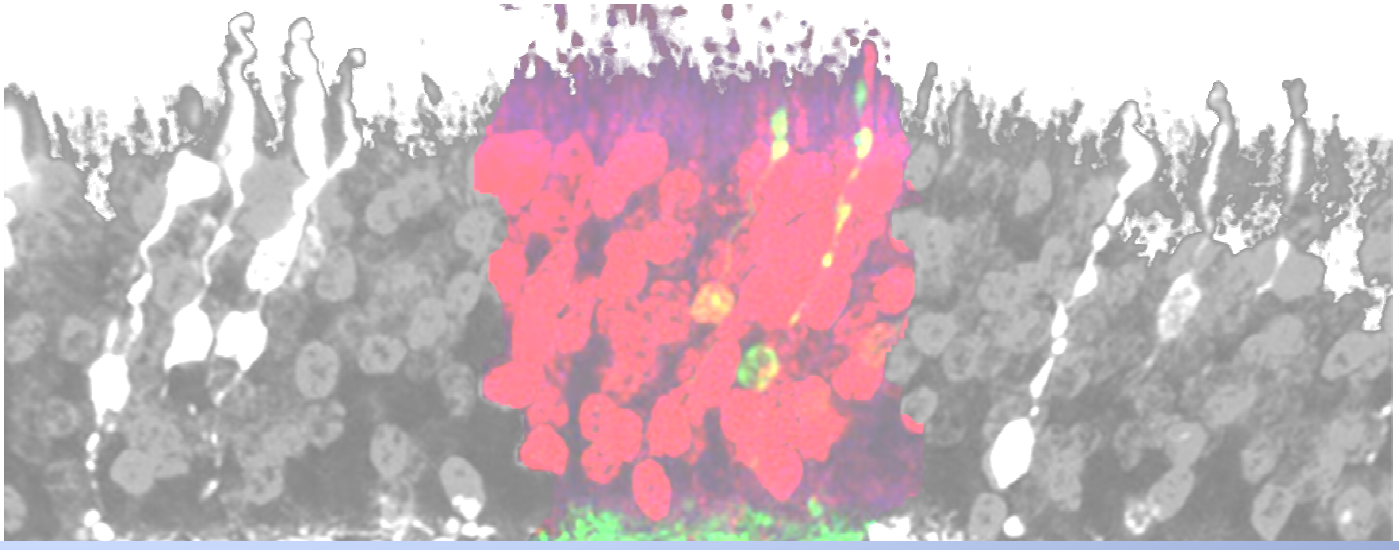
University of Crete
Department of Basic Science, Laboratory of Pharmacology
School of Medicine, Heraklion, Greece



Investigation of new therapeutic targets for the treatment of Diabetic Retinopathy: Neurosteroids/Microneurotrophins

Μελέτη Νέων Θεραπευτικών Στόχων για την αντιμετώπιση της Διαβητικής Αμφιβληστροειδοπάθειας: Νευροστεροειδή/Μικρονευροτροφίνες

Ph.D. Thesis

Ruth Ibán-Arias, MSc.

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Thesis Coordinator and Mentor:
Kyriaki Thermos, Ph.D.
Professor of Pharmacology

Members of the Ph.D. Committee:

Kyriaki (Kiki) Thermos, Professor of Pharmacology
Achilleas Gravanis, Professor of Pharmacology
Miltiadis Tsilimbaris, Professor of Ophthalmology
Domna Karagogeos, Professor of Molecular Biology-Developmental Neurobiology
George Liapakis, Associate Professor of Pharmacology
Ioannis Charalampopoulos, Assistant Professor of Pharmacology
Vassiliki Nikoletopoulou, Researcher, Associate Professor Grade

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La Retina es la Puerta de la Luz a nuestro Entendimiento y, sobre todo, a nuestro Mundo Emocional

Santiago Ramón y Cajal

TABLE OF CONTENTS

Abbreviations

ALS: amyotrophic lateral sclerosis; AMD: Age-related Macular Degeneration; AMPA: α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid; BBB: Blood Brain Barrier; BDNF: Brain Derived Neurotrophic Factor; bNOS: brain Nitric Oxide Synthetase; BRB: Blood Retina Barrier; BSA: Bovine Serum Albumin; CGNs: Cerebellar Granule Neurons; CNS: Central Nervous System; CREB: cAMP response element-binding; DAG: DiAcylGlycerol; DHEA: DeHydroEpiAndrosterone; DM: *Diabetes Mellitus*; DME: Diabetic Macular Edema; DMSO: Dimethyl Sulfoxide; DR: Diabetic Retinopathy; DTT: DiThioThreitol; ECD: extracellular domain fragment; ERG: Electroretinogram; ERK: Extracellular-signal Regulated Kinase; FDA: U.S. Food & Drug Administration; GABA: Gamma-AminoButyric Acid; GAPDH: GlycerAldehyde 3-Phosphate DeHydrogenase; GCL: Ganglion Cell Layer; GFAP: Glial fibrillary acidic protein; H-E: Hematoxylin-Eosin; HPLC-MS/MS: High-Performance Liquid Chromatography (HPLC) with tandem Mass Spectrometric (MS/MS); i.p.: intraperitoneal; Iba-1: Ionized calcium Binding Adaptor molecule 1; IC_{50}: half maximal Inhibitory Concentration; ICD: intracellular domain fragment; IHC: ImmunoHistoChemistry; IL: interleukin; INL: Inner Nuclear Layer; IP: Immunoprecipitation or Immunoprecipitate; IP_3: Inositol TriPhosphate; IPL: Inner Plexiform Layer; IR: Immunoreactivity; Kd: Dissociation constant; MAPK: Mitogen Activated Protein Kinase; MCP-1: Monocyte Chemoattractant protein-1; MEK: MAPK/ERK Kinase; MMP: Matrix Metalloproteinases; MS: Multiple Sclerosis; NFL: Nerve Fiber Layer or Neurofilament; NFκB: Nuclear Factor κappa B; NGF: Nerve Growth Factor; NGS: Normal Goat Serum; NT-3: Neurotrophin 3; NT-4/5: Neurotrophin 4; NTs: Neurotrophins; O/N: OverNight; OCT: Optimal Cutting Temperature compound; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; OPs: Oscillatory Potentials; $p75^{NTR}$: p75 neurotrophin receptor; PARP: Poly (ADP-ribose) polymerase; PB: Phosphate Buffer; PBS: Phosphate-Buffered Saline; PBS: Phosphate-Buffered Saline; PDGF: Platelet-Derived Growth Factor; PDR: Proliferative Diabetic Retinopathy; PI3K: PhosphatidylInositol-3 Kinase; PKC: Phospho Kinase C; PLC-*γ*: PhosphoLipase C-*γ*; PNS: Peripheral Nervous System; POS: Photoreceptor Outer Segment; proNGF: pro Nerve Growth Factor; RD: Retinal Detachment; RGC: Retinal Ganglion Cell; RIP: Receptor-Interacting Protein; RPE: Retinal Pigment Epithelium; RT: Room Temperature; SAPK/JNK: Stress-Activated Protein Kinase or c-Jun N-amino-terminal Kinase; SD: Standard Deviation; SDS-PAGE: Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis; SEM: Standard Error Deviation; STZ: Streptozotocin; T1D: Diabetes Type 1; T2D: Diabetes Type 2; TBST: Tris-Buffered Saline with Polysorbate 20 (Tween20); TH: Tyrosine Hydroxylase; TIMP-1: Tissue Inhibitor of MetalloProteinase 1; TNF-α: Tumor Necrosis Factor -α; TrkA, TrkB, TrkC: Tropomyosin related kinase A, B, C; TrkAR: Tropomyosin related kinase A Receptor; VEGF: Vascular Endothelial Growth Factor; Vps10p: Vacuolar protein-sorting 10 protein; WB: Western Blot

Abstract

Diabetic Retinopathy (DR) is a major ocular complication of diabetes that leads to suboptimal visual acuity and blindness. This disease has been characterized by three pathogenic processes that underlie the mechanisms leading to retinal cell death, namely, inflammation, neurodegeneration and microangiopathy. Standard therapies for DR rely on laser surgery and intravitreal injections of corticosteroids or anti-angiogenic factors like anti-vascular endothelial growth factor (VEGF) agents, which inhibit and/or reverse the vascular damage. These therapies are effective but have various side effects. New pharmacological approaches are essential in order to treat the inflammatory and neurodegenerative components of the disease.

In the present study, we investigated the putative use of the neurosteroidal microneurotrophin BNN27, a 17-spiro DHEA analogue, as a potential therapeutic agent for the treatment of DR. BNN27 has been described as a microneurotrophin given that it is a small-sized molecule and acts as a Nerve Growth Factor (NGF) mimetic that binds specifically to its receptors, TrkA and p75 neurotrophin receptors. Furthermore, it is highly lipophilic, thus able to penetrate the blood brain barrier and it is not metabolized to estrogens or androgens. Employing the STZ-rat model of DR, BNN27 was administered at three doses, 2, 10 and 50mg/kg, either intraperitoneally or by eye-drops, 4 weeks post-STZ-injection for 7 days. We demonstrated the ability of BNN27 to exert its neuroprotective properties to the diabetic retina despite sustained high glucose levels in diabetic rats.

BNN27 restored the STZ-induced attenuation of bNOS- and TH-immunoreactivity (IR; retinal amacrine cell markers) and NFL-IR (ganglion cell axon marker) when administered both intraperitoneally and as eye drops. This was further substantiated by the BNN27-dependent activation/phosphorylation of the NGF TrkA receptor. Moreover, a specific TrkA receptor antagonist reversed BNN27's actions. These data strongly suggest that BNN27 mediates its pharmacological pro-survival actions in the diabetic rat retina by activating specifically the NGF TrkA receptor. BNN27 also attenuated the expression of $p75^{NTR}$ death receptor in the diabetic retina when administered intraperitoneally. However, it did not reverse the diabetes-induced increase in $TUNEL^{+}$ retinal cells but it did attenuate the diabetes-induced activation of cleaved caspase-3. BNN27 also reduced the diabetes-induced up-regulation of the GFAP protein, expressed in Müller macroglial cells and the number of Iba-1-expressing microglia. Finally, BNN27 restored retinal homeostasis after the diabetes-

induced imbalance between the two isoforms of NGF, mature NGF and immature proNGF, in the diabetic rat retina.

In conclusion, the microneurotrophin BNN27 exerts neuroprotective actions to the diabetic rat retina when administered either intraperitoneally or by eye drops. BNN27 was shown to specifically target retinal neurons and glial cells, acting as a pro-survival and anti-inflammatory agent, respectively. It displays the pharmacological profile needed to address the neurodegenerative and inflammatory parameters that characterize DR. Our results advocate that this novel microneurotrophin, BNN27, has the potential to become a "lead" molecule for the treatment for DR.

Περίληψη

Η διαβητική αμφιβληστροειδοπάθεια (ΔΑ) αποτελεί κύρια και συχνά εμφανιζόμενη επιπλοκή του σακχαρώδη διαβήτη που οδηγεί στη μείωση της οπτικής οξύτητας και στην τύφλωση. Η ΔΑ χαρακτηρίζεται από τρεις παθολογικές παραμέτρους τη μικροαγγειακή βλάβη, τη νευροεκφύλιση και την φλεγμονή. Οι μέχρι σήμερα καθιερωμένες θεραπείες για την αντιμετώπιση της ΔΑς είναι η φωτοπηξία με λέϊζερ και οι ενδοϋαλοειδικές εγχύσεις κορτικοστεροειδών ή αντι-αγγειογενετικών παραγόντων όπως του αντί-Αγγειο-Ενδοθηλιακού Αυξητικού Παράγοντα (VEGF), οι οποίες στοχεύουν στη μείωση της μικροαγγειακής βλάβης. Οι θεραπείες αυτές είναι αποτελεσματικές, αλλά παρουσιάζουν σοβαρές ανεπιθύμητες ενέργειες. Νέες φαρμακολογικές προσεγγίσεις απαιτούνται για την αντιμετώπιση και των τριών παραμέτρων της ΔΑς.

Στην παρούσα μελέτη, ερευνήσαμε την πιθανή χρήση της νευροστεροειδούς-προέλευσης μικρονευροτροφίνης BNN27, ενός συνθετικού σπιρο-αναλόγου (17-spiro-DHEA) της Διϋδροεπιανδροστερόνης (Dehydroepiandrosterone, DHEA), ως δυνητικού θεραπευτικού παράγοντα για τη θεραπεία της ΔΑς. Η BNN27 χαρακτηρίστηκε ως μικρονευροτροφίνη δεδομένου ότι είναι μικρού μεγέθους μόριο, μιμείται τη δράση του Νευρικού Αυξητικού Παράγοντα (NGF) κι επομένως δρα ως αγωνιστής των υποδοχέων του, δεσμευόμενη ειδικά με τους NGF TrkA και $p75^{NTR}$υποδοχείς. Επιπλέον, είναι ιδιαίτερα λιπόφιλο μόριο, κι ως εκ τούτου είναι ικανό να διεισδύσει στον αιματοεγκεφαλικό φραγμό, και στερείται οιστρογονικών και ανδρογονικών δράσεων. Χρησιμοποιώντας το ζωϊκό μοντέλο στρεπτοζοτοκίνης (streptozotocin, STZ) για την πρόκληση διαβήτη στον αρουραίο, χορηγήθηκε η BNN27 σε τρεις δοσολογίες, των 2, 10 και 50mg/kg, είτε ενδοπεριτοναϊκά είτε με τοπική έγχυση οφθαλμικών σταγόνων, 4 εβδομάδες μετά τη χορήγηση STZ, για 7 ημέρες. Η BNN27 εμφάνισε νευροπροστατευτικές δράσεις στον διαβητικό αμφιβληστροειδή παρά τα διατηρούμενα υψηλά επίπεδα γλυκόζης στους διαβητικούς αρουραίους.

Η BNN27 αποκατέστησε την προκαλούμενη από τη STZ μείωση της bNOS και TH-ανοσοδραστικότητας (δείκτες αμακρινικών κυττάρων του αμφιβληστροειδούς) και την NFL-ανοσοδραστικότητα (δείκτης αξόνων των γαγγλιακών κυττάρων) μετά από ενδοπεριτοναϊκή ή τοπική υπό μορφή οφθαλμικών σταγόνων χορήγηση. Επιπροσθέτως, ο ειδικός ανταγωνιστής του TrkA υποδοχέα αντέστρεψε τις προστατευτικές δράσεις του BNN27. Αυτά τα δεδομένα υποδεικνύουν ότι η BNN27 διεκπεραιώνει τις φαρμακολογικές προ-επιβιωτικές δράσεις της στο διαβητικό αμφιβληστροειδή αρουραίου μέσω της

ενεργοποίησης ειδικά του NGF TrkA υποδοχέα. Αυτό επιβεβαιώθηκε περαιτέρω από την BNN27 προκαλούμενη ενεργοποίηση / φωσφορυλίωση του NGF TrkA υποδοχέα. Η BNN27 χορηγούμενη ενδοπεριτοναϊκά μείωσε την έκφραση του υποδοχέα θανάτου $p75^{NTR}$ στο διαβητικό αμφιβληστροειδή. Ωστόσο, το BNN27 δεν ανέστρεψε την επαγόμενη από το διαβήτη αύξηση στα $TUNEL^{+}$ κύτταρα του αμφιβληστροειδούς, αλλά εξασθένησε την επαγόμενη από το διαβήτη ενεργοποίηση της κασπάσης-3. Η BNN27 μείωσε επίσης την επαγώμενη από το διαβήτη ανοδική ρύθμιση της πρωτεΐνης GFAP, που εκφράζεται στα μακρογλοιακά κύτταρα Müller, και τον αριθμό των ενεργοποιημένων μικρογλοιακών κυττάρων (Iba-1 ανοσοδραστικότητα). Εντέλει, ο διαβήτης προκάλεσε ανισορροπία μεταξύ των επιπέδων των δύο ισομορφών του NGF, της ώριμης (NGF) κι ανώριμης (proNGF) ισομορφής, στον αμφιβληστροειδή αρουραίου. Η χορήγηση της BNN27 αποκατέστησε την ισορροπία αυτή δρώντας ομοιοστατικά στον αμφιβληστροειδή.

Συμπερασματικά, η μικρονευροτροφίνη BNN27 ασκεί νευροπροστατευτικές δράσεις στον διαβητικό αμφιβληστροειδή αρουραίου όταν χορηγείται είτε ενδοπεριτοναϊκά είτε με οφθαλμικές σταγόνες. Η BNN27 έδειξε ότι στοχεύει ειδικά τους νευρώνες και τα γλοιακά κύτταρα του αμφιβληστροειδούς, δρώντας ως νευροπροστατευτικός κι αντιφλεγμονώδης παράγοντας, αντίστοιχα. Διαθέτει το φαρμακολογικό προφίλ που απαιτείται για την αντιμετώπιση των νευροεκφυλιστικών και φλεγμονωδών παραμέτρων που χαρακτηρίζουν τη ΔΑ. Τα αποτελέσματά μας υποστηρίζουν ότι η καινοφανής νευροτροφίνη BNN27 θα μπορούσε δυνητικά να χαρακτηριστεί ως πρωτότυπο μόριο-οδηγός για τη θεραπεία της ΔΑς.

1. INTRODUCTION

1.1. The Retina

The vision is a sense of capital importance thanks to which we can perceive the world around us. Vision is defined as the perception of physical reality through sight. It provides us information about how everything is: colour, shape, distance, height, etc. The retina is part of the central nervous system and responsible for the visual process. In the early 20th century, the Spanish neuroanatomist Santiago Ramón y Cajal, the father of the modern Neuroscience, along with his scientific adversary, the Italian Camillo Golgi shared the Nobel Prize in Physiology or Medicine in 1906, in recognition of their work on the structure of the nervous system. Cajal investigated the neuroanatomy of the visual system using the Golgi method to impregnate nerve tissue. As shown in Fig. 1 we can see a drawing taken from a Cajal's book of the distribution of retinal neurons in vertebrates. He illustrated the different morphologies of the main cell types on the retina: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. Even though there might be some misinterpretation in the retinal architecture and function in Cajal studies, his interest and pioneer contribution at that time, shed light on the understanding of the retinal structure and represents a milestone in the first steps of neuroscience. From then on, many studies were undergone to better understand retinal organization and function and their importance in the visual system.

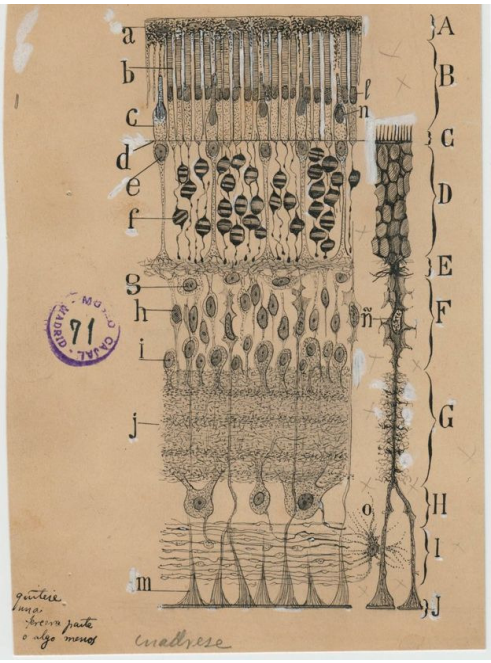


Figure 1. Cajal's diagram showing the important classes of cells and structural layers in the retina. Cajal Legacy (Instituto Cajal, Madrid).

Retinal cell populations

The retina is composed of a set of 6 different cells: neurons, retinal pigmented cells, macroglia, microglia, vascular endothelial cells and pericytes, as described below:

Neurons: The five main retinal neuronal types are:

- Photoreceptors: There are two types of sensory photoreceptors: rods and cones. The rods are responsible of the black-and-white vision as all of them contain the same light-sensitive pigment, rhodopsin. Whereas each cone expresses only one opsin pigment so different light wavelengths pertaining to the wide range of vision colours can be detected (Masland, 2012).
- Bipolar cells: are vertically oriented cells that participate in the signal transfer from photoreceptors to ganglion cells.
- Horizontal cells: are interneurons horizontally oriented and they take part in the modulation of the photoreceptors function and in the information transmission from photoreceptors to bipolar cells. Together with amacrine cells they operate the lateral information processing.
- Amacrine cells: These cells are interneurons, essential to integrate the retinal information in a vertical direction mainly, and modulate the visual message delivered from bipolar cells to the ganglion cells. There are more than 20 different amacrine cells depending on the classic or non-classic neurotransmitter (e.g. acetylcholine, dopamine, serotonin, GABA, glycine, somatostatin, nitric oxide synthetase) that they express, and their morphology. Dopamine, somatostatin and nitric oxide are important in the modulation of light adaptation (Witkovsky, 2004).
- Ganglion cells: they integrate the ganglion cell layer forming one cell layer thickness except close to macula and optic disc, where more than one cell is present (Remington, 2012). We can find around 30 different types of ganglion neurons in

retinas of mammals with distinct dendritic morphology, cell body and dendritic tree size and stratification patterns besides their particular functional and molecular features (Marshak, 2009; Münch, 2010; Sanes and Masland, 2015). The long axons of retinal ganglion cells (RGCs) converge in the fiber layer to finally form the optic nerve. These large cells are responsible for the transmission of the visual information from amacrine cells to higher structures in the brain through the optic nerve.

Pigmented epithelial cells: these cells are arranged in a monolayer named the retinal pigment epithelium (RPE), which integrates the outer blood-retina barrier (BRB) (Rizzolo, 2007; Sparrow *et al*., 2010). The RPE mediates several processes to maintain the photoreceptor excitability after absorbing light. It also plays a discriminating filter role to deliver nutrients from the blood stream to the photoreceptors preserving the integrity of both the endothelium cells and the photoreceptors (Strauss, 2005).

Macroglia: Among macroglia we can find two cell types in the mammalian retina: Müller cells and astrocytes (Holländer *et al*., 1991). Müller cells are the most representative glial cell in the retina. It is thought that each Müller cell exerts the function of nutrition, support and maintenance of the neuronal function to the immediate surrounding neurons through the regulation of extracellular substrates such as K^+, glutamate, GABA and H^+ (Newman *et al*., 1996). They also envelop the blood vessels so they could be involved in the BRB function (Reichenbach *et al*., 1995). Under pathological conditions such as diabetes, the retina undergoes gliosis turning Müller cells into an activated form (Bringmann *et al*., 2009). Within the set of complex signals involved in gliosis we can highlight the up-regulation of the glial acidic fibrillary protein (GFAP) in Müller cells whereas under normal conditions this protein is barely present (Mizutani *et al*., 1998; Belecky-Adams *et al*., 2013).

The astrocytes are also macroglial cells but they are not produced in the retina as Müller cells, instead, they migrate into the retina from the optic nerve (Holländer *et al*.,

1991). Generally, astroglial cells provide support and maintenance to either neurons or blood retinal vessels.

As a response to persistent detrimental conditions in the retina, either Müller cells or astrocytes may produce different components compromising the integrity of the BRB and promoting neurodegeneration (Kim *et al*., 2006).

Microglia: these cells show different morphology depending on the state of activation as a response of physiologic or pathological stimuli (Kettenman *et al*., 2011). Commonly, the cells are small and ramified when at a quiescence state. However, they become ameboidal with surface protrusions when activated (Davis *et al*., 1994). Nonetheless, microglia may undergo a wide range of physiological intermediate states (Cherry *et al*., 2014). The main function of microglia is defend and preserve the retinal immune integrity from invading pathogens by secreting anti-inflammatory cytokines and pro-survival factors or by inducing phagocytosis of apoptotic neurons (Rivest *et al*., 2009). But also, the interaction with other cells, such as neurons, helps to the homeostasis of neural networks (Kettenman *et al*., 2013). As the astrocytes, they may contribute to neuronal cell death if the deleterious signals are persistent (Zeng *et al*., 2008).

Vascular endothelial cells and pericytes: the endothelial cells constitute the inner BRB and they protect the neighbouring neuronal cells from potentially damaging molecules in the bloodstream (Chou *et al*., 2014). Pericytes are embedded in the basement membrane of blood vessels as coverage of capillaries (Gerhardt and Betsholtz, 2003). Among the functions of these last cells include the sustainability of the BRB and control of the vascular network (Armulik *et al*., 2005). Pericytes may be the first vascular cells affected by DR and as a result the endothelial cells undergo changes in their development and function inducing dysregulated angiogenesis (Hammes, 1995; Gerhardt and Betsholtz, 2003).

Retinal cell organization

In a micrograph of a vertical section of a retina we can observe an anatomical organization thickness of three layers containing the cell bodies and two layers holding the synapses (Fig. 2). The set of all different retinal neurons along with astrocytes, Müller cells and microglia shape a complex neural network named neuroretina which contacts the inner and outer areas of the retina.

The outermost layer is the choroid, formed by blood vessels and from which the oxygen and nutrients are delivered to the outer retinal layers. Over the choroid is the sclera or the white of the eye formed essentially by collagen. Below the choroidal is situated the RPE and adjacent to its basal membrane, the photoreceptors. The outer segment of the photoreceptors, which contains the colour pigments, is located in the photoreceptor outer segment (POS) layer while the inner segments are in the outer nuclear layers (ONL) and the axons in the outer plexiform layer (OPL). Also, in the OPL, the synapses among rods, cones, bipolar and horizontal cells take place. Below this last layer, we can find the inner nuclear layer (INL) which contains the cell bodies pertaining to bipolar, horizontal and amacrine cells. Their axons are contained in the inner plexiform layer (IPL) but here, we can also find displaced amacrine cells. In the IPL there are interactions among all different amacrine cell populations with ganglion cells. The ganglion cell layer (GCL) is the innermost layer that includes the cell bodies of ganglion cells and also displaced amacrine cells. Ganglion cell axons are contained in the nerve fiber layer (NFL) making the optic nerve that runs to the visual areas in the brain. The optic nerve not only contains the ganglion cell axons but also blood vessels in order to provide vascularization to the inner retinal layers. Microglia is distributed along all the retina width (GCL, IPL, INL and OPL). Astrocytes can be found as starry shaped cells associated to blood vessels dispersed in the innermost retinal layers: nerve fiber and ganglion cell layer. Müller cells are wide spread through the whole thickness

of the retina. They present a radial disposition starting from the outer retinal layers until the nerve fiber layer where the end-feet lie.

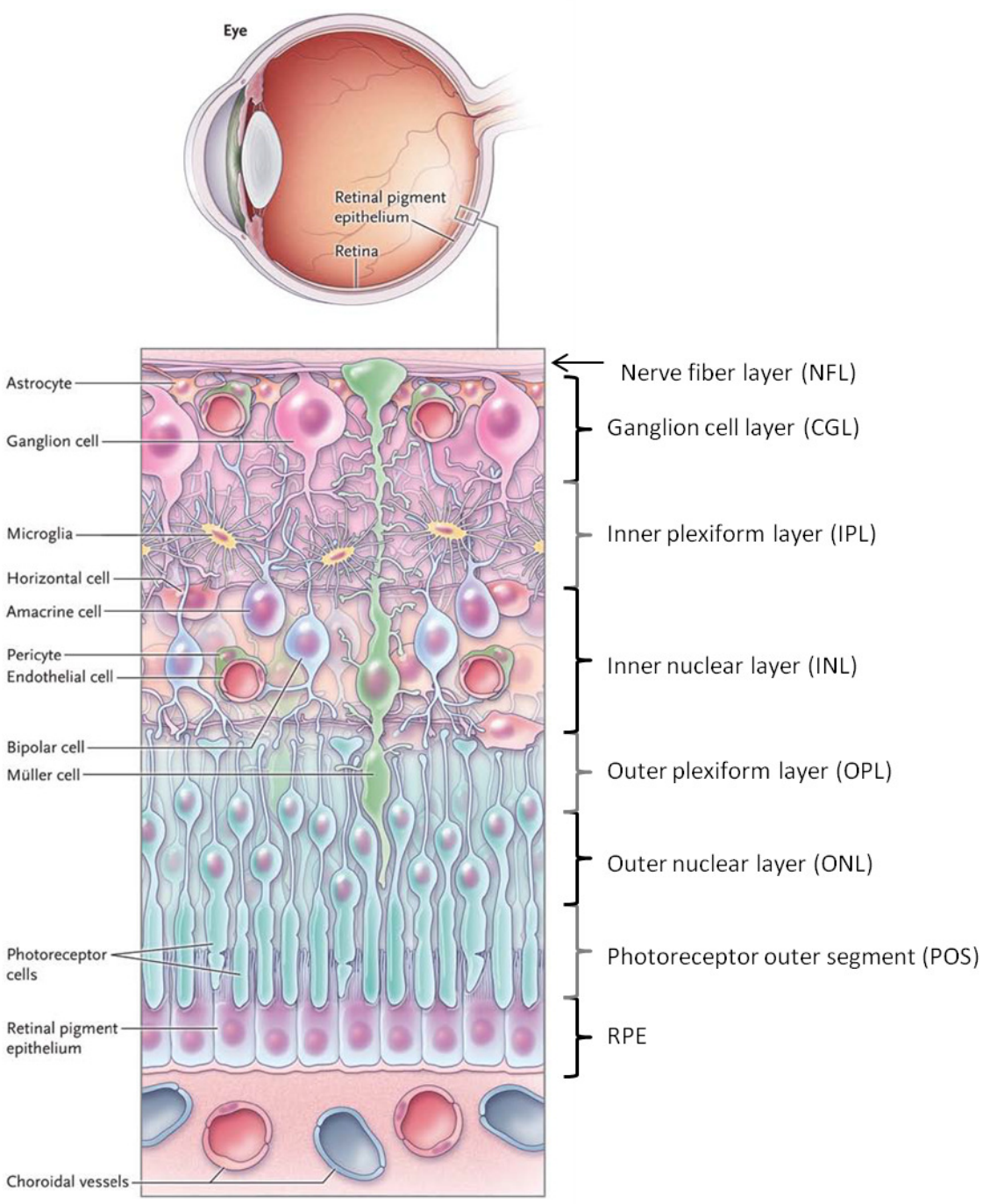


Figure 2. The Neurovascular Unit of the Retina. Under normal conditions, blood-vessel endothelial cells and pericytes, astrocytes, Müller cells, and neurons are intimately connected to establish the BRB to control nutrient flow to the neural retina affording energy balance, to maintain the proper ionic environment for neural signaling, to regulate synaptic transmission, and to provide adaptable responses to the environment to allow vision. *Image adapted from Antonetti et al., 2012.*

Retinal function

The process of the formation of the visual information is quite particular. The incoming light has to travel through all the layers before reaching the photoreceptors, located at the bottom of the eye. These are the only retinal cells capable to perceive the light signals. Interestingly, the retinal neurons are transparent, so the light can transit with no hindrance through the retinal thickness. However, the visual information is integrated in the opposite direction to how it was received (Fig. 3). The cones and rods are activated upon absorbance of the light and they convert it into electric signal which is transmitted and spread to the inner layers through synapses in the retinal neuronal network. The ganglion cells are responsible for discharging the biochemical signal to the visual cortex, located in the occipital lobe of the brain, along their axons comprised in the optic nerve. Hence, the maintenance of the functional integrity is essential in order to provide a pro-survival environment to all retinal components and to preserve the BRB for a proper visual system function (Antonetti *et al*., 2006).

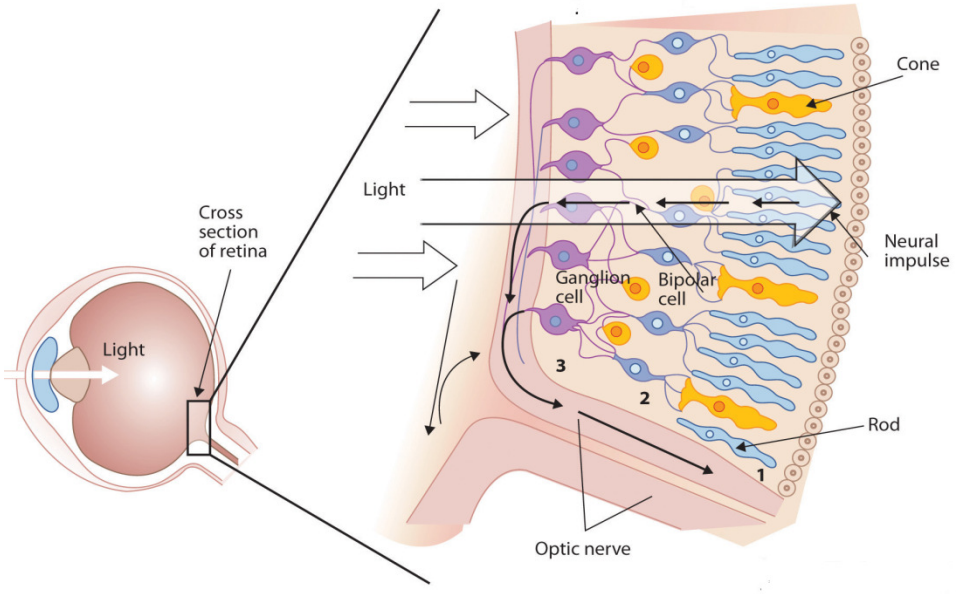


Figure 3. Light travel through retina. When light enters the eye, it first reaches the back of the retina in photoreceptors (1). Then, they transmit a chemical signal to horizontal, bipolar, amacrine and ganglion cells (2). Next, the biochemical message is sent through the optic nerve to the visual cortex via thalamus (3). *Digital image from: http://open.lib.umn.edu/intropsyc/chapter/4-2-seeing/.*

1.2. Diabetic Retinopathy

The global prevalence of diabetes mellitus (DM) has been estimated to be 9% among adults aged 18+ years old, and it will be the 7th cause of death in 2030 (WHO, 2015) leading to a "diabetes epidemic" (Antonetti *et al*., 2012). There are two categories of diabetes: Diabetes type 1 (T1D), characterized by a deficiency of insulin due to autoimmune destruction of beta cells. Usually, this disease is developed during childhood or adolescence however it may also appear in adults. Diabetes type 2 (T2D) is characterized by insulin resistance. The organism produces insulin but it can't be used by the cells to neutralize the high blood sugar levels. The progression of this disease will lead to impaired insulin production. T2D occurs due to a combination of unhealthy lifestyle and genetic risk factors, therefore it is partially preventable. People suffering from either T1D or T2D for long time, will develop concomitant metabolic complications affecting other organs such as kidney, heart, adipose tissue, peripheral nerves or eyes. The adaptation of the retina to this molecular stress may be hampered by its particular structure and physiology (Antonetti *et al*., 2006). According to Romero-Aroca *et al*. (2010), after 20 years with diabetes, approximately the 100% of patients with T1D and the 80% with T2D will have some degree of retinopathy. Diabetic retinopathy (DR) is the most common complication of both T1D and T2D, and the leading cause of blindness worldwide in adult diabetic patients (Yu *et al*., 2001; Cheung *et al*., 2010). Yau *et al*. (2012) provided an accurate study of global prevalence of DM, taking into account several factors including the geographic area, race, type and duration of DM, hypertensive and metabolic control. This investigation showed that 35% of diabetic patients had some form of DR, a 7% had proliferative diabetic retinopathy (PDR), 7% had diabetic macular edema (DME) and a 10% showed some of the previously mentioned vision loss stages. Moreover, the expected future increase of people suffering from diabetes would involve an important and serious world socioeconomic impact in the healthcare

system (Heintz *et al*., 2010). It has been estimated that the number of patients with DR will increment from 127 million in 2010 to 191 million by 2030 (Zheng *et al*., 2012). In summary, these studies depict the importance of Diabetic Retinopathy, and the imperative need to prevent and/or treat the deleterious effects caused in the diabetic retina.

Stages of Diabetic Retinopathy

Diabetic Retinopathy is classified as proliferative and non-proliferative (Fig. 4). The non-proliferative Diabetic Retinopathy corresponds to an early stage of DR. The retina has been slightly affected by the high glucose levels over several years. It is characterized by the deterioration of capillaries or microangiopathy. These small vessels become thick and weak, so they may bleed leading to the formation of microhemorrhages and microaneurysms. These microvascular changes along with the occurrence of hard exudates or cotton-wool spots, made up of extracellular lipid leaked from abnormal retinal capillaries, often indicate retinal edema, and development of DME. Proliferative Diabetic Retinopathy is an advanced stage of DR defined by the capillary sprout from pre-existing blood vessels or angiogenesis, and development of abnormal new blood vessels or neovascularization. The emergence of new vessels may pull the retina away from the back of the eye resulting in retinal detachment and serious loss of sight or even blindness (Antonetti *et al*., 2012). As a consequence of an advanced grade of the disease, people experience physical symptoms in both eyes such as spots or dark strings floating in the vision, blurred and fluctuating vision, impaired colour vision, dark or empty areas in the vision. This proliferative stage of DR integrates a crucial healthcare threat and only these patients will undergo serious vision loss (Stitt *et al*., 2016) (Fig 5).

