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Neuroprotective effects of PACAP, VIP and NAP against hyperglycaemic retinal damage

PhD thesis

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LIST OF ABBREVIATIONS

AC	Adenylate Cyclase
ADNF	Activity-Dependent Neurotrophic Factor
ADNP	Activity-Dependent Neuroprotective Protein
ARVO	Research In Vision And Ophthalmology
AS	Angiostatin
BRB	Blood-Retinal Barrier
CAT	Catalase
DMO	Diabetic Macular Oedema
DR	Diabetic Retinopathy
EPO	Erythropoietin
ERK 1/2	Extracellular Signal-Regulated Kinase 1/2
GABA	γ-aminobutyric acid
GCL	Ganglion Cell Layer
GPCR	G-Protein Coupled Receptor
GSH	Glutathione
IDDM	Insulin-Dependent Diabete Mellitus
IL-1α	Interleukin -1α
IL-1β	Interleukin -1β
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RI	Interleukin -1 receptor type I
IL-1RII	Interleukin-1 receptor type II
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
JNK	c-Jun-N-terminal kinase
MAPKs	Mitogen-Activated Protein Kinases
NAP	Davunetide
NF-k β	Nuclear Factor-Kappa B

NIDDM	Non-Insulin-Dependent Diabete Mellitus
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OS/IS	Outer and inner segments of rods and cones
PACAP	Pituitary adenylate cyclase-activatingpeptide
PDGF	Platelet-derived growth factor
PDR	Proliferative diabetic retinopathy
PEDF	Pigment epithelium-derived factor
PHI	Peptide histidine-isoleucine
PKA	Protein kinase A
PKC	Protein kinase C
RGCs	Retinal ganglion cells
ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
SOD	Superoxide dismutase
STZ	Streptozotocin
TNF- α	Tumor necrosis factor-α
VIP	Vasoactive intestinal polypeptide
WHO	World Health Organization

ABSTRACT

Diabetic retinopathy (DR), one the most significant and disabling chronic complications of diabetes mellitus, has mainly been regarded as a microcirculatory disease of the retina. However, there is emerging evidence to suggest that a primary and early neurodegenerative process characterizes DR. The reason for the retinal degeneration resides in the metabolic changes caused by hyperglycemia and in the decreased capability of the retina to adapt to this environment. This situation leads to altered expression patterns of neuropeptides, growth and transcription factors, apoptosis, increased oxidative stress, inflammatory response, angiogenesis and disruption of the blood-retinal barrier (BRB). All major cell types of the retina are affected: neuronal as well as the glial cells and pigment epithelial cells.

Pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal peptide (VIP) and davunetide (NAP) are three pleiotropic related neuropeptides also expressed in the retina. Their protective role has been shown in different retinal injuries, but little is known about the relationship between these peptides and DR.

The aim of the present thesis was to investigate, using both in vitro and in vivo model of DR, the role of these peptdides on retinal layers by monitoring apoptotic events by western blot and confocal microscopy analysis and measuring the integrity of the outer blood retinal barrier with permeability and transepithelial electrical resistance assay. The underlying signal transduction pathways activated by each peptide and the impact of hyperglicaemia on the expression and distribution at retinal levels of the inflammatory IL-1 mediators were also evaluated. Diabetes was mimicked in adult rats by intraperitoneal injection of streptozotocin (STZ) and human retinal pigment epithelial cells

(ARPE19) were cultured for 26 days in high glucose (25mM of D-glucose) and IL-1β.

The results confirmed that hyperglycemia induced early apoptotic death in the cellular components of the neuroretina, breakdown of the outer blood retinal barrier (BRB) and dysregulation of several components of the metabolic and signaling pathways. The neuropeptides tested activate promising pathways useful for the treatment of this retinal degenerative disease. In particular, PACAP and VIP promote the integrity of the outer BRB, possibly through the modulation of proteins related to tight junctions. Davunetive is able to reduce apoptosis in the diabetic retina by activating the anti-apoptotic p-Akt, p-ERK1, p-ERK2, Bcl-2 and decreasing levels of the pro-apoptotic elements, such as cleaved caspase-3. These results raise the opportunity for the use of these peptides as a possible therapeutic or preventive methods in treating diabetes.

GENERAL INTRODUCTION

Diabetic retinopathy and visual impairment

The prevalence of diabetes is increasing worldwide. According to the World Health Organization (WHO), the total number of people with diabetes is expected to rise to an estimated 300 million cases by the year 2025 [1]. The onset of diabetes immediately increases the possibility for the patient to develop a broad spectrum of irreversible complications [2].

The term diabetes mellitus indicates a group of metabolic disorders characterized by hyperglycaemia and impaired insulin signalling. The disease is usually classified into type 1 diabetes, type 2 diabetes, and other specific types including gestational diabetes. Type 1 diabetes, also known as insulin-dependent diabetes, constitutes about 5-10% of all cases of diabetes. It is characterized by the destruction of β-pancreatic cells, leading to absolute insulin deficiency. This failure to produce insulin can be autoimmune-mediated or idiopathic. Type 2 diabetes, also known as non-insulin-dependent diabetes, constitutes 90-95% of all diabetes cases and results from insulin resistance, inadequate compensatory insulin secretory response, or both. It is characterized by reduced responsiveness of the cells in the body to insulin and consequently less ability to transfer glucose out of the circulation [3]. Complications resulting from diabetes can be largely divided into microvascular or macrovascular categories. Microvascular complications include diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy. Macrovascular complications include

cerebrovascular disease, coronary heart disease, and peripheral vascular disease.

Diabetic retinopathy (DR) is one of the most significant and disabling chronic complications of diabetes mellitus [4]. DR can be divided into two clinical stages: nonproliferative and proliferative diabetic retinopathy (PDR). During thr nonproliferative stage of DR, altered permeability of capillaries leads to the formation of microaneurysms, the earliest visible sign of retinal damage. Abnormal capillary permeability results in leakage of fluid and solutes into the surrounding retinal tissue, which accumulate around the macula; causing macular oedema (DMO). As the severity of DR increases, the growth of new capillaries on the surface of the retina from preexisting vascular beds (neovascularization) causes vascular irregularities in the retina, like the occlusion of capillaries and ischaemia. This process defines PDR. However, these vessels are fragile and haemorrhage easily, and the resulting accumulation of blood in the vitreous cavity causes serious visual impairments in diabetic patients. It has been estimated that without treatment for PDR, 50% of all patients will become blind within 5 years following the diagnosis [5]. About one-third of the diabetic population has signs of DR, and one-tenth has vision-visual impairments form DMO and PDR. Tight control of blood glucose levels and blood pressure are essential in preventing or arresting the progression of DR. However, these therapeutic objectives are difficult to achieve, even with strict glycemic control, and as a consequence, PDR and DMO still appears at proportionately high rates in patients with both type I (insulin-dependent diabete mellitus, IDDM) and type 2 (non-insulin-dependent diabete mellitus, NIDDM) diabetes during the evolution of the disease [6]. Population studies indicate that DR afflicts approximately 93 million people worldwide and this number is

expected to increase as the prevalence of type 2 diabetes continues to climb, representing a significant socioeconomic cost for healthcare system worldwide [7].

The global prevalence of DR highlights the importance of searching for new approaches beyond the current standards of treatment for diabetes. Furthermore, there is abundant data to suggest that diabetes not only affects the entire neurovascular unit of the retina, but causes an early loss of neurovascular function, gradual neurodegeneration, gliosis, and neuroinflammation before any observable vascular damages. Therefore, the study of the underlying mechanisms that lead to neurodegeneration will be essential for identifying new therapeutic targets in the early stages of DR.

Current treatments for Diabetic Retinopathy

E maintenance and restoration of functions of the eye, the main sense organs by which people receive 80% of their information about theenvironment, is among the central issues of modern medicine. Visual functions are integral fo most professional and everyday activities, and thus, out of all the senses, their decline or loss has the largest negative impact on the quality of life. Furthermore, DR is the third leading cause of visual impairment overall. Loss of vision due to diabetic retinopathy results from DMO, vitreous hemorrhage and fibrous tissue leading to retinal detachment.

In its earliest stages, DR usually has no symptoms. However, some retinal lesions indicate a risk of progression. Laser photocoagulation, vitreoctomies, intravitreal injections of corticosteroids and anti vascular endothelial growth factor (VEGF) drugs are the present standard

strategies used in the treatment of DR and DMO. However, intravitreal injections are invasive procedures and can have adverse effects like infection, glaucoma, retinal detachment and cataract formation. While anti-VEGF treatments have not proven wholly succeddful, this strategy encourages the development of alternative treatmentsfocusing the other factors that are altered in the vitreous fluid of DMO and PDR patients: increased proangiogenic factors [*e.g.* platelet-derived growth factor (PDGF), erythropoietin (EPO)] or decreased antiangiogenic factors [*e.g.* angiostatin (AS), pigment epithelium-derived factor (PEDF)] [8, 9].

The goal for the future is to better understand early pathophysiological changes that lead to visual loss in diabetic patients, and develop new, less-invasive pharmacological to maintain good vision. Above all, retinal neurodegeneration may be the most promising new target, and intensive research is needed to further elucidate the underlying mechanisms that lead to retinal degeneration and its relationship with microvasculature impairment.

Neurodegeneration in the diabetic retina

The retina is a complex structure with several layers of neurons interconnected by synapses (neuroretina) and a monolayer of pigmented cells called the retinal pigmented epithelium (RPE). The RPE is situated between the neuroretina and the choroids, which are essential for the functionality of the neuroretina [10]. The photoreceptors cells, rodes and cones, are the only neurons that are directly sensitive to lights. Their role is to facilitate the process of phototransduction, the translation of light information into neural signals. The somata of second-order neurons (bipolar and horizontal cells), the so-caled

amacrine celle, and the main glial element (Muller cells) constitute the inner nuclear layer. The innermost cellular layer contains the ganglion cells and amacrine cells and is termed the ganglion cell layer. Photoreceptors form synapses with bipolar and horizontal cell processes in the outer plexiform layer. The inner plexiform layer consist of the axon of bipolar cells and the dendrites of the amacrine and ganglion cells. The choroid and the retinal circulation provide oxygen and nutrients to the human retina and most mammals. Retinal neural tissue is protected by harmful molecules by the inner and outer BRB, which are respectively constituted by endothelial cells and RPE cells.

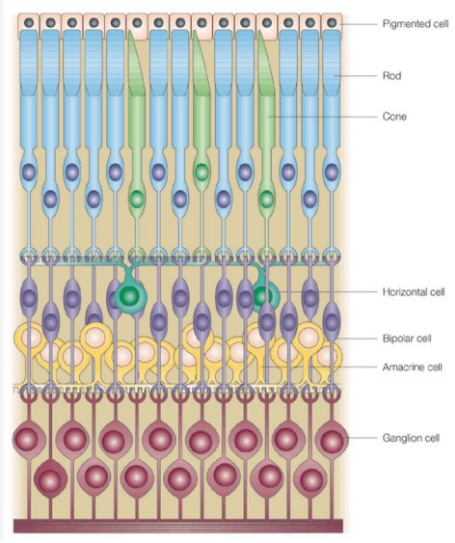

Figure 1 **Schematic of the mammalian retina. a** There are seven main cell types in the mammalian retina: pigmented cells, rods, cones, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells. They have a laminar distribution Figure adapted from Livesey FJ and Cepko CL (2001) and Wässle H (2004).

The retinal pathway carrying processed visual information to the brain can be described as a chain of neurons and their synapses. Neural signals, from rodes and cones, undergo processing in the retina and action potentials are generated in retinal ganglion cells (RGCs), the axons of which form the optic nerve. These synapses use glutamate as their neurotransmitter, while the horizontal and amacrine cell modulate the cascade and use glycine and γ-aminobutyric acid (GABA) as their main transmitters [11-14].

For many decades, DR has been considered a microangiopathic disease of the retina with key clinical features: vascular leakage and pre-retinal neovascularization, resulting from breakdown of the blood retinal barrier (BRB) [15]. However, there is a mounting evidence to suggest that the pathogenesis of DR may also comprise neuroinflammatory and neuropathic processes which contribute to visual impairment [16]. Loss of neuroretinal adaptation to the diabetic metabolic environment and neural apoptosis may occur in DR prior to any clinically detectable microvasculopathy, in both human and animal models [17].

There are clear indications that retinal function, which depends on a complex integral network of multiple neuronal subtypes, is disturbed soon after the onset of diabetes, and that neurodegeneration is an ongoing component of DR.

Several interconnected factors induce retinal neuronal degeneration. Among these, hyperglicaemia is considered the main culprit compromising neural and vascular function. The numerous metabolic pathways triggered by hyperglicaemia such as the polyol pathway, the hexosamine pathway, the DAG-PKC pathway, lead to extracellular glutamate accumulation, oxidative stress and reduction of neuroprotective factors synthesized by the retina [18]. All of these mediators are involved in pathways that lead to neuronal apoptosis and

glial dysfunction, hallmarks of retinal neurodegeneration, and to BRB breakdown and altered microvascular system, which are the main features of early microvascular abnormalities (Figure 2).

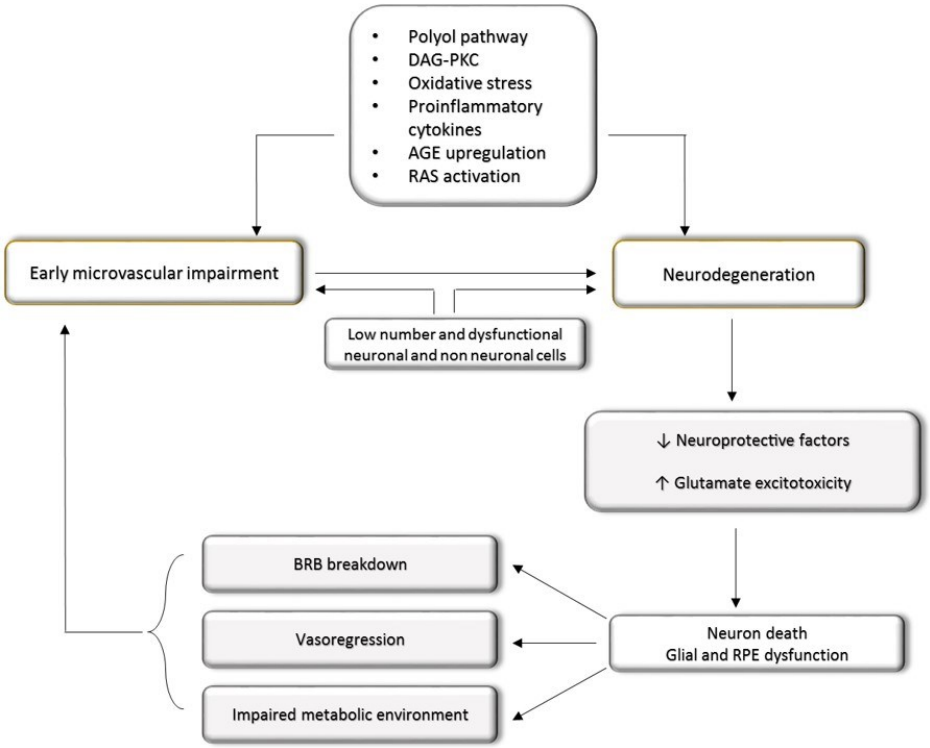

Figure 2. Diagram of the main mechanisms leading to diabetic retinopathy (DR). Figure adapted from Simò R and Hernàndez C (2014).

Furthermore, the major excitatory neurotransmitter in the retina, glutamate was found elevated in the extracellular space in experimental model of diabetes, as well as in the vitreous fluid of diabetic patients with PDR. This extracellular glutamate accumulation, known as excitotoxicity, is deleterious on retinal neurons [19].

Increased generation of reactive oxygen species (ROS) in the retina of patients with DR has also been described. Although the retina contains a robust pool of antioxidant molecules (GSH, Vitamin C and E) and endogenous enzymes (SOD, CAT and Glutathione peroxidase) able to

quench ROS and maintain normal cellular homeostasis, during diabetes an imbalance between pro-oxidants and anti-oxidants is evident and leads to oxidative stress. [20]. Oxidative stress has been associated with cellular inflammation and the release of important inflammatory cytokines [e.g. tumor necrosis factor-α (TNF- α), interleuikin-1β (IL-1β)] that can interact with transcription factor Nuclear factor-kappa β (NF-k β) able to initiate the transcription of many genes involved in apoptosis, like Caspase-3 in the retina [21].

All neuronal cell types in the retina seem to be susceptible to hyperglicemia-induced apoptosis. Over time, neurons in retinal layers lose their capacity to adapt to diabetic metabolic alterations of the environment and succumbs to cellular stress, as evidenced by reduced axonal and dendritic branching, apoptotic cell death, neural and vascular inflammation, cell loss and retinal layer thinning [9].

Apoptosis is a distinct form of cell death, regulated by genetic programs and characterized by morphological changes including cell shrinkage, plasma membrane blebbing and nuclear and cytoplasmic condensation, with apoptotic body formation. It consists of two distinct signalling pathways, the extrinsic and intrinsic one, both of which have a common final stage, the execution pathway- This final step is which is mediated by the activation of caspases. Caspases belong to the conserved cysteine protease family and catalyze the cleavage of their downstream targets at sites on the carboxyl side of aspartic acid residues. Fourteen caspases have been identified in mammals; they are classified into initiators and effectors. Among the effectors, Caspase-3 has been described as a pivotal molecular player in the execution-hase of apoptosis [22]. It has been demonstrated that, an imbalance between proapoptotic and survival signalling exists in the neuroretina of diabetic patients in the early stages of DR. Increased numbers of apoptotic neurons and up-

regulated expression of caspases-3, Bax and p53 have been identified in STZ rat retinas and in post mortem human diabetic retinas when compared to controls [23]. The proposed apoptotic pathway in DR is summarized in Fig. 3.

