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Retinal protection: new therapeutical perspectives

PhD thesis

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Big or small, life and work success become more significant when shared with people you love!

LIST OF ABBREVIATIONS

A_1R: ADENOSINE A_1 RECEPTOR

$A_{2A}R$: ADENOSINE A_{2A} RECEPTOR

A2E: N-RETINYLIDENE-N-RETINYLETHANOLAMINE

AD: ALZHEIMER'S DISEASE

AMD: AGE-RELATED MACULAR DEGENERATION

AMPA: AMMINO-3-IDROSSI-5-METIL-4-ISOSSAZOL-PROPIONIC ACID

APP: AMYLOID PRECURSOR PROTEIN

AREDs: AGE-RELATED EYE DISEASES

ARPE-19: HUMAN RETINAL PIGMENT EPITHELIAL CELLS

ASC: APOPTOSIS-ASSOCIATED SPECK LIKE PROTEIN

ATP: ADENOSINE TRIPHOSPHATE

Aβ: AMYLOID BETA

BBB: BLOOD BRAIN BARRIER

BDNF: BRAIN-DERIVED NEUROTROPHIC FACTOR

bFGF: BASIC FIBROBLAST GROWTH FACTOR

BRB: BLOOD RETINAL BARRIER

CD200: CLUSTER OF DIFFERENTIATION-200

CD200R: CLUSTER OF DIFFERENTIATION-200 RECEPTOR

CNS: CENTRAL NERVOUS SYSTEM

CNTF: CILIARY NEUROTROPHIC FACTOR

CNV: CHOROIDAL NEOVASCULARIZATION

$COCL_2$: COBALT (II) CHLORIDE

CX3CL1: C-X3-C MOTIF CHEMOKINE LIGAND 1

CX3CL1: C-X3-C MOTIF CHEMOKINE LIGAND 1

CX3CR1: C-X3-C MOTIF CHEMOKINE RECEPTOR 1

DA: DOPAMINE

DAMPs: DAMAGE-ASSOCIATED MOLECULAR PATTERNS

DR: DIABETIC RETINOPATHY

DR5: DEATH RECEPTOR 5

ERKs: EXTRACELLULAR SIGNAL-REGULATED KINASES

GCL: GANGLION CELL LAYER

GDNF: GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR

GFAP: GLIAL FIBRILLARY ACIDIC PROTEIN

H_20_2: HYDROGEN PEROXYDE

HIF-1α: HYPOXIA-INDUCIBLE FACTOR 1 SUBUNIT ALPHA

HMGB1: HIGH MOBILITY GROUP BOX 1

I/R: ISCHEMIA-REPERFUSION

IBA-1: IONIZED CALCIUM BINDING ADAPTOR MOLECULE 1

ICAM-1: INTRACELLULAR ADHESION MOLECULE 1

IL: INTERLEUKIN

ILM: INNER LIMITING MEMBRANE

INL: INNER NUCLEAR LAYER

IOP: INTRAOCULAR PRESSURE

LPS: LIPOPOLYSACCHARIDE

MIO-M1: MOORFIELDS/INSTITUTE OF OPHTHALMOLOGY-MÜLLER 1

NFL: NERVE FIBER LAYER

NFTs: NEUROFIBRILLARY TANGLES

NF-κB: NUCLEAR FACTOR KAPPA-LIGHT-CHAIN-ENHANCER OF ACTIVATED B CELLS

NGF: NERVE GROWTH FACTOR

NLRP3: NUCLEOTIDE-BINDING LEUCINE-RICH REPEAT CONTAINING RECEPTOR 3

NLRs: NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN (NOD)-LIKE RECEPTORS

NMDA: N-METHYL-D-ASPARTATE

NO: NITRIC OXIDE

NOS2: NITRIC OXIDE SYNTHASE 2

NPDR: NON-PROLIFERATIVE DIABETIC RETINOPATHY

NT-3/4/5: NEUROTROPHIN 3/4/5

NTFs: NEUROTROPHIC FACTORS

OBRB: OUTER BRB

OLM: OUTER LIMITING MEMBRANE

ONL: OUTER NUCLEAR LAYER

P2X7: PURINERGIC P2X7 RECEPTOR

PAMPs: PATTERN-ASSOCIATED MOLECULAR PATTERNS

PD: PARKINSON'S DISEASE

PDEs: PHOSPHODIESTERASES

PDR: PROLIFERATIVE DIABETIC RETINOPATHY

PERG: PATTERN ELECTRORETINOGRAM

PI3K: PHOSPHATIDYLINOSITOL 3-KINASE

P-TAU: HYPERPHOSPHORYLATED TAU

R&D: RESEARCH AND DEVELOPMENT

RGCs: RETINAL GANGLION CELLS

RNFL: RETINAL NERVE FIBER LAYER

ROS: REACTIVE OXYGEN SPECIES

RPE: RETINAL PIGMENT EPITHELIUM

STZ: STREPTOZOTOCIN

TGF-β: TRANSFORMING GROWTH FACTOR BETA

TLRs: TOLL-LIKE RECEPTORS

TNF: TUMOR NECROSIS FACTOR

TRAIL: TNF-RELATED APOPTOSIS-INDUCING LIGAND

TRkB: TROPOMYOSIN-RELATED KINASE B

TRK-MAPK: TROPOMYOSIN RELATED KINASE-MITOGEN-ACTIVATED PROTEIN KINASE

VEGF: VASCULAR ENDOTHELIAL GROWTH FACTOR

WNT: WINGLESS/INTEGRATED

α-SYN: ALPHA-SYNUCLEIN

ABSTRACT

Neuroprotection represents a challenge in the research and development (R&D) of drugs aimed to treat retinal degenerative diseases. Neuroprotective therapeutic strategies may address neuroinflammation since it is considered a crucial therapeutic target to control neurodegenerative processes. Retinal neurodegeneration has been linked to neurotrophins deprivation, such as brain-derived growth factor (BDNF) or nerve growth factor (NGF). Neuroprotective strategies may include neurotrophins administration or drug treatments able to increase neurotrophins expression. However, neurotrophins need to be delivered to the back of the eye and several efforts have been carried out to develop innovative eye drop formulations to deliver neurotrophins and other biologic drugs, without success so far. Thereby, R&D of drugs able to increase retinal neurotrophins expression may be a suitable strategy for retinal neuroprotection, taking into account that nanotechnological systems are potential able to provide sustained release of drugs on retinal tissue. Besides, a fast-track approach to develop molecules is the drug repurposing, which is aimed to identify and validate the efficacy of drugs already approved for different indications. Accordingly, this work of thesis was aimed to investigate the anti-inflammatory and neuroprotectanct activity of two already approved drugs in *in vitro* and *in vivo* models of retinal inflammation/neurodegeneration, that represent shared pathological features in glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy (DR). Glaucoma is an optic neuropathy characterized by retinal ganglion cells (RGCs) degeneration, then leading to cell death and subsequent progressive vision loss. High intraocular pressure (IOP) is the main risk factor of glaucoma, which is cronically treated with ocular hypotensive eye drops. However, these pharmacological treatments do not target neurodegeration, and glaucoma generally progresses to blindness. As regard as AMD and DR,

current treatments are targeting retinal neovascularization, although neurodegeneration and neuroinflammation are pathogenic mechanisms of these diseases, no neuroprotective strategies have been already approved for AMD and DR. Chapter I shows the results of the first study aimed to investigate the neuroprotective effect of brimonidine in a mice model of ischemia reperfusion (I/R) damage, which resembles glaucomatous neurodegeneration. Brimonidine is an α2 agonist drug appoved for the treatment of increased intraocular pressure in patients with open-angle glaucoma or ocular hypertension. In my study I demonstrated that brimonidine protected RGCs from retinal neuroinflammation induced by I/R. Moreover, brimonidine counteracted the downregulation of retinal BDNF mRNA levels in I/R eyes. These results demonstrated the anti-inflammatory and neuroprotective action of brimonidine. In Chapter II, the potential neuroprotective effect of caffeine has been investigated. Caffeine, one of the most consumed central nervous system (CNS) stimulants, is indicated for treatment of apnea, bronchopulmonary and hypoxic consequences, in preterm newborns. I investigated the effects of caffeine in *in vitro* and *in vivo* models of retinal inflammation/neurodegeneration, such as ARPE-19 exposed to lipopolysaccharide (LPS) challenge, and I/R injured mice. In these models, caffeine was able to reduce inflammation and increased BDNF mRNA levels. Additionally, caffeine restored the integrity of the retinal pigment epithelium (RPE) monolayer *in vitro*, as well as preserved RGCs function in mice, after eye topical administration. Indeed, my results showed the anti-inflammatory and neuroprotective action of caffeine in the retina suggesting an involvement of the adenosine A_{2A} receptor ($A_{2A}R$), since the selective $A_{2A}R$ agonist, CGS 21680, counteracted the caffeine effects in my *in vitro* model. In conclusion, brimonidine and caffeine exterted neuroprotective effects in models of retinal neurodegeneration, counteracting inflammation and neurotrophins

deprivation. These findings are very promising because can open the way to further development and clinical trials.

GENERAL INTRODUCTION

NEUROPROTECTION

Neuroprotection is a therapeutic strategy aimed to preserve or improve the neuronal function and structure, through the inhibition of one or more pathophysiological steps involved in neurodegeneration. Practically, neuroprotection consists of pharmacological agents or gene therapies to prevent or treat a specific pathological condition [1]. Neuroprotective strategies are generally considered as a disease-modyfing approach, given that neurodegenerative diseases are currently treated with symptomatic approaches.

NEURODEGENERATIVE DISEASES: COMMON FEATURES BETWEEN RETINA AND CENTRAL NERVOUS SYSTEM

Central nervous system (CNS) neurodegenerative diseases are leading causes of morbidity and mortality, affecting millions of people in the world, and aging is considered the major risk factor [2]. Retina shares the same embryological origin and is an extension of CNS, as demonstrated even by the close association and the tight similarities between brain neurodegenerative pathologies like Alzheimer's disease (AD) and Parkinson's disease (PD), and retinal neurodegenerative conditions, e.g., glaucoma, age-related macular degeneration (AMD) and diabetic retinopathy (DR), main causes of blindness in industrialized world. The main causes of neurotoxicity in AD are the extracellular accumulation of amyloid beta (Aβ),

produced by abnormal processing of the amyloid precursor protein (APP), and the neurofibrillary tangles (NFTs), which are aggregates of the hyper-phosphorylated form of the microtubule-associated protein tau (p-tau), promoted by Aβ peptides [3,4]. Furthermore, neuroinflammatory process due to the chronic activation of astrocytes and microglia, mainly induced by the presence of Aβ, characterizes AD brains. Subsequently, substained inflammatory response in turn enhances Aβ damage and tau hyperphosphorylation, suggesting that inflammation plays a central role in the onset as well as in the progression of AD [5]. Indeed, inflammatory signaling exacerbates the pathology by triggering a cascade of events, as well as reactive oxygen species (ROS) production and related toxicity partly induced by iron dyshomeostasis, which contribute to neuronal damage and cell death [6]. Noteworthy, AD affects different structures of the visual system, leading to manifestation of prominent ocular synthoms and distinct signs, which are connected to progression, cognitive decline and disease severity; indeed, these vision-related changes develop before cognitive deterioration. Therefore, the visual system could support to diagnose the early stages of AD, highlithing the revelance of the ophthalmological examinations, less invasive and expensive than other biomarkers [7]. AMD is a slow and progressive retinal disease which affects the macula and represents the leading cause of irreversible blindness during aging. AMD can be classified into two forms: “dry” or atrophic form and “wet” or neovascular form. About 15 % of patients switch to the wet form, that is responsible for 90% of acute vision loss due to AMD [8–10]. Retinal degeneration in AMD shares several features with AD. Indeed, this retinal pathology is associated with the accumulation of drusen, between retinal pigment epithelium (RPE) cell layer and the Bruch’s membrane, which are constituted prominently by Aβ aggregates [11]. Aβ triggers inflammation, induces mitochondrial and

lysosomal dysfunction, as well as leads to oxidative stress [12], which play an important role in disease progression, as well as the iron accumulation, suggesting a strong correlation with AD [13]. Glaucoma is an optic neuropathy characterized by high intraocular pressure (IOP), as well as retinal ganglion cells (RGCs) death, which leads to irreversible blindness. Glaucoma is globally widespread and, after cataracts, represents the second leading cause of vision loss [14]. It can be classified into two wide categories: open-angle glaucoma and angle-closure glaucoma and both can be primary disease. Whereas secondary glaucoma can be caused by tumor, trauma, inflammation or medications [15]. Evidence suggests that chronic elevation of IOP leads to accumulation of Aβ and p-tau in RGCs, causing cells loss and suggesting an overlap with AD [16,17]. Moreover, in glaucomatous degeneration, Aβ and p-tau deposits in RGCs trigger an inflammatory cascade, by increasing cytokine levels and accumulation of iron, which in turn lead to oxidative stress, pointing out other common pathogenetic mechanisms with AD [18]. In addition, specific microRNAs were found similarly dysregulated in glaucoma and AD, as well as in AMD and AD, highlithing common biochemical pathways, mainly associated with inflammation [19]. RGCs death is responsible for thinning of the retinal nerve fiber layer (RNFL) and atrophy of the optic nerve, resulting lastly in blindness. Indeed, glaucoma is considered mainly neurodegenerative disease, also evidenced by the fact that RGCs axon degeneration spreads into the brain, extending from the retina to the visual cortex (anterograde effect), leading to CNS degeneration at multiple levels, related to the disease severity [20,21]. Moreover, glaucoma has been clinically reported as AD comorbidity [22,23], evidencing a retrograde neurodegeneration from CNS to the eye [24]. Currently, pharmacological treatments of glaucoma include the administration of ocular topical agents, which aim to decrease aqueous humor production or increase

its outflow with a mechanical action, like alpha-adrenergic agonists, beta-adrenergic antagonists, parasympathomimetic agents, prostaglandin analogs and carbonic anhydrase inhibitors. However, independently of their effect linked to IOP, some of these agents are able to prevent RGCs death, suggesting a neuroprotective action [25,26]. Therefore, in some glaucoma patients, the disease still progresses despite the IOP reduction [27], suggesting that the pathophysiology of glaucoma is not completely understood and IOP reduction may not be sufficient. DR is a microvascular secondary complication of diabetes mellitus, characterized by progressive retinal vascular abnormalities. DR can be classified in two types, non-proliferative diabetic retinopathy (NPDR), which refers to early stages of the disease; and proliferative diabetic retinopathy (PDR) which represents the andvanced form of the pathology. DR is typically asympthomatic in the early stages, whereas it is associated with a prominent risk of blindness in the later stage. Acute vision loss is often a consequence of microvascular complications, however permanent visual loss after resolution of these abnormalities, underlines that neurodegeneration contributes to the loss of function. Hence, neurodegeneration plays a prominent role in the pathogenesis of DR linked with the development of microvascular abnormalities, and is considered an early component of the disease [28]. The concept of neurovascular unit has been adopted from the brain, to indicate the functional and structural interactions between neurons, glial cells and vascular cells also in the inner retina, which control blood flow in response to metabolic demand; indeed, the neurovascular unit is crucial to understand the link between retinal degeneration and vascular dysfunctions [29]. However, until now there are no treatments which address the neurodegenerative alterations of DR [1]. DR shares similar pathophysiological pathways with PD, which is second most common CNS neurodegenerative pathology. Specifically, the close

correlation between DR and PD is evidenced by the disrupted dopamine (DA) activity in brain and retina, since both express D1-like and D2-like dopamine receptors. Indeed, dopamine is a critical neurotransmitter involved in the cognitive, motor and visual function and alterations in the dopaminergic system are connected with neurological pathologies [30]. Increased expression of alpha-synuclein (α-Syn), which is a key protein involved in PD, has been reported even in DR and this is associated with loss of dopaminergic neurons [30,31]. Moreover, in PD patients, accumulation of phosphorylated α-Syn both in brain and retina is an early event, considering that it precedes the PD onset. Besides, early PD patients reported impairment in the retinal microvasculature and reduced visual acuity [32]. Therefore, ocular changes in PD represent promising biomarkers for an early diagnosis, as well as to evaluate the disease progression [33]. Abnormalities of neurotrophic factors (NTFs) levels, like brain-derived neurotrophic factor (BDNF) [34], nerve growth factor (NGF) [35] and glial cell line-derived neurotrophic factor (GDNF) [36], have a critical role in the pathophysiological process of both DR and PD [30], evidencing a tight association between retina and brain degenerative conditions. Specifically, BDNF retinal proteins levels were found decreased in diabetic mice 5 weeks after induction of diabetes [37]. Moreover, pharmacological or genetic therapy which targets BDNF, owns a therapeutic potential for PD patients [38]. Some therapeutic drugs benefit both DR and PD highlighting again a close association between these two diseases [39,40]. Furthermore, chronic inflammation, as well as oxidative stress represent further common features in DR and PD [41,42]. In particular, a dysregulation of the canonical wingless/integrated (Wnt) signaling pathway which leads to increased inflammation and oxidative stress, has been described in the pathogenesis of either DR and PD and contributes to the development of the diseases [30].

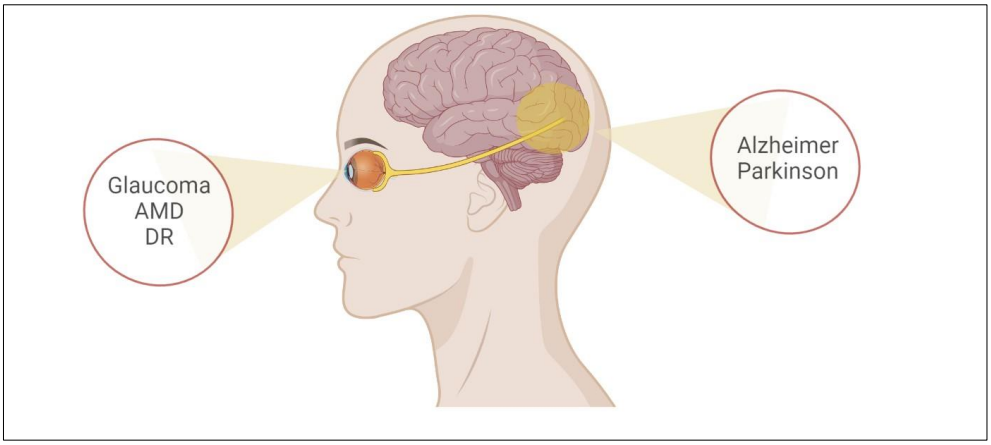

FIGURE 1: eye: window to the brain. Tight association between retinal and brain neurodegenerative diseases. AMD: age-related macular degeneration; DR: diabetic retinopathy.