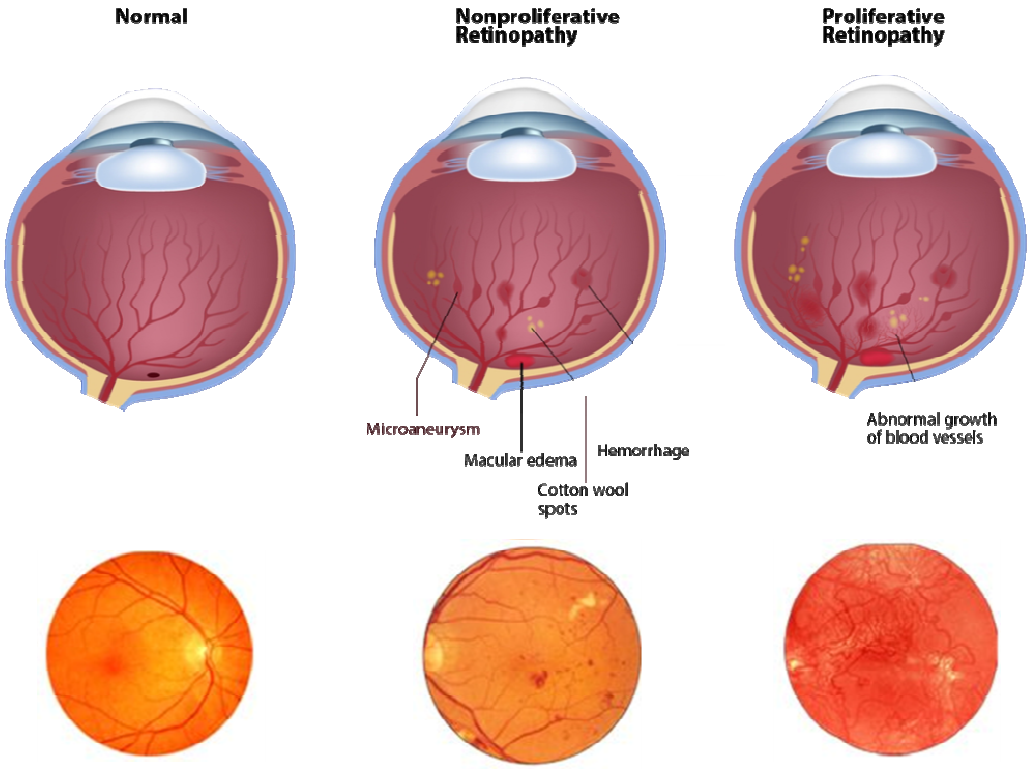


Figure 4. Comparison of a normal eye with two stages of Diabetic Retinopathy: non-proliferative and proliferative. *Adaptation of two digital images from: https://www.eyedoctorophthalmologistnyc.com/procedures/diabetic-eye-exam/ and https://gr.pinterest.com/explore/diabetic-retinopathy/?lp=true*

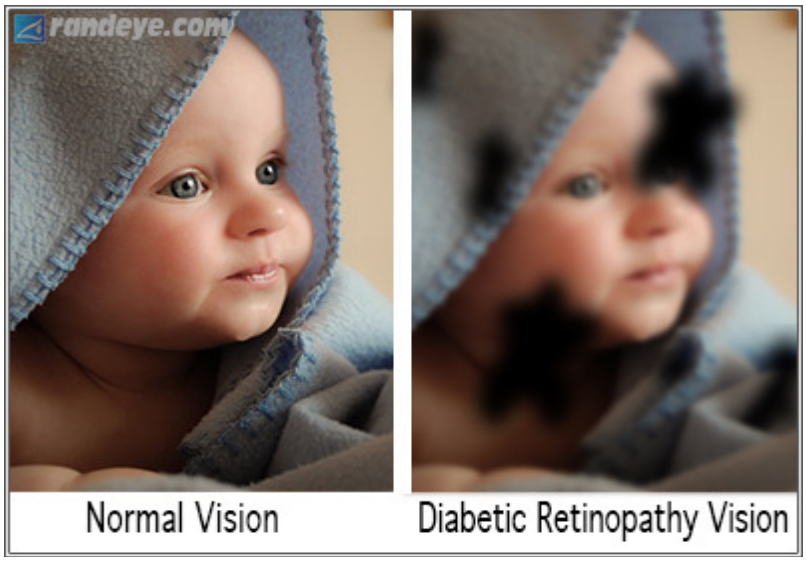


Figure 5. Comparison of sight perception between a person with a normal vision and a person with Diabetic Retinopathy. *Digital image from: https://www.randeye.com/diabetic-retinopathy-diabetes/*

Effect of Diabetic Retinopathy on the three pathogenic edges:

Inflammation-Neurodegeneration-Microangiopathy

A proper interaction among neurons, glia and endothelial cells in the retinal network determines a suitable environment for a correct retinal functionality (Antonetti *et al*., 2006, 2012). Under normal and healthy conditions in the retina, endothelial cells and pericytes together with glial cells and neurons, cooperate to maintain a homeostatic equilibrium (Fig. 6A). However, when diabetic retinopathy is developed, a crescent set of dysfunctional changes on the neuronal-vascular tandem takes place in the retina (Mysona *et al*., 2014). As a result of persistent abnormal metabolic changes in the retina, macroglia is vastly activated disturbing the retinal capillary permeability which contributes to the BRB breakdown. Additionally, the neuronal network is deteriorated and the release of proinflammatory cytokines is increased, mainly by microglia. These deleterious effects will induce the degeneration of retinal neurons, release of pro-inflammatory cytokines and formation of new blood vessels, leading to retinal damage and eventually, to cell death reaching irreversible visual impairment or blindness (Lieth *et al*., 2000; Antonetti *et al.,* 2012) (Fig. 6B).

Therefore, three components encompass all previously mentioned processes and thus play a key role in the pathophysiology of DR: neurodegeneration (Barber *et al*.,1998, 2003; Villarroel *et al*., 2010; Simó *et al*., 2014), inflammation (Kern, 2007; Tang and Kern, 2011; Coucha *et al*., 2015) and microvascular damage (Gupta *et al*., 2013; Rodrígues *et al*., 2013). Even though we know that DR is the result of the combination of these three processes, the actual molecular mechanisms underlying the pathological changes in DR are not fully elucidated. Also, the time course of the interplay among all three of them is a widely debated subject. Along with this, Kizawa *et al*., (2006) detected retinal dysfunction in patients with early stage of DR, represented by abnormal oscillatory potentials (OPs) in the

electroretinogram (ERG), sooner than important vascular damage occurred. Several studies have contributed to the principle that neurodegeneration may precede microvascular lesions (Barber *et al*., 1998; Park *et al*., 2003; Carrasco *et al*., 2007, 2008). Similarly, Kern *et al*. (2007) showed the potential of Nepafenac, a commercial drug used after cataract surgery, administered with eye drops in STZ-diabetic animals for 9 months, in reversing the diabetes-induced vascular decay. However, it did not have any effect on ganglion cell loss, suggesting that microangiopathy might progress oblivious to neurodegeneration, and then they can be considered as independent mechanisms of the disease. It has also been demonstrated that the death of retinal neurons in humans emerges after approximately 2 years from the onset of DM while the vascular damage takes place 10-15 years after diabetes is developed (Aizu *et al*., 2002). Retinal neurodegeneration was proposed as an event in the early stages of the disease and stated the importance to be treated at an initial phase (Simó *et al*., 2014). The interplay among the microvascular, neurodegenerative and inflammatory components of diabetic retinopathy is still not clear, yet their involvement in the pathophysiology of the disease is fully accepted.

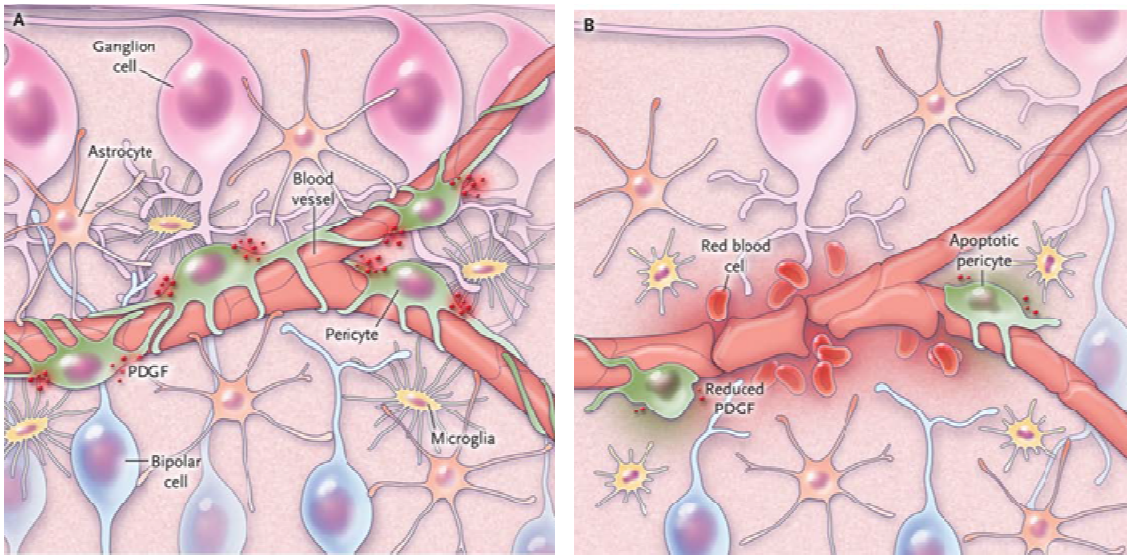


Figure 6. Disruption of the Neurovascular Unit of the Retina by Diabetes. A) It depicts the neurovascular unit in the retina. Pericytes and glial cells promote formation of the BRB in the vasculature, helping to create the environment for proper neural function. Microglial processes monitor the retinal environment. **B)** It

shows how normal cellular communication is altered in diabetes, with elevated VEGF from glial cells, combined with increased inflammatory cytokines, in part from activated microglia and adherent leukocytes (not shown), and the loss of platelet-derived growth factor (PDGF) signaling in pericytes, contributing to the breakdown of the BRB and, in some cases, to angiogenesis.. In addition to microvascular complications, the loss of insulin receptor signaling and damage from inflammatory cytokines may contribute to synaptic degeneration and neuronal apoptosis and impairment of visual function in patients with diabetes. *Image from Antonetti et al., 2012.*

Histopathological changes of Diabetic Retinopathy

The diabetic-induced deleterious effects to the retina are firstly detected by Müller cells, astrocytes and microglia in the inner layers, the most vulnerable layers (Tang and Kern, 2011; Rungger-Brändle *et al*., 2000). The continuous exposure to hyperglycemia induce hypoxia or ischemia in the retina and, as a consequence, Müller cells turn into an active state phenotype inducing gliosis, one of the hallmarks and early feature of DR (Rungger-Brändle *et al*., 2000). In normal rat retinas, either Müller glial cells or astrocytes express little or no GFAP, the main constituent of intermediate filaments in these glial cells (Bringmann *et al*., 2006, 2009). On the contrary, it has been observed overexpression of this protein in the retina under different retinal diseases or injury such as ischemia (Larsen and Osborne, 1996), glaucoma (Xue *et al*., 2006), kainate-induced neurotoxicity (Chang et al., 2007) and DR (Mizutani *et al*., 1998; Agardh *et al*., 2001). The upregulation of Müller cells contribute to maintained high levels of the principal neurotransmitter in the retina, glutamate, given that they are unable to convert it into glucosamine (Lieth *et al*., 1998; Gowda *et al*., 2011). As a consequence, glutamate is accumulated extracellularly stimulating the increase of intracellular calcium in postsynaptic neurons leading to excitotoxicity cell death (Lieth *et al*., 1998).

The function impairment in Müller glial cells will affect the retinal network. In a like manner, ganglion cell neurons are highly sensitive to prolonged exposure to abnormal high levels of extracellular glutamate, causing therefore excitotoxicity (Kuehn *et al*., 2005). It is thought that amacrine and ganglion cells are the first retinal neurons to be significantly

affected by diabetic retinopathy in both humans (Barber *et al*., 1998) and rats (Zeng *et al*., 2000; Seki *et al*., 2004). The early neuronal dysfunction in amacrine and ganglion cells might be due to the fact that the inner retinal layers, location of these cells, are more compromised than the outer cells (kohzaki *et al*., 2008) and this occurs because the inner BRB is more vulnerable to vascular insults than the outer BRB (Do Carmo *et al*., 1998). It has been reported that bipolar cells and photoreceptors are also affected by the metabolic changes associated to DR (Park *et al*., 2003), yet its relevance to the retinal features in DR is not clear (Kern, 2007).

The abnormal DR-induced activation of Müller cells promotes the overexpression of VEGF by the same cells (Wang *et al*., 2015). The increase of VEGF will promote the formation of new vessels, first in the inner nuclear layers and lately in deeper layers (Stone *et al*., 1995). The vascular damage becomes more pronounced over the years in humans or months in experimental models, when the disease is more advanced and clinically settled. Retinal capillaries get deteriorated by occlusion and eventually undergo cell death leading, as a consequence, to the ischemia-induced formation of new or secondary blood vessels (Antonetti *et al*., 2012). Neovascularization or angiogenesis give rise to the proliferative stage of DR. The emergence of abnormal blood vessels may pull the retina away from the back of the eye resulting in retinal detachment and serious loss of sight or even blindness.

The relevance of inflammation in the pathogenesis of early and late DR has also been established (Kowluru *et al*., 2004). The DR-induced release of inflammatory cytokines is mediated by activated microglial cells (Rungger-Brändle et al., 2000; Krady et al., 2005; Zeng et al., 2008), activated Müller cells (Wang *et al*., 2010), and pericytes (Kowluru *et al.,* 2010a) and less clear by endothelial cells (Busik *et al*., 2008). Pro-inflammatory interleukins -1β, -6 and -8 or TNF-α, were detected in the vitreous of patients with PDR (Abu el Asrar *et al*., 1992; Yuuki *et al*., 2001; Demircan *et al*., 2006) and in retinal lysates from STZ-induced diabetic rats (Carmo *et al*., 1999). Hernández *et al*. (2005) not only showed high levels of the

pro-inflammatory mediators IL-8 and the monocyte chemoattractant protein-1 (MCP-1) in the vitreous fluid of patients with PDR, but also, attenuation on the anti-inflammatory IL-10 levels. More studies confirm the correlation of elevated concentration of inflammatory proteins with PDR (Elner *et al*., 1998; Cicik *et al*., 2003). The expression of the inflammatory mediators is determinant regulated through transcription factors such as the nuclear factor kappa B (NFκB), among others (Rahman, 2002), and this factor was shown to be up-regulated in a STZ-model of DR (Zheng *et al*., 2004).

The increase of inflammatory mediators (IL-1 and TNF-α) in the diabetic retina may compromise the endothelial cells and pericytes function by damage on the gap junctions disrupting the BRB permeability (Claudio *et al*., 1994). It has been reported that T2D patients showed chronic inflammatory events in the first place before vascular damage emerged (Stehouwer *et al*., 2002). Schram *et al.* (2005), stated the association between inflammation and microvascular lesions in T1D, however, the emergence of the two processes throughout diabetes development is not clear.

Cell death in Diabetic Retinopathy

Apoptosis is considered to be the major form of cell death in diseases such as age-related macular degeneration (AMD) (Dunaief *et al*., 2002), retinitis pigmentosa (Wong, 1994), glaucoma (Kerrigan *et al*., 1997) and experimental retinal detachment (Cook *et al*., 1995) or retinal detachment in humans (Chang *et al*., 1995). Barber *et al*. (1998) reported apoptosis in retinal neurons just after 1 month of diabetes in a STZ-animal model of DR as well as in retinas of patients with diabetes for six years. Diabetes raises cell death in neurons and vascular cells. Furthermore, accumulating evidence suggests that another cell death pathway named programmed necrosis or necroptosis is also important several diseases including retinal degeneration (Vandenabeele *et al*., 2010). Apoptosis or programmed cell

death implicates chromatin condensation and nuclear fragmentation, cells tend to round up, plasmatic membrane is maintained and it happens not only upon cell damage but also during development (Nikoletopoulou *et al*., 2013). On the other hand, necroptosis is an event characterized by overall cellular disruption, maintaining the nucleus unharmed though as response of injury or pathological conditions only (Leist *et al*., 2001). These two routes of cell death can be induced by the same insult and take place simultaneously albeit the magnitude of the stimulus will determine the prevalence of one via or the other (Shimizu *et al*., 1996: Bonfoco *et al*., 1995). Hyperglycemia might induce oxidative stress increasing the levels of noxious superoxide species (Nishikawa *et al*., 2000; Ceriello, 2003). As a consequence, the prompt release of cytochrome *c* to the cytosol takes place, which is a crucial event in the activation of the Caspase-3 signaling, hence in the initiation of apoptosis (Kowluru *et al*., 1996, 2001; Cai *et al*., 2002). There are several studies that strongly support the Caspase-3 induced-cell death pathway through which apoptosis and necrosis occur (Eguchi *et al*., 1997; Leist *et al*., 1997; Palomba *et al*., 1996). However, whether the same Caspase-3-like protease is activated or not in both cell death mechanisms remain elusive (Higuchi *et al*., 1998). Murakami *et al*., (2014) demonstrated the necrosis-induced cell death of RPE and photoreceptors in a mouse model of retinal degeneration. Necroptosis involves the receptor-interacting protein 3 (RIP3) which interacts with receptor-interacting protein 1 (RIP1) to form a pro-necrotic complex (Christofferson and Yuan, 2010). Notably, inhibition of RIP1 by necrostatin-1 or the lack of RIP3, have been shown to hinder the necrotic disturbances and alleviate the cell damage (Trichonas *et al*., 2010; Xie *et al*., 2013). Recent studies have shown that when caspase pathways are blocked, receptor interacting protein (RIP) kinases promote necroptosis and overcome apoptosis inhibition (Hanus *et al*., 2016; Wu *et al*., 2012; Nikoletopoulou *et al*., 2013).

Nevertheless, the loss of function of certain set of neurons is not always the result of neuronal cell loss (Gorman, 2008). In the early stages of DR, there is relatively little loss of

neurons, so the loss of function or dysfunction might be on account of processes other than cell death (Barber *et al*., 2011). One of the reasons why the retinal function might be affected is by alteration of synaptic transmission due to loss of the synaptic proteins. This event can be linked to overexpression of glutamate receptors and calcium-binding proteins, thus disturbing the glutamate and calcium metabolism in the diabetic retina (Ng *et al*., 2004). Kurihara *et al*. (2008) showed the diminished levels of synaptophysin, a presynaptic vesicle protein that participates in the synaptic transmission, in the diabetic retina. This effect was also observed in Alzheimer's and Parkinson's disease (Zhan *et al*., 1993) or retinal detachment (Kurihara *et al*., 2006). VanGuilder *et al*. (2008) measured different presynaptic proteins from diabetic rat retinas in a STZ-model of DR, and they came to the conclusion that DR inflicts the synaptic function in the retina by reducing the levels of presynaptic components. Since the synaptic transmission is disrupted, so will the neuronal signaling, leading to retinal function impairment and subsequent vision decline. These events seem to appear in the first place and to build the way up to the progressive loss of neurons in degenerative diseases (Bredesen *et al*., 2006). Moreover, proteins may undergo diabetes-induced changes in the biochemistry and physiology, adopting other structural conformation that might trigger cell death processes (Barber *et al*., 2011).

1.3. Therapeutic approaches in Diabetic retinopathy

Diabetic Retinopathy is a disease resulting from the combination of three molecular mechanisms, as previously described. It should be mentioned that a rigorous control of blood glucose levels (DCCT, 1993), blood pressure (Kohner *et al*., 1995) or even dislipidemia (Chowdhury *et al*., 2002), is fundamental to maintain stable the evolution of the disease. However, therapeutic treatments are imperatively needed to arrest the progressive detrimental insults in the diabetic retina. Currently, the available clinical therapies treat the

microvascular damage, and to a lesser extend the inflammatory. However, no available clinical means exist to address neurodegeneration. Some of the therapeutic approaches utilized nowadays to combat the deleterious mechanisms from microangiopathy rely on laser photocoagulation in specifically affected areas in the retina (Mohamed *et al*., 2007). It has been reported that laser treatment improves substantially the vision threatening in about the 50% of patients with DME (Aiello *et al*., 2010). Pharmacologically, one way to treat the inflammatory deleterious effects of DR is the intravitreal administration of corticosteroids, such as triamcinolone, dexamethasone and fluocinolone. Nonetheless, several sessions are needed leading to potentially serious side effects as cataract or glaucoma (Simó *et al*., 2009). The utilization of both laser and corticosteroids administration in conjunction has been seen to be successful in some people with DME (Stitt *et al*., 2016). The efficacy of anti-VEGF drugs, such as ranibizumab (Heier *et al*., 2006), bevacizumab (Michaels *et al.,* 2006), pegabtanib (Gragoudas *et al.,* 2004), and aflibercept (Trichonas and Kaiser, 2013) to treat neovascularization in AMD has been demonstrated. More recently, approval from FDA was received to use these drugs as treatment for DME and DR. The administration of these drugs is intravitreal and several sessions are needed in the first three years of the treatment of DME (around 12-15) (Diabetic Retinopathy Clinical Research *et al*., 2012). Usually, these therapies are well tolerated, and they seem to preserve vision much more than the laser does (Do *et al*., 2013). However, it should be taken into account the aggravating systemic complications that the anti-VEGF treatment may entail due to the possibility to pass into the systemic circulation (Simó *et al*., 2008). Nevertheless, it turns out that the main obstacle could be that the treatment is considerably expensive and the need for several repetitions. Vitreoretinal surgery is another clinical treatment predominantly used in the late stages of the disease, when the vision has already been significantly affected. However, it can be rarely used in the early stages. This therapy is the most expensive, then not available to all DR patients (Joussen and Joeres, 2007). In addition, a set

of anti-inflammatory drugs, firstly utilized for conjunctivitis, has been tried in patients with DR and DME. However, some research is ongoing in order to provide an efficient drug delivery and improve the potential as therapeutics to treat the disease (Kim *et al*., 2010; Sahoo *et al*., 2015; Semeraro *et al*., 2015).

In summary, most of the current therapies for DR requires invasive procedures at a high cost, and fails to address the neuronal damage of the disease. Therefore, there still are some limitations regarding the available treatments for DR. The development of novel pharmacological drugs for DR with the ability to prevent the onset and progression of the disease, require a broaden knowledge of the pathophysiology in order to identify relevant molecular targets. Moreover, the current pharmacological approaches are aimed at the late stages of DR (DME and PDR), when the clinical outcome is well established and the retina has been highly damaged. These facts along with neurodegeneration, which is established as an early event in DR, make it urgent to find a therapeutic strategy to prevent or arrest not only the neuronal-glial degeneration but also the diabetes-induced inflammatory responses with the use of pharmacological agents providing high efficacy and fewer side effects (Vasilaki and Thermos, 2009).

1.4. Neurotrophins and their receptors

Neurotrophins (NTs) pertain to a family of evolutionarily well-conserved growth factors (Hallböök *et al*., 1991; Götz and Schartl, 1994). These large peptides exhibit a wide range of cellular functions inside and outside the nervous system such as neuronal survival, differentiation, axonal and dendritic outgrowth or maintenance of synapses among others (Ichim *et al*., 2012). More specifically, NTs are involved in retinal development and play a pivotal role as regulators of neural function in the visual system during retinal injury or retinal neurodegeneration (von Bartheld, 1998). Four different types of neurotrophins are

described in mammals: Neurotrophic Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT-3) and Neurotrophin 4 (NT-4/5). NGF is the most well known neurotrophin, isolated by Rita Levi-Montalcini and Stanley Cohen at Washington University in St. Louis, USA, for which they received the Nobel Prize of Physiology or Medicine in 1982, 30 years after the discovery.

All neurotrophins are synthesized in the endoplasmic reticulum as biologically active precursors, and subsequently undergo intracellular and extracellular proteolytic cleavage to become mature proteins (Rafieva *et al*., 2015). This proteolytic cleavage is selectively regulated by the serin protease plasmin or furin and different matrix metalloproteinases (MMP-3 and -7), releasing the mature active form of the neurotrophin (Feng *et al*., 2010; Lee *et al*., 2001). Pro-neurotrophins have a molecular mass of 30-35 kDa. They have also been detected in retinal lysates by western blot (WB) analysis at 25, 37 and 53 kDa as a consequence of glycosylation or alternative splicing processes (Bradshaw *et al*., 2015; Wei *et al*., 2012). In regard to the mature neurotrophins, they have been observed by WB at 12-14 kDa. Similarly, due to the use of non-reducing conditions their dimeric conformation is maintained resulting in another band at ≈26 kDa (Yang and Duan, 2013; Barcelona *et al*., 2016).

Neurotrophins exert their actions by binding to two different transmembrane cell surface receptors: the high-affinity tyrosine receptor kinase (Trk) (Tropomyosin Related Kinases) and the low-affinity p75 pan-neurotrophin receptor ($p75^{NTR}$), which pertains to the Tumor Necrosis Factor (TNF) receptor superfamily (Nag *et al*., 1999). There are three types of Trk receptors: TrkA, TrkB and TrkC, with distinct ligand binding affinity and signaling to generate diverse biological responses either in the peripheral or the central nervous system (Obianyo *et al*., 2013). Each mature neurotrophin binds with high affinity to a specific receptor: NGF preferably binds to TrkA, BDNF and NT4 bind to TrkB receptor and NT-3 has affinity toward Trk A, B and C receptors (Fig. 7). The mature neurotrophins have low affinity

for the $p75^{NTR}$. In contrast, all pro-neurotrophins bind to $p75^{NTR}$ with high affinity (Hempstead, 2002). Furthermore, the mature (NGF) and non-processed (proNGF) peptides exert diametrically opposite biological functions when activating TrkA or $p75^{NTR}$, respectively. Thus, Trk receptors display well-characterized trophic pro-survival actions (Huang and Reichardt, 2003), while $p75^{NTR}$ mainly play a role in cell death, Schwann cell migration, myelination, axonal growth or under specific conditions in survival and regeneration (Reichardt, 2006). Hence, the proteolytic cleavage that pro-neurotrophins undergo depicts a crucial event that will drive the direction of action of neurotrophins (Chao and Bothwell, 2002; Ibáñez, 2002; Lu, 2003) and the outcome will depend on the cellular environment.

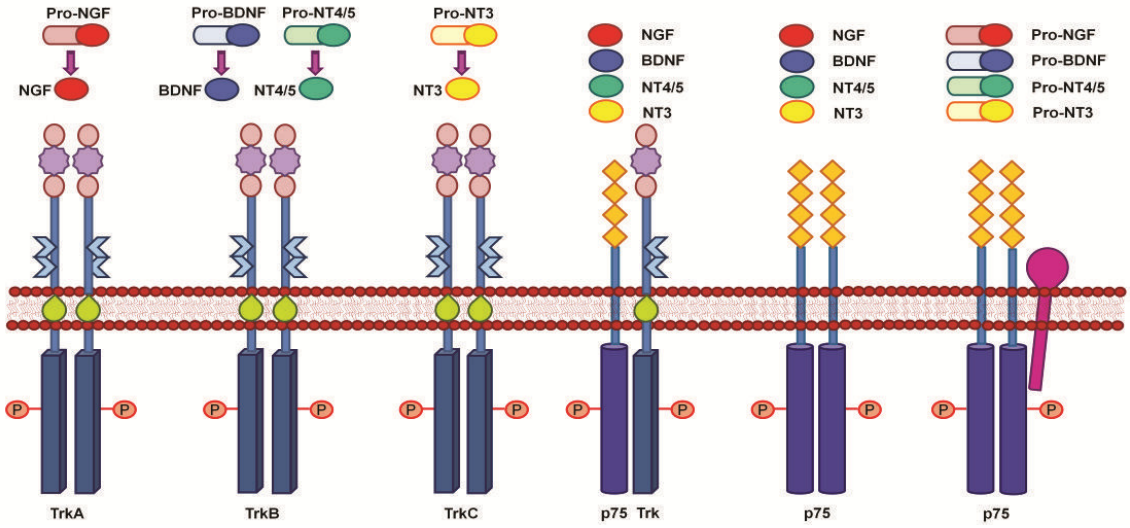


Figure 7. Neurotrophin binding affinity to neurotrophin receptors. Mature neurotrophins bind to their preferred Trk receptors: TrkA for NGF, TrkB for BDNF and NT4/5 and TrkC for NT3. $P75^{NTR}$ enhances the ligand binding to mature neurotrophins when associated to Trk receptors. However, when $p75^{NTR}$ alone, shows los affinity binding to all mature neurotrophins. Unprocessed neurotrophins are higher specificity ligands for $p75^{NTR}$ when associated with sortilin. *Image from Bucci et al., 2014.*

Functional role of TrkA and p75 neurotrophin receptors

TrkA receptor

TrkA receptors are glycoproteins (≈140 kDa) with similar molecular architecture as the rest of Trk receptors and present a ligand-binding domain in the extracellular region, a single transmembrane domain and a cytoplasmic part which contains the tyrosine kinase catalytic activity (Lemmon and Schlessinger, 2010) (Fig. 8). Under quiescence conditions these receptors may be presented as monomers or oligomers. However, they undergo ligand-induced dimerization of the extracellular regions, becoming active after subsequent autophosphorylation on specific tyrosine(s) residues within the intracellular domain. There have been identified the Trk tyrosine residues Y490, Y670, Y674, Y675 and Y785 as substrates for autophosphorylation (Stephens *et al*., 1994). Depending on which of these becomes phosphorylated or phosphorylated to a greater extent, different signaling pathways will be triggered including cell survival, differentiation, axonal and dendritic growth, synapses or plasticity. Three major TrkA signal transduction pathways have been identified in response to neurotrophins: a) the phosphatidylinositol-3 kinase (PI3K)-Akt pathway, b) *Ras*-*Raf*-MAPK pathway and c) the phospholipase C (PLC-*γ*) pathway (Roux and Barker, 2002; Kaplan and Miller, 2000) (Fig. 8). Phosphorylation of the Tyr490 residue activates different adaptor proteins which can be recruited to the receptor activating the PI3K-Akt pathway signaling (Lemmon *and Schlessinger,* 2010). As a result, survival, proliferation and growth events are stimulated as well as suppression of apoptotic proteins (Cantley, 2002; Vivanco and Sawyers, 2002). Pro-survival signaling could be exerted through Akt-mediated activation of NFκB (Romashkova and Makarov, 1999; Foehr *et al*., 2000). The *Ras*-*Raf*-MAPK (mitogen-activated protein kinases) signaling pathway is the most well known. As a response of TrkA receptor activation by NGF, *Ras* and *Raf* are recruited and subsequently MAPK/ERK kinase (MEK) is phosphorylated which then activates the

extracellular signal-regulated kinases ERK1 and ERK2 (Avruch *et al*., 1994; Hill and Treisman, 1995). These kinases phosphorylate a number of transcription factors such as CREB or NFκB, which will produce changes in the gene expression involving regulation of axonal and dendritic growth, differentiation, plasticity or cell death suppression (Riccio *et al.,* 1997, 1999).

In addition, the phosphorylation of Tyr785 induces the PLC-*γ* pathway which in turn activates two different signaling cascades*:* inositol triphosphate (IP_3) and diacylglycerol (DAG) (Obermeier *et al*., 1993) (Fig. 8). As a result, the Calcium-calmodulin-regulated kinases will be stimulated in order to control the intracellular Ca^{+2} content. IP3 and DAG may induce activation of phospho kinase C (PKC) and as a result, the MAPK/ERK1 signaling pathway will be activated through *Raf* proteins (Corbit *et al*., 1999).

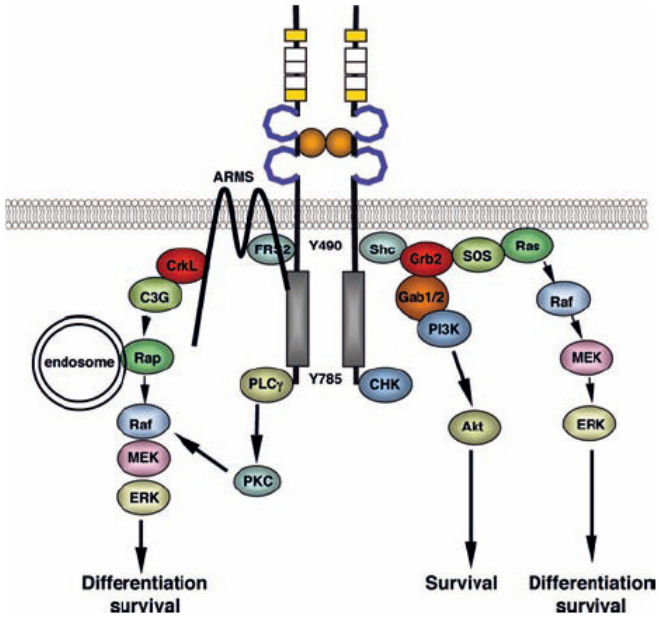


Figure 8. Trk receptor-mediated signaling pathways. Neurotrophin binding to Trk receptors leads to their activation and to the recruitment of different proteins that associate with specific phosphotyrosine residues in the cytoplasmic domain of Trk receptors. These interactions trigger the activation of various signaling pathways, such as the MAPK, PI3K, and PLCγ pathways, which result in survival, neurite outgrowth, gene expression, and synaptic plasticity. Tyrosine residue nomenclature is based on the human sequence of TrkA. *Image from Arévalo and Wu, 2006.*

P75 neurotrophin receptor

$P75^{NTR}$ displays a particular molecular behaviour. This receptor (≈75 kDa) consists structurally of three domains: a ligand-binding extracellular domain, a transmembrane domain and the intracellular domain with a type II death domain which lacks of catalytic or kinase activity (Fig. 9). The $p75^{NTR}$ is able to bind both mature and immature neurotrophins. Pro-neurotrophins are high-affinity ligands for $p75^{NTR}$ only when associated with the co-receptor sortilin, a vacuolar protein-sorting 10 protein (Vps10p) receptor, that regulates the intracellular trafficking of multiple ligands, inducing cell death processes (Nykjaer *et al*., 2004). On the contrary, $p75^{NTR}$ binds with low affinity to all mature neurotrophins leading to neuronal growth, proliferation or myelination mechanisms (Schecterson *et al*., 2010).

When $p75^{NTR}$ is activated by either non-processed or mature neurotrophins, it undergoes intramembrane proteolysis by sequential α-secretase and γ-secretase-catalyzed cleavage of extracellular domain (ECD) and intracellular domain (ICD) respectively, to release the pro-apoptotic p75ICD (Frade, 2005; Zampieri *et al*., 2005; Blöchl and Blöchl, 2010). The $p75^{NTR}$ does not have kinase activity or catalytic domain, thus the pathway signaling proceeds through ligand-induced recruitment and association of the p75ICD with cytoplasmic interactor proteins (Gentry *et al*., 2004) (Fig. 9).

Cell death processes triggered upon $p75^{NTR}$ activation involve the phosphorylation of the stress activated MAP kinase, stress-activated protein kinase or c-jun amino-terminal kinase (SAPK/JNK) pathway (Harrington *et al*., 2002). As a consequence, a number of interacting proteins is recruited (Fig. 9). Interestingly, the activation of Trk-mediated induction of the pro-survival Akt and ERK pathways inhibits the $p75^{NTR}$-induced cell death processes via JNK (Kaplan and Miller, 2000). Furthermore, when proNGF interacts simultaneously with $p75^{NTR}$ and its co-expressed co-receptor sortilin, a ternary complex is formed which promotes pro-apoptotic mechanisms (Nykjaer *et al*., 2004).

On the other hand, $p75^{NTR}$ has also the capability to induce pro-survival mechanisms. A well-characterized pro-survival pathway is the activation of the transcription factor NFκB which has been observed upon $p75^{NTR}$ interaction with specific adaptor proteins (Khursigara *et al*., 2001; Yeiser *et al*., 2004; Casademunt *et al*., 1999; Mamidipudi *et al*., 2004) (Fig. 9). It has been shown that $p75^{NTR}$ induces survival by NFκB activation in a Schwannoma cell line and primary Schwann cells that express only $p75^{NTR}$ (Gentry *et al*., 2000; Khursigara *et al*., 2001). The $p75^{NTR}$ has also the dexterity to induce survival through the activation of PI3K-Akt pathway in PC12nnr5 cells and rat hippocampal neurons that barely express TrkA receptor (Bui *et al*., 2002). On the contrary, other studies showed that $p75^{NTR}$ prompts cell death processes when expressed alone, in the absence of Trk signaling or in cells that do not express Trks (Roux and Barker, 2002).

Moreover, it is known that $p75^{NTR}$ can associate with TrkA receptor resulting in a tetrameric/dimeric complex with high affinity for the mature form of NGF, boosting pro-survival machinery (Makkerh *et al*., 2005). There seems that $p75^{NTR}$ directly affects the NGF binding site conformation in the TrkA receptor and its presence could be essential to provide high-affinity binding and specificity of TrkA receptor activation by NGF, regardless the binding of ligands to $p75^{NTR}$ (Segal, 2003). It has been found in several studies using p75 null mice that the presence of this receptor is vital for neuronal survival in neocortical subplate neurons in the developing cortex or in sensory neurons (DeFreitas *et al*., 2001; Lee *et al*., 1992) and this happens because the neurotrophic signaling resulting from the complex Trk-$p75^{NTR}$ might be reduced.

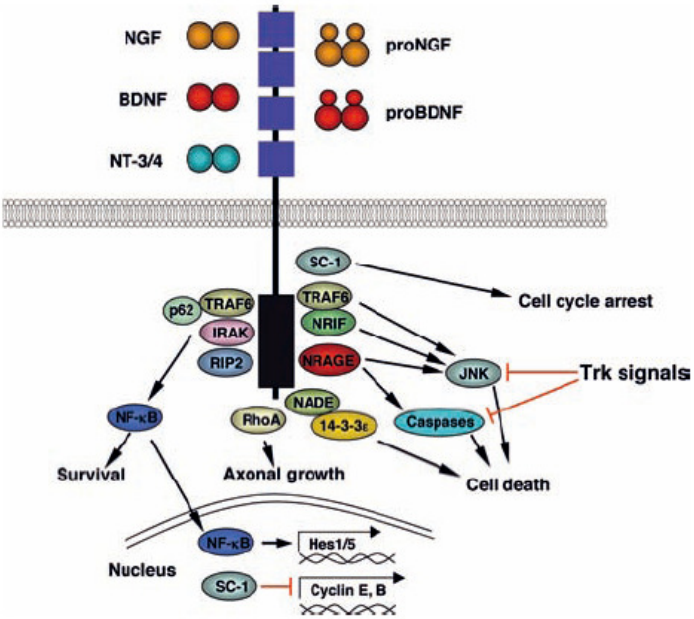


Figure 9. p75^{NTR} receptor-mediated signaling pathways. Binding of mature neurotrophins or pro-neurotrophins to p75NTR triggers the activation of different signaling pathways through different intracellular adaptors that result in diverse and at times opposite outcomes like survival, apoptosis, axonal growth, axonal collapse, and cell cycle arrest. *Image from Arévalo and Wu, 2006.*

Localization of proNGF, NGF, TrkA and p75^{NTR} in adult rat retina

The presence of all NTs and their specific receptors in the retina has been reported in a number of different species such as fish (Caminos *et al.*, 1999; Vecino *et al.*, 1998a), lizard (Santos *et al.*, 2008), frog (Cohen-Cory *et al.*, 1996), chick (Harvey *et al.*, 2009), porcine (García *et al.*, 2003), rat (Carmignoto *et al.*, 1991; Vecino *et al.*, 1998b, c, 2002), monkey (Schatteman *et al.*, 1988) and humans (Nag *et al.*, 1999). More specifically, vast studies in the rodent retina stated the importance of these molecules in retinal damage or degeneration models (Ali *et al.*, 2011; Barcelona *et al.*, 2016). They can be transported in a retrograde fashion (from the axon terminus to the cell somata) by RGCs, as p75^{NTR}, and act in an autocrine manner (Acheson *et al.*, 1995), or use anterograde trafficking and act in a paracrine manner (Carmignoto *et al.*, 1991; Conner *et al.*, 1997).

In order to perform an accurate study with regard to the cellular location or expression of NGF, García *et al*. (2017) proposed some features to take into consideration such as differentiation between precursor proNGF and mature NGF, distinction between NGF mRNA and NGF protein, NGF expression in adult healthy or damaged retina or even NGF expression variability amongst different rodent strains. Therefore, it is of capital importance the use of specific markers against the NGF and proNGF to have rigorous information about the levels and the immunolocalization for each neurotrophin separately in the retina of adult rodents (Santos *et al*., 2012; Al-Gayyar *et al*., 2011). Even so, due to the tight cell assembly between the different neuronal populations and glia in the retina, it would be convenient to perform a double staining by immunohistochemical (IHC) studies or the use of isolated retinal cell cultures to ascertain the specific cellular location of either NGF or a neurotrophin receptor.

