Treatment of Diabetic Retinopathy through Stabilizing Neurovascular Microenvironment

Inaugural dissertation

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been accurately cited and indicated in the thesis.

Since my research project is subject to an international cooperation project titled "Improvement of neurovascular microenvironment through neuropeptides in diabetic retinopathy", it was planned to perform part of the work at the Laboratory of the Department of Ophthalmology at the University of Düsseldorf, however due to the change of Universities of Prof. Stefan Schrader, all experiments were completed in the laboratory of the Academic Unit of Ophthalmology at University of Bristol. My co-supervisors Dr. Lei Liu and Prof. Andrew Dick directly supervised me with their expertise in the field of diabetic retinopathy during my time in the UK.

Contents

List of Abbreviations

ANGPTL-4	Angiopoietin-like 4
ARVO	Association for Research in Vision and Ophthalmology
AGEs	Advanced glycation end-products
BRB	Blood-retina barrier
CNS	Central nervous system
DR	Diabetic retinopathy
DM	Diabetes mellitus
EB	Evans Blue
ERK1/2	Extracellular signal-related kinases 1/2
ERG	Electroretinography
fERG	Focal Electroretinogram
GCL	Ganglion cell layer
GLUT1	Glucose transporter 1
GFAP	Glial fibrillary acidic protein
HP	Hypoxic preconditioning
HIF-1α	Hypoxia-inducible factor-1alpha
HIF-1	Hypoxia-inducible factor 1
HRMECs	Human Primary Retinal Microvascular Endothelial cells
HUVECs	Human Umbilical Vein Endothelial Cells
ILM	Inner limiting membrane
IPL	Inner plexiform layer
INL	Inner nuclear layer
MAPK	Mitogen-activated protein kinase
MFI	Mean Fluorescent Intensity
mTOR	Mammalian target of rapamycin
MRMECs	Mouse Primary Retinal Microvascular Endothelial cells
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NMDA	N-methyl-D-aspartate
NFL	Nerve fiber layer
NPDR	Non-proliferative diabetic retinopathy

List of Abbreviations

NK1R	Neurokinin 1 receptor
OPL	Outer plexiform layer
ONL	Outer nuclear layer
OLM	Outer limiting membrane
OCR	Oxygen consumption rate
OCT	Optical coherence tomography
PEDF	Pigment epithelium-derived factor
PDR	Proliferative diabetic retinopathy
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PKC	Protein kinase C
PKCζ	Protein kinase C zeta
PL	Photoreceptor layer
RGC	Retinal ganglion cells
ROS	Reactive oxygen species
RPE	Retinal pigmented epithelium
RT	Room temperature
SP	Substance P
STZ	Streptozotocin
SDHD	Succinate dehydrogenase complex subunit D
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
WHO	World Health Organization
ZO-1	Zonula occludens-1

Abstract

Diabetic retinopathy (DR) is one of the most severe clinical manifestations of diabetes and a major cause of vision loss in worldwide working-age adults. The hallmarks of diabetic retinopathy include the damage and subsequent reduction of pericytes and endothelial cells numbers, followed by thickening of the vascular basement and further to disrupt the blood-retina barrier (BRB). The main known contributors to the breakdown of the BRB are pro-angiogenic factors, especially, vascular endothelial growth factor (VEGF), mainly secreted by upregulation from the hypoxia-induced activation of retinal Müller cells. However, there are early stages of diabetes-induced retinal neurodegeneration, which include reactive gliosis with subsequent impairment of retinal neuronal function. These changes are observed before the onset of vascular abnormalities in human and mouse diabetic retinal tissue. Therefore, DR is widely recognized as a neurovascular disease, which is in contrast to its conventional perception as a merely vascular disease. In the recent years, considerable efforts have been made to find treatments for this condition. Studies have shown that hypoxic preconditioning (HP) can stabilize hypoxia-inducible factor-1alpha (HIF-1α) to regulate the hypoxic retinal disease. It has also been reported that neuropeptides exert functions through their multiple receptors are potentially important for consideration in developing new drug strategies.

The aim of this thesis was to investigate HP of retinal Müller cells and adjunctive Neuropeptide Y (NPY) and Substance P (SP) to stabilize and improve the health of neurovascular microenvironment. The effect of HP was assessed in detail, using several methods, including *in vitro* angiogenesis and vascular integrity models. In parallel, metabolism of Müller cells was assessed using Seahorse XF Cell Mito Stress Test. In the next series of my experiments, to interrogate the protective effect of SP and NPY in maintaining the retinal neurovascular unit, tight junction proteins expression and Vascular Permeability Image Assay were used to determine vascular integrity *in vitro*. The protective effect of SP and NPY on retinal ganglion cells (RGC) was also analyzed in *ex vivo* retinal explants. More importantly, the protective effect of the neuropeptides was further investigated utilizing *in vivo* murine diabetic models.

Abstract

The experiments revealed that the hypoxic response of retinal Müller cells promotes vascular permeability and angiogenesis through HIF-dependent upregulation of pro-angiogenic factors VEGF and angiopoietin-like 4 (ANGPTL4). HP had a robust effect decreasing HIF-1α stabilization in response to hypoxia. In turn, HIF-mediated transcription and secretion of pro-angiogenic factors were reduced. This protective mechanism was linked to the change in the retinal Müller cells metabolic status. It was also demonstrated that SP and NPY are neuroprotective, suppressing apoptosis of RGC induced by N-methyl-D-aspartate (NMDA), and protect against VEGF-induced microvascular leakage of the retina. Moreover, NPY showed neurovascular protection of DR in type 1 diabetic mice.

These effects demonstrate the significant role of HP and that the modulation of neuropeptide transmitters (SP and NPY) levels can be a potential novel approach of treatment in maintaining neurovascular-unit homeostasis, and open up new therapeutic avenues for DR.

1. Introduction

1.1 The Retina

1.1.1 Architecture of the Retina

The retina is a light-sensitive layer of tissue located in the posterior chamber of the eyeball of most vertebrates (Figure 1A). The retina consists of 10 layers (Figure 1B).

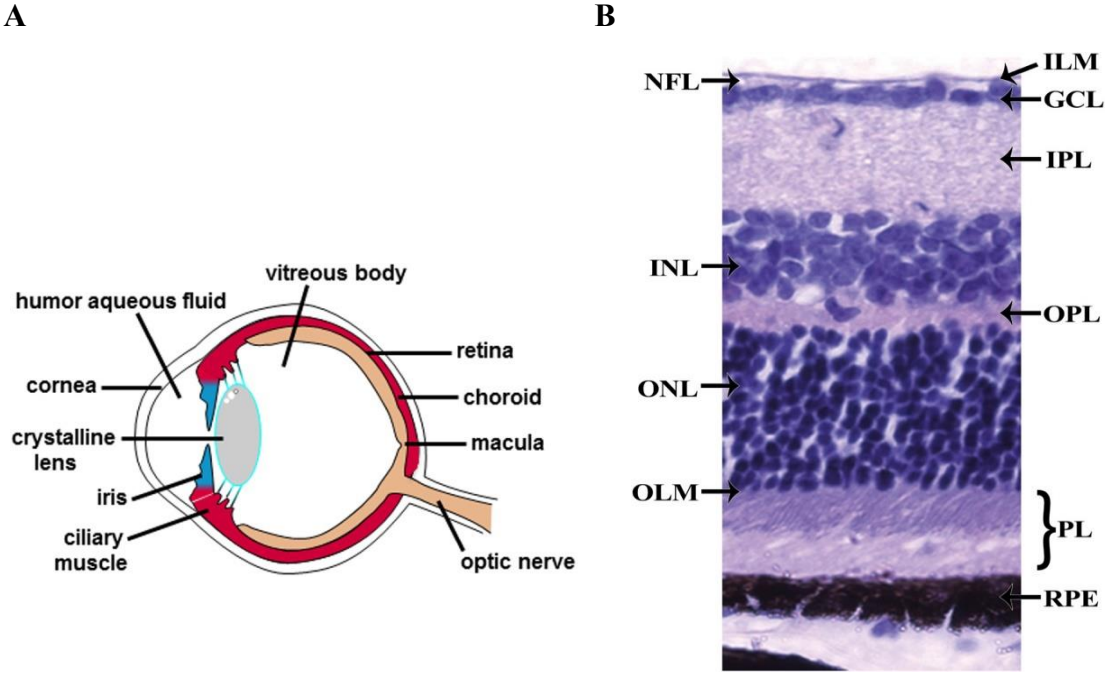

Fig. 1. The retina. (A) Anatomical structure of the human eye. (B) The 10 layers of retina including (1) the inner limiting membrane (ILM); (2) the nerve fiber layer (NFL); (3) the ganglion cell layer (GCL); (4) the inner plexiform layer (IPL); (5) the inner nuclear layer (INL); (6) the outer plexiform layer (OPL); (7) the outer nuclear layer (ONL); (8) the outer limiting membrane (OLM); (9) the photoreceptor layer (PL), and (10) the retinal pigmented epithelium (RPE) monolayer [1].

1.1.2 Retinal cells

Histologically, more than 10 diverse kinds of cells are embedded in the retinal layers (Figure 2). The Müller cells span across multiple layers in the retina, from the ILM to the distal end of the ONL [2]. Most of the retinal cells are located in different retinal layers. Astrocytes are located in the ILM, axons of retinal ganglion cells (RGC) and part of glial cells are found in the NFL. Nuclei of RGC, glial cells, and part of amacrine cells are found in the GCL. Bipolar and amacrine cells are found in the IPL, bipolar and horizontal cells are in the INL. The interconnecting neurons of the photoreceptor cells

(rods and cones), the horizontal and bipolar cells are located in the OPL. The ONL and OLM consists mainly of photoreceptor cells. The cones and rods are found in the PL. The pigment epithelium layer is formed by RPE cells [1,3].

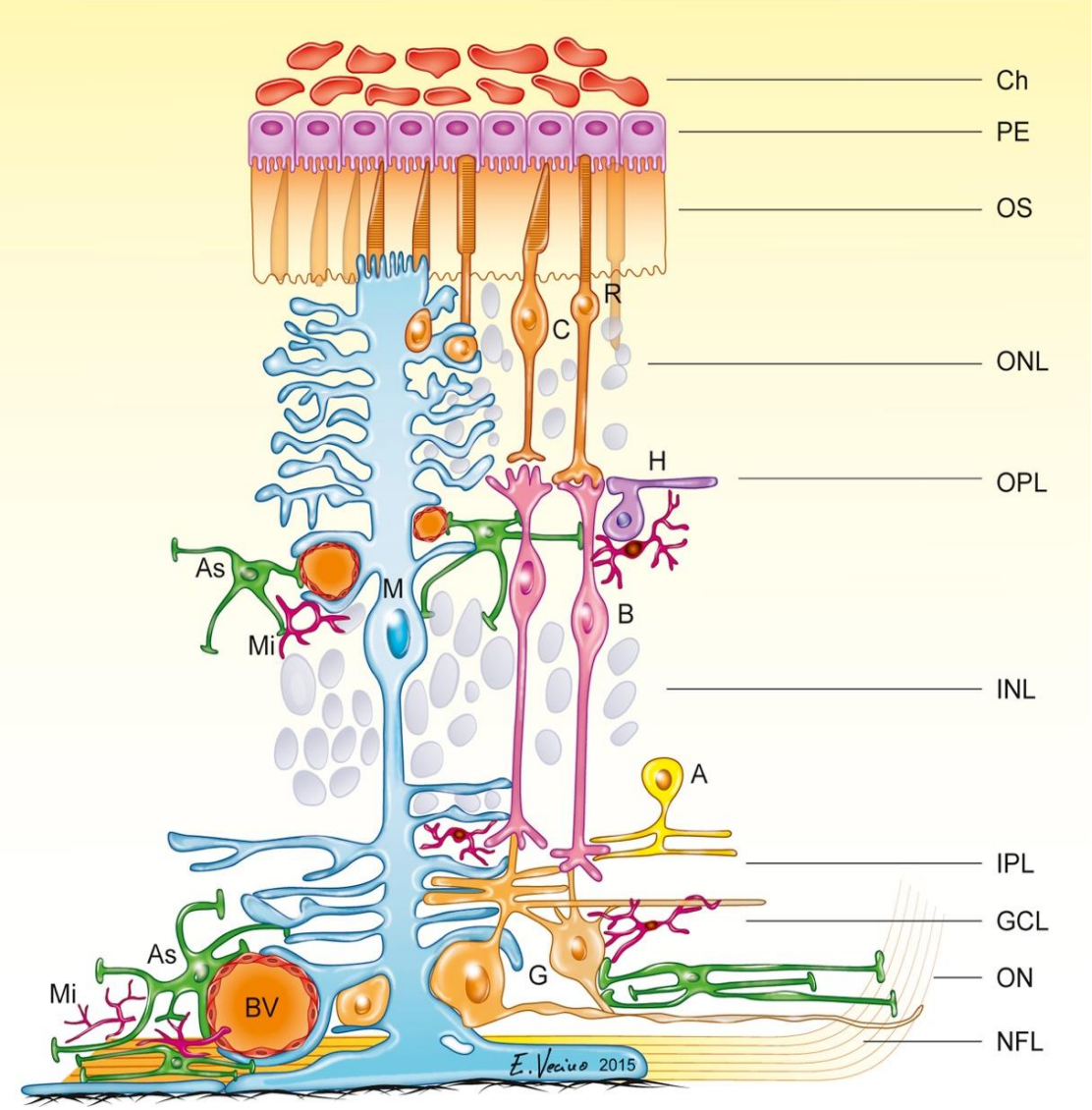

Fig. 2. Schematic drawing of the retinal cellular components [3]. Amacrine cells (A), astrocytes (As, green), blood vessels (BV). bipolar cells (B), cones (C), RGC (G), horizontal cells (H), Müller cells (M), microglia cells (Mi, red), rods (R), optic nerve (ON), outer segment layer (OS), pigment epithelium (PE), choroid (Ch).

1.2 Diabetes Mellitus (DM)

There is a significant increasing prevalence of diabetes worldwide, especially in western countries [4]. The World Health Organization (WHO) estimates that the number of DM patients is expected to significantly increase to 592 million by 2035 [5]. DM is not only a disease found in industrialized countries, but also spreading into the developing countries due to lack of healthy diet habits and physical activity [6].

1.2.1 Diabetes Classification

Diabetes can be divided into two broad pathogenetic categories: type 1 and type 2. Type 1 diabetes, also called the juvenile diabetes, is an autoimmune disease, where the immune system attacks beta cells in the pancreas, leading to a reduction or complete inability of the pancreas to produce insulin. However, the underlying mechanisms are still unclear [7]. On the other side, type 2 diabetes, is based on insulin resistance or a disturbed insulin production in the pancreas. Type 2 diabetes accounts for around 90-95% of diagnosed DM in adults [8]. There is also a range of atypical types of diabetes. For example, monogenics, infection-induced and iatrogenic (caused by certain medications or surgery or hormonal imbalances) diabetes. These atypical types of diabetes account for around 2% of the whole diabetic population [9].

1.3 Diabetic Retinopathy (DR)

DM patients suffer from many serious life-threatening complications, including microvascular-related neuropathy [10], nephropathy [11], retinopathy [12] and macrovascular-related cardiovascular diseases, such as ischemia [13], stroke [14] and peripheral artery disease [15]. Among these, patients who have suffered with diabetes for longer than 10 years are at a high-risk of developing diabetic retinopathy (DR) [16]; type 1 diabetic patients have a higher risk of developing DR compared to those with type 2 DM [17,18]. Currently, approximately 93 million people in the world have DR, a quarter of whom have vision-threatening DR [19]. This number is predicted to rise along with the increasing incidence of diabetes [5].

1.3.1 DR classification

Clinically, DR is classified into two main types: proliferative and non-proliferative [18]. The key criterion is whether the retina starts growing abnormal blood vessels (Figure 3) [20].

1.3.1.1 NPDR

Non-proliferative diabetic retinopathy (NPDR) is the early stage of DR without the occurrence of neovascularization. NPDR can be subdivided into mild, moderate and severe stages. Mild NPDR is characterized by a few microaneurysms in the retina. Moderate NPDR is identified by various microaneurysms, hemorrhages and hard exudates. Severe NPDR presents severe intraretinal hemorrhages, many more cotton wool spots, capillary occlusion and hard exudates [21,22], all of which can be detected by fundus examination.

1.3.1.2 PDR

Proliferative diabetic retinopathy (PDR) is a more advanced form of DR, identified by significant retinal neovascularization. However, the abnormal blood vessels are fragile and leaky [23]. They may have misdirected growth into the vitreous, resulting in vitreous hemorrhage followed by vision loss. In addition, the abnormal blood vessels may scar and lead to tractional retinal detachment, ultimately resulting in vision loss [22,24].

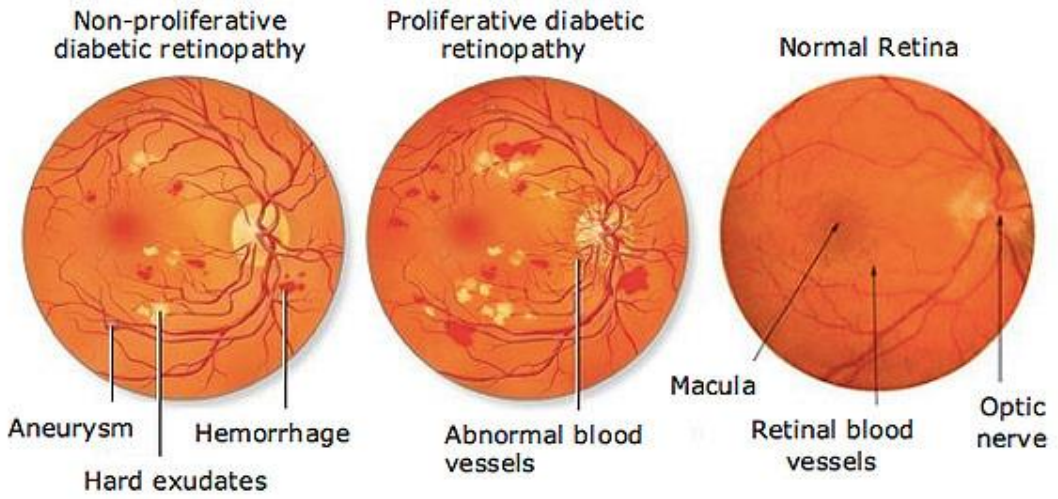

Fig. 3. Progression of retinal dysfunction in diabetes. The left image illustrates mild NPDR. The middle image shows examples of PDR, the right image is a normal retina [25].

Figure: Reproduced with permission from https://www.retinamd.com/diseases-and-treatments/retinal-conditions-and-diseases/diabetic-retinopathy/

1.3.2 Pathological Mechanism of Diabetic retinopathy

Numerous studies have suggested that hyperglycemia plays an essential role in the development of DR via the following biochemical mechanisms: increased polyol pathway flux [26], activation of protein kinase C (PKC) [27], increased hexosamine pathways flux [28], formation of reactive oxygen species (ROS) [29]and advanced glycation end products formation (AGEs) (Figure 4) [30].

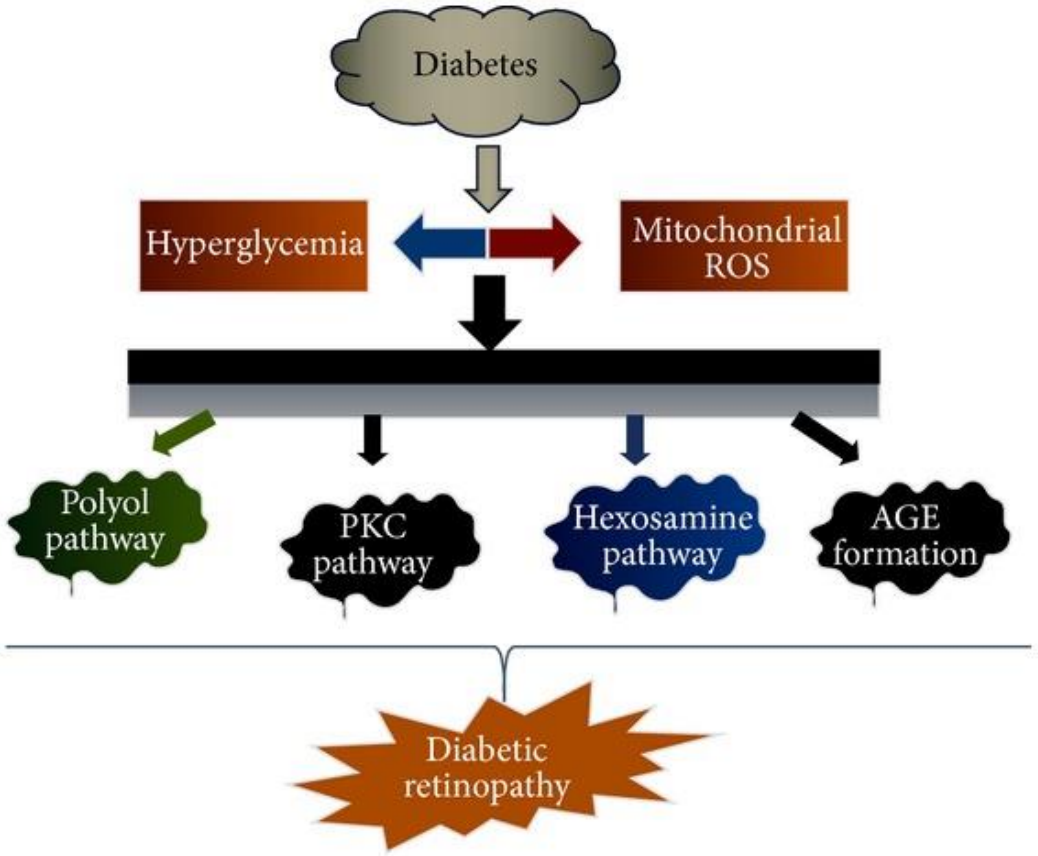

Fig. 4. The four main pathways associated with DR [31].

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1.3.2.1 Hyperglycemia and Retinal Microvasculopathy

Hyperglycemia is a major factor that links with the formation and development of DR [32]. The primary responses to hyperglycemia lead to retinal vessel dilation and blood

flow increase, as hyperglycemia suppresses the supply of energy substrates to the retina [33,34]. These changes might represent retinal autoregulation as an attempt to balance the changes in metabolism. Meanwhile, under hyperglycemic conditions, studies have shown the loss of pericytes in the diabetic retina both *in vitro* and *in vivo* [35]. The loss of pericytes is thought to initiate structural damages to retinal capillary walls, leading to microaneurysm formation which is the onset of NPDR [36]. Previous studies reported that retinal capillary coverage with pericytes is associated with the survival of endothelial cells. The loss of pericytes also impairs endothelial-pericyte interactions and subsequently causes retinal leakage, which is crucial for blood-retina barrier (BRB) [37]. In addition, hyperglycemia leads to apoptosis of retinal endothelial cells and disrupts the BRB [38]. Furthermore, apoptotic endothelial cells are deprived of their replicative ability. This ultimately results in capillary occlusion which leads to ischemia and hypoxia [39]. Retinal ischemia/hypoxia magnifies the effect of multiple pro-angiogenic factors through the activation of HIF-1. This may disrupt the integrity of BRB and leads to further visual impairment in DM [40].

1.3.2.2 Neurovascular Changes in DR

However, in addition to existing research on microvascular changes in the diabetic retina, more recent pre-clinical studies demonstrated that components of the neurovascular-unit, including the inner and outer neurosensory retina, are disrupted in diabetic patients (Figure 5) [41,42]. Disturbed neuronal function is reflected by damaged glutamate and dopamine neurotransmitter systems [43]. In contrast to the enlargement of dendritic fields [44], the decreased expression of synaptic protein has been documented [45]. These changes ultimately lead to apoptosis of neurons alongside persistent uncontrollable diabetes [46]. Additional diabetic changes include altered glial cell activation, demonstrated by damaged interaction between glutamate and glutamine [47], reduced potassium channels [48], and subsequent markedly altered the expression of intermediary filament proteins [49]. The transformation of M ller glia cells from a quiescent to active phenotype increases the expression of glial fibrillary acidic protein (GFAP) in the human diabetic retina [50,51]. Studies have also shown an increased GFAP expression of rat M ller glia cells at 2 to 5 months in a diabetic model [52]. In addition, diabetes causes the dysfunction of retinal astrocytes, reduction of connexin expression as well as cell apoptosis with the progression of DR [53], though the

underlying mechanism needs to be explored. Microglial cells, the resident immune cells in the retinal, function to monitor the retinal microenvironment, react to local insults and translocate their positions in the retina to maintain homeostasis [54]. Nonetheless, microglial cells may lead to retinal damage associated with chronical stimulation [55]. In some diabetic patients, the alteration of retinal electrophysiology and neurodegeneration have also been observed [56,57]. The physiological and biochemical dysfunction of diabetic rat retina occurred is associated with the onset of hyperglycemia [58]. In contrast, significant alterations of the retinal microvascular system such as vascular leakage and angiogenesis occur after years of developing diabetes in humans or around 6 months later in diabetic animal models [59,60]. Therefore, diabetes-induced glia-neuronal abnormalities prior to vascular diseases may contribute to the pathogenesis of retinal vessels [61]. Hence, DR cannot be considered merely as a microvascular disease but more a neurovascular-unit disease.

