ACCELERATED RETINAL AGING IN PACAP KNOCK-OUT MICE

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Abstract—Pituitary adenylate cyclase activating polypeptide (PACAP) is a neurotrophic and neuroprotective peptide. PACAP and its receptors are widely distributed in the retina. A number of reports provided evidence that PACAP is neuroprotective in retinal degenerations. The current study compared retina cell type-specific differences in young (3–4 months) and aged adults (14–16 months), of wild-type (WT) mice and knock-out (KO) mice lacking endogenous PACAP production during the course of aging. Histological, immunocytochemical and Western blot examinations were performed. The staining for standard neurochemical markers (tyrosine hydroxylase for dopaminergic cells, calbindin 28 kDa for horizontal cells, protein kinase Cα for rod bipolar cells) of young adult PACAP KO retinas showed no substantial alterations compared to young adult WT retinas, except for the specific PACAP receptor (PAC1-R) staining. We could not detect PAC1-R immunoreactivity in bipolar and horizontal cells in young adult PACAP KO animals. Some other age-related changes were observed only in the PACAP KO mice only. These alterations included horizontal and rod bipolar cell dendritic sprouting into the photoreceptor layer and decreased ganglion cell number. Also, Müller glial cells showed elevated GFAP expression compared to the aging WT retinas. Furthermore, Western blot analyses revealed significant differences between the phosphorylation state of ERK1/2 and JNK in KO mice, indicating alterations in the MAPK signaling pathway. These results support the conclusion that endogenous PACAP contributes to protection against aging of the nervous system. © 2017 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Key words: pituitary adenylate cyclase activating polypeptide, immunocytochemistry, Western blot, retina neurons, Müller glia.

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) is a widely distributed neuropeptide, first isolated from hypothalamic extracts (Miyata et al., 1989). PACAP belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family, and its receptors are expressed throughout the nervous system and in peripheral organs (Vaudry et al., 2009). In the retina, PACAP immunoreactivity is present in amacrine and horizontal cells, in the inner plexiform layer (IPL), in the ganglion cell layer (GCL), and in the nerve fiber layer (Seki et al., 2000a). There are two types of PACAP receptors: the PAC1 receptor, which binds to PACAP with much higher affinity than to VIP, and VPAC1 and VPAC2 receptors, which bind VIP and PACAP with similar affinities (Laburthe et al., 2007; Vaudry et al., 2009). In the retina, the selective PAC1 receptor is predominant and its mRNA is present in the ganglion cells, amacrine cells and in some other cells of the inner nuclear layer (INL) (Seki et al., 1997, 2000b). The neurotrophic and neuroprotective effects of this peptide are now well established (Waschek, 2002; Shioda et al., 2006; Vaudry et al., 2009; Reglodi et al., 2011, 2015; Seaborn et al., 2011; Szabadfi et al., 2014a; D'Amico et al., 2015). PACAP protects neurons against different toxic agents *in vitro* and provides neuroprotection in several models of brain pathology (Shioda et al., 2006; Botia et al., 2011; Reglodi et al., 2015).

Based on the important neurotrophic effects of PACAP during neuronal development, the involvement of the peptide in endogenous restorative processes was hypothesized (Waschek, 2002). Indeed, several studies have demonstrated that endogenous PACAP level increases upon nervous injury and PACAP-deficient mice respond to insults with more severe deficits and a lower level of regeneration (Zhang et al., 1996; Larsen et al., 1997; Vaudry et al., 2005; Armstrong et al., 2008). These

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[1] Dr. Krisztina Szabadfi has passed away unexpectedly during the course of this study. This paper is dedicated to her memory.
Abbreviations: GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; KO, knock out; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; PACAP, pituitary adenylate cyclase activating polypeptide; PAC1-R, PACAP receptor; PBS, phosphate-buffered saline; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; TBS-T, Tris-buffered saline; VIP, vasoactive intestinal peptide; WT, wild type.

studies support the hypothesis that PACAP plays a role in the natural defense mechanism against nervous injuries and it seems to be a juvenescent factor (Atlasz et al., 2010b; Reglodi et al., 2012).

Detailed information on the roles of PACAP in retinal aging, however, is lacking. During the normal course of aging, the mean lifespan of the parent line of the PACAP knock-out (KO) animals is around 850 days (~28 months). In these old wild-type (WT) animals, the histological appearance of the retina does not change with advanced age. A statistically significant decrease in photoreceptor length was observed in extremely old animals (between 930 and 1000 days (~31–33 months); Trachimowicz et al., 1981). PACAP KO animals were generated on a CD1 background, which lacks pigment granules. None of the PACAP KO animals lived longer than 700 days (23 months). This was an indication that endogenous PACAP may have a pivotal role in anti-aging processes. Aging may be associated with a weakening of the phagocytic properties of the cells, resulting in increased lipofuscin formation (Dylewski et al., 1983).

Subtle age-related changes in the neural retina of rodents have been reported. There is an overall decline in nuclear densities from age of 1 through 27 months. Retinal thickness significantly decreases with age. The retinal ganglion cells (RGC) seem to be more vulnerable to age-related loss than other retinal cells. The intercellular connections between photoreceptors and the number of synaptic connections of the bipolar cells also decrease significantly with age (Cavallotti et al., 2001). The number of retinal capillaries is diminished with age. In the RPE, age-related ultrastructural changes (a substantial accumulation of lipofuscin and an apparent thickening of the basement membrane as well as greatly enlarged pleiomorphic basal infoldings) were described. Also there was a –two- to threefold increase in thickness of the capillary basement membrane (Weisse et al.,

1990). Many of these changes were noted in young adult animals under disease conditions, especially in diabetes (Szabadfi et al., 2014b). Not only synaptic but also vesicular proteins have been found to diminish staining in rodents during retinal aging (Szabadfi et al., 2015).

