Master Program of Neurosciences

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BNN27 THERAPEUTIC EFFECTS ON OPTIC NERVE INJURY

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ABBREVATIONS

a7nAChRs: a7 nicotinic acetylcholine receptors a7nAChRs

AAD: Acute Axonal Degeneration

AAV2: Adenoassociated serotype 2

AIS: Axonal Initial Segment

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB: Blood Brain Barrier

BDNF: brain-derived neurotrophic factor

BSA: Bovine Serum Albumin

Ca^{2+}: calcium

cAMP: cyclic AMP

CGS: Collagen- GAG Scaffold

CNS: Central Nervous System

COI: Controlled Orbital Impact

Cslo: Confocal Scanning Laser Ophalmoscope

CSPGs: Chondroitin Sulfate Proteoglycans

CTB: Cholera Toxin B

DB: Die Back

DHEA: Dehydroepiandrosterone

Dpi: days post injury

DR: Diabetic Retinopathy

DRG: Dorsal Root Ganglia

ECM: Extracellular Matrix

FG: Fluorogold

GAGs: glycosaminoglycans

GAP-43: Growth Associated Protein- 43

GCL: Ganglion cell layer

GFAP: Glial Fibrillary Acidic Protein

GS: glutamine synthetase

HRP: Horseradish Peroxidase

i.p: intraperitoneally

i.v: intravitreally

IBA-1: Ionized Calcium-Binding Adaptor molecule 1

IHC: Immunohistochemistry

INL: Inner Nuclear Layer

IOP: Intraocular Pressure

IPL: Inner Plexiform Layer

JNK: c-Jun N-terminal kinase

K^+: potassium

KO: Knock Out

LGN: Lateral Geniculate Nucleus

MAG: Myelin Associated Glycoprotein

MAIs: Myelin Associated Inhibitors

MAPK: mitogen-activated protein kinase

Mnt: mature Neurotrophin

MNTS: microneurotrophins

MRI: Magnetic Resonance Imaging

MS: Multiple Sclerosis

MSC: Mesenchymal Stem Cells

Na^+: sodium

nAchR: nicotinic Acetylholine Receptor

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NFL: Neurofilament

NGF: Nervous Growth Factor

NgR1: Nogo Receptor 1

NMDA: N-methyl-D-aspartate receptor

NSPC: Neural Stem Progenitor Cells

NT: neurotrophin

OBI: Ocular Blast Injury

OCT: Optimal Cutting Temperature

OEC: Oldfactory Ensheathing Cells

OL: oligodendrocytes

OMGp: Oligodendrocyte Myelin Glycoprotein

ON: Optic Nerve

ONC: Optic Nerve Crush

ONH: Optic Nerve Head

ONL: Outer Nuclear Layer

ONT: Optic Nerve Transection

OPC: Oligodendrocyte Progenitor Cells

OPL: Outer Plexiform Layer

p75NTR: pan neurotrophin receptor 75

PB: phosphate buffer

PD-1: Progremmed Cell Death receptor 1

PFA: paraformaldehyde

PI3-K: phosphoinositide 3-kinase

PKC: protein kinase C

PLCy1: phospholipase C gamma one

PNS: peripheral nervous system

ProNT: Premature Neurotrophin

RAGs: Regeneration Associated Genes

RAPD: Relative Afferent Pupillary Defect

RBMPS: RNA- binding protein with multiple splicing

RD: Retinal Detachment

RGC: Retinal Ganglion Cells

RGL: Retinal Ganglion cell Layer

RGM: Repulsive Guidance Molecule

rhNGF: recombinant human Nerve Growth Factor

RPE: Retinal Pigment epithelium

RTG: Reverse Thermal Gel

SCI: Spinal Cord Injury

SCN: suprachiasmatic nucleus

Sema 4D: Semaphorin 4D

SI-TON: Sonication- Induced Traumatic Optic Neuropathy

SOX-11: SRY-box containg gene 11

SPF: Specific Pathogen Free

SRTG: Sulfonated Reverse Thermal Gel

SsDNA: single stranded DNA

TFs: transcription factors

TNFR: Tumor Necrosis Factor Receptor

TON: Traumatic Optic Neuropathy

Trk: Tropomyosin receptor kinase

TUNEL: Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling

VF: visual field

WD: Wallerian Degeneration

WT: Wild Type

ABSTRACT

Traumatic optic neuropathy (TON) is a condition, induced when an injury occurs within the optic pathway and results in complete vision loss. TON is caused by falls, car accidents or even attacks and affects thousands of people every year, mainly young people aged between 11-30 years old. The optic pathway consists of the retina, optic nerve, optic tracts, optic radiation and finally of the primary visual cortex. At a cellular level TON leads to Retinal Ganglion Cell (RGC) death, inflammation/astrogliosis, demyelination and axon degeneration. Due to CNS incapacity to promote axon regeneration, TON tends to be an irreversible situation. There is still no appropriate treatment that promotes CNS axons to regenerate effectively, reversing the vision loss effect caused by TON. Clinical treatments emphasize mainly to corticosteroid administration, whereas experimental approaches include: eye drops, injections, biomaterials' implantation, Peripheral Nervous System grafts, cell transplantation and also gene therapy. Neurotrophins secreted from the neurons or from the glial cells within the retina and the optic nerve are promising molecules for TON therapy, as generally exert neuroprotective properties to neurons, increase their proliferation and their differentiation. In this study, a synthetic analog of DHEA, a microneurotrophin, named BNN27 that mimics NGF is administered in Optic Nerve Crushed mice either 2 weeks or 10 weeks after injury. BNN27 is administered by two strategies: 1. Eye drops (1 eye drop/day or 2 eye drops/day) and 2. through biomaterial-implantation. In the latter approach, BNN27 at the day of the surgery, is entrapped in a scaffold/peptide that is placed around the lesion site of the Optic Nerve. This strategy assures steady release of the drug in the lesioned tissue. Our results prove that unilateral Optic Nerve Crush model is reproducible as it results in a 40% RGC loss within the first week after injury and a 60% RGC death within 2 weeks after injury. The results of the 2- weeks study prove that BNN27 delivered either by eye drops or by biomaterial reduces significantly Retinal Ganglion cell death caused by Optic Nerve Crush. Furthermore, BNN27 does not exert anti-inflammatory properties to Muller cells and does not inhibit demyelination in the Optic Nerve. Concerning the 10 -week study, BNN27 does not minimize Retinal Ganglion Cells loss and does not promote axon regeneration within the Optic Nerve. Taking these results into account, an important finding of this research is that BNN27 delays RGC death in the retina 2 weeks after injury, whereas it does not exert any neuroprotective properties in RGCs within 10 weeks after injury, resulting in RGC loss and eventually in lack of axon regeneration.