RETINAL NEUROINFLAMMATION

Acute inflammation in the brain is primarily a defense mechanism which protects from infection, toxins, and injury. However, when the equilibrium between anti-inflammatory and pro-inflammatory mediators is unbalanced, chronic inflammation occurs [5]. Therefore, the term neuroinflammation refers to the chronic inflammatory processes in the CNS and represents a common feature of retinal and brain neurodegenerative diseases. Specifically, uncontrolled and substained inflammation is a common pathological mechanism, caused by the chronic activation of the immune system, that then leads to neuronal damage. The chronic activation of immune response is mediated by immune-competent cells. In particular, resident glial cells, as

well as microglia, astrocytes and Müller cells are involved in the immune surveillance and mediate the inflammatory response. Because of a damage, glial cells become reactive, produce cytokines and chemokines and recruite blood-derived immune cells, amplifying and sustaining the inflammatory response [43]. Moreover, the damage-activated immune response is also mediated by the activation of inflammosomes, which play a detrimental role in the pathogenesis of neurodegenerative diseases [44]. Neuroinflammation contributes to neuronal damage and death, prompting the diseases progression; accordingly, monitoring neuroinflammation is a rising strategy to block neuronal damage in the retina and CNS diseases.

Retinal microglial cells

Microglia-mediated neuroinflammation is involved in several retinal degenerative diseases, however it's unclear whether microglia reactivity is the cause of neurodegeneration or a direct consequence. Indeed, microglial cells own a pivotal role in the activation of the inflammatory response but overactivation of these cells leads to an excessive production of inflammatory mediators which can damage neurons, resulting in retinal neurodegeneration [45]. Microglial cells represent the mononuclear phagocyte population in the CNS and retina and are involved in immune defence [46] and neuronal homeostasis [47]. In physiological condition, microglia are in a "resting" or inactive state, morphologically characterized by ramifications and small somas. In this state, they act as motile cells in order to constantly supervise their microenviroment and clear metabolic products and tissue debris through phagocytosis [48]. Additionally, microglial cells communicate with neurons and other glial cells, which control their activation status and the phagocytosis of cellular debris [49]. Specifically, soluble factors, as well as nucleotides

[50], transforming growth factor-beta (TGF-β) [51], NGF [52] and the C-X3-C motif chemokine ligand 1 (CX3CL1) [53], released from neurons, astrocytes and retinal pigment epithelium, constitute important regulatory signals involved in the communication between microglia, neurons and other glial cells and in the regulation of retinal homeostasis. Moreover, even direct contacts between neurons and microglia, as well as the interactions between the cluster of differentiation-200 (CD200), expressed on the neurons surface and the CD200 receptor (CD200R), expressed on the microglial cells surface, regulate microglia activation [54]. In response to modifications in the neuronal microenvironment, microglial cells are activated and shift to a more reactive phenotype, characterized by changes in cells morphology (amoeboid cell shape), expression and release of inflammatory factors and chemokines, that in turn promote immune cells recruitment and migration [55,56]. In particular, microglia can assume a neurotoxic phenotype, characterized by the production of proinflammatory factors as well as IL-1β, TNF-α, and IL-6 and cytotoxic mediators like ROS, or can be polarized into a neuroprotective and anti-inflammatory phenotype, in which cells release trophic and protective factors, prompting anti-inflammatory responses [56]. Indeed, neurotrophic factors released by microglia, as well as BDNF, ciliary neurotrophic factor (CNTF), GDNF, NGF, neurotrophin-3 (NT3), and basic fibroblast growth factor (bFGF) contributes to neuronal physiology and survival and they also have a key role in regulation and protection of photoreceptor survival [57,58]. Hence, initially the balanced activity of microglia between pro- and anti-inflammatory states, contributes to neuronal protection and tissue regeneration; indeed, microglia prompt repair mechanisms as well as glial scar formation [59]. However, protracted microglia activation leads to chronic inflammation, and the prolonged release of proinflammatory mediators enhances neuroinflammation, further activating microglia, which release

neurotoxic and proinflammatory factors, in turn contributing to neuronal dysfunction [45]. Noteworthy, in different animal models of eye diseases, microglial activation often anticipates retinal degeneration and photoreceptor apoptosis [60,61]. Microglia establish connections with Müller cells, which constitute the predominant glia in the vertebrate retina. The functional interaction between microglial cells and Müller cells may be bidirectional and it is important to regulate photoreceptor cells survival in retinal degeneration. Indeed, photoreceptors degeneration stimulates the microglia migration from the inner to the outer retina and modifies the production of trophic factors which control survival of photoreceptor cells. Moreover, microglia-derived factors alter the production of secondary trophic factors in Müller cells, which can enhance photoreceptor rescue or induce photoreceptor apoptosis. Therefore, to prevent neurodegenerative diseases, strategies which strengthen survival pathways or impair apoptotic pathways can be worthwhile [62]. Neuroinflammation plays a leading role in the pathogenesis of glaucoma [63] and increased evidence suggests its involvement either in the onset or progression of the disease. Noteworthy, blocking proinflammatory pathways may deliver neuroprotective effects. Indeed, increased levels of inflammatory cytokines, as tumor necrosis factor (TNF) [64,65], interleukin (IL)-6 [63,66,67], IL-1β[63] and nitric oxide (NO) [68], have been observed in the retina of experimental animal models of glaucoma and in the aqueous humor of glaucoma patients. Noteworthy, microglial cells are strictly involved in the glaucoma inflammatory environment; specifically, microglia showed a reactive phenotype in experimental glaucoma models, in the early stage of the disease, contributing to its onset and progression [69,70]. In particular, Lazzara et al., demonstrated a prominent retinal microglial activation in glaucomatous DBA/2J mice retina, evaluated through the immunohistochemistry quantification of ionized calcium binding adaptor

molecule 1 (IBA-1) [63], marker of microglia activation [71]. Moreover, as early changes observed in animal models of glaucoma, microglia become reactive and redistribute in the retina, optic nerve and optic tract, in correlation with axonal damage [72]. The abnormal reactivity and distribution of microglia in animal model of RGCs degeneration, as retinal ischemia [73], suggests that microglia are activated by degeneration of RGCs. However, these cells can contribute directly to the loss of RGCs in glaucoma, as evidenced by the recruitment and activation of microglia cells prior to RGCs death [70]. As regard as AMD, the immune system and inflammatory responses are strictly involved in its development and progression [74]. Indeed, plasma levels of soluble TNF receptor II are increased in AMD patients [75]; moreover, intracellular adhesion molecule 1 (ICAM-1) and IL-6 were found overexpressed in an *in vivo* model of choroidal neurovascularization, and were prevented by administration of an anti-inflammatory carotenoid, highiliting the contribution of inflammatory reponse in AMD [76]. The inflammatory cytokines IL-1β, IL-6 and TNF-α were also found increased in an *in vitro* paradigm of AMD and they were counteracted by vitamin D_3 and meso-zeaxanthin, which own anti-inflammatory properties [77]. Recruitment of microglia is a detrimental factor for AMD progression and severity; indeed, accumulation of pro-inflammatory microglia cells in the subretina has been found in a mouse model of AMD [78]. Moreover, during the initial stages of the disease, drusen deposits attract macrophages [79]. In AMD patients, activated microglial cells have been found in outer nuclear layer (ONL) and this has been linked with photoreceptor degeneration [80]. Another evidence concerning the contribution of microglia to AMD is the correlation between polymorphisms in the C-X3-C motif chemokine receptor 1 (CX3CR1) gene, which encodes the C-X3-C motif chemokine ligand 1 (CX3CL1) receptor, and the disease

pathogenesis [81]. Indeed, it has been showed that CX3CR1 mutations are involved in the recruitment of monocytes/microglia into the subretinal space of AMD patients [82], displaying a role in the development of both dry and wet AMD [83,84]. The inappropriate activation of the complement system is an additional prominent factor which drives and excacerbates degeneration in age-related conditions, as AMD [85]. Specifically, accumulation of the bisretinoid N-retinylidene-N-retinylethanolamine (A2E) in retinal microglia leads to an immune dysregulation which contributes to AMD progression [86]. Chronic inflammation characterizes even DR, likewise the other retinal neurodegerative diseases [87]. Elevated levels of proinflammatory cytokines and chemokines and microglia reactivity are key hallmarks in DR. Indeed, DR patients displayed increased vitreous levels of proinflammatory cytokines, as well as TNF-α, IL-1β and IL-6 [88–90]; this inflammatory response is correlated with functional alterations, as blood retinal barrier (BRB) breakdown, increased endothelial cell permeability and apoptosis [91–93]. Moreover, activated pro-inflammatory microglial cells have been identified in the retina of DR patients [94] and in animal models of DR [95], before electroretinographic alterations [96], suggesting that microglia reactivity arises early in DR pathology. Despite the role of microglia reactivity and the molecular link with the progression of the disease are not clearly understood in DR, pharmacological treatments aimed to regulate microglia activity have been investigated in this pathology [97,98] and can be considered as a promising strategy in DR management.

Retinal macroglia activation and reactive gliosis

Retinal macroglia activation and reactive gliosis are important hallmarks of many CNS and retinal neurodenerative diseases. In the retina, there are two types of macroglia, astrocytes and Müller cells. Astrocytes, in most mammals, are mainly located in the nerve fiber layer (NFL) and in ganglion cell layer (GCL) and exert structural support to the retina [99]. Müller cells represent radially oriented cells, which span the entire retinal depth, from the outer limiting membrane (OLM) to the inner limiting membrane (ILM) [99], creating an anatomical link between the subretinal space, blood vessels, vitreous body and the retinal neurons [100]. Indeed astrocytes, together with Müller cells, integrate the vascular and neuronal activity of the retina [101], through regulation of ionic balance, neurotransmission and synaptic plasticity [102]. Astrocytes defend the retina through a process called *reactive gliosis*. Reactive gliosis represents a complex defense mechanism, triggered in response to polyetiological insults [99], such as ischemic damage, trauma, neurodegeneration or neuroinflammation. Primarly, this process aims to restore retinal homeostasis and to limit tissue damage, showing a direct neuroprotective effect on the retina. Nevertheless, dysfunctional and chronic gliosis can become detrimental since it inhibits regenerative response and facilitates the disease progression [103]. Indeed, astrocytes become reactive in response to injurious stimuli, such as elevated intraocular pressure, excitotoxicity or retinal ischemia [104,105], bursting in number (hyperplasia/proliferation), manifesting enlarged soma (hypertrophy) with increased number and length of processes; therefore, they migrate to the damage site in order to remove dying cells and cellular debris [99]. In addition, astrocytes upregulate glial fibrillary acidic protein (GFAP), which is a hallmark of astrocyte activation in neurodegenerative disorders [106]. Astrocytes can be pro-inflammatory and neuroprotective, similarly to

microglia cells. The pro-inflammatory reactive astrocytes induce pro-inflammatory mediators, as well as IL-1β, IL-6, TNF-α, and NO and promote RGCs death [105,107]; moreover, they upregulate the expression of genes involved in the complement cascade. Noteworthy, astrocytes act synergistically with microglia. Actually, inflammatory mediators, released by pro-inflammatory microglia, can induce a secondary inflammatory response, activating pro-inflammatory astrocytes [108]. Moreover, inflammatory mediators produced by astrocytes, may exacerbate chronic microglial activation, facilitating neuronal death [109]. Whereas the neuroprotective reactive astrocytes upregulate neurotrophic factors to promote neurons survival, and release the anti-inflammatory cytokines IL-4, IL-10, and also TGF-β [110]. Reactive gliosis has been described in several retinal degenerative pathologies and exacerbates the diseases progression. Indeed, in AMD, activated and hypertrophic astrocytes have been found [99,111], as well as increased expression of GFAP in *in vivo* models of DR and glaucoma [63,112,113]. Moreover, it has been demonstrated that reactive astroglia increases vascular permeability and neovascularization in AMD and DR, through vascular endothelial growth factor (VEGF) [114]. In DR, the astrocytes release of inflammatory cytokines induced by hyperglicemia, led to activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*κ*B) pathway and enhanced oxidative stress [115]. As regard as Müller cells, gliosis is characterized by increased expression of GFAP and by the activation of the extracellular signal-regulated kinases (ERKs) [100] and owns both neuroprotective and toxic effects on retinal neurons [116]. In particular during early stage of retinal injury, Müller cells support neurons survival and restrict tissue damage, through release of neurotrophic and antioxidant factors; and this is described as conservative or non proliferative gliosis [117]. Indeed, in DR, Müller glia activation sustains neuroprotection

by releasing angiogenic and neurotrophic factors, through an ERK1/2-dependent mechanism [118]. On the contrary, sustained Müller cells gliosis is detrimental to the retina and leads to neuronal death [100]. Interestingly, a possible event involved in the transition from conservative to uncontrolled gliosis, is the breackdown of BRB [119]. Moreover, Müller cells are activated by immune system cells and express TNF-α, ILs, interferon, and ICAM-1 [117]. Other retinal diseases are associated to gliosis of Müller cells, such as glaucoma and AMD. In fact, in an experimental model of glaucoma, expression of GFAP in Müller cells has been observed [120]. While in AMD pathology, Müller cells GFAP overexpression seems to be associated with disruption of BRB and RPE cells [121].

NLRP3 inflammasome

The nucleotide-binding leucine-rich repeat containing receptor 3 (NLRP3) inflammasome is a component of the innate immune system, which activation contributes to the pathogenesis of retinal degenerative diseases, expecially in chronic age-related eye diseases (AREDs), such as AMD, DR and glaucoma. In inflammatory pathologies, the activation of inflammasome is an early event, which constantly persists through the late stages of the disease, amplifying and perpetuating itself, leading to chronic inflammation and cell death. The process defined "inflammaging" highlights the rigorous connection between aging and the inability to control systemic inflammation, in which NLRP3 inflammasome is centrally involved [122]. Noteworthy, during aging, physiological alterations in the immune system occur, indicated as immunosenescense, leading to its decreased efficacy and its inability at preserving immune homeostasis [123]. NLRP3 inflammasome belongs to the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

cytosolic proteins family, which responds to infective and non infective antigenic stimuli. Among all, NLRP3 is the most studied and characterized in the eye and its activation, with few exceptions, consists of two-step process. In particular, in the first priming step, NLRP3 protein levels are increased and the NF-κB pathway is activated. Indeed, NF-κB translocates into the nucleus and stimulates the transcription of NLRP3 itself, as well as of the inactive pro-inflammatory cytokines pro-IL-1β and pro-IL-18. NF-κB activation can be induced by pattern-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and their interaction with pattern recognition receptors such as toll-like receptors (TLRs) [124]. For example, in glaucoma, the high mobility group box 1 (HMGB1) protein can act as a DAMP, activating the inflammasome [125], as well as ROS and amyloid beta [126,127]. Whereas, in AMD, drusen components, ROS, complement proteins and also nucleic acid, have been described as inflammasome activators [128,129], as well as ROS and adenosine triphosphate (ATP) in DR pathology [130,131]. Moreover, the interaction of proinflammatory cytokines such as TNF-α and IL-1β with their specific receptors, can activate NF-κB [132]. In the second step, the inflammasome complex formation requires an activation signal, which triggers the complexing of the NLRP3 inflammasome. Therefore, behind the activation stimuli, NLRP3 constitutes a multimeric inflammasome complex, recruiting apoptosis-associated speck-like protein (ASC) and procaspase-1, which in turn leads to the autolytic cleavage of procaspase-1 to its active form and then, to the cleavage and activation of IL-1β and IL-18. Specifically, ATP (through the activation of purinergic P2X7 receptor), potassium efflux and calcium signaling, ROS, mitochondrial dysfunction and lysosomal rupture have been reported as key inflammasome activators [133]. As regards as AMD, the lipofuscin fluorophore A2E activates the NLRP3 inflammasome pathway in retinal

pigment epithelial cells, which release inflammatory chemokines and cytokines [134]. Moreover, it is well-known that extracellular ATP is strictly linked with CNS neurodegeneration [135], as well as purinergic signaling is tightly associated with retinal degeneration [136]. Noteworthy, in glaucoma, DR and AMD, blocking the purinergic P2X7 receptors has been described as potential therapeutic treatment [137–139]. Incidentally, targeting P2X7 receptors in the eye prevents IL-1β and IL-18 release, through blocking the inflammasome pathway [140]. Therefore, the activation of P2X7R-NLRP3 pathway in retinal microglial cells induces inflammatory cytokines release, which promotes RGCs death [141]. Indeed, therapeutics which target NLRP3 signaling pathways directly or indirectly offer prominent hope for future treatments of chronic eye diseases, associated with aging.

