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96 Abstract

Retinoprotective effects of pituitary adenylate cyclase activating polypeptide (PACAP) are well-known and have been demonstrated in various pathological conditions, such as diabetic retinopathy,

		excitotoxic retinal injury, UV light-induced degeneration, and ischemic retinal lesion. The neuronal degeneration observed in the different retinal layers under the above pathological conditions can be successfully decreased by PACAP; however, whether this morphological improvement is also reflected in functional amelioration remains unknown. Therefore, our purpose was to investigate the protective effect of PACAP on the rat retina after bilateral common carotid artery occlusion (BCCAO) with electroretinography (ERG) to parallel the functional data with the previous morphological and neurochemical observations. Control eyes received saline treatment while PACAP was injected into the vitreous space of the other eye immediately after the induction of ischemia. Retinal damage and protective effects of PACAP were quantified by the changes in the wave forms and amplitudes. On postoperative days 2 and 14, several parameters were assessed with special attention to the changes of *b* wave. The results confirm that the previously described morphological protection induced by PACAP treatment is reflected in functional improvement in ischemic retinal lesions.
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PACAP Application Improves Functional Outcome of Chronic Retinal Ischemic Injury in Rats—Evidence From Electroretinographic Measurements

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Abstract Retinoprotective effects of pituitary adenylate cyclase activating polypeptide (PACAP) are well-known and have been demonstrated in various pathological conditions, such as diabetic retinopathy, excitotoxic retinal injury, UV light-induced degeneration, and ischemic retinal lesion. The neuronal degeneration observed in the different retinal layers under the above pathological conditions can be successfully decreased by PACAP; however, whether this morphological improvement is also reflected in functional amelioration remains unknown. Therefore, our purpose was to investigate the protective effect of PACAP on the rat retina after bilateral common carotid artery occlusion (BCCAO) with electroretinography (ERG) to parallel the functional data with the previous morphological and neurochemical observations. Control eyes received saline treatment while PACAP was injected into the vitreous space of the other eye immediately after the induction of ischemia. Retinal damage and protective effects of PACAP were quantified by the changes in the wave forms and amplitudes. On postoperative days 2 and 14, several parameters were assessed with special attention to the changes of *b* wave. The results confirm that the previously described morphological protection induced by PACAP treatment is reflected in functional improvement in ischemic retinal lesions.

Keywords Rat · Electroretinography · BCCAO · PACAP · Functional retinoprotection

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide that belongs to the VIP/glucagon/secretin neuropeptide family (Vaudry et al. 2009). PACAP has, among others, very potent neuroprotective effects that have been proven in several in vitro and in vivo models of neuronal injuries (Shioda et al. 2006; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2000). Numerous data support the protective effects of PACAP in ischemic lesions. Among others, PACAP treatment results in smaller infarct size in focal cerebral ischemia, it leads to less extensive hippocampal damage in global cerebral ischemia, and it decreases postischemic endothelial dysfunction (Lenti et al. 2009; Ohtaki et al. 2008; Reglodi et al. 2002). Mice deficient in endogenous PACAP have larger infarct size in models of stroke (Chen et al. 2006; Ohtaki et al. 2006). PACAP is also protective in ischemic retinal lesions (Atlasz et al. 2010a), in addition to the various other types of retinopathies in animal models, such as diabetic retinopathy, excitotoxic retinal injury, UV light-induced degeneration (Nakamachi et al. 2012; Szabadfi et al. 2010, 2012a). The lack of endogenous PACAP causes a more

severe ischemic retinal lesion, which can be counteracted by exogenous PACAP treatment (Szabadfi et al. 2012b).

