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The role of neurotrophins and VEGF in the maintenance of ocular homeostasis

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ABSTRACT

Neurotrophins (NTs), the proteins involved in survival and growth of neuronal and no neuronal cell types, and vascular endothelial growth factor (VEGF), which mediates blood vessel growth in healthy and pathological tissues, play a central role in ocular homeostasis. Deregulation of NTs and VEGF signalling has been observed in many eye disorders characterised by angiogenesis, which are commonly treated by intraocular anti-VEGF drug administration. Several investigations evaluated the safety profile of anti-VEGF drug ocular treatment, also showing adverse effects like a reduced cell viability, but a very few studies addressed the molecular mechanisms at the basis of anti-VEGF actions. To fill the gap in the literature about this topic, the present PhD project explored the hypothesis that the observed adverse effects following intraocular VEGF blockade might be related to the alteration of neurotrophic pathways. With this aim, the effects of intracameral (IC) or intravitreal (IVT) injections of the anti-VEGF drugs aflibercept and ranibizumab were performed in rabbits to investigate their effects on the cornea (Study I) and the retina (Study II).

In summary, the results of molecular and histological analysis revealed a decreased cell viability and increased levels of apoptosis and autophagy markers in the corneal endothelium and retina. These effects were associated with an unbalance in the expression of both pro/mature NTs, and the receptors p75 neurotrophin receptor (p75NTR) and tropomyosin receptor kinase A/B (TrkA/B). These structural and molecular changes were stronger after aflibercept injection than following the ranibizumab one, probably due to the difference related to drug structures and molecular targets. These data suggest that anti-VEGF-dependent impairment in neurotrophic signalling could be responsible for the activation of death pathways in ocular tissues, and corroborate the integrate activity of VEGF and the NTs nerve growth factor/brain derived neurotrophic factor (NGF/BDNF) in ocular homeostasis. To further characterise the NGF/VEGF mechanism, a study addressed to evaluate the effects of treatment with NGF in retina degeneration has been part of the PhD project. Since today, the recombinant human NGF (rhNGF) produced by Dompé Farmaceutici - the industrial partner of this Industrial PhD course – is the first approved drug to treat neurotrophic keratitis (NK), a rare disorder of the eye. This molecule has been used to perform Study III, where different routes of administration of rhNGF, such as ocular and systemic, intraperitoneal and subcutaneous, were tested in an animal model of glaucoma. Study III is actually ongoing and the related results are Dompé Farmaceutici's intellectual property.

KEY WORDS: eye, NGF, BDNF, VEGF, aflibercept, ranibizumab.

SECTION I – INTRODUCTION

1. Neurotrophins

Neurotrophins (NTs) are the best-known proteins mainly acting as trophic factors in the nervous system (NS). There are four well-characterized NTs in mammals: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) [1,2]. These factors have a common ancestor gene, thus sharing similarities in sequences and structures [3,4]. NTs are firstly synthesized as precursor polypeptides containing a signal sequence, a pro-domain and a mature growth factor sequence. The precursors are then cleaved by furin or pro-convertases, enzymes that process pro-molecules into their biologically active form, in order to release soluble and mature NTs [1,5–9]. Despite the production of mature NTs, these growth factors are also released in their immature forms called proneurotrophins (proNTs), which were discovered to have their own biological effect [10].

NTs play an important role in neuronal related events in the peripheral and central nervous system (PNS and CNS), not only during the development of the NS but also when it reaches its adult form. During NS development, NTs are released from target tissue in limited amounts to let the potential neuronal cells compete for interacting with them to trigger specific intracellular signals such as cell survival, axonal and dendritic growth, and guidance [4,7]. This competition brings to a selection of the surviving cells becoming neurons, and to the development of their neurites towards the target tissue that has released the NTs [7]. The responsiveness of the potential neuronal cells towards these growth factors is mainly due to the NT-specific receptors the cells express on their surface. There are two classes of NTs interacting receptors: tropomyosin receptor kinase (Trk) and p75 neurotrophin receptor (p75NTR) [1,7]. The Trk family is composed of several members such as TrkA, TrkB and TrkC that respectively bind NGF, BDNF and NT-3. TrkB can also bind NT-4/5 and even interact with NT-3 as well as TrkA [11]. Trk activation is leading by the binding to the specific ligand homodimer, which promotes the dimerization of Trk monomers to form a receptor composed by two Trk units. This event induces the trans-phosphorylation of the tyrosine residues on the cytoplasmic tail of each receptor, thus leading to conformational changes in order to initiate the downstream signalling [12].

P75NTR is a member of the tumour necrosis factor (TNF) superfamily that binds all the NTs with almost the same affinity [13]. P75NTR does not possess a catalytic domain as the Trk tyrosine kinase sequence but it is able to interact with other proteins to transduce the downstream signal after its activation by the ligands [14]. While Trk dimer receptors mostly bind mature NTs, p75NTR mainly interact with proNTs, recruiting several co-factor proteins and inducing an apoptotic signal rather than a survival one. The different release

of mature NTs and their proforms, together with the different expression of Trks and p75NTR combination on cell membranes, leads to the activation of specific intracellular pathways deciding cell fate [7] (Figure 1). NTs exert also their action in the adult NS. In the normal adult CNS, both neurons and glia express NTs, which are involved in synaptic plasticity, eliciting neurite outgrowth, and in neuronal survival, morphology and differentiation [15]. They also play further roles outside the NS in the events such as cardiac development, neovascularization and immune system function [1]. NTs seem to participate also in behavioural circuits such as learning and memory, regulating both long-term potentiation and depression (LTP and LTD, respectively), and, moreover, the growth factors exert a specific activity in neurodegenerative pathologies like Alzheimer's and Parkinson's Disease (AD and PD, respectively) [16].

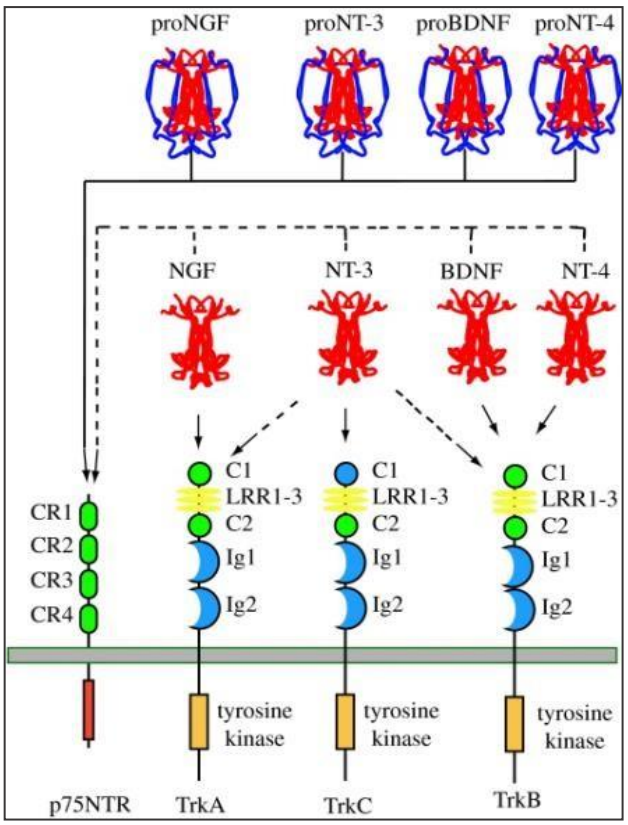

Figure 1. Neurotrophins and their receptors. CR = cysteine rich repeats, C = cystein rich cluster, LRR1-3 = leucine rich repeat, Ig = immunoglobulin-like domain [1].

1.1 Nerve Growth Factor (NGF) and its Signalling Pathway

NGF was discovered by Rita Levi-Montalcini and colleagues in the middle of the nineties [17]. During first experiments on chicken embryo and sarcomas, it was shown that a specific factor, initially named as nerve growth promoting factor and later known as NGF, could stimulate neuronal growth [18]. In collaboration with Stanley Cohen, NGF was isolated from cobra venom and mouse salivary glands, where it was found to be released in huge quantities, and characterized as a protein factor rather than a nucleic one [19–21]. The discovery of a new factor implicated in central and peripheral neurons growth, trophism and differentiation, brought Rita Levi-Montalcini to win the Nobel Prize, shared with Stanley Cohen, in 1986. NGF is initially synthesized as a proform polypeptide, known as proNGF, which goes under cleavage by furin-convertases, to be transformed in its 13 kDa mature form named mature NGF (mNGF or NGF). mNGF contains three hairpin loops followed by twisted and antiparallel β strands and a C-terminal region, hosting a cysteine-knot motif that includes three disulfide bonds [22].

NGF specifically interacts with TrkA receptor. This receptor shares the typical structure of a tyrosine kinase receptor with a large extracellular domain containing two cysteine rich clusters, three leucine repeats, followed by another cysteine cluster and two immunoglobulin-like domains. The receptor contains also a trans-membrane sequence and an intracellular part, which is mainly composed of a kinase domain. After glycosylation, the 110 kDa TrkA protein undergoes further post-translational modifications like the addition of sialic acid, generating the 140 kDa mature form. TrkA possesses important amino acidic residues that can be phosphorylated such as tyrosine 490 (Y490) and 785 (Y785), which work as major docking sites for the early adaptor protein Src homology 2 domain-containing (Shc) and phosphoinositide phospholipase C γ (PLCγ) respectively. To initiate a downstream signalling, TrkA interacts with NGF, causing the receptor phosphorylation at Y490, which brings to the activation of a pro-survival and pro-differentiation pathway. When Shc is recruited, it is in turn phosphorylated allowing the binding of the adaptor protein Grb2. The first steps of this intracellular cascade bring to the activation of Ras, which stimulates phosphatidylinositol 3-kinase (PI3K) and Raf downstream pathways. PI3K activates Akt, which phosphorylates the BCL2 associated agonist of cell death (Bad), inhibiting its pro-apoptotic activity. Raf activation brings to gene expression involved in neuronal differentiation and survival [23,24]. Akt phosphorylation through NGF-TrkA mediated pathway leads to the activation of the 40-kDa pro-rich Akt-substrate (PRAS40) and of mammalian target of rapamicin (mTOR), which promote pro-survival events [25].

While TrkA has a kinase activity, p75NTR lacks any catalytic activity [26]. P75NTR has four cysteine-rich repeats localized in the extracellular region that are all required for ligand binding [27], while the juxtamembrane domain together with the death domain (DD) and the Ser-Pro-Val (SPV) domains are situated in the intracellular region [28]. P75NTR can interact with mNGF and its precursor proNGF, acting as

a “molecular signal switch” to determine the kind of signal to be activated: cell survival or death. When the receptor binds mNGF, it can induce neuronal survival, even this is a low affinity interaction. Instead, p75NTR and proNGF mediate cell apoptosis pathways (Figure 2) by recruiting several downstream factors like the co-receptor Sortilin and the neurotrophin receptor-interacting factor (NRIF) [29]. This event promotes a signalling cascade involving the phosphorylation of c-Jun N-terminal kinase (JNK) and the consequent activation of p53, which has multiple pro-apoptotic targets like the *bax* gene [1]. After p75NTR interaction with a ligand, the receptor can undergo several cleavage steps. Due to the activity of the secretases, enzymes that cut trans-membrane proteins, there is the production of several p75NTR fragments such as p75NTR extracellular domain (p75NTR ECD) in the extracellular matrix (ECM), p75NTR intracellular domain (p75TR ICD) in the intracellular milieu, and p75NTR C-terminal fragment (CTF), which remains located in the plasmatic membrane. The p75NTR CTF, if not processed, keeps containing the p75NTR ICD sequence. Each p75NTR fragment was observed to independently initiate its own action that can be beneficial or detrimental depending upon p75NTR co-receptor interactions and cellular environment [30].

Sometimes, p75NTR-mediated apoptosis can be accompanied by autophagy, a programmed cell death event that helps the cell to get rid of damaged cellular components [31]. This process involves several proteins such as Akt and mTOR, which are capable of inhibiting it in physiological conditions while, during pathologies, their phosphorylation and related survival activities are impaired, thus leading to the potential activation of autophagy. To exert a proper function, autophagy needs the formation of the autophagosome, which is a double membrane vesicle, where the damaged cellular components are targeted and isolated to be later degraded and eventually recycled. To induce and assemble the autophagosome, some factors are recruited such as Beclin-1 and the microtubule associated protein 1 light chain 3 (LC3), which can be modified to produce a cytosolic form (LC3I) and a membrane-bound one (LC3II) [32].

NGF is a factor that can participate in several processes, through different routes, in both PNS and CNS. In CNS, NGF supports the survival of the cholinergic neurons belonging to the basal forebrain circuit (BFC) and it affects other processes as synaptic re-arrangement and dendritic arborisation. NGF plays also a role in other physiological systems such as immune, endocrine and cardiovascular [26]. NGF seems to be involved in different pathologies such as Down Syndrome (DS), which shows traits in common with AD, in Niemann Pick Disease (NPD) and hyperalgesia, through the activation of PLCγ downstream signalling [33]. In addition, the overexpression of NGF and TrkA seems to be associated with breast cancer [26]. Since NGF exerts its functions also outside the NS, it can be produced and utilized by some different cell types such as endothelial cells, epithelial cells, lymphocytes and granulocytes [34].

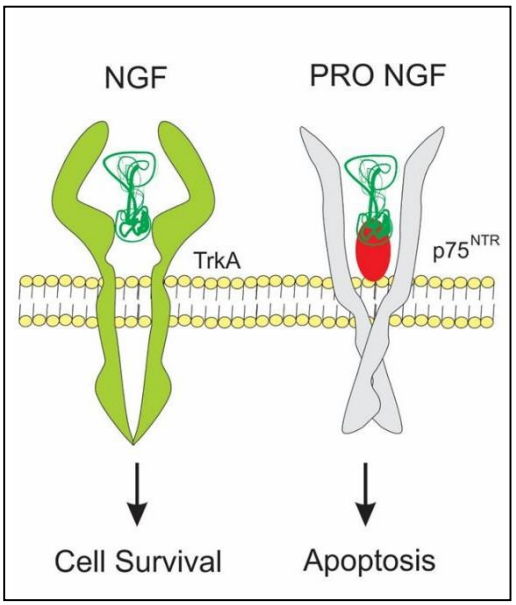

Figure 2. Effects of NGF and its receptors on cell death and survival. NGF interaction with TrkA mediates cell survival signalling while proNGF binding to p75NTR promotes apoptosis activation [35].