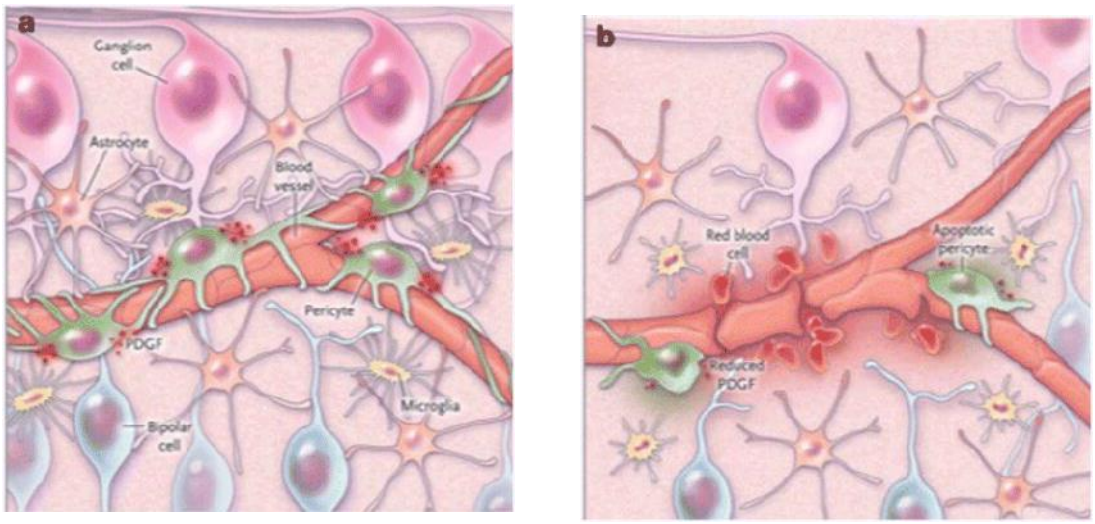

Fig. 5. The retinal neurovascular interaction. (a) The integrity of normal retinal microenvironment. (b) The disruption of neurovascular-unit in the DR [62].

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1.4 Retinal Hypoxia and Müller Cells

1.4.1 Retinal Hypoxia and HIF-1

Retinal ischemia and hypoxia are the main causes of DR complications and visual loss.

Hypoxia, or low-oxygen conditions, will active hypoxia-inducible factor-1 (HIF-1) in eukaryotic cells [63]. HIF-1 is an oxygen-regulated heterodimer transcription factor formed by HIF-1α and HIF-1β subunit [64]. Under normoxic conditions, HIF-1α subunits are degraded by proteasomes due to its oxygen sensitivity. Under hypoxic conditions, the degradation of HIF-1α is reduced, thus the transcriptional activity is enhanced [65,66]. This leads to an accumulation of active HIF-1α subunits that bind to HIF-1β subunits, forming HIF-1 [67]. Activated HIF-1 is capable of regulating specific downstream genes; it induces gene expression to facilitate the adaptation of cells, tissues and organisms to hypoxic conditions [68].

1.4.2 HIF-1 and VEGF

A variety of HIF-1–target genes have been reported to stimulate neovascularization and vascular permeability in the retina. VEGF is the most critical pro-angiogenic factor among the HIF-1–dependent factors identified in retinopathies including DR [69,70]. VEGF or vascular permeability factor (VPF) consists of 8 highly conserved cysteines, and they belong to the platelet-derived growth factor (PDGF) supergene family [71]. The family of VEGF includes 7 members, 5 of which (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF) exist in mammals [72]; however, VEGF-E and *Trimeresurus flavoviridis* sv VEGF do not [73]. VEGF is found to drive the angiogenesis and the formation of new vessels in DR [70], which lead to the development of anti-VEGF therapy[74]. VEGFs exert their biological functions through targeting VEGF receptors, the currently known VEGF receptors are: VEGFR1 (Flk-1), VEGFR2 (KDR/Flk-1in mice) and VEGFR3 (Flt-4) [75]. VEGF-A is also called VEGF, VEGF-A pre-mRNA can be spliced into multiple isoforms including VEGF121, VEGF165, VEGF189 and VEGF206 in humans (VEGF120, VEGF164, VEGF188 in mouse) [76]. The predominant expression VEGF-A isoforms in human and mouse tissues are VEGF165 and VEGF164 respectively [77]. Intensive investigations of the mechanisms of hypoxic retinal diseases suggest that oxygen levels are a crucial regulator of VEGF-A gene expression [78] and that VEGF induced by HIF-1 plays a major role in inducing vascular leakage and retinal neovascularization in DR [79,80].

1.4.3 VEGF and Hypoxic Müller cells

1.4.3.1 Müller Cells

Müller cells account for 90% of the retinal glia and spans across the entire the retina [3]. Anatomically and functionally Müller cells constitute a link among the retinal vitreous body, neurons, blood vessels (forming glial limitans) and the subretinal space [81].

1.4.3.2 Müller Cells Function

Müller cells display various shapes in different species, but they have a considerable number of common features. The morphological localization of the Müller cells is designed to provide structural stabilization, which plays an essential supporting role in maintaining the homeostasis of retina [82,83]. Müller cells also provides direction for neurons and neurites to migrate in processes, such as during the early stages of retinal development, the recycling of neurotransmitter and information processing [84]. Apart from their neuroprotective effect, Müller cells are participated in regulating retinal vascularization. In physiological conditions, factors secreted by Müller cells, such as pigment epithelium-derived factor (PEDF), neurturin and thrombospondin-1, are able to enhance the BRB's integrity [85,86]. However, in response to injuries such as retinal ischemia/hypoxia caused by DR, the factors secreted by Müller cells become imbalanced. In particular, the increased secretion of VEGF can result in degraded tight junctions and breakdown of the BRB, ultimately leading to increased vessel leakage [87,88].

1.4.3.3 VEGF and Müller Cells

Currently observations show that there are five retinal cell types have the ability to generate and release VEGF, RPE [89], astrocytes [90], Müller cells [91], vascular endothelial [92] and RGC[93]. Amongst these cell types, the Müller glia cells are the primary source of VEGF in the retina [94]. It has been demonstrated that the conditional ablation of VEGF in mouse Müller glia cells has resulted in reduction of the total retinal VEGF by almost half [87][95]. Similar effects were observed in a Streptozotocin (STZ)-induced type 1 DM model [96]. However, the Müller glia-derived VEGF is a double-edged sword. Although it is essential for vasculogenesis and angiogenesis in the development of retina, it has also been found to be critical in the molecular pathogenesis of several retinal diseases [83]. Studies have revealed that Müller glia-derived VEGF is a key contributor to neovascularization and vascular leakage in DR, and the up-

regulation of VEGF in hypoxic Müller cells leads to the breakdown of the BRB in both *in vitro* and *in vivo* models [97]. Therefore, the stabilization of VEGF secretion in hypoxia-induced Müller cells needs to be explored.

1.5 Preconditioning

Preconditioning is a phenomenon where an entity is briefly exposed to a variety of stress and insults to subsequently induce resilience against lethal injuries in the future. Preconditioning stimuli like hypoxia [98], ischemia [99], hypothermia [100], endotoxin pretreatment [101] and light [102] have been shown to activate multiple pro-survival pathways and provide robust protection. Moreover, a number of preconditioning methods have been investigated in the kidney [103], liver [104] and retina [105], as well as other organs [106].

1.5.1 The Protective Effects of Hypoxic Preconditioning (HP)

HP or hypoxia-induced tolerance refers to the exposure of cells, tissues or organs to mild hypoxia leading to accumulated tolerance towards subsequent hypoxia or severe insults that occurs minutes, hours, or even days later [107,108]. Sublethal hypoxia serves as a stimulatory "warning" signal that we term HP. Initial research confirmed the endogenous protective functions of HP in animals and later in various cell types and organs [109,110]. HP has been described in the heart [111], brain [112] and retina [113] as well as other tissues [114]. The cytoprotection of HP can be divided into two stages: firstly, an immediate protective effect that appears quickly and is short-lived after the stimulus, lasting only a few hours; secondly, a stronger protection occurring a few hours after hypoxia that lasts for many days or even several weeks [109]. The later stage of HP is mediated by HIF through regulating the transcription factor, HIF-1α, thereby targeting the downstream synthesis of genes and proteins such as VEGF [115]. Recently, many studies have reported the ability of HP to provide tissue protection and neuroprotection through the stabilization of HIF-1α [116,117]. HP also strongly protects the retina against cell death [118]. This encourages further exploration into the mechanisms of how Müller cells stabilize the retinal microvascular environment via the process of HP.

1.6 Neuropeptides

1.6.1 Neuropeptides in The Retina

Neuropeptides are small amino acids, primarily generated by neurons, which modulate neuronal communication by acting on cell surface receptors [119]. Studies have shown that different neuropeptides participate in a range of physiological regulations [120] to include metabolism [121], stress control [122] and pain perception [123]. Neuropeptides are neural signaling molecules and evoke biological responses via specific receptors [124]. There are more than 10 neuropeptides expressed within the INL of the retina and RGC [125], of which Substance P (SP) and Neuropeptide Y (NPY) are the most highly-expressed [126,127].

1.6.2 SP

SP is a neuropeptide that is 11 amino acids long; it belongs to the tachykinin neuropeptide family. SP is not only produced by neuronal cells, but also non-neuronal cells such as immune cells [128]. SP performs its biological function through high affinity neurokinin receptors: endogenous neurokinin 1 receptor (NK1R; which has the highest affinity interaction), NK2R and NK3R [129,130]. SP also performs a variety of neuroprotective functions, such as in Alzheimer's disease [131,132]. In addition, the effect of SP are observed in wound healing [133], cell proliferation [134], angiogenesis [135] and ocular homeostasis [136].

1.6.3 NPY

NPY is the most highly-expressed neuropeptide (under physiological conditions) within the central nervous system (CNS) of mammals; it is involved in numerous physiological processes [137]. NPY consists of 36 amino acids and performs its biological function through G-protein-coupled receptors, Y1-6 [138]. The expression of NPY in several regions of the eye and in the retinal cells of different species have been demonstrated by NPY-immunoreactivity [139]. It has been shown that NPY exists in large amount within retinal neurons, especially the human RGC [140]. Likewise, the expression of NPY in non-neural elements has also been discovered in some species, such as within the Müller cells in humans [141,142]. NPY has a crucial effect on metabolism, and the dysregulation of NPY has been observed in multiple pathophysiological processes [143][144]. It has also been shown that NPY has a neuroprotective effect against excitotoxicity in the CNS [145]. Furthermore, Cavadas C *et al.* reported that NPY

protects retinal neurons from glutamate-induced excitotoxicity in cultured rat retinal neural cells [146]. Meanwhile, increased NPY levels in plasma were observed under several stress conditions in humans and animals, such as hypoxia, tissue injury and ischemia [147,148].

1.6.4 Neuropeptides (NPY/ SP) and DR

In recent years, studies have shown that elevated levels of NPY and SP observed in the retina are associated with acute stress, yet both levels are markedly reduced in diabetes [149,150]. A current opinion is that NPY and SP are neuroprotective, and that retinal neural apoptosis is attenuated when the neuropeptides are elevated along with preventing the loss of retinal functions in animals [151,152]. In addition, SP and NPY promote the proliferation, differentiation and migration of vascular endothelial cells [153,154], while NYP also stimulates vascular smooth muscle cells [155].

2. Aim of the thesis

The primary aim of the thesis was to interrogate the impact of HP in modifying Müller cells in DR. The secondary aims were to investigate the function of neuropeptides (SP and NPY) in regulating the neurovascular-unit in DR.

2.1 The first hypothesis: Müller cells stabilize microvasculature through HP

According to the protective effect of HP though stabilizing the HIF-1α in the retina. The beneficial effects of HP might involve counter-regulation of Müller cell activation and negate subsequent secretion of pro-angiogenic factors, including VEGF and angiopoietin-like 4 (ANGPTL-4).

This study aimed to answer the following scientific questions:

1) Can Müller cells under hypoxic conditions increase retinal vascular leakage and angiogenesis?
2) Can HP stabilize Müller cells in hypoxic conditions to protect vascular integrity in retinal endothelial models?
3) What is the potential mechanism of HP in stabilizing HIF-1α in Müller cells?

2.2 The second hypothesis: Adjunctive neuropeptides (SP and NPY) therapy sustain neurovascular health thereby attenuating DR

Based on the known functions of SP and NPY, it is worth assessing whether NPY and SP can exhibit neurovascular protective properties in the retina, and specifically in a model of DR.

This study aimed to answer the following scientific questions:

1) Are neural dysfunction and microvascular abnormality interdependent in DR?
2) Can improvement of neuronal health through neuropeptides (SP and NPY) prevent or rescue vasculopathy in DR?

3. Results

3.1 Müller Cells Stabilize Microvasculature through Hypoxic Preconditioning

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Abstract

Background/Aims: Hypoxia of the retina is a common pathogenic drive leading to vision loss as a result of tissue ischemia, increased vascular permeability and ultimately retinal neovascularisation. Here we tested the hypothesis that Müller cells stabilize the neurovascular unit, microvasculature by suppression of hypoxia-inducible factor-1alpha (HIF-1α) activation as a result of hypoxic preconditioning. ***Methods:*** Tube Formation Assay and *In Vitro* Vascular Permeability Image Assay were used to analyze angiogenesis and vascular integrity. Seahorse XF Cell Mito Stress Test was used to measure mitochondrial respiration. Gene and protein expression were examined by qRT-PCR, ELISA and western blot. ***Results:*** Hypoxic insult induces a significant induction of pro-angiogenic factors including vascular endothelial growth factor (VEGF) and angiopoietin-like 4 (ANGPTL-4) resulting in angiogenesis and increased vascular permeability of vascular endothelial cells. Hypoxic preconditioning of a human retinal Müller glia cell line significantly attenuates HIF-1α activation through the inhibition of mammalian target of rapamycin (mTOR) and concomitant induction of aerobic glycolysis, stabilizing endothelial cells. ***Conclusions:*** Hypoxic preconditioning of Müller cells confers a robust protection to endothelial cells, through

the suppression of HIF-1α activation and its downstream regulation of VEGF and ANGPTL-4.

Running title

Microvasculature Stabilization through Hypoxic Preconditioning

Key words: Müller cells, angiogenesis, vascular permeability, hypoxia, hypoxic preconditioning

1. Introduction

Retinal hypoxia is recognized in a number of sight-threatening disorders including central retinal artery occlusion, ischemic central retinal vein occlusion, diabetic retinopathy and occlusive retinal vasculitis [156–158]. Hypoxic environments induce expression of Hypoxia-inducible factor 1-alpha (HIF-1α), which modulates target genes such as vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) in many tissues [159]. Under conditions of hypoxic stress, degradation of the oxygen-sensitive HIF-1α subunit is reduced, whereas its transcriptional activity is enhanced [65,66,160]. The increased amount of active HIF-1α protein localizes to the nucleus by binding to HIF-1β, forming a heterodimer (HIF-1) that is capable of binding to the DNA of specific (hypoxia-inducible) genes and inducing broad changes in gene expression, which mediate acclimation of cells, tissues, and the organism to conditions of low oxygen tension [68]. The HIF family consists of three identified members, with HIF-1 being the best-characterized protein [161]. The action of HIF-1 and associated downstream regulated genes, particularly VEGF, are essential for retinal development, vasculature stability, proper retinal function, and vision maintenance [162]. Nevertheless, the HIF-1 targeted genes represent a double-edged sword [163]. Under hypoxic conditions, overexpression of these target genes alters the tissue homeostasis and contribute to ocular disease, through increased retinal angiogenesis and vascular permeability [164].

Müller cells account for 90% of the retinal glia [3]. Müller cells span across the entire thickness of the retina, from the inner limiting membrane of the retina to the distal end of the outer nuclear layer. Anatomically and functionally Müller cells constitute a link between the retinal neurons, the vitreous body, the retinal blood vessels (forming glial limitans) and the subretinal space [165]. However, reactive Müller cell gliosis often occurs in the mammalian retina in response to injury. It has been demonstrated that the release of several proinflammatory factors, cytokines and proangiogenic growth factors from Müller cells can accentuate retinal damage [83]. As an example, evidence demonstrates that VEGF and ANGPTL-4 are up-regulated by HIF-1 in hypoxic retinal Müller cells *in vitro* and the ischemic inner retina *in vivo* [69]. Increased expression of VEGF and ANGPTL-4 promotes angiogenesis and vascular permeability resulting in disruption of the blood retinal barrier [69,166].

3. Results

Hypoxic preconditioning (HP) or hypoxia-induced tolerance, refers to a brief period of hypoxia that induces protection against subsequent lethal insult occurring minutes, hours, or even days later [108]. HP represents a fundamental adaptive response to environmental stress, whereby cells adapt to stress by upregulating defense mechanisms and switching to a protective phenotype [167]. Reports have shown that HP stabilizes HIF-1α in the retina and protects photoreceptors against light-induced cell death [168]. Whilst the underlying pro-survival mechanisms are not fully understood, the cellular response to hypoxia is characterized by the differential regulation of genes. Therefore, we hypothesized that the beneficial effects of HP might involve counter-regulation of Müller cell activation and negate subsequent secretion of pro-angiogenic factors. In this *in vitro* study, we demonstrate that HP of Müller cells stabilizes microvasculature via HIF-1 regulation.

2. Materials and Methods

2.1 Cell Culture and Reagents.

Spontaneously immortalized human Müller glia cell line (MIO-M1) purchased from UCL Business PLC (London, UK), were cultured in DMEM medium containing high glucose and stable glutamine, supplemented with 10% fetal bovine serum (FBS) (Life Technologies, UK). Human Primary Retinal Microvascular Endothelial cells (HRMECs, H6065, Generon, UK) were cultured in Complete Human Endothelial Cell Medium (H1168, Generon, UK), Human Umbilical Vein Endothelial Cells (HUVECs) (C-12200, Promocell, UK) were cultured in Endothelial Cell Growth Medium 2 (C-39211, Promocell, UK). Rapamycin (R8781, working concentration 100 nM) and Digoxin (D6003, working concentration 100 nM) were obtained from Sigma-Aldrich. Apoptosis/ Necrosis Detection Kit (ab176749, Abcam, UK) was used to assess HP-induced cell viability. MIO-M1 were seeded at a density of 3×10^4 in 96 well plate, then cultured in normoxic and hypoxic incubator for 3 days. Cells were then stained with Apoptosis/ Necrosis Detection Kit as described by the manufacturer's protocol. MIO-M1 cell viability was quantified by counting the number of apoptosis and necrosis cells account for the total number of cells under a fluorescence microscope (Nikon, Japan).

Hypoxic conditions was used according to previous report [69]. In brief, MIO-M1 cells were cultured in normal conditions to confluency and then transferred to hypoxic chamber supplied with 1% O_2, 94% N_2, and 5% CO_2. Control cultures were incubated under standard normoxic conditions for the same duration. In HP experiments, MIO-M1 cells are transferred to hypoxic culture conditions for a defined duration (1 hour, 2 hours and 4 hours) before returning and reoxygenation in the normoxic incubator (95% air, 5% CO_2) for 24 hours. Cultures are then re-incubated for a further 48 hours within the hypoxic environment. After the re-incubation time, the supernatant and cells were collected for the quantitative assays.

2.2 Quantitative Real time RT-PCR

Total RNA extraction was carried out using TRIzol™ Reagent (15596026, Life Technologies, UK) according to the manufacturer's instructions. Total RNA was quantified by Nanodrop and treated with RQ1 RNase-free DNase before cDNA synthesis using the ImProm-II™ Reverse Transcription System (A3800, Promega, UK). cDNA was amplified using the Power SYBR® Green PCR Master Mix Reagent

(4367659, Life Technologies, UK) on a StepOne™ Applied Biosystems Real-Time PCR System. Primer sequences used were: β-Actin, forward 5'-gggaaatcgtgcgtgacattaag, reverse 5'-tgtgttggcgtacaggtctttg; HIF-1α, forward 5'-gaaagcgcaagtcctcaaag, reverse 5'-tgggtaggagatggagatgc; ANGPTL-4, forward 5'-ggacacggcctatagcctg, reverse 5'-ctcttggcgcagttcttgtc. succinate dehydrogenase complex subunit D (SDHD), forward 5'-atggcggttctctggaggctg, reverse 5'-gagcttccacgcatggcaac; glucose transporter 1 (GLUT1), forward 5'-tcactgtgctcctggttttctg, reverse 5'-cctgtgcctcctgagagatcc. The equation fold change = $2^{-\Delta\Delta ct}$ was used for calculation relative changes in expression levels. All measurements were performed at least in duplicates and the experiments were performed at least three times independently.

2.3 SDS_PAGE analysis

For analysis of protein levels, cultured cells were rinsed twice with ice-cold PBS and then lysed with ice-cold CelLytic™ MT Cell Lysis Reagent (C2978, Sigma-Aldrich, UK). Cell lysates were centrifuged at 14,000 g for 10 min at 4°C, the supernatant was transferred to fresh tubes and Pierce™ BCA Protein Assay Kit (23225, Thermo Fisher Scientific, UK) used to determine the protein concentration in each sample. Then the equal protein was mixed with 4× SDS sample buffer, boiled for 10 min at 90°C, and resolved using 4% to 20% NuPAGE gels (XV04200PK20, Invitrogen, UK). After electrophoresis, the proteins were transferred to PVDF membranes (IB24001, Invitrogen, UK) by electrophoretic transfer. The membranes were blocked with 5% skimmed milk for 2 hours, rinsed, and incubated overnight at 4°C with the following primary antibodies: Anti-HIF-1 alpha (ab2185; Abcam, UK; 1: 1000) and mTOR (7C10) Rabbit mAb (2983; Cell Signaling Technology, UK; 1:1000). Excess antibody was then removed by washing the membrane in PBS/ 0.1% Tween 20, and the membranes were incubated for 2 hours with horseradish peroxidase-conjugated secondary antibodies (A0545, Abcam, UK; 1:2000). Following further washes in PBS/ 0.1% Tween 20, the signals were developed with ECL reagent (GERPN2209, Sigma, UK) and captured by an electronic imaging system (Konica Minolta).

2.4 ELISA

To quantify levels of secreted VEGF and ANGPTL-4, culture supernatants from hypoxic conditioned MIO-M1 cells were assayed using DuoSet human VEGF

(DY293B, Bio-Techne, UK) or ANGPTL-4 ELISA (DY3458, Bio-Techne, UK) kits according manufacturer´s instructions. Three samples for each experimental condition were used. In each experiment, all samples and standards were measured in duplicate.

2.5 Tube formation

Tube formation assay was performed using growth factor-reduced Matrigel (No.354320, BD Biosciences, UK). Fifty microliters of Matrigel was added into a prechilled 96-well plate and placed in a 37°C CO_2 incubator for 30 min. HRMECs were counted and seeded 2×10^4 cells in 100 μl of culture medium which were mixed with 50 μl of test sample, then added onto the matrigel surface. Eighteen hours later, phase contrast photos were taken by Wide field microscope (Leica DMI6000). The whole area of tube length was quantified by ImageJ1.46r (National Institutes of Health, USA).

2.6 Permeability Assay

Permeability visualization experiments were performed on 18×18 mm square Rinzle plastic coverslips with *In Vitro* Vascular Permeability Imaging Assay (17-10398, Merck, UK). HUVEC cells were cultured for 48-72 hours on biotinylated gelatin coated coverslips in 35 mm culture dishes in a density of 4.5×10^5 cells/dish. The culture medium was then changed to EGM-2 containing 2% FBS for 2 hours prior to stimulation. Then culture medium was mixed with test samples at 1∶3 ratio and incubated for 30 min at 37°C before evaluation of HUVEC cell monolayer permeability. Fluorescein-streptavidin (25 μg/ml) was directly added to the culture medium for 5 min, followed by two washing steps (3 ml of PBS, pH 7.4, 37°C). The cells were fixed (3.7% formaldehyde in PBS for 10 min) and subjected to immunofluorescence staining for VE-cadherin according to the manufacturer's instructions. Overlay of Fluorescein and VE-cadherin staining demonstrates reciprocal relations between VE-cadherin peripheral localization and increased local permeability of Fluorescein-streptavidin. Local permeability change induced by conditioned medium was visualized in cells grown on biotinylated gelatin plastic coverslips. The pattern of Fluorescein-streptavidin binding to the biotinylated gelatin underlying the cell monolayer was examined under Leica SP5-AOBS confocal laser microscope. Images were processed with ImageJ software.

