month-old rats were subjected to BCCAO and treated with intravitreal VIP injection. Their retinas were processed for histology after 2 weeks of survival. We measured the number of the cells/100 μm of the ganglion cell layer and the thickness of each layer such as the outer nuclear, outer plexiform, inner nuclear, and inner plexiform layers as well as that of the whole retina. We found that treatment with 1,000 pmol VIP, but not 100 pmol VIP, had significant protective effects in BCCAO-injured retina, as shown by the morphometric analysis. Comparing the neuroprotective effects of VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) in BCCAO-operated retinas, PACAP was more effective, already protective at 100-pmol doses. Similar to other studies, we found that VIP must be given at least in 10 times more concentration than PACAP to achieve a similar degree of neuroprotection in the retina.

65 Keywords separated by ' - '

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Protective Effects of Vasoactive Intestinal Peptide (VIP) in Ischemic Retinal Degeneration

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Abstract Vasoactive intestinal peptide (VIP) is a pleiotropic neuropeptide, acting as a neuromodulator and neuroprotective peptide in the CNS after injuries. We have previously described that pituitary adenylate cyclase-activating polypeptide, another member of the same peptide family, is retinoprotective in ischemic lesions. The aim of this study was to investigate the protective potential of VIP in bilateral common carotid artery occlusion (BCCAO)-induced ischemic retinal lesion. Two-month-old rats were subjected to BCCAO and treated with intravitreal VIP injection. Their retinas were processed for histology after 2 weeks of survival. We measured the number of the cells/100 μm of the ganglion cell layer and the thickness of each layer such as the outer nuclear, outer plexiform, inner nuclear, and inner plexiform layers as well as that of the whole retina. We found that treatment with 1,000 pmol VIP, but not 100 pmol VIP, had significant protective effects in BCCAO-injured retina, as shown by the morphometric analysis. Comparing the neuroprotective effects of VIP and pituitary adenylate cyclase-activating polypeptide (PACAP) in BCCAO-operated retinas, PACAP was more effective, already protective at 100-pmol doses. Similar to other studies, we found that VIP must be given at least in 10 times more concentration than PACAP to achieve a similar degree of neuroprotection in the retina.

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Introduction

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 pituitary adenylate cyclase-activating polypeptide (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 (Ganea and Delgado 2002; Laburthe et al. 2007; Masmoudi-Kouki et al. 2007). VIP has also been shown to exert neuroprotective effects in various in vitro and in vivo injury models (Gozes et al. 2003; Dejda et al. 2005; Pilzer and Gozes 2006).

Chronic cerebral ischemic hypoperfusion injury can be mimicked by permanent bilateral carotid artery occlusion in rats, producing white matter lesion in the brain along with ischemic lesion of the retina (Farkas et al. 2007). Previously, we have provided evidence for the efficacy of various putative protective agents in this model, including PARP inhibitors (Mester et al. 2009), the mitochondrial ATP-sensitive K^+ channel opener diazoxide (Atlasz et al. 2007a), and urocortin 2 (Szabadfi et al. 2009). More importantly, PACAP, which belongs to the same peptide family as VIP and shares receptors with VIP, has also been shown to have retinoprotective effects in various retinal lesions. The protective effects of PACAP in the retina have been proven in excitotoxicity-induced retinal degeneration (Tamas et al. 2004; Babai et al. 2006; Seki et al. 2006; Atlasz et al. 2008; Endo et al. 2011), optic nerve transection (Seki et al. 2008), UV-induced retinal damage (Atlasz et al.

2011), and diabetic retinopathy (Szabadfi et al. 2012a). Regarding ischemic lesions, PACAP has been proven to provide protection in hypoperfusion induced by high intraocular pressure (Seki et al. 2011) and bilateral carotic artery occlusion (Atlasz et al. 2007b; Szabadfi et al. 2012b). Based on these studies, the retinoprotective effects of PACAP are well established (Atlasz et al. 2010a). 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 (Yang et al. 2011). In the retina, it has been demonstrated that VIP protects against lipid peroxidation following ligation of ophthalmic vessels (Tuncel et al. 1996).