Accumulating evidence showed the presence of proNGF and NGF proteins in the retina tissue of adult rodents by WB and IHC (Bronzetti *et al*., 2007; Braunger *et al*., 2013; García *et al*., 2014) and revealed concentrations of NGF in the order of 77-87pg/g (Coassin *et al*., 2008; Colafrancesco *et al*., 2011a). The location of NGF has been observed in RPE, ONL, INL, IPL, GCL and more specifically in amacrine and ganglion cell neurons and glial Müller cells (Vecino *et al*., 1998b) (Fig. 8). As to the proNGF location, it has been detected in RPE, ONL, INL, IPL, ganglion and Müller cells (Chakrabarti *et al*., 1990; Ali *et al*., 2011; Wei *et al*., 2012) (Fig. 10). It remains less clear if these two neurotrophin isoforms are present in photoreceptors of rodent retina due to contradictory studies. Microglia is responsible for producing and releasing the proNGF (Vecino *et al*., 1998b). However, NGF has not been found in these cells, agreeing with the theory that the cleavage to form the mature NGF occurs extracellularly (Srinivasan *et al*., 2004).

On the other hand, TrkA neurotrophin receptor is expressed in bipolar cells and amacrine cells (Wexler *et al*., 1998; García *et al*., 2014), ganglion cells (Vecino *et al*., 1998b;

Kokona *et al*., 2012), Müller cells (Sun *et al*., 2008; Kokona *et al*., 2012) and astrocytes (Kim *et al*., 2013) (Fig. 10). Whether the TrkA receptor is present in photoreceptors is not yet elucidated and further studies of co-immunostaining with rods and cones are necessary to resolve this issue.

Regarding $p75^{NTR}$ location, immunohistochemical studies have shown that this receptor is mainly expressed in photoreceptors (Santos *et al*., 2012; Sheedlo *et al*., 2002) and Müller cells (Mohamed and El-Remessy, 2015) (Fig. 10). It seems that $p75^{NTR}$ could be expressed in amacrine cells during retinal development but not in the adult retina (García *et al*., 2017). Moreover, several studies confirm its presence in ganglion cells (García *et al*., 2014; Vecino *et al*., 1998b). Nevertheless, there are some controversial results from other authors who did not show $p75^{NTR}$ expression in these cells (Hu *et al*., 1998, 1999; Xu *et al*., 2009). García *et al*., (2017) suggested the possibility that two different ganglion cell populations exist due to their ample molecular variability in retinas of mammals: those that express $p75^{NTR}$ and those that do not.

It should be mentioned that Lebrun-Julien *et al.*, (2009) claimed that $p75^{NTR}$ and TrkA receptors are expressed in different cell populations in the adult rodent retina. The co-presence of these four elements (TrkA, $p75^{NTR}$, proNGF, NGF) in same or neighbouring retinal cell populations suggests the interconnection amongst them to either trigger pro-survival or cell death signaling pathways (García *et al.* 2017). However, the physiological state of the cell will affect the proNGF/NGF ratio and expression level and activity of TrkA and $p75^{NTR}$ receptors leading to these two different neurotrophin receptors to have independent, synergistic, or antagonistic functions (Masoudi *et al*., 2009; Blöchl and Blöchl, 2010; Feng *et al*., 2010).

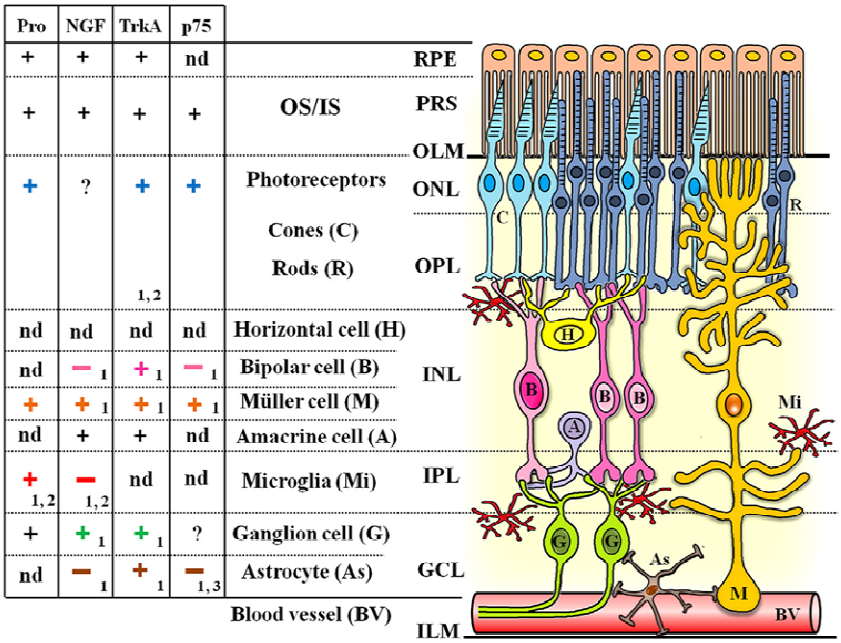


Figure 10. Localization of proNGF (Pro), NGF, and the receptors TrkA and p75^{NTR} in the RPE and cells of the neural retina from adult rats and mice. RPE, retinal pigment epithelium; PRS, segments of photoreceptors; OS, outer segments; IS, inner segments; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ILM, inner limiting membrane. (+), mRNA or protein expression; (-) mRNA or protein expression was not detected; (1), data derived from immunolabeling analysis using a specific cell marker in retinal sections or expression analysis in cell cultures; (2) Retinas from RCS rats. (3) One recent report found a weak labeling for p75^{NTR} in astrocytes from control retinas of adult mice; (nd), not determined; (?) contradictory data regarding the localization of NGF in photoreceptors and p75^{NTR} in RGCs. Note that the data regarding the expression of NGF may represent the total NGF (proNGF plus mature NGF) because the authors used antibodies which recognize both molecules. *Image from García et al., 2017.*

Imbalance of proNGF/NGF and their receptors in Diabetic Retinopathy

Alteration either in the proNGF/NGF balance or in the ratio of their receptors TrkA/p75^{NTR} have been manifested in patients with diverse pathological conditions such as Alzheimer's disease (Pedraza *et al*., 2005), amyotrophic lateral sclerosis (ALS) (Lowry *et al*., 2001), multiple sclerosis (MS) (Dowling *et al*., 1999), brain injury (Harrington et al., 2004) or retinal dystrophy (Srinivasan *et al*., 2004). Such functional impairment has been reproduced and profoundly investigated in several rodent models of diabetic retinopathy (Al-Gayyar *et al*., 2011; Mysona *et al*., 2013; Ola *et al*., 2015; Barcelona *et al*., 2016).

Diabetes contributes to the homeostasis impairment of proNGF/NGF ratio as shown in the diabetic milieu resulting in abnormal increase of proNGF expression and lack of optimal amounts of mature NGF to maintain neuronal survival (Mohamed and El-Remessy, 2015). The progression of diabetic retinopathy was seen to induce an increase in the levels and accumulation of proNGF in the retina of diabetic animals from 3 up to 6 weeks of diabetes (Al- Gayyar *et al*., 2011; Ali *et al*., 2011; Mysona *et al*., 2013). This abnormal increase was also observed in glucose-stimulated retinal Müller cells cultures (Gayyar *et al*., 2011; Ali *et al*., 2011). Activation of microglial cells is known to happen before the onset of neuronal cell death in diabetic rat retinas (Krady *et al*., 2005). On the contrary, NGF protein levels seem to be downregulated in rodent diabetic retinas from 4 up to 8 weeks of diabetes (Ali *et al*., 2011; Ola *et al*., 2015; Colafrancesco *et al*., 2011a). In addition, Matragoon *et al*., (2012) saw that those diabetic rat retinas with up-regulation of proNGF showed a high increase in the number of TUNEL positive ($^{+}$) retinal cells in GCL, INL, Brn3a-positive ganglion cell loss or loss of neurons not only in the central retina but also in the peripheral retina, compared to control rat retinas.

The imbalance of proNGF/NGF is linked to alteration in the expression or activation of TrkA and p75 neurotrophin receptors. It appears that diabetes affects the NGF/TrkA receptor activation, inducing a decrease on its phosphorylation. However, no alteration was observed on its expression in either diabetic human or rat retinas (Marsh *et al*., 2003; Ali *et al*., 2008). The increase in the levels of the proNGF favors the stimulation of the proNGF/$p75^{NTR}$ axis which involves up-regulation of $p75^{NTR}$ expression (Mysona *et al*., 2013, 2015). This connection was also demonstrated in another study where the imbalance of proNT3/NT3 was associated with high levels of $p75^{NTR}$ expression, leading to photoreceptors degeneration in a model of Müller cell ablation (Shen *et al*., 2013). Furthermore, since diabetes changes the TrkA/$p75^{NTR}$ ratio by increasing the expression of $p75^{NTR}$, there might be a decrease in the responsiveness to NGF (García *et al*., 2017). The boost of

proNGF/p75^{NTR} pathway has been connected to the release of pro-inflammatory factors such as TNF-α and IL-1β in Müller cells which contributes to pro-cell death mechanisms (Lebrun-Julien *et al*., 2010). It has been shown in several models of proNGF overexpression, that the increase of proNGF and p75^{NTR} in glial Müller cells is accompanied by increased GFAP expression (Gardner *et al*., 2002; Al-Gayyar *et al*., 2011; Ali *et al*., 2011; Mysona *et al*., 2013, 2014). In response to diabetic retinopathy, retinal Müller cells undergo gliosis as shown by up-regulated GFAP expression and increase in the production of proNGF in diabetic rat retinas. Moreover, arisen levels of GFAP were related to alteration in the expression of endogenous NGF in an acute model of retinal degeneration (Jian *et al*., 2015). GFAP and proNGF were seen to be colocalized in the end feet of retinal Müller cells linking this effect to RGCs death (Al-Gayyar *et al*., 2011; Ali *et al*., 2011). In addition, it has been well established the involvement of p75^{NTR} in retinal ganglion cells death in several experimental model of glaucoma (Bai *et al*., 2010) or diabetic retinopathy (Mysona *et al*., 2013; Barcelona *et al*., 2016).

The apoptotic function exerted by proNGF/p75^{NTR} involves phosphorylation of the SAPK/JNK, the p38 subgroups of MAPK (p38MAPK) and cleaved-Poly (ADP-ribose) polymerase (PARP) activity as seen in oligodendrocytes, RGCs of STZ-treated rat animals and sympathetic neonatal neurons (Casaccia-Bonnefil *et al*., 1996; Al-Gayyar *et al*., 2011; Bamji *et al*., 1998). Shanab *et al*. (2015) utilizing proNGF overexpression *in vitro* and *in vivo* models, demonstrated for the first time that proNGF induced the release of the p75ICD, which recruited the neurotrophin receptor interacting factor (NRIF) to promote apoptotic mechanisms through JNK activation. In the same study, silencing of p75^{NTR} expression restored the ratio proNGF/NGF, suppressed the proNGF/p75^{NTR} signals and stimulated the NGF/TrkA pathway activation.

Given that neurotrophins are known to be essential for growth, differentiation and survival in the developing and mature retina, boosting the neurotrophic actions in diabetic

retina in order to restore the balance of proNGF/NGF and targeting the silencing of proNGF/$p75^{NTR}$ axis while favouring the TrkA-mediated pro-survival signaling, may be a potential therapeutic strategy to prevent early signs of DR.

Therapeutic treatment of NGF in retinal injury or disease

The potential of NGF as a therapeutic agent in the treatment of numerous neurodegenerative diseases has been broadly investigated in distinct experimental models (Micera *et al*., 2004; Aloe *et al*., 2012). NGF plays an important role as a neuroprotective molecule in neurodegeneration, inflammation, vascular permeability and apoptosis, all important processes in the aetiology and pathogenesis of DR (Covaceuszach *et al*., 2008). NGF (5mg/kg, 3 times per weeks, 14 weeks) showed protective activity for the survival of RGCs and Müller cells, prevented loss of pericytes and development of occluded vessels when administered to diabetic rats (Hammes *et al*., 1995). More recent studies showed that topical ophthalmic administration of NGF protected RGCs from apoptosis in an animal model of glaucoma (200µg/ml, 4 times, daily, 7 weeks), in patients with glaucoma (200µg/ml, 3 months) (Lambiase *et al*., 2009) and in the STZ model of DR (7 or 11 weeks) (Mantelli *et al*., 2014). This last group observed early increase of NGF levels after induction of diabetes. This effect might occur due to an attempt of the retina to protect from degeneration by increasing the endogenous NGF levels. Similarly, RGCs were preserved from detrimental apoptotic events when NGF was administered by eye drops 8 weeks after induction of diabetes and glaucoma in rat animals (20µl, 0.2µg/µl, twice a day, 3 weeks) (Colafrancesco et al., 2011b). Kokona *et al*., (2012) showed that NGF administered intravitreally (60pg/eye; single injection) prevented the retinal damage induced by the excitatory amino acid AMPA, reversing the attenuation of the number of amacrine and horizontal retinal cells and decreasing the number of $TUNEL^{+}$ cells in the INL, compared to non-treated animals. More

recent studies showed that exogenous NGF administration using a rat model of inherited retinitis pigmentosa delayed and protected isolated photoreceptors from degeneration by expressing high levels of the anti-apoptotic Bcl2 protein and low levels of the pro-apoptotic protein Bax (Rocco *et al*., 2015). Furthermore, in a model of retinal detachment (RD), intravitreal injection of NGF (1µg/µl, every four days after injury, 0.5-32 days) prevented the abnormal expression of GFAP (Sun *et al*., 2008).

Important differences have been observed regarding the neuroprotection afforded by exogenous NGF to injured or diseased retinas depending on the frequency of the treatment. Apparently NGF administered once does not prevent from retinal degeneration (Lebrun-Julien *et al*., 2009; Sivilia *et al*., 2009), in contrast to the studies using a longer-term treatment (Colafrancesco *et al*., 2011a, 2011b; Lenzi *et al*., 2005). This effect might be due to the short half-life of this neurotrophin, thus it should be applied several times in order to activate the relevant survival mechanisms to preserve the functional integrity of retinal cells. Yet, adverse side effects may appear because the need to employ several injections. Therefore, the use of less eye-invasive routes of administration such as eye drops could be an adequate alternative method to provide neuroprotection to the damaged retina (Lambiase *et al*., 2009).

Despite the therapeutic potential of exogenous NGF administration in different models of retinal injury or disease, the pharmacological properties of this molecule impede its clinical use. NGF is a large peptidic molecule (26.5kDa) not able to cross the blood brain barrier (BBB) and susceptible to proteolysis (Yi *et al*., 2014). Additionally, it has been reported that NGF administration induced Müller cell differentiation and overproduction of VEGF. These events could contribute to glial scar formation or to solidify the neovascularization mechanisms in DR turning the NGF treatment in a disadvantageous therapy for the injured or diseased retina (García *et al*., 2017).

1.5. BNN27: A DHEA-derived, synthetic microneurotrophin

The neurosteroid Dehydroepiandrosterone (DHEA) is an endogenous steroid hormone mainly secreted by adrenal glands and gonads, as well as in brain (neurosteroid), and it acts as a metabolic intermediate in the biosynthesis of androgen and estrogen sex steroids (Baulieu *et al.,* 1997). However, it has also been described as a neurotrophic molecule with trophic effects and neuroprotective activity not only to the retina but also to other structures of the central and peripheral nervous system (CNS and PNS, respectively) (Cascio *et al*., 2007). In agreement with this, Kimonides *et al*. (1998) showed the ability of DHEA to protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. This neurosteroid has also been examined as a beneficial therapeutic drug for the treatment of spinal cord and cerebral ischemia acting on GABAergic receptors (Lapchak *et al*., 2000). Moreover, the therapeutic potential of DHEA-S has been determined in the prevention and treatment of cerebellar granule cell culture against oxygen-glucose deprivation-induced injury (Kaasik *et al*., 2001). DHEA also improved the metabolic state of surviving neurons and glial cells after ischemia-reperfusion-induced retinal injury (Bucolo *et al*., 2004). More studies show us the ability of this neurosteroid to promote axonal growth and neuronal differentiation (Compagnone *et al*., 2000). In addition, Charalampopoulos *et al*. (2004) showed the anti-apoptotic and survival effect of DHEA and its sulfate ester DHEAS (at low concentrations of 1mM) on PC12 rat sympathoadrenal cells using a model of serum deprivation-induced apoptosis, inducing the activation of the anti-apoptotic Bcl-2 proteins and the transcription factors NFκB and CREB. Later, the same group demonstrated the DHEA-induced subsequent activation of downstream pro-survival signaling pathways like MAPKs and PI3K/Akt kinases (Charalampopoulos *et al*., 2006, 2008).

More recently, DHEA was shown to exert anti-apoptotic activity on neuronal cells by directly interacting with both NGF receptors TrkA and $p75^{NTR}$ at nanomolar levels (Lazaridis

et al., 2011). Furthermore, DHEA provided neuroprotective actions to amacrine, horizontal and cone bipolar retinal cells in the rat *in vivo* model of AMPA excitotoxicity by the activation of the NGF-TrkA receptor (Kokona *et al*., 2012). Additionally, Pediaditakis *et al*., (2015) contributed to the DHEA mechanism characterization by showing a similar and high affinity binding of this neurosteroid not only to the TrkA receptor but also to the other two mammalian Trk receptors: TrkB and TrkC, as well as their ancestral isoforms.

Despite the fact that DHEA could have a therapeutic potential for the treatment of retinal disease including DR, its pharmacological use has some limitations due its endocrine activity and the corresponding consequences that this entails (Fourkala *et al*., 2012).

This constraint was overcome by the synthesis of a novel spiro-epoxy DHEA derivative: BNN27 (20*R*)-3*β*,21-dihydroxy-17α,20-epoxy-5-pregnene). This molecule was shown to mimic the activity of endogenous neurotrophins by inducing pro-survival mechanisms in serum deprived PC12 cells with an IC_{50} on the order of 6.4±0.3 nM (Calogeropoulou *et al*., 2009).

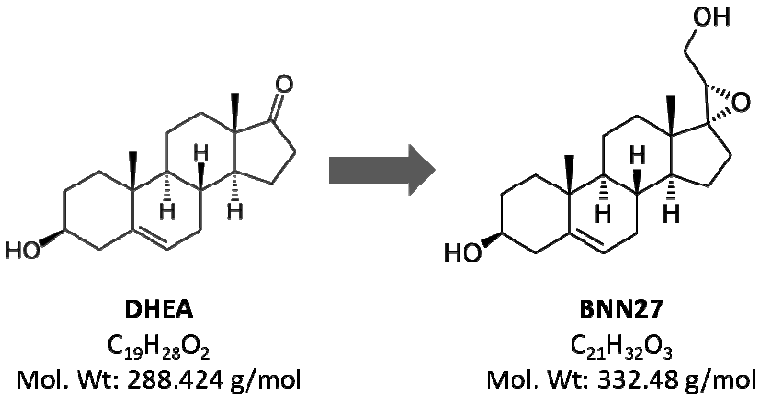
BNN27 has been described as a small neurosteroidal microneurotrophin, highly lipophilic, thus capable to penetrate the BBB (Bennett *et al*., 2016), and unable to stimulate the classical steroid receptors, therefore its administration would not cause hormonal side effects (Calogeropoulou *et al*., 2009).

Recently, several studies using BNN27 were published showing the wide range of beneficial biologic functions that this novel molecule can exert and therefore the therapeutic potential that could be applied to neurodegenerative diseases. Bennett *et al*. (2016) showed the potential of BNN27 to mimic neurotrophins actions in two human derived motor neurons cell lines, as a model of ALS, demonstrating the capacity of BNN27 to cross the BBB. BNN27 was recently reported to activate TrkA signaling in neuronal (sympathetic and sensory primary neurons, PC12 cell line) and microglial (BV2 mouse cell line) cells and protect TrkA-positive and NGF-dependent sympathetic and sensory neurons from apoptosis

(Pediaditakis *et al*., 2016a). To extend and complement the previous studies, further investigation was done to characterize this molecule: it has high affinity for TrkA but not TrkB or TrkC receptors, and it anchors to different binding sites to those that NGF specifically recognizes; it has a synergetic action with NGF, potentiating the effect of low levels of NGF or it induces faster recycling of TrkA receptor into the cell surface allowing the maintenance of the signaling, among others (Pediaditakis *et al*., 2016a). The same group, reported BNN27 to reverse the serum deprivation-induced apoptosis of cerebellar granule neurons (CGNs which do not express TrkA) by activating the low affinity neurotrophin receptor $p75^{NTR}$ (Pediaditakis *et al*., 2016b).

Moreover, BNN27 has been used by other research groups utilizing different ALS models. Thus, Glajch *et al*. (2016) showed that BNN27 attenuated loss of motor neurons co-cultured with astrocytes derived from human ALS patients and Bonetto *et al*. (2017) showed that BNN27 preserves mature oligodendrocyte during myelin degeneration in the cuprizone mouse model of MS.

This synthetic microneurotrophin has more beneficial features over DHEA and NGF, such as, it is a small-sized molecule, highly lipophilic and thus it possesses the ability to cross the BBB/BRB. The structural and chemical characteristics of BNN27 allow it to selectively interact with the NGF neurotrophin receptors: TrkA and p75 (IC_{50} at nanomolar levels), thus mimicking the neurotrophic properties of NGF. Hence, these studies strongly suggest that BNN27 represents a potential therapeutic drug for the treatment of neurodegenerative diseases (Gravanis *et al*., 2017).



DHEA
$C_{19}H_{28}O_2$
Mol. Wt: 288.424 g/mol

BNN27
$C_{21}H_{32}O_3$
Mol. Wt: 332.48 g/mol

Figure 11. Scheme shows the different chemical structures of BNN27 designed from DHEA

1.6. Aims of the study

The main goal of this doctoral thesis was to investigate the therapeutic potential of the novel neurosteroidal microneurotrophin BNN27 as a neuroprotective molecule against the neurodegenerative and inflammatory components of diabetic retinopathy employing the experimental animal STZ-model of DR.

The research aims of the thesis were to investigate:

1. The dose-dependent effect of BNN27 administered intraperitoneally and as eye drops in the diabetic rat retinal neurons.
2. The involvement of the NGF TrkA and $p75^{NTR}$ in the BNN27-induced neuroprotective effects in the diabetic retina.
3. The effect of BNN27 on the proNGF/NGF ratio.
4. The effect of BNN27 on $TUNEL^+$ cells and cleaved caspase-3 levels, induced by DR.
5. The effect of BNN27 on macro- and micro glia in the diabetic rat retina.

2. MATERIALS AND METHODS

2.1. Laboratory animals

Female and male of Sprague-Dawley rats (≃2 months old; weight ≃180-300g) were utilized for this project. All the animals employed in this study were treated in accordance to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in Ophthalmic and Vision Research, in compliance with Greek National (Animal Act, P.D. 160/91) and EU (directive 2010/63/EU) legislation. All protocols were approved by local Veterinarian Authorities.

2.2. Induction of diabetes

Diabetes was induced by a single dose of Streptozotocin intraperitoneally (i.p.) injected (STZ; 70 mg/kg; Sigma-Aldrich, St. Louis, MO). STZ was weighed on a microscale taking into account the initial weight of the animal and under dark conditions due to its sensitivity to light. STZ was diluted in a final volume of 500µl of Citrate buffer for each animal (Citric acid 0,1M, sodium citrate 0,1M, water for injection, pH 4.7). In order to perform the intraperitoneal (i.p.) injection, insulin syringes of 1 ml with 27-G needles were utilized. STZ administration was performed after a fasting period of 8-12 hours. Right after the injection, animals were provided with 10% sucrose water for protecting them from the sudden hypoglycaemic period that occurs immediately after the lysis of the pancreatic islet cells by STZ. Animals were housed one per cage in a room maintained at 22-25ºC, with an alternating 12-h light-dark cycle. Food and water were available *ad libitum*.

Mechanism of action of STZ in the pancreas: the administration of STZ to rats induces diabetes. This is due to the destruction of the pancreatic β-cells or islets of Langerhans that release insulin. Insulin is the hormone that regulates glucose levels and its

lack leads to diabetes mellitus (Type 1). The toxicity of STZ is attributable to intracellular oxidative stress characterized by the generation of highly harmful reactive oxygen species (´OH, $O^{-}_{2,}$ H_2O_2) and nitric monoxide which will contribute to the β-cells damage (Corbett and McDaniel, 1992; Rabinovitch & Suarez-Pinzón, 1998; Lau *et al.,* 1998; Flodström *et al.,* 1999). It seems that STZ has the potential to trigger the mitochondrial respiratory chain reaction of reactive species formation (Gille *et al*., 2002). Additionally, the levels of intracellular glutathione peroxidase, an enzyme responsible of catalyzing the reduction of peroxides, are low. All the previous events together with the increase of monocyte/macrophages due to STZ administration will increment the inflammatory cellular response that lead to β-cells deterioration.

2.3. Measurement of blood glucose levels

Blood glucose levels were monitored before STZ administration and 4 and 5 weeks post STZ injection. The range of normal blood glucose levels is between 60 to 100 mg/dl and levels higher than 250 mg/dl indicate diabetic hyperglycaemia. Rats were covered with a towel to minimize the stress during the blood collection. A superficial cut with a scalpel on the base of the tail and pressing around it, allowed the appearance of a drop of blood. To collect it, a glucometer and blood glucose test strips (Contour, Bayer Consumer Care AG, Basel, SW) were employed. Once the drop of blood was superficially evident, the end of the strip already inserted in the glucometer was approached and the drop rose by capillarity. Next, the blood glucose level (milligrams per deciliter) was seen on the glucometer screen and was noted.

2.4. Administration of BNN27: Neuroprotection studies

Animals were randomly assigned to three experimental groups, namely Control, Diabetic non-treated and Diabetic treated group. BNN27 doses of 2, 10 and 50mg/kg were utilized for the study. After 4 weeks post-STZ injection, the microneurotrophin treatment was administered daily, about the same time and for seven days, by two different routes of administration: intraperitoneally (i.p.) or eye drops. BNN27 (M_W = 332.48g/mol) was weighed for everyday treatment taking into account the particular dose of BNN27 and the weight of the animal.

2.4.1. Intraperitoneal Administration

BNN27 was completely dissolved in 100µl of absolute ethanol (10% v/v in water for injection). The higher concentration of 50 mg/kg needed to be warmed in order to reach a proper dissolution. Control and diabetic non-treated groups received the vehicle alone. BNN27 (100µl) was mixed with water for injection (900µl) in an insulin syringe of 1ml with 27-G needle, and injected i.p. smoothly and quickly to avoid crystallization. The puncture was done in the lower quadrant either right or left and the midline was avoided in order to not damage vital organs, such as the bladder. The animals were immobilised and calmed by a lab partner. The help of a lab partner was needed due to the volume of the animals and the speed that the process requires.

2.4.2. Eye drop Administration

BNN27 was diluted in 80µl of Dimethyl Sulfoxide (DMSO) (per eye) at amounts used for the corresponding doses of 2, 10 and 50mg/kg administered intraperitoneally. Animals were placed in a suitable calm environment. They were immobilized and the upper eyelid

pulled up in order to gently pour a drop with a pipette above the rat eye surface. The eye was gently closed by the experimenter a few times to make sure that the drop got inside the eye.

2.5. Intravitreal administration of a NGF TrkA receptor inhibitor

To examine the involvement of the NGF TrkAR in the neuroprotective effect of the neurosteroidal microneurotrophin BNN27, a specific TrkAR inhibitor (10^{-3}M, Calbiochem, Cat. No. 648450) was administered intravitreally according to Kokona and Thermos (2015). Each intravitreal injection was performed with a flow rate of 1 µl/min for 5 min at days 1, 4 and 6 in conjunction with BNN27 treatment (10mg/kg, i.p. or eye drops). The dose for BNN27 was chosen as the optimum dose since it was the lowest dose that led to the activation/phosphorylation of the TrkAR. The TrkAR inhibitor concentration was chosen taking into account the calculated molarity of the BNN27 dose of 10mg/kg (i.p.). DMSO [10%, v/v in phosphate-buffered saline (PBS, 50mM K_2HPO_4/NaH_2PO_4, 0.9% NaCl, pH 7.4)] was used as the solvent for the inhibitor and as vehicle in the other experimental groups. Animals received DMSO in the absence or presence of TrkAR inhibitor. Both eyes received the same treatment.

2.6. Immunohistochemical and histological studies

2.6.1. Retinal tissue preparation

Animals were euthanized by inhalation of diethyl ether, 24 hours after the last treatment and their eyes collected. After the eyes were removed, they were placed in buffer PBS 0.1M. Lens and vitreous were carefully removed with the help of some tweezers and the

eyecups kept. Retinal tissue was fixed using 4% paraformaldehyde (PFA) in Phosphate buffer (PB 0.1M) for 1 h and cryopreserved in 30% sucrose solution in PB 0.1M overnight at 4°C. Subsequently, eyecups were embedded in optimal cutting temperature compound (OCT compound, Prolabo, Leuven, Belgium) and submersed in isopentane for 1 min, reaching a temperature from -45°C to -50°C in dry ice. Frozen tissues were vertically sectioned at 10 µm thickness by cryotome (-25°C) and the initial 1.5 mm of tissue was discarded. Superfrost Plus slides (Thermo Fisher Scientific, Gerhard Menzel GmbH, Braunschweig, Germany) were utilized. Nine retinal sections from each eyecup (each animal) were placed in each slide in order to have a sufficient number of appropriate viable slices. Sections were placed in a consecutive fashion on a total of 6 slides (first section on the first slide, second section on the second slide, etc.). This way, sections pertaining to the same retinal area, including the optic nerve, were thoroughly present in the six slides utilized for each retina (different animal). After the sectioning procedure, slides were stored at -20°C until further use.

2.6.2. Histological staining with Hematoxylin/Eosin

In order to study the effect of diabetes on retinal thickness a Hematoxylin-Eosin (H-E) histological staining was performed. Defrosted sections were immersed in 100% ethanol for 2 min, rinsed with distilled water (dH_2O) and stained with Mayer's Hematoxylin solution (Sigma). After 5 minutes, slides were washed with dH_2O and soaked in 0.25% Eosin solution (Sigma). Subsequently, more washes in dH_2O were done followed by a dehydration process by which slides were immersed in an increasing order of a series of ethanol solutions (70%, 80%, 95% and 100%). Tissues were rehydrated in xylene for 5 min and coverslipped with Entellan New Rapid Mounting Medium for light microscopy (Millipore, Cat. No. 107961).

2.6.3. Immunohistochemistry procedure

Defrosted tissues were washed with Tris buffer saline (TBS) 0.1M (2 x 10 min). Slides were always drained and wiped around the sections with tissue paper after last washing. Retinal sections were blocked with TBS 0.1M containing 3.3% Normal Goat Serum (NGS) for 30 min. The use of NGS will minimize cross-reaction of the secondary antibody (raised also in Goat) with endogenous immunoglobulins present in the retinal tissue. It also reduces non-specific binding by eliminating Fc receptor binding to both primary and secondary antibodies. Subsequently, retinas were washed (3 x 5 min) in TBS 0.1M and the primary antibody diluted in TBS 0.1M containing 0.3% Triton-X-100 and 0.5% NGS was applied overnight at room temperature. Slices were subsequently rinsed in TBS 0.1M (3 x 5 min), and incubation with the appropriate fluorescence secondary antibody for 1-2 h was performed in dark conditions to avoid photobleaching. Finally, after some more rinse in TBS 0.1M (3 x 5 min), slides were cover slipped using EverBrite Mounting Medium with DAPI (Biotium, Cat. No. 23002) for fluorescence microscopy. Coverslips were immobilized by nail polish and were let dried. Slides were kept at 4°C until further use.

Overnight incubation was chosen to ensure maximum antibody-antigen binding in the tissue. The use of Triton-X-100 reduces surface tension, allowing reagents to cover the whole tissue section with ease and also reduces non-specific binding since it dissolves Fc receptors. All primary antibodies utilized were raised in a species different from the rat tissue avoiding high background.

Antibodies utilized for the study of amacrine retinal cells and ganglion cell axons: the effect of the STZ on retinal cell viability in rats, 1-5 weeks following its administration was assessed utilizing a rabbit polyclonal antibody raised against brain nitric oxide synthetase (anti-bNOS, 1:2000, Sigma, Cat. No. N7280). This antibody was used as a marker for neuronal nitric oxide synthetase-expressing amacrine retinal cells. A rabbit polyclonal

antibody raised against Tyrosine Hydroxylase (anti-TH, 1:1000, Sigma, Cat. No. T8700) was employed to study the immunoreactivity of dopaminergic amacrine retinal cells. A mouse antibody raised against neurofilament (anti-NFL, 1:500, Chemicon, Cat. No. MAB1615) was used as a marker for the labeling of neurofilament in ganglion cell axons.

Antibody utilized for the study of macroglia *and microglia*: In order to evaluate the neuroprotective effect of BNN27 on modulating glial activation, a mouse antibody raised against glial fibrillary acidic protein (anti-GFAP, 1:2000, Millipore, Cat. No. G3893) was utilized. Anti-GFAP was chosen as a cell specific marker for Müller cells, since it is the main constituent of intermediate filaments in these glial cells. An antibody against Iba-1 (anti-Ionized calcium Binding Adaptor molecule 1, 1:500, Biocare, Cat. No. CP290A) was employed as marker for retinal microglia.

Antibodies utilized for the study of proNGF and NGF neurotrophins**:** Examination of proNGF and NGF neurotrophins immunoreactivity location in the retina was performed by the utilization of a rabbit polyclonal antibody against the unprocessed NGF neurotrophin proNGF (anti-proNGF, 1:100, Alomone, Cat. No. ANT005) and against the mature neurotrophin NGF (anti-NGF, 1:200, Abcam, Cat. No. GR323234-2). Immunohistochemical experiments without primary antibody were done in order to make sure of specific binding of this antibody.

Antibody utilized for the study of the NGF-p75 neurotrophin receptor**:** NGF p75 neurotrophin receptor immunoreactivity was studied by the employment of a rabbit polyclonal antibody against $p75^{NTR}$ (anti-$p75^{NTR}$, 1:500, Promega, Cat. No. G3231).

Antibody utilized for the study of apoptosis**:** the antibody against Caspase-3 (Caspase-3 (Asp175), 1:300, Cell Signaling, Cat. No. 9661; Anti-Caspase 3, active (cleaved) form, 1:100, Millipore, Cat. No. 2376423) was employed for the study of apoptosis.

Secondary Antibodies utilized: A goat anti-rabbit secondary antibody (CF543, Goat Anti-Rabbit IgG (H+L), 1:1000, Biotium, Cat. No. 20309; CF488A, Goat Anti-Rabbit IgG (H+L),

1:800, Biotium, Cat. No. 20010) was utilized against the previously mentioned primary antibodies produced in rabbit. A goat anti-mouse secondary antibody (CF488A, Goat Anti-Mouse IgG (H+L), 1:400, Biotium, Cat. No. 20012) was employed against the previously mentioned primary antibodies produced in mouse.

2.7. TUNEL analysis

Defrosted sections were washed in PBS 0.1M (2 x 5 min) and incubated with permeabilization solution containing Sodium Citrate TriBasic (0.1%) and Triton-X-100 (0.1%) in PBS 0.1M for 30 minutes. Sections were rinsed again in PBS 0.1M (3 x 5 min) and subsequently, incubated with TUNEL reaction mixture containing Label and Enzyme Solutions (V_{final}=50µl/each slide) in a humidified atmosphere for 1 h at +37ºC under dark conditions. A homogenous spread of TUNEL reaction mixture across cell monolayer and avoidance of evaporative loss were ensured. After more rinses (3 x 5 min) with PBS 0.1M, slides were cover slipped using EverBrite Mounting Medium with DAPI (Biotium, Cat. No. 23002) and observed with fluorescence microscopy. Slides were stored at 4ºC until further use. To perform the TUNEL studies the *In Situ* Cell Death Detection Kit was utilized (TMR red, Roche Diagnostics GmbH, Cat. No. 12156792910).

2.8. Microscopy

Fluorescence images mages were obtained using an Axioskop Plan-Neofluar (Carl Zeiss, Oberkochen, Germany) or HC PL Fluotar, Leica DMLB (X20 or X40/0.75 lens), and Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Photos adjustments were processed with the use of Adobe Photoshop software (version 7.0, Adobe Systems, San Jose, CA) and Image J 1.43m (Java 1.8.0_31).

2.9. Quantification of Immunohistochemical Images

Quantification by the number of retinal neurons: the count of bNOS and TH immunoreactive retinal neurons was performed according to Kiagiadaki and Thermos (2008). Three sections were employed for the quantification studies in each slide examined. The total number of immunoreactive neurons (somata) was counted in each section by microscopic observation. The mean of those three values pertaining to the three sections, was utilized as the number of total bNOS or TH-expressing retinal cells found on each particular slide.

Quantification by the density of immunofluorescence in retina: Several markers (mentioned below) were quantified by the evaluation of immunoreactivity (IR). For that, the total retinal fields were counted by observation under fluorescence microscopy with a X40/0.75 magnification, and only the two central fields were considered for quantification analysis. Two microphotographs were collected from the two central fields in each retinal section. Three sections were examined for the studies in each slide (6 total images per slide/different retina). A specific area, taking into account the immunoreactivity observed for each marker, was delineated using the public domain Image J 1.44 software. The mean gray value [integrated density (fluorescence density)/delineated area] of this region was calculated in each image and expressed as a percentage of the mean gray value of the control retinas (100%). The different markers and the specific delineated areas considered for the quantification are mentioned below:

- ***NFL and $p75^{NTR}$ –IR***: was quantified delineating the area starting from the GCL until the IPL.
- ***GFAP, proNGF and NGF –IR***: the area starting from the GCL until the ONL was evaluated. For GFAP antibody, the magnification chosen for the analysis of images was X20. ProNGF and NGF data were expressed as fold-increase, setting as 1 the controls.

- ***Caspase-3 –IR***: the density area of the INL was delineated in each image.

Quantification for TUNEL analysis was performed on two central micrographs from 3 retinal sections per slide (6 total images per slide/different retina), taken with a X40/0.75 magnification. $TUNEL^+$ cells counted with the Image J 1.43m; Java 1.8.0_31. Each microphotograph field (at X40) represents an area in the order of 320.99 x 254.32 μm^2 (81.63 mm^2) while retinal areas range was 28.81 - 65.65 mm^2. Final data was normalized to 1mm^2 of retinal area as to express the number of $TUNEL^+$ cells per area. Also, $TUNEL^+$ cells and DAPI nuclei were counted from the ONL to GCL (total retinal thickness) and individual layers (ONL, INL, GCL) using Image J 1.43m software, as above. Data were normalized and expressed as the number of $TUNEL^+$ cells per area (mm^2). The percentage of the ratio of TUNEL/DAPI cells was calculated in rat retinas 4weeks post-STZ injection and after BNN27 treatment. Some representative images for diabetic rat retinas were taken using a Leica SP8 confocal microscope (Leica Microsystems, Germany).

Iba-1 quantification: the number of Iba-1 positive cells was manually counted; data normalized to the total counting area (from GCL to INL) and expressed as percentage of the control retinas (100%).

Quantification for Hematoxylin-Eosin for histochemical studies: Slides stained with Hematoxylin-Eosin (H-E) were observed with light microscope using a X20/0.75 magnification (Leica). Two fields at the central retina of 3 retinal sections per slide (6 total images per slide/different retina) were pictured and retinal layers and the whole retina width were measured with the Image J 1.43m; Java 1.8.0_31.

