Pituitary Adenylate Cyclase Activating Polypeptide (PACAP1–38) Exerts Both Pro and Anti-Apoptotic Effects on Postnatal Retinal Development in Rat

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Abstract—PACAP1–38, a ubiquitous and multifunctional regulator has been in the focus of neurotoxicity research due to its impressive neuroprotective potential. Although the literature extensively demonstrated its repressive effect on the apoptotic machinery in neurodegenerative models, there is a striking absence of analysis on its role in normal development. We performed quantitative analyses on caspase activity in developing retina upon 100, 50, 25 or 1 pmol intravitreal PACAP1–38 injection from postnatal day 1 (P1) through P7 in Wistar rats. Retinas were harvested at 6, 12, 18, 24 or 48 h post-injection. Apoptotic activity was revealed using fluorescent caspase 3/7 enzyme assay, western blots and TUNEL assay. Unexpectedly, we found that 100 pmol PACAP1–38 increased the activity of caspase 3/7 at P1 and P5 whereas it had no effect at P7. At P3, as a biphasic effect, PACAP1–38 repressed active caspase 3/7 at 18 h post-injection while increased their activity in 24 h post-injection. Amounts, smaller than 100 pmol, could not inhibit apoptosis whereas 50, 25 or 1 pmol PACAP1–38 could evoke significant elevation in caspase 3/7 activity. TUNEL-positive cells appeared in the proximal part of inner nuclear as well as ganglion cell layers in response to PACAP1–38 treatment. The fundamental novelty of these results is that PACAP1–38 induces apoptosis during early postnatal retinogenesis. The dose as well as stage-dependent response suggests that PACAP1–38 has a Janus face in apoptosis regulation. It not only inhibits development-related apoptosis, but as a long-term effect, facilitates it. © 2018 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: PACAP, pro-apoptotic effect, retina, postnatal development.

INTRODUCTION

PACAP1–38 has been proved to be an ubiquitous and pleiotropic regulator involved in various physiological processes in mammals (i.e. secretion of hormones, saliva secretion, glucose and lipid metabolism, blood pressure regulation, circadian rhythm, food intake) (Vaudry et al., 2009; Dickson and Finlayson, 2009). In addition to mature tissues, PACAP1–38 and its receptors are expressed in prenatal as well as early postnatal stages, indicating a disparate array of developmental functions in a wide range of vertebral nervous systems from fish to human (Olianas et al., 1997; Erhardt et al., 2001; Ciarlo et al., 2007; Alexandre et al., 2011). The list of functions includes neurite outgrowth, neurogenesis, migration, differentiation, and last but not least, neuroprotection (Falluel-Morel et al., 2005; Meyer, 2006; Ogata et al., 2015). Evidently, the versatile effects correlate with differential expression of PACAP receptors and their isoforms. To date, PACAP1–38 can signal through three major receptors (PAC1, VPAC1 and VPAC2). Moreover, insertion or deletion of cassettes into the third intracellular loop and/or the N-terminal region of PAC1-R results in 16 isoforms in mammals characterized with various affinities and signaling pathways (Blechman and Levkowitz, 2013). More precisely, intracellular loop variants (Null, Hip, Hop1, Hop2, Hiphop1, Hiphop2) have been identified based on the presence of one or two 28 amino acid cassettes (Dickson and Finlayson, 2009). In rat retina, PACAP1–38 can signal via VPAC1, VPAC1 and VPAC2. Moreover, insertion or deletion of cassettes into the third intracellular loop and/or the N-terminal region of PAC1-R results in 16 isoforms in mammals characterized with various affinities and signaling pathways (Blechman and Levkowitz, 2013).
No doubt, the most noteworthy feature of PACAP1–38 is its significant anti-apoptotic effect. Apoptosis, the highly regulated and evolutionarily conserved program of cellular suicide, plays a key role in elimination of unwanted cells in order to establish proper cell number during histogenesis. In addition to normal development, dysregulated and excess apoptosis contributes to neurodegenerative diseases, thus investigation of apoptotic process has been in the focus of neurotoxicity research (Endres et al., 1998; Daemen et al., 1999; Barber, 2003). The apoptotic machinery is composed of both pro-apoptotic and anti-apoptotic factors, at ratios that determine cellular survival or death. Members of cysteiny1 aspartate specific proteinase (caspase) family create a multiple, cross-linked cascade system that plays the central role in both initiation (i.e. caspase-2, -8, -9, -10, -11 and -12) and execution (i.e. caspase-3, -6 and -7) of apoptosis (Nicholson, 1999; Mattson and Chan, 2003; Deniaud et al., 2008). There are three ways to induce apoptosis. While external cues activate death receptors, internal cues trigger the apoptotic cascade through mitochondria or endoplasmic reticulum (Ashkenazi and Dixit, 1999; Breckenridge et al., 2003; Jeong and Seol, 2008). Either way, executioner caspase-3, the hub of all apoptotic pathways, undergoes cleavage and proceeds the demolition phase of cell death along with caspase-7 and 6 (Slee et al., 2001).