However, it is not known whether VIP has protective effects on the retinal morphology in ischemic lesion induced by permanent bilateral carotid artery occlusion. 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.

Materials and Methods

Bilateral Common Carotid Artery Occlusion and VIP Treatment

Adult male Wistar rats (n=17) weighing 250–300 g were subjected to permanent bilateral common carotid artery occlusion (BCCAO). Animal housing and care and application of experimental procedures were in accordance with institutional guidelines under approved protocols (no BA02/2000-24/2011, University of Pecs). Animals were maintained under 12-h light/dark cycle with free access to food and water.

Under isoflurane anesthesia, both common carotid arteries were ligated with a 3.0 filament through a midline cervical incision. 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 1,000 pmol). These animals served as sham-operated animals (n=6).

Histological Analysis

Two weeks after the carotid occlusion, rats were sacrificed under isoflurane anesthesia. The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4 % paraformaldehyde dissolved in 0.1 M phosphate buffer (Sigma, Hungary). Tissues were embedded in Durcupan ACM resin (Fluka, Switzerland), cut at 2 µm, and stained with toluidine blue (Sigma, Hungary). Sections were mounted in DPX medium (Sigma, Hungary) 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 between 1–2 mm from the optic nerve head. Files were then further processed with Adobe Photoshop 7.0 program. Samples for measurements were derived from at least six tissue blocks (n=4–5 measurements from one tissue block). The following parameters were measured: (1) cross section of the retina from the outer limiting membrane to the inner limiting membrane, (2) the width of the outer and inner nuclear and outer and inner plexiform layers (ONL, INL, OPL, IPL, respectively), (3) the number of cells/100-µm section length in the ganglion cell layer (GCL). Results are presented as mean±standard error of the mean (SEM). Statistical comparisons were made using the ANOVA test followed by Tukey's B post hoc analysis (GraphPad Prism5.01).

Results

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 each present (Figs. 1a and 2a). VIP (100 or 1,000 pmol) treatment in sham-operated animals did not cause any morphological alteration in retinal structure (Figs. 1b and 2b).

BCCAO resulted in severely reduced thickness of retinal layers compared to sham-operated controls (Figs. 1c and 2c). 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 (Figs. 1e and 2e). Several empty cell body-shaped spaces were seen in the ONL and INL which layers intermingled with the OPL (Figs. 1c and 2c). Numerous cells in the GCL displayed severe degeneration, which was well reflected in the reduced number of cells in the GCL (Figs. 1f, 2f).

Intravitreal treatment with 100 pmol VIP following BCCAO caused no visible improvement in the degenerated retina structure (Fig. 1c, d). Significant differences could not be observed between the BCCAO and BCCAO+100 pmol VIP groups by morphometrical analysis (Fig. 1e). Quantitative analysis demonstrated that 100 pmol VIP