Immunohistochemical experiments for each marker were replicated at least three times using different retinas.

2.10. Western blot and Immunoprecipitation

2.10.1. TrkA neurotrophin receptor

Retinas were gently homogenized in 500µl of lysis buffer containing 50mM Tris-HCl (pH 7.5), NaCl (150mM), Triton-X-100 (1%) and protease/phosphatase inhibitor cocktail (Thermo Sci., Waltham MA). Lysates were centrifuged (10000rpm, 20 min, 4^0C). Supernatants were split in two eppendorfs: one was employed for immunoprecipitation (IP) and the other for lysates. Supernatants for IP were immunoprecipitated with mAb total TrkA (1:60) overnight (O/N) at 4^0C under rotary agitation. Lysates were incubated in methanol, to pull all protein down (O/N) at -20^0C). Lysates were centrifuged (10000rpm, 20 min, 4^0C) and the pellet was kept and resuspended in SDS-sample buffer with DTT. In parallel, protein G Plus-Agarose (Santa Cruz Biotechnology) beads (1:8 dilution) were added to the IPs and incubated for 2h at 4^0C under rotary agitation. The protein-antibody complex bound to protein G-agarose was sedimented by centrifugation (2000g, 2 min, 4^0C). Pellets were washed in lysis buffer and resuspended in SDS-sample buffer. Bound proteins were eluted by boiling (95^0C, 5min). Precipitates and lysates were loaded (10µl), analyzed by SDS-PAGE (7,5-10% of acrylamide) (70V until samples reach the separation gel and 120V) and transferred onto nitrocellulose membranes (Macherey-Nagel, Düren, Germany) (310mA, ≈1.30h). Two to three different retinas for each group were run on each gel. Membranes were blocked with 5% BSA TBST 0.1M (RT, 1h). Next, blots with IPs were incubated with the phosphorylated variant of the primary antibody (phospho TrkA, 1:1000) and blots with lysates incubated with GAPDH (1:1000) to normalize the protein content (O/N, 4^0C, gentle shaking). Antibodies were diluted in 3% BSA TBST in a final volume of 1ml. Membranes were washed (3 x 10 min) with TBST and the subsequent incubation with the peroxidase-conjugated secondary antibody HRP-goat anti rabbit IgG (1:5000; Invitrogen, Waltham

Massachusetts, US) in 3% BSA TBST (final volume of 10ml) (1h under shaking). Membranes were washed (3 x 10 min) with TBST and the proteins visualized utilizing ECL western blotting kit (supersignal west pico chemiluminescent substrate Thermo Scientific, Rockford, USA) and the quantitative densitometry of the protein bands was performed using Image-Lab 5.0 software (Bio-Rad). Blots with IPs were stripped using mild stripping buffer (15 min, RT). Blots were washed with PBS (10 min) and with TBST (2 x 5 min). Subsequently, blocking was done by the use of 5% BSA TBST (1h under shaking, RT) and incubation with the total TrkA antibody was done (O/N, 4^0C, gentle shaking). Same procedure was followed as for the phospho TrkA secondary antibody incubation and development.

2.10.2. P75 neurotrophin receptor

The same procedure as mentioned above was followed for $p75^{NTR}$ immunoblots, with some modification as stated below. Samples were immunoprecipitated with $p75^{NTR}$ (1:60), and blots with IPs were incubated with total TrkA (O/N, 4ºC, gentle shaking). Membranes were subsequently stripped with mild stripping buffer and incubated with $p75^{NTR}$ antibody (1:500). Blots with lysates were incubated with GAPDH, to normalize the protein content.

All antibodies used for western blot analysis and the relevant description, are shown in the table below:

Antibodies used for Western blot (WB) studies		
Antibody	**Working dilution**	**Company/Code**
Primary Antibody		
Phospho Tyr490 TrkA	1:1000	Cell Signaling/9141

Total TrkA	1:1000	Millipore/2382158
$p75^{NTR}$	1:500	Promega/G3231
GAPDH	1:1000	Cell signaling/2118
Secondary Antibody		
Goat anti-Rabbit IgG, Peroxidase Conjugated, H+L	1:5000	Invitrogen/1428689A

[Western blot and immunoprecipitation studies were performed by Dr. Silvia Lisa]

2.11. Determination of BNN27 levels: HPLC-MS/MS analysis

Healthy control rats were administered i.p. daily with BNN27 at the optimum dose of 10mg/kg for 7 days. After treatment, rats were euthanized and retinas collected. To each retinal tissue, methanol (495µl) and 5µl d6-DHEA (2500ng/µl) were added, and the samples were homogenized for 1.5min on ice, sonicated for 30s, and centrifuged at 13500rpm for 1h at 4°C. The supernatants were stored at -20°C until HPLC-MS/MS analysis. Chromatographic separation was performed on a UniverSil UHS18 column (150x2.1 mm, 3µm) with a gradient elution system consisting of methanol and water, both with 0.1% formic acid (Total flow rate 200µl/min). Tandem mass spectral analysis was performed with a Thermo Fisher Scientific TSQ triple quadrupole mass analyzer equipped with an electrospray ionization source operating in positive mode using selected reaction-monitoring detection. For BNN27, 315.3 to 297.1 ion pair was used for quantitation, and 315.3 to 255.2 was used for confirmation. D6-DHEA was used as the internal standard (selected reaction-monitoring detection 295.3 to 277.2).

[The analysis of the BNN27 levels by HPLC-MS/MS was performed by Dr. Antonis Kouvarakis, the supervision of Prof. H.E. Katerinopoulos, Dept. of Chemistry, University of Crete].

2.12. Statistical analysis

All data are expressed as mean ± SEM or mean ± SD (scatter plots). Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad, San Diego, CA, USA), and differences between groups were evaluated by one-way ANOVA with Tukey posthoc analysis or unpaired *t* test (two tailed). Statistical significance was set at $P < 0.05$. The number of animals used in each group (n values) is shown in the relevant figures or in the legend.

3. RESULTS

3.1. Streptozotocin administration affects females and males differently

The aim of our study focused on the effect of BNN27 in the neurodegenerative component of DR involving female and male rat animals in order to examine a more inclusive and representative population of the actual human community who suffers from this disease. Higher morbidity and mortality was observed in male STZ-treated rats. After collecting all animals utilized for this thesis, we saw that the 45.35±7.11% of the STZ-injected male animals died during the first week post injection, while the female rate of mortality was 20.22±4.35% ($^{**}p<0.01$) (Fig. 12). On the other hand, the survival output observed in males was in the order of 54.65±7.11% while the female rats showed a much higher survival rate of 79.78±4.35% ($^{**}p<0.01$) (Fig. 12). Male pancreatic islet β-cells are more prone than female to STZ-induced cytotoxicity (Furman, 2015). Therefore, male rats may die due to islet β-cell necrosis. Lower mortality rates were observed in later time points after STZ injection.

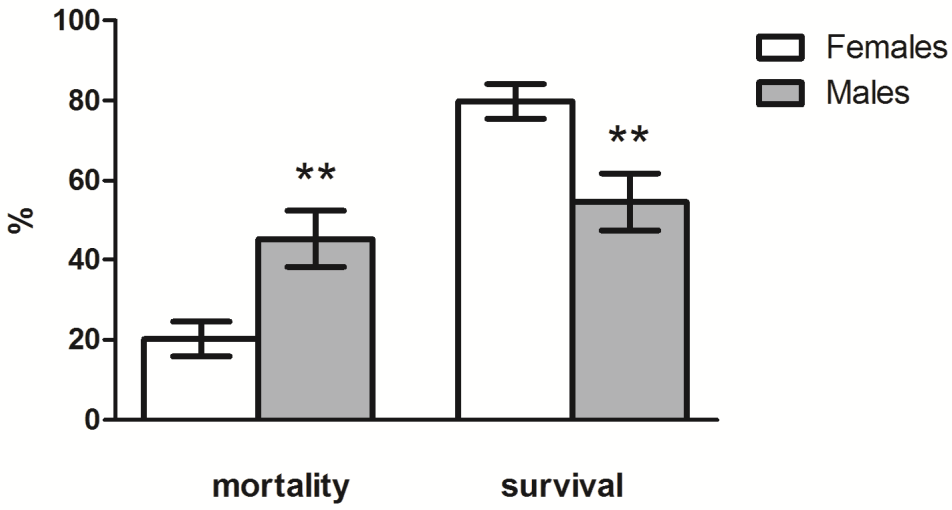


Figure 12. Representative graph showing the mortality versus survival for female and male rats after STZ administration.

3.2. Glucose levels are not affected by the administration of BNN27

Glucose levels were monitored before the STZ injection, at week 4 and after BNN27 treatment. The initial Glucose levels of Control (77.35±4.05), Diabetic (73.79±2.92) and Diabetic + BNN27 10mg/kg (76.79±2.92) were very close (Fig. 13). Control animals had about the same magnitude at the end of the study (93.11±2.43). Glucose levels were higher than 400 mg/dl in all experimental rats 7 days after the STZ injection and around 600 mg/dl the following weeks and up to the end of the experiment. Final levels of glucose in Diabetic and Diabetic treated animals increased considerably (552.6±12.26; 574.9±10.86, respectively), and there were not significant differences between these two groups (Fig. 13). Also, no differences were observed in glucose levels between male and female rats.

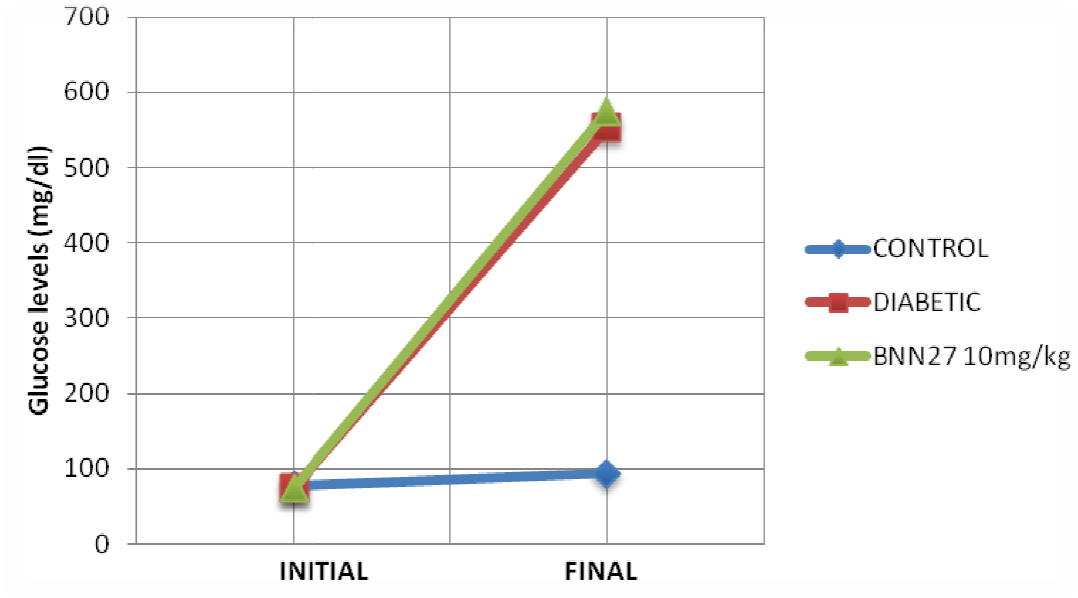


Figure 13. Glucose levels of Control, Diabetic and Diabetic + BNN27 10mg/kg, at the beginning (before the STZ injection) and at the end of the study (after last treatment with BNN27).

3.3. Diabetes does not affect the retinal thickness in the STZ-model of Diabetic Retinopathy

In order to evaluate the repercussion of diabetes on retinal width and thickness of each layer that comprises it, staining with Hematoxylin-eosin (H&E) was performed and shown in Fig. 14. Quantification of the H&E staining in whole retina (total thickness) showed no statistically significant differences between diabetic (124.9±12.02; n=5) and control rat retinas (131.9±8.6; n=6) (Fig. 15A), using t- test analysis. Similarly, no significant differences were observed in diabetic versus control rat retinas for the different retinal layers: ONL (Control:48.8±4.1, n=3; Diabetic: 52.02±1.4; n=3) (Fig. 15B), INL (Control:29.4±0.3, n=3; Diabetic: 34.6±2.1, n=3) (Fig. 15C), IPL (Control:31.9±1.9, n=3; Diabetic: 36.6±1.6; n=3) (Fig. 15D) and GCL (Control:15.1±1.9, n=3 Diabetic: 17.6±0.5; n=3) (Fig. 15D).

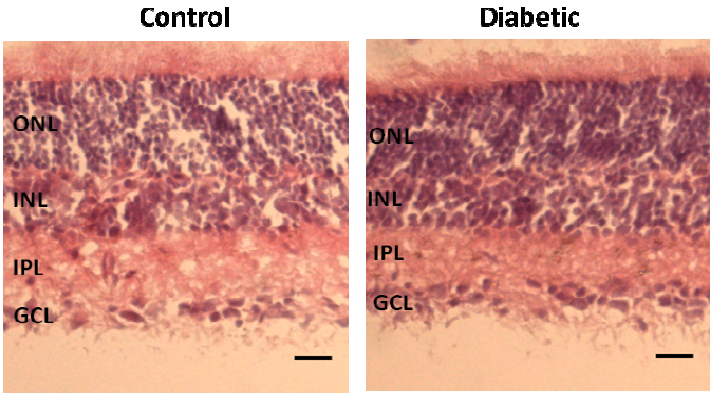


Figure 14. Representative images of Control and Diabetic rat retina sections stained with Hematoxylin & Eosin (H&E). (Ganglion cell layer (GCL), Inner plexiform layer (IPL), Inner nuclear layer (INL), Outer nuclear layer (ONL). X20 magnification. Scale bar 20µm.

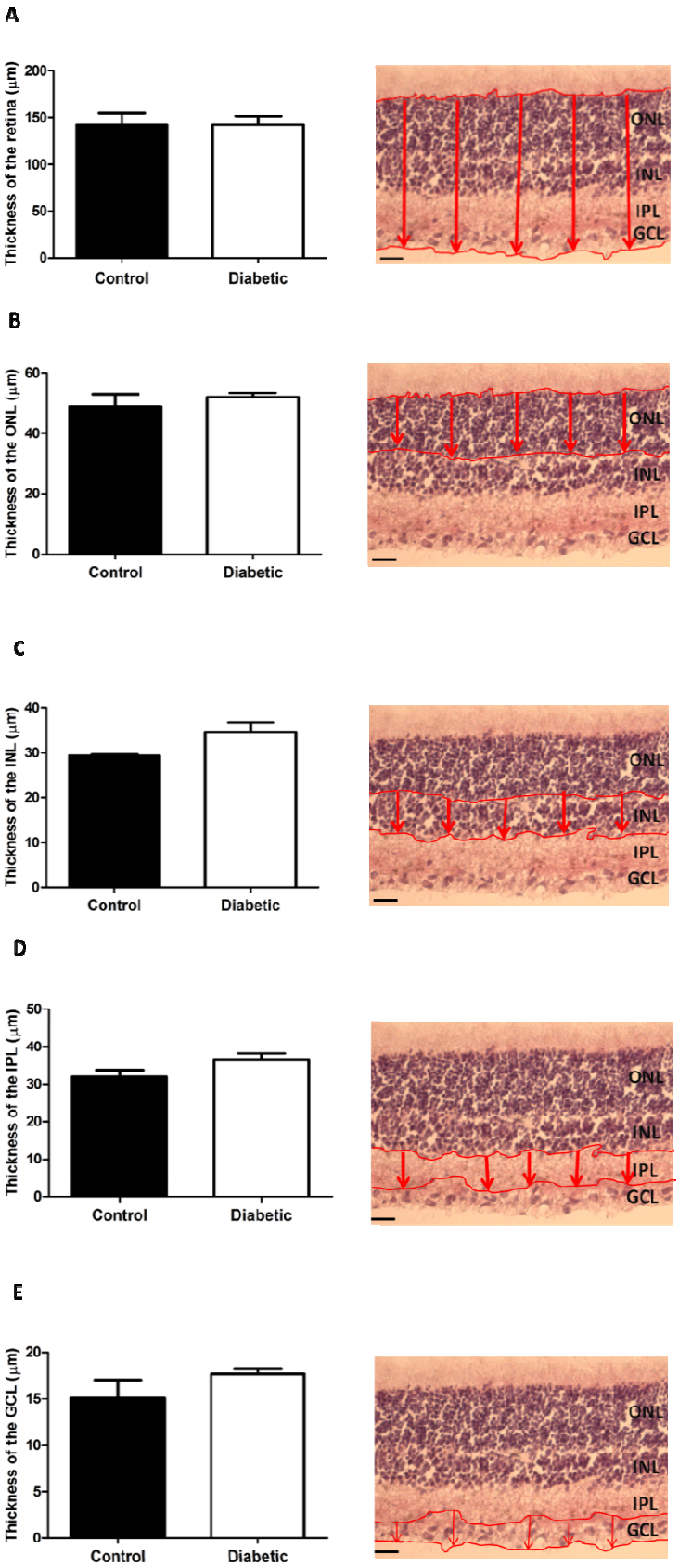

A
Thickness of the retina (µm)
200
150
100
50
0
Control
Diabetic
ONL
INL
IPL
GCL
B
Thickness of the ONL (µm)
60
40
20
0
Control
Diabetic
ONL
INL
IPL
GCL
C
Thickness of the INL (µm)
40
30
20
10
0
Control
Diabetic
ONL
INL
IPL
GCL
D
Thickness of the IPL (µm)
50
40
30
20
10
0
Control
Diabetic
ONL
INL
IPL
GCL
E
Thickness of the GCL (µm)
20
15
10
5
0
Control
Diabetic
ONL
INL
IPL
GCL

Figure 15. Quantification studies and representative figure showing the retinal area taken into account for A) the whole retina, B) ONL (Outer nuclear layer), C) INL (Inner nuclear layer), D) IPL (Inner plexiform layer) and E) GCL (Ganglion cell layer) in Control and Diabetic non treated rat retinas 5 weeks after induction of diabetes. No changes were observed between control and diabetic rat retinas, neither in the retinal thickness nor the different layers that compose it. 20x magnification. Scale bar 20µm. Data are expressed as Mean ± S.E.M.

PART I. Study of the effect of BNN27 on retinal cell viability and the mechanisms involved in its neuroprotective actions when administered intraperitoneally

3.4. Diabetic retinopathy affects the retinal viability of bNOS and TH-expressing amacrine cells and ganglion cell axons and increases the number of TUNEL positive cells

Qualitative immuhistochemical studies were performed to assess the effect of the STZ-administration on retinal cell viability in rats, 1 to 4 weeks following STZ administration. No differences were observed between the control and diabetic retinas in the first 3 weeks in the staining pattern of bNOS-IR, TH-IR and NFL-IR (data not shown). The bNOS-IR was localized in amacrine cell somata in the inner nuclear layer (INL), displaced amacrine cell somata in the ganglion cell layer (GCL) and amacrine cell processes in the inner plexiform layer (IPL) (Fig 16A). TH-IR was localized in amacrine cell somata in the INL (Fig 16A). NFL-IR was localized in ganglion cell axons in the GCL and in the IPL (Fig 16A). However, 4 weeks post STZ-injection a significant decrease in NFL- (≈39%), bNOS- (≈63%), and TH-IR (≈66%) was observed in diabetic rat retinas (61.17±7.44, n=5; 36.38±10.94, n=5; 33.75±10.69, n=5, respectively) as confirmed by the quantitative analysis of the immunofluorescence images, compared to those control rat retinas (100±18.12, n=6; 100±25.5, n=6; 100±22.71, n=6, respectively; $^{**}p<0.01$, $^{***}p<0.001$, $^{***}p<0.001$, respectively). (Fig. 16B, C, D).

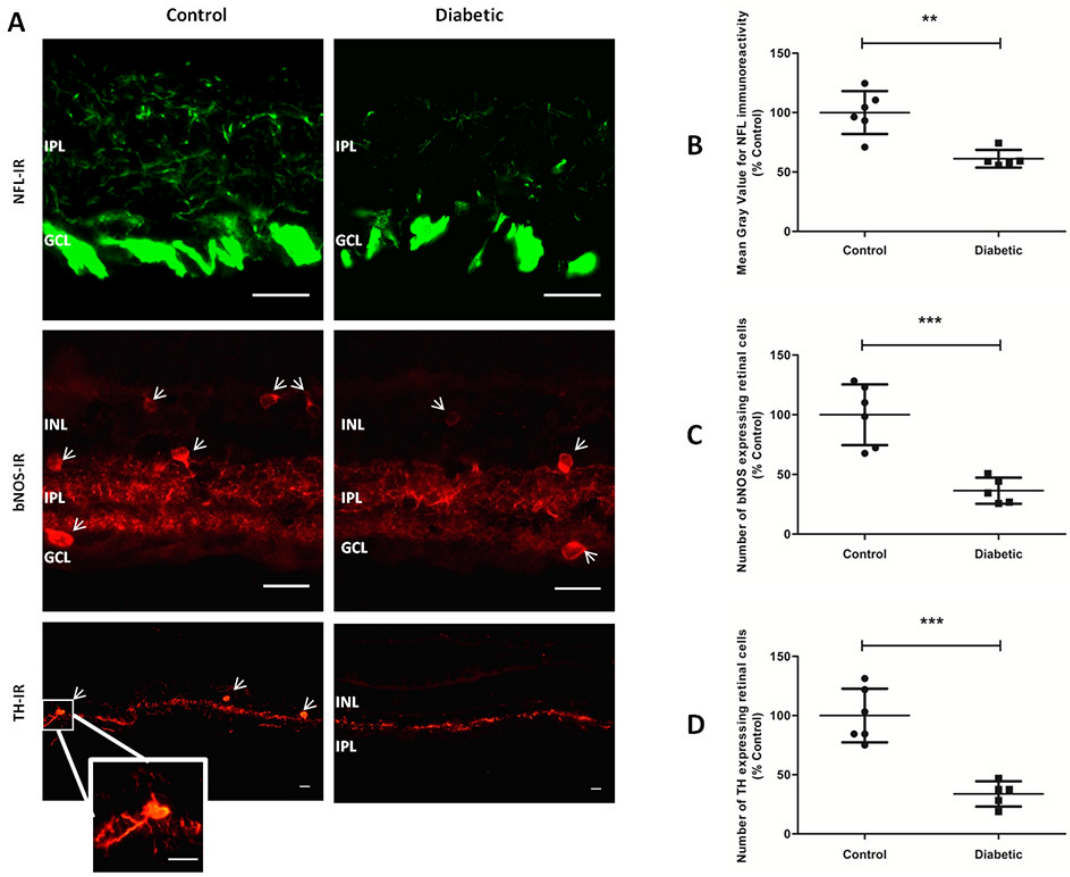


Figure 16. Effect of Diabetes on retinal neurons after 4 weeks of STZ injection. (A) Representative images of NFL-, bNOS-, and TH- IR in Control and Diabetic rat retinas after 4 weeks post STZ-injection**.** NFL and bNOS-IF images: X40 magnification; TH-IR images: X20 and X40 magnification. Scale bar 20µm. Quantification of the immunohistochemical images of NFL-IR **(B),** bNOS-IR **(C)** and TH-IR **(D)** in Control and Diabetic and Diabetic rat retinas. Diabetes promoted a decrease in the Mean Gray value obtained for NFL-IR and in the number of bNOS and TH expressing amacrine cells compared to those Control rat retinas. ($^{**}p<0.01$, $^{***}p<0.001$, $^{***}p<0.001$, respectively). Arrows depict bNOS or TH expressing amacrine cells. Mean ± SD is shown.

TUNEL analyses were performed to examine retinal cell death after 4 weeks of STZ-injection (Fig. 17A). Two TUNEL representative photos of Control and Diabetic rat retinas are shown (Fig. 17A). To differentiate retinal layers, mounting medium with DAPI was used (seen in blue). Control rat retinas barely exhibited apoptotic neurons while diabetic rat retinas displayed a high number of neurons with DNA damage (seen in red) mainly along the GCL, INL and ONL. Quantification of the TUNEL^{+} cells per area (mm^{2}) in the total retinal thickness showed an approximate 10-fold increase of diabetic non-treated rat retinas after 4 weeks of STZ-injection (2.9±0.6, n=6 compared to the number of apoptotic retinal cells

found in control retinas 0.3±0.1, n=6; $^{**}p<0.01$) (Fig. 17B). The TUNEL/DAPI ratios show the percent of retinal cell death in total retina and its layers (GCL, INL, ONL), the highest being in the GCL (Fig. 17C).

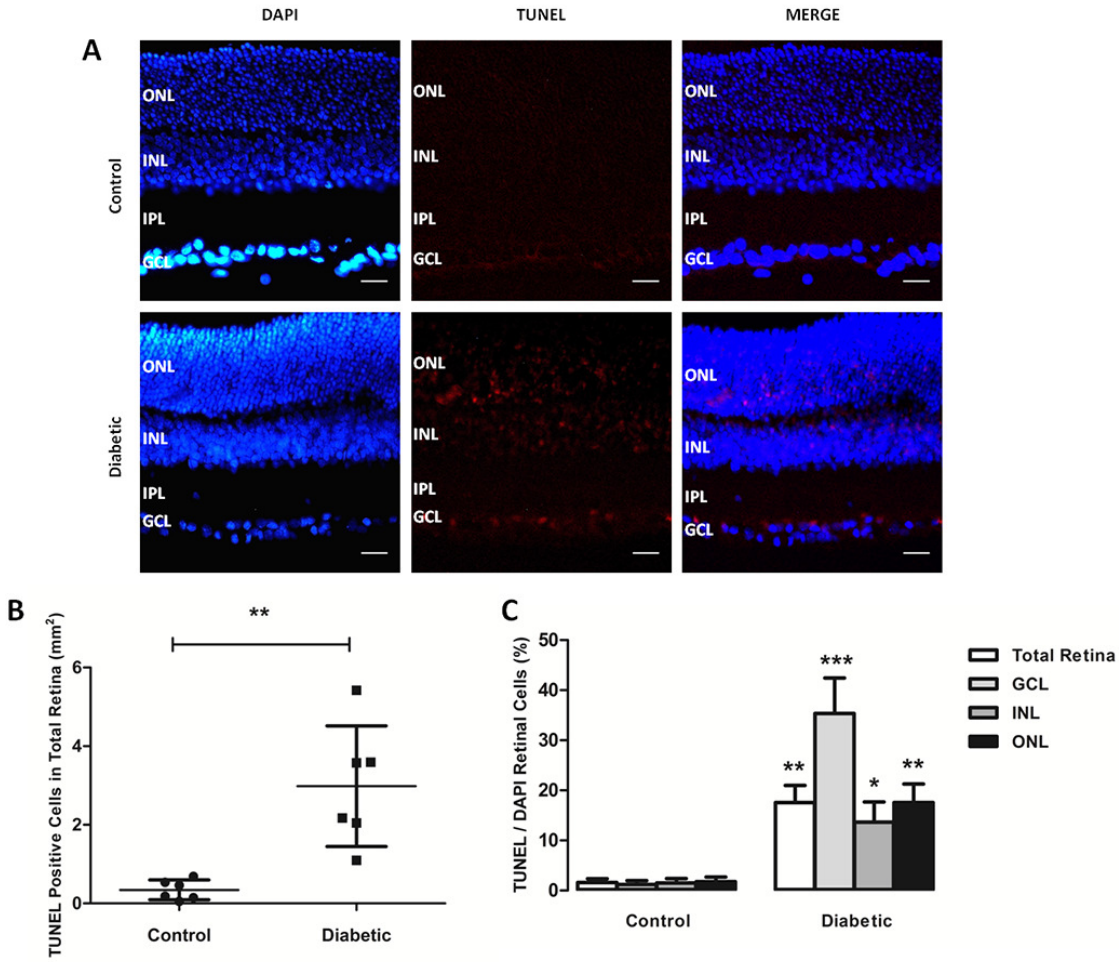


Figure 17. Evaluation of retinal cell death after 4 weeks of STZ injection by TUNEL analysis. **(A)** Representative images of DAPI, TUNEL staining, and merge in Control and Diabetic rat retinas 4 weeks post-STZ injection. TUNEL^{+} cells were observed in ONL, INL and GCL in Diabetic retinas. X40 magnification. Scale bar 20µm. **(B)** Quantification of the TUNEL^{+} cells per area (mm^2) in Control and Diabetic rat retinas ($^{**}p<0.01$ compared to Control). Mean ± SD is shown. **(C)** Quantification of the percentage of TUNEL/DAPI cells ratio in whole retina and its different layers for Control and Diabetic rat retinas (n=6 utilized for each experimental group; Total Retina: $^{**}p<0.01$, GCL: $^{***}p<0.001$, INL: $^{*}p<0.05$, ONL: $^{**}p<0.01$ compared to Control)**.** Mean ± SEM is shown.

3.5. BNN27 affords neuroprotection to bNOS and TH-expressing retinal amacrine cells and to ganglion cell axons in a dose-dependent manner

In the rat model of STZ-induced diabetic retinopathy, BNN27 administered i.p. 4 weeks post STZ-injection, daily for 7 days, reversed in a dose-dependent manner the diabetes-induced reduction in the NFL-, bNOS- and TH-IRs (Fig. 18A). Quantitative analysis of the immunofluorescence images showed an approximately 41% reduction in the NFL-IR in ganglion cell axons in diabetic rat retinas [58.69±5.77 percentage of mean gray area per section per section, compared to control rat retinas (100.0±4.06 percentage of mean gray area per section per section; $^{***}p<0.001$)]. BNN27 reversed the deleterious effects of DR on ganglion cell axons only at the doses 10 (87.51±6.67 percentage of mean gray area per section per section; $^{\#}p<0.05$) and of 50mg/kg (92.57±7.3 percentage of mean gray area per section per section; $^{\#\#}p<0.01$) compared to the non-treated diabetic retinas. No neuroprotective effect was observed at the concentration of 2mg/kg (67.56±4.29 percentage of mean gray area per section per section; $p>0.05$) (Fig. 18B).

The number bNOS-expressing amacrine cells underwent an approximately 46% decrease [53.70±2.86 percentage of number of cells per section, compared to control rat retinas (100.0±2.37 percentage of number of cells per section; $^{***}p<0.001$)]. Intraperitoneal administration of BNN27 at 10mg/kg (87.55±9.34 percentage of number of cells per section) and 50mg/kg (83.62±6.35 percentage of number of cells per section) provided neuroprotection to the diabetic retinas in a dose-dependent manner ($^{\#\#\#}p<0.001$ and $^{\#\#}p<0.01$, respectively), compared to the non treated diabetic retina. No significant differences were seen with BNN27 at 2mg/kg (70.61±5.95 percentage of number of cells per section; $p>0.05$) (Fig. 18C).

Similarly, the number of TH-expressing amacrine cells was decreased an approximately 65% due to the development of DR in diabetic animals [35.16±6.42

percentage of number of cells per section; compared to control animals (100.0±5.16 percentage of number of cells per section; $^{***}p<0.001$)]. Dose-dependent neuroprotective effects were seen when BNN27 was administered at the highest doses of 10 and 50mg/kg (81.27±6.59 and 82.44±8.87 percentage of number of cells per section, respectively; $^{###}p<0.001$) compared to the non-treated diabetic retinas. No significant differences were seen with BNN27 at 2mg/kg (42.86±3.54 percentage of number of cells per section; $p>0.05$) (Fig. 18B).

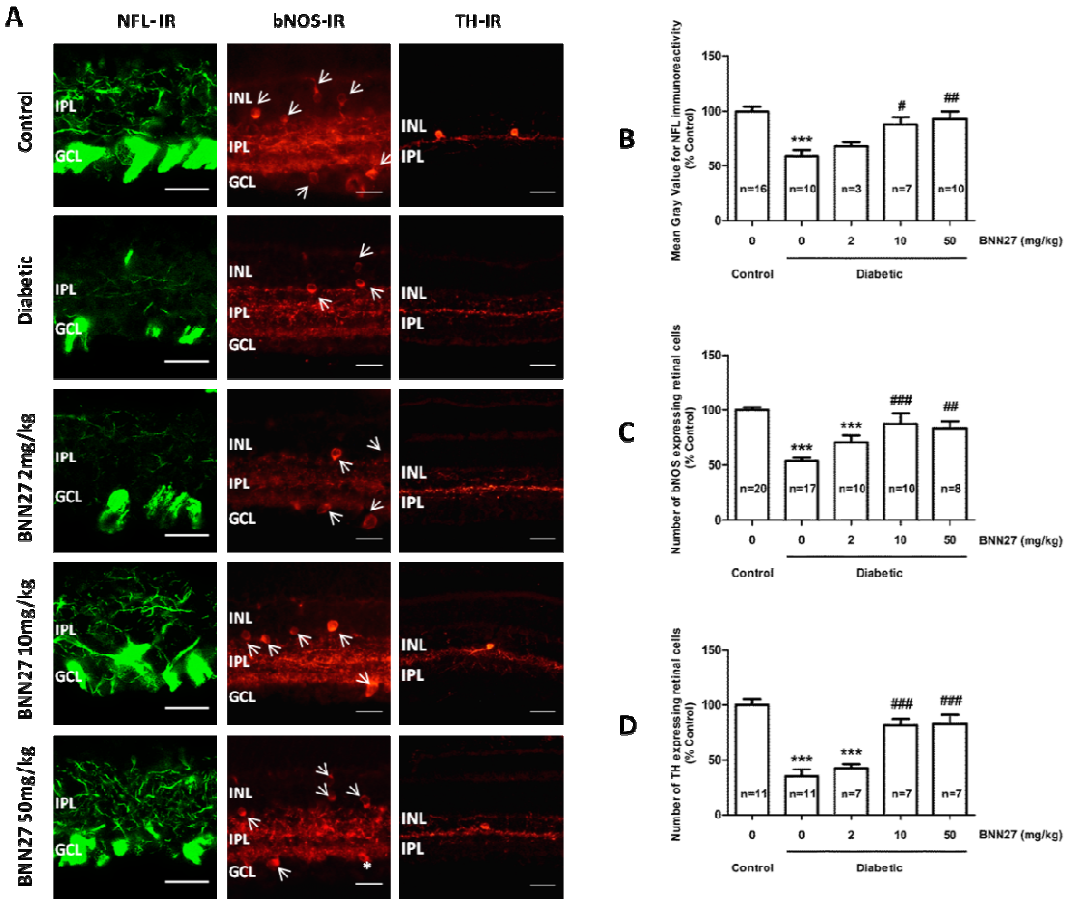


Figure 18. Dose-dependent effect of BNN27 on retinal cell neurons in the STZ model of DR. **(A)** Representative images of NFL-, bNOS-, and TH- IR in Control, Diabetic and Diabetic +BNN27 (2, 10, 50 mg/kg, i.p.) rat retinas after 5 weeks after STZ-injection (*depicts artifact and arrows depict bNOS-expressing cell bodies). Magnification X40 for NFL and bNOS; X20 for TH. Scale bar 20µm. Quantification of the immunohistochemical images of NFL-IR **(B),** bNOS-IR **(C)** and TH-IR **(D)** in Control, Diabetic and Diabetic +BNN27 (2, 10, 50mg/kg, 7d, i.p) rat retinas ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to Diabetic). Data are expressed as Mean ± S.E.M.

3.6. BNN27 properties afforded to the diabetic retina are TrkA receptor-mediated

In order to elucidate the involvement of the NGF TrkAR in the neuroprotective actions of BNN27, the effect of TrkAR inhibitor (10^{-3}M) on the BNN27-induced actions on the bNOS and TH-expressing amacrine retinal cells and ganglion cell axons in the diabetic retina, was examined. Quantitative analysis of the immunofluorescence images (not shown) suggests that the intravitreal administration of TrkAR inhibitor reversed the BNN27 (10mg/kg) actions on NFL- bNOS- and TH-IR (58.38±3.89 percentage of mean gray area per section per section; 54.23±3.14 percentage of number of cells per section; 41.24±9.65 percentage of number of cells per section, respectively) compared to diabetic retinas treated with BNN27 alone (95.06±8.28 percentage of mean gray area per section per section; 90.46±8.78 percentage of number of cells per section; 78.52±532 percentage of number of cells per section, respectively; $^{++}p<0.01$, $^{+}p<0.05$, $^{+}p<0.05$, respectively). The TrkAR inhibitor had no effect when administered alone compared to those non-treated diabetic retinas ($p>0.05$) (Fig. 19).

These data suggested that the neuroprotection observed in NFL-, bNOS, and TH-immunoreactive retinal neurons was mediated by BNN27 activation of the NGF TrkAR. To substantiate this finding further, the effect of BNN27 on the phosphorylation of the TrkAR was examined.

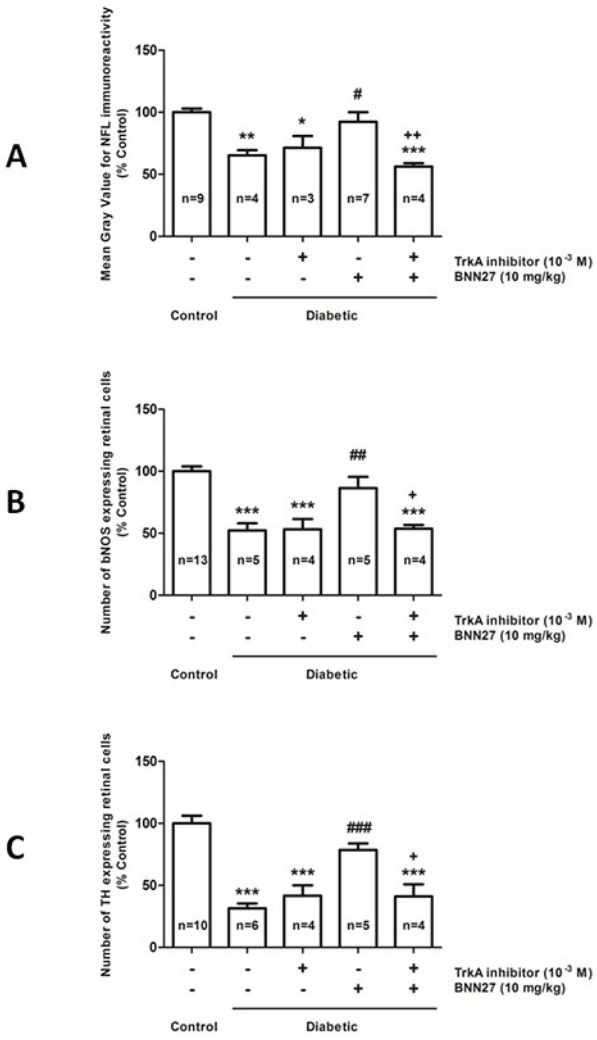


Figure 19. Effect of TrkA inhibitor on the neuroprotective actions of BNN27 (10mg/kg, i.p.) on retinal neurons in the diabetic retina. The TrkAR inhibitor reversed the BNN27 (10mg/kg, 7d, i.p, Paradigm A) effect on **(A)** NFL-, **(B)** bNOS- and **(C)** TH-IR ($^{++}$p<0.01, $^{+}$p<0.05, $^{+}$p<0.05, respectively, compared to BNN27 treated Diabetic rat retinas). No statistically significant differences were observed between Diabetic non-treated retinas in the absence or presence of the TrkAR for all three markers utilized (p>0.05). Data are expressed as the Mean ± SEM.