2.7. Extracellular flux analysis

Cell metabolism was assessed using a Seahorse XFp Extracellular Flux Analyzer (Agilent, UK). MIO-M1 cells were seeded at a density of 3×10^4 per well and were treated with appropriate reagents. Real time measurements of oxygen consumption rate (OCR) were normalized to total protein content using a BCA assay. Pre-optimized injections of the reagents specific for each assay were used. Cell Mito Stress kit (103015-100, Agilent, UK) injections: oligomycin, FCCP and antimycin A/rotenone.

2.8 Statistical Analysis.

Results from cell culture and animal models are shown as mean ± SD from a minimum of three independent experiments. Statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons, while unpaired Student's *t* test and Mann-Whitney test for two individual comparisons where indicated. Statistical analysis was performed using Prism 6.0 software (GraphPad). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

3. Results

To determine whether HP can alter MIO-M1 cells responses in low-level oxygen environments, we initially established the hypoxic response profile of the MIO-M1 cell line through *in vitro* assays. Consistent with previous reports [69,169], MIO-M1 cells remain viable when cultured in a hypoxic (1% O_2) environment, with increased protein expression of HIF-1α, and gene expression and secretion of VEGF and ANGPTL-4 over the 48 hour time-period examined (Figure 1). Pretreatment of MIO-M1 cells with digoxin to block hypoxic induction of HIF-1α, suppressed VEGF and ANGPTL-4 production (Figure 1).

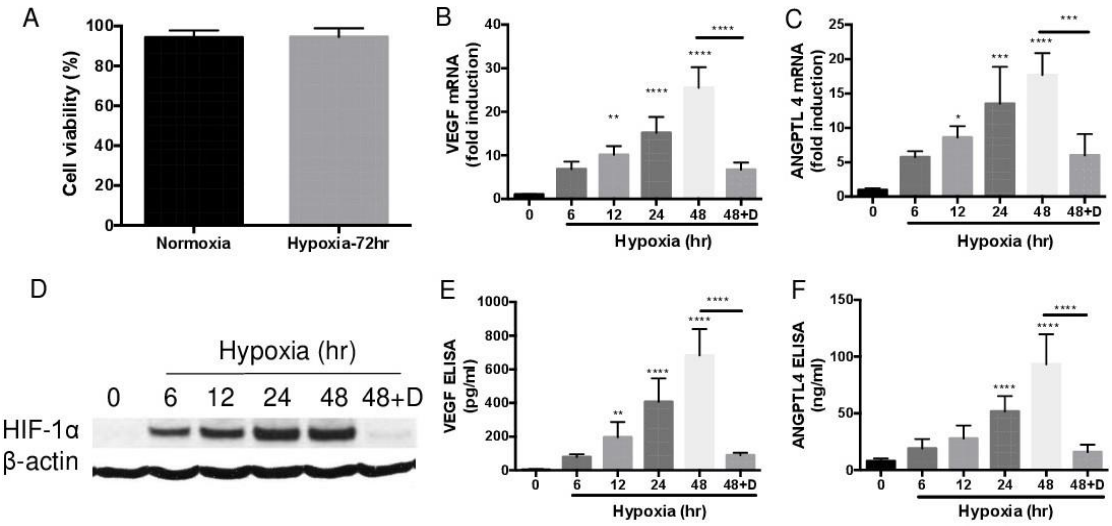

Fig. 1. Hypoxia insult induces HIF-1-dependent up-regulation of VEGF and ANGPTL-4 in cultured MIO-M1 cells without reducing cell viability. (A). Prolonged 1% O_2 hypoxia (72 hours) did not alter the cell viability staining in MIO-M1 cells culture (n=4 per group). (B-F) Exposure of MIO-M1 cells to hypoxia (indicated time) resulted in an increase of HIF-1α protein and a corresponding increase in VEGF and ANGPTL-4 mRNA and protein level, which can be inhibited by pretreatment with the HIF inhibitor, digoxin (100 nM; 2 hours before hypoxia, 48+D) (n=4 per group). Data represents means ± SD of relative values vs control from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons, while unpaired Student's t test and Mann-Whitney test for two individual comparisons.

To confirm a potential contribution of secreted factors elicited by hypoxia-treated MIO-M1 cells to angiogenesis and vascular leakage, HRMECs were treated with conditioned medium from the MIO-M1 cells exposed to hypoxia for 48 hours (Hypoxia-CM). Endothelial cell proliferation and permeability was determined by tube formation and Fluorescein-positive intercellular signal respectively. Hypoxia-CM increased HRMEC

tube length compared with normoxic conditioned medium (Normoxia-CM). Conditioned medium from hypoxic MIO-M1 cells pre-treated with digoxin resulted in reduced overall tube length, comparable to Normoxia-CM (Figure 2A-B). Furthermore, exposure of a monolayer of HRMECs to Hypoxia-CM from the MIO-M1 cells promoted endothelial cell permeability and was perturbed by addition of digoxin (Figure 2C-D). Collectively, these results support a HIF-1α-mediated up-regulation and functional effect of pro-angiogenic factors secreted by MIO-M1 cells.

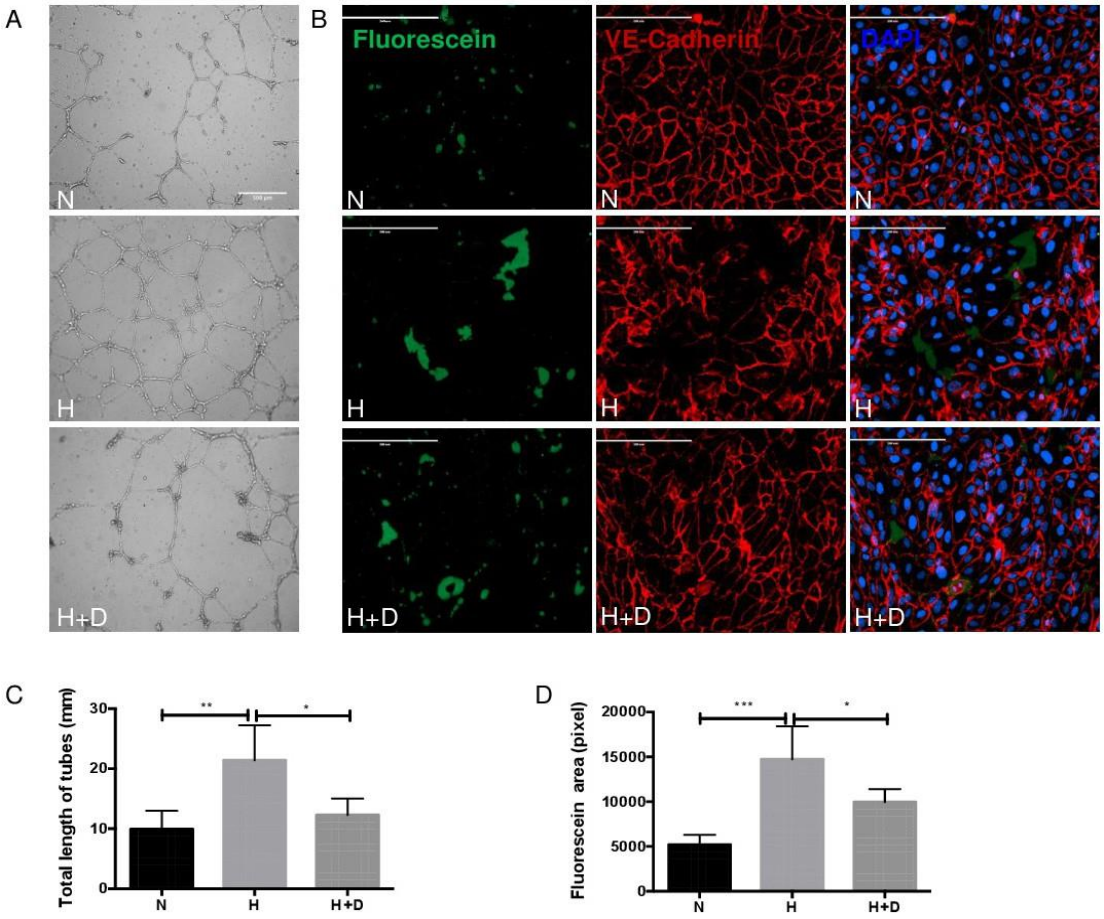

Fig. 2. Conditioned medium from hypoxic MIO-M1 cells promotes angiogenesis and vessel leakage. (A-B) Conditioned media from MIO-M1 cells exposed to 1% O_2 hypoxia 48 hours (H) stimulated HRMECs tube formation compared to 20% O_2 normoxic condition (N) (n=12 per group). The total length of tubes decreased by pretreatment with digoxin (H+D). Images was performed with Leica DMI6000 microscope (×5). Scale bar, 500 μm. (C-D) The area of Fluorescein-positive intercellular spots increased conditioned medium from hypoxic MIO-M1 cells and decreased by pretreatment with digoxin (n=3 per group). Scale bar, 200 μm. Data represents means ± SD of relative values vs control from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Extending these observations further to determine a suitable hypoxic stimulus period for evaluating HP responses, we wished to confirm the early hypoxic response kinetics of HIF expression. MIO-M1 cells were similarly cultured in low O_2 conditions, mRNA and protein expression levels were quantified at several early time points, ranging from 1 to 6 hours. Expression of HIF, VEGF and ANGPTL-4 increased in a time-dependent response; elevated by 2.6 folds by 2 hours, and further increased by 3.9 folds by 4 hours, as compared to cells under normoxic conditions (Figure 3A-E). Based on the concept of HP (brief hypoxic exposure), we determined that the cellular response within the first 6 hours following hypoxic exposure elicit induced HIF & proangiogenic expression, but not to a detrimental level. On this basis, we examined the effect of 1, 2 & 4 hours HP exposure to assess the potential beneficial effect of HP.

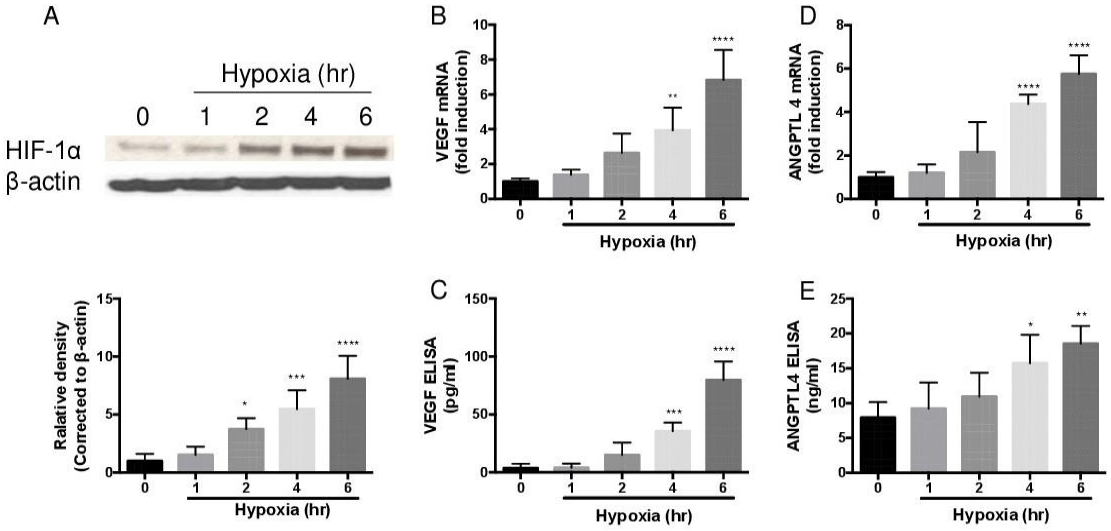

Fig. 3. HIF-1α protein accumulation and VEGF expression in cultured hypoxic MIO-M1 cells. (A) Exposure of MIO-M1 cells to 1% O_2 hypoxia (indicated time) induces HIF-1α protein stability. (B-E) Hypoxic conditions lead to increase expression of VEGF and ANGPTL-4 mRNA and protein. All the samples were normalized to their β-actin expression (n=4 per group). Data represents means ± SD of relative values vs control from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Hypoxic Preconditioning Suppresses Hypoxia Induced Expression of HIF-1α, Angiopoietin-Like 4 and VEGF in MIO-M1 cells

The HP protocol involves an initial short exposure of MIO-M1 cells to hypoxia (1, 2 or 4 hours), then a 24 hours reoxygenation period under standard normoxic conditions, before a return to the hypoxic environment for a further 48 hours (Supplementary 1).

3. Results

We compared the effect of the HP duration on VEGF and ANGPTL-4 expression to MIO-M1 cells without return to normoxia. Following HP for 2 hours, the level of mRNA and secreted protein were both significantly reduced. HP for 1 hour and 4 hours did not alter expression (Figure 4A-D). To interrogate whether the protective effect of HP in MIO-M1 cells in hypoxia was mediated via altered expression/regulation of HIF-1α, we performed SDS-PAGE on cell lysates from HP and hypoxia alone samples. Consistent with our angiogenic markers expression, HP-2 hours resulted in the maximal attenuation of HIF-1α protein accumulation (Figure 4E).

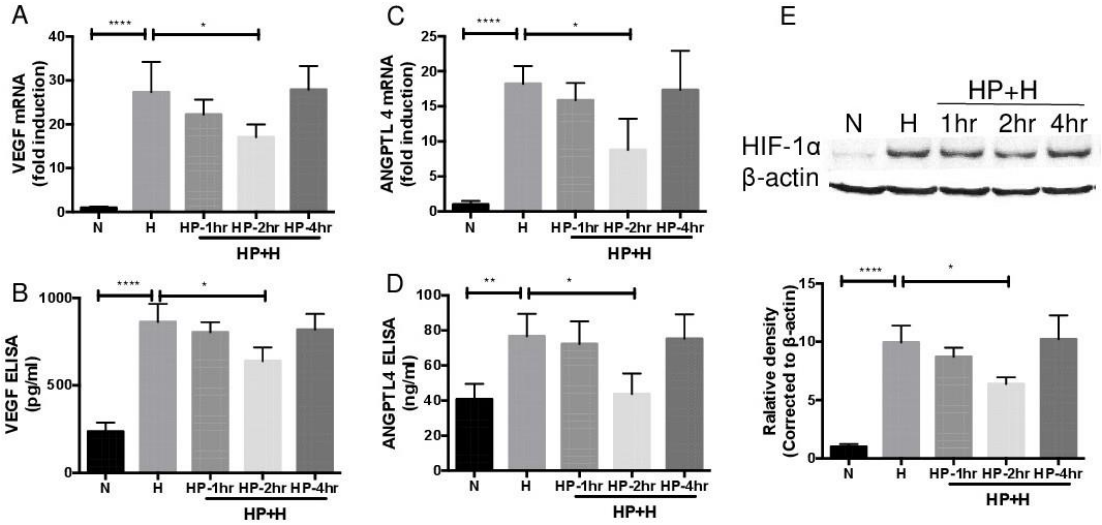

Fig. 4. Hypoxic preconditioning suppresses prolonged hypoxia-induced VEGF and ANGPTL-4 in MIO-M1 cells culture. (A-E) The expression of VEGF and ANTPGL-4 as well as HIF-1α protein remarkably increased with exposure of MIO-M1 cells to 1% O_2 Hypoxia 48 hours (H) in comparison with 20% O_2 normoxic conditions (N). Brief hypoxia exposure for 2 hours before prolonged hypoxia (HP+H) insult significantly reduced the expression of VEGF and ANGPTL-4. The induction of HIF-1α was also significantly suppressed by brief HP for 2 hours (n=4 per group). Data represents means ± SD of relative values vs control (N) from 3 independent experiments. *P<0.05; ***P<0.001; ****P<0.0001, statistical analysis was performed with oneway ANOVA with Dunn's test for multiple comparisons.

Hypoxic Preconditioning Suppresses Hypoxia Induced Angiogenesis and Vascular Permeability in MIO-M1 cells

The data demonstrates the potential beneficial effect of HP that suppresses HIF mediated upregulation of proangiogenic factors in response to hypoxia. Thus, we evaluated how altered cellular responses following HP may regulate endothelial cell responses. Monolayers of HRMECs were treated with Hypoxia-CM or conditioned medium from hypoxic preconditioning (Hypoxic preconditioning-CM) and tube

formation and vascular permeability assessed. Whilst Hypoxia-CM promoted endothelial cell proliferation, Hypoxic preconditioning-CM from MIO-M1 cells subjected to the HP-2 hours protocol demonstrate significantly reduced HRMEC tube length and junction formation by 35% and 64% respectively (Figure 5A-C). The HP-1 hour and HP-4 hours conditions failed to significantly reduce angiogenic potential. Similarly, assessment of vascular permeability corroborates the anti-angiogenic potential that HP confers to MIO-M1 cells. Conditioned medium from the MIO-M1 cells exposed at HP-2 hours demonstrate reduced by 44% endothelial cell permeability with decreased Fluorescein-positive intercellular signal across the HRMEC monolayer (Figure 5D-E). Whilst the extent of vascular leak was slightly reduced with conditioned medium from HP-1 hour and 4 hours, this was not statistically significant.

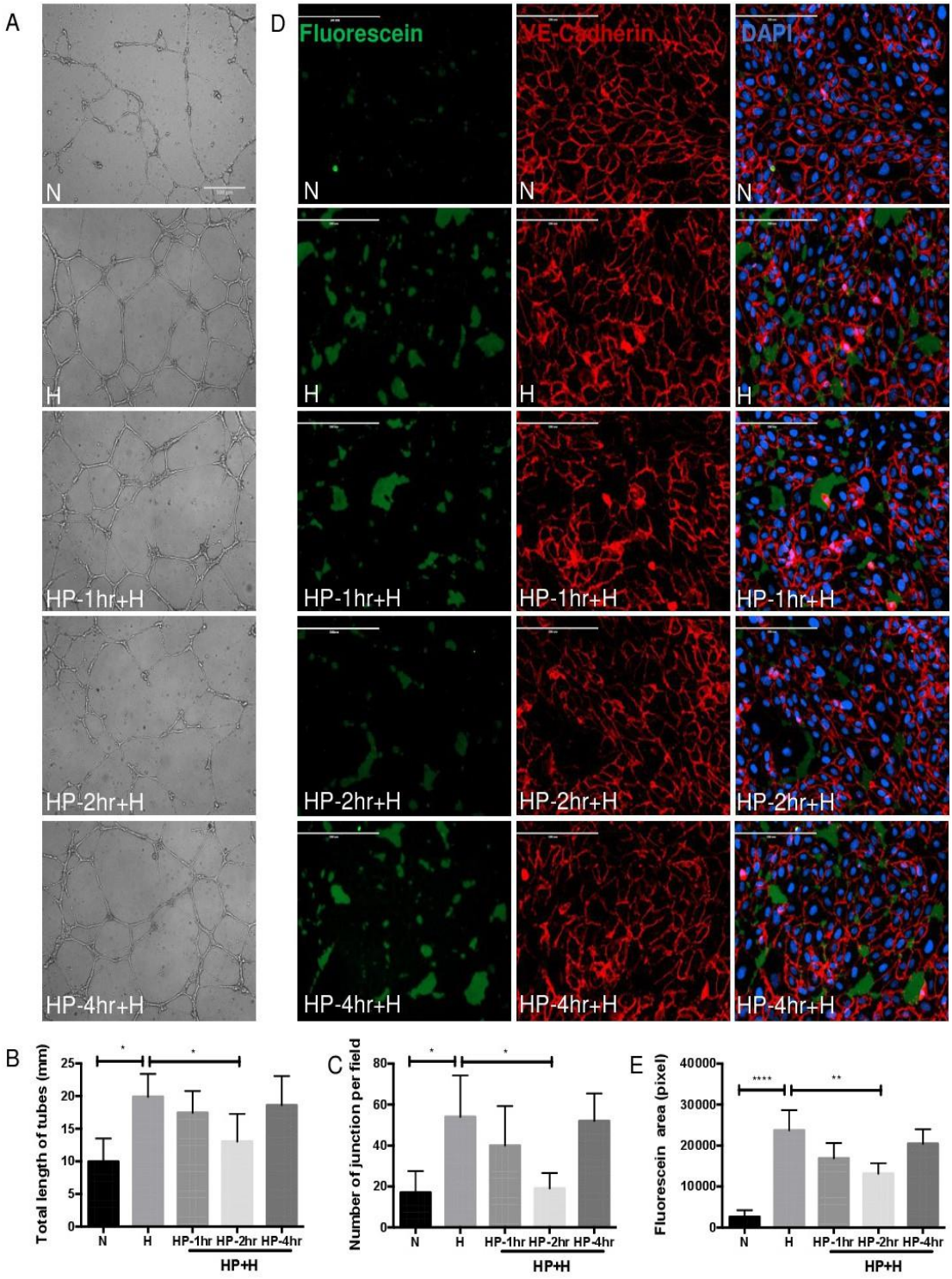

Fig. 5. Hypoxic preconditioning decreases HIF-1-dependent up-regulation of angiogenesis and vascular permeability. (A-C) Stimulation of HRMECs tube formation by conditioned media from MIO-M1 cells exposed to 1% O_2 hypoxia 48 hours (H) compare to 20% O_2 normoxic condition (N). Supernatant from HP-2 hours (HP-2hr) reduced both the total length and junction number of tubes compared to Hypoxic group (n=12 per group). Images was performed with Leica DMI6000 microscope (×5). Scale bar, 500 μm. (D, E) HUVECs were plated on coverslips coated with biotinylated gelatin and grown to confluence.

then treated for 10 min with conditioned medium from cultured Müller cells. Fluorescein-avidin was added for 3 min, cells were fixed and subjected to immunofluorescence staining for VE-cadherin (red) to visualize cell-cell contacts. Green fluorescence depicts areas permeable for Fluorescein-labeled avidin. Conditioned medium from hypoxic MIO-M1 cells increase area of Fluorescein-positive intercellular spots and decreased by HP-2 hours (n=3 per group). Scale bar, 200 μm. Data represents means ± SD of relative values vs control from 3 independent experiments. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Hypoxic Preconditioning Suppresses Hypoxia-Induced MIO-M1 cells Activation through Regulation of Metabolic Signaling.

As a key transcription factor, HIF-1α also upregulates genes involved in glycolytic energy metabolism and cell survival, including GLUT1 and several glycolytic enzymes [170]. Stabilization of HIF-1α under normoxia impairs the TCA cycle and mitochondrial activity [171]. We therefore hypothesized that HP could protect mitochondrial function by modulating oxidative metabolism, and thus preventing the HIF-1a mediated glycolysis as a rapid energy source.

To understand how HP may inhibit stabilization of HIF-1α, we examined changes to the mammalian target of rapamycin (mTOR) signaling pathway that is known to regulate HIF [172]. Accordingly, we noted that mTOR and HIF are upregulated in response to hypoxia in MIO-M1 cells, and expression of both is diminished upon pretreatment with rapamycin (mTOR inhibitor) (Figure 6A). A similar reduction in mTOR and HIF expression is observed in cells subjected to the HP-2 hours protocol (Figure 6B).

To this end we explored how hypoxia vs HP influenced cellular metabolism. In response to prolonged hypoxia, MIO-M1 cells switch to aerobic glycolysis, shown by the increased expression of GLUT1, and decreased expression of SDHD (Figure 6C-D). Therefore, switch to aerobic glycolysis under hypoxia is associated with the upregulation of HIF-1α. Interestingly, HP-2 hours prevents this switch to glycolysis as shown by reduced GLUT1 and increased SDHD expression.

Using the mitochondrial stress test confirmed that cells under hypoxia have reduced basal and maximal respiration, as shown with OCR results. This demonstrates that cells

have increased aerobic glycolysis compared to mitochondrial activity, which is consistent with the expression profile of metabolic genes GLUT1 and SHDH (Figure 6E). Interestingly, HP-2 hours partially protected cells' mitochondrial function, as cells after 2 hours HP measured significantly increased basal and maximal respiration, as well as spare respiratory capacity compared to cells not exposed to preconditioning/ under hypoxia (Figure 6F-H).