NEUROTROPHINS

Deprivation of neurotrophins is crucially involved in the pathophysiology of CNS and even in retinal diseases associated with neuroinflammation and neurodegeneration. Indeed, optic neuroprotective strategies aim to preserve and rescue retinal neurons and the axonal projections. BDNF, as well as NGF or neurotrophin-3/4/5 (NT-3/4/5) play a key role in the RGCs development, differentiation and survival [142,143]. Accordingly, neurotrophins can be used directly (through exogenous administration) or indirectly (through compounds that enhance neurotrophins expression) to slow down vision loss [144–146]. In particular, among the neurotrophins, BDNF is prominently involved in glaucoma pathology and low serum levels of this neurotrophic factor were found in the early stage of glaucoma [147,148]. Indeed, BDNF through the activation of the specific tropomyosin-related kinase B (TrkB) receptor, induces downstream signaling cascades which preserve RGCs

survival by inhibiting apoptotic pathways [149]. Moreover, BDNF regulates synaptic plasticity [150], and it has been found decreased in the hippocampus of type 1 and type 2 diabetic rats [151,152]; as well as low serum BDNF levels have been associated with cognitive deficits in diabetic patients [153]. In diabetic mice, BDNF attenuated the hyperglicemia-induced neuroinflammation in the hippocampus, by blocking the increase in HMGB1 levels, as well as the inflammatory factors TNF-α and IL-6. Moreover, BNDF reversed the increased protein levels of p-NFκB and the hyperglicemia-induced microglial activation [154]. Noteworthy, BDNF levels were found decreased in serum, as well as in the aqueous humor of diabetic patients, before the clinical manifestation of DR, suggesting that this neurotrophic factor could be a marker for the early stages of DR [155]. Regarding AMD, low BDNF levels in the aqueous humor of AMD patients have been found, correlated with a thinning of the ONL, suggesting an insufficient protection of photoreceptors [156]. Moreover, the reduction of BDNF expression and its function might be one of the mechanisms which explains the negative impact of pro-inflammatory cytokines on neuroplasticity, creating a link between neuroinflammation and neuronal damage [157].

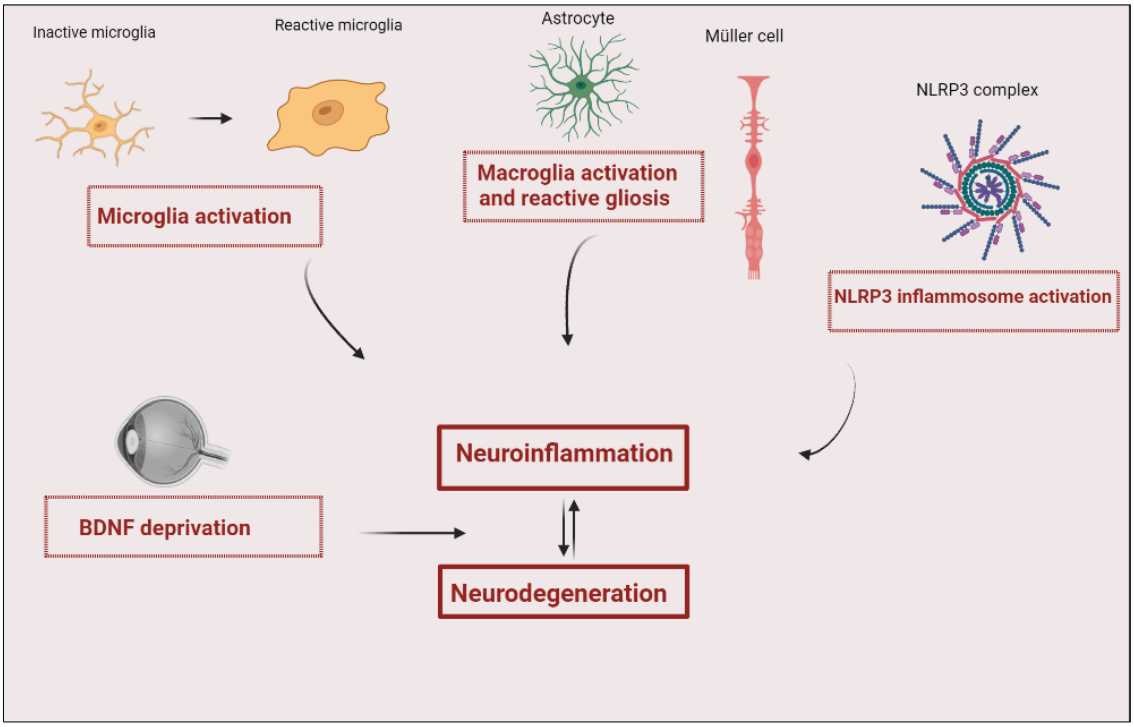

FIGURE 2: Detrimental factors involved in retinal neuroinflammation and neurodegeneration.

IN VITRO MODELS OF RETINAL INFLAMMATION/NEURODEGENERATION

In vitro models of retinal diseases are useful to identify and validate potential new signaling pathways involved in the disease initiation and progression, as well as to screen pre-clinically new therapies or to perform toxicology and permeability studies [158]. *In vitro* cell-based models, expecially those based on animal and human immortalized cell lines, are relatively cheap, reproducible, easy and quick to set up [159]. Neuroinflammation represents a common feature in retinal degenerative diseases pathophysiology which contributes to neurodegeneration; indeed, utilize an *in vitro* model which reproduces inflammatory environment can be useful to study these retinal diseases and to test new potential neuroprotective drugs. RPE is one cell layer in thickness, interposed between the choroid and the neural retina. RPE preserves the structure and the physiological activity of the close tissues and it is crucial for vision function [160]. Indeed, RPE cells usually release neurotrophic factors, such as BDNF, which is a crucial factor for survival and function of RGCs and photoreceptors. Moreover, in normal physiological conditions, RPE cells preserve the neural retina by secreting and balancing the levels of different growth factors and cytokines. In addition, RPE constitutes the outer BRB (oBRB), regulating nutrients, ions and water between the neural retina and the choriocapillaris, in order to maintain the retinal homeostasis [161]. Accordingly, protection of RPE is a strategic approach to prevent the impairment of retinal degenerative diseases. A well-established and widely used *in vitro* model of retinal inflammation/degeneration [162–165] is represented by lipopolysaccharide (LPS)-mediated TLR-4 activation, in the immortalized human retinal pigment epithelial cells (ARPE-19). LPS leads to the activation of NF-κB signaling pathway, with the subsequently overexpression of inflammatory cytokines,

such as IL-6, IL-1β, or TNF-α. Retinal inflammation/degeneration in RPE cells can be induced directly even by treatment with inflammatory cytokines, like TNF- α or IL-1β or by the challenge with Aβ (1–42) oligomers [77,166]. Moreover, RPE cells can be exposed to hydrogen peroxyde (H_2O_2) in order to induce retinal oxidative stress, which is strictly associated with retinal inflammation and neurodegeneration [77]. Along with RPE cells-based *in vitro* models, human immortalized Müller cells, as Moorfields/Institute of Ophthalmology-Müller 1 (MIO-M1), represent useful tools to study retinal physiological and pathological events under different stress conditions, as well as to test pharmacological tools. Retinal hypoxia and oxidative stress are both causative and/or consequential factors in retinal degenerative diseases [167,168], contributing to retinal inflammation and neurodegeneration [169]. Accordingly, Müller cells can be exposed to hypoxic insult through cobalt chloride ($CoCl_2$) treatment (chemical hypoxia) or through low percentage of O_2 (with hypoxic chambers) [170,171]. This challenge induces the expression of the transcription factor hypoxia-inducible factor 1 subunit alpha (HIF-1α) [172], which leads to the transcriptional induction of many genes, involved in oxygen homeostasis. In addition, Müller cells can be stimulated with other types of insult, as well as H_20_2, to mimic oxidative stress environment [173] or with Aβ (1–42) oligomers to induce neurotoxicity [174], depicting ulterior crucial *in vitro* models of retinal degeneration.

IN VIVO MODELS OF RETINAL INFLAMMATION/NEURODEGENERATION

In vivo animal models of retinal degeneration are salient to investigate the pathophysiology of retinal degenerative diseases and to study the potential effect of neuroprotective compounds, since they represent the biological complexity of integrated systems. Retinal ischemia-reperfusion (I/R) animal model is a well-established paradigm of retinal injury and inflammation, described in many rodent species [175–178]. Retinal I/R damage is performed by cannulating the eye anterior chamber with a needle, using a saline reservoir placed at a specific height, in order to raise IOP above systolic arterial blood pressure, leading to a temporary arrest of the inner retinal blood flow (ischemia). This ischemia phase produces a condition of retinal hypersensitivity to oxygen and to other nutrients. By removing the cannula, the circulation is subsequently restored (reperfusion) and prominent oxidative and inflammatory injury occurs, resulting in retinal neurodegeneration [179]. Retinal I/R is a pathological feature of different retinal diseases like glaucoma and even DR and AMD [180,181]; indeed, this model is a valuable tool to investigate the neuronal pathogenesis, as well as therapy, in eye degenerative diseases. Another well-established model used to elicit retinal cells damage is the Ammino-3-idrossi-5-Metil-4-isossazol-Propionic Acid (AMPA)-induced excitotoxicity in rat retina, performed by intravitreal injection of this excitatory amino acid. AMPA activates the non-N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors, which overactivation leads to retinal toxicity, affecting mainly retinal horizontal and amacrine cells [182,183]. Furthermore, retinal neurotoxicity can be induced in rats even by intravitreal administration of NMDA [184], which causes the overactivation of NMDA ionotropic receptors, prompting a cascade of events leading to RGCs death [181]. Moreover, intravitreal administration of Aβ (1-42)

oligomers in rats or in mice [185,186] represents a widely used *in vivo* model of inflammatory/neurodegenerative damage. Therefore, together with these disease non-specific *in vivo* paradigms, there are even models which resemble specific retinal pathologies. For example, the DBA/2J mouse strain is a well-established model of spontaneous glaucoma, characterized by progressive RGCs loss and optic disc excavation, which are the hallmarks of glaucoma [187]. Moreover, in DBA/2J mice, inflammatory response and immune dysfunction occur, evidenced by the increase of pro-inflammatory citokines, as well as microglia and glial activation [63]. The mouse model of laser-induced choroidal neovascularization (CNV) has been widely used to mimic the wet form of AMD. This model consists in the laser-induced rupture of Bruch's membrane, leading to new vessels growth from the choroid to the subretinal space, a hallmark of neovascular AMD, together with retinal inflammation [188]. Several *in vivo* models for the dry form of AMD have been developed, which mimic the early features of the disease. For example, one of them is represented by the *Alu* transposons RNA accumulation, due to DICER 1 (ribonuclease III) deficiency [189], which activates the NLRP3 inflammasome. As regard as DR, several animal paradigms have been employed; among the induced model, the most used is the streptozotocin (STZ)-induced diabetes, in rats or in mice, obtained by the disruption of pancreatic islets of Langerhans and loss of β cells, and subsequent hyperglicemia. DR STZ- phenotypes comprises retinal gliosis, RGCs loss, thinning of the retinal inner nuclear layer (INL) and ONL, neovascularization and loss of perycites [190]. Moreover, in STZ model, retinal inflammation, oxidative stress damage and retinal microglia activation occur [191]. Another paradigm of DR is the $Ins2^{Akita}$ genetic mice model. This strain contains a mutation in the insulin 2 gene, which leads to β-cell death. Retinal complications include increased vascular permeability, reactive gliosis,

decreased number of RGCs, cholinergic and dopaminergic amacrine cells, as well as inflammation [190].

NEUROPROTECTANTS

Neuroprotective compounds aimed to treat retinal degenerative diseases have been investigated in several *in vivo* and *in vitro* models, and even though they have shown preclinically efficacy, none of them have been translated to the clinical use. This failure could be related to different issues: (i) low bioavailability of the drugs candidates, (ii) late diagnosis of retinal degenerative diseases, (iii) clinical trials design and improper clinical endpoints chosen for the efficacy evaluation. All these issues contribute to low success rate, and high costs of clinical trials for research and development (R&D) in the field of neurosciences. An example of lack of functional endpoints in clinical trials is the memantine study for glaucoma treatment. Memantine, an NMDA-receptor antagonist, did not show significant difference between the placebo and the drug patients, resulting in clinical trial failure [192,193]. Therefore, new therapeutic treatments focused on neuroprotection and disease-modifying therapies are needed, as well as new strategies aimed at translating the potential drugs into clinical practice. Noteworthy, repurposing of already approved drugs is an attractive approach in drug discovery and development, since it leads to a reduced costs and timelines of drugs development.

Brimonidine: potential candidate for retinal degenerative diseases

Brimonidine is an α2A-adrenergic receptor agonist, approved for lowering intraocular pressure (IOP) in patients with open-angle glaucoma, working mechanically through a reduction of aqueous production and enhancement of uveoscleral outflow [194,195]. However, different preclinical studies have demonstrated that brimonidine possesses an IOP-independent neuroprotective action in the retina [196]. Specifically, Lambert et al, showed the brimonidine protective effect against axonal and somatic degeneration of RGCs, in rats with elevated IOP [197]. Moreover, brimonidine decreased RGCs apoptosis through reduction of Aβ production and its precursor APP, in a rat model of Aβ-induced neurotoxicity [198]. In addition, this compound blocked oxidative stress induced by glutamate excitotoxicity in a rat model of transient ischemia [199] and inhibited VEGF expression and BRB brackdown in diabetic rats, suggesting a role for the treatment of ocular pathologies with BRB impairment [200]. Nevertheless, despite the preclinical potential of brimonidine, clinical trials have failed to translate these results into the clinical practice. Moreover, although α2A receptors have been identified in the RGCs [201], the mechanisms by which α2A agonists exert neuroprotection are not well-established. Multiple pathways have been linked with brimonidine mediated neuroprotection, including the direct or indirect modulation of neurotrophic factors, as well as BDNF [202,203]. Considering the reported evidence, brimodine could be employed in the treatment of the eye neurodegenerative disease, as potential neuroprotective agent.

Caffeine: retinal neuroprotection mediated by anti-inflammatory and antioxidant action

Caffeine, a methylxanthine derivative, is a non-selective adenosine receptor (AR) antagonist, with a high affinity for the adenosine A_1 receptor (A_1R) and the adenosine A_{2A} receptor ($A_{2A}R$). Moreover, caffeine is a non-selective inhibitor of phosphodiesterases (PDEs) [204]. This compound represents the most consumed CNS stimulants [205], mainly through consumption of coffee, tea, and soft drinks enriched with caffeine. Indeed, in the adult population, its daily average assumption is within 100–400 mg per capita [205,206]. Caffeine is already clinically approved as therapy in preterm infants [207]. Actually, it has shown protective effect in CNS neurodegenerative diseases [208,209], as well as in several chronic pathologies, including diabetes [210]. Neuroprotective action of caffeine has been associated with its well-known anti-inflammatory and antioxidant properties in different systems. Specifically, Madeira et al., showed that caffeine administration attenuates loss of RGCs and prevents retinal microglia activation and neuroinflammatory response, in an *in vivo* model of glaucoma [211], as well as in a transient retinal ischemic model [212]. Therefore, caffeine inhibited LPS-induced oxidative stress and neuroinflammation in a mouse model of neurodegeneration and synaptic impairment [213]. The anti-inflammatory effect of caffeine has been shown even *in vitro*, in retinal microglia and monocyte/macrophage-like cells challenged with LPS [214,215]. Caffeine neuroprotective effect seems to be linked with modulation of BDNF [216,217] and with the blockade of $A_{2A}R$ [209,212]. Indeed, the antagonism of this receptor has been strongly associated with attenuation of microglia-mediated neuroinflammation in different degenerative brain and retinal conditions [218]; accordingly, $A_{2A}R$ is a significant drug target in CNS, as well as in the retina, since its widely

expression in the eye tissues [219–221]. Indeed, caffeine represents a promising candidate for treatment of retinal degenerative diseases and further pre-clinical and clinical studies are needed to explore it.

Chapter I

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Brimonidine is Neuroprotective in Animal Paradigm of Retinal Ganglion Cell Damage

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Abstract

To investigate the neuroprotective effect of brimonidine after retinal ischemia damage on mouse eye. Glaucoma is an optic neuropathy characterized by retinal ganglion cells (RGCs) death, irreversible peripheral and central visual field loss, and high intraocular pressure. Ischemia reperfusion (I/R) injury model was used in C57BL/6J mice to mimic conditions of glaucomatous neurodegeneration. Mouse eyes were treated topically with brimonidine and pattern electroretinogram were used to assess the retinal ganglion cells (RGCs) function. A wide range of inflammatory markers, as well as anti-inflammatory and neurotrophic molecules, were investigated to figure out the potential protective effects of brimonidine in mouse retina. In particular, brain-derived neurotrophic factor (BDNF), IL-6, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its death receptor DR-5, TNF-α, GFAP, Iba-1, NOS, IL-1β and IL-10 were assessed in mouse retina that underwent to I/R insult with or without brimonidine treatment. Brimonidine provided remarkable RGCs protection in our paradigm. PERG amplitude values were significantly ($p < 0.05$) higher in brimonidine-treated eyes in comparison to I/R retinas. Retinal BDNF mRNA levels in the I/R group dropped significantly ($p < 0.05$) compared to the control group (normal mice); brimonidine treatment counteracted the downregulation of retinal BDNF mRNA in I/R eyes.

Retinal inflammatory markers increased significantly ($p < 0.05$) in the I/R group and brimonidine treatment was able to revert that. The anti-inflammatory IL-10 decreased significantly ($p < 0.05$) after retinal I/R insult and increased significantly ($p < 0.05$) in the group treated with brimonidine. In conclusion, brimonidine was effective in preventing loss of function of RGCs and in regulating inflammatory biomarkers elicited by retinal I/R injury.