Western blot analyses showed (Fig. 20A) that administration of BNN27 increased the phosphorylation of TrkAR on the Y^{490} residue in a dose-dependent manner, compared to the diabetic non-treated retinas. No statistically significant effect was observed at the dose of 2mg/kg (0.16±0.03 phospho/total TrkA ratio, p>0.05) compared to the diabetic non-treated retinas, whereas 10mg/kg and 50mg/kg increased the activation/phosphorylation of the receptor (0.32±0.02 phospho/total TrkA ratio, $^{\#}$p<0.05); (0.34±0.01 phospho/total TrkA

ratio, $^{\#}p<0.05$, respectively). There were not significant differences in the phospho/total TrkA ratio between control and diabetic non-treated rat retinas (0.18±0.02 and 0.20±0.03 phospho/total TrkA ratio, respectively, $p>0.05$). The increase in the phosphorylation of TrkA receptor was reversed by the TrkAR inhibitor (0.14±0.04 phospho/total TrkA ratio; $^{+++}p<0.001$) compared to the diabetic treated retinas (0.55±0.07 phospho/total TrkA ratio), bringing the phospho/total TrkA ratio levels to those observed in control retinas (0.16±0.01 phospho/total TrkA ratio, $p>0.05$) (Fig. 20B). The TrkAR inhibitor had no effect when administered alone compared to those non-treated diabetic retinas ($p>0.05$).

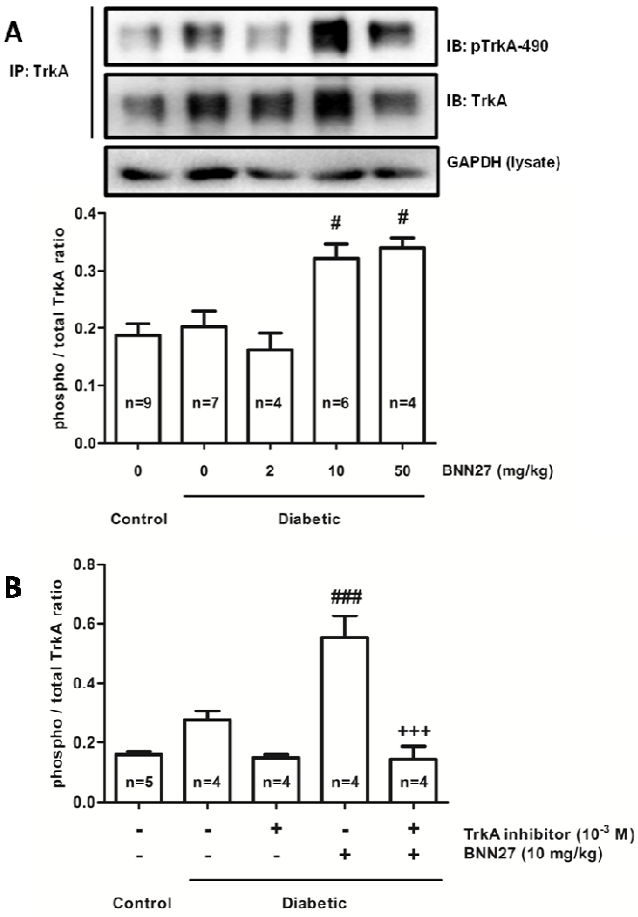


Figure 20. **Representative images and quantification of phospho/total TrkA Receptor ratio and effect of TrkAR inhibitor.** **(A)** BNN27 increased the phosphorylation of TrkAR in diabetic retinas in a dose dependent manner ($^{\#}p<0.05$ compared to retinas of diabetic non-treated animals). **(B)** The TrkA inhibitor reversed the BNN27 (10mg/kg) effect ($^{+++}p<0.001$ compared to diabetic treated animals). No statistical significant difference was observed in the phosphorylation of TrkA in diabetic non-treated animals in presence of TrkA inhibitor ($p>0.05$ compared to diabetic non-treated animals). Data are expressed as Mean ± S.E.M.

3.7. BNN27 hinders the activation of the NGF-p75 receptor in diabetic rat retinas

BNN27 was also shown to bind to $p75^{NTR}$ (Pediaditakis *et al*., 2016b). Therefore, we examined the effect of BNN27 on the expression of the $p75^{NTR}$ in our model. As shown in Fig. 21A, diabetic retinas displayed more spread $p75^{NTR}$ staining extending from the ganglion cell layer until the inner nuclear layer in the retina, while in control rat retinas the staining was fairly diminished. BNN27 reversed this increase in a dose dependent manner. The quantitative analysis of the integrated density obtained from the $p75^{NTR}$ immunofluorescence images, depicted an increase in $p75^{NTR}$-IR (180.1±48.89 percentage of mean gray value) in diabetic retinas compared to the control rat retinas (100±7.98 percentage of mean gray value; $^{***}p<0.001$) (Fig. 21B). Diabetic rat animals treated with BNN27 at 10mg/kg (109.4±15.49 percentage of mean gray value) and 50mg/kg (116.7±26.11 percentage of mean gray value) underwent a marked neuroprotective effect to the retina compared to the diabetic non-treated animals ($^{\#\#}p<0.01$). $P75^{NTR}$-IR obtained in diabetic rat retinas treated with BNN27 was very close to those levels in control rat retinas (Fig. 22B).

Similarly, western blot analysis showed an increase in the expression of $p75^{NTR}$ in diabetic rat retinas (7313±439) compared to the control rat retinas (2452±529; $^{***}p<0.001$) (Fig. 21C). BNN27 administration (10mg/kg; i.p.) reversed the $p75^{NTR}$ expression levels reaching the control levels (2276±442.5; $^{\#\#\#}p<0.001$ compared to diabetic retinas).

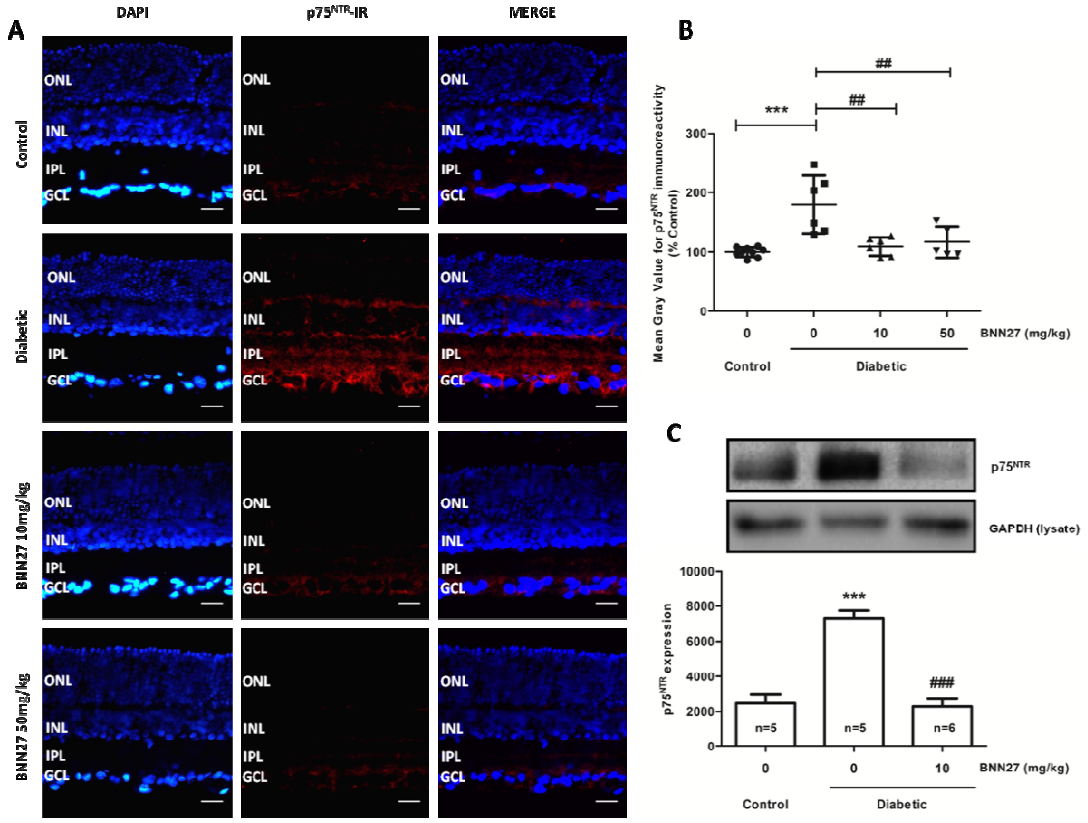


Figure 21. Dose-response effect of BNN27 on p75^{NTR} immunoreactivity and expression. (A) Representative images of p75^{NTR} immunoreactivity rat retinal sections, Control (n=9), Diabetic (n=6) and Diabetic +BNN27 [(10 (n=6) and 50 (n=5) mg/kg, 7d, i.p., Paradigm A]. X40 magnification. Scale bar 20µm. Corresponding DAPI and merge is also shown. **(B)** Quantification of the p75^{NTR}–IR images. BNN27 (10 and 50mg/kg) attenuated the diabetes induced increase in p75^{NTR}–IR (***p<0.001 compared to Control rat retinas, $^{\#\#}$p<0.01 compared to Diabetic rat retinas). Data are expressed as Mean ± SD. **(C)** Representative blots and quantification of the expression of p75^{NTR} in Control, Diabetic and Diabetic +BNN27 (10 mg/kg) rat retinas. BNN27 reversed the diabetes induced increase in p75^{NTR} expression (***p<0.001 compared to Control, $^{\#\#\#}$p<0.001 compared to Diabetic non-treated rat retinas). Data are expressed as Mean ± S.E.M.

3.8. BNN27 restores proNGF and NGF immunoreactivity to a balanced level

The effect of BNN27 treatment on the natural neurotrophins, proNGF and NGF levels was also evaluated. ProNGF and NGF markers showed similar staining patterns, extending from the GCL until the outer layers (Fig. 22A). ProNGF staining was increased in diabetic retinas in contrast to the NGF staining. Development of DR caused an imbalance in the ratio proNGF/NGF. As observed in Fig. 22B, an approximately 1-fold increase in proNGF-IR and 0.5-fold decrease in NGF were observed in diabetic non-treated retinas (1.93±0.18;

0.62±0.02, respectively) compared to control rat retinas (1±0.05; 1±0.07, respectively; $^{**}p<0.01$). Treatment with BNN27 at 10mg/kg i.p. depicted a 0.60-fold decrease on proNGF-IR (1.34±0.16; $^{\#}p<0.05$) and a 0.23-fold increase on NGF-IR (0.85±0.05; $^{\#}p<0.05$) compared to the non-treated diabetic rat retinas, returning the proNGF/NGF ratio to control levels.

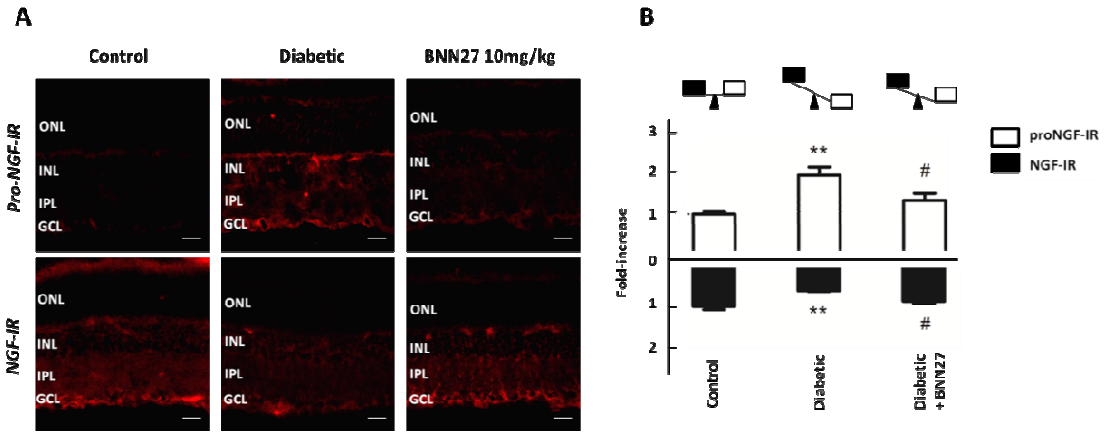


Figure 22. Evaluation of BNN27 effect on proNGF and NGF. (A) Representative images of rat retinal sections stained with proNGF and NGF antibodies. X40 magnification. Scale bar 20µm**. (B)** Comparison of the fold increase for both proNGF and NGF-IR in Control (n=6; n=5, respectively), Diabetic (n=6; n=4, respectively) and Diabetic +BNN27 (10m/kg) (n=6; n=4, respectively), 7d, i.p. An increase in pro-NGF IR is observed in Diabetic retinas ($^{**}p<0.01$ compared to Control rat retinas). BNN27 reduces proNGF-IR ($^{\#}p<0.05$ compared to Diabetic non-treated rat retinas). However, NGF was shown to be decreased in diabetic non-treated rat retinas ($^{**}p<0.01$ compared to Control rat retinas). This effect was reversed by BNN27 ($^{\#}p<0.05$ compared to Diabetic non-treated rat retinas). Data are expressed as Mean ± S.E.M.

3.9. BNN27 does not have any effect on the number of TUNEL positive cells in diabetic rat retinas

Retinal cell death due to the progress of diabetes was examined using dUTP-Terminal Nick-End Labeling in rat retina cryosections of 5 weeks after STZ injection. One important feature of late stage apoptosis is the formation of 3´-hydroxyl termini of DNA ends due to nuclear chromatin fragmentation. TUNEL method allows the identification of damaged DNA. More specifically, the enzyme Terminal deoxynucleotidyl transferase (TdT) catalyzes blunt ends of double-stranded DNA breaks and then incorporation of deoxyuridine

triphosphates (dUTPs) to the TdT reaction serves to label the break sites. Detection is done by using an anti-dUTPs antibody and labeled apoptotic cells were visualized by fluorescence microscopy.

$TUNEL^+$ cells were observed in the 5 week diabetic retinas. With the use of confocal microscopy we observed $TUNEL^+$ cells (light and darker stain) colocalized with DAPI (Fig. 23). We considered as $TUNEL^+$ cells all cells that were observed to be colocalized with DAPI. BNN27 had no effect on the total number of $TUNEL^+$ cells in the diabetic retina [3.23±0.96 $TUNEL^+$ cells in total retina per area (mm^2)] compared to the non-treated diabetic retinas [2.99±0.91 $TUNEL^+$ cells in total retina per area (mm^2); $p>0.05$] (Fig. 24A, C). Statistical significant differences of the diabetic and diabetic+BNN27 compared to control were seen ($^*p<0.05$). No colocalization of TUNEL and the bNOS amacrine cell marker was observed (Fig. 24B).

These results suggested that BNN27 does not protect the retinal from the apoptosis induced by the diabetic insult.

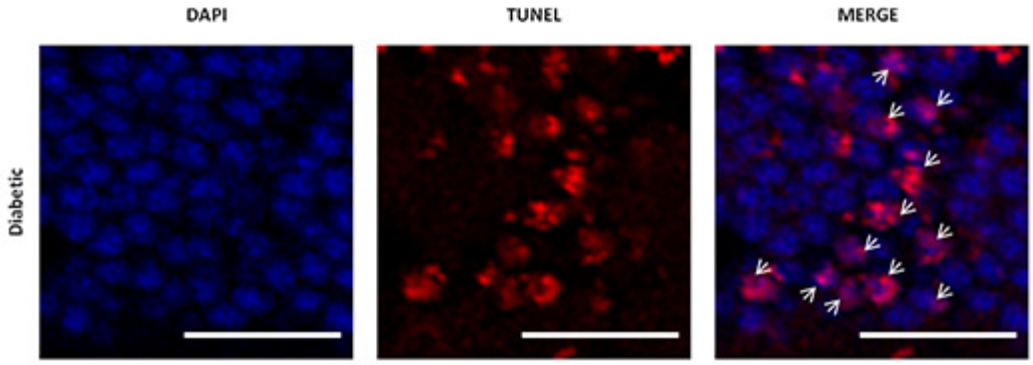


Figure 23. TUNEL images for Diabetic non-treated rat retinas after 5 weeks of STZ injection. DAPI, TUNEL and the merge images were taken by Confocal microscopy. The merge shows in some cases the colocalization of a $TUNEL^+$ retinal cell with a DAPI nuclei as pointed out by the arrows, depicting a retinal neuron undergoing cell death. X40 magnification. Scale bar 20µm.

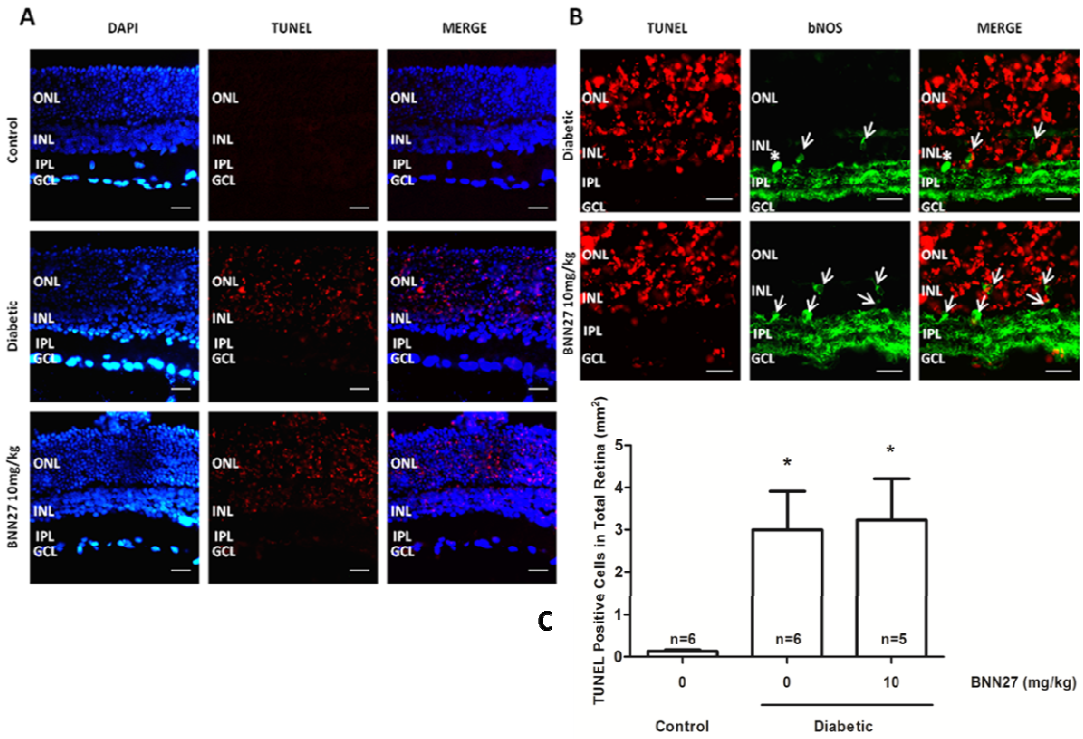


Figure 24. Evaluation of BNN27 (10mg/kg, i.p.) effect on retinal cell death by TUNEL analysis. **(A)** Representative images of DAPI, TUNEL and the corresponding merge for Control, Diabetic and Diabetic rat retinas treated with BNN27 at 10mg/kg (i.p.) 5 weeks post-STZ administration. X40 magnification. Scale bar 20µm. **(B)** Representative images for bNOS/TUNEL co-immuno staining in Diabetic and Diabetic+BNN27 (10mg/kg) rat retinas. No colocalization was observed (*depicts artifact, and arrows depict bNOS-expressing cell bodies). **(C)** Quantification of the $TUNEL^+$ cells per area (mm^2) in total retina of Control, Diabetic and Diabetic+BNN27 (10mg/kg, i.p.) rat retinas. Statistically significant differences were seen in Diabetic non-treated and Diabetic treated retinas compared to Control ($^*p<0.05$). No significant differences were observed in the $TUNEL^+$ retinal cells between Diabetic and Diabetic treated rat retinas ($p>0.05$).

3.10. BNN27 decreases Cleaved Caspase-3 immunoreactivity in diabetic rat retinas

In order to assess further the neuroprotective/antiapoptotic effect of BNN27 we examined its effect on the expression of cleaved caspase-3 apoptosis marker (Fig. 25A). The staining of this marker was predominantly observed in the innermost layers: GCL, IPL and INL (Fig. 25A). Quantitative analysis of the immunofluorescence images showed a BNN27-induced statistically significant decrease in cleaved caspase-3 IR (67.07±3.2 percentage of mean gray value) compared to diabetic rat retinas (100±5.65 percentage of mean gray value; $^{**}p<0.01$) (Fig. 25B).

These findings were instrumental in the elucidation of the neuroprotective effect of BNN27 in the diabetic retina.

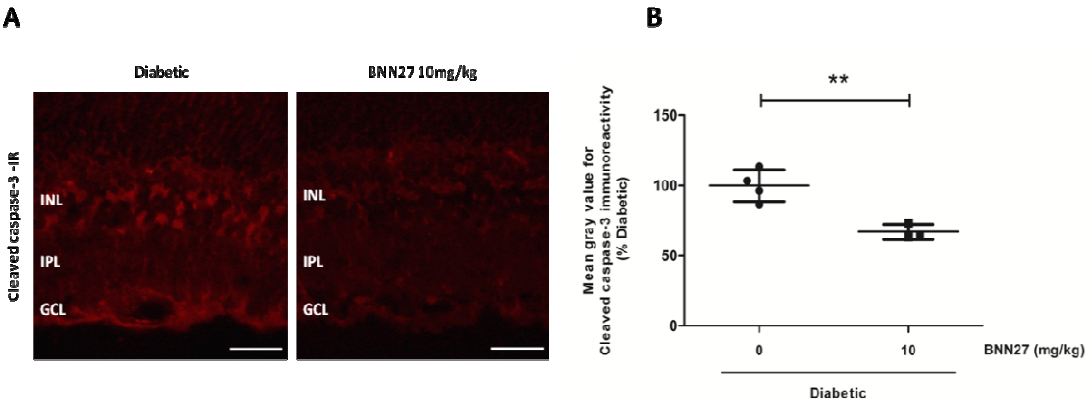


Figure 25. Evaluation of BNN27 effect on Cleaved Caspase-3-IR. (A) Representative images and **(B)** quantification for Cleaved Caspase-3 staining in Diabetic (n=4) and Diabetic+BNN27 (10mg/kg, i.p., n=3) rat retinas 5 weeks post-STZ administration**.** X40 magnification. Scale bar 50µm.

3.11. BNN27 prevents macroglia and microglia over expression in diabetic rat retinas

To study the effect of BNN27 on retinal glial cells in the STZ-model of DR, the macroglia and microglia markers, GFAP and Iba-1, respectively, were examined. Diabetes induced an increase of approximately 49% in macroglial activation (GFAP-IR, 149.0±20.43 percentage of mean gray area compared to control retinas, 100.0±11.75 percentage of mean gray area; $^{***}p<0.001$) as depicted in the large Müller cells by high GFAP-IR staining extending from the nerve fiber layer until the outer nuclear layer (Fig. 26A, B). BNN27 prevented glial activation (10mg/kg: 111.0±10.13; 50mg/kg: 109.5±10.42 percentage of mean gray area; $^{###}p<0.001$, compared to diabetic non treated retinas).

In addition, immunofluorescence images depicted cells stained with Iba-1 marker within the innermost layers: GCL, IPL and INL. The quantification of the number of microglial

cells in the whole retina showed a statistically significant increase in diabetic rat retinas [255±91 percentage of the number of Iba-1 positive cells per are (mm^2)] compared to the control retinas [100±29.01 percentage of the number of Iba-1 positive cells per are (mm^2); $^{***}p<0.001$] (Fig. 27A, B). BNN27 also reduced the number of Iba-1 positive microglial cells [10mg/kg: 67±20.53, 50mg/kg: 113±34 percentage of the number of Iba-1 positive cells per are (mm^2)] compared to diabetic non treated retinas ($^{###}p<0.001$, $^{##}p<0.01$, respectively).

These proteins are known to play an important role in inflammation and thus suggest that BNN27 may treat the inflammatory component of DR.

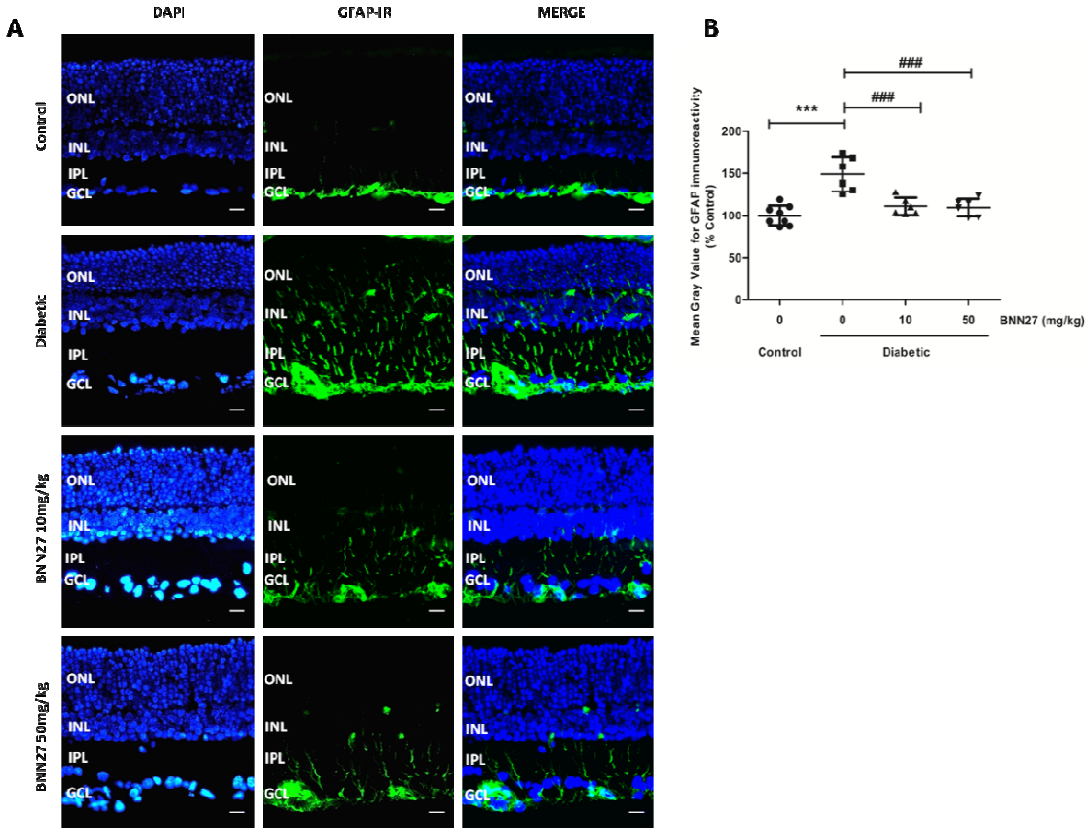


Figure 26. Dose-response effect of BNN27 on retinal glial modulation. **(A)** Representative images of rat retina sections stained with anti-GFAP antibody in Control, Diabetic and Diabetic +BNN27 (10, 50mg/kg, 7d, i.p) rat retinas. X20 magnification. Scale bar 20µm. Corresponding DAPI and merge is also shown. **(B)** Quantification of the immunofluorescence images for GFAP-IR. An increase in GFAP-IR is observed in Diabetic retinas (n=6, $^{***}p<0.001$ compared to Control rat retinas, n=8). BNN27 at 10 (n=6) and 50 (n=6) mg/kg reduces GFAP-IR ($^{###}p<0.001$ compared to Diabetic rat retinas).

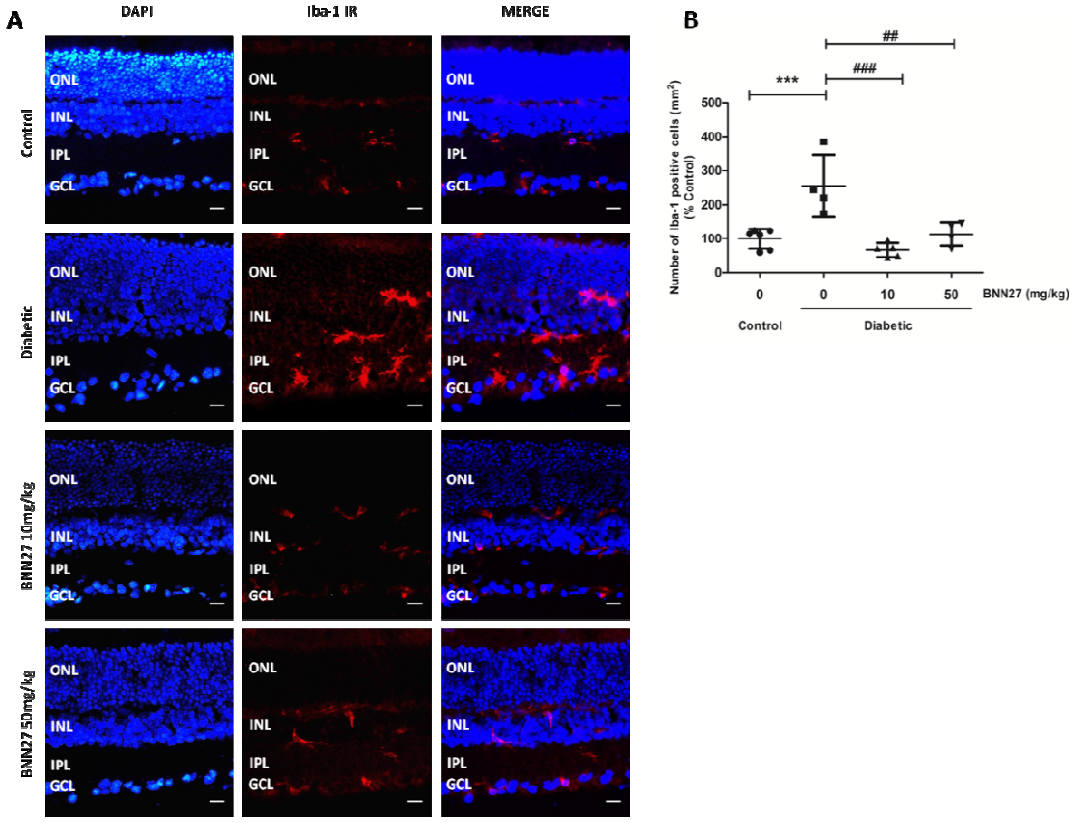


Figure 27. Evaluation of the dose-response effect of BNN27 (10 and 50mg/kg, 7d, i.p) on retinal microglia modulation. **(A)** Representative images of rat retina sections stained with anti-Iba-1 antibody in Control, Diabetic and Diabetic rat retinas treated with BNN27 at 10 and 50mg/kg. **(B)** Quantification of the Iba-1 immunofluorescence images. An increase in the number of Iba-1 positive cells is observed in Diabetic retinas compared to Control ($^{***}p<0.001$). Administration of BNN27 at 10 and 50mg/kg prevented microglia activation by decreasing significantly the number of Iba-1 positive cells compared to non treated Diabetic rat retinas ($^{###}p<0.001$, $^{##}p<0.01$, respectively). X20 magnification. Scale bar 20µm. Mean ± SD is shown.

3.12. BNN27 levels in retina after intraperitoneal administration in control rats

To examine if the neuroprotective effect of BNN27 in our model coincided with the levels of BNN27 reaching the retina after i.p administration, HPLC-MS/MS analysis of retinal samples of control animals (non diabetic) that received BNN27 (10mg/kg, i.p., 7days) was performed. Our results show that BNN27 levels reaching the retina were in the order of 227±95nM (n=6).

PART II. Study of the effect of BNN27 on retinal viability and the mechanisms involved in its neuroprotective actions when administered with eye drops

3.13. BNN27 affords neuroprotection to the bNOS and TH-expressing retinal amacrine cells and ganglion cell axons

The results presented above strongly suggest that BNN27 protects the retina from the diabetic insult when administered intraperitoneally, by activating the NGF TrkAR, reducing the expression of the $p75^{NTR}$, reducing macro and microglia activation.

We also examined the neuroprotective effect of BNN27 when administered with eye drops, another route of administration, more relevant to the therapeutics of retinal disease.

As mentioned in Methods, the amount of BNN27 administered as eye drops was correlated according the doses administered intraperitoneally. Therefore in Figure 30 the doses of BNN27 administered as eye drops are shown not in Molarity units but mg/kg in order to have a direct comparison to the data obtained after the intraperitoneal administration. An approximate 52% reduction in NFL-IR was observed in diabetic retinas (48.16±2.91 percentage of number of mean gray area) compared to control rat retinas (100.0±13.74 percentage of mean gray area; $^{***}p<0.001$). A prominent dose-dependent neuroprotection on ganglion cell axons was afforded by BNN27 administered with eye drops (10mg/kg: 84.83±7.3 percentage of mean gray area, 50mg/kg: 98.98±5.21 percentage of mean gray area; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, respectively) compared to non-treated diabetic rat retinas. The lowest dose of 2mg/kg (62.54±9.19 percentage of mean gray area) did not provide neuroprotection to the ganglion cell axons compared to non-treated diabetic retinas ($p>0.05$) (Fig. 30A). The intravitreal administration of a TrkAR reversed the BNN27-induced increase (10mg/kg) of NFL-IR to diabetic levels ($^{++}p<0.01$ compared to retinas treated with

BNN27 alone). No significant differences were seen between diabetics in the presence and absence of the TrkAR inhibitor (Fig. 30B).

The number of bNOS-IR cells showed an approximately 50% decrease in non-treated diabetic animals (50.85±2.27 percentage of number of cells per section) compared to control retinas (100.0±3.1 percentage of number of cells per section; $^{***}p<0.001$). BNN27 afforded a dose-dependent reversal in the number of bNOS-expressing cells (10mg/kg: 84.69±3.72 percentage of number of cells per section, 50mg/kg: 89.03±3.45 percentage of number of cells per section; $^{###}p<0.001$). No significant differences were observed at the lowest dose of 2mg/kg (50.44±7.39 percentage of number of cells per section) compared to the diabetic non-treated animals ($p>0.05$) (Fig. 30C). BNN27 (10mg/kg) neuroprotective activity in the bNOS-expressing retinal cells was reversed in the presence of TrkAR inhibitor ($^{+}p<0.05$ compared to retinas treated with BNN27 alone). No significant differences were seen between diabetics in the presence and absence of the TrkAR inhibitor (Fig. 30D).

Diabetes strongly affected the dopaminergic amacrine cells, reducing the number of TH-expressing amacrine cells in an approximately 65% in diabetic animals (34.63±2.87 percentage of number of cells per section) compared to control animals (100.0±13.74 percentage of number of cells per section; $^{***}p<0.001$). Animals treated with BNN27 depicted an increase in the number of dopaminergic cells at two highest doses of 10mg/kg (82.79±4.96 percentage of number of cells per section) and at 50mg/kg (75.0±3.63 percentage of number of cells per section; $^{###}p<0.001$, $^{#}p<0.05$ respectively) compared to the non-treated diabetic retinas. No significant differences were seen for the concentration of 2mg/kg (25.0±5.27 percentage of number of cells per section) compared to the retinas of the non-treated diabetic animals ($p>0.05$) (Fig. 30E). When a TrkAR inhibitor was applied, BNN27 (10mg/kg) effect on TH-expressing amacrine cells was reversed compared to those retinas treated with BNN27 alone ($^{+}p<0.05$). No significant differences were seen between diabetics in the presence and absence of the TrkAR inhibitor (Fig. 30F).

These findings are in agreement to those obtained when BNN27 was administered intraperitoneally.

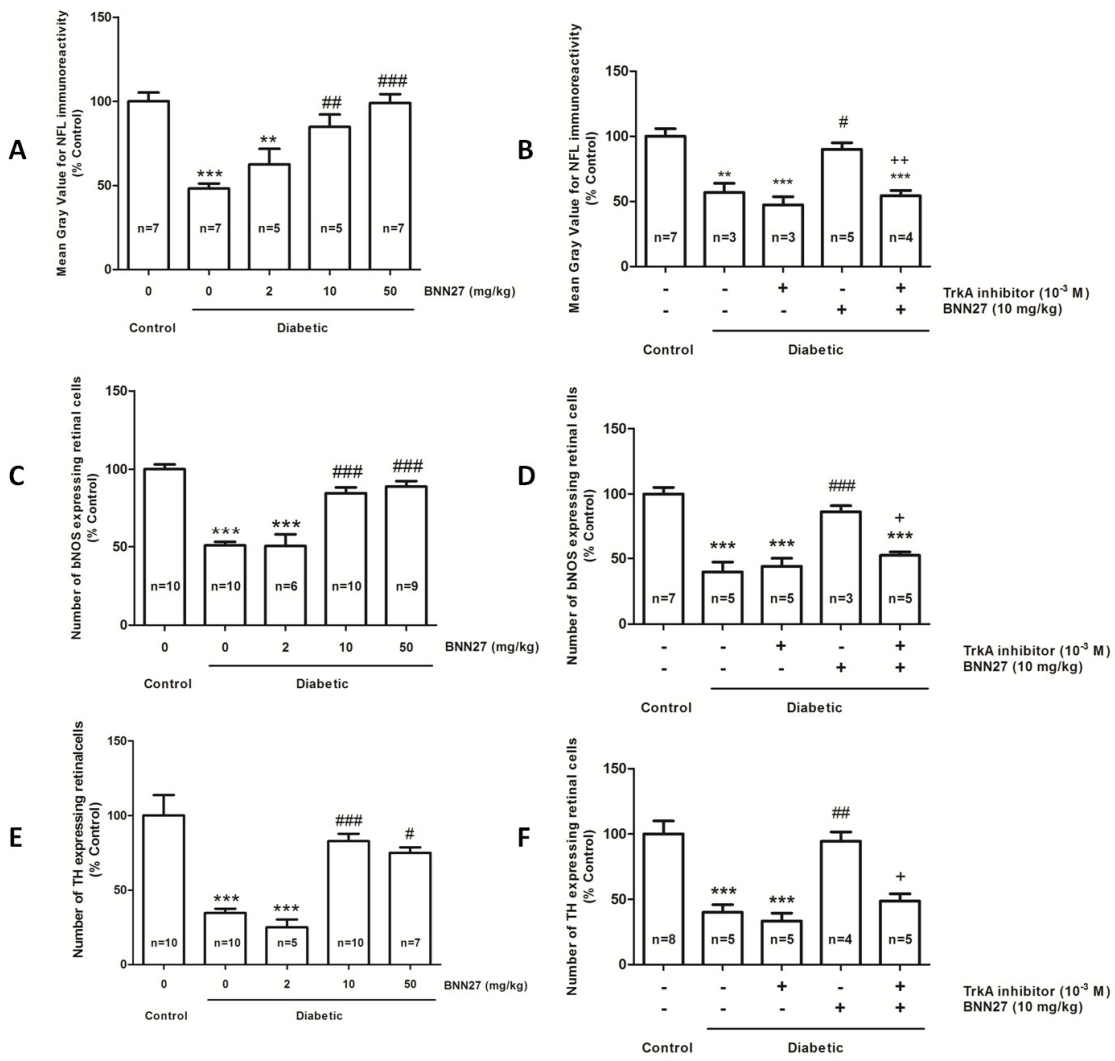


Figure 28. Quantification studies of (A, C, E) Neuroprotective effect of BNN27 (2, 10, 50mg/kg) administered with Eye drops in the diabetic retina: (B, D, F) Effect of TrkAR inhibitor. (A) bNOS-IR. BNN27 afforded neuroprotection to bNOS expressing amacrine cells against the diabetic insult in rat retinas at the two higher doses 10 mg/kg and 50mg/kg ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, respectively) compared to diabetic rat retinas**. (B)** The effect of BNN27 (10mg/kg) was reversed by the TrkAR inhibitor. **(C) TH-IR.** BNN27 protected the TH expressing amacrine cells in diabetic rat retinas at the doses of 10 and 50mg/kg ($^{\#\#\#}p<0.001$) compared to diabetic rat retinas. Diabetic retinas showed a reduction in bNOS and TH-IR ($^{***}p<0.001$) compared to Control. **(D)** The effect of BNN27 (10mg/kg) was reversed by the TrkAR inhibitor. **(E) NFL-IR** BNN27 afforded neuroprotection to NFL-IR ganglion cell axons at the two highest doses of 10 and 50mg/kg ($^{\#\#\#}p<0.001$, $^{\#}p<0.05$, respectively) compared to diabetic rat retinas**. (F)** The effect of BNN27 (10mg/kg) was reversed by the TrkAR inhibitor.

