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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.

97	Keywords separated by ' - '	Rat - Electroretinography - BCCAO - PACAP - Functional retinoprotection
98	Foot note information	

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

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

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

64 Whether the morphological ameliorative effects are also
65 reflected in functional improvement is an essential question
66 for determining the efficacy of PACAP. Electroretinography
67 (ERG) is a suitable method for making such assessments. An
68 entire record of ERG consists of a negative wave (*a* wave)
69 followed by a positive wave (*b* wave) with two to five oscil-
70 latory potentials on the rising slope (Imai et al. 1990). There is
71 a largely variable *c* wave, the origin of which is attributed to
72 the pigment epithelium or the glial (Müller) cells of the retina
73 (Noell 1963; Gouras 1970; Perlman 2009). The amplitude of
74 each component of the ERG varies from animal to animal,
75 whereas they are very stable within the same animal (Perlman
76 1995; Imai et al. 1990). ERG is useful for the detection of
77 early-stage retinal dysfunction (Gouras 1970; Perlman 2009;
78 Shahar et al. 2012). The gross physiological response of the
79 retina to ischemia is commonly measured using the ERG,
80 whose *b* wave component has attributes from the interaction
81 between photoreceptors and ON bipolar cells (Gurevich and
82 Slaughter 1993; Osborne et al. 2004). Recordings of ERG
83 showed reduction of the *b* wave during the bilateral common
84 carotid artery occlusion (BCCAO) (Block et al. 1992; Barnett
85 and Osborne 1995; Osborne et al. 1999) indicating an effect at
86 the level of the photoreceptors, bipolar cells, and Müller cells.

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

91 Retinal ischemia/hypoperfusion is one of the leading
92 causes of retinal degeneration and blindness, which can be
93 modelled by permanent BCCAO in rats. A severe ischemic
94 insult affects retinal ganglion cells first; additional damage to
95 most of the inner retina develops slower and finally photore-
96 ceptors can also be affected (Hughes 1991; Osborne et al.
97 2004). Blood flow in the retina is severely reduced after
98 BCCAO (Gurevich and Slaughter 1993; Osborne et al.
99 2004). Although numerous data prove that PACAP has mul-
100 tiple retinoprotective effects in excitotoxic retinal degenera-
101 tion, it is still not known whether PACAP treatment-induced
102 morphological and neurochemical protection in ischemia cor-
103 relate with functional amelioration. Therefore, our purpose
104 was to investigate the protective effect of PACAP on the rat
105 retina after BCCAO with ERG and parallel the functional data
106 with the previously described morphological and neurochem-
107 ical results (Atlasz et al. 2007, 2010b).

108 Materials and Methods

109 In order to induce hypoperfusion of the retina, BCCAO was
110 carried out on Wistar rats ($n=8$; 3 months of age, weighing
111 350 g). Carotid arteries on both sides were permanently

112 ligated under isoflurane anesthesia, according to previous
113 descriptions (Atlasz et al. 2007). The operation was immedi-
114 ately followed by intravitreal PACAP treatment (100 pmol in
115 5 μ l vehicle/eye) into the right eye, while saline was injected
116 into the other eye as we have previously described.
117 Experimental procedures were carried out in accordance with
118 approved protocols (University of Pecs; no: BA02/2000-
119 15024/2011).

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

133 ERG potentials arise in the retina after light stimulation and
134 they are detectable all around the eye, being the largest in the
135 center of the cornea (Gouras 1970; Perlman 1995). ERGs
136 were recorded by surface electrodes from the center of the
137 cornea with the negative electrode placed under the skin of
138 the cheek and the ground electrode stuck under the skin
139 of the neck. All procedures were performed under a dim
140 red LED light (632 nm; Zhang et al. 2013). The res-
141 sponses to light flashes (5.0 cd/m², 0.25 Hz, 503 nm
142 green LED light) were pre-amplified, amplified (2,000 \times ,
143 Bioamp SbA4-V6, Supertech, Hungary), and recorded
144 with an A/D converter (Ratsoft-Solar Electronic;
145 Jacobs et al. 2001; Szabo-Salfay et al. 2001).
146 Responses ($n=150$) were averaged with the software of
147 the A/D converter to draw the graph of each measure-
148 ment. The values of the selected parameters were ana-
149 lyzed by two-way ANOVA with Bonferroni post hoc
150 analysis ($p<0.05$; $p<0.01$; $p<0.001$).

