

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

**Short title:** Role of PACAP in retinal inflammation

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**Keywords:** retina, PACAP, knock out, LPS, inflammation, neuroprotection

## **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<sup>th</sup> 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  $\mu$ m 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 compared). 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  $\mu$ m 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 DCT<sup>TM</sup> 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/m<sup>2</sup>, 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  $\pm$  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- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , 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-1 $\alpha$ , MIP-1 $\beta$ , MIP-2, RANTES, SDF-1, TARC, TNF- $\alpha$ , 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 (TNF $\alpha$ ) 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 excitotoxicity [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

**Acknowledgements:**

Supported by NKFIH FK129190 (Budapest, Hungary) to *T.A.*, NAP 2017-1.2.1-NKP-2017-00002 (Budapest, Hungary) to *D.R.*, Bolyai Scholarship (MTA, Budapest, Hungary) to *T.A.*, GINOP-2.3.2-15-2016-00050 “PEPSYS” (Budapest, Hungary) to *D.R.*, MTA-TKI 14016 (Budapest, Hungary) to *D.R.*, EFOP-3.6.3-VEKOP-16-15 2017-00008 “The role of neuro-inflammation in neurodegeneration: from molecules to clinics” (Budapest, Hungary) to *D.R.*, EFOP-3.6.3-VEKOP-16-2017-00009 (Budapest, Hungary) to *D.R.*, EFOP-3.6.1.-16-2016-00004 Comprehensive Development for Implementing Smart Specialization Strategies at the University of Pécs (Budapest, Hungary) to *D.R.*, UNKP-18-4 (Budapest, Hungary) to *T.A.*, UNKP-18-2 (Budapest, Hungary) to *P.K.*, UNKP-16-4 (Budapest, Hungary) to *T.A.*, UNKP-17-2-II New National Excellence Program of the Ministry of Human Capacities (Budapest, Hungary) to *P.K.*, Centre for Neuroscience (Pecs, Hungary) to *D.R.*, PTE AOK Research Grant KA-2017-15 (Pecs, Hungary) to *T.A.*, Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the 20765-3/2018/FEKUTSTRAT (PTE, Pecs Hungary) to *D.R.*,

**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



## References

- [1] Padua D, Vu JP, Germano PM, et al. The Role of Neuropeptides in Mouse Models of Colitis. *J Mol Neurosci* 2016; 59(2):203–10.
- [2] Krajcs N, Hernádi L, Pirger Z, et al. PACAP Modulates Acetylcholine-Elicited Contractions at Nicotinic Neuromuscular Contacts of the Land Snail. *J Mol Neurosci* 2015; 57(4):492–500.
- [3] Hashimoto H. [Physiological significance of pituitary adenylate cyclase-activating polypeptide (PACAP) in the nervous system]. *Yakugaku Zasshi* 2002; 122(12):1109–21.
- [4] Sherwood NM, Krueckl SL, McRory JE. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 2000; 21(6):619–70.
- [5] Miyata A, Arimura A, Dahl RR, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* 1989; 164(1):567–74.
- [6] Arimura A, Somogyvari-Vigh A, Weill C, et al. PACAP functions as a neurotrophic factor. *Ann N Y Acad Sci* 1994; 739:228–43.
- [7] Bourgault S, Vaudry D, Dejda A, et al. Pituitary adenylate cyclase-activating polypeptide: focus on structure-activity relationships of a neuroprotective Peptide. *Curr Med Chem* 2009; 16(33):4462–80.
- [8] Brenneman DE. Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Peptides* 2007; 28(9):1720–26.
- [9] Dejda A, Jolivel V, Bourgault S, et al. Inhibitory effect of PACAP on caspase activity