1.2 Brain Derived Neurotrophic Factor (BDNF) and its Signalling Pathway

BDNF belongs to the NT protein family and was discovered by Theonen and Co. at the end of the nineties [36]. This protein was observed to promote survival of dorsal root ganglion (DRG) neurons subpopulation [37], having a role in the regulation of inflammatory injury in peripheral nerves [38], and in the clinical condition known as "neuropathic pain" [39]. BDNF plays an important role in the adult tissue but also during development like the one of the visual system, being a critical factor for the correct organization of the ocular dominance columns in the visual cortex [40]. The growth factor is indeed studied because of the correlation between its decreased expression and the onset of neurodegenerative pathologies such as PD and Huntington's Disease [41]. BDNF seems to participate also in the "neurotrophin hypothesis of depression" because many studies reported that the depressive behaviour related to stress is characterized by a BDNF level decrease in hippocampus and prefrontal cortex that contributes to their cellular atrophy [42]. As for NGF, BDNF is initially synthesized in the endoplasmic reticulum of neuronal cells as an immature protein, a pre-proBDNF, and it is later cleaved in multiple sites by pro-convertase furin in Golgi apparatus becoming a proBDNF. Then, the precursor undergoes a further cleavage to reach the mature form known as mature BDNF (mBDNF or BDNF) [43]. In the end, the factor is secreted where it can be eventually modified by metallo-proteinases [44]. BDNF is usually released into the synapse where it is able to interact with its high affinity receptor TrkB, activating several intracellular pathways that are involved in critical events for connections, structure, neurotransmitter release and plasticity regulation [23].

TrkB shares a similar structure with TrkA, containing a large extracellular domain with two cysteine rich clusters, three leucine repeats, followed by another cysteine cluster and two immunoglobulin-like domains. A trans-membrane sequence is also a part of the receptor structure and it is followed by an intracellular region, which mostly contains a kinase domain.

When TrkB is activated by its ligand, it can dimerize leading to a process of autophosphorylation of amino acidic residues, tyrosines and serines, located in the receptor tail. The main intracellular pathways activated are similar to the NGF aforementioned ones, including cell survival, differentiation and plasticity, and involving several proteins as Shc, PI3K, Ras and Akt. BDNF and TrkB are both expressed in many CNS regions such as the hippocampus [45,46], the cortex [4] and the eye [47]. While the NT mature form preferentially interacts with TrkB, proBDNF, which can be released outside the cell as well as mBDNF, can act as a ligand through its interaction with p75NTR, thus activating signalling cascades through the recruitment of adaptor proteins like Sortilin [47] and including JNK as one of the major intracellular pro-apoptosis transducers [1,48] (Figure 3). As for NGF, when p75NTR is activated, it goes towards a series of cleavage steps, thus producing fragments like p75NTR CTF and p75NTR ICD, which can have their own function [49].

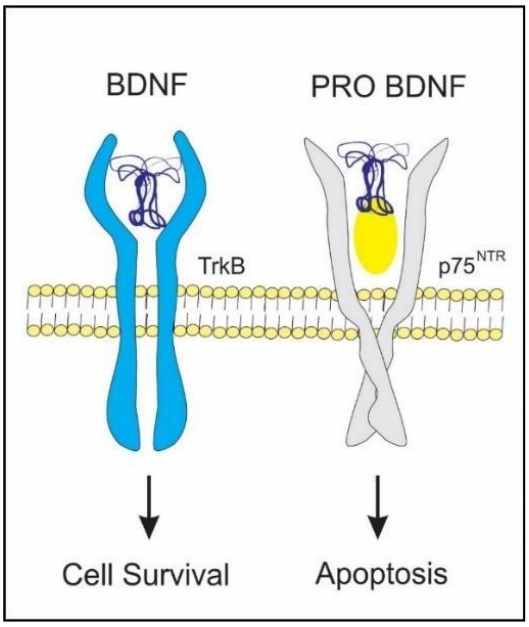

Figure 3. Effects of BDNF and its receptors on cell death and survival. BDNF interaction with TrkB mediates cell survival signalling while proBDNF binding to p75NTR promotes apoptosis activation [35].

2. Vascular Endothelial Growth Factor (VEGF)

Vascular Endothelial Growth Factors (VEGFs) are proteins belonged to a family mainly involved in vascular development and neovascularization in both physiological and pathological conditions. VEGF commonly participates in vasculogenesis and angiogenesis, which consist respectively in the generation of new blood vessels *de novo* in the embryo and from pre-existing ones in the adult. VEGF family is composed of several homodimeric glycoproteins named as VEGFA, B, C, D, E, and placental growth factor (PlGF) [50] (Figure 4). VEGFA was the first VEGF to be discovered and, initially, it was known as vascular permeability factor (VPF) [51]. VEGFA can be found in four different isoforms due to alternative splicing [52], containing respectively 121, 165, 189 and 201 amino acids. Each isoform has its own features. In fact, VEGFA121 is freely diffusible while VEGFA165 and VEGFA189 can be retained onto the cellular surface. VEGFA expression is mainly regulated by hypoxia-inducible factor (HIF), stimulating blood vessel sprouting, modifications and regeneration, during both physiological and pathological tissue growth [53].

VEGFB, also called VEGF-related factor (VRF), can be usually found in myocardium and skeletal muscle in two different isoforms, VEGFB167, which is non-glycosylated and located in the ECM, and VEGFB186 that is the diffusible glycosylated isoform [54].

While VEGFA and VEGFB derived from VEGF mRNA alternative splicing, VEGFC and VEGFD resulted from a proteolytic cleavage after protein synthesis. Isoforms C and D mainly promote mitogenesis, migration and survival of endothelial cells during lymphoangionesis both in physiological and pathological conditions [55].

VEGFE is secreted as native protein in mammalian cells, acting as a heat-stable dimer and having similar actions to those of VEGFA. When compared to the A isoform, VEGFE exerts the mitogenic activity towards endothelial cells with a different affinity for its related receptors, and it has a different capability to induce vascular permeability [56].

The VEGF forms mostly interact with three tyrosine kinase receptors known as VEGFR1, VEGFR2 and VEGFR3.

When VEGF binds VEGFR, the interaction induces a receptor homo or heterodimerization and the phosphorylation of tyrosine residues as well as the recruitment of adaptor proteins to initiate the intracellular response. A downstream cascade is activated, thus leading to several events such as cell proliferation, migration and arrangement to form vascular vessels [52].

VEGFR1 (fms-like tyrosine kinase - Flt1 - in mouse) can be found in a trans-membrane full-length form or in a soluble one (sFlt1), which derives from an alternative splicing [57], and it is mainly expressed in monocytes and macrophages. VEGFR1 binds the VEGF isoforms A and B, and also PlGF. VEGFR1 does not act as a kinase receptor due to its poor kinase activity but, by sequestering VEGFA, which can bind VEGFR2, it plays a role in the negative regulation of VEGFR2 activity during angiogenic events [58]. Through VEGFB

interaction, VEGFR1 participates to the myocardium revascularization after ischemia and to the fatty acid uptake in heart endothelial cells [59].

VEGFR2, also known also as kinase insert domain receptor (KDR) in human and as fetal liver kinase-1 (Flk-1) in mouse, is mainly expressed in vascular endothelial cells during both physiological events such as vasculogenesis and angiogenesis, and pathological ones like tumour angiogenesis [60]. VEGFR2 mainly engages interaction with VEGFA but it can also bind VEGFC when circulating in its soluble form (sVEGFR2). This event helps to inhibit the binding between VEGFC and VEGFR3, thus preventing lymphatic endothelial cells proliferation [61]. Through the interaction between VEGFR2 and VEGFA, more than one intracellular cascade can be induced depending on the amino acidic residue phosphorylation and the effect needed. To regulate angiogenesis, the pathway involves PLCγ, which hydrolyzes phospholipid phosphatidylinositol (4,5)-biphosphate (PIP_2) generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Their production is followed by protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) proteins activation [62]. When VEGFA binds VEGFR2, it can lead to the activation of heterotrimeric G-proteins signalling and phosphoinositide phospholipase C β3 (PLCβ3), which results in an essential event for endothelial cell migration during angiogenesis. Furthermore, VEGFR2 interaction with VEGFA promotes survival by activating Akt, which in turn phosphorylates the BCL-2 associated death promoter (BAD), thus inhibiting apoptosis [63].

VEGFR3, also named as Flt4, engages interaction with VEGFC and VEGFD. VEGFR3 is expressed in vascular endothelial cells while in adult VEGFR3 localization is restricted to lymphatic endothelial cells [64].

In addition, VEGF can also bind neuropilin receptor 1 (NP1) and 2 (NP2), which are trans-membrane proteins lacking catalytic activity. They are involved in neuronal guidance through semaphoring binding and can also act as co-receptors for VEGFRs, contributing to the modulation of the downstream response after ligand binding [65].

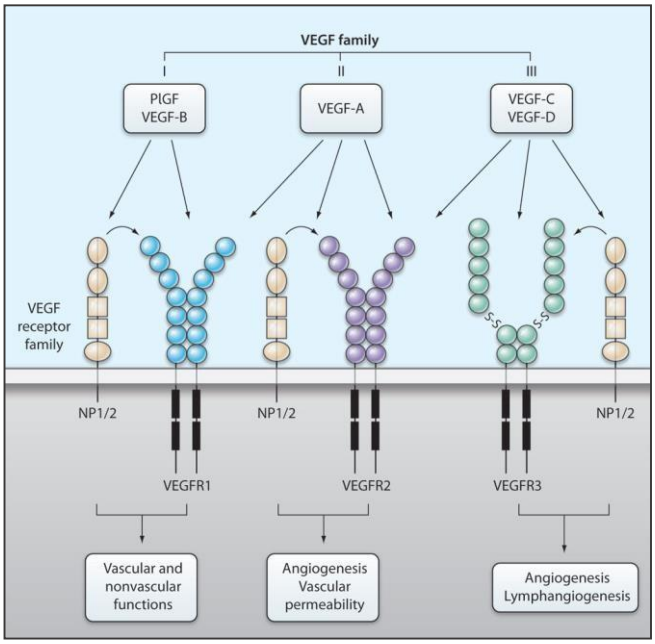

Figure 4. VEGFs and their receptors VEGFR2 and neuropilin 1 and 2 (NP1/2). After VEGFs binding to VEGFRs, different downstream signalling can be activated [66].

3. The Eye

The eye is the primary organ of vision, projecting towards the brain with its connection, represented by the optic nerve, to form the visual system. The eye, including the retina and the optic nerve, is part of the CNS, evolving embryonically from evaginations of the anterior tube lateral portions that form the optic vesicles. The main function of the eye is the transition of the external world visual information towards the brain to be cognitively processed [67]. In clinical field, the eye can be divided in the anterior and posterior segments, which are filled respectively with fluids named aqueous and vitreous humour, primary involved in nourishing the internal structures and generating pressure to maintain the eyeball shape. The anterior segment contains the cornea, the iris, the pupil, the ciliary body, and the lens. The anterior segment, in turn, can be divided into two chambers, the anterior one, which is comprised between the cornea and the iris, and the posterior chamber located between the lens and the iris. Instead, the retina, the choroid, the sclera and the optic nerve are located in the posterior segment [68] (Figure 5).

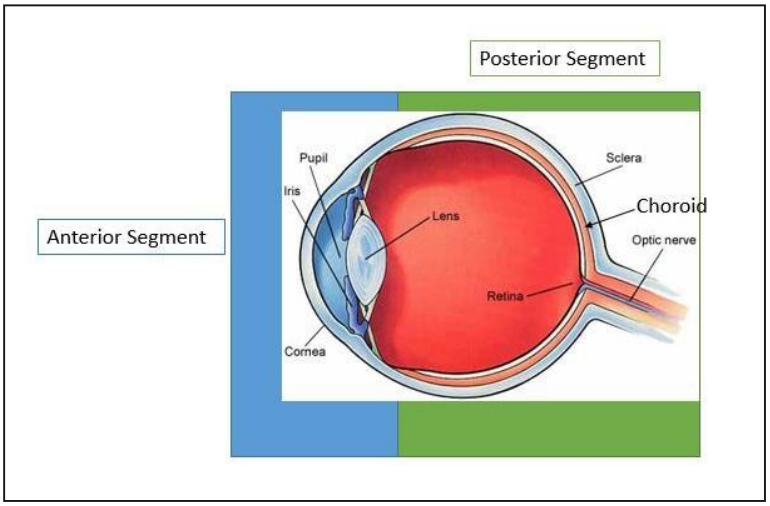

Figure 5. Schematic representation of ocular segments. Anterior segment: cornea, iris, pupil, lens. Posterior segment: sclera, choroid, retina, optic nerve (Copyright 2020 Retina Consultants of Boston).

The cornea (Figure 6) is the transparent part of the eye through which the light enters the ocular bulb. The cornea is continuously kept hydrated, because of the direct contact with the air, by the blinking of the lids. The cornea is one of the most innervated and sensitive tissue, mainly acting as a protection from foreign substances and possessing a great refractive function, which promotes, together with the lens, a correct focusing of the image [69].

The cornea consists in the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium. The epithelium is the outer part of the cornea that works to prevent the entrance of foreign substances and to accommodate the absorption of nutrients [70]. Bowman's layer and Descemet's

membrane are mostly composed of laminin and collagen, which can be also greatly found in the stroma, where they contribute to let the layer providing ocular mechanical strength and refractive power. The endothelium is the innermost layer of the cornea, playing an important role in maintaining its clarity. Because endothelial cells do not seem to appreciably regenerate, their damage can cause lasting dysfunctions. Even though the cornea is an avascular tissue, it receives blood supply from small loops of the anterior ciliary vessels, which invade its periphery for about 1 mm. Actually, these loops are not in the cornea but in the subconjunctival tissue that overlaps the cornea. Other nutrients are supplied *via* diffusion from tear fluid at the outside and from aqueous humour from the inside [71].