Chapter 1: Introduction

Retina constitutes of variable layers, each of them containing a different cell population. Axons of Retinal Ganglion Cells which are found in the inner part of the retina, form the Optic Nerve (ON) which eventually leads to the Superior Colliculus in the brain. An injury to the Optic Nerve results in Traumatic Optic Neuropathy (TON) which leads in vision loss. TON is characterized by a cascade of events that begin within the first hours after injury and progress as the time passes. Some major events that characterize TON are: RGC death, inflammation/astrogliosis, demyelination and lack of axon regeneration. Neurotrophins are key molecules of the aforementioned procedure and therefore their role in TON is examined.

1.1 Introduction to Traumatic Optic Neuropathy

1.1.1 Anatomy and physiology of the Retina and the Optic Nerve

Sight is a specialized method for most animals to navigate through their surroundings. Photons enter the eye, access the visual pathway and transfer the information of an image to the brain. This phenomenon is called visual perception (Armstrong, Cubbidge, 2019). The visual pathway includes the anatomical structures that manage to convert the energy of the light into action potentials producing a nerve pulse. The visual pathway while moving from the retina to the brain consists of the optic nerve (ON), the optic chiasm, the optic tracts, the lateral geniculate nucleus (LGN) of the thalamus, the optic radiations and finally the primary visual cortex and more specifically Broadmann area 17 (Forrester et al. 2015). The visual field (VF) and the retina have an inversed relationship. Each eye is mentally divided in the inferior and posterior retina and also in the temporal and nasal retina. The upper VF falls to the inferior retina and the lower VF to the posterior, respectively. Also, the nasal VF falls on the temporal retina and the temporal VF to the nasal one (Kline & Bajandas, 2008).

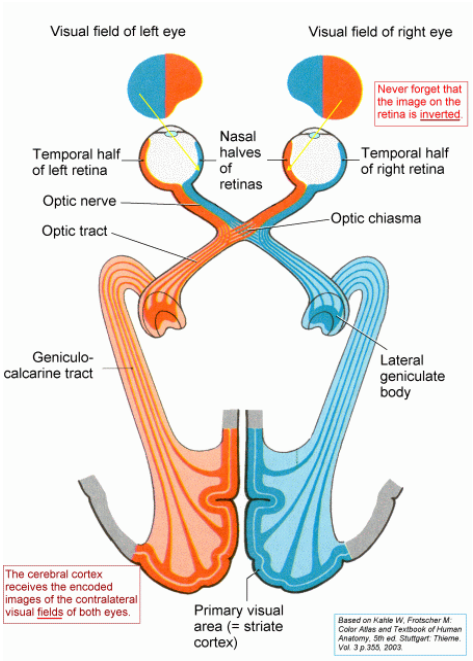


Fig. 1-1: The visual pathway of the human eye consisted of the eyes, optic nerve, optic chiasm, LGN, optic radiations and primary visual cortex (Kahle & Frotscher, 2015).

In the mammalian **retina** there are seven major cell types which are divided into three groups: cells of association, glial and neurons. Cells of association include horizontal and amacrine cells, glial cells include Muller cells, astrocytes and microglia and neurons include photoreceptors, retinal ganglion cells (RGCs) and bipolar cells (Kolb, 2011b). In the central retina (fovea) there are up to seven layers of ganglion cell bodies (60-80μm thickness), whereas in the peripheral retina there is only one cell layer (10-20μm thickness) (Forrester et al., 2015). There are 500.000 and 1.2 million GCs per retina (Trattler et al., 2012) and 100 rods and 4-6 cones per GC (Forrester et al., 2015).

Light signals from the retina are translated into chemical signals that are sent to the brain (Hoon et al., 2014). This process requires cell- to-cell contact and begins with photoreceptors (rods and cones), while ends with RGCs in the retina.

Retina is organized into several layers. Moving from the frontal part of the eye to the back, the first layer is the retinal pigment epithelium that is comprised of the pigment epithelial cells and then there is the outer limiting membrane. Deeper of that membrane is located the outer nuclear layer which consists of the cell bodies of the photoreceptors. Photoreceptors synapse with bipolar cells in the outer plexiform layer, while bipolar cell bodies are located in the inner nuclear layer, together with the cell bodies of amacrine and horizontal cells. In the inner plexiform layer takes place the synapse of bipolar cells with the dendrites of RGCs, whose cell bodies are found in retinal ganglion cell layer. The axons of the RGCs are connected at the optic disc that leaves the eye, and form the optic nerve that projects

to higher brain centers. (Kolb, 2011). Each cell type plays a distinct role in signal transduction. Photoreceptors use the glutamate in order to communicate with bipolar cells. Horizontal cells modulate this communication by contributing to contrast enhancement and preservation of spatial information (Chapot et al., 2017). Bipolar cells are responsible for signal transmission to the RGCs, while amacrine cells which are found between the beforementioned cell types are inhibitory cells that modulate the communication of bipolar and RGCs. Regarding glial cells, Muller cells are retina-specific cells that support metabolically the neurons (Lukowski et al., 2019), while microglia participate to immune response (Boycott and Hopkins, 1981) and astrocytes to retinal homeostasis and glucose provision (Kolb, 2013).

Regarding neurotransmission, photoreceptors use glutamate that is received by metabotropic (mGluR6) and ionotropic (AMPA) receptors of bipolar cells. At their axonal endings, bipolar cells express GABA and glycine receptors, while RGCs express all the aforementioned receptor kinds (mGluR6, AMPA, GABA, glycine) along with acetylholine (Kolb et al., 2011). Finally, RGCs, bipolar cells, photoreceptors and cells of association express beyond the other types of receptors also dopamine receptors (D1) which can act both excitatory and inhibitory (Akaike et al., 1987).