All the above data indicate that aging affects retinal cells and circuitry and that exogenous and endogenous anti-aging compounds influence the time course of aging. In the present study we examined how the lack of one of those endogenous substances, PACAP influences retinal aging. Our main conclusion is that, in the absence of PACAP, retinal aging is accelerated.

EXPERIMENTAL PROCEDURES

Animals

Generation and maintenance of PACAP KO mice with a CD1 background have been described previously (Hashimoto et al., 2001, 2009). Animals were back-crossed for ten generations with the CD1 strain, all were routinely genotyped and only homozygous knockouts were used in our experiments. Mouse tail samples were used for genotyping with the Phire Animal Tissue Direct PCR Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences for the detection were 5′-ACC GAA AAC AAA TGG CTG TC-3′ (sense) and 5′-GGT CCA CAA AGT ATA TCT GTG CAT TCT-3′ (antisense) for PACAP WT, and 5′-ATC TCC TGT CAT CTC ACC TTG CTC CT-3′ (sense) and 5′-GAA GAA CTC GTC AAG AGA GGC GAT AG-3′ (antisense) for KO mice. The PCR was run at 98 °C for 5 min; followed by 36 cycles of 98 °C 5 s; 61 °C 5 s; 72 °C 20 s; and finally 72 °C 1 min. After agarose gel-electrophoresis gels were stained with Sybr Green I (Thermo Fischer Scientific, Waltham, MA, USA). DNA bands on gel photos were evaluated using confirmed

Table 1. Antibodies used in immunocytochemical and western blot experiments

Methods	Primary antibodies	Company	Raised in	Dilution	Secondary antibodies	Company	Dilution
Immunhistochemistry	Anti-Brn3a	Santa Cruz, Hungary	Mouse	1:50	Alexa Fluor "488"	Thermo-Fisher, Hungary	1:1000
	Anti-calbindin	Sigma-Aldrich, Hungary	Mouse	1:1000			
	Anti-CtBP2	BD Transduction, Germany	Mouse	1:2000			
	Anti-GFAP	Sigma-Aldrich, Hungary	Rabbit	1:500	Alexa Fluor "568"		
	Anti-PAC1R	Sigma-Aldrich, Hungary	Rabbit	1:100			
	Anti-PKCα	Santa Cruz, Hungary	Mouse	1:200	Cy3.5	AbCam, Hungary	
	Anti-vGLUT1	AbCam, Hungary	Rabbit	1:500	Alexa Fluor "568"	Thermo-Fisher, Hungary	
	PNA	Vector Laboratories, USA		1:500			
Western blot	Anti-GAPDH	Sigma-Aldrich, Hungary	Mouse	1:5000	Horseredish peroxidase-conjugated secondary Antibody	BioRad, Hungary	1:3000
	Anti-GFAP	Sigma-Aldrich, Hungary	Rabbit	1:500		BioRad, Hungary	1:3000
	Anti-vGLUT1	AbCam, Hungary	Rabbit	1:500		Invitrogen, Hungary	1:10,000
	Anti-PKCα	Santa Cruz, Hungary	Mouse	1:200		Invitrogen, Hungary	1:10,000
	Anti-pJNK	Cell Signaling, USA	Rabbit	1:1000		BioRad, Hungary	1:3000
	Anti-pERK1					BioRad, Hungary	1:3000
	Anti-PAC1R	Sigma-Aldrich, Hungary	Human	1:1000		Thermo-Fisher, Hungary	1:10,000

controls in the PCR reaction (heterozygous control, no template control).

Animals were fed and watered ad libitum, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-15024/2011). Altogether 36 mice (3–4 and 14–16 months of age) from both sexes were used in this study. Animals were sacrificed with an overdose of anesthetic (120 mg/kg pentobarbital, Nembutal, Sanofi-Phylaxia, Budapest, Hungary) and the eyes were immediately dissected into phosphate buffered saline (PBS).

Histology

Retinas were processed for histological analysis as described previously (Atlasz et al., 2010a). Briefly, eyes (young adult WT, aging WT, young adult PACAP KO and aging PACAP KO, $n = 5$ in each group) were dissected immediately after sacrifice. The cornea was cut away, the lens removed and one of the resulting posterior eyecup pairs was used for histology, the other for immunohistochemistry (as described below). The eyecups intended for histological processing were fixed in 4% paraformaldehyde (PFA) in phosphate buffer (PB) for 2 h, dehydrated in an alcohol series and embedded in Durcupan ACM resin (Merck, Budapest, Hungary). Embedded tissues were cut at 2 μm, stained with 1% toluidine blue (Sigma, Budapest, Hungary), and examined with a Nikon Eclipse 80i microscope. For histological measurements a 40× objective (NA 0.75) was used. The images were captured by the SPOT Basic program. Files were then further processed with Adobe Photoshop 7.0 program. Six tissue blocks from at least three animals were prepared from each group. Central retinal areas within 1–2 mm from the optic disk were used for measurements ($n = 2$–5 measurements from one tissue block). The following parameters were measured: (i) cross-section of the retina from outer limiting membrane to the inner limiting membrane (OLM-ILM); (ii) the width of individual retinal layers; (iii) the number of ganglion cells/100-μm section length in the GCL. Sections in which the INL was composed of more than four rows, or the GCL appeared thicker than a single cell row, were excluded from evaluation.