Keywords: neuroprotection, retinal ganglion cells, ischemia-reperfusion, brimonidine, PERG

Introduction

Glaucoma is an optic neuropathy characterized by retinal ganglion cells (RGCs) death, irreversible peripheral and central visual field loss and high IOP (Bucolo and Drago, 2011). Currently, six main classes of topical drugs are available; they include beta-blockers, carbonic anhydrase inhibitors, prostaglandin derivatives, sympathomimetics, miotics, and Rho-kinase inhibitors. For neovascular glaucoma the therapeutic approach could be different, on this regards it is worth of note that anti-VEGF agents, used in clinical practice, such as ranibizumab, bevacizumab and aflibercept are considerably different in terms of molecular interactions when they bind with VEGF (Platania et al., 2015). Brimonidine is an α2A-adrenergic receptor agonist, approved for lowering intraocular pressure (IOP) in patients with open-angle glaucoma. Although α2A receptors have been identified in the RGCs, the mechanisms by which α2A agonists exert neuroprotection are not well-established. There are many controversial studies on brimonidine and its effects to preserve retinal tissue. Some non-clinical findings have demonstrated that brimonidine possess retinal protective action (Lambert et al., 2011; Nizari et al., 2016; Marangoz et al., 2018). However, to date, clinical trials have failed to translate into similar efficacy in humans. Recently, a Cochrane systematic review (Sena and Lindsley, 2017) showed that although one clinical trial found less visual field loss in the brimonidine-treated group, the evidence was of such low certainty that it is not possible draw conclusions from this only finding. Incidentally, the authors concluded that further clinical research is needed to determine whether brimonidine may be beneficial for individuals with glaucoma. More recently, a systematic review and meta-analysis concluded that the clinical evidence of neuroprotective effect of brimonidine is inconclusive and needs stronger support maybe with large double-blind randomized clinical trials (Scuteri et al., 2020). To shed light on these controversial studies we aimed to investigate topical brimonidine on a well-known in vivo paradigm of retinal damage. The neurodegenerative

process in several eye diseases is characterized by progressive death of RGCs, optic nerve degeneration, and sometime blindness (Chou et al., 2020). RGCs degeneration is often associated to ischemia in central retinal artery occlusion and ischemic optic neuropathies (Kunimi et al., 2019). Remarkable insights in therapy for retinal ischemia have arisen through the investigation of rodent models of ischemia-reperfusion. Retinal ischemia–reperfusion (I/R) is an experimental model that triggers an inflammatory process eliciting a large number of detrimental molecules such as TNF, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and ILs (Osborne et al., 2004; Wei et al., 2011; Dibas et al., 2018). Gliosis, another critical event contributing to glaucoma pathogenesis, is a hallmark of retinal degeneration. Retinal reactive glia cells increased glial fibrillary acidic protein (GFAP)-immunoreactivity and ionized calcium binding adaptor molecule 1 (Iba1). It is well known that injury-induced gliosis in the optic nerve head and retina promote the death of RGCs due to over-release of pro-inflammatory mediators (Ganesh and Chintala, 2011). TRAIL mediates different neuroinflammatory responses (Huang et al., 2011). TRAIL and its receptors were found up-regulated in brain ischemia-reperfusion (Cui et al., 2010). The unmet medical need in glaucoma is mainly related to disease progression (RGC death) despite IOP control. In fact, glaucoma progression could be related to neurotrophins deprivation; interestingly, low serum levels of BDNF and nerve growth factor (NGF) were associated to early moderate stages of glaucoma. It is worth of note that the potential therapeutic value of neurotrophins to manage glaucoma is important, however the main point that damper the development of these factors as eye drops is related to the drug delivery issues (Bucolo et al., 2018). On this regards it could be useful develop a biodegradable deliver system in order to sustain prolonged pharmacological levels of drug into the back of the eye (Conti et al., 1997) even though topical formulation is ideal. Aim of the present study was to investigate the neuroprotective effects of brimonidine eye drops in a mouse model of retinal I/R damage. Pattern electroretinogram (PERG) analysis, the most specific non-invasive technique for electrophysiological assessment of RGCs activity, was used to evaluate the in vivo protection of RCGs function. Further, the retinal inflammatory profile after I/R insult with or without brimonidine treatment was investigated.

Material and methods

Animals

Male C57BL6/J mice (Charles River Laboratories, Italy) were housed in a temperature-controlled environment with free access to food and water during a 12-h light–dark cycle. All animals were treated according to the Principles for the Care and Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology. University of Catania (Italy) Ethics Committee approval #343.

Ischemic-Reperfusion Retina Damage

Retinal ischemia/reperfusion has been used as a model of retinal injury and has been described in many rodent species (Osborne et al., 2004; Gustavsson et al., 2008; Ulbrich et al., 2017; Stankowska et al., 2019). A validated modified I/R model (Hartsock et al., 2016) (Hartsock et al., 2016) was used in the present study. Mice were anesthetized by intraperitoneal injection with tiletamine + zolazepam (60 mg/kg) and medetomidine (40 μg/kg) plus a topical instillation of 0.4% oxybuprocaine (Novesina®, Laboratoires Thea, Clermont-Ferrand, France). The animals were placed on a heating pad to prevent hypothermia during the experiment. A 32-gauge needle, connected with a reservoir containing PBS, was introduced into the anterior chamber through the cornea to increase intraocular pressure (up to 90 mm Hg). Retinal ischemia was confirmed by an observation of blanching of the anterior segment and arteries in the eye. Following 60 min of ischemia, the needle was removed to allow rapid reperfusion. Ocular formulation of brimonidine tartrate (2 mg/ml) was instilled (10 μL) 60 min before I/R and after reperfusion, twice in 2 h. The effect of brimonidine was evaluated after 72 h from I/R insult. Mice were euthanized after 72 h from I/R insult, the eyes were enucleated, and the retinas collected.

Pattern Electroretinogram

As a sensitive measure of RGCs function we used the PERG (Chou et al., 2018). Anesthetized mice were transferred on a heating plate with the mouse superior incisor teeth hooked to a bite bar and the head gently restrained by two ear knobs. Body was kept at a constant temperature of 37°C using a feedback-controlled heating pad (TCAT-2LV, Physitemp Instruments, Inc., Clifton, NJ, United States). Two microliters of balanced salt solution (BSS) were topically applied to prevent corneal dryness. Simultaneous recordings of PERG response from both eyes were obtained using a common subcutaneous needle in the snout with a commercially available instrument (Jorvec Corp., Miami, FL, United States). Figure

1, panel B, shows the mouse PERG recording layout. Visual stimuli consisted of black-white horizontal bars generated on LED tablets and presented independently to each eye at 10 cm distance (56° vertical × 63° horizontal field; spatial frequency, 0.05 cycles/deg; 98% contrast; 800 cd/sqm mean luminance; left-eye reversal rate, 0.992 Hz; right-eye reversal rate, 0.984 Hz). Electrical signals recorded from the common snout electrode were averaged (>1,110 epochs), and PERG responses from each eye isolated by averaging at stimulus-specific synchrony. As previously described [17], PERG waveforms consisted of a positive wave (defined as P1) followed by a slower negative wave with a broad trough (defined as N2). Therefore, each waveform has been analyzed by measuring the peak-to-trough (P1-N2) amplitude defined as PERG amplitude and the time-to-peak of the P1 wave as PERG latency (Porciatti, 2015).

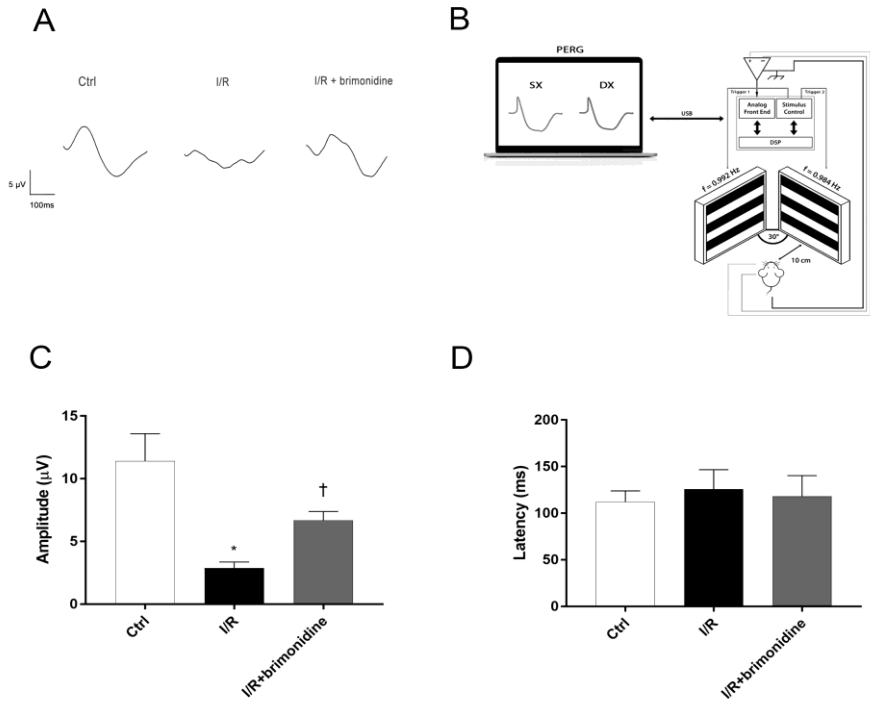

Figure 1. RGCs function assessment. (A) Representative PERG waveforms in C57BL6/J mice control, I/R and I/R plus brimonidine. (B) Mouse PERG recording layout. (C) Comparison between PERG amplitude values (µV) and latency values (D) of control, I/R and brimonidine treated mice. Brimonidine significantly counteracted RCGs loss of function induced by I/R injury, after 72 h, in mice retina. In each panel, bars represent the mean values

and corresponding standard errors (±SD). One-way ANOVA analysis was performed followed by the Tukey post-hoc test. *$p < 0.05$ vs. Ctrl; † $p < 0.05$ vs. I/R.

Ribonucleic Acid (RNA) Extraction and Complementary Deoxyribonucleic Acid (cDNA) Synthesis

Mice were sacrificed after 72 h from I/R and brimonidine treatment by cervical dislocation, eyes were enucleated, and retinas were isolated. The extraction of total RNA from mice retina samples was performed by using TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The A260/A280 ratio of the optical density of RNA samples (measured with Nanodrop spectrophotometer ND-1000, Thermofisher) was 1.95–2.01. cDNA was synthesized from 500 ng of RNA with a reverse transcription kit (SuperScript™ II Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Carlsbad, CA, United States).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed with the Rotor-Gene Q (Qiagen). The amplification reaction mix included Master Mix Qiagen (Qiagen QuantiNova SYBR Green Real Time-PCR Kit) and cDNA. For each sample, were made forty-five amplification cycles, in triplicate. Melting curve analysis confirmed the specificity of the amplified products. Results were analysed with the $2^{-\Delta\Delta Ct}$ method and expressed as fold change vs. control. Quantitative PCR experiments followed the MIQE guidelines. BDNF and IL-6 genes were analyzed by using specific primers purchased from Eurofin Genomics (Milan, Italy) and Qiagen (Milan, Italy) respectively. Gene expression levels were normalized with levels of a constitutively expressed gene (18S, Eurofin Genomics). Primer sequences are listed in Table 1.

Primer murine sequence/Catalogue number
Forward: 5'-GTTCCGACCATAAACGATGCC-3' Reverse: 5'-TGGTGGTGCCCTTCCGTCAAT-3'
Forward: 5'-GTTCGAGAGGTCTGACGACG-3' Reverse: 5'-AGTCCGCGTCCTTATGGTTT-3'
Cat. No. QT00098875

Table 1. Primers used for RT-PCR

Tissue Homogenization and Protein Extraction

Proteins were extracted from the retina samples with RIPA lysis buffer containing protease inhibitor cocktail, EDTA-free (Sigma, Inc.) by first sonicating for 20 s, and then centrifuging for 15 min at 14,000 rpm at 4°C. The supernatant was collected in new tubes and placed on ice. The protein concentration was measured using the Pierce™ Coomassie Protein Assay Kit (ThermoFisher, Monza, Italy).

Western Blot

Equal amounts of protein (30 µg) were resolved by 8–12% SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, United Kingdom). Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in phosphate-buffered saline plus 0.1% Tween 20 (PBS-T) and were then probed overnight with the following appropriate primary antibodies: rabbit anti-TRAIL (1:200, ab2435; Abcam, Cambridge, United Kingdom); rabbit anti-DR5 (1:500, ab8416; Abcam Cambridge, United Kingdom); mouse anti-GFAP (1:500, ab3670; Cell Signaling Technology, Inc., Danvers, MA, United States); rabbit anti-Iba1 (1:1000, PA5-27436; Thermo Fisher Scientific Italy, Rodano, Milan, Italy); rabbit anti-TNF-α antibody (1:1000, NB600-587; Novus Biologicals, Milan, Italy); rabbit anti-IL10 antibody (1:500, 250,713; Abbiotec, San Diego, CA, United States); rabbit NOS2 (1:250, sc-651; Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States); mouse anti-IL-1β (1:250, sc-52012; Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States). Then, the membranes were washed with PBS-T, and probed with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (GENA934, GNENA931; Amersham Life Science, Buckinghamshire, United Kingdom) for 1 h at RT. Beta-Tubulin (1:500, sc5274; Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) was used as control to validate the amount of protein loaded in the

gels. After washing with PBS-T, protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Milan, Italy) and scanned with the iBright FL1500 Imaging System (Thermo Fisher Scientific, Milan, Italy). Densitometric analysis of band intensity was done on immunoblots by using IMAGE J software (https://imagej.nih.gov/ij/).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software, version 8 (GraphPad Software, Inc., San Diego, CA, United States). PERG amplitude and latency were analyzed for significance with one-way ANOVA followed by Tukey test for multiple comparisons. For single comparisons, Student's t test was applied. p values ≤ 0.05 were considered statistically significant. Data are plotted as mean ± SD.

Results

Retinal Ganglion Cells Function was Ameliorated by Brimonidine Treatment

Figure 1 shows that 72 h after I/R, RGCs function, measured with PERG, was reduced by more than 50%. This effect was significantly attenuated by brimonidine treatment. Representative PERG waveforms recorded from the eyes in each group are shown in Figure 1A. PERG amplitudes of control group, I/R group, and I/R plus brimonidine group were compared as shown in Figure 1C. The average value of control PERG amplitude was 13.8 μV in agreement with previous studies on wild type mice (Romano et al., 2020). No significant changes were observed in terms of latency in all groups (Figure 1D) as expected considering the short time after the injury, whereas the average PERG amplitude of I/R mice was significantly ($p < 0.05$) reduced compared to the control retina. Worth of note, the average value of PERG amplitude of I/R brimonidine–treated mice, was significantly ($p < 0.05$) higher when compared with I/R, suggesting a protection of RGC function.

Neuroprotective and Anti-inflammatory Effect of Brimonidine in I/R-Injured Mice

I/R injury significantly ($p < 0.05$) downregulated the mRNA expression of BDNF in mice retina, while treatment with brimonidine maintained BDNF mRNA levels close to the control group values, with a significant difference ($p < 0.05$) compared to I/R group (Figure 2A). Furthermore, I/R insult elicited significant ($p < 0.05$) increase of IL-6 mRNA levels, that was significantly ($p < 0.05$) reduced by brimonidine treatment (Figure 2B). To better investigate the anti-inflammatory effect of brimonidine treatment on mice retina, we analyzed different

inflammatory mediators. In particular, we found that protein levels of TRAIL and its receptor DR-5, were significantly ($p < 0.05$) higher in I/R injured retina, while brimonidine treatment significantly ($p < 0.05$) reduced the expression of both proteins (Figure 3A). In consideration of the well-known involvement of retinal activated microglia, astrocytes and Muller glial cells in glaucoma, we assessed retinal Iba1 and GFAP expression, which were significantly ($p < 0.05$) increased after I/R injury (3-fold and 5-fold, respectively) compared to control (Figure 3B). The effect of brimonidine was demonstrated by the remarkable reduction of Iba1 and GFAP levels in the retinal tissue (Figure 3B). Furthermore, I/R insult significantly ($p < 0.05$) increased retinal levels of pro-inflammatory cytokines such as TNF-α, and reduced protein levels of IL-10, an anti-inflammatory molecule (Figure 3B). Protein levels of IL-1β and NOS2 were found significantly ($p < 0.05$) higher after I/R damage in comparison with control mice, and treatment with brimonidine significantly ($p < 0.05$) counteracted the expression of these proteins (Figure 3C).

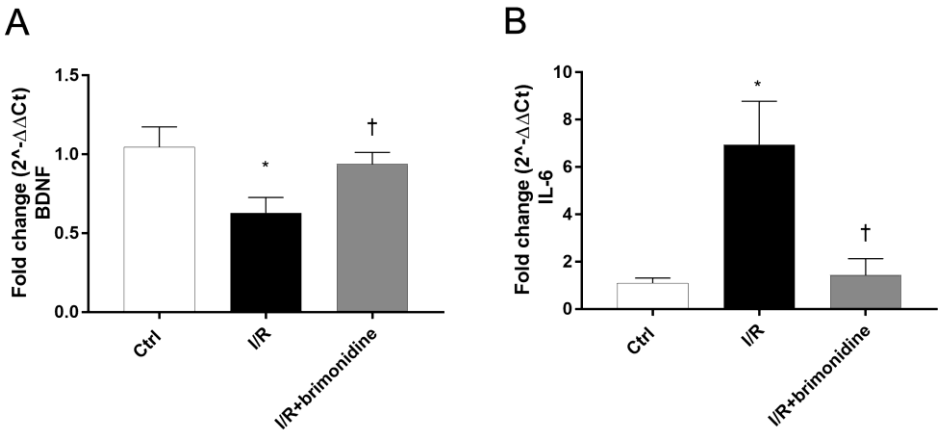

Figure 2. BDNF and IL-6 mRNA expression in mice retina. Brimonidine treatment maintained BDNF (A) mRNA levels close to control group, in comparison to I/R injured mice. Furthermore, brimonidine reverted the up-regulation of IL-6 (B) elicited by I/R injury. The mRNA levels were evaluated by RT-PCR; values represent the mRNA fold changes relative to 18 S used as housekeeping gene. Values are reported as a mean ± SD (n = 5). One-way ANOVA analysis was performed followed by the Tukey post-hoc test. *$p < 0.05$ vs. Ctrl; † $p < 0.05$ vs. I/R.

