



PACAP and NAP: Effect of Two Functionally Related Peptides in Diabetic Retinopathy

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a peptide involved in physio-pathological processes of the eye. It exerts multiple effects directly through activation of its related receptors and indirectly through increases in the synthesis of activity-dependent neuroprotective protein (ADNP). To study the role of ADNP and protect against ADNP deficiencies, a small peptide called NAP was synthesized. It includes an eight amino acid active site sequence of ADNP. In this review, we summarize the knowledge regarding the neuroprotective function played by PACAP and NAP in retinal tissue and provide an overview of the correlation between PACAP and ADNP in the context of diabetic retinopathy.

Keywords Diabetic retinopathy · PACAP · ADNP · NAP · Hyperglycemia

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide encoded by the ADCYAP1 gene that belongs to the vasoactive intestinal polypeptide (VIP)-secretin-glucagon peptide superfamily (Harmar et al. 1998; Arimura and Shioda 1995). This neuropeptide was first isolated from ovine hypothalamus by Miyata and coworkers in 1989. PACAP and its related receptors are expressed in the central nervous system (CNS) and in most peripheral organs (Ghatei et al. 1993; Arimura and Shioda 1995; Vaudry 2009; Moody et al. 2020; Girard et al. 2020; Toth et al. 2020). This peptide exists in two isoforms: PACAP38, which includes 38 aminoacids, and PACAP27, which is PACAP-38 truncated in C-terminal form and includes 27 aminoacids. It shows a high

sequence homology with vasoactive intestinal polypeptide (VIP) (Miyata et al. 1989; 1990; Segre and Goldring 1993).

PACAP is a pleiotropic peptide, as it is involved in a wide array of physiological processes such as neuromodulation of immune response, neuroprotection, and stimulation of cell proliferation. It also plays a protective effect in several pathologies affecting the CNS and eye, including retinopathies (Canonico et al. 1996; Arimura et al., 1998; Vaudry et al. 2003; 2009; Giunta et al. 2010; Nackamachi et al. 2011, 2012, 2016; Atlasz et al. 2010a and 2010b; Varga et al. 2011; Scuderi et al. 2013; Castorina et al. 2014; Maugeri et al. 2018; 2019a; Kovacs et al. 2020; Martínez-Rojas et al. 2020; Józsa et al. 2001).

PACAP carries out its functions in tissue-specific manner by binding to different G protein-coupled receptors including PAC1 (PAC1-R), VPAC-1, and VPAC-2 receptors (VPAC1-R and VPAC2-R) (Vaudry 2009). The PAC1-R was first isolated in rat pancreatic acinar carcinoma cell line, whereas VPAC receptors were isolated initially in rat lung and olfactory bulb (Hosoya et al. 1993; Morrow et al. 1993; Svoboda et al. 1993; Lutz et al. 1993; Laburthe et al. 2002). The cDNA of the rat VPAC-1 receptor encodes for a protein of 459-amino acid and shows a sequence identity of the 50% to the rat PAC1-R (Pisegna and Wank 1993). PAC1-R receptor binds to PACAP with higher affinity than VPAC1 and 2 receptors (Vaudry 2009; Harmar et al. 2012). PAC1-R exists in diverse splice variants (Null, Hip, Hop1, Hop2, Hiphop1, Hiphop2, short and very short isoforms)

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that differ at the N-terminal domain and/or in the third intracellular loop (Blechman 2013). These isoforms can activate two different signaling cascades: the adenylate cyclase (AC) pathway activating cAMP and phospholipase C (PLC) pathway stimulating protein kinase C (PKC) formation (Lack et al. 2015). By binding to VPAC-1 and VPAC-2 receptors, PACAP induces the activation of AC as well as some other signaling cascades independently by cAMP (Somogyvari-Vigh and Reglodi, 2004; Ohtaki et al., 2008; Shioda et al. 2006; Waschek et al. 2002). Therefore, multiple effects of PACAP depend on the concentration of the peptide and receptor splice variant expressed in the specific tissue and cell (Ashur-Fabian et al. 1997; Pilzer et al. 2006).