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

165 **Results**

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

172 Control average ERG waveforms were similar in both left
 173 and right eyes (Fig. 1a, b). Our results show that BCCAO
 174 caused a severe functional damage reflecting the previously
 175 described alteration of the histological structure (Atlasz et al.
 176 2007). BCCAO-induced functional retinal degeneration was
 177 already observed on postoperative days 2 and lasted through-
 178 out the entire observation period. Intravitreal injection of
 179 PACAP immediately after BCCAO resulted in a more
 180 retained retinal function as assessed by average ERG wave-
 181 forms compared to the BCCAO-operated groups on both
 182 postoperative 2nd and 14th days (Fig. 1). The same tendencies
 183 could be observed on postoperative day 6 and 10 (data not
 184 shown). Average amplitudes of waves decreased with the
 185 duration of ischemia in BCCAO eyes (Figs. 1 and 2).

186 Differences were found in the average amplitudes of the *a*
 187 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 con-
 trols 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 postopera-
 tive 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 param-
 eters are in accordance with our previous observation that
 PACAP treatment ameliorated the BCCAO-induced retinal
 degeneration (Atlasz et al. 2007, 2010a, b; Fig. 4).

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 morphologi-
 cal and neurochemical protection in BCCAO-induced retinal
 degeneration (Atlasz et al. 2007, 2010b). However, functional
 outcome measures, such as ERG, were missing. Electroretino-
 gram 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 with-
 in 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 demon-
 strate 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