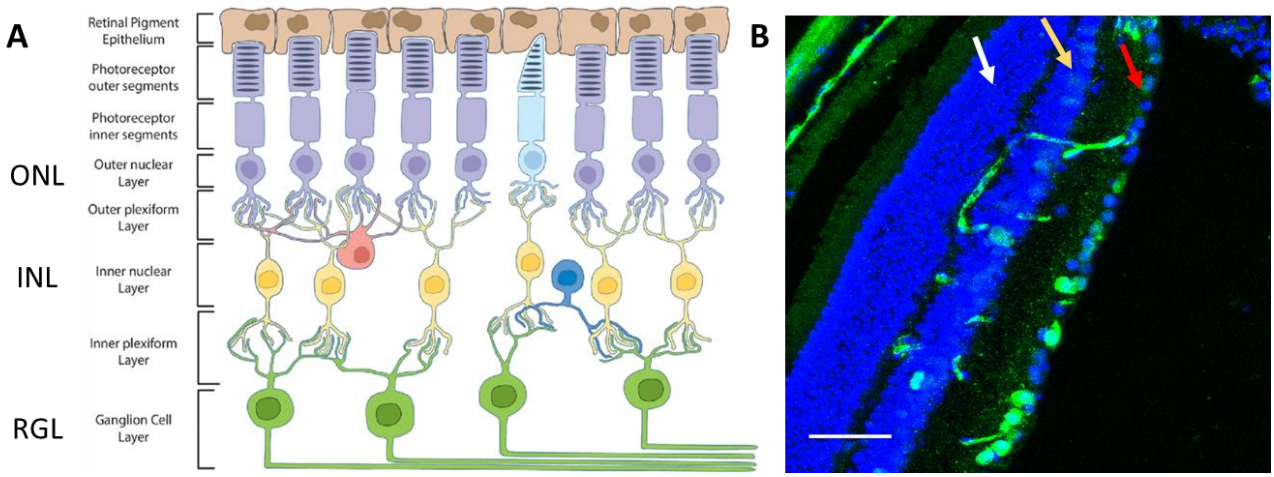


Fig. 1-2: A. Distribution of glial cells and neurons in the vertebrate retina. There are 2 limiting membranes: the outer and the inner and 5 layers: ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer) and GCL (ganglion cell layer), moving from the outer part of the retina to the inner one. Also, retina is characterized by the presence of 6 cell types: photoreceptors (purple and light blue), horizontal (red), amacrine (dark blue), bipolar (yellow) cells, RGCs (green) and Muller cells (not depicted) (Sengillo et al., 2016). B. Representative image of murine retina's structure. The red arrow indicates the GCL, the yellow arrow indicates the INL and the white arrow indicates the ONL. Scale bar=50μm

The **Optic nerve** (ON) connects the retina to the brain. It is formed by the convergence of the RGC axons that exert the eye, at the optic disc (Lee et al., 2020). Optic disc or papilla is the part of the eye that has no photoreceptors and is known as blind spot (Salazar et al., 2018). RGC axons in the retina are unmyelinated and become myelinated by oligodendrocyte progenitor cells (OPCs) at the optic nerve head (ONH), (Yazdankhah et al., 2020; Rea 2014). In humans there are between 770.000-1.7 million fibers that form the ON (Jonas et al., 1992) and they are divided into fascicles by connective tissue and glial septa, surrounded by cerebrospinal fluid. ON is separated into four main parts: intraocular nerve head (1mm), intraorbital (24mm), intracanalicular (9mm) and intracranial part (16mm) (Forrester et al., 2015; Kumaran&Sundar, 2014).

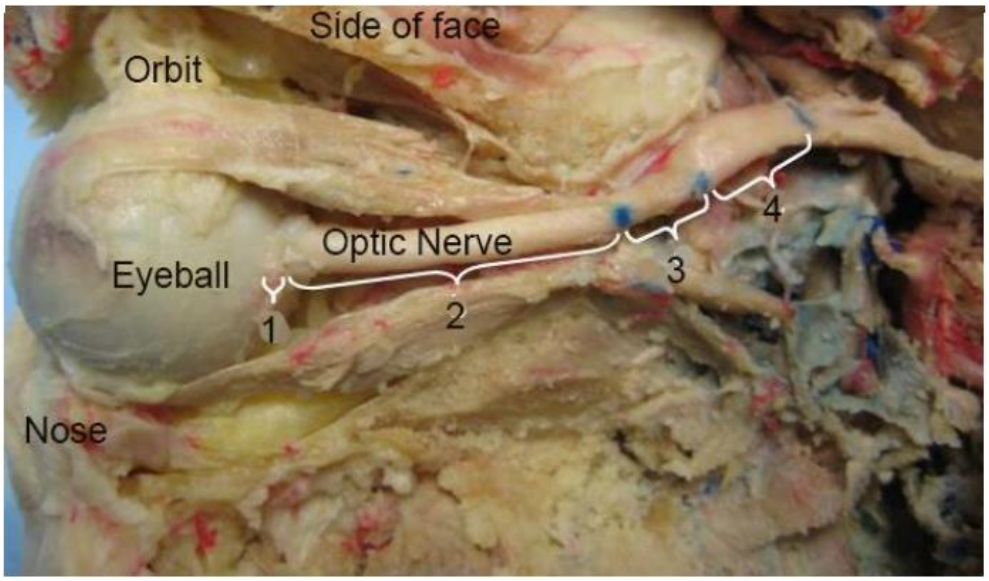


Fig. 1-3: Illustration of the ON and its four anatomical parts: intraocular nerve head (1), intraorbital (2), intracanalicular (3) and intracranial (4) part (Anatomyczar).

ON in characterized also by the presence of glial cells whose general role is to maintain the neuronal activity, structural stability and face situations such as inflammation, injury and ischemia that are destructive for its function (Vecino et al., 2016; Berry et al., 2002). The glial cell types encountered in the ON are: macroglia (astrocytes and Muller cells), oligodendrocytes (OLs), microglia and NG2 cells (Yazdankhah et al., 2020; Cuenca et al., 2014).

Astrocytes: Astrocytes provide neurons with nutrients by connecting them to the blood vessels (Bouzier-Sore&Pellerin, 2013) and release growth factors and cytokines (Schwab et al., 2000). They are also necessary for potassium homeostasis, axon-glial communication and maintenance of blood - brain barrier and blood-retina barrier integrity (Khakh & Deneen, 2019). Beyond all the aforementioned roles, astrocytes participate in glial scar formation (Anderson et al., 2016) and maintain RGC mitochondria in a healthy state (Munemasa & Kitaoka, 2015). Normally, astrocytes are

in a quiescent situation but upon inflammation or ischemia they turn into a reactive state. They are distinguished in two types: A1 and A2. A1-type astrocytes are known to harm the synapses whereas A2 contribute to synapse repair owing to the upregulation of thrombospondins (Liddelow et al., 2017; Liddelow & Barres, 2017; Li et al., 2019).

Muller cells: Muller cells are radial glia cells and account for the largest glial cell type in the vertebrate retina (Cuenca et al., 2014). They are characterized by large cell bodies and complex morphology, as their bodies are found in the INL whereas their branches extend between photoreceptors bodies and the ONL, forming two boundaries in the retina: outer limiting membrane (apically) and inner limiting membrane (basically-where their end feet are) (Lewis & Fischer, 2013). They are responsible for maintaining retinal homeostasis, phagocytosing cellular debris (Kolb, 2013), regulating synaptic activity in the inner retina (Barnett & Pow, 2000; Izumi et al., 1999) and secreting neurotrophins and cytokines so as to protect the RGCs. In case of injury, Muller cells are the first glial cell type to detect such changes and respond by altering their morphology and biochemistry (Bringmann et al., 2006, 2009). They also increase their proliferation and become hypertrophic during CNS trauma (Goldman 2014).

Oligodendrocytes (OLs) are the mature OPCs and are responsible for the formation of myelin sheaths around the axons of RGCs (Butt et al., 2004). Myelin sheaths are necessary for the conduction of axon potentials in retinal neurons (Stadelmann et al., 2019). Another key function of OLs is the provision of metabolic support to neurons such as the OL-derived lactate for the RGC axons (Funfschilling et al., 2012). OLs are highly sensitive to CNS insults as their apoptosis leads to demyelination which impairs ON functional role (Butt et al. 2004).

NG2 cells: are OL progenitors (Yazdankhah et al., 2020) that participate in axon-glial signaling and glial scar formation upon CNS insults (Parolisi & Boda, 2018).

