Title: Protective role of endogenous PACAP in inflammation-induced retinal degeneration

Short title: Role of PACAP in retinal inflammation

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Abstract

Purpose. Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuroprotective peptide that has been shown to exert protective effects in different models of neurodegenerative diseases, including retinal degenerations. Data obtained from PACAP-deficient (PACAP KO) mice provide evidence that endogenous PACAP has neuroprotective role in different pathologies. PACAP KO mice show enhanced sensitivity to different insults, such as oxidative stress, hypoxia and inflammation. The aim of the present study was to investigate the protective effects of endogenous PACAP in retinal inflammation.

Methods. Endotoxin-caused eye inflammation was induced by intraperitoneal injection of lipopolysaccharide (LPS) in PACAP KO and wild type (Wt) mice. After LPS treatment, retinas were processed for histological examination. To detect the alterations of different proteins and cytokines, immunohistochemical, western blot and cytokine array were used. We also performed dark-adapted electroretinography (ERG) to detect the functional differences.

Results. The thickness of nearly all layers was significantly less in LPS-injected PACAP KO mice compared to Wt animals. Increased expression of glial fibrillary acidic protein (GFAP) was induced in Müller glial cells after LPS treatment, which was more intense in PACAP KO mice. The levels of pAkt and pGSK were decreased in PACAP KO group during inflammation. LPS treatment significantly increased cytokines (sICAM-1, JE, TIMP-1) in both treated groups, but it was more expressed in PACAP KO animals. Furthermore, ERG responses were disturbed after LPS injection in PACAP KO mice.

Conclusion. Our results showed that endogenous PACAP has a protective role in LPS-caused retinal inflammation.

Text:

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is the most conserved member of the vasoactive intestinal peptide (VIP)/PACAP/glucagon superfamily [1–4]. Since its discovery in 1989 by Miyata and co-workers [5] in hypothalamus, numerous functions have been attributed to PACAP in addition to its adenylate cyclase activation in pituitary cells. Five years after the peptide isolation, Arimura and co-workers [6] published its neurotrophic and neuroprotective effects. PACAP is now considered to be a potent neuroprotective and cytoprotective peptide with potential therapeutic use in numerous diseases [7–13]. The neuroprotective effects of PACAP have been shown in several different cell types in vitro against various toxic agents, such as oxidative stress, glutamate or 6-hydroxydopamine [14–16]. In vivo descriptions have also proven that PACAP is protective in global and focal cerebral ischemia [15, 17, 18], traumatic brain injury and neurodegenerative diseases [19, 20]. PACAP also has cytoprotective effects in different non-neuronal cells, such as endothelial cells, intestinal cells or pinealocytes [11, 16, 21, 22].

The important role of PACAP as a modulator in immunity has long been recognized in acute and chronic inflammatory conditions [4, 23–25]. Kong and coworkers found that lipopolysaccharide (LPS)-induced release of nitric oxide and lactate dehydrogenase into the culture medium, indicative of cell injury, was decreased by PACAP and the protective effects were blocked by the potent PACAP antagonist, PACAP6-38 [26]. Using neuron-glia cultures, Yang and colleagues showed that PACAP38 and PACAP27 were neuroprotective against LPS-induced dopaminergic neurotoxicity [27]. Moreover, PACAP dose-dependently attenuated the LPS-caused inflammation in dopaminergic cells, reducing caspase activation and increasing BDNF expression as well as CREB phosphorylation [28]. Similarly, several

studies have shown the neuroprotective roles of PACAP in the retina [29]. Protective actions of PACAP have been proven in different pathological conditions, such as excitotoxic retinal injury [30, 31], diabetic retinopathy [32–34], UV-A light-induced degeneration [35], ischemic damages [36, 37] and oxygen-induced retinopathy [38]. PACAP is upregulated upon numerous harmful stimuli, supporting its endogenous protective effects in restorative processes [11]. As it has been shown in numerous models, PACAP knockout (KO) mice are more vulnerable to different types of injuries, from hypoxia to oxidative stress, compared to wild type (Wt) mice [39–41]. PACAP KO mice had significantly greater retinal damage in ischemia compared to Wt mice [42]. Furthermore, several degenerative changes were observed at an earlier age in PACAP KO mice retina [43].

