The Neuroprotective Peptide PACAP1-38 Contributes to Horizontal Cell Development in Postnatal Rat Retina

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Submitted: September 12, 2018
Accepted: January 18, 2019

Citation: Denes V, Hideg O, Nyisztor Z, et al. The neuroprotective peptide PACAP1-38 contributes to horizontal cell development in postnatal rat retina. *Invest Ophthalmol Vis Sci*. 2019;60:770–778. https://doi.org/10.1167/iovs.18-25719

Purpose. PACAP1-38, a member of the secretin/glucagon superfamily, is expressed in the developing retina with documented neuroprotective effects. However, its function in retinal cell differentiation has yet to be elucidated. Our goals, therefore, were to identify PAC1 expressing cells morphologically, investigate the PACAP1-38 action functionally, and establish PACAP1-38 regulated events developmentally during the first postnatal week in rat retina.

Methods. P1 retinal sections or whole mounts of Wistar rats were used to reveal PAC1 and calbindin immunoreactive structures. P1, P3, or P7 pups were injected intravitreally with 100 pmol PACAP1-38. Tissues were harvested 24 hours post-treatment, then processed for calbindin immunohistochemistry to determine horizontal cell number, or 6, 12, 24 hours post-treatment for real-time PCR and immunoblots to detect PCNA expression. To localize proliferating cells, anti-PCNA antibody was applied.

Results. We showed various PAC1 expressing cells in RPE, NBL, and GCL in P1 retina including calbindin positive horizontal cells. We found that PACAP1-38 induced a marked cell number increase at P3 and P7 and showed upregulated cell proliferation as its mechanism; however, it was ineffective at P1. PACAP1-38 induced proliferative cells localized in the NBL, and double-marker studies demonstrated that the induced proliferative cells were horizontal cells.

Conclusions. PACAP1-38 appears to act in retinal differentiation by inducing mitosis selectively in a time and cell specific manner through PAC1. The control of horizontal cell proliferation raises the novel possibilities that (1) PACAP1-38 may be a major player in retinal patterning and (2) PACAP signaling may be critical in retinoblastoma.

Keywords: PACAP, horizontal cells, proliferation, retina, postnatal development

Pituitary adenylate cyclase-activating polypeptide (PACAP1-38), the highly conserved member of secretin/glucagon/VIP family, was first described as a hypothalamic hormone composed of 38 amino acids.[1] Since its discovery, the peptide has emerged as a pivotal regulator with great impact on various developmental processes such as neurite outgrowth, neurogenesis, migration, and differentiation.[2–4] Disparate effects of PACAP1-38 are mediated via three fundamental receptor types, namely PAC1, VPAC1, and VPAC2.[5] As the result of alternative splicing, 16 isoforms of PAC1 receptor (PAC1-R) have been identified in mammals that have been characterized with various affinities and multiple biases toward downstream signaling pathways. One group of the PAC1-R variants (Null, Hip, Hop1, Hop2, Hiphop1, Hiphop2) differs in its third intracellular loop, due either to the absence or the insertion of two 28 amino acid cassettes; the other group differs in its N-terminal region (PAC1-d5, PAC1-short, PAC1-very short).[6]

Of all the functions, the repressive effect of PACAP1-38 on the apoptotic machinery has been an area of active research that confers a significant neuroprotective potential on this peptide. A remarkable number of studies suggest its importance in the etiology of neurodegenerative disorders.[7–14] PACAP1-38 effect on the adult retina has also been elaborately studied in terms of neuroprotection. PACAP1-38 efficiently protects neurons in ischemia, diabetic retinopathy, glutamate-induced excitotoxicity or UVB-caused cell death, to name a few.[15] In spite of these critical roles in neuroprotection and neural development, studies unveiling the role of PACAP1-38 as an endogenous regulator in retinal development is strikingly limited. The fact that developing human and rat retinas initiate PACAP1-38 synthesis very early (embryonic weeks 13 and 19, respectively) strongly suggests that PACAP1-38 is involved in retinal differentiation.[16,17] Furthermore, we found that in postnatal day 1 (P1), four PAC1-R isoforms (i.e., Null, Hop1, Hip, Hiphop1) are present in rat retina. Each of them displays unique expression pattern from marked increases to baseline downregulation during the first three postnatal weeks (i.e., retina shifts from Hip to Hop1 isoform between P6 and P7).[18,19] In newborn retinas, PACAP1-38 also induce a set of crucial morphogens (Fgf1, Bmp4, Wnt1, Gdf3) with a broad developmental repertoire in newborn retina, supporting its role as a potential secretagouge. Consequently, the diversity of PACAP1-38 actions through its versatile receptors is further enhanced by its interactions with other regulators and their effects on the

retinal microenvironment.[20] With respect to the development of particular retinal cell types, data on the role of PACAP1-38 are very limited. Exogenous PACAP1-38 increased the number of dopaminergic amacrine cells through PAC1-R, whereas PAC1-R overexpressing retina was reported to contain significantly less GABAergic amacrine cells.[21,22] It is also documented that PAC1-R is expressed in ganglion cells, in some amacrine cells, horizontal, and rod bipolar neurons, and Müller glia from P5 to P10 in rats.[19] Functional studies have demonstrated the contribution of PACAP1-38 to cell number establishment—not solely by reducing retinal progenitor proliferation (via cyclin D1 downregulation, thereby reducing proliferation of retinal progenitors[17,23]) but also by exerting pro-apoptotic effect at P1.[24] Further, PAC1 expressing elements of the newborn rat retina are not adequately defined (i.e., progenitor and postmitotic cells) in the critical early phase of postnatal development. The main purposes of the present study are, therefore, (1) to identify PAC1-R immunoreactive cells of P1 retina and (2) investigate the morphological consequences of PACAP1-38 injection in P1, P3, and P7 rat retinas.