Whether the morphological ameliorative effects are also reflected in functional improvement is an essential question for determining the efficacy of PACAP. Electroretinography (ERG) is a suitable method for making such assessments. An entire record of ERG consists of a negative wave (*a* wave) followed by a positive wave (*b* wave) with two to five oscillatory potentials on the rising slope (Imai et al. 1990). There is a largely variable *c* wave, the origin of which is attributed to the pigment epithelium or the glial (Müller) cells of the retina (Noell 1963; Gouras 1970; Perlman 2009). The amplitude of each component of the ERG varies from animal to animal, whereas they are very stable within the same animal (Perlman 1995; Imai et al. 1990). ERG is useful for the detection of early-stage retinal dysfunction (Gouras 1970; Perlman 2009; Shahar et al. 2012). The gross physiological response of the retina to ischemia is commonly measured using the ERG, whose *b* wave component has attributes from the interaction between photoreceptors and ON bipolar cells (Gurevich and Slaughter 1993; Osborne et al. 2004). Recordings of ERG showed reduction of the *b* wave during the bilateral common carotid artery occlusion (BCCAO) (Block et al. 1992; Barnett and Osborne 1995; Osborne et al. 1999) indicating an effect at the level of the photoreceptors, bipolar cells, and Müller cells.

Indeed, earlier we have described by ERG, which measures electric potentials of the retina indicative of visual function, that PACAP improves the functional outcome after excitotoxic retinal lesions (Varga et al. 2011).

Retinal ischemia/hypoperfusion is one of the leading causes of retinal degeneration and blindness, which can be modelled by permanent BCCAO in rats. A severe ischemic insult affects retinal ganglion cells first; additional damage to most of the inner retina develops slower and finally photoreceptors can also be affected (Hughes 1991; Osborne et al. 2004). Blood flow in the retina is severely reduced after BCCAO (Gurevich and Slaughter 1993; Osborne et al. 2004). Although numerous data prove that PACAP has multiple retinoprotective effects in excitotoxic retinal degeneration, it is still not known whether PACAP treatment-induced morphological and neurochemical protection in ischemia correlate with functional amelioration. Therefore, our purpose was to investigate the protective effect of PACAP on the rat retina after BCCAO with ERG and parallel the functional data with the previously described morphological and neurochemical results (Atlasz et al. 2007, 2010b).

Materials and Methods

In order to induce hypoperfusion of the retina, BCCAO was carried out on Wistar rats (n=8; 3 months of age, weighing 350 g). Carotid arteries on both sides were permanently ligated under isoflurane anesthesia, according to previous descriptions (Atlasz et al. 2007). The operation was immediately followed by intravitreal PACAP treatment (100 pmol in 5 μl vehicle/eye) into the right eye, while saline was injected into the other eye as we have previously described. Experimental procedures were carried out in accordance with approved protocols (University of Pecs; no: BA02/2000-15024/2011).

ERG measurements were performed to assess retinal function in each group, after an overnight dark adaption. The animals were anesthetized by intraperitoneal injection of ketamine 5 % (*w*/*v*, Calypsol, Richter Gedeon, Hungary, 90 mg/BW kg) and xylazine 20 % (*w*/*v*, Sedaxylan, Dechra, Netherlands, 10 mg/ kg) during the electrophysiological measurements (Sharp and La Regina 1998). The pupils were dilated with 0.5 % cyclopentolate (*w*/*v*, Humapent-Teva, Hungary). Oxybuprocaine 0.4 % eye drops (Humacain-Teva, Hungary) were used for the topical anesthesia. Flash ERG was recorded before BCCAO as nontreated controls and on postoperative days 2, 6, 10, and 14 in each group.

ERG potentials arise in the retina after light stimulation and they are detectable all around the eye, being the largest in the center of the cornea (Gouras 1970; Perlman 1995). ERGs were recorded by surface electrodes from the center of the cornea with the negative electrode placed under the skin of the cheek and the ground electrode stuck under the skin of the neck. All procedures were performed under a dim red LED light (632 nm; Zhang et al. 2013). The responses to light flashes (5.0 cd/m^2, 0.25 Hz, 503 nm green LED light) were pre-amplified, amplified (2,000×, Bioamp SbA4-V6, Supertech, Hungary), and recorded with an A/D converter (Ratsoft-Solar Electronic; Jacobs et al. 2001; Szabo-Salfay et al. 2001). Responses (n=150) were averaged with the software of the A/D converter to draw the graph of each measurement. The values of the selected parameters were analyzed by two-way ANOVA with Bonferroni post hoc analysis ($p<0.05$; $p<0.01$; $p<0.001$).