The number of reports proving the repressive effect of PACAP1–38 on the apoptotic machinery in various neurodegenerative models is impressive. It is important to point out, however, that PACAP1–38 has been investigated exclusively when apoptosis had been induced either by an exogenous agent, such as ethanol, nitroprusside, tumor necrosis factor-alpha, ketamine or glutamate (Tamas et al., 2004; Sanchez et al., 2009; Botla et al., 2011; Bian et al., 2017; Mansouri et al., 2017) or by pathological conditions (e.g. diabetes, ischemia, osteoarthritis) (Dohi et al., 2002; Gabriel, 2013; Giunta et al., 2015). A number of studies demonstrated the neuroprotective potential of PACAP1–38 including the retina (Shioda et al., 2016). However, the role of PACAP1–38 in normal apoptosis accompanying retinal development is far from being elaborately investigated. Developmental apoptosis sweeps through the retina in two waves coinciding first with neurogenesis in early prenatal phase then with synaptogenesis in the postnatal phase (Bähr, 2000). To be strictly defined, ganglion cells undergo dramatic loss due to programmed cell death during the first postnatal week of retinal development in rats as well as mice (Cunningham et al., 1981; Perry et al., 1983; Young, 1984). The multi-layered ganglion cell layer (GCL) of newborn rats is dramatically extenuating by the age of postnatal day 8 (P8) and develops into a single-layered structure by P14 (Vrolyk et al., 2018). Therefore, the aim of the present study is to provide data on the role of this peptide in retinal apoptosis during the first postnatal week. We report here for the first time that PACAP1–38 has a stage-dependent and opposing effect on developmental apoptosis. Consistent with its neuroprotective reputation, PACAP1–38 evokes anti-apoptotic effect but only in P3 retinas using high dosage. In the same, earlier or later developmental stages, PACAP1–38 also appeared to facilitate apoptosis in dosage as low as 1 pmol.

EXPERIMENTAL PROCEDURES

Animals, treatments, tissue preparation

Postnatal day 1 (P1)-, three (P3)-, five (P5) and seven (P7)-days old albino Wistar rats, equal number of both males and females were used for this study. Animal handling, housing and experimental procedures were reviewed and approved by the ethics committee of University of Pécs (PTE/43902/2016). All efforts were made to minimize pain. Animals were anesthetized by inhalation using Forane prior to treatment or sacrifice. PACAP1–38 (Bio Basic Canada Inc., Markham, Canada) was injected intravitreally (i.v.) into one eye of the animals meanwhile the paired eye was injected with the same volume of 0.9% saline. To prevent rapid degradation of PACAP1–38, N-terminally acetylated PACAP1–38 was used (Bourgault et al., 2008). Eyes were removed and retinas were dissected in cold, phosphate-buffered saline. Upon dissection, tissues were frozen on dry ice and stored at −80 °C until processed.