Materials and Methods

Animals, Treatments, Tissue Preparation

P1-, P3-, and P7 old albino Wistar rats in equal numbers of males and females were used for this study. Animal handling, housing, and experimental procedures followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the ethical committee of University of Pécs (BA/35/51-58/2016). All efforts were made to minimize pain. Animals were anesthetized by inhalation of Forane (Abbott Laboratories, Budapest, Hungary) prior to treatment or euthanasia. In general, 2.5 µl 0.2 µg/µl N-terminally acetylated PACAP1-38 (100 pmol) (Bio Basic Canada, Inc., Markham, Canada) was injected intravitreally into one eye of the animals; the paired eye was injected with the same volume of 0.9% saline. Eyes were removed and dissected in cold, phosphate-buffered saline (PBS). Upon dissection, tissues were either fixed or frozen on dry ice depending on the downstream application.

PAC1-R and Calbindin Immunohistochemistry on Sections

Experiments were performed on P1 retinas. The eyecups were immersion fixed in 2% phosphate buffered paraformaldehyde (PFA) for 2 hours at room temperature. PFA was removed by washing the eyecups in PBS for 3 × 10 minutes. For cryotomy, tissues were sequentially immersed into 10-20-30% sucrose solution, then embedded in O.C.T. compound mounting media (VWR International, Budapest, Hungary). Eyecups were cut into 10–12 µm thick sections and processed for immunohistochemistry. Using optic discs as reference points, immunohistochemistry was carried out on the central part of both control and treated retinas. For membrane-permeabilization, 0.3% Triton X-100 in PBS was used (Sigma-Aldrich, Budapest, Hungary) for 20 to 30 minutes. To reduce fluorescence due to nonspecific antibody binding, sections were incubated in 5% normal goat serum and 1% bovine serum albumin dissolved in PBS for 30 minutes. Thereafter, sections were incubated in anti-PAC1-R antibody (rabbit, 1:1000) (ThermoScientific, Waltham, MA, USA), mouse or rabbit anti-calbindin (1:1000) (Sigma-Aldrich, Budapest, Hungary, Swant, Marly, Switzerland, respectively) overnight. Incubation with Alexa Fluor 568 conjugated goat anti-rabbit IgG (1:500) and Alexa Fluor 448 conjugated goat anti-mouse IgG (Life Technologies, Grand Island, NY, USA) was carried out for 4 to 6 hours. Sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) by mounting with ProLong Gold antifade reagent (Life Technologies, Budapest, Hungary). Omission of the primary antibodies in both the single- and double-labeling experiments resulted in no staining. Cross-reactivity of the non-corresponding primary and secondary antibodies was not detected. Reactions were carried out on tissues derived at least from three different animals. Samples were examined using an Olympus FV-1000 laser scanning confocal fluorescence microscope.

Calbindin Immunohistochemistry on Whole Mounts

Three experimental groups were created, each composed of three animals. In the first group, pups were treated with 100 pmol PACAP1-38 twice, at P1 and P3, then sacrificed at P5. In the second group, pups were treated a single time at P3, then dissected at P5. The third experimental group was composed of pups treated at P7 with eyes removed at P8. Fixation was performed as described previously. Whole retinas were carefully detached from the sclera and removed. having made four radial incisions, tissues were incubated in dimethyl sulfoxide (VWR International, Budapest, Hungary) for 20 minutes followed by incubation in PBS containing 0.3% Triton X-100 (Sigma-Aldrich, Budapest, Hungary) for 20 minutes to increase the permeability of the tissue. To avoid non-specific binding, sections were incubated in 5% normal goat serum and 1% bovine serum albumin dissolved in PBS for 60 minutes. Thereafter, sections were incubated in anti-calbindin antibody (mouse, 1:1000) (Sigma-Aldrich, Budapest, Hungary) for 48 hours followed by incubation in Alexa 448 conjugated goat anti-mouse IgG (Life Technologies, Grand Island, NY, USA) for 24 hours. Retinas were flat-mounted onto microscope slides and covered.

Calbindin and Proliferating Cell Nuclear Antigen (PCNA) Double Labeling on Sections

PACAP1-38 intravitreal injections were performed at P3 and P7 (time points when upregulation of PCNA was detected). Retinas were removed in 24 hours and processed for immunohistochemistry, as described, except that for PCNA labeling, slides were incubated in ice-cold methanol prior to 0.3% Triton X-100 solution. Sections were incubated in a mixture of anti-calbindin and anti-PCNA antibodies (Cell Signaling Technology, Danvers, MA) diluted to 1:1000 and 1:2000, respectively.

Quantitative Real-Time PCR (Q-PCR) to Assess PCNA Gene Expression

Total RNA was extracted from retinas treated at P1, P3, and P7 then dissected 6, 12, 24 hours post-injection. RNeasy Plus Mini Kit (Qiagen, Germantown, MD, USA) were used following the manufacturer's instructions. Two µg of total RNA was converted into first-strand cDNA using oligo(dT) primer (ThermoScientific, Waltham, MA, USA) and RevertAid H minus Reverse Transcriptase (ThermoScientific). Q-PCR was performed in a 20-µl reaction mixture containing 2-2.5 µl cDNA, forward primer, reverse primer, and SYBR Green PCR Master Mix (ThermoScientific). Along with *PCNA*, the gene of interest, two constitutively expressed genes were amplified as endogenous controls (i.e., RPL13a, lactate dehydrogenase). The following primer pairs were designed, respectively: PCNA: ctcatgaggtcgggtggaagta; tgggttcacaccaagagtcc, RPL13a: cacccctcaccaaacatgccta; cacttgagtggttggttccatca, lactate dehydrogenase: aaagccactccactgtctcc; ggcatctcagaagcgatcgt. Gene