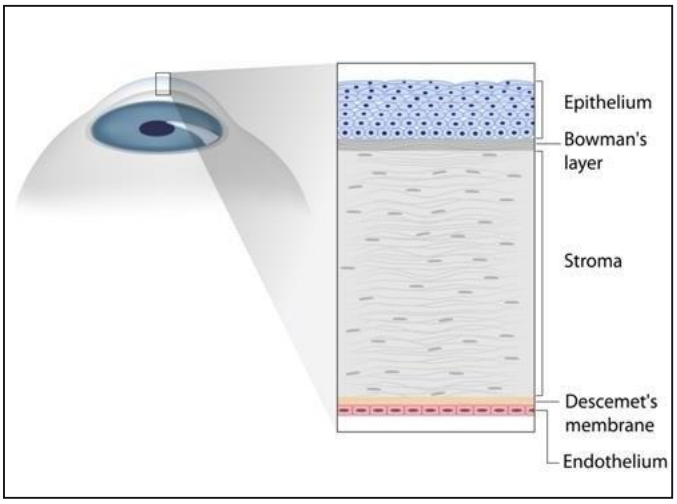

Figure 6. Cornea. Schematic representation of corneal layers of the eye: epithelium, Bowman's layer, stroma, Descemet's membrane, endothelium (https://www.occhioallaretina.it/ cornea/website).

The retina (Figure 7) is the inner part of the eye. It is composed of several layers where neuronal cell bodies and synapses are distributed and interconnected. The outer nuclear layer (ONL) contains neuro-epithelial cells named photoreceptors, highly specialised in photo-transduction, a process able to convert light energy into electrical signals [72], which are codified into optic nerve impulses by retinal interneurons and then elaborated by the brain [73]. There are two types of photoreceptors: rods and cones. Rods are more numerous than cones and specifically sensitive to half-light vision, responding to black and white vision. Instead, cones are activated in response to bright light, allowing the perception of colours, and they are highly concentrated in a specific part of the retina named macula, where it is located the centre of visual acuity named fovea [72]. Adjacent to the ONL, there is the retinal pigmented epithelium (RPE) that is essential for the maintenance of rods and cones. RPE is also a source of retinal pluripotent cells able to generate new photoreceptors, glial cells and neurons. The inner nuclear layer (INL) contains different retinal classes of interneurons such as bipolar cells, which transfer the visual information from photoreceptors to retinal ganglion cells (RGCs), the inhibitory interneurons between bipolar cells and RGCs

called amacrine cells, and the horizontal cells, which regulate the activity of photoreceptors and transfer the information to bipolar cells, by parallel connections, to retinal layers [74]. The ganglion cell layer (GCL) comprises the RGCs cell bodies whose axons form the nerve fiber layers, converging in the optic disk forming the optic nerve. Together with the optic nerve, the central artery enters the bulb to bring blood supply to the inner retina [75]. The synaptic contacts between RGCs, bipolar and amacrine cells are localized in the inner plexiform layer (IPL), which is located between INL and GCL, while the outer plexiform layer (OPL) can be found between ONL and INL, containing the interactions between the axons of photoreceptors and the dendrites of horizontal and bipolar cells [73]. Besides neurons and photoreceptors, other representative retinal cells are the Müller cells, which belong to the microglia population, and astrocytes, passing through all the retinal layers and responsible for homeostatic and metabolic support of the retina. Müller cell bodies are located in the INL, whereas their processes expand throughout the neural retina. Activated astrocytes and Müller cells constitute a process called reactive gliosis, which has an acute neuroprotective effect but, as it persists chronically after injury, it also limits regeneration and exacerbates neurodegeneration, causing direct and indirect damage to neurons [73]. The eye and brain areas receiving retinal inputs constitute the visual system. In all mammals, including humans, vision is guaranteed by the integrity of retinofugal pathways that transfer information from the eye to the brain visual areas and by survival and regulatory inputs retrogradely directed to the retina [76].

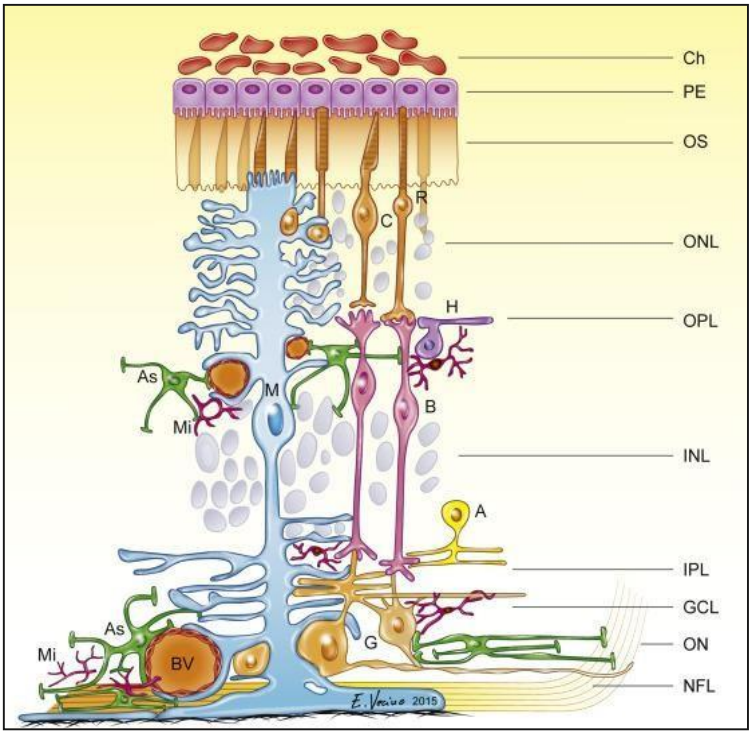

Figure 7. Schematic representation of retina layers. Abbreviations: Amacrine cells (A), astrocytes in green (AS), bipolar cells (B), cones (C), ganglion cells (G), horizontal cells (H), Müller cells (M, in blue), microglia (Mi, in red), rods (R), cones (C), optic nerve (ON), nerve fibre layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), outer segment layer (OS), pigment epithelium (PE), choroid (Ch), interactions between the cells and blood vessel (BV) [69].

4. Neurotrophins and VEGF in the Eye

The growth factors NTs and VEGF play an important role in the ocular milieu. NTs and their related receptors are expressed from a variety of tissues in the anterior segment of the eye such as corneal epithelium, stroma and endothelium [77] as well as in the vitreous, choroid and retina, located in the posterior segment, thus regulating the development and differentiation of several cellular types [78].

NGF and BDNF proteins are synthesized and released in the highly innervated corneal tissues, promoting growth, proliferation and survival, during different conditions as corneal healing from ulcers or scars, and physiological ones [77].

NTs result to be very important regulators of retinal development but also major players in regeneration of neural circuits in the visual system during retinal degenerative diseases. As expected, the lack of neurotrophic support was associated with retinal neurodegeneration and in particular with RGCs loss [79].

VEGF isoforms are greatly produced by several cellular types in the eye posterior segment such as those belonging to RPE, endothelial cells, pericytes, astrocytes, Müller cells, amacrine, and RGCs [80]. VEGFs are found to be responsible not only for vascular-related processes but also for contributing to normal retinal function and neuroretinal cell apoptosis limitation [81].

Even though the cornea is not vascularized because it has to be maintain clear to ensure light entrance in the eye, its cells can produce VEGFs and their specific receptors, especially during pathological conditions characterized by corneal neovascularisation (CNV) [82].

NTs and VEGF can act in a combined and concerted manner in order to mediate neuroprotection in the eye. In fact, increasing evidence suggests that these factors possess reciprocal angiogenic and neurotrophic properties, acting on neurons and blood vessels in a paracrine or autocrine fashion [83].

5. Eye Pathologies and Treatments

Vision is often considered as the most important of senses, and the one that most people fear losing. Because of their specialized structures, cornea and retina are vulnerable to a broad range of physical, chemical and pathological injures. In fact, both these ocular tissues can be interested from eye pathologies that can cause vision impairment such as keratitis, CNV, diabetic retinopathy (DR), glaucoma (GC) and aged-related macular degeneration (AMD) [84,85].

CNV consists in an abnormal growth of blood vessels in the cornea, which is usually avascular, from pre-existing pericorneal vascular structures. This event, characterised by a disequilibrium between pro-angiogenic and anti-angiogenic factors, is related to a non-specific corneal response to several traumas such as allergies, hypoxia and congenital or autoimmune diseases. CNV, when it is not properly treated, can compromise visual acuity and can eventually lead to visual loss [86].

Keratitis is an eye disease mainly characterised by an inflammatory condition of the cornea, which can be related to an infection brought by virus, bacteria or fungi other than be a result of pathological complications. If non-properly treated, the disease can cause corneal scars and perforation, thus promoting visual impairments, and even the loss of the eye. Among the various types of keratitis, it is relevant to note the neurotrophic keratitis (NK), which is a rare and chronic degenerative corneal condition caused by a lesion of the trigeminal nerve, as well as a consequence of corneal surgeries and of complications of diabetes mellitus and multiple sclerosis [87].

DR is the most common form of diabetic eye disease and one of the major cause of reduced vision in working age population. DR is a retinal disorder, characterized by different stages of progression including pericyte/vascular dysfunction, which leads to the loss of blood retina barrier (BRB) proper functionality, inflammation, neurodegeneration and the potential onset of diabetic macular edema (DME), thus decreasing visual acuity [88]. One of the distinct trait of DR is angiogenesis, which is mediated by a deregulated VEGF expression, caused by the hypoxic environment that characterises the stages of the pathology, and possibly resulting in the onset of proliferative diabetic retinopathy (PDR) [89].

GC is a term used to describe a group of conditions with a chronic and progressive optic neuropathy, characterized by gradual RGCs degeneration and morphological changes in the optic nerve, constituted by optic nerve axons, together with progressive deficits of the peripheral and central visual field [90]. It remains a leading cause of irreversible blindness throughout the world and one of the most common neuropathies. Although the aetiology of GC is still a matter of intense investigation, the risk factors that accelerate progression are the elevated intraocular pressure (IOP), age, thinner corneal thickness, vasculature dysregulation and genetic background [91].

AMD is a degenerative disease that affects the macula, located in the central part of the retina, causing a

progressive vision lost. There are some AMD types such as dry AMD, which is characterised by yellow deposits under the retina (drusen), and wet AMD, involving choroidal neovascularization [92].

To counteract the onset and the progression of eye related pathologies, many treatments that take advantage of drug ocular administrations, are performed. Intravitreal (IVT), subconjunctival and retrobulbar injections, together with topical drops, represent the most common routes of ocular drug administration. These methods can be used to treat different ocular diseases affecting the anterior and posterior segments of eye (Figure 8). In general, IVT injection, which is performed in the eye posterior segment near the retina, and topical application as eye drop (ed), are at opposite ends in terms of bioavailability and efficacy on the retina, being conceived for direct drug delivery to the posterior segment and treatment of the anterior segment respectively [93]. Recently, it was introduced a new route of intraocular administration named intracameral (IC) injection, through which the drug is directly released in the fluid of the anterior chamber of the eye, immediately behind the cornea. IC injection seems to offer an alternative to IVT injection in order to reduce certain risks related to the IVT procedure like the increase of IOP in the vitreous chamber [94].

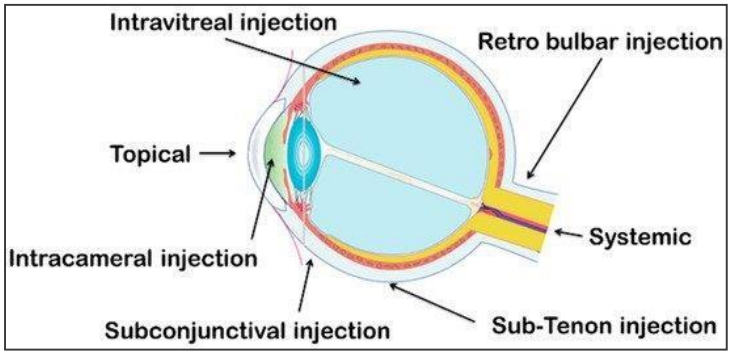

Figure 8. Different routes for ocular drug administration [95].

5.1 Anti-VEGF drugs injections

Since many eye related disorders involved a deregulation of blood vessel growth such as DR, AMD, and PDR, IVT injection of anti-VEGF drugs is known to be the gold standard treatment to counteract the process of angiogenesis, which is one of the main feature of the aforementioned pathologies. In fact, anti-VEGF drugs inhibit the action of VEGF that it is primarily involved in abnormal blood vessel growth during related pathological conditions in the eye [96]. Through the years, several anti-VEGF drugs were developed and approved to be used as successful treatments, which are mainly intravitreally, and recently even intracamerally, injected in the patient eye.

The first anti-VEGF drug to be approved by the Food and Drug Administration (FDA) was pegaptanib, an oligonucleotide ligand that selectively bound the 165 isoform of VEGFA to treat neovascular AMD [97]. Immediately after, bevacizumab, whose commercial name is Avastin, was produced as a full-length humanized monoclonal antibody of 148 kDa, capable of binding all VEGF forms. At the beginning, Avastin was only used as a medication for various type of cancers such as colorectal, lung and renal but, because of its characteristics, bevacizumab has started to be also used as an off-label drug to treat CNV, DME, and macular edema due to retinal vein occlusions [98].

From bevacizumab structure, two more anti-VEGF drugs were produced, ranibizumab and aflibercept. Ranibizumab (commercial name Lucentis) is a smaller anti-VEGF agent, which corresponds to the Fab fragment of bevacizumab antibody, able to easily penetrate into the ocular tissue and allowing a faster systemic clearance. Ranibizumab is capable of binding all the different VEGFAs and it is approved to treat patients affected by macular edema following retinal vein occlusion, DME and DR [99].