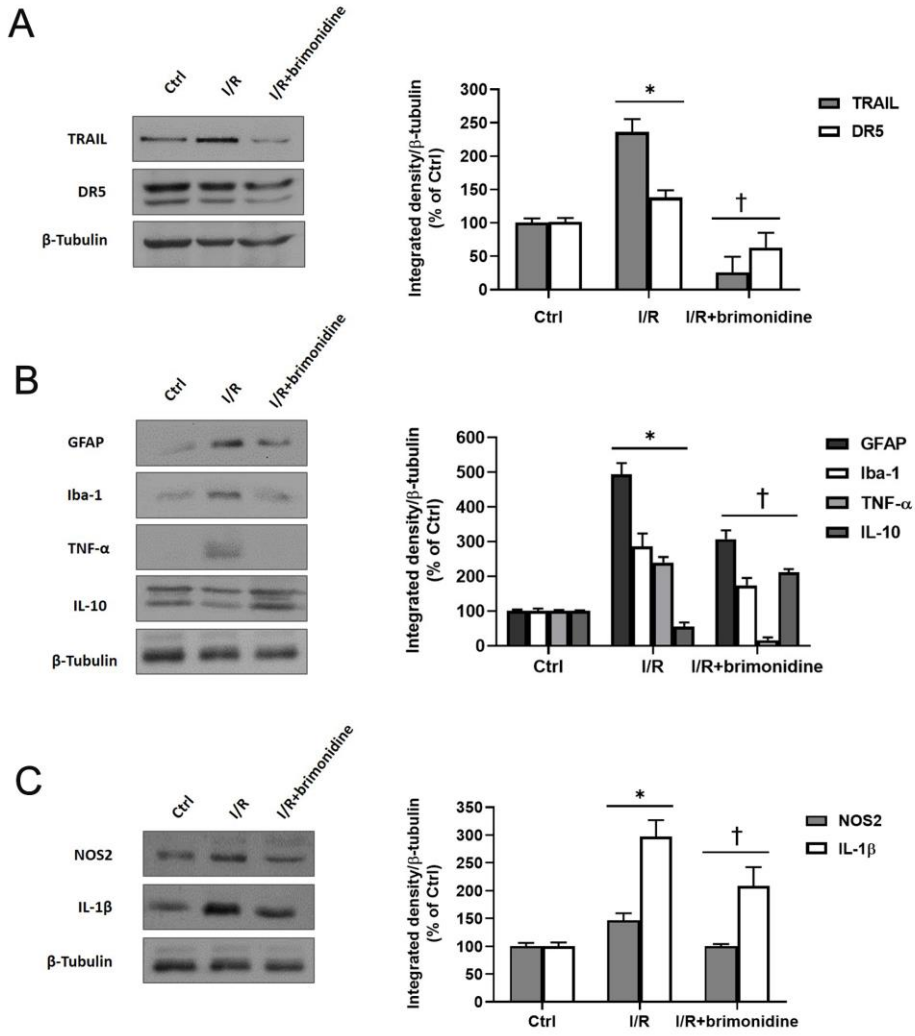

Figure 3. Western Blot. (A) TRAIL and DR5 protein levels in control, I/R and brimonidine-treated mice retina; (B) GFAP, Iba-1, TNF-α and IL-10 proteins in mice retina w or w/o brimonidine; (C) NOS2 and IL-1β proteins in mice retina w or w/o brimonidine. Values represent protein expression relative to β-tubulin, used as housekeeping protein. Values are reported as mean ± SD (n = 5). One-way ANOVA analysis was performed followed by the Tukey post-hoc test. *$p < 0.05$ vs. Ctrl; † $p < 0.05$ vs. I/R.

Discussion

Glaucoma is a progressive neurodegenerative disease, and the major unmet medical need in this condition is the protection of retinal ganglion cells. In fact, it is well known that pharmacological interventions intended to only lower IOP are not always effective in preventing visual field loss, even though IOP represents the major risk factor for glaucoma progression. Neuroprotective treatment for glaucoma endeavors to preserve vision by preventing the death of RGCs. Different experimental models of ocular hypertension and different electrophysiological measurements of RGCs function have shown that cell dysfunction occurs in the early phases preceding cell death (Chou et al., 2014; Porciatti, 2015). The time lag between RGC dysfunction and death may be related both on the magnitude of IOP elevation and the susceptibility to IOP stress. In the present study we carried out retinal I/R in mouse eye, showing that ischemic insult elicited a significant impairment of RGCs function and a remarkable expression of several inflammatory markers, such as TNF and ILs, in the retina. We also found a significant glial cells activation as demonstrated by GFAP and Iba1 upregulation. We showed that topical treatment with brimonidine preserved RGCs function and reverted the inflammatory profile elicited by I/R injury. Further, brimonidine was able to maintain physiological levels of BDNF in the retinal tissue of I/R mice group. Relevant non-clinical studies (Yoles et al., 1999) demonstrated that brimonidine has neuroprotective properties in optic nerve degeneration and retinal ischemia (Wheeler et al., 1999) even though the authors did not figure out the mechanism of that effect. It has been hypothesized that the neuroprotection of brimonidine is related to modulation of BDNF, this latter is a potent neurotrophic factor that prevent RGCs death after axotomy in the optic nerve (Mansour-Robaey et al., 1994). Gao et al. (2002) demonstrated that brimonidine was able to up-regulate the BDNF in retinal rat after 48 h from drug treatment. How the brimonidine up-regulate retinal BDNF remains to be elucidated, in fact the authors speculated that α2 receptor activation can result in the regulation of multiple signaling pathways directly or indirectly related with BDNF expression. It has been also demonstrated that brimonidine was able to upregulate several growth factors such as BDNF, NT3 and CTNF in ischemic rat retina (Lonngren et al., 2006). Recently, it has been demonstrated (Ortin-Martinez et al., 2014) that topical brimonidine protects retinal tissue in a light-emitting diode-induced phototoxicity. More recently, Yukita et al. (2017) showed that brimonidine enhances the electrophysiological response of RGCs through the Trk-MAPK/ERK and PI3K pathways in axotomized rat eye, hypothesizing that these pathways regulate BDNF. Beside these important proofs, another inflammatory marker, called TRAIL, has been recently

highlighted. TRAIL is a member of the TNF superfamily and it is constitutively expressed in retina (Lee et al., 2002). TRAIL acts mostly through the death receptor DR5, and it is a potent mediator of prominent neuronal loss induced in both chronic and acute neurodegenerative processes, including those related to brain ischemia (Martin-Villalba et al., 1999; Cantarella et al., 2014). Upon injury, disease or inflammation, healthy neurons may get damaged eliciting an environment alteration that activate resting microglia with a release of pro-inflammatory molecules. In addition to its pro-inflammatory pattern, microglia can also stimulate an alternative activation pathway, associated with increased production of anti-inflammatory cytokines such as IL-10 and neurotrophic factors such as BDNF to promote neuronal recovery (Di Polo et al., 1998; Gallego et al., 2012; Gonzalez et al., 2014). Privation of oxygen and nutrients during ischemia, generates reactive oxygen species production leading to inflammation. I/R injury elevates the retinal expression of several inflammatory markers such as ILs, TNF-α, TRAIL and nitric oxide (NO) (Dreyer et al., 1996; Kawasaki et al., 2000; Tezel and Wax, 2000; Wang et al., 2005). These results are in accordance with the findings generated in the present study; moreover, we observed that RGCs damage elicited the upregulation of GFAP and Iba1, demonstrating glial cells activation (Mao and Yan, 2014). In conclusion, the ocular topical brimonidine treatment showed retinal protection in an acute model of RCGs death, reducing the expression of inflammatory cytokines, enhancing the expression of BDNF, and preserving retinal function.

Data Availability Statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by the IACUC, University of Catania #343. All animals were treated according to the Principles for the Care and Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology.

Author Contributions

CB, FC, GLR, and GC made substantial contributions to conception, design, and interpretation of data. FC, GLR, GD, FL, and MDT carried out formal analysis of data. CB, FL, GC, GD, CME, RR, RB, FD, and MDT wrote initial draft of the manuscript. CB, GC,

CME, RR, RB, and FD reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter II

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Caffeine Protects Against Retinal Inflammation

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Abstract

Caffeine, one of the most consumed central nervous system (CNS) stimulants, is an antagonist of A1 and A2A adenosine receptors. In this study, we investigated the potential protective effects of this methylxanthine in the retinal tissue. We tested caffeine by using in vitro and in vivo paradigms of retinal inflammation. Human retinal pigment epithelial cells (ARPE-19) were exposed to lipopolysaccharide (LPS) with or without caffeine. This latter was able to reduce the inflammatory response in ARPE-19 cells exposed to LPS, attenuating the release of IL-1β, IL-6, and TNF-α and the nuclear translocation of p-NFκB. Additionally, caffeine treatment restored the integrity of the ARPE-19 monolayer assessed by transepithelial electrical resistance (TEER) and the sodium fluorescein permeability test. Finally, the ischemia reperfusion (I/R) injury model was used in C57BL/6J mice to induce retinal inflammation and investigate the effects of caffeine treatment. Mouse eyes were treated topically with caffeine, and a pattern electroretinogram (PERG) was used to assess the retinal ganglion cell (RGC) function; furthermore, we evaluated the levels of IL-6 and BDNF in the retina. Retinal BDNF dropped significantly ($p < 0.05$) in the I/R group compared to the control group (normal mice); on the contrary, caffeine treatment maintained physiological levels of BDNF in the retina of I/R eyes. Caffeine was also able to reduce IL-6 mRNA levels in the retina of I/R eyes. In conclusion, these findings suggest that caffeine is a good candidate to counteract inflammation in retinal diseases.

Keywords: caffeine, inflammation, retina, BDNF, retinal pigment epithelial cells

Introduction

Caffeine is the 1,3,7 trimethylxanthine and represents one of the most consumed central nervous system (CNS) stimulants, with an average assumption within 100–400 mg per day (Sc and Muralidhara, 2016), through consumption of coffee, tea, and soft drinks enriched with caffeine (Mitchell et al., 2014), along with caffeine supplements, generally used as metabolism boosters (Gurley et al., 2015). Caffeine is endowed with anti-inflammatory and antioxidant properties, as reported by several studies on different models of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (Dall'lgna et al., 2003; Carman et al., 2014; Xu et al., 2016; Hosny et al., 2019). Moreover, caffeine has shown beneficial effects in several pathologies, such as chronic stress, diabetes, attention deficit, and hyperactivity disorders (Park et al., 2007; Alves et al., 2020; Ibrahim et al., 2020). Caffeine is a non-selective adenosine receptor (AR) antagonist, although it has a higher affinity for the adenosine A1 receptor (A1R) and the adenosine A2A receptor (A2AR); furthermore, caffeine is a non-selective inhibitor of phosphodiesterases (PDEs) (Jacobson et al., 2020). A1R and A2AR are G-protein–coupled receptors (GPCRs), and they are expressed in human retinal pigment epithelial (RPE) cells (Wan et al., 2011) and in other layers of the retina (Fredholm et al., 2011; Wurm et al., 2011; Liu et al., 2018).

Caffeine has provided neuroprotective action through the blockade of A2AR (Xu et al., 2016; Boia et al., 2017). In consideration of the complex pharmacological profile of this drug, the effects of caffeine are not straightforward to be predicted (Dai and Zhou, 2011), and although the current literature provides some evidence regarding the effects of caffeine in the CNS, few studies were carried out regarding its actions in the eye. Considering the similarities between neurodegenerative diseases of the brain and retina (Romano et al., 2015, Romano et al., 2017; Platania et al., 2017), we investigated caffeine by using in vitro and in vivo paradigms of retinal inflammation. The retinal inflammatory process occurs in several ocular diseases such as age-related macular degeneration (AMD) and diabetic retinopathy (DR). This latter is one of the leading causes of irreversible vision loss in industrialized countries and represents a severe retinal degenerative disease (Van Lookeren Campagne et al., 2014). AMD is mainly characterized by accumulation of the pigment lipofuscin in the RPE cells (Katz, 2002) and by retinal ischemia (Rivera et al., 2017). About 15% of AMD patients switch to the wet form, characterized by choroidal neovascularization. Currently, approved pharmacological treatments such as anti-VEGF agents and steroids are available only for the wet form of AMD and DR (Bucolo et al., 2005; Sarao et al., 2014; Giurdanella et al., 2015; Amadio et al., 2016; Campochiaro et al., 2016; Bucolo et al., 2018); no treatments have been

approved yet for the dry form of the disease where the inflammation is prevalent, and it is considered a hallmark of the early phase of this condition. Protection of RPE cells and retinal ganglion cells (RGCs), along with blood retinal barrier (BRB) preservation, can be considered as a new strategy to prevent the devastating damage of retinal degenerative diseases. It has been widely demonstrated that the activation of toll-like receptor 4 (TLR-4), induced by LPS, stimulates the nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and, as a direct consequence, the over-expression of inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β), or tumor necrosis factor-alpha (TNF-α) (Holtkamp et al., 1998; Izumi-Nagai et al., 2007; Salminen and Kaarniranta, 2009; Do et al., 2020). It is noteworthy that AMD patients have increased vitreous levels of IL-1β (Tang and Kern, 2011; Zhao et al., 2015; Dabouz et al., 2020) and plasmatic tumor necrosis receptor 2 (TNF-R2) (Krogh Nielsen et al., 2019). Usually, RPE cells release neurotrophic factors such as the brain-derived growth factor (BDNF), which is a key factor for survival and function of RGCs and photoreceptors (Ponnalagu et al., 2017; Bahrami et al., 2019). Furthermore, low BDNF levels have been found in the aqueous humor of AMD patients, causing an insufficient protection of the retinal tissue (Inanc Tekin et al., 2018). The aim of the present study was to explore the neuroprotective and the anti-inflammatory effects of caffeine in two models of retinal inflammation by using human RPE cells and C57BL/6J mice, respectively.

Material and Methods

Cell Culture

Human retinal pigment epithelial cells (ARPE-19) were purchased from ATCC® (Manassas, Virginia, United States). Cells were cultured at 37°C (humidified atmosphere with 5% CO2) in ATCC-formulated DMEM:F12 medium (ATCC number 30–2006, Manassas, Virginia, United States) with 100 U/mL penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). After reaching confluence (~70%), ARPE-19 were pretreated for 24 h with caffeine at concentrations of 1, 10, 100 and 1000 µM (Sigma-Aldrich, Cat.No. C0750, St Louis, MO) and/or 1 µM of CGS 21680 hydrochloride (Tocris Bioscience, Cat.No. 1063, Bristol, United Kingdom) (Wang et al., 2014) in DMEM:F12 supplemented with only 5% FBS to starve cells. In control cells (untreated), only fresh medium has been added. After pretreatment, ARPE-19 were challenged with 150 ng/ml, 2 µg/ml, or 10 µg/ml of lipopolysaccharide E. coli (LPS) (Enzo Life Sciences ALX-581–010-L001, Farmingdale,

NY) to simulate inflammation and also with different concentrations of caffeine (1–100 µM) and/or 1 µM of CGS 21680.

MTT Assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrasodium bromide (MTT; Chemicon, Temecula, CA) was used to assess cell viability. Optimal cell density was obtained by seeding 3 × 104 cells/well in 96-well plates (Costar, Corning, New York). After 24 h of culture, ARPE-19 were treated with caffeine (1–1000 µM), in medium containing FBS 10% for 48 h. At the end of the treatment, ARPE-19 were incubated at 37°C with MTT (0.5 mg/ml) for 2 h; then, DMSO 100 µL per well was added, and absorbance was measured at 570 nm in a plate reader (VariosKan, Thermo Fisher Scientific, Waltham, MA). Results were reported as the percentage of control.

LDH Assay

Lactate dehydrogenase (LDH) cell release was measured using the Cytotoxicity Detection KitPLUS (LDH) (Roche Diagnostics 04744934001, Basel, Switzerland). ARPE-19 cells were seeded at 3 × 104 cells/well in 96-well plates (Costar, Corning, New York). After reaching confluence, cells were treated for 48 h with caffeine (1–1000 µM), in medium containing FBS 10%. After treatment, according to the manufacturer's protocol, lysis solution was added to positive control wells (non-treated cells) for 15 min. After transferring 100 µL of the medium in a new multi-well, 100 µL of the working solution was added. After 10–15 min at room temperature, 50 µL of the stop solution was added lastly. The absorbance values were measured at 490 nm using a plate reader (VarioSkan, Thermo Fisher Scientific, Waltham, MA). LDH release is reported as LDH (% control): (absx ÷ absctrl+) × 100. In the equation, absx is absorbance in the x well, and absctrl+ is the average absorbance of positive control cells (untreated lysed cells). Absorbance values were edited by removing the blank.

Extraction of Total RNA and cDNA Synthesis

Extraction of total RNA, from ARPE-19 and mouse retinas, was performed with TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, United States). The A260/A280 ratio of the optical density of RNA samples (measured with Multimode Reader Flash di Varioskan™) was 1.95–2.01; this RNA purity was confirmed by electrophoresis in the non-denaturing 1% agarose gel (in TAE). cDNA was synthesized from 2 µg (ARPE-19) and 500 ng (mice retinas) of RNA with a reverse transcription kit (SuperScript™ II Reverse transcriptase, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, United States).

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR was carried out with the Rotor-Gene Q (Qiagen, Germantown, MD, United States). The amplification reaction mix included the Master Mix Qiagen (10 µL) (Qiagen QuantiNova SYBR Green Real-Time PCR Kit, Germantown, MD, United States) and cDNA (1 µL,100 ng). Forty-five amplification cycles were carried out for each sample. Results were analyzed with the 2−ΔΔCt method (Leggio et al., 2019). Quantitative PCR experiments followed the MIQE guidelines. Gene expression levels were normalized with levels of housekeeping gene (18S). Primers were purchased from Eurofins Genomics (Milan, Italy) and Qiagen (Milan, Italy). Forward and reverse primer sequences (for human and mouse genes) and catalogue numbers are herein listed: human IL-1β (Forward: 5′-AGCTAC GAATCTCCGACCAC-3'; Reverse: 5′-CGTTATCCCATGTGTCGAAGAA-3′), human IL-6 (Catalogue Number QT00083720), human TNF-α (Forward 5′-AGCCCATGTTGTAGC AAACC-3'; Reverse 5′-TGAGGTACAGGCCCTCTGAT-3′), human 18S (Forward 5′-AGT CCCTGCCCTTTG-3'; Reverse 5′-GATCCGAGGGCCTCACTAAAC-3′), human BDNF (Catalogue Number QT00235368), mice 18S (Forward: 5′-GTTCCGACCATAAACGAT GCC-3′; Reverse: 5′-TGGTGGTGCCCTTCCGTCAAT-3′), mice BDNF (Forward: 5′-GTT CGAGAGGTCTGACGACG-3′; Reverse: 5′-AGTCCGCGTCCTTATGGTTT-3′), and mice IL-6 (Cat. No. QT00098875).

