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Neuroprotective Approaches in Experimental Models of Retinal Degeneration

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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Abstract

BNN27, a blood-brain barrier-permeable, C17-spiroepoxy derivative of dehydroepiandrosterone (DHEA) has shown promising neuroprotective activity through interaction with nerve growth factor receptors, tropomyosin receptor kinase A (TrkA) and pan-neurotrophin receptor $p75^{NTR}$. In this study, we administered systemically BNN27 in two murine models of acute retinal degeneration/injury; experimental retinal detachment (RD) that results in photoreceptor cell death and N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity that results in cell death primarily of the inner retina and the retinal ganglion cells (RGCs). $TUNEL^{+}$ (Terminal deoxynucleotidyl transferase -TdT- dUTP Nick-End Labeling) photoreceptors were significantly decreased 24 hours post RD after a single administration of 200 mg/kg BNN27. Furthermore, BNN27 increased inflammatory cell infiltration, as well as, two markers of gliosis 24 hours post RD. However, single or multiple doses of BNN27 were not able to protect the overall survival of photoreceptors 7 days post injury. Additionally, BNN27 did not induce the activation/phosphorylation of $TrkA^{Y490}$ in the detached retina although the mRNA levels of the receptor were increased in the detached photoreceptors. In NMDA-mediated retinal injury, BNN27 was able to reduce $TUNEL^{+}$ cell death only in the photoreceptors and not in the RGCs or the inner retina in any of the three doses that we tested. Furthermore, it did not induce any changes in macrophage/microglia cell infiltration or in NMDA-mediated retinal gliosis. Moreover, similarly to RD injury, BNN27 did not induce the activation/phosphorylation of $TrkA^{Y490}$ in the NMDA-mediated injured retina although TrkA was downregulated following NMDA insult. Together, these findings, do not demonstrate neuroprotective activity of BNN27 in experimentally-induced RD or NMDA-mediated retinal excitotoxicity. Further studies are needed in order to elucidate the paradox/contradiction of these results and the mechanism of action of BNN27 in these models of acute retinal cell damage.

Περίληψη

Το BNN27, [(R)-3β, 21-dihydroxy-17R, 20-epoxy-5-pregnene] είναι ένα συνθετικό ανάλογο της δεϋδροεπιανδροστερόνης (dehydroepiandrosterone, DHEA), το οποίο έχει δείξει σημαντική αντί-αποπτωτική δράση (είδος κυτταρικού θανάτου) σε καλλιέργειες πρωτογενών νευρώνων, μέσω της πρόσδεσης του στους υποδοχείς της νευροτροφίνης nerve growth factor (NGF), tropomyosin kinase receptor A (TrkA) και pan-neurotrophin receptor $p75^{NTR}$. Το καινοτόμο αυτό στεροειδές είναι ένα μικρό, λιπόφιλο μόριο, το οποίο διαπερνάει τον αιματοεγκεφαλικό φραγμό και κατ' επέκταση μπορεί να χορηγηθεί συστηματικά. Σκοπός της παρούσας εργασίας ήταν η συστηματική χορήγηση του μορίου BNN27 σε δύο διαφορετικά πειραματικά μοντέλα οξείας νευροεκφύλισης του αμφιβληστροειδή σε τρωκτικά (επίμυες). Το πρώτο προκλινικό μοντέλο το οποίο εξετάστηκε, και στο οποίο δόθηκε και η μεγαλύτερη βαρύτητα, ήταν η πειραματική αποκόλληση του αμφιβληστροειδή, η οποία οδηγεί στον κυτταρικό θάνατο του έξω αμφιβληστροειδή λόγω της απομάκρυνσης των φωτοϋποδοχέων (φωτοαισθητήρια κύτταρα του οφθαλμού) από το μελάγχρουν επιθήλιο και τα χοριοτριχοειδή, τα οποία είναι η μοναδική πηγή μεταβολισμού τους. Το δεύτερο μοντέλο το οποίο εξετάστηκε ήταν αυτό της N-Methyl-D-aspartate (NMDA) διεγερσιτοξικότητας, το οποίο έχει ως αποτέλεσμα τον κυτταρικό θάνατο, πρωτίστως, του έσω αμφιβληστροειδή και συγκεκριμένα των γαγγλιακών κυττάρων (τα κύτταρα των οποίων οι άξονες σχηματίζουν το οπτικό νεύρο) και κάποιων υποτύπων αμακρυνικών κυττάρων (ενδιάμεσοι νευρώνες του αμφιβληστροειδή).

Τα αποτελέσματα της μελέτης μας έδειξαν ότι μία χορήγηση BNN27 (200 mg/kg) μία ώρα μετά την επαγωγή της πειραματικής αποκόλλησης, μειώνει σημαντικά τον κυτταρικό θάνατο των $TUNEL^{+}$ (Terminal deoxynucleotidyl transferase -TdT- dUTP Nick-End Labeling) φωτοϋποδοχέων 24 ώρες αργότερα. Επίσης, το ίδιο σχήμα χορήγησης, αύξησε σημαντικά τον αριθμό των $CD11b^{+}$ φλεγμονωδών κυττάρων στον υπαμφιβληστροειδικό χώρο και στον αμφιβληστροειδή στις 24 ώρες μετά την αποκόλληση. Συγκεκριμένα, όχι μόνο αυξήθηκε ο αριθμός των κυττάρων τα οποία εξέφραζαν τον κυτταρικό δείκτη CD11b (μακροφάγα και μικρογλοία), αλλά παρατηρήθηκε και αυξημένος αριθμός συσσωματωμάτων κυττάρων φλεγμονής στον υπαμφιβληστροειδικό χώρο. Παρομοίως, υπήρξε αυξημένη έκφραση σε δύο δείκτες γλοίωσης (GFAP και vimentin) στις 24 ώρες στην ομάδα των πειραματόζωων που είχαν λάβει μία χορήγηση BNN27 (200 mg/kg). Στη συνέχεια, οι μελέτες για την έκφραση των υποδοχέων έδειξαν ότι ο TrkA δεν εκφράζεται σε υγιείς φωτοϋποδοχείς ενώ αυξάνεται

δραστικά η έκφραση του 24 ώρες μετά την αποκόλληση, σε αντίθεση με τον υποδοχέα $p75^{NTR}$ όπου η έκφραση του παρέμεινε σταθερή πριν και μετά τον τραυματισμό. Σχετικά με τη φωσφορυλίωση του υποδοχέα (phospho-TrkA), δεν παρατηρήθηκε κάποια σημαντική διαφορά ανάμεσα στους αποκολλημένους αμφιβληστροειδείς της ομάδας ελέγχου και της ομάδας που της είχε χορηγηθεί BNN27 (200 mg/kg), όπως δεν παρατηρήθηκε και καμία διαφορά στα επίπεδα φωσφορυλίωσης των πρωτεϊνών (Akt και ERK1/2) οι οποίες βρίσκονται downstream του TrkA υποδοχέα και επάγουν νευροπροστατευτική σηματοδότηση. Τέλος, διερευνήθηκε η μακροχρόνια πιθανή νευροπροστασία μέσω του BNN27; Μία χορήγηση 200 mg/kg δεν μείωσε σημαντικά τον $TUNEL^+$-κυτταρικό θάνατο αλλά ούτε ήταν αρκετή για να αλλάξει σημαντικά το πάχος της στιβάδας των φωτοϋποδοχέων 7 ημέρες μετά την αποκόλληση. Παρομοίως, 7 επαναλαμβανόμενες χορηγήσεις της ίδιας δόσης (200 mg/kg) δεν επέφεραν νευροπροστασία στον αμφιβληστροειδή. Επιπροσθέτως, έγινε διερεύνηση με τρεις ακόμα δόσεις (10, 50 και 100 mg/kg) με 7 επαναλαμβανόμενες χορηγήσεις αλλά ούτε αυτές οι προσεγγίσεις έφεραν κάποια σημαντική αλλαγή στη διατήρηση του πάχους του αμφιβληστροειδή.

Στη συνέχεια, διερευνήσαμε την πιθανή νευροπροστατευτική δράση του BNN27 στο μοντέλο της NMDA διεγερσιτοξικότητας. Μία ενδοϋαλοειδική ένεση 100 nmoles NMDA είχε ως αποτέλεσμα τον οξύ και μαζικό θάνατο των κυττάρων του αμφιβληστροειδή σε όλες του τις στιβάδες 24 ώρες αργότερα. Μία χορήγηση BNN27 σε τρεις διαφορετικές δόσεις (40, 100 και 200 mg/kg), μία ώρα μετά την ενδοϋαλοειδική ένεση, δεν κατάφερε να μειώσει (καμία από τις τρεις δόσεις) τον $TUNEL^+$-κυτταρικό θάνατο των γαγγλιακών κυττάρων και των αμακρυνικών κυττάρων του έσω αμφιβληστροειδή, αλλά μείωσε σημαντικά (και οι τρεις δόσεις) τον $TUNEL^+$-κυτταρικό θάνατο των φωτοϋποδοχέων. Σε αυτό το μοντέλο δεν καταφέραμε να δούμε καμία διαφορά στη διήθηση των $CD11b^+$ φλεγμονωδών κυττάρων, στη γλοίωση του αμφιβληστροειδή (GFAP και vimentin) και στη φωσφορυλίωση του TrkA υποδοχέα ανάμεσα στην ομάδα των πειραματόζωων που είχαν λάβει BNN27 (100 mg/kg) και στην ομάδα ελέγχου. Παρ' όλα αυτά, παρατηρήσαμε για πρώτη φορά ότι στο μοντέλο της NMDA διεγερσιτοξικότητας η έκφραση του TrkA υποδοχέα είναι σημαντικά μειωμένη 24 ώρες μετά τον τραυματισμό.

Η πολύ σημαντική μείωση του κυτταρικού θανάτου των φωτοϋποδοχέων στις 24 ώρες μετά την επαγωγή της πειραματικής αποκόλλησης υποδηλώνει ότι το BNN27 πιθανόν να έχει σημαντική δράση στον οξύ τραυματισμό των κυττάρων αυτών, κάτι το οποίο ενισχύθηκε και από τα αποτελέσματα μας από το μοντέλο της NMDA διεγερσιτοξικότητας στο οποίο το

BNN27 μείωσε δραστικά τον κυτταρικό θάνατο αποκλειστικά των φωτοϋποδοχέων και όχι των άλλων κυττάρων του αμφιβληστροειδή. Ακόμα, στο μοντέλο της αποκόλλησης, το BNN27 αύξησε σημαντικά τη φλεγμονή και τη γλοίωση, ενώ δεν φάνηκε να έχει κάποια επιρροή στις δύο αυτές συνιστώσες στο μοντέλο της διεγερσιτοξικότητας. Είναι σημαντικό να αναφερθεί ότι ο ρόλος των κυττάρων της φλεγμονής και της γλοίωσης στα νευροεκφυλιστικά νοσήματα παραμένει εν μέρει κατανοητός με τους πληθυσμούς αυτούς να έχουν και ευεργετικό αλλά και βλαβερό ρόλο, οπότε περαιτέρω μελέτες χρειάζονται για την αποσαφήνιση της δράσης του BNN27 σε αυτούς τους πληθυσμούς κυττάρων. Ακόμα, σε αντίθεση με αποτελέσματα από άλλα νευροεκφυλιστικά νοσήματα, στον οξύ τραυματισμό του αμφιβληστροειδή δεν φαίνεται να ενεργοποιείται και κατ' επέκταση να επάγεται η φωσφορυλίωση του TrkA υποδοχέα, υποδεικνύοντας ότι το BNN27 μπορεί να δρα μέσω άλλων υποδοχέων στα συγκεκριμένα δύο μοντέλα. Η έλλειψη ενεργοποίησης του TrkA και της downstream νευροπροστατευτικής σηματοδότησης με τη σειρά της μπορεί να είναι υπεύθυνη και για την απουσία μακροχρόνιας νευροπροστασίας. Συνοψίζοντας, η παρούσα εργασία δεν ήταν ικανή να καταλήξει αν το BNN27 έχει νευροπροστατευτική δράση σε πειραματικά μοντέλα οξέων τραυμάτων του αμφιβληστροειδή. Περεταίρω ερευνητικές προσεγγίσεις είναι απαραίτητες για να αξιολογηθεί η δυνατότητα του BNN27 ως νευροπροστατευτικό μόριο σε αμφιβληστροειδοπάθειες οι οποίες περιλαμβάνουν αποκόλληση του αμφιβληστροειδή ή διεγερσιτοξικότητα.

Introduction

1.1 Vitreoretinal diseases

Vitreoretinal diseases comprise a group of retinal and vitreous disorders and can be initiated by several different stimuli. They can cause vision loss at varying degrees and ultimately, irreversible blindness. Millions of people around the world suffer from retinopathies, which are the leading cause of moderate to severe vision loss in industrialized countries and the third leading cause worldwide (13%), following uncorrected refractive errors and un-operated cataract[1]. In addition to the devastating health condition and the difficulties to perform daily living tasks, impairment of vision loss is also a huge global economic burden, especially recently, when the average life expectancy has been significantly increased.

Vitreoretinal disorders are classified into inherited retinal degenerations (IRDs) or retinal dystrophies such as retinitis pigmentosa (RP), Stargardt macular dystrophy (STGD) and Leber's congenital amaurosis (LCA) and acquired retinopathies, which are multifactorial entities such as glaucoma, diabetic retinopathy (DR) and age-related macular degeneration (AMD). IRDs can be syndromic or non-syndromic with more than 260 genes identified to date[2]. Vision loss is usually progressive and most of the times starts early in childhood, sometimes even in birth, or adolescence and severity increase with age because of the continuous loss of photoreceptors, the light sensing cells of the retina. Vision impairment varies from poor peripheral vision to complete blindness and occurs due to defects in the structure of photoreceptors that consequently lead to distortion of their function, which is no other than phototransduction. Retinal dystrophies have been associated with most types of inheritance; autosomal (both recessive and dominant), X-linked (both recessive and dominant) and mitochondrial, as well as, with sporadic mutations. Acquired retinal diseases have genetic and environmental influences and the onset of the disease is usually after the 6th decade of life; however, it is impossible to group all of them as an entity due to the very complex nature of each disease and the profound heterogeneity. Glaucoma, a group of diseases, often but not always, associated with increased intraocular pressure (IOP), results in cell death of retinal ganglion cells (RGCs) and subsequent damage of the optic nerve that is formed from their

axons. It has an estimated prevalence of 76 million people affected worldwide in 2020 (64.3 million in 2013)[3] and left untreated can lead to irreversible complete vision loss. The mechanisms underlying the pathophysiology of glaucoma are still not fully understood, although the largest risk factors are elevated IOP, age and genetics. Age-related macular degeneration (AMD) is a degeneration of the central area of the retina (macula) and is categorized into early, intermediate and advanced. Early and intermediate stages of AMD are nonneovascular or nonexudative and are referred as 'dry AMD', while advanced stages of the disease are referred as geographic atrophy (GA) and lead to complete macular atrophy (loss of photoreceptors) combined with loss of the choroid and the retinal pigment epithelium (RPE; the nourishing tissue of the retina) in the macular region. 'Wet AMD' is the term describing neovascular or exudative AMD, which in advanced stages leads to rapid vision loss. AMD has been correlated with smoking, genetic background, high blood pressure and obesity (among others) and has a predicted prevalence of 196 million people worldwide in 2020 living with the disease[4]. Along with glaucoma and AMD, another very common and severe retinal disease is diabetic retinopathy (DR), a microangiopathy caused secondary to diabetes (both type I and II). DR leads to vascular complications and accumulation of fluid in the macula (diabetic macular edema) and ultimately to retinal degeneration and cell death of the RGCs as well as other neurons of the inner retina. Among the diabetic patients, one third have signs of DR and among them one third have an advanced, vison-threatening stage of the disease, defined as severe non-proliferative diabetic retinopathy, proliferative diabetic retinopathy and diabetic macular edema[5]. Given that, in 2010 an estimated of 285 million people were living worldwide with diabetes[5] and this number was risen to 451 million in 2017[6] with a projection of 693 million affected by 2045[6], it is understandable why DR is the fifth most common cause of severe visual impairment worldwide[7]. Finally, it is important to add that along with the aforementioned entities, other insults can also lead to severe retinal damage like retinal detachment (RD) and trauma.

Nevertheless, despite the heterogeneity of vitreoretinal disorders, common to all, is the dysfunction and subsequent cell death of one or more types of retinal neurons and/or the RPE. Primary or secondary to neuronal cell death there is also local neuroinflammation as well as significant changes in retinal glial cells (macro- and microglia), which in turn lead to remodeling of the very well stratified structure of the retina. In many retinopathies, along with the neural cellular changes, there is also a significant vascular component which leads to severe retinal complications. Normal retina receives its blood supply from two sources; the outer

retina (photoreceptors and the RPE) from choroid's choriocapillaris and the inner retina from branches of the central retinal artery. Under pathological conditions the outer retina becomes hypoxic and ischemic, and thus new choroidal vessels are formed, penetrate the subretinal space (the space between the RPE and the retina), form membranes and most importantly leak serous fluid and/or blood.

1.2 Current treatments for retinopathies

Treatment options for retinopathies vary, and, of course, different approaches are necessary in order to cover different pathologic elements. In terms of retinal degenerative diseases, for the last several years, the primary goals of treatment were to control and eliminate vascular complications and inflammation. Indeed, during the last couple of decades, the field of pathological retinal neovascularization has been truly revolutionized with the discovery and remarkable results of intravitreal administration of inhibitors of vascular endothelial growth factor (VEGF). Anti-VEGFs prevent VEGF-A from binding to VEGF receptor 1 and/or 2 (VEGFR-1 and VEGFR-2 respectively), a key driver of angiogenesis, and are either monoclonal antibodies such as bevacizumab (Avastin) or fragments of humanized monoclonal antibodies (Fabs) such as ranibizumab (Lucentis) and brolucizumab. Alternatively, other anti-VEGFs act as a decoy receptor and bind altogether to VEGF-A, VEGF-B and PIGF (placental growth factor) such as aflibercept (Eylea) or act as an aptamer and bind to isoform $VEGF_{165}$ of VEGF-A such as pegaptanib (Macugen). Currently, multiple Phase III have been conducted for several anti-VEGFs that are available in the clinic with very good vision outcomes for patients with neovascular AMD[8–11]. Along with neovascular AMD, patients with diabetic macular edema (DME) were also particularly benefitted from anti-VEGF treatment[12,13]. However, it is important to note that controlling neovascularization can only slow the progression of the vascular component of the disease and not affect the degenerative process that is simultaneously happening. Long-term studies of anti-VEGF therapy for neovascular AMD have shown that almost all patients (up to 98.2%) had macular atrophy and 48% to 97% of patients had active disease in a follow-up of seven years[14,15].