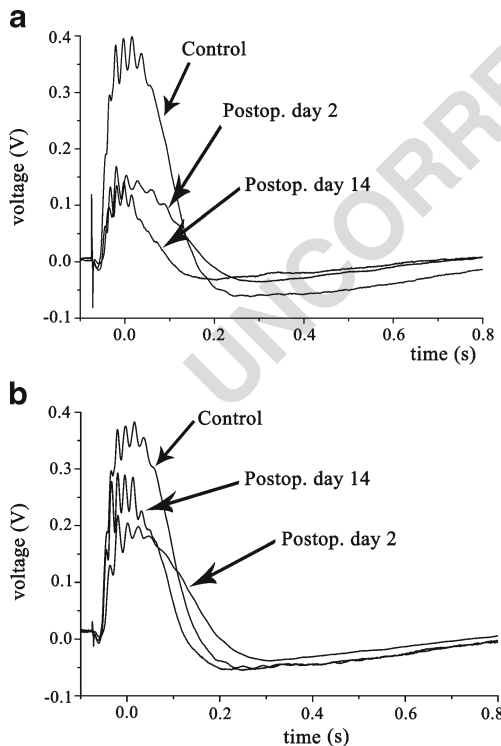
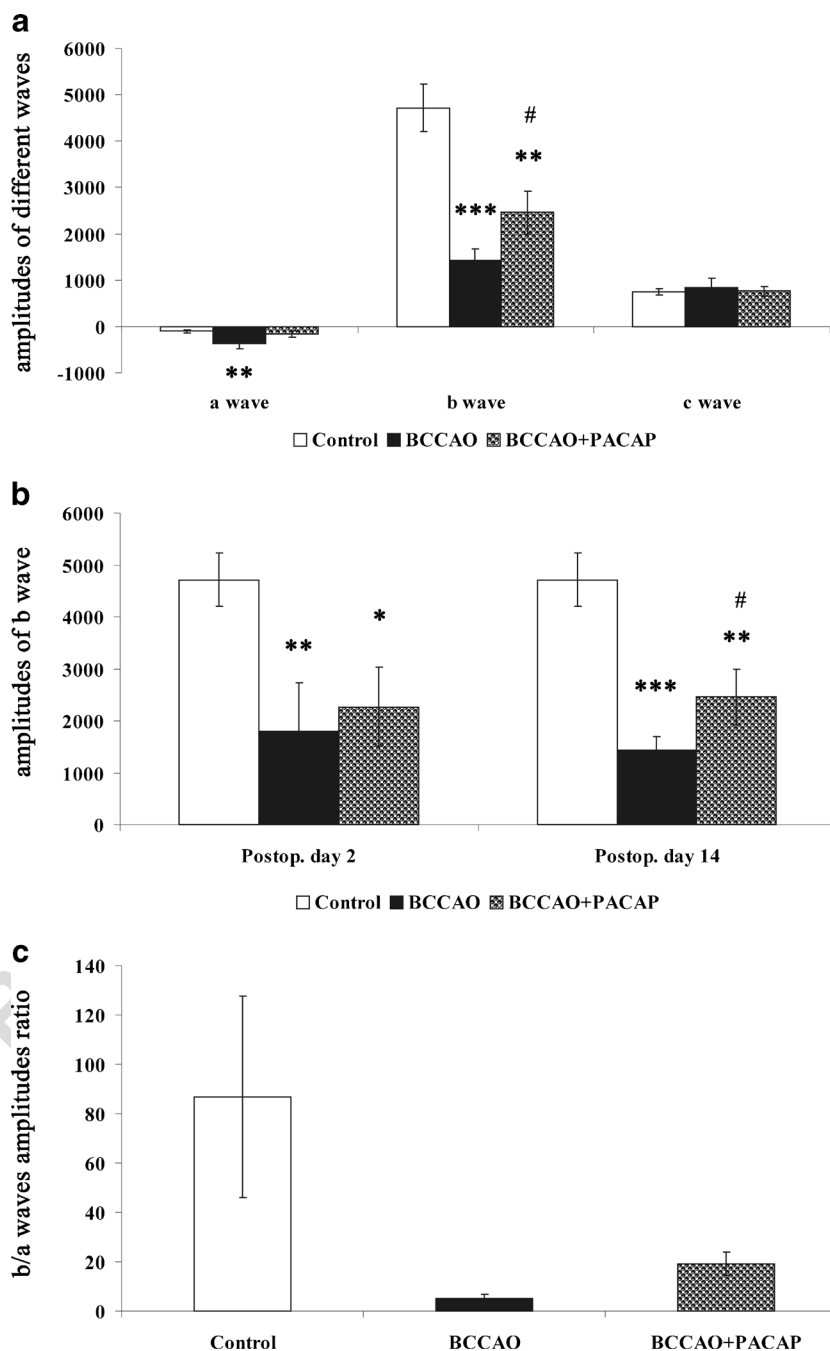


Fig. 1 Representative average ERG recordings of BCCAO (a) and PACAP-treated BCCAO (b) groups on postoperative days 2 and 14. The BCCAO-induced retinal degeneration was first observed in the alteration of the amplitudes of the waves on postoperative day 2 (a). Partial recovery of the ischemia-induced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

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



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

240 Although retinal ganglion cells are the most vulnerable to
 241 severe ischemia of the retina (Osborne et al. 2004) and their
 242 damage was found most prominent after BCCAO (Yamamoto
 243 et al. 2006), it has been shown that 2 weeks after the operation,
 244 other cellular layers are also seriously degenerated (Atlasz
 245 et al. 2007; Farkas et al. 2007). Therefore, deterioration of
 246 the ERG can be expected. Former studies have identified the
 247 OPs of the flash-elicited ERG *b* wave as sensitive indices of
 248 abnormalities within the retinal circulation (Kergoat and

249 Lovasik 1990). The OPs are quantitative and objective index
 250 for dysfunction and manifested earliest not only in ischemic
 251 conditions but also in diabetic retinopathy (Yonemura et al.
 252 1962; Galloway et al. 1971), which also involves worsened
 253 circulation and production of reactive oxygen species
 254 (Fernandes et al. 2011).