PACAP's activity on cell proliferation and differentiation in the CNS is also carried out through the stimulation of an intracellular astrocyte-derived neurotrophic factor known as activity-dependent protein (ADNP) (Bassan et al. 1999; Zamostiano et al. 2001). ADNP is a neuroprotective protein of 123.56 kDa largely distributed throughout the body, including the CNS, where it is involved in brain development and cognition (Gozes 1999; Gozes et al. 2002; Pinhasov et al. 2003). This protein is an essential factor during embryogenesis, as it has been demonstrated that ADNP-knockout mice have impairment in neural tube closure (Pinhasov et al. 2003). It was demonstrated that PACAP increases ADNP level in young astrocytes and in co-cultures of neurons and glial cells (Zusev et al. 2004; Li et al. 2005), suggesting that some intracellular activity of PACAP could be mediated by ADNP-induction. This evidence was corroborated by other studies demonstrating that ADNP is a mediator of some PACAP neuroprotective activity. It was proven that PACAP-38 induced ADNP expression in a bimodal manner depending on its concentration. More specifically, subpicomolar concentration of PACAP stimulates ADNP release via PAC1-R, whereas nanomolar concentration of peptide induces ADNP expression mediated by VPAC1-R (Nakamachi et al., 2006). Furthermore, Nakamachi et al. (2008) revealed that ADNP and PAC1-R co-localized in different areas of mouse brain, suggesting that ADNP expression in neurons and astrocytes is regulated by PACAP. Interestingly, in an *in vivo* mouse model of ADNP deficiency, PACAP provided protection (Sragovich et al. 2019).

To identify the ADNP neuroprotective active site, small peptides were synthesized and a small peptide known as NAP was discovered. This small active element exerts protective activity at femtomolar concentration (Bassan et al. 1999; Pinhasov et al. 2003). The protective action of NAP was demonstrated in many *in vitro* and *in vivo* studies; it prevents apoptotic cell death in neurons exposed to different kinds of stress such as glucose deprivation and β -amyloid or tetrodotoxin treatment, and in a rat model of cerebral ischemia and severe head injury (Bassan et al. 1999; Leker et al. 2002; Beni-Adani et al. 2001; Zaltzman

et al. 2005; Zemlyak et al. 2007; Jehle et al. 2008; Gozes et al. 2008; Belokopytov et al. 2011). Noteworthy, the chemical structure of this small fragment peptide allows it to penetrate into cell membrane and by binding to microtubules it protects astrocytes and neurons (Divinski et al. 2004; Oz et al. 2012; 2014; Ivashko-Pachima et al. 2017; 2018; 2019a and 2019b; 2020; Gozes et al. 2015; 2018; 2019; Grigg et al. 2020). Furthermore, it has been demonstrated that NAP provides significant neuroprotection also in a diabetes rat model (Idan-Feldman et al. 2011) and it has been also showed that NAP regulates PAC1 expression (Kapitansky et al. 2020).

Retinal Expression of PACAP and ADNP

To date, various studies have provided detailed descriptions of the retinal distribution of PACAP and related receptors in different mammalian species and described retinoprotective functions in response to different insults (Onali et al. 1994; Wang et al. 1995; D'Agata and Cavallaro 1998; Cavallaro et al 1996; Silveira et al. 2002; Borba et al. 2005). *In situ* hybridization and immunohistochemical studies have revealed that PAC1-R is strongly expressed in the ganglion cell layer (GCL), inner nuclear layer (INL), and nerve fiber layer (NFL), while a weak expression was found in the inner plexiform layer (IPL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor layer (Seki et al. 1997, 1998, 2000a, 2000b). Furthermore, other papers have described the retinal distribution of PAC1-R splice variants, Null, Hip, Hop1, Hop2, and Hiphop1, as well as VPAC-1 and VPAC-2 receptors (Lakk et al. 2012; Zhang et al. 2005). The presence of PACAP has been found in mammalian, teleost, turtle, and chicken retina using immunohistochemistry (Reglodi et al. 2001). The peptide expression was identified in rat retinohypothalamic tract and amacrine and horizontal cells, the GCL, and the NFL, whereas it was absent in photoreceptor layer and in retinal pigmented epithelium (Seki et al. 1997; 1998; Hannibal 1997 and 2002). At the ultrastructural level, PACAP was detected in the plasma membranes, rough endoplasmic reticulum, and cytoplasmic matrix in retinal INL neurons (Seki et al. 1997; 2000a, b).