Immunohistochemistry and peanut-agglutinin (PNA) labeling

The eyecups destined for immunocytochemistry ($n = 5$ in each

group) were fixed in 4% PFA for 20 min (for synaptic proteins) or 1 h at room temperature. Tissues were then washed in PBS and cryoprotected in 20% sucrose (Sigma, Budapest, Hungary) at 4 °C. For cryostat sectioning, retinas were embedded in tissue-freezing medium (Tissue-Tek, OCT Compound, Sakura Finetech, Alphen aan den Rijn, Netherlands), cut radially into 10-μm-thick sections. Primary antibodies (Table 1.) were applied overnight at room temperature followed by 6× washes in PBS. Sections were then incubated for 2 h with the corresponding secondary fluorescent antibodies (Table 1), then cover-slipped using Fluoromount-G (Southern Biotech, Birmingham, USA). For control experiments, primary antibodies were omitted, and cross-reactivity of the non-corresponding secondary antibodies with the primaries was also checked. Photographs were taken in an Olympus Fluorview FV-1000 Laser Confocal Scanning Microscope (Olympus, Japan) and further processed with the Adobe Photoshop 7.0 program. Images were
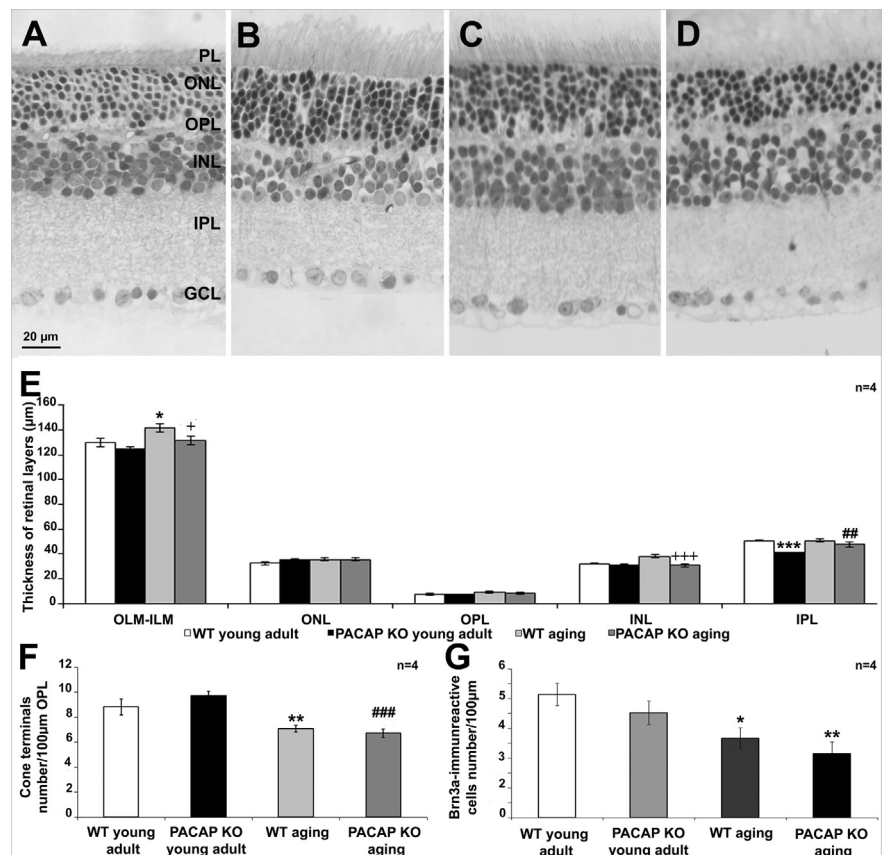

Fig. 1. Comparison of retinal layers and cell numbers. Abbreviation: PL – plexiform layer; OLM – outer limiting membrane; ILM – inner limiting membrane; ONL – outer nuclear layer; OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer. Representative histological sections in young adult WT (A), PACAP KO mice (B), aging WT (C) and aging PACAP KO mice (D). Aged WT and aged KO retinas were slightly thicker than samples from young adult animals. This is reflected strongly in the IPL width (E). A consequently smaller cone terminal density was observed in both WT and KO aging animals compared to young adults (F) while these changes were more prominent in the density of ganglion cells (G) in aging versus young adults. Data are presented as mean ± SEM. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ compared to young adult WT; ###$p < 0.001$ and ##$p < 0.01$ compared to young adult PACAP KO; +++$p < 0.001$ and +$p < 0.05$ compared to aging WT retina.

adjusted for contrast only, aligned, arranged, and labeled using the functions of the above program. Images were evaluated by an examiner blinded to the experimental treatment.

The length and the number of calbindin-immunoreactive and PKCα-processes sprouting into the outer nuclear layer (ONL) were measured. We identified 100 sproutings in each section, from at least two sections in each slide and at least in four identically processed slides. The number of Brn3a-positive RGCs/100 μm retina length was also counted (Xiang et al., 1995; Nadal-Nicolas et al., 2009). Counting was done from at least four identically processed slides (at least two sections in each examined section at least four fields). Results are presented as mean ± SEM. Statistical comparisons were made using Student's t test and one-way ANOVA ($p < 0.05$).

Double immunofluorescence labeling was performed for PKCα and vesicular glutamate transporter 1 (vGLUT1). The antibodies and the protocol used in this study were the same as described above. The two primary antibodies were applied simultaneously, followed by incubation with the corresponding secondary antibodies. As a control, we omitted the primary antibodies and we also ruled out the cross-reactivity of the primary antibodies with the non-corresponding secondary antibodies.

PNA labeling was carried out on cryostat sections. The sections were treated with PNA-fluorescein isothiocyanate conjugate (1:200, 3 h at room temperature), then washed and mounted in Fluoromount. Labeled terminals were counted systematically. From at least four slides processed in parallel, at least two sections were examined. In each examined section at least four fields (each 100 μm long) was imaged. Statistical analysis was done as described above.