In addition to neovascularization and vascular leakage, local retinal inflammation represents another therapeutic target of recent years. Intravitreal administration of steroids, results in significant reduction of inflammation both in pure inflammatory retinal diseases such

as posterior uveitis and in diseases with a significant inflammatory component such as diabetic retinopathy. Several Phase III have shown particularly favorable outcomes especially when corticosteroids were administered as an implant of sustained release. Administration of fluocinolone acetonide and dexamethasone implants (Iluvien and Ozurdex respectively) resulted in gaining three or more lines (15 letters) in visual acuity on a Snellen chart for patients with diabetic macular edema (DME) over a period of three years[16,17]. Today, multiple formulations of corticosteroids are available in the clinic for posterior uveitis or as a second-line treatment for patients with DME after anti-VEGF injections. However, there are important side effects, with most common the development of cataract in the majority of patients as well as rise of intraocular pressure in considerable percentage of patients.

Last but not least, another substantial revolution in the battle of retinopathies was the advancement of vitrectomy as a surgical intervention. First pars plana vitrectomy was performed at 1970 by Robert Machemer and is based on the principle to cut and aspirate the vitreous from the center of the eye. The surgical technique has since then been further advanced and can be combined with many others surgical approaches and/or interventions, however it remains today, by far, the most common and successful approach to treat a variety of retinal disorders including retinal detachment, advanced diabetic retinopathy, proliferative vitreoretinopathy, macular holes and trauma.

Nonetheless, when it comes to retinal dystrophies either inherited/primary or secondary to other retinopathies, until very recently, no cure was available, neither treatment options to slow the progression of the disease. However, within the last two decades, remarkable advancements have been made and new therapeutic approaches seem now more and more realistic for this group of retinopathies together with the end-stage retinal degenerations.

One promising therapeutic approach is stem cell replacement therapy with either embryonic stem cells (ESCs) or adult stem cells. In the first two Phase I/II studies a total of 18 patients with Stargardt macular dystrophy and geographic atrophy, received human ESC (hESC)-derived RPE cells that were administered subretinally[18]. There were no signs of side effects regarding the safety of the transplantation or rejection, while 7 out of 14 patients who had a 12 months follow-up, had an improvement of 15 letters in their best corrected visual acuity (BCVA)[18]. The safety of hESC-derived RPE was confirmed later on with another Phase I/II study[19]. Apart from ESCs, adult stem cells, either mesenchymal stem cells (MSCs) or induced pluripotent stem cells (iPSCs) were also FDA approved. In a prospective Phase I, bone marrow derived MSCs were administered intravitreally in patients with retinitis pigmentosa

(RP) and rod-cone dystrophy and there were also no side effects regarding the safety of the transplantation[20]. Nonetheless, 12 months after the administration the initial improvement in BCVA was lost[20,21]. Furthermore, a single patient with advanced stage of exudative AMD received iPSCs but that approach was discontinued due to the development of a genetic mutation[22]. Unfortunately, despite the optimistic outcomes, as applications of stem cells increased so did ocular complications[23,24]. Further, more extensive studies are necessary in order to eliminate the ocular side effects following transplantation and prolong the effect of the therapy.

Another potential advancement for patients with complete vision loss is artificial vision provided by electronic retinal implants. The very first patient who experienced artificial perception of light was back at 1968 with a device inserted into his occipital cortex[25], however, it took more than 30 years for the first implant to be inserted epiretinally into a patient with RP[26]. Today there are at least five different devices, with different potent location in the retina (subretinal or epiretinal)[27]. Among them, Argus II retinal prosthesis system (the only one FDA-approved) has been implanted into 30 patients with bare or no light perception and the clinical trial is estimated to be concluded in 2019. Early results from 2016, after a 5 years follow-up showed that the implant (also called the bionic eye) was able to increase patients' ability to recognize characters or small worlds, perform visual activities of daily living and succeed in orientation and mobility tasks[28]. However, there was a 6% adverse effect of conjunctival erosion among the patients[29].

The last, and perhaps currently, most promising novel approach for inherited degenerative retinopathies is gene therapy. Delivery of functional complementary DNA (cDNA) by adeno-associated virus (AAV) vectors was first successfully achieved in a Phase I study in 3 patients with Leber's congenital amaurosis (LCA) with no adverse effects and thus it opened the way for further gene replacement/augmentation studies[30,31]. Furthermore, 6 more patients received gene therapy for choroideremia, a disease that is characterized by slow degeneration of the retina, the PRE and the choroid, and gained 3 to 8 letters, however with the negative side effect of retinal detachment[32]. Nonetheless, the medical breakthrough happened on 2015 when for the first time in gene therapy, a Phase III study was completed. The trial was carried out in patients with LCA and RP and showed that voretigene neparvovec, a gene therapy product targeting the *Rpe65* gene, resulted in significant and clinically meaningful gain in vision[33]. Today, Luxturna (voretigene neparvovec-rzyl), the gene replacement drug for patients with genetic mutations in both copies of *Rpe65* gene, is the first ever gene replacement drug

approved from the FDA and has been administered to the first patient post FDA-approval in early 2018. However, a prerequisite for treatment with Luxturna is the sufficient number of remaining photoreceptors and the cost is quite extravagant. Currently numerous Phase I/II are ongoing for at least 11 genes associated with inherited retinal dystrophies.

Although outstanding progress has been made thus far, there are still much that need to be addressed. It is always important to acknowledge the wide heterogeneity and the multifactorial nature of many retinal diseases. In regards of the commonly used approaches, even when the vascular and/or the inflammatory component are controlled, or successful surgical management has been achieved, the final visual outcome is not always the desired for a significant number of patients, primarily because of the degenerative process that takes place due to the extreme sensitivity of the neural retinal tissue. The aforementioned degenerative component ends up being the most crucial step in the pathophysiology of most retinal diseases and leads to cell death and permanent loss of retinal function. Hence, control of retinal degeneration represents a very hot topic in retinal therapeutics. Taking all into consideration, new strategies are required in order to address neural dysfunction and degeneration in combination with the other components of retinal diseases. Therefore, approaches that can intervene in the degenerative process of the retinal neurons and can enhance neuronal survival and function, could intensify the outcomes of already existing therapies and thus be particularly beneficial for retinopathies.

1.3 Cell death pathways in retinal degeneration and targets for retinal neuroprotection

Retinal neuroprotection is defined as any approach that reduces cell death of retinal neurons or the optic nerve and has been thus far an unmet need. Numerous groups have been constantly trying to understand the mechanisms of the pathophysiology underlying retinal cell death and thus identify potent targets for neuroprotection. An integral part of this process is the employment of preclinical models of retinal degeneration that result in cell death of photoreceptors or of the inner retina and/or the retinal ganglion cells (RGCs). Early studies in animals revealed the involvement of programmed cell death (apoptosis)[34–36] through activation of both initiator and executioner caspases and consequent cleavage of Bid (B-cell lymphoma-2-homology domain 3 interacting-domain death agonist) in photoreceptor cell death[37,38].

Furthermore, death receptors/ligands that activate the extrinsic pathway such as tumour necrosis factor α (TNFα) and Fas ligand are elevated in retinal injury/degeneration and inhibition of those pathways results in amelioration of neuronal cell death in the retina[38–41]. On the other hand, inhibition of the intrinsic pathway by directing the balance between the pro-survival and pro-death members of the Bcl-2 (B-cell lymphoma-2) protein family, and thus regulating the mitochondrial outer membrane permeabilization (MOMP) and consequent release of the intermembrane space (IMS) proteins, also diminished retinal cell death in animal models of retinal detachment (RD)[42–44], optic neuropathy[45], light injury[46,47] and retinitis pigmentosa (RP)[46], although for the last two models results are contradictory[48,49] and *Bim* (Bcl-2-like protein 11) deficiency failed to prevent RGCs in the DBA/2J mouse of glaucoma[45]. Nonetheless, somewhat interestingly, intravitreal administration of the pan-caspase inhibitor Z-VAD-FMK did not reduce apoptotic cell death in various animal models of photoreceptors' degeneration, including experimental RD[42,50], light injury[51], RP[52] and the double-stranded RNA (dsRNA) model of AMD[53], in which a subretinal injection of polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analogue of dsRNA, results in cell death of the photoreceptors and the inner retina, as well as loss of the retinal pigment epithelium (RPE). Additionally, Z-VAD-FMK was not able to rescue RGCs and the inner retina in N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity, an animal model that mimic traumatic optic neuropathy, a condition in which acute injury to the optic nerve and the RGCs from trauma leads to vision loss (Tsoka, P. *et al.*, unpublished data, Angiogenesis Lab, Retina Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School).

Based on that evidence, it has been further examined if other cell death modalities participate in retinal degeneration. Indeed, RD-induced cell death was associated for the first time with RIP (receptor-interacting proteins) kinases-mediated regulated necrosis (also termed necroptosis)[50]. It was shown that when apoptosis is blocked in experimental RD, there is a significant increase in necrotic detached photoreceptors, suggesting that alternate cell death pathways coexist and contribute to the degenerative process of a certain type of cells under specific stimuli[50]. Furthermore, in the same study, intravitreal co-administration of pan-caspase inhibitor (Z-VAD-FMK) and necrostatin-1, an inhibitor of receptor-interacting protein 1 (RIP1)[54–56], significantly reduced cell death in photoreceptors as well as increased the overall protection in the long term, providing strong evidence that both cell death pathways have to be blocked simultaneously in order to have significant neuroprotection in this experimental model of retinal neurodegeneration[50]. These findings were later confirmed in the dsRNA model of

AMD[53], in which intravitreal co-administration of Z-VAD-FMK and Nec-1 prevented both photoreceptor and RPE loss[53]. Electron microscopy analysis further showed that in this model of retinal degeneration, photoreceptors die primarily from apoptosis while RPE die primarily from necrosis[53]. Similar findings for alternate cell death pathways between different subsets of retinal neurons were also reported in a model of inherited retinitis pigmentosa, where it was shown that Nec-1 administration and/or *RIP* deficiency protect the cones and not the rods[57]. Moreover, intravitreal administration of Nec-1 was able to protect the inner retina and improve functional characteristics of the electroretinogram (ERG) in the retinal ischemia-reperfusion injury model[58], as well as, to reduce cell death in NMDA-induced retinal excitotoxicity (Tsoka, P. *et al*., unpublished data, Angiogenesis Lab, Retina Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School).

In addition to necroptosis, another type of cell death has been proved to be involved in retinal degenerations. It was shown in recent studies that autophagy occurs in dying photoreceptors and that Atg5 and LC3-II (autophagy-related 5 and microtubule-associated protein light chain 3 respectively, both essential for autophagy) are elevated in the detached retina in a Fas-dependent manner[59]. Furthermore, it was reported that activation and prolongation of autophagy diminishes apoptosis-mediated photoreceptor cell death in experimental retinal detachment[59,60]. In addition, autophagy is necessary in the rods, in order to degrade phototransduction proteins and thus prevent retinal degeneration[61]. On the contrary, inhibition of autophagy was beneficial in an experimental model of retinitis pigmentosa that was caused by protein misfolding[62].

Moreover, caspase-independent cell death is another promising target for retinal neuroprotection through apoptosis-inducing factor (AIF). AIF is a flavoprotein at the mitochondrial intermembrane space, which during cell death, translocates to the nucleus and mediates non-caspase mediated apoptosis or necrosis[63,64]. Inhibition of AIF resulted in less photoreceptor cell death in experimental RD[42,44] and translocation of AIF from the inner segments of the photoreceptors to their shrunken nucleus[42]. Similar neuroprotective results and translocation of AIF have also been reported in animals models of RP[52,65–67].

More recently, new evidence came to light and additionally linked the inflammatory caspase-1-mediated cell death (also termed pyroptosis) with AMD[68,69] and later on with experimental retinal detachment[70], experimental retinitis pigmentosa[71] and experimental traumatic optic neuropathy; in the animal model of optic nerve crush[72] and NMDA-induced retinal excitotoxicity[73]. NLRP3 (nucleotide-binding domain, leucine-rich-repeat containing

family, pyrin domain containing-3) inflammasome, a cytoplasmic molecular platform that upon activation leads to the cleavage of caspase-1 and subsequent secretion of the mature forms of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 (IL-18)[74–77], was reported that can be activated by drusen[69] (yellow deposits made up of lipids) and Alu RNA[68] (transposable elements of non-coding RNA), which in turn can be found in the retina and the RPE (respectively) of patients with AMD. Nonetheless, NLRP3 inflammasome-mediated IL-1β and IL-18 driven inflammation has until today controversial implications regarding retinal cell death and survival[68–72,78–80]. Inhibition of NLRP3 has led to reduced cell death in a mouse model of retinal detachment[70] and to increased cone survival in a mouse model of retinitis pigmentosa[71]. Moreover, deficiency of *Nlrp3* led to significant delay of RGCs' loss in an experimental model of optic neuropathy[72]. On the contrary, NLRP3-mediated IL-18 induction was neuroprotective in experimental models of AMD in rodents and non-human primates[69,78,79].

Neuroprotection can either block cell death or enhance cell survival. Therefore, another promising target for retinal neuroprotection are pro-survival signalling pathways that counteract the cell death cascades in the retina. Enhancement of endogenous molecules that can activate beneficial cell survival and differentiation pathways such as JAK/STAT (Janus kinase/signal transducer and activator of transcription protein), MAPK/ERK (mitogen-activated protein kinases/extracellular signal-regulated kinases) and Akt (RAC serine/threonine-protein kinases) could be particularly favorable as pharmaceutical neuroprotectants.

One such category of molecules is the family of neurotrophins. Neurotrophins are secreted factors that are essential for development, differentiation, function, survival and death of certain neuronal populations of the mammalian central and peripheral nervous system (CNS and PNS respectively) and play a significant role in various other systems such as the immune and the endocrine. There are four mammalian neurotrophins; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 and 5 (NT-4/5). They are responsible for axonal regulation, dendritic growth and guidance, synaptic structure, modulation of synaptic connections, neurotransmitter release and synaptic plasticity. Neurotrophins exert their function through selective binding to their receptors. Each neurotrophin binds with high affinity to a particular tropomyosin receptor kinase (Trk) and with lower affinity to the pan-neurotrophin receptor $p75^{NTR}$. NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB and NT-3 binds to TrkC. Alteration in neurotrophins' levels have

profound effects on a wide variety of phenomena including myelination, regeneration, pain aggression, depression and pain abuse, and thus, neurotrophins have been widely associated with degenerative and psychiatric disorders such as chronic or neuropathic pain, Alzheimer's, Huntington's and Parkinson's disease, stroke and brain trauma. The potential therapeutic effect of neurotrophins on neurodegenerative diseases has been extensively studied in animal models with very optimistic results (for review[81–83]). However, neurotrophins are large polypeptidic molecules that cannot cross the blood-brain barrier (BBB) and thus administration can be problematic (e.g. intracerebroventricular or intraputaminal). Indeed, despite the encouraging preclinical data, the majority of clinical trials have failed. Identifying new molecules that mimic neurotrophic action and have better pharmacokinetic profiles would be particularly promising as potent neuroprotective molecules.

1.4 BNN27

BNN27 is a novel synthetic C17-spiroepoxy [(R)-3β, 21-dihydroxy-17R, 20-epoxy-5-pregnene] steroid derivative of dehydroepiandrosterone (DHEA)[84]. DHEA and its sulfate derivative/ester (DHEAS), the most abundant steroids in the plasma, are produced in adrenals, in gonads and in brain (in both neurons and glia). They are well-characterized neurosteroids[85,86] and notable neuroprotective molecules due to their ability to prevent neuronal cell death on various experimental neurodegenerative models both *in vivo* and *in vitro*[87–92]. It has been now been well-established that some of those neuroprotective and neuromodulatory effects of DHEA and DHEAS are mediated through neurotransmitter receptors[93] such as gamma-aminobutyric acid A ($GABA_A$)[94–97], N-methyl-D-aspartate (NMDA)[98–101] and sigma 1 (σ1)[100,102–104]. Lately, the scientific interest regarding the neuroprotective potency of DHEA has been intensified because of the discovery that DHEA acts as an ancestral ligand to neurotrophins' receptors[105–108]; and thus, it binds to and activates all tropomyosin receptor kinases (Trk), as well as, the pan-neurotrophin receptor $p75^{NTR}$.

However, among all neurotrophins' receptors, DHEA binds with the highest affinity to TrkA receptor (K_D: 7.4 nM)[105], which is preferiantially activated by NGF. NGF was discovered in the 1950s by Rita Levi Montalcini in collaboration with Stanley Cohen and was the first characterized neurotrophin. Mature NGF is derived from its proform, proNGF, after proteolytic cleavage and is mainly characterized as a pleiotropic factor utilizing its effects in both

development and adult life. The potential neuroprotective ability of NGF has been widely assessed on a variety of experimental models of neurodegeneration with very favorable outcomes. However, administration of NGF in experimental retinal degeneration has thus far been contradictory. Exogenous administration of NGF protected the retinal neurons in various animal models, including retinal detachment[109], retinitis pigmentosa[110,111], retinal ischemia-reperfusion injury[112], diabetic retinopathy[113] and AMPA-mediated retinal excitotoxicity[106]. On the other hand, administration of NGF alone was not able to rescue degenerating retinal ganglion cells in experimental models of increased intraocular pressure (IOP)[114,115] and optic nerve axotomy[115,116]. On the contrary, in the above-mentioned studies, only agonists of NGF were able to offer significant neuroprotection, either alone or synergistically with NGF through TrkA signalling[114–116].

In regards of clinical use, a handful phase I clinical trials have been conducted with NGF treatment on patients with Alzheimer's disease, as well as, several case reports and/or studies for other neurodegenerative diseases of the brain that resulted in improvement of several characteristics of the pathophysiology of each condition (for review[83,117]). In retinal degenerative diseases, thus far, there have been one pilot study, one case study and one case report for patients with retinitis pigmentosa[118], glaucoma[119] and AMD[120] respectively, in all which, NGF was administered through eye drops with no serious adverse effects and minor improvements in a cluster of patients[118–120].

Similar to NGF, DHEA induces TrkA phosphorylation as well as downstream molecules of NGF-mediated TrkA signalling and has anti-apoptotic effects on $TrkA^+$ superior cervical ganglia in NGF-deprived induced apoptosis[105] (superior cervical ganglia, which are primarily consisted from sympathetic neurons, is a well-characterized NGF/TrkA mammalian neuronal system in which, deprivation of NGF leads to apoptotic cell death). Furthermore, DHEA was able to rescue $TrkA^+$ sensory neurons of dorsal root ganglia from apoptotic cell death in NGF null embryos[105]. In another study, in a rat model of AMPA-induced (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) retinal excitotoxicity, intravitreal administration of DHEA provided significant neuroprotection to brain nitric oxide synthetase (bNOS)- and choline acetyltransferase (ChAT)- expressing amacrine cells as well in $calbindin^+$ amacrine and horizontal cells of the inner retina[106]. In the same study, inhibition of TrkA reversed the neuroprotective effect of DHEA[106]. In addition, DHEA exerts neuromodulatory effects through TrkA signalling as it was shown in an acute experimental model of neuro-inflammation in the CNS induced by lipopolysaccharide (LPS)[108]. In LPS-mediated neuro-inflammation, DHEA

was able to reduce microglia-mediated inflammation through phosphorylation of TrkA and subsequent activation of Akt1/Akt2-CREB-Jmjd3 pathway (cyclic-AMP response element binding protein and Jumonji d3 respectively), which has been associated with genes that regulate inflammation and microglia polarization[108].