All these above results indicate the function of endogenous PACAP as a stress-response peptide that is necessary for endogenous protection against different retinal insults, however, the possible protective role of endogenous PACAP in retinal inflammation is yet unclear. The aim of the present study, using morphological, immunological, biochemical and functional techniques, was to investigate the protective and anti-inflammatory effects of endogenous PACAP in PACAP KO- and Wt mice in LPS-induced retinal inflammation.

Methods

Animals

Adult male three-month-old (CD1 strain) Wt and PACAP KO mice (n=100 in 4 groups) were used in the experiments. Generation and maintenance of PACAP KO mice with a CD1 background was previously described [44]. Animals were backcrossed for ten generations with the CD1 strain, all were genotyped with PCR and only homozygous knockouts were used to the experiments. Mice were maintained in a temperature- and humidity-controlled room under 12h light/dark cycle with free access to food and water. All animal protocols were approved by the institutional ethical guidelines (permission number: BA02/2000-38/2017).

LPS treatment

Mice received a single intraperitoneal injection of 6.0 mg/kg body weight of LPS from Escherichia coli (n=50) in phosphate-buffered saline (PBS). Control groups were injected PBS intraperitoneally (n=50). Mice were killed and investigated 24h after injections. This time point was chosen for immunohistochemical, cytokine array, western blot and ERG analyses, as most of the pathological changes in the retina were detectable at this time-point. The morphological changes of the retina were measured on the 14^{th} day after injections.

Histological analysis

Mice were anesthetized with isoflurane and sacrificed 14 days after LPS treatment (n=6 animals/ each conditions). Both eyes were removed and dissected in 0.1 M PBS and fixed in

4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB) (Sigma, Hungary). Eye-cup tissues were embedded in resin (Durcupan ACM resin, Fluka, Switzerland). Retinas were cut at 2 μm and stained with toluidine blue (Sigma, Hungary). Sections mounted in DPX medium (Sigma, Hungary) and examined in a Nikon Eclipse 80i microscope, measured with Q-Capture Pro7 program (Q-Imaging, USA). Central retinal areas within 2 mm from the optic nerve were used for measurements (n=5 measurements from one tissue block and the blocks were compered). Images were further processed with Adobe Photoshop CS6 program. The following parameters were measured: (i) retinal cross section between the outer and inner limiting membranes (OLM-ILM), as well as (ii) the width of individual retinal layers (ONL, INL, OPL, IPL).

Glial fibrillary acidic protein (GFAP) immunohistochemistry in Müller cells

Animals were sacrificed 24h after LPS or vehicle (PBS) injections (n=5 animals/ each conditions). Immunohistochemistry was performed following the procedure described previously [36]. For measurement of glial fibrillary acidic protein (GFAP) activity in the Müller glial cells, eyes were dissected in ice-cold PBS and postfixed in 4% PFA dissolved in 0.1M PB (pH 7.4) for 4h at room temperature. Tissues were washed in 0.1M PB, followed by dehydration procedures with graded sucrose solutions (2 h in 10%, 20% and overnight in 30%; Sigma, Budapest, Hungary) at 4°C. The eyecups were vertically sectioned in tissue freezing medium (Cryomatrix, Shandon, USA) at 16 μm thickness on a freezing microtome (Leica, Nussloch, Germany). Sections were collected on chrome–alum–gelatin coated slides and stored at −20 °C until use then samples were rinsed in PBS, permeabilized with 0.1% Triton X-100 (Sigma, Budapest, Hungary), and incubated in PBS containing 3% normal donkey serum and 0.1% Na-azide for 1 h to block the nonspecific binding sites. The samples