In our previous work, we have demonstrated that a significant reduction of mRNA expression of PACAP and its related receptors occurs in diabetic rat retinas (Giunta et al. 2012). Furthermore, we have also proven that ADNP mRNA retinal expression is significantly downregulated in diabetic rats compared with control animals (Scuderi et al. 2014). This confirms that ADNP expression is affected by hyperglycemic condition similarly to PACAP, suggesting that hyperglycemic insult on retinal functions is at least in part linked to impairment of endogenous PACAP and ADNP levels.

PACAP and NAP Retinal Protection

The protective effect of PACAP has been largely demonstrated in different *in vivo* and *in vitro* models of retinopathy, including UVA-induced retinal damage (Atlasz et al. 2011), retinal ischemia (Atlasz et al. 2007; 2010a, b; Seki et al. 2011; Ye et al. 2019), glutamate toxicity (Atlasz et al. 2008; 2009), and diabetic retinopathy (Szabadfi et al. 2016). Moreover, its activity has been also demonstrated during retinal development, as it interferes with retinal progenitor cells proliferation (Njaine et al. 2010). It has also been suggested that PACAP counteracts retinal aging process since PACAP knock-out mice showed an early degeneration of the retina (Kovács-Valasek et al. 2017).

Numerous studies have demonstrated that PACAP exerts its retinoprotective effects in a dose-dependent manner. In fact, this peptide modulates apoptotic cell death occurring after retinal monosodium glutamate lesion (Racz et al. 2006a and 2006b) by inducing a cAMP/PKA signaling cascade at micromolar or nanomolar concentration (Silveira et al. 2002; Racz et al. 2007) or PLC pathway at a picomolar dose (Lakk et al. 2015). The PACAP administration counteracts retinal hypoperfusion after bilateral common carotid occlusion (BCCAO) through modulation of inflammatory cytokines, induction of MAPKs phosphorylation, and concomitant reduction of apoptotic related genes, including JNK and p38 (Szabo et al. 2012). Furthermore, it has been demonstrated that PACAP crosses the ocular barriers and is able to exert neuroprotective effect even given in eye drops in rat chronic retinal ischemia (Werling et al. 2016, 2017). The protective role is also associated to functional improvement, as demonstrated by measuring electrical activity after retinal hypoxia (Danyadi et al. 2014). In this context, by using the PAC1-selective agonist maxadilan, it has been suggested that PACAP's effect is mediated through PAC1 receptor activation (Vaczy et al. 2016). The protective role of PACAP was also demonstrated in a rodent model of retinopathy of prematurity (Kvarik et al. 2016). In this model, the intravitreal injections of PACAP have significantly reduced the oxygen-induced damage in the retinal tissue by increasing vascularized area and counteracting the expression of proinflammatory cytokines. In accord to these evidences, studies conducted by using *in vitro* models of oxidative stress demonstrated that PACAP reduces expression of inflammatory cytokines in human retinal pigment epithelial cells and also counteracts cellular apoptotic death interfering with the balance between pro- and anti-apoptotic genes (Zhang et al. 2005; Mester et al. 2011; Fabian et al. 2012). More recently, Fabian and coworkers (2019) have demonstrated the efficacy of PACAP in counteracting morphological changes occurring in

human retinal pigmented epithelium cells (ARPE-19) exposed to hyper-osmosis and oxidative stress. They also demonstrated that PACAP inhibits VEGF release in these cells. Furthermore, PACAP counteracts glutamate-induced excitotoxicity by reducing glutamate, decreasing proinflammatory factors, and concomitantly normalizing glutathione levels that play an important role as free radical scavengers (D'Alessandro et al. 2014).