Aflibercept, whose commercial name is Eylea, was instead generated by the combination of the second domain of VEGFR1 and the third domain of VEGFR2 with a human ImmunoglobulinG (IgG) Fc fragment. Aflibercept recognizes all the different VEGFAs as well as PlGF and VEGFB. The drug is mainly used to treat neovascular AMD and DME. Aflibercept is known also as “VEGF-trap” because of its unique ability to bind both sides of the VEGF dimer and to interact with PlGF, “trapping” these factors and blocking their potential interactions with the related receptors [100].

5.2 recombinant human NGF (rhNGF) and related drug administration

Due to its role in the ocular tissues, NGF has been investigated for its potential use as clinical application to treat eye diseases [87]. Several *in vivo* studies have been already performed testing murine NGF when injected intraocularly in animal models of ocular hypertension, optic nerve crush and ischemia, showed the ability to inhibit RGCs degeneration [101]. Furthermore, NGF was also tested as eye drops in rat model of GC, evidencing the neuroprotective function in the optic nerve, after being capable of reaching the retina [102]. NGF based drug formulations, which can contain the factor of murine origin or also human NGF, were also used to perform clinical studies on patients with corneal neurotrophic ulcers, moderate and severe NK, promoting a restoration of corneal integrity [103]. Recently, a new human NGF named recombinant human NGF (rhNGF), produced in *Escherichia coli*, has been introduced as part of an eye drop formulation. It was successfully tested in a phase I clinical trial showing a good safety and tolerability profile [104]. European Medicine Agency (EMA) licensed a full market authorisation for the use of rhNGF eye drops (Cenegermin) to treat moderate and severe NK, a rare ocular disease characterised by impairment of corneal sensitivity, healing and tear production (EMA/351805/2017; Committee for Medicinal Products for Human Use) [105].

SECTION II - MATERIALS AND METHODS

6. Study I

6.1 Animals and Pharmacological Treatment

To evaluate the effects of anti-VEGF treatment on corneal endothelium, 25 µl of ranibizumab, aflibercept, or vehicle were injected in the anterior chamber of adult male eyes of New Zealand White rabbits. Two concentrations of each drug were used: ranibizumab at concentrations of 10 and 5 mg/ml and aflibercept at concentrations of 40 and 20 mg/ml (n = 8 for each group). As a further control for biochemical studies, two additional groups of animals were treated with intravitreal (IVT) of 25 µl of ranibizumab (10 mg/ml) or aflibercept (40 mg/ml; n = 8 for each group). Both the intracameral (IC) or IVT injections of anti-VEGFs were performed in the left eye, whereas the same amount of isotonic buffer was injected in the right eye of each rabbit (internal control, CTR). All intraocular procedures were performed under sterile conditions using an operating microscope for visualization. Before injections, topical anaesthesia was induced by tetracaine (1%) eye drops. Povidone-iodine was applied to the eyelid margins and the lashes. After application of a sterile drape, a lid speculum was inserted. Povidone-iodine (5%) was applied to the conjunctiva bulbi and the fornices for at least 3 min. IVT injection was performed using a 30-G needle attached to a tuberculin syringe, inserted bevel up approximately 2 mm posterior to the limbus. IC was performed through a 30-G needle attached to a tuberculin syringe at the limbus, in the inferior peripheral cornea, between the 4 and 8 o'clock position. Slit-lamp and funduscopic examinations were performed, and the animals were observed in the post-injection period for signs of infection, inflammation, or toxicity. After treatment, animals were returned to their cages and were monitored for the next 4 hr to verify the recovery from anaesthesia and surgery. Rabbits were killed 1 week after treatment, eyes were removed, and the cornea was dissected and processed for histology, immunohistochemistry (IHC), and biochemical analyses. All research animals were obtained and used in compliance with National and Institutional regulations. Animals were maintained in accordance with the guidelines of the European Communities Council regulatory guidelines. This study was approved by Animal Care and Use Committee and Italian Ministry of Health (Authorization n.668/2016-PR). A veterinary also checked the health status before starting the experiments. All rabbits were kept untouched for 2 weeks in their cages to recover the stress from transport and to get habituated to the conditions of the new animal facility, the Interdipartimental Service Centre for Animal Technology, located in Tor Vergata University of Rome.

6.2 Endothelial Cell Viability Evaluation

Fresh corneas were cut in two parts. One part was used to quantify the effects of treatment on corneal endothelial cell viability by evaluating Trypan Blue (TB) incorporation. Specifically, the half fresh corneas placed endothelial side-up were covered with TB solution (Sigma Aldrich, Milan, Italy) for 5 min and then washed with phosphate buffered saline (PBS) to remove dye excess. Stained tissues were visualized by phase contrast microscopy, equipped with digital camera and connected to a computer; two images per cornea were taken at ×10 magnification. Cells incorporating TB were counted on binary images by computer morphometric program (Nikon NIS-Elements AR 2.30 Nikon Instruments Europe BV, Amsterdam, The Netherlands), which discriminates stained dead cells by area size and gray levels. Data are presented as means of TB cells/mm^2 per group of treatment ± standard deviation (SD).

6.3 Immunohistochemistry and Immunofluorescence Evaluation

The other part of the cornea was fixed in 4% paraformaldehyde in PBS solution for 24 hr and then dehydrated in 20–30% sucrose solution. The cornea were sectioned at 20 µm thicknesswith a cryostat (Leica Microsystems GmbH, Wetzlar, Germany), mounted on superglass slides and processed for IHC and IF. IF was performed on cornea slices by using the primary antibodies against p75NTR (1:10, MAb192; Millipore, Milan, italy) and cleaved caspase3 (c-Casp3; 1:100, #9661; Cell Signaling Technology, The Netherlands). After the overnight 4°C incubation with the specified antibodies, Alexa Fluor anti-rabbit 594 or Alexa Fluor anti-mouse 488 (Invitrogen Corporation, Carlsbad, CA) was used as fluorochrome-conjugated secondary antibodies. They were washed with PBS and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, CA) 5 min at room temperature (RT). After several washes, coverslips were mounted on glass slides (Superfrost Plus; Thermofisher, Carlsbad, CA) with the antifading mounting medium Prolong Gold (Life Technologies). Images were acquired through a confocal laser scanning microscope (CLSM; SP5 Leica Microsystems, Wetzlar, Germany) equipped with four laser lines. The figures were generated by adjusting only image brightness and contrast and composed by using Photoshop CS6. Immunohistochemistry was performed on cornea slices using a primary antibody against mouse monoclonal Rho-A (1:100, sc-418; Santa Cruz Biotechnology, Dallas, Texas, USA) and ABC Vectastain Kit (Vector Lab. Inc., Burlingame, USA) for reaction development. The IHC preparations were visualized on the Zeiss Axiophot phase contrast microscopy, equipped with a digital camera (KY-F558 JVC Camera).

6.4 Western Blot and Pathscan Analysis Evaluations

For biochemical analysis, the endothelium was removed from the stroma by gentle scraping, using a surgical razor blade and then processed for cornea protein extraction. Proteins were extracted in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 8 M urea, 100 mM NaCl, and 10 mM dithiothreitol (DTT) and measured by using Bio-Rad protein assay. Sample proteins were used for western blot (WB) analysis and PathScan Intracellular Signaling assay kit (Cell Signaling Technology, The Netherlands). In WB analysis, sample protein extracts (20–50 µg of total protein) were dissolved in loading buffer (0.1 mol/L Tris–HCl buffer, pH 6.8, containing 0.2 mol/L DTT, 4% sodium dodecyl sulfate, 20% glycerol, and 0.1% bromophenol blue), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes. The membranes were incubated for 1 hr at RT with 5% nonfat-dried milk dissolved in tris-buffered saline with tween 20 (TBS-T; 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl and 0.1% Tween-20), washed three times for 10 min each in TBS-T, and then incubated overnight at 4°C with primary antibodies mouse monoclonal anti-p75NTR (1:1000, sc-271708; Santa Cruz Biotechnology); mouse monoclonal anti-phospho TrkA (1:1000, sc-271708; Santa Cruz Biotechnology) and anti-mouse TrkA (1:1000, sc-7268; Santa Cruz Biotechnology); mouse monoclonal proNGF (1:1000, sc-365944; Santa Cruz Biotechnology), mouse monoclonal RhoA (1:1000, sc-418; Santa Cruz Biotechnology); anti-VEGF-R2 (1:1000, Flk-1 (D-8): sc-393163; Santa Cruz Biotechnology) and anti-phospho VEGFR2 (1:1000 Mabs191; Millipore-MERCK, Italy). After incubation, membranes were washed two times for 10 min each in TBS-T and incubated 1 hr at RT with horseradish peroxidase–conjugated anti-mouse IgG (1:5000; Cell Signaling Technology, Danvers, MA) secondary antibodies. The blots were developed with ECL Chemiluminescent HRP Substrate (Millipore) as the chromophore. The public-domain ImageJ software (http://rsb.info.nih.gov/ij/) was used for gel densitometry. The band quantification was performed following the tutorial (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-/) created to standardize the gel band quantification to eliminate sample-to-sample/gel-to-gel variability. The integrated density of anti-Actin (1:5000, Santa Cruz; sc-47778 HRP) served as normalizing factor. Eight corneas were used for each group (CTR, IC aflibercept or IC ranibizumab treatment). Data are expressed as relative optical density (arbitrary units) and presented as the means ± SD.

The PathScan Intracellular Signaling assay kit (Cell Signalling Technology) was used according to the manufacturer's instructions to detect the effect of IC or IVT administration of aflibercept and ranibizumab on signalling pathways in rabbit corneas. The kit allows the simultaneous detection of

18 activated signalling molecules involved in cell cycle progression, growth, and survival, including ERK1/2 (Thr102/Tyr204), STAT1 (Tyr701), STAT3 (Tyr705), AKT (Thr308), AKT (Ser473), AMPKa (Thr172), S6 Ribosomal Protein (Ser235/236), mTOR (Ser2448), HSP27 (Ser78), Bad (Ser112), p70S6K (Thr389), Pras40 (Thr246), p53 (Ser15), p38 (Thr180/Tyr182), Sap/JNK (Thr183/Tyr185), GSK-3β (Ser9), PARP (Asp214) and caspase 3 (Asp175). Protein samples were diluted in array diluent buffer to 1 mg/ml. Glass slides with antibody-spotted nitrocellulose pads were connected with a multiwell gasket for blocking each pad with 100 µl array blocking buffer per well for 15 min, followed by 16 hr incubation at 4°C with 75 µl of diluted samples/well. After four washing steps with 100 µl of array wash buffer, the pads were incubated with 75 µl of the detection antibody cocktail for 1 hr at RT. LumiGLO reagent was used to reveal the bound detection antibody by chemiluminescence. The images were captured with the UVItec gel documentation system (UVItec Limited, Cambridge, UK) and the spot intensities were quantified using the software Nikon NIS-Elements AR 2.30 (Nikon Instruments Europe BV, The Netherlands). A fixed threshold over the background and feature restriction functions were applied to define the measurable spot area and intensity. Measurements were standardized between the experimental groups using the same calibration system and threshold. Data are expressed as mean optical density (arbitrary units) and presented as the mean ± SD.

6.5 Statistical Analysis

Statistical analysis was performed by GraphPad Prism software (GraphPad Software Inc.) and StatPlus Software for Mac (Analyst-Soft Inc.). Cell count and biochemical data were compared with analysis of variance using the treatment as variables. Significance between groups was evaluated by Tukey–Kramer's post hoc test: $p<0.05$ was considered statistically significant.

7. Study II

7.1 Animals and Pharmacological Treatment

All procedures involving animal care or treatments were approved by the Italian Ministry of Health (Authorization n.668/2016-PR) and performed in compliance with the guidelines of the Italian Ministry of Health (according to Legislative Decree 116/92), the Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. Adult male New Zealand White (NZW) rabbits were maintained under controlled temperature, humidity and illumination, in the Interdipartimental Service Centre for Animal Technology, located in Tor Vergata University of Rome. Food and water were provided *ad libitum*. A veterinary also checked the health status before and during the experiments. All rabbits were kept untouched for 2 weeks in their cages to recover the stress from transport and to be habituated to new animal facility conditions. In the attempt to evaluate the prospective role of VEGF on neurotrophin signalling pathways and retinal cell viability, 25 µl of ranibizumab, aflibercept or vehicle were administered through IVT injection in the eyes of male rabbits. Ranibizumab was used at a concentration of 10 mg/ml, and aflibercept at the concentration of 40 mg/ml. The same volume of isotonic buffer was injected in the eyes of animals, which served as controls (vehicle group). A disposable syringe with a 30-gauge needle was used for ranibizumab, aflibercept or vehicle injection. The needle was introduced into the vitreous cavity, 1.0–1.5 mm posterior to the superotemporal limbus. The volume of solution was administered slowly and, to avoid reflux from the entry site, the needle was held in place for 10 s before its gentle extraction. Binocular indirect ophthalmoscopy was performed before and after each injection to exclude the presence of potential iatrogenic lesions. After treatment, rabbits were returned to their cages, and were monitored for the next four hours to verify the recovery from anaesthesia and surgery. Rabbits were killed 1 week (n = 8 for each experimental group) and 4 weeks (n = 8 for each experimental group) after anti-VEGF administration, the eyes were enucleated, and the retina removed from the rest of the eye structures and stored at −80°C for subsequent biochemical analyses.

7.2 Lysate Preparation from Retina Samples

Tissue lysates were prepared according to the previously described protocol [106]. Briefly, retinas were homogenized in a homogenization buffer (0.01 M Tris-HCl, 0.001 M CaCl2, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, phosphatase inhibitor cocktail, pH 7.5), and the homogenate was then lysed by sonication. Samples were then centrifuged at 10,000 g for 10

min, to remove tissue debris. Protein concentration was evaluated by the method of Lowry, Rosebrough, Farr, and Randall (1951). All lysate samples were boiled for 3 min before loading to the SDS-PAGE for subsequent western blot analysis.