Microglia are characterized as the macrophages of the CNS, as their main role is to regulate the immune response by engulfing cellular and myelin debris (Dissing-Olesen et al., 2007). They also support neurons by secreting insulin-like growth factor 1 (IGF-1) (Frade &Barde,1998) and regulate the neurogenesis by phagocytosing apoptotic neural cells (Fantin et al., 2010). In the human ON there are 1140 microglial cells per mg (Yazdankhah et al., 2020). Normally, microglia are quiescent but in case of injury they become active. Activation of microglia during acute injury is protective, but in chronic disease becomes destructive and leads to ON degeneration (Yazdankhah et al., 2020; De Hoz et al., 2013; Gallego et al., 2012).

1.1.2 Traumatic Optic Neuropathy Overview

Traumatic Optic Neuropathy (TON) is a condition in which a direct or indirect acute injury to the ON results in vision loss. The severity of TON varies between a simple contusion to complete avulsion of the ON (Gupta et al., 2021).

TON has severe implications in the quality of life (Kumaran et al., 2014; Pirouzmand, 2012; Lee et al., 2010; Upadhyay, 2017) and can result in visual loss, deficits in visual filed or even color perception (Lee et al., 2010; Liu et al., 2020) TON affects young males between 11–30-year-old (58,3%) and most usual causes are motor vehicle accidents (83.3%), blunt trauma (12.5%) and fall (4.2%) (Lee et al., 2010).

TON can be classified depending on two parameters: the site (portions of the ON) and the mode (direct or indirect) of the injury (Sarkies 2004; Ford et al., 2012). Direct TON refers to the application of stress to the ON directly which leads to severe anatomical disruption and it includes optic nerve transection, avulsion, optic nerve sheath hemorrhage, orbital hemorrhage and orbital emphysema (Sarkies 2004; Kumaran et al., 2014; Yu-wai-man, 2015). It can be caused by the induction of a sharp object, missiles and also bony fragments (Nazir et al., 2010). Direct TON can be diagnosed by neuroimaging such as computed tomography and magnetic resonance imaging (MRI). However, it is rare because of the protection provided by the bony orbit (Jang, 2018). On the other hand, indirect TON is induced when a stress stimulus is transmitted through soft oculofacial tissues and skeleton, damaging the integrity of the ON, leading to a reduction of the vascular supply of the ON and finally causing mid-to- severe visual loss, as a result of orbitofacial or cranial trauma (Atkins et al., 2008; Sarkies, 2004; Gross et al., 1981; Kumaran et al., 2014;Glaser, 1999; Beretska & Rizzo, 1994).Commonest site of induction of indirect TON is the optic nerve canal, whereas the most susceptible portions of the ON are the intracanalicular and the intracranial. (Steinsaspir et al., 1994; Sarkies, 2004) Indirect TON is most usual and it underlies the following clinical features: positive relative afferent pupillary defect sign (RAPD), impairment of color vision and visual field defects (Yu-Wai-Man, 2015).

There are many proposed therapies for TON, but each of them presents some drawbacks and cannot be applied in every individual suffering from TON, as it depends on the time window after TON, but also of the severity and the portion of the ON that is injured. Suggested therapeutic interventions include: observation, corticosteroid therapy, surgery, neuroprotection and neuroregeneration therapies and also ocular transplantation. Observation of the patient's state is the first therapy to be applied but is not always very efficient (Lee et al., 2010). Corticosteroids are applied from the early 1980s and seem to minimize the damage by exerting their neuroprotective effects,

reducing swelling and improving metabolic processing and microcirculation at the site of the lesion (Kumaran et al., 2014). Surgeries in the ON include removal of the optic canal when injury takes places in optic canal fracture and this results to decompression of the ON. This type of therapy can be combined with administration of steroids (Agrawal et al., 2013; Li et al., 1999). Other surgical approaches include intracranial, transethmoidal, endonasal and sublabial interventions and also endoscopic analyses such as open surgery (Fukado, 1975; Fujitani et al., 1986). Another beneficial therapeutic strategy is the administration of neuroprotective treatment, in order to promote RGC survival and regeneration of their axons (Kumaran et al., 2014). A quite invasive therapeutic strategy is transplantation of the whole eye, so as to entirely restore vision. However, this is very difficult to succeed because of the incidence of immune rejection and of the inability of axons to maintain their circulation after TON (Ellenberg et al., 2009).

1.1.3 Molecular and Cellular Biology of TON

CNS Injury Phases

CNS injury following a neurotraumatic event consists of two phases: primary and secondary injury (Pearn et al., 2017; Alizadeh et al., 2019). The primary phase of a CNS injury includes direct structural damage in the tissue and vasculature, which leads to immediate cell death, hemorrhage, ischemia and edema. Usually, primary injury phase consists the initial injury suffered upon impact (Quraishe et al., 2018), whereas the consequent secondary damage takes place over the subsequent weeks and months (Hay et al., 2015; Feng et al., 2017; Quraishe et al., 2018) and refers to the surrounding -of the injury- tissue which is highly vulnerable to further damage (Alizadeh et al., 2019; Kumar et al., 2012). Secondary injury phase includes breakdown of Blood Brain Barrier (BBB) and leakage of various compounds from the degenerating axons. These compounds are normally found in the CNS microenvironment but their concentrations are altered upon injury and as a result they become toxic and can lead to inflammation, metabolism alterations, oxidative stress, calcium influx and excitoxicity. Released compounds include excitatory amino acids (Glu), opioids, ions such as K^+ and Ca^{2+}, free radicals and proteases (Schwartz et al., 2004; Park et al., 2004; Werner & Engelhard, 2007; Li et al., 2014; Alizadeh et al., 2019). The secondary phase of injury is followed by degeneration of neuronal axons, leading to a variety of intracellular signaling events and finally to apoptosis.

Primary injury

The first minutes after an injury in the ON structural changes take place in the axon that lead to the formation of dystrophic axons which lack filopodia, are vacuolated, move backwards over time

and are characterized by a bulb at the site of the injury, which is known as retraction bulb (Hawthorne et al., 2011). Specifically, 30 minutes following injury axonal swelling, misalignment of neurofilaments and fragmentation of microtubules take place. Also, there is an increase in Ca^{2+} intracellularly, which tends to normalize afterwards. In the next 60 minutes there is an alteration in the axonal integrity between the most proximal and the most distal parts of the axon. Initiation of autophagy occurs 90 minutes after trauma and persists until the 6^{th} day after injury. Meanwhile, the diameter of the axon is increased proximally to the lesion site which is correlated with a progressive increase in g-ratio. All this time, axons continue to disintegrate and are characterized by a cytoplasmic accumulation of mitochondria and vacuoles in the retraction bulb (Knoferle et al., 2010).

Axon degeneration

Axon degeneration occurs prior to cell apoptosis (Knoferle et al., 2010; Ribas et al., 2016) and can be classified as Wallerian degeneration (WD), Acute Axonal Degeneration (AAD) or Die Back (DB). WD is encountered in severely damaged axons, causing atrophy and rapid loss of their cytoskeletal structure along the whole axon. The cell whose axon is degenerated remains alive for several days before it undergoes apoptosis (Berkelaar et al., 1994; Saxena & Caroni, 2007; Budak & Akdogan, 2011). AAD takes place immediately after axonal lesion (Ribas et al., 2016; Knoferle et al., 2010). DB takes place after moderate injury and is characterized by slower retrograde degeneration. Die-back can occur over several months (Budak & Akdogan, 2011).