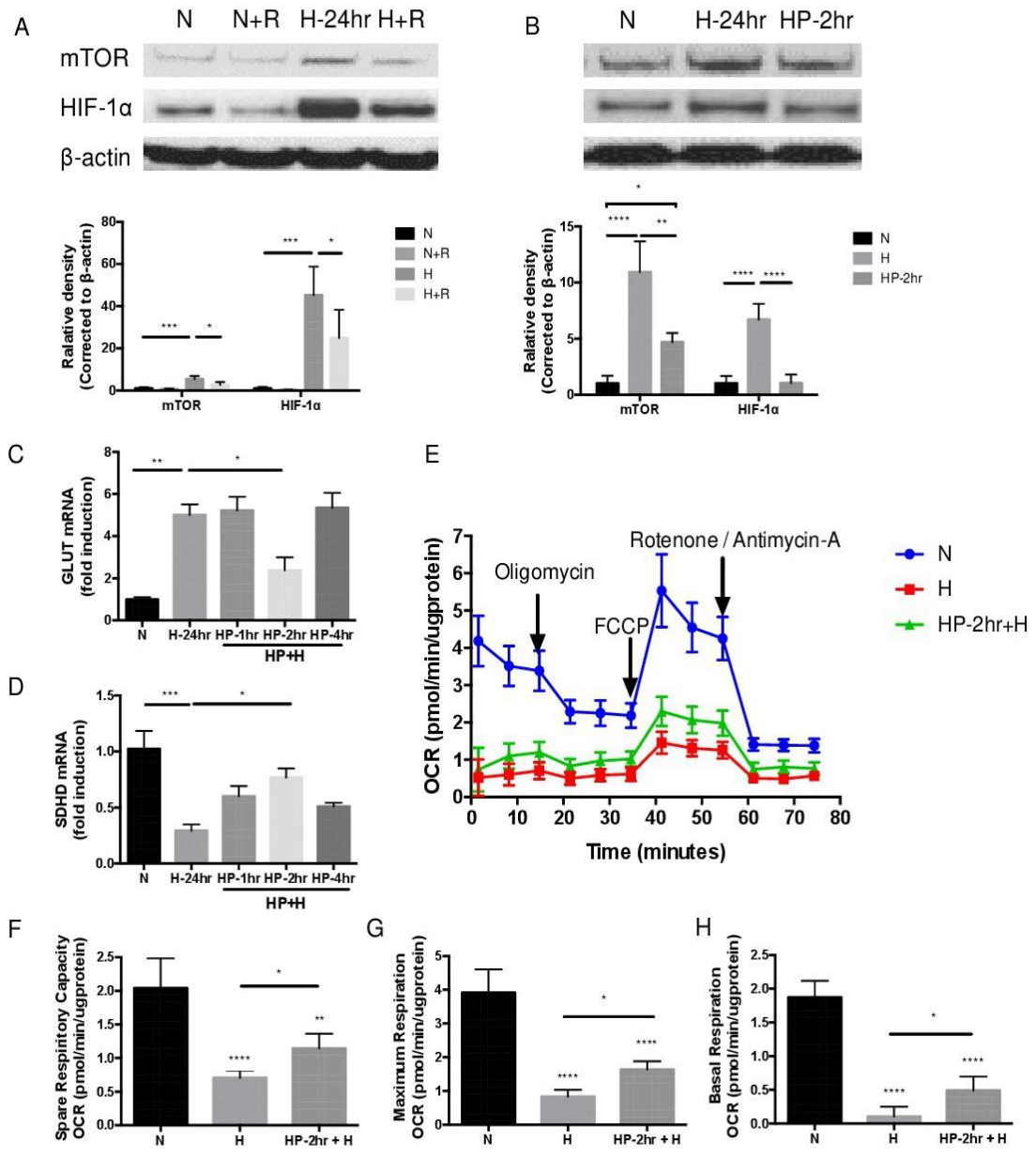

Fig. 6. Hypoxic preconditioning inhibits HIF-1α activation by modulating mTOR and decreasing glycolysis in MIO-M1 cells. (A) Exposure of MIO-M1 cells to 1% O_2 hypoxia 24 hours (H-24hr), the increased expression of mTOR in accordance with HIF-1α compare to normoxia (N) and both (Normoxia with Rapamycin: N+R; Hypoxia with Rapamycin: H+R) were inhibited through pretreatment with

rapamycin (100 nM) at protein level (n=3 per group). (B) HP-2 hours (HP-2hr) could inhibit the expression of HIF-1α through suppressing mTOR expression, decreased glycolysis and improves mitochondrial function in comparison with hypoxic treatment (n=3 per group). (C, D) Exposure MIO-M1 cells to hypoxia increased gene expression of GLUT and SDHD compared to normoxic treatment. However, HP-2 hours partially reversed the expression in comparison with hypoxia (n=4 per group). (E) Representative mitochondrial stress test measured with sequential injections of oligomycin, FCCP and rotenone/antimycin A. (F) Spare-respiratory capacity was calculated as the difference between maximal and basal OCR. (G) Maximal respiration was calculated as difference between maximal OCR value post-injection of FCCP and non-mitochondrial OCR. (H) Basal respiration was calculated as the difference between first OCR measurement and non-mitochondrial OCR. Data represents means ± SD of relative values vs control from 3 independent experiments. $P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Therefore, the mitochondrial stress showed the increased mitochondrial activity after hypoxic preconditioning, which supports the reduced glycolytic function observed by the reduced mRNA expression of key glycolytic enzyme GLUT1.

Discussion

The protective role that HP offers to tissue, including the retina, has been previously highlighted [117]. Whilst the hypoxic response of MIO-M1 cells promotes vascular permeability through HIF-dependent upregulation of pro-angiogenic factors (e.g ANGPTL4) [69], the potential protective effects of HP have not previously been investigated in those cells. In the current study, we demonstrate that HP has a robust effect decreasing HIF-1α stabilization in response to hypoxia. In turn, HIF-mediated transcription and secretion of pro-angiogenic factors are reduced. This protective mechanism is linked with a change in the MIO-M1 cells' metabolic status.

Consistent with previous reports [69,169], we have shown that conditioned medium from hypoxic MIO-M1 cells upregulates HIF-1α and promotes endothelial cell angiogenesis and vascular permeability in HUVEC cultures. Whilst the endothelial cells are from different origins it provides principal of an endothelial non-specificity as well as regulation of tight junctions in common between the two cell types. Furthermore, our data demonstrate a protective effect of HP in hypoxic MIO-M1 cells. The protective effect is consistent with other data showing that hypoxia-preconditioned cells delay apoptosis in response to subsequent hypoxia [173], hypoxia-preconditioned bone marrow stem cells promote nerve regeneration in cerebral ischemic rats [174], HP treated astrocytes are tolerant to severe hypoxia [167] and HP treated neurons were protected against the effects of subsequent oxygen and glucose deprivation [175]. In our experiments, we show that cells subjected to HP demonstrate improved tolerance to a subsequent severe hypoxic exposure. However, HP as a brief period of hypoxia to induce protection against subsequent severe insult is ill-defined. We assessed the time-course of a cellular response under hypoxia (Figure 3B-E), and MIO-M1 cells start showing a response at 2 hours which exaggerates at 4 hours. The protective effect of HP on the permeability and angiogenesis assays was optimal for 2 hours, implying the importance of selecting the appropriate duration of HP.

It has been suggested that metabolic products, specifically fumarate and succinate, regulate the expression of hypoxia inducible genes including HIF-1α in cancer pathology [176]. We therefore assessed metabolic pathways as a potential mechanism through which HP-2 hours exerts the protective anti-angiogenic effect. We found that transcriptional activity of HIF during hypoxia and HIF-1α stabilization is dependent on

the activation of mTOR. Although the mechanism of how mTOR modulates HIF within cells remains elusive, our results confirm previous cancer studies that showed that mTOR activation potently enhances the activity of HIF-1α and VEGF-A secretion during hypoxia, and this is reversed with rapamycin [172]. In our experiments, HP significantly perturbed mTOR expression suggesting that HP could provide a suitable strategy for the treatment of hypoxic retinopathy by suppressing mTOR-mediated pro-angiogenic factors through HIF-1α.

Furthermore, Lu et al. demonstrated that the upregulation of end products of glycolytic metabolism under hypoxia in cancer – the well-known Warburg effect –promotes HIF-1α protein stability and activation of HIF-1-inducible genes [170]. In our experiments, we found reduced oxidative metabolism of retinal MIO-M1 cells under hypoxia, which was partially prevented after HP, as shown by OCR results at the mitochondrial stress test (Figure 6E-H). Furthermore, gene expression of glycolytic proteins was reduced by HP, while SHDH expression was significantly increased, suggesting the usage of the TCA cycle. We observed a correlation of GLUT expression levels with HIF-1α expression in MIO-M1 cells, with HP preventing the upregulation of GLUT, and subsequently of HIF-1α. Overall, we demonstrate that HP regulates the cellular metabolism of MIO-M1 cells, stabilizing the cells, preventing the switch to aerobic glycolysis, and therefore preventing angiogenesis (Figure 6C-D). Although we propose a mechanism for the protective effect of HP on angiogenesis, future studies are required to investigate further how HP specifically influences all metabolic pathways and the HIF regulation.

The inhibition of HIF-1α and HIF-1α - mediated genes expression has emerged as a major target for cancer treatment. Additionally, the beneficial effects of HP in reducing tissue damage from cerebral ischemia are also considered to involve HIF-1α- mediated genes expression [177]. Our data on retinal MIO-M1 cells and *in vitro* angiogenesis models further demonstrated that treatments that regulate aerobic glycolysis may also target HIF-1α, with HP representing a potential therapeutic strategy in hypoxic retinopathy. In conclusion, HP induced significant protection against hypoxic insults and suppressed HIF-1α activation. Pretreatment of MIO-M1 cells with sublethal exposure to hypoxia inhibited the expression of proangiogenic factors reducing the

promotion of endothelial cell proliferation and leakage. These results further indicate the importance of HP in preventing hypoxic injury.

3.2 Restoring Retinal Neurovascular Health via Substance P

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Abstract

Regulation of vascular permeability plays a major role in the pathophysiology of visually threatening conditions such as retinal vein occlusion and diabetic retinopathy. Principally, several factors such as vascular endothelial growth factor (VEGF), are up-regulated or induced in response to hypoxia thus adversely affecting the blood-retinal barrier (BRB), resulting in retinal edema and neovascularisation. Furthermore, current evidence supports a dysregulation of the inner retinal neural-vascular integrity as a critical factor driving retinal ganglion cell (RGC) death and visual loss. The principal objective of this study was to interrogate whether Substance P (SP), a constitutive neurotransmitter of amacrine and RGC, may protect against N-methyl-D-aspartate (NMDA)-induced excitotoxic apoptosis of RGC and VEGF-induced vessel leakage in the retina. Tight junctional protein expression and a Vascular Permeability Image Assay were used to determine vascular integrity *in vitro*. The protective effect of SP on RGC was established in *ex vivo* retinal explants and *in vivo* murine models. After NMDA administration, a reduction in TUNEL+ cells and a maintained number of Brn-3a+ cells were found, indicating an inhibition of RGC apoptosis mediated by SP. Additionally, SP maintained endothelial

tight junctions and decreased VEGF-induced vascular permeability. In conclusion, administration of SP protects against NMDA apoptosis of RGC and VEGF-induced endothelial barrier breakdown.

Key words
Substance P, retinal ganglion cell, vascular permeability, ZO-1

Introduction

The retina is one of the most metabolically active tissues and its demand for oxygen is higher than many other tissues including the brain [178,179]. Optimal function of the retina is dependent on a continuous supply of oxygen from the circulation [180]. A number of retinal disorders are propagated as a result of loss of retinal vascular integrity and subsequent retinal cell apoptosis. For example, in conditions such as retinal artery or vein occlusions and diabetic retinopathy, ischemia causes an up-regulation hypoxia-inducible factors such as HIF-1 alpha which elevate pro-angiogenic factors including vascular endothelial growth factor (VEGF). As a result the blood-retinal barrier (BRB) is disrupted which leads to increased vascular permeability. With respect to the inner retinal barrier the resulting leakage of fluid into the inner retina is a critical response implicated in retinal ganglion cell (RGC) death and loss of vision [181]. RGC are integral to processing of visual information and thus critical for vision [182]. Due to RGCs and other central nervous system (CNS) neurons failure to regenerate after injury, the loss of RGCs observed in many retinal diseases further contribute to visual loss [183]. Chronic diseases such as glaucoma and diabetic retinopathy are associated with RGC death [156,184] and associated with persistent ischemia in glaucoma [185,186], either by mechanical compression of retinal blood vessels around the optic nerve head caused by increased intraocular pressure or by ineffective vascular autoregulation [187]. The implication is that additional therapeutic options should be considered addressing the retinal neurovascular unit [188]. Consequently, therapeutic strategies for enhancing RGC viability (neuroprotection) and preventing or reversing retinal neuronal dysfunction as well as vascular leakage remain a goal of basic and translational research.

Substance P (SP) is a neuropeptide secreted by neurons and is involved in many biological processes via specific receptors [189]. More than 10 neuropeptides are expressed in the inner nuclear layer and the RGC of the retina, of which SP is the most abundant [125,126,190]. SP modulates a diverse set of pathways, including those involved in vasodilatation, cell proliferation, apoptosis, and inflammation [191], as well as promoting the migration and differentiation of vascular endothelial cells [192]. Animal models have provided insights into the biology of this peptide, and compelling evidence highlights the importance of SP in cell-to-cell communication by either paracrine or endocrine signaling [189]. Moreover, higher levels of SP are observed in retina in response to acute stress [193]. A corollary observed is that SP is markedly

reduced in diabetes [149,194], supporting a notion that SP may be important for the retinal health and its loss contributes to disease progression.

Based on these functions, we hypothesized that SP is neuroprotective, attenuating retinal neural apoptosis and maintains inner retinal vascular integrity. In this study, the effect of SP was assessed by evaluating endothelial and ganglion cell viability, apoptosis, and vascular permeability *ex vivo,* as well as in an animal model *in vivo.*

Materials and methods

Intravitreal administration of VEGF and N-methyl-D-aspartate (NMDA)

All procedures were conducted under the regulation of the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, and were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The methods were carried out in accordance with the approved University of Bristol institutional guidelines and all experimental protocols under Home Office Project Licence 30/3045 and 30/3281 were approved by the University of Bristol Ethical Review Group.

In vivo retinal vascular permeability was assessed as previously described [195]. Eight-week-old C57BL/6 mice were purchased from Charles River. Briefly, adult mice were deeply anesthetized by intraperitoneal injection of Vetelar (ketamine hydrochloride 100 mg/mL, Pfizer, UK) and Rompun (xylazine hydrochloride 20 mg/mL, Bayer, UK) mixed with sterile water in the ratio 0.6:1:84, and the pupils of mice were dilated using topical 1% tropicamide (0.5% w/v, Bausch & Lomb, Aubenas, France), before induction of anesthesia. Mice received the following intravitreal injections (2 µl) with a 33-gauge needle into one eye: VEGF (v7259, Sigma-Aldrich, in distilled water) (100 ng); SP (ab120170, Abcam, in distilled water) (20 nmol); VEGF plus SP together; 0.9% saline vehicle. 48 hours post the first injection mice received tail vein injections of Evans Blue (200 µl, 2%). Mice were killed 10 minutes later and eyes were fixed with 4% paraformaldehyde (PFA) for 2 hours. Retinal flatmounts were mounted in antifading medium (Vector Laboratories, Inc., Burlingame, CA, UAS), and images were captured by Leica SP5-AOBS confocal laser microscope.

NMDA (M3262, Sigma-Aldrich, in distilled water) was used to induce excitotoxicity of RGC in the retina, under experimental conditions described above. Mice received the following intravitreal injections (2 µl) with a 33-gauge needle into one eye: NMDA (10 nmol); SP (20 nmol); NMDA with SP together; 0.9% saline vehicle control. Mice were killed 24 hours following NMDA injection, and retinal flatmounts from eyes prepared. Eyes were fixed in 2% PFA for 2 hours for TUNEL labeling as well as immunofluorescence staining.

Retinal Explant Culture *ex vivo* model

Retinal explant culture was performed as per protocol [196], Han Wistar rat (20-old-day) with healthy, untreated eyes were killed by exposure to a rising concentration of carbon dioxide followed by cervical dislocation. The retina was dissected radially into four equal-sized pieces, and individual explants positioned onto 12-mm diameter filters (0.4 μm pore, Millipore) with the RGC side facing up. The filters were placed into the wells of a 24-well plate, each of which contained 800 μl of retinal explant media, consisting of neuronal growth medium (Neurobasal A) supplemented with 2% B27 (Invitrogen Ltd.), 1% N2 (Invitrogen Ltd.), L-glutamine (0.8 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Retinal explant cultures were maintained in humidified incubators at 37°C and 5% CO_2. Half of the media were changed on day 1 and every second day thereafter.

Cell culture and viability

Mouse Primary Retinal Microvascular Endothelial cells (MRMECs, Generon, UK) were cultured in Complete Mouse Endothelial Cell Medium (Generon, UK). Cell viability was assessed using the MTT assay. A total of 2×10^4 cells were seeded in each well of a 48-well plate and cultured for 48 hours to confluency. Following serum starvation for 24 hours, fresh medium supplemented with different concentrations of VEGF (25-200 ng/ml) or SP (1-80 μM) were added for a further 24 hours. To assess cell viability, the MTT assay was performed according to manufacturer's instructions. In brief, 50 μl of MTT solution (Thermo Fisher Scientific, UK) was added to each culture well, and the plate was incubated for a further 2 hours at 37 °C with 5% CO_2. The MTT solution was replaced with HCl/ isopropyl alcohol to dissolve formazan, and the optical density (OD) of the wells was measured at 562 nm on a microplate reader and was calculated using linear regression analysis.

***In Vitro* Vascular Permeability Assay**

Permeability visualization experiments were performed using 18×18 mm glass coverslips coated with biotinylated gelatin and placed in 35 mm culture dishes. MRMECs were seeded (4.5×10^5 cells/dish) and cultured for 72 hours to reach 100% confluency. Culture medium was changed to serum free medium supplemented with treatment (VEGF, SP or SP+VEGF) for a further 24 hours. To evaluate permeability of the MRMEC monolayer, Fluorescein conjugated streptavidin (25 μg/ml final concentration) was directly added to the culture medium for 5 mins, washed twice with

PBS, before cell fixation with 3.7% formaldehyde in PBS 10 mins, room temperature (RT). Overlay of Fluorescein and Zonula occludens-1 (ZO-1) staining demonstrates reciprocal relations between ZO-1 peripheral localization and increased local permeability of Fluorescein-streptavidin. The pattern of Fluorescein-streptavidin binding to the biotinylated gelatin underlying the cell monolayer was examined under Leica SP5-AOBS confocal laser microscope. Images were processed with ImageJ software. The percentage expression of fluorescein intensity was taken from areas with associated DAPI staining only, therefore excluding non-cellular area of fluorescence.

Immunofluorescence staining

Rat retinal explants were fixed with 4% PFA and plus 4% sucrose and snap frozen in OCT (VWR Chemicals, UK). Cryosections were prepared, washed in PBS and permeabilized with 0.1% Triton X-100. Non-specific staining was blocked with 5% BSA in PBS for 30 min at RT followed by immunostaining with goat anti-mouse GFAP (Cell Signaling Technology, UK, 1:100), rabbit anti-mouse Brn-3a (Santa Cruz Biotechnology, Inc., UK, 1:50), goat anti-mouse Rhodopsin (Abcam, UK, 1:100) antibodies. The secondary antibodies Alexa Fluor 549-labeled donkey anti-goat IgG (Invitrogen, CA) and Alexa Fluor 488-labeled donkey anti-rabbit IgG (Invitrogen, CA), both with 1:200 dilution in 2% BSA in PBS at room temperature (RT) for 1 hour in the dark. Exclusion of the primary antibody acted as the negative control.

TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the instructions provided by the manufacturer (Roche; Indianapolis, IN). In brief, fixed retinal explant were washed in PBS, retinas were incubated in 0.1% Triton X-100 for 1 hour to permeabilize the cells, rinsed three times with PBS, before incubation with the TUNEL reaction mixture for 1 hour at 37 °C. Retina were mounted using anti-fading mounting medium (Vector Laboratories, Inc., Burlingame, CA), and apoptotic cells were observed using Leica SP5-AOBS confocal laser microscope. The number of TUNEL+ cells in retinal explant was counted in five random fields on each retinal explant (four per condition), and the average number of TUNEL+ cells per random field was determined for each condition tested. *In vivo* TUNEL staining was quantitatively analyzed by determining the intensities of TUNEL+ cells from dissected whole retina tissues (n=6 per group) using ImageJ.

Retinal flatmount

The whole retina was carefully dissected from the eyecup, permeabilized with 0.2% Triton X-100 and non-specific binding blocked by 5% normal goat serum in PBS for 2 hours RT. The retinas were then incubated with the primary antibody (purified rabbit anti-Brn-3a (1:100)) for 24 hours at 4°C. Tissues were washed before incubation with secondary Cy3 conjugated goat anti-rabbit IgG (1:250) for 2 hours at RT. For TUNEL staining, retinas were incubated with the reaction mixture for 2 hours at 37°C, before being flat mounted onto microscope slides and covered with anti-fading mounting medium for confocal microscopy. The number of Brn-3a+ cell bodies was determined from four separate fields of view (the same distance from the optic nerve) per retinal flatmount and expressed as an average number of Brn-3a+ cells per field.

SDS-PAGE analysis

Cultured MRMEC cells were washed twice with ice-cold PBS before lysis with ice-cold CelLytic™ MT Cell Lysis Reagent (Sigma-Aldrich, UK). Cell lysates were centrifuged at 14,000 g for 10 min at 4°C, the supernatant was transferred to fresh tubes and protein concentration of samples determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK). Equal amounts of protein were mixed with 4× SDS sample buffer, boiled for 10 mins at 90°C, and resolved using 4% to 20% NuPAGE gels (Invitrogen, UK). Proteins were transferred to PVDF membranes (Invitrogen, UK), blocked with 5% skimmed milk for 2 hours, and incubated overnight at 4°C with primary antibodies: Anti-ZO-1 (Merck, UK; 1:1000), anti-p38 MAPK (Cell Signaling Technology, UK; 1:1000), anti-p44/42 MAPK (Cell Signaling Technology, UK; 1: 1000) and β-actin (Cell Signaling Technology, UK; 1:1000). Excess antibody was removed by washing the membrane in PBS/ 0.1% Tween 20, and the membranes were incubated for 2 hours with horseradish peroxidase-conjugated secondary antibodies (Abcam, UK; 1:2000). Following further washes in PBS/ 0.1% Tween 20, the signals were developed with ECL reagent (Sigma, UK) and captured by an electronic imaging system (Konica Minolta). Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, UK) was used to re-blot the membranes. The density of protein was quantified using the ImageJ.

Statistics

All experiments were repeated at least three times. Results are therefore presented as

means ± standard deviation (S.D.). Comparisons of two individual experimental groups were performed by unpaired Student's t test and Mann-Whitney test. For multiple comparisons, nonparametric analysis was performed using one-way ANOVA test with Dunn's test. All the analysis was performed using GraphPad Prism 6 (GraphPad Software, version 6.01, La Jolla, USA). Two-tailed tests were used throughout. The significant differences were considered at $P \leq 0.05$.

Results

SP regulates ZO-1 expression in cultured MRMECs, through MAP-kinase inactivation.

Toward initial understanding of role of SP in regulation of vascular barriers we examined the effects of SP on the tight junction proteins in primary retinal microvascular endothelial cells (MRMECs). We assessed the response of MRMECs following exposure to VEGF, SP or a combination of VEGF and SP. At all concentrations tested, VEGF had no effect on cell viability, while we established that SP demonstrated toxicity at the high dose of 80 (μM) *in vitro* (Figure 1A-B), which we therefore omitted from further experiments. VEGF resulted in a dose-dependent reduction of ZO-1 in MRMECs as shown by western blot, whereas SP at non-toxic doses increased significantly ZO-1 expression in a dose-dependent manner (Figure 1C-D). Treatment of cultured MRMECs with VEGF led to a significant (52%) reduction of ZO-1 expression as compared to untreated control. Incubation of the cells with SP (20 μM), applied 6 hours before VEGF treatment, showed an increase in ZO-1 expression by 43% compared to VEGF alone. (Figure 1E).

To understand the potential mechanism for the observed SP protective effect, we examined expression of MAP-kinase which is recognised to modulate the cellular expression of tight junction proteins in response to various stimuli [197]. In MRMECs, we showed that SP treatment significantly reduced the phosphorylated forms, p38-MAPK and p44/p42 MAPK. The data supported that treatment of MRMECs with SP led to down-regulation of activated MAPK isoforms, accompanied by increased expression of ZO-1, compared to VEGF alone (Figure 1F-G).