255 Compared to nonischemic (control) values, the ERG *a* and
 256 *b* wave amplitudes were strongly reduced after the BCCAO
 257 operation. PACAP treatment alleviated the functional conse-
 258 quences of BCCAO-induced ischemia. In our previous stud-
 259 ies, 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

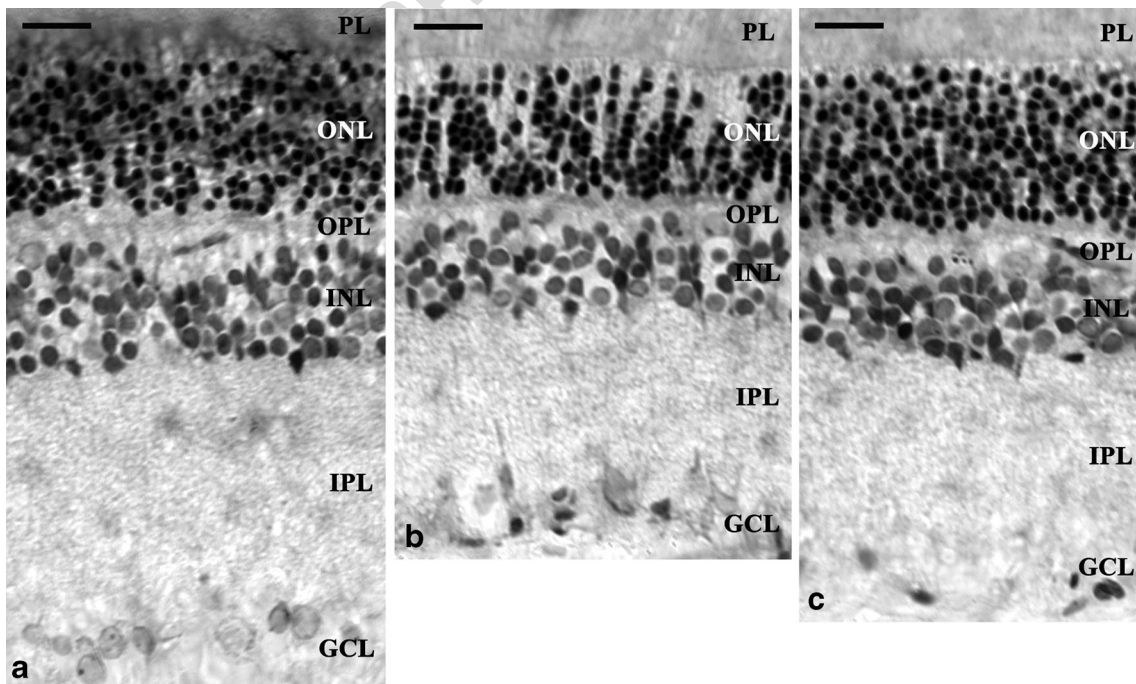
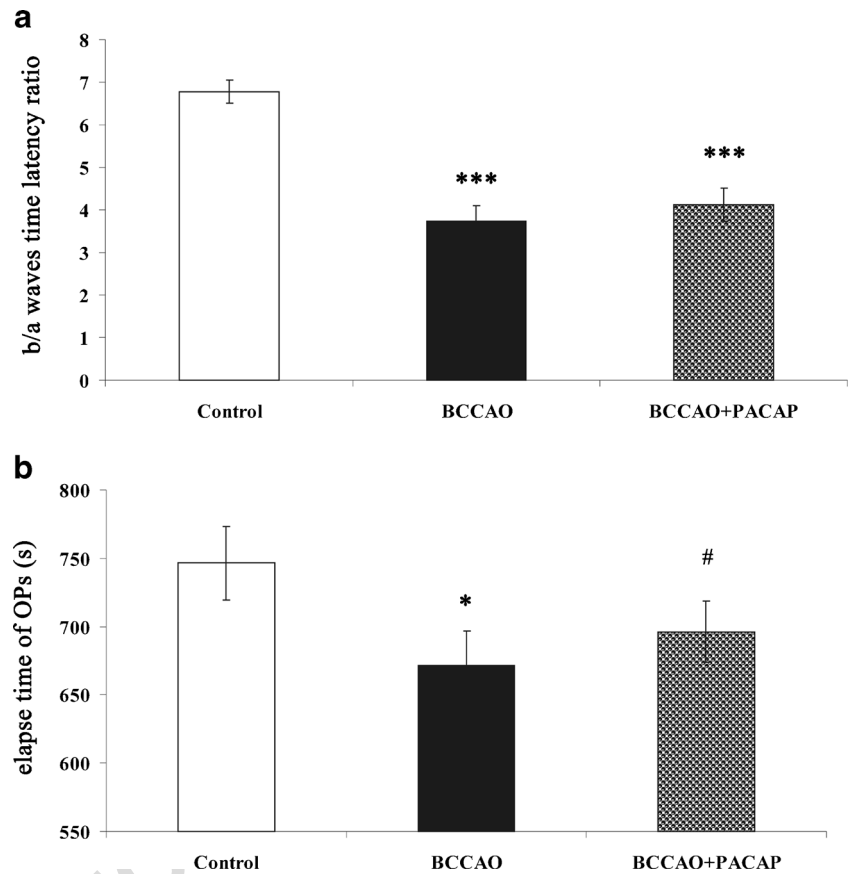