were incubated with polyclonal antibodies against anti-GFAP antibody at 4°C overnight. On the following day, the appropriate second fluorescent anti-rabbit antibody Alexa Fluor 488 (donkey anti-rabbit, 1:200, Life Technologies, Budapest, Hungary) was added in a dark room for 2h. After washing, propidium iodide (PI, 1:500, Sigma, Budapest, Hungary) was used to detect the nuclear components. Preparations were mounted with Fluoroshield (Sigma, Budapest, Hungary) and detected by a fluorescent microscope (Nikon Eclipse 80i). Central retinal areas were used for immunohistochemical analysis. All images were further analyzed under masked conditions using Adobe Photoshop CS6 program and ImageJ software (NIH). Photographs were transferred into grayscale, the background was subtracted and upper and lower thresholds were set. The percentage of GFAP labeled area was measured in each picture using an ImageJ macro (NIH).

Cytokine array analysis

One day after the administration of LPS (n=4 animals/ each conditions), retinas were dissected and kept at -80 °C until tested. Proteome Profiler Mouse Cytokine Array Kit, Panel A from R&D System (Biomedica, Budapest, Hungary) was used for the analysis. The array is based on antibodies binding with nitrocellulose membranes and it was performed as described by the manufacturer. Samples were pooled and homogenized in PBS with protease inhibitors. After homogenization, Triton X-100 was added to a final concentration of 1%. The nitrocellulose membranes were blocked and incubated with reconstituted detection antibody cocktail. Membranes were incubated overnight with 400 µg protein containing homogenates. After washing and streptavidin-horseradish peroxidase addition to the membranes, plates were spread to a chemiluminescent detection reagent (Amersham Biosciences, Hungary).

Developed films were scanned and the mean intensities of the dot blots of the different cytokines were calculated by ImageJ software (NIH).

Western blot measurements

For western blot experiments retinas were removed 24h after LPS injection (n=4 animals/ each conditions) and stored at -80 C until analysis. Samples were processed for western blot as described earlier [45]. Frozen tissues were homogenized (50 mM TRIS, 50 mM EDTA, 50 mM sodium metavanadate, 0.5% protease inhibitor cocktail, 0.5% phosphatase inhibitor cocktail, pH = 7.4) and 300 μg protein concentration was determined with a DC™ Protein Assay kit (Bio-Rad, Hercules, CA). Membranes were probed overnight at 4°C with the primary antibodies: phospho-specific anti-Akt-1 Ser473 (pAkt; 1:1000; R&D Systems, Hungary), phospho-specific glycogen synthase kinase-3β Ser9 (pGSK; 1:1000; Cell Signaling Technology, Beverly, USA). Non-phosphorylated total-Akt (tAkt; 1:1000) antibody was used as internal control as described by Pitre et al. [46]. Membranes were washed six times for 5 min in Tris buffered saline (pH = 7.5) containing 0.2% Tween prior to addition of goat anti-rabbit or anti-mouse horseradish peroxidase- conjugated secondary antibody (1:3000; BioRad, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. For quantification of blots, band intensities were quantified by NIH ImageJ program.

Electroretinography

Scotopic ERGs were performed to assess retinal function in Wt- and PACAP KO groups. ERG flashes were recorded before LPS treatment and 24h after LPS-induced inflammation

(n=6 animals/ each conditions). Mice were dark adapted for at least 12h and prepared under dim red illumination (632 nm) [47], anesthetized with 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/BW kg) [48]. Mice were placed on a heating pad throughout the experiment and pupils were dilated with one drop of 1 % homatropine (w/v, Humapent- Teva, Hungary). ERGs were recorded by surface electrodes from the center of the cornea [49, 50]. The reference electrode was placed subcutaneously between the eyes, and the ground electrode was used subcutaneously under the skin of the back. The light pulses intensity (5cd s/m2, 0.25 Hz, 503 nm green LED light) were preamplified, amplified (2.000×, Bioamp SbA4-V6, Supertech, Hungary) and recorded with an A/D converter (Ratsoft-Solar Electronic) [51, 52]. Responses (n=50/eye) were averaged with Ratsoft software. The graphs were analyzed with OriginPro 2016 (Macasoft, Hungary). The following parameters were measured: amplitude of the a-wave (from baseline to the trough of the a-wave), amplitude of the b-wave (from the trough of the a-wave to the peak of the b-wave).