7.3 Western Blot Analysis

Western blot was performed as previously reported [107]. Briefly, proteins (30 μg) were resolved by 7–15% SDS-PAGE at 30 mA (constant current) for 60 min. Protein transfer onto nitrocellulose membrane was carried out by using trans-blot turbo transfer system (Bio-Rad Laboratories, Milan, Italy) for 10 min at RT. The nitrocellulose membrane was blocked with 5% fat-free milk in Tris-buffered saline (0.138M NaCl, 0.027M KCl, 0.025M Tris-HCl, and 0.05% Tween-20, pH 6.8) at RT for 1 hr, and probed at 4°C overnight with primary antibodies against p-TrkA (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8058), TrkA (Santa Cruz Biotechnology; sc-118), p-TrkB (Santa Cruz; sc-135465), TrkB (Cell Signaling Technology, Danvers, MA; 4603S), p75NTR (Santa Cruz; sc-271708), BDNF (Santa Cruz; sc-546), NGF (Santa Cruz; sc-365944), LC3 (Sigma; L7543), Beclin1 (Santa Cruz; sc-48341), and caspase-3 (Santa Cruz; sc-7272). Subsequently, membranes were incubated for 1 hr with horseradish peroxidase-conjugated secondary IgG antibodies (Bio-Rad Laboratories). The nitrocellulose membrane was then reprobed with anti-Actin (Santa Cruz; sc-47778 HRP). Bound antibodies were visualized using Clarity Western ECL substrate (Bio-Rad Laboratories) and image acquisition was performed through using enhanced chemoluminescence detection (GE Healthcare, Little Chalfont, UK) and exposure to Amersham Hyperfilm ECL (GE Healthcare). Images derived from western blot were analysed with ImageJ (National Institute of Health, Bethesda, MD) software for Windows. All samples were normalized for protein loading by using actin, which serves as protein loading control. Values were obtained from the ratio between arbitrary units derived by the protein band and the respective actin band.

7.4 Statistical Analysis

Results are expressed as mean ± SD. Data were analyzed with oneway analysis of variance followed by the Tukey's post-hoc test. Values of $p<0.05$ were considered to indicate a significant difference. Statistical analysis was performed using GraphPad InStat3 (GraphPad, La Jolla, CA) for Windows.

8. Study III

8.1 Animals

All animal experiments were in accordance with the regulations of UK Home Office and the statement of Association for Research in Vision and Ophthalmology for the use of animals in research and were performed under general anaesthesia. In total, 33 adult male Dark Agouti (DA) rats (150–200 g, Harlan Laboratories, UK) aged 8 weeks were used in this study. The rats were housed in a temperature (21°C) and humidity-controlled environment with a 12 hr light-dark cycle (140–260 lx) in the Biological Research Unit (BRU), University College of London (UCL) – Institute of Ophthalmology (IoO) (PPL 708787). Water and food were available *ad libitum*. *In vivo* experiments were conducted under general anaesthesia with a mixture of 37.5% Ketamine, 25% Domitor and 37.5% sterile water, at 2 ml/kg administered intraperitoneally.

8.2 Treatments

Animals were divided in several groups. Group 1 was left untreated (naïve) for normal control. Animals in groups 2 and 3 received topical treatment either placebo or rhNGF eye drops, two drops daily for 3 weeks following intraocular pressure (IOP) elevation. Animals in groups 4 and 5 received a subcutaneous administration either vehicle or rhNGF containing solution, a single injection daily for 3 weeks following IOP elevation. Animals in groups 6 and 7 received an intraperitoneal administration either vehicle or rhNGF containing solution, a single injection daily for 3 weeks following IOP elevation.

8.3 Rat Model of Glaucoma

In this study, a well-established rat model of chronic ocular hypertension (OHT) was used. Chronic OHT was surgically induced in rats aged at 8 weeks using Morrison's method, as previously described and characterized [108]. Briefly, IOP was elevated in the left eye of each animal by the injection of 50 µl of hypertonic saline solution into the episcleral veins, using a syringe pump (50 µl/min, UMP2, World precision Instruments, Sarasota, FL, USA). A propylene ring with a 1 mm gap cut out of its circumference was placed around the equator to prevent injected saline outflow from other aqueous veins. Contralateral, nonsurgical eyes acted as the control [109].

8.4 IOP measurements

Under inhalational anaesthesia of a mixture of oxygen and isoflurane (0.4% isoflurane in oxygen, Merial; Animal Health, Ltd., Essex, UK), with a handheld tonometer (Tonopen XL; Medtronic Solan, Jacksonville, FL), the IOP of both eyes in each rat was measured. IOP measurements were taken the day of glaucoma induction before surgery and the day after, and at regular weekly intervals after surgery (7, 14 and 21 days).

8.5 Tissue Preparation

At the end of the treatments, each animal was euthanized by cervical dislocation and the eyes were enucleated. Some eyes/each group were dissected to collect the retina, while the remaining eyes/each group were placed in PFA 4% for successive histology.

8.6 Western Blot, Immunofluorescence and IOP analysis

Western Blot, Immunofluorescence and IOP analysis, defined as the integral of IOP elevation over time (mm Hg/day), are currently on going. Dompé Farmaceutici owns the intellectual property of the results.

SECTION III – RESULTS

9. Study I: Effects of anti-VEGF intracameral (IC) injection on corneal endothelial cells

9.1 The inhibitory effect of anti-VEGF drugs on VEGFR2 and its activation

In order to validate the inhibitory effect of the drugs used on VEGF activity in rabbit cornea, the expression of VEGFR2 and its phosphorylation levels were analysed by WB technique. As reported in Figure 1 a,b, IC injection of aflibercept (A) 40 mg/ml and ranibizumab (R) 10 mg/ml results in undetectable levels of p-VEGFR2 when compared to control, without affecting the expression of VEGF receptor. In line with this, a significant reduction of the p-VEGFR2/total VEGFR2 ratio is found after anti-VEGF treatment (A *vs.* CTR, *p<0.05; R *vs.* CTR, *p<0.05). These data support the efficacy of aflibercept and ranibizumab to inhibit VEGF activity in rabbit cornea after their IC injection.

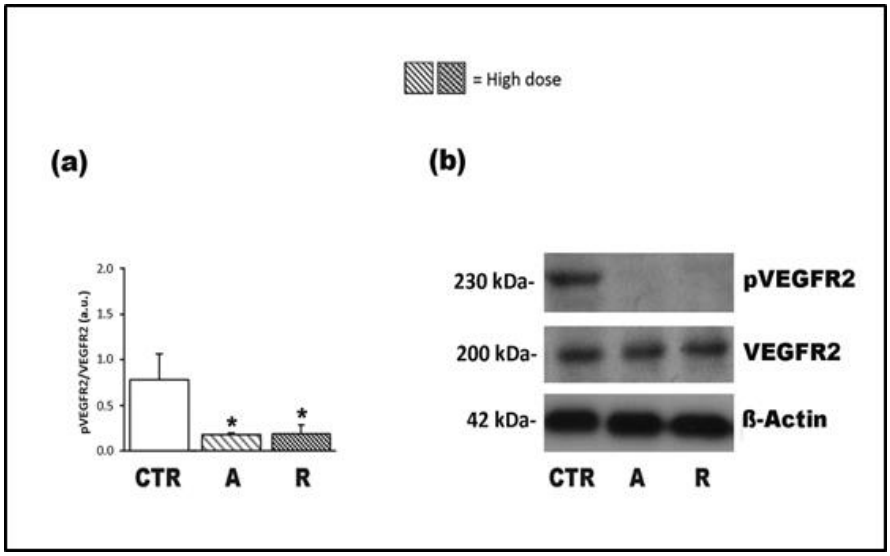

Figure 1. Western blot analysis of total and phosphorylated VEGFR2 (VEGFR2, pVEGFR2) in rabbit corneal endothelial cells. The effect of anti-VEGF compounds is shown in (a) and a representative western blot reporting the band size of each protein is shown in (b). Data are expressed as optical density (arbitrary units) and presented as mean ± SD. Statistically difference: *p<0.05, *vs.* CTR.
CTR: control, A: aflibercept high dose, R: ranibizumab high dose.

9.2 Analysis of corneal endothelial cell viability

To investigate the potential side effects of aflibercept (A) or ranibizumab (R) IC treatments, in terms of cell viability of the corneal endothelial layer, a Trypan Blue (TB) assay was performed comparing treated animals with the control group (CTR). Each anti-VEGF drug was used at two different concentrations: A at 40 and 20 mg/ml and R at 10 and 5 mg/ml following indicated as high and low dose respectively. The assay shows a significant increased number of TB stained endothelial cells after IC administration of both doses of

anti-VEGF drugs compared to the control group (A *vs.* CTR, **p<0.01; R *vs.* CTR, **p<0.01). The TB incorporation was higher in the A treated groups when compared with the R treated ones for both the doses used (A *vs.* R, §p<0.05), thus showing that VEGF inhibition reduces cellular viability (Figure 2).

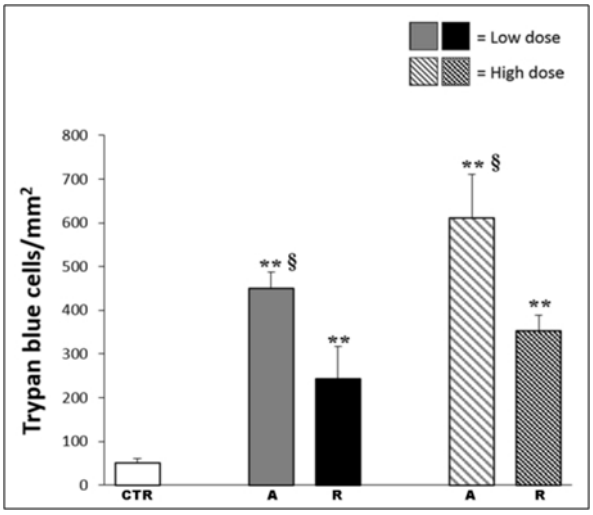

Figure 2. Effect of IC injection of anti-VEGF drugs on the viability of corneal endothelial cells. The graph shows that both doses of aflibercept (A) and ranibizumab (R) induce a significant increase in the number of endothelial cells incorporating TB when compared with controls (A *vs.* CTR; **p<0.01; R *vs.* CTR; **p<0.01). A treated groups seem to greater affect cell viability when compared with the R treated ones (A *vs*. R, §p<0.05).

9.3 Expression of apoptotic markers in rabbit cornea

To support the TB assay data, expression of the apoptotic markers p75NTR and c-Casp3 in rabbit corneal sections was analysed by immunofluorescence (IF) technique. The IF revealed that in control group p75NTR is expressed in all corneal layers, while c-Casp3 is modestly detected in corneal stroma (Figure 3a). After both doses of aflibercept (A) or ranibizumab (R) IC injection, c-Casp3 expression is increased in endothelial cells, and a co-expression of p75NTR and c-Casp3 is observable. The apoptotic markers are more expressed following A than R treatment (compared panels b and c in Figure 3).

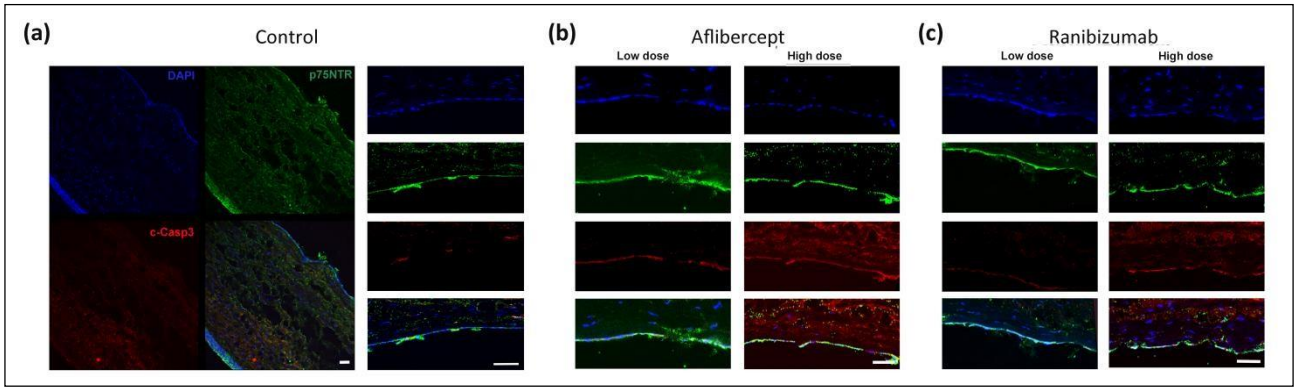

Figure 3. (a–c) Expression and localization of c-Casp3 (red), p75NTR (green) and DAPI (blue) in the control cornea (a), and in the corneal endothelial layer of rabbit receiving aflibercept (b) or ranibizumab (c) at low and high doses (white scale bar: 100 µm).

Rabbit corneal sections were also used to investigate the distribution of the apoptotic marker RhoA by immunohistochemistry. As it is shown in Figure 4a, RhoA distribution in control cornea is detected in corneal epithelium and slightly in the stroma and endothelium. An increase of RhoA immunoreactivity in epithelial and endothelial layers is observable following IC injections of both anti-VEGF drugs. Similar to what observed for the other apoptotic markers, enhanced RhoA expression is found in corneal endothelium of aflibercept (A) treated group than in ranibizumab (R) treated one (Figure 4b *vs*. 4c).

The microscopy observations are confirmed by the WB analysis (Figure 5e,f). Indeed, the expression of RhoA is increased after anti-VEGF when compared to control. Specifically, both doses of A affect R (*p<0.05) while only low dose of R resulted in RhoA change (*p<0.05) (Figure 5e,f).