Although PACAP exerts numerous effects acting through the signaling pathways mentioned above, its activity is also mediated by induction of neuroprotective molecules from glial cells, including microglia and macrophages, as well as Müller glial cells (Nakatani et al. 2006; Wada et al. 2013; Werling et al. 2016). In a rodent model of ischemia, it has been demonstrated that PACAP counteracts the morphological retinal changes by reducing GFAP expression occurring in Müller cells (Atlasz 2010b), representing the retinal cells activated first following an insult. Thus, the modulatory effect of PACAP on glial cells could underlie to its neuroprotective action. The glial cells in the retina are involved in maintaining the correct retinal microenvironment in neurons and vessels. These cells are primarily responsible for regulation of retinal ions, levels of glutamate, and counteracting retinal impairments due to stress caused by free radicals or hypoxia, by modulating glutathione synthesis. Any retinal damage leads to microenvironmental alterations with consequent hyper-activation of Müller cells. This event leads to increased expression of glial fibrillary acidic protein (GFAP) in Müller cells, which in turn may be accompanied by hypertrophy and cellular proliferation in damaged tissue. Among insults able to induce retinal alterations, hyperglycemia triggers enhanced expression of GFAP in Müller cells in the early phase of diabetes in both humans and animal experimental models (Gabriel et al. 2013, 2019). Therefore, it was suggested that activation of Müller glial cells could represent the first scenario in this pathology. In the context of increased glucose levels, these glial cells activate ion-regulatory machinery and induce the release of different molecules including vascular endothelial growth factor (VEGF), and inflammatory cytokines such as interleukins or tumor necrosis factor, which are responsible for triggering the degeneration process. In this context, it has been demonstrated that PACAP is able to ameliorate pathological Müller glial induction during diabetic retinopathy (Szabadfi et al. 2014, 2016).

Among glial mediators of PACAP' actions, the astrocytes-released neuroprotective protein, ADNP, has also been suggested (Gozes et al. 2003). The smallest active element of this protein is an octapeptide named NAP that has shown to exert beneficial effects in different retinal pathologies. It protects neuroretinal cells from hypoxic damage because it induces neurite growth of retinal ganglion cell (RGC) in retinal explant of rat pups and counteracts RGC injuries

following optic nerve crush and retinal ischemia (Jehle et al. 2008). Furthermore, Zheng et al. (2010) have demonstrated that NAP transfection into Müller cells protects them and surrounding retinal neurons from hypoxia. Intravitreal administration of NAP is also efficacious in counteracting laser-induced retinal damage (Lagrèze et al. 2005; Belokopytov et al. 2011).

Effect of PACAP and NAP in Diabetic Retinopathy

Among ocular pathological conditions, diabetic retinopathy (DR) is the most common disease affecting patients with Type 1 or Type 2 diabetes (Yau et al. 2012; Gabriel et al. 2019). It represents a microvascular complication of diabetes leading to blindness. The retinal impairments during diabetic retinopathy can be ascribable to metabolic changes caused by hyperglycemia leading to microvascular alteration, retinal hypoxia, inflammation, impairments of retinal architecture, and consequent general tissue dysfunction. The micro environmental changes that followed DR lead to thinning of retinal layers, loss of GCL, a decreased number of amacrine cells, and rods and cones (Holopigian et al. 1997; Gastinger et al. 2006). It also leads to activation of Müller cells and astrocytes at the onset of diabetes (Zeng et al. 2000, 2008; Puro et al. 2002). It was also demonstrated that rat retinal Müller cells are impaired by hypoxic insult causing neuroretinal dysfunction (Bringmann et al. 2001; Puro 2002). This scenario is additionally characterized by apoptosis of neuronal cells, synapse loss among retinal layers, and significant loss of ganglion cells that occurs at the early stage of diabetes (Szabadfi et al. 2016).