Data analysis

Data are expressed as mean ± standard error (SEM). Data were analyzed using Kolmogorov-Smirnov normality test followed by ANOVA test and Fisher LSD's post hoc analysis (OriginPro 2016, Macasoft, Hungary). Significant differences were considered at p values below 0.05.

Results

Effects of LPS treatment on histological changes of the retina

No differences were observed between the control retinas of Wt (Fig. 1 A) and PACAP KO mice (Fig. 1 B). Wt retinas in the LPS-treated group (Fig. 1 C) did not show remarkable differences (except in INL layer; Fig. 2 B) compared to control groups (Fig. 1 A, B). Retinal layers in LPS-treated PACAP KO group (Fig. 1 D) showed signs of severe degeneration compared to PBS-treated controls (Fig. 1 A, B) and the LPS-treated Wt (Fig. 1 C) groups.

In LPS-treated KO animals, all retinal layers were significantly thinner than in the control and LPS-treated Wt groups (Fig. 2 A, B). Marked reduction was observed in the ONL, but significant changes were also found in the INL, OPL and IPL. We found severe reduction of the whole retinal thickness between OLM-ILM in this group (Fig. 1 D, Fig. 2 A, B).

Analysis of glial fibrillary acidic protein in Müller glial cells

Under control conditions the retinas did not show any remarkable immunofluorescent changes in either vehicle-treated Wt or PACAP KO groups (Fig. 3 A, B, E). GFAP was markedly upregulated following LPS treatment in the retinas of Wt and PACAP KO mice (Fig. 3 C, D, E). Expression was more intense in the entire cell from the OLM to ILM in LPS-treated PACAP KO animals compared to the LPS-injected Wt mice (Fig. 3 C, D, E).

Effects of LPS treatment on cytokine expression profile of the retina

The expression level of several cytokines was increased after LPS treatment (Fig. 4). The activation of sICAM-1 (soluble intercellular adhesion molecule-1), TIMP-1 (tissue inhibitor of metalloproteinase-1) and JE (monocyte chemoattractant protein-1) was increased in the retinas that underwent LPS inflammation compared to control groups (Fig. 4). The expression level of these three cytokines was significantly stronger in the LPS-treated PACAP KO group compared to the LPS-injected Wt group (Fig. 4). Other spots, where no significant changes were observed are (from upper left corner, without numbers): BCL, C5/C5a, G-CSF, GM-CSF, I-309, Eotaxin, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12p70, IL-16, IL-17, IL-23, IL-27, IP-10, I-TAC, KC, M-CSF, MCP-5, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, SDF-1, TARC, TNF-α, TERM-1.

Western blot analysis

No differences were detected between the control groups, however, marked alterations were observed in retinas 24h after LPS injection (Fig. 5). Inflammation itself induced a decreased pAkt expression, which was more severe in the LPS-treated PACAP KO group compared to the LPS-injected Wt group (Fig. 5 A, B). Similar changes were observed in the glycogen synthase kinase (GSK)-3, the downstream target of Akt (Fig. 5 A, C).

Protective effect of endogenous PACAP on visual responses after retinal inflammation

Representative ERG was recorded 12h after dark adaptation (Fig. 6 A, B). In control situations, ERG waveforms were similar in Wt and PACAP KO mice (Fig. 6 A). Luminance-responses were reduced 24h after inflammation in both LPS-treated groups, but responses were more preserved in the Wt animals compared to the PACAP KO mice (Fig. 6 B).

Amplitudes of a-wave and b-wave were significantly decreased after inflammation, but those changes were more severe in the LPS-treated PACAP KO group (Fig. 6 C, D). The latency of a/b waves was significantly decreased in both treated groups compared to their controls, but no differences could be observed between the LPS-treated Wt and PACAP KO mice (data not shown).