Caspase3/7 assays

To monitor the changes in Casp-3/7 enzymatic activity in response to 2.5 µl 0.2 µg/µl (100 pmol) i.v. PACAP1–38 injection, P1, P3, P5 and P7 pups were treated. Retinas were collected at 6, 12, 18, 24 and 48 h following PACAP1–38 treatment. At P3, two further experimental groups were created. In both groups, pups were treated with either 50 or 25 or 1 pmol PACAP1–38 but one group was sacrificed in 18 h post-injection meanwhile the other group was terminated in 24 h post-injection. Tissues were processed according to the instructions of Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) except that tissue lysis was performed in hypotonic lysis buffer (20 mM TRIS–HCl, 10 mM NaCl, 3 mM MgCl₂, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin). Homogenized samples were centrifuged at 4 °C and supernatants were used for the assay. Caspase substrate, Z-DEVD-R110 was added to samples and incubated for 30–90 min. Casp-3/7 activity was reflected in the intensity of fluorescent signal released by Z-DEVD-R110 upon cleavage. Protein concentration of the samples was determined with BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) in order to normalize fluorescence signal to protein concentration and express it as fluorescence/mg protein. Using control samples as references, ratios were calculated and relative change in caspase activity in each experimental group was expressed as mean per centages ± SD. For statistical analysis, first, normality was tested using Shapiro-Wilk test. Then, to identify groups whose means were significantly different from the mean of the reference groups we used independent sample t-test. A value of $p \leq 0.05$ was considered statistically significant.
Western blot

To detect changes in the amount of initiator caspases, retinas were treated at P3 with 2.5 μl 0.2 μg/μl (100 pmol) PACAP1–38 but harvested in 24 h following 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% Nonidet P-40, 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 micropetostes on ice for 5 min. Thereafter, samples were centrifuged at 4°C and the supernatants saved. Protein concentration was determined using BCA™ Protein Assay Kit (Pierce, Rockford, IL). Sample preparation, buffer preparation, gel electrophoresis and blotting were carried out according to NuPAGE Instruction Manual (Invitrogen, Carsbad, CA, USA). Approximately 20–25-μg protein/sample was loaded and run in 4–12% gradient polyacrylamide gel. For activated Casp-9 and 12 detection, membranes were probed against anti-cleaved Casp-9 antibody (Cell Signaling Technology, Danvers, MA, USA) or anti-cleaved Casp-12 antibody (BioVision, Mountain View, CA, USA) diluted to 1:1000. Proteins of 3 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 either on Kodak X-OMAT Blue Autoradiography Film (Sigma-Aldrich, Budapest, Hungary) or by FluorChem Q Imaging system (ProteinSimple, San Jose, CA, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test

TUNEL test was performed on P4 retinas (n = 3) dissected in 24 h post-injection. Eyecups were fixed in 2% buffered paraformaldehyde (PFA) for 2 h at room temperature followed by washing in phosphate buffered saline (PBS). For cryotomy, tissues were immersed into 10–20–30% sucrose solution then embedded in O.C.T. compound mounting media (VWR International, Bridgend, UK). Tissues were cut at 10–12-μm sections and were rehydrated with 100–95–70% ethanol series and permeabilized by Cytoxin solution, a protease free, saponin-based buffer provided by the manufacturer. Sections were covered and incubated in labeling reaction mix (TdT dNTP, Mn²⁺, TdT enzyme and TdT buffer) for 60 min at 37°C in a humidity chamber. Reaction was terminated in TdT Stop buffer then sections were incubated in fluorescein-conjugated streptavidin. For negative control, TdT enzyme was omitted from the reaction mix whereas TACS-nucleate treated slides served as positive control. Samples were examined using an Olympus FV-1000 laser scanning confocal fluorescence microscope (Olympus, Hamburg, Germany). To assess the number of apoptotic cells of NBL and GCL, TUNEL-positive cells were counted in 100 μm² area or along 100 μm length, respectively, in three PACAP1–38 treated retinas.

RESULTS

PACAP1–38 regulates caspase 3/7 activity on a developmental stage-dependent manner

We analyzed the effect of i.v. injected PACAP1–38 on retinal apoptosis on a precise developmental time course covering the first postnatal week from P1 through P7. In our previous study, we proved that 100 pmol PACAP1–38 could affect gene expression as early as 3 h post-injection with a sustained down or upregulation up to 24 h (Lakk et al., 2017). Therefore, we investigated Casp 3/7 activity upon PACAP1–38 treatment at 6, 12, 18, 24, 48 h time points.

When PACAP1–38 was administered to P1 retina, no changes in Casp-3/7 activity could be detected at 6 (n = 5) or 12 h (n = 5) (Fig. 1A). As PACAP1–38 was reported to repress apoptosis (Dejda et al., 2008), a surprising elevation (125.8 ± 18, n = 4, p = 0.048) was observed at 18 h post-injection in response to PACAP1–38 treatment. The effect was only transient, however, as at 24 (n = 5) or 48 h (n = 5) post-injection the same caspase activity was measured in both treated and control retinas.