3.14. BNN27 stimulates the NGF-TrkA receptor activation in diabetic rat retinas

To ascertain whether the BNN27 effects in the diabetic retina, when administered with eye drops, were TrkAR-mediated, the ratio of phospho/total TrkA was examined. We chose the optimum dose of 10mg/kg for the examination of the BNN27 signaling when applied with eye drops, since it was the lowest dose that afforded full protection in the i.p. route of administration. Treatment of BNN27 (10mg/kg) with eye drops to diabetic animals increased the phosphorylation of TrkA (pTrkA^{490}) receptor as seen in the phospho/total ratio (0.22±0.03 phospho/total TrkA ratio, compared to the diabetic non-treated retinas (0.13±0.02 phospho/total TrkA ratio, $^{\#}p<0.05$) (Fig. 31). No statistically significant differences were observed between control (0.12±0.01 phospho/total TrkA ratio) and diabetic non-treated rat retinas ($p>0.05$).

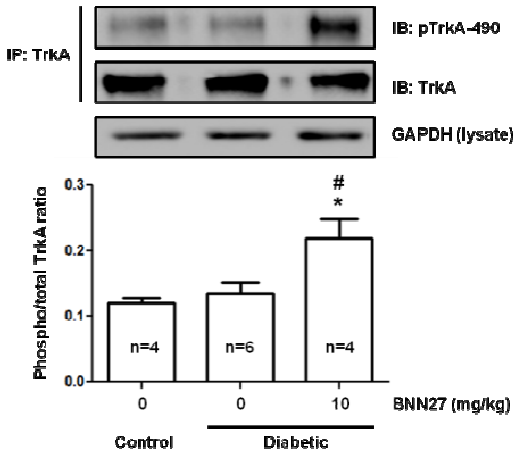


Figure 29. Representative western blot and quantification analysis of the effect of BNN27 administered with Eye drops on the phosphorylation of the Tyr490 of NGF TrkA receptor in diabetic rats. BNN27 at 10 mg/kg increased the phosphorylation of Tyr490 of NGF TrkA receptor ($^{\#}p<0.05$) compared to retinas of diabetic non-treated animals.

4. DISCUSSION

Diabetic Retinopathy is a major ocular complication derived from diabetes development among adult individuals in developed countries. This disease has been characterized by three pathogenic mechanisms namely: neurodegeneration, inflammation and vascular damage. Nowadays, despite the vast progress at the clinical level, the only therapies that exist treat mainly the diabetes induced-microangiopathy, and to a lesser extent the inflammation. However, the treatment for the diabetes-induced emergence of neurodegeneration is still on hold. In the present study, we advocate the use of the novel neurosteroidal microneurotrophin BNN27, a synthetic derivative of DHEA, as a putative therapeutic agent for the treatment of neurodegeneration and inflammation in DR. The STZ-rat model of DR was used and hyperglycemia was detected as early as one-day post-STZ injection, keeping glucose blood levels high up to 5 weeks (>600mg/dl). It has been reported that DHEA, the mother compound of BNN27, improved hyperglycemia in the STZ-model of DR, by activating glucose metabolism-related signaling pathways in skeletal muscle (Sato *et al*., 2009). In this study, blood glucose levels remained high in those diabetic rats treated with BNN27 (10mg/kg, i.p.) and no statistically differences were observed compared to non-treated diabetic animals. The examination of the effect of DR on retinal neuron viability was of capital importance to set up the starting time point for the BNN27 treatment. As was mentioned earlier, amacrine and ganglion cells were shown to be the first retinal neurons affected by diabetic retinopathy in both humans (Barber *et al*., 1998) and rats (Zeng *et al*., 2000; Seki *et al*., 2004). We chose to assess the effect of diabetic retinopathy on amacrine and ganglion neurons, and subsequently the effect of BNN27, given that they are neurons essential to integrate and modulate the visual message from bipolar to ganglion cells, and to transfer the visual information to higher brain centres, respectively. The evaluation of the integrity of these specific retinal neurons would provide important information about the overall state of the retina system under diabetic conditions. Thereby, after 3 weeks of STZ-injection, we observed that DR did not affect the NFL-expressing ganglion cell axons and

bNOS and TH-expressing amacrine cells (data not shown). However, a manifested detrimental effect was detected on these specific retinal neurons 4 weeks after the onset of diabetes, as shown by the loss of the immunoreactivity of NFL, bNOS and TH markers (Fig. 16).

Measurement of retinal thickness was employed as a reliable indicator to assess the grade of retinal neurodegeneration. There are several studies that have observed loss of retinal thickness as a result of DR progression. It has been reported that people suffering from diabetes for approximately 15 years, exhibited loss of ganglion axons as shown by a decrease in the thickness of the nerve fiber layer (Lopes de Faria *et al*., 2002). Also, a reduction in the thickness of the IPL (22%) and inner nuclear layers (14%) was detected in retinal sections from STZ-diabetic rats after 7.5 months of diabetes (Barber *et al*., 1998). Sohn *et al*. (2016) stated that patients at the first stages of DR undergone a total retinal thinning in the set of NFL+GCL+IPL layers in the order of 0.53µm per year and STZ-diabetic mice lost 1.57µm (17.5%) and 2.53µm (39.2%) in NFL+GCL layers after 6 and 20 weeks, respectively, of diabetes induction compared to the control retinas. In our study, the histological staining with Hematoxylin-Eosin was performed to assess the effect of DR on the retinal thickness of rats after 4 weeks of diabetes onset. The values obtained showed that the retinal thickness in diabetic rats remained unaltered and did not present statistically significant differences in comparison with control rat retinas, same as in any of the individual retinal layers (Fig. 15). These data suggest that the shrinkage of retinal thickness due to DR progression may become evident at a later than the 4-week period tested.

At this time point of the disease, diabetic rat retinas start undergoing a cascade of deleterious effects, destabilizing the homeostasis of the survival versus detrimental events. The progress of DR comes along with the rise of cell death processes as seen in retinas of experimental rats after 1-month of STZ-induced diabetes as well as in retinas of diabetic patients after 6 years with DM (Barber *et al*., 1998). In the current study, TUNEL analysis on

retinal cryosections of 4 weeks post-STZ injection, showed a 10-fold increase in the number of TUNEL^{+} cells per area (mm^2), observed across the whole retinal thickness, with remarkable appearance of apoptotic cells in the GCL, INL and ONL (% TUNEL/DAPI, Fig. 17). In support of these results, increased retinal cell death have been reported by several research groups that employed the experimental STZ-model of DR (Asnaghi *et al*., 2003; Martin *et al*., 2004; El-Remessy *et al*., 2006; Ali *et al*., 2008; Hernández *et al*., 2013).

The administration of BNN27 either i.p. or with eye drops for 7 days, after 4 weeks of STZ-injection, reversed the diabetic-induced decline of NFL-, bNOS- and TH-IR in a dose-dependent manner, suggesting a restorative role of this agent against the diabetic insult to these specific retinal neurons (Figs. 18, 28). BNN27 was shown to bind and activate solely the TrkAR and not TrkB or TrkC neurotrophin receptors, to protect TrkA positive and NGF-dependent sympathetic and sensory neurons from apoptosis (Pediaditakis *et al*., 2016a). To ascertain the involvement of this neurotrophin receptor on the BNN27-induced actions on the retinal cells markers a TrkAR inhibitor (10^{-3}M) was applied intravitreally. The TrkAR inhibitor reversed the neuroprotective effect of BNN27 (10mg/kg) on NFL-IR in ganglion cell axons and bNOS- and TH-IR expressing amacrine cells, when administered either i.p. or with eye drops (Fig. 19, 28, respectively). Interestingly, this inhibitor did not show any effect on those diabetic non-treated rat retinas when administered alone suggesting that BNN27 restores the diabetic-induced phenotype of these specific retinal neurons via NGF TrkAR. These data consolidate the previous findings regarding BNN27's mechanism of action (Pediaditakis *et al*., 2016a), so we can conclude that the TrkAR is responsible for the BNN27-induced neuroprotection of the retina against the diabetic insult.

TrkAR seems to be expressed in bipolar and amacrine (Wexler *et al*., 1998; García *et al*., 2014) and ganglion cells (Vecino *et al*., 1998c; Kokona *et al*., 2012) in addition to its expression in Müller cells (Sun *et al*., 2008; Kokona *et al*., 2012) and astrocytes (Kim *et al*., 2013) (Fig. 10). Therefore, the neuroprotection afforded by BNN27 to ganglion cell axons

and amacrine cells might be due to the direct activation of the TrkAR by this microneurotrophin located in these neurons.

BNN27 activates the TrkAR by inducing the phosphorylation of the tyrosine residues Y490, Y674/675 and Y785 at the intracellular domain as shown in CHO cells transfected with TrkAR cDNA, as well as the phosphorylation of the Y490 in TrkA-positive BV2 mouse glial cells (Pediaditakis *et al*., 2016a). In the current study, among all tyrosine residues examined in diabetic rat retinas, Y490 was shown to be phosphorylated in a statistically significant manner after BNN27 treatment either i.p. (10, 50mg/kg) or with eye drops (10mg/kg) (Figs. 20, 29, respectively). Additionally, the expression of the TrkAR was not altered in the diabetic compared to control rat retinas, in agreement with Ali *et al*. (2008) and Mysona *et al*. (2013). This might occur in an attempt to preserve a basal survival activity in the cells under pathological circumstances.

It has been well described that upon BNN27 binding, TrkAR phosphorylation of Y490 residue, leads to activation of the signal transduction pathways*:* Ras-Raf-MAPK and PI3K-Akt pathways in NGF-dependent sympathetic neurons after 15 minutes treatment (100nM) (Pediaditakis *et al*., 2016a). In this regard, the investigators saw an increase in the phosphorylation levels of the ERK1/2 and Akt kinases. Several reports have described that ERK1/2 kinases play an important role in the neuroprotection against RGC death in DR (Akiyama *et al*., 2002; Nakazawa *et al*., 2002).

In a study by Lisa *et al*. (unpublished data) in our group, it was shown that TrkAR activation by BNN27 led to the phosphorylation of ERK1/2 kinases in a dose-dependent manner either i.p. or with eye drops. The effect of BNN27 (10mg/kg, i.p.) was reversed in the presence of a TrkAR inhibitor. Diabetic non-treated rat retinas showed a slight increase of ERK1/2 phosphorylation compared to controls. This might be due to a survival response against stress stimuli that may be activated by different mechanisms other than those stimulated by TrkAR neurotrophin binding. These findings support the involvement of TrkAR

activation and the subsequent phosphorylation of the downstream signaling pathway (ERK1/2 kinases) in the prosurvival effects of BNN27 in diabetic retinas.

On the other hand, the PI3K/Akt downstream signaling cascade is commonly involved in survival mechanisms although it has also been shown to be activated as a response to pro-inflammatory cytokine production (Yoo *et al*., 2005). More specifically, Cahill and Rogers (2008) demonstrated upregulation of the Akt kinase in colon carcinoma cells and discovered that PI3K/Akt pathway activation through IL-1, leads to the prompt release of IL-6. Additionally, other studies confirmed the Akt-induced phosphorylation by IL-18 in rheumatoid arthritis synovial tissue fibroblasts, and this effect was hindered in the presence of a PI3-kinase inhibitor (Morel *et al*., 2002). On the contrary, in STZ-diabetic animals after 4 weeks of the diabetes onset, it was shown that the phosphorylation of the Akt kinase was attenuated (Reiter *et al*., 2006). In addition, insulin administration promoted retinal survival by the activation of PI3K/Akt pathway which led to a caspase-3 expression decrease (Barber *et al*., 2001). Interestingly, in our paradigm the phosphorylation levels of Akt in diabetic rat retinas, were highly increased compared to controls (Lisa *et al*., unpublished data). This event was reversed by BNN27 (i.p.) in a dose-dependent manner affording a neuroprotective role in the occurrence of Akt upregulation, returning the Akt phosphorylation to baseline levels. These data support that the activation of the PI3K/Akt signaling pathway in our STZ-model of DR is involved in the detrimental mechanisms arisen in the diabetic retina instead of being part of the pro-survival system.

During healthy conditions NGF is expressed in higher levels than proNGF to elicit survival, and a proper physiological function to the nervous system by binding to Trk receptors. However, the neurotrophin balance between NGF and proNGF has been shown to be switched in several neurodegenerative diseases, including DR, suggesting a ligand-induced cell death role for proNGF (Hempstead, 2009). Augmented levels of proNGF promote the proNGF/p75 axis upregulation which will spur detrimental actions in the

principal source of neurotrophins: neurons and glia (Bhakar *et al*., 2003; Kenchappa *et al*., 2006; Barde, 2004). Thus, increased proNGF in rat and human brain with Alzheimer's disease was linked with cholinergic neuronal loss (Fahnestock *et al*., 2001). Moreover, Alzheimer's-induced activation of $p75^{NTR}$ in cortical neurons was also reported (Mufson and Kordower, 1992). In a like manner, $p75^{NTR}$ activation in oligodendrocytes was seen to be proNGF-mediated and both were induced after spinal cord injury (Beattie *et al*., 2002). The intraocular injection of proNGF in rodent retinas was observed to induce retinal ganglion cell death and this effect was not detected in $p75^{NTR}$ null retinas, suggesting the involvement of the proNGF/p75 axis in the death of RGCs (Lebrun-Julien *et al*., 2010). Our data reflected the proNGF/NGF diabetes-induced imbalance showing higher levels of proNGF in diabetic non-treated rat retinas compared to control retinas (Fig. 22). However, BNN27 (10mg/kg, i.p.) re-established the disproportion between the mature and the non-processed neurotrophin restoring the levels to a healthy state. ProNGF/NGF dysfunctional imbalance has been attributed to posttranslational disturbances in the NGF biosynthesis (Fahnestock *et al*., 2001). The most accepted explanation for this event has been referred that some alteration occurred in the expression or activity of the MMP-7, enzyme responsible of the proNGF cleavage, due to the diabetes milieu (Lee *et al*., 2001; Ali *et al*., 2011). In fact, several studies have reported that the proteolytic activity of MMPs contribute to the pathophysiology of DR, either in the early stages facilitating endothelial cell and pericytes loss (Yang *et al*., 2007; Kowluru *et al*., 2009, 2010b, 2011) or when the disease has seriously evolved (PDR) promoting neovascularization processes (Kowluru *et al*., 2012). Consistent with these results, low levels of MMP-7 were detected after kainic acid-induced seizure in rat hippocampus (Le *et al*., 2012). Also, mRNA and protein levels of the MMP-7 inhibitor named tissue inhibitor of metalloproteinase 1 (TIMP-1), were increased in forebrain and hippocampus after seizure (Rivera *et al*., 1997). These events coincide with the attenuation of the cleaving activity leading to accumulation of proNGF, and $p75^{NTR}$ activation. Therefore

BNN27 could be involved in the reinforcement of the proper functioning on the way of proNGF to NGF by the rescue of the MMP-7 faculties or other components implicated in this process such as the TIMP-1, enhancing the proNGF/NGF balance in the diabetic retina.

As previously mentioned, the accumulation of proNGF is found to be within the cluster of detrimental molecular mechanisms due to DR progression, which will induce the upregulation of $p75^{NTR}$ leading to neuronal cell death (Ali *et al*., 2008, 2011; Al-Gayyar *et al*., 2011). It has been reported that after CNS axotomy, proNGF secretion is increased and it promotes $p75^{NTR}$ activation by ligand-binding in corticospinal neurons (Harrington *et al*., 2004). Furthermore, these neurons seemed to be recovered after proNGF-$p75^{NTR}$ binding was interrupted, proving the capital relevance of proNGF in the pathophysiology conditions after CNS injury. $P75^{NTR}$ overexpression has also been related to many other neurodegenerative diseases such as MS (Dowling *et al*., 1999), ALS (Lowry *et al*., 2001), and Alzheimer's disease (Mufson *et al*., 2002). Indeed, data from our study support this concept by showing high levels of $p75^{NTR}$-IR and $p75^{NTR}$ expression in diabetic rat retinas (Figs. 21). Treatment with BNN27 (10mg/kg, i.p.) downregulates either the $p75^{NTR}$-IR or the expression of the $p75^{NTR}$, suggesting the neuroprotective potential of this agent in the diabetic retina. Immunohistochemical studies have shown that $p75^{NTR}$ is located in photoreceptors (Santos *et al*., 2012; Sheedlo *et al*., 2002), Müller cells (Vecino *et al*., 1998c; Mohamed and El-Remessy, 2015) and, most controversial, in ganglion cells (García *et al*., 2014). Thereby, the attenuation of $p75^{NTR}$ expression by BNN27 may diminish the diabetes-induced deleterious effects in these retinal cells, and so extend its beneficial effect to the entire diabetic retina. According to the effects triggered by the proNGF/$p75^{NTR}$ tandem, it would be plausible to come to the conclusion that inhibiting this neurotrophin receptor may abolish the deleterious mechanisms caused by its activation via proNGF-binding. Nevertheless, it is known that $p75^{NTR}$ can be present in partnership with TrkAR modulating pro-survival pathways (Barker and Shooter, 1994; Verdi *et al*., 1994). More importantly, there are several

studies with $p75^{NTR}$ knock-out mice which exhibited sympathetic neuron impairment suggesting that the time course of natural occurring apoptosis depends upon the presence of $p75^{NTR}$ to ensure a proper cell-to-cell interaction (Bamji *et al*., 1998).

Several reports have stated that $p75^{NTR}$ enables cell death mechanisms involving stimulation of the SAPK/JNK downstream signaling pathway (Aloyz *et al*., 1998; Yoon *et al*., 1998; Roux *et al*., 2002). More specifically, Shanab *et al*. (2015) showed that the proNGF binding to $p75^{NTR}$ induced phosphorylation of the JNK kinase in endothelial cells of rat retina. This effect was reversed by using shRNA and siRNA for *in vivo* and *in vitro* analysis, respectively, to silence $p75^{NTR}$ expression. In support of these data, an increased phosphorylation/activation of the pro-apoptotic SAPK/JNK kinases was observed in our model of DR and this effect was attenuated by BNN27 at the optimum dose of 10mg/kg administered either i.p. or with eye drops (Lisa *et al*., unpublished data). This is in agreement with the study that demonstrated the ability of BNN27 to decrease apoptosis in mouse cerebellar granule neurons (CGNs) under NGF serum deprivation conditions, by reducing the $p75^{NTR}$-mediated activation of the JNK kinase (Pediaditakis *et al*., 2016a).

Taking together these findings, we can affirm that the pharmacological actions of BNN27 involve both TrkA and p75 neurotrophin receptors. These results are in agreement with Lazaridis *et al*. (2011), who demonstrated that DHEA, the parent molecule of BNN27, directly interacts with TrkAR and $p75^{NTR}$ in isolated membranes from HEK293 cells.

While the above mentioned data suggest BNN27 as a potential neuroprotectant in the STZ-model of DR, we decided to explore the role of this microneurotrophin as an anti-apoptotic agent. As mentioned earlier, bNOS-immunoreactivity was attenuated in the diabetic rat retina. However, bNOS-TUNEL co-immunostaining did not show colocalization of the two markers (Fig. 24B). These data provided some insight into the cell state of the bNOS-expressing amacrine cells, suggesting that this type of retinal neuron did not undergo apoptosis but most probably functional impairment. This could explain the reason why we

see an increase in the number of bNOS-expressing amacrine cells after treatment with BNN27, discarding the hypothesis of cell regeneration. It has been documented that the loss of function in retinal neurons is not always the result of neuronal cell death (Gorman, 2008). This event may be due to the loss of neurotransmitters which disrupts synaptic transmission leading to retinal function impairment, and subsequent vision decline (Van Guilder *et al*., 2008). Also, diabetes may induce changes in the biochemistry and physiology of proteins (Barber *et al*., 2011). Therefore, these results suggest that the overall reduction of the neuronal markers utilized in our study, may not be due to retinal cell death, but as a result of diabetes-induced changes in the protein synthesis or metabolism, in which BNN27 plays a restorative role.

In contrast to our expectations, TUNEL analysis showed that BNN27 had no effect on the total number of $TUNEL^{+}$ cells in the diabetic rat retina (Fig. 24A, C). Nevertheless, BNN27 was shown to protect TrkA positive and NGF-dependent sympathetic and sensory neurons from apoptosis (Pediaditakis *et al*., 2016a), in addition to reversing the serum deprivation-induced apoptosis in CGNs (which do not express TrkA) by activating the low affinity neurotrophin receptor $p75^{NTR}$ (Pediaditakis *et al*., 2016b). It is known that DR induces activation of caspases and more specifically, caspase-3 in endothelial cells and pericytes (Kowluru *et al*., 2002; Mohr *et al*., 2002). Taking into consideration the previous data and the fact that the TUNEL assay fails to discriminate among apoptosis, necrosis or other type of cell death, we chose to investigate the effect of BNN27 on the apoptotic enzyme cleaved caspase-3. Research done by others saw an upregulation of this death protease in STZ-rat retinas and this effect was hindered by the eye drop treatment with somatostatin (Hernández *et al*., 2013). In the current study, it turned out that BNN27 (10mg/kg, i.p.) protected the diabetic rat retina against apoptosis by reducing the diabetes-induced increase of cleaved caspase-3-IR (Fig 25). In agreement with our results, BNN27 was shown to decrease the number of apoptotic caspase-3 positive cells in serum deprived PC12 cells

transfected with shRNAs for TrkAR, as well as, *in vivo* analysis, reducing the loss of apoptotic TrkA positive neurons in dorsal root ganglia of ngf-/- mouse embryos (Pediaditakis *et al*., 2016a). Furthermore, the cuprizone-induced increase of cleaved caspase-3 expression in a primary cell line of oligodendrocytes was attenuated by BNN27 treatment (Bonetto *et al*., 2017). Taking together the previous findings of BNN27 as an anti-apoptotic agent along with the fact that caspase-3 is a specific marker for early apoptosis in tissue sections (Duan *et al*., 2003), it appears that BNN27 selectively prevented the caspase-3-mediated cell death, having no effect on other apoptotic or necrotic pathways, in contrast to its lack of effect on TUNEL staining that labels all dead cells. Pro-neurotrophin binding to $p75^{NTR}$ triggers cell death mechanisms involving caspase-3 activation, among other caspases, in hippocampal rat neurons *in vitro* and *in vivo* (Troy *et al*., 2002). Nevertheless, despite the $p75^{NTR}$ expression and cleaved caspase-3 increase observed in diabetic non-treated animals, we cannot suggest the causal relationship of $p75^{NTR}$ activation and stimulation of this specific caspase in our model of DR.

The stimulation of the proNGF/$p75^{NTR}$ axis triggers deleterious effects in the diabetic retina not only in neurons or vascular cells but also in glial cells (Lebrun-Julien *et al*., 2010; Mysona *et al*., 2013). More specifically, the diabetes-induced release of inflammatory cytokines can be mediated by Müller macroglial cells (Wang *et al*., 2010) and activated microglial cells (Tang and Kern, 2011). Progression of DR induces the activation of Müller glial cells and as a consequence, the abnormal production of GFAP. This was shown in our GFAP immunofluorescence images by the conspicuous radial elongations of Müller cells, extended along the whole retina (Fig. 26). This is in agreement with observations in human retinas after 10±4 years of diabetes, and in STZ-diabetic rat retinas after 3 months of the onset of diabetes where GFAP was largely increased (Mizutani *et al*., 1998; Lieth *et al*., 1998). Treatment with BNN27 (i.p.) provided neuroprotection to the diabetic rat retina by reducing the GFAP-IR in a dose-dependent manner. Given that Müller cells are responsible

for maintaining a suitable environment for neurons under normal conditions (Bringmann *et al.*, 2006) BNN27 may exert a neuroprotective effect on Müller cells so they can provide a proper metabolic and supportive function, and therefore, promote retinal neuron viability.

With regards to microglia, these cells migrate to inflamed areas in the retina as a response to retinal axotomy insults in order to degrade damaged neurons (Thanos *et al.*, 1991). As a response to acute inflammatory stimuli they change from a non-active to an active state shifting their morphology from ramified to ameboid, respectively (Kettenman *et al.*, 2011). Commonly, after this resolution phase, microglia returns to a quiescent phase. However, this step seems to be disturbed if inflammation is chronically persistent leading to neurodegeneration processes (Glass *et al.*, 2010). In the $Ins2^{Akita}$ mouse model of diabetes reactive Iba-1^{+} retinal microglia was observed after 8 weeks of the hyperglycemia onset (Barber *et al.*, 2005). In our studies, we observed a high increase in the number of Iba-1^{+} cells in rat retinas after 5 weeks of diabetes induction and this effect was reversed by BNN27 (i.p.) in a dose-dependent manner (Fig. 27). Microglial cell distribution was restricted to the inner retinal layers (GCL, IPL and INL) in agreement with data from studies of retinal degeneration utilizing either a renitis pigmentosa or STZ-induced DR experimental models (Zeng *et al.*, 2000; Noilles *et al.*, 2014). The Iba-1 protein has been identified to be specifically expressed in microglia and macrophages *in vivo* or *in vitro* (Ito *et al.*, 1998) both of which are present in the retina due to the diabetes-induced BRB disruption. Therefore, the increase in Iba-1^{+} retinal cells does not necessarily indicate the activation of microglia alone. Nevertheless, the morphology of Iba-1^{+} cells was examined and it was noticed that these cells in control and diabetic retinas treated with BNN27 were smaller and with thin ramifications. However, the Iba-1^{+} cells in diabetic retinas untreated presented swollen protrusions. The features observed in our rat retinal sections indicated that microglia shift from a quiescent state in control retinas to an activated or intermediate state in diabetic

retinas, and BNN27 rearranges this shift returning the activated microglia to a control or resting state.

The effect of BNN27 on GFAP and Iba-1 expression suggest its role as an anti-inflammatory agent. These findings are further strengthened by data in our lab showing the ability of BNN27 to reduce the levels of the pro-inflammatory components TNF-α and IL-1β in the diabetic retina and increase the production of the anti-inflammatory cytokines IL-4 and IL-10 (Ibán-Arias *et al*., 2018). Moreover, these events were in accordance to our expectations given that DHEA, the mother compound of BNN27, was also shown to diminish pro-inflammatory mediator release in a mouse model of lipopolysaccharide-induced brain inflammation (Alexaki *et al*., 2017). Pro-inflammatory interleukins such as IL-1β or TNF-α, among others, have also been detected in the vitreous (Abu el Asrar *et al*., 1992; Yuuki *et al*., 2001; Demircan *et al*., 2006) and aqueous (Wu et al., 2017) humor of patients with proliferative DR as well as in retinal lysates from STZ-induced diabetic rats (Carmo *et al*., 1999). Notably, the production of the anti-inflammatory cytokine IL-10 has been related to downregulation of VEGF expression in the vitreous of PDR patients (Mao and Yan, 2014). Similarly, IL-4 release, where appropriate, has been linked with an anti-angiogenesis function in synovial fibroblasts from patients with rheumatoid arthritis (Hong *et al*., 2007) in addition to inhibiting the expression of pro-inflammatory cytokines (Cilensek *et al*., 2011). As mentioned above, BNN27 treatment increases the IL-4 and IL-10 anti-inflammatory cytokine levels, thus contributing not only to blocking the prompt release of nocuous interleukins but also in an indirect fashion to hinder the diabetes-induced angiogenesis.

Moreover, the expression of inflammatory cytokines is regulated through the transcription factor NFκB, among others (Rahman, 2002). In a study by Lisa *et al*. (unpublished data) in our group, the upregulation of NFκB in diabetic rat retinas was observed, in agreement with Zheng *et al*. (2004). It has been shown that upregulation of NFκB is induced by TNF-α-increase during diabetes resulting in leukocyte adhesion to the

retinal capillaries (Joussen *et al*., 2009) and leading to BRB disruption (Behl *et al*., 2008). Jacot and Sherris (2011) manifested the importance of NFκB in the diabetes-induced neovascularization, inflammation and apoptosis. They also hypothesized that the inhibition of NFκB through the PI3K/Akt/mTOR pathway would have anti-inflammatory effect in DR. Nevertheless, since the involvement of this signaling pathway in the pathology of DR is rather controversial, as mentioned above, further investigation is imperative to assess the role of the PI3K/Akt pathway in the regulation of the inflammatory mechanisms as an adaptive response to DR progression.

The specific reactive phenotype change of glial Müller cells may be due to the increase in $p75^{NTR}$ expression during DR progression, since $p75^{NTR}$ is located in these cells. Certainly, the upregulation of this neurotrophin receptor is also supported by the diabetes-induced increase of proNGF levels. Within the concomitant mechanisms triggered by the exposure of these cells to hyperglycemia is the aberrant production of inflammatory mediators such as IL-1β and TNF-α (Gerhardinger *et al*., 2005; Walker and Steinle, 2007). Moreover, investigations utilizing the glaucoma and the *in vivo* optic nerve transection models to study RGC degeneration, reported the implication of increased TNF-α and TNF-α_2-microglobulin levels in the NGF-activation of $p75^{NTR}$ and glial upregulation that led to RGC toxicity (Bai *et al*., 2010). This detrimental environment in the diabetic milieu is also supported by the microglial-induced release of noxious cytokines as seen in diabetic rat (Krady *et al*., 2005) and human retinas (Zeng *et al*., 2008). Additionally, the increase of IL-1β in rat Müller cells induced apoptotic processes by the activation of Caspase-3 and Caspase-6 and, as a consequence, the disruption of the retinal-neuron homeostasis and viability (Yego *et al*., 2009). The overall cascade of damaging events in the diabetic retina has been shown to be attenuated when $p75^{NTR}$ is blocked in Müller cells as shown by the prevention of photoreceptor apoptosis in a model of light-induced retinal degeneration (Harada *et al*., 2006). Similarly, Lebrun-Julien *et al*. (2009) indicated that the inhibition of $p75^{NTR}$ after RGC

axotomy showed an important increase in RGC survival. Our data support these studies and demonstrate the importance of Müller glial cells in the $p75^{NTR}$-induced inflammation features developed during the progression of DR. Since glial cells operate to provide physical support and nourishment to neurons, in order to maintain the functional homeostasis, it is valid to suggest that the deterioration of Müller retinal cells will consequently affect the state of neurons. Therefore inflammation may precede neurodegeneration in DR.

Given that the potential neuroprotective and anti-inflammatory effect of BNN27 in the diabetic retina was demonstrated, we were also interested in examining that BNN27 actually reaches the retina. In the present study, BNN27 (10mg/kg) was administered (i.p.) to control rats daily for 7 days and 24h after the last treatment, retinas were analyzed by HPLC-MS/MS. The dose of 10mg/kg was chosen as the optimum dose since it was the minimum dose at which we saw TrkAR activation. BNN27 levels detected were in the order of 227.3±95.7nM. Basal levels of NGF found in rat retina were reported to be 147±52 pg/g tissue (Lambiase *et al*., 2005). The fact that BNN27 was shown to pass the BRB and reached the retina corroborates our findings regarding the BNN27 TrkAR-mediated neuroprotection of the diabetic retina. These data are in agreement with Bennett *et al*. (2016) who reported that BNN27 is able to pass the BBB, in contrast with NGF, which cannot crosses the BBB due to its polypeptidic nature.

BNN27 exposure of TrkAR transfected CHO cells at the optimum dose of 1nM for 15 min was shown to phosphorylate the Y490 of the TrkAR reaching a plateau at the concentration of 100nM (Pediaditakis *et al*., 2016a). Furthermore, TrkAR activation remained up to 6 h in CHO cells and 60 min in PC12 cells, in contrast to NGF (100ng/ml) activity that ended after 60 and 15 min, respectively.

While it is difficult to compare *in vivo* and *in vitro* data regarding saturation doses and drug function based on all the above mentioned BNN27 studies, we can suggest that

BNN27 in our experimental *in vivo* model of DR reaches a plateau and can exert a beneficial effect on the retinal machinery to preserve the tissue against DR progression.

The findings of the present study suggest that BNN27 exhibits a pharmacological profile of a potential therapeutic for the treatment of DR. Therefore, what are the reasons to suggest that this microneurotrophin should be employed as therapy to overcome the diabetes-induced retinal damage in lieu of either DHEA or NGF? Several investigations have addressed the potential of these molecules in the treatment of retinal impairment in different models of retinal degeneration. In this regard, it has been reported that NGF topically administered in eyes (200µg/ml, 4drops/day, x7 weeks) resulted in RGCs prevention loss in an animal model of glaucoma and improved visual function in three patients with advanced glaucoma (200µg/ml, 50µl/drop, 4drops/day, x3 months) (Lambiase *et al*., 2009). DHEA was also shown to protect rat retinal neurons from AMPA excitotoxicity, mimicking the actions of NGF via a NGF TrkAR mechanism (Kokona *et al*., 2012). Notwithstanding the foregoing studies that endorse the use of NGF and DHEA as therapeutic agents in neurodegenerative retinal diseases, these molecules show important molecular limitations. Regarding NGF, it is a large peptidic molecule (26.5kDa) not able to cross the blood-brain barrier (BBB) and susceptible to proteolysis (Yi *et al*., 2014). DHEA is also restricted due to its metabolism to androgens and estrogens. Its long-term use in humans runs the risk of increasing hormone-dependent tumours, particularly in genetically predisposed patients (Fourkala *et al*., 2012). BNN27 was synthesized to overcome the limitations of NGF and DHEA.

This small lipophilic molecule is able to cross the BRB in order to protect the retina from the STZ-induced diabetic insult acting as a microneurotrophin. The neurotrophic prosurvival actions of BNN27 have also been shown in other experimental models of neurodegeneration, such as: the G93A SOD1 mouse *in vivo* model (Glajch *et al*., 2016) as well as *in vitro* models of ALS, utilizing human stem cell-derived (iPSC) motor neurons

(Bennett *et al*., 2016) or co-cultures of mouse motor neurons with astrocytes derived from ALS patients (Glajch *et al*., 2016); the scopolamine-induced recognition memory deficit model of Alzheimer's disease (Pitsikas and Gravanis, 2017); the cuprizone mouse model of demyelination (Bonetto *et al*., 2017) as model of MS.

5. CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

In the present investigation using the STZ-model of DR, we have demonstrated the ability of BNN27 to exert its neuroprotective properties when administered by two different routes, namely intraperitoneally and by eye drops. The utilization of eye drops was chosen as a non-invasive means of administration that may avoid or minimize the stress of drug administration improving the lab animal welfare and to adopt a better clinical strategy for prospective patients. Furthermore, BNN27's actions in the diabetic retina are clearly independent of any improvement of hyperglycemia. On the contrary and most importantly, BNN27 is able to provide a beneficial effect despite sustained high glucose levels in diabetic rats. BNN27 acts as an NGF agonist and restores the STZ-induced imbalance to the diabetic rat retina, performing its neuroprotectant role on bNOS and TH-expressing amacrine retinal neurons, ganglion cell axons and glial cells. Moreover, these data strongly suggest that BNN27 mediates its pharmacological prosurvival actions in the diabetic rat retina by activating specifically the NGF TrkAR and $p75^{NTR}$, thus being more advantageous compared to DHEA that binds to all Trk receptors (Pediaditakis *et al.*, 2015). The fact that BNN27 can exert its actions in retinal neurons and glia that are highly affected by the diabetes-induced damage makes it a possible "lead" molecule for the treatment of neurodegenerative and inflammatory diseases such as Diabetic Retinopathy.

FUTURE DIRECTIONS

Despite the large amount of information compiled in this thesis, there are further studies that need to be performed in order to complement the present preclinical data and provide more information needed to promote BNN27 towards clinical trials and to understand further the role of BNN27 in the neurobiology of DR.

1. *Evaluation of the retinal functional changes by ERG.* The examination of the diabetes-induced functional changes in retinal neurons would be of great importance since it would provide essential information regarding the functional state of the retina.
2. *Determination of BNN27 levels in diabetic rat retinas when administered either i.p. or with eye drops by HPLC/MS-MS analysis*. The measurement of BNN27 levels in diabetic rat retinas would demonstrate whether BNN27 is accumulated in larger amounts given that we expect these retinas to undergo diabetes-induced BRB disruption and therefore, BNN27 would reach easier the retina.
3. *Further pharmacokinetic analysis of BNN27 in the diabetic retina.* Identification of the metabolites of BNN27 when administered i.p. or with eye drops and their possible side toxicity.
4. *Investigation of BNN27 actions on MMP-7/ TIMP-1 and correlation with proNGF/NGF balance.*
5. *Further characterization of the neuron-vascular interplay in the diabetic retina*

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7. PUBLISHED ARTICLES

The Synthetic Microneurotrophin BNN27 Affects Retinal Function in Rats With Streptozotocin-Induced Diabetes

Ruth Ibán-Arias,[1] Silvia Lisa,[1] Niki Mastrodimou,[1] Despina Kokona,[1] Emmanuil Koulakis,[1] Panagiota Iordanidou,[1] Antonis Kouvarakis,[2] Myrto Fothiadaki,[1] Sofia Papadogkonaki,[1] Aggeliki Sotiriou,[1] Haralambos E. Katerinopoulos,[3] Achille Gravanis,[1,4] Ioannis Charalampopoulos,[1] and Kyriaki Thermos[1]

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BNN27, a C17-spiroepoxy derivative of DHEA, was shown to have antiapoptotic properties via mechanisms involving the nerve growth factor receptors (tropomyosin-related kinase A [TrkA]/neurotrophin receptor p75 [$p75^{NTR}$]). In this study, we examined the effects of BNN27 on neural/glial cell function, apoptosis, and inflammation in the experimental rat streptozotocin (STZ) model of diabetic retinopathy (DR). The ability of BNN27 to activate the TrkA receptor and regulate $p75^{NTR}$ expression was investigated. BNN27 (2,10, and 50 mg/kg i.p. for 7 days) administration 4 weeks post–STZ injection (paradigm A) reversed the diabetes-induced glial activation and loss of function of amacrine cells (brain nitric oxide synthetase/tyrosine hydroxylase expression) and ganglion cell axons via a TrkA receptor (TrkAR)-dependent mechanism. BNN27 activated/phosphorylated the $TrkA^{Y490}$ residue in the absence but not the presence of TrkAR inhibitor and abolished the diabetes-induced increase in $p75^{NTR}$ expression. However, it had no effect on retinal cell death ($TUNEL^+$ cells). A similar result was observed when BNN27 (10 mg/kg i.p.) was administered at the onset of diabetes, every other day for 4 weeks (paradigm B). However, BNN27 decreased the activation of caspase-3 in both paradigms. Finally, BNN27 reduced the proinflammatory (TNFα and IL-1β) and increased the anti-inflammatory (IL-10 and IL-4) cytokine levels. These findings suggest that BNN27 has the pharmacological profile of a therapeutic for DR, since it targets both the neurodegenerative and inflammatory components of the disease.