Inflammation

Later in the secondary phase of injury, inflammatory cells are infiltrated in the lesion site and remove the damaged tissue. Activated microglia, reactive astrocytes and leukocytes from the bone marrow, translocate to the lesion site and secrete cytokines and chemokines, creating a proinflammatory environment (Berry et al., 1999; Anwar et al., 2016; Feng et al., 2017; Pearn et al., 2017). Astroglial and microglial proliferation occurs between 2-7 dpi (Ohlsson et al., 2014). Microglia infiltration precedes astrocytes infiltration into the injury site. Phagocytes, which are microglia/macrophages infiltrate in the lesion site on 2dpi and phagocytose the myelin debris from injured axons. One week after crush, microglia reach its peak and 3 weeks post crush, have retreated. However, microglia that reaches sites distally from the lesion, become activated a few days later, at 6 dpi (Frank & Wolburg, 1996). The infiltration of activated astrocytes, oligodendrocyte progenitor cells (OPCs) and also microglia around the lesion site gives birth to the formation of the glial scar, whose role is controversial as it protects the injured tissue from further secondary damage, but it is also considered a barrier for axonal regeneration and remodeling of the neural circuits (Kawavo et al., 2012;

Silver&Miller. 2004; Li et al., 2020). Pericytes proliferate, leave the blood vessel wall and then differentiate into scar-forming fibroblasts, which contribute to the core of fibrotic scar. Fibrotic scar is surrounded by glial scar and is formed around 3 days post injury (Liu et al., 2020; Williams et al., 2020). Glial scar is formed around 10 dpi (Liu et al., 2020). However, the kinetics of migration of the glial cells that from the glial and fibrotic scar are not well-established, as some studies support that is starts around 7 days post injury (dpi), whereas others mention that on 3 dpi glial scar has already been created (Pearson et al., 2020). Simultaneously, Muller cells and reactive gliosis hypertrophy take place along with extended swelling of axon processes (Mamane et al., 2016) and shortening of their axonal initial segments (AIS) which leads to their significant loss on 7 dpi (Marin et al., 2016). Microglia reach its peak on 14 dpi (Nadal-Nicolas et al., 2017) and until 31 dpi they are present in the ON at higher numbers than in the retina (Heuss et al., 2018). 21 days after injury astrocytes are still present in the lesion site but microglia and macrophages withdraw, whereas $NG2^{+}$ oligodendrocyte Precursor Cells (OPCs) are observed in the cavity (Pearson et al., 2020).

RGC apoptosis

RGC apoptosis takes place within the first days-weeks post injury. Levkovitch -Verbin et al., 2000 revealed that RGC survival was significantly decreased 2 weeks after ONC in transgenic mice. Another study, examined the effect of ONC on RGCs in mice, and found that RGC loss was about 65% in the first 7 days. (Sanchez-Migallon et al., 2016). Overall, RGC survival is usually assessed within the first 2-4 weeks post injury (Berhelaar et al., 1994; Kwong et al., 2011; Rodriguez et al., 2014). Few studies have examined long-term RGC survival after ONC (some months post injury). Templeton et al., 2009 observed that 30 days post-ONC 54% of RGCs had survived. Another study assessed RGC survival after 6 months and demonstrated contradictory results. Only 1-4% of RGCs survived after ONC or ONT. (Nadal-Nicolas et al., 2015b).

Following injury, there is a decrease in mTOR pathway activation which is related to cell growth, survival and proliferation. A progressive loss of myelin sheaths that surround the axons leads to impaired conduction velocities (Marin et al., 2016; Bei et al., 2016). For instance, the first 5-7 dpi there is a small number of cells in the GCL that is clumped and is characterized by fragmented nuclei (Berkelaar et al., 1994). However, other studies support that until 7 dpi, 50% of RGCs are lost and RGC death starts on 3 dpi (Marin et al., 2016; Ueno et al., 2018; Galan et al., 2014). Another study reports that there is a 65% decrease in RGC survival within the first 7 days after crush and an additional 4% decrease over the next 3 days (Sanchez-Migallon et al., 2016). On 14 dpi about 80% of RGCs received Casp2 apoptotic signals (Berkelaar et al., 1994; Marin et al., 2016; Park et al., 2008; Puyang et al., 2016). At 28 dpi there is a complete loss of RGCs in the GCL (Sharma et al., 2014). Furthermore,

following injury there is increased expression of genes related to apoptosis, immune system processes, organization of actin cytoskeleton and signal transduction whereas there is a decrease in those related to visual perception, ion transport and synaptic transmission (Ueno et al. 2018).

Axonal Elongation

After ON injury, CNS microenvironment is inhibitory to axon regrowth, compared to PNS (David & Aguayo, 1981; Smith et al., 1986) due to both intrinsic and extrinsic factors. Intrinsic factors refer exclusively to neurons and include the decline of regeneration-associated genes (RAGs) such as c-Jun, ATF-3, growth-associated protein-43 (GAP-43), SRY-box containing gene 11 (SOX11) etc (Raivich et al., 2004; Huebner & Strittmatter, 2009; Bonilla et al., 2002; Bomze et al., 2001). On the other hand, extrinsic factors refer to the microenvironment of the damaged tissue, which consists of Myelin Associated Inhibitor proteins (MAIs) such as Nogo, Oligodendrocyte Myelin glycoprotein (OMgp) and Myelin- Associated glycoprotein (MAG), whose expression level is altered upon injury (McKerracher et al., 1994; Caroni et al., 1988; Kottis et al., 2002; Chen et al., 2000). Furthermore, upregulation of Chondroitin Sulfate Proteoglycans (CSPGs), which are inhibitory molecules found in the glial scar function as a barrier to axon regeneration after injury (Hueber &Strittmatter, 2009; Asher et al., 2000; Silver&Miller, 2004). The synthesis and secretion of glycosaminoglycans (GAGs) into the extracellular matrix (ECM) by astrocytes, neurons, macrophages and oligodendrocytes (Uhlin-Hansen et al., 1993; Jones & Jones, 2000; Asher et al., 2002) that contribute to the CNS inhibitory environment peaks on 7 dpi. GAGs are still present in the lesion site until 21dpi (Pearson et al., 2020). Obstacles to axon regeneration include chemorepellent proteins such as the repulsive guidance molecule (RGM) (Wang et al., 2018) and Semaphorin 4D (Sema4D) (Moreau-Fauvarque et al., 2003). When all these barriers are overcome, axon regeneration is potent, but it usually takes more time to occur. Luo et al., 2013 observed that 1 month after injury less than 120 axons per ON reach the optic chiasm in mice, whereas a similar pattern was observed after 6 weeks in another study (Kurimoto et al., 2010). Midline crossing of the chiasm in transgenic mice takes place almost at 8 weeks (Pernet et al., 2013) or at 10 weeks (de Lima et al., 2012), whereas at 3 months post injury (mpi) axons can reach the SCN which is the nearest brain target. However, 4 months following injury the growth pattern of the regenerating axons changes continuously, until new neuronal circuits are completely reformed (Li et al., 2015).