Histological examination was carried out to confirm the severity of the retinal lesions and correlate functional changes with morphological alterations induced by ischemia and PACAP treatment. After the termination of the ERG measurements, rats were decapitated under isoflurane anesthesia and eyes were immediately dissected in ice-cold phosphate buffered saline (PBS; VWR International, Hungary) and fixed in 4 % paraformaldehyde (PFA; Merck, Hungary). Retina histology was performed on Durcupan ACM resin (Sigma, Hungary) embedded tissues. Sections were cut at 2 μm, stained with toluidine blue (Sigma), and examined in a Nikon Eclipse 80i microscope as we have previously described (Atlasz et al. 2007).

Results

We investigated the possible effects of PACAP treatment in BCCAO-induced ischemic retinal degeneration on neuronal cell activity by recording ERGs at various time points before (control measurements) and 2, 6, 10, and 14 days after the induction of ischemia. Representative ERG recordings of the untreated and PACAP-treated eyes are shown in Fig. 1.

Control average ERG waveforms were similar in both left and right eyes (Fig. 1a, b). Our results show that BCCAO caused a severe functional damage reflecting the previously described alteration of the histological structure (Atlasz et al. 2007). 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 (Fig. 1). The same tendencies could be observed on postoperative day 6 and 10 (data not shown). Average amplitudes of waves decreased with the duration of ischemia in BCCAO eyes (Figs. 1 and 2).

Differences were found in the average amplitudes of the *a* waves and *b* waves between control and vehicle-treated BCCAO eyes, but no differences could be observed in the case of *c* wave on postoperative day 14 (Fig. 2a). 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 (Fig. 2a). Amplitudes of the *b* wave decreased in BCCAO (33 % of controls). Significant difference could be detected between BCCAO and PACAP-treated BCCAO groups on postoperative day 14 (53 % of control; Fig. 2b). The same tendency could be observed on postoperative day 2 in BCCAO- and BCCAO + PACAP-treated groups in the *b* wave amplitudes (Fig. 2b). A slight but not significant decrease in the *b* wave/*a* wave amplitude ratio (*b*/*a*) was observed in the BCCAO and PACAP-treated BCCAO retinas (Fig. 2c).

The latency of *b*/*a* waves was significantly decreased in both BCCAO- and PACAP-treated BCCAO groups compared to their controls, but no differences could be observed between the treated and untreated ischemic groups (Fig. 3a). The elapse time of the five major oscillatory potentials (OPs) was reduced in the BCCAO ischemic group, but PACAP treatment led to significant protection (Fig. 3b). The morphological parameters are in accordance with our previous observation that PACAP treatment ameliorated the BCCAO-induced retinal degeneration (Atlasz et al. 2007, 2010a, b; Fig. 4).