It has been proven that alterations characterizing DR progression are attenuated or counteracted by treatment with peptides, including PACAP and NAP. As summarized in Table 1, the neuroprotective effect of these peptides has been

widely demonstrated in several studies. For instance, the intravitreal injection of PACAP has been shown to play a significant ameliorative effect against retinal degenerations in streptozotocin-induced diabetic rats. More specifically, PACAP preserves cone photoreceptors damages, counteracts the reduction of ganglion and dopaminergic amacrine cells (Szabadfi et al. 2012), and it is able to rescue neurons from apoptotic death (Szabadfi et al. 2014).

In line with this evidence, our research group has demonstrated that PACAP plays a neuroprotective activity in the retina during the early phase of DR. In fact, PACAP and its related receptors are downregulated after three weeks of hyperglycemia, suggesting their involvement in retinal dysfunction during diabetes. In this model, a single injection of PACAP provided neuroprotection in the diabetic retina by inducing modulation of apoptotic genes expression such as p53 and Bcl2 levels (Giunta et al. 2012).

To deepen our knowledge on the molecular mechanisms underlying PACAP's effect in this pathology, we investigated whether its activity was directly linked ADNP induction in retinal tissue. We tested the effect of ADNP's smallest active element, NAP, in a model in vivo of rat DR. We demonstrated that both NAP and PACAP are able to improve retinal morphology, counteract its thinning, and reduce loss of GCL at early phase of hyperglycemia (D'Amico et al. 2017a). We have also shown that a single dose of NAP was able to decrease the rate of apoptotic cell death by activating the MAPK/ERK pathway in diabetic retina (Scuderi et al. 2014). Furthermore, both NAP and PACAP interfere with hypoxia signaling pathway occurring 3 weeks from the beginning of hyperglycemia. In particular, both peptides modulate the expression of some hypoxic inducible factors (HIFs). The latter are heterodimeric transcription factors representing the first biological response to cellular hypoxia. Among these, HIF-1 α and HIF-2 α under hypoxia elude the proteasome degradation system, translocate into the nucleus and activate many target genes. These genes include VEGF, the main

Table 1 Summary of knowledge regard PACAP and NAP effect in diabetic retinopathy

Key findings	Reference
Treatment of PACAP prevents cone photoreceptor damage and reduction of ganglion and dopaminergic amacrine cells	Szabadfi et al. (2012)
PACAP provides neuroprotection in diabetic retina by inducing modulation of apoptotic genes, reducing p53 and increasing Bcl2 levels	Giunta et al. (2012)
PACAP counteracts neural apoptotic death, by inducing expression of antiapoptotic p-Akt, p-ERK1-2, PKC, and Bcl-2	Szabadfi et al. (2014)
NAP treatment reduces apoptotic retinal cell death and improves cell survival by inducing MAPK/ERK	Scuderi et al. (2014)
PACAP interferes with hypoxia inducible factors (HIFs)	D'Amico et al. (2015)
PACAP treatment saves ribbon synapses and pigment epithelial cell morphology	Szabadfi et al. (2016)
PACAP treatment reduces expression levels of IL-1 β , VEGF, and VEGFRs	D'Amico et al. (2017a)
NAP treatment modulates Hypoxia signaling cascade by reducing expression of HIF-1 α , HIF-2 α , and VEGF	D'Amico (2017b)
PACAP treatment induces EGFR phosphorylation via PKA-signaling cascade activation	Maugeri et al. (2019a, b)

factor responsible to microvasculature system dysfunction. In diabetic rat retinas, HIF-1 α and HIF-2 α are aberrantly expressed since they are upregulated in different retinal layers including INL and ONL. PACAP and NAP administration significantly downregulates HIF-1 α and HIF-2 α expression and, at the same time, enhances HIF-3 α levels in diabetic retina (D'Amico et al. 2015, 2017b; Maugeri et al. 2017a). This finding was relevant because HIF-3 α is well-known as a negative regulator of the other two hypoxic inducible factors and inhibits their activity. Noteworthy, PACAP and NAP also significantly reduced VEGF levels in diabetes-affected retina and abrogated its expression in the GCL, including ganglionic cells, the axons of which

form the optic nerve (D'Amico et al. 2017a and b). Therefore, these two peptides represent two factors able to act as upstream regulators of VEGF expression. This data is in agreement with the ameliorative effect played by PACAP in counteract vascular dysfunction hyperglycemia-induced (Solyman et al. 2018).