Injection of PACAP1–38 in a later developmental stage, at P3, resulted in a biphasic effect (Fig. 1B). No changes in Casp-3/7 activity were observed until 18 h post-injection, when a significant drop was detected (49.5 ± 21.3, n = 4, p = 0.006). Thereafter, Casp-3/7 activity showed a significant and transient increase (120 ± 6.7, n = 3, p = 0.014) at 24 h following treatment and it was again normalized at 48 h (n = 5).

When PACAP1–38 was injected into eyes of 5-day-old rats (Fig. 1C), anti-apoptotic effect could not be observed. Nevertheless, a significant elevation of Casp-3/7 activity (131.18 ± 15.75, n = 5, p = 0.002) was measured at 18 h post-injection. In the two remaining time points (i.e., 24 h, 48 h, n = 4, n = 4, respectively), no changes were induced by PACAP1–38.

Finally, PACAP1–38 appeared to have no effect on apoptosis at P7 (Fig. 1D). Measuring Casp3/7 activity in 6–12–18–24–48 h (n = 4 in all groups) upon PACAP1–38 injection, no changes could be detected.

Gradually decreasing concentration does not affect pro-apoptotic but diminishes anti-apoptotic action of PACAP1–38

Two prominent time points were chosen in P3 animals (P3–18 h and P3–24 h) in order to further analyze the effects of the peptide on apoptosis. In Fig. 2A, it is
clearly demonstrated that PACAP1–38 could block apoptosis only in a dosage as high as 100 pmol at 18 h (n = 3). Decreasing amount of PACAP1–38 (i.e. 50, 25, or 1 pmol, n = 4, n = 4, n = 4, respectively) could not exert inhibition on the apoptotic cascade. Interestingly, reduction of PACAP1–38 concentration did not affect its pro-apoptotic action. Fig. 2B shows that 50, 25, or even 1 pmol (n = 3, n = 4, n = 3, respectively) PACAP1–38 could increase significantly the Casp-3/7 activity in P3 retinas at 24 h.

Fig. 1. Temporal dissection of PACAP1–38 effect on Casp3/7 activation. (A) At P1, 100 pmol PACAP1–38 induces a significant change of Casp-3/7 activity in the 18 h time points following injection. (B) At the age of P3, PACAP1–38 shows a biphasic effect by suppressing Casp-3/7 activity at 18 h but inducing it 24 h post-injection. (C) In P5 retina, PACAP1–38 could stimulate Casp-3/7 enzyme activity only at the 18 h time point following treatment. (D) When PACAP1–38 was injected in P7 retina, no effect was observed on Casp-3/7 activation. Asterisks indicate statistically significant changes (p < 0.05).

Fig. 2. Correlation of Casp3/7 activity with decreasing doses of PACAP1–38 in P3 retina. (A) The diagram shows that PACAP1–38 fails to inhibit Casp-3/7 activity at amounts lower than 100 pmol at 18 h following treatment. (B) Injection of lower amount of PACAP1–38 (50, 25, 1 pmol) can lead to elevation of Casp-3/7 function at 24 h following treatment. Asterisks indicate statistically significant changes (p < 0.05).
PACAP1–38 turns on the intrinsic route of apoptosis by activating both Casp-9 and Casp-12

Results obtained from the caspase assays clearly indicated that PACAP1–38 has a pro-apoptotic potential in 24 h upon treatment in P3 retinas. Subsequently, the expression of initiator Casp-9 and Casp-12 were measured. As a consequence of PACAP1–38 injection, both Casp-9 (Fig. 3A) and Casp-12 (Fig. 3B) showed a remarkable increase in 24 h.