Western Blot

ARPE-19 were cultured in 60 mm petri dishes at a density of 1,3 × 106. After 24 h of pretreatment with caffeine (1–100 µM) and/or CGS 21680 (1 µM) and co-treatment with 10 µg/ml of LPS for 2 h, cytoplasmic and nuclear proteins were extracted by using the CER/NER kit (NE-PER Nuclear and Cytoplasmic extraction reagents,78,833, Invitrogen, Life Technologies, Carlsbad, United States) according to the manufacturer's protocol. The protein content was determined by using the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, United States). Extracted proteins (20 µg) were loaded on the NuPAGE TM 10% Bis-Tris mini protein gel (Invitrogen, Life Technologies, Carlsbad, CA, United States). After electrophoresis, proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA). Membranes were blocked with milk 5% in Tris-buffered saline 0.2% Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated overnight (4°C) with appropriate primary phospho-NFκB p65 (Ser536; mouse mAb #3036 Cell Signaling Technology, MA, United States, 1:500 dilution), anti-β-Actin (Rabbit mAb #A2066 Sigma-Aldrich, St Louis, MO; 1:1000 dilution), and anti-lamin B (Mouse monoclonal IgG2b, sc-365214 Santa Cruz Biotechnology, INC, CA, United States; 1:1000 dilution) antibodies. After overnight incubation, the membranes

were then incubated with secondary chemiluminescent antibodies (ECL anti-mouse, NA931 and ECL anti-rabbit, NA934, 1:2000 dilution) for 1 h at room temperature. After secondary antibodies, membranes were incubated with ECL (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, 34,577, Thermo Fisher Scientific, Carlsbad, CA, United States) and were detected through I-BrightTM 1500 (A43679, Invitrogen, Life Technologies, Carlsbad, CA, United States) by chemiluminescence. Densitometry analyses of blots were performed at non-saturating exposures and analyzed by ImageJ software (NIH, Bethesda, MD). The values were normalized to β-actin and lamin B, which were used as housekeeping control for cytoplasmic and nuclear fraction, respectively.

Transepithelial Electrical Resistance (TEER) and Permeability Test

Transepithelial electrical resistance was measured by using a Millicell-Electrical Resistance System (ERS2) (Merck, Millipore, Burlington, MA, United States) as previously described (Giurdanella et al., 2017). TEER values were reported as ω×cm2 and were calculated as (average resistance of well–average resistance of the blank well) × 0.33 (the area of the membrane). ARPE-19 cells were seeded (1 × 105 cells/well) in 24-well plates on cell culture transwell inserts (FalconTM 24 well 0.4 μm pore size, #353095, Becton Dickinson Labware, Bedford, MA, United States). After reaching confluence, cells were pretreated with caffeine (1–100 μM) for 24 h in DMEM:F12 supplemented with 5% FBS and next with LPS 2 μg/ml and caffeine (1–100 μM) for 24 h. To evaluate the BRB permeability, cell culture inserts were transferred in new 24-well plates, and a solution of sodium fluorescein (Na-F) (10 mg/ml) was added. After 5, 15, and 30 min, the quantification of fluorescence (Na-F: excitation 480 nm, emission 535 nm) was carried out using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, United States). Values were reported as previously described (Fresta et al., 2020).

Immunocytochemistry

ARPE-19 cells were seeded at a density of 7 × 104/well on 24-well glass chamber slides. After 3 days, cells were pretreated with caffeine (1–100 μM) for 24 h in DMEM F12 containing 5% of FBS. Subsequently, cells were subjected to LPS stimulus (10 μg/ml) for 72 h and different concentrations of caffeine. At the end of the treatment, cells were fixed with acetone for 15′ at -20°C and subsequently with methanol for 20′ at -20°C. After washing with PBS 1X, cells were permeabilized with Triton 0.2% for 5′ at 4°C. After permeabilization, cells were incubated with the primary antibody (Rabbit anti-ZO-1, 617300, Invitrogen, Life Technologies, Carlsbad, CA, United States; 1:100 in triton 0.1%) overnight at 4 °C. Then, cells were washed and incubated with the secondary antibody (Goat anti-rabbit, ab96899,

Abcam, Cambridge, United Kingdom; 1:300 in triton 0.1%) for 1 h at room temperature in the dark. After washing again, the slides were mounted using Fluoroshield™ with DAPI (F6057-29ML Sigma-Aldrich, St Louis, MO). Images were acquired by using the Zeiss Observer Z1 microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). A semi-quantitative evaluation of the ZO-1 expression was carried out analyzing images from slides of each condition (n = 4) (Ctrl, LPS, LPS + caffeine 1 μM, LPS + caffeine 10 μM, LPS + caffeine 100 μM). The images (n = 4 per group) were analyzed, and ImageJ was used for measurements of the average gray scale.

Animals

Male C57BL/6J mice (3 months of age) (Charles River Laboratories, Italy) were housed in a temperature-controlled environment with free access to food and water during a 12 h light–dark cycle. All animals were treated according to the Principles for the Care and Use of Animals in Ophthalmic and Vision Research approved by the Association for Research in Vision and Ophthalmology. University of Catania (Italy) Ethics Committee approval #343.

Ischemic/Reperfusion Retina Damage

Retinal ischemia/reperfusion (I/R) has been used to induce retinal injury, as previously described in many rodent species (Osborne et al., 2004; Gustavsson et al., 2008; Ulbrich et al., 2017; Stankowska et al., 2019). Mice were anesthetized by tiletamine + zolazepam (60 mg/kg) and medetomidine (40 μg/kg) administered through intraperitoneal injection; moreover, 0.4% oxybuprocaine (Novesina®, Laboratoires Thea, Clermont-Ferrand, France) has been administered topically. The animals were placed on a heating pad to prevent hypothermia during the experiment. A 32-gauge needle, connected with a reservoir containing phosphate-buffered saline, was introduced into the anterior chamber through the cornea to increase the intraocular pressure (up to 90 mmHg). Retinal ischemia was confirmed by the observation of blanching of the anterior segment and arteries in the eye. After 60 min, the needle was removed to allow reperfusion. Ocular formulation of 1.9% caffeine was instilled (10 μL) 60 min before I/R and after reperfusion, twice a day for 72 h. Mice were euthanized after 72 h from I/R insult, the eyes were enucleated, and the retinas were collected to assess IL-6 and BDNF mRNA expressions.

Pattern Electroretinogram (PERG)

PERG has been used as a sensitive measure of RGC function (Chou et al., 2018). Anesthetized mice were transferred on a heating plate with the mouse superior incisor teeth hooked to a bite bar and the head gently restrained by two ear knobs. The body was kept at a constant temperature of 37 °C using a feedback-controlled heating pad (TCAT-2LV,

Physitemp Instruments, Inc, Clifton, NJ, United States). Two microliters of topical balanced salt solution (BSS) were applied to prevent corneal dryness. Simultaneous recordings of PERG response from both eyes were obtained using a common subcutaneous needle in the snout (jorvec Corp, Miami, FL, United States). To obtain PERG records, visual stimuli (black–white horizontal bars generated on LED tablets) are presented independently to each eye at 10 cm distance (56° vertical × 63° horizontal field; spatial frequency, 0.05 cycles/deg; 98% contrast; 800 cd/sqm mean luminance; left-eye reversal rate, 0.992 Hz; right-eye reversal rate, 0.984 Hz). Electrical signals recorded were averaged (>1,110 epochs), and PERG responses from each eye were isolated by averaging at stimulus-specific synchrony. PERG waveforms consist of a positive wave (defined as P1) followed by a slower negative wave with a broad trough (defined as N2). Therefore, each waveform has been analyzed by measuring the peak-to-trough (P1-N2) amplitude defined as the PERG amplitude and the time-to-peak of the P1 wave defined as PERG latency (Porciatti, 2014).

Statistical Analysis

Statistical analysis was performed with GraphPad prism 7 (GraphPad software La Jolla, California). The data generated by all experiments are reported as mean ± SD (n = 4). One-way analysis of variance (ANOVA) was carried out, and Tukey's post hoc test was used for multiple comparisons. Differences between groups were considered statistically significant for p-values < 0.05.

Results

Effects of Caffeine on ARPE-19 Cell Viability

Preliminary studies were carried out to evaluate cell viability and cytotoxicity after treatment with caffeine (1–1000 μM). At concentrations of 1–10 and 100 μM, caffeine did not reduce cell viability compared to control cells (Figure 1A). Moreover, as shown in Figure 1B, caffeine did not increase the LDH release, compared to untreated (control) cells, whereas treatment with caffeine 1000 μM led to a significant ($p < 0.05$) reduction of cell viability and to a significant ($p < 0.05$) increase of the LDH release (Figure 1A, B). For this reason, we excluded caffeine 1000 μM for all subsequent experiments.

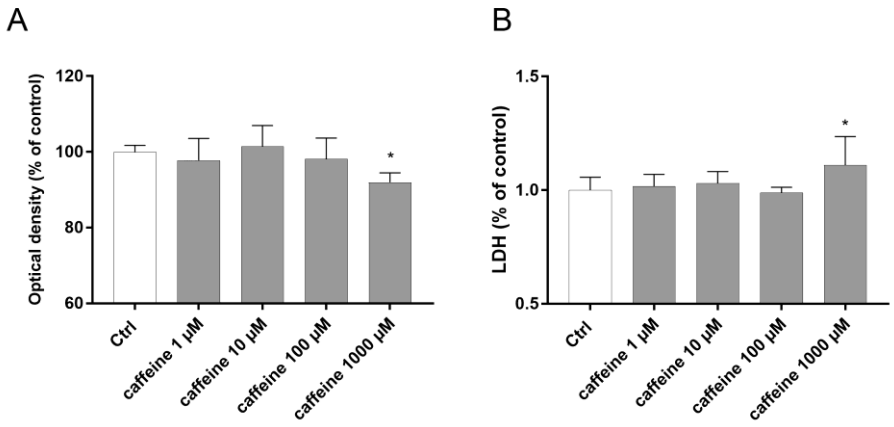

Figure 1. Caffeine 1–100 µM is tolerated by ARPE-19 cells. Caffeine (1–10–100 µM) after 48 h did not reduce cell viability (A) and did not increase LDH release (B). Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control.

Effects of Caffeine on Inflammatory Markers and BDNF

Treatment with LPS (150 ng/ml) led to a significant ($p < 0.05$) increase of TNF-α, IL-6, and IL-1β mRNA levels, compared to control cells. Caffeine, at all tested concentrations, significantly ($p < 0.05$) reduced the expression of these inflammatory cytokines, in comparison with LPS-treated cells (Figure 2A–C). Moreover, LPS treatment significantly ($p < 0.05$) reduced the BDNF expression in ARPE-19 cells, compared to untreated cells. This effect was significantly ($p < 0.05$) counteracted by caffeine as demonstrated by the BDNF mRNA expression in ARPE-19 cells, damaged by LPS (Figure 2D). We found that caffeine upregulated the BDNF expression in ARPE-19 cells exposed to LPS, even though the intermediate concentration (10 µM) did not have effect (Figure 2D). Furthermore, as shown in Figure 3 (A, B, and C), the A2A selective receptor agonist, CGS 21680 (1 µM), led to a significant ($p < 0.05$) increase in mRNA levels of inflammatory cytokines, compared to cells treated with caffeine 100 µM. Moreover, while caffeine (100 µM) was able to restore significantly ($p < 0.05$) the BDNF mRNA levels, the A2A selective receptor agonist CGS (1 µM) counteracted the effect of caffeine on the BDNF expression (Figure 3D).

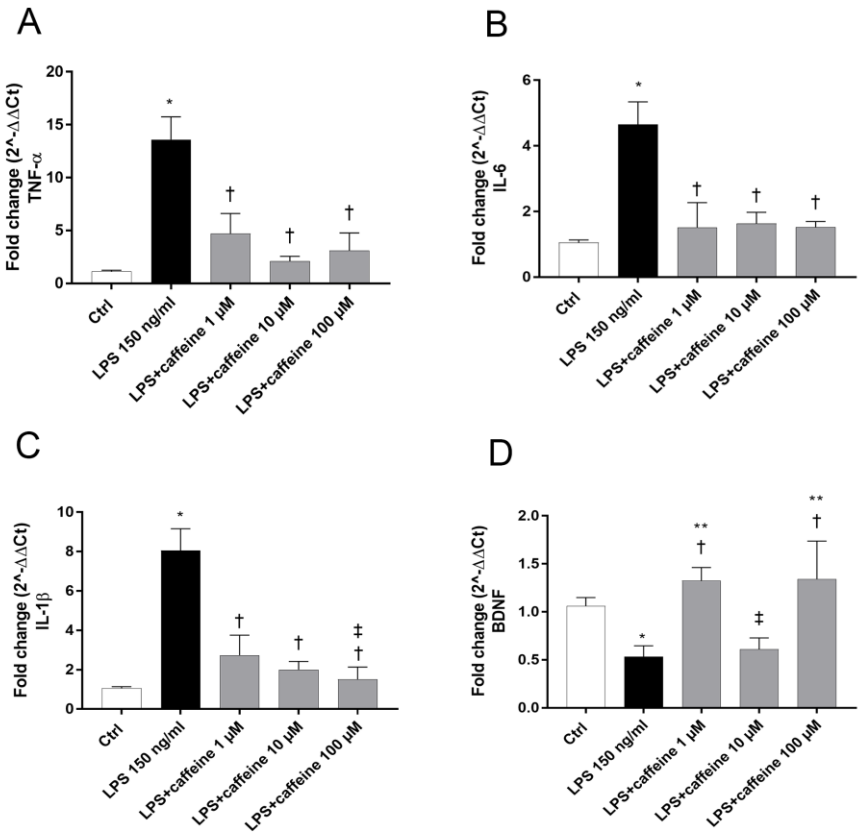

Figure 2. Caffeine counteracts inflammation and elicits the BDNF expression after LPS treatment. ARPE-19 were pretreated with caffeine (1–10–100 µM) for 24 h and then co-treated with LPS (150 ng/ml) for 2 h. Caffeine downregulated TNF-α (A), IL-6, (B) and IL-1β (C) mRNA levels, up-regulated by the LPS challenge. Caffeine 1 and 100 µM increased BDNF mRNA levels, reduced by LPS (D). Values were reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; †$p < 0.05$ vs. LPS 150 ng/ml; ‡ $p < 0.05$ vs. LPS + caffeine 1 µM; **$p < 0.05$ vs. LPS + caffeine 10 µM.

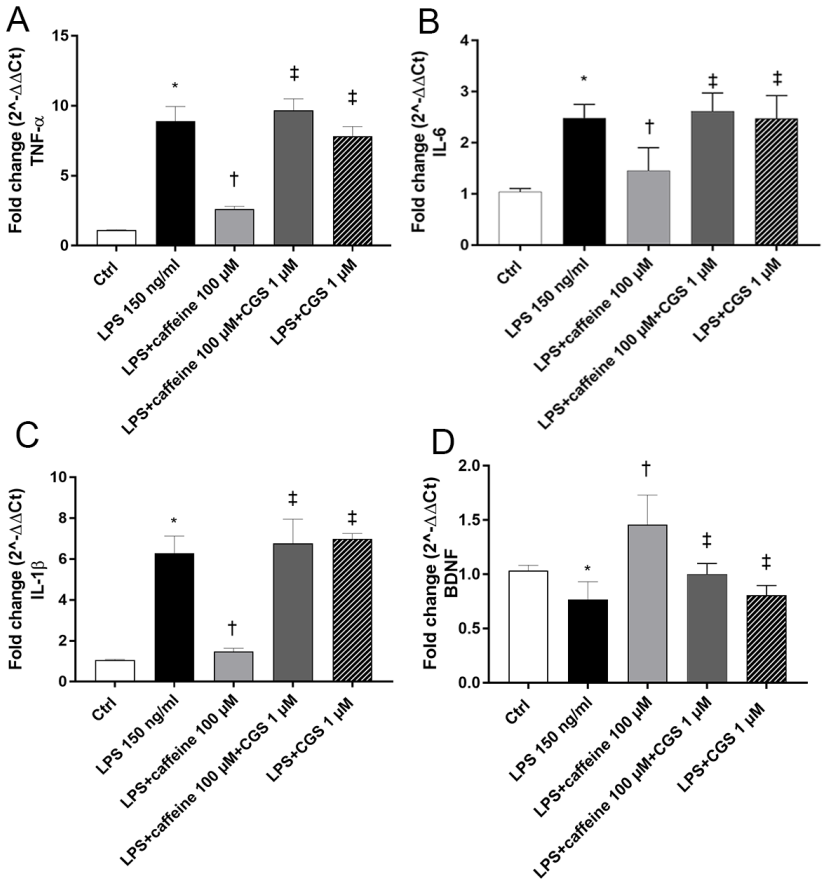

Figure 3. The selective A2A agonist CGS 21680 counteracts the effects of caffeine. Cells were pretreated with caffeine (100 µM) and/or CGS (1 µM) for 24 h and then co-treated with LPS (150 ng/ml) for 2 h. The treatment with CGS (LPS + CGS; LPS + caffeine + CGS) did not counteract LPS-mediated effects as regard as inflammatory cytokines (A–C) or BDNF (D). Contrarily, pretreatment with caffeine (LPS + caffeine) reduced inflammatory cytokines mRNA expression and increased BDNF mRNA levels. Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; † $p < 0.05$ vs. LPS 150 ng/ml; ‡ $p < 0.05$ vs. LPS + caffeine 100 µM.

Effects of Caffeine on p-NFκB p65 Nuclear Translocation

After 2 h, LPS (10 μg/ml) exposure significantly ($p < 0.05$) increased the nuclear translocation of p-NFκB p65, compared to control. Pretreatment for 24 h with caffeine (1 and 100 μM) significantly ($p < 0.05$) reduced the nuclear translocation of p-NFκB p65, confirming the anti-inflammatory effect of this compound in retinal pigment epithelial cells, challenged with LPS. However, caffeine 10 μM did not counteract the activation of NFκB (Figure 4A,B). As shown in Figure 4 (C and D), the selective A2A agonist CGS (1 μM) counteracted the anti-inflammatory effects of caffeine on ARPE-19 cells damaged by LPS, as regards as p-NFκB p65 nuclear translocation.