This is relevant evidence because anti-VEGF-therapy represents an elective tool in DR treatment, although it is efficacious only in 50% of treated-patients. The identification of molecules able to modulate the expression of this angiogenic growth factor could represent a new therapeutic strategy to prevent angiogenesis in non-responders. In our studies, we have demonstrated that PACAP and NAP are

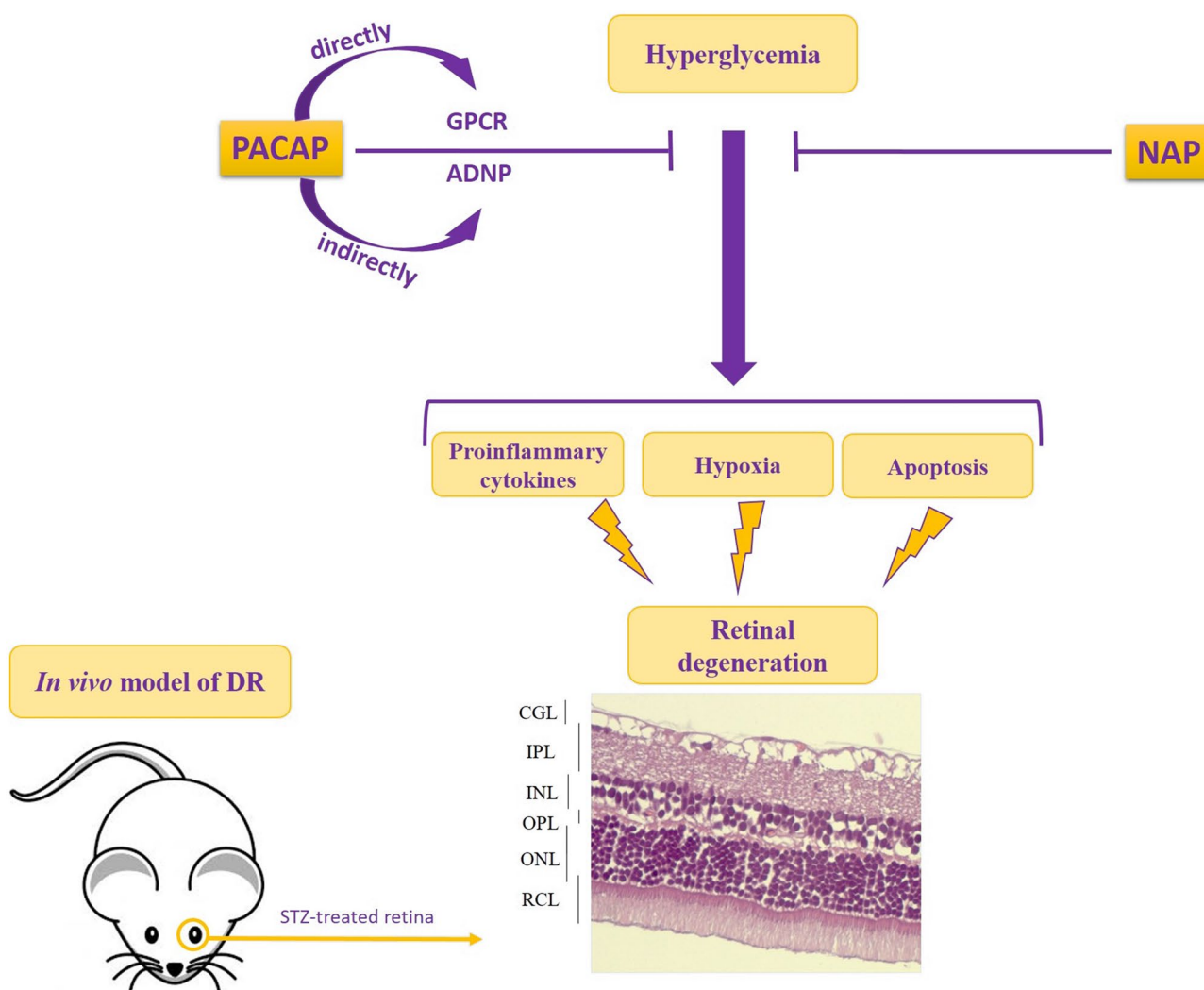


Fig. 1 Graphical representation of PACAP and NAP effect in hyperglycemic retina. Hyperglycemia induces retinal damage during diabetes, by promoting tissue inflammation, hypoxia, and apoptosis. This event leads to alterations of retinal architecture and consequent general dysfunction mirroring loss of ganglion cell layer (GCL) and thinning of inner plexiform layer (IPL), inner nuclear layer (INL), outer

plexiform layer (OPL), and outer nuclear layer (ONL). PACAP exerts its neuroprotective action on diabetic retina directly by binding to its related receptors or indirectly by inducing ADNP secretion. Exogenous administration of NAP, the smallest active element of this neurotrophic protein, and PACAP counteracts the hyperglycemia-induced metabolic changes

able to interfere with the HIF-VEGF signaling cascade at the early stage of hyperglycemia.

As mentioned above, hyperglycemia/hypoxia also stimulates release of inflammatory cytokines. Among these, IL-1 β was described as a mediator of retinal tissue damage at early stages of the disease. In line with this evidence, we demonstrated that this cytokine and its related receptors, IL-1R1 more than IL-1R2, were upregulated in diabetic rat retinas. During hyperglycemia, IL-1 β and IL-1R1 are overexpressed in the photoreceptor layer and in the ONL, highlighting the induction of inflammatory process in specific retinal regions as well as their involvement in blood retinal barrier breakdown (BRB) during DR progression (Scuderi et al. 2015). For the first time, we have demonstrated that PACAP and NAP counteract this inflammatory event, by downregulating IL-1 β and IL-1R1 aberrant expression, and maintaining BRB integrity (D'Amico et al. 2018, 2019; Maugeri et al. 2017b; Fabian et al. 2019). The maintenance of BRB integrity is fundamental for visual function. The impairment of this structure in macular region