Remyelination

In order to entirely reform the neuronal circuits remyelination is necessary. Myelin sheaths that surround the neuronal axons are produced by mature oligodendrocytes (OLs) and facilitate rapid

electrical conduction of axon potentials (Butt et al., 2019). An injury in the ON damages not only the axon, but also the myelin sheaths, which are phagocytosed by macrophages in order to ‘’clean’’ the injury site from myelin debris (Kopper & Gensel, 2018). When inflammation stops and axon regeneration begins, remyelination process can start. Remyelination depends on the extent of OL death after axotomy and the availability of growth factors that can drive OPC proliferation and differentiation into mature OLs (LIoyd & Miron, 2016). After injury, mature OLs cannot contribute to remyelination. Instead, remyelination depends mainly on OPC differentiation and Neural Stem Progenitor Cells (NSPCs). Oligodendrogenesis starts the first few months after injury (Quraishe et al., 2018).

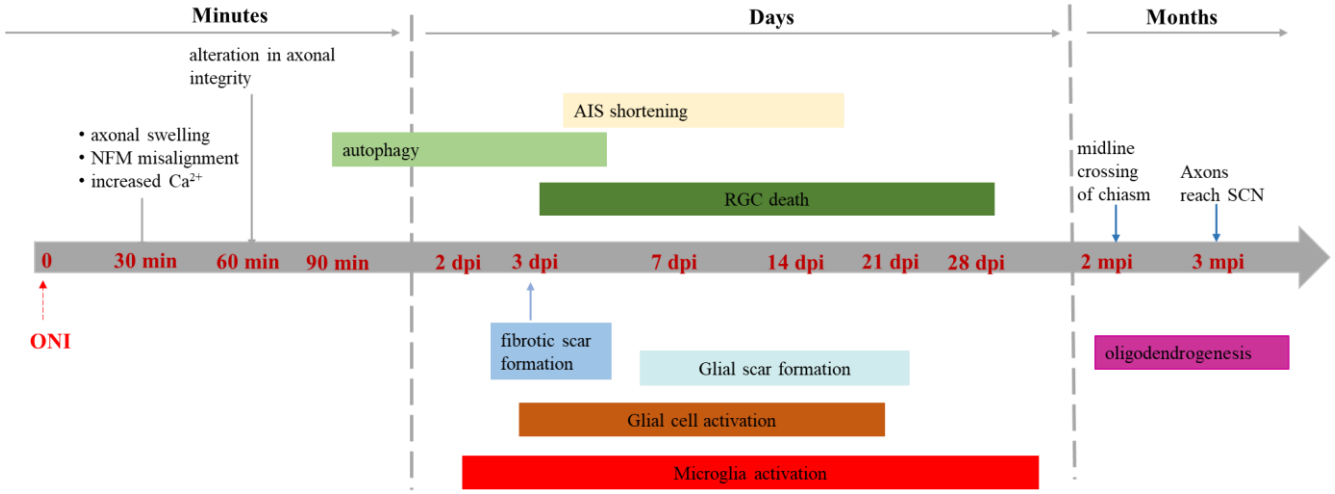


Fig 1-4: Illustration of the timeline of elementary events that take place after Optic Nerve Injury (ONI). The primary phase is short and consists of ischemia and edema. The secondary phase takes longer and is characterized by a cascade of cellular and molecular events that begin immediately after injury and progress until several months post injury. NFM: neurofilament, RGC: retinal ganglion cells, GAGs: glycosaminoglycans, AIS: axonal initial segment, SCN: suprachiasmatic nucleus.

1.2. Models of Traumatic Optic Neuropathy

1.2.1 TON Models

Appropriate animal models are crucial for mimicking a disease, understanding its mechanism and also evaluating the efficacy of candidate treatments. Regarding TON, there have been established five different models that refer to direct and also indirect type of injury (Bastakis et al., 2019; Ibrahim et al., 2018). Direct TON includes optic nerve transection (ONT) (Vidal-Sanz, et al.,1987; Villegas-Perez et al., 1993; Solomon et al., 1996; Magharious et al., 2011) and optic nerve crush (ONC) (Selles-Navarro et al., 2001; Tang et al., 2011; Templeton & Geisert, 2012) whereas indirect TON includes

controlled orbital impact (COI) (Ibrahim et al., 2018), ocular blast injury (OBI) (Hines-Beard et al., 2012) and sonication- induced traumatic optic neuropathy (SI-TON).

Optic Nerve Crush

Optic nerve crush is a well-established model for studying vision – related neurodegenerative diseases such as glaucoma (Schwartz, 2004). After trauma, RGC number decreases progressively. One week after crush there is a 47% RGC survival, two weeks after crush RGC survival drops to 27%, while 8 weeks after crush RGC survival drops to 8% (Levkovitch-Verbin et al., 2000).

There are two ways to perform ONC: intra-orbitally (Levkovitch-Verbin, 2004) and intracranially (Chierzi et al., 1999), using tools such as forceps, balloons or other devices. Forceps are most commonly used in the intra-orbital ONC (Villegas-Perez et al., 1993; Yoles et al., 1999). A conjunctival peritomy is made behind the limbus and the blunt is dissected above the eyeball and posteriorly. Forceps are used to crush the optic nerve. The ON is exposed after retracting the superior ocular muscles and then crushed 0.5 - 1 mm behind the optic disc, taking care not to injure retinal vessels and disrupt blood supply (Liu et al., 2019). The duration applied to induce ONC varies between 1 sec (Levkovitch-Verbin, 2004) and 10 sec (Chierzi et al., 1999; Liu et al., 2020). This technique presents some drawbacks. It is hard to estimate the applied force to achieve a standard result of optic nerve crush, so it depends on the individual performing (Levkovitch-Verbin, 2004; Xue et al., 2016; Tao et al., 2017). For this reason, there have been developed microinjury devices that measure and control the parameters of force and time during the experiment (Gellrich et al., 2002).

Optic Nerve Transection (axotomy)

Optic nerve transection (ONT) is considered a highly invasive model of TON (Bastakis et al., 2019). An incision is made in the skin above the orbital rim and supero-external orbital contents are dissected. The superior and external rectus muscles are sectioned and the left ON is exposed and transected completely (Parilla-Reverter et al., 2009). Afterwards, the eye fundus of each eye is checked in order to verify that the retinal blood supply is normally maintained (Levkovitch-Verbin, 2004; Migallon et al., 2016).