- in neuronal apoptosis: a better understanding towards therapeutic applications in neurodegenerative diseases. *J Mol Neurosci* 2008; 36(1–3):26–37.
- [10] Shioda S, Ohtaki H, Nakamachi T, et al. Pleiotropic functions of PACAP in the CNS: neuroprotection and neurodevelopment. *Ann N Y Acad Sci* 2006; 1070:550–60.
- [11] Somogyvári-Vigh A, Reglodi DD, Somogyvari-Vigh A, et al. Pituitary adenylate cyclase activating polypeptide: a potential neuroprotective peptide. *Curr Pharm Des* 2004; 10(23):2861–89.
- [12] Vaudry D, Gonzalez BJ, Basille M, et al. Pituitary adenylate cyclase-activating polypeptide stimulates both c-fos gene expression and cell survival in rat cerebellar granule neurons through activation of the protein kinase A pathway. *Neuroscience* 1998; 84(3):801–12.
- [13] Vaudry D, Falluel-Morel A, Bourgault S, et al. Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 2009; 61(3):283–357.
- [14] Kasica N, Podlasz P, Sundvik M, et al. Protective Effects of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Against Oxidative Stress in Zebrafish Hair Cells. *Neurotox Res* 2016; 30(4):633–47.
- [15] Ohtaki H, Nakamachi T, Dohi K, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) decreases ischemic neuronal cell death in association with IL-6. *Proc Natl Acad Sci U S A* 2006; 103(19):7488–93.
- [16] Reglodi D, Kiss P, Lubics A, et al. Review on the protective effects of PACAP in models of neurodegenerative diseases in vitro and in vivo. *Curr Pharm Des* 2011; 17(10):962–72.

- [17] Chen Y, Samal B, Hamelink CR, et al. Neuroprotection by endogenous and exogenous PACAP following stroke. *Regul Pept* 2006; 137(1–2):4–19.
- [18] Reglodi D, Somogyvari-Vigh A, Vigh S, et al. Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. *Stroke* 2000; 31(6):1411–17.
- [19] Mao SS, Hua R, Zhao XP, et al. Exogenous administration of PACAP alleviates traumatic brain injury in rats through a mechanism involving the TLR4/MyD88/NF-kappaB pathway. *J Neurotrauma* 2012; 29(10):1941–59.
- [20] Feher M, Gaszner B, Tamas A, et al. Alteration of the PAC1 Receptor Expression in the Basal Ganglia of MPTP-Induced Parkinsonian Macaque Monkeys. *Neurotox Res* 2017;
- [21] Horvath G, Reglodi D, Oppert B, et al. Effects of PACAP on the oxidative stress-induced cell death in chicken pinealocytes is influenced by the phase of the circadian clock. *Neurosci Lett* 2010; 484(2):148–52.
- [22] Illes A, Oppert B, Reglodi D, et al. Effects of pituitary adenylate cyclase activating polypeptide on small intestinal INT 407 cells. *Neuropeptides* 2017; 65:106–13.
- [23] Delgado M, Abad C, Martinez C, et al. PACAP in immunity and inflammation. *Ann N Y Acad Sci* 2003; 992:141–57.
- [24] Ganea D, Delgado M. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) as modulators of both innate and adaptive immunity. *Crit Rev Oral Biol Med* 2002; 13(3):229–37.
- [25] Delgado M, Martinez C, Pozo D, et al. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal

- endotoxemia through the inhibition of TNF- $\alpha$  and IL-6. *J Immunol* 1999; 162(2):1200–05.
- [26] Kong LY, Maderdrut JL, Jeohn GH, et al. Reduction of lipopolysaccharide-induced neurotoxicity in mixed cortical neuron/glia cultures by femtomolar concentrations of pituitary adenylate cyclase-activating polypeptide. *Neuroscience* 1999; 91(2):493–500.
- [27] Yang S, Yang J, Yang Z, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP) 38 and PACAP4-6 are neuroprotective through inhibition of NADPH oxidase: potent regulators of microglia-mediated oxidative stress. *J Pharmacol Exp Ther* 2006; 319(2):595–603.
- [28] Brown D, Tamas A, Reglodi D, et al. PACAP protects against inflammatory-mediated toxicity in dopaminergic SH-SY5Y cells: implication for Parkinson's disease. *Neurotox Res* 2014; 26(3):230–39.
- [29] Tamas Atlasz, Alexandra Vaczy, Dora Werling, Peter Kiss, Andrea Tamas, Krisztina Kovacs, Eszter Fabian, Timea Kvarik, Barbara Mammel, Bese Danyadi, Emese Lokos DR. Protective Effects of PACAP in the Retina. In: Dora Reglodi AT, Ed. *Pituitary Adenylate Cyclase Activating Polypeptide — PACAP 11th ed.* Springer, Cham: New York 2016; pp. 501–27.
- [30] Endo K, Nakamachi T, Seki T, et al. Neuroprotective Effect of PACAP Against NMDA-Induced Retinal Damage in the Mouse. *J Mol Neurosci* 2011; 43(1):22–29.
- [31] BABAI N, Babai N, Atlasz T, Tamás A, Reglodi D, Tóth G, Kiss P GR. Search for the Optimal Monosodium Glutamate Treatment Schedule to Study the Neuroprotective Effects of PACAP in the Retina. *Ann N Y Acad Sci* 2006; 1070(1):149–55.
- [32] D'Amico AG, Maugeri G, Reitano R, et al. PACAP Modulates Expression of Hypoxia-