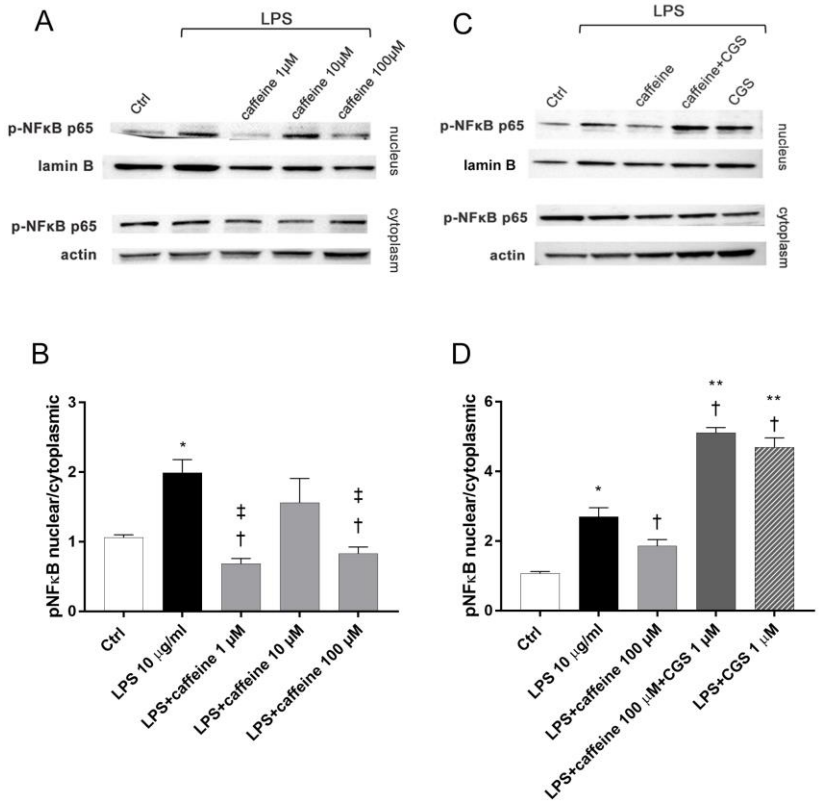

Figure 4. Caffeine reduces nuclear translocation of p-NFκB elicited by LPS. ARPE-19 were pretreated with caffeine (1–10–100 μM) and/or CGS (1 μM) for 24 h and then co-treated with LPS (10 μg/ml) for 2 h (A–C) Representative blots of nuclear and cytoplasmic proteins (B–D) Densitometry of the p-NFκB p65 nuclear translocation in treated cells (ratio of nuclear p-NFκB p65/lamin B and cytoplasmic p-NFκB p65/actin). Each bar represents the mean value ±SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; †$p < 0.05$ vs. LPS 10 μg/ml; ‡$p < 0.05$ vs. LPS + caffeine 10 μM, **$p < 0.05$ vs LPS + caffeine 100 μM.

Effects of Caffeine on BRB Integrity

To investigate the effect of caffeine on BRB integrity, we assessed the transepithelial electrical resistance (TEER) and immunostaining of ZO-1 tight junction, in ARPE-19. After 24 h, the LPS challenge (2 μg/ml) significantly ($p < 0.05$) decreased TEER values, in comparison to untreated cells (control) (Figure 5A). Caffeine, at all tested concentrations, significantly ($p < 0.05$) increased TEER values, in comparison to LPS-treated cells, meaning a restored BRB integrity (Figure 5A). These data were also confirmed by measurement of the apical-to-basolateral permeability of sodium fluorescein (Na-F). Treatment with caffeine (1–100 μM), at all considered time points (5′-15′ and 30'; 5′ and 30′ Supplementary Figure S1, Supplementary Material), led to a significant ($p < 0.05$) reduction of cell permeability, significantly ($p < 0.05$) increased by the LPS challenge (Figure 5B). According to the instrumental and spectroscopic evaluation of ARPE-19 monolayer integrity, after 72 h, LPS (10 μg/ml) significantly ($p < 0.05$) decreased the ZO-1 expression (Figure 6B), compared to control cells (Figure 6A). The treatment with caffeine (1, 10, 100 μM) reverted this LPS-related damage, reestablishing the ZO-1 expression and BRB integrity (Figure 6C–E).

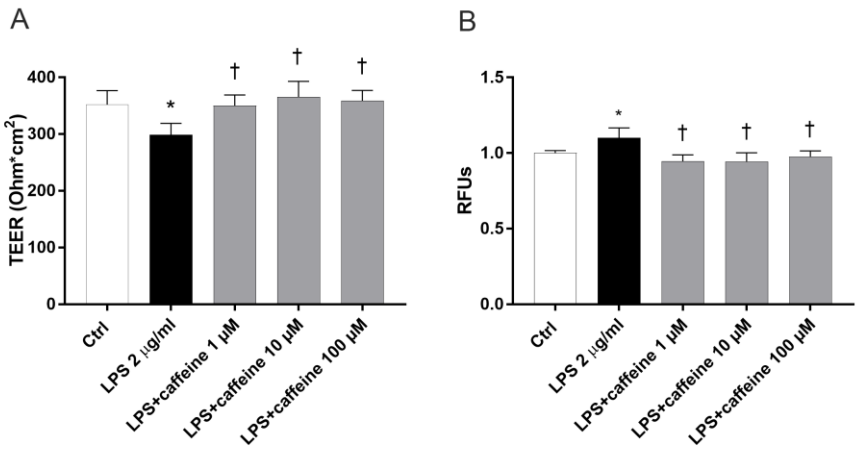

Figure 5. Caffeine protects the ARPE-19 cell monolayer from LPS-induced damage. ARPE-19 were pretreated with caffeine (1–10–100 µM) for 24 h and co-treated with LPS (2 µ/ml) for 24 h (A) Caffeine, at all concentrations, increased TEER values, which were reduced by LPS (B) Measurement of apical-to-basolateral Na-F permeability. Representative Na-F permeability measured after 15 min. Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; †$p < 0.05$ vs. LPS 2 µg/ml.

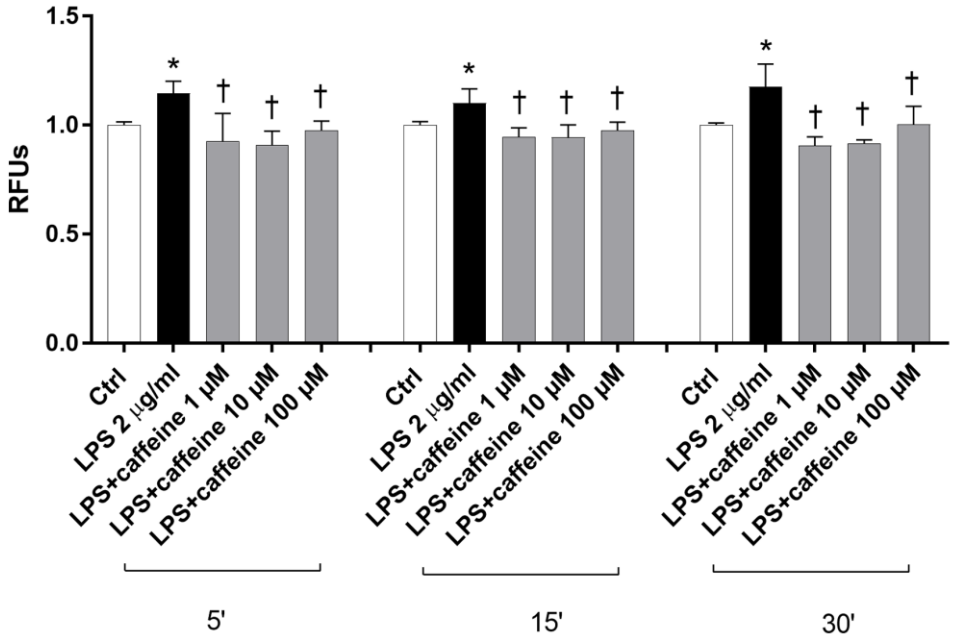

Figure S1. Measurement of apical-to-basolateral Na-F permeability. Na-F permeability was measured after 5, 15, and 30 min through fluorescence measurement. At all time points, caffeine (1-100 µM) was able to reduce permeability, increased by LPS. Values are reported as mean ± SD; n=4. Data were analyzed by one-way ANOVA and Tukey post-hoc test for multiple comparisons. *p<0.05 *vs.* control; † p < 0.05 *vs.* LPS 2 µg/ml.

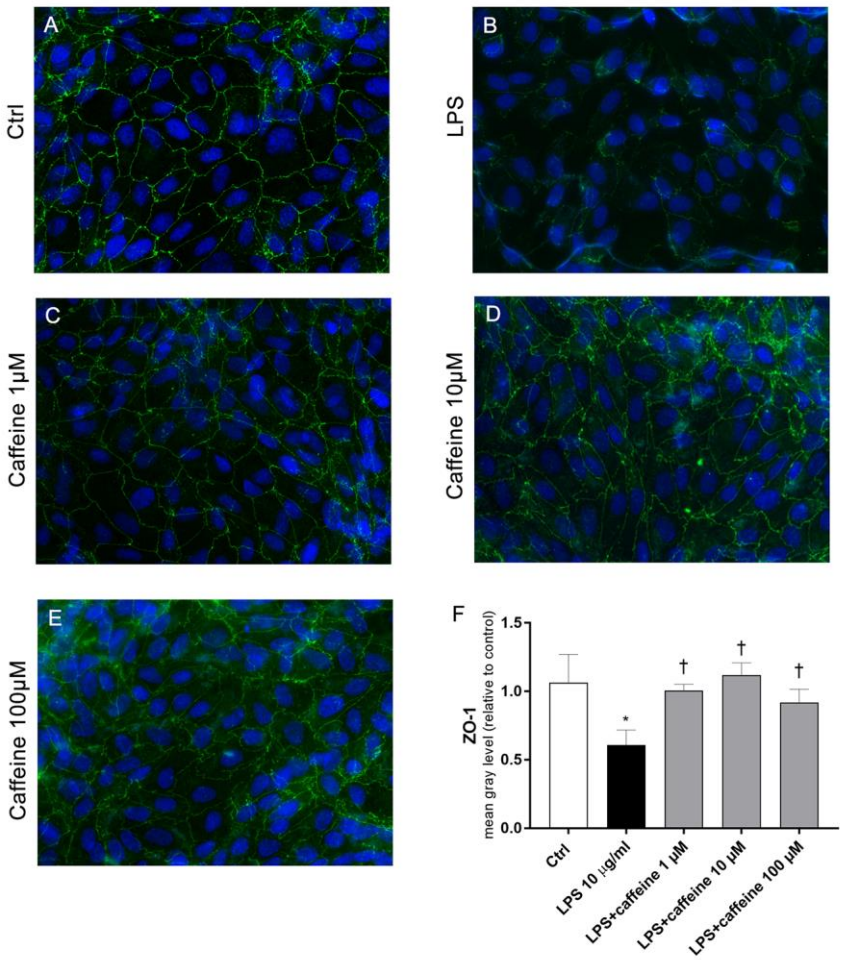

Figure 6. Caffeine re-establishes the BRB integrity through modulation of ZO-1. ARPE-19 were pretreated with caffeine (1–10–100 µM) for 24 h and subsequently co-treated with LPS (10 µg/ml) for 72 h. Caffeine, at all concentrations, increased the expression of ZO-1 protein, which was significantly reduced by LPS (A–E) Representative images for the ZO-1 expression in ARPE-19 after treatment with LPS and caffeine. ZO-1 was labeled with FITC (green); nuclei were labeled with DAPI (blue). Images were acquired at 20× magnification (F) Fluorescence semi-quantification of ZO-1 protein (mean gray levels). Values are reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; † $p < 0.05$ vs. LPS 10 µg/ml.

Effects of Caffeine in Retinal I/R-Injured Mice

We analyzed the effect of caffeine on the RGC function in I/R mice after 72 h, by means of PERG measurements (Figure 7 A, representative retinal waveforms). As expected, the PERG amplitude decreased (∼50%) in I/R-injured mice, in comparison to control mice, while caffeine-treated mice showed a PERG amplitude significantly ($p < 0.05$) higher than I/R-injured mice (Figure 7B). Indeed, the average value of the PERG amplitude was 11.41 μV in the control group, in agreement with previous studies (Porciatti et al., 2010; Romano et al., 2020), while the average value of the PERG amplitude of I/R mice was significantly ($p < 0.05$) reduced to 4.51 μV, compared to the control retina. It is noteworthy that the average value of the PERG amplitude of I/R caffeine-treated mice was 9.41 μV, suggesting a protective effect of caffeine in terms of RGC function (Figure 7B). No significant changes were observed in PERG latency in all experimental groups, as expected, considering the short time after the injury (Figure 7C). As shown in Figure 8A, I/R injury elicited significant ($p < 0.05$) increase of the IL-6 mRNA expression, that was significantly ($p < 0.05$) counteracted by caffeine treatment. Furthermore, I/R damage significantly ($p < 0.05$) downregulated the mRNA expression of BDNF in mouse retinas, while caffeine significantly induced ($p < 0.05$) the BDNF mRNA expression compared to the I/R group (Figure 8B).

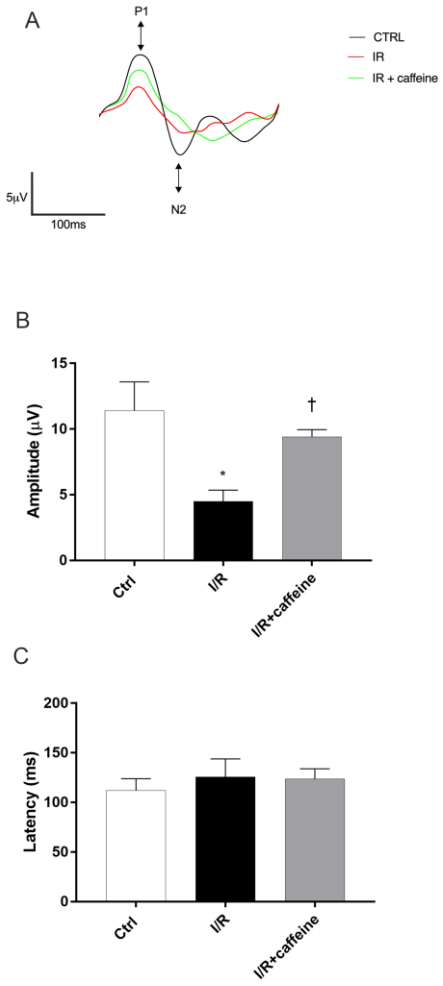

Figure 7. Caffeine counteracts RGC loss of function induced by I/R injury in mouse retinas. RGC function was assessed by the pattern electroretinogram (PERG). (A) Representative waveforms of PERG of all experimental groups (C57BL/6J mouse control (Ctrl), I/R, and I/R + caffeine). Each waveform, deriving from PERG recordings, was analyzed for the peak-to-trough amplitude (P1-N2). (B) Comparison between PERG amplitude values (μV) and latency values (ms). (C) of control, I/R, and I/R + caffeine. In each panel, bars represent the mean values ±SD; n = 4. One-way ANOVA analysis was performed followed by the Tukey post hoc test. *$p < 0.05$ vs. Ctrl; † $p < 0.05$ vs. I/R.

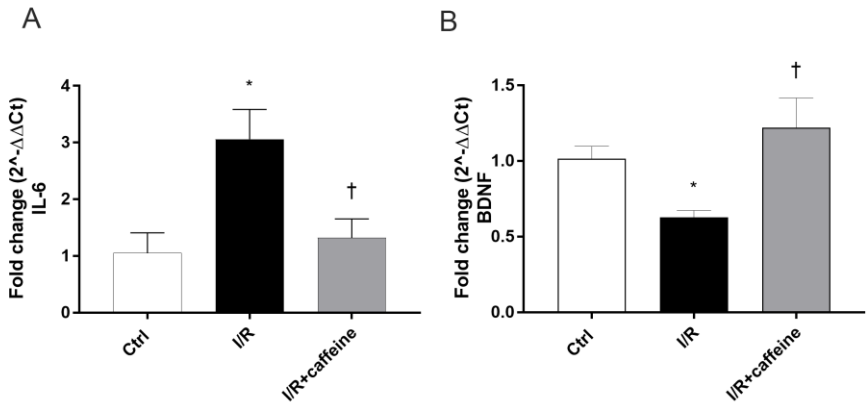

Figure 8. Caffeine counteracts inflammation and elicits the BDNF expression in I/R mice. After 72 h, caffeine was able to revert the upregulation of IL-6 mRNA levels (A) elicited by I/R damage in mouse retinas. BDNF mRNA levels were upregulated by caffeine, compared to I/R mice (B). Values were reported as mean ± SD; n = 4. Data were analyzed by one-way ANOVA and the Tukey post hoc test for multiple comparisons. *$p < 0.05$ vs. control; † $p < 0.05$ vs. I/R.