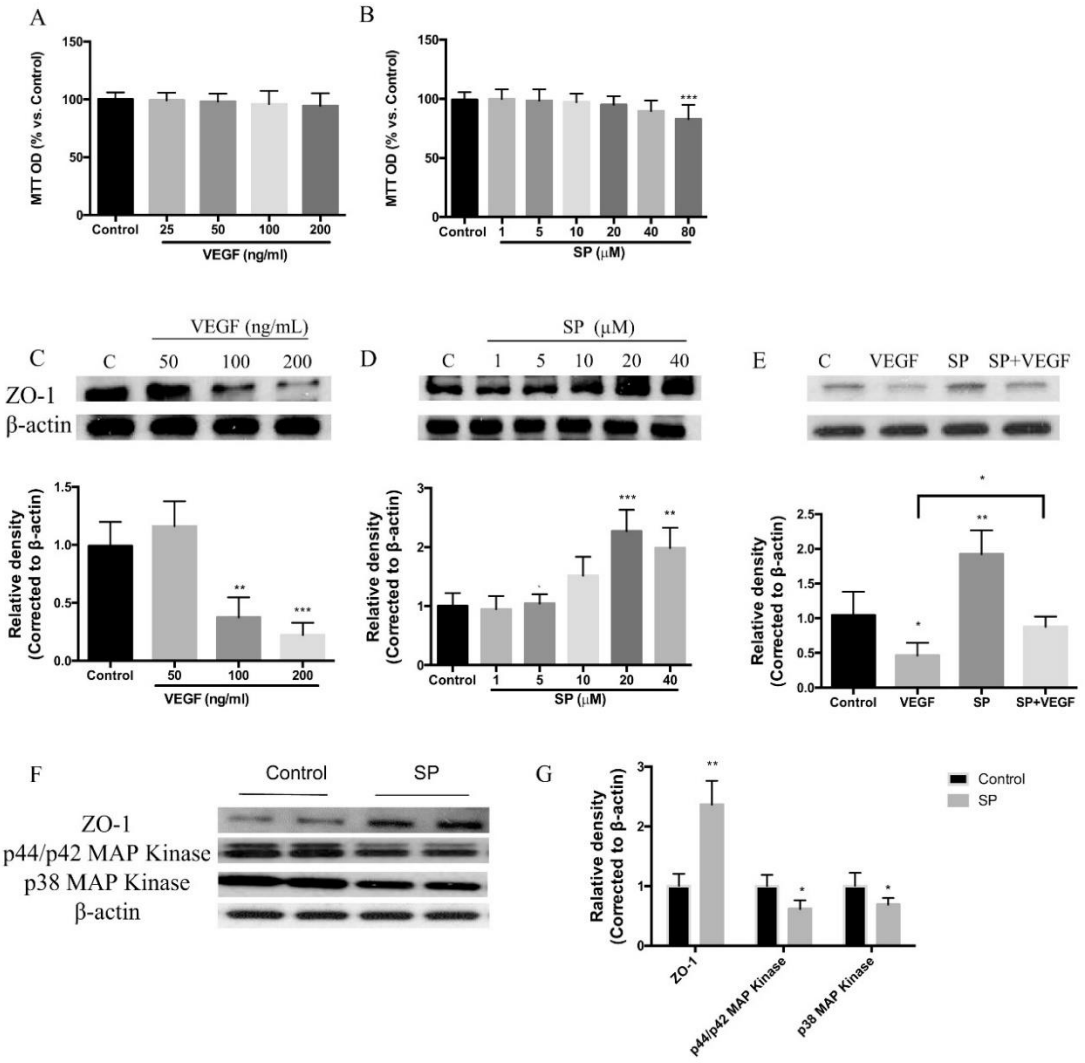


Fig. 1. Effects of Substance P on the expression of tight junction protein ZO-1 in MRMEC cells. (A, B) Cells were deprived of serum for 24 h and then incubated in the presence of SP or VEGF. MRMEC viability was determined in each group using the MTT assay. Data were normalized by control. (C, D) ZO-1 expression in MRMECs deprived of serum for 24 h, upon treatment with SP (0–40 μM) or VEGF (0–200 ng/ml). (E) ZO-1 expression in MRMECs deprived of serum for 24 h and then incubated in the presence of SP (20 μM), VEGF (100 ng/ml) or SP with VEGF (pre-incubated with SP for 6 h before addition of VEGF). (F, G) p44/p42 MAPK, p38 MAPK and ZO-1 expression in MRMECs treated with SP (20 μM) or VEGF (100 ng/ml). Data represents means ± SD of relative values vs control from 3 independent experiments. $*P < 0.05$; $***P < 0.001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

SP inhibits VEGF-induced vascular permeability by preventing dissociation of endothelial ZO-1.

To determine how the SP-mediated increase in ZO-1 expression may influence the barrier function of MRMEC cells, we exploited an *in vitro* vascular permeability assay.

This approach permitted dual assessment of endothelial cell integrity (tight junction expression of ZO-1) and extent of vascular leakage (fluorescence intensity of fluorescent-positive intercellular signal) in response to SP and VEGF treatments.

When MRMEC cells monolayer were treated with VEGF (100 ng/ml, 24 hours), a 6-fold increase in fluorescence intensity compared to control (untreated cells) was observed indicating increased permeability. SP partially protected against the increased permeability through preservation of the cell junction integrity demonstrated by attenuation of fluorescence intensity by 36% compared to VEGF alone (p<0.001) (Figure 2A-B). Furthermore, ZO-1 immunostaining confirmed the loss of tight junction (TJ) integrity following VEGF treatment, which was again attenuated by SP pre-treatment (Figure 2A).

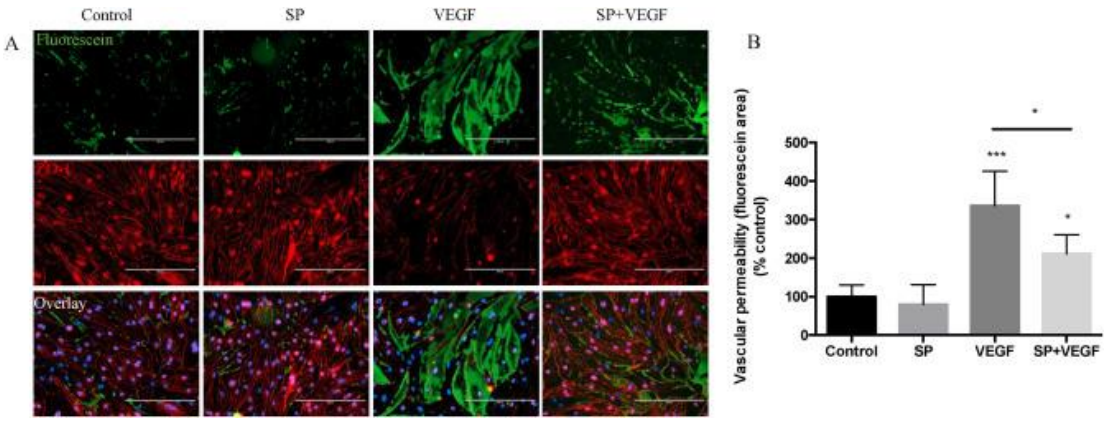


Fig. 2. Substance P inhibits VEGF-induced retinal vascular permeability *in vitro*. (A) Immunostaining of MRMECs for Fluorescein-streptavidin to detect permeable areas and for ZO-1 to show cell-cell contact; cells deprived of serum for 24 h and incubated in the presence of SP (20 μM), VEGF (100 ng/ml) or SP with VEGF (pre-incubated with SP for 6 h before addition of VEGF) for 24 h (n = 4 per group). (B) Fluorescein intensity associated with DAPI expression was calculated by Image J in all treatment groups. Scale bar, 200 μm. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

To support the *in vitro* data, we investigated the protective effect of SP on VEGF-induced vessel leakage *in vivo*. Groups of C57BL/6J mice were treated with a single intravitreal injection of VEGF, SP, SP+VEGF or vehicle control. Vascular leakage was measured at 48 hours post-injection by extravasation of intravascular Evans Blue dye.

3. Results

Flat-mount retinal preparations showed that intravitreal injection of VEGF induced a 70% increase in Evans blue extravasation as determined via fluorescent intensity compared to control. Intravitreal injection of SP together with VEGF suppressed the VEGF-induced leakage in retinal vessels by 47%. By contrast, only minimal vascular leakage was observed in animals receiving normal saline or SP alone (Figure 3A-B).

Deploying ZO-1 immunofluorescence staining, we assessed the spatial relationship between TJ protein expression and changes in paracellular vascular permeability. Classical staining of intact vasculature exposes the demarcated lateral membranes of retinal microvascular endothelial cells. Localization and integrity of junctional complexes were not altered following exposure to SP or saline alone. However, intravitreal injection of VEGF resulted in a dramatic loss of staining of the ZO-1 junctional protein network. Combined administration of SP with VEGF led to a significant increase in the expression of ZO-1 protein (Figure 3C). Collectively this data supports a protective effect of SP on retinal vascular integrity.

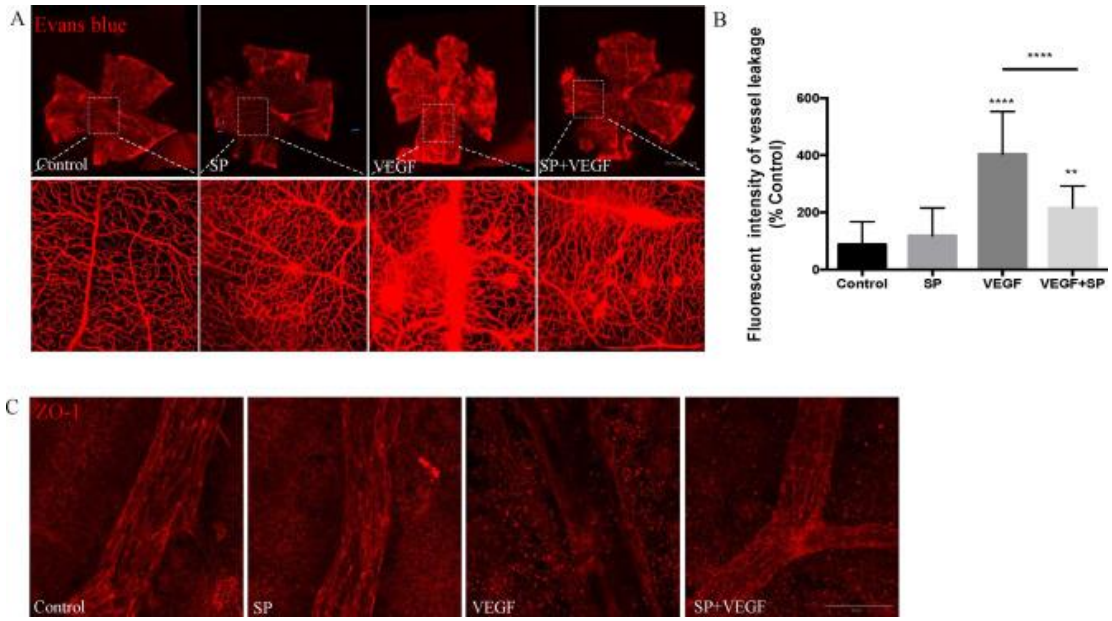


Fig. 3. Substance P prevents vascular leakage in the mouse retina. Groups of C57BL/6J mice received intravitreal injections of SP (20 nmol), VEGF (100 ng), SP in combination with VEGF or vehicle control (n = 6 per group). (A) Representative images 48 h following treatment demonstrate differences in the vasculature (leakage of Evans blue dye, which appears as red). Scale bar = 50 μm. (B) The fluorescent intensity of Evans blue was quantified by ImageJ. (C) Representative confocal images of retinal vessels immunostained with ZO-1. Scale bar = 20 μm. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

SP protects retinal ganglion cell against apoptotic cell death induced by NMDA *ex vivo* retinal explants

Given the distribution of neurotransmitter SP expression in the inner retina, next we wished to determine whether SP conferred a homeostatic role and delivered a protective effect leading to preservation of retinal microarchitecture and other cell types, specifically retinal ganglion cells (RGC). *Ex vivo* retinal explants cultured for 2 weeks maintained the laminar structure of the retina, with no loss of cells from the outer and inner retinal layers (Figure 4A). Furthermore, immunostaining demonstrated major cell types were preserved after 2 weeks in culture. Brn-3a+ RGC axon bundles were observed in the retinal nerve fiber layer, but also GFAP+ astrocytes and Müller cells and Rhodopsin+ photoreceptors were retained (Figure 4B).

To evaluate the role of SP on RGC health, we employed the well-established NMDA-induced model of excitotoxic damage in retinal explants [25]. Quantification of TUNEL+ cells shows NMDA treatment results in a 10-fold increase in the number of apoptotic cells. Pre-treatment of the explant with SP for 6 hours prior to NMDA exposure significantly reduced the cytotoxic effects, as shown by a reduction in the number of TUNEL+ cells by 27% compared to NMDA alone, SP treatment alone did not lead to any apoptosis or retinal toxicity, with comparable number of TUNEL+ cells to control explants (Figure 4D and E). To confirm with an *in vivo* correlate we analyzed wholemounts obtained from eyes exposed to NMDA for 24 hours (10 nmol, intravitreal injection) where upon following incubation with NMDA for 24 hours RGC NMDA-induced excitotoxic damage of RGC cells undergoing apoptosis was noted. (Figure 4C).

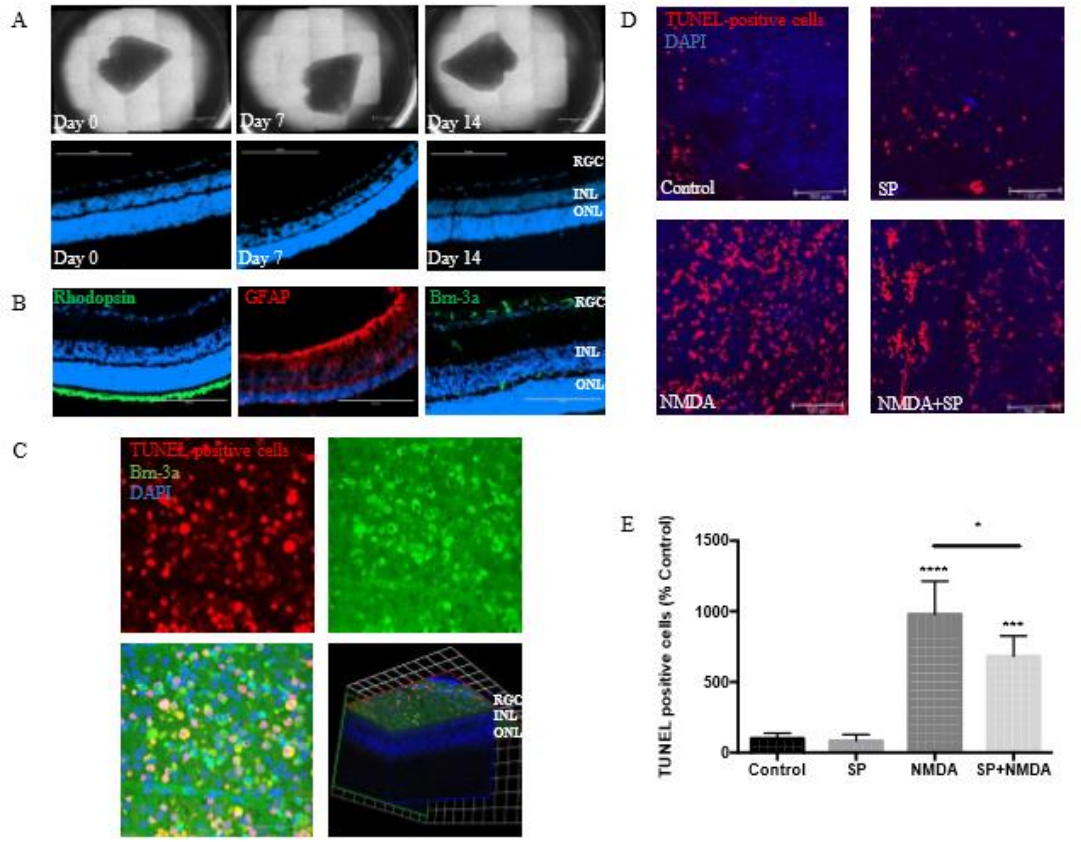


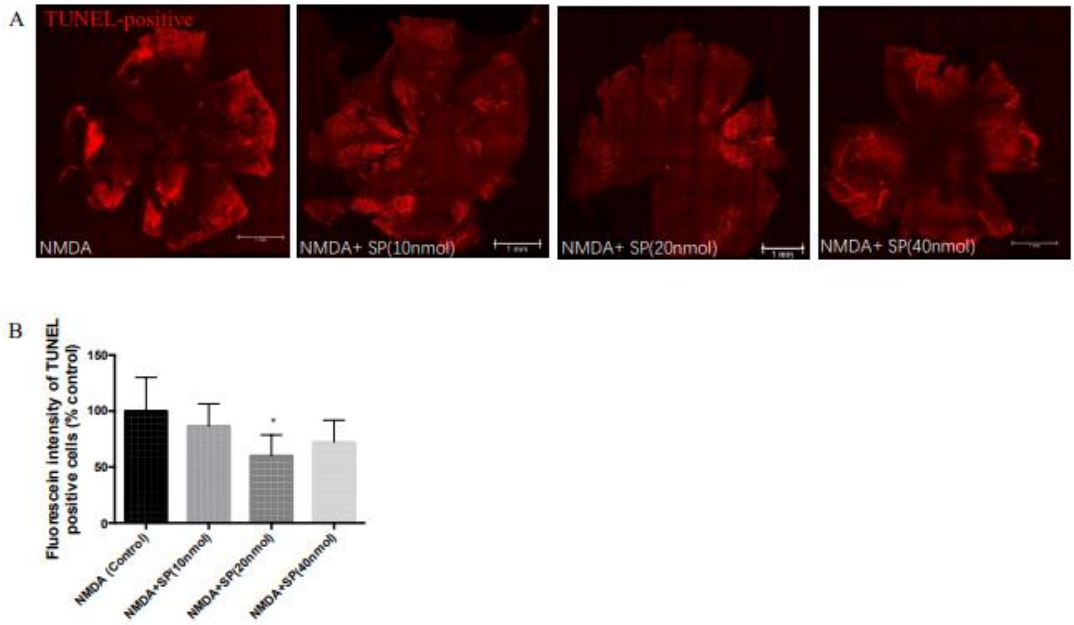
Fig. 4. Substance P protects against apoptotic cell deathin the retinal explants induced by NMDA. Gross morphology and tissue viability of rat retinal explants, cultured in B27/N2 and maintained to Day 14. (A) Wholemount photographs. Scale bar, 2 mm. Sectioned explants, stained with DAPI to visualize nuclei, demonstrated good survival of cells in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). Scale bar, 200 μm. (B) Immuno-staining of retinal sections from day 14 explant for different cell markers (Rhodopsin, Brn-3a, GFAP), counterstained with DAPI (blue). Scale bar, 200 μm. (D, E) Representative images showing TUNEL-positive cells in rat explants exposed to NMDA (10 μM), SP (20 μM) or pretreated with SP 6 h before NMDA exposure (n = 6 per group). Scale bar, 100 μm. Quantification of TUNEL positive cells expressed as percentage of control. (C) Representative images of retinal wholemount obtained from eyes exposed to NMDA for 24 h (10 nmol, intravitreal injection), showing TUNEL+ (red), Brn-3a+ (green), and cell nuclei stained with DAPI (blue). 3D image indicates NMDA specifically induced TUNEL+ in GCL. Scale bar, 50 μm. Data represents means ± SD of relative values vs control from 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

SP protects ganglion cells from NMDA excitotoxicity *in vivo*

To validate the protective effect of SP in suppressing NMDA-induced RGC apoptosis, we assessed *in vivo* efficacy. In these experiments, intravitreal administration of NMDA (10 nmol) was used to induce neuronal excitotoxic damage in the eyes of mice.

3. Results

A dose-response was undertaken previously to ascertain the optimal dosing of SP (10nmol to 40 nmol). We found that 20 nmol of SP demonstrated the optimal protective effect in NMDA-induced ganglion cell apoptosis model (Supplemental figure 1A-B). The extent of NMDA-induced RGC apoptosis was quantified by immuno-staining (TUNEL and Brn-3a positivity) of *ex vivo* retinal whole-mounts (Figure 5A-D). We observed a significantly higher fluorescence in the NMDA-injected eyes than PBS-injected contralateral eyes. Combined administration of SP and NMDA, resulted in a significant reduction (34%) in the fluorescence intensity of TUNEL+ cells in the retina as compared to NMDA alone. Of note, no significant differences were detected between the SP-injected experimental eyes and the respective control (Figure 5A, C).



Supplementary Figure 1. Optimizing the dose of Substance P with NMDA-induced ganglion cell apoptosis model (A) Representative images of retinal whole-mounts prepared for TUNEL assay, 24 hours following a single intravitreal injection of NMDA (10 nmol), different dose of SP (10 nmol, 20 nmol and 40 nmol) combined with NMDA (n=4 per group). (B) The fluorescence intensity of TUNEL+ was quantified by Image J, showing that SP (20 nmol) significantly decreases the intensity of apoptotic RGC induced by NMDA. $*P < 0.05$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

The number of Brn-3a+ cells was also evaluated from the retinal flat mount, and showed that NMDA reduced decreased the number of Brn-3a+ cells to 45% of control. Conversely, after SP was injected along with NMDA injection in the mouse vitreous,

there was a statistically significant protective effect as larger number of Brn-3a+ cells remained (from 45% to 68% of control) (Figure 5B, D).

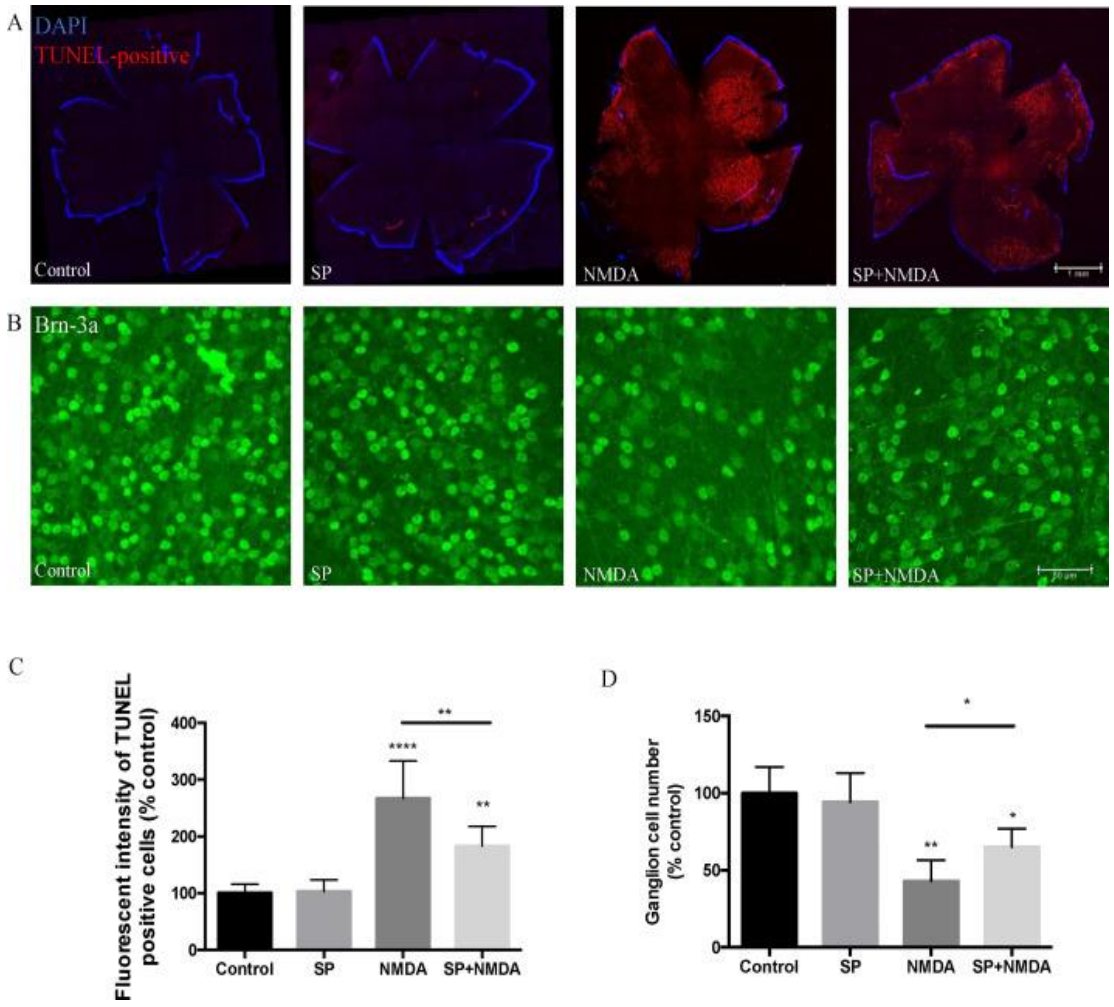


Fig. 5. Substance P protects neuronal cell death induced by NMDA in murine retina. Representative images of retinal whole-mounts prepared for TUNEL assay (A) and Brn-3a immunostaining (n = 6 per group). (B) 24 h following a single intravitreal injection of SP (20 nmol), NMDA (10 nmol), SP combined NMDA or vehicle control (n = 6 per group). (C) The fluorescence intensity of TUNEL+ was quantified by ImageJ, showing that SP decreases the intensity of apoptoticRGC induced by NMDA. (D) Brn-3a+ were counted by ImageJ, showing that SP protects against the loss of RGC. Data represents means ± SD of relative values vs control from 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Discussion

In this study, we demonstrated a protective role of SP against NMDA-induced retinal ganglion cell death *in vitro* and *in vivo*. Furthermore, our results revealed that SP suppresses VEGF-induced increase in vascular permeability. These findings suggest that modulating SP levels can be a potential new avenue of treatments to preserve the neurovascular unit in retinal neurodegenerative and vascular diseases.