Retinal ganglion cells (RGCs), located in the inner retina are the first apoptotic cells detected in DR. Among all the retinal neuronal cell types, RGCs seem to be the most susceptible to hyperglyceamic stimulation and the most sensitive to cellular damage and neurotoxicity [24]. This results, in a reduced thickness of the retinal nerve fiber layer, and has been detected in STZ-induced diabetes animal models, and diabetic patients without DR [25, 26]. BRB disruption is another core element of retinal dysfuntion in DR patients. It involves both structural and functional changes to the barrier. The tight junction of RPE cells are an important component of the outer BRB, and can be distrupted by inflammatory and oxidative changes associated with hyperglycaemia.

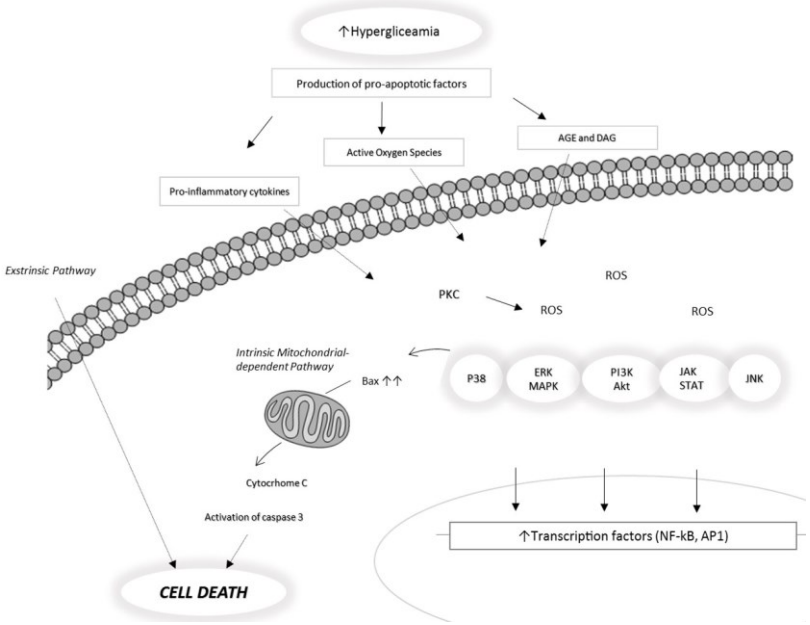

Figure 3. Proposed apoptotic pathway in diabetic retinopathy. Figure adapted from Zhang X et al., (2013).

Neuropeptides and diabetic retinopathy

The presence of neuropeptides in the human retina has mostly been studied through immunohistochemical and chromatographic assays. Around 20 neuropeptides have been identified in the human retina (Table 1). Neuropeptides are produced from both neural and non-neural cells, and some peptides are produced by extra-retinal cell sources. In particular, among retinal nerve cells, amacrine cells are the only cells able to release their products within the retina, because ganglion cells, send their axons towards the brain [27]. It has become clear that the neuropeptides described in the retina can be divided into two categories: peptides that promote the development of DR symptoms, and others that are able to prevent, delay or eliminate them. Disturbed retinal balance between these neurotoxic and neuroprotective factors is crucial in accounting for neuronal cell death in the diabetic retina. Downregulation of neuroprotective factors may compromise natural neuroprotection against neurotoxic executors involved in neurodegeneration [28].

Table 1
Neuropeptides in the human retina

Neuropeptide	Neuronal	Müller cell	Pigment epithelium	Receptors
Angiotensin II	U	+	+	AT1R and AT2R
Bradykinin		unknown	+	B1R
Cortistatin	U	—	++	SST 1, 2 and 4 receptors
Enkephalins	A	—	—	sigma
Erythropoietin	unknown	+	unknown	EPOR
Neurokinin A and B	A, G	—	—	NK1R and NK3R
Neuropeptide Y	A, G	—	—	Y1, Y2, Y4 and Y5
Neurotensin/LANT6	A, G	—	—	Not known
Orexin A and B	A, G	—	+	OXR1
Pituitary adenylate cyclase activating peptide	A, G	—	—	PAC1R, VPAC1 and 2
Secretoneurin	A, G	—	—	Not known
Somatostatin	A, dA	—	—	SST1, 2 and 4 receptors
Substance P	A, G	—	—	NK1R and NK3R
Thyrotrophinreleasing hormone	A	—	—	TRHR1 and R2
Urocorintin I, II and III	unknown	—	+	CRF1R
Vasoactive intestinal polypeptide	A, dA	—	—	VPAC1 and 2

A, amacrine cell; dA, displaced amacrine cell; G, ganglion cell; U, unidentified cell type; +, present; ++, present in high quantity; unknown, not certified. Table adapted from Gabriel R., (2013).

Pituitary adenylyl cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP), two similar neuropeptides that belong to the secretine, glucagon and peptide histidine-isoleucine (PHI) superfamily, are widely present in the retina. The sequences of VIP and the PACAP peptides are highly conserved across species. Two PACAP isoforms have been identified, a 38 amino acid form (PACAP38) and a C-terminally truncated 27 amino acid form (PACAP27). Analysis of retinal tissue, by electron and light microscopy, reveals the presence of PACAP and VIP, as well as their respective receptors. In particular, PACAP immunopositivity was observed in amacrine, horizontal and ganglion cells while VIP immunopositivity was observed in amacrine and displaced amacrine cells. Ultrastructural studies show that PACAP immunoreactivity is visible near the plasma membrane, in the rough endoplasmic reticulum, and the cytoplasmic matrix [29-31].

The receptors for PACAP and VIP are G protein-coupled receptors, and can be classified into two main groups PAC1, which binds PACAP with high affinity and VPAC, which bind both peptides with high affinity (includingVPAC1 and VPAC2 subtypes). Through adenylate cyclase (AC) activation, they elevate cyclic 3,5-adenosine monophosphate (cAMP), and activates protein kinase A (PKA), which can activate the mitogen activated protein kinase (MAPK) pathway [32]. PAC1 receptor binding can stimulate various downstream executors of PKA and and protein kinase C (PKC), ion channels and β-arrestin. These, and other pathways regulated by PAC1 receptors are different in distinct cell types depending on the expressed splice variant, the peptides concentration and other factors present in the cells. VPAC receptors couple to Gs proteins resulting in activation of AC; other signaling pathways downstream of cAMP or independent of cAMP are

associated with VPAC receptor activation depending on the tissues in which they are expressed [33-36].

One of the many functions of PACAP and VIP is their strong cytoprotective role, promoting survival in many type of neuronal and non-neuronal cell and tissue, including lymphocytes and endothelial cell; in the liver, lungs and ovaries [37, 38]. Both peptides influences apoptotic signalling at various levels, from initiation to downstream cytosolic and mitochondrial pathways and finally affecting executor caspases [39, 40]. Findings demonstrated that neuroprotection by subpicomolar levels of PACAP38 involves ERK type MAPK, whereas neuroprotection by nanomolar levels PACAP38 is mediated by the activation of cAMP [41].

Furthermore PACAP and VIP neuroprotective actions are mediated by two different glial derived proteins: activity-dependent neurotrophic factor (ADNF) [42], and activity-dependent neuroprotective protein (ADNP) [43, 44]. Davunetide (AL-108, NAP) is the acetate salt of an eight aminoacid peptide (NAPVSIPQ) derived from ADNP, a growth factor released from glia in response to exposure to VIP and PACAP. In cell cultures, femtomolar concentrations of davunetide have potent neuroprotective effects on cell death and microtubule disruption from a variety of toxic insults [45-49]. NAP has been shown to be involved in microtubule re-organization and in transient increases of non-phosphorylated tau levels [46]. It has also been demonstrated to promotes neuronal growth and differentiation, and its protective actions are mediated by the activation of MAPK/ERK and PI-3K/AKT [50].

The role of the above peptides has been investigated in various models of retinal injuries. Previous studies have shown that PACAP and VIP protect the retina from excitotoxic, ischemic, and UV-A-induced retinal degeneration [51-53]. Changes in the retinal expression of PACAP/VIP

and their receptors have been reported during the earliest phases of STZ-induced diabetes [54]. Furthermore PACAP treatment could protect the retina against the harmful effects of diabetes, especially through its well-known caspase-inhibiting effect [55]. However, whether these peptides are also involved in maintenance of outer BRB function during DR remains to be clarified. NAP protects retinal ganglion cells against damage induced by retinal ischemia and optic nerve crush [56], it prevents hypoxia-induced injuries to rat retinal Muller cells, and promotes retinal neuronals growth [57]. Although it prevents some diabetes-related brain pathologies in the STZ injected rat model [58], to date there are not evidences in literature regarding the effect of NAP in DR.

Models for testing Neuroprotective drugs

There are several different species of animal models used to study DR from tiny zebrafish to monkeys. Different rodent models of DR have been used for the investigation of the mechanisms of retinal damage and preclinical drug screening. The advantage of using rodent models is the similarity of their genetic background to humans. Currently, the most used model to study early neurodegeneration in the diabetic retina is a rat receiving intraperitoneal injections of streptozotocin (STZ). Rats have been used more often than mice as an experimental model of DR and retinal degeneration because rats are less resistant to the STZ effect and they have higher eyecups and a higher degree lesion of lesions compared to mice (mice need from 3-5 doses of STZ to induce diabetes compare to rats where one dose is sufficient) [59]. STZ, a toxic glucose analogue compound, derived from *Streptomyces achromogenes*

is used clinically as a chemotherapeutic agent in the treatment of pancreatic β cell carcinoma. STZ has been used in both type 1 and type 2 diabetes animal models. STZ damages pancreatic β cells, accumulating in them via the GLUT2 glucose transporter, resulting in hypoinsulinemia and hyperglycemia, and greatly affecting the β-cell mass. Similarly, a variation for β-cell mass in both type 1 and type 2 diabetes exists in humans [60-62].

Although the above method is often used to make a diabetes model, pathological neovascularization caused hyperglicaemia, which is a typical finding in severe diabetic patients, does not appear in these animals. However, since neurodegeneration is an early event in the pathogenesis of DR, it is not necessary to use animal models, with subsequent microangiopathic complications, to test the efficacy of neuroprotective factors [63, 64]. Furthermore, STZ-induced diabetes model shows apoptosis of the inner retinal neurons, such as ganglion cells and amacrine cells, and the activation of the Müller glial cells in the retina [10, 11].

In addition, many models have been generated *in vitro* mimicking the diabetic metabolic environment using human or nonhuman tissues [65]. The *in vitro* culture systems allow easier manipulation of the retina and a immediate variation of the experimental conditions.

AIMS

Chapter I To characterize the *in vivo* model of DR used in this work, focusing on the inflammatory process. We study the effect of hyperglicaemia on the IL-1 family elements expression and distribution in retinal layers of STZ-induced diabetic rats.

Chapter II To investigate the possible ameliorative effect of NAP in streptozotocin-induced diabetic retinopathy and to evaluate its anti-apoptotic effect in this model of early retinal neurodegeneration. NAP has been shown to have protective effects against different retinal injuries, but its role has not been investigated in DR.

Chapter II To provide evidence that PACAP and VIP are also outer BRB protective peptides. A large body of evidence establishes the retinoprotective effects of both peptides. However, little is known about the relationship between them and one of the most common DR complications: the disruption of the outer BRB. In this study, an *in vitro* model of outer BRB was used.

Chapter I

submitted

Different retinal expression patterns of IL-1 α, IL-1 β, and their receptors in a rat model of type 1 STZ-induced diabetes

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Abstract

Diabetic retinopathy (DR), a common complication of diabetes, remains a major cause of blindness among working-age population. Considerable amounts of evidences suggest that DR resembles an inflammatory disease. Increased levels of pro-inflammatory cytokines, including interleukin-1β (IL-1 β), were found in the vitreous of diabetic patients and in the retina of diabetic rats. However, in this context, no attention has been given to the other main IL-1 family members: IL-1α, two transmembrane receptors IL-1RI and IL-1RII, and the natural antagonist receptor IL-1Ra. Despite they actively participate in the IL-1 mediated-inflammation process, their role in DR has not been described. Thus, we investigated by western blot and confocal laser scanning microscopy analysis the effect of hyperglycaemia on the IL-1

molecules regulation in retinal layers, using an *in vivo* model of type 1 diabetes. Diabetes was induced in adult rats by intraperitoneal injection of streptozotocin (STZ). Exposure to hyperglycaemia causes changes in the expression and distribution levels of all IL-1 family members studied. It induces a significantly increase in the proteins expression of IL-1β, IL-1RI, IL-RII and IL-1Ra but not of IL-1α. Moreover, high glucose alters their distribution pattern in the rat retina. The compromised layers, with an upset IL-1 inflammatory scenario, are the photoreceptor, the inner plexiform and ganglion cell layers. These findings point to all of these IL-1 molecules, as key elements in the pro-inflammatory cascade triggers by hyperglycaemia in the early phase of DR.

Keywords: interleukin-1α; interleukin-1β; , IL-1 Receptor I; IL-Receptor II; IL-1 Receptor antagonist; diabetic retinopathy; streptozotocin

1. Introduction

Diabetic retinopathy (DR) is a major cause of visual impairment and acquired blindness among patients with type 1 and type 2 diabetes [66, 67]. DR has mainly been considered as a microvascular disease, caused by a range of hyperglycemia-linked pathways [68] and characterized by basement membrane thickening of retinal vessels, loss of pericytes and endothelial cells, blood-retinal barrier breakdown (BRB) [69, 70]. However, there are emerging evidences to suggest that neurodegenerative processes may occur prior any detectable microvascular alterations of the retina. All neuronal cell types are susceptible to hyperglicaemia-induced biochemical alterations that lead to oxidative stress, apoptosis and inflammation [54, 71]. Inflammation has been recognized as a trigger element in the early and late stages of DR [72, 73]. In fact, varieties of physiological and molecular abnormalities, consistent with inflammation, have been found in the retinas or vitreous humor of diabetic animals and patients. These include macroglial and microglial activation, leukostasis, increased vascular permeability, acute phase proteins, vasoactive peptides [74-76] and increased levels of inflammatory cytokines, such as Interleukin-1beta (IL-1β) and tumor necrosis factor-α (TNF-α) [77-80].

Interesting reports on the correlation of dysfunctional IL-1β signalling with incident of many pathologies, including DR [21, 75, 81], have diverted our attention to reveal whether the other members of this family play a role during early phase of diabetes.

The IL-1 family includes seven ligands with agonist activity (IL-1α and IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38), and an anti-inflammatory cytokine (IL-37). Members of the IL-1 Receptor (IL-1R) family include six receptor chains forming four

signaling receptor complexes, two decoy receptors (IL-1R2, IL-18BP), and two negative regulators (TIR8 or SIGIRR, IL-1RAcPb) [82]. The best studied are IL-1α and IL-1β, both pro-inflammatory cytokines, and the endogenous receptor antagonist IL-1Ra [83].

All IL-1 ligands are produced as pro-peptides, and while IL-1β requires proteolytic cleavage for its activity, IL-1α possesses limited activity prior to processing [84]. The IL-1Ra is released by the cell, as an active molecule of 25 KDa without a requirement for processing. Once released and cleaved, IL-1 ligands elicit a multitude of effects on target cells, binding to two different types of transmembrane receptors (IL-1R type I and IL-1R type II) [85] that lead to tissue damage [86]. IL-1RII is a decoy receptor, which lacks the cytoplasmatic domain and cannot signal, but binds to IL-1RI and thus limits its biological availability. IL-1Ra competes with IL-1 for the binding to IL-1 receptor, but unlike IL-1, this binding does not induce any signal transduction [87].

In the present work, we used an *in vivo* model of streptozotocin-induced diabetes type I and by immunohistochemistry and Western blotting analysis, three weeks after the onset of hyperglicaemia, we investigated the expression patterns of IL-1α, IL-1β, IL-receptor antagonist and their receptors (IL-1RI e IL-1RII) both in nondiabetic and diabetic rat retina.

2. Materials and Methods

2.1 Rats

Male Sprague–Dawley rats, three months old, weighing approximately 200 g each, obtained from Charles River (Calco, Italy) were used for the present study. All the animals were treated according to the Association

for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were fed with standard laboratory chow and were allowed free access to water in an air–conditioned room with a 12 h light / 12 h dark cycle. Final group sizes for all measurements were n = 6–9.

2.2 Induction of Diabetes

Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma – Aldrich, St. Louis, MO, USA) as previously described [78]. Briefly, after twelve hours of fasting, a group of animals received a single injection of STZ (60 mg/kg body weight) in 10 mM sodium citrate buffer, pH 4.5 (1 mL/kg dosevolume). Nondiabetic animals were fasted and received citrate buffer alone. Twenty four hours post STZ injection blood glucose levels was measured and rats showing blood glucose levels greater than 250 mg/dL were considered as diabetic [88] and selected for the study. We monitored throughout the study the diabetic state by evaluating glycemia daily using a blood glucose meter (Accu–CheckActive; Roche Diagnostic, Milan, Italy) and by the weight loss. All experiments were performed three weeks after the induction of diabetes. For subsequent experiments, rats were killed with a lethal i.p. dose of sodium pentobarbital. Retinas were immediately removed and homogenized in ice cold buffer for Western blot analysis and others were fixed in 4% paraformaldehyde for histological and immunohistochemical analysis.