Discussion

Caffeine is approved for clinical use, and it is indicated for the treatment of the apnea of prematurity (Abdel-Hady, 2015). Increasing interest on caffeine has risen from several in vitro and in vivo evidence of neuroprotective effects in the model of brain neurodegenerative diseases (Chen et al., 2001, Chen et al., 2008a; Singhal et al., 2015; Akomolafe et al., 2017). Moreover, some clinical trials are focusing on the therapeutic potential of this natural stimulatory compound in Alzheimer's disease and Parkinson's disease (NCT01190735; NCT05009199; NCT01190735; NCT04570085). Previous studies reported the anti-inflammatory, anti-oxidative, and neuroprotective properties of caffeine (Jung et al., 2017; Kolahdouzan and Hamadeh, 2017; Metro et al., 2017). Only few studies have investigated the effects of caffeine on retinal diseases. On this regards, the Coimbra Eye Study, an epidemiological cross-sectional study, evidenced an inverse correlation between consumption of caffeine and AMD progression. The authors concluded that caffeine could be a promising nutritional supplement for slow-down of the AMD progression (Campochiaro

et al., 2016; Raimundo et al., 2018), highlighting the need for further pre-clinical pharmacological studies on caffeine and retinal diseases. Based on this evidence, we evaluated, for the first time, the anti-inflammatory and neuroprotective effect of caffeine, in human RPE cells and mouse retinas challenged with LPS (Ozal et al., 2018) and ischemia/reperfusion (Conti et al., 2021), respectively. LPS elicits retinal inflammation through activation of TLR-4, which is expressed in RPE cells, leading to inflammatory cytokine release and causing several degenerative processes (Kumar et al., 2004; Chen et al., 2017; Klettner et al., 2020). It has been demonstrated that caffeine suppresses the LPS-induced inflammatory response, reducing the expression of several inflammatory mediators in different types of cells, such as microglia and monocyte/macrophage-like cells (Kang et al., 2012; Hwang et al., 2016). In accordance with these findings, we demonstrated that caffeine exerted anti-inflammatory and neuroprotective effects also in RPE cells and in the retina of mice after LPS and ischemia insults, respectively. Caffeine reduced the mRNA expression of TNF-α, IL-6, and IL-1β in RPE cells after LPS exposure. Furthermore, caffeine significantly counteracted the p-NFκB p65 nuclear translocation in RPE cells exposed to LPS, showing a biphasic effect, already observed in other systems (Su et al., 2013) and with other compounds (Calabrese, 2016; Kurano et al., 2016). Finally, caffeine protected RPE cells also through the upregulation of BDNF, as already reported in different systems (Costa et al., 2008; Sallaberry et al., 2013; Lao-Peregrín et al., 2017). Moreover, retinal BDNF was also upregulated by caffeine in our retinal I/R in vivo model; in this paradigm, we also demonstrated that caffeine reduced IL-6 mRNA levels in comparison to I/R mice. BDNF is strongly reduced in several neurodegenerative processes both in the brain (Howells et al., 2000; Leyhe et al., 2008) and in the retina (Johnson et al., 2009; Kimura et al., 2016; Oddone et al., 2017; Platania et al., 2019; Conti et al., 2021). The overexpression of BDNF, and the reduced expression of IL-6, elicited by caffeine treatment in the retinal I/R model, could explain the protection of RGCs showed by PERG analysis in mice. Moreover, several studies demonstrated that some retinal diseases such as AMD are characterized by the abnormal expression and irregular distribution of tight junction proteins in RPE cells (Du et al., 2013). Hence, it is well known that LPS affects the epithelial integrity (Zheng et al., 2018; He et al., 2019), reducing the expression of ZO-1 protein and diminishing TEER values, in ARPE-19 cell monolayers (Chen et al., 2017; Zou et al., 2018; Hernandez et al., 2021). In this study, we confirmed that the LPS insult reduced the ARPE-19 monolayer integrity, as shown by instrumental (TEER measurements), spectroscopic (NaF permeability assays), and immunocytochemistry analyses. The pretreatment with caffeine brought TEER values and

NaF permeability to levels shown by ARPE-19-negative control cells. Furthermore, caffeine restored the ZO-1 expression, in ARPE-19 exposed to LPS treatment. The present findings are in line with the previous studies, which demonstrated that caffeine is able to prevent cell–cell interaction network disruption, not only as regard as the retinal barrier but also in the blood–brain barrier (BBB) (Chen et al., 2008a, Chen et al., 2008b; Maugeri et al., 2017). Caffeine has an interesting pharmacological profile, and several studies demonstrated that the antagonism of A2A receptors modulates neuroinflammation in retinal ganglion cells (Madeira et al., 2016; Boia et al., 2017), in microglia (Madeira et al., 2018), and in neuronal cells (Rebola et al., 2011). Based on this evidence, we supported the hypothesis that caffeine exerts its neuroprotective and anti-inflammatory effects through A2A receptor signaling because the agonist (CGS 21680) counteracted the effects of caffeine in RPE cells exposed to LPS. In conclusion, we demonstrated that caffeine was able to protect RPE cells and RGCs from damage elicited by LPS and ischemia, respectively, showing a key role of BDNF. These findings suggest that caffeine may be a potential candidate for retinal degeneration treatment.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics Statement

The animal study was reviewed and approved by the University of Catania (Italy) Ethics Committee.

Author Contributions

CB made substantial contributions to conception, design, and interpretation of data. FC, FL, and GLR carried out experiments. FC, FL, and CBMP carried out formal analysis of data. FC, FL, CBMP, and CB wrote the initial draft of the manuscript. CB and FD reviewed the manuscript critically for important intellectual content and gave final approval of the version to be submitted.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.824885/full#supplementary-material

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DISCUSSION AND CONCLUSIONS

As evidenced by the tight association between CNS and eye degenerative diseases, retina is considered the "window to the brain", since brain damage can affect the retina and its physiological function, leading to retinal alterations [222]. Neurodegeneration and neuroinflammation are pathological features involved both in CNS and retinal degenerative diseases. Currently, research is focused on the development of new neuroprotective and anti-inflammatory strategies, aimed to treat the early event of retinal pathologies, to slow-down disease's progression, with particular interest in the drug repurposing approach. In chapter I, I analyzed the neuroprotective effects of brimonidine in a mice model of I/R damage, which mimics early events of the glaucomatous neurodegeneration. Glaucoma is a progressive neurodegenerative disease characterized initially by RGCs dysfunctions and then by cells death [223]; currently, glaucoma therapies do not target the progressive RGCs loss of function. This highlights an unmet clinical need for the development of new treatment paradigms, regardless IOP-related treatments. In the present study (chapter I), retinal ischemic insult led to a significant impairment of RGCs function, elicited the overexpression of several inflammatory markers (TNF-α, ILs, and nitric oxide synthase 2 (NOS2) [68,181,224,225], and reduced anti-inflammatory cytokines, like IL-10 [226–228]. Together with this inflammatory mediators' modulation, I demonstrated even that I/R damage activated the entire inflammatory machine with the increased expression of TNF-related apoptosis-inducing ligand (TRAIL) and death receptor 5 (DR5), in addition to the activation of retinal microglia and astrocyte cells, evidenced by IBA-1 and GFAP increased levels [229]. Within this degenerative environment I/R-induced, brimonidine was able not only to ameliorate RGCs function, as demonstrated by the pattern electroretinogram (PERG), but also to revert the inflammatory

process activation. In addition, brimonidine maintained physiological BDNF levels in the retina of damaged mice, significantly reduced by the insult. Some pre-clinical studies have demonstrated the neuroprotective effect of brimonidine in retinal ischemia, as well as in optic nerve degeneration [230,231]; however, the mechanism of action was not clearly explained. Nevertheless, Gao H. et al., showed a brimonidine-induced increased expression of BDNF in a rat model of optic nerve crush, in accordance to my results showed in chapter I, and as already demonstrated even in ischemic rat retina [232]. Moreover, the authors speculated that α2 receptor activation can lead to the regulation of multiple signaling pathways directly or indirectly linked with BDNF expression, but how brimonidine acts exaclty remains to be elucidated [202]. The involvement of BDNF in the neuroprotective action of brimonidine was pointed out, more recently, by Yukita et al., which showed that brimonidine preserves RGCs survival and electrophysiological activity through the tropomyosin related kinase-mitogen-activated protein kinase/ERK (Trk-MAPK/ERK) and phosphatidylInositol 3-Kinase (PI3K) pathways after axotomy in rats, hypothesizing that these pathways regulate BDNF [203]. As reported in chapter I, my results support the neuroprotective and anti-inflammatory action of brimonidine, after eye topical treatment, in an acute model of RCGs death. Subsequently, in chapter II, I explored the neuroprotective action of another widespread compound, caffeine, already approved for clinical use in preterm infants [207]. Caffeine possesses anti-inflammatory, antioxidant and neuroprotective properties [233–235]. Indeed, the current literature highlights the neuroprotective effects of caffeine in the CNS, but few studies were carried out regarding its action in the eye [236–238]. Therefore, based on this evidence, I analyzed for the first time, the caffeine effect by using *in vitro* and *in vivo* models of retinal inflammation, as human RPE cells challenged with LPS and mice exposed to I/R injury.

Considering that LPS elicits inflammatory reponse in RPE cells [239,240] and causes the disruption of the BRB integrity [239,241], I used this *in vitro* model to mimic the retinal inflammatory environment. In chapter II, I demonstrated that caffeine was able to reduce the mRNA expression of TNF-α, IL-6, and IL-1β, counteracting the activation of NFκB in RPE cells exposed to LPS, as already demonstrated in other retinal cells [214,215]. In addition, caffeine restored the RPE monolayer integrity injured by LPS stimulus, and my results are in line with other studies regarding the protective effects of caffeine in damaged blood brain barrier (BBB) and BRB [242,243]. Moreover, I showed that caffeine protected RPE cells also through the upregulation of BDNF and these results corroborate with the modulation of this important neurotrophic factor exterted by caffeine even in other systems [216,217]. Besides, in chapter II, I further demonstrated that caffeine upregulated BDNF even in my *in vivo* model of I/R, as well as reduced retinal IL-6 mRNA levels in I/R mice. This could explain the protective effect of caffeine of RGCs proved by PERG measurement, after eye topical treatment. Furthermore, considering the complex pharmacological profile of caffeine and the reported evidence about the involvement of the $A_{2A}R$ blockade in its neuroprotective action, I used a selective $A_{2A}R$ agonist, which counteracted the effects of caffeine in RPE cells treated with LPS. My results indicate that caffeine exerts its actions through A_{2A} receptor signaling. Indeed, chapter II provides new insights about the neuroprotective and anti-inflammatory effect of caffeine in the eye. In the present PhD thesis, a repurposing approach of two drugs, brimonidine and caffeine, has been carried out as potential candidates for the treatment of retinal degenerative diseases. These compounds are already approved for open-angle glaucoma and apnea of prematurity, respectively. Brimonidine and caffeine share the ability to attenuate retinal inflammatory response that represents a prominent pathological feature in retinal degenerative diseases,

expecially in the early phases. Moreover, either brimonidine and caffeine modulate BDNF expression in the retina, which is a key factor involved in retinal and CNS neuroprotection. Tested compounds showed protective effects in *in vitro* and *in vivo* models of retinal degenerative diseases, which only partially resemble pathological mechanisms behind neurodegeneration, such as early phases of inflammation or ischemic events. Thereby, future perspective studies will be aimed at investigating the action of caffeine and brimonidine even in other pathological models, which mimic further retinal disease-specific mechanisms. In conclusion, this thesis points out the importance of neuroinflammation and neurotrophins deprivation in retinal degenerative diseases and laying the basis for further development in repurposing drugs for clinical treatment.

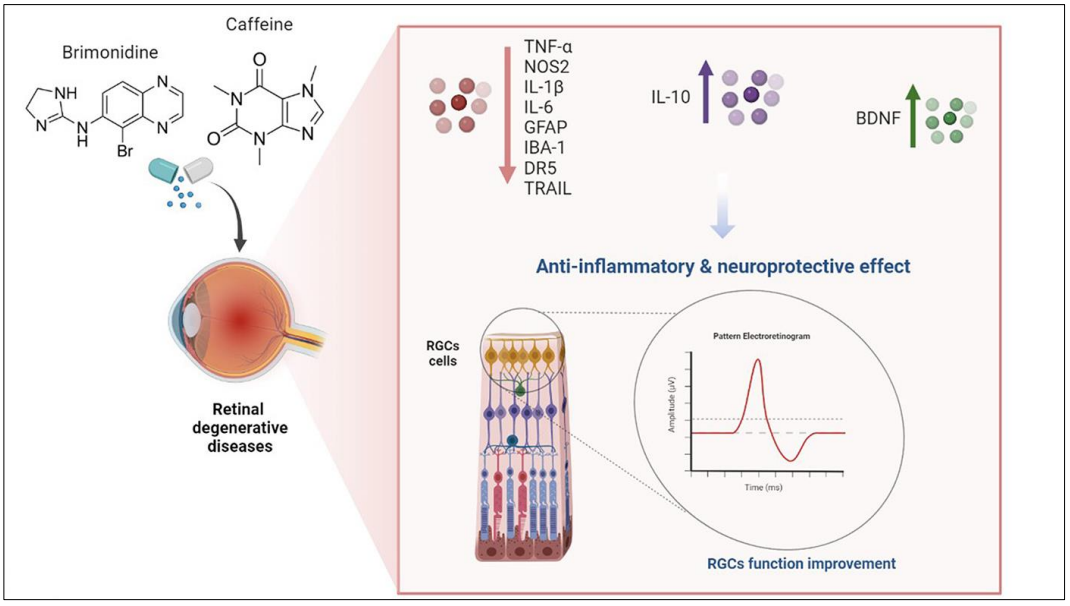

FIGURA 3: Brimonidine and caffeine anti-inflammatory and neuroprotective effects.

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LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

- *Front Pharmacol*. 2022;12:824885. Published 2022 Jan 6. doi:10.3389/fphar.2021.824885.

  Caffeine Protects Against Retinal Inflammation.

  Conti F, Lazzara F, Romano GL, Platania CBM, Drago F, Bucolo C.

- *Front Pharmacol*. 2021 Nov 5;12:778165. doi: 10.3389/fphar.2021.778165. PMID: 34803719; PMCID: PMC8602342.

  Effects of Vitamin D_3 and Meso-Zeaxanthin on Human Retinal Pigmented Epithelial Cells in Three Integrated *in vitro* Paradigms of Age-Related Macular Degeneration.

  Lazzara F, Conti F, Platania CBM, Eandi CM, Drago F, Bucolo C.

- *Cell Death Dis*. 2021 Oct 5;12(10):905.doi: 10.1038/s41419-021-04165-x. PMID: 34611142; PMCID: PMC8492692.

  Targeting the miRNA-155/TNFSF10 network restrains inflammatory response in the retina in a mouse model of Alzheimer's disease.

  Burgaletto C, Platania CBM, Di Benedetto G, Munafò A, Giurdanella G, Federico C, Caltabiano R, Saccone S, Conti F, Bernardini R, Bucolo C, Cantarella G.

- *J Neuroinflammation*. 2021 Sep 16;18(1):206. doi: 10.1186/s12974-021-02263-3. PMID: 34530842; PMCID: PMC8444391.

  1α,25-dihydroxyvitamin D3 protects retinal ganglion cells in glaucomatous mice.

  Lazzara F, Amato R, Platania CBM, Conti F, Chou TH, Porciatti V, Drago F, Bucolo C.

- *Front Pharmacol*. 2021 Jul 21;12:705405. doi: 10.3389/fphar.2021.705405. PMID: 34366858; PMCID: PMC8333612.

  Brimonidine is Neuroprotective in Animal Paradigm of Retinal Ganglion Cell Damage. Conti F, Romano GL, Eandi CM, Toro MD, Rejdak R, Di Benedetto G, Lazzara F, Bernardini R, Drago F, Cantarella G, Bucolo C.

- *Int J Mol Sci*. 2020 Dec 15;21(24):9558. doi: 10.3390/ijms21249558. PMID: 33334029; PMCID: PMC7765505.

  TGF-β Serum Levels in Diabetic Retinopathy Patients and the Role of Anti-VEGF Therapy.

  Bonfiglio V, Platania CBM, Lazzara F, Conti F, Pizzo C, Reibaldi M, Russo A, Fallico M, Ortisi E, Pignatelli F, Longo A, Avitabile T, Drago F, Bucolo C.
- *World J Stem Cells*. 2020 Oct 26;12(10):1152-1170. doi: 10.4252/wjsc.v12.i10.1152. PMID: 33178398; PMCID: PMC7596446.

  Pericyte-like differentiation of human adipose-derived mesenchymal stem cells: An in vitro study.

  Mannino G, Gennuso F, Giurdanella G, Conti F, Drago F, Salomone S, Lo Furno D, Bucolo C, Giuffrida R.
- *Int J Mol Sci*. 2020 Oct 13;21(20):7528. doi: 10.3390/ijms21207528. PMID: 33065984; PMCID: PMC7589177.

  Activation of the VEGF-A/ERK/PLA2 Axis Mediates Early Retinal Endothelial Cell Damage Induced by High Glucose: New Insight from an In Vitro Model of Diabetic Retinopathy.

  Giurdanella G, Lupo G, Gennuso F, Conti F, Lo Furno D, Mannino G, Anfuso CD, Drago F, Salomone S, Bucolo C
- *Biochem Pharmacol. 2019;168:249-258. doi:10.1016/j.bcp.2019.07.010*

  Blood-retinal barrier protection against high glucose damage: the role of P2X7 receptor.

  Platania CBM, Lazzara F, Fidilio A, Fresta C, Conti F, Giurdanella G, Leggio GM, Salomone S, Drago F, Bucolo C.

CONFERENCE PROCEDEEDINGS

- Poster: ***"Caffeine protects against retinal inflammation"***. Federica Conti, Francesca Lazzara, Giovanni Luca Romano, Chiara Bianca Maria Platania, Filippo Drago, Claudio Bucolo. 41st National congress SIF 2022-Rome.

- Poster: **"*Pericyte-like differentiation of human adipose-derived mesenchymal stem cells: an in vitro study*"**. Federica Conti, Giuliana Mannino, Florinda Gennuso, Giovanni Giurdanella, Rosario Giuffrida, Debora Lo Furno, Salvatore Salomone, Claudio Bucolo, Filippo Drago. 40th National congress SIF 2021- Digital edition.

- Poster: **"*Effects of ototoxic drugs on a new in vitro model of stria vascularis"***. Federica Conti, Giovanni Giurdanella, Florinda Gennuso, Claudio Bucolo, Filippo Drago, Salvatore Salomone. 39th National congress SIF 2019- Florence.

ABROAD EXPERIENCE

- January-June 2022: **"Evaluation of neuroprotective compounds in an *in vivo* model of retinal excitotoxicity"**, pharmacology laboratory of Professor Kyriaki Thermos, School of Medicine, University of Crete, Greece.