Western blot analyses

Separate set of animals were used for Western blot experiments. Retinas ($n = 4$ in each group) were removed as described above. Retinas were homogenized in 150-μl hypotonic lysis buffer (1% NP40, 1% Na-deoxycholate, 0.1% SDS, 15 mM NaCl, 10 mM phosphate buffer, 2 mM EDTA, 2 μg/ml aprotonin, 0.5 μg/ml leupeptin, 2 mM sodium-vanadate, 20 mM sodium-fluoride, 0.5 mM DTT, 1 mM PMSF) with micropestles for 3 min. The lysate was centrifuged at 13,000 rpm for 30 min at 4 °C, and the supernatant was collected. Protein concentration was determined with BCA™ Protein Assay Kit (Thermo-Fisher, Budapest, Hungary). 20–25-μg protein per

sample was loaded on 4–12% NuPAGE SDS–polyacrylamide gels (Thermo-Fisher, Budapest, Hungary), then transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk and 1% BSA containing in Tris-buffered saline (TBS-T; 20 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween-20). The membranes were sequentially probed overnight at 4 °C with primary antibodies (Table 1.). Membranes were washed 6 × 5 min in TBS-T (pH = 7.5) containing 0.2% Tween prior to addition of appropriate secondary antibody. Anti-GAPDH (1:10,000; Sigma, Budapest, Hungary) antibodies were used as loading controls. The binding of the primary antibodies was quantified using horse-radish peroxidase-conjugated secondary antibody at a 1:10,000 dilution (Thermo-Fisher, Budapest, Hungary). The blotted proteins were developed using Western Lightning Chemiluminescence reagent Plus detection system (PerkinElmer, Waltham, MA, USA) and detected on Kodak X-OMAT Blue Autoradiography Film (Sigma, Budapest, Hungary) or by FluorChem Q Imaging system (ProteinSimple, Santa Clara, CA, USA). The chemiluminescence signal was quantified by densitometric analysis in AlphaView Q software. The data were presented by pixel density in arbitrary units ± SEM (one-way ANOVA, Tukey-B posthoc analysis; $p < 0.05$).
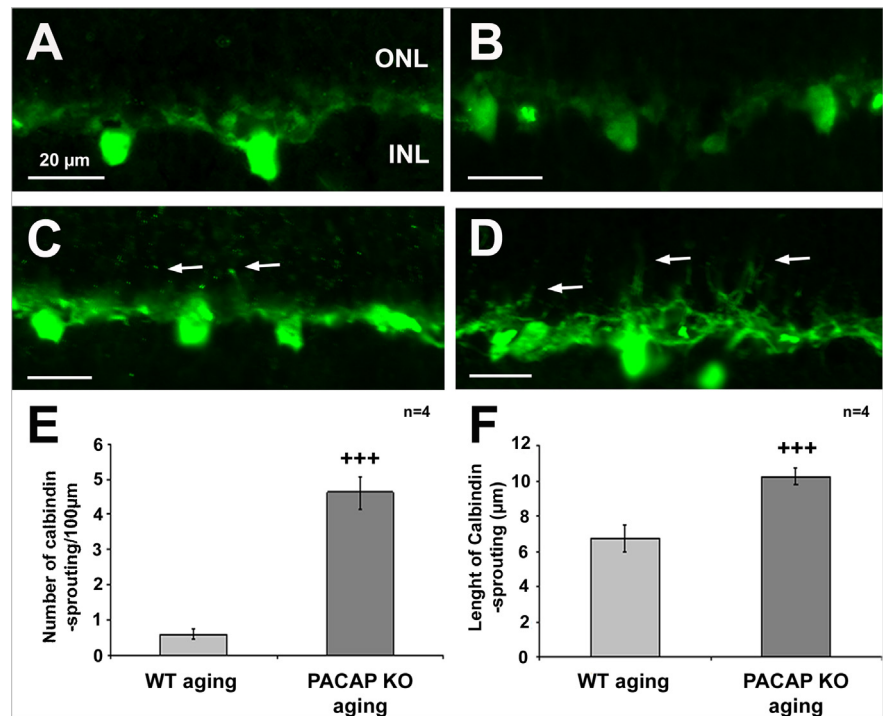