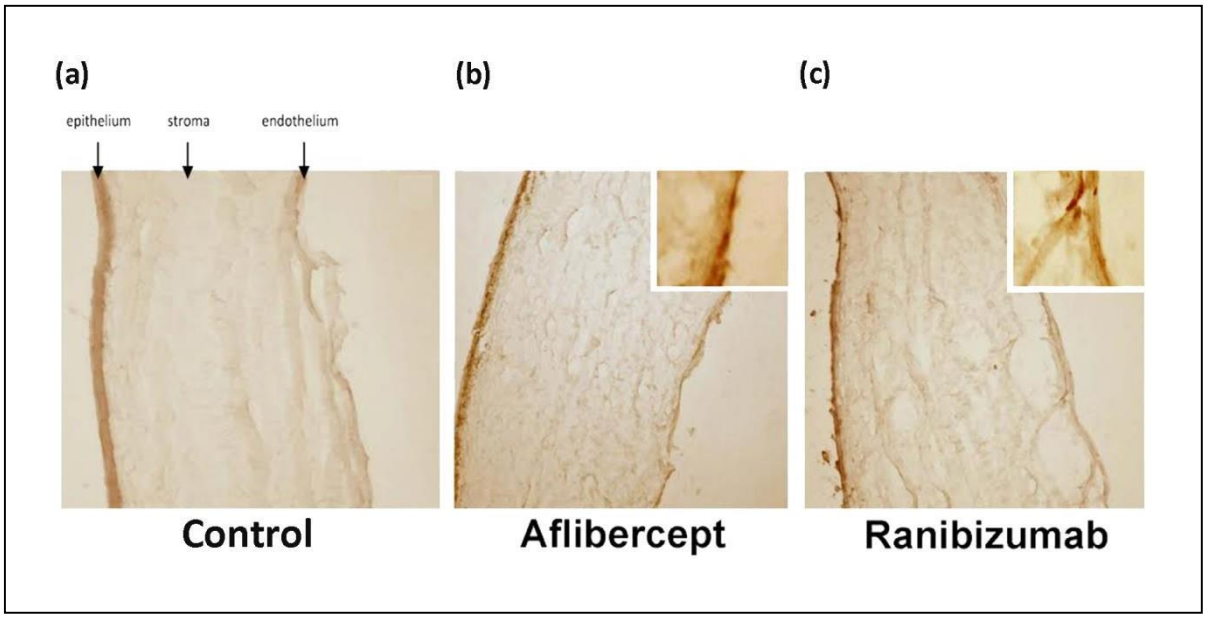

Figure 4. (a-c) Immunohistochemical detection of RhoA–expressing cells in the control rabbit cornea (a), and following IC injection with high dose of aflibercept (b) or ranibizumab (c). Insets in (b) and (c) show higher magnification of the endothelial layer (white scale bar: 100 µm).

9.4 Expression of NGF and NGF receptors

Western Blot (WB) analysis was performed to evaluate the expression of TrkA, its phosphorylated form phosphor-TrkA (p-TrkA), p75NTR and proNGF. No differences in p-TrkA and TrkA expressions are observed after anti-VEGF IC injection compared to the CTR group (Figure 5a,b). Aflibercept (A) and ranibizumab (R) IC injections induce a p75NTR expression increase when high doses are used (A *vs.* CTR, *p<0.05; R *vs*. CTR, **p<0.01; Figure 5c,f). The proNGF expression increase is found following both doses of A (A low dose *vs.* CTR, **p<0.01; A high dose *vs.* CTR, *p<0.05), while no changes are observable after R injection (Figure 5d,f).

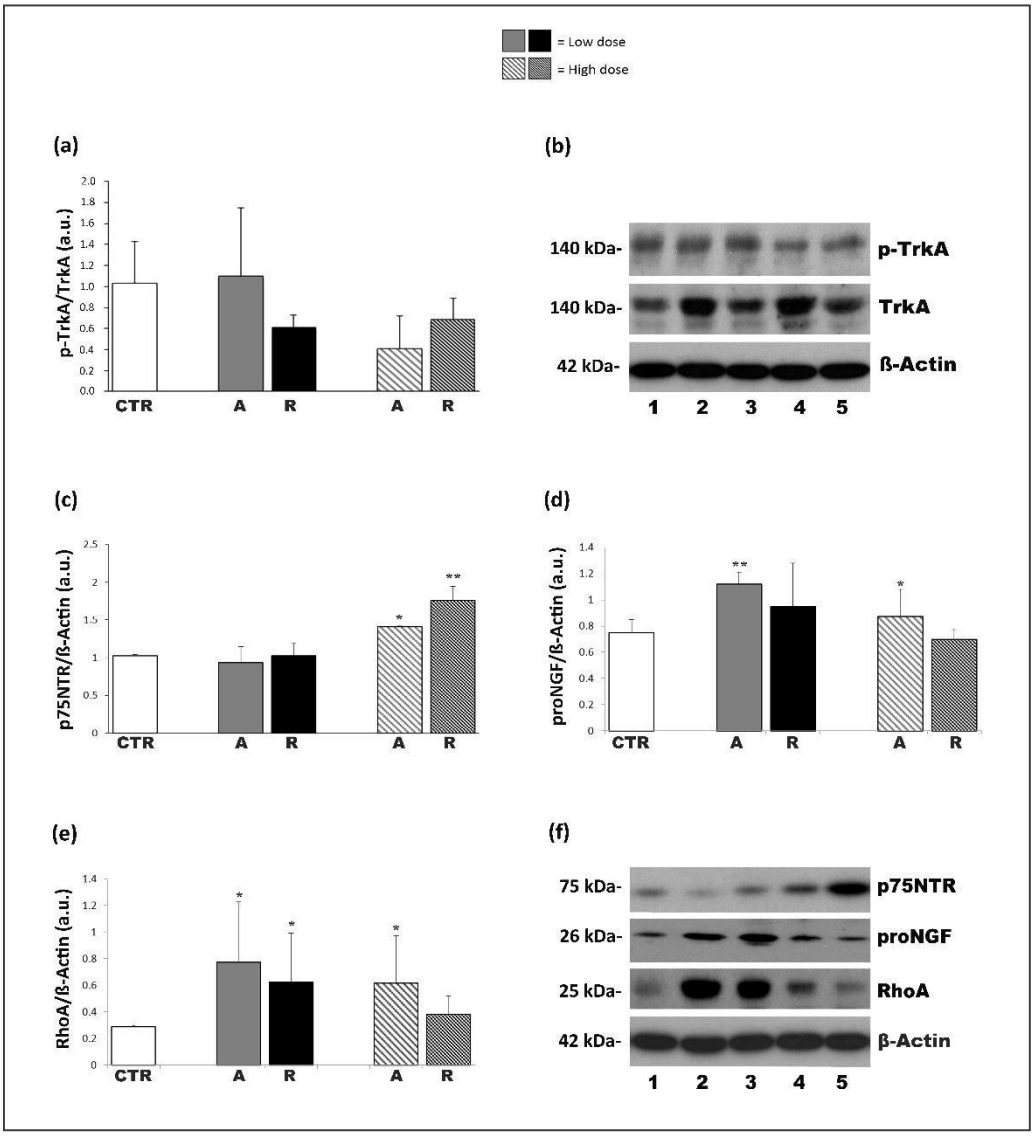

Figure 5. (a–f) Expression of apoptotic markers in corneal endothelium. The levels of pTrkA (a,b), p75NTR (c,f), proNGF (d,f), and RhoA (e,f) were quantified by western blot analysis in protein extracted from the corneal endothelium of rabbit following intracameral injection with vehicle (CTR), aflibercept (A), or ranibizumab (R). Representative western blots reporting the band size of each protein, and the expression trend in the different experimental groups are shown in (d). Data are expressed as optical density (arbitrary units) and presented as mean ± SD. Statistically difference: *p<0.05, *vs*. CTR; **p<0.01, *vs*. CTR. CTR: lane 1, aflibercept low dose: lane 2, ranibizumab low dose: lane 3, aflibercept high dose: lane 4 and ranibizumab high dose: lane 5.

9.5 Evaluation of intracellular pathway activation in corneal endothelium following intraocular anti-VEGF drugs

PathScan Assay was used to evaluate the activation of intracellular pathway after IC injections of both aflibercept (A) and ranibizumab (R) doses in corneal endothelium. IVT injection of single dose of anti-VEGF drugs was used as internal control in this experiment. As reported in Table 1, low and high doses of A and R activate several intracellular pathways (x) in extract of cornea endothelium, while IVT injection does not affect the activation of the same analysed molecules (ns = not significant and na = non activated). Since anti-VEGF drugs cannot reach the cornea when intravitreally injected, the changes of intracellular markers following IC treatment support the validity of the VEGF inhibition in the cornea.

TABLE 1 Activation of intracellular signaling molecules in the cornea after anti-VEGF injection

	IC injection					
	Low dose		High dose		IVT injection	
	Aflibercept	Ranibizumab	Aflibercept	Ranibizumab	Aflibercept	Ranibizumab
ERK1/2	ns	ns	ns	ns	ns	ns
STAT1	x	x	x	x	na	na
STAT3	x	x	x	x	ns	ns
AKT (Thr308)	x	x	x	x	ns	ns
AKT (Ser473)	x	x	x	x	ns	ns
AMPKa	ns	ns	ns	ns	na	na
S6 Rib.Prot.	na	na	na	na	na	na
mTOR	x	x	x	x	ns	ns
HSP27	x	x	x	x	ns	ns
Bad (Ser112)	x	x	x	x	ns	ns
p70 S6 Kinase	ns	ns	ns	ns	ns	ns
Pras40	x	x	x	x	na	na
p53	na	na	na	na	na	na
P38	ns	ns	ns	ns	ns	ns
Sap/JNK	x	x	x	x	ns	ns
PARP	ns	ns	ns	ns	ns	ns
c-Caspase 3	x	x	x	x	na	na
GSK3	ns	ns	ns	ns	ns	ns

Table 1. Intracellular signals phosphorylated or cleaved at the specified residues detectable with PathScan Assay are reported in the table. Molecules that are activated but do not result significantly different from the related control are indicated as ns = not significant and na = not activated. The letter "x" indicates molecules significantly activated by IC anti-VEGF injection.

Since qualitative data can be obtained by the PathScan Assay analysis, some intracellular markers were also analysed by WB technique (Figure 6).

It was found that A and R IC treatment differently affected Akt phosphorylation, as well as Akt related downstream molecules such as proline-rich Akt substrate of 40 kDa (Pras40) (Figure 6c) and mammalian target of rapamicin (mTOR) (Figure 6d). Chiefly, a high dose of R significantly decreases Akt phosphorylation on Ser473 (R *vs.* CTR, *$p<0.05$) (Figure 6a) and on Thr308 (R *vs.* CTR, **$p<0.01$) (Figure 6b), while only a decrease of Akt phosphorylation on Thr308 is observed after high dose of A (A *vs*. CTR, *$p<0.05$) (Figure 6a).

A decreased activation is also shown when analysing phosphorylation of the Akt downstream signalling molecules mTOR and Pras40 (Figure 6c,d). mTOR phosphorylation on Thr2448 is significantly decreased after the high dose of anti-VEGF drugs (A *vs.* CTR, **p<0.01; R *vs.* CTR, **p<0.01) but not at low doses (Figure 6c). At variance, a reduced phosphorylation on Thr389 of Pras40 is observable at both high and low dose as seen in Figure 6d. The phosphorylation of the apoptotic related markers Bad Ser122 and c-Casp3 are also affected, resulting decreased and increased compared to CTR, respectively (Figure 6e,f). Differences are found by comparing the high and low doses anti-VEGF drugs. It is worth to note that, with the exception of mTOR, a significant difference between high dose A and R is found in all intracellular signals analysed (*p<0.05) (Figure 6).

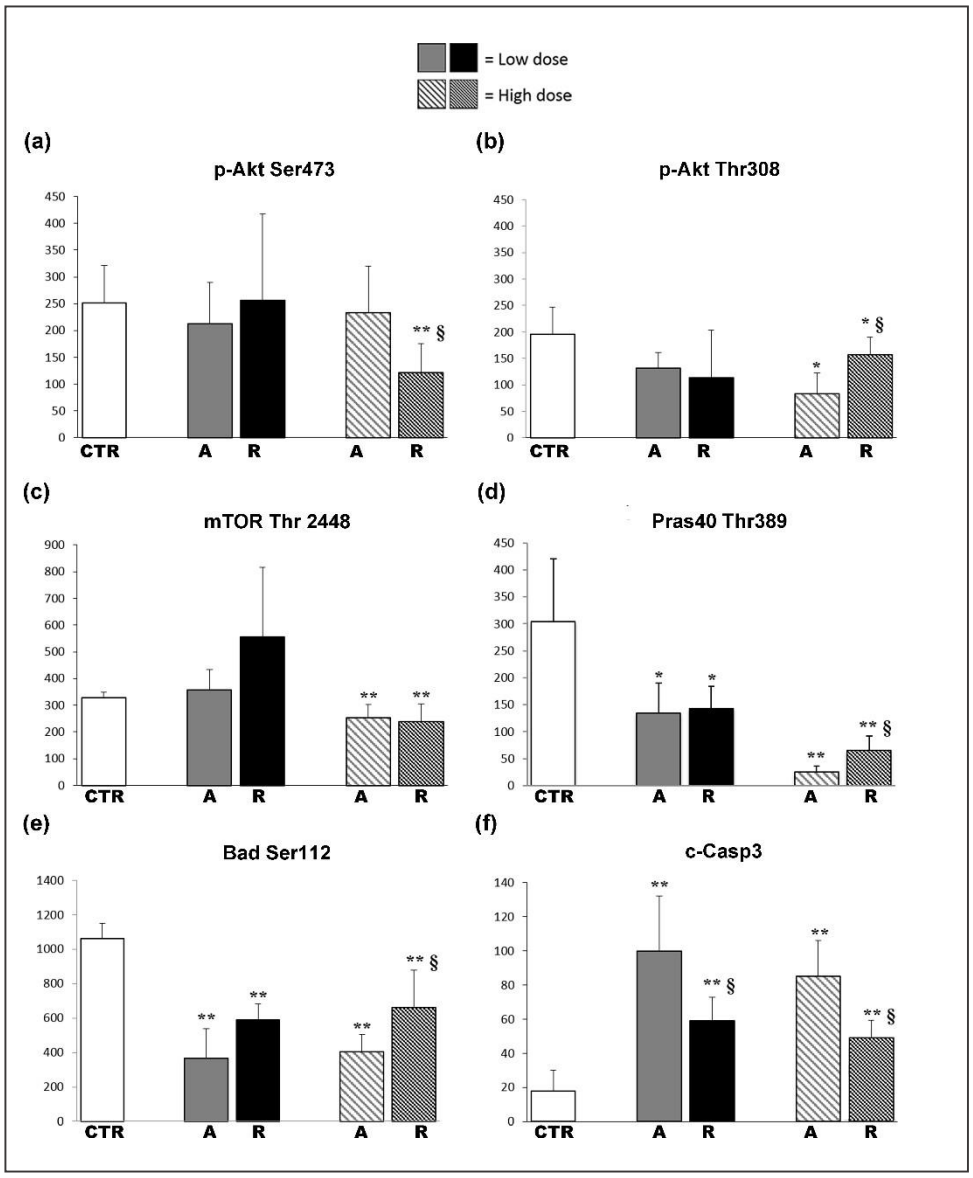

Figure 6. (a-f) Activated intracellular components of survival signalling in corneal endothelium by intracameral injection with vehicle (CTR), aflibercept (A) or ranibizumab (R) at low or high doses. The levels of p-Akt Ser473 (a), p-Akt Thr308 (b), mTOR Thr2448 (c), Pras40 Thr389 (d), Bad Ser112 (e) and c-Casp3 (f), Data are expressed as optical density (arbitrary units) and presented as mean ± SD. Statistically difference: *p<0.05, *vs*. CTR; **p<0.01, *vs*. CTR; §p<0.05, A *vs*. R.