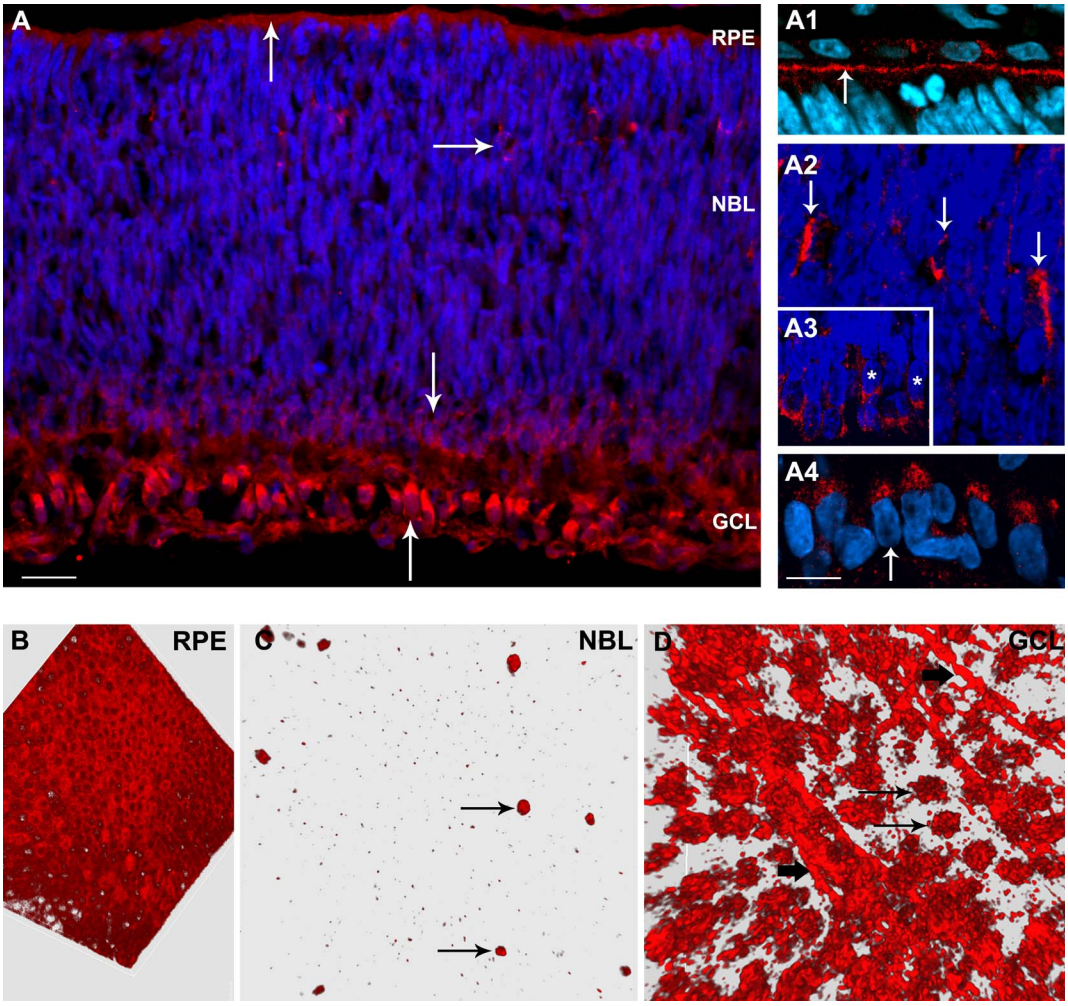

Figure 1. Confocal fluorescence micrographs of PAC1-R expressing elements in P1 retina. (**A**) Immunostaining on P1 cross section reveals PAC1-IR cells in the RPE, outer and inner NBL and GCL (*arrows*). (**A1**) Higher magnification view of the PAC1-IR cells of RPE (*arrow*). (**A2**) PAC1-IR distinct cell populations of the outer NBL (*arrows*). **(A3)** PAC1-IR cell population of the inner NBL adjacent to IPL (*asterisks*). (**A4**) Intensive PAC1 immunoreactivity was observed in ganglion cells (*arrow*). (**B**) 3D reconstruction of PAC1-R immunopositive RPE. (**C**) 3D reconstruction of PAC1-R immunopositive cells of the outer NBL. (**D**) 3D reconstruction of PAC1-R immunopositive ganglion cells (*thin arrows*) and axons (*thick arrows*). *Scale bar*: 25 μm in **A**. *Scale bar*: 15 μm in **A1, A2, A3, A4.**

expressions measured in treated retinas were compared to three control retinas pooled and used as reference. To calculate relative fold changes in mRNA expression levels, the $2^{-\Delta\Delta Ct}$ method was used. Results are shown as mean ± SD of fold changes that were adjusted according to the efficiency of each primer pair.

Western Blots to Assess PCNA Protein Level

To detect changes in PCNA levels, five pups were treated at P1 or P3 or P7 with 2.5 μl 0.2 μg/μl (100 pmol) PACAP1-38. Retinas were harvested in 12 or 24 hours following the PACAP1-38 injection. For protein extraction, tissues were homogenized in 300 μl RIPA buffer (10 mM phosphate buffer pH 7.2, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 mM sodium vanadate, 20 mM sodium fluoride, 0.5 mM DTT, 10 mM PMSF) with micropestles on ice for 5 minutes. Thereafter, the samples were centrifuged at 4°C and the supernatants collected. Protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Sample preparation, buffer preparation, gel electrophoresis and blotting were carried out according to NuPAGE Instruction Manual (Invitrogen, Carlsbad, CA, USA). Approximately 20 to 25 μg protein/sample was loaded and run in 4% to 12% gradient polyacrylamide gel. For PCNA detection, membranes were probed against anti-PCNA (1:2000) (Cell Signaling Technology, Danvers, MA, USA). Proteins of 5 control samples were pooled and used as a reference. To normalize loading, β-tubulin was detected using mouse anti-β-tubulin antibody (1:10,000) (Sigma-Aldrich, Budapest, Hungary). Anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were diluted to 1:10,000. For signal detection, we used WesternBright Chemiluminescence Detection reagent (Advansta, Menlo Park, CA, USA). The chemiluminescent signal was captured and processed by ChemiDoc MP System and Image Lab software, respectively (Bio-Rad Laboratories, Budapest, Hungary).