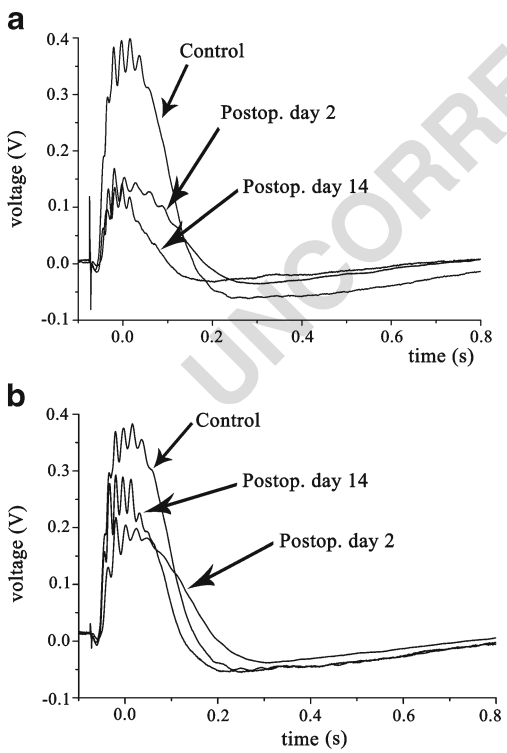

Fig. 1 Representative average ERG recordings of BCCAO (**a**) and PACAP-treated BCCAO (**b**) groups on postoperative days 2 and 14. The BCCAO-induced retinal degeneration was first observed in the alteration of the amplitudes of the waves on postoperative day 2 (**a**). Partial recovery of the ischemia-induced changes in the PACAP-treated animals was evident by postoperative day 14 (**b**). Control measurements were made before BCCAO from the same animals

Discussion

The present results show that the morphological protection exerted by PACAP in ischemic retinal lesion is accompanied by functional amelioration, as reflected by electrical activity. We have previously described that PACAP exerts morphological and neurochemical protection in BCCAO-induced retinal degeneration (Atlasz et al. 2007, 2010b). However, functional outcome measures, such as ERG, were missing. Electroretinogram displays a composite retinal electric activity which depends upon different cells, extending from the pigment epithelium to the innermost retinal layers (Noell 1953; Brown 1968), allowing differential participation of cells within different layers (Grozdanic et al. 2003; Vilela et al. 1998; Arden et al. 1982). This method is attractive in assessing retinal neurodegeneration because it is not invasive and can thus be used in longitudinal assessments. The ERGs for all control eyes in our present study were well within the range of previously published normative data (Bui and Fortune 2004; Szabo-Salfay et al. 2001). The results of this study demonstrate that the *b* wave is the most sensitive detectable ERG parameter of functional abnormalities in this rat model of BCCAO-induced retinal ischemia. Our observations suggest that BCCAO and PACAP treatment also led to changes in neuronal cell activity of the retina. The main functional