Fig. 4 Representative sections of control (a), BCCAO (b), and PACAP-treated BCCAO (c) retinas stained with toluidine blue. Morphological differences were found between the examined groups; PACAP treatment could ameliorate the ischemia-induced retinal degeneration. Scale bar:

20 μ m. Abbreviations: *PL* photoreceptor layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer

260 histological and neurochemical levels (Atlasz et al. 2007,
 261 2010b; Szabo et al. 2012). The present results indicate the
 262 PACAP treatment induced a significant recovery of the wave-
 263 form and amplitudes of *a* wave and *b* wave as seen in ERG.
 264 PACAP treatment did not completely prevent retinal dysfunc-
 265 tion; however, the ERG recovery was a sustained effect noted
 266 as early as 2 days after BCCAO.

267 There are two further interesting results in the ERG record-
 268 ings. One is that after BCCAO, the *a* wave amplitudes in-
 269 crease slightly, possibly reflecting a relatively late and low
 270 level of photoreceptor damage and the lack of intraretinal
 271 negative feedback. This is supported by the fact that the
 272 thickness of the outer nuclear layer did not change as much
 273 as the inner retinal layers and the photoreceptors look rela-
 274 tively healthy in the histological preparations. The second
 275 observation is that the *b/a* wave latency ratio did not improve
 276 significantly after PACAP treatment. A possible explanation
 277 for that is that the time after BCCAO was not sufficient for the
 278 degenerations to reach the photoreceptors retrogradely. Our
 279 present results confirm our earlier findings in excitotoxic
 280 retinal damage, where PACAP treatment led to functional
 281 amelioration parallel with protection reflected in morpholog-
 282 ical structure (Varga et al. 2011).

283 In conclusion, the results reported in the present study
 284 demonstrate a functional protective effect of PACAP against
 285 retinal ischemic injury caused by BCCAO. Since this is the
 286 second metabolically induced retinal degeneration model
 287 where we can demonstrate such protection, data are convinc-
 288 ing that morphological and neurochemical protection go par-
 289 allel with functional protection.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

- 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.

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