Discussion

The neuropeptide PACAP exerts anti-inflammatory and protective effects in several organs, such as brain, immune system and eye. In the present study, we showed, for the first time, that endogenous PACAP is protective in LPS-induced ocular inflammation in the retina using PACAP KO mice. Based on our results, no major differences were found in the histological structure, cytokine expressions, or in visual function between the retinas of Wt and PACAP KO mice under normal conditions, whereas we detected several differences during inflammation. Earlier studies have proven that exogenously applied PACAP is retinoprotective in excitotoxic injury induced by glutamate [53, 54], N-methyl-D-aspartate (NMDA) [55], kainate [56], hypoperfusion-induced degeneration after carotid artery ligation [36, 37], UV-A light radiation [35], optic nerve transection [57], and streptozotocin-induced diabetic damages in the retina [33], as well as in retinopathy of prematurity [38].

Numerous studies have proven that endogenous PACAP plays an important role in several physiological functions such as regulation of body temperature [58] and fertility [59–61]. Furthermore, PACAP KO mice display behavioral abnormalities, altered pain and inflammatory reactions [44, 62–66]. Endogenous PACAP suppresses dry eye signs by regulation of tear secretion [67] and protects the retina during ischemia [42]. However, the role of endogenous PACAP in the LPS-induced retinal inflammation had not been tested yet.

In our present study, we detected dramatic changes of the retinal layers after LPS-induced inflammation in the PACAP KO groups compared to the Wt ones. These findings correlated with results of other research groups, where PACAP KO mice showed increased severe retinal abnormalities in aging or ischemia [42, 43].

We showed irregularity in Müller glial cells during LPS-induced inflammation, which was more intense in the PACAP KO group. The decreased uptake of GABA and glutamate results in accumulations of these proteins and causes abnormalities in the retinal neurons [68–72]. PACAP is retinoprotective on Müller glial cells, and stimulates the release of interleukin-6, which has been confirmed in ischemic and excitotoxic brain lesions [73–75].

In our study, retinal inflammation induced changes in several cytokines (TIMP-1, sICAM-1 and JE regulatory proteins). Members of the TIMP family play an important role in cell proliferation and apoptosis and they also have an inhibitory effect on matrix metalloproteinases (MMPs), which are able to degrade the extracellular matrix [76]. During inflammatory events, the transcription of MMPs inhibitor TIMP-1 is induced by pro-inflammatory mediators [77]. In our experiment, TIMP-1 level showed a strong activation 24h after the LPS treatment in both treated groups, but was more severe in the PACAP KO mice. Our present findings are in accordance with earlier studies, where increased expression of TIMP-1 was associated with many pathological conditions, such as diabetic nephropathy [78], ischemia-induced kidney injury [79], mesenteric ischemia [77], glaucoma [80] or ischemic retinopathy [45]. Furthermore, exogenously administered PACAP attenuated the activation of TIMP-1 expression in diabetes-induced nephropathy [78], ischemia-induced kidney damage [79], small bowel [77, 81] and retinal injury [36]. In the present study, sICAM-1 activation was detectable in PACAP KO mice in inflammation. Upregulation of sICAM-1 is enhanced by inflammatory cytokines, including tumor necrosis factor alpha (TNFa) and it produces pro-inflammatory effects such as recruited leukocytes into the site of

the inflammation [82, 83]. High concentrations of sICAM-1 are described in patients in vitreoretinopathy [84, 85], in uveitis [86] or in sickle cell retinopathy [87]. Increased expression of sICAM-1 was also observed in ischemia/reperfusion (I/R)-induced injury in several organs, and PACAP treatment partially or totally blocked this cytokine [45, 77, 78, 88].