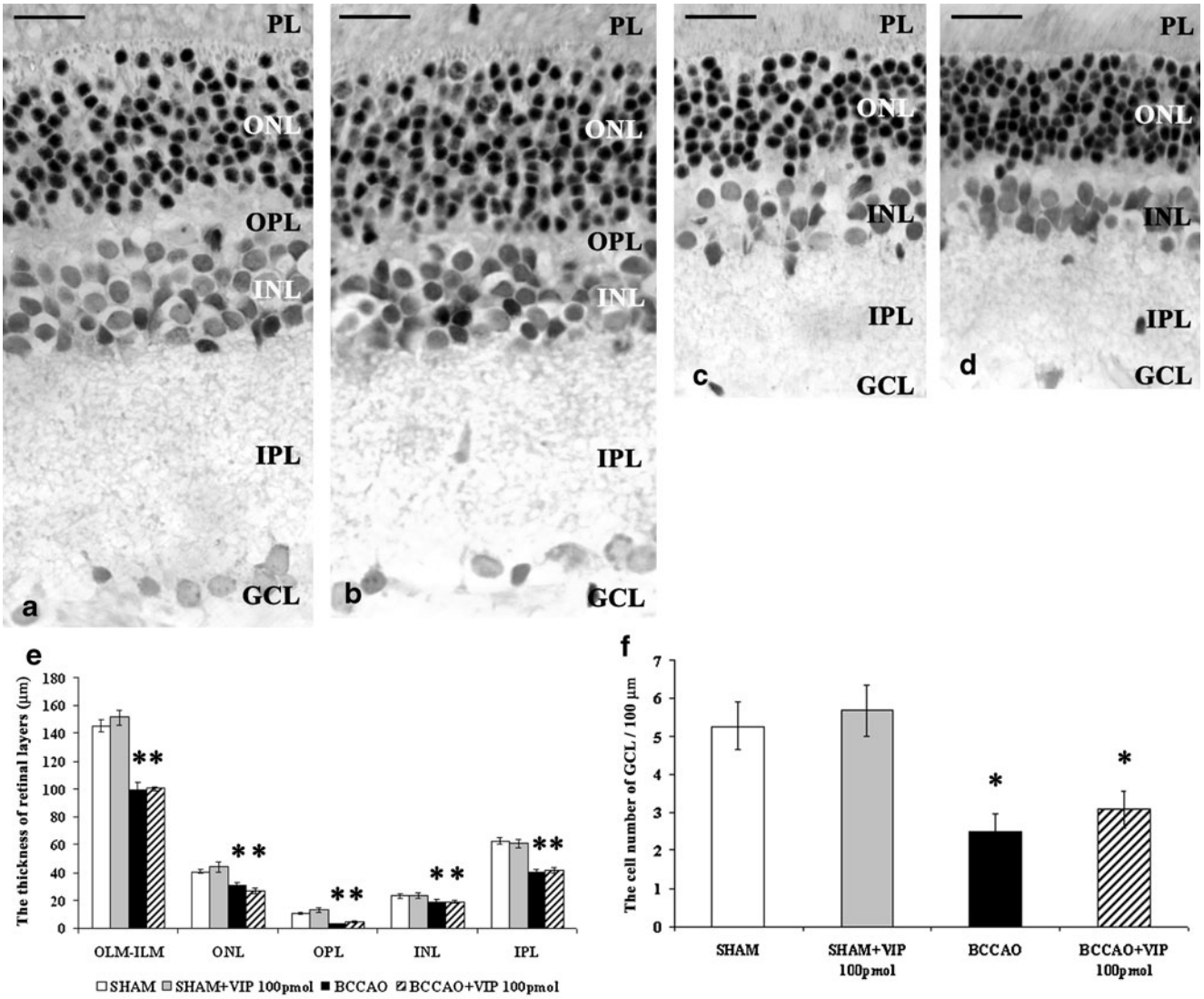


Fig. 1 Light microphotographs of representative retinal sections: sham-operated (**a**), sham+100 pmol VIP-treated (**b**), BCCAO-damaged (**c**), and BCCAO+100 pmol VIP-treated retina (**d**). Morphometric analysis of the whole retina and thickness of individual retinal layers (**e**). Cell number in 100-µm GCL length (**f**) in each treated group. Retinal tissue from BCCAO shows severe degeneration compared to retinas of sham-operated animals. The total thickness and the thickness of individual layers are significantly reduced. The degeneration is not ameliorated by the intravitreal 100 pmol VIP treatment. *Scale bar*, 20 µm. *OLM-ILM*, cross section of the retina from the outer limiting membrane to the inner limiting membrane; *PL*, photoreceptor layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; GCL, ganglion cell layer. Data are expressed as mean± SEM. $*p<0.001$, compared to sham-operated retinas; $^{\#}p<0.001$, compared to BCCAO-damaged retinas

administration could not protect the cells in the GCL (Fig. 1f). However, 1,000 pmol 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 well-visible OPL and INL with three cell rows (Fig. 2d). However, the differences between BCCAO- and BCCAO+1,000 pmol VIP-treated retinas were statistically significant in almost all retinal layers, except for the OPL (Fig. 2e). The number of cells in the GCL was higher in the BCCAO+1,000 pmol VIP-treated group compared to the BCCAO group (Fig. 2f).