2.3 Measurement of Blood Retinal Barrier Breakdown

In a separate set of animals the Blood Retinal Barrier breakdown was measured using Evans blue dye (Sigma-Aldrich, St. Louis, MO) three weeks post STZ injection as previously reported [89]. Rats were killed and

the retinas were carefully dissected and thoroughly dried in a concentration/drying system (SpeedVac; Thermo Fisher Scientific, Milan, Italy). The dry weight was used to normalize the quantitation of Evans blue leakage. Evans blue was extracted from each retina and the samples used for spectrophotometric measurements. The background-subtracted absorbance was determined by measuring each sample at 620 nm (the maximum absorbance for Evans blue in formamide) and 740 nm (the minimum absorbance). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. BRB breakdown was calculated using the following equation, with results being expressed in µL plasma × g retina dry weight^{-1} × h^{-1}.

$$\frac{Evans\ blue\ (\mu g)\ /Retina\ dry\ weight\ (g)}{Time\ averaged\ Evans\ blue\ concentration\ (\mu g\ /plasma\ \mu l/\text{circulation time (h)})}$$

We expressed results as percentage of control.

2.4 Western blot analysis

Western blot analysis was performed to determine the relative levels of the Interleukin-1 family ligands and receptors using specific antibodies. Analysis was performed as previously described by D'Amico et al. (2013b). Briefly, proteins were extracted with buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. The Quant-iT Protein Assay Kit (Invitrogen) determined protein concentrations. Sample

proteins (30 µg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (BIO-RAD) by electrophoresis and then transferred to a nitrocellulose membrane (BIO-RAD). Blots were blocked using the Odyssey Blocking Buffer (LI-COR Biosciences) and probed with appropriate antibodies: rabbit anti-β-tubulin (sc-9104, Santa Cruz Biotechnology; 1:500), rabbit anti-IL-1α (sc-7929, Santa Cruz Biotechnology; 1:200); goat anti-IL-1 β (sc-1250, Santa Cruz Biotechnology; 1:200), rabbit anti-IL-1RI (sc-688, Santa Cruz Biotechnology; 1:200), rabbit anti- IL-1RII (sc-292522, Santa Cruz Biotechnology; 1:200), mouse anti-IL-Ra (sc-376094, Santa Cruz Biotechnology; 1:200). The secondary antibody goat anti-rabbit IRDye 800CW (#926-32211; LI-COR Biosciences), donkey anti-goat IRDye 800CW (#926-32214 LI-COR), goat anti-mouse IRDye 680CW, (#926-68020D; LI-COR Biosciences) were used at 1:20000. Blots were scanned using an Odyssey Infrared Imaging System (Odyssey). Densitometric analysis of Western blot signals was performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

2.5 Immunolocalization

Eyes were enucleated and fixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.6). Analysis was performed as previously described by [54]. Before immunofluorescence staining, paraffin-embedded retinas sections (14µm) mounted on glass slides, were dewaxed in xylene and rehydrated through graded alcohols. Sections were put into Rodent Decloaker 1X retrieval solution (RD913, BIO-CARE MEDICAL) in a slide container and then incubated with the anti IL-1 β, IL-1α, IL-1R type I and II, IL-1Ra primary antibodies. Tissue sections were then incubated with Alexa fluor 488 goat anti-rabbit and Alexa fluor 488 donkey anti-goat secondary antibodies,

respectively for 1.5 h at room temperature and shielded from light. DAPI (diamidino-2-phenylindole) was used to stain nuclei (#940110 Vector Laboratories). Images of the central retina were taken with a confocal laser scanning microscope (CLSM) (Zeiss LSM700 with a x20, x40, and x63 objectives, as previously described [90] and analysed using the ZEN 2011 software. Experiments were repeated at least three times to confirm results.

2.6 Statistical analysis

Statistical analyses were performed using specific software GraphPad Prism-5. Paired t tests were used to determine whether differences were significant. The level of significance for all statistical tests was $p \leq 0.05$

3. Results

3.1 Changes in the body weight and blood glucose levels after 3 weeks of STZ administration

Table 1 shows the effects of STZ treatment on body weight and non-fasting blood glucose levels in rats after 3 weeks. Body weight and glycemia of rats before treatments were approximately 200g and 100mg/dL respectively. The statistical analysis revealed a significant decrease in body weight 3 weeks after STZ injection ($p<0.01$ *vs.* nondiabetic control group). Three weeks after onset of diabetes, blood glucose values in diabetic rats were significantly ($p<0.01$) higher than corresponding values in non diabetic rats (391 ± 35 and 98 ±18 mg/dL respectively).

Groups	Body weight (g)	Non fasting blood glucose (mg/dl)
Control	270 ± 27	98 ± 18
Diabetic	205 ±20*	391 ± 35*

Data are expressed as mean ± SD.

*$p<0.01$ *vs.* control.

Table 1. Effects of STZ-induced diabetes on body weight and blood glucose levels after 3 weeks. Control (non-diabetic) group represents normal rats injected with the vehicle used to dissolve STZ. Diabetes was induced by 60 mg/kg STZ injection.

3.2 Blood Retinal Barrier Breakdown

Increased BRB permeability is an early event in rats with hyperglycemia. Evans Blue permeability was measured 3 weeks after STZ-diabetes induction.

Figure 1 shows that Evans blue leakage from retinas of diabetic rats was 2-fold higher than that from non diabetic rats ($p< 0.01$).

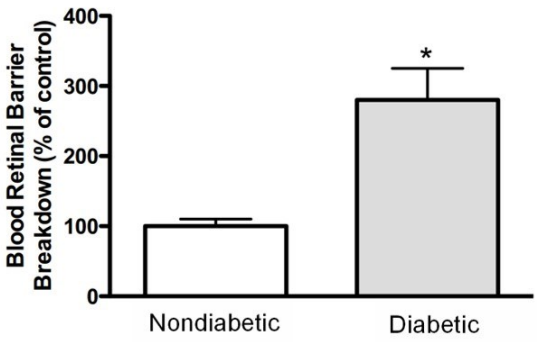

Figure 1 BRB breakdown The Evans Blue leakage increases 2-fold in diabetic rats compared to nondiabetic (*$p<0.01$), 3 weeks after STZ hyperglycemia induction.

3.3 Effects of STZ–induced diabetes on IL-1 family member protein expression

The expression of IL-1 family members was examined by western blot analysis. Figure 2 showed that IL-1 receptor type I and IL-1β levels were significantly increased in diabetic rats retina, compared with nondiabetic (***$p<0.001$). STZ administration resulted also in a small but significant induction of the IL-1 receptor type II and of the receptor antagonist IL-Ra (respectively *$p<0.05$ and **$p<0.01$ *vs.* retina from nondiabetic rats) but no effect was observed on IL-1α expression (Figure 2 A, B).

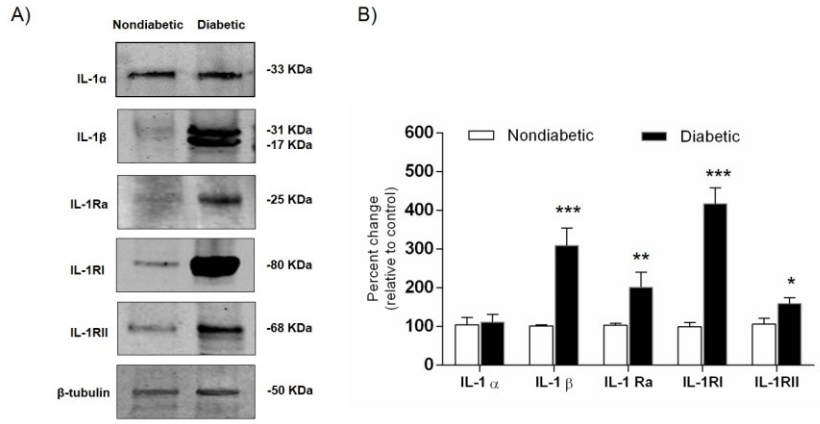

Figure 2 - Hyperglycemia increases the proinflammatory IL-1 signalling, in rat retina. Rats were injected with STZ (60mg/kg, i.p.) and levels of Interleukin-1 alpha (IL-1α), Interleukin-1beta (IL-1β), Interleukin-1 receptor antagonist (IL-1Ra), Interleukin receptor type I (IL1-RI) and type II (IL1-RII) were determined by Western blot analysis. Levels of total IL-1α, IL-1β, IL-1Ra, IL1-RI and IL1-RII were determined to control (β-tubulin) for loading differences. (A) Representative Western blot images for each protein are shown. (B) Results were quantified and are the mean ± SEM, percent of control (n = 6 animals; *p <0.05, **p<0.01 and ***p<0.001 compared with control, Student t test).

3.4 Regional distribution of IL-1 family members

The distribution of the IL-1α, IL-1β, IL-1Ra, IL-1RI, and IL-1RII was detected, using specific antibodies, in the retina of rats after three weeks of STZ injection. The IL-1α is widely distributed in the retina (Figure 3). IL-1α immunoreactivity was mostly found in the photoreceptor layer (layer 1), inner nuclear layer (layer 4), inner plexiform layer (layer 5) and ganglion cell layer (layer 6) but differences are not appreciable between groups, nondiabetic and diabetic retina (Figure 3 A, and B). In a more detail, in the case of the photoreceptor layer, IL-1α is located in the more peripheral part of the outer cone/rod segments. Moreover, in the case of the inner nuclear layer, the fluorescent signals were also observed inside the nuclei of the some cells (Fig. 3 C, and D).

The IL-1β expression and the distribution in retinal layers is different in the nondiabetic respect to the diabetic retina (Figure 4). In the nondiabetic retina, IL-1β is prevalently located in the photoreceptor layer (layer 1), and in a lesser extent in the inner plexiform and in the ganglion cell layers (layer 5 and 6, respectively). In the diabetic retina, the fluorescent signals for IL-1β increased in all layers except in the outer nuclear layer (Fig. 4, A and E). The hybridization signals observed in the photoreceptor layer (layer 1) are present in both inner and outer segments of the cone/rods, and the location seems to be along the cytoplasmic membrane (see Figure 4 B, and F).

IL-1Ra was observed only in the photoreceptor (layer 1) and in the outer plexiform (layer 5) layers, but an increased expression was detected in diabetic retina respect to the non diabetic one (Fig. 5 A, and E). In a more detail, in the plexiform layer (layer 5) we observed IL-1Ra immunoreactivity in the proximal area of the outer nuclear layer (see Fig. 5 G).

A different expression pattern is evident for the IL-1RI (Figure 6A, and B) and IL-1RII (Figure 6C, and D) in nondiabetic retina. IL-1RI (Figure 6A) is distributed in all retinal layers, being almost absent in the outer segment of the photoreceptor layer (layer 1). Instead, IL-1RII is particularly evident in the entire photoreceptor layer (layer 1), in the ganglion cell layer (layer 6), and in the region of the plexiform layer (layer 5) close to the inner nuclear layer (layer 4). The hyperglycemic condition induced by STZ, determines an overall expression of both IL-1RI, and IL-1RII in all the retina layers (Figure 6B, and 6D), with a larger amount of the former respect to the second (see also figure 2A). In the case of IL-1RI, the increase is very evident in the outer nuclear layer (layer 2) on the contrary to that observed for IL-1RII whose increment in the outer nuclear layer (layer 2) is lesser massive than IL-1RI (Figure 6B, and 6D).

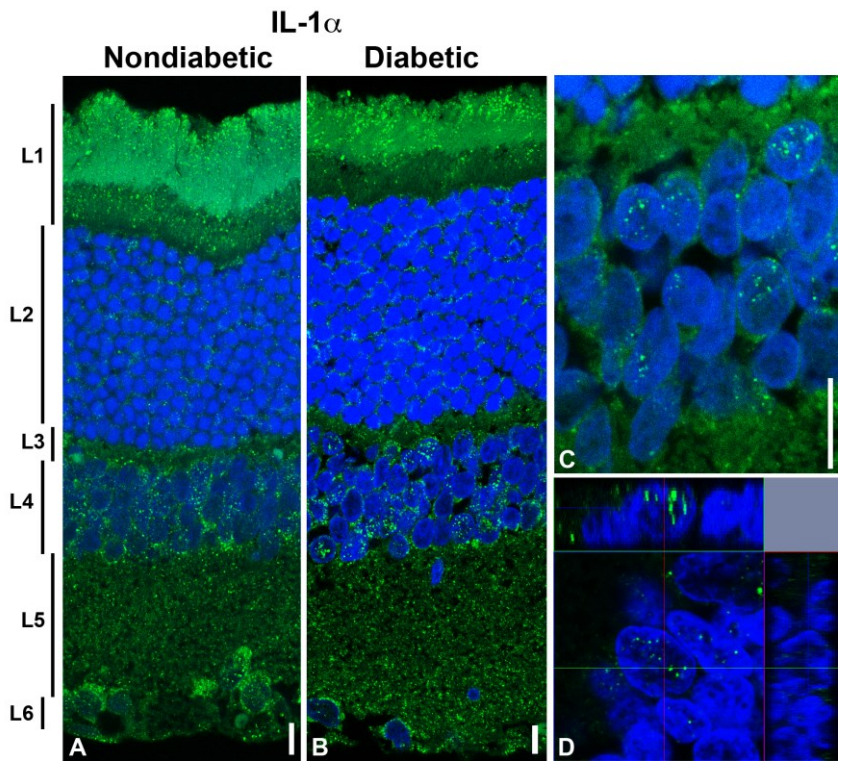

Figure 3 - Distribution of IL-1 α in the normal and diabetic rat retina

Representative images showing distribution of IL-1α in retinal layers of control (A), diabetic rats (B). Retinal distribution of IL-1α was detected using a rabbit anti-IL-1α primary antibody revealed with Alexafluor488 goat anti rabbit (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left part of the figure, as follows: L1: photoreceptors outer and inner segment; L2: outer nuclear layer; L3: outer plexiform layer; L4: inner nuclear layer; L5: inner plexiform layer; L6: ganglion cell layer. C, and D: cells from the inner nuclear layer (diabetic retina) where fluorescence signals are visible in some nuclei. Orthogonal vision of cells from the same layer is shown in panel D. Scale bars: 10 μm.

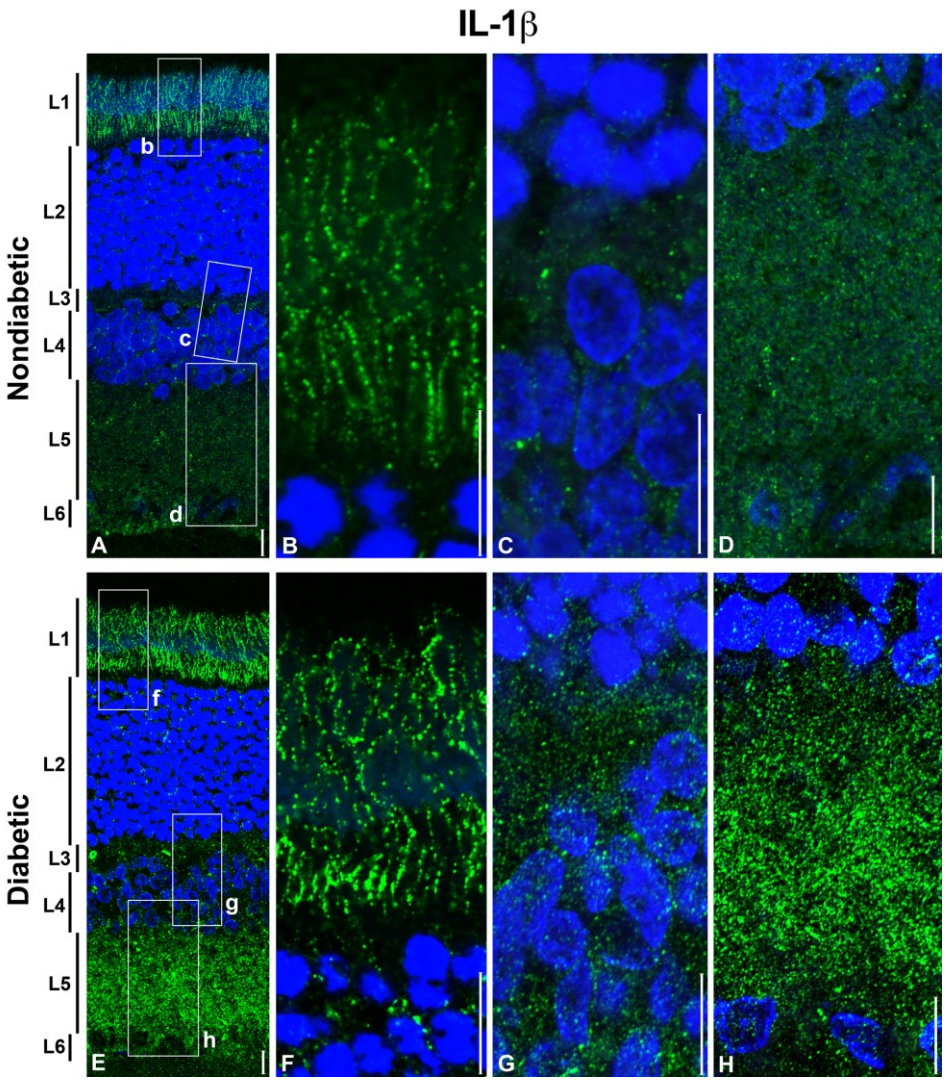

Figure 4 - Distribution of IL-1β in the normal and diabetic rat retina

Representative images showing distribution of IL-1β in the retinal layers of control rats (A, B, C, and D), and diabetic rats (E, F, G, and H). IL-1β was detected using a rabbit anti- IL-1 β primary antibody revealed with Alexafluor488 mouse anti goat (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the figure, as follows: L1: photoreceptors outer and inner segment; L2: outer nuclear layer; L3: outer plexiform layer; L4: inner nuclear layer; L5: inner plexiform layer; L6: ganglion cell layer. B, C, and D show the enlarged sectors indicated in the panel A. F, G, and H show the enlarged sectors indicated in the panel E. Scale bars: 10μm.