Fig. 2. Horizontal cells. Abbreviation: ONL – outer nuclear layer; INL – inner nuclear layer. Calbindin labeling was observed in the cell bodies and also in the arbor of the horizontal cell dendrites in young adult WT (A), PACAP KO mice (B), aging WT (C) and aging PACAP KO mice (D). Dendrites sprouting into the ONL (arrowheads) were observed in aging WT (C) and PACAP KO mice (D). Scale bar = 20 μm. Comparison of the number (E) and the length (F) of calbindin-positive sprouts into the ONL. [+++]$p < 0.001$ compared to aging WT retina.
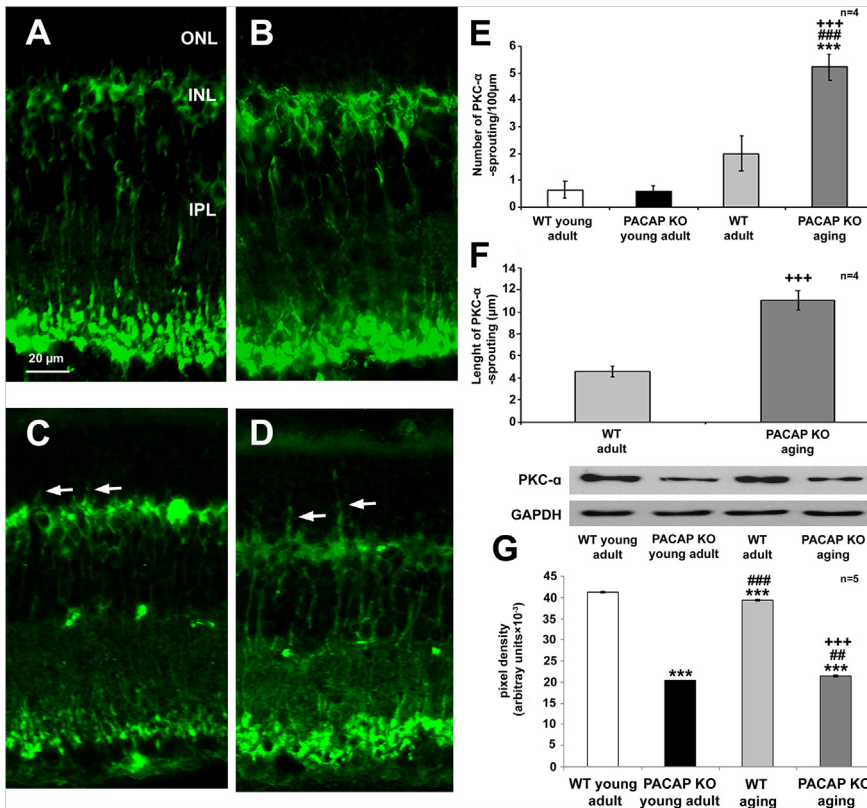
Fig. 3. Rod bipolar cells stained for PKC. Abbreviation: ONL – outer nuclear layer; INL – inner nuclear layer; IPL – inner plexiform layer. PKCα-staining of rod bipolar cells in young adult WT (A), young adult PACAP KO (B), aging WT (C) and aging PACAP KO (D) mice. Sprouting of PKCα-positive dendrites into the ONL (arrows) could be seen in the retina of aging WT (C) and even more prominently in aging PACAP KO (D) mice (arrow). Scale bar = 20 μm. Comparison of the number (E) and the length (F) of PKCα-immunopositive sprouts into the ONL. PKCα levels detected by Western blotting were consistently lower in KO animals than in WT (G). GAPDH served as a normalization control. $^{***}p < 0.001$ compared to young adult WT; $^{###}p < 0.001$ and $^{##}p < 0.01$ compared to young adult PACAP KO; $^{+++}p < 0.001$ compared to aging WT retina.

RESULTS

General morphology and cell numbers

We observed no large-scale differences in the layering between WT and PACAP KO retinas (Fig 1A–D). The OLM-ILM distance was slightly greater in aging retinas than in young adults. The layer thickness of the IPL was thinner in KO animals (Fig. 1E). There was also a slight difference between young adult and aging retinas regarding the density of the cone terminals (labeled with peanut agglutinin; Fig. 1F). The number of RGCs decreased in aging PACAP KO animals compared to young adult WT, but did not differ significantly from aging WT mice (Fig. 1G).

Neurochemical observations

Calbindin-staining was observed in the cell bodies and also in the arbor of the horizontal cells of young adult WT (Fig. 2A) and PACAP KO mice (Fig. 2B). The number and staining intensity of horizontal cells did not show any difference between WT and PACAP KO animals. At the same time, size differences in dendrites sprouting into the ONL were observed between aging

WT (Fig. 2C) and aging PACAP KO retinas (Fig. 2D). That is, both the number (Fig. 2E) and the length (Fig. 2F) of these sprouts were found to be larger in PACAP KO aging mice compared to their respective controls.

The general pattern of PKCα staining of rod bipolar cells did not differ significantly between groups (Fig. 3A–D). However, the appearance of rod bipolar cell dendrites sprouting into the ONL of both WT and KO aging animals (Fig. 3C, D) was observed, but was more prominent in aging KO animals compared to all other conditions (Fig. 3E, F). It is noteworthy that the amount of PKCα protein was significantly lower in KO animals (both the young adult and aging animals; see Fig. 3G), than in WT mice. We further checked if there was any correspondence between differences of PKCα levels and the major transmitter-related transporter, the vesicular glutamate transporter 1 (vGLUT1 – Sherry et al., 2003) in these cells. Our results indicate that vGLUT1 immunoreactivity was always closely apposed to PKCα-positive dendrites in the outer plexiform layer (OPL) and displayed a closer match with PKCα in the IPL of young adult animals (Fig. 4WT: A, E, I; M; N; KO: B, F, J) than in aging specimens (Fig. 4WT: C, G, K; KO: D, H, L). As seen from Western blots, vGLUT protein levels differ significantly between ages (young adult vs. aging) but not between conditions (WT vs. KO) (Fig. 4. P).

Elevated GFAP expression in the Müller glial cells is a stress marker in the retina. Alteration in the GFAP expression pattern could be observed only in the aging PACAP KO group (Fig. 5D), whereas in the remaining groups only the endfeet of Müller cells showed GFAP-immunopositivity, with progressively increasing intensities in the young adult WT, young adult KO and aging WT mice (Fig. 5A–C). These immunocytochemical results were corroborated by Western blotting. The lowest amount of GFAP was found in the young adult WT animal, somewhat stronger staining was seen in the young adult PACAP KO mice, while in the aging groups this level was even higher, with the aging PACAP KO group being the highest (Fig. 5E).