- Inducible Factors in Streptozotocin-Induced Diabetic Rat Retina. *J Mol Neurosci* 2015; 57(4):501–09.
- [33] Szabadfi K, Atlasz T, Kiss P, et al. Protective effects of the neuropeptide PACAP in diabetic retinopathy. *Cell Tissue Res* 2012; 348(1):37–46.
- [34] Szabadfi K, Reglodi D, Szabo A, et al. Pituitary Adenylate Cyclase Activating Polypeptide, A Potential Therapeutic Agent for Diabetic Retinopathy in Rats: Focus on the Vertical Information Processing Pathway. *Neurotox Res* 2016; 29(3):432–46.
- [35] Atlasz T, Szabadfi K, Kiss P, et al. Effects of PACAP in UV-A Radiation-Induced Retinal Degeneration Models in Rats. *J Mol Neurosci* 2011; 43(1):51–57.
- [36] Werling D, Reglodi D, Banks WA, et al. Ocular Delivery of PACAP1-27 Protects the Retina From Ischemic Damage in Rodents. *Investig Ophthalmology Vis Sci* 2016; 57(15):6683.
- [37] Vaczy A, Reglodi D, Somoskeoy T, et al. The Protective Role of PAC1-Receptor Agonist Maxadilan in BCCAO-Induced Retinal Degeneration. *J Mol Neurosci Journal of Molecular Neuroscience* 2016; 60(2):186–94.
- [38] Kvarik T, Mammel B, Reglodi D, et al. PACAP Is Protective in a Rat Model of Retinopathy of Prematurity. *J Mol Neurosci Journal of Molecular Neuroscience* 2016; 60(2):179–85.
- [39] Nemeth A, Szabadfi K, Fulop B, et al. Examination of calcium-binding protein expression in the inner ear of wild-type, heterozygous and homozygous pituitary adenylate cyclase-activating polypeptide (PACAP)-knockout mice in kanamycin-induced ototoxicity. *Neurotox Res* 2014; 25(1):57–67.
- [40] Lacaille H, Duterte-Boucher D, Vaudry H, et al. PACAP Protects the Adolescent and

Adult Mice Brain from Ethanol Toxicity and Modulates Distinct Sets of Genes Regulating Similar Networks. *Mol Neurobiol* 2017; 54(9):7534–48.

- [41] Ohtaki H, Satoh A, Nakamachi T, et al. Regulation of oxidative stress by pituitary adenylate cyclase-activating polypeptide (PACAP) mediated by PACAP receptor. *J Mol Neurosci* 2010; 42(3):397–403.
- [42] Szabadfi K, Atlasz T, Kiss P, et al. Mice deficient in pituitary adenylate cyclase activating polypeptide (PACAP) are more susceptible to retinal ischemic injury in vivo. *Neurotox Res* 2012; 21(1):41–48.
- [43] Kovács-Valasek A, Szabadfi K, Dénes V, et al. Accelerated retinal aging in PACAP knock-out mice. *Neuroscience* 2017; 348:1–10.
- [44] Hashimoto H, Hashimoto R, Shintani N, et al. Depression-like behavior in the forced swimming test in PACAP-deficient mice: Amelioration by the atypical antipsychotic risperidone. *J Neurochem* 2009; 110(2):595–602.
- [45] Szabo A, Danyadi B, Bognar E, et al. Effect of PACAP on MAP kinases, Akt and cytokine expressions in rat retinal hypoperfusion. *Neurosci Lett Elsevier Ireland Ltd* 2012; 523(2):93–98.
- [46] Pitre A, Davis N, Paul M, et al. Synemin promotes AKT-dependent glioblastoma cell proliferation by antagonizing PP2A. *Mol Biol Cell* 2012; 23(7):1243–53.
- [47] Zhang X-Y, Xiao Y-Q, Zhang Y, et al. Protective effect of pioglitazone on retinal ischemia/reperfusion injury in rats. *Invest Ophthalmol Vis Sci* 2013; 54(6):3912–21.
- [48] Danyadi B, Szabadfi K, Reglodi D, et al. PACAP Application Improves Functional Outcome of Chronic Retinal Ischemic Injury in Rats-Evidence From Electroretinographic Measurements. *J Mol Neurosci* 2014; 54(3):293–99.