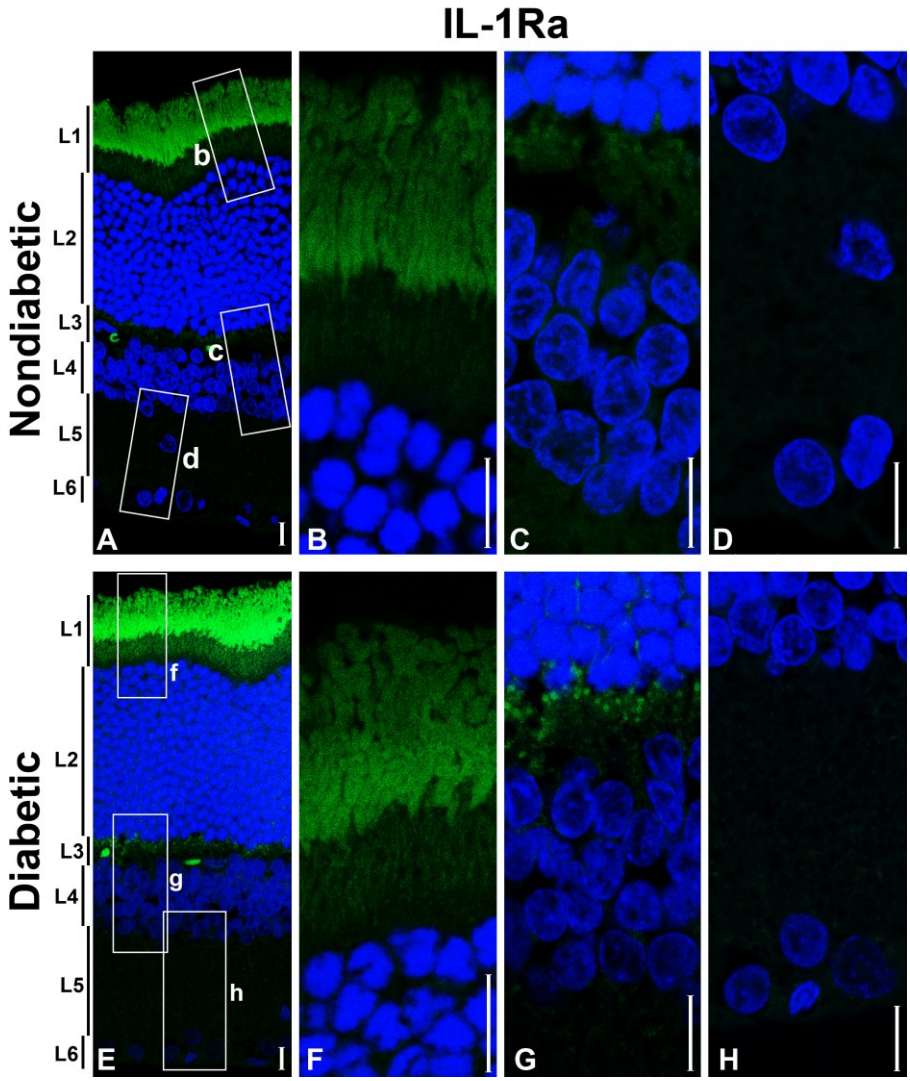

Figure 5 - Distribution of IL-1Ra in the normal and diabetic rat retina

Representative images showing distribution of IL-Ra in the retinal layers of control rats (A, B, C, and D), and diabetic rats (E, F, G, and H). IL-Ra was detected using a rabbit anti- IL-1 β primary antibody revealed with Alexafluor488 mouse anti goat (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the figure, as follows: L1: photoreceptors outer and inner segment; L2: outer nuclear layer; L3: outer plexiform layer; L4: inner nuclear layer; L5: inner plexiform layer; L6: ganglion cell layer. B, C, and D show the enlarged sectors indicated in the panel A. F, G, and H show the enlarged sectors indicated in the panel E. Scale bars: 10μm.

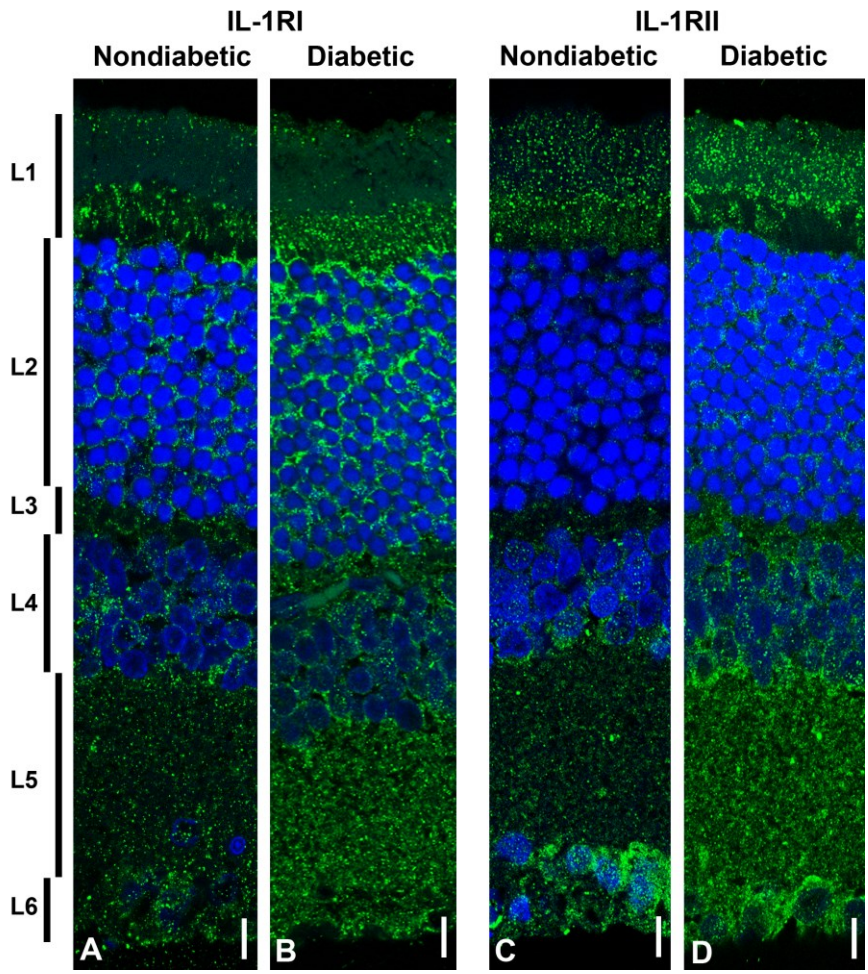

Figure 6 - Distribution of IL-1RI and IL-1RII in the normal and diabetic rat retina

Representative images showing the distribution of IL-1RI (A, and B), and IL-1RII (C, and D), in the retinal layers of control rats (A, and C) and diabetic rats (B, and D) after three weeks of hyperglycaemia. Retinal distribution of IL-1RI and IL-1RII was detected using a rabbit anti- IL-1 RI and anti- IL-1 RII primary antibody revealed with Alexa fluor 488 goat anti rabbit (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Retinal layers are indicated, on the left of the figure, as follows: L1: photoreceptors outer and inner segment; L2: outer nuclear layer; L3: outer plexiform layer; L4: inner nuclear layer; L5: inner plexiform layer; L6: ganglion cell layer. Scale bars: 10μm.

4. Discussion

A conceivable contribution of inflammation to the pathogenesis of DR developed out when diabetic patients treated with salicylates for rheumatoid arthritis had a lower-than-expected incidence of DR [91]. Since then, varieties of physiological and molecular abnormalities that, are consistent with inflammation, have been found altered in the retinas or in the vitreous humor of diabetic animals and patients (Yuuki, et al., 2001). Hyperglycaemia has recognized as the main committer of both neural and vascular inflammation in DR. Thus, during hyperglycemic conditions excessive oxidative stress and reduce neutralizing mechanisms lead to generation of important inflammatory molecules. Increased proinflammatory cytokines, such as interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα) and interleukin-1-β (IL-1β) have been found in DR and other ocular diseases [79, 91, 92]. Although IL-1β has been well described in literature, as a mediator of early retinal damage in DR [21, 75, 81], in this context, no attention has been given to the other IL-1 family members, whom all together, participate and modulate the inflammatory cascade. In the present study, we show that hyperglycaemia affects most of the IL-1 molecules analyzed, and we point out their importance, in the inflammatory scene of DR. Diabetic retinas showed a significant increased levels of IL-β and IL-1RI ($p<0.001$) and in lesser extent of IL-Ra and IL-1RII ($p<0.01$ and $p<0.05$ respectively) compared to nondiabetic ones (Figure 1). IL-1β results are consistent with previous data, that have also demonstrated that once released, IL-β triggers other inflammatory mediators and lead to tissue damage [86]. Notable, IL-1β is able to induce its own synthesis via autocrine/paracrine auto stimulation, acting as an amplifier of inflammation [93]. The observed up regulation of IL-1RI, essential IL-1β transduction element, apparently seems to be in contrast with a previous work [94] in which the authors described a decrease

of IL-1RI under diabetic conditions as well as its nuclear translocation. However, it is important to consider the experimental model used, an *in vitro* model of retinal endothelial cells, a condition reasonable more different if compared to the whole scenario of the retina, analyzed here.

Hyperglycaemia alters also the expression of the other IL-1 antagonist, IL-1Ra and IL-1RII. Since its discovery, IL-1Ra was considered the major natural inhibitor of IL-1 action and an important *in vivo* regulator of IL-1 action [82]. Furthermore, it has been demonstrated that IL-1Ra protects retinal endothelial cells against the degeneration induced by nitric oxidative stress and cell death induce by glucose [21]. However, our results demonstrate that, its expression even if follow the trend of the other inflammatory trigger elements, seems to be not sufficient to counteract their overwhelming effects. This is supported also, by our earlier works in which, soon after establishment of diabetes, we demonstrated that features of retinal neurodegeneration (e.g. apoptosis) are already present together with impaired signalling survival pathways and overexpression of activated caspase 3 and p53 [54, 95]. As expected, apoptotic retinal cell death resulting from the inflammatory cascade, causes a reduction in the thickness of various layers i.e. ganglion cell layer, inner and outer nuclear layer. This observation were made both in animal models of DR and clinically [96, 97]. Similarly, we found decreased thickness of retina especially in the ganglion cell layer of diabetic retina (Fig 3).

This is the first detailed immunohystochemical study on the distributions of IL-1 family members in the retina. All receptors examined (IL-1RI and IL-1RII) are present in the retina and show partially overlapping distribution, between retinal layers. IL-1RI is almost absent in the outer segment of the photoreceptor layer where instead IL-1RII is particularly abundant. IL-1α is widely distributed in the retina, especially in the photoreceptor layer, inner

nuclear layer, inner plexiform layer and ganglion cell layer, while IL-1β is prevalently located in the photoreceptor layer, and in a lesser extent in the inner plexiform and in the ganglion cell layers.

The hyperglycemic condition determines not only changes at protein expression levels, but also affect the regional distribution of these inflammatory elements. In fact, in the diabetic retina, IL-1RI become detectable in the outer nuclear layer, and this change is concomitant with the increased expression of IL-1β in the same retinal layer. This highlights the hyper-activation of an important inflammatory pathway, in an anatomical region strictly connected with the BRB, seriously compromised during DR.

Moreover, nuclear retention of IL-1α was observed in the inner nuclear layer of the diabetic retina. The finding that IL-1α can be immobilized in nuclear was previously demonstrated [98], and the same authors proposed it as a mechanism for attenuating inflammation caused by the death of IL-1α-expressing cells.

Taken together, these findings indicate that all of these IL-1 molecules may play distinct roles in the context of retinal damage and homeostasis during DR. The inflammatory scenario is clearly compromised by hyperglycaemia even though are present mechanisms that attempt to negatively regulate the inflammatory response in DR.

Our studies offer a new rational approach to develop new IL-1β receptor antagonists (already being tested for other inflammatory and immune disorders) capable to cross the blood retinal barrier and inhibit the development of inflammatory process of DR in each specific retinal layers.

Conclusions

Intensive and systemic glycemic control remains the most effective therapy for DR. However, localized treatment of the retina is required for vision threatening retinopathy and certainly, the IL-1 signaling system is an attractive therapeutic target that might be implemented.

6. Acknowledgements

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Chapter II

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Davunetide (NAP) protects the retina against early diabetic injury by reducing apoptotic death

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Abstract

NAP (davunetide) is an eight amino acid peptide that has been shown to provide potent neuroprotection. In the present study we investigated the neuroprotective effect of NAP in diabetic retinopathy using an in vivo streptozotocin (STZ)-induced diabetic model. A single intraocular injection of NAP (100μg/ml) or vehicle was administered 1 week after STZ injection. Three weeks after diabetes induction we assessed the retinal expression and distribution of apoptosis markers, cleaved caspase-3 and Bcl2, by using Western blot and immunofluorescent analysis. Furthermore, we evaluated the activation of MAPK/ERK and/or PI-3K/Akt pathways by measuring the protein levels of p-ERK and p-AKT with or without NAP treatment. Results demonstrated that NAP treatment reduced apoptotic event in diabetic retina, it restored increased cleaved caspase-3 expression levels in the retina of STZ-injected rats as well as decreased Bcl2. NAP treatment improved cellular survival through the activation of MAPK/ERK pathway. Taken together,

these findings suggested that NAP might be useful to treat retinal degenerative diseases.

Keywords: davunetide; NAP; diabetic retinopathy; streptozotocin; retina; apoptosis; MAPK/ERK pathway

1. Introduction

Diabetic retinopathy (DR), is one of the leading causes of blindness among the working age population in western countries (Pan et al., 2008; Yamagishi and Matsui 2011). The pathogenesis of DR is highly complex owing to the involvement of multiple interlinked mechanisms leading to cellular damage and adaptive changes in the retina (Frank 2004). Several evidences indicate that retinal complications of diabetes are a composite of functional and structural alterations in both the microvascular and neuroglial compartments (Curtis et al., 2009; Villaroel et al., 2010). One of the early manifestations of DR is persistent apoptosis of vascular and neural cells in the retinal tissue (Barber et al.,2011; Giunta et al., 2012a); glial reactivity, loss of ganglion cell bodies and reduction in thickness of the inner retinal layers have also been described (Van Dijk et al.2001). In the late stages of DR can also occur diabetic macular edema as consequence of blood-retinal barrier breakdown which cause vision loss (Hu et al., 2012; Scuderi et al., 2013).

However the exact mechanisms by which hyperglycemia initiates the vascular or neuronal alterations in retinopathy have not been completely defined, it is widely accepted that hyperglycemia-induced oxidative stress due to an imbalance between production of reactive oxygen species (ROS) and their neutralization by antioxidants (Barber et al.,2011). Increased oxidative stress has been shown to promote the activation of both apoptotic (Barber et al.1998, Kowluru et al., 2004, Park et al., 2003) and inflammatory mechanisms (Yuuki et al., 2001), as well as variations in growth factor levels (Paques et al.,1997; Schlingemann et al.,2004; Grant et al.,2004).

Recently, we demonstrated (Giunta et al., 2012a) that hyperglycemia induces the over-expression of two apoptotic–related genes in the retina, in an *in vivo* model of diabetes induced by systemic administration of streptozotocin (STZ). Furthermore we showed significant changes in the retinal expression

of two peptides, PACAP and VIP suggesting a role for both peptides in the pathogenesis of DR (Giunta et al., 2012a).

It has been shown that the neuroprotective actions of both peptides, PACAP and VIP, are mediated by two different glial derived proteins: activity-dependent neurotrophic factor (ADNP) (Brenneman and Gozes, 1996), and activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Nakamachi et al., 2006). The peptide NAPVSIPQ (NAP, also known as davunetide or AL-108) is an octapeptide that was identified as the smallest active element of ADNP. This peptide has neuroprotective effect at femtomolar concentration both in cells cultures and in animal models (Steingart et al. 2000; Gozes et al.,2000, Ashur-Fabian et al.2003; Gozes et al., 2004; Toso et al. 2007). NAP was shown to be involved in microtubule re-organization and in transient increases of non-phosphorylated tau levels (Gozes and Divinski, 2004). It was also demonstrated that this octapeptide promotes neuronal growth and differentiation and its protective actions are mediated by the activation of MAPK/ERK and PI-3K/AKT (Pascual and Guerri, 2007).

NAP has demonstrated protective effects in various models of retinal injuries, it protects retinal ganglion cells against damage induced by retinal ischemia and optic nerve crush (Jehle et al.,2008); it prevents hypoxia-induced rat retinal Muller cells injuries and promotes retinal neurons growth (Zheng et al., 2010). NAP has also ameliorative effect on laser-induced retinal damage (Belokopytov et al.,2011). Importantly, NAP penetrates the BBB and is non-toxic (Leker et al., 2002; Gozes et al., 2004).

Although it has been suggested that NAP prevents some diabetes-related brain pathologies in the STZ injected rat model (Idan-Feldman et al.,2011), to date there are not evidences in literature regarding the effect of this peptide in DR.

