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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.

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, VPAC2 and 4 PAC1 receptor isoforms (Null, Hip, Hop1 and Hiphop1), each of them displaying a unique time-curve of expression (Lakk et al., 2012; Denes et al., 2013).

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Abbreviations: PACAP1–38, pituitary adenylate cyclase activating polypeptide; i.v., intravitreal; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; NBL, neuroblast layer; GCL, ganglion cell layer.

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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 cysteinyl 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; Botia et al., 2011; Bian et al., 2017; Mansouri et al., 2017) or by pathological conditions (e.g. diabetes, ischemia, osteoarthritis) (Dohi et al., 2002; Gábriel, 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 $MgCl_2$, 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 percentages ± 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

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