PACAP receptor (PAC1-R) labeling was observed in INL, IPL and GCL in young adult WT (Fig. 6A), PACAP KO mice (Fig. 6B), aging WT (Fig. 6C) and PACAP KO mice (Fig. 6D). Fewer cells expressed PAC1-R in young adult PACAP KO retinas than in WT (Fig. 6A, B). Decreased PAC1-R expression was found in aging
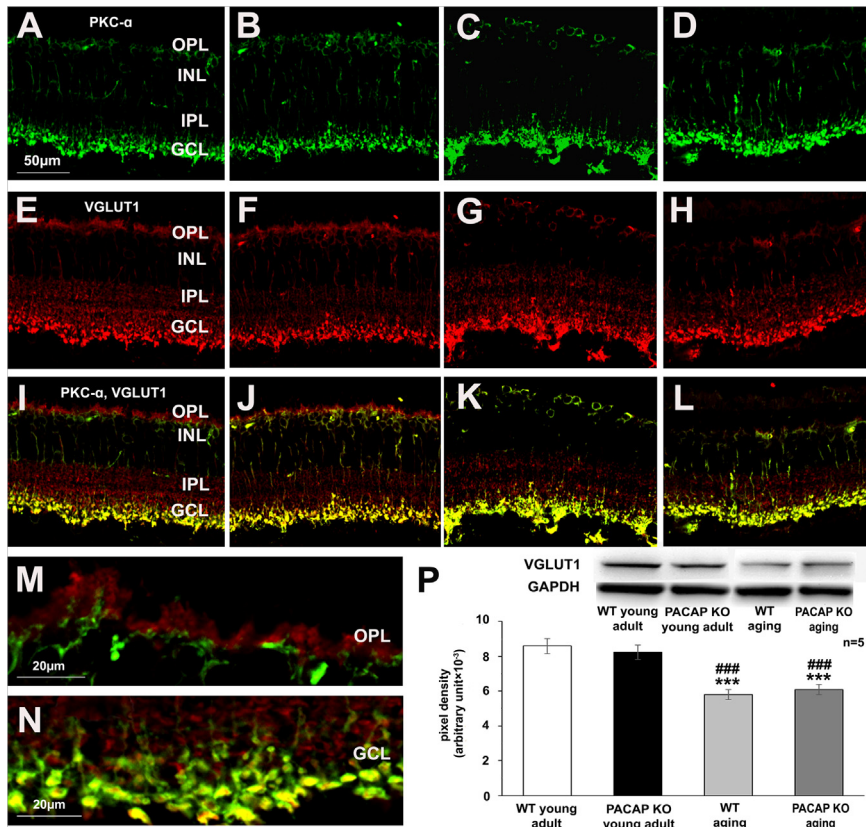
Fig. 4. Double immunofluorescence for PKCα and vGLUT1. Abbreviation: OPL – outer plexiform layer; INL – inner nuclear layer; IPL – inner plexiform layer; GCL - ganglion cell layer. PKCα matched more perfectly with vGLUT1-immunoreactivity in the IPL of young adult animals (WT:A, E, I, M, N, KO: B, F, J) than in aged specimens (WT: C, G, K, KO: D, H, L). vGLUT1 immunoreactivity was always presynaptic to PKCα-positive dendrites in the OPL (M) and colocalized in the rod bipolar terminals in the IPL (N) in young adult WT. Scale bar = 50 μm (A–L), 20 μm (M, N). Immunoblots and relative quantities of vGLUT1 protein expression are presented as mean ± SEM (P). GAPDH served as a normalization control. $^{***}p < 0.001$ compared to young adult WT; $^{###}p < 0.001$ compared to young adult PACAP KO retina.

PACAP KO retinas (Fig. 6D). PAC1-R protein level, to our surprise, was increased significantly in PACAP KO young adult animals compared to young adult WT. In contrast, PAC1-R displayed the same expression in young adult WT and aging WT, but severe down-regulation was detected (Fig. 6E).

When the phosphorylated form of the members of the mitogen activated protein kinases (MAPK) signaling pathway, ERK and JNK, were analyzed we found that two p-JNK isoforms were the highest in aging KO animals, followed by aging WT specimens while decreased in young KO animals (Fig. 7A). Nevertheless, p-ERK1, p-ERK2 levels were to be found the highest in aging WT animals, followed by aging KO mice (Fig. 7B).

DISCUSSION

The generation and characterization of PACAP and VPAC/PACR transgenic mice have proven to be extremely useful in delineating some of their physiological roles (Dickson and Finlayson, 2009). PACAP is implicated as an important pleiotropic and neuroprotective substance in numerous physiological and pathological situations. This is also true for the several different roles played by PACAP in the eye from controlling tear secretion (Nakamachi et al., 2016) to providing protection against experimental diabetic retinopathy (Shioda et al., 2016; Szabadfi et al., 2014a,b, 2015, 2016).

In this study we have tested the combined effect of aging and the lack of PACAP in the retinal tissue. Based on the results presented here, we can state that there are no major differences in the histological structure and expression of different immunohistochemical markers between young adult WT and PACAP KO mice, whereas we noted several differences in aging mice. Aging is associated with a reduced cone terminal number, a process not significantly enhanced in the PACAP-KO mouse. We confirmed KO-specific difference in PKC-alpha level in rod bipolar cells and at the same time PKC-alpha level did not seem to depend on age. In analogy with pathological conditions, we also expected changes in GFAP level in Müller cells and this could be confirmed both by histology and Western blot experiments. A somewhat different situation was found for VGLUT1 immunoreactivity: instead of detecting difference between WT and KO animals we saw alteration with aging. An interesting pattern seemed to emerge regarding PAC1-R, where young adult KO animals showed a higher level of expression than WT animals. This situation turned around with increasing age. At the same time, ganglion cell number decreased with age, and knocking out the PACAP gene enhanced the severity of ganglion cell loss. Functionally, it means that fewer or less strong signals leave the retina through the optic nerve. Indeed, reduced ERG signals were observed in aging retinas, particularly in albino animals (Charng et al., 2011) and retinas with degenerative disorders (Jansson et al., 2015; Pescosolido et al., 2015; Tzekov 2015; Liu et al., 2016; Rösch et al., 2016), but the same has not been reported yet for PACAP KO animals. One possible explanation for the enhanced cell loss could be a more active state of the pro-apoptotic marker pJNK protein and lower level of the anti-apoptotic pERK molecule in aging KO animals than in WT mice. Similar alterations in pro-apoptotic proteins have been observed in retinodegenerative diseases, which could be rescued by PACAP-treatment by the increasing levels of anti-apoptotic markers (Atlasz et al., 2010a; Szabadfi et al., 2014a; D'Amico et al., 2015).