Diabetic retinopathy (DR) is a serious complication of diabetes that leads to loss of visual acuity and blindness. It has been estimated that by 2030 the number of people with DR globally will rise to 191 million from 127 million in 2010 (1). The healthy retina provides a homeostatic equilibrium between all its cellular components, namely, neurons, vascular cells, and glial cells (Müller cells, astrocytes, and microglia). An imbalance of this homeostatic equilibrium may play a major role in the pathophysiology of DR (2).

Proliferative DR is defined as a microvascular disease characterized by neovascularization. An earlier study (3) reported the increase of apoptotic cells in retinas of rats 1 month after treatment with streptozotocin (STZ). TUNEL-positive ($TUNEL^+$) cells were detected in the ganglion cell layer (GCL) but not in vascular endothelial cells (3). These findings suggested for the first time that DR has a neurodegenerative component, since retinal neural cell death occurred in diabetes prior to the vascular insult. Subsequent studies contributed to this principle (4–6). Also, electroretinograms in patients with diabetes and in diabetic rats showed abnormalities that preceded the vascular features associated with DR (7).

The imbalance between prosurvival neurotrophic factors and inflammatory components is believed to lead to apoptosis and proinflammatory responses (2,8). Indeed, proinflammatory markers were detected in the vitreous of patients with proliferative DR (9,10). Standard therapies for DR, such as photocoagulation and intraocular injection

[1]Department of Pharmacology, School of Medicine, University of Crete, Heraklion, Crete, Greece
[2]Laboratory of Environmental Chemical Processes, Department of Chemistry, University of Crete, Heraklion, Crete, Greece
[3]Organic Chemistry, Department of Chemistry, University of Crete, Heraklion, Crete, Greece
[4]Institute of Molecular Biology and Biotechnology, Foundation for Research & Technology-Hellas, University of Crete, Crete, Greece
Corresponding author: Kyriaki Thermos, thermos@uoc.gr.
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S.L. is currently affiliated with the Department of Cell Biology and Pathology, Instituto de Neurociencias de Castilla y León, University of Salamanca, and Institute of Biomedical Research, Salamanca, Spain. D.K. is currently affiliated with the Department of Ophthalmology, Inselspital, Bern, Switzerland.

of anti-VEGF agents, are efficacious but target only the neovascular component of the disease. New research strategies are essential in order to identify new therapeutic targets to combat the neurodegenerative and proinflammatory components of DR (11).

The neurosteroid dehydroepiandrosterone (DHEA) (12) has provided neuroprotection in different central nervous system paradigms, including excitotoxicity, ischemia, and ischemia-reperfusion injury (13–15), and against serum deprivation in PC12 cells (16). Most recently, DHEA was shown to mimic the actions of the prosurvival neurotrophin nerve growth factor (NGF) in protecting neuronal cell types from apoptosis (17).

NGF binds with high affinity to the prosurvival tropomyosin-related kinase (Trk)A receptor (TrkAR) and with a lower affinity to neurotrophin receptor p75 [$p75^{NTR}$], member of the TNF receptor superfamily. DHEA was shown to activate both TrkAR and $p75^{NTR}$ via which it mediates its neurotrophic effects (17). DHEA also binds to the other two mammalian Trk receptors (TrkB and TrkC), as well as their ancestral isoforms, with high affinity (18).

Neurosteroids are expressed in the retina (19), as are NGF and its receptors, TrkAR and $p75^{NTR}$ (20). NGF prevented ganglion cell death when administered to diabetic rats (21). More recent studies showed that topical ophthalmic administration of NGF protected retinal ganglion cells (RGCs) in an animal model of glaucoma, in patients with glaucoma (22), and in the STZ model of DR (23). DHEA was also shown to protect rat retinal neurons from AMPA excitotoxicity, mimicking the actions of NGF via an NGF TrkAR mechanism (24).

These studies support the use of NGF and DHEA as a therapeutic in neurodegenerative retinal disease. However, NGF is a large peptidic molecule (26.5 kDa) not able to cross the blood-brain barrier (BBB) and susceptible to proteolysis (25). DHEA is also restricted owing to its metabolism to androgens and estrogens. Its long-term use in humans runs the risk of increasing hormone-dependent tumors, particularly in genetically predisposed patients (26).

Recently, BNN27, a novel C17-spiroepoxide derivative of DHEA, that has no affinity for the classic steroid receptors, was synthesized. This agent is a small, highly lipophilic neurosteroidal molecule that crosses the BBB (27,28). BNN27 was recently reported to activate TrkAR signaling in neuronal (sympathetic and sensory primary neurons [PC12 cell line]) and microglial (BV2 mouse cell line) cells and to protect TrkA-positive and NGF-dependent sympathetic and sensory neurons from apoptosis. It had no effect on TrkB or TrkC receptors (29). We recently reported that BNN27 reversed the serum deprivation–induced apoptosis of cerebellar granule neurons (that do not express TrkA) by activating $p75^{NTR}$ (30). These data strongly suggest that BNN27 mediates its pharmacological prosurvival actions by activating specifically the NGF TrkAR and $p75^{NTR}$, thus being more advantageous compared with DHEA, which binds to all Trk receptors (18).

We hypothesized that smaller-than-NGF, highly lipophilic molecules that cross the BBB/blood retina barrier (BRB), such as BNN27, that mimic the neurotrophic prosurvival properties of NGF, may be more efficacious as therapeutics in DR. The aim of this study was to investigate how BNN27 administration affects the diabetic retina. Our findings support that BNN27 reverses the diabetes-induced retinal damage by activating the NGF TrkA receptor, reducing $p75^{NTR}$, cleavage of caspase-3, glial activation, and anti-inflammatory cytokine levels.

RESEARCH DESIGN AND METHODS

Animals

Animal experiments followed the *Guide for the Care and Use of Laboratory Animals*, 8th edition (2011), and were in compliance with Greek national legislation (Animal Act, P.D. 160/91). Both male and female Sprague-Dawley rats (180–300 g) were used in the current study. The animals were maintained on a 12-h light-dark cycle at 22–25°C. Food and water were available ad libitum.

Induction of Diabetes

Diabetes was induced by a single dose of STZ (70 mg/kg i.p.; Sigma-Aldrich, Tanfkirchen, Germany) dissolved in sodium citrate (0.1 mol/L) buffer (diabetic group) after a fasting period of 8–12 h. Animals with blood glucose levels >350 mg/dL, at 72 h post–STZ injection, were considered diabetic (31). Both male and female Sprague-Dawley rats were used in the current study. Higher morbidity and mortality were observed in male STZ-administered rats. Specifically, 70% of the STZ-injected animals died during the first week post-injection. Of these animals, 76.2% were male and the rest (23.8%) were female. Male pancreatic islet β-cells are more prone than female to STZ-induced cytotoxicity (32). Therefore, male rats may die as a result of islet β-cell necrosis. Lower mortality rates were observed at later time points after STZ injection. Glucose levels were >400 mg/dL in all experimental rats 7 days after the STZ injection and >600 mg/dL the following weeks and until the end of the experiments. No differences were observed in glucose levels between male and female rats. Body mass was monitored every week after the STZ injection. Loss of 11.47 ± 1.47% (mean ± SEM) of body mass was observed in male rats, while female rats lost 1.75 ± 4.0% of body mass at day 7 after the STZ treatment. Small fluctuations of body mass were observed the following weeks in both male and female rats. The sex difference (in mortality and body mass) noted in the present study and in other studies (33) has not been explicitly defined; yet, estrogens are known to have beneficial effects on hyperglycemia and islet β-cell functions in the STZ-induced diabetic rat and mouse models (34,35).

Neuroprotection Studies

Three experimental groups were used, namely, a control group, a diabetic group, and a diabetic treated group in two different paradigms: in *paradigm A*, BNN27 (2, 10, and 50 mg/kg i.p.) was administered daily for 7 days at 4 weeks post–STZ injection, and in *paradigm B*, BNN27 (10 mg/kg i.p.) was administered 48 h after STZ injection every second day for 4 weeks. The intraperitoneal route was selected to

assure active levels of the above agent in the retina. BNN27 was dissolved in absolute ethanol (10% v/v in water for injection). Appropriate vehicles were administered to control and diabetic nontreated animals.

For examination of the involvement of the NGF TrkAR in the actions of BNN27, a specific TrkAR inhibitor (10^{-3} mol/L, category no. 648450; Calbiochem) was administered intravitreally (flow rate of 1 μL/min for 5 min) at days 1, 4, and 6 in conjunction with BNN27 administration (10 mg/kg i.p.) (24). The TrkAR inhibitor concentration was chosen taking into account the calculated molarity of the BNN27 dose of 10 mg/kg i.p. DMSO (10% v/v in PBS [50 mmol/L K_2HPO_4/NaH_2PO_4 and 0.9% NaCl, pH 7.4]) was used as the solvent for the inhibitor and as vehicle in the other experimental groups. Both eyes received the same treatment.

Glucose levels were monitored in the diabetic treated animals to ascertain the putative effect of BNN27 (10 and 50 mg/kg i.p. for 7 days [paradigm A]). The results obtained did not suggest any BNN27 effect on glucose levels (diabetic, mean ± SEM 573 ± 14 [n = 23]; diabetic+BNN27 [10 mg/kg], 575 ± 13 [n = 23]; and diabetic+BNN27 [50 mg/kg], 564 ± 29 [n = 10]). No statistical significant differences were observed between the diabetic treated and nontreated animals or between treated groups ($P > 0.05$). Control animals did not show any significant alteration in glucose levels during the 5-week period.

Tissue Preparation

Animals were euthanized with ether inhalation 24 after the last treatment, their eyes were removed, and retinas were obtained and prepared for immunohistochemical, Western blot, ELISA, and high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) studies.

Immunohistochemical Studies

The eyes were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 1 h at 4°C. After fixation and cryoprotection, tissues were embedded in optimal cutting temperature compound (Prolabo, Leuven, Belgium) and frozen in isopentane for 1 min. The eyecups were sectioned vertically, and the initial 1.5 mm tissue was discarded. Serial 10-μm-thick sections were placed onto six slides, and nine sections were collected on every slide. This way, every slide contained a representative part of retinal tissue, including the optic nerve head (24).

Immunohistochemical studies using antibodies raised against retinal cell markers for ganglion cell axons (nerve fiber layer [NFL]) and brain nitric oxide synthetase (bNOS)- and tyrosine hydroxylase (TH)-expressing amacrine cells were performed to assess the effect of the STZ on retinal cell viability in rats 1–5 weeks after its administration (24). Moreover, antibodies raised against glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule-1 (Iba-1), cleaved caspase-3, and $p75^{NTR}$ were also used (Supplementary Table 1). Cryostat sections were treated overnight with the appropriate primary antibody in 0.1 mol/L tris-buffered saline containing 0.3% Triton-X-100 and 0.5% normal goat serum and subsequently with a fluorescence secondary antibody for 1–2 h. Slides were cover slipped using EverBrite Mounting Medium with DAPI (Biotium).

TUNEL Assay

The terminal deoxynucleotidyl transferase (TDT)-mediated TUNEL assay (Roche, Grenzach-Wyhlen, Germany) was employed to assess retinal cell death in control and diabetic retinas 4 and 5 weeks post–STZ administration, as well as at the onset of diabetes. Retinal tissues were incubated with the TUNEL reaction mixture containing Label and Enzyme Solutions according to the directions of the manufacturer. Slides were cover slipped using EverBrite Mounting Medium with DAPI (Biotium). Colocalization studies (bNOS immunoreactivity [bNOS-IR] and TUNEL) were performed in order to examine the viability of bNOS-immunoreactive cells in retinas of diabetic animals and diabetic animals treated with BNN27 (10 mg/kg).

Western Blot and Immunoprecipitation

Retinal lysates were prepared in Tris-HCl buffer (50 mmol/L, pH 7.5) containing NaCl (150 mmol/L), NP40 (1%), sodium deoxycholate (0.1%), and phenylmethylsulfonyl fluoride (0.1 mmol/L) and protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). For immunoprecipitation, retinas were suspended in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, and protease inhibitor cocktail [pH 7.5]). Lysates were centrifuged (10,000g for 20 min) and the supernatants incubated with TrkA monoclonal antibody (Millipore) (1:60 dilution) overnight at 4°C. Protein G PLUS-Agarose (Santa Cruz Biotechnology) beads were added (1:8 dilution) and incubated for 2 h at 4°C. Beads were collected by centrifugation (2,000g for 2 min) and pellets were washed in lysis buffer and resuspended in SDS sample buffer. Precipitates and lysates were analyzed by SDS-PAGE (12.5% and 7.5% acrylamide, respectively) and incubated with specific antibodies (Supplementary Table 1). The GAPDH antibody was used to normalize protein content in lysates. Two to three different retinas for each group were run on each gel. The proteins were visualized using an ECL Western blotting kit (Thermo Scientific, Rockford, IL), and quantitative densitometry of the protein bands was performed using Image Lab, version 5.0, software (Bio-Rad) (36).

ELISA Assay

ELISA kits (Abcam) were used to evaluate the levels of TNFα, IL-1β, IL-10, and IL-4 in retinas according to the manufacturer's instructions. The samples were analyzed in duplicates using an ELISA reader (450 nm, model 680; BioRad). Protein concentration was determined by NanoDrop 2000 (Thermo Fisher Scientific).

Determination of BNN27 Levels: HPLC-MS/MS Analysis

Rats were administered BNN27 (10 mg/kg) for 7 days. This dose was chosen because it was the lowest dose that led to the activation/phosphorylation of the TrkAR. To each retina, methanol (495 μL) and 5 μL d6-DHEA (2,500 ng/μL) were added, and the samples were homogenized

for 1.5 min on ice, sonicated for 30 s, and centrifuged at 13,500 rpm for 1 h at 4°C. The supernatant was stored at −20°C until HPLC-MS/MS analysis (37). Chromatographic separation was performed on a UniverSil UHS18 column (150 × 2.1 mm, 3 μm) with a gradient elution system consisting of methanol and water, both with 0.1% formic acid (total flow rate 200 μL/min). Tandem mass spectral analysis was performed with a Thermo Fisher Scientific TSQ triple quadrupole mass analyzer equipped with an electrospray ionization source operating in positive mode using selected reaction-monitoring detection. For BNN27, 315.3–297.1 ion pair was used for quantitation, and 315.3–255.2 was used for confirmation. d6-DHEA was used as the internal standard (selected reaction monitoring detection 295.3–277.2).

Quantification Studies

The total number of bNOS- and TH-IR somata was manually counted in three sections/retina. The density area starting from the GCL until the inner plexiform layer (IPL) for NFL and $p75^{NTR}$ and from GCL until the outer nuclear layer (ONL) for GFAP and the inner nuclear layer (INL) for cleaved caspase-3 was delineated in each image (2 images from 3 sections/retina) using the public domain ImageJ, version 1.43m, software. The mean gray value [integrated density (fluorescence density)/delineated area] of this region was calculated and expressed as a percentage of the mean gray value of the control or diabetic retina (100%). The number of Iba-1–positive cells was manually counted, with data normalized to the total counting area (from GCL to INL) and expressed as percentage of the control retinas (100%). $TUNEL^+$ cells and DAPI nuclei were counted from the ONL to GCL (total retinal thickness) and individual layers (ONL, INL, and GCL) using ImageJ 1.43m software as described above. Data were normalized and expressed as the number of $TUNEL^+$ cells per area (mm^2). The percentage of the ratio of TUNEL to DAPI cells was calculated in retinas of animals 4 weeks post–STZ injection. Each experiment was replicated a minimum of three times. Images were obtained using an Axioskop Plan-Neofluar (Carl Zeiss, Oberkochen, Germany) or HC PL Fluotar, Leica DMLB (×20 or ×40/0.75 lens), and Leica SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

Data are expressed as mean ± SEM or mean ± SD (scatter plots). Statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad, San Diego, CA), and differences between groups were evaluated by one-way ANOVA with Tukey post hoc analysis or unpaired *t* test (two tailed). Statistical significance was set at $P < 0.05$. The number of animals used in each group (*n* values) is shown in the relevant figures or in the legend.

RESULTS

Induction of Diabetic Retinopathy

Qualitative immuhistochemical studies were performed to assess the effect of the STZ administration on retinal cell viability in rats 1–4 weeks after STZ administration. No differences were observed between the control and diabetic retinas in the first 3 weeks in the staining pattern of bNOS-IR localized in amacrine cell somata in the INL, displaced amacrine cell somata in the GCL and amacrine cell processes in the IPL, TH-IR localized in amacrine cell somata in the INL, or NFL-IR localized in ganglion cell axons in the GCL and in the IPL (data not shown). However, 4 weeks post–STZ injection, a significant decrease in NFL-, bNOS-, and TH-IR was observed in the diabetic retina (Supplementary Fig. 1*A*). Quantitative data analysis of the images confirmed the immunohistochemical data (Supplementary Fig. 1*B–D*).

TUNEL analysis was performed to examine retinal cell death after 4 weeks of STZ injection (Supplementary Fig. 2*A*). Quantification of the $TUNEL^+$ cells per area (mm^2) in the total retinal thickness showed an approximate 10-fold increase (2.9 ± 0.6, $n = 6$, compared with the number of apoptotic retinal cells found in control retinas [0.3 ± 0.1, $n = 6$] [$P < 0.01$]) (Supplementary Fig. 2*B*). The TUNEL-to-DAPI ratios (Supplementary Fig. 2*C*) show the percent of retinal cell death in total retina and its layers (GCL, INL, and ONL), with the highest in the GCL.

Dose-Dependent Effect of BNN27 in the Diabetic Retina

BNN27 administered intraperitoneally 4 weeks post–STZ injection, daily for 7 days (paradigm A), reversed in a dose-dependent manner the diabetes-induced reduction in the NFL-, bNOS- and TH-IRs (Fig. 1*A*). Quantitative analysis of the immunohistochemical data gave the following results for the control and diabetic retinas: NFL-IR (mean gray area/image), control 25,837 ± 1,373 and diabetic 15,613 ± 2,077; bNOS-IR (cell somata/section), control 85 ± 2 and diabetic 40 ± 1; and TH-IR (cell somata/section), control 3.5 ± 0.3 and diabetic 1.4 ± 0.3. BNN27 reversed the diabetes-induced decrease in the expression of the three retinal markers at the doses of 10 and 50 mg/kg ($P < 0.001$, $P < 0.01$, and $P < 0.05$ compared with the nontreated diabetic retina and $P > 0.05$ compared with control) (Fig. 1*B–D*). No statistically significant difference was observed at the dose of 2 mg/kg ($P > 0.05$ compared with the diabetic nontreated retinas).

Involvement of TrkA Receptor in the Reversal of the Diabetic Actions by BNN27

The effect of TrkAR inhibitor (10^{-3} mol/L) on the BNN27-induced actions on NFL bNOS- and TH-IR and in the diabetic retina was examined (Fig. 2). Quantitative analysis of the immunohistochemical images (not shown) suggests that the intravitreal administration of TrkAR inhibitor reversed the BNN27 (10 mg/kg) actions ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively, for NFL-IR, bNOS-IR, and TH-IR, compared with diabetic retinas treated with BNN27 alone). The TrkAR inhibitor had no effect when administered alone ($P > 0.05$ compared with the diabetic retinas). These results suggest that the NGF TrkAR mediates the restoration of the phenotype of bNOS-, TH-, and NFL-positive cells in the diabetic retina.

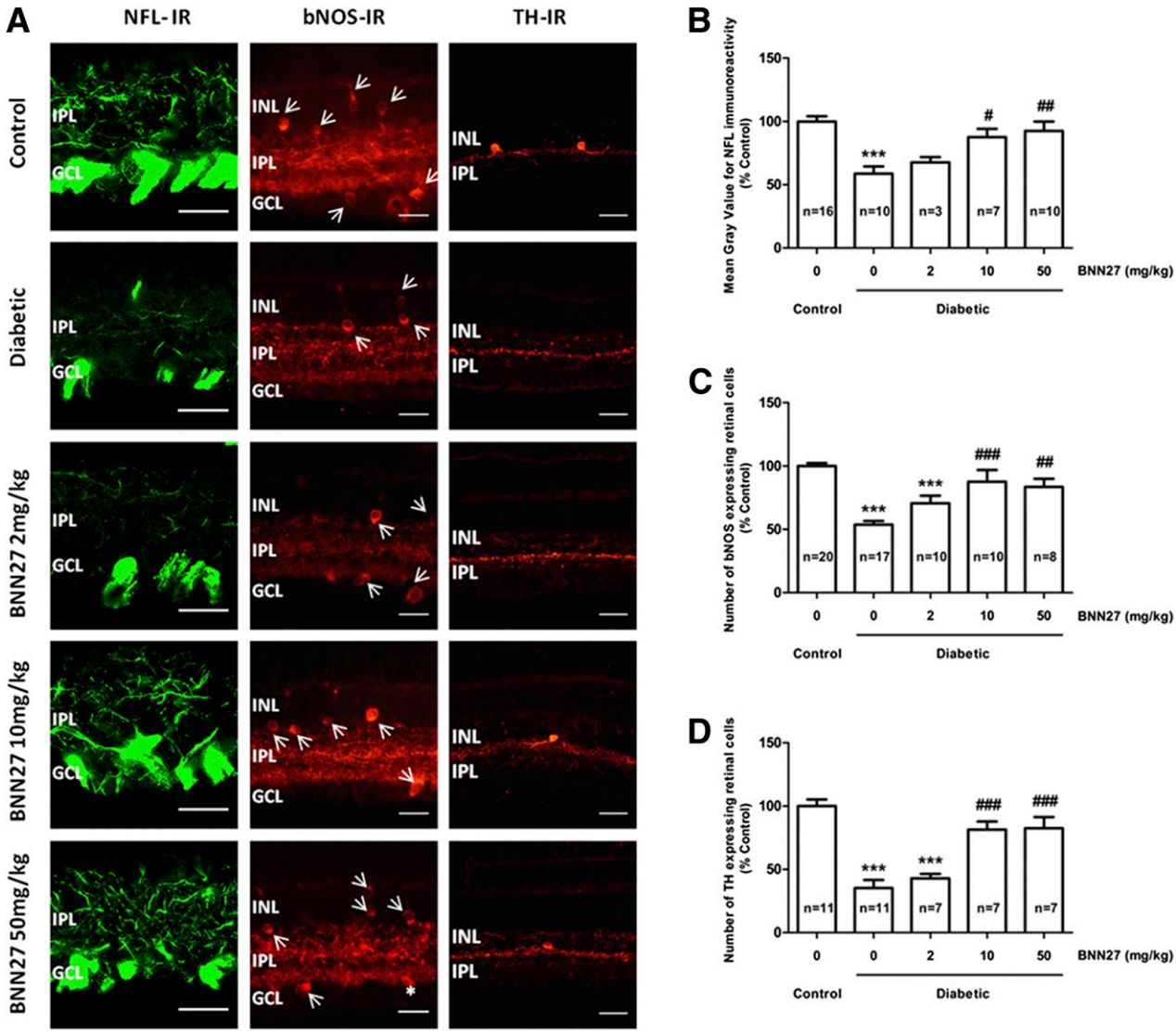

Figure 1—Dose-dependent effect of BNN27 on retinal neurons in the STZ model of DR. *A*: Representative images of NFL-, bNOS-, and TH-IR in control, diabetic, and diabetic+BNN27 (2, 10, and 50 mg/kg i.p.) rat retinas 5 weeks after STZ injection (* depicts artifact, and arrows depict bNOS-expressing cell bodies). Magnification ×40 for NFL and bNOS and ×20 for TH. Scale bar = 20 μm. Quantification of the immunohistochemical images of NFL-IR (*B*), bNOS-IR (*C*), and TH-IR (*D*) in control, diabetic, and diabetic+BNN27 (2, 10, and 50 mg/kg i.p. for 7 days) rat retinas (***$P < 0.001$ compared with control; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with diabetic).

BNN27 (10 and 50 mg/kg) increased TrkAR phosphorylation on Y^{490} residue to similar levels ($P < 0.05$ compared with the diabetic nontreated retinas, with no statistically significant difference between the two doses [$P > 0.05$]). No statistically significant effect was observed at the dose of 2 mg/kg ($P > 0.05$ compared with the diabetic nontreated retinas) (Fig. 3*A*). The phosphorylated-to-total TrkA ratio in retinal samples showed no statistically significant difference between control and diabetic nontreated animals (0.18 ± 0.02 and 0.20 ± 0.03 phosphorylated-to-total TrkA ratio, respectively, $P > 0.05$). The TrkAR inhibitor reversed the BNN27 -dependent (10 mg/kg) increase in phosphorylation of $TrkA^{Y490}$ ($P < 0.001$ compared with the diabetic treated retina and $P < 0.001$ compared with the diabetic retina) (Fig. 3*B*).

Effect of BNN27 on $p75^{NTR}$ Expression

Representative images from immunohistochemical studies depict an increase in $p75^{NTR}$ expression in diabetic retinas (Fig. 4*A*). Quantitative analysis of the images show that BNN27 (10 and 50 mg/kg i.p.) attenuated the diabetes-induced increase in $p75^{NTR}$-IR ($P < 0.001$ compared with control and $P < 0.01$ compared with the diabetic nontreated retina) (Fig. 4*B*). $p75^{NTR}$ expression was increased in the diabetic retina ($P < 0.001$ compared with control), and BNN27 (10 mg/kg i.p.) led to its reversal ($P < 0.001$) (Fig. 4*C*).

TUNEL Staining

$TUNEL^+$ cells were observed in the 5-week diabetic retinas (paradigm A). With the use of confocal microscopy, we

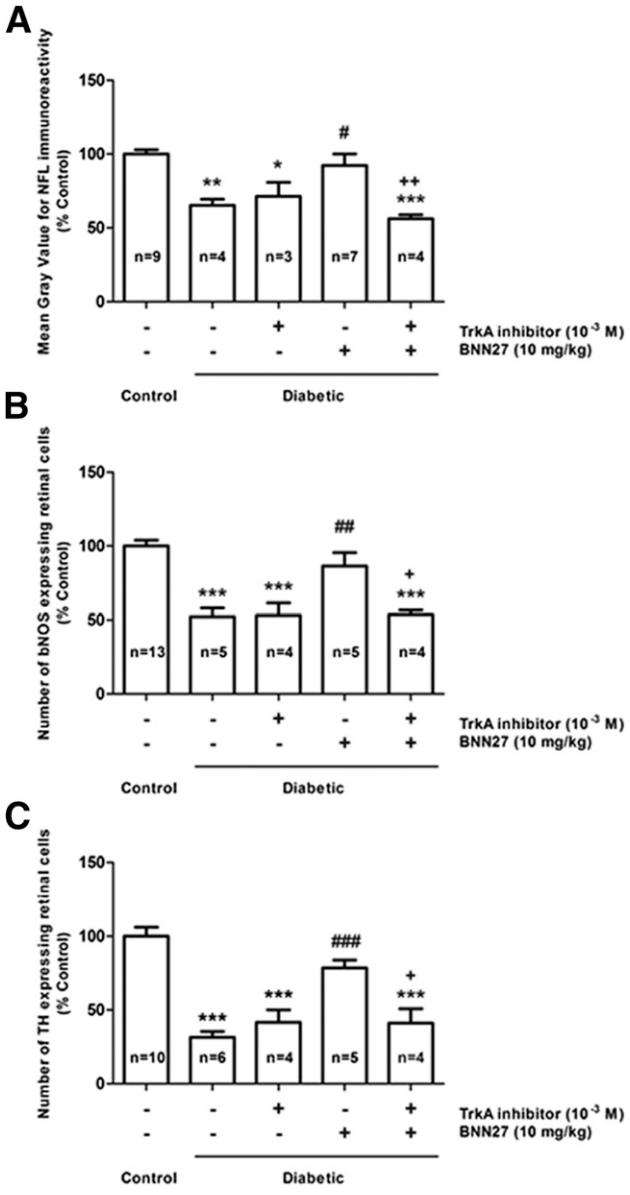

Figure 2—Effect of a TrkA inhibitor on BNN27 actions in rat retinas. The TrkAR inhibitor reversed the BNN27 (10 mg/kg, i.p. for 7 days) (paradigm A) effect on NFL-IR (*A*), bNOS-IR (*B*), and TH-IR (*C*) (++$P <$ 0.01, +$P < 0.05$, and +$P < 0.05$, respectively, compared with BNN27-treated diabetic rat retinas). No statistically significant differences were observed between diabetic nontreated retinas in the absence or presence of the TrkAR for all three markers used ($P >$ 0.05). Data are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control retinas; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with diabetic retinas.

observed TUNEL$^+$ cells (light and darker stain) colocalized with DAPI (Supplementary Fig. 3). We considered as TUNEL$^+$ cells all cells that were observed to be colocalized with DAPI. BNN27 had no effect on the total number of TUNEL$^+$ cells in the diabetic retina (Fig. 5*A*), but there was a statistical significant difference of the diabetic and diabetic+ BNN27 compared with control ($P < 0.05$). No colocalization of TUNEL or one of the retinal markers (bNOS) was observed (Fig. 5*B*).

TUNEL$^+$ cells were observed in retinas of animals that were administered BNN27 (10 mg/kg) every other day for 4 weeks (paradigm B) (Supplementary Fig. 4). In this paradigm, as in paradigm A, BNN27 was not able to reduce the number of TUNEL$^+$ cells per area (mm^2) ($P > 0.05$ compared with the diabetic retina). However, BNN27 did reverse the diabetes-induced attenuation of the bNOS and TH immunoreactive cells (data not shown).

Effect of BNN27 on Cleaved Caspase-3 Immunoreactivity

For assessment of the effect of BNN27 on caspase-3–induced apoptosis, immunohistochemical studies were performed in diabetic and diabetic+BNN27 retinas in both paradigms (Fig. 6*A* and *C*). Quantitative analysis of the images showed that BNN27 induced a statistically significant decrease (paradigm A, $P < 0.01$ compared with diabetic, and paradigm B, $P < 0.05$ compared with diabetic) in cleaved caspase-3–IR (Fig. 6*B* and *D*).

Effect of BNN27 on Macroglia and Microglia

The effect of BNN27 was also examined on macroglia and microglia markers for proteins, GFAP and Iba-1, respectively, known to play an important role in inflammation. Diabetes induced an increase in macroglial activation (GFAP-IR, 149.0 ± 8.0 percent of mean gray area, compared with control retinas, 100.0 ± 4.0 percent of mean gray area; $P < 0.001$) (Fig. 7*A* and *B*), whereas BNN27 prevented glial activation (10 mg/kg, 111.0 ± 4.1 percent of mean gray area; 50 mg/kg, 109.5 ± 4.2 percent of mean gray area; $P < 0.001$ compared with diabetic nontreated retinas). BNN27 also reduced the number of Iba-1–positive cells per area (mm^2) (10 mg/kg, 67 ± 20; 50 mg/kg, 113 ± 34; $P < 0.001$ and $P < 0.01$, respectively, compared with diabetic nontreated retinas [255 ± 91], and $P < 0.001$ compared with control [100 ± 29]) (Supplementary Fig. 5).

Effect of BNN27 in the Levels of Pro- and Anti-Inflammatory Cytokines

Diabetes induced an increase in the levels of proinflammatory cytokines (TNFα and IL-1β). BNN27 (10 mg/kg i.p. [paradigm A]) reversed this increase in TNFα ($P < 0.001$ compared with the diabetic), but only the higher dose of 50 mg/kg reduced the diabetes-induced increase in IL-1β levels ($P < 0.05$). BNN27 also increased the levels of anti-inflammatory cytokines IL-10 and IL-4 ($P < 0.05$ compared with the levels observed in the diabetic retina) (Fig. 8 and Supplementary Table 2).

Quantification of BNN27 Levels in Rat Retina Using HPLC-MS/MS

HPLC-MS/MS analysis of retinal samples of control animals (nondiabetic) that received BNN27 (10 mg/kg i.p. for 7days) showed that BNN27 levels reaching the retina were in the order of 227 ± 95 nmol/L (n = 6).

DISCUSSION

In the current study, we identified a putative therapeutic for the treatment of DR, the neurosteroidal microneurotrophin

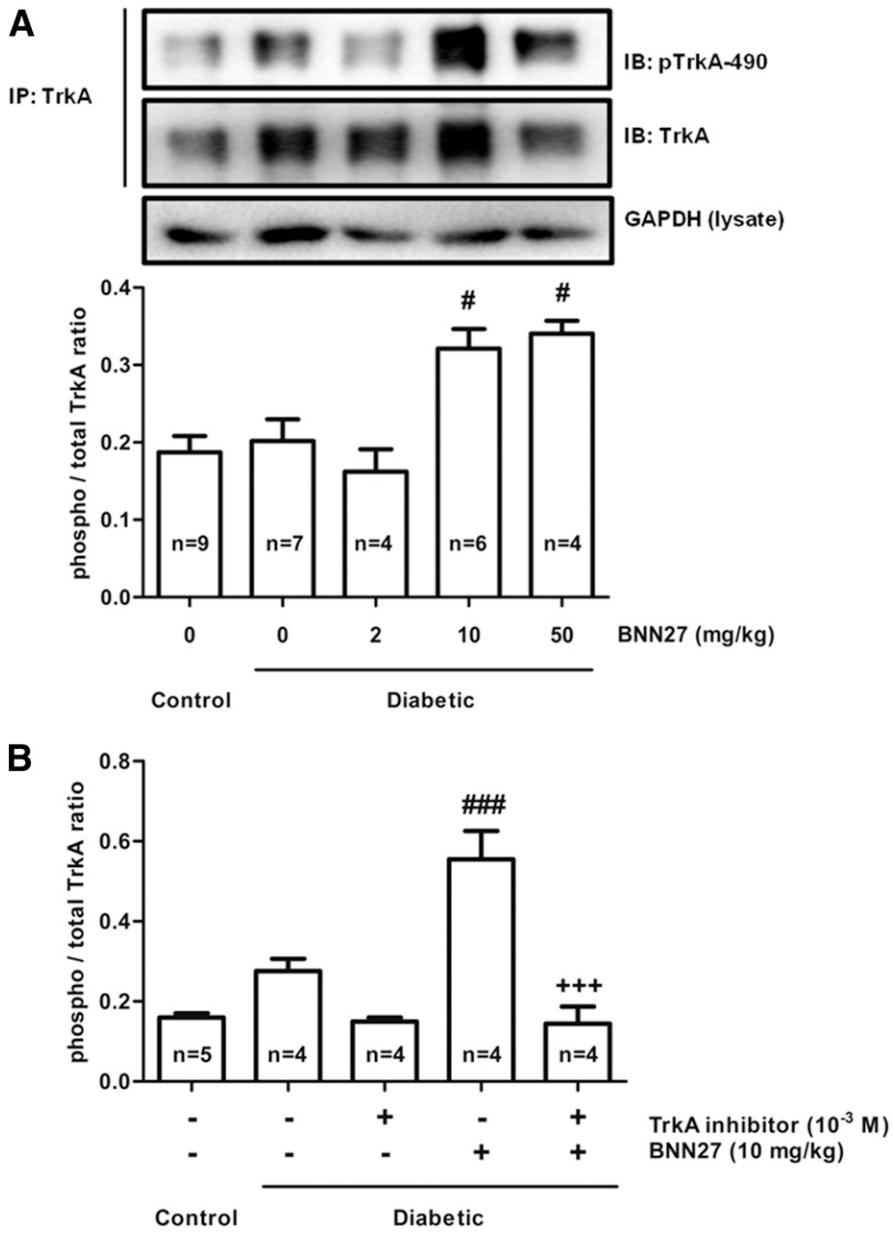

Figure 3—Representative images and quantification of BNN27 regulation of TrkAR: effect of TrkA inhibitor. *A*: BNN27 increased the phosphorylation of TrkAR in diabetic retinas in a dose-dependent manner (#$P < 0.05$ compared with retinas of diabetic nontreated animals). *B*: The TrkA inhibitor reversed the BNN27 (10 mg/kg) effect (+++$P < 0.001$ compared with diabetic treated animals and ###$P < 0.001$ compared with the diabetic nontreated animals). No statistical significant difference was observed in the phosphorylation of TrkA in diabetic nontreated animals in the presence of TrkA inhibitor ($P > 0.05$ compared with diabetic nontreated animals). IB, immunoblot; IP, immunoprecipitation; phospho, phosphorylated; pTrkA-^{490}, phosyphorylated TrkA^{Y490}.

BNN27. This novel spiroepoxy derivative of DHEA was shown to reverse the diabetes-induced retinal damage by activating the NGF TrkAR and reducing the expression of p75^{NTR} and glial activation.

Hyperglycemia was detected as early as 1 day post–STZ injection, with glucose levels remaining high for up to 5 weeks (>600 mg/dL). Its effect on retinal neurons was evident 4 weeks post–STZ injection, as shown by the reduction of retinal markers bNOS, TH, and NFL (Supplementary Fig. 1). A 10-fold increase in the number of TUNEL$^+$ cells per area (mm^2) was observed across the whole retina thickness, with significant increases in apoptotic cells in layers GCL, INL, and ONL (% TUNEL/DAPI) (Supplementary Fig. 2). These results are in agreement with other studies that used the experimental STZ model of DR (38,39).