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family and a potent chemotactic factor for monocytes [89]. MCP-1 is identical to JE in mice, where the upregulation of this cytokine has been implicated in a number of acute and chronic inflammatory diseases, such as atherosclerosis [90], glomerulonephritis [91], diabetic retinopathy [92], Eales' Disease [93], ischemic retinopathy [94] or LPS-induced uveitis [95]. Elevated level of this cytokine was observed in several models such as hypoxia-induced injury in the kidney [88] or acute ileitis [96], where exogenous PACAP administration ameliorates acute inflammation in the above mentioned diseases. Akt is a kinase downstream phosphatidylinositol 3-kinase (PI3K), it is an important molecule that promotes cell survival in response to extracellular signals such as retinal ischemia [36, 45, 97, 98]. GSK-3 acts downstream of PI3K pathway/Akt and is involved in regulation of inflammation [99]. Inhibition of Akt activation by harmful stimuli, such as LPS-induced inflammation, prevents the inhibitory phosphorylation of GSK-3, promotes its kinase activity and increases the degree of organic injury [100]. Consistent with results generated from other studies, our observation showed decreased level of phosphorylated Akt and GSK during LPS-induced inflammation [101–103]. In the PACAP KO animals we detected slightly lower levels of pGSK. The reason for this phenomenon might be that exogenously applied PACAP induces pGSK [104] and thus the lack of endogenous PACAP results in lower baseline levels in untreated PACAP KO mice. The reduction of pAKT and pGSK was more severe in the LPS-treated PACAP KO group. This study tested the hypothesis that endogenous PACAP

plays an anti-inflammatory role in LPS-induced retinal damage through preservation of PI3K/Akt functional activity. Previous studies have shown the functional protective effects of exogenously applied PACAP in different kinds of retinal injuries, like excitotoxity [105] or ischemia [48]. Response of the retina to harmful stimuli is measured by ERG, where an a-wave (initial negative deflection) followed by a b-wave (positive deflection) can be distinguished. The a-wave is produced by the photoreceptors, while the b-wave is produced mainly by ON-bipolar neurons, and also from amacrine, ganglion and Muller glial cells [106]. Similarly to earlier studies [68, 107] we demonstrated severe disturbance of visual function in the inflamed retinas by ERG. Endogenous PACAP successfully prevented pathologic changes, prevented the a-wave amplitude of ERG, thus protecting the photoreceptor cell function in LPS-induced retinal inflammation. The malfunction of Müller glial cells involved in the decreased responses of b-wave in ERG, which was also preserved in the presence of endogenous peptide.

Our findings further suggest that endogenous PACAP represents an important part of the natural defense mechanism against retinal inflammation.

Conflict of interest:

Alexandra Vaczy: no conflict of interest, ***Petra Kovari***: no conflict of interest, ***Krisztina Kovacs***: no conflict of interest, ***Kinga Farkas***: no conflict of interest, ***Edina Szabo***: no conflict of interest, ***Timea Kvarik***: no conflict of interest, ***Bela Kocsis***: no conflict of interest, ***Balazs Fulop***: no conflict of interest, ***Tamas Atlasz***: no conflict of interest, ***Dora Reglodi***: no conflict of interest

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Authors' contributions are:

Participated in research design: Vaczy, Atlasz and Reglodi,
Conducted experiments: Vaczy, Kovari, Kovacs, Farkas, Szabo, Kvarik, Atlasz and Reglodi
Contributed new reagents or analytical tools: Kocsis and Fulop
Collected data: Vaczy, Kovari, Kovacs, Farkas, Szabo, Kvarik and Atlasz
Performed data analysis: Vaczy, Kovari and Atlasz.
Wrote or contributed to the writing of the manuscript: Vaczy, Atlasz and Reglodi

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Figure legends

FIGURE 1. (**A**–**D**) Light microphotographs showing toluidine blue-stained representative retinal sections (2 μm). The retinal damage is shown by the width of retinal layers and cell profiles. (**A**) Control Wt retina, treated with intraperitoneal PBS. (**B**) Control PACAP KO retina, treated with intraperitoneal PBS. (**C**) Wt + intraperitoneal LPS and (**D**) PACAP KO + intraperitoneal LPS treatment. LPS-induced retinal degeneration showed more apparent damage in PACAP KO group compared to the treated Wt and control groups. Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer. (scale bar: 30 μm)

FIGURE 2. (**A**) Cross section of the retina from the outer limiting membrane to the inner limiting membrane (OLM-ILM) and (**B**) quantitative comparison of the different retinal layers. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer. Data are expressed as mean ± SEM. *$p< 0.05$ compared to the control Wt retinas; #$p< 0.05$ compared to LPS-treated retinas. Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide; OLM-ILM: whole retina thickness; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer.