10. Study II: effects of intravitreal (IVT) injection of anti-VEGF on rabbit retina

10.1 Expression of NGF and BDNF and their receptors

In order to evaluate the potential modulation induced by VEGF on neurotrophin system, the effects of aflibercept (A) and ranibizumab (R) IVT injections in the retina, the expression of NGF and BDNF mature and precursor forms, and their receptor expression and activation were evaluated. Compared to vehicle treated animals, after 1 (Figure 7a) and 4 weeks from IVT treatment (Figure 7b), both anti-VEGF drugs induced a decrease in proNGF expression (A *vs*. Veh, ***p<0.001; R *vs*. Veh, *p<0.05, Figure 7a on the left; and A *vs*. Veh, **p<0.01; R *vs*. Veh, **p<0.01, Figure 7b on the left), and an increase of proBDNF expression (A *vs*. Veh, *p<0.05; R *vs*. Veh, *p<0.05; Figure 7a,b). A difference between the two anti-VEGF drugs used was observed only after 1 week IVT treatment (A *vs*. R, *p<0.05; Figure 7a on the left). Mature NGF and BDNF forms could not be detected in our WB conditions.

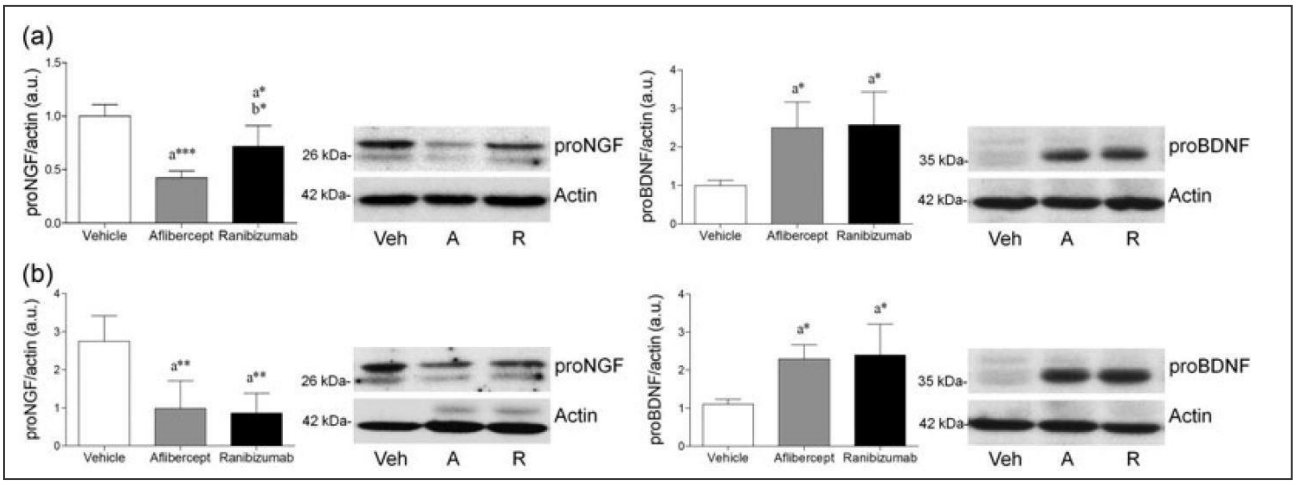

Figure 7. VEGF blockade alters proNGF and proBDNF expression levels in rabbit retina. (a) Densitometric analysis and representative western blot analysis of proNGF and proBDNF 1 week after treatment. (b) Densitometric analysis and representative western blot of proNGF and proBDNF 4 weeks after treatment. Animals per group: n = 8. Data represent means ± SD. *p<0.05; **p<0.01; ***p<0.001. "a" indicates statistical significance compared with vehicle; "b" indicates statistical significance compared with aflibercept. Statistical analysis was performed by using one-way ANOVA followed by Tukey's post-hoc test.

As far as the expression and phosphorylation of TrkA and TrkB are concerned, the data of the WB analysis are reported in Figure 8 and 9 respectively. With the exception of a slightly increased of TrkA observable after 1 week of treatment with A (Figure 8 line a), IVT with anti-VEGF does not affect the expression of both TrkA and TrkB.

Differences are found by analysing the expression levels of the phosphorylated receptors, and thus the ratio of phosphor-Trks and TrkA or TrkB, which indicate the levels of receptor activation. Indeed, a significant decrease of TrkA phosphorylation is found at both 1 and 4 weeks after A and R treatment (Figure 8a and b), while a decrease of TrkB activation is observable only after 4 week (Figure 9b).

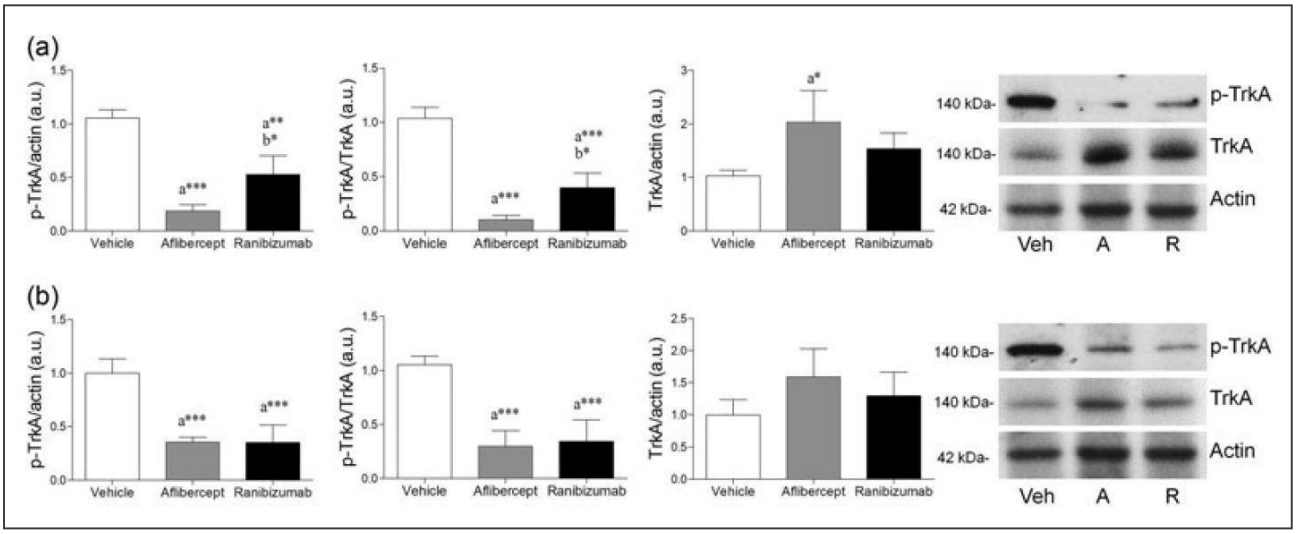

Figure 8. VEGF inhibition suppresses TrkA activating phosphorylation in rabbit retina. (a) Densitometric analysis and representative western blot of p-TrkA and total TrkA, 1 week after treatment. (b) Densitometric analysis and representative western blot of p-TrkA and total TrkA, 4 weeks after treatment. n = 8 animals per group. Data are expressed as means ± SD. *p<0.05; **p<0.01; ***p<0.001. "a" indicates statistical significance compared with vehicle; "b" indicates statistical significance compared with Aflibercept. Statistical analysis was assessed by ANOVA followed by Tukey's post-hoc test.

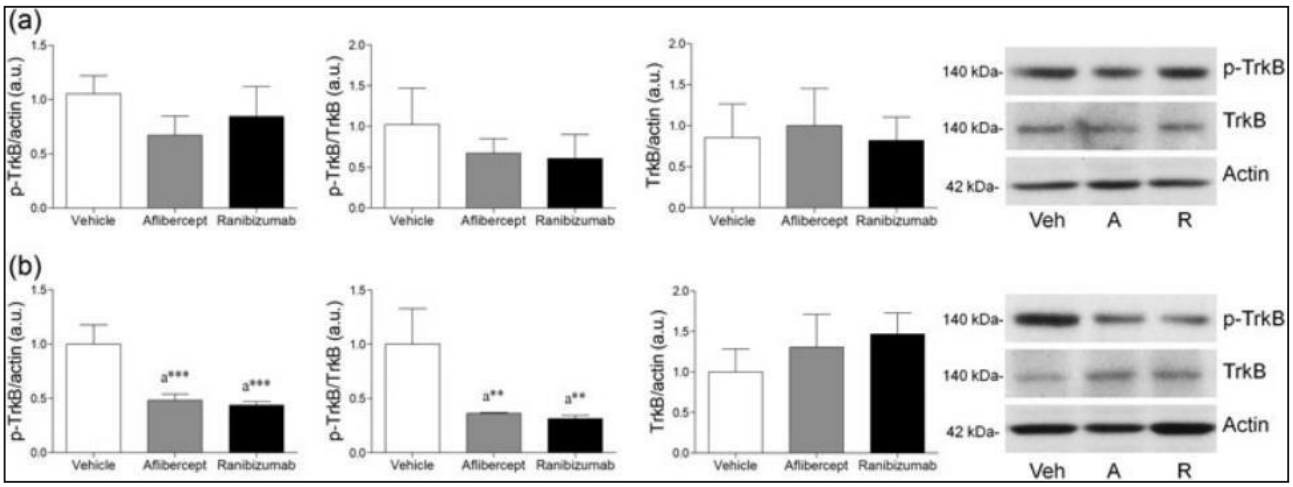

Figure 9. Anti-VEGF treatment inhibits TrkB activating phosphorylation. (a) Densitometric analysis and representative western blot analysis of p-TrkB and total TrkB, 1 week after treatment. (b) Densitometric analysis and representative western blot of p-TrkB and total TrkB, 4 weeks after treatment. Animals per group: n = 8. Data represent means ± SD. **p<0.01; ***p<0.001. "a" indicates statistical significance compared with vehicle. Statistical analysis was performed by using one-way ANOVA followed by Tukey's post-hoc test.

The effects of anti-VEGF drugs intravitreal injections on the expression of p75NTR receptor full length (p75NTR FL) and its cleavage products: the C-terminal fragment (CTF) and the intracellular domain (ICD) is shown in Figure 10. Reduced levels of p75NTR FL and fragments expression are found at 1 and 4 weeks after treatments with anti-VEGF drugs (1 week: A *vs*. Veh, *p<0.05; Figure 10a; and 4 weeks: A *vs*. Veh, **p<0.01; R *vs*. Veh, *p<0.05; Figure 10b). No significant changes of the ICD expression is detected by A and R at 1 and 4 weeks (Figure 10a,b).

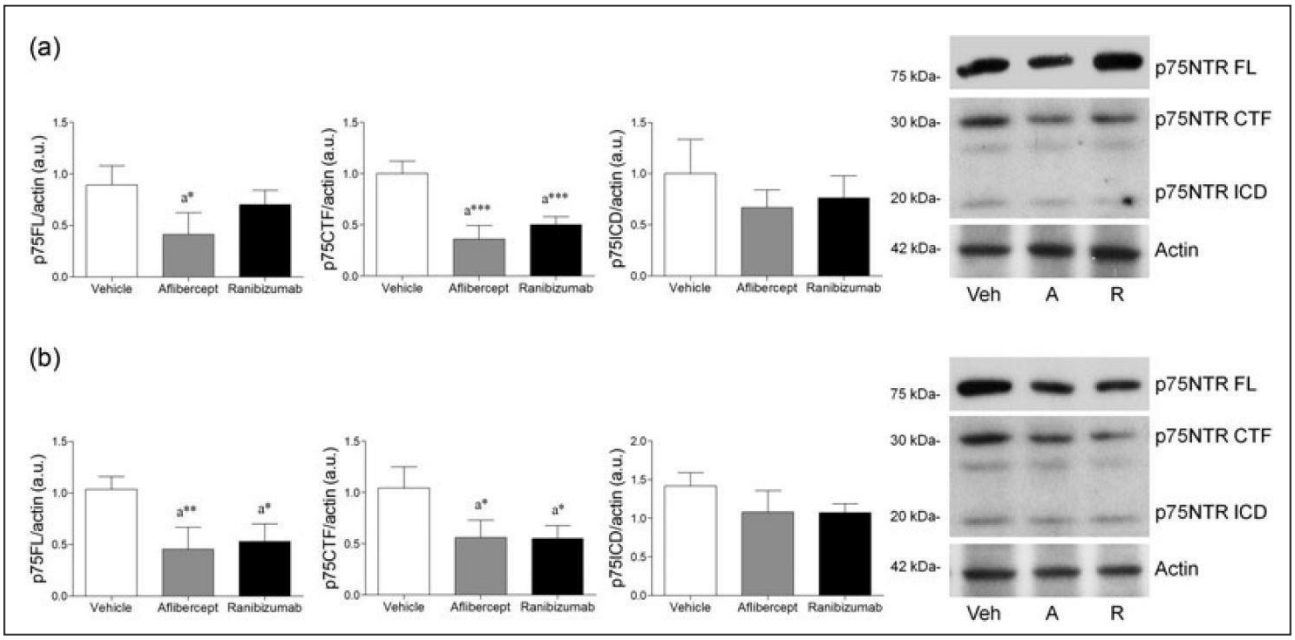

Figure 10. Anti-VEGF agents alters the expression of FL and CTF p75NTR in rabbit retina. (a) Densitometric analysis and representative western blot analysis of p75NTR (FL, CTF and ICD), 1 week after treatment; (b) Densitometric analysis and representative western blot analysis of p75NTR (FL, CTF and ICD), 4 weeks after treatment. n = 8 animals per group. Data are expressed as means ± SD. *p<0.05; **p<0.01; ***p<0.001. "a" indicates statistical significance compared with vehicle. Statistical analysis was assessed by one-way ANOVA followed by Tukey's post-hoc test. CTF: C-terminal fragments; FL: full length; ICD: intracellular domain.