PACAP1–38 induces a systematic apoptosis in the developing retina

Once pro-apoptotic action of PACAP1–38 was proven, we investigated the localization of dying cells in the retina. Based on the results of the caspase assay, cells undergoing apoptosis upon PACAP1–38 treatment were mapped in P4 retinas at 24 h post-injection. In P4 control retinas, TUNEL-positive cells could hardly be observed (Fig. 4, panel A) thus for better orientation DAPI was used as a counter staining (Fig. 4, panel A'). PACAP1–38 injection had a dramatic effect on the number of apoptotic cells (Fig. 4, panel B). Fig. 4 panel B' clearly shows that PACAP1–38 evoked apoptosis in the lower portion of the neuroblast layer (NBL, arrows) but predominantly in the ganglion cell layer (GCL, arrows). TUNEL-positive cells of the NBL are shown at higher magnification in panel C and C' whereas panel D–D' provides a closer view of dying cells in the GCL. Cell counting revealed that approximately 3.64 ± 3.9 cells/100 μm² were detected in the proximal NBL meanwhile 15.06 ± 0.94 cells/100 μm showed TUNEL positivity in the GCL following PACAP1–38 injection. In control tissues, there was a negligible number of TUNEL-positive cells in this developmental period. Taking into account that apoptosis is a kinetic process, also, the quick clearance of the dying cells (Galli-Resta and Ensini, 1996), it is crucial to note that the above numbers of apoptotic cells reflect only a momentary physiology.

DISCUSSION

It is unquestionable that PACAP1–38 has earned a remarkable neuroprotective reputation based on its anti-apoptotic action in various degenerative models (Dejda et al., 2008). It is also well-established that PACAP1–38 exerts its powerful anti-apoptotic effect through activation of multiple signaling pathways involving ERK or P13-kinase/Akt or blocking of potassium channels (Bhave and Hoffman, 2004; Mei et al., 2004; Li et al., 2005). It is important to point out, however, that its effects on apoptotic pathways have been examined under pathological conditions when cell death was induced by various stresses (e.g. growth factor withdrawal, ethanol, oxidative stress, etc) (Vaudry et al., 2002; Scharf et al., 2008; Masmoudi-Kouki et al., 2011). Very few papers have intended to provide evidence how PACAP1–38 contributes to regulation of developmental apoptosis. Vaudry et al. (2000) reported that PACAP1–38

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inhibited caspase activity and increased viability of developing cerebellar granule cells in vitro. Furthermore, apoptosis of chick neuroblasts was increased by blocking PAC1 receptors, however, exogenously administered PACAP1–38 appeared to be ineffective (Erhardt and Sherwood, 2004).

Likewise, conspicuous number of reports has been providing data on the retinoprotective functions of PACAP1–38 (Shioda et al., 2016). Nevertheless, the present study is the first, which aimed to investigate in vivo the role of PACAP1–38 in normal apoptosis in the postnatal mammalian retina. Here, we examined on a precise developmental time scale how and when PACAP1–38 affects developmental apoptosis in the rat retina. Surprisingly, we detected anti-apoptotic action only when PACAP1–38 was injected into P3 eyes. In P1, P5 or P7 retinas, PACAP1–38 did not exert anti-apoptotic action. In reflection of that vast amount of data proving anti-apoptotic role of the peptide, it seems that PACAP1–38 does not interfere with normal apoptotic processes but suppresses their activation induced at pathological levels. A similar phenomenon was reported by Hamelink et al. (2002), who demonstrated that although PACAP is evidently a sympathoadrenal co-transmitter, basal adrenalin secretion in PACAP KO mice did not appear to be altered. Nevertheless, animals were vulnerable to prolonged metabolic stresses since adrenaline secretion could not be stimulated in absence of PACAP when it was needed.

Furthermore, anti-apoptotic effect was exerted only by 100 pmol PACAP1–38. Lower amount (i.e. 50, 25, 1 pmol) was truly ineffective, although PACAP1–38 was reported to inhibit ethanol or anisomycin-induced apoptosis at concentration as low as 1 or 0.1 pM, respectively (Silveira et al., 2002; Vaudry et al., 2002). In the present experiments, only a relatively high dosage could evoke anti-apoptotic effect. Numerous pharmacological studies provided evidence that PACAP1–38 has potency to signal via VPAC1 and VPAC2 receptors and exert neuroprotective effects (Dejda et al., 2005). Considering that PACAP1–38 has a much lower potency to stimulate either cAMP or Ca²⁺ response through VPAC1 and VPAC2 receptors (Dickson et al., 2006), it is reasonable to assume that the high dosage of PACAP1–38 could signal via VPAC1 causing a subsequent inhibition of caspase activity. Both the expression of VPAC1 in the postnatal rat retina and its well-established inhibitory effect on apoptosis give the rationale to this assumption (Zupan et al., 2000; Calafat et al., 2009; Lakk et al., 2012; Yu et al., 2017). Interestingly, PACAP1–38 exerted anti-apoptotic action only at P3. Stage-dependent action of any regulators can be explained by either differential expression of their receptors or by the sensitivity of the target cell population. The exclusive anti-apoptotic effect cannot be explained by differential expression of PAC1 or VPAC1 receptors, since they do not display any changes from P0 through P5 (Lakk et al., 2012). It has been indicated by several reports that sensitivity of retinal cells to cell death depends on intracellular pathways activated in distinct stages of differentiation (Chiarini et al., 2003; Campos et al., 2006). Therefore, the matter, why retinal cells are responsive to the anti-apoptotic effect of PACAP1–38 only at P3 definitely prompts further studies.