In the present study we investigated the potential neuroprotective role of the neuropeptide NAP in a model of DR. We observed that hyperglycemia induced changes in ADNP mRNA retinal expression level in STZ injected rats after 3 weeks. Based on these findings, it would appear that the protective potential of endogenous ADNP is compromised in diabetic retinas, we therefore hypothesized that NAP treatment might have protective role in DR ameliorating in part dysfunction associated with ADNP reduction. To this end we evaluated the effect of NAP treatment on expression of ADNP and two apoptotic–related genes, Bcl-2 and cleaved caspase-3, in diabetic rat retina.
Finally, to test the signalling cascade which mediated the protective effect of NAP we monitored the activation of mitogen activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and of the phosphatidylinositol-3kinase (PI-3K)/AKT.

2. Materials and Methods

2.1 Animals

Three-months-old Male Sprague–Dawley rats weighing approximately 200 g were obtained from Charles River (Calco, Italy). All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were fed with standard laboratory chow and were allowed free access to water in an air–conditioned room with a 12 h light / 12 h dark cycle. Final group sizes for all measurements were n = 6–9 except as noted.

2.2 Induction of Diabetes

Induction of diabetes was performed as previously described (Bucolo et al., 2012). After 12 h of fasting, the animals received a single 60 mg / kg

i.v. injection of STZ (Sigma – Aldrich, St. Louis, MO, USA) in 10 mM sodium citrate buffer, pH 4.5 (1 mL / kg dosevolume). Control (nondiabetic) animals were fasted and received citrate buffer alone. After 24 h, animals with blood glucose levels greater than 250 mg / dL were considered diabetic and randomly divided into groups of six animals each. We performed all experiments 1 week and 3 weeks after the induction of diabetes. We confirmed the diabetic state by evaluating glycemia daily using a blood glucose meter (Accu–CheckActive; Roche Diagnostic, Milan, Italy). For subsequent experiments, rats were killed with a lethal i. p. dose of sodium pentobarbital. Eyes were rapidly enucleated. Retinas were harvested, frozen in liquid nitrogen and stored at −80 °C until use.

2.3 Intravitreal administration of NAP

A single intraocular injection of 100 µg/ml of NAP (New England Peptide, UK) dissolved in PBS solution (final volume = 4 µL), as previously described by Jehle et al.(2008), was administered 1 week after STZ injection or sodium citrate buffer under general anesthesia with diethylether and topical anesthesia with a drop of 2 % lidocaine applied to the eyes. NAP was injected into the vitreous space of one eye chosen at random. An equal volume of vehicle (PBS) was injected in the other eye as control. Retinas were dissected 2 weeks after the intraocular injection.

2.4 Real-time quantitative PCR analysis

Single stranded cDNAs were synthesized and amplified as previously described (Giunta et al. 2012b). Aliquots of cDNA were amplified using specific primers for ADNP as well as for the S18 ribosomial subunit. Oligonucleotide sequences are listed in Table 2.

Aliquots of cDNA (400 ng) from nondiabetic (control) and 1 or 3 weeks diabetic retinas with vehicle or with NAP, were amplified in parallel reactions

with external standards at known amounts (purified PCR products, ranging from 10^2 to 10^8 copies) using specific primer pairs listed in Table 2. To control samples mRNA integrity and any eventual error attributable to experimental mishandling, mRNA levels of the 18s ribosomal RNA subunit (reference gene) were measured in each amplification. Each amplification was carried out as previously described (Castorina et al. 2012).

To assess the different expression levels we employed the well–established ΔCt comparative method (Schmittgen and Livak 2008). For quantification of each gene we considered healthy retinas from uninjected rats as our positive samples (calibrator sample). Retinas from vehicle– or STZ–injected rats after 1 or 3 weeks were considered as unknown samples, respectively.

2.5 Western blot analysis

Western blot analysis was performed to determine the relative levels of the two apoptotic-related genes Bcl2 and cleaved caspase-3, and to evaluate the activation of mitogen activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK) and phosphatidylinositolo-3-kinase (PI-3K)/Akt pathways using specific antibodies. Analysis was performed as previously described by Scuderi et al. 2013. Briefly, proteins were extracted with buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose and a protease inhibitor cocktail (Roche Diagnostics) using a Teflon-glass homogenizer and then sonicated twice for 20 sec using an ultrasonic probe, followed by centrifugation at 10.000 g for 10 min at 4 °C. Protein concentrations were determined by the Quant-iT Protein Assay Kit (Invitrogen). Sample proteins (27 μg) were diluted in 2X Laemmli buffer (Invitrogen, Carlsbad, CA, USA), heated at 70°C for 10 min and then separated on a Biorad Criterion XT 4-15% Bis-tris gel (BIO-RAD) by electrophoresis and then transferred to a nitrocellulose membrane (BIO-

RAD). Blots were blocked using the Odyssey Blocking Buffer (LI-COR Biosciences) and probed with appropriate antibodies: rabbit anti-βtubulin (sc-9104, Santa Cruz Biotechnology; 1:500), goat anti-caspase-3 (sc-1225, Santa Cruz Biotechnology; 1:100); rabbit anti-Cleaved Caspase-3 (#9661, Cell Signaling; 1:500), mouse anti-Bcl2 (IS614, DAKO; 10µl/ml), mouse anti-ERK (sc-135900, Santa Cruz Biotechnology; 1:200), goat anti-p-ERK (sc-16982, Santa Cruz Biotechnology; 1:200), mouse anti-AKT(sc-5298, Santa Cruz Biotechnology; 1:200), mouse anti-p-AKT(#4051, Cell Signaling; 1:1000). The secondary antibody goat anti-rabbit IRDye 800CW (#926-32211; LI-COR Biosciences), donkey anti-goat IRDye 800CW (#926-32214 LI-COR), goat anti-mouse IRDye 680CW, (#926-68020D; LI-COR Biosciences) were used at 1:20000. Blots were scanned using an Odyssey Infrared Imaging System (Odyssey). Densitometric analysis of Western blot signals was performed at non saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

2.6 Tissue preparation for immunofluorescence analysis

Eyes were enucleated and fixed overnight with 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.6). Prior to immunofluorescence staining, paraffin-embedded retinas sections mounted on glass slides, were dewaxed in xylene and rehydrated through graded alcohols. Sections were put into Rodent Decloaker 1X retrieval solution (RD913, BIO-CARE MEDICAL) in a slide container and then incubated with the anti-Cleaved Caspase-3 or the anti-phosphorylated ERK primary antibodies. Tissue sections were then incubated with Alexa fluor 488 goat anti-rabbit and Alexa fluor 488 donkey anti-goat secondary antibodies, respectively for 1.5 h at room temperature and shielded from light. DAPI was used to stain nuclei (#940110 Vector Laboratories). Sections were examined under Confocal Laser Scanning

Microscopy (CLSM) (Zeiss LSM700) and images were taken at ×40 magnification. Experiments were repeated at least three times to confirm results. Relative quantification of immunofluorescence staining was performed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) as previously described by Arqués et al., 2012.

2.7 Statistical analysis

Data are reported as mean ± S.E.M. Statistical analyses were performed using specific software GraphPad Prism-5. One-way analysis of variance (ANOVA) was used to compare differences among groups and statistical significance was assessed by Tukey-Kramer *post-hoc* test. The level of significance for all statistical tests was $p \leq 0.05$.

3. Results

3.1 Effects of STZ–induced diabetes on body weight and blood glucose levels 3 weeks

Table 1 shows the effects of STZ treatment on body weight and non-fasting blood glucose levels in rats after 3 weeks. Body weight and glycemia of rats before treatments were approximately 200 g and 100mg/dL respectively. The statistically analysis revealed a significant decrease in body weight 3 weeks after STZ injection ($p<0.01$ *vs.* nondiabetic control group). Twenty days after onset of diabetes, blood glucose values in diabetic rats were significantly ($p<0.01$) higher than corresponding values in nondiabetic rats (391 ± 35 and 98 ±18 mg/dL respectively).

Groups	Body weight (*g*)	Nonfasting blood glucose (*mg/dl*)
Control (normal)	285 ± 30	98 ±18
Nondiabetic (sham)	280 ± 25	101 ±25
Diabetic (STZ)	210 ± 20**	391 ± 35**
Diabetic+ NAP	230 ± 35	380 ± 20

Table 1.

Effects of STZ-induced diabetes on body weight and blood glucose level after three weeks. Control (normal), vehicle injected (sham non-diabetic), STZ injected (diabetic), STZ + NAP injected (diabetic + NAP). Diabetes was induced by 60mg/Kg (i.p) injection of STZ. Data are expressed as mean ±S.D.

**$p<0.01$ vs nondiabetic control rats

3.2 Effects of STZ–induced diabetes on ADNP mRNA expression in rat retinas after 1 and 3 weeks

To establish whether administration of STZ affected early retinal expression of ADNP mRNA, quantitative real–time PCR analyses were carried out using rat retina cDNAs obtained from uninjected (which was used as internal calibrator), vehicle– or STZ–injected animals after 1 and 3 weeks.

Induction of diabetes by STZ administration caused a statistical significant ($p<0.01$) decrease of ADNP mRNA at 3 weeks compared to nondiabetic control retinas ($p<0.01$ *vs.* nondiabetic control retina) (Fig.1)., no changes were observed after 1 week. NAP treatment restores ADNP transcript levels to control values in retinas of diabetic rats after 3 weeks (###$p<0.001$ *vs* diabetic retinas) (Fig.1).This result suggest that some diabetes effects on retinas degeneration are, at least in part, mediated by diabetes-induced impairment of the levels of ADNP.

Gene	Forward	Reverse	bp length
Activity-dependent neuroprotective protein ADNP Acc# NM_022681.2	AGGCTGGGGCTAGGTGGCAA	GGGGCCTCTGCTCAGCACCT	118
Ribosomal protein S18 Acc# NM_213557.1	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	110

Table 2. Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column.

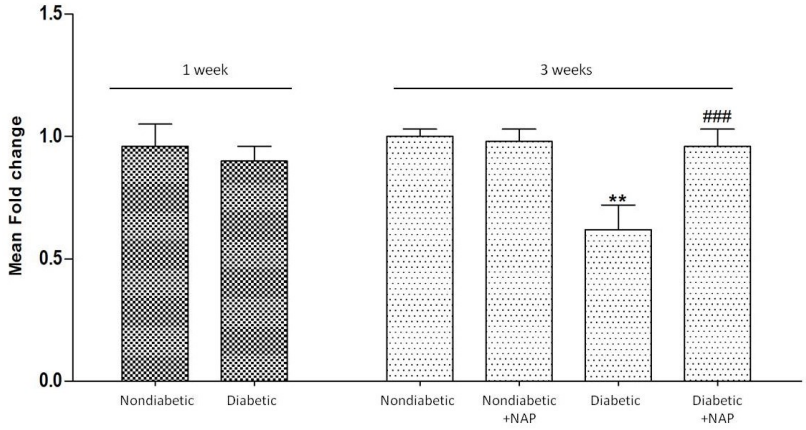

Fig. 1 ADNP mRNA expression levels in rat retinas after 1 or 3 weeks streptozotocin injection. Results are presented as mean fold changes of gene expression in retinas of nondiabetic (control) and diabetic rats intravitreally injected either with vehicle or NAP (100µg/ml) at the indicated times (n=6) ± S.E.M. Relative fold changes of ADNP expression level were normalized to the endogenous ribosomal protein S18 (housekeeping gene) and then calculated using the comparative ΔCt method. Baseline expression levels were set to 1. Results are representative of at least three independent experiments. **p<0.01 *vs* non diabetic retinas ###p<0.001 *vs* diabetic retinas at the indicated times; One way ANOVA followed by Tukey's Multiple Comparison Test.

3.3 NAP up-regulated the expression of Bcl2 and downregulated the expression of cleaved caspase-3 in the rat retinas after 3 weeks of STZ-injection

Apoptosis can contribute to the neuronal loss in the diabetic retina. In order to determine whether NAP plays a preventive role in diabetes-induced apoptosis, the expression of Bcl2 and cleaved caspase-3, two apoptosis-related proteins, were examined by western blot analysis. Fig.2 panel a. shows that Bcl2 levels, an anti-apoptotic cell death protein, are reduced in diabetic rats retina, compared to nondiabetic control (0.46 ± 0.02 *vs* 0.25± 0.08 respectively, p<0.001 *vs.* retinas from nondiabetic rats injected

intravitreally with vehicle alone). Caspase-3 is a cysteine protease that plays a critical role in the induction of apoptosis, activation of caspase-3 indicates that cells entered an apoptotic pathway. Cleaved caspase-3 expression level increased ($p<0.001$) in the diabetic rats as compared to nondiabetic (2.68±0.10 *vs* 1.88 ± 0.04 respectively) (Fig.2 panel b). NAP administration decreased the diabetes-induced cell death in retina. In fact NAP treatment upregulated the expression of Bcl2 (0.43±0.06 *vs* 0.25±0.08, $p<0.01$) and downregulated cleaved caspase-3 (2.20±0.16 vs 2.68±0.10, $p<0.001$) compared with diabetic rats injected intravitreally with vehicle alone respectively (Fig.2 panel a and b).

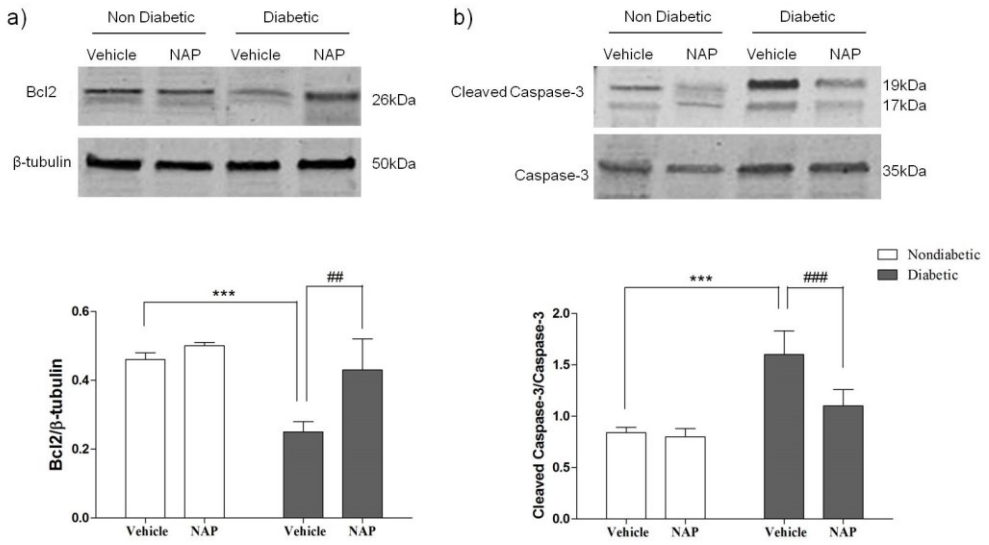

Fig. 2 Effect of intravitreal injection of NAP on Bcl-2 and Cleaved Caspase 3 protein expression in the retina of STZ-injected rats after 3 weeks. The Bcl2 and Cleaved Caspase-3 protein expression levels was evaluated in retinas of nondiabetic or diabetic rats intravitreally injected either with vehicle or NAP (100µg/ml) after 3 weeks by western blot analysis (n=6 for each group). Representative bands shown indicate that intravitreal NAP is effective in restoring

STZ- induced changes on retinal Bcl2 and Cleaved Caspase-3 expressions to nondiabetic levels. β-tubulin was used as loading control in each experiment. Relative bands density were quantified using the ImageJ software and normalized values were plotted in the histograms shown in the lower. Bar graph shows the quantification of gels of three independent experiments. Data are expressed as mean ± S.E.M. (***p<0,001 vs retinas from nondiabetic rats injected intravitreally with vehicle alone; ##p<0,01 or ###p<0,001 vs retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test).

3.4Regional distribution of cleaved caspase-3 proteins diabetic rat retinas after 3 weeks

Using a well characterized antibody we demonstrated the presence and localization of cleaved caspase-3 in the retina of nondiabetic or diabetic rats intravitreally injected either with vehicle or NAP (100µg/ml) after 3 weeks. Immunohistochemical analysis showed that cleaved caspase-3 immunoreactivity was evident in the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL) and especially in the outer plexiform layer (OPL) of diabetic rats (Fig. 3, panel b and e). NAP treatment seems to induce a reduction of cleaved caspase-3 immunosignal in these layers (Fig.3, panel c and f). Data were validated by relative quantification of immunofluorescence (*p<0.05; **p<0.01 and ***p<0,001 vs retinas from nondiabetic rats injected intravitreally with vehicle alone; #p<0.05, #p<0,01; ###p<0.001 vs retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test (Fig. 3 g) . Fluorescence of outer photoreceptor segment is in part an artifact caused by autofluorescence detected in control sections (data not shown), activated by the 488 laser line with emissions collected between 400 and 500 nm.