10.2 Activation of apoptosis and autophagyc markers after IVT anti-VEGF drugs administration

The results of WB analysis are shown in Figure 11. The autophagyc markers Beclin-1 and LC3 expression are differently affected by the two anti-VEGF drugs, so that a significantly increased of Beclin-1 and LC3II/LC3I ratio are found in the retina of rabbit at 1 and 4 weeks after treatment with aflibercept (A), while ranibizumab (R) result in marker changes only at 4 weeks (compare Figure 11 a and b). The upregulation of c-Casp3 is also significantly induced by A at 1 and 4 weeks from injection (A *vs*. Veh, **p<0.01), while no alteration are found following R (Figure 11a,b).

The WB evaluation of Akt activation, through its phosphorylation on Ser473, also shows difference between the two anti-VEGFs. Indeed, although both drugs result in a decrease of phopsho-Akt (p-Akt), a more pronounced effect of A, which induce an about 70% of reduction of Akt activation respect to control.

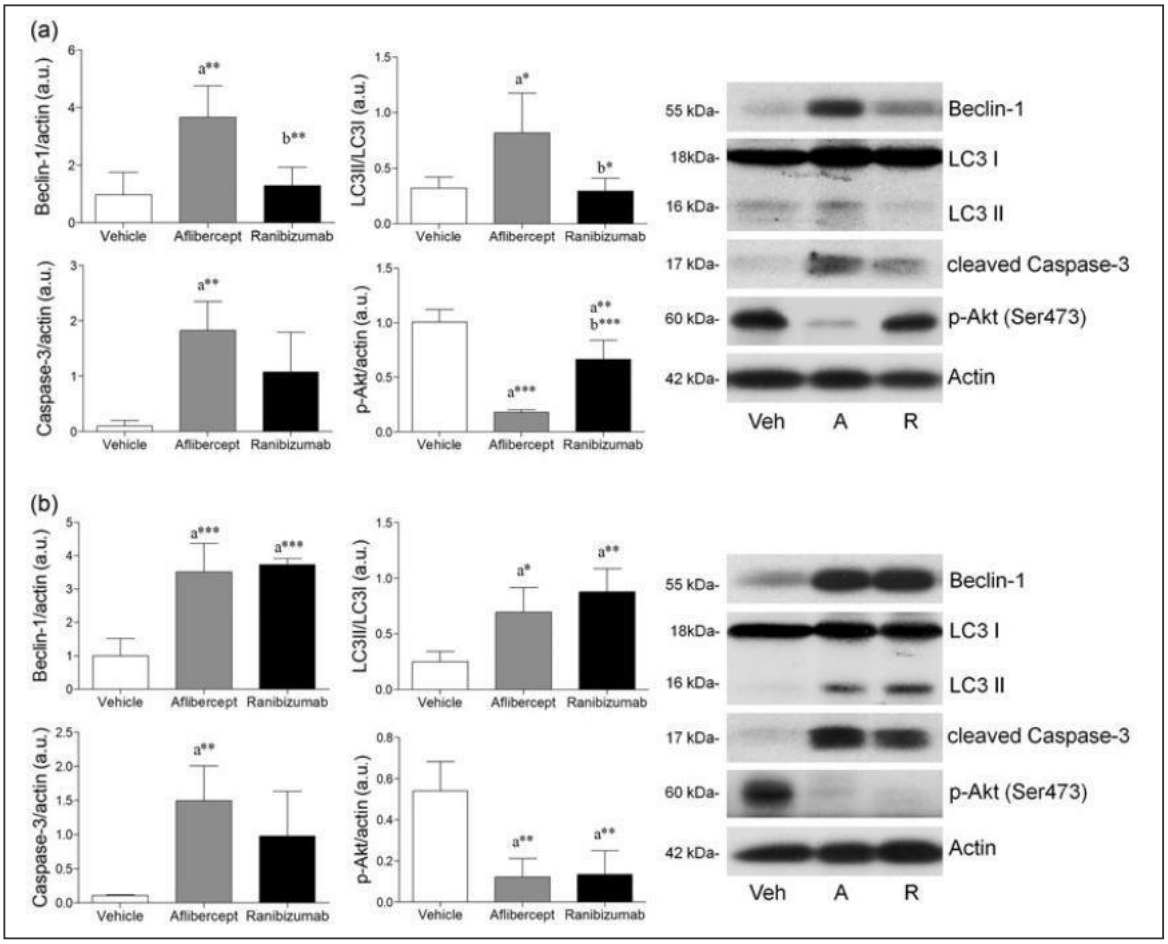

Figure 11. VEGF inhibition induces apoptotic and autophagyc markers and alters Akt phosphorylation in rabbit retina. (a) Densitometric analysis and representative western blot of Beclin-1, LC3II/LC3I ratio, cleaved Caspase-3 and p-Akt (Ser473) 1 week after treatment. (b) Densitometric analysis and representative western blot of Beclin-1, LC3II/LC3I ratio, cleaved Caspase-3 and p-Akt (Ser473), 4 weeks after treatment. Animals per group: n = 8. Data represent means ± SD. *p<0.05; **p<0.01; ***p<0.001. "a" indicates statistical significance compared with vehicle; "b" indicates statistical significance compared with Aflibercept. Statistical analysis was performed by using one-way ANOVA followed by Tukey's post-hoc test.

SECTION IV – DISCUSSION

VEGF is the main factor regulating ocular blood vessels growth related events in physiological and pathological conditions [110,111]. Common treatments for VEGF-related eye disorders take advantage from VEGF blockade agents' administration like IVT injection of aflibercept (A) and ranibizumab (R) [112]. Although IVT injection is considered the gold standard for ocular treatments, in the last decades the IC injection has been proposed as a new route of administration for neo-vascular ocular pathologies [113]. At the present, there is a limited amount of information about potential adverse effects of anti-VEGF drugs following IC administration, and only some reports investigated the toxic actions of IVT injection towards retinal cells [114,115]. These questions were covered in the present Thesis by evaluating whether VEGF blockade performed by IC and IVT injections of commercial anti-VEGF drugs might affect corneal endothelium (Study I) and retina cells (Study II). Since altered NGF and BDNF maturation, and/or an unbalance of their receptor expression and activation featured eye pathologies [116], and that the crosstalk between NTs and VEGF contributes to homeostasis and neuronal recovery in the eye [117,118], the effects of anti-VEGF treatment on NT expression and signalling were analysed in the present studies.

Extending the previous observations that VEGF blockade by anti-VEGF drugs can promote apoptosis in the retina [119], we demonstrate that A and R lower cell viability in corneal endothelium when IC injected (Study I). This observation is supported by the increased expression and the activation of intracellular signals promoting apoptosis. Indeed, our immunohistological and biochemical analysis show that A and R induce increase of the apoptosis markers RhoA and c-Casp3 in corneal endothelium, suggesting that this ocular tissue can be also a target of anti-VEGF drugs. Our findings that A and R inhibit VEGFR2 in endothelial cells when IC injected, and that intracellular pro-apoptotic signal activation in endothelial cells extracts is not influenced by these drugs when IVT administrated but only when IC administrated, support our suggestion. Further, since RhoA is known to mediate the pro-apoptotic signalling, through the cleavage and activation of Caspase-3, a process contributing to the c-Casp3 production, and c-Casp3 was observed in retinal cell lines following VEGF blockade [120], our data are consistent with the pro-apoptotic action of A and R. The reported altered phosphorylation of Akt and its related signalling molecules PRAS40 and mTOR also suggest a drug- and dose-dependent activation of specific intracellular pathways. The effects of A and R on the level of Ser112 phosphorylation of Bad2, which is a signal of apoptosis suppression [121] might further support this suggestion. More, the different effects of A and R on Akt phosphorylation on Ser473 and Thr389 after IC injections, as well as on the reduction of PRAS40 and mTOR activation - which are known to affect cell growth and balance [122] – indicate that the potential pro-apoptotic activity is dependent on the type of VEGF blockade. In fact A, containing both VEGFR1 and VEGFR2 interacting ligand

domains, can bind VEGFB and PlGF as well as VEGFA, while R acts blocking only VEGFA [123,124]. It is therefore likely that the higher proapoptotic effect of A [125] may depend on its larger ligand domains.

In line with this explanation, Study I also shows that, while both anti-VEGF drugs IC injection induces p75NTR expression enhancement, only A increases proNGF at both low and high doses. Although no alteration of TrkA expression and activation is observed, the increase of p75NTR leads to an unbalance in TrkA/p75NTR ratio, whose equilibrium is essential for promoting cellular survival [1]. In this receptor condition the presence of high levels of proNGF favours the activation of the apoptotic pathway through p75NTR binding [126], suggesting that after A IC injection, a proNGF/p75NTR mediated mechanism of toxicity rises inducing cell loss in corneal endothelium.

The involvement of NTs in the mechanism of action of anti-VEGF drugs is also suggested by Study II on the retina. Previous studies reported the effects of IVT injections on retinal cell viability [127], but few information on NTs are available. We found that IVT injection of A and R affects the precursor forms of NTs proNGF and proBDNF at both 1 week and 4 weeks post injection. The finding that IVT anti-VEGF administration downregulates NGF mRNA levels in rabbit retina [128] supports our observation that proNGF levels are decreased by both anti-VEGF drugs, and suggests that VEGF blockade influences the synthesis of NGF. On the contrary, an increase of proBDNF is observable after treatment with A and R. To the best of our knowledge, no other study reported this data, and therefore the significance of the diverse NT trend after A and R might be only speculated at the present. The anti-VEGF blockade induced-increase of proBDNF might indicate an increased synthesis, as well as a defective mechanism of maturation and/or cleavage of BDNF molecule. Even if the anti-BDNF antibody used in this study is able to recognize both the pro- and mature form, no signals for mature BDNF was detected in the retina in our experimental conditions. The proNTs are the predominant NTs forms in neuronal tissues while mature NGF and BDNF are usually expressed at low levels sometimes almost undetectable [129,130]. Thus, it is not possible to define if the increased levels of proBDNF in retina follow anti-VEGF drugs might reflect an enhancement of synthesis or a block of maturation.

Although this fact needs further investigations, it is worth to note that the unbalance between NGF and BDNF (reduced NGF synthesis and enhanced proBDNF levels) is associated with an increase of apoptotic and autophagyc markers confirming that the alteration of mature NTs or an increase of their precursors are trigger factors for cell death in the retina [131].

The findings on the effects of anti-VEGF drugs on Trk receptor expression and activation, and p75NTR cleavage might support this hypothesis. The decrease of TrkA and TrkB phosphorylation in absence of a reduction of their expression following A and R is indeed a marker of cell impairment, which might reflect the reduced availability of NGF and BDNF, and the intracellular changes due to the block of VEGF, including the reduction and cleavage of p75NTR. Extending the observation by Rocco and co-authors [132], we found

that the reduction of p75NTR FL upon anti-VEGF blockade in retinal cells is associated with decreased level of its fragments. Since it has been observed that one of the function of P75NTR CTF and ICD is to potentiate Trk-initiated trophic signalling [133,134], it is likely that the reduction of p75NTR contributes, together with the deactivation of Trks, to decrease the pro-survival signalling mediated by NTs in the retina.

Consistent with these alterations, in Study II the impairment of NT signalling activation is accompanied by the significant increased level of the apoptotic marker c-Casp3 and of the autophagyc markers Beclin-1 and LC3 I/LC3 II. The causality between NTs' impairment and the activation of apoptosis and autophagy can be explained by deregulation of Akt activation, because in pathological conditions, Akt suppression mediates c-Casp3, thus contributing to sustain the aforementioned cell death related events [135].

In addition, we found that the occurrence of retinal cell impairment/death after IVT injection of anti-VEGF drugs is greater after A administration than R one, and at 4 weeks. The different effect of the two drugs might be due to the difference in their structures and targets, as reported for Study I, and as it was also found in the retina by Ferrara 2016 [136]. The evidence that the anti-VEGF agents bioavailability persists for approximately 1 month in rabbit retina when IVT injected [137,138] and that the anti-VEGF compounds administration-induced progressive molecular and cellular changes increase the chance of ocular adverse effects [139], is line with our observations at 4 weeks post A and R injection, and suggest a prolonged effects of the anti-VEGF drugs on retina.

SECTION V – CONCLUSIONS

Collectively, morphological and molecular data obtained using anti-VEGF drugs in rabbit cornea and retina are consistent with the hypothesis that NT/VEGF system is strongly involved in ocular homeostasis. Any interference of this regulatory network could alter the physiological trophic support provided by NTs leading to the onset of apoptotic and autophagyc events.

Even if A and R are indiscriminately used in clinical practice because their biological efficacy is considered equal [140], our results show that A and R have a different impact on NT pathway and cell viability in the cornea and retina. Considering the importance of NTs in the trophic support maintenance of ocular tissues, the data collected in Study I and II suggest that the drug choice and dosage should be carefully evaluated as well as the route of the administration and the timing.

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