Based on our previous data on the protective potential of PACAP in BCCAO-induced injury (Atlasz et al. 2007a; 2010b; Szabadfi et al. 2010), we compared the protective potential of PACAP and VIP. PACAP was more effective, already protective at 100-pmol 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 1,000 pmol VIP and 100 pmol PACAP treatment (Fig. 3a). However, PACAP was more effective in preserving cells in the GCL where significant differences could be observed between the protective potential of 1,000 pmol VIP and 100 pmol PACAP (Fig. 3b).

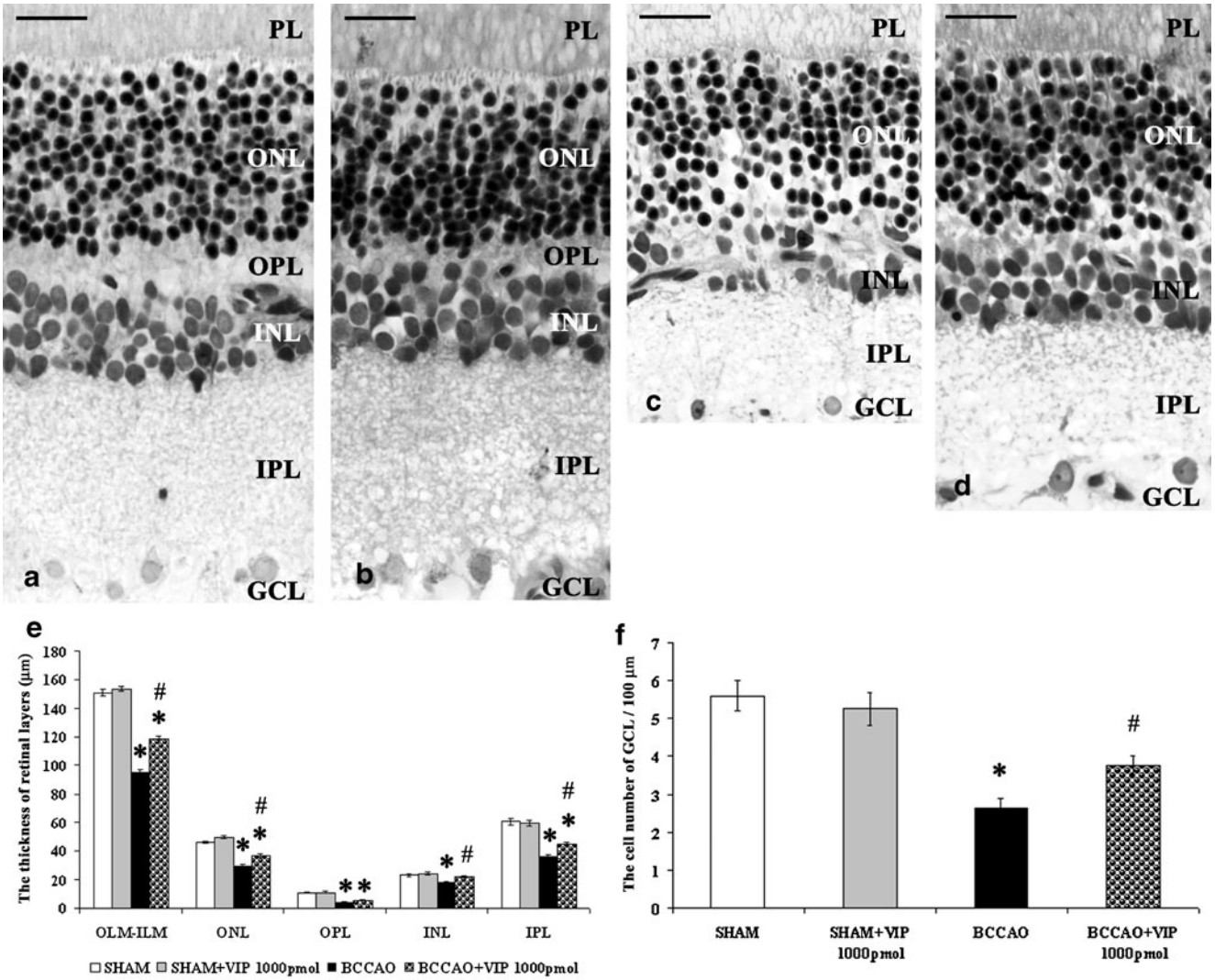
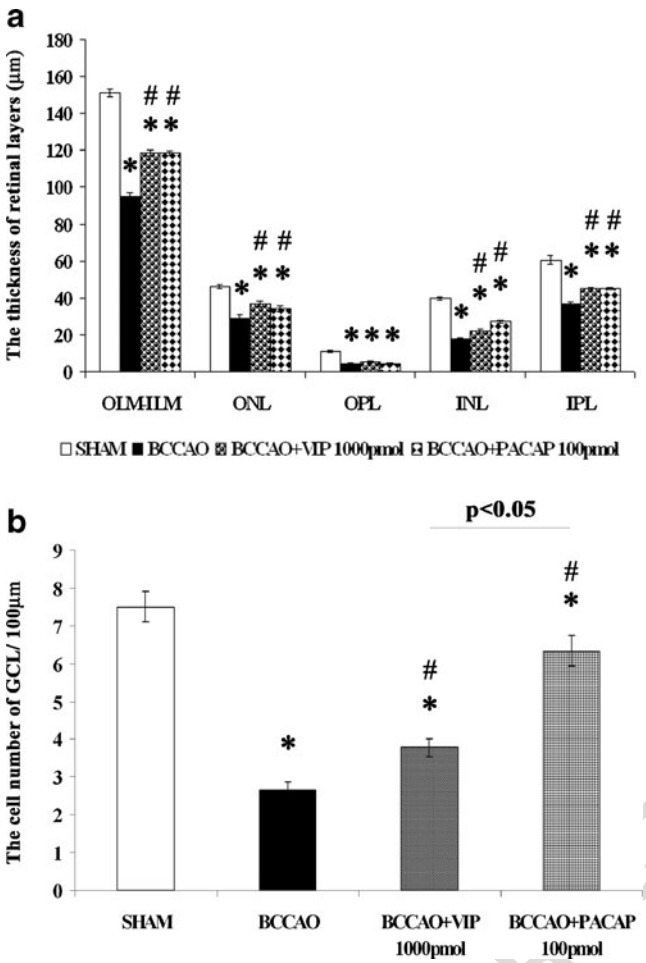