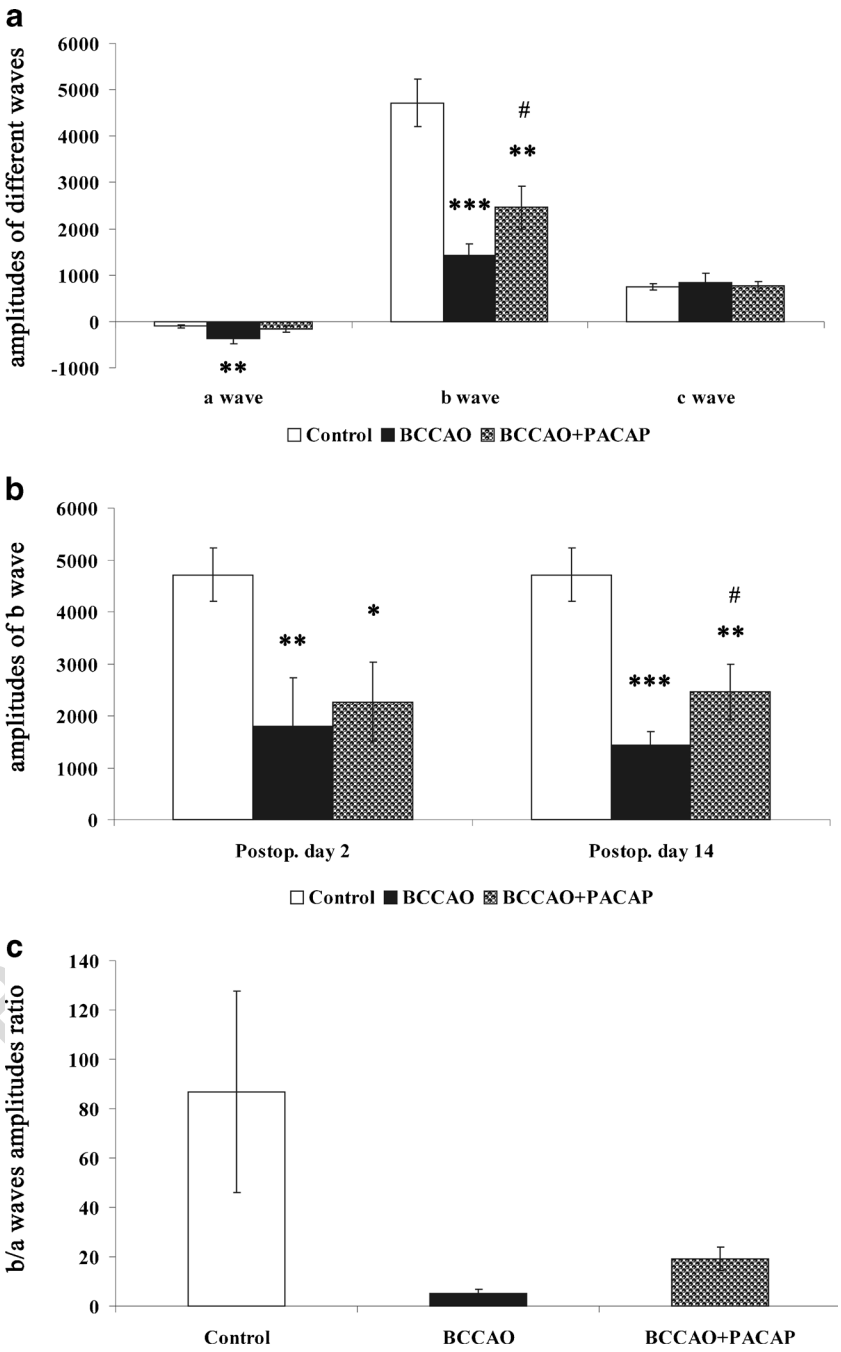

Fig. 2 Comparative analysis of the average amplitudes of different waves (**a**), the amplitude ratio of *b*/*a* waves (**c**) on postoperative day 14, and the alteration of amplitudes of *b* waves on postoperative days 2 and 14 (**b**). Ischemia induced significant alterations in the amplitudes of *a* waves and *b* waves compared to the controls. PACAP treatment reduced the deterioration and patterns were similar to the control group. No significant differences could be registered in the amplitude of *c* wave between the three groups (**a**, $^{**}p<0.01$; $^{***}p<0.001$ vs. control; $\#p<0.05$ vs. BCCAO). The changes of the amplitudes of different waves were recognized on postoperative days 2 and 14. Significant alteration could be observed in the amplitudes of *b* waves between the control, ischemic, and PACAP-treated BCCAO groups on day 14 (**b**, $^{*}p<0.05$; $^{**}p<0.01$ vs. control). No significant differences could be observed in the amplitude ratio of *b*/*a* waves between the control, ischemic and the PACAP-treated ischemic group (C, $^{*}p<0.05$ vs. control; $\#p<0.05$ vs. BCCAO). $n=8$ in each group

protective effects of PACAP were showed by the alteration of the *b* wave amplitudes.

Although retinal ganglion cells are the most vulnerable to severe ischemia of the retina (Osborne et al. 2004) and their damage was found most prominent after BCCAO (Yamamoto et al. 2006), it has been shown that 2 weeks after the operation, other cellular layers are also seriously degenerated (Atlasz et al. 2007; Farkas et al. 2007). Therefore, deterioration of the ERG can be expected. Former studies have identified the OPs of the flash-elicited ERG *b* wave as sensitive indices of abnormalities within the retinal circulation (Kergoat and Lovasik 1990). The OPs are quantitative and objective index for dysfunction and manifested earliest not only in ischemic conditions but also in diabetic retinopathy (Yonemura et al. 1962; Galloway et al. 1971), which also involves worsened circulation and production of reactive oxygen species (Fernandes et al. 2011).