Regulation of vascular permeability plays a major role in the pathophysiology of neovascular diseases, with numerous VEGF-inhibitory strategies to reduce retinal vascular permeability [198]. The integrity of endothelial cell–cell junctions and vascular barrier function is regulated by a series of adhesion molecules that form three types of junctions, tight, gap and adherens [199–201]. Among the various protein components of tight junctions, ZO-1 is a phosphoprotein that participates in multiple protein–protein interactions and has been implicated in the regulation of tight junction integrity [202–204]. Furthermore, VEGF disrupts tight junctions by altering phosphorylation of ZO-1 and occludin through a Src-dependent pathway [205,206]. In our experiments, and in agreement with previous studies, we were also able to demonstrate the inhibitory effect of VEGF on the expression of tight junction protein ZO-1 in endothelial cells. However here, we demonstrated that VEGF and SP have opposite effects on the expression of ZO-1 in the MRMEC monolayers. SP increased ZO-1 expression, which supported our initial hypothesis that SP maintains the vascular integrity.

To further assess the mechanism of action of SP, we assessed vascular permeability both *in vitro* and *in vivo*. We observed that SP can attenuate VEGF-induced vascular leakage of microvascular retinal endothelial cell monolayer. These results suggest that SP improves barrier function to maintain homeostasis of retinal endothelial cells. Our results support other data demonstrating that SP up-regulates the tight-junction protein ZO-1 in various cell types, including epithelial cells [207], and also promotes wound healing and tissue integrity [133,208].

During pathological conditions, many components may interplay to determine either death or survival of RGCs [187], but there are many mechanisms for RGC death that are shared among different diseases [209]. Changes in electrical activity [210] and

growth factor deprivation [211] likely contribute to RGC death after optic nerve injury, while other mechanisms, such as excitotoxicity [212], ischemia and hypoxia [156], oxidative stress [213] and abnormal protein trafficking, induce RGC death. Xie's [126] results from studies in diabetic rats showed that the levels of SP in the retina and serum were significantly reduced, with an associated increase in apoptosis and caspase-3 activity in the retina. More importantly, restoration of endogenous SP paralleled the inhibition of the apoptosis of the RGC and the caspase-3 activity in the diabetic animals. This indicates its potential use in the development of therapeutic strategies to fight retinal disease.

Here we extend the known beneficial effect to show that SP exerts neuroprotective properties on RGC in NMDA-induced neuro-excitotoxicity apoptotic cell death both *in vitro* and in an animal model. For SP neuroprotective property we speculated that SP exerts its protective function mainly through MAPK signaling, as MAPK has been shown to regulate tight junction function. In other tissues, p38 MAPK is activated by tumor necrosis factor α and interferon γ, leading to down-regulation of occludin, ZO-1 and claudin-2, which contributes to the increase in paracellular permeability [214]. In our experiments, we similarly found that both phosphorylated forms of MAPK (p38 and p44/42) were reduced upon SP treatment, alongside ZO-1 upregulation, supporting that SP inactivated MAPK to maintain vascular integrity. Furthermore, several cytokines such as IL-10 and IL-12 have been observed to mediate partially the effect of SP in several organs/tissues [215,216]. There appear to be opposing data on the role of SP on endothelial cells. For example, SP has been reported to regulate angiogenesis directly by inducing endothelial cells to produce nitric oxide [217], or indirectly via its interactions with mast cells and granulocytes [135], while VEGF blockade does not abolish the proangiogenic property of SP [218]. On the other side, in support of our findings, SP can reportedly prevent laser-induced retinal degeneration *in vivo*, by suppressing inflammation and reducing neovascularization [219]. These differences are likely attributable to different animal models studied, the type and kinetics of retinal insult, selective mechanisms potentially affecting only some subtypes of RGC or experimental methods that select some types of RGC for observation over others (e.g. central retina versus peripheral) [187]. Nevertheless, as we continue to decode the molecular mechanisms of SP, other important potential therapies may be incorporated

into a multifaceted approach. It will be important moving forward to transition as many of these are possible into well-structured clinical trials in humans.

In conclusion, we have shown that SP is neuroprotective, suppressing apoptosis of RGC induced by NMDA, and SP also protects against VEGF-induced microvascular leakage. Both effects demonstrate the significant role that the neuropeptide transmitter plays in maintaining neurovascular unit homeostasis.

3.3 Treatment of Diabetic Retinopathy through Neuropeptide Y-mediated Enhancement of Neurovascular Microenvironment

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Abstract

Diabetic retinopathy (DR) is one of the most severe clinical manifestations of diabetes mellitus (DM) and a major cause of blindness. The progress of DR induces neuronal and glial activation resulting in disruption of the neurovascular unit and regulation of the microvasculature. The objective of this study was to determine a potential protective role of neuropeptide Y (NPY) using an established model of DR permissive to N-methyl-D-aspartate (NMDA)-induced excitotoxic apoptosis of retinal ganglion cells (RGC) and vascular endothelial growth factor (VEGF) induced vascular leakage. *In vitro* evaluation using primary retinal endothelial cells demonstrates that NPY promotes vascular integrity, evidenced by maintained tight-junction protein expression and reduced permeability in response to VEGF treatment. Furthermore, *ex vivo* assessment of retinal tissue explants shows that NPY can protect RGC from excitotoxic induced apoptosis. *In vivo* clinical imaging and *ex vivo* tissue analysis in the diabetic model permitted assessment of NPY treatment in relation to neural and endothelial changes. The neuroprotective effects of NPY were confirmed by attenuating NMDA-induced RGC apoptosis and able to maintain inner retinal vascular integrity. These findings

could have important clinical implications and offer novel therapeutic approaches for the treatment in the early stages of DR.

Key words

Diabetic retinopathy, NPY, vascular permeability, ZO-1, retinal ganglion cells, neurovascular unit

Introduction

Diabetic Retinopathy (DR) is a major cause of visual loss in worldwide working-age adults [220]. This number of DR is predicted to rise along with the increasing incidence of diabetes mellitus (DM) [18]. Conventional clinical assessment and classification is based on classical microvascular features including: haemorrhage, lipid exudate, cotton wool spots and neovascularization; all observed predominantly in the inner retina [221]. However, preclinical studies also demonstrate that components of the neurovascular unit, including the inner and outer neurosensory retina are disrupted in diabetes [41,42]. Perturbed neuronal function reflected by impaired glutamatergic and dopaminergic neurotransmitter signaling [43]. In contrast to the enlargement of dendritic fields [44], the decreased expression of synaptic protein has been documented [45]. These changes ultimately leading to apoptosis of neurons alongside persistent uncontrolled diabetes [46]. Additional diabetic changes include altered Glial cell activation, demonstrated by impaired interconversion of glutamate and glutamine [47], reduced potassium channels [48], and subsequent markedly altered expression of the glutamate-aspartate transporter and intermediary filament proteins such as glial fibrillary acidic protein (GFAP). Diabetes also induces changes to retinal astrocytes, which located in the retinal nerve fibre layer and aligned with blood vessels provide contact with synapses [222], and where connexin expression is reduced in the early course of diabetes prior to astrocyte loss [52]. Collectively, diabetes causes components of the retinal neurovascular unit to "dis-integrate", and accordingly DR should be recognized as a neurovascular degeneration and not solely a microvascular disease [223,224]. Nevertheless, recognition that disruption of the neurovascular unit presents opportunities for new therapeutic strategies and/or molecular targets that may offer potential for treating in the early stages of disease.

Neuropeptide Y (NPY) is one of the most highly-expressed neuropeptides (under physiological conditions) within the central nervous system (CNS) of mammals [225]. NPY consists of 36 amino acid peptide and performs its biological function through G-protein-coupled receptors Y1 – 6 [226], expressed in the retina of several species including mice [227–230]. Studies demonstrate putative neuroprotective effects of NPY in different CNS regions [145], including inhibition of glutamate release in rat hippocampus and striatum [231]. Selective activation of NPY receptors has also been shown to protect mouse hippocampal cells from excitotoxic lesions [232], and NPY

receptor signalling suppresses glutamate-induced necrotic and apoptosis in retinal neural cells [146]. Although no direct evidence in retinal endothelium, NPY also can induce migration and proliferation of endothelial cells via its receptors, and regulate their function [233].

In light of the reported observations of NPY function, we examined whether administration of NPY would extend and offer protection of the neurovascular multicellular complex in the diabetic retina. Using *in vitro* platforms, we first assessed whether neuroprotective actions of NPY could reduce the sensitivity of retinal neurons to glutamate-induced excitotoxicity. Additionally, we evaluated the potential intracellular signalling pathways of NPY suppression of vascular endothelial growth factor (VEGF)-induced vascular leakage in retinal endothelial cells. Finally, we established an *in vivo* model of DR, permissive to accelerated glutamate excitotoxicity and VEGF-induced vascular permeability to evaluate whether NPY is neuroprotective, attenuating retinal ganglion cell (RGC) apoptosis and able to maintain inner retinal vascular integrity.

Materials and methods

Cell culture and viability

Primary Retinal Microvascular Endothelial cells from mouse (MRMECs) or human (HRMECs) were cultured in Complete Mouse and Human Endothelial Cell Medium respectively (Generon, UK). Using MTT assay to assess cell viability.

In Vitro Vascular Permeability Assay

Permeability visualization experiments were performed as previously described[234]. For assays, serum free culture medium supplemented with the treatment (combinations of NPY, N-methyl-D-aspartate (NMDA), VEGF) was added for a further 24 hours. Then Fluorescein-streptavidin was directly added to the culture medium for 5 mins, washed twice with PBS before cell fixation with 3.7% paraformaldehyde (PFA) in room temperature (RT) subjected to immunofluorescence staining according to the manufacturer's instructions.

SDS-PAGE analysis

The equal protein from cultured MRMECs cells determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK), and boiled for 10 mins at 90°C with 4× SDS sample buffer. Then resolved using 4% to 20% NuPAGE gels (Invitrogen, UK). Proteins were transferred to PVDF membranes (Invitrogen, UK), blocked for 2 hours with 5% skimmed milk and incubated overnight at 4°C with the primary antibodies: Anti-ZO-1 (Merck, UK; 1: 1000) and β-actin (Cell Signaling Technology, UK; 1:1000). Then wash the membrane in PBS/ 0.1% Tween 20, subsequently incubated for 2 hours with horseradish peroxidase-conjugated secondary antibodies (Abcam, UK; 1:2000). Following further washes in PBS/ 0.1% Tween, protein signals were developed with ECL reagent (Sigma, UK) and captured by an electronic imaging system (Konica Minolta). Membranes were re-blotted by Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, UK), and the density of protein was quantified using the ImageJ.

Retinal Explant Culture *ex vivo* model

Retinal explant culture was performed as previously described [196,234]. Healthy Han Wistar rat's retina (20-old-day) was dissected into four equal-sized pieces, and the explants were separately transferred onto 12-mm diameter filters (0.4 μm pore,

Millipore) with the RGC side facing up. The filters were placed into the wells of a 24-well plate, each contained 800 µl of culture media. Retinal explant cultures were maintained in humidified incubators at 37°C and 5% CO_2. Half of the media was refreshed on day 1 and every second day thereafter.

Immunofluorescence staining

Eyes from 6-month-diabetic mice and rat retinal explants were fixed with 4% paraformaldehyde (PFA) and snap frozen in OCT (VWR Chemicals, UK). For immunostaining, 8 µm cryosections were washed in PBS and blocked in 5% donkey serum, 3% BSA and 0.3% Triton X-100 in PBS for 30 min at RT followed by incubating with the following primary antibodies– mouse anti-GFAP (#3670, Cell Signaling Technology, UK, 1:200), mouse anti-Brn-3a (sc-8429, Santa Cruz Biotechnology, Inc., UK, 1:50), rabbit anti-Rhodopsin (ab3424, Abcam, UK, 1:100)– overnight at 4°C. The secondary antibodies Cy3 conjugated goat anti-mouse IgG (Merck, UK) and Alexa Fluor 488-labeled donkey anti-rabbit IgG (Invitrogen, CA), both with 1:200 dilution in 2% BSA in PBS at room temperature (RT) for 1 hour in the dark. DAPI (Vector Laboratories, Peterborough, UK) was used to show nuclei in sections. Retinal flatmounts were mounted in antifading medium. Images were captured by Leica SP5-AOBS confocal laser microscope and processed with ImageJ software.

For retinal flatmount, the whole retina was permeabilized with 0.2% Triton X-100 and blocked in PBS with 5% NGS for 2 hours RT. The retinas were then incubated with the primary antibodies (anti-Brn-3a (1:50), anti-GFAP (1:200)) for 48 hours at 4℃. Tissues were washed before incubation with secondary Alexa Fluor 549-labeled donkey anti-mouse IgG (1:200, Invitrogen, CA) for 2 hours at RT. Then washed in PBS, flat mounted onto microscope slides and covered with anti-fading mounting medium (with DAPI) for confocal microscopy.

Quantitative Real time RT-PCR

Total RNA extraction was performed using TRIzolTM Reagent (Life Technologies, UK). Total RNA was quantified and used to synthesize cDNA. Using the Power SYBR® Green PCR Master Mix Reagent to amplify cDNA. Primer sequences used were: β-Actin, forward 5'-gggaaatcgtgcgtgacattaag, reverse 5'-tgtgttggcgtacaggtctttg; NPY, forward 5'-actccgctctgcgacactacat, reverse 5'-gcgttttctgtgctttccttca; VEGF,

forward 5'-ttactgctgtacctccacc, reverse 5'-acaggacggcttgaagatg; ANGPTL-4, forward 5'-ggacacggcctatagcctg, reverse 5'-ctcttggcgcagttcttgtc. The equation fold change = 2-ΔΔct was used for calculation relative changes in expression levels.

TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Roche; Indianapolis, IN). In brief, retinal explants were fixed with 4% PFA and washed in PBS, retinas were permeabilized with 0.1% Triton X-100 for 1 hour, rinsed three times with PBS, before incubation with the TUNEL reaction mixture for 1 hour at 37 °C. Retinal explants were mounted using anti-fading mounting medium, and apoptotic cells were observed using Leica SP5-AOBS confocal laser microscope.

Type 1 Diabetic mouse model

All procedures were conducted under the regulation of the United Kingdom Home Office Animals (Scientific Procedures) Act 1986, and were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

Adult (6-8 weeks) C57BL/6 strains of male mice were obtained from Charles River Laboratories (Margate, UK). Type I diabetes was induced by 5 daily intraperitoneal injections with streptozotocin (STZ) (Sigma-Aldrich, UK) (40 µg/g body weight in 0.1 M citrate buffer), a pharmacological agent that is toxic to pancreatic insulin secreting beta islet cells[235]. Control mice were injected with citrate buffer alone. Diabetes was confirmed by measuring urine glucose (14 days after the onset of diabetic induction, >300 mg/dl considered diabetic).

Intravitreal administration of NPY, VEGF and NMDA

In vivo retinal vascular permeability was assessed as previously described [236]. Briefly, the pupils of 3-month-old diabetic mice were dilated using topical 1% tropicamide before induction of anaesthesia. Mice received the following intravitreal injections (2 µl) with a 33-gauge needle into one eye: VEGF (100 ng); NPY (10 nmol); VEGF with NPY combined; PBS vehicle. 48 hours post the first injection mice received tail vein injections of Evans Blue (200 µl, 2%). Mice were killed 10 minutes later and eyes were

fixed with 4% PFA for 2 hours. Retinal flatmounts were mounted in antifading medium, and images captured by Leica SP5-AOBS confocal laser microscope and processed with ImageJ.

NMDA (Sigma-Aldrich) was used to induce excitotoxicity of RGC in the retina [234], under experimental conditions described above. A dose-response was undertaken previously to ascertain the optimal dosing of NPY (5 nmol to 20 nmol), then mice received the following intravitreal injections (2 µl) with a 33-gauge needle into one eye: NMDA (10 nmol); NPY (10 nmol); NMDA with NPY together; PBS vehicle. Mice were killed in 2 days following NMDA injection, and eyes as well as optic nerves were isolated. Eyes were fixed in 2% PFA for 2 hours for immunofluorescence staining.

Electroretinography (ERG) and Optical coherence tomography (OCT) measurements

In vivo imaging was performed in anaesthetized mice with the Micron IV retinal imaging system. Image-guided spectral domain OCT and focal electroretinogram (fERG) were used to investigate retinal structure and function. Responses to focal light stimuli (1mm diameter; spot size D) were recorded at luminance ranging from −0.9 to 3.9 log cd*sec/m^2. Data is displayed as the mean amplitude of the a-wave (indicates photoreceptor function) and b-wave (indicates bipolar cell function).

Statistics

All experiments were repeated at least three times. Results are therefore presented as means ± standard deviation (S.D.). Comparisons of two individual experimental groups were performed by unpaired Student's t test and Mann-Whitney test. For multiple comparisons, nonparametric analysis was performed using one-way ANOVA test with Dunn's test. All the analysis was performed using GraphPad Prism 6 (GraphPad Software, version 6.01, La Jolla, USA). Two-tailed tests were used throughout. The significant differences were considered at $P \leq 0.05$.

Results

Diabetes drives neurovascular dysfunction, including Müller cell activation and ganglion cells loss.

To understand how diabetes influences the neurovascular unit in the eye, we first confirmed what alterations to vasculature and neural retina occur in STZ-induced diabetic model. *In vivo* monitoring of STZ injected mice and controls permitted longtitudinal assessment of retinal function by ERG. There was a steady decline of ERG function in diabetic eyes from 6 weeks to 25 weeks, and by 6 months a significant suppression of the B wave, indicative of dysfunctional Müller and bipolar cells (Figure 1A&B). *Ex vivo* assessment of vascular integrity using retinal flatmount demonstrates leakage of Evans Blue dye is remarkably increased in the retina of diabetes (219.6±29%) compared to control retina (100.0±9.4%, $P \leq 0.001$; Figure 1C&D). The number of Brn3a+ cells, a specific marker of RGC was quantified on tissue sections and was significantly decreased by 43.6±12.8% of normal controls (Figure 1E&F). In the healthy retina, Müller cells do not express high levels of GFAP, a common marker of reactive gliosis [237], however in the diabetic retina an increase mean fluorescent intensity (MFI) of 76.7±17.1% of GFAP expression was observed (Figure 1G, H). Next, we assessed whole retinal tissues for altered expression of gene transcripts encoding NPY and vasoproliferative factors, VEGF and ANGPTL-4. In 6-month diabetic retinas there was significantly reduced NPY mRNA levels detected (Figure 1I), with corresponding increased expression of the pro-angiogenic VEGF and ANGPTL-4 (Figure 1J).

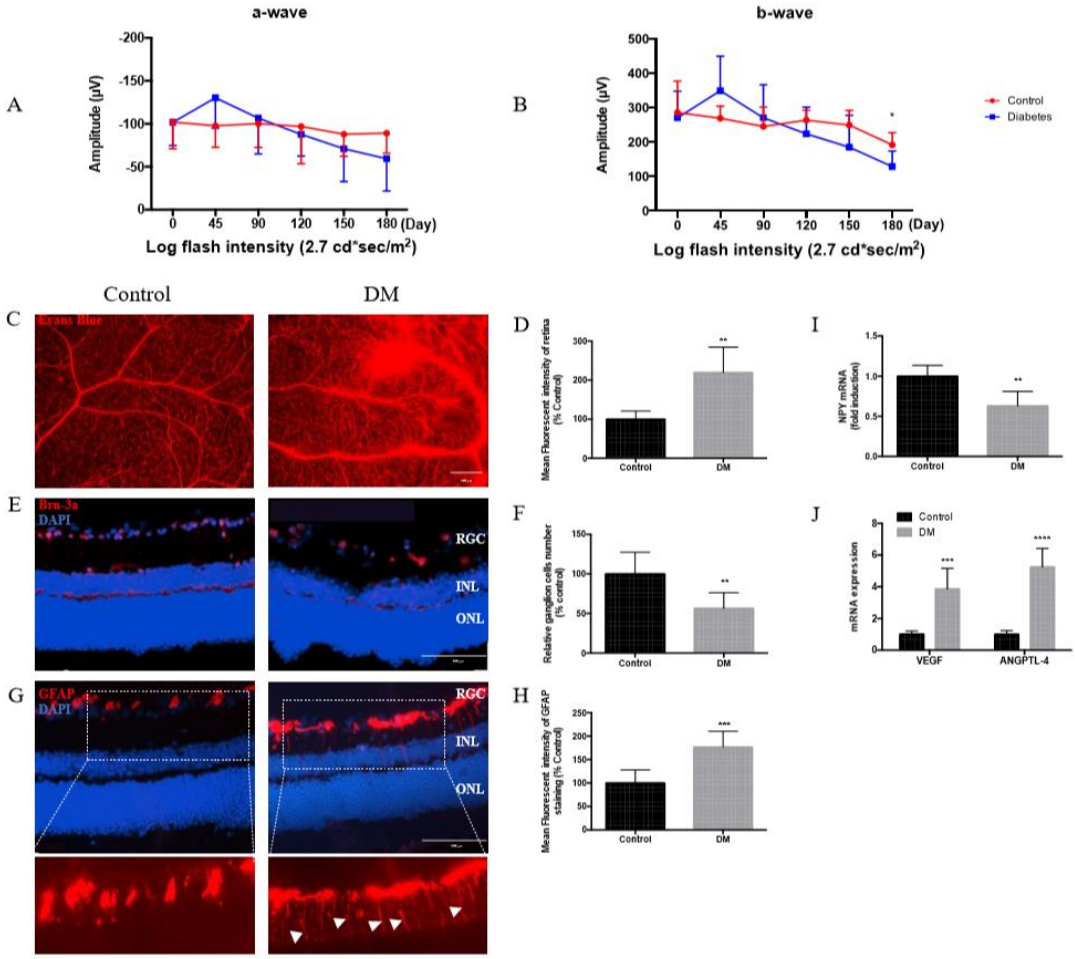


Fig. 1. Diabetic retinas exhibit neurovascular dysfunction. Representative images captured from retinal flatmounts and sections obtained from age-matched wild-type and 6-month-old diabetic mellitus (DM). (A&B) ERG recorded a- and b- waves in scotopic conditions show photoreceptor and inner retina electroretinogram data respectively. (C) Fluorescent images of retina demonstrated differences in vascular integrity (leakage of Evans blue dye, which appears as red). (D) The mean fluorescence intensity of Evans blue was quantified using ImageJ and shown to be significantly increased in the DM group. (E) Immuno-staining of tissue sections RGC marker Brn-3a (red) and DAPI (blue). (F) Quantification of Brn-3a+ expression as percentage of control. (G) Immuno-staining of retinal sections with retinal macroglial cell marker GFAP (red) counterstained with DAPI (blue). Arrows indicate activated Müller cells in diabetic mice. Scale bar, 100 μm. (H) The mean fluorescence intensity of GFAP staining was quantified using Image J and shown to be significantly increased in the DM group. Retinal ganglion layer (RGC), inner nuclear layer (INL) and outer nuclear layer (ONL). (I&J) Gene expression in control and diabetic retina demonstrates significant reduction in NPY and increase of pro-angiogenic factors (VEGF and ANGPTL-4). *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, statistical analysis was performed with unpaired student's t-test.

NPY regulates the tight junctions of retinal endothelium through MAP-kinase.

3. Results

The observed reduction in NPY gene expression in the diabetic retina correlates with an increase in vascular permeability and pro-angiogenic factors. We therefore wished to interrogate the potential role of NPY in maintenance and regulation of the retinal vascular barrier. Using an *in vitro* approach, we examined the effects of NPY in terms of maintaining the tight junctions in cultured MRMECs. First, we established the response profile of MRMECs following incubation for 24 hours with a range of VEGF and NPY doses (Figure 2A&B). VEGF had no effect on cell viability, and NPY was toxic only at the high dose of 40 μM. SDS-PAGE analysis of MRMEC cell lysates demonstrated that VEGF resulted in a dose-dependent reduction of ZO-1, whereas NPY increased significantly ZO-1 expression (Figure 2C&D). For subsequent *in vitro* assessment the following doses were used: VEGF 100ng/ml and NPY 10 μM. At these concentrations, MRMEC expression of ZO-1 following incubation with VEGF resulted in a significant (43%) reduction, whereas NPY increased expression by 40% compared to untreated controls. When cells were pre-treated with NPY, applied 6 hours before VEGF, there was a significant increase in ZO-1 expression (33% higher than VEGF alone) equivalent to expression of the control cells. (Figure 2E). To understand the potential mechanism for the protective effect of NPY, we next examined expression of MAP-kinase (MAPK), recognized to regulate the cellular expression of tight junction proteins[238]. Our results indicate that treatment of MRMECs with NPY increased expression of ZO-1, in contrast to down-regulate MAPK isoforms p38-MAPK and p44/p42 MAPK, compared to VEGF alone ($P \leq 0.05$; Figure 2F-G).