However, DHEA is an intermediate in the biosynthesis of androgens and estrogens and thus treatment with this steroid can be problematic due to potential endocrine side effects[121–124]. For this reason, effort has been made to develop analogues that will retain the anti-apoptotic properties of DHEA without converting to estrogens or androgens. Indeed, the chemical library of spiro-analogs of DHEA (BNNs) have been tested and reported to have neuroprotective activity, while they are not metabolized into sex steroids[84]. Indeed, BNN27 (among others BNNs) was unable to activate estrogen receptor alpha and beta in a micromolar range of concentrations as well as to interfere with estrogen-dependent gene expression[84]. Furthermore it was not able to stimulate the growth of various cancer-related cell lines[84].

The small lipophilic BNN27 can cross the BBB and can be detected in the mouse brain 30 minutes after intraperitoneal administration[125]. Furthermore, BNN27 given systemically can be detected by LC-MS chromatography in the rat retina two hours after intraperitoneal injection with a peak at four hours post administration[126,127]. BNN27 selectively binds to TrkA receptor[128–130], induces its phosphorylation[128,130–132] and upregulates the expression of phospho-Erk [128,131,132] and phospho-Akt[128], while in the absence of TrkA receptor, BNN27 binds to and activates $p75^{NTR}$ receptor and consequently protects the murine cerebellar granule neurons from serum deprivation-induced apoptosis[133]. BNN27 was able to protect the neural crest-derived pheochromocytoma (PC12) cells from serum deprivation-induced apoptosis[84,128], reduced $TUNEL^{+}$ (Terminal deoxynucleotidyl transferase -TdT- dUTP Nick-End Labeling) cell death in superior cervical ganglia following NGF deprivation[128] and diminished caspase-3 mediated cell death in dorsal root ganglia of NGF null embryos[128], in serum-deprived PC12 cells[128] and in cuprizone[129]- and diabetes[130]-induced apoptosis. Furthermore, BNN27 was able to protect mature oligodendrocytes in an animal model of multiple sclerosis (MS)[129] and reverse the diabetes-induced loss of immunoreactivity of retinal amacrine cells and ganglion cell axons' markers in an experimental model of diabetic retinopathy (DR)[130].

Taken all into consideration, BNN27 could be a potent neuroprotective agent in acute retinal injury and retinal degeneration. The aim of the present study was to investigate whether systemically administered BNN27 can protect photoreceptors from cell death in the murine model of experimental retinal detachment, as well as, the inner retina and the retinal ganglion

cells in the murine model of N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity. Furthermore, to assess how BNN27 affects the injured/degenerating retina and particularly inflammation and gliosis and to determine if the effects of BNN27 on the above-mentioned paradigms of retinal degeneration are mediated through TrkA signalling.

1.5 Retinal Detachment

Retinal detachment (RD) is characterized by the separation of the photoreceptors from the underlying nourishing tissue, the retinal pigment epithelium (RPE) and the choriocapillaris. Photoreceptors' sole metabolic supply is the choroidal vasculature and the choriocapillaris, and thus, once separation occurs, photoreceptors cannot receive sufficient oxygen and nutrients. Detachment of the photoreceptors from the RPE results in significant consequences for the retina, including crosstalk of cell death, immune cell infiltration, gliosis and remodeling.

The most common type of RD is rhegmatogenous RD (RRD) in which the separation of the retina from the RPE occurs due to a retinal tear (break). The retinal tear allows fluid from the vitreous into the subretinal space and is usually created (80-90%) because of vitreoretinal traction as a result of posterior vitreous detachment (PVD), a very common eye condition caused by natural changes in the vitreous (liquefaction) during aging. Along with retinal tears due to PVD, breaks in the retina are also caused by degenerative causes (retinal holes), inflammation and/or trauma. Besides RRD, there are two more types of retinal detachment, the tractional and the exudative. Both tractional and exudative RDs are present in combination with other retinal diseases, usually with a strong inflammatory component such as diabetic retinopathy (DR) and age-related macular degeneration (AMD) or secondary to choroidal tumors respectively. Asymptomatic and non-progressive chronic RDs can also occur and are usually correlated to retinal cysts or subretinal fibrosis.

The prevalence of RRD alone in phakic patients (people who have intact their natural crystalline lens) is about 1 in 10.000 people a year (based on observation studies in random places in Europe and the U.S., for review[134]) with a peak in the 60- to 70-year-old age group, following the peak of PVD, and is more common in men than women. Nonetheless, there are several factors that can induce to a variable extent the risk of RRD with most common among them; myopia, cataract surgery, family history and history of fellow-eye with RRD (for review[134]). Short-sighted (over three diopters of myopia) people have a 10-fold increased

incidence of RRD[135], while people with history of fellow-eye with RRD have a 2% to 10% risk of progressing to bilateral RRD (for review[134]). Moreover, people who have had previous cataract surgery also have an increased incidence of RRD (for review[134]). Finally, people with symptomatic pre-existing retinal disorders or lesions, people who underwent trauma, as well as, people with specific genetic disorders such as Marfan, Ehlers-Danlos, Wagner and Stickler syndromes have also a higher risk of developing RRD (for review[134]).

Initial detachment might be small and local but without treatment in a timely manner, bigger parts of the retina might detach and subsequently lead to vision loss and blindness. Currently, there are several surgical approaches to treat RD, which include scleral buckling, pneumatic retinopexy and vitrectomy and are all based on the same principle; Find and seal the retinal breaks as well as relieve present and potential vitreoretinal traction. Each method has characteristic advantages and the choice of the procedure is based on a variety of aspects such as the size of the break, the age of the patient together with resources and experience of the surgeon. In all cases, the success rate of a re-attachment of the retina is around 80%. In combination with the side effects, the photoreceptor cell loss that ensues, results in suboptimal visual outcome in many cases and only two fifths of the patients with macula-off RRD (the fovea is detached) regain 20/40 or better vision (for review[134]). Furthermore, a large percentage of patients have a secondary detachment due to complications of proliferative vitreoretinopathy (PVR) and subsequently undergo multiple surgeries in order to establish an attached retina and even then, not always with a good visual prognosis (for review[134]). Therefore, intense research is taking place around the world in order to elucidate the cell death pathways in retinal detachment and indicate targets that can enhance photoreceptor survival.

The experimental model of RD was first described in 1968 by Machemer and Norton in owl monkeys [136] and since then it has been used extensively in the laboratory as a trustworthy and highly repetitive animal model to elucidate retinal degeneration. However, it is important to note that due to variation in the experimental microsurgical technique of the RD and consequently in the size of the detached retina, there are various outcomes in regards of the time course of cell death and the natural history of the detachment[42,50,137]. Furthermore, very interestingly, various strains of the same animal species often used in the lab, result in different levels of cell death, as well as, crosstalk between the different cell death pathways following experimental RD [137]. In 1995, Cook and his colleagues showed for the first time that RD results in internucleosomal DNA fragmentation (a landmark of apoptosis) in the outer nuclear layer (ONL) of the retina (the layer that is consisted of the photoreceptor nuclei)[36] and later on these

findings were also confirmed in human detached retinas and it was shown that photoreceptor cell death starts as early as 12 hours post detachment[138]. Since then, as it was discussed in detail above, retinal detachment-induced photoreceptor cell death has been associated with caspase-dependent cell death[38,40,41], caspase-independent cell death[42,44,65], receptor interacting protein (RIP) kinases-mediated programmed necrosis (necroptosis)[50,70,137], inflammation-mediated cell death (pyroptosis)[70] and autophagy[59,60]. Taken all together, mechanical separation of the retina from the RPE results in a signaling cascade and a perplexed cross-talk of cell death pathways and simultaneous inhibition seems necessary in order to achieve neuroprotection.

Inflammation affects the formation and progression of various vitreoretinal diseases. Thus far, it is not clear if the inflammatory response is secondary to cell death or to the pathogenic changes of the retina (including angiogenesis and fibrosis) or is a leading component of some diseases. In retinal detachment, in addition to increased photoreceptor cell death, there is an intense inflammatory response and an accumulation of immune cells in the retina; the vast majority of those cells are activated macrophages, which infiltrate the subretinal space (the space that has been created between the retina and the RPE because of the detachment)[41,70,137,139,140] and play a pivotal role in a strong interplay between cell death/survival, inflammation and clearance. In addition to the accumulation of infiltrating cells into the retina, there is an increase in the production and secretion of pro-inflammatory cytokines and chemokines. Interleukin-1β (IL-1β) is highly elevated in the subretinal fluid of patients with RRD compared to the levels of IL-1β in their vitreous or in the vitreous of healthy subjects[70]. In addition, interleukin-6 and -8 (IL-6 and IL-8 respectively) together with monocyte chemoattractant protein 1 (MCP-1) are significantly elevated in the vitreous of patients with RRD similarly to other vitreoretinal diseases[141]. Furthermore, *Mcp1* deficiency led to significant attenuation of dying photoreceptors in experimental RD[142] suggesting that MCP-1 mediates photoreceptor cell death. Recently, new evidence came to light and provided strong evidence that the source of IL-1β in the detached retina (and thus probably of all pro-inflammatory cytokines) are the infiltrating macrophages/microglia located in the subretinal space and not resident macrophages/microglia of the retina or dying photoreceptors[70]. The definitive role of immune cells in the detached retina has not yet been fully elucidated. Traditionally, and from a clinical aspect, increased inflammation in the retina has been linked to poor visual outcomes, however, recent evidence shows that although activated macrophages might be detrimental for the survival of the photoreceptors, activated microglia is beneficial towards the dying photoreceptors[143]. Furthermore, it is still unclear what is the role of the

polarization of macrophages in the detached retina (M0, M1 and M2 macrophages' subtypes) along with other types of immune cells that might contribute such as T cells, that have been very recently indicated to prolong retinal ganglion cells' degeneration in experimental glaucoma[144].

RD triggers also the activation and proliferation of retinal glial cells, astrocytes and Müller glial (MG) cells (radial glial cells of the retina), a response known as reactive gliosis. Upon activation of these cells, there is an increased production of the intermediate filament proteins, glial fibrillary acidic protein (GFAP) and vimentin and also characteristic alterations in their morphology[145]. Following retinal detachment many MG cells (their nuclei) migrate into the outer retina (photoreceptor layer), undergo mitosis and eventually reside in subretinal glial scars. Another portion of MG cells, following RD, stop expressing well-accepted MG cell marker proteins indicating that these subset of MG cells undergo dedifferentiation. Analogous to the perspective regarding the immune cells and their subsequent role in retinal detachment, gliosis has been traditionally considered harmful for the retina. Scar tissue in the retina results in membranes formation and worse visual outcomes combined with increased surgical difficulty. Moreover, deletion of *Gfap* and/or *Vim* (vimentin) resulted in attenuation of photoreceptor cell death in experimental model of retinal detachment[146]. However, on the contrary, reactive astroglia has been reported to have a neuroprotective phenotype and thus support cell survival[147,148], while MG cells have the ability of reprogramming and thus becoming reparative towards injury[149,150].

In addition to MG cells, RPE cells also proliferate and migrate into the inner retina and the vitreous cavity; pigmented cells visible in the vitreous cavity during fundus ophthalmoscopy is a well-established sign in the diagnosis of RRD. Proliferation of the RPE starts early at 24 hours post retinal detachment/tear/hole and appears to be local and not involve other retinal areas[151]. The RPE cells that migrate into the vitreous many times dedifferentiate into fibroblast-like cells and might be responsible for membranes formation and development of PVR.

Mechanisms that can protect photoreceptors from dying while they are still detached could be particularly beneficial for many retinopathies that involve photoreceptor separation from the RPE, prolong the time window for surgical intervention and increase the chance for a positive outcome.

1.6 Retinal Excitotoxicity

The term 'excitotoxicity' has been first described in the 1970s by John Olney based on his work regarding the detrimental effect of glutamate and its analogues on neurons[152–154]. A decade later, Olney proposed that the primary excitatory neurotransmitters of the CNS, in particular glutamate and aspartate, mediate the excitotoxic cell death of neurons[155]. Later on, it was proved that glutamate activates the N-Methyl- D-aspartate receptors (NMDARs), which upon activation allow influx of Ca^{2+}[156]. Furthermore, antagonists of NMDARs were able to protect cortical neurons *in vitro* as well as hippocampal neurons in an experimental model of transient forebrain ischemia[157,158]. Since then, it has been well-established that excessive stimulation of NMDARs allows massive intracellular influx of Ca^{2+}, which in turn activates two Ca^{2+}-dependant enzymes, calpain 1 and neuronal nitric oxide synthase (nNOS), that subsequently lead to mitochondrial damage, oxidative stress and cell death, a process termed excitotoxicity.

NMDARs belong to the ionotropic glutamate receptors and play key roles in neuronal communication. Until recently, it was believed that synaptic activity of NMDARs mediates excitotoxic cell death; in particular, excessive release of glutamate from the pre-synaptic neurons activates a superfluous number of post-synaptic NMDARs, and thus allowing massive intracellular influx of Ca^{2+} through receptor-operated cation channels and hence triggering excitotoxicity and neuronal cell death[159]. However, in 2002 it was shown by Hardingham and colleagues that extrasynaptic NMDARs were responsible for the excitotoxic insult, while synaptic NMDARs were neuroprotective[160]. It is now well-accepted, though still not fully understood, that cellular responses to NMDARs activation are based on the receptor's location; synaptic or extrasynaptic (for review[161]). Stimulation of synaptic NMDARs promotes neuroprotection and neuronal health by initiating transcriptional changes that promotes resistance to various traumatic stimuli (for review[161]). Synaptic NMDARs, primarily through nuclear Ca^{2+} signalling, suppress transcriptional expression of molecules of the intrinsic cell death pathway, while upregulate transcriptional expression of antioxidant and regulatory factors that in turn reduce oxidative stress (for review[161]), as well as pro-survival signalling through Akt and ERK1/2. On the other hand, stimulation of extrasynaptic NMDARs promotes neuronal cell death and/or vulnerability to trauma (for review[161]). Extrasynaptic NMDARs inactivate Akt and ERK1/2, though contradictory[162], while import into the nucleus the pro-death transcription factor FOXO (forkhead box protein O) (for review[161]). Recent evidence has

shown that different types of calpain might be responsible for survival synaptic and neurodegenerative extrasynaptic NMDARs signalling[162,163]. NMDARs are tetrameric protein complexes usually comprised by two obligatory NMDA receptor 1 (GluN1) subunits and two modulatory NMDA receptor 2/3 (GluN2/3) subunits. GluN1 has in turn 8 different subunits generated by alternative splicing from a single gene. GluN2 has 4 different subunits (A-D) and GluN3 has 2 (A and B). Recently, the dichotomy regarding NMDARs signalling has been also correlated with the different subunits of the receptor[164,165]. Depending on the subunit of the receptor that each molecule interacts, the cell can either be led to cell death or survival.

Excitotoxicity has been associated with all types of acute brain injury, including prolonged epileptic seizures, stroke, cerebral ischemia, traumatic brain and spinal cord injuries and hypoglycemia. Furthermore, certain chronic neurodegenerative diseases have been also associated with noncanonical NMDARs activity, such as Alzheimer's and Huntington's disease (for reviews[161,166]). A shift in the normal balance between synaptic and extrasynaptic NMDARs activity might play a crucial role in the aetiology of neurodegenerative diseases.

In the retina, NMDARs are expressed in both the outer and inner retina with most robust expression in the latter. NMDARs has been found to be diffusively distributed in the outer plexiform layer (OPL; the layer that is consisted of the synapses of photoreceptors, horizontal and bipolar cells of the retina), as well as, throughout the whole inner plexiform layer (IPL; the layer that is consisted of the synapses of bipolar, amacrine and ganglion cells of the retina) (for review[167]). Furthermore, immunoreactivity of different subtypes of the NMDA receptor was found and co-localized with cholinergic, dopaminergic, GABAergic and glycinergic amacrine cells, as well as with $Brn3a^{+}$ (brain-specific homeobox/POU domain protein 3A; cell-specific marker, expressed from the vast majority of RGCs' subsets) retinal ganglion cells[168] (for review[167]). In addition, NMDARs have also been detected in both rod and cone bipolar cells (for review[167]). Of course, positive results for expression of one of the subunits of the receptor does not necessarily means that the receptor itself is functional. Horizontal cells and photoreceptors do not seem to have functional NMDARs and current responses failed to be induced. However, the possibility remains still open, with several studies using immunohistochemistry and/or immunofluorescence resulting in contradictory data (for review[167]).

Retinal excitotoxicity has been correlated with a wide spectrum of retinopathies that result in cell death of the inner retina and the retinal ganglion cells (RGCs) such as optic neuropathies, diabetic retinopathy (DR) and glaucoma.

Glaucoma, as mentioned above, is a group of diseases that gradually leads to optic nerve degeneration, RGCs' death and damage to the microglia surrounding the optic nerve. It is usually, though not always, associated with increased intraocular pressure (IOP). The etiopathogenesis of glaucoma has thus far been associated with oxidative stress, impairment in axonal transport, neuroinflammation and excitotoxicity (for review[169]). *Myoc* (myocilin) and *Optn* (optineurin) are two genes related and often mutated in primary open angle glaucoma (POAG) and normal tension glaucoma (NTG) respectively[170]. POAG is the most common subtype of glaucoma and is a chronic multifactorial degenerative disease with slow progression and decreased aqueous humor outflow. POAG can occur with either elevated or normal IOP. Cases of POAG with normal IOP, are called NTG. The cause of optic nerve damage in NTG is still unclear given that the IOP is never elevated in those patients. Both myocilin and optineurin proteins play a key role in endocytic pathway, which is responsible for recycling of vesicles and has been tightly linked to glutamate excitotoxicity.

Diabetic retinopathy (DR) has been classically considered a microcirculatory disease of the retina. However, the last couple of decades new evidence came to light and showed RGCs apoptotic cell death in experimental models of DR, indicating that DR has a strong neurodegenerative component (first study was by Barber and colleagues[171], for review[172]). It is now widely-accepted that retinal degeneration in DR precedes vascular impairment. Elevated levels of glutamate and Ca^{2+}, oxidative stress, overexpression of the renin-angiotensin system, excitotoxicity as well as upregulation of RAGE (receptor for advanced glycation end-products) might all be contributory factors to the degenerative diabetic retina (for review[173]). The relationship between retinal excitotoxicity and VEGF (vascular endothelial growth factor) is one of the most interesting pathways linking neurodegeneration and vascular impairment. NMDARs exert a significant inhibition of VEGF secretion on primary cultures of Müller glial (MG) cells[174], suggesting a neuroprotective role of NMDARs signalling in the healthy retina, while on the contrary, elevation of VEGF expression and BBB breakdown in experimental DR are reversed by brimonidine, an alpha2 adrenergic agonist and neuromodulator of NMDA receptor[175,176].