It was also a surprise that PACAP KO condition reduced PKC levels at both ages but was not reflected in cell numbers. In this case we can only infer that signal transduction within individual rod bipolar cells suffers considerable changes. Indeed PKCα-signaling influences GABAA receptor function by phosphorylation (Brandon et al., 2002) by increasing synaptic efficacy. GABAergic inhibition of rod bipolar cell terminals will decrease glutamate release, which in turn will reduce ganglion cell responses and thereby reduce the electrical output of the retina. Both the peak amplitude and temporal properties will be affected (Xiong et al., 2015), which may cause light responses to deteriorate under scotopic conditions.

Lack of PACAP may also act as stress signal in the retina, since we detected increased GFAP levels in the retina. GFAP is accounted as a strong aspecific metabolic stress signal in the retina (Lundkvist et al., 2004; Bringmann et al., 2006; Szabadfi et al., 2014b). Indeed, protection from glial reaction seems to be a key event that protects the retina from damage (Duarte et al., 2015) by mobilizing the sirtuin pathway.

Despite the lack of the ligand in PACAP KO animals, PAC1-R is expressed in PACAP-deficient young adult mice, but the level decreases in aging PACAP KO samples. This process may be an adaptation to the conditions, i.e. in the continuous lack of PACAP the expression of PAC1 receptor is downregulated. To understand the underlying biological processes, gene expression studies and examinations at several time points are needed. Also we shall examine the PAC1-R isoform changes in light of the results we have obtained during early postnatal development (hip isoform is gradually disappearing while hop1 isoforms become dominant between P5-P20: Lakk et al., 2012). PAC1-R containing retinal neuron populations in all four conditions should also be identified. Downregulation may affect certain neuron populations earlier and stronger than others.

Besides neurochemical changes of second- and third-order retinal neurons, their connectivity may also change in the absence of PACAP. Dendrites of rod bipolar cells show sprouting during normal aging (Liets et al., 2006; Terzibasi et al., 2009), but this phenomenon was more prominent in PACAP KO animals. There have also been reports of horizontal cell dendritic sprouting but this was seen only after kainic acid treatment (Peichl and Bolz, 1984). These findings suggest that at least in the case of certain retinal cell types, biological properties and processes characteristics of advanced age in WT animals appear early in young adult life in PACAP KO animals. Besides the PACAP KO model presented in this study several other molecular deficiencies lead to a similar
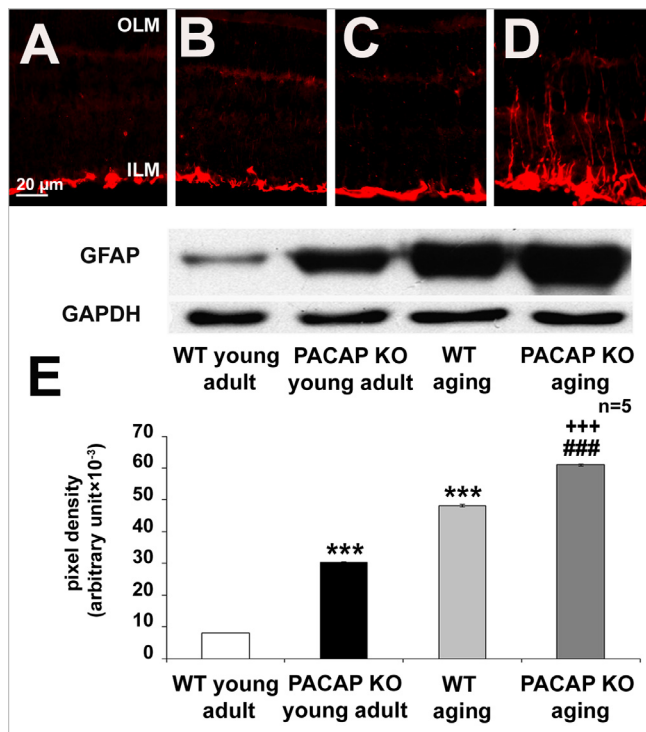

Fig. 5. GFAP immunoreactivity in glial cells. Abbreviation: OLM – outer limiting membrane; ILM – inner limiting membrane. GFAP- immunoreactivity are restricted to the endfeet of Müller cells in young adult WT (A) and young adult PACAP KO mice (B), aging WT (C) and PACAP KO mice (D). Scale bar = 20 μm. Immunoblots and relative quantities of GFAP protein expression are presented as mean ± SEM (E). GAPDH served as a normalization control. $^{***}p < 0.001$ compared to young adult WT; $^{###}p < 0.001$ compared to young adult PACAP KO; $^{+++}p < 0.001$ compared to aging WT retina.
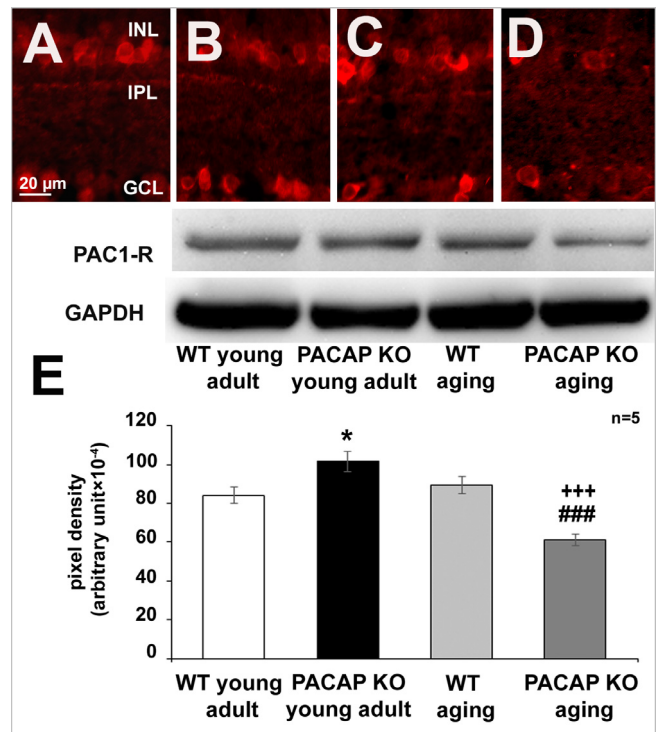