To demonstrate how NPY-mediated increase in expression of ZO-1 influenced the barrier function in MRMECs, we next exploited an *in vitro* vascular permeability assay. This approach permits dual assessment of tight junction expression and extent of monolayer leakage in response to NPY and VEGF treatment. When treat MRMEC cell monolayers with VEGF (100 ng/ml, 24 hours), a 7-fold increase in fluorescence area (fluorescent-positive intercellular signal) compared to control (untreated cells) was observed, indicating increased permeability. NPY attenuated permeability through preservation the integrity of cellular tight junction, demonstrated reduction of fluorescent area by 30% compared to VEGF alone ($p<0.05$) ($p<0.05$) (Figure 2H&I). In addition, ZO-1 immunostaining confirmed the breakdown integrity of tight junction following VEGF treatment, which was similarly partially prevented by NPY pre-treatment (Figure 2H). To assess whether NPY modulation of murine endothelial cells

could be extended to human and thus bring a translational understanding of the role of NPY, experiments were also performed using HMRECs, and demonstrated similar protective effects and maintained ZO-1 expression (Supplementary 1A). To model microvascular disturbance observed in the hyperglycemic retina [239], we determined the angiogenic potential of HRMECs in response to NPY under high glucose (HG) conditions. Utilizing the *in vitro* tube formation assay, quantitative analysis showed that HRMEC tube length was significantly reduced with high glucose exposure (50 mM) compared with control group (5.5 mM glucose), while NPY co-treatment under high glucose conditions maintained the angiogenic capacity (p<0.05; Supplementary 1B& C).

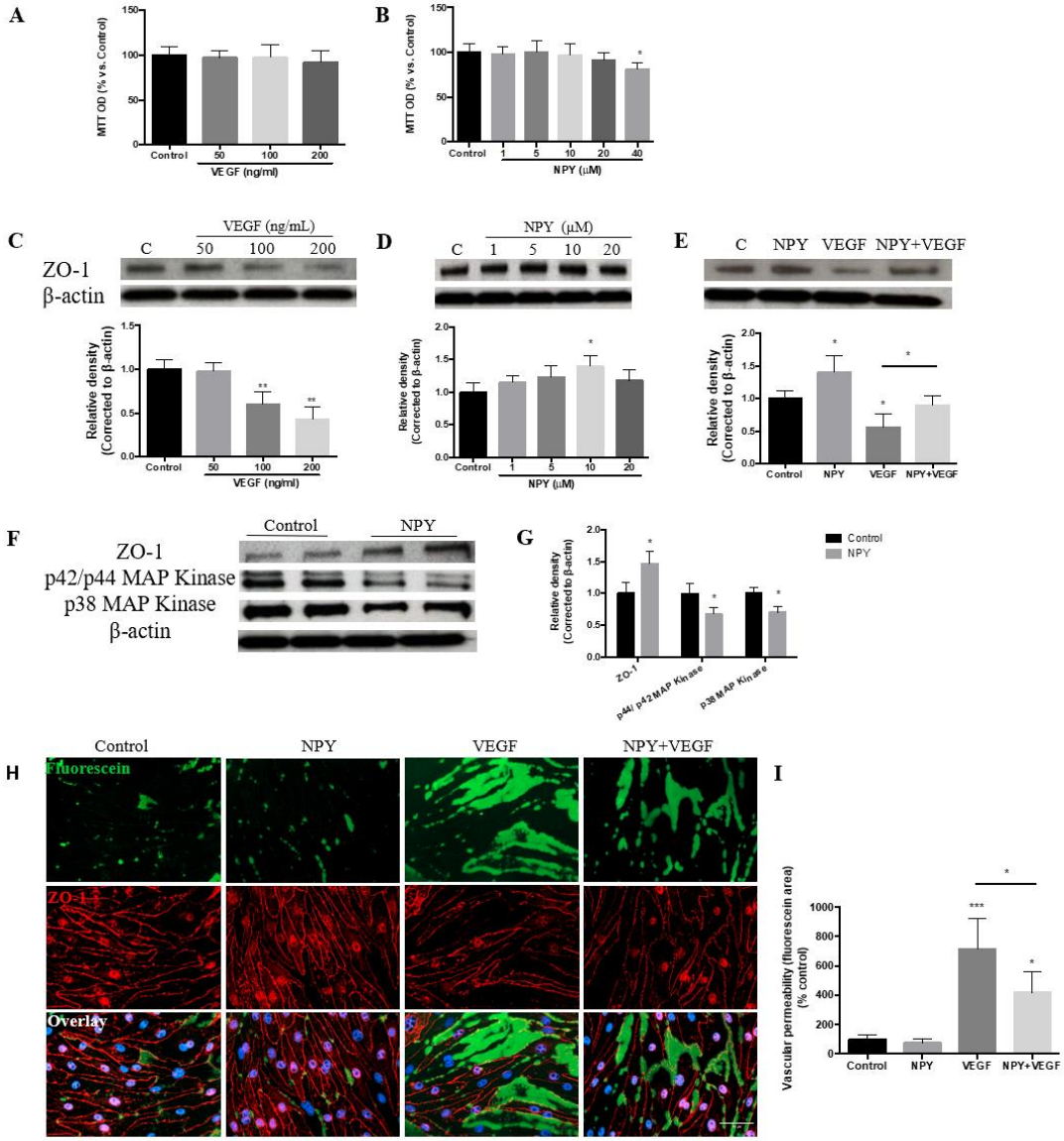
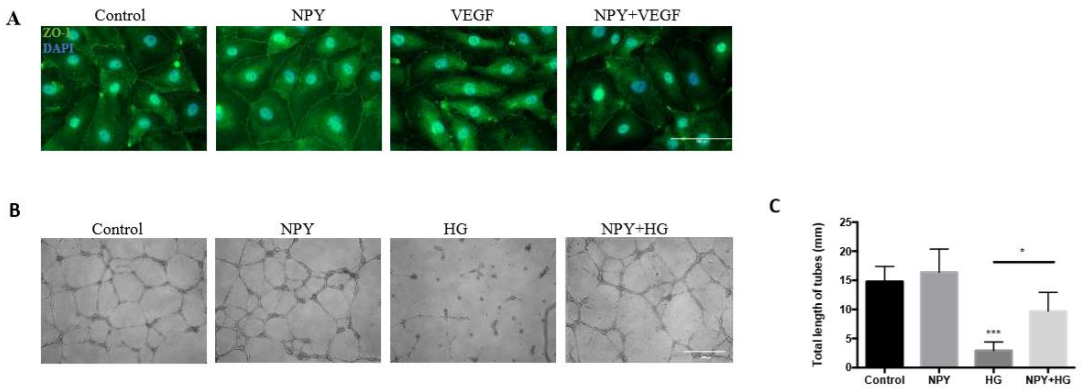


Fig. 2. NPY regulates the tight junctions of retinal endothelium through MAPK. (A, B) MRMEC cell monolayers were serum starved for 24 hours, before incubation with complete medium supplemented

with NPY or VEGF for a further 24 hours. Cell viability was determined in each group using the MTT assay. Data were normalized by control. (C, D) SDS-PAGE analysis demonstrating altered ZO-1 expression in MRMEC cell lysates treated with NPY (0 – 20 µM) or VEGF (0 – 200 ng/ml). (E) ZO-1 expression in MRMECs stimulated with NPY (10 µM), VEGF (100 ng/ml) or NPY+VEGF (pre-incubated with NPY for 6 hours before addition of VEGF). (F, G) ZO-1, p44/p42 MAPK and p38 MAPK protein expression in MRMECs treated with NPY (10 µM) or VEGF (100 ng/ml). (H) Immunostaining of MRMECs for Fluorescein-streptavidin to detect permeable areas and for ZO-1 to show cell-cell contact; cells deprived of serum for 24 hours and incubated in the presence of NPY (10 µM), VEGF (100 ng/ml) or NPY+VEGF (pre-incubated with NPY for 6 hours before addition of VEGF) for 24 hours (n=4 per group). (I) Fluorescein intensity associated with DAPI expression was calculated by ImageJ in all treatment groups. Scale bar, 50 µm. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.



Supplementary 1. (A) NPY stabilizes ZO-1 expression *in vitro* following VEGF-induced permeability. Representative pictures of confluent HRMEC cultures at 24 hours following treatment with NPY (10 µM), VEGF (100 ng/ml) or NPY followed by VEGF 6 hours later stained for ZO-1 (green) and nuclei (DAPI). Scale bar 200 µm. (B&C) Representative bright-field images showing HRMEC tube formation. Addition of HG (50 mM) alone inhibited tube formation, whereas the addition of NPY (10 µM) in combination with HG only partially inhibits. Scale bar, 500 µm. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; *** $P < 0.001$; statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

NPY inhibits VEGF-induced vascular permeability in diabetic mice

The *in vitro* data demonstrates that NPY can modulate VEGF-induced vascular changes, so we next investigated whether this protective effect on vascular integrity translated to an *in vivo* setting. Groups of 6-month aged diabetic mice were treated with a single intravitreal injection of VEGF (100 ng), NPY (10 nmol) or NPY+VEGF and clinically assessed at 48 hours post-injection. Clinical OCT assessment demonstrated that VEGF

or the combined VEGF+NPY treatments did not alter structural integrity as measured by retinal thickness (Figure 3A&B).

Vascular leakage was then assessed by extravasation of intravascular Evans Blue (EB) dye in the different treatments. Flatmount retinal preparations showed that intravitreal injection of VEGF results in a 4.8-fold increase in extravasation of EB as determined via MFI compared to control. Intravitreal injection of NPY together with VEGF decreased the leakage in retinal vessels by 36% compared to VEGF alone (Figure 3C&D). On the contrary, minimal vascular permeability of EB was observed in animals receiving vehicle (PBS control) or NPY alone injections (p<0.01).

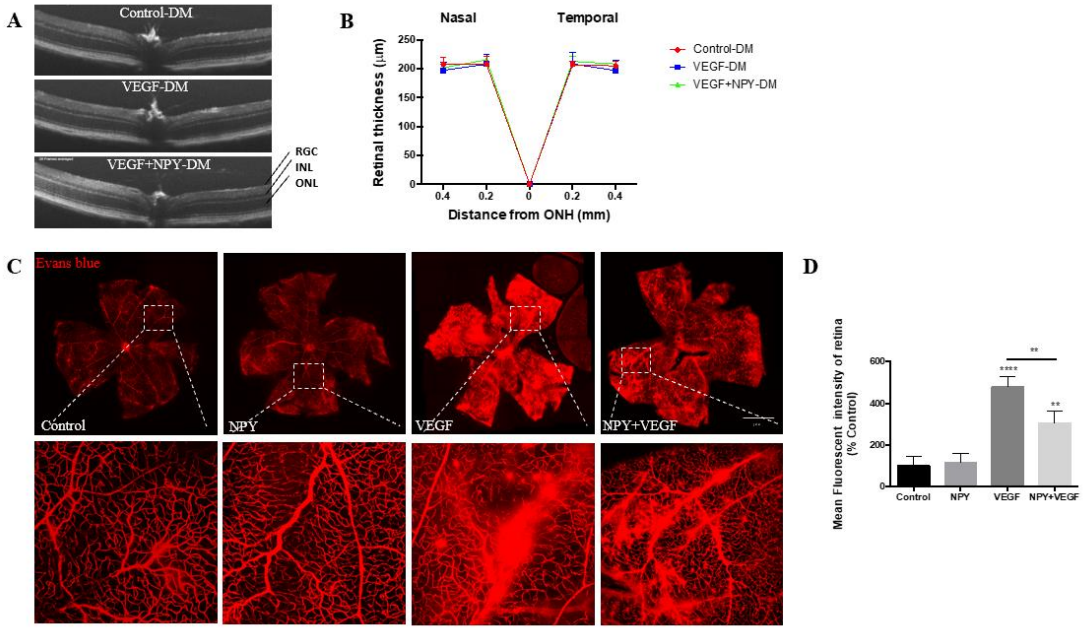


Fig. 3. NPY inhibits VEGF-induced vascular permeability in diabetic mice. Groups of 3-month-old diabetic mice received intravitreal injections of NPY (10 nmol), VEGF (10 nmol), or NPY in combination with VEGF (n=6 per group). (A&B) At 24 hours post-injection, clinical OCT images were acquired, and retinal thickness quantified. (C) Representative *ex vivo* retinal flatmount images prepared 48 hours following treatment demonstrate differences in the vasculature (leakage of Evans blue dye, which appears as red). Scale bar=1 mm. (F) The fluorescent intensity of Evans blue was quantified by Image J. Data represents means ± SD of relative values vs control from 3 independent experiments. *P < 0.05; **P < 0.01; **** P < 0.0001, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

NPY protects retinal ganglion cells against apoptotic cell death induced by NMDA *ex vivo* and *in vivo.*

As NPY receptors are expressed and distributed in the inner retina, and recognition that RGC are perturbed in diabetes [59], we then wished to determine whether NPY played a homeostatic role and exerted a protective effect to preserve RGC. To investigate this further, the NMDA-induced RGC excitotoxic model were employed in retinal explants [234].

Quantification of TUNEL+ cells indicates NMDA treatment leads to a 4.8-fold increase in the number of apoptotic cells. Using NPY (10 μM) treated the explant for 6 hours prior to exposure to NMDA remarkedly decreased the cytotoxic effects, as presented by a significant reduction in the number of TUNEL+ cells by 38% as compared to NMDA alone ($p<0.05$; Figure 4A&B). NPY treatment result in comparable low numbers of TUNEL+ cells to control explants.

To further elucidate with an *in vivo* correlate, we analyzed flatmounts of diabetic eyes exposed to NMDA for 24 hours as a model of induced neuronal excitotoxic damage. A dose-response was tested prior to confirm the optimal dosing of NPY (5 nmol to 20 nmol), which showed that a 10 nmol NPY dose provided the optimal protective effect in perturbing NMDA-induced RGC apoptosis model (Supplementary Figure 2A&B). In diabetic eyes, administration of NMDA alone, reduced the overall total retinal thickness by 23% compared to disease controls. However, the combined administration of NPY and NMDA the extent of cell loss is significantly reduced by 16% compared to NMDA alone ($p<0.05$; Figure 4C&D). Similarly, ERG responses demonstrate that eyes receiving a combined NPY and NMDA injection have improved functional responses, with increased a- and b-waves, compared to NMDA alone, although these were not statistically significant (Figure 4E&F). Retinal wholemount assessment from diabetic eyes receiving NMDA show a 7.9-fold increase in the number of TUNEL+ cells compared to control. The combined NPY injection led to a significant reduction in the number of TUNEL+ cells (from 7.9-fold to 4.1-fold of control) ($p<0.05$; Figure 4 G&H).

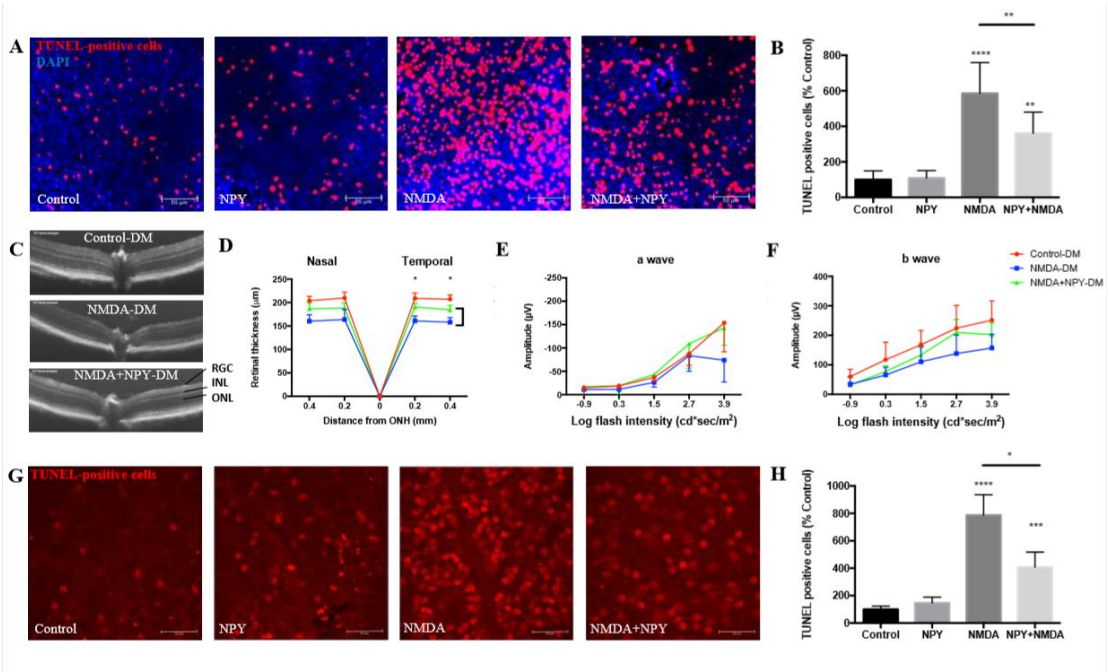
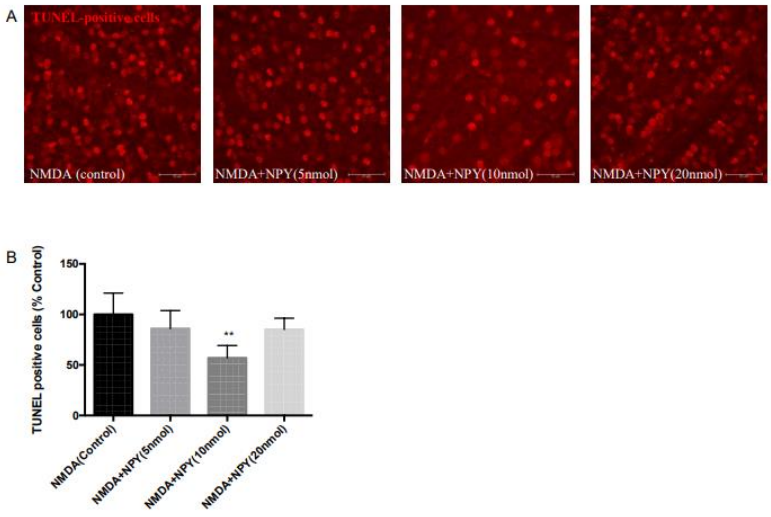


Fig. 4. NPY protects RGC against apoptotic cell death induced by NMDA. (A&B) Representative images showing TUNEL+ cells in rat retinal explants exposed to NMDA (10 µM), NPY (10 µM) or pre-treated with NPY 6 hours before NMDA exposure, showing TUNEL+ (red) and cell nuclei (blue). Scale bar, 50 µm. Quantification of TUNEL+ cells expressed as percentage of control. Groups of 3-month-old diabetic mice received intravitreal injections of NPY (10 nmol), NMDA (10 nmol), NPY in combination with NMDA (n=6 per group). (C) At 48 hours post-injection OCT images show intravitreal NMDA results in reduced retinal thickness and NPY partially protects. ONH = optic nerve head. Retinal ganglion layer (RGC), inner nuclear layer (INL) and outer nuclear layer (ONL) (E, F) ERG a- and b-wave responses represented by mean values of amplitudes in scotopic conditions. (G&H) Representative images of retinal whole-mounts prepared for TUNEL staining and Image J analysis of confocal images. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.



Supplementary 2. (A) Representative images of retinal whole-mounts prepared for TUNEL assay, 24 hours following a single intravitreal injection of NMDA (10 nmol), different dose of NPY (5 nmol, 10 nmol and 20 nmol) combined with NMDA (n=4 per group). (B) TUNEL+ was quantified by ImageJ, showing that NPY (10 nmol) significantly decreases the intensity of apoptotic RGC induced by NMDA. Scale bar, 1mm. $^{**}P < 0.01$, statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

NPY restored neurovascular function in diabetic retinas *in vivo*

To determine whether the positive effects of NPY extended to offer long-term protection in the diabetic retina, NPY was administered via intravitreal injection at 3- and 5-months post-induction of diabetes. Clinical assessment by OCT to measure retinal thickness and ERG to determine changes in retinal function were performed at regular intervals throughout the experimental time-course. We observed no difference of retinal thickness in the NPY-injected eyes compared to age-matched control eyes, whereas the total retinal thickness in DM groups was reduced by 15% compared to control group, with cell loss occurring predominantly in the inner plexiform layer (IPL) (Figure 5A-C). ERG assessment also demonstrates that NPY administration increased a- and b-wave amplitudes to levels equivalent to age-matched non-diabetic controls ($p<0.05$; Figure 5D). These data support a notion that diabetic eyes injected with NPY had sustained neuronal cell function. To confirm whether the protective effect of NPY was evident in RGC and astrocytes, retinal flatmounts were prepared for *ex vivo* immunohistochemistry assessment. Quantification of Brn-3a+ RGC numbers in the diabetic retina show a 66% reduction in cell numbers compared to age-matched controls. By contrast, DM eyes treated with NPY demonstrate a significant increase in the number of Brn-3a+ cells (from 34% to 59% of control) ($p<0.05$; Figure 5E&F). Retinal glial coverage was evaluated by staining with GFAP, showing widespread loss of glial coverage in the retina of diabetes (Figure 5G). The relative glial coverage in the diabetic retina was significantly reduced by 55% compared to control retina. The diabetic eyes treated with NPY demonstrated a significant increase by 49% in glial cell coverage compared to untreated diabetic eyes ($p<0.05$; Figure 5H). Vascular integrity assessment by extravasation of EB dye showed a 3-fold increase in the diabetic retina, and this was remarkably reduced in the NPY group. ($p<0.05$; Figure 5I&J).

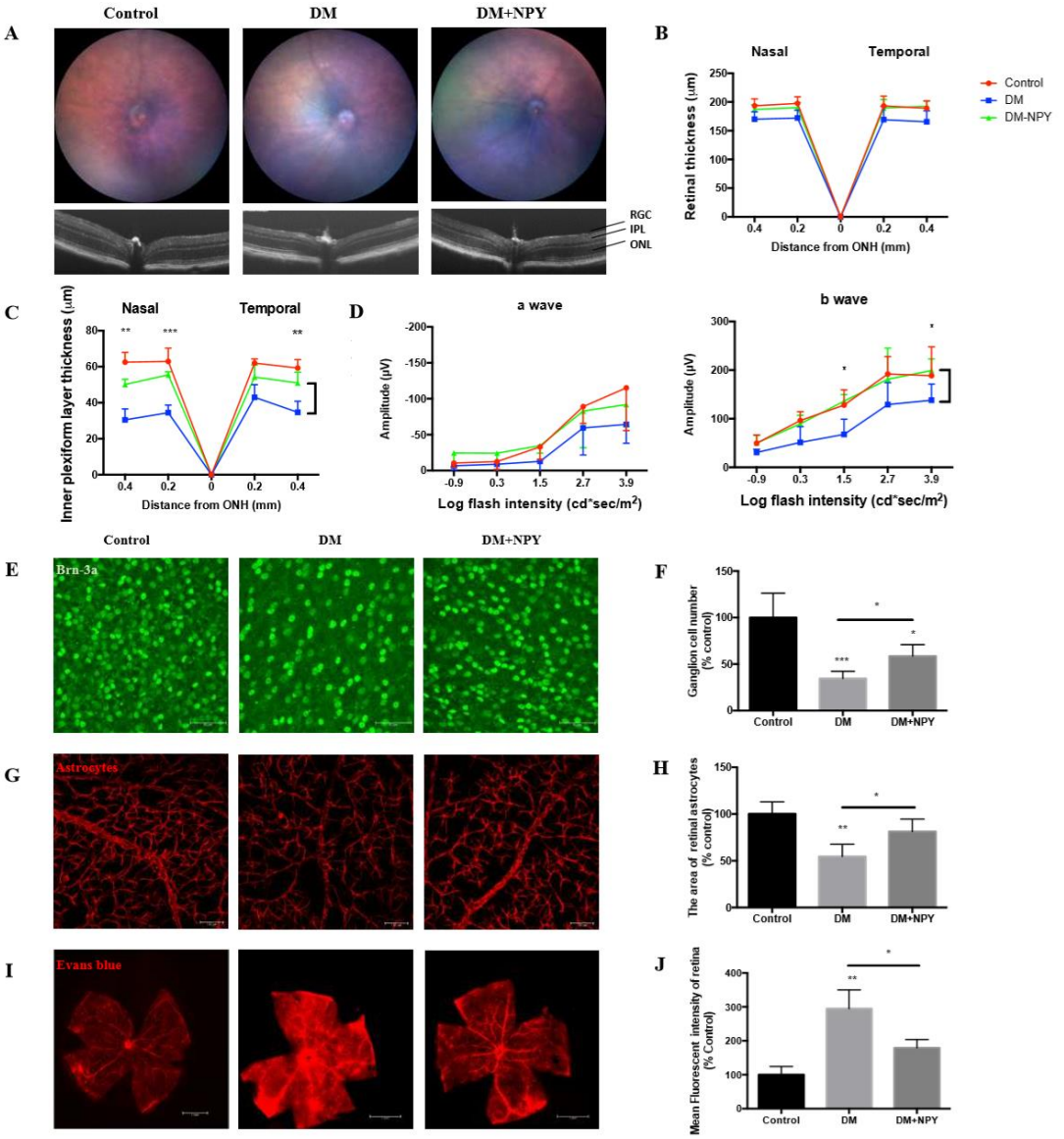


Fig. 5. NPY restores neurovascular function in diabetic retinas. Groups of diabetic mice received intravitreal injections (NPY; 10 nmol) at 3- and 5-months. (A-C) Representative fundal and OCT images captured at 6-months, showing no difference in retinal thickness between NPY-injected eyes and age-matched control eyes. The total retinal thickness showed significant reduction in the diabetic retina, primarily in inner plexiform layer. (D) ERG a- and b-wave responses represented by mean values of amplitudes in scotopic conditions. (E&F) Representative confocal images of retinal flatmount and quantification of Brn-3a immunostaining, showing that NPY prevents the loss of RGC in diabetic mice. (G&H) Representative images and quantification of GFAP immunostaining, showing that NPY also protects against the loss of retinal astrocytes (I&J) Representative images 48 hours following treatment demonstrate differences in the vasculature (leakage of Evans blue dye, which appears as red). Scale bar=50 μm. The fluorescent intensity of Evans blue was quantified by ImageJ. Data represents means ± SD of relative values vs control from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; statistical analysis was performed with one-way ANOVA with Dunn's test for multiple comparisons.