Confocal Imaging (Z-Stack, Three-Dimensional Reconstruction)

Flat mounts labeled with either PAC1-R or calbindin antibodies were the subject of Z-stacking using an Olympus FV-1000 laser

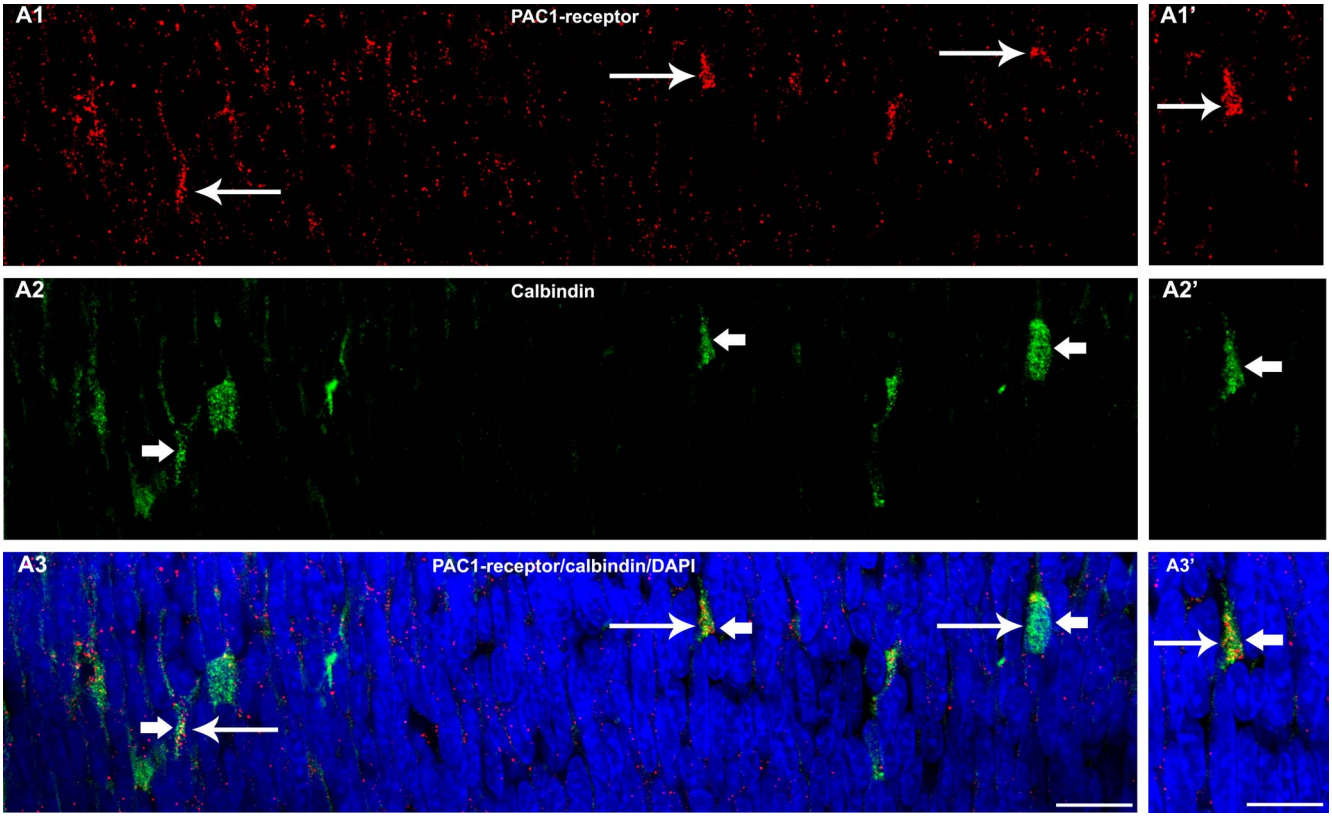

FIGURE 2. Confocal fluorescence micrographs of a P1 retinal section demonstrate that PAC1-R is expressed by CALB-immunoreactive horizontal cells. (**A1**) *Thin arrows* point to PAC1-IR cells in the outer NBL. (**A2**) CALB-IR cells are indicated by *thin arrows*. (**A3**) Both *thin* and *thick arrows* indicates cells co-labeled for PAC1 and calbindin. *Scale bar*: 20 μm

scanning confocal fluorescence microscope. Imagies recorded at different focal planes were taken by incrementally stepping through the flat mounts. Optical sections of the immunoreactive cells were z-stacked to form a composite three-dimensional (3D) image. 3D visualization was obtained using Imaris software (BitplaneAG, Zurich, Switzerland).

Cell Counting

The number of calbindin-IR horizontal cells was analyzed in whole mount preparations. Squares were selected equally from both peripheral and central parts of the retinas. In total, 0.5 mm^2 up to 0.7 mm^2 areas were examined in control as well as in treated whole mounts.

RESULTS

PAC1-R Is Expressed in Distinct Layers of P1 Rat Retina

PAC1-R immunoreactive (PAC1-IR) structures were observed in the retinal pigment epithelium (RPE), the neuroblast layer (NBL), and ganglion cell layer (GCL) in native uninduced P1 retina (Fig. 1A, arrows). The pigment epithelial cells in the RPE are shown at higher magnification in panel A1 demonstrating PAC1-R colocalization with the cell membrane. The continuous PAC1-IR layer is depicted by 3D reconstruction (Fig. 1B). In the neuroblast layer, two cell populations appeared to be PAC1-IR. In the outer region of the NBL, spare cells were seen (A2) that are identical with the round cells shown in Fig. 1C, arrows) while in the inner part, PAC1-IR cells could be identified based on their location as future amacrine cells (A3, asterisks). Robust PAC1 expression was detected in the GCL, where a conspicuous number of PAC1-IR cells were found (A4 and D, arrows). It is noteworthy that not only the cell bodies of ganglion cells showed immunoreactivity for PAC1 but also their axons (Fig. 1D, thick arrows).

Calbindin-IR Horizontal Cells Express PAC1-R in P1 Rat Retina

We could morphologically identify most PAC1 positive cells in the developing retina, except the small round cells in the outer NBL layer. The position and morphology of these PAC1 positive cells were reminiscent of horizontal cells based on our earlier observations that their processes express PAC1 receptors.[19] We used calbindin, a specific marker of horizontal cells,[25] to positively identify the PAC1-IR cells in this layer of NBL. Calbindin/PAC1 double labeling revealed that PAC1 immunoreactivity co-localized with calbindin immunostaining (Fig. 2). All PAC1-IR cells of the outer NBL seemed to be calbindin-IR.