BNN27 administered (intraperitoneally) for 7 days, 4 weeks post–STZ injection, reversed the diabetes-induced attenuation of NFL-, bNOS-, and TH-IR in a dose-dependent manner, suggesting a restorative role of this agent against

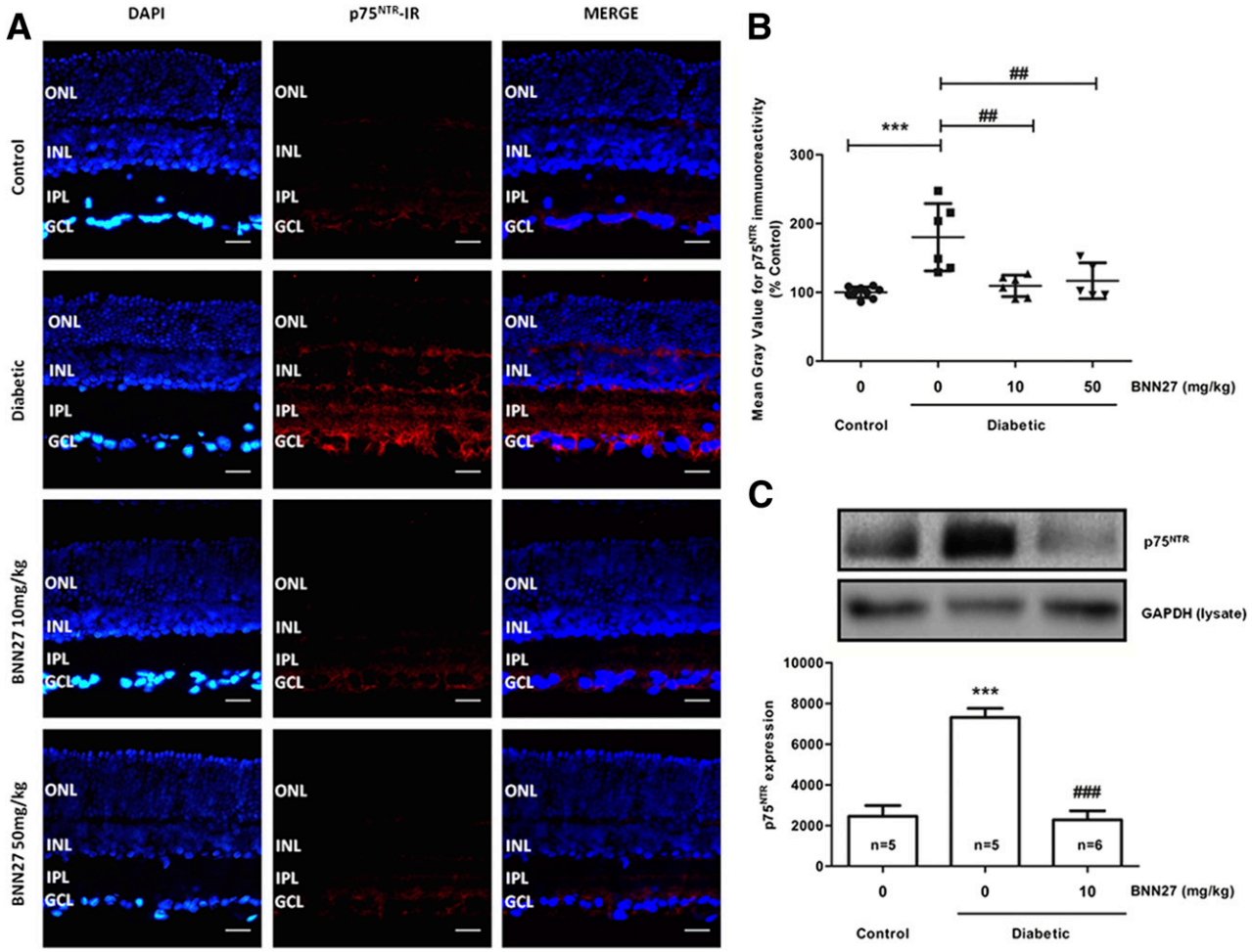

Figure 4—Dose-response effect of BNN27 on p75^{NTR} immunoreactivity and expression. *A*: Representative images of p75^{NTR} immunoreactivity rat retinal sections. Control (n = 9), diabetic (n = 6), and diabetic+BNN27 (10 [n = 6] and 50 [n = 5] mg/kg i.p. for 7 days [paradigm A]) (×40 magnification). Scale bar = 20 μm. Corresponding DAPI and merge are also shown. *B*: Quantification of the p75^{NTR}-IR images. BNN27 (10 and 50 mg/kg) attenuated the diabetes-induced increase in p75^{NTR}-IR (***$P < 0.001$ compared with control rat retinas; ##$P < 0.01$ compared with diabetic rat retinas). *C*: Representative blots and quantification of the expression of p75^{NTR} in control, diabetic, and diabetic+BNN27 (10 mg/kg) rat retinas. BNN27 reversed the diabetes-induced increase in p75^{NTR} expression (***$P < 0.001$ compared with control; ###$P < 0.001$ compared with diabetic nontreated rat retinas).

the diabetic insult. The TrkAR inhibitor reversed the BNN27-induced protective effects on bNOS- and TH-IR amacrine cells and NFL-IR ganglion cell axons but had no effect when administered alone (Fig. 2). Thus, BNN27 restores the phenotype of these retinal cells.

The TrkAR is located in ganglion cells, in the INL, and processes of the IPL (40). Therefore, the neuroprotection of ganglion cell axons by BNN27 may be due to the direct activation of the TrkAR located in these neurons. However, the identity of the amacrine cells in the INL expressing the TrkAR has not been reported. The activation of the TrkAR in the GCL may initiate a cascade of events and provide neuroprotection to amacrine cells via an indirect mechanism.

The aforementioned effects of BNN27 are mediated by the activation/phosphorylation of the TrkA^{Y490} in the diabetic retina (Fig. 3*A*). NGF induces the phosphorylation of several tyrosine residues located intracellularly, leading to the activation of downstream prosurvival signaling cascades. Among the three residues described to mediate TrkAR activation (490, 674/675, and 785) (41), only Y490 was phosphorylated in our paradigm. The expression of the TrkAR was not altered in the diabetic retinas, which is in agreement with the report of Ali et al. (42).

In a recent report, BNN27 was shown to bind and activate solely the TrkAR receptor and not TrkB or TrkC receptors and to protect TrkA-positive and NGF-dependent sympathetic and sensory neurons from apoptosis (29). These results complement the present data that show that the BNN27-induced phosphorylation of TrkAR is reversed by the intravitreal injection of the TrkAR inhibitor (Fig. 3*B*). Therefore, we conclude that the TrkAR is responsible for the BNN27-induced neuroprotection of the retina against the diabetic insult.

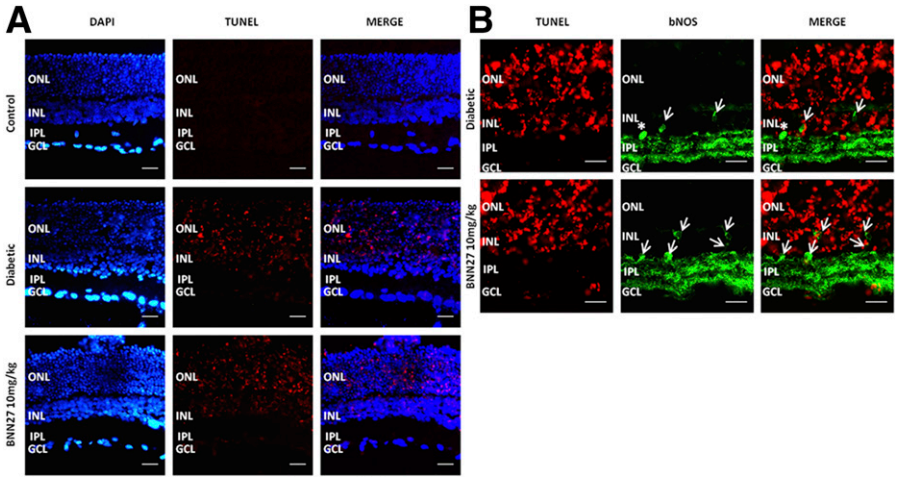

Figure 5—Evaluation of BNN27 effect on retinal cell death. *A*: Representative images of TUNEL staining, DAPI, and merge in control, diabetic, and diabetic+BNN27 (10 mg/kg i.p. for 7 days) rat retinas 5 weeks post–STZ administration (paradigm A) (×40 magnification). *B*: Representative images for bNOS/TUNEL coimmunostaining in diabetic and diabetic+BNN27 (10 mg/kg) rat retinas. No colocalization was observed (* depicts artifact, and arrows depict bNOS-expressing cell bodies). Scale bars = 20 μm.

The p75^{NTR} is also located on ganglion cells in the INL and Müller cells. Therefore, the attenuation of its expression by BNN27 may also affect its downstream signaling and provide neuroprotection to ganglion and amacrine cells. In the current study, our data support that BNN27 downregulates the expression of the p75^{NTR} death receptor. BNN27 significantly attenuated the diabetes-induced increase of p75^{NTR} protein expression in a dose-dependent

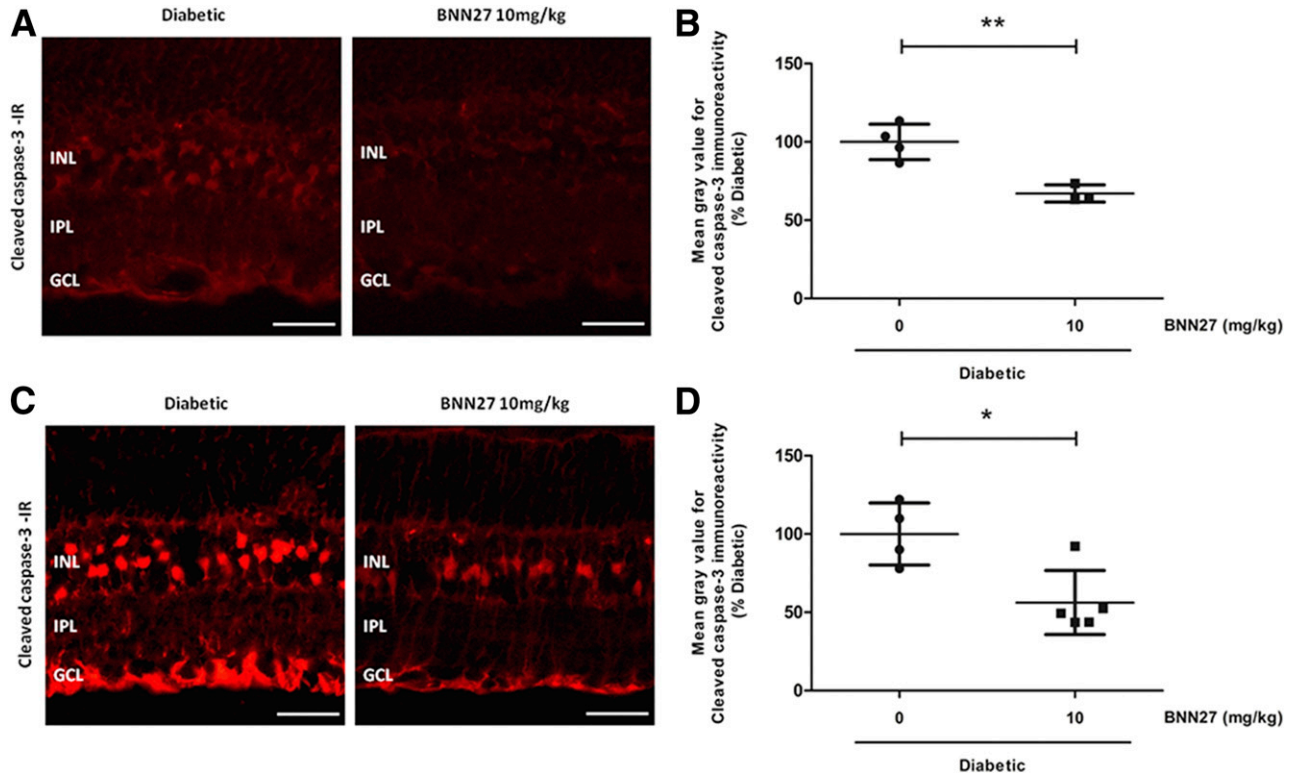

Figure 6—Evaluation of BNN27 effect on cleaved caspase-3–IR. Representative images (*A*) and quantification (*B*) for cleaved caspase-3 staining in diabetic (n = 4) and diabetic+BNN27 (10 mg/kg i.p. for 7 days) (n = 3) rat retinas 5 weeks post–STZ administration (paradigm A) (×40 magnification). Scale bar = 50 μm. Representative images (*C*) and quantification (*D*) for cleaved caspase-3 staining in diabetic (n = 4) and diabetic+BNN27 (10 mg/kg i.p.) (n = 5) rat retinas treated every second day for 4 weeks post–STZ administration (paradigm B). Paradigm A, $^{**}P < 0.01$ compared with diabetic, and paradigm B, $^{*}P < 0.05$ compared with diabetic.

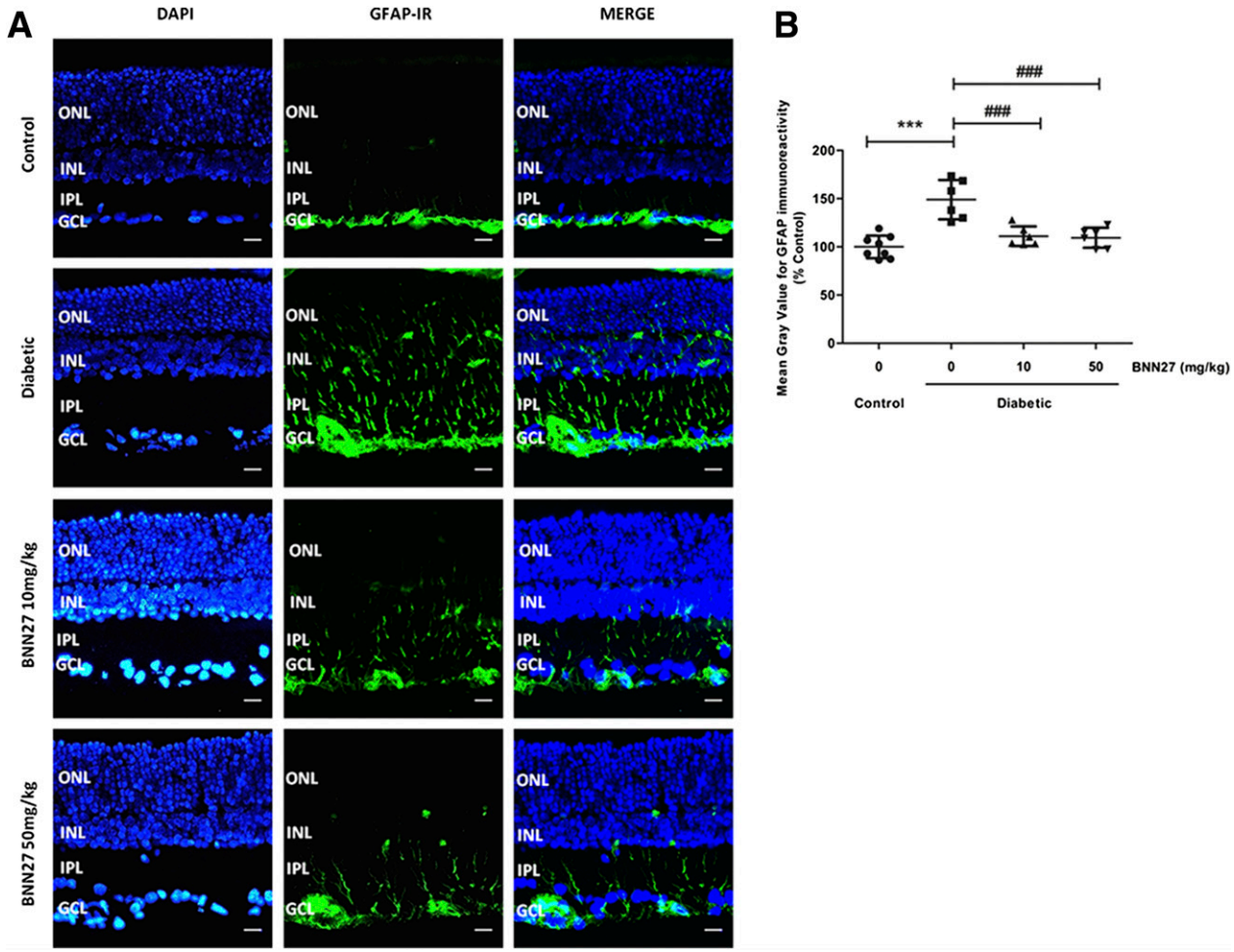

Figure 7—Dose-response effect of BNN27 on retinal glial modulation. *A*: Representative images of rat retina sections stained with anti-GFAP antibody in control, diabetic, and diabetic+BNN27 (10 and 50 mg/kg i.p. for 7 days) rat retinas (×20 magnification). Scale bar = 20 μm. Corresponding DAPI and merge are also shown. *B*: Quantification of the immunohistochemical images for GFAP-IR. An increase in GFAP-IR is observed in diabetic retinas ($n = 6$, $^{***}P < 0.001$ compared with control rat retinas, $n = 8$). BNN27 at 10 ($n = 6$) and 50 ($n = 6$) mg/kg reduces GFAP-IR ($^{###}P < 0.001$ compared with diabetic rat retinas).

manner. These findings suggest that the pharmacological actions of BNN27 involve both NGF receptors (TrkA and $p75^{NTR}$), in agreement with the report of Lazaridis et al. (17), who showed that the parent molecule DHEA decreased $p75^{NTR}$ levels in serum-deprived PC12 cells.

While the above-mentioned data suggest a neuroprotective role of BNN27 in the DR model, the TUNEL analysis performed on the tissues obtained from paradigm A showed that BNN27 had no effect on the total number of $TUNEL^+$ cells in the retina. In order to ascertain whether the amacrine cells in the INL shown in the current study to be affected by diabetes undergo apoptosis, we performed costaining for TUNEL and one of the two retinal markers used (bNOS). $TUNEL^+$ cells and bNOS staining were clearly not colocalized. These results suggest that the reduction of specific neural markers in the diabetic retina is not due to retinal cell death but may be a result of diabetes-induced protein synthesis or metabolism dysfunction, in which BNN27 plays a restorative role. The correlation, and lack thereof, between a neuronal marker and caspase-3–IR or $TUNEL^+$ cells at different time points has been also reported in a model of focal ischemia in human hippocampus (43). It was also reported that patterns of retinal cell immunoreactivity in transmissible spongiform encephalopathies are not correlated with retinal degeneration (44). The mechanism via which this phenomenon is mediated has not been elucidated, but it may be a result of protein synthesis or metabolism dysfunction. This may hold true in our paradigm, with BNN27 playing an important role in restoring retinal cell function.

To examine the preventive properties of BNN27 on DR, we administered 10 mg/kg i.p. every other day for 4 weeks starting at the onset of diabetes (paradigm B). BNN27 had no statistical significant effect on TUNEL staining, but it reversed the diabetes-induced decrease of bNOS and TH immunoreactive cells (data not shown) similarly to what was observed in paradigm A.

At this point, we chose to investigate the effect of BNN27 on cleaved caspase-3, since cleavage of caspase-3 more than TUNEL labeling alone is suggested to be a more

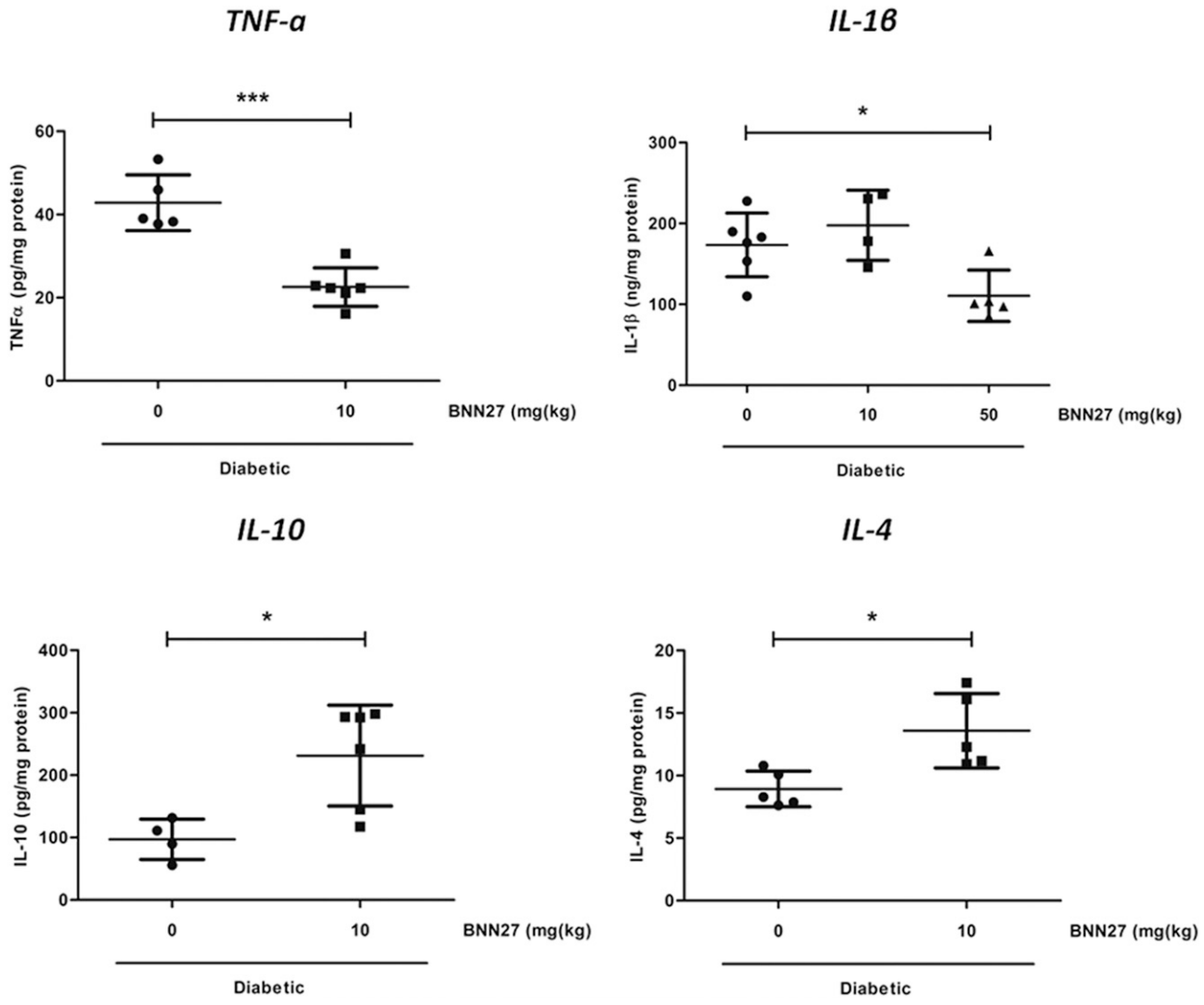

Figure 8—Effect of BNN27 on the levels of pro- and anti-inflammatory cytokines in diabetic rat retinas (paradigm A). BNN27 (10 mg/kg) decreased the levels of TNFα in the diabetic rat retinas (n = 6) but only at 50 mg/kg reduced the levels of IL-1β (n = 5) (***$P < 0.001$, *$P < 0.05$, compared with the diabetic nontreated) (n = 5 and n = 6 for TNFα and IL-1β, respectively). BNN27 (10 mg/kg) raised the levels of the anti-inflammatory cytokines IL-10 (n = 6) and IL-4 (n = 5) in diabetic rat retinas (*$P < 0.05$) compared with diabetic nontreated animals (n = 4 and n = 5 for IL-10 and IL- 4, respectively).

specific marker for early apoptosis in tissue sections (45). BNN27 reduced the diabetes-induced increase of cleaved caspase-3 in both paradigms, suggesting that it provides neuroprotection to the diabetic retina specifically against caspase-3–mediated cell death. Thus, BNN27 seems to selectively prevent the caspase-3–mediated cell death, having no effect on other apoptotic or necrotic pathways, in contrast to its lack of effect on TUNEL staining that labels all dead cells.

Diabetes was shown to induce an increase in peroxynitrite concentration leading to TrkAR tyrosine nitration, the attenuation of its activation by NGF, and the increase in the expression of $p75^{NTR}$. These events reversed the NGF/TrkA prosurvival actions leading to retinal neurodegeneration (38). Investigations using glaucoma and optic nerve transection models (in vivo RGC degeneration) reported that NGF activation of $p75^{NTR}$ and increases in glia activation led to RGC toxicity. This was shown to be mediated by increases in TNFα and $TNF\alpha_2$-microglobulin levels (46). In the current study, we show that BNN27 reduces the diabetes-induced increase in *1*) $p75^{NTR}$-IR and its expression and *2*) macroglial (GFAP-IR) and microglial (Iba-1–IR) activation. Increase in Iba-1^+ cells does not necessarily indicate microglia activation, since macrophages may also be present in the DR retina (BRB is not intact in DR [8]) and are also Iba-1^+. However, the morphology of the Iba-1^+ cells indicates that they are in an activated (or, rather, intermediate) state. The actions of BNN27 on GFAP and Iba-1 expression suggest its role as an anti-inflammatory agent. This is further strengthened by its ability to reduce the levels of the proinflammatory agents TNFα and IL-1β in the diabetic retina and increase the levels of the

anti-inflammatory cytokines, IL-10 and IL-4 (Fig. 8 and Supplementary Table 2).

Basal levels of NGF in rat retina were reported to be 147 ± 52 pg/g tissue (47). Topically applied NGF (10 μL drop of 200 μg/mL) resulted in a twofold increase in NGF levels in retina 6 h after treatment (47). NGF was shown to protect RGCs in an animal model of glaucoma and improved visual function in three patients with advanced glaucoma, as well as in the STZ model of DR (22,23). In the current study, BNN27 levels (227.3 ± 95.7 nmol/L) were detected in control rat retinas, after its intraperitoneal administration, with HPLC MS/MS analysis. These results suggest that BNN27 crosses the BRB and provides neuroprotection to the retina, in agreement with Bennett et al. (28), who reported that BNN27 crosses the BBB.

In total, these findings suggest that BNN27 protects the retina from the STZ-induced diabetic insult acting as a microneurotrophin. Sato et al. (48) reported that DHEA improved hyperglycemia in the STZ model of DR by activating glucose metabolism–related signaling pathways in skeletal muscle. In our paradigm, BNN27 had no effect on rat hyperglycemia at the doses examined. (See RESEARCH DESIGN AND METHODS.) Thus, the BNN27 effects in the diabetic retina are clearly independent of any improvement of hyperglycemia. On the contrary, and most importantly, BNN27 exerts its protective effects despite sustained high glucose levels.

BNN27 can be recommended as a therapeutic for DR, having the following advantages over NGF. BNN27, like NGF, binds to the high-affinity receptor (TrkA) in a nanomolar concentration (29), propagating prosurvival signaling. Moreover, it attenuates the expression of $p75^{NTR}$ death receptor in the diabetic retina. In addition, BNN27 unlike NGF is a small, lipophilic molecule that crosses the BRB.

In conclusion, BNN27 has the pharmacological profile of a therapeutic for DR, since it targets both the neurodegenerative and inflammatory components of the disease. More preclinical data are essential for further assessment of the pharmacodynamic and pharmacokinetic properties of BNN27 that will recommend its investigation at the clinical level. Studies are in progress examining the effect of BNN27 on proNGF/NGF levels and proNGF/ $p75^{NTR}$ signaling in the diabetic retina.

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Duality of Interest. A.G. is the cofounder of Bionature E.A. LTD, proprietor of compound BNN27 (patented with the WO 2008/1555 34 A2 number at the World Intellectual Property Organization). I.C. has patent WO 2008/1555 34 A2 with royalties paid. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. R.I.-A. performed experiments, analyzed and interpreted data, revised the manuscript, and was instrumental in the resubmission. S.L. performed experiments, analyzed and interpreted data, and contributed to the drafting and revision of the original manuscript. N.M., E.K., P.I., M.F., S.P., and A.S. performed experiments, analyzed and interpreted data, and revised the manuscript. D.K. performed experiments, analyzed and interpreted data, revised the manuscript, and was instrumental in the resubmission. A.K. contributed to the design, performed the HPLC-MS/MS analysis of BNN27, and interpreted data. H.E.K. contributed to the design and interpretation of the HPLC-MS/MS data and revised the manuscript. A.G. contributed to the conception of the study and revised the manuscript. I.C. contributed to the study design, interpreted data, and revised the manuscript. K.T. conceived and designed the experiments, analyzed and interpreted data, wrote and revised the manuscript, and supervised the project. All authors read the final version of the manuscript. K.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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8. CURRICULUM VITAE

CURRICULUM VITAE Ruth Ibán-Arias

Name: Ruth
Surnames: Ibán Arias
Address: Stavrakia-Voutes, 70013, School of Medicine, Pharmacology Department, Heraklion, Greece
Telephone: +306944996096, +34677130650
E-mail: ruthibarias@gmail.com

researchgate.net/profile/ • ruthbio1 (Skype)

EDUCATION

2018

- **Ph.D. in Medicine**
- University of Crete (Greece), School of Medicine, Department of Basic Sciences, Pharmacology laboratory
- Title of the Thesis:"Investigation of new therapeutic agents for the treatment of Diabetic Retinopathy: Neurosteroids/Microneurotrophins"
- Mentor: Kyriaki Thermos, Ph.D., Professor of Pharmacology

2012

- **Master in Neuroscience and Pain**
- University of Granada (Spain), School of Medicine, Department of Pharmacology and Institute of Neuroscience *Federico Olóriz*
- Title of Master Thesis: "Antioxidant effect of melatonin on mitochondrial dysfunction due to obesity in Zucker rat´s liver (ZDF)"
- Supervisor: Dr. Ahmad Agil

2011

- **Bachelor in Biology**
- Faculty of Biological and Environmental Sciences, University of León (Spain)

RESEARCH EXPERIENCE

February 2014 - Now

- PhD candidate in Medicine, molecular and cellular research.
- Department of Basic Sciences, Pharmacology laboratory, School of Medicine, University of Crete (Greece).
- "Investigation of novel synthetic neurosteroidal microneurotrophins (BNN20, BNN27) for the treatment of Retinal Neurodegeneration and Inflammation in the STZ-model of Diabetic Retinopathy", led by Prof. Kyriaki Thermos.

November 2011 - September 2012

- Co-investigator, molecular and cellular research.
- Department of Pharmacology and Institute of Neuroscience "Federico Olóriz", School of Medicine at the University of Granada (Spain).
- "Effects of melatonin at mitochondrial level in the complications associated to metabolic syndrome (obesity, diabetes, hypertension, proinflammatory state, oxidative stress, etc.) observed in the animal model of obesity *Zucker diabetic Fatty Rats*", led by Prof. Ahmad Agil.

August 2009

- Research assistant, molecular and cellular research.
- Research Unit at the Hospital of León (Spain)
- "Cellular and molecular analysis of the innate immune response to tuberculosis", led by Dr. Miguel Octavio Rivero Lezcano.

PUBLICATIONS

- Ibán-Arias R, Lisa S, Mastrodimou N, Kokona D, Koulakis E, Iordanidou P, Kouvarakis A, Fothiadaki M, Papadogkonaki S, Sotiriou A, Katerinopoulos H, Gravanis A, Charalampopoulos I, Thermos K (2018) The Synthetic Microneurotrophin BNN27 affects retinal function in streptozotocin-induced diabetic rats. Diabetes. 67(2):321-333. doi: 10.2337/db17-0391.

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PARTICIPATION IN SCIENTIFIC MEETINGS

- **R. Ibán-Arias**, S. Lisa, N. Mastrodimou, I. Charalampopoulos, A. Gravanis, K. Thermos. **Neuroprotective and anti-inflammatory effects of the microneurotrophin BNN27 in the STZ-model of Diabetic Retinopathy.** 47th Annual Meeting of the Society for Neuroscience (SfN), Washington DC, USA. November 2017.
- **R. Ibán Arias**, S. Poulaki, S. Lisa, N. Mastrodimou, A. Gravanis, I. Charalampopoulos, K. Thermos. **The Dehydroepiandrosterone spiro-epoxy derivative, BNN27, provides neuroprotection to retinal neurons in the STZ-model of Diabetic Retinopathy after eye drop administration**. FENS Featured Regional Meeting (FFRM) 2015, Thessaloniki, Greece. October 2015.
- **R. Ibán Arias**, S. Lisa, S. Poulaki, N. Mastrodimou, D. Kokona, M. Boumpouli, A. Gravanis, I. Charalampopoulos, K. Thermos. **The microneurotrophin BNN27 provides neuroprotection to retinal neurons through NGF TrkA receptor, when it is administered either intraperitoneally or by eye-drops**. 16th Congress of the Spanish Society of Neuroscience (SENC), Granada, Spain. September 2015.
- **Ibán-Arias R**, Lisa S, Kokona D, Mastrodimou N, Gravanis A, Charalampopoulos I, Thermos K. *The* **novel microneurotrophins BNN27 and BNN20 prevent retinal neurodegeneration in the in vivo STZ-model of Diabetic Retinopathy through NGF TrkA receptor activation.** European Neuroscience Conference by Doctoral Students (ENCODS) for doctoral students, Sesimbra, Portugal. April 2015.
- **Ibán-Arias R**, Jiménez-Aranda A, Castillo-Martínez M, Agil A. **Melatonin effect on fatty liver: Mitochondrial dysfunction characterization by using swelling assay**. IV Meeting of young pharmacologists of Andalucia. Granada. Spain. June 2012.
- **Ibán-Arias R**, Jiménez-Aranda A, Castillo-Martínez M, Agil A. **Efectos beneficiosos de la melatonina en la peroxidación lipídica y estado pro-inflamatorio en ratas ZDF.** IV Matchday of the Institute of Neuroscience "Federico Olóriz". Granada. Spain. June 2012.
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- Lisa S, **Ibán-Arias R**, Mastrodimou N, Kokona D, Charalampopoulos I, Gravanis A, Thermos K. **EFFECTS OF NOVEL SYNTHETIC MICRONEUROTROPHINS IN DIABETIC RETINOPATHY**. Conference of the *European Society for Neurochemistry* (ESN) in Molecular Mechanisms of Regulation in the Nervous System. Tartu, Estonia. June 2015.
- N. Mastrodimou, S. Lisa Ferrer, **R. Ibán-Arias**, D. Kokona, I. Charalampopoulos, A. Gravanis, K. Thermos. **The novel microneurotrophin BNN27 protects retinal neurons in the in vivo STZ-model of Diabetic Retinopathy by activating NGF TrkA receptor**. Annual Meeting of the *Association for Research in Vision and Ophthalmology* (ARVO), Denver, USA. May 2015.
- K. Thermos, N. Mastrodimou, S. Lisa, **R. Ibán-Arias**, S. Poulaki, P. Iordanidou, D. Giannogonas, D. Kokona, M. Kamaratou, E. Volitaki, I. Charalampopoulos, A. Gravanis. **Effect of the DHEA spiro-epoxy derivatives, BNN20 and 27, in an ex vivo model of chemical ischemia and the in vivo STZ-model of Diabetic Retinopathy: Neuroprotection via TrkA signaling**. 44th annual meeting of the Society for Neuroscience (SFN), Washington, DC, USA. August 2014.

- Agil A; Jiménez-Aranda A; **Ibán-Arias R**; Fernández-Vázquez G. **Melatonin effect on thermogenic gene expression profile in adipose tissues (brown & visceral) of Zucker Diabetic fatty (ZDF) rats**. 6th European congress of Pharmacology. Granada. Spain. July 2012.
- Jiménez-Aranda A, Fernández-Vázquez G, **Ibán-Arias R**, Agil A. **Melatonin increases brown adipose tissue mass and its functionality in young Zucker diabetic fatty (ZDF) rats**. 6th European congress of Pharmacology. Granada. Spain. July 2012.
- Jiménez-Aranda A, **Ibán-Arias R**, Castillo-Martínez M, Agil A. **Melatonin increases brown adipose tissue mass and its functionality in young Zucker diabetic fatty (ZDF) rats**. IV Meeting of young pharmacologists of Andalucía. Granada. Spain. June 2012.
- Jiménez-Aranda A, **Ibán-Arias R**, Navarro-Alarcón M, Agil A. **Melatonin induces thermogenic gene expression in white adipose tissue in Zucker diabetic fatty (ZDF) rats**. IV Meeting of young pharmacologists of Andalucía. Granada. Spain. June 2012.

ACADEMIC AWARDS

As undergraduate student:

- Scholarship with the International mobility program "AMICUS" for training internships in foreign universities awarded by the University of León (Spain) for the academic year 2009-2010 at the University of Colima (Colima, Mexico)

As MSc student:

- Scholarship for postgraduate studies awarded by the Spanish Ministry of Education for the academic year 2011-2012 at the University of Granada (Spain)

As PhD candidate:

- November 2015-October 2017: Scholarship for Ph.D. candidates, awarded by the Special Account for Research of the University of Crete, Greece (ELKE, KA4371).

Travel Grant awards:

- Travel grant awarded by the Hellenic Society for Neurosciences (HSfN) to attend the FENS Featured Regional Meeting (FFRM), October 2015, Thessaloniki, Greece.
- Travel grant awarded by the Spanish Society of Neurosciences to attend the 16th Congress of the Spanish Society of Neuroscience (SENC), September 2015, Granada, Spain.

SCIENTIFIC AFFILIATION

April, 2015. Member of the *Hellenic Society for Neurosciences* (HSfN)
May, 2015. Member of the *Sociedad Española de Neurociencia* (SENC)
May, 2017. Member of the *Society for Neuroscience* (SfN)

LABORATORY SKILLS

Animal handling

- Animal model of obesity (*Zucker Diabetic Fatty rats*: ZDF) • Rat streptozotocin model of diabetic retinopathy (Sprague-Dawley rats)

Administration of drugs (pharmacodynamics-pharmacokinetics)

- Intravitreal injections • Intraperitoneal • Intramuscular • Eye drop administration

Behavioral assays

- Behavioral tests of nociception: Von Frey test (Dynamic Plantar Aesthesiometer) and Hot plate test • Behavioral test of locomotion: Open field test

Tissue handling

- Animal surgery in rodents • Stereotaxic surgery • Histological preparation of brain and retina tissue • Extraction of different tissue: muscle, liver, white and brown adipose tissue, heart, retina • Blood extraction from heart • Histological sectioning of brain and eye with cryotome • Endothelial cell extraction from umbilical cord

Cell culture

- Bacterial cultures • Endothelial cells (from human umbilical cord) • HeLa cells

Cellular and Molecular assays

- Cloning • Electrophoresis • ELISA • Transfection by electroporation *in vitro* of monocytes and neutrophils with *Mycobacterium tuberculosis* • Gene and protein expression analysis in experimentally-induced infected monocytes and neutrophils • Fluorimetry (measurement of mitochondrial viability against Calcium ions input; ATP levels in mitochondria) • Histological studies (Hematoxylin-Eosin) • Immunohistochemistry • Mitochondrial extraction and isolation • Nucleic acids extraction • Oxygraphy (measurement of respiratory chain complexes state) • PCR and Real time PCR • Protein quantification with Bradford • Spectrophotometry • Western Blot

Microscopy

- Confocal • Fluorescence • Light

Computer skills

- *Statistical software*: Graph Pad Prism 5 • *Programming*: currently learning MATLAB / R • *Imaging programs and data analysis:* Image J (1.43m), GIMP, Adobe Illustrator, Adobe Photoshop (version 14.1 x32)