Not only that anti-apoptotic effect was restricted to a single developmental stage, but the suppression of Casp-3 and 7 was rather a transient action followed by the activation of the executioner caspases. What counts more here is the evidence that PACAP1–38 appears to have a remarkable stage-dependent pro-apoptotic action throughout the first postnatal week. As TUNEL test demonstrated, PACAP1–38 triggers apoptosis in the GCL and the inner part of the NBL where ganglion cells and putative newborn amacrine cells develop, respectively. Vrołyk et al. (2018) investigating the postnatal development of ocular structures in rats, described developmental apoptosis in the GCL between P1 and P8 whereas did not find notable apoptotic activity in the NBL during maturation. Consequently, PACAP1–38 might very well contribute to the normal cell death of ganglion cell population, meanwhile it might act as a selective regulator in NBL eliminating non-preferred cells that are sensitive for such external cues.

Another key thing to discuss is the concentration-dependent manner of PACAP1–38 activities. That is to say, once injected, only 100 pmol PACAP could evoke inhibition of Casp-3 and 7 at P3, lower amount were ineffective, whereas significant apoptotic action could be induced even by 1 pmol PACAP1–38. One can speculate that after longer times, the peptide is subject to dilution or/and the action of tissue peptidases and could not signal through low affinity receptors such as VPAC1. High affinity receptors, namely PAC1, however could still initiate signaling pathway that can result in activation of apoptotic enzymes. Since apoptotic effects were rather delayed in 18 or 24 h post-injection, the other possible explanation of the apoptotic effect is that PACAP1–38 acted indirectly through the induction of another secreted regulator. Our recent work (Lakk et al., 2017) also supports mechanism of the hysteretic way of PACAP1–38. We showed that the development of the newborn retina is orchestrated by interaction between all PACAP receptors and critically important proteins such as members of the fibroblast growth factor and bone morphogenetic protein families (Lakk et al., 2017). The findings suggest that PACAP1–38 can play multiple roles in a variety of developmental processes as a secretagogue, and furthermore, this may also include apoptotic action.

The only developmental stage when PACAP1–38 seemed to be ineffective was the end of the first postnatal week (i.e. P7). A reasonable explanation may relate to the significant expression changes PAC1 receptor isoforms undergo during this period. By dramatically down regulating the Hip isoform, the retina switches to Hop1 receptors. More specifically, we showed that Hip expression declined at P6 and remained down regulated through P10 (Denes et al., 2014). Supporting this scenario of the changing outcome of PACAP1–38 regulation is the observation that PACAP1–38 can act as both a mitogenic and anti-mitogenic factor depending on the differential expression of PAC1 receptor isoforms (Lu et al., 1998; Yan et al., 2013).
All things considered, it seems very likely that multiple PACAP1–38 receptors with different affinities or with different sets of downstream mediator(s) are involved in the opposing effects. The unique, biphasic biology of PACAP1–38 regulation is puzzling and warrants further studies to identify the receptors and signaling pathways mediating the pro-apoptotic effect.

In summary, this is the first report on the role of PACAP1–38 in developmental apoptosis and we provide experimental evidence of the pro-apoptotic function of PACAP1–38 in the mammalian retina. Our results elucidate a new feature of this multifaceted peptide and suggest that PACAP1–38 contributes to the establishment of the proper retinal architecture by controlling ganglion and amacrine cell numbers in postnatal development.

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