Optic neuropathies are a group of diseases with multiple aetiologies including demyelinating, inflammatory, ischemic, traumatic, compressive, toxic/nutritional and hereditary causes that lead to damage of the optic nerve and cell death of the RGCs. Mitochondrial dysfunction, excitotoxicity, impairment in mitophagy, vulnerability to cell death signalling and impairment in axonal transport are the key players underlying the degenerative

process in those conditions (for review[177]). Dominant optic atrophy is caused by mutations in the optic atrophy gene 1 (*Opa1*) which has been strongly associated with mitochondrial abnormalities and overexpression of NMDARs[178]. Furthermore, mutations in mitochondrial DNA (mtDNA) accounted for Leber hereditary optic neuropathy (LHON) have been found to be associated with disruption of glutamate transport resulting in glutamate excess and thus excitotoxicity[179].

N-Methyl-D-aspartate (NMDA), the agonist molecule that binds selectively to NMDARs, has been extensively used in the experimental model of NMDA-induced retinal excitotoxicity, which is an acute form of retinal injury. NMDA is delivered intravitreally in the range of nanomolar concentrations and results in severe and rapid cell death of RGCs and the inner retina. NMDA-induced retinal excitotoxicity upregulates tumor necrosis factor α (TNFα) expression and downstream signalling of its death receptor TNFR[180–182] and has been extensively correlated with endoplasmic reticulum (ER) stress[182–184]. However, deficiency in proteins downstream of the above-mentioned pathways did not offer neuroprotection in RGCs according to a study published last year[185], similarly to the results following administration of a pan-caspase inhibitor (discussed in details earlier). Recently, high-mobility group box-1 (HMGB1), a damage-associated molecular pattern (DAMP) molecule secreted only from necrotic cells, has also been found to be involved in NMDA-mediated retinal cell death[186] and to induce nuclear factor kappa B (NF-kB) activation and NF-kB-mediated excitotoxicity[187], confirming unpublished data showing that administration of an inhibitor of programmed necrosis (necrostatin-1, mentioned analytically above) results in significant reduction of $TUNEL^+$ RGCs and cells of the inner retina (Tsoka, P. *et al*., unpublished data, Angiogenesis Lab, Massachusetts Eye and Ear Infirmary, Harvard Medical School). Furthermore, it has been reported that NMDA-mediated injury activates resident microglia [188] and Müller glial cells [189] and mediates $CD45^+$ cell infiltration [190] in the rodent retina.

Inhibition of NMDARs has been neuroprotective in numerous *in vivo* models of experimental RGCs' death[191–196]. However, unfortunately, one of the biggest failures of neuroprotection thus far was a clinical trial of memantine (a well-studied antagonist of NMDARs) for treatment of glaucoma. The second Phase III trial concluded after 9 years and $80 million dollars that there were no significant benefits of memantine over placebo[197].

Therefore, understanding the mechanisms of NMDA-mediated cell death and identifying new potential therapeutic targets could be particularly beneficial for many neurological and retinal disorders in which excitotoxicity is considered to be contributory in their pathology.

Materials and Methods

2.1 Animals

All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Massachusetts Eye and Ear Infirmary. C57BL/6 male mice (7-10 weeks) were purchased from Charles River Laboratories (Wilmington, MA, USA) and had free access to food and water in an air-conditioned room with a 12-hour light/12-hour dark cycle.

2.2 Experimental Model of Retinal Detachment (RD)

Thus far, the vast majority of the experimental models for RD result in a peak of photoreceptor cell death 3 days post detachment and in reattachment of the retina at 7 days[42,50]. For all the experiments of this study we have used a recently modified experimental model that results in a bullous RD of approximately three quarters of the neuroretina with the peak of cell death at 24 hours post RD[137,198]. Moreover, with the usage of surgical glue at the conjunctival incision reattachment of the retina is prevented for at least 30 days post detachment[198]. Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg, Ketaved; Ketamine HCL 100 mg, Vedco Inc., Saint Joseph, MO, USA) and xylazine (6 mg/kg, Anased Injection 20 mg; Lloyd Inc., Shenandoah, IA, USA) and proparacaine drops (0.5% Proparacaine Hydrochloride Ophthalmic Solution; Sandoz Inc., Princeton, NJ, USA) were also applied for topical anesthesia. Pupils were dilated with a topical applied mixture of phenylephrine (5%) and tropicamide (0.5%) (Massachusetts Eye and Ear Infirmary Pharmacy, Boston, MA, USA). Next, a conjunctival incision was made over the temporal aspect of the eye and a sclerotomy was created with a 30-gauge needle with the bevel pointed up approximately 3-4 mm to the limbus. Subsequently, a corneal paracentesis was made with the same needle but with the bevel pointed down to lower intraocular pressure. Finally, a 10-μl syringe (NANOFIL; WPI,

Sarasota, FL, USA or Hamilton, 701RN SYR, #7635-01; Hamilton Company, Reno, NV, USA) connected to a 34-gauge needle (34 G beveled NanoFil needle, #NF34BV-2; WPI, Sarasota, FL, USA) or to a 33-gauge needle (Hamilton Custom Needles: Length: 10.00mm/Point Style: 4/Angle: 20, #7803-05; Hamilton Company, Reno, NV, USA) respectively was inserted with the bevel pointed down into the subretinal space and 4 µl of 1% sodium hyaluronate (Provisc; Alcon, Fort Worth, TX, USA) were injected gently to detach the retina from the underlying retinal pigment epithelium (RPE). Approximately 60% of the temporal-nasal neurosensory retina was detached. At the end of the procedure, cyanoacrylate surgical glue (WebglueTM; Patterson Companies, Mendota Heights, MN, USA) was applied on the scleral wound to prevent leaking and keep the conjunctiva attached to the original position. Special care was given to avoid hitting the lens. Eyes with subretinal hemorrhage or cataract were excluded from the analysis. Antibiotic ointment (Bacitracin Zinc Ointment; Fougera Pharmaceuticals Inc, Melville, NY, USA) was applied topically as a last step to prevent microbial infection.

2.2 Experimental Model of N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity

Mice were anesthetized with an intraperitoneal injection of Tribromoethanol (125 mg/kg, Avertin; A stock solution of 100% Tribromoethanol -Avertin- was prepared by mixing 2,2,2-Tribromoethanol in 10 ml of 2-Methyl-2-butanol, #T48402; and #240486; respectively, MilliporeSigma, Burlington, MA, USA), followed by proparacaine eye drops (0.5% Proparacaine Hydrochloride Ophthalmic Solution; Sandoz Inc., Princeton, NJ, USA) for topical anesthesia. Pupils were dilated by instillation of 5% phenylephrine and 0.5% tropicamide eye drops (Massachusetts Eye and Ear Infirmary Pharmacy, Boston, MA, USA). Consequently, a conjunctival incision was made temporally and a sclerotomy was created with a 30-gauge needle with the bevel pointed up 3-4 mm from the limbus. A 33-gauge needle (Hamilton Custom Needles: Length: 10.00mm/Point Style: 4/Angle: 20, #7803-05; Hamilton Company, Reno, NV, USA) connected to a 10-µl Hamilton syringe (Hamilton, 701RN SYR, #7635-01; Hamilton Company, Reno, NV, USA) was then inserted with the bevel pointed down into the intravitreal cavity and 2 µl of 100 nmoles of NMDA (#M3262; MilliporeSigma, Burlington, MA, USA) in phosphate buffer saline (PBS) were injected slowly into the intravitreal space. To avoid drug reflux the needle remained into the vitreal cavity for 10 sec

and then was removed slowly. Special care was given to avoid hitting the lens. Eyes with subretinal hemorrhage or cataract were excluded from the analysis. Antibiotic ointment (Bacitracin Zinc Ointment; Fougera Pharmaceuticals Inc, Melville, NY, USA) was applied topically as a last step to prevent microbial infection. NMDA stock solution (100 mM) was prepared in water for injection (WFI) and kept at -20 °C.

2.4 BNN27 Injections

BNN27 was obtained from Bionature E.A. Ltd (Nicosia, Cyprus) and kept at -20 ℃. The stock solution (150 mg/ml) was prepared by diluting 60 mg of BNN27 in 400 μl of absolute ethanol at 57-60 ℃ until the solution was clear. Prior to injection, the alcoholic solution of BNN27 was added to heated WFI (37 ℃) and administered to the animal immediately (due to increased coagulation in water), intraperitoneally, with a 25-gauge needle (due to decreased solubility in water). Animals that underwent experimental RD received either one injection of BNN27 (200 mg/kg, diluted in 6% absolute ethanol in water)/vehicle (6% absolute ethanol in water) one-hour post RD or seven injections of BNN27 (10, 50, 100 or 200 mg/kg, diluted in 6% absolute ethanol in water)/vehicle (6% absolute ethanol in water) starting one-hour post RD and then administered once daily. Animals that received an intravitreal injection of NMDA, did also receive one injection of BNN27 (40, 100 or 200 mg/kg, diluted in 6% absolute ethanol in water) or one injection of vehicle (6% absolute ethanol in water) one hour later (treated vs. control groups respectively).

2.5 TUNEL (TdT-dUTP terminal nick-end labeling) assay

Mice were euthanized 24 hours or 7 days post RD or NMDA injection and eyes were enucleated, embedded in O.C.T. Compound (Tissue-Tek #4583; Sakura Finetek, Torrance, CA, USA) and fresh-frozen at -80 ℃. Serial sections were cut in the sagittal plane at 10 μm-thickness on a cryostat (Leica CM1850; Leica Biosystems, Buffalo Grove, IL, USA) and fixed in 4% paraformaldehyde (PFA), followed by TUNEL assay analysis according to the manufacturer's protocol, omitting post-fixation (ApopTag Fluorescein In Situ Apoptosis Detection Kit #S7110; MilliporeSigma, Burlington, MA, USA). Finally, sections were counterstained with TO-PRO®-3 Iodide (642/661) (Life Technologies #T3605; Thermo Fisher Scientific, Waltham, MA) and mounted with Fluoromount-G (SouthernBiotech, Birmingham,

ALA, USA). Images were taken with an upright AXIO Imager.M2 Zeiss fluorescence microscope and were analyzed using the Zeiss ZEN software (Carl Zeiss Inc., Thornwood, NY, USA).

2.6 Immunofluorescence

Animals were euthanized 24 hours post RD or NMDA intravitreal administration, eyes were enucleated and serial sections were taken as described above. Subsequently, sections were fixed either in 4% PFA, blocked with 5% normal goat serum (NGS) and incubated overnight at 4 °C with anti-Vimentin (1:200, Millipore #AB5733; MilliporeSigma, Burlington, MA, USA) and anti-Glial Fibrillary Acidic Protein (GFAP) antibodies (1:200, Dako #Z0334; Agilent Technologies, Santa Clara, CA, USA) in 0.1% Triton-X-100 in PBS to study retinal gliosis or fixed in acetone, blocked in 5% milk and incubated overnight at 4 °C with anti-CD11b antibody (1:50, BD Pharmingen #550282; BD Biosciences, San Jose, CA, USA) in PBS to study immune cell infiltration or blocked with 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with anti-inducible nitric oxide synthase (iNOS), anti-Arginase-1 and anti-F4/80 (1:200, #ab15323; #ab60176; and #ab16911; respectively, Abcam, Inc., Cambridge, MA, USA) in 0.1% Triton-X-100 in Tris-buffered saline (TBS) to study M1/M2 macrophage polarization. Following the primary antibody incubation, the sections were stained with either goat anti-chicken 647 or goat anti-rabbit 488 or goat anti-rat 488 or donkey anti-goat 568 (1:500, Alexa Fluor® 647 goat anti-chicken #A-21449; Alexa Fluor® 488 goat anti-rabbit #A-11034; Alexa Fluor® 488 goat anti-rat #A-11006; Alexa Fluor® 568 donkey anti-goat #A-11057; respectively, Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). Finally, sections were counterstained with TO-PRO™-3 Iodide (642/661) (Life Technologies #T3605; Thermo Fisher Scientific, Waltham, MA) or DAPI and mounted as described above. Images were taken with an upright AXIO Imager.M2 Zeiss fluorescence microscope and were analyzed using the Zeiss ZEN software (Carl Zeiss Inc., Thornwood, NY, USA).

2.7 Laser Capture Microdissection (LCM)

Mice were euthanized 24 hours post RD, eyes were enucleated, embedded in O.C.T. Compound (Tissue-Tek #4583; Sakura Finetek, Torrance, CA, USA) and fresh-frozen at -80 ℃. Eyes were then cut in the sagittal plane at 20 μm-thickness on a cryostat (Leica CM1850; Leica Biosystems, Buffalo Grove, IL, USA) and serial sections were collected on polyethylene terephthalate-membrane (PET) frame slides (PET FrameSlide #0010, steel frames, RNase-free, material number #11505190; Leica Microsystems, Wetzlar, Germany). Sections were fixed in 75% ethanol (30 sec), washed with nuclease-free water (30 sec), stained with 0.02% toluidine blue solution for 20 sec and washed again as described above. Finally, sections were dehydrated with 75%, 95% and 100% ethanol (30 sec, 30 sec and 2 x 30 sec respectively). LCM was performed with the Leica LMD7000 system and LMD application version 7.5 (Leica Microsystems, Wetzlar, Germany). Photoreceptors' layer was cut by laser and collected into 0.5 ml tubes containing RNAlater stabilization solution (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

2.8 RNA extraction and RT-PCR

RNA extraction was achieved with RNeasy plus micro kit (Qiagen, Germantown, MD, USA) according to manufacturer's protocol. cDNA was synthesized with SuperScript III Reverse Transcriptase and Oligo$(dT)_{20}$ Primer following manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was carried out by StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Reactions were performed with TaqMan Fast Universal PCR Master Mix, no AmpErase UNG (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan primers [18s rRNA: Mm03928990_g1; TrkA: Mm01219406_m1; $p75^{NTR}$: Mm00446296_m1; TaqMan Gene Expression Assay (FAM), Thermo Fisher Scientific, Waltham, MA, USA)]. The relative quantity of mRNA expression was calculated by ΔΔ Ct method normalized to 18s rRNA as endogenous control.

2.9 Western Blotting

Animals were euthanized 24 hours post RD or NMDA injection, retinas were dissected and immediately immersed in ice-cold lysis buffer containing 20 mM NaHEPES, 20 mM KCl,

20 mM NaF, 20 mM glycerophosphate, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% Triton-X-100 and a cocktail of protease inhibitors (cOmplete, Mini #11836170001; Roche, MilliporeSigma, Burlington, MA, USA). Total retinal lysates (each lysate contained two retinas) were sonicated (20% amplitude, 5 seconds, 2 times at 4 °C) and centrifuged (17,000 x g, 20 minutes at 4 °C). Supernatants were electrophoresed onto 4-12% Bis-Tris polyacrylamide gels (NuPage #NP0321; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and proteins were transferred on a 0.45 μm PVDF membrane (Immobilon-P #IPVH00010; Millipore, MilliporeSigma, Burlington, MA, USA). After blocking with 5% BSA in 1% Triton-X-100-TBS the membranes were incubated overnight at 4 °C with primary antibodies [TrkA (1:1500, #ab76291; Abcam, Cambridge, MA, USA) phospho-TrkA (Tyr490), p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Akt, phospho-Akt (Ser473) and β-actin (1:1000, #9141; #4695; #4370; #4691; #4060; #4970; respectively, Cell Signaling, Danvers, MA, USA)]. Following primary antibody incubation, the membranes were incubated with HRP-labeled secondary antibodies. Bands were detected by a chemiluminescent reagent (Amersham ECL Select Western Blotting Detection Reagent #RPN2235; GE Healthcare Life Sciences, Chicago, IL, USA) and images were taken with ChemiDoc MP (Bio-Rad Laboratories, Hercules, CA, USA).

2.10 Evaluation of Outer Nuclear Layer (ONL)/Inner Nuclear Layer (INL) Ratio

Mice were euthanized 7 days post RD, eyes were enucleated and serial sections were taken as described above. Following fixation in 4% PFA, sections were stained with Hematoxylin solution, Gill No. 2, counterstained with 0.25% Eosin Y solution and mounted with VectaMount Permanent Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Images were taken and analyzed as described previously.

2.11 Quantification Analysis

2.11.1 Quantification Studies in RD

For the quantification of TUNEL$^+$ cells each section was examined under a 20x/0.8 lens (Zeiss PLAN-APOCHROMAT, Carl Zeiss Inc., Thornwood, NY, USA). To evaluate the TUNEL$^+$ cell density, the total number of TUNEL$^+$ cells in the ONL was counted and the area (of the ONL) was measured by Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). It has been previously reported that the center of RD had less variability of TUNEL$^+$ cells, therefore sections were collected around 1000 μm from the injection site. Shrunk part of the retina was excluded from the counting because mechanical stress can accelerate photoreceptor cell death. The average of two parts of the retina, one from either side of the detached retina, was calculated as the representative TUNEL$^+$ photoreceptor cell density per section.

For the quantification of CD11b$^+$ cells each section was examined under a 10x/0.3 lens (Zeiss EC-PLAN NEOFLUAR, Carl Zeiss Inc., Thornwood, NY, USA). To calculate the CD11b$^+$ cell density, the total number of CD11b$^+$ cells in the retina and in the subretinal space was counted and the whole area (retina and subretinal space) was measured by Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

For the calculation of GFAP and Vimentin intensity, each section was examined under a 20x/0.8 lens (Zeiss PLAN-APOCHROMAT, Carl Zeiss Inc., Thornwood, NY, USA). To assess the intensity per area, the gray mean value and the area of interest [ganglion cell layer (GCL) and inner plexiform layer (IPL)] were calculated and measured by Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

For the evaluation of the ONL/INL ratio, the outer nuclear layer (ONL) and the inner nuclear layer (INL) thickness of the retina was measured by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) at 2 points at each section and ONL/INL ratio was calculated.

The average of three consecutive sections (with a step of 150 μm) was estimated as the representative measurement of each eye.

2.11.2 Quantification Studies in NMDA-Induced Retinal Excitotoxicity

For the quantification of TUNEL$^+$ cells each section was examined under a 20x/0.8 lens (Zeiss PLAN-APOCHROMAT, Carl Zeiss Inc., Thornwood, NY, USA). To evaluate the

TUNEL$^+$ cell density, the total number of TUNEL$^+$ cells in all three nuclear layers of the retina (ganglion cell layer-GCL, INL, ONL) was quantified and TUNEL$^+$ cell density was calculated as the ratio of TUNEL$^+$ cells/mm of the length of the layer of interest, which was measured by using Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). In order to include the area of optic nerve in the analysis, sections were collected around 1500 μm from the injection site. Shrunk part of the retina was excluded from the counting because mechanical stress can accelerate cell death. For each layer of interest, the average of two parts of the retina peripapillary, was calculated as the representative TUNEL$^+$ cell density per layer per section.