In ONT, RGC death occurs more quickly compared to ONC (Migallon et al., 2016). RGC death is not an instantaneous process, but rather it lasts for several days. It can be distinguished in two exponential phases: most RGCs die via apoptosis 3 to 7 days after lesion. Between 9dpi and 21dpi, the number of RGCs that survive reaches a plateau (Migallon et al., 2016). Overall, RGC death is extensive after ON

transection and reaches more than 90% in 2 weeks (Li et al., 2017). RGCs in axotomized ONs die due to withdrawal of neurotrophic factors from their axonal terminals (Reynolds et al., 2000). Apoptosis can be triggered by two ways: death receptor activation (extrinsic pathway) and mitochondrial release of cytochrome C (intrinsic pathway) (D' Amelio et al., 2012).

Optic nerve transection is useful for studying injury-induced loss of RGCs (Lafuente et al., 2002; Lindqvist et al., 2002; Lindqvist et al., 2004), protection of RGCs' from apoptosis using specific compounds (Lafuente et al., 2002; Aviles-Trigueros et al., 2003; Mayor-Torroglosa et al., 2005) and molecular and functional alterations in RGCs upon injury (Salvador-Silva et al., 2000; Chidlow et al., 2005; Sobrado-Calvo et al., 2007; Agudo et al., 2008; Agudo et al., 2009; Nadal-Nicolás et al., 2009).

Sonication-Induced TON

SI-TON is a non-invasive TON model that uses ultrasonic pulses to create the lesion. SI-TON significantly decreases the number of RGCs in the middle and central retina, in those mice that survived the injury during the first week. Moreover, there is a progressive decrease in RGCs survival over time and also NFL and IPL seem thinner in the injured retinae, another evidence that indicates loss of RGCs owing to the injury (Tao et al., 2017).

To trigger TON, a laboratory sonifer with microtip (3 mm diameter) is placed on the supraorbital ridge at 2mm medial and 2mm caudal to the vertical mid-pupillary line of each anaesthetized mouse. The ultrasound pulse can be delivered directly through the bones of the optic canal and absorbed by the optic nerve. The sound energy is concentrated focally at the entrance of the optic canal (Tao et al., 2017). However, SI-TON presents some serious drawbacks: pulses can also damage the contralateral ON due to the scattering of ultrasound energy, therefore the contralateral eye cannot be used as a control. Furthermore, the microtip used to create the injury can target inaccurately and cause ocular rupture (Bastakis et al., 2019).

Controlled Orbital Impact

Controlled Orbital Impact (COI) is a novel model of TON which provides simplicity, precision control and flexibility, provides reproducible results, and is considered a good model to study neuroprotection and neurorestoration. Regarding RGCs' survival, it is observed a progressive deficit in RGC electrophysiological functions, as RGC loss is significantly decreased 3-5 days post injury. Simultaneously, there is also microglia activation which may contribute to RGCs' death.

In order to establish the COI model, the mouse is put in a stereotactic frame and using forceps and scissors is induced an incision at the medial canthus, taking care to avoid bleeding. The eyeball is retracted from the orbital margin and the extraocular tissues remain exposed to an impactor tip. The injury site is located 2-3 mm away from the posterior pole of the globe and the necessary velocity is 2-3m/sec, whereas the contusion depth and time are 0.6 mm and 10 ms respectively.

COI presents some important benefits such as no indications of mortality and ocular comorbidity including cataract and edema. Furthermore, there are no occasions of tissue damage as in ONT and ONC and there is a wide range of TON severities depending on the velocity of the impactor. Lastly, the contralateral eye can be safely used as a control (Ibrahim et al., 2018).

Ocular Blast Injury

OBI model is a very useful model to study the molecular mechanisms of TON and also to identify and test potential therapeutic compounds. After OBI, there is observed a photoreceptor cell loss, a physical damage to the choroid and also optic nerve avulsion (Hines- Beard et al., 2012).

This model implies the exposure of the eye to an open waveform primary blast of known pressures. This can be controlled by regulating the input pressure or by increasing the distance between the eye and the barrel. The rest of the body is protected from blast and usually the contralateral eye remains unaffected. However, there is a very low incidence of cataract and corneal edema in the injured eye (Hines- Beard et al., 2012).

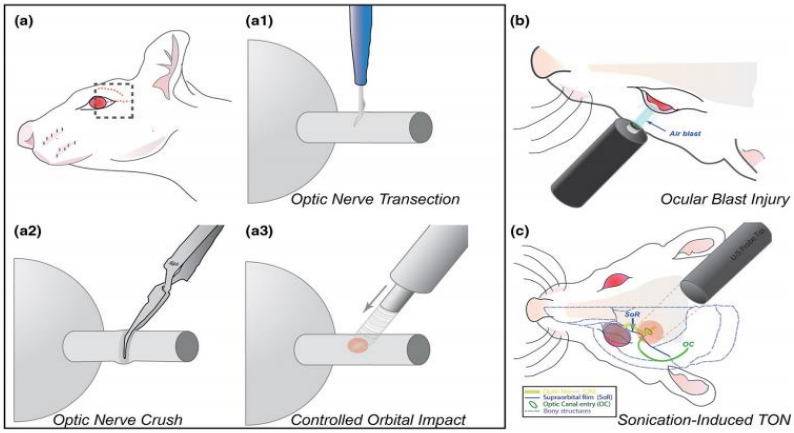


Fig. 1-5: Schematic of TON animal models. a: invasive TON models such as: ONT (a1), ONC (a2) and COI (a3). (b): non-invasive TON models such as: OBI (b) and SI-TON (c) (Bastakis et al., 2019).

1.2.2 Tissue Assays Utilized in TON Studies

RGC survival

RGCs, located in the GCL together with astrocytes and displaced amacrine cells, comprise 40-50% of GCL cells (Bunt & Lund, 1974; Perry, 1981; Schlamp et al., 2013; Nadal-Nicolas et al., 2015b). RGC survival is evaluated immunohistochemically using specific markers that stain RGCs such as NeuN, Brn3 family, tubulin β3, Thy-1 and Y-synucleins.

NeuN is a common marker (Buckingham et al., 2008; Templeton et al., 2009; Yang et al., 2012; Zhu et al., 2013) as it is expressed by approximately 68% of cells in the mouse retina. However, NeuN also stains displaced amacrine cells located in the GCL (Raymond et al., 2008). Another marker used for RGC survival is the Brn3 family of transcription factors that are highly important to RGC development (Mead et al., 2016). This family includes Brn3a (Pou471), Brn3b and Brn3c. Brn3a is expressed by approximately 46% of GCL cells, whereas Brn3b is expressed by 54% of RGC (Nadal-Nicolas et al., 2009; Fernades et al., 2012; Zhu et al., 2012). Other studies revealed that 80-90% of RGCs are Brn3a positive (Nadal-Nicolas et al., 2009; Rodriguez et al., 2014; Mead et al., 2014). RBPMS is an RNA-binding protein, predominantly located in the nucleus and is involved in post-transcriptional modifications of mRNA (Kwong et al., 2011). It is an authoritative marker of RGCs as 99.5% of RGCs are RBPMS positive (Mead et al., 2016). Moreover, RGC survival can also be assessed by tubulin β3 staining (Leibinger et al., 2013; Jiang et al., 2015). Tubulin β3 stains both RGCs and amacrine cells located in the GCL (Mead et al., 2014). Y-synucleins are small, unfolded proteins (Surguchov et al., 2001) that are expressed in the RNFL and the GCL in the murine retina. They stain mainly the cytoplasm of RGCs but also other cells that are in GCL and are not affected by ONC (Sun et al., 2014).