leads to diabetic macular edema (DME), a serious complication leading to vision loss. BRB is constituted by an inner compartment, known as inner BRB, that is represented by vascular endothelium and an outer compartment, known as outer BRB, characterized by retinal pigmented epithelium (RPE) (Simó et al. 2010). These cells play a protective role in survival of photoreceptor exposed to different insults. Therefore, the identification of molecules able to preserve RPE survival represents a further therapeutic approach in the retinal diseases.

Our research group has demonstrated the ameliorative effect played by PACAP and NAP in maintenance of outer BRB integrity. More specifically, we showed that PACAP and NAP preserve barrier integrity by reducing hyper-permeability of pigment epithelium exposed to various insults accounting for diabetic macular edema (i.e., high glucose and interleukin 1 β). In these cells, PACAP and NAP preserve retinal pigment epithelial cell monolayer by inhibiting HIFs-VEGF signaling cascade and reducing pro-inflammatory pathway. Recently, we have demonstrated that PACAP exert its activity also through induction of EGFR phosphorylation in DR. (Maugeri et al. 2019b).

Overall, these evidences suggest that the neuroprotective effect of PACAP is mediated, at least in part, by ADNP induction in the diabetic retina, summarized in Fig. 1.

Conclusion

In conclusion, PACAP and NAP play a key role in retinal physiopathology; however, while PACAP's half-life is a minute or shorter, NAP's half-life is relatively long for a peptide, half an hour. NAP has previously been used in clinical trials

showing safety and tolerability as well as efficacy increasing cognitive functions and protecting activities of daily living in mild cognitive impairment patients and schizophrenia patients, respectively (Gozes 2020). Overall, the evidences reported suggest that PACAP and NAP could be considered good candidates for therapeutic approach to DR.

Authors' contributions A.G.D and G.M.: writing original draft preparation, V.D and D.R: writing—review and editing, G.M: visualization, V.D: supervision, D.R: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no conflict of interest.

Consent for Publication The authors agree to publication.

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