For the quantification of CD11b$^+$ cells each section was examined under a 10x/0.3 lens (Zeiss EC-PLAN NEOFLUAR, Carl Zeiss Inc., Thornwood, NY, USA). To calculate the CD11b$^+$ cell density, the total number of CD11b$^+$ cells in the retina, in the vitreous and in the optic nerve was counted and the whole area (retina, vitreous, optic nerve) was measured by Image J software using Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

For the calculation of GFAP and Vimentin intensity, pictures were examined and calculations were made as described above.

The average of three consecutive sections (with a step of 150 μm) was estimated as the representative measurement of each eye.

2.12 Statistical Analysis

Statistical analysis was performed using Student's t-test for all figures, except Fig. 3.1.5 A and 3.2.1 B in which one-way ANOVA was performed, followed by post analysis with Tukey HSD test. Data are presented as the mean value ± SEM. The significance level was set at $P < 0.05$ (* in figures), $P < 0.01$ (** in figures) and $P < 0.001$ (*** in figures).

2.13 Data Availability

The datasets generated and/or analyzed during the current Thesis are available from the author and/or the supervisor of this study on reasonable request.

Results

3.1 BNN27 and Retinal Detachment (RD)

3.1.1 BNN27 reduces TUNEL$^+$ photoreceptors following RD

To determine the potential neuroprotective effect of BNN27 on the photoreceptors after experimental retinal detachment (RD), we examined the RD-induced cell death in the outer nuclear layer (ONL) by TUNEL assay. Photoreceptor cell death peaks at 24 hours post RD and wanes by day 7[41,137,139]. A single intraperitoneal injection of BNN27 (200 mg/kg), 60 minutes post RD, decreased TUNEL$^+$ cells by 65% on day 1 (RD + Vehicle: 1068 ± 99 cells/mm^2, RD + BNN27: 346 ± 102 cells/mm^2, $^{**}P < 0.01$, $n = 15$) but did not result in statistically significant difference on day 7, n = 6-7 (Fig. 3.1.1 A and B).

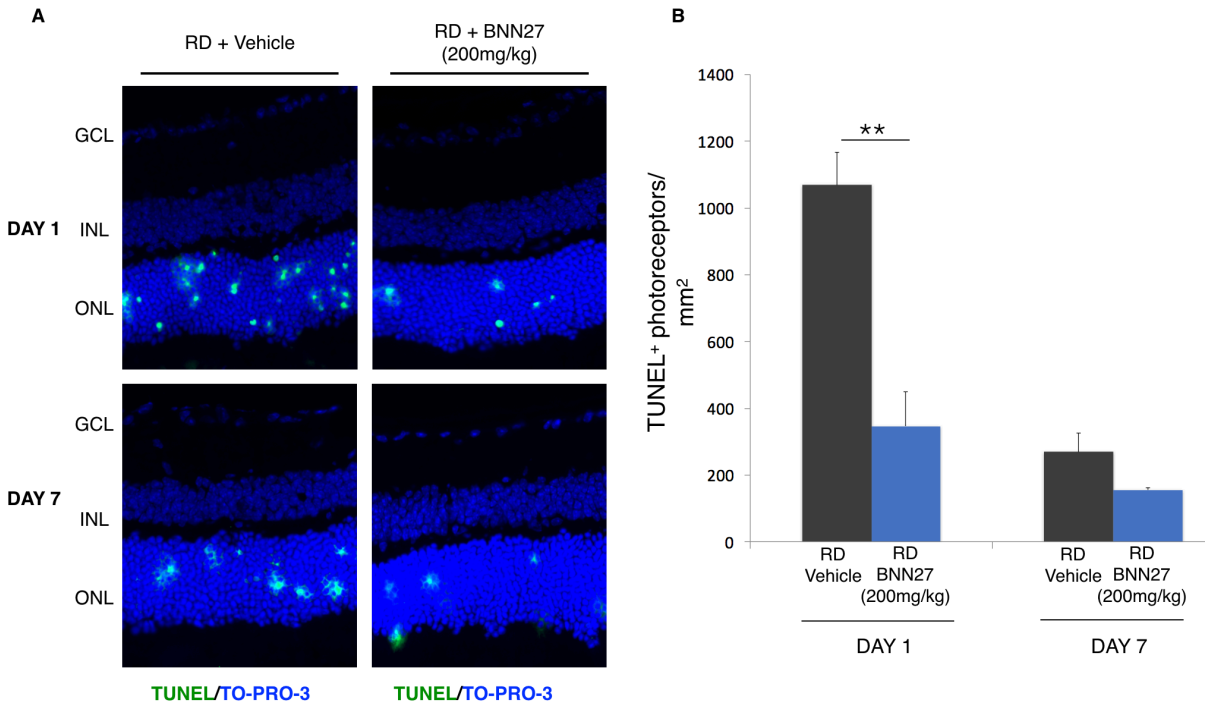


Figure 3.1.1 Effect of BNN27 on RD-induced cell death. (**A**) TUNEL (green) and TO-PRO-3 (blue) staining of retinal sections from untreated (vehicle) and BNN27-treated eyes,

24 hours and seven days post RD. (**B**) 24 hours post RD, BNN27-treated group showed significantly lower numbers of TUNEL$^+$ photoreceptors (cells/mm^2), $n = 15$, $^{**}P < 0.01$. On the contrary, 7 days post RD, BNN27 treatment did not reach a statistically significant level of reduction of TUNEL$^+$ photoreceptors (cells/mm^2), $n = 6\text{-}7$. Scale bar: 100µm. The graph shows mean ± SEM. RD; Retinal Detachment, ONL; Outer Nuclear Layer, INL; Inner Nuclear Layer, GCL; Ganglion Cell Layer.

3.1.2 BNN27 induces macrophage/microglia infiltration post RD

Retinal detachment promotes an accumulation of CD11b$^+$ macrophages and activated microglia in the retina and more specifically in the subretinal space[41,70,137,139,142,199]. We have previously reported that in our model the peak of the infiltration of CD11b$^+$ cells into the subretinal space coincides with the peak of photoreceptor cell death 24 hours after RD[137]. Thus, we examined the effect of BNN27 on macrophage/microglia infiltration by detecting the macrophage/microglial marker CD11b by immunofluorescence 24 hours post RD. BNN27-treated group displayed a significant increase of the CD11b$^+$ cells compared to vehicle-treated (RD + BNN27: 52 ± 7 cells/mm^2 vs. RD + Vehicle: 27 ± 5 cells/mm^2, $^{*}P < 0.05$, $n = 12$, Fig. 3.1.2 A and C). In addition to individual CD11b$^+$ cells, clusters of CD11b$^+$ cells were also found in both groups. Again, BNN27-treated animals had more and larger clusters (Fig. 3.1.2 B).

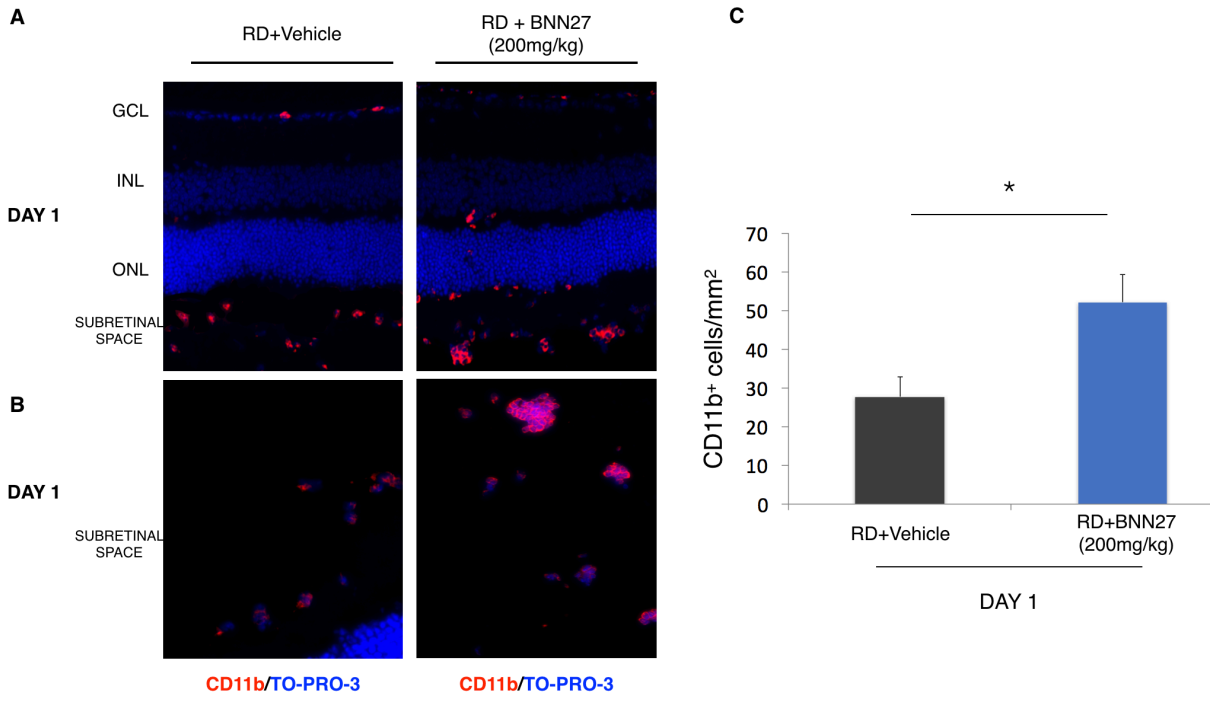


Figure 3.1.2 Effect of BNN27 on RD-induced macrophage/microglia infiltration. (**A**) CD11b (red) and TO-PRO-3 (blue) staining 24 hours post RD, scale bar: 500 µm (**B**) CD11b (red) and TO-PRO-3 (blue) staining. Large aggregates of $CD11b^+$ cells in the subretinal space of the BNN27 treated eyes, scale bar: 100 µm (**C**) Infiltration of $CD11b^+$ cells was significantly higher in the group which received the BNN27 treatment 24 hours after RD, $n = 12$, $^*P < 0.05$. The graph shows mean ± SEM. RD; Retinal Detachment, ONL; Outer Nuclear Layer, INL; Inner Nuclear Layer, GCL; Ganglion Cell Layer.

3.1.3 BNN27 increases RD-induced gliosis

RD triggers the activation and proliferation of glial cells, a response known as reactive gliosis[200]. Reactive gliosis is characterized by morphological alterations in astrocytes and Müller cells and by increased expression of glial fibrillary acidic protein (GFAP) and vimentin[146,200–202]. To investigate the action of BNN27 on RD-induced gliosis, retinal sections were stained with anti-GFAP and anti-vimentin antibodies. Both GFAP and vimentin intensity/mm^2 were significantly increased in the BNN27-treated group 24 hours post detachment (RD + Vehicle: 209 ± 38 mean gray value/mm^2, RD + BNN27: 487 ± 102 mean gray value/mm^2, $n = 9$, $^*P < 0.05$ for GFAP and RD + Vehicle: 578 ± 20 mean gray value/mm^2,

RD + BNN27: 952 ± 67 mean gray value/mm^2, $n = 9$, ***$P < 0.001$ for vimentin, Fig. 3.1.3 A, B and C).

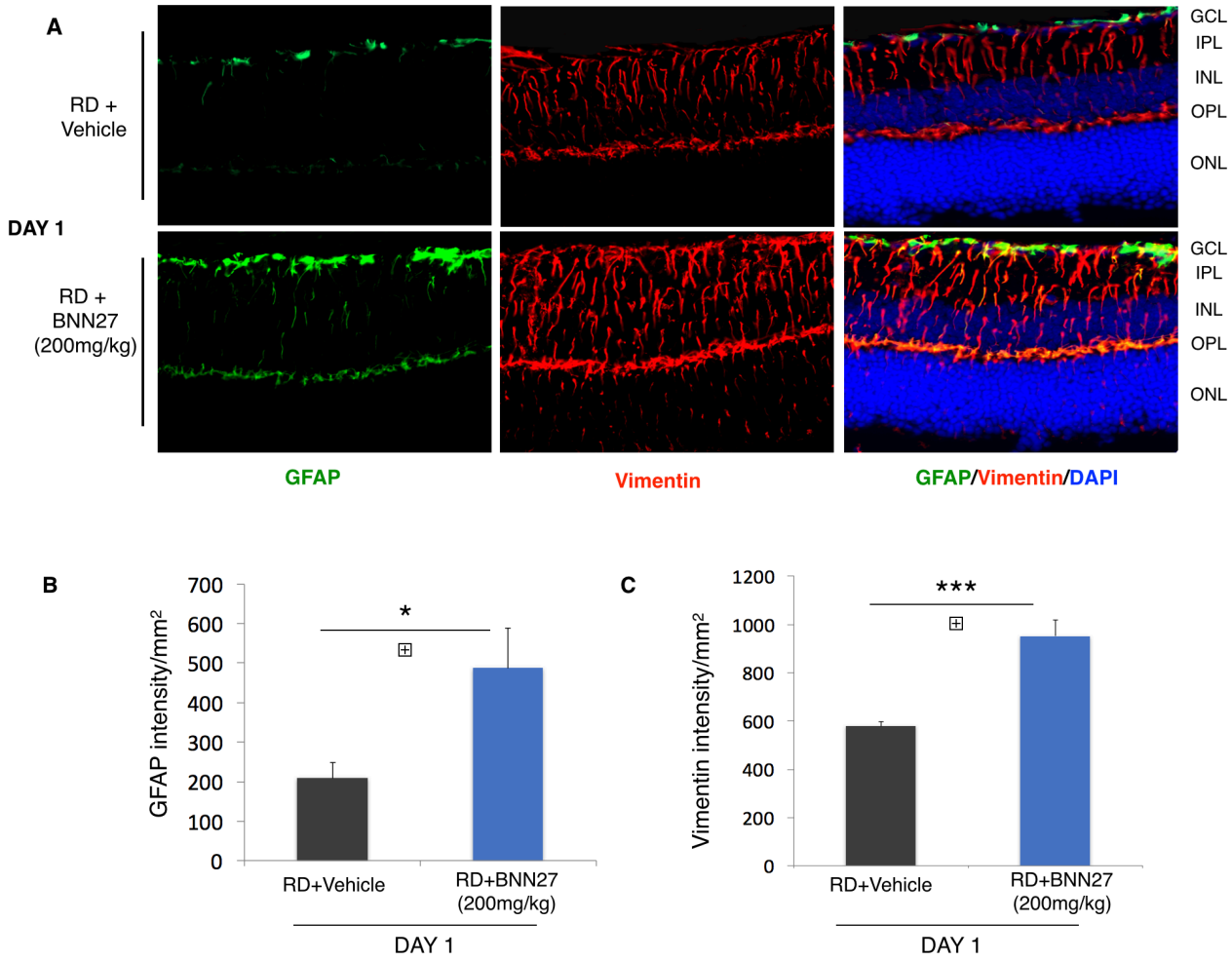
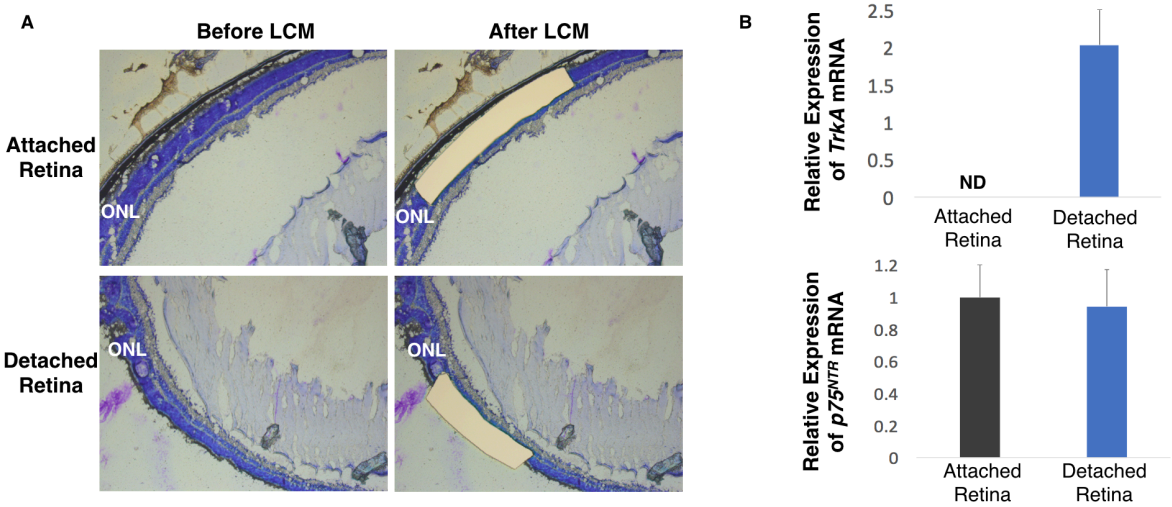


Figure 3.1.3 Effect of BNN27 on RD-induced gliosis. (**A**) Representative images of vimentin (red), GFAP (green) and DAPI (blue) staining 24 hours post RD. (**B** and **C**). GFAP and vimentin Intensity were significantly higher in the BNN27-treated group 24 hours post RD (*$P < 0.05$ and *$P < 0.001$ respectively), $n = 9$. Scale bar: 100 µm. The graphs show mean ± SEM. RD; Retinal Detachment, ONL; Outer Nuclear Layer, OPL; Outer Plexiform Layer, INL; Inner Nuclear Layer, IPL; Inner Plexiform Layer, GCL; Ganglion Cell Layer.

3.1.4 BNN27 does not induce TrkA phosphorylation, although the mRNA levels of the receptor are elevated in the detached photoreceptors.

NGF has been extensively studied in retinal degenerations, however, the expression of its receptors, TrkA and p75^{NTR} in healthy and degenerated photoreceptors has not been fully elucidated (for review[82]). To clarify this point, we examined the mRNA levels of TrkA and p75^{NTR} in the outer nuclear layer (ONL) in both healthy and detached retina by laser capture microdissection (LCM) (Fig. 3.1.4 A). TrkA mRNA was not detected in the ONL before injury while it was robustly increased 24 hours post RD (Fig. 3.1.4 B, n = 4-5). On the contrary, there was no significant change in the mRNA levels of p75^{NTR} before and after injury (Fig. 3.1.4 B, n = 4-5), indicating that at least in the photoreceptors p75^{NTR} does not play an instrumental role following RD. BNN27 selectively binds to TrkA receptor leading to its phosphorylation and promoting neuroprotection in a TrkA-dependent manner[128]. We have previously shown that phosphorylation, thus activation of TrkA, is elevated following experimental RD[137]. To assess if BNN27 can further upregulate TrkA activation, we examined the phosphorylation of the receptor on Y^{490} residue and the downstream signaling which leads to neuronal survival and differentiation in BNN27-treated and untreated detached retinas. Interestingly, phosphorylation of TrkA was not significantly increased in the BNN27-treated group and consequently neither was phosphorylation of Akt or Erk (phosphorylated-to-total ratio, Fig. 3.1.4 C, n = 4).