Discussion

In this study, we demonstrate a protective role of NPY in the retina, preventing loss of RGC and vascular leakage in diabetic mice *in vivo*. These findings are supported by *in vitro* and *ex vivo* evidence of NPY mediated protection of MRMECs and RGC. The data supports how modulation of neurovascular unit may offer therapeutic opportunities to prevent progression of DR.

The pathogenesis of DR is highly complex and a multifactorial process. Hyperglycemia perturbs the metabolic and haemodynamic equilibrium, altering the molecular signature of multiple different cell types, principally retinal endothelial cells and pericytes. Diabetes elicits the formation of advanced glycation end-products and reactive intermediates of altered metabolism, increased oxidative stress, which ultimately drive progressive neural and vascular damage [30,240,241]. There is also reported data suggesting that DR results from changes to the neurovascular unit rather than isolated neuroglial or vascular alterations [242,243]. The *in vivo* clinical assessment detailing the changes in ERG response and OCT retinal thickness in the DM-mice supports previous reports which demonstrate a progressive neuronal injury at 6 weeks after diabetic induction [244]. Furthermore, besides vascular disruption we have shown there is significant neuronal impairment (reduced numbers of Brn-3a+ RGC and activation of GFAP+ retinal glial) at 6 months, which supports that dysfunction of retinal neurons occurs early following the onset of diabetes. Considering the current data together with previous reports, there is compelling evidence to support that DR is a consequence of neurovascular-unit disruption. Reduction of NPY expression (as we demonstrate in DM mice) may be associated with increased retinal neuron apoptosis and development of DR. This would suggest that that normal levels of NPY are critical to the homeostasis and maintenance of neurovascular unit.

Increased vascular permeability in diabetes is recognized as a complex process involving multiple signaling pathways, mediated principally by VEGF. This alters the integrity of endothelial tight junctions by altering phosphorylation of ZO-1 and occludin proteins [40,245]. Our results are consistent with previous work describing the disruptive effect of VEGF on endothelial cell integrity [206,246]. Moreover, we demonstrate that VEGF and NPY have opposite effects modulating the expression of ZO-1 *in vitro* using the MRMEC cell monolayers. Treatment with NPY increased

expression of ZO-1, accompanied by reduction of the phosphorylated MAPK isoforms, supporting our initial hypothesis that NPY maintains vascular integrity, and this is dependent on MAPK inhibition. In addition, NPY has been shown to inhibit nuclear translocation of NF-κB and activation of microglia challenged with IL-1β suggesting and a further mechanism to protect and regulate the endothelial barrier function [247–250].

Our previous studies using *in vitro* vascular permeability assay clearly demonstrate VEGF mediated increase in retinal endothelial cell monolayer permeability [234], and when the assay was deployed here, results show that NPY suppressed VEGF-induced monolayer leakage and permeability. In addition, NPY treatment enhances stability of ZO-1 proteins at cell-cell contacts in human retinal endothelial monolayer, coincident with leakage of fluorescence. Our data suggests the protective effect of NPY on the integrity of the retinal endothelial barrier is likely due to its ability to induce stabilization of tight junction complexes. Furthermore, and relevant to DR, our observations from the diabetic mice demonstrate the protective role of NPY against VEGF-induced microvascular permeability *in vivo*. The protective effects of NPY are similar to another neuropeptide, Substance P [207], which we previously demonstrated can maintain and support retinal vascular integrity.

Other recognised alterations in the diabetic retina include an increased frequency of apoptosis of RGC, demonstrated in experimental models of diabetes and human tissue samples from individuals with DM [59]. Similarly, we report that in the retinas of STZ-induced DM, there is a significant decrease of retinal thickness in DM compared with controls. Interestingly, segmentation analysis of retinal OCT images indicates cell loss occurs primarily in the retinal IPL, a layer comprising a dense reticulum of fibrils predominantly formed by interlaced dendrites of RGC. RGC death in DR by glutamate-related toxicity [251]. Glutamate is the primary excitatory neurotransmitter in the retina, utilized by RGC, photoreceptors and bipolar cells. However, when glutamate levels are elevated this is recognized to contribute to RGC death as in diabetes [251–256]. Expression of NPY in the mouse retina affords neuroprotective effects [146], confirmed by *in vitro* observations that rat retinal cells are protected from NMDA-induced excitotoxicity [257]. In the current study, we extend understanding of the beneficial effects and show that NPY can protect RGC against NMDA-induced apoptosis both *in*

vitro (using the retinal explant model) and *in vivo* following NMDA administration in diabetic eyes. The intracellular mechanisms underlying the neuroprotective function of NPY, are likely linked to the inhibition of glutamate release, as reported in the CNS [231,258]. Previous studies using cultures of retinal neurons from rats demonstrate that NPY inhibits the increase in intracellular [Ca^{2+}] through activation of NPY receptors [146,259]. Different intracellular signaling pathways are also reported to facilitate NPY-mediated neuroprotection; the involvement of extracellular signal-related kinases 1/2 (ERK1/2) and Akt pathways are reported in animal model of Parkinson's disease [260], as well as PKA and p38K in retinal neural cells [146]. In terms of neuroprotection, we speculate that NPY similarly exerts a protective effect through MAPK signaling pathways, as MAPK has been shown to regulate tight junction function. Further work in this area will be required to confirm.

Taken together, these studies highlight the regulatory and homeostatic role of the neurovascular unit that is disrupted in DR. The current data supports that adjunctive NPY therapy sustains neuronal health and can attenuate at least in models the downstream effects of diabetic retinal vasculopathy, notably vascular leakage and neuronal death, as well as the loss of Glia cells.

4. General Discussion

4.1 Müller Cells Stabilize Microvasculature through Hypoxic Preconditioning

One of the initial aims of this thesis demonstrated that HP has a robust effect on HIF-1α stabilization in response to hypoxia. I found that HIF-mediated transcription and pro-angiogenic factors are downregulated following exposure to HP. This protective mechanism is associated with the regulation of the retinal Müller cells' mitochondrial metabolism.

Diabetes leads to distinct retinal microvascular complications including vascular leakage and neovascularisation [261]. Numerous clinical and experimental observations have shown that retinal ischemia/ hypoxia, one of major factors driving retinal neovascularization, vessel leakage and macular oedema, leads to vision loss in DR [92,262]. Studies have shown that retinal Müller cell-derived VEGF contributes to a significant increase of neovascularization and vascular permeability under retinal ischemia/ hypoxia, suggesting that Müller glial cells play an essential role in maintaining retinal microvascular homeostasis [263–265]. In heathy conditions, Müller cells regulate the integrity of BRB, by balancing the secretion of pro- and anti-angiogenic factors, such as VEGF [266], PEDF [267] and thrombospondin-1[268] etc.. In DR, the homeostasis of Müller cells is disrupted in retinal hypoxia, leading to increased secretion of pro-angiogenic factors, particularly VEGF, subsequently causing angiogenesis and vessel leakage [97,263,269].

VEGF is essential to the development of retinal vessels, whereas, overexpression of VEGF can disrupt integrity of BRB [270]. Recently, Sodhi et al., has identified that hypoxic retinal Müller cells induce vascular permeability despite inhibiting the expression of VEGF [69], suggesting that there are other HIF-dependent factors involved in retinal vascular disease [69]. ANGPTL-4 has been identified as a novel pro-angiogenic factor. Hypoxic Müller cell-derived ANGPTL-4 expression leads to vessel leakage and angiogenesis *in vitro* and *in vivo* models [271]. Consistent with previous studies, in this thesis it was shown that the protein level of HIF-1α and the expression of pro-angiogenic factors (VEGF and ANGPTL-4) were significantly increased in hypoxic Müller cells. In support, it was found that up-regulation of VEGF and

ANGPTL-4 in conditioned medium from hypoxic Müller cells significantly promotes tube formation and permeability in HUVEC cultures. The corollary is the expression of VEGF and ANGPTL-4 was attenuated when applying digoxin to block HIF-1α translation in hypoxic Müller cells.

In addition to pro-angiogenic factors, previous studies have shown that multiple hypoxia-induced cytokines and other growth factors contribute to retinal pathogenesis in DR [272,273]. These cytokines are activated during hypoxia through HIF-1. Therefore, HIF-1 is a key target to regulate hypoxic retinal microvascular disease. Through inhibiting HIF-1α and thereby modifying the downstream target genes expression, HIF-1α has emerged as a major target for cancer treatment [274]. However, a systemic knock out of the oxygen dependent regulated component HIF-1α is lethal [275]. Meanwhile, multiple studies have focused on developing specific therapeutic targets, such as proteins [276], small molecules [277] and RNAs [278] to inhibit HIF-1α locally in the tissue. Although, there are not any selective HIF-1α inhibitors currently approved for clinical use, future studies will need to develop a specific stabilize HIF-1α in hypoxic retinal disease.

Preconditioning which is the exposure of a tissue or an organism to sub- lethal stress conditions, has recently been demonstrated to be an effective strategy to attenuate neuro- and retinal degeneration in animal models [279]. This induces pro-survival pathways to provide protection against future injury. Additionally, the beneficial effects of HP in reducing tissue damage from cerebral ischemia are also considered to involve in HIF-1α- mediated genes expression [177]. Therefore, the connection between preconditioning and HIF-1α expression was analyzed in this thesis.

In the current study, for the first time, the potential protective effects of HP in retinal Müller cells was explored, and it was demonstrated that HP has a robust effect decreasing HIF-1α stabilization in response to hypoxia. In turn, HIF-mediated transcription and secretion of pro-angiogenic factors was reduced. This leads to noted suppression of both vascular tube formation *in vitro* and vascular permeability with HP pretreated Hypoxic Müller cells' medium. The protective effect is consistent with other data, showing that HP can delay the apoptosis of cells in response to further hypoxic insults [173], the HP of bone marrow stem cells can stimulate nerve regeneration in

cerebral ischemia of rats [174] and HP pre-treated astrocytes are tolerant to severe hypoxia [167]. In these experiments, it has been shown that cells subjected to HP display an improved tolerance to a subsequent severe hypoxic exposure.

Thiersch et al. [280] have shown that photoreceptors of normoxic (21% O_2) control mice undergo apoptosis when exposed to light. In contrast, retinal morphology and function of mice preconditioned by hypoxia (6% O_2) for 6 hours were completely maintained when exposed to light after 4 hours of reoxygenation. When retinas of mice are exposed to oxygen concentration of 6 to 10%, it can protect retina from light induced degeneration completely, 14% oxygen showed an intermediate level of protection, while 18% oxygen did not display any protection. Thereby, the oxygen level of HP correlates with the level of retinal neuroprotection. Meanwhile, the current study has shown that the protective effect of HP lies within a narrow time window, through assessing the time-course of a cellular response under hypoxia. The protective effect of HP on the permeability and angiogenesis assays was optimal for 2 hours, implying the importance of selecting the appropriate duration of HP. Moreover, the potential protective factors derived from HP may be short-lived and rapidly degraded or inactivated during reoxygenation.

It has been demonstrated that metabolic products, specifically fumarate and succinate, regulate the expression of hypoxia inducible genes including HIF-1α in cancer pathology [176,281]. The metabolic pathways have been assessed in this present study, as a potential mechanism through which HP- 2-hours exerts the protective anti-angiogenic effect. Further it was shown that transcriptional activity of HIF and HIF-1α stabilization is dependent on the activation of mTOR under hypoxia. These data confirm previous cancer study that showed that mTOR activation potently enhances the activity of HIF-1α and VEGF-A secretion during hypoxia, and can be reversed with rapamycin [172]. HP significantly perturbed mTOR expression, suggesting that HP could provide a suitable strategy for the treatment of hypoxic retinopathy by suppressing mTOR-mediated pro-angiogenic factors through HIF-1α.

Furthermore, Lu et al. demonstrated that the up-regulation of end products of glycolytic metabolism under hypoxia in cancer – the well-known Warburg effect –promotes HIF-1α protein stability and activation of HIF-1-inducible genes [170]. In this study, the

data also shows that reduced oxidative metabolism in hypoxic retinal Müller cells was partially prevented after HP, as supported by OCR results from the mitochondrial stress test. Furthermore, gene expression of glycolytic proteins was reduced by HP, while SDHD expression was significantly increased, suggesting deployment of the TCA cycle. The association of GLUT expression levels with HIF-1α expression in Müller cells have been observed, with HP preventing the upregulation of GLUT, and subsequently of HIF-1α. Overall, the results obtained demonstrate that HP regulates the cellular metabolism of Müller cells, stabilizes the cells, prevents the switch to aerobic glycolysis, and prevents angiogenesis. The data implies that HP may represent a clinically applicable approach to improve the microvascular environment in DR.

However, more recently, numerous studies have shown that the dysfunction of Glia-neurons was disturbed prior to the pathogenic changes of microvessels in DR [62,282]. Therefore, it is arguably more important to maintain the integrity of neurovascular-unit in DR, rather than merely control vasculopathy.

4.2 Restoring retinal neurovascular health via SP and NPY

In the neuropeptides project of this thesis, results have shown a protective function of SP and NPY against NMDA-induced RGC death *in vitro* and *in vivo* models. Moreover, SP and NPY directly suppressed VEGF expression, which lead to the reduction of vascular permeability in experimental models. Furthermore, NPY prevented loss of RGC and Glia cells, as well as vessel leakage in diabetic mice *in vivo*.

The pathogenesis of DR is highly complex and a multifactorial process. Hyperglycemia perturbs the metabolic and haemodynamic equilibrium, altering the molecular signature of multiple different cell types, principally endothelial cells and pericytes in the retina [37,283]. Diabetes elicit the formation of AGEs, elevate reactive intermediates because of altered metabolism and increase oxidative stress, which ultimately drive progressive neural and vascular damage [30,240,241]. There is also reported data that DR results from changes to the neurovascular-unit rather than isolated neuroglial or vascular alterations [242,243]. The continual change in ERG and OCT data in STZ-mice supports previous reports, demonstrating the development of neuronal damage in mice after 6 weeks of diabetic induction [244]. Current studies have shown significant

neuronal impairment at 6 months, which further supports that the dysfunction of retinal neurons occurs in the early start of diabetes. Thereafter, the vascular impairment in the diabetic retina has been observed, which increases the vessel leakage, activation of Glia cells and eventually leads to the loss of RGC. Taking the data together, there is compelling support that DR is a consequence of neurovascular-unit disruption. Reduction of SP and NPY expression (as observed in diabetic mice) may be associated with the increased retinal neuron apoptosis and development of DR. Therefore, the assumption is that normal levels of SP and NPY are critical to maintenance of neurovascular-unit.

The progress of DR causes vascular permeability is complicated. The principal reason is that VEGF and other pro-angiogenic factors disrupt the integrity of endothelial cell–cell junctions [40]. Among the various protein components of tight junctions, ZO-1 is a phosphoprotein that participates in multiple protein–protein interactions regulating tight junction integrity [245]. VEGF disrupts tight junctions by altering phosphorylation of ZO-1 and occludin through a Src-dependent pathway [246]. Accordingly with previous studies [206], our results are also able to demonstrate the inhibitory effect of VEGF on the expression of tight junction protein ZO-1 in retinal endothelial cells. Moreover, it has been demonstrated that VEGF and NPY have opposite effects on the expression of ZO-1 in the MRMEC monolayers. SP and NPY increased ZO-1 expression, which supported the initial hypothesis that SP and NPY maintains the vascular integrity and additionally that this is dependent on MAPK inhibition. These results are consistent with Aveleira CA et al. [247], who showed that PKC–MAPK–NF-κB pathway modulates the level of tight junction proteins, further to regulate the blood-barrier in various organs or cells. Furthermore, using a peptide or a chemical inhibitor to block protein kinase C zeta (PKCζ) resulted in reducing NF-κB activation, subsequently inhibiting the alterations in cell-cell tight junction [248].

Furthermore, several cytokines such as IL-10 and IL-12 have been observed to mediate partially the effect of SP in several organs/tissues [215,216]. There appears to be opposing data on the role of SP on endothelial cells. For example, SP has been reported to regulate angiogenesis directly by inducing endothelial cells to produce nitric oxide [217], or indirectly via its interactions with mast cells and granulocytes [135], while VEGF blockade does not abolish the proangiogenic property of SP [218]. On the other

side, in support of our findings, SP can reportedly prevent laser-induced retinal degeneration *in vivo*, by suppressing inflammation and reducing neovascularization [219]. These differences are likely attributable to different animal models studied, the type and kinetics of retinal insult.

In addition, studies have shown that using NPY inhibits nuclear translocation of NF-κB in microglia against IL-1β-induced nitric oxide release, and protects the endothelial barrier function [249,250]. Current work was consistent with previous studies that verified VEGF increases retinal endothelial cell monolayer permeability [234]. Notably, these results have also shown that SP and NPY blocked VEGF-induced monolayer leakage. Furthermore, NPY treatment also enhanced stability of ZO-1 proteins at cell-cell contacts in human retinal endothelial monolayer. These findings indicated that the protective effects of SP and NPY are likely due to their ability to induce stabilization of tight junction complexes at cell contact. In terms of relevance to DR, present study highlights a potential role of NPY to protect against VEGF-induced vessel leakage as demonstrated by *in vivo* findings in diabetic mice.

Whereas, although VEGF is identified as a crucial inducer of vascular permeability and neovascularization, ANGPTL-4 and other pro-angiogenic factors also contribute to the induction of angiogenesis and vessel leakage in diabetes. In term of clinical, controlling VEGF alone has limitations [69], additionally, some patients also have suboptimal response or develop resistance to anti-VEGF therapy [284]. Therefore, the protective regulation of SP and NPY in other pro-angiogenic factors still need to be explored.

Another very dramatic alteration of the retina in diabetes is the increased frequency of apoptosis of retinal cells, which has been demonstrated that in experimental diabetes of rats and DM [59]. In this study, it has shown the same phenomenon in the retinas of STZ-induced diabetic model, the cell number of diabetic retinas reduced a lot compared to control. There is evidence that glutamate-mediated toxicity is one of pathways resulting in the RGC death in DR [251]. It has been reported that glutamate level is elevated in the retina of experimental diabetes [254], especially increased significantly in the first 3 months of STZ-induced rat model [251]. It also confirmed the increase of glutamate is also associated with human diabetic patients [285]. Glutamate is considered to be the primary excitatory neurotransmitter in the retina, most of retinal

cells use glutamate to transmit signals, including RGC, photoreceptors and bipolar cells etc. [252]. Glutamate is also a well-known excitotoxin associated with a number of pathologic conditions, increased level of glutamate involved in neurodegeneration has been demonstrated [255,256,286]. Xie's results from studies in diabetic rats showed that the levels of SP in the retina and serum were significantly reduced, with an associated increase in apoptosis and caspase-3 activity in the retina [126]. More importantly, restoration of endogenous SP paralleled the inhibition of the apoptosis of the RGC and the caspase-3 activity in the diabetic animals. Studies have also shown that NPY exerts neuroprotective effect in the mouse retina [146]. Moreover, it has been shown that NPY protected rat retinal cells against NMDA-induced toxicity [257]. This indicates that the potential use of NPY in the development of therapeutic strategies to fight retinal disease. Here, this study extended the known beneficial effect to show that SP and NPY exerted neuroprotective properties on RGC in NMDA-induced neuro-excitotoxicity apoptotic cell death both *in vitro* and in an animal model, particularly in a diabetic animal model with NPY. Overall, current studies have shown that SP and NPY were neuroprotective, suppressing apoptosis of RGC induced by NMDA, and also protect against VEGF-induced microvascular leakage, further investigated in a diabetic model. Both effects demonstrate the significant role that the neuropeptide transmitter plays in maintaining neurovascular-unit homeostasis.

5. Conclusion

In conclusion, HP induced significant protection against hypoxic insults through the suppression of HIF-1α activation in the retina. Pretreatment of retinal Müller cells with sublethal exposure to hypoxia, inhibits the expression of proangiogenic factors, thereafter, reduces the promotion of endothelial cell proliferation and leakage. These results further indicate the importance of HP in stabilizing retinal Müller cells under hypoxic condition.

In addition, my results have shown that SP and NPY are neuroprotective by suppressing apoptosis of RGCs induced by NMDA, and protect against VEGF-induced microvascular leakage of the retina. Moreover, NPY attenuates the destruction of DR to retinal neurovascular-unit in diabetic mice.

Overall, these findings demonstrate that HP and neuropeptide (SP and NPY) exert significant protective role in maintaining neurovascular-unit homeostasis in DR, and can potentially provide the new therapeutic avenues for DR.

6. Future Work

6.1 To interrogate the protective effect of preconditioned hypoxic Müller cells in RGC

Müller cells are the main macro-glia cells in the retina, play an essential role in retinal development via maintaining its microenvironment, structure and physiology. Studies have shown that Müller cells contribute to the integrity of the BRB [264], and support neurons and their functions in the retina [287,288]. In contrast, selectively ablation of Müller cell in the retina leads to breakdown of the BRB as well as neovascularization. It also induces retinal dysplasia and abnormal neurotransmitters recycle, further to neuronal cell apoptosis and retinal degeneration [289–291].

It has been shown that HP activates multiple pro-survival pathways and prevents neuronal degeneration in mouse and human [108,292]. Hypoxic and ischemic preconditioning strongly protects the retina against cell death [118]. Therefore, HP should be an excellent candidate for regulating retinal neuroprotection. Meanwhile, HIF-1 is associated with bone marrow mesenchymal stem cells cell survival after preconditioning [293], and HIF-1 controls the expression of genes involved in apoptosis [294]. Current results demonstrated that the level of HIF-1 is reduced in the hypoxic Müller cells after HP. Future studies are required to evaluate whether HP of retinal Müller cells has a protective effect in RGC, prevents the loss of RGC or maintains the normal function of the retina.

6.2 To investigate the potential mechanism of SP and NPY in maintaining the survival of RGC

In terms of the underlying mechanisms of the neuroprotective effect of SP and NPY against glutamate-induced RGC death, the protective function of NPY is considered to be associated with inhibition of glutamate release in the CNS, specifically the hippocampus [231,258]. Various intracellular signaling pathways are also reported to facilitate NPY-mediated neuroprotection. ERK1/2 and Akt pathways were involved in a disease model of Parkinson [260]. PKA and p38K pathways were participated in retinal neural cells [146]. Interestingly, it has been shown that SP-NK1R coupling translocates signals to MAPK via the activation of phospholipase C and adenylate

cyclase, generating DAG/IP_3 and cAMP, respectively [295]. Subsequently, MAPK activates ERK1/2 to regulate the expression of cytokines by the serine/threonine protein kinase, mTOR and the transcription factors [296]. Therefore, future study speculates whether the protective effect of SP and NPY in preventing or attenuating the loss of RGC through the MAPK signaling pathway.

7. References

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