FIGURE 3. Representative vertical retinal sections stained by GFAP antibody showing the effect of LPS in control Wt (**A**), control PACAP KO (**B**) retina and LPS-treated Wt (**C**) and LPS-injected PACAP KO (**D**) sample. PI (red) was used to detect the nuclear components. GFAP-immunoreactivity (green) was restricted onlyto the GCL and nerve fiber layer in control conditions (**A, B**). Retinal degeneration induced by LPS showed strong upregulation

of immunoreactivity (**C, D**). GFAP immunopositivity was stretched into IPL, INL and OPL layers in LPS-treated PACAP KO retina (**D**). Quantitative comparison of GFAP immunoreactivity in control Wt, control PACAP KO retinas and LPS-treated Wt and LPS-injected PACAP KO samples (**E**). Data are expressed as mean ± SEM. *p< 0.05 compared to the control Wt retinas; #p< 0.05 compared to LPS-treated Wt retinas; Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer; PI: propidium iodide; GFAP: glial fibrillary acidic protein. (scale bar: 30 μm)

FIGURE 4. (**A**) Representative panels show cytokine arrays from homogenates of control Wt, PACAP KO samples, LPS-treated Wt, and PACAP KO retinas. The panels show the examined cytokines in each box, highlighting changes after LPS-treatment. (**B**) The table indicates the examined cytokines in each box, highlighting changes after LPS-treatment. (**C**, **D**, **E**). Quantification of cytokine levels of control Wt, PACAP KO, LPS-injected Wt, and PACAP KO groups. Each cytokine was measured by Protein Array Analyzer for ImageJ. (**C**) TIMP-1, (**D**) sICAM-1 and (**E**) JE demonstrate the effects of LPS-induced retinal inflammation. Graph values are given as means ± SEM. *p< 0.05 compared to control Wt retinas; #p< 0.05 compared to LPS-treated Wt samples. Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide; TIMP-1: tissue inhibitor of metalloproteinase-1; sICAM-1: soluble intercellular adhesion molecule-1; JE: mouse monocyte chemoattractant protein-1.

FIGURE 5. (**A**) Representative panels show the results of western blot analysis (1-Wt, 2-PACAP KO, 3-LPS+Wt, 4-LPS+PACAP KO samples). (**B**) pAkt and (**C**) pGSK levels in

control Wt, PACAP KO and LPS+Wt, LPS+PACAP KO retinas. tAkt was used as control for pAkt and pGSK. Data are given as mean ± SEM. *p <0.05 compared to control Wt retinas; #p< 0.05 compared to LPS-treated Wt samples. Statistical analysis of protein levels was measured by ImageJ software. Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide; pAkt: phosphorylation of Akt; pGSK: phosphorylation of GSK; tAkt: total Akt.

FIGURE 6. Representative panels show ERG responses after 24h of dark adaptation. (**A**) ERG response was similar in Wt and PACAP KO mice under healthy condition. (**B)** Abnormalities was detected during inflammation in both treated groups. ERG recording of LPS-injected PACAP KO mice was more reduced compared to the LPS+ Wt. (**C)** Comparative analysis of the average amplitudes of a-waves and (**D**) b-waves. The wave amplitudes were significantly altered during inflammation which were more severe in LPS+PACAP KO group. Data are given as mean ± SEM. *p <0.05 compared to control Wt retinas; #p< 0.05 compared to LPS-treated Wt samples. Abbreviations: Wt: wild type; PACAP KO: PACAP knock out; LPS: lipopolysaccharide.