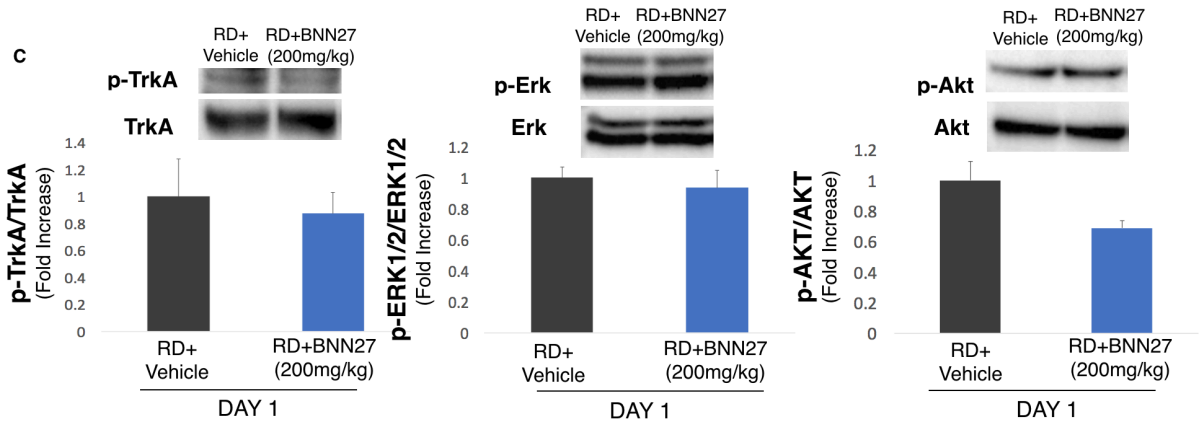
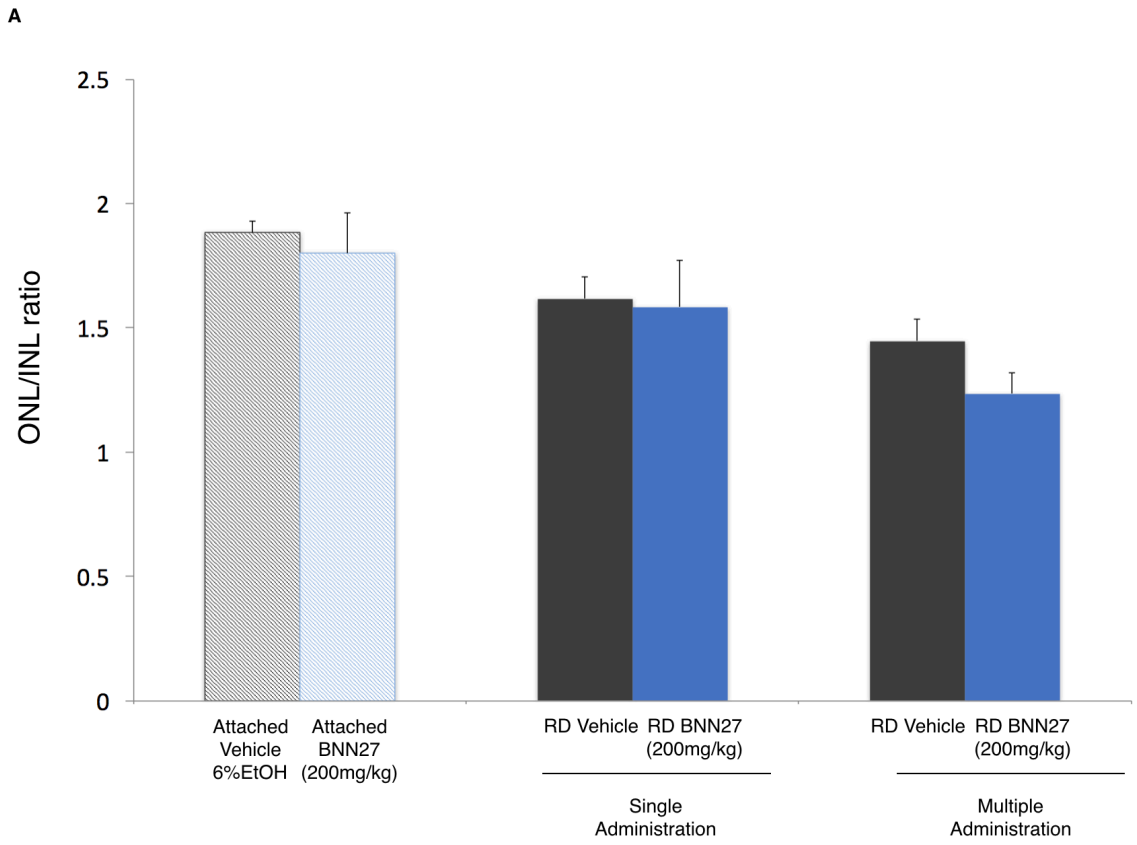


Figure 3.1.4 Expression of TrkA and p75^{NTR} in photoreceptors and effect of BNN27 on TrkA phosphorylation and downstream signaling following RD. **(A)** Representative pictures of retinal sections before and after cutting the ONL with LCM from attached and detached retina. Nuclei were stained with toluidine blue. **(B)** TrkA and p75^{NTR} mRNA expression in the ONL following isolation of the photoreceptors' nuclei with LCM. TrkA mRNA levels were not detected in the attached retina while they were significantly elevated in the detached, *n* = 4-5. On the contrary, p75^{NTR} mRNA levels were not altered before and after injury, *n* = 4-5. **(C)** Western blotting images and densitometry analysis of phosphorylated TrkA, total TrkA, phosphorylated Erk, total Erk, phosphorylated Akt and total Akt of detached retinas between untreated and BNN27-treated eyes. BNN27 did not further induce phosphorylation of TrkA, Erk or Akt, *n* = 4**.** Scale bar: 100μm. The graphs show mean ± SEM. RD; Retinal Detachment, ONL; Outer Nuclear Layer, LCM; Laser Capture Microdissection, ND; Not Detected.

3.1.5 BNN27 does not protect the outer nuclear layer (ONL) thickness.

Given the opposing effects of BNN27 on TUNEL positivity, inflammatory/gliotic markers and lack of activation of TrkA downstream signalling following RD, we wanted to evaluate what is its overall impact on survival of photoreceptor nuclei (ONL) at day 7 post injury. As depicted in Fig. 1, a single systemic administration of BNN27 led to a significant reduction in TUNEL^{+} cells at day 1 post RD but did not prevent the loss of photoreceptors (ONL thickness) by day 7, *n* = 6-7 (Fig. 3.1.5 A). To examine if more frequent administration of BNN27 could lead to rescue of ONL, the experiment was repeated with seven daily administrations of BNN27, *n* = 6-7. However, even the frequent dosing did not lead to rescue of the ONL (Fig.

3.1.5 A). To evaluate further if a different dosing regimen is needed for optimal effects of BNN27, we administered BNN27 at three more doses (10 mg/kg, 50 mg/kg and 100 mg/kg) daily for seven days. However, we were not able to see any differences between the treated and the untreated eyes (n = 6-9 for each of the three groups, Fig. 3.1.5 B).



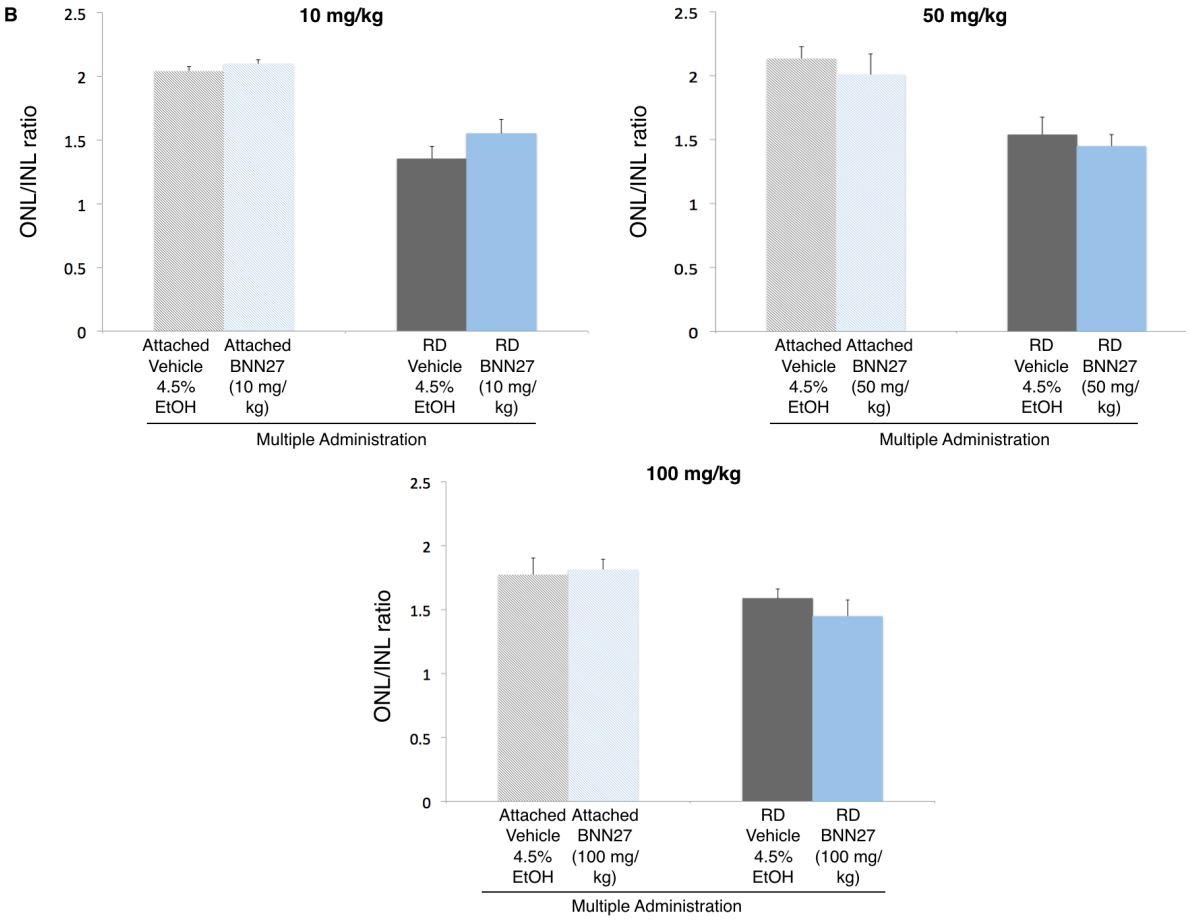
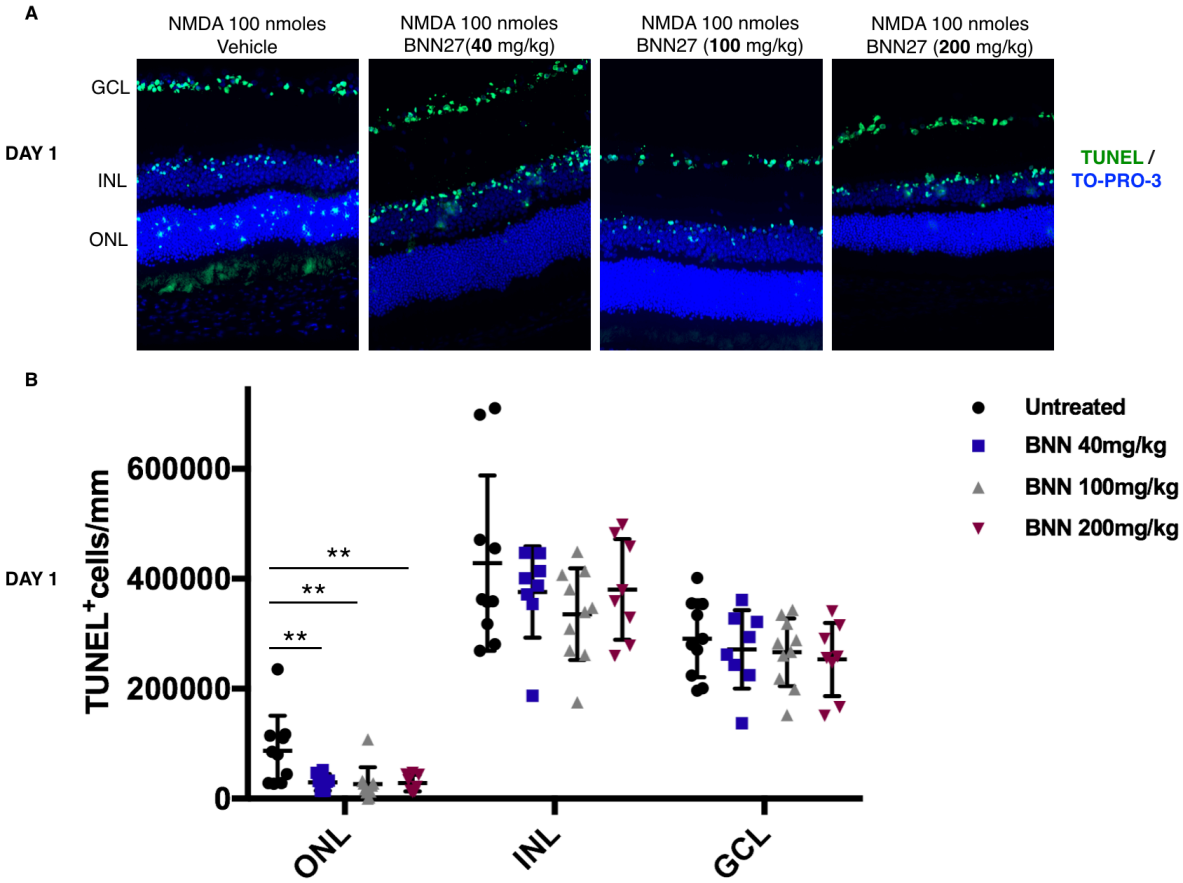


Figure 3.1.5 Effect of BNN27 on outer nuclear layer (ONL) thickness. **(A)** Ratio of ONL/INL in the attached and the detached retina following RD. At day 7, single or multiple (daily administration, 7 injections total) doses of BNN27 (200 mg/kg) were not able to protect the overall thickness of the ONL of the detached retina, *n* = 6-7. **(B)** Ratio of ONL/INL in the attached and the detached retina following RD. At day 7, multiple (daily administration, 7 injections total) doses of BNN27 (10, 50 or 100 mg/kg) were not able to protect the overall thickness of the ONL of the detached retina, *n* = 6-9. The graphs show mean ± SEM. RD; Retinal Detachment, ONL; Outer Nuclear Layer, INL; Inner Nuclear Layer.

3.2 BNN27 and N-methyl-D-aspartate (NMDA) excitotoxicity

3.2.1 BNN27 reduces TUNEL$^+$ photoreceptors but not TUNEL$^+$ RGCs or TUNEL$^+$ cells of the inner retina in NMDA-induced retinal excitotoxicity

An intravitreal injection of 100 nmoles of NMDA resulted in significant cell death of the RGCs and of a small portion of the inner retina and of the photoreceptors at 24 hours. A single intraperitoneal injection of BNN27 in 3 different doses (40, 100 or 200 mg/kg), 60 minutes post NMDA, decreased TUNEL^{+} photoreceptors by an average of 83% on day 1 (NMDA + Vehicle: 93.172 ± 846 photoreceptors/mm, NMDA + BNN27 40 mg/kg: 61.384 ± 109 photoreceptors/mm, $^{**}P < 0.01$, NMDA + BNN27 100 mg/kg: 59.028 ± 432 photoreceptors/mm, $^{**}P < 0.01$, NMDA + BNN27 200 mg/kg: 60.756 ± 88 photoreceptors/mm, $^{**}P < 0.01$, n = 9-11 for all groups) but did not result in statistically significant differences for the RGCs or the inner retina in any of the 3 doses that we examined, n = 9-11 (Fig. 3.2.1 A and B). Given that all 3 doses resulted in similar levels of cell death, we decided to proceed to our experiments with the medium dose (NMDA + Vehicle: 112.957 ± 630 photoreceptors/mm, NMDA + BNN27 100 mg/kg: 49.308 ± 109 photoreceptors/mm, $^{***}P < 0.001$, n =15, Fig. 3.2.1 C).



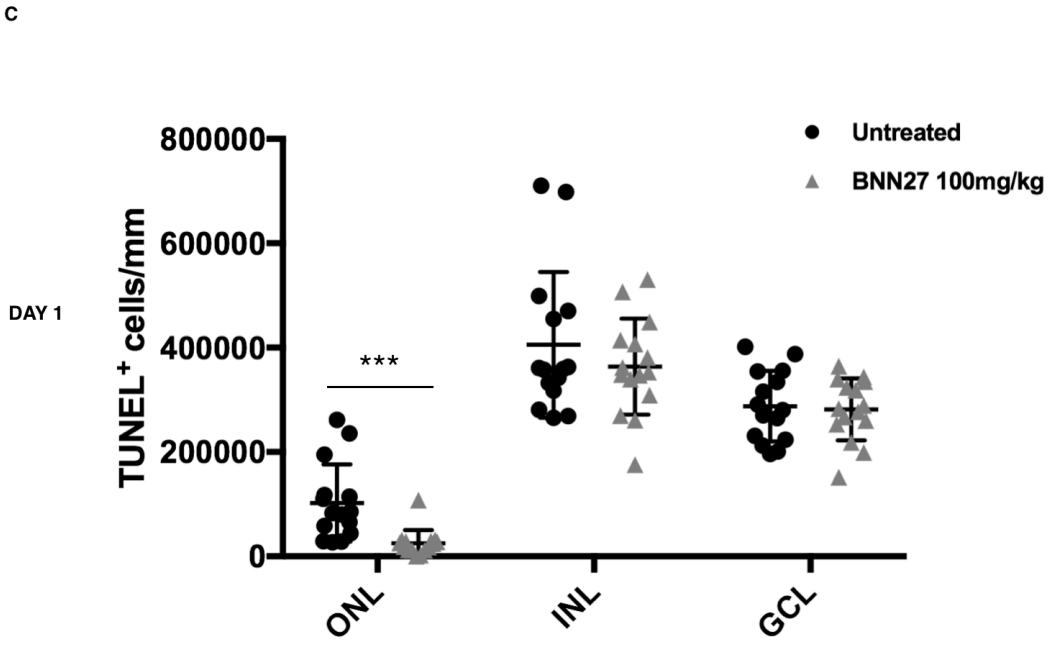


Figure 3.2.1 Effect of BNN27 on NMDA-induced cell death (A) TUNEL (green) and TO-PRO-3 (blue) staining of mouse retinal sections from NMDA (100 nmoles)-treated eyes 24 hours post injury after a single administration of BNN27 40 mg/kg, 100 mg/kg and 200 mg/kg or vehicle. **(B)** Quantification of $TUNEL^+$ cells/mm per retinal layer 24 hours post NMDA injury in BNN27-treated (40 mg/kg, 100 mg/kg and 200 mg/kg) and untreated animals. BNN27 (all 3 doses) resulted in significant reduction of $TUNEL^+$ cell death in the photoreceptors but not in the inner retina or in the RGCs 24 hours after NMDA intravitreal injection (** $P < 0.01$, $n = 9$-11). **(C)** Quantification of $TUNEL^+$ cells/mm per retinal layer 24 hours post NMDA injury in BNN27-treated (100 mg/kg) and untreated animals. BNN27 (100 mg/kg) resulted in significant reduction of $TUNEL^+$ cell death in the photoreceptors but not in the inner retina or in the RGCs (*** $P < 0.001$, $n = 15$). NMDA; N-methyl-D-aspartate, ONL; outer nuclear layer, INL; inner nuclear layer, GCL; ganglion cell layer.