PACAP1-38 Intravitreal Injection Results in a Remarkable Increase in Horizontal Cell Numbers

Immunolabeling results that PAC1-R was expressed by horizontal cells indicated that PACAP1-38 might very well have an effect on horizontal cells. Pursuing our investigation, we injected 100 pmol PACAP1-38 intravitreally in different developmental stages and examined the morphological consequences. First, PACAP1-38 was applied twice, at P1 and P3. To exert any effect, 24 hours were allowed, then retinas were dissected at P5 and processed. PACAP1-38 caused marked increases in cell numbers (Fig. 3A and A'), which varied from

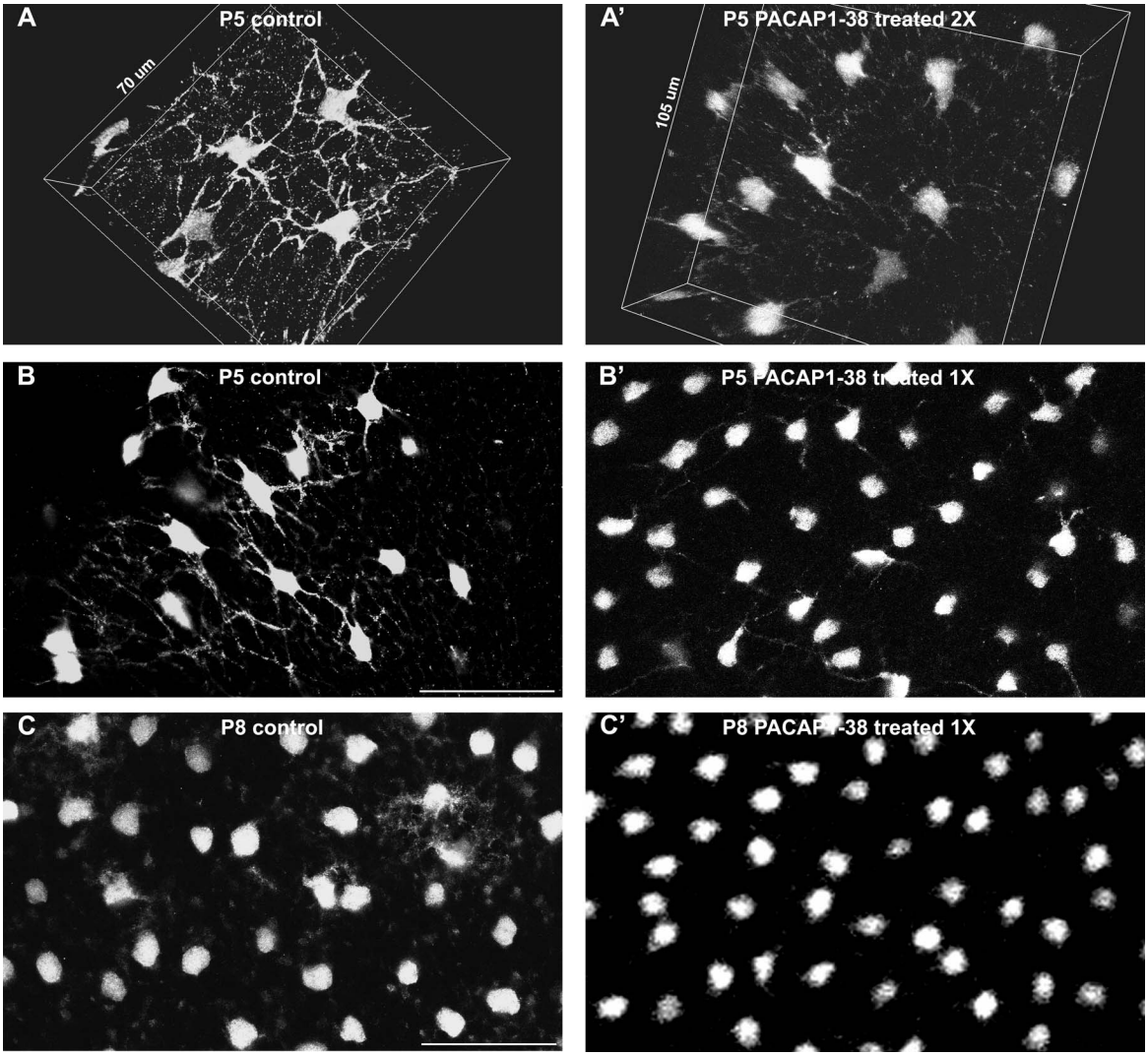

FIGURE 3. Effect of intravitreal injection(s) of PACAP1-38 on horizontal cell number. (**A**, **A'**) 3D view of CALB-IR horizontal cells in P5 control and PACAP1-38 retina injected both at P1 and P3. PACAP1-38 injection appeared to result in cell number elevation. (**B**, **B'**) Number of CALB-IR horizontal cells in P5 retina also seems to be increased following a single PACAP1-38 injection at P3. (**C**, **C'**) CALB-IR horizontal cells in P8 retina are upregulated following PACAP1-38 treatments at P7. *Scale bar*: 50 μm

5% up to 33%. However, Figure 3B and B' demonstrate that the same effect could be obtained by a single injection at P3, which caused 12% up to 30% elevation. Injecting at P7, PACAP1-38 also increased the size of calbindin-IR cell population (Fig. 3C and C') approximately a quarter-fold in two retinas out of three. The results of cell counting are summarized in the Table.