Fig. 6. Immunohistochemical localization of PAC1-R and Western blots showing PAC1-R protein expression. Abbreviation: INL – inner nuclear layer; IPL – inner plexiform layer; GCL – ganglion cell layer. PAC1-R labeling in INL, IPL and GCL in young adult WT (A), PACAP KO mice (B), aging WT (C) and PACAP KO mice (D). Scale bar = 20 μm. PAC1-R protein levels (E). GAPDH served as a normalization control. Data are presented as mean ± SEM. $^{*}p < 0.05$ compared to young adult WT; $^{###}p < 0.001$ compared to young adult PACAP KO; $^{+++}p < 0.001$ compared to aging WT retina.
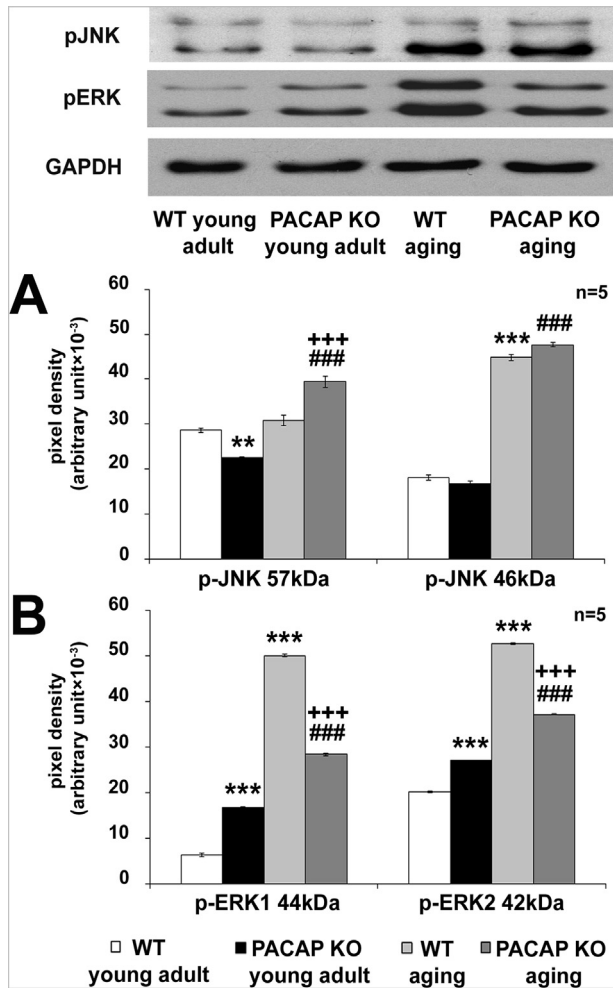
Fig. 7. Protein expression of p-ERK1, p-ERK2 and pJNK isoforms. Analysis of p-ERK1, p-ERK2 and pJNK in young adult WT, young adult PACAP KO, WT aging and PACAP KO aging retina. GAPDH served as a normalization control. Blots and relative quantities (arbitrary unit) are presented of p-JNK in panel A and p-ERK1 and p-ERK2 in panel B. Data are presented as mean ± SEM. $^{***}p < 0.001$ and $^{**}p < 0.01$ compared to young adult WT; $^{###}p < 0.001$ compared to young adult PACAP KO; $^{+++}p < 0.001$ compared to aging WT retina.

phenotypic appearance (e.g. Bassoon KO, Dystroglycan KO: Hoon et al., 2014; nob2: Chang et al., 2006; Down syndrome cell adhesion molecule KO: de Andrade et al., 2014; Crx mutant: Morrow et al., 2005). Ultrastructural studies have documented that marked degenerative changes appear at an earlier age in mutant animals lacking one or more of the above molecules, including in the PACAP-deficient mice studied in the present report, which is more prominently reflected in structural damage of bipolar cell ribbons (Gábriel R., unpublished).

The results presented in this paper altogether support the endogenous protective role of PACAP in maintaining normal neural activity and in counteracting aging processes in the retina, and more generally, in the nervous system. PACAP may be continuously present as a juvenescence factor in the retina and its absence may accelerate aging.

CONTRIBUTIONS OF AUTHORS

Andrea Kovács-Valasek, writing the manuscript, immunocytochemistry and Western blots;
Krisztina Szabadfi, writing the manuscript, immunocytochemistry;
Viktória Dénes, revising the manuscript, Western blots, Bálint Szalontai, genotyping;
Andrea Tamás, Péter Kiss, revising the manuscript, animal husbandry;
Aliz Szabó, revising the manuscript, Western blots,
Gyorgy Setalo Jr, revising the manuscript, confocal microscopy;
Dóra Reglődi and Robert Gábriel, conceptualization, writing and revising the manuscript.

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