- [49] Gouras P. Electoretinography: Some basic principles. Invest Ophthalmol 1970; 9(8):557–69.
- [50] Perlman I. The Electoretinogram: ERG,. Webvision: The Organization of the Retina and Visual System 1995[Online] 1995.
- [51] Jacobs GH, Fenwick JA, Williams GA. Cone-based vision of rats for ultraviolet and visible lights. J Exp Biol 2001; 204(Pt 14):2439–46.
- [52] Szabó-Salfay O, Pálhalmi J, Szatmári E, et al. The electoretinogram and visual evoked potential of freely moving rats. Brain Res Bull 2001; 56(1):7–14.
- [53] Atlasz T, Szabadfi K, Reglodi D, et al. Effects of pituitary adenylate cyclase activating polypeptide and its fragments on retinal degeneration induced by neonatal monosodium glutamate treatment. Ann N Y Acad Sci 2009; 1163:348–52.
- [54] Babai N, Atlasz T, Tamás A, et al. Degree of damage compensation by various PACAP treatments in monosodium glutamate-induced retinal degeneration. Neurotox Res 2005; 8(3–4):227–33.
- [55] Cheng H, Ding Y, Yu R, et al. Neuroprotection of a novel cyclopeptide C???HSDGIC??? from the cyclization of PACAP (1-5) in cellular and rodent models of retinal ganglion cell apoptosis. PLoS One 2014; 9(10):1–11.
- [56] SEKI T. Neuroprotective Effect of PACAP against Kainic Acid-Induced Neurotoxicity in Rat Retina. Ann N Y Acad Sci 2006; 1070(1):531–34.
- [57] Seki T, Itoh H, Nakamachi T, et al. Suppression of Ganglion Cell Death by PACAP Following Optic Nerve Transection in the Rat. J Mol Neurosci 2008; 36(1–3):57–60.
- [58] Gray SL, Yamaguchi N, Vencová P, et al. Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 2002;

143(10):3946–54.

- [59] Reglodi D, Tamas A, Koppan M, et al. Role of PACAP in Female Fertility and Reproduction at Gonadal Level - Recent Advances. *Front Endocrinol (Lausanne)* 2012; 3:155.
- [60] Reglodi D, Cseh S, Somoskoi B, et al. Disturbed spermatogenic signaling in PACAP deficient mice. *Reproduction* 2017;
- [61] Isaac ER, Sherwood NM. Pituitary adenylate cyclase-activating polypeptide (PACAP) is important for embryo implantation in mice. *Mol Cell Endocrinol* 2008; 280(1–2):13–19.
- [62] Sándor K, Kormos V, Botz B, et al. Impaired nocifensive behaviours and mechanical hyperalgesia, but enhanced thermal allodynia in pituitary adenylate cyclase-activating polypeptide deficient mice. *Neuropeptides* 2010; 44(5):363–71.
- [63] Hashimoto H, Shintani N, Tanaka K, et al. Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proc Natl Acad Sci U S A* 2001; 98(23):13355–60.
- [64] Kawaguchi C, Isojima Y, Shintani N, et al. PACAP-deficient mice exhibit light parameter-dependent abnormalities on nonvisual photoreception and early activity onset. *PLoS One* 2010; 5(2):1–9.
- [65] Kemény Á, Reglodi D, Cseharovszky R, et al. Pituitary adenylate cyclase-activating polypeptide deficiency enhances oxazolone-induced allergic contact dermatitis in mice. *J Mol Neurosci* 2010; 42(3):443–49.
- [66] Matsuyama S, Matsumoto A, Hashimoto H, et al. Impaired long-term potentiation in vivo in the dentate gyrus of pituitary adenylate cyclase-activating polypeptide