Fig. 2 Representative retinal cross sections stained with toluidine blue: sham-operated (**a**), sham+1,000 pmol VIP-treated (**b**), BCCAO-injured (**c**), and BCCAO+1,000 pmol VIP-treated (**d**) retinal sections. Comparison of the whole retinal thickness and thickness of each retinal layer (**e**). Number of cells/100-µm GCL length (**f**) in different groups. Retinas undergoing hypoperfusion induced by BCCAO show severe damage compared to the retinas of sham-operated animals. BCCAO-induced retinal degeneration is ameliorated with 1,000 pmol VIP. The retinal structure is retained, showing similarity to that of the sham-operated retina. *Scale bar*, 20 µm. *OLM-ILM*, cross section of the retina from the outer limiting membrane to the inner limiting membrane; *PL*, photoreceptor layer; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. Data are expressed as mean ±SEM. $^{*}p<0.001$, compared to sham-operated retinas; $^{\#}p<0.001$, compared to BCCAO-induced ischemic retinas

Discussion

In the present study, we showed that intravitreal VIP exerted neuroprotective effects in the retina in ischemic retinal lesion, given at 1,000-pmol (1 nmol) 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 (Pilzer and Gozes 2006). 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 (Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009). A recent study showing that VIP protected the ischemic brain in focal cerebral ischemia found decreased number of apoptotic



Q10 **Fig. 3** Comparison of the protective potential of 1,000 pmol VIP and 100 pmol PACAP in BCCAO-operated retinas. The thickness of the whole retina and each retinal layer (**a**); cell number in GCL/100-µm retina length (**b**). Protection of the individual layers is not significantly different between VIP- and PACAP-treated retinas, but the neuronal cell number is significantly higher after PACAP treatment. Note that the *bars* showing the effect of 1,000 pmol VIP treatment are identical to those in Fig. 2. *OLM-ILM*, cross section of the retina from the outer limiting membrane to the inner limiting membrane; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. Data are expressed as mean ±SEM. *$p<0.001$, compared to sham-operated retinas; $^{\#}p<0.001$, compared to BCCAO-induced ischemic retinas

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 (Gozes and Brenneman 2000; Gozes et al. 2003). 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 (Gozes et al. 2003; Lagreze et al. 2005).