PACAP1-38 Induces Cell Proliferation at P3 and P7 But Not P1

The demonstrated increase in cell numbers could be the result of a variety of mechanisms, but for reasons outlined later in "Discussion," we focused on proliferation. We used a consensus cell proliferation marker, PCNA essential for DNA synthesis and repair. P1, P3, and P7 pups were intravitreally treated with PACAP1-38, with subsequent tissue harvest occurring in 6, 12, and 24 hours. Examination of PCNA message levels revealed no changes at any time points as is demonstrated by the diagrams of Figure 4 (panel A). Further, when PACAP1-38 was administered to P1 retina, no changes in PCNA protein level could be detected. Injection of PACAP1-38 in a later developmental stage, at P3 and P7, resulted in no changes in PCNA protein expression detected at 6 or 12 hours post-injection (not shown). PCNA protein level, however, displayed upregulation at 24 hours post-injection, shown in Figure 4 (panel B), clearly indicating increased cell proliferation.

Horizontal Cells Are PCNA-IR in P3 and P7 Rat Retina

We demonstrated that the cell number increases were the results of induced proliferation by PACAP1-38 treatment at P3 and P7. Next, we wanted to identify the localization of dividing cells in those developmental stages. Accordingly, cells undergoing mitosis were mapped in P4 as well as P8 retinas at 24 hours post-injection using anti-PCNA antibody (Fig. 5). In both P4 control and PACAP1-38 treated retinas, a vast number of PCNA-IR cells counterstained with DAPI could be observed in the outer as well as the middle part of NBL, where a strong immunoreactive seam of dividing cell bodies was seen (A and A'). In respect of PCNA-IR cells of the outer NBL, calbindin (B1) and PCNA (B2) co-labeling revealed that some faint PCNA-positive cells are calbindin-IR; thus, they were classified to be

Table. Effect of PACAP1-38 Injection on Horizontal Cell Density

Horizontal Cells/mm²			Horizontal Cells/mm²			Horizontal Cells/mm²		
P5 Control retina	P5 P1–38 Treated retina (2×)	Relative Increase (%)	P5 Control retina	P5 P1–38 Treated retina (1×)	Relative Increase (%)	P8 Control retina	P8 P1–38 Treated retina (1×)	Relative Increase (%)
1184	1291	8%	1247	1460	17%	1286	1585	23%
1370	1446	5%	1392	1811	30%	1344	1684	25%
979	1301	33%	1267	1425	12%	1404	1356	N/Ch

horizontal cells (B3). At P8, micrograph C and C' showed that the former NBL was divided into three layers (i.e., outer nuclear, outer plexiform, and inner nuclear layers). In both control (C) and PACAP1-38 injected (C') retinas, PCNA immunoreactivity is seen in horizontal cells (arrows in C and C') and cells in the middle part of inner nuclear layer (INL) (asterisks in C and C'). The former ones could be identified due to their position and shape as horizontal cells; meanwhile, the latter ones were identified as Müller cells for their glutamine synthetase immunopositivity (not shown). PCNA-IR cells of the INL are shown at higher magnification in panel C". Interestingly, PCNA immunoreactivity seemed that it was not restricted to the nuclei but it also appears in the processes and cytoplasm of horizontal cells as indicated with arrowheads in B2, C', C".

Discussion

In addition to an outstanding neuroprotective role,[26] PACAP1-38 can also be characterized by versatile developmental functions. Especially, proliferative versus anti-proliferative actions of PACAP1-38 on progenitors have been meticulously studied,[3] as it might implicate another potential protective mechanism mediated by PACAP1-38 in neurodegeneration:

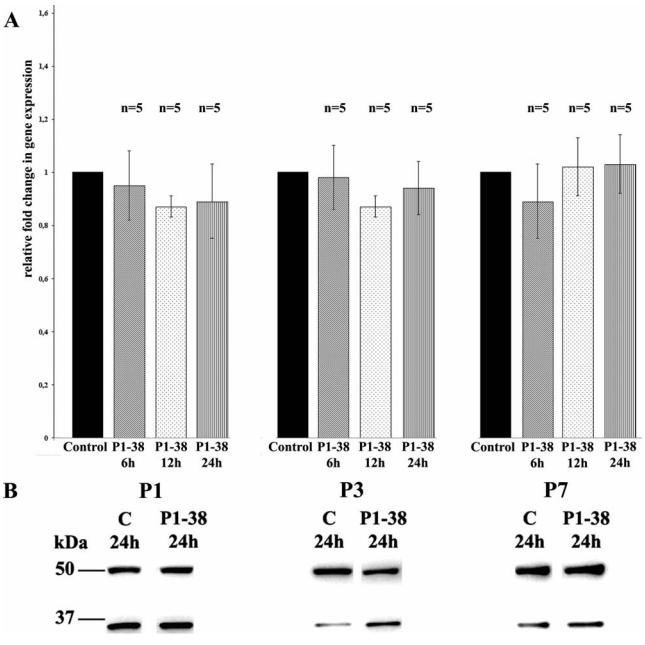

Figure 4. Measurement of PCNA expression in P1, P3 and P7 retinas. (**A**) Gene expression analysis revealed that PACAP1-38 does not control PCNA transcription as message levels do not alter 6-, 12- or 24-hour post-injection in P1, P3, or P7 retinas. (**B**) Amount of PCNA protein was unaltered in P1 retina but increased in P3 and P7 retinas in 24 hours after PACAP1-38 treatment.

induced progenitor-mediated regeneration. A wealth of information has been provided that PACAP1-38 induces stem or progenitor proliferation[27–30] as well as cell cycle arrest[31] or provided evidence that PACAP1-38 is capable of both depending on concentration or type of PAC1-R isoform.[32–34] With this in mind, it is understandable why PACAP1-38 is anti-proliferative in cultured retinal explants,[17,23] whereas the peptide causes proliferation applied intravitreally in the same tissue and developmental stage. With respect of retinal cell types, the number of retinal ganglion cells and dopaminergic amacrine cells are positively regulated by PACAP1-38,[21,29] whereas GABAergic amacrine cells are reduced if PAC1-R is overexpressed.[22] According to our observation, the results we present in this paper strengthen the conclusion of the former as exogenous PACAP1-38 administration increased the number of retinal horizontal cells.