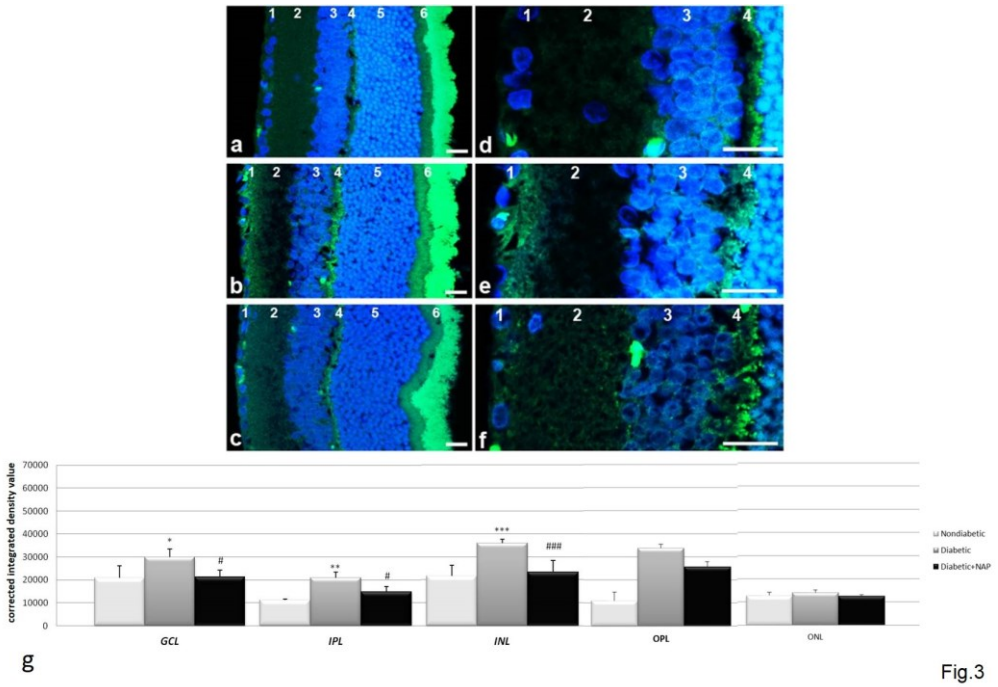

Fig.3

Fig. 3 Localization of activated Caspase-3 in the retina of STZ–injected rats after 3 weeks

Representative images showing distribution of Cleaved Caspase-3 positive cells in the different retinal layers of control rats (a, d), diabetic rats injected with vehicle (b, e), and diabetic rats injected with NAP (c, f) after three weeks. Retinal distribution of cleaved Caspase-3 was detected using a rabbit anti-Cleaved Caspase-3 primary antibody revealed with Alexafluor488 goat anti rabbit (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Relative fluorescence were quantified using the ImageJ software and normalized values were plotted in the histograms shown in the lower (g). Bar graph shows the quantification of different fields from randomly selected slides. Data are expressed as mean ± S.D. (*$p<0.05$; **$p<0.01$ and ***$p<0,001$ vs retinas from nondiabetic rats injected intravitreally with vehicle alone; #$p<0.05$, ###$p<0.001$ vs retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test).

Retinal layers are indicated as follows: 1: ganglion cell layer; 2: inner plexiform layer; 3: inner nuclear layer; 4: outer plexiform layer; 5: outer nuclear layer; 6: photoreceptors outer and inner segment. Scale bars: 20µm.

3.5Effect of intravitreal injection of NAP on p-ERK and p-AKT protein expression in the rat retinas after 3 weeks of STZ-injection

To figure out the molecular mechanism mediating the protective effect of NAP, the protein levels of ERK1/2 (both total and phosphorylated) and AKT (both total and phosphorylated) were measured in diabetic retina. Modest changes of p-ERK/total ERK ratio was observed in diabetic retina compared to control (0.89±0.3 *vs* 1.43±0.09, $p<0.05$), demonstrating that the survival pathway of MAPK is partially compromise in hyperglycemic rats retina. Treatment with NAP induced the activation of MAPK pathway both in control, even if not significant, and in diabetic group in a very significant manner compared to control animals (3.04±1.43±0.09, $p<0.001$ Fig.4 a)

Whereas p-AKT/total AKT ratio was significantly decreased in diabetic retina compared to nondiabetic rats injected intravitreally with vehicle alone (0.15±0.06 *vs* 0.52±0.03, $p<0.01$). but no significant changes was found in the retina of diabetic rats treated with NAP (0.13±0.08 *vs* 0.15±0.06, ns) (Fig. 4, panel b).

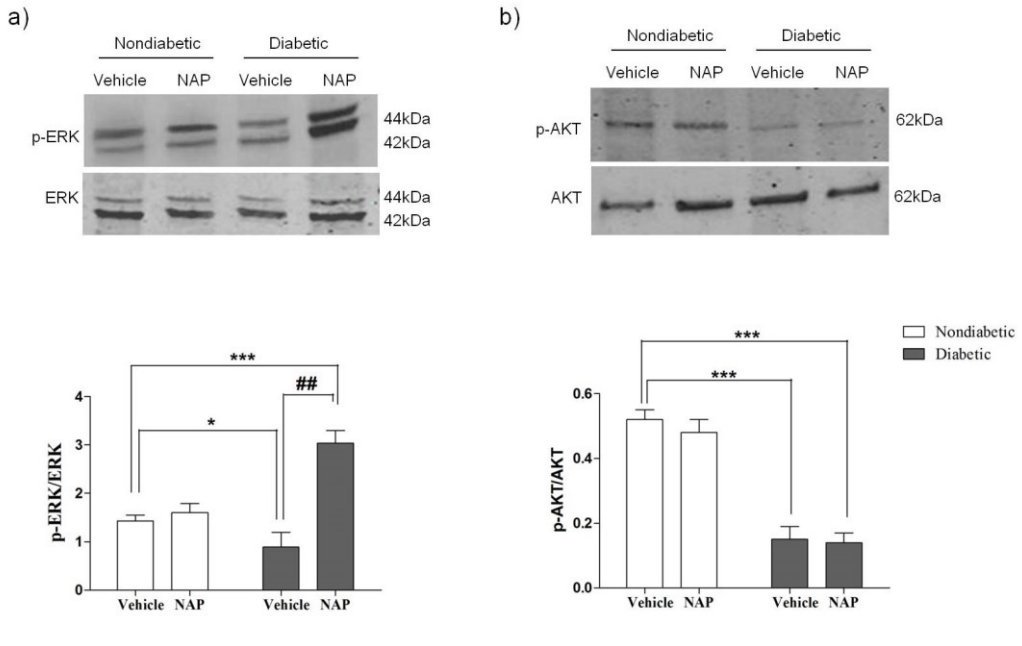

Fig.4

Fig. 4 Effects of NAP on p-ERK and p-AKT proteins expression in the retina of STZ-injected rats after 3 weeks. The expression of p-ERK and p-AKT proteins was evaluated in retinas of non diabetic and diabetic rats intravitreally injected either with vehicle or NAP (100µg/ml) after 3 weeks by Western blot analysis (n=6 for each group). β-tubulin was used as loading control in each experiment. Relative bands density were quantified using the ImageJ software and normalized values were plotted in the histograms shown in the lower. Bar graph shows the quantification of gels of three independent experiments. Data are expressed as mean ± S.E.M. (*p<0,05 *vs* retinas from nondiabetic rats injected intravitreally with vehicle alone ***p<0,001 *vs* retinas from nondiabetic rats injected intravitreally with vehicle alone ##p<0,001 *vs* retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test).

3.6 Regional distribution of pERK proteins in diabetic rat retina after 3 weeks

Three weeks after the induction of diabetes, p-ERK immunoreactivity seems to be lower in the outer plexiform layer (OPL) as compared to control (Fig. 5, panel b and e). NAP treatment induces an induction of p-ERK immunosignal

in the GCL, IPL, INL and OPL as compared to controls (Fig. 5 panel c and f). Data were validated by relative quantification of immunofluorescence (*p<0.05 and ***p<0,001 vs retinas from nondiabetic rats injected intravitreally with vehicle alone; #p<0,01 and ###p<0.001 vs retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test).

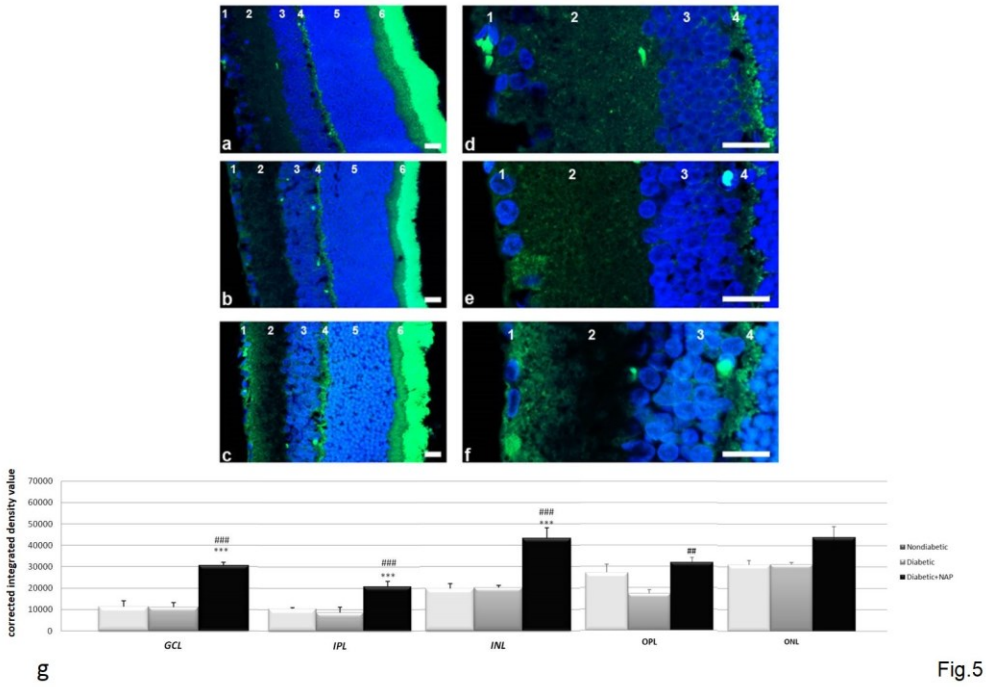

Fig.5

Fig. 5 pERK expression in the retina of streptozotocin-induced diabetic rats. Representative images showing distribution of p-ERK in the different retinal layers of control rats (a, d), diabetic rats injected with vehicle (b, e), and diabetic rats injected with NAP (c, f) after three weeks.Retinal distribution of p-ERK was detected using a goat anti- p-ERK primary antibody revealed with Alexafluor488 donkeyanti goat (green fluorescence). Cell nuclei were stained with DAPI (blue fluorescence). Images shown are representative results taken from different fields from randomly selected slides and scanned by CLSM. Relative fluorescence were quantified using the ImageJ software and normalized values were plotted in the histograms shown in the lower (g). Bar graph shows the quantification of different fields from randomly selected slides. Data are expressed as mean ± S.D. (**p<0.01

and ***p<0,001 vs retinas from nondiabetic rats injected intravitreally with vehicle alone; ###p<0.001 vs retinas from diabetic rats injected intravitreally with vehicle alone; One-way ANOVA followed by Tukey's Multiple Comparison Test).Retinal layers are indicated as follows: 1: ganglion cell layer; 2: inner plexiform layer; 3: inner nuclear layer; 4: outer plexiform layer; 5: outer nuclear layer; 6: photoreceptors outer and inner segment. Scale bars: 20μm.

Discussion

The present study demonstrated that NAP exerts a protective action against early retinal neurodegeneration induced by hyperglycemia in an *in vivo* model of DR. It has been well established that NAP is the smallest member identified within the ADNP family, having potent neuroprotective activity also against different type of retinal injuries (Pascual et Guerri 2007; Jehle et al.,2008; Belokopytov et al.,2011;). It has been recently discovered that peptidergic mechanisms are potentially important in drug development strategies. One of the most promising peptidergic pathways for which treatment strategies may be developed at present is pituitary adenylyl cyclase-activating polypeptide (PACAP)-related pathway (Gabriel, 2013).

In a recent study (Giunta et al. 2012a) we demonstrated that PACAP and VIP, pleiotropic peptides acting as neurotransmitter, neuromodulator and neurotrophic factor (Arimura et al., 1994; Gressens et al., 1999) are modified in the retina during early stages of diabetes, and that intraocular PACAP treatment provided protection in the diabetic retina. These data suggest that both peptides, PACAP and VIP, play an important contribute in the pathogenesis of DR (Giunta et al. 2012a). It is well known that PACAP's and VIP's neuroprotective action is mediated by two different glial derived proteins: activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996) and activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999; Nakamachi et al. 2006; Castorina et al., 2012). According with our previous paper (Giunta et al. 2012a), we observed that retinas exposed to hyperglycemia display a decrease in the mRNA levels of the protective mediator ADNP. This demonstrates that the protective potential role of endogenous ADNP is dampened in diabetic retinas in the early phase of experimental diabetes, confirming that ADNP expression is modulated by

hyperglycemia as PACAP and VIP, and suggesting that some hyperglycemia insults on retinal functions are, at least in part, mediated by impairment of ADNP.

ADNP expression has been shown to be regulated by other injury, such as different kind of brain damage, head trauma (Zaltman et al., 2004; Gozes et al., 2005;), exposure to anesthetic gas xenon (Cattano et al.,2008), exposure to alcohol in fetal alcohol syndrome (Poggi et al.,2003; Pascual and Guerri, 2007). Interestingly, the octapeptide NAP, derived from ADNP, has shown protection against the consequences of ADNP deficiency/mis-metabolism in ADNP+/-mice (Vulih-Shultzman et al.,2007), in models of closed head injury (Beni-Adana et al., 2001; Zaltman et al.,2005) and in models of fetal alcohol syndrome (Sari and Gozes, 2006; Pascual and Guerri, 2007; Spong et al.,2001). Therefore, NAP was tested into our *in vivo* model of DR to investigate its efficacy in counteract some aspects associated with retinal neurodegeneration.

It is well-documented that hyperglycemia leads to apoptotic death of both retinal neurons and vascular cells, immediately after the onset of diabetes (Barber et al., 1998; Li et al., 2002; Mohr, 2002; Kusner et al., 2004; Xi et al., 2005; Liu et al., 2013). Retinal cell death has been widely held as a central event that leads to retinal neurodegeneration, vascular dysfunction and eventually irreversible blindness in ocular diseases such as DR (Jing et al., 2011). Neurotrophic and neuroprotective actions of ADNP/NAP are well documented in numerous pathologies, including retinal injuries (Jehle et al., 2008; Belokopypov et al., 2011) and previous studies have shown NAP protection against apoptotic death (Busciglio et al., 2007; Zemlyak et al., 2009, Idan-Feldman et al., 2012).

In order to assess retinal damage we analyzed the protein expression levels of two apoptotic related genes, Bcl2 and caspase-3. Western blot analysis

indicates that high glucose levels increased the expression of cleaved caspase3 and downregulated Bcl2, in retina from STZ rats (Fig.2.a and b).

The upregulation of cleaved caspase3 levels was confirmed also by confocal microscopy analysis. Accordingly to previous studies (Kim et al., 2010; Qin et al., 2011) we observed sparse cleaved caspase-3 signals in the INL, GCL and OPL of diabetic retina (Fig.3, b and e). Based on these findings it is possible to hypothesize that in our *in vivo* model of DR most of the retinal cells are involved in apoptotic process, although cells morphology did not show evident alterations. In fact activation of caspase-3 plays an extremely important role in neuronal apoptosis and is considered the terminal event preceding cell death Snigdha et al., 2012). Cleaved caspase 3 as executioner proteases once activated degrade structural proteins, signaling molecules and DNA repair enzyme (Nicholson, 1999). Our results demonstrate NAP protective role in early diabetic retinal injuries and underpin the hypothesis that NAP acts inhibiting the early events of apoptosis, preventing caspase-3 activation.

By western blot analysis, was observed a quite significant MAPK/ERK and a very significant PI-3K/AKT pathways inactivation in the retina of diabetic rats ($p<0.05$ and $p<0.001$ respectively) compared to non-diabetic animals.

These results are in agreement with other data (Qin et al.,2011; Liu et al.,2013) and it is possible to hypothesize that the modest downregualtion of p-ERK and AKT induced by hyperglycemia is a consequence of the increased level of cleaved caspase 3, in fact it is well known that caspase-dependent cleavage of specific proteins inactivates survival pathways, such as the MAPK/ERK and the PI-3K/AKT pathways (Snigdha et al 2012). NAP treatment acts upregulatating MAPK/ERK, in a very significant manner in the diabetic retina as compared to control (Fig. 4A and 4B). Immunofluorescent analysis confirmed the increased expression of p-ERK, induced by NAP treatment, in GCL, IPL, INL and OPL (Fig. 5. panel c, f and g).

Despite these evidences we cannot rule out that NAP acts by reducing major apoptotic triggers such as inflammatory cytokines production (Beni-Adani et al., 2001) and oxidative stress (Steingart et al., 2010) that could justify the over-expression of p-ERK in the diabetic retina.

In summary, our findings demonstrate that intravitreal injection of NAP attenuates STZ-induced retinal damages and restores ADNP transcript levels to control values in retinas of diabetic rats after 3 weeks. Immunoblotting data indicates that NAP treatment leads in the diabetic group to a significant increase in Bcl2 and reduces the cleaved caspase-3 activation compared to nondiabetic rats.

In conclusion, our findings suggest that the octapeptide NAP represents an interesting pharmacological tool potentially useful to treat degenerative retinal disease such as DR. However, further studies, particularly clinical trials, may be needed to explore this possibility.

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Chapter III

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Ameliorative effect of PACAP and VIP against increased permeability in a model of outer blood retinal barrier dysfunction

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Abstract

Breakdown of outer blood retinal barrier (BRB) due to the disruption of tight junctions (TJs) is one of the main factors accounting for diabetic macular edema (DME), a major complication of diabetic retinopathy. Previously it has been shown that PACAP and VIP are protective against several types of retinal injuries. However, their involvement in the maintenance of outer BRB function during DME remains uncovered. Here, using an in vitro model of DME, we explored the effects of both PACAP and VIP. Human retinal pigment epithelial cells (ARPE19) were cultured for 26 days either in normal glucose (5.5 mM, NG) or in high glucose (25mM, HG). In addition, to mimic the inflammatory aspect of the diabetic milieu, cells were also treated with IL-1β (NG + IL-1β and HG + IL-1β). Effects of PACAP or VIP on cells permeability were evaluated by measuring both apical-to-basolateral movements of fluorescein isothyocyanate (FITC) dextran and transepithelial electrical resistance (TEER). Expression of TJ-related proteins were evaluated by immunoblot. Results demonstrated that NG + IL-1β and, to a greater extent, HG + IL-1β significantly increased FITC-dextran diffusion, paralleled by decreased TEER. PACAP or VIP reversed both of these effects. Furthermore, HG + IL-1β-induced reduction of claudin-1 and ZO-1 expression was reversed by PACAP and VIP. Occludin expression was not affected in any of the conditions tested. Altogether, these finding show that

both peptides counteract HG + IL-1β-induced damage in ARPE19 cells, suggesting that they might be relevant to the maintenance of outer BRB function in DME.