Compared to nonischemic (control) values, the ERG *a* and *b* wave amplitudes were strongly reduced after the BCCAO operation. PACAP treatment alleviated the functional consequences of BCCAO-induced ischemia. In our previous studies, the beneficial effects of PACAP were observed at

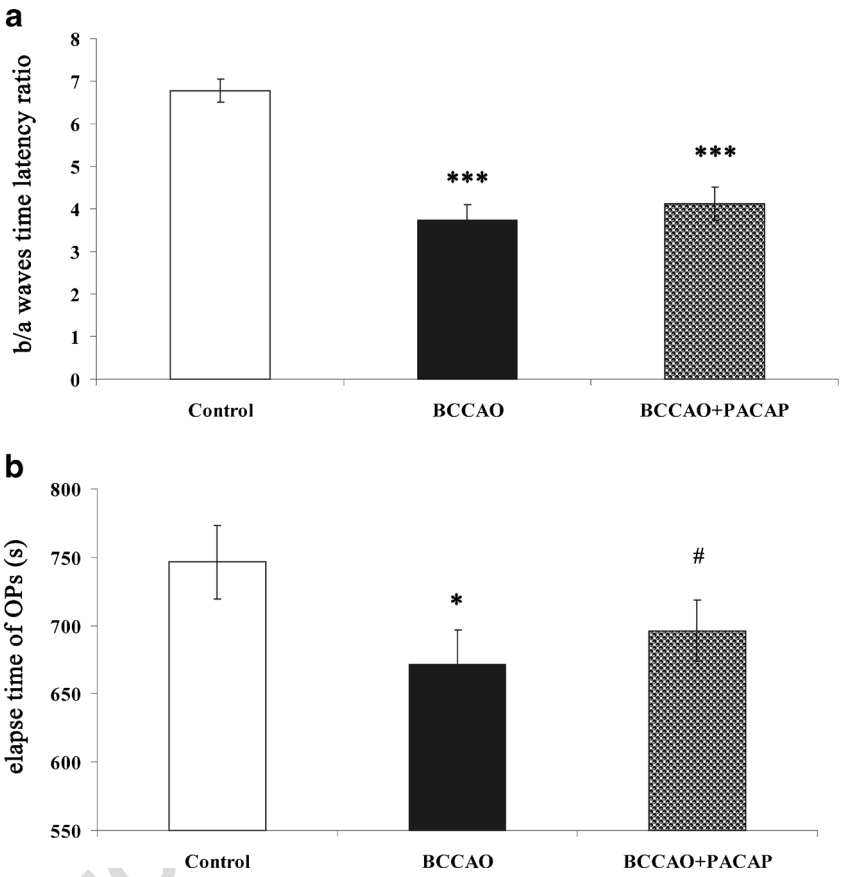

Fig. 3 Comparative analysis of time latency ratio of *b*/*a* waves (**a**) and differences in elapse time of OPs (**b**) between the three groups on postoperative day 14. Significant differences could be observed in the time latency ratios of *b*/*a* waves between the ischemic and the PACAP-treated ischemic groups. However, there are no significant differences between the BCCAO- and PACAP-treated BCCAO groups (**a**, ***$p<0.001$ vs. control). Significant differences could be observed in the elapse time of OPs between control and BCCAO animals, but no significant differences were seen between the control and PACAP-treated BCCAO groups. PACAP treatment led to significant retention of the elapse time of OPs (**b**,*$p<0.05$ vs. control; #$p<0.05$ vs. BCCAO). $n=8$ in each group