Underlying Mechanism of Horizontal Cell Number Upregulation

Speculatively, the mechanisms of cell number increase may use four methods: (1) inhibition of apoptosis, (2) promotion of cell differentiation, (3) proliferation, or (4) migration. As for the first, the known mechanism of neuroprotection would suggest that attenuation of cell death is the underlying mechanism in horizontal cell gain. Nevertheless, recent study of our laboratory served with an unexpected conclusion that PACAP1-38 either did not affect or even evoked developmental apoptosis during the first postnatal week. Although PACAP1-38 caused a transient decrease in caspase 3/7 level in P3 retina, it was followed by a significant increase.[24] It is interesting to register that the pro-apoptotic action coincides with the PCNA elevation described in the present study. Importantly, however, these two opposite actions take place in different cell populations. Apoptosis is induced by PACAP1-38 in the inner NBL and GCL where post-mitotic cells reside, whereas PCNA immunopositivity were detected in horizontal cells and Müller glia in the middle and outer NBL, for example. The phenomenon that PCNA, a nuclear factor was detected in the horizontal cell processes raises an interesting question to discuss. PCNA is an essential scaffold proteins for DNA polymerase[35]; thus, it has been ubiquitously applied to label proliferating cells. However, interacting with cytoplasmic enzymes, cytoplasmic appearance of PCNA has been reported binding oncoproteins, metabolic, and other factors outside of the nucleus.[36,37] Our result suggests that PACAP1-38 may take other actions, as well as inducing proliferation through upregulation of PCNA expression. The results indicate that attenuation of cell death is probably not involved in PACAP-induced cell number elevations. Alternatively, higher cell numbers can also be caused by promoting cell differentiation or migration. Horizontal cells are one of the earliest cell types that are born during retinogenesis. Coupled with amacrine cells, horizontal cells emerge between embryonic day 11 (E11) and 16.[38,39] Expression of a specific set of transcriptional

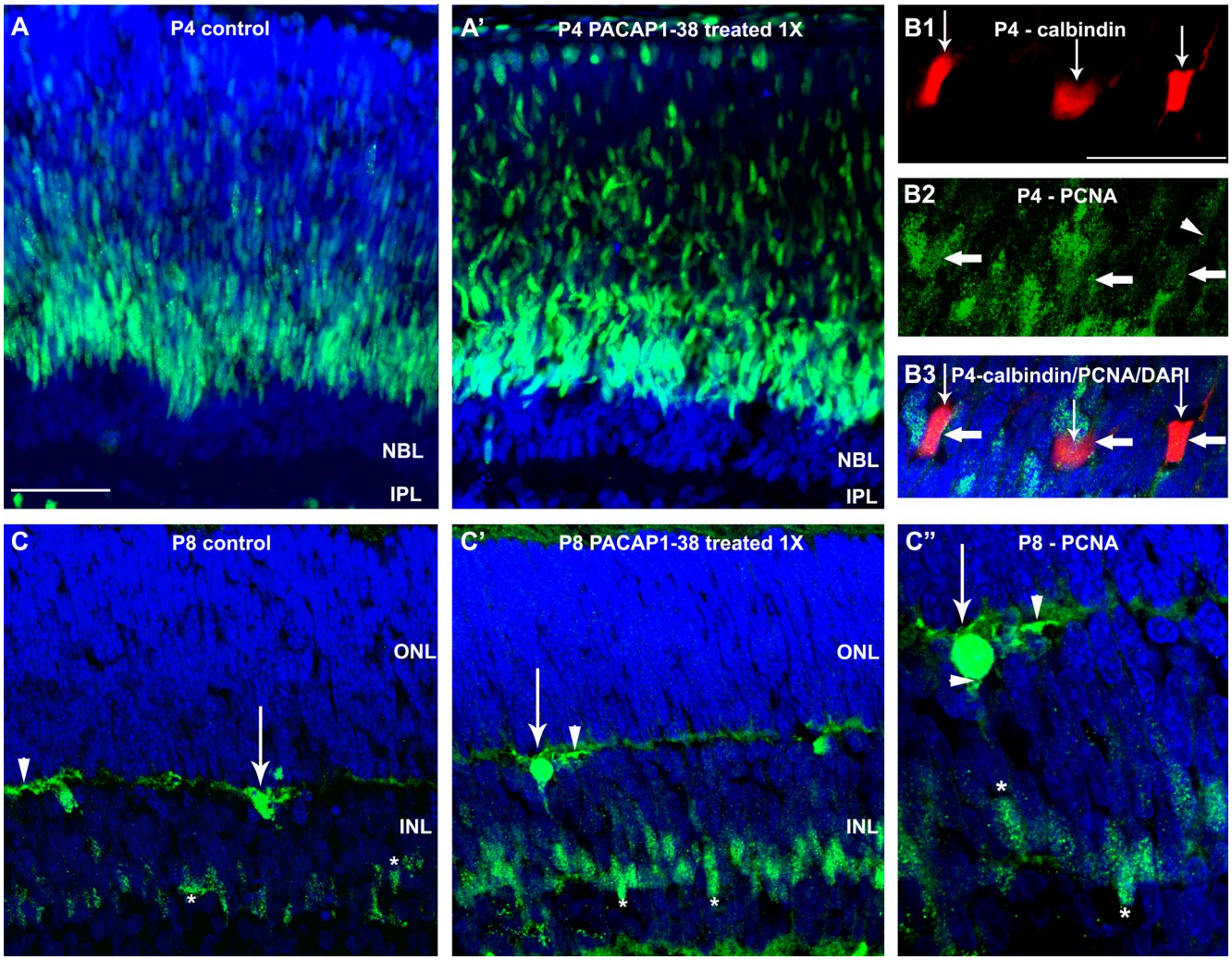

Figure 5. Confocal fluorescence micrographs of P4 and P8 retinal sections labeled for PCNA and calbindin. (**A**, **A'**) Conspicuous number of PCNA-IR cells was detected in the NBL of control as well as PACAP1-38 treated retinas at P4. Nevertheless, more PCNA-IR cells were seen in PACAP1-38 retina. (**B1**–**B3**) Co-localization of calbindin and PCNA proved that horizontal cells were proliferating in P4 retina. (**C**, **C'**) PCNA-IR cells in the INL of P8 control and PACAP1-38 treated retinas. Horizontal cells and Müller glia are indicated by arrows and asterisks, respectively. (**C''**) At higher magnification, a PCNA immunopositive horizontal cell (*arrow*), its processes (*arrowheads*), and PCNA-immunopositive Müller glia cells (*asterisks*) are shown in P8 retina. *Scale bar*: 50 μm in **A** refers also to **A'**; **C**, **C'** *Scale bar*: 30 μm in **B1** refers also to **B2**, **B3**, **C''.**