Keywords: PACAP, VIP, tight junction, outer blood retinal barrier, diabetic retinopathy, macular edema

1. Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes and despite advancements in the delivery of ophthalmological care, it remains a major cause of blindness in the working age population [11]. Diabetic macular edema (DME) is a prominent clinical manifestation that frequently leads to severe loss of central vision in patients with diabetes. The breakdown of the blood retinal barrier (BRB) due to the disruption of the tight junctions appears to be one of the main factors accounting for DME [17].

The BRB is composed of two elements: the inner BRB (retinal capillary walls) and outer BRB which is formed by retinal pigment epithelium (RPE). The RPE has been shown to play critical roles in the physiology of the underlying photoreceptors, controlling the flow of solutes and fluid from the choroidal vasculature into the outer retina [30]. However, while extensive work has been carried out to identify factors involved in the disruption of the inner BRB during DME events, the mechanisms implicated in outer BRB regulation have been poorly uncovered.

PACAP and VIP belong to a family of peptides which include secretine, glucagon and peptide histidine-isoleucine (PHI), all involved in the modulation of numerous biological functions in vertebrates. There are two bioactive forms of PACAP, namely PACAP-38 and PACAP-27, that act through G protein-coupled receptors: the specific PAC1 receptor as well as the VPAC1 and VPAC2 receptors, which also bind VIP with similar affinity. Detailed localization studies have revealed that PACAP, VIP and their receptors occur throughout the nervous system, including the retinal neuronal layers [6, 14, 15, 16, 23, 25, 26], where they exert protective effects in response to insults of different nature. However, their delivery to brain seems to complicated by the blood brain barrier (BBB). Recently Dogrukol-Ak et al. (2009) [7] have characterized, isolated, and sequenced for the first time a

BBB trasporter for PACAP, a component of peptide transport system 6 (PTS-6), which was identified as a β-F1 ATPase.

Both peptides have been shown to possess retinoprotective potential against different types of lesions, including kainic acid- and glutamate-induced excitotoxicity [2, 4, 28, 33], optic nerve transection [22], anisomycin-induced cell death in the neuroblastic layer of newborn retinas [3, 22, 24, 29, 34] and streptozotocin (STZ)-induced diabetes in rats [4].

In a recent work, we have demonstrated the occurrence of changes in the retinal expression of PACAP/VIP and their receptors during the earliest phases of STZ-induced diabetes and that a single intravitreal injection of PACAP38 is sufficient to provide protection in retina of early diabetic rats [12]. However, whether these peptides are also involved in maintenance of outer BRB function during DME remains to be clarified.

The aim of the present study was to explore the effects of PACAP and VIP on outer BRB permeability in cultures of human RPE (ARPE19). Given that pro-inflammatory cytokines and, in particular, IL-1β, plays a key role in the pathogenesis of DME and increases outer BRB permeability [30, 31], cells were grown in media containing both IL-1β and high glucose concentrations (HG, 25mM) in order to mimic the diabetic milieu. Dextran-permeability, transepithelial electrical resistance (TEER) and the expression of tight junctions-related proteins (zonula occludens-1, claudin-1 and occludin, respectively) were evaluated. Results showed that either PACAP or VIP treatment were able to recover the RPE barrier hyperpermeability and the reduced TEER induced by HG+IL-1β treatment, and this effect appeared to be associated with augmented expression of claudin-1 and ZO-1, suggesting that both peptides might be involved in the maintenance of tight junction integrity during DME.

2. Materials and Methods

2.1 Human RPE cell culture

ARPE-19 is a spontaneously immortalized human RPE cell line, purchased from the American Type Culture Collection (Rockville, MA, USA), that has become a good alternative model of outer BRB *in vitro.* This cell line, extensively characterized, form stable monolayers and exhibit several phenotypic characteristics similar to those of primary RPE [8, 19, 20]. Cells were cultured in T_{75} flasks using DMEM-F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, USA), 1 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Lonza, Italy). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and sub-cultured once a week. Cells were cultured for 7 days on transwell-clear permeable supports (Corning Costar, Cambridge, MA) at a density of 1.5 x 10^5 cells/cm^2 in normal glucose condition (NG, 5.5mM D-glucose). Subsequently, half of the transwells were maintained with 5.5mM D-glucose and the other half were switched to high glucose conditions (HG, 25mM D-glucose) for a further 7 days. On the last week of the experiments, cells were subjected to serum starvation (1% FBS) and treated either with IL-1β (10ng/ml) alone or in combination with PACAP (100nM) or VIP (100nM) (Sigma Aldrich, USA). To rule out a potential bias by an osmotic effect, the experiment was also performed using mannitol (5.5 mM D-glucose + 19.5 mM mannitol vs. 25 mM D-glucose) as an osmotic control agent.

2.2 Permeability assay

Permeability experiments with filter-cultured ARPE-19 cells were performed using transwell-clear permeable supports (Corning Costar, Cambridge, MA) at 21 days by measuring the apical-to-basolateral movements of FITC-dextran (Chemicon, Millipore). First, cells were allowed to equilibrate in phosphate-buffered saline (PBS), and then the solution in the apical compartment was

replaced by the FITC-dextran solution. The samples were collected from the receiver compartment at 15, 30, 60 min. Absorbance was measured using a microplate reader (BioRAD 680) using 480nm for excitation and 535nm for emission.

2.3 Measurement of trans-epithelial-electrical resistance

The progress of epithelial barrier formation and polarization was monitored by measuring trans-epithelial-electrical resistance (TEER) using a Millicel-Electrical Resistance System (ERS2, Millipore, Epithelial Volt-Ohm Meter). TEER measurements were performed in ARPE19 and were commenced after a 15 min equilibration period at room temperature. Values are expressed as Ω/cm^2. The combined resistance of the filter was subtracted from the values of filter-cultured ARPE-19 cells in order to calculate the resistance of the cell layer. Measurements were performed every 3 days on 3 different wells for each experimental condition.

2.4 Analysis of mRNA expression by RT-PCR

Total RNA was extracted using TRI-Reagent® (Invitrogen), 0.2 ml chloroform and precipitated with 0.5 ml isopropanol. Pellet was washed with 75 % ethanol and air dried. Single stranded cDNAs were synthesized incubating total RNA (10 µg) with SuperScript III RNase H-reverse transcriptase (200 U/µl) (Invitrogen); Oligo-(dT)20 primer (100 nM) (Invitrogen); 1 mM dNTP mix (Invitrogen), dithiothreitol (DTT, 0.1 M), Recombinant RNase-inhibitor (40 U/µl) at 42 °C for 1 h in a final volume of 20 µl. Reaction was terminated by incubation of samples at 70 °C for 10 min [18]. Aliquots of cDNA were amplified using specific primers for PACAP, VIP, PAC1 VPAC1, VPAC2, zonula occludens-1 (ZO-1), claudin-1, occludin and S18 ribosomial subunit. Oligonucleotide sequences are listed in Table 1. Each PCR reaction contained 0.4 µM specific primers, 200 µM dNTPs, 1.25

U AmpliTaq Gold DNA polymerase and GeneAmp buffer containing 2.5 mM $MgCl^{2+}$ (Applied Biosystem). PCR was performed using the following three cycle programs: (1) denaturation of cDNA (1 cycle: 95 °C for 12 min); (2) amplification (40 cycles: 95 °C for 30 sec, 60 °C for 30 sec, 72 °C for 45 sec); (3) final extension (1 cycle: 72 °C for 7 min). Amplification products were separated by electrophoresis in a 1.8 % agarose gel in 0.045 M Tris-borate / 1 mM EDTA (TBE) buffer.

2.5 Western Blot analysis

Western blot analysis was performed to determine the relative levels of tight junction-related proteins ZO-1, occludin and claudin-1, using specific antibodies. Proteins were extracted from cell culture in a cold buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, 0.5 mM EGTA; 50 mM mercaptoethanol, 0.32 mM sucrose in the presence of protease inhibitors (Sigma) and then sonicated thrice for 20 sec with an ultrasonic probe, followed by centrifugation at 10.000 g for 10 minutes at 4°C. The supernatant was collected, aliquoted and stored at −80°C until use [13]. Samples containing about 10 µg of protein homogenate, as determined by the Quant-iT Protein Assay Kit (Invitrogen), were diluted in 2X Laemmli buffer (Biorad, Carlsbad, CA, USA), heated at 70°C for 10 min and then loaded in a Biorad Criterion XT Bis-Tris 4-15% gel for protein fractionation by electrophoresis and then electro-transferred to a nitrocellulose membrane (Biorad). Blots were blocked using the Odyssey Blocking Buffer (LI-COR Biosciences) and probed with appropriate antibodies: rabbit anti-ZO-1 (cat n. 61-7300, Invitrogen; 2 µg/ml), rabbit anti-claudin-1 (cat n. 51-9000, Invitrogen; 2 µg/ml), rabbit anti-occludin (cat n. 71-1500, Invitrogen; 2 µg/ml). The immunologic detections were determined using IRDye Secondary Antibody (goat anti-rabbit IRDye 800 nm (827-06905; LI-COR)

according to manufacturer's instructions. Blots were scanned using an Odyssey Infrared Imaging System (Odyssey). Densitometric analysis of Western blot signals was performed at non saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

2.6 Statistical analysis

Data are reported as mean ± S.D. One-way analysis of variance (ANOVA) was used to compare differences among groups and statistical significance was assessed by Tukey-Kramer *post-hoc* test. The level of significance for all statistical tests was $p \leq 0.05$.

3. Results

3.1 RT-PCR analysis of PACAP, VIP and related receptors in ARPE19 cells

In order to identify mRNA expression of PACAP and VIP peptides, as well as, PAC1 / VPAC type receptors in ARPE19 cells, a RT–PCR analysis was performed. As shown in Fig. 1, ARPE19 cells express both PACAP and VIP peptide mRNAs. Amplification products obtained using specific primers recognizing PAC1, VPAC1 and VPAC2 receptors mRNAs produced bands of the predicted length. All primer sequences are shown in Table 1. The S18 ribosomal subunit was used as control in each PCR amplification and generated a band of the expected length.

Gene	Forward primer	Reverse primer	Accession	size (bp)
PACAP	AGCACCTGCAGTCGCTCGTG	ATCCCGTCCGAGTGGCGCTT	NM_001099733.1	100
PAC1 receptor	GCTGGGCTCCTTCCAGGGCT	CGGTTCACCTTCCAGCTTCGCC	NM_001199635.1	105
VIP	AGAAATAAGGCCCAGCTCCTTGTGC	ACCTGAGAGCAGAAGGTGCCCTGTA	NM_194435.2	100
VPAC1 receptor	AAGTGGACTGGCCCCTGGGT	GCATTCGCTGGTGGCTGCCT	NM_004624.3	104
VPAC2 receptor	CCGCTGTTCGGCGTCCACTAC	CCCTGGAACGACCCGAGGCA	NM_003382.4	101
Ribosomal protein S18	GAGGATGAGGTGGAACGTGT	GGACCTGGCTGTATTTTCCA	NM_213557.1	115

Table 1. Primer sequences
Forward and reverse primers were selected from the 5' and 3' region of each gene mRNA. The expected length of each PCR amplification product is indicated in the right column.

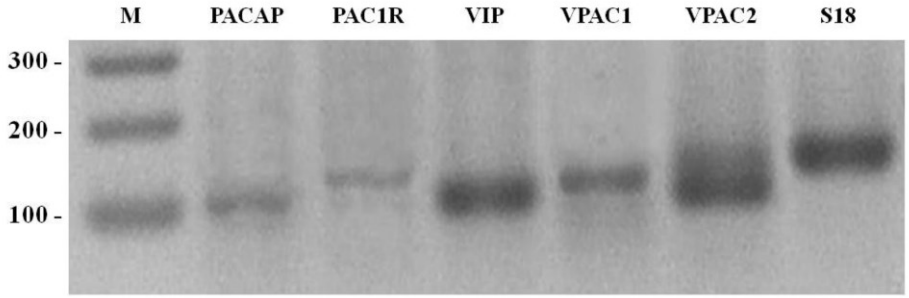

Figure 1. RT-PCR analysis of PACAP/VIP peptides and related receptors mRNAs in human retinal pigment epithelial cell line, ARPE19

Amplification products obtained using specific primers, which recognized PACAP, and VIP peptides as well as their related receptors generated bands of the expected length (Table 1). Ribosomal protein S18 was included as control. A 100-bp DNA ladder is shown on the left side of the gel (lane M) with bands labelled in bp units.

3.2 PACAP and VIP improve hyperpermeability induced by high glucose plus IL-1β in RPE cells

To determine whether PACAP or VIP treatment was able to recover RPE cells from the hyperpermeability induced by high glucose plus IL-1β, apical-to-basolateral movements of FITC-dextran were measured in ARPE19 cells grown either in normal glucose (NG), NG+IL-1β, high glucose (HG), HG+IL-1β alone and in combination with 100nM of PACAP or VIP. Results are displayed in Fig. 2.

Cells grown in HG showed increased permeability to FITC-dextran diffusion, although it did not reach statistical significance ($F_{5,29}$=28.70; $p>0.05$) whereas addition of IL-1β (10 ng/ml) significantly increased barrier leakage both in NG grown cells (520.5±29.65 *vs* 409.3±9.6, *$p<0.05$) and, to a greater extent, in HG cultures (672.75±51.11 *vs* 409.3±9.6, ***$p<0.001$ vs NG; 672.75±51.11 *vs* 465.5±43.55, ###$p<0.001$ vs HG), suggesting a synergistic

effect of HG in outer BRB dysfunction in the latter condition. Interestingly, a significant reduction in permeability was observed when cells grown in HG+IL-1β were treated either with 100nM PACAP (421.4±35.41 *vs* 672.75±51.11; §§§p<0.001 *vs* HG+IL-1β) or VIP (396.2±21.26 *vs* 672.75±51.11; §§§p<0.001 *vs* HG+IL-1β), reaching values comparable to controls (NG).

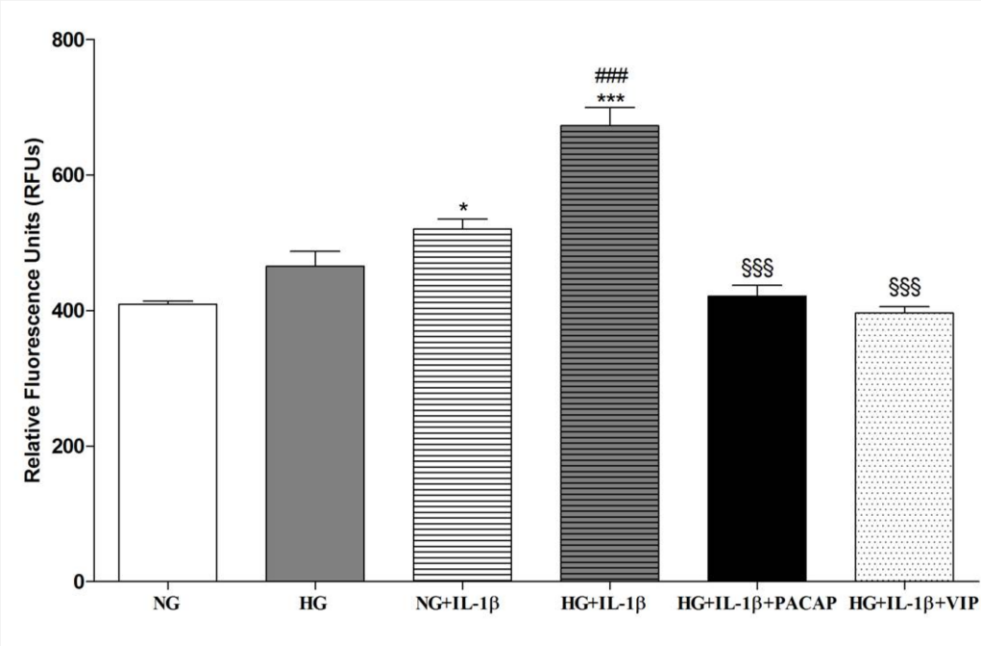

Figure 2. Effect of PACAP and VIP on ARPE-19 cell monolayer permeability
PACAP or VIP ability to recover ARPE-19 cells from the hyperpermeability induced by high glucose plus interleukin-1β (IL-1β) was evaluated by measuring apical-to-basolateral movements of FITC-dextran in cells grown on transwell-clear permeable supports either in the presence of normal glucose (NG, white bar), high glucose (HG, gray bar), NG+IL-1β (striped white bar), HG+IL-1β alone (striped gray bar) and in combination with 100nM of PACAP (black bar) or VIP (dotted white bar). Results are presented as relative fluorescence units (RFUs) ± SD obtained from measurements on 3 different wells x treatment in three separate experiments (n=3). *p<0.05 or ***p<0.001 *vs* NG; ###p<0.001 *vs* HG; §§§p<0.001 *vs* HG+IL-1β, as determined by One-Way ANOVA followed by Tukey-Kramer *post-hoc* test

3.3 *Effect of PACAP and VIP on trans-epithelial electrical resistance in RPE cells exposed to hyperglycemia and IL-1β*

In order to confirm permeability data, TEER values of ARPE19 cells grown under the same experimental conditions described above were also measured. Measurements were performed every 3 days on 3 different wells x treatment starting from 5 DIV (5^{th}, 8^{th}, 11^{th}, 14^{th}, 17^{th}, 20^{th}, 23^{rd} and 26^{th} day, respectively). TEER measures the integrity of the barrier. In the presence of paracellular permeability enhancers, TJ-related protein dysfunction, deficient monolayer, cannaliculi, or formation of fenestrae through the cells the TEER of the retinal epithelium will be reduced because of the increasing flow of ions through the intercellular spaces [5]. Therefore, TEER is inversely correlated to cellular permeability values. In agreement with measurements of FITC-dextran diffusion (Fig. 2), TEER values of cells treated with interleukin-1β alone (IL-1β) were significantly decreased already after 3 days ($F_{29,89}$= 56.16, **p<0.01 *vs* NG at the 17^{th} day) and progressively diminished in a time-dependent manner (***p<0.001 *vs* NG at the 20^{th}, 23^{rd} and 26^{th} day, respectively) (Fig. 3). The effect of treatment with IL-1β appeared to be more pronounced in HG than in NG grown cells starting at 3 days (***p<0.001 and **p<0.01 *vs* NG, respectively), supporting a role of glucose in potentiating cytokine-induced outer BRB disruption. Treatment with either 100nM PACAP or VIP significantly increased TEER as compared to cells exposed to HG+IL-1β alone within 6 days (###p<0.001 *vs* HG+IL-1β at the 20^{th} day), with PACAP being more efficacious at the last experimental time tested (###p<0.001 *vs* HG+IL-1β at the 26^{th} day).