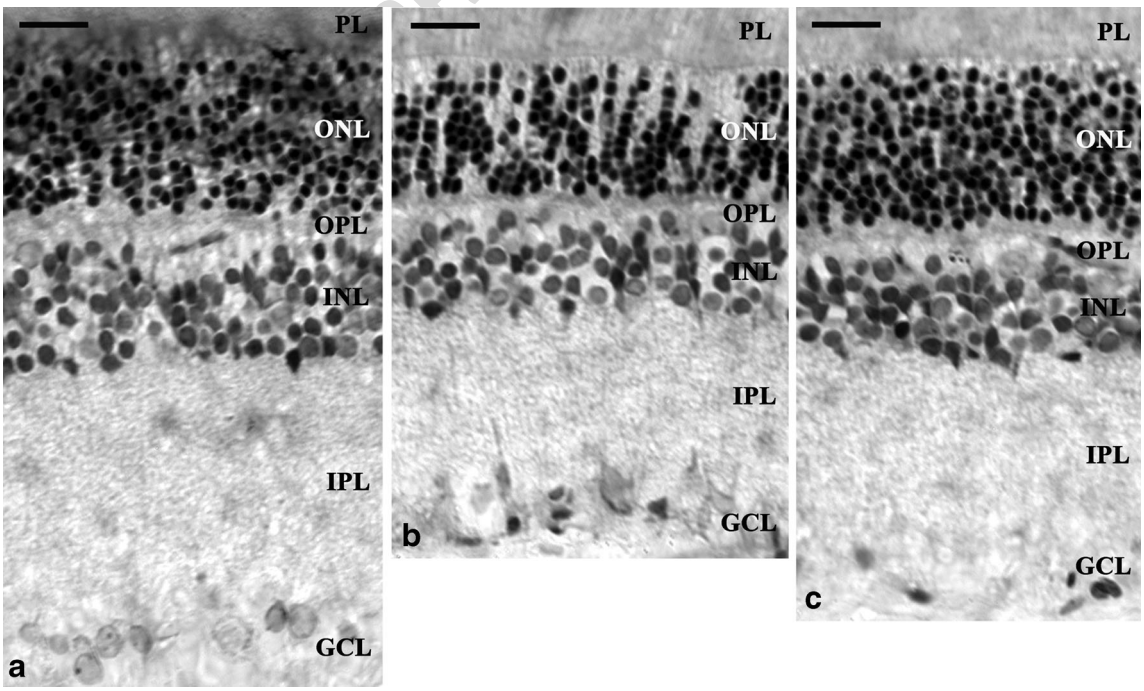

Fig. 4 Representative sections of control (**a**), BCCAO (**b**), and PACAP-treated BCCAO (**c**) retinas stained with toluidine blue. Morphological differences were found between the examined groups; PACAP treatment could ameliorate the ischemia-induced retinal degeneration. *Scale bar*: 20 μm. Abbreviations: *PL* photoreceptor layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer

histological and neurochemical levels (Atlasz et al. 2007, 2010b; Szabo et al. 2012). The present results indicate the PACAP treatment induced a significant recovery of the waveform and amplitudes of *a* wave and *b* wave as seen in ERG. PACAP treatment did not completely prevent retinal dysfunction; however, the ERG recovery was a sustained effect noted as early as 2 days after BCCAO.

There are two further interesting results in the ERG recordings. One is that after BCCAO, the *a* wave amplitudes increase slightly, possibly reflecting a relatively late and low level of photoreceptor damage and the lack of intraretinal negative feedback. This is supported by the fact that the thickness of the outer nuclear layer did not change as much as the inner retinal layers and the photoreceptors look relatively healthy in the histological preparations. The second observation is that the *b*/*a* wave latency ratio did not improve significantly after PACAP treatment. A possible explanation for that is that the time after BCCAO was not sufficient for the degenerations to reach the photoreceptors retrogradely. Our present results confirm our earlier findings in excitotoxic retinal damage, where PACAP treatment led to functional amelioration parallel with protection reflected in morphological structure (Varga et al. 2011).

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

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AUTHOR QUERIES

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Q1. Please check if the affiliations and contact details are presented correctly.
Q2. Please check if the acknowledgments section is presented correctly.
Q3. References "Imai et al. 1990" (2x) based on original manuscript we received were identical. Hence, the latter was deleted and reference list and citations were adjusted. Please check if appropriate.
Q4. Please check provided publisher location “USA” in reference entry “Sharp and La Regina (1998)” if appropriate.