- (PACAP) or PACAP type 1 receptor-mutant mice. *Neuroreport* 2003; 14(16):2095–98.
- [67] Nakamachi T, Ohtaki H, Seki T, et al. PACAP suppresses dry eye signs by stimulating tear secretion. *Nat Commun* Nature Publishing Group 2016; 7(May):12034.
- [68] Kurihara T, Ozawa Y, Shinoda K, et al. Neuroprotective Effects of Angiotensin II Type 1 Receptor (AT1R) Blocker, Telmisartan, via Modulating AT1R and AT2R Signaling in Retinal Inflammation. *Investig Ophthalmology Vis Sci* 2006; 47(12):5545.
- [69] Lewis GP, Fisher SK. Up-regulation of glial fibrillary acidic protein in response to retinal injury: its potential role in glial remodeling and a comparison to vimentin expression. *Int Rev Cytol* 2003; 230:263–90.
- [70] Li Q, Puro DG. Diabetes-induced dysfunction of the glutamate transporter in retinal M $\mu$ ller cells. *Investig Ophthalmol Vis Sci* 2002; 43(9):3109–16.
- [71] Ambati J, Chalam K V, Chawla DK, et al. Elevated gamma-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. *Arch Ophthalmol* (Chicago, Ill 1960) 1997; 115(9):1161–66.
- [72] Ishikawa A, Ishiguro S, Tamai M. Changes in GABA metabolism in streptozotocin-induced diabetic rat retinas. *Curr Eye Res* 1996; 15(1):63–71.
- [73] Liu Z, Qiu YH, Li B, et al. Neuroprotection of interleukin-6 against NMDA-induced apoptosis and its signal-transduction mechanisms. *Neurotox Res* 2011; 19(3):484–95.
- [74] Ohtaki H, Nakamachi T, Dohi K, et al. Role of PACAP in ischemic neural death. *J Mol Neurosci* 2008; 36(1–3):16–25.
- [75] Nakatani M, Seki T, Shinohara Y, et al. Pituitary adenylate cyclase-activating peptide (PACAP) stimulates production of interleukin-6 in rat M $\mu$ ller cells. *Peptides* 2006;

27(7):1871–76.

- [76] Reichenstein M, Reich R, Lehoux JG, et al. ACTH induces TIMP-1 expression and inhibits collagenase in adrenal cortex cells. *Mol Cell Endocrinol* 2004; 215(1–2):109–14.
- [77] Nedvig K, Weber G, Nemeth J, et al. Changes of PACAP immunoreactivities and cytokine levels after PACAP-38 containing intestinal preservation and autotransplantation. *J Mol Neurosci* 2012; 48(3):788–94.
- [78] Banki E, Degrell P, Kiss P, et al. Effect of PACAP treatment on kidney morphology and cytokine expression in rat diabetic nephropathy. *Peptides* 2013; 42:125–30.
- [79] Laszlo E, Varga A, Kovacs K, et al. Ischemia/reperfusion-induced Kidney Injury in Heterozygous PACAP-deficient Mice. *Transplant Proc* 2015; 47(7):2210–15.
- [80] Guo M-S, Wu Y-Y, Liang Z-B. Hyaluronic acid increases MMP-2 and MMP-9 expressions in cultured trabecular meshwork cells from patients with primary open-angle glaucoma. *Mol Vis* 2012; 18(May):1175–81.
- [81] Nedvig K, Szabó G, Csukás D, et al. [Examination of cytoprotective and anti-inflammatory effect of PACAP-38 on small bowel autotransplantation]. *Magy Seb* 2013; 66(5):250–55.
- [82] Vollmar B, Menger MD. Intestinal ischemia/reperfusion: microcirculatory pathology and functional consequences. *Langenbeck's Arch Surg* 2011; 396(1):13–29.
- [83] Etienne-Manneville S, Chaverot N, Strosberg AD, et al. ICAM-1-coupled signaling pathways in astrocytes converge to cyclic AMP response element-binding protein phosphorylation and TNF- $\alpha$  secretion. *J Immunol* 1999; 163(2):668–74.
- [84] Limb GA, Chignell AH. Vitreous levels of intercellular adhesion molecule 1 (ICAM-1)

- as a risk indicator of proliferative vitreoretinopathy. *Br J Ophthalmol* 1999; 83(8):953–56.
- [85] Webster L, Stanbury RM, Chignell AH, et al. Vitreous intercellular adhesion molecule 1 in uveitis complicated by retinal detachment. *Br J Ophthalmol* 1998; 82(4):438–43.
- [86] Visser L de, H. de Boer J, T. Rijkers G, et al. Cytokines and Chemokines Involved in Acute Retinal Necrosis. *Investig Ophthalmology Vis Sci* 2017; 58(4):2139.
- [87] Cruz PRS, Lira RPC, Pereira Filho SAC, et al. Increased circulating PEDF and low sICAM-1 are associated with sickle cell retinopathy. *Blood Cells Mol Dis Elsevier Inc.* 2015; 54(1):33–37.
- [88] Reglodi D, Kiss P, Horvath G, et al. Effects of pituitary adenylate cyclase activating polypeptide in the urinary system, with special emphasis on its protective effects in the kidney. *Neuropeptides* 2012; 46(2):61–70.
- [89] Deshmane SL, Kremlev S, Amini S, et al. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *J Interf Cytokine Res* 2009; 29(6):313–26.
- [90] Lu B, Rutledge BJ, Gu L, et al. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 1998; 187(4):601–08.
- [91] Chen S, Bacon KB, Li L, et al. In vivo inhibition of CC and CX3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar-Kyoto (WKY) rats by vMIP-II. *J Exp Med* 1998; 188(1):193–98.
- [92] Murugeswari P, Shukla D, Kim R, et al. Angiogenic potential of vitreous from proliferative diabetic retinopathy and Eales’ disease patients. *PLoS One* 2014; 9(10):1–8.