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 (Shoge et al. 1999; Atlasz et al. 2009; 2010a), and the same Q11 has been shown for VIP in vitro (Shoge et al. 1998). 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 (Atlasz et al. 2011), while the VIP-mediator NAP protects against laser-induced retinal damage as reported by others (Belokopytov et al. 2011). 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 (Troger et al. 2001). Recently, we have shown that PACAP effectively prevents several morphological changes in the same model (Szabadfi Q12 et al. 2012a).

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 (Atlasz et al. 2007b; Seki et al. Q13 2011). Similarly, intraperitoneal injection of NAP protects retinal ganglion cells in high intraocular pressure-induced retinal ischemia (Jehle et al. 2008). Another study has demonstrated that NAP in retinal Muller glial cells prevents hypoxia-induced injury and promotes neuron growth (Zheng et al. 2010). An earlier study reported that VIP protected the retina against ischemia/reperfusion injury induced by ligation of ophthalmic vessels (Tuncel et al. 1996). Q14 The authors showed that both systemic and intravitreal VIP significantly decreased malondialdehyde levels, indicating decreased oxidative stress. Lipid peroxidation is a characteristic for the reperfusion period of this type of injury. VIP administration also prevented histological alterations of the retina analyzed after a 90-min ischemia and 3-h 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 (Tuncel et al. 1996). 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 (Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009). However, opposite effects have also been reported: while neonatal white matter lesion is

reduced by VIP, PACAP was not found to be effective (Rangon et al. 2005). 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.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. Szabadfi K. has been set as the corresponding author. Please check and advise if correct.
Q2. The sentence "We found that treatment with 100 pmol VIP did not, but 1000 pmol had significant protective effects in BCCAO–injured retina, as shown by the morphometric analysis." was modified to "We found that treatment with 1,000 pmol VIP, but not 100 pmol VIP, had significant protective effects in BCCAO–injured retina, as shown by the morphometric analysis." for clarity. Please check if its intended meaning has been retained.
Q3. "mitochondrial ATP–sensitive K^+ channel opener diazoxid" was changed to "mitochondrial ATP–sensitive K^+ channel opener diazoxide." Please check if this is appropriate.
Q4. The citation "Atlasz et al. 2007" (original) has been changed to "Atlasz et al. 2007a". Please check if appropriate.
Q5. The citation "Atlasz et al. 2007" (original) has been changed to "Atlasz et al. 2007b". Please check if appropriate.
Q6. The citation "Atlasz et al. 2010" (original) has been changed to "Atlasz et al. 2010a". Please check if appropriate.
Q7. Please check if the expnaded form, "standard error of the mean," provided for SEM is correct.
Q8. The citation "Atlasz et al. 2007" (original) has been changed to "Atlasz et al. 2007a". Please check if appropriate.
Q9. The citation "Atlasz et al. 2010" (original) has been changed to "Atlasz et al. 2010b". Please check if appropriate.
Q10. Figure 3 contains poor quality text. Please provide replacement otherwise, please advise if we can proceed with the figure as is.
Q11. The citation "Atlasz et al. 2010" (original) has been changed to "Atlasz et al. 2010a". Please check if appropriate.
Q12. The citation "Szabadfi et al. 2012" (original) has been changed to "Szabadfi et al. 2012a". Please check if appropriate.
Q13. The citation "Atlasz et al. 2007" (original) has been changed to "Atlasz et al. 2007b". Please check if appropriate.
Q14. The sentence "An earlier study reported that VIP protected against ischemia/reperfusion injury induced by ligation of ophthalmic vessels (Tuncel et al., 1996)." was modified to "An earlier study reported that VIP protected the retina against ischemia/reperfusion injury induced by ligation of ophthalmic vessels (Tuncel et al. 1996)." for clarity. Please check if its intended meaning has been retained.