3.2.2 BNN27 does not alter macrophage/microglia infiltration following NMDA-mediated retinal injury

It has been recently reported that NMDA-mediated retinal excitotoxicity recruits macrophage/microglia peripapillary and at the optic nerve head[73]. Therefore, and also because of our findings in experimental RD, we examined the effect of BNN27 (100 mg/kg) on macrophage/microglia infiltration by detecting the macrophage/microglial marker CD11b by immunofluorescence 24 hours post NMDA. However, in this type of acute retinal injury we were not able to detect any differences between the BNN-treated and the untreated group (Fig. 3.2.2 A and B, n = 11-15).

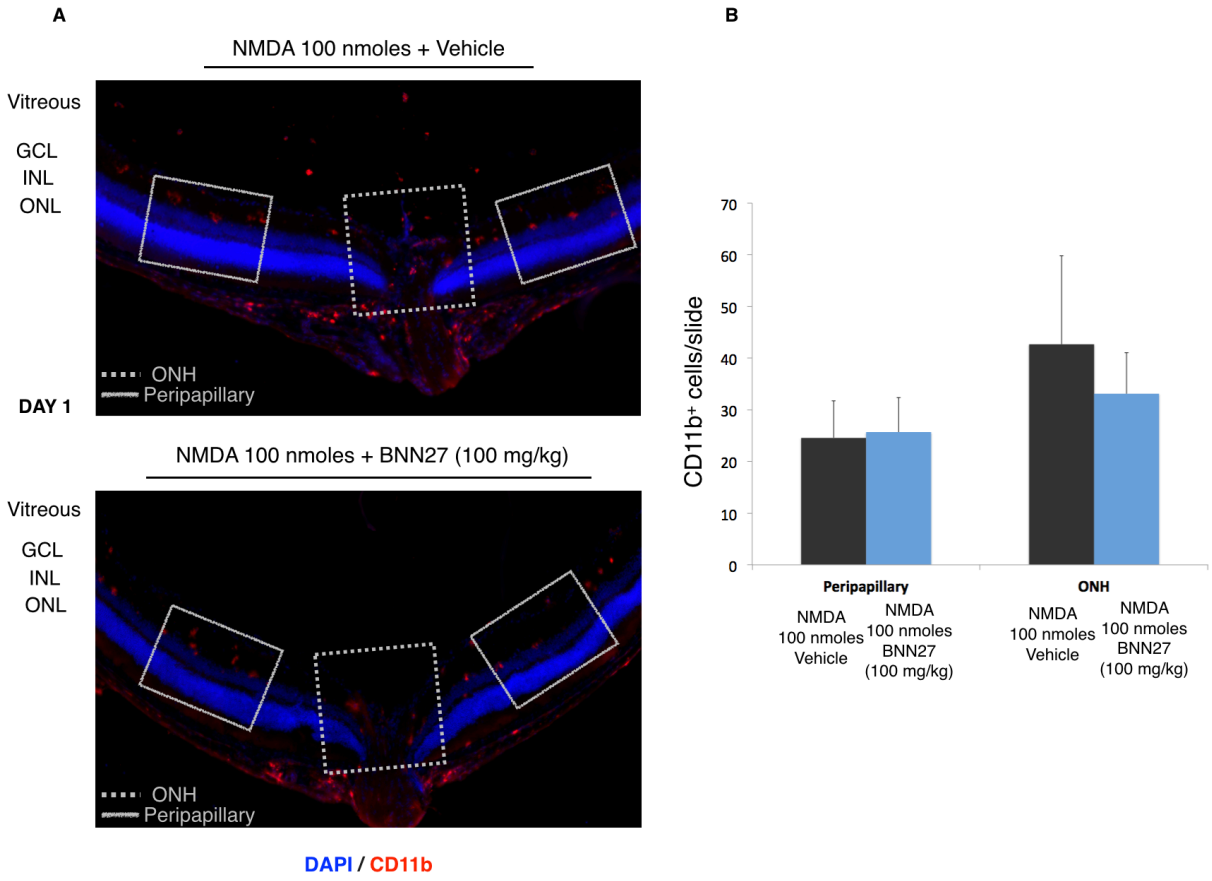


Figure 3.2.2 Effect of BNN27 on NMDA-induced macrophage/microglia infiltration. (**A**) CD11b (red) and TO-PRO-3 (blue) staining 24 hours post NMDA, scale bar: 500 µm (**B**) Quantification of $CD11b^+$ cells/slide peripapillary and at the ONH 24 hours post NMDA injury in BNN27-treated (100 mg/kg) and untreated animals. There were no significant differences in infiltration of $CD11b^+$ macrophage/microglia between the BNN27-treated (100 mg/kg) and the untreated group 24 hours after NMDA injury (n = 11-15). The graph shows mean ± SEM. NMDA; N-methyl-D-aspartate, ONL; Outer Nuclear Layer, INL; Inner Nuclear Layer, GCL; Ganglion Cell Layer, ONH; optic nerve head.

3.2.3 BNN27 does not alter retinal gliosis on NMDA-induced retinal excitotoxicity

NMDA retinal injury has been reported to upregulate Müller glial cells activity. This finding and our data in the experimental model of RD, prompted us to assess the effects of BNN27 on NMDA-induced retinal gliosis. Intravitreal administration of BNN27 resulted in upregulation of gliosis, however BNN treatment did not result in significant differences compared to the untreated group for both GFAP and vimentin (Fig. 3.2.3 A and B, n = 11-15).

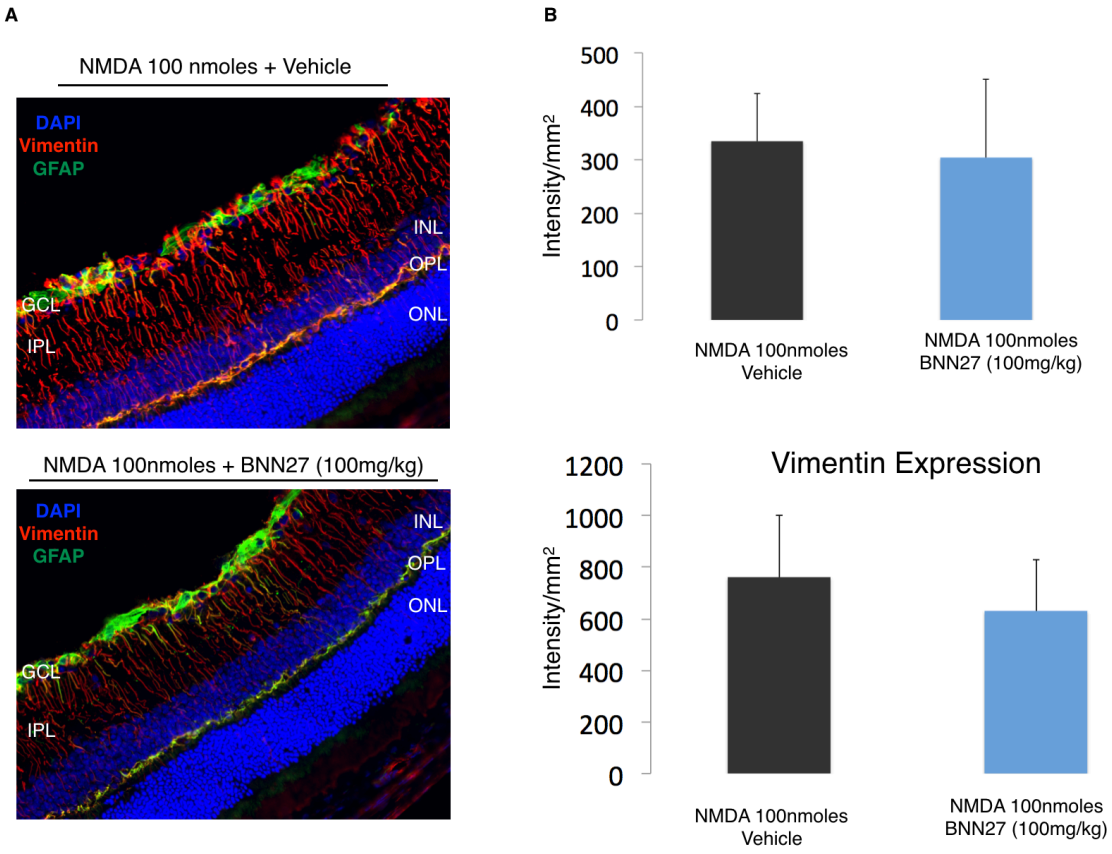


Figure 3.2.3 Effect of BNN27 on NMDA-induced gliosis. (**A**) Representative images of vimentin (red), GFAP (green) and DAPI (blue) staining 24 hours post NMDA. (**B**) GFAP and vimentin intensity were not altered in the BNN27-treated group (100 mg/kg) 24 hours after NMDA injury, n = 11-15. Scale bar: 100 µm. The graphs show mean ± SEM. NMDA; N-methyl-D-aspartate, ONL; Outer Nuclear Layer, OPL; Outer Plexiform Layer, INL; Inner Nuclear Layer, IPL; Inner Plexiform Layer, GCL; Ganglion Cell Layer.

3.2.4 BNN27 does not induce TrkA phosphorylation, although TrkA is downregulated following NMDA injury

Alterations in levels of expression of TrkA and potential phosphorylation as the result of injury, has thus far been extensively-documented in experimental models that result in cell death of the RGCs and the inner retina (for review[82]). However, the expression levels of TrkA following NMDA insult in the retina have not been examined. NMDA-mediated excitotoxicity resulted in significant downregulation of TrkA in the total retina (n = 3-6, $^{**}P < 0.01$, Fig. 3.2.4).

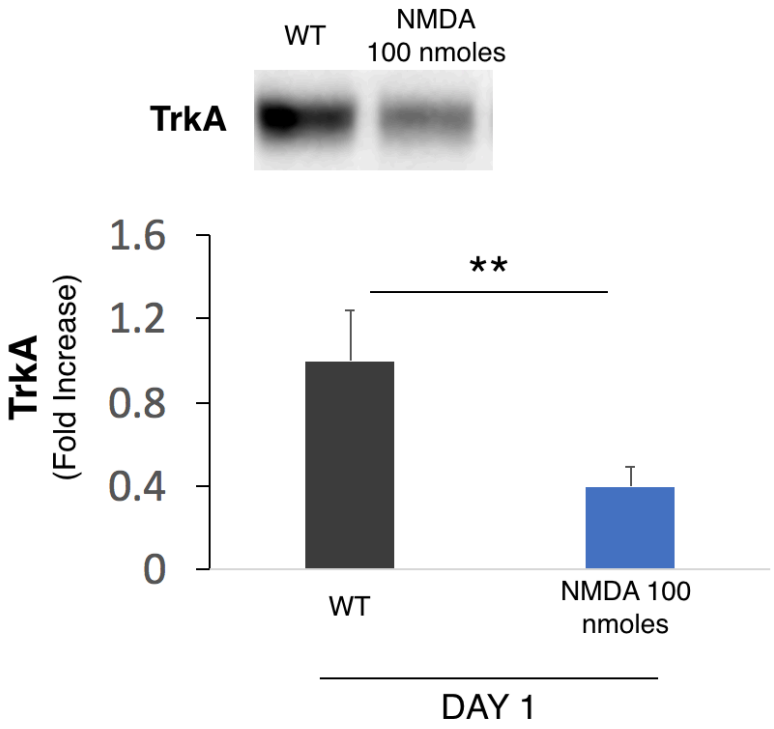


Figure 3.2.4 Expression of TrkA following NMDA-mediated retinal excitotoxicity. Western blotting images and densitometry analysis of total TrkA of healthy retinas and NMDA-treated retinas at 24 hours. Total TrkA was significantly downregulated 24 hours after NMDA insult ($^{**}P < 0.01$, n = 3-6)**.** The graph shows mean ± SEM. WT; wild type, NMDA; N-methyl-D-aspartate.

To further examine the effects of BNN27 on TrkA receptor we examined the phosphorylation of the receptor on Y^{490} residue in BNN27-treated and untreated retinas following NMDA injury. NMDA-mediated injury does not seem to induce TrkA phosphorylation nor does BNN27 administration in the NMDA-injured retina.

Discussion

In the present study, we administered systemically BNN27 in two mouse models of acute retinal degeneration/injury. BNN27 is a spiroepoxy-analogue of dehydroepiandrosterone (DHEA) that binds to and activates tropomyosin receptor kinase A (TrkA), which is also preferentially activated by the neurotrophin nerve growth factor (NGF). The two *in vivo* models that we used are α) the experimental retinal detachment (RD) that results in photoreceptor cell death and b) N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity that results in cell death of the inner retina and the retinal ganglion cells (RGCs). We demonstrated that a single administration of BNN27 alters the levels of cell death in both models as well as inflammation and gliosis in RD but not in NMDA-excitotoxicity. Moreover, we found that the effects of BNN27 in acute retinal injury of either photoreceptors or of the inner retina and the RGCs are not mediated by TrkA signalling and that TrkA receptor is not expressed in healthy photoreceptors. Finally, we showed that BNN27 did not offer neuroprotection in RD-induced damage in the retina regardless of the dose or the paradigm of administration.

Separation of photoreceptors from the underlying/supporting retinal pigment epithelium (RPE) results in photoreceptor cell loss and visual dysfunction and can be seen in many disorders such as rhegmatogenous RD (RRD), age-related macular degeneration (AMD)[203], diabetic retinopathy (DR)[171] and retinopathy of prematurity (ROP)[204]. In case of RD, surgical re-apposition of the retina to the RPE is a well-established therapeutic approach, however, visual acuity is not always restored[205]. Understanding the cellular mechanisms of photoreceptor cell loss will aid in identifying potential therapeutic targets for effective neuroprotection and improved visual function.

In our study a single dose of BNN27 (200 mg/kg) given 60 minutes after RD injury significantly reduced TUNEL$^+$ (Terminal deoxynucleotidyl transferase -TdT- dUTP Nick-End Labeling) photoreceptor cell death at 24 hours (the peak of cell death in this model) but not at 7 days. To the best of our knowledge this is the first time that administration of BNN27 in an in vivo model of neurodegeneration results in significant elimination of TUNEL$^+$ cell death in line with previous studies in which BNN27 reduced TUNEL$^+$ cell death in primary cultures of superior cervical ganglia following NGF deprivation. Conversely, BNN27 did not reduce the

TUNEL$^+$ cell death of the inner retina and the RGCs in the streptozotocin-induced model of diabetic retinopathy in two different paradigms of administration[130]. TUNEL detects extensive DNA degradation by labelling blunt ends of double-stranded DNA fragments. Although it has been long considered as a gold-standard method to identify late apoptosis, it is now widely-accepted that TUNEL detects DNA fragmentation in cells damaged by other cell death modalities as well. Therefore, given that several cell death pathways have been reported in experimental RD[50,60,70,137] and that when one pathway is blocked another one is augmented[50], we do not know what type of cell death BNN27 reduces in our model. On the other hand, BNN27 was able to diminish caspase-3-mediated cell death in primary cultures[128] and in the cuprizone-induced experimental model of multiple sclerosis (MS)[129], as well as, interestingly, in the above-mentioned model of DR[130], suggesting that BNN27 might block only the extrinsic pathway of caspase-dependent cell death. Future studies investigating the effects of BNN27 on programmed necrosis and/or different cell death pathways would be particularly explanatory for the mechanism of action of BNN27.

Because cell death is associated with inflammation, and because activators of TrkA have reported immunomodulatory effects[108] (for review[206]), we examined the effects of BNN27 in the inflammatory response seen after RD by using immunofluorescence for the cell-surface marker CD11b. Systemic administration of BNN27 (200 mg/kg) significantly increased the number of CD11b$^+$ infiltrating macrophages/microglia in the subretinal space and to a lesser extent in the retina. Not only the number of the CD11b$^+$ cells was significantly higher in the treated group but also their distribution was altered with noticeable increase in the presence of large aggregates of CD11b$^+$ cells. It has been previously reported that NGF mRNA levels are elevated following experimental RD[137]. NGF increases microglial migration[207] and can induce macrophage-mediated tumor necrosis factor α (TNFα) production[208], interleukin-1β (IL-1β) secretion and inflammasome activation[209,210]. Our data indicate that BNN27, probably by mimicking NGF or cooperating with the endogenous, enhances the immune response of RD by increasing the numbers of infiltrating macrophages and microglia. Our results are, however, in contrast with two recent studies showing that BNN27 administration can reduce Iba-1$^+$ microglia and pro-inflammatory cytokines in a mouse model of MS or of DR[129,130]. At the same time, BNN27 increased anti-inflammatory cytokine, interleukin-10 and -4 (IL-10 and IL-4 respectively), levels in diabetic retinas[130] and reduce the pro-inflammatory interleukin-6 (IL-6) in microglial cultures[128].