- [93] Atmaca LS, Batioglu F, Atmaca Sonmez P. A long-term follow-up of Eales' disease. *Ocul Immunol Inflamm* 2002; 10(3):213–21.
- [94] Yoshida S, Yoshida A, Ishibashi T, et al. Role of MCP-1 and MIP-1alpha in retinal neovascularization during postischemic inflammation in a mouse model of retinal neovascularization. *J Leukoc Biol* 2003; 73(1):137–44.
- [95] Barbour M, Allan D, Xu H, et al. IL-33 attenuates the development of experimental autoimmune uveitis. *Eur J Immunol* 2014; 44(11):3320–29.
- [96] Heimesaat MM, Dunay IR, Schulze S, et al. Pituitary adenylate cyclase-activating polypeptide ameliorates experimental acute ileitis and extra-intestinal sequelae. *PLoS One* 2014; 9(9):e108389.
- [97] Nakazawa T, Shimura M, Tomita H, et al. Intrinsic activation of PI3K/Akt signaling pathway and its neuroprotective effect against retinal injury. *Curr Eye Res* 2003; 26(1):55–63.
- [98] Dreixler JC, Hemmert JW, Shenoy SK, et al. The role of Akt/protein kinase B subtypes in retinal ischemic preconditioning. *Exp Eye Res Elsevier Ltd* 2009; 88(3):512–21.
- [99] Wang H, Brown J, Martin M. Glycogen synthase kinase 3: a point of convergence for the host inflammatory response. *Cytokine* 2011; 53(2):130–40.
- [100] Gong JH, Gong JP, Li JZ, et al. Glycogen synthase kinase 3 inhibitor attenuates endotoxin-induced liver injury. *J Surg Res Elsevier Ltd* 2013; 184(2):1035–44.
- [101] Williams DL, Ozment-Skelton T, Li C. Modulation of the Phosphoinositide 3-Kinase Signaling Pathway Alters Host Response To Sepsis, Inflammation, and Ischemia/Reperfusion Injury. *Shock* 2006; 25(5):432–39.
- [102] Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits

- lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J Biol Chem* 2002; 277(35):32124–32.
- [103] Yin H, Tan Y, Wu X, et al. Association between TLR4 and PTEN Involved in LPS-TLR4 Signaling Response. *Biomed Res Int Hindawi Publishing Corporation* 2016; 2016.
- [104] Zhang W, Smith A, Liu J ping, et al. GSK3 $\beta$  modulates PACAP-induced neuritogenesis in PC12 cells by acting downstream of Rap1 in a caveolae-dependent manner. *Cell Signal* 2009; 21(2):237–45.
- [105] Varga B, Szabadfi K, Kiss P, et al. PACAP improves functional outcome in excitotoxic retinal lesion: an electroretinographic study. *J Mol Neurosci* 2011; 43(1):44–50.
- [106] Gurevich L, Slaughter MM. Comparison of the waveforms of the ON bipolar neuron and the b-wave of the electroretinogram. *Vision Res* 1993; 33(17):2431–35.
- [107] Sasaki M, Ozawa Y, Kurihara T, et al. Neuroprotective effect of an antioxidant, lutein, during retinal inflammation. *Investig Ophthalmol Vis Sci* 2009; 50(3):1433–39.

## Figure legends

**FIGURE 1. (A–D)** Light microphotographs showing toluidine blue-stained representative retinal sections (2  $\mu$ m). 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  $\mu$ m)

**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  $\pm$  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 only to 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  $\pm$  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  $\mu$ m)

**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  $\pm$  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  $\pm$  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  $\pm$  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.