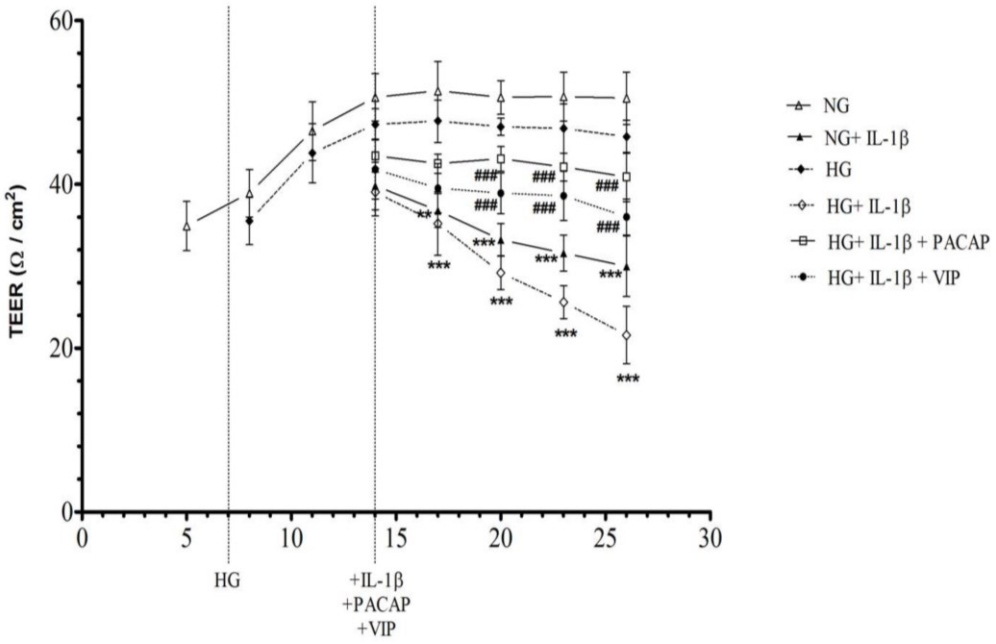

Figure 3. Effect of PACAP and VIP on TEER of ARPE-19 cell monolayer
Effect of PACAP and VIP on TEER values in ARPE-19 cells. Vertical axis represents the TEER, expressed in Ohm x cm^2, and the horizontal axis is the time (day). Results are expressed as the mean ± SD (n = 3). **p<0.01 or ***p<0.001 *vs* NG; ###p<0.001 vs HG + IL-1β as determined by One-Way ANOVA followed by Tukey-Kramer *post-hoc* test

3.4 *Effect of PACAP and VIP on high glucose- and IL-1β-induced claudin-1, occludin and ZO-1 protein expression*

To evaluate the effect of either PACAP or VIP on outer BRB integrity, we analyzed the expression of different tight junction-related proteins, which have been previously demonstrated to play a key role in the maintenance of epithelial barrier function [27]. Western blot analyses using specific antibodies recognizing both claudin-1, occludin and ZO-1 proteins were carried out in ARPE19 cells cultured either in the presence of normal glucose (NG), which was used as positive control, high glucose + interleukin-1β (HG+ IL-1β, negative control) alone or in combination with 100nM PACAP or VIP (HG+ IL-1β+PACAP or HG+ IL-1β+VIP, respectively). As depicted

in Fig. 4A, claudin-1 expression was significantly reduced in cells exposed to HG+IL-1β as compared to NG cultured cells ($F_{3,17}$=8.301 *p<0.05 *vs* NG). Treatment with either PACAP and, to a minor extent, with VIP, significantly increased claudin-1 expression (§§p<0.01 *vs* HG+IL-1β for PACAP and §p<0.05 for VIP), reaching values comparable to controls (NG). Similarly, ZO-1 protein expression was reduced by HG+IL-1β exposure ($F_{3,15}$=9.775 *p<0.05 *vs* NG) (Fig. 4B). Addition of PACAP, but not VIP, significantly increased ZO-1 protein levels in cells exposed to HG+IL-1β (§§p<0.01). Occludin expression was slightly reduced in cells grown in HG+IL-1β ($F_{3,11}$=0.6741, p>0.05) and was not affected neither by PACAP nor by VIP treatment (Fig. 4C). β-tubulin was employed in each experiment and was used as loading control.

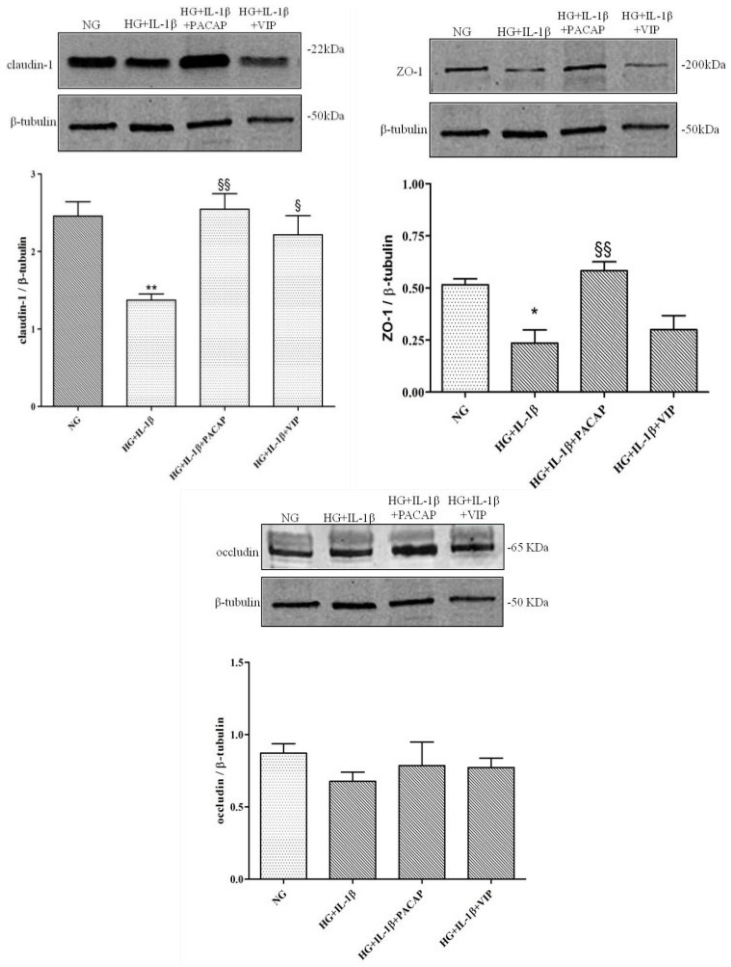

Figure 4. Effect of PACAP and VIP on claudin-1, Zo1 and occludin expression in ARPE-19 cell monolayer

Representative immunoblots obtained using 10 µg of homogenates from ARPE-19 cells cultured on transwell permeable supports in the presence of 5.5 mM D-glucose + 19.5 mM mannitol (NG) or 25 mM D-glucose + IL-1β (HG+IL-1β), HG + IL-1β + PACAP (100 nM), HG + IL-1β + VIP (100 nM). The bar graph shows the results of three independent experiments. Protein levels are expressed as arbitrary units obtained after normalization with β-tubulin, which was used as loading control. *p<0.05 *or* **p<0.01 *vs* NG, §p<0.05 or §§p<0.01 *vs* HG+IL-1β, as determined by One-Way ANOVA followed by Tukey-Kramer *post-hoc* test No significant differences were found among groups tested for occludin expression.

4. Discussion

The present study showed, for the first time, that PACAP and its structurally related peptide VIP exert an ameliorative effect against the increased permeability induced by diabetic conditions in a model of outer blood retinal barrier (BRB).

For this purpose, retinal pigment epithelial cells (ARPE19) were cultured on transwell-clear permeable supports in the presence of high glucose and then treated with IL-1β as previously described by Trudeau et al.(2010) [32], in order to mimic the major pathological events (i.e. hyperglycemia and inflammatory response) that accompany diabetic macular edema (DME), a prominent clinical manifestation that frequently leads to severe loss of central vision in patients with diabetes, which is mainly characterized by the breakdown of the BRB due to the disruption of tight junctions [17].

PACAP and VIP are both known for their retinoprotective potential against several types of injuries both *in vitro* and *in vivo*, including diabetic retinopathy [3]. Moreover, the protective efficacy of PACAP and VIP has also been proven in retinal pigment epithelial cells [9, 21, 35], suggesting that both peptides might play an active role in the maintenance of outer BRB integrity. However, whether PACAP or VIP could be effective in restoring the integrity of RPE after diabetic injury remains to be clarified.

Here we show that following hyperglycemia and IL-1β treatment permeability of ARPE19 cells was significantly increased, as demonstrated by the augmented diffusion of FITC dextran solution from the apical to the basolateral compartment of the transwells (Fig. 2), as well as by the reduction of TEER values, a measure of tight junction integrity between adjacent retinal epithelial cells [5] (Fig. 3). Treatment with either PACAP or VIP significantly reduced the hyperpermeability and the increased TEER values induced by glucose and IL-1β (Fig. 2 and 3), suggesting an ameliorative

effect on barrier integrity. These results are in agreement with that observed using fenofibric acid, a hypolipemiant drug [32], or erythropoietin, a major regulator of erythropoiesis in ARPE19 cells exposed to diabetic conditions [10], implying that restoring of stress-induced outer BRB dysfunction might rely on common mechanisms activated by apparently unrelated molecules. One such mechanism could involve the regulation of tight junction-related proteins content or expression [10, 32, 34]. Among these, claudin-1, ZO-1 and occludin cooperate in the sealing function of epithelium [27].

In the present study we observed that exposure of cells to HG+IL-β reduces both claudin-1 and ZO-1 expression, while levels of occludin remain unchanged (Fig. 4-6). PACAP treatment restored both claudin-1 and ZO-1 expression to control levels (NG) whereas VIP seemed to be less effective since it was able to upregulate claudin-1 expression only. However, we were not able to determine to which extent each of these proteins participate in epithelial barrier integrity. In our opinion, it is possible that both claudin-1 and ZO-1 act cooperatively to reduce barrier permeability following injury. Alternatively, each of these tight junction-related proteins, independently, should be regulated in order to restore barrier integrity after diabetic insult and/or that PACAP and VIP activate protective signaling pathways other than those related to tight junction protein expression, including anti-apoptotic pathways.

Indeed, converging evidences pointed out that altered amount of tight junctions proteins may not necessarily be the unique cause of BRB dysfunction and that correct protein distribution may also be a critical factor for proper barrier functioning [1, 34]. As such, further studies to determine the exact mechanisms by which PACAP and VIP contribute to the overall increase of outer BRB function after combined treatment with high glucose and IL-1β and how these peptides affect the distribution of tight junction proteins in DME should be warranted.

In summary, our study demonstrated that PACAP, and to a lesser extent VIP, can partially prevent the breakdown of outer BRB induced by diabetic conditions in an *in vitro* model of DME, possibly through the modulation of tight junctions expression, suggesting that both peptides may have a protective effect on retinal edema.

Acknowledgements

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DISCUSSION AND CONCLUSION

Diabetic retinopathy and diabetic macular oedema are among the most significant complications of diabetes. Even though mechanisms of DR are widely studied, a definite treatment has not yet been established probably because of the vast number of factors involved in its pathophysiology. Maintenance and restoration of functions of the eye, being one of the main sense organs, is a vital issue of modern medicine.

The first-line of management for diabetes mellitus remains the tight control of glucose levels, blood pressure, and lipids. In clinical practice, the primary treatment for DR is most often surgery (e.g. photocoagulation, vitrectomy). However, in the last 15 years surgery has often been supplemented by drug treatment. Injectables have shown some promise in exploratory studies; however, none have been effective in treating all pathologies with persistent DME. However, reinjection is often necessary and there are side effects such as infection, glaucoma and cataract formation. Also, intravitreal anti VEGF agents are emerging as a new treatment, but this procedure is highly invasive with local and or systemic complications due to its capacity to pass into systemic circulation [99]. Therefore, new pharmacological treatments for the early stages of the disease are needed.

In this scenario, neuropeptides emerge as potential therapeutic agents, especially considering that they are already synthesized by the retina in normal physiological conditions.

Among these neuroprotective factors, pituitary adenylyl cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide

(VIP) seem most relevant. These neuropeptides with highly potent neuroprotective and general cytoprotective effects are located with their receptors in the retina [29, 30]. Several studies have demonstrated the PACAP/VIP protective role in many retinal pathologies, like excitotoxic, ischemic and UV light-induced retinal degeneration [44, 51]. Early changes of these neurotrophic factors and their receptors in DR have been described previously, suggesting their higher importance in the pathogenesis of DR.

In this study, we found that PACAP/VIP-induced pathways are able to attenuate the disruption of the outer BRB, a core element of retinal dysfunction in DR patients [100]

PACAP and VIP restored the high glucose and inflammatory injuries of retinal pigmented epithelium (ARPE 19 cells) modulating the expression of two elements of the tight junction component: ZO1 and claudin-1. The tight junctions are an important component of the BRB, and they are often the targets of inflammatory and oxidative changes associated with hyperglycemia. With other factors such as Fenofibrate, Sitagliptin and EPO, PACAP and VIP may be effective in DR and BRB breakdown, but their optimal delivery method and dosage need to be studied further.

Targeting the apoptotic events is another promising strategy in the treatment of DR. Apoptosis is an early and persistent event in the diabetic retina. It can be observed even after termination of hyperglycaemia and as early after the induction of diabetes in rats [101]. Apoptosis of retinal neurons has been recognized as a critical event and a prominent pathological feature of diabetic retinopathy. In this work, we proposed the peptide NAP as a new anti-apoptotic factor. *In vivo* NAP treatment is protective in diabetic retinopathy and this ameliorative effect is at least partly due to the anti-apoptotic effect of

NAP. Our observations increase the list of retinal pathologies against which NAP has protective effects.

Further investigation on the mechanisms involved in neurodegeneration and its relationship with microvasculature impairment is urgent in order to elucidate potential therapeutic strategies based on neuroprotection that could be effective in preventing or arresting DR development. The standardization of methods for monitoring neuroprotection is also necessary so that further clinical trials to determine the long term effectiveness and safety of this approach can be pursued.

However, the peptidergic therapeutic strategy is tangible and other peptides like Normoftal, Pancragen, Vesugen, Crystagen, and Pinealon are already widely used in the medical practice as the means for prevention and treatment of different eye diseases [102].

Neuroprotective drugs could open up a new strategy for the treatment of early stages of DR. Targeting prevention may be more cost-effective than assuming the considerable costs related to DR treatment.

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LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

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Professional Honors & Recognitions

2014: Research Fellowship, Italian Society of Pharmacology (SIF)

Conference procedeedings

Oral communication

Soraya Scuderi and Velia D'Agata "Retinal protection: role of PACAP and NAP" *IT-ARVO Chapter meeting* – Catania, 3-4 Febbraio 2014

Poster

Soraya Scuderi, Rossella Imbesi and Alessandro Castorina "*PACAP and VIP increase the expression of myelin-markers in rat schwannoma cells"* 67th National Congress of The Italian Society of Anatomy And Histology September 20-22, 2013 Brescia, Italy

S. Scuderi, A.G. D'Amico, S. Saccone, A. Castorina, P. Castrogiovanni, M.L. Carnazza, V. D'Agata *"NAPmodulates the expression and distribution of cleaved caspase-3 in retina of streptozotocin-injected rats"* 35th National Congress of The Italian Society of Histochemistry, June 12-14, 2013 S. Margherita di Pula – Cagliari, Italia

A.G. D'Amico**, S. Scuderi,** S. Saccone, A. Castorina, R. Imbesi, M.L. Carnazza, V. D'Agata *"PACAP and VIP affect nestin expression in serum deprived glioma cells*" 35th National Congress of The Italian Society of Histochemistry, June 12-14, 2013 S. Margherita di Pula – Cagliari, Italia