Neuroinflammation plays a pivotal role in many neurodegenerative diseases and retinal detachment is definitely among them. A future direction for our study and for further understanding of the mechanism of action of BNN27 could be a detailed examination of its effect on the different subtypes of inflammatory cells. The M1 versus M2 polarization of macrophages is a switch between neuronal cell death and survival. The classically activated pro-inflammatory macrophages (termed M1) lead to inflammation and neurodegeneration, while alternatively activated anti-inflammatory macrophages (termed M2) lead to neuroprotection (for review[211]). Increased profile of one out of two subtypes has been reported in advanced cases of AMD[212] and thus examination of the subtypes of macrophages following RD is necessary. Therefore, it must be ascertained if the observed increase in the infiltrating cells following BNN27 administration in RD is beneficial or not given that M2 macrophages can induce anti-inflammatory cytokines' production and secretion such as IL-10 and IL-4 (for review[211]) that have been found elevated in experimental DR[130]. Moreover, CD11b and Iba-1 that have been used as markers of detection of immune cells on this and previous studies, are both expressed by macrophage and microglia populations and each marker alone cannot discriminate resident microglia from infiltrating macrophages[213–215] so perhaps BNN27 has an opposite effect on these two populations. Within the last year, new evidence came to light and revealed a neuroprotective role of microglia on experimental RD[143]. By using newly identified markers, exclusively expressed in microglia, it was shown that microglia are rapidly activated following RD and are closely associated with infiltrating macrophages[143]. In addition, it was demonstrated, that when microglia reached the photoreceptor layer, it became responsible of phagocytosis of the dying/injured photoreceptors[143]. Moreover, depletion of microglia resulted in impairment of infiltrating macrophages and attenuated photoreceptor cell death, indicating that microglia has a neuroprotective role in RD[143]. In addition, in a study published also last year, it was reported that NGF can steer microglia into a neuroprotective and anti-inflammatory phenotype[148]. Taken all into account, it could be possible that BNN27 results in an increase in microglia cells instead of increase of macrophages or steer macrophages or microglia or both towards a beneficial phenotype for the retinal neuronal cells and thus decreases $TUNEL^+$ cell death. Extensive further research is necessary in order to characterize the effect of BNN27 on those two distinct types of inflammatory cells, as well as, on other types of immune responses.

Retinal detachment injury results in upregulation of retinal macro-and astroglia (Müller glial cells and astrocytes respectively), a process termed retinal gliosis. Hypertrophy of Müller glia (MG) cells, the major type of glia in the retina that cannot be found anywhere else in the

CNS, has been traditionally related with poor visual acuity, membrane formation and neurodegeneration. Extensive proliferation and dedifferentiation of MG cells can lead to the development of proliferative vitreoretinopathy (PVR), a vision threatening disease secondary to RD, or to massive retinal gliosis (MRG), a rare disease in which the retina is almost completely filled with glial cells causing severe vision loss, secondary to other neurodegenerative cause or trauma. NGF has been found to modulate retinal gliosis and decrease glial fibrillary acidic protein (GFAP) levels in different models of retinal degeneration or injury[109,216,217]. On the other hand, NGF acts as a mitogenic signal for MG cells and thus increases their proliferation and dedifferentiation[217–220], hence the effect of NGF treatment in the injured retina might be detrimental. In our study, intraperitoneal administration of BNN27 significantly increased the production of both GFAP and vimentin, two intermediate filament proteins that are elevated during reactive gliosis and further altered the morphology of the $GFAP^+$ glial cells. On the contrary, BNN27 was able to reduce the GFAP-mediated astrogliosis in experimental DR[130], in which diabetes affects the inner retina, the Müller cells and the retinal ganglion cells (RGCs). Interestingly, photoreceptors, Müller cells and RGCs have different patterns of NGF/pro-NGF and/or TrkA expression during degeneration (for review[82]). In addition, BNN27 was able to reduce GFAP-mediated astrogliosis in the cuprizone-induced demyelinated corpus callosum[129]. Nonetheless, it is important to note that overactivation of MG cells can also be a potential therapeutic target due to their ability of reprogramming and thus becoming reparative towards injury[149,150]. Future studies are necessary in order to elucidate if BNN27-induced overactivation of retinal glial cells is beneficial or detrimental to the retina, secondary to the primary injury.

Expression of TrkA and $p75^{NTR}$ has been extensively studied in the healthy rodent retina, as well as, in different models of inherited retinal degenerations and retinal injuries[106,109,111,116,137,217,221–224] (for review[82]). However, thus far, it is uncertain if TrkA is expressed in healthy rodent photoreceptors[106,109,116,221,224] (for review[82]). Although previous studies have shown immunoreactivity of TrkA in the outer nuclear layer (ONL; the retinal layer that is consisted by the nuclei of photoreceptors)[109,224], the specificity of the antibody was questioned[106,116,217,224] (for review[82]). In our study, we demonstrated that mRNA levels of TrkA are not detectable in healthy photoreceptors, isolated by laser capture microdissection (LCM), in agreement to a single previous study which used the same method in rats[221]. Also, we showed for the first time, that 24 hours post RD, mRNA levels of TrkA are significantly elevated in the photoreceptors, in line with a previous study in which TrkA was detected by

immunohistochemistry in the detached retinas[109]. On the contrary, in another model of experimental retinal degeneration that also leads to photoreceptor cell death, only the mRNA levels of TrkC were altered after intense light exposure and no difference was observed in the levels of TrkA or TrkB[221]. In contrast to TrkA, $p75^{NTR}$ mRNA levels were detectable in the attached healthy retina, in accordance to previous studies that have verified the expression of $p75^{NTR}$ in healthy photoreceptors by various methods[222,223]. However, there was no significant upregulation following RD injury. Likewise, $p75^{NTR}$ mRNA levels were not altered in photoreceptors after light injury as was detected by LCM[221], although, in another study $p75^{NTR}$ was significantly elevated in photoreceptors in the same type of injury as was detected by in situ hybridization and immunostaining[223]. Furthermore, elevated levels of $p75^{NTR}$ in photoreceptors were also detected by electron microscopy in an experimental model of retinal dystrophy[222]. The heterogeneity in the expression of Trk and $p75^{NTR}$ in photoreceptors following injury and/or degeneration implicates that various detrimental stimuli to photoreceptors result in different modulation of Trk and/or $p75^{NTR}$ receptors.

BNN27 specifically binds to TrkA receptor[128–130] and induces its phosphorylation[128,130–132] and subsequent TrkA-mediated survival signalling through ERK1/2[128,131,132] and Akt[128]. Given the elevated mRNA levels of TrkA in detached photoreceptors[137] and the reported induction of TrkA phosphorylation following RD[137], we examined if BNN27 can further activate the TrkA receptor and its downstream neuroprotective signalling. Surprisingly, BNN27 was not able to significantly induce the phosphorylation of TrkA, Akt or Erk proteins. A possible explanation is that BNN27 does only slightly upregulate TrkA phosphorylation in this type of injury and primarily in other cells (e.g. Müller cells) and not in photoreceptors. In that case, given that photoreceptors significantly outnumber any other retinal cell population, western blotting might lack sensitivity. Single cell western blotting or other techniques might be required to detect such slight differences, if any. The different way of how each retinal cell population reacts to degeneration/trauma should also be considered. BNN27 significantly induced TrkA and Erk phosphorylation in experimental DR[130–132], a chronic metabolic disease of the inner retina. However, our data strongly indicate that BNN27 might have a different mechanism of action in photoreceptors and/or in acute trauma in CNS through interaction with other DHEA's receptors or other pathways that have not been correlated with BNNs yet.

We were discouraged to check downstream signalling of $p75^{NTR}$ due to the absence of increase in the $p75^{NTR}$ mRNA levels in the detached photoreceptors and also due to the fact that BNN27 binds to $p75^{NTR}$ only in the absence of TrkA[133]. Nonetheless, further research is

needed in order to elucidate the possible upregulation of $p75^{NTR}$ in the total detached retina (potential upregulation of $p75^{NTR}$ from different cell populations rather than photoreceptors).

Given the opposing effects of BNN27 on $TUNEL^+$ cells, inflammation/gliosis and activation of TrkA signalling, we wanted to see its overall effect on preservation of photoreceptors in the outer nuclear layer (ONL; the retinal layer that is consisted by the nuclei of photoreceptors) by day 7. Despite the reduction in $TUNEL^+$ cells at 24 hours (the peak of cell death in our model[41,137,139]), we were not able to detect any differences in the ONL thickness between the BNN27-treated and the untreated group seven days post RD either after a single or after multiple administrations of BNN27 (200mg/kg). To evaluate further if a different dosing regimen is needed for optimal effects of BNN27, we administered BNN27 at three more doses (10 mg/kg, 50 mg/kg and 100 mg/kg) daily for seven days. However, we were not able to see any differences between the treated and the untreated eyes. Measurements regarding the retinal thickness are widely used by investigators in order to assess overall neuroprotection of potential therapeutic compounds. However, this approach has certain limitations that are based on the quality of the sections, the subjectivity of the measurement (which parts of the retina are measured each time), the lack of repeatability and the condition of the retinal tissue. Therefore, future assessments of BNN27's overall ability of neuroprotection in the retina should also include electrophysiological and behavioural tests.

To the best of our knowledge, our results indicate that BNN27 could not offer neuroprotection in any paradigm or dose of administration in the RD-induced injured retina. These results could suggest that there is an overall shift in the cell death kinetics and/or that BNN27 significantly reduced one type of cell death and then because of that inhibition, additional cell death pathways are augmented. Indeed, in experimental RD when apoptosis is inhibited, programmed necrosis is notably elevated[50]. As was discussed in details earlier, BNN27 has thus far been proven to reduce caspase-3-mediated cell death[128–130] and TUNEL[128]. Effects of BNN27 on programmed necrosis and/or autophagy or perhaps potential combination of inhibitors of those pathways together with BNN27 might increase its therapeutic potential.

N-methyl-D-aspartate receptors (NMDARs)-mediated signalling plays a pivotal role in synaptic plasticity. Excessive stimulation of NMDARs from glutamate or its agonists, leads to severe mitochondrial dysfunction and subsequent cell death. This process, known as excitotoxicity, has been strongly implied to play a contributory role in many chronic and acute neurodegenerative diseases with most significant implications in the latter. Intravitreal administration of N-methyl-D-aspartate (NMDA) results in robust and acute cell death of the

retina and is an experimental model widely used to study cell death pathways and neuroprotective approaches related to the retinal ganglion cells (RGCs) and the inner retina.

In our study, intravitreal administration of 100 nmoles of NMDA resulted in massive $TUNEL^+$ cell death of the RGCs (almost 70% of the retinal ganglion cell layer), significant cell death in the inner retina and in particular in the cells located towards the IPL (inner plexiform layer; the retinal layer consisted of the synapses of amacrine, bipolar cells and RGCs) and also sufficient cell death in the photoreceptors 24 hours post injury (the peak of cell death in this model). Unfortunately, a single dose of BNN27 given 60 minutes after NMDA injury in three different doses (40, 100, 200 mg/kg) was not able to notably reduce $TUNEL^+$ cell death in the RGCs or in the inner retina, while it offered significant decrease in the $TUNEL^+$ photoreceptors (each layer was quantified separately) at 24 hours. Our results indicate that BNN27 cannot protect $NMDARs^+$ retinal neurons from the excitotoxic insult. It is also indicated that NMDA (100 nmoles) is a very intense stimulus and irreversibly aggressive to the retinal neurons, such that cell death can be induced even in the presence of BNN27. A wide range of NMDA doses has been thus far intravitreally administered in small rodents in many investigations[181,187,196,225,226]. We performed a pilot dose dependence study in order to decide the preferable NMDA dose for our experiments. Thus, we examined $TUNEL^+$ cell death after an intravitreal injection of 10, 20, 100 and 200 nmoles of NMDA at 24 hours. The two lower doses (10 and 20 nmoles) resulted in a very limited number of $TUNEL^+$ cell death, while the two higher (100 and 200 nmoles) resulted in similar $TUNEL^+$ cells (no statistically significant differences). Our results showed most consistent TUNEL positivity and least variability at the dose of 100 nmoles, and thus we chose this dose for our further studies. However, it is important to note that 100 nmoles of NMDA, intravitreally administered, resulted in massive retinal cell death, contrary to lower doses (e.g. 40 nmoles). Perhaps BNN27 could lead to protection in a mild NMDA-mediated insult.

Our study also highlights that NDMA injury is not restricted to the RGCs and the inner retina and also affects the outer retina. It has been well established that NMDARs are robustly expressed in the RGCs and in some subsets of amacrine cells (for review[167]). Furthermore, recently, functional NMDARs have also been reported in rod and cone bipolar cells (for review[167]). However, only a handful number of studies have shown evidence that photoreceptors, and specifically cones, might express NMDARs and those results are still contradictory[227,228]. Nonetheless, NMDARs' expression in photoreceptors has been so far only poorly understood, and thus we do not know if photoreceptor cell death in our model is due to

overactivation of NMDARs that are expressed in those cells or, most likely, secondary to the released harmful endogenous products of the NMDARs^{+} dying cells. Indeed, in pilot studies we conducted in order to determine the preferable dose of intravitreal administration of NMDA and the time course of cell death in NMDA-induced retinal excitotoxicity, 10 or 20 nmoles of NMDA did not result in photoreceptor cell death and photoreceptors started dying hours after the inner retina and the RGCs, confirming our hypothesis that photoreceptors are dying secondary to the massive cell death of the inner retina and the RGCs. In addition, photoreceptor cell death as a result of intense NMDA insult has been confirmed by another recent study[73]. Very interestingly, BNN27 offered significant decrease in TUNEL^{+} photoreceptors in any of the 3 doses that we examined, suggesting that although BNN27 failed to reduce cell death following the first noxious stimulus, it might be overall beneficial for the NMDA-injured retina and/or for secondary death stimuli resulting in further damage of the retina. Our results suggest that BNN27 might have further mechanisms of action that are potentially beneficial for the fate of certain types of cell.

All three different doses of BNN27 that we administered, resulted in similar levels of cell death/protection, and thus we decided to proceed to our experiments with the medium dose. Following our studies regarding inflammation and gliosis in experimental RD, we repeated those experiments in NMDA-induced excitotoxicity. Previous study has reported increased CD45^{+} cell infiltration in the retina following administration of 200 nmoles of NMDA[190], while several other studies have shown that NMDA-mediated retinal cell death induces the activation of resident microglia[188,190] and Müller glial (MG) cells[188,189]. Moreover, a recent study has shown that NMDA insult (100 nmoles) results in increased infiltration of CD11b^{+} cells both peripapillary and at the optic nerve head (ONH), contrary to the vehicle-treated eyes in which there were only a few CD11b^{+} cells throughout the different retinal layers and none in the vitreous or ONH, suggesting that the latter is the primary route of immune cell infiltration in that type of injury and that CD11b^{+} immune cell infiltration is activated post NMDA injury[73]. Systemic administration of BNN27 (100 mg/kg) one hour after NMDA intravitreal injection did not result in any significant differences in CD11b^{+} macrophage/microglia infiltration or in the upregulation of GFAP and vimentin, indicating that the effects of BNN27 in the NMDA injured retina are not due to altered macrophage/microglia recruitment or mediated by gliosis. However, as was discussed in details earlier, macrophage and microglia might play different roles as well in this type of injury, therefore further studies are needed in order to elucidate the prominent immune cells in NMDA-mediated excitotoxicity and the potential effects of BNN27

on each different immune cell subset. It is important to note that in our study, we did not examine the effects of NMDA cell death on optic nerve (ON) and hence perhaps BNN27 has an effect on the oligodendrocytes or astrocytes that surround it which are particularly the cell subsets that have been reported to be damaged in glaucoma. Assessment of BNN27 action on optic nerve injury, neuroprotection and regeneration would be especially interesting as a future research direction.

Within the different subsets of retinal neurons, expression of TrkA in the retina has been examined more extensively in the RGCs and in experimental models that result in their degeneration (for review[82]). However, results thus far have been contradictory; TrkA is expressed in healthy RGCs,[106,116,229] though according to some studies, only in very low levels[109,116,230]. Moreover, TrkA mRNA was found elevated in experimental models of glaucoma[229,231,232] and optic nerve (ON) injury[116,230] but was also found decreased in ischemic injury models causing RGC death[233,234]. Similarly, expression of TrkA and/or phosphorylation of the receptor has been reported both upregulated or downregulated in different models of glaucoma[229,231,232], ischemia[233,234], ON axotomy[116,230] and diabetic retinopathy (DR)[130,235–237] but was also unable to be detected in several studies[237–241]. In our study, TrkA was downregulated in NMDA injured retinas, while we were not able to detect any differences in the phosphorylation of the receptor between healthy retinas, NMDA-injured retinas and BNN27-treated retinas. Our results strongly indicate that BNN27 does not act through TrkA receptor in NMDA-mediated excitotoxicity or if it does it phosphorylates TrkA only in very small subsets of cells. Taken into consideration that BNN27 did not further induce phosphorylation of TrkA in experimental RD, it might be possible that BNN27 does not induce TrkA signalling in acute injuries and acts through different receptors. Nonetheless, the lack of BNN27-induced TrkA phosphorylation might be responsible for the lack of the overall protection.

In our study, BNN27 was able to reduce cell death in two models of acute retinal injury but it was not able to offer overall neuroprotection. Similarly, BNN27 was able to rescue mouse motor neurons co-cultured with human astrocytes from patients with amyotrophic lateral sclerosis (ALS) with mutations in the *Sod1* gene (superoxide dismutase 1), however, it failed to show an overall effect on neuropathological markers in an SOD1 mutant mouse model[242]. On the contrary, systemic administration of BNN27 was able to protect the brain nitric oxide synthase (bNOS)- and tyrosine hydroxylase (TH)- expressing amacrine cells as well as preserve the ganglion cell axons in a rat model of diabetic retinopathy (DR), though it did not

reduce TUNEL-mediated cell death[130]. Furthermore, in a different study, BNN27 reduced cuprizone-induced apoptosis in mature oligodendrocytes in experimental multiple sclerosis (MS) but did not prevent demyelination in the same challenge[129]. Taken all together, BNN27 seems to have a very divergent effect on different models of CNS neurodegeneration. In summary, each deleterious stimulus activates distinct cell signalling combinations and perhaps RD and NMDA-mediated injury have a very different nature given the acute ischemic trauma to the retina compare to the chronic metabolic DR or the inflammatory demyelinating MS. Furthermore, important differences in the CNS between mice and rats have been reported several times in the past including different patterns of neurogenesis[243], response upon stimuli/trauma[244,245] and pharmacology[246]. Differences between the two species could be another possible explanation for the contradictory results of BNN's potential neuroprotective activity in different models of retinal neurodegeneration along with the nature of the disease.

The paradox of decreased $TUNEL^+$ cells with the increased macrophage infiltration and gliosis markers, concurrently with the lack of TrkA activation, appears to be quite complex and could explain why overall ONL thickness was unaltered despite a drastic reduction in observed $TUNEL^+$ cell death in the experimental model of RD. Furthermore, neuroinflammation and gliosis are not necessarily neurotoxic; they include both neuroprotective and neurotoxic signals. The shift between these two signals is still unclear and the mechanism of action of BNN27 on the inflammatory cells and the glial cells of the retina remains to be elucidated. On the other hand, BNN27 seems to have a secondary neuroprotective effect on the animal model of NMDA-induced retinal excitotoxicity without affecting any of the parameters that we examined and without being able to induce TrkA phosphorylation or to offer overall protection towards excitotoxicity. In summary, our study was not able to conclude if BNN27 has overall neuroprotective activity in the two acute models of retinal injury affecting the outer and inner retina. More extensive studies with different dosing and/or models are needed to assess the potential therapeutic role of this novel molecule in diseases like RD affecting the outer retinal layers or in diseases in which excitotoxicity is a contributing factor.

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