factors (Foxn4, Ptf1a, Prox1, Lim1) orchestrating their neurogenesis, cell fate commitment, migration, and differentiation follow a strict temporal sequence in rodents (E11 through P5).[40–42] The early postnatal NBL contains progenitors that express Foxn4 and Ptf1a; thus, there was a chance that they could be induced by PACAP1-38. Our preliminary data, however, indicate that Foxn4 and Ptf1a expression is impervious to PACAP1-38 at P1 then downregulates both of them at P3 (unpublished data). Furthermore, the unique bi-directional migration of horizontal cells also gets terminated in the prenatal period as horizontal cells form a monolayer from E18.5 through P0 in their final destination.[42,43] Thus, cell birth or migration are all but eliminated as potential targets for PACAP1-38 to change horizontal cell numbers in P1, P3, or P7 retinas. The above results ruled out all possible mechanisms except proliferation as the only candidate for the observed PACAP1-38 induced changes. Investigation of PCNA expression revealed that mRNA levels and protein levels do not correlate as PCNA message level did not change, yet we detected protein abundance. One can conclude, therefore, that PACAP1-38 did not control the transcription of PCNA but did affect protein expression. The regulatory processes contributing to PCNA upregulation might involve microRNA mechanisms, other translational controls or protein degradation regulation. PACAP1-38 injected twice caused a marked increase in horizontal cell numbers in P5 retinas, raising the question whether it is the number or the timing of the treatments that matters. Since the same stimulatory effect was shown as consequence of single PACAP1-38 injection at P3, in addition, PCNA was not affected by injections at P1 but strongly upregulated at P3, one can conclude that the timing of injections counts and PACAP1-38 affects proliferation in a stage-dependent manner. Similarly, PACAP1-38 induced horizontal cells to proliferate in later stage, at P7, per elevated cell numbers and PCNA level. Mitogenic effect of PACAP1-38 was proved to be mediated via Hop1 isoform in cortical and sympathetic precursors, while the anti-mitogenic action was relayed by the Null isoform.[32,33] Considering that the Hip isoform is completely downregulated from P6 through P8,[19] one can speculate that the proliferative

action of PACAP1-38 is mediated by the Hop1 receptor in the postnatal rat retina as well.

Although horizontal cells become PCNA-negative by E15.5 in murine retina,[42] PCNA immunoreactivity reappears in P1 rat retina and PACAP1-38 could evoke proliferation in them. The explanation must be sought within that rather unique developmental program that horizontal cells follow.[44] One of the developmental characteristics that deviates from the standard is that committed horizontal precursors are able to re-enter cell cycle and, subsequently, divide.[45,46] It seems that PACAP1-38 could be involved in this process, but to elucidate the mechanism, further studies are required.

Horizontal Cell Number Upregulation Is Induced Presumably via PAC1 Receptor

As it was first evidenced in the present study, RPE, horizontal, amacrine, and ganglion cells express PAC1-R; thus, these cell types of the newborn rat retina might well be affected by PACAP1-38 in the early postnatal period. It is interesting to note that in a later period of postnatal development (i.e., P5–P10), the Müller glia also becomes PAC1-R positive.[19] Although other PACAP1-38 receptors, VPAC1, or VPAC2 receptors might also be involved to induce cell division, no information is available on the proliferative effect of VPAC1 or -2 receptors in retinal tissue. Nevertheless, it is well documented that both receptors stimulate growth of cancer cells but exert anti-proliferative effect on smooth muscle cells.[47,48] Furthermore, no data have been reported as to whether horizontal cells express VPAC receptors. Therefore, the question—whether VPAC receptors are also involved in the mediation of the horizontal cell proliferation—definitely prompts further studies.

In conclusion, this study opens an avenue to better understand the neuroprotective effect of PACAP and its roots in retinal development. The morphological and functional studies we present demonstrate a novel interaction between PACAP1-38 and retinal horizontal cells in the early critical postnatal developmental phase. We showed that PACAP1-38 contributes to the normal patterning of the retina by positively regulating horizontal cell numbers. Their responsiveness, however, appears to change over time since PACAP1-38 could induce them at P3 and P7 but not at P1. This instability of horizontal cell precursors is consistent with previous observations that committed horizontal precursors can re-enter the cell cycle, indicating a uniquely non-stringent terminal differentiation program. On one hand, this can be a mechanism to readily resurrect much needed retinal progenitors on short notice in case of retinal damage. This is a potential novel pathway of PACAP-induced neuroprotection, one of the novel conceptual conclusions of our studies. On the other hand, there is compelling evidence that horizontal cells are the cell-of-origin for retinoblastoma, a tumorous fatal childhood disease.[49] This is not unrelated to the non-stringency of its terminal differentiation that can easily transition into cancer stem cells. Our results that PACAP1-38 and its receptor specifically target horizontal cells raises the novel possibility that PACAP signaling is critical in retinoblastoma. This represents another conceptual novelty of our results, a striking new development to search for potential PACAP-based therapeutic modalities in cancer.

Acknowledgments

Supported by the National Research, Development and Innovation Office (KTIA_NAP_20017-1.2.1-NKP-2017-0002 and NKFIH 119 289) and by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the 20765-3/2018/FEKUTSTRAT 5 thematic programme.

Disclosure: **V. Denes**, None; **O. Hideg**, None; **Z. Nyisztor**, None; **M. Lakk**, None; **Z. Godri**, None; **G. Berta**, None; **P. Geck**, None; **R. Gabriel**, None

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