Fluorogold (FG) is a fluorescent retrograde tracer useful for labeling RGC morphometry after insults (Selles-Navarro et al., 1996; Jehle et al., 2008). FG is injected intracranially either before ONC or 5-7 days before sacrifice (Huang et al., 2014). FG injection prior to ONC leads to overcounting because FG also labels engulfing macrophages and microglia (Sorensen et al., 1996; Levkovitch-Verbin, 2003; Blairet et al., 2005; Grieshaber et al., 2010).

RGC death

RGC apoptosis is studied via various apoptosis-specific assays including TUNEL. The deoxynucleotidyl transferase Dutp (TUNEL) assay probes DNA fragmentation that takes place during

programmed cell death. Fragmentation enables a modified Dutp (BrdUTP or EdUTP) to bind to the 3-OH of the DNA as the TUNEL reaction indicates. Evaluation of apoptotic cells takes place after incubation with specific antibodies (Kyrylkova et al., 2012). The ideal time to study RGC apoptosis via TUNEL assay are the first 1-3weeks after crush, as almost 90% RGC die between 7-14 dpi (Thomas et al., 2017). Zhang studied RGC death in an IOP glaucoma model using the TUNEL assay together with the Brn3a RGC marker at 1,2,4 and 6 weeks after glaucoma introduction (Zhang et al., 2019). TUNEL assay revealed that RGC death peaked 7 dpi after ONC (Li et al., 1999).

Real-time imaging of RGC death is a novel technique that measures RGC death *in vivo* within 10 minutes. It is a minimally invasive and quick method and requires a confocal scanning laser ophthalmoscope (cSLO) and a photo-switching, cell-impermeant and fluorescent nucleic acid dyeing compound, SYTOX orange (SO). SO is injected in the vitreous and 10 minutes later, RGC death is evaluated *in vivo*. The result is that RGCs die by apoptosis between 4-6 dpi and reach their peak (almost 53%) on 5 dpi, (Tsuda et al., 2016).

As RGC death is mediated through caspase – dependent apoptosis, immunohistochemistry against caspase-3 can be used to study RGC death. Cheung et al., 2004 showed that on 3 dpi, activated caspase 3 was present in the majority of cells with fragmented nuclei in axotomized retinas. Another study showed that the amount of cleavaged caspase-3 peaks on 5 dpi (Wang et al., 2015). Wang et al., 2015 studied the expression of Programmed cell death (PD-1) receptor, which is expressed by T- and B- cells by Real Time PCR on 0, 1, 3, 7 and 10 days after ONC. On 3dpi PD-1 mRNA was found significantly elevated, whereas on 10dpi PD-1 mRNA was increased 8 folds compared to controls. Immunohistochemistry against PD-1 proved that PD-1 in large RGCs was increased by 82% in the injured retina, compared to controls.

RGC apoptosis takes place within the first days-weeks post injury. Levkovitch -Verbin et al., 2000 revealed that RGC survival was significantly decreased 2 weeks after ONC in transgenic mice. Another study, examined the effect of ONC on RGCs in mice, and found that RGC loss was about 65% in the first 7 days (Sanchez-Migallon et al., 2016). Overall, RGC survival is usually evaluated within the first 2-4 weeks post injury (Berhelaar et al., 1994; Kwong et al., 2011; Rodriguez et al., 2014). Few studies have examined long-term RGC survival after ONC (some months post injury). Templeton et al., 2009 observed that 30 days post-ONC 54% of RGCs had survived. Another study assessed RGC survival after 6 months and demonstrated contradictory results. Only 1-4% of RGCs survived after ONC or ONT. (Nadal-Nicolas et al., 2015b).

#	Publication	Model of injury	Timepoint (days post injury)	Treatment	Result
1	Grinblat et al., 2018	ONC in mice (1s)	5	No	50% RGC survival
2	Sanchez-Migallon et al., 2016	ONC in mice (10s)	7	No	40% RGC survival
3	Wang et al., 2015	ONC in mice (3sec)	7	No	17% RGC survival
4	Li et al., 2007	ONC in C57BL/6J mice (3s)	14	No	68% RGC survival
5	Templeton et al., 2009	ONC in mice (10s)	30	No	54% RGC survival
6	Mesentier-Louro et al., 2019	ONC in rats (15s)	60	MSC transplantation	50% RGC survival
			240		50% RGC survival

Table 1.2-1: Indicative publications that study RGC survival and apoptosis.

Assays of inflammation/astrogliosis

Inflammation takes place within the first hours/days after ONC and concerns three types of retinal cells: microglia, Muller cells and astrocytes (Cuenca et al., 2014). Microglia phagocytose useless and toxic products in human retina (Graeber & Streit, 1990) and are responsible for cleaning myelin and cellular debris after injury. Microglia secrete anti-inflammatory cytokines and Growth Factors such as BDNF, CNTF, GDNF etc (Nimmerjahn et al., 2005; Neumann et al., 2009; Cuenca et al., 2014). Normally, microglia are at resting state, express Ionized Calcium – Binding Adaptor molecule 1 (IBA-1) (Mori et al., 2000), whereas upon injury they become activated and present elevated expression of IBA-1(Wan et al., 2020). Furthermore, Glial Fibrillary Acidic Protein (GFAP) expression is increased in astrocytes and Muller cells upon injury and therefore is used as an indicator of retinal stress (Craft et al., 1985; Ramirez et al., 1994; Wu et al., 2003; Luna et al., 2010). GFAP expression is modulated by cytokines and growth factors found near to the lesion site (Wu et al., 2003). GFAP expression in retinal astrocytes is difficult to observe because astrocytes' baseline GFAP expression is high (Lewis & Fisher, 2003). On the other hand, Muller cells do not normally show

GFAP immunoreactivity (Molnar et al., 1984; Hiscott et al., 1984). Upon injury, Muller cells express GFAP positive processes across the entire length of the retina (Wu et al., 2003). Another important marker of inflammation is the intermediate filament vimentin. Vimentin expression is increased in both astrocytes and Muller cells after reactive gliosis caused by ONC (Lewis & Fisher, 2003; Cuenca et al., 2014).

Assays of Axon Degeneration and Regeneration

Axon degeneration takes place during the first days after ONC and causes loss of connectivity between the axons and as a result lead to loss of neuronal circuits function and loss of vision. Cholera Toxin B (CTB) is a very useful marker to study immunohistochemically the axon degeneration. CTB is injected intraocularly and labels anterogradely axons that begin from the RGC somata and project to the brain. It can be applied either some days before ONC (Kole et al., 2020) or some days before animal sacrifice (Mendoca et al., 2020). CTB staining quantification is based on counting the number of CTB positive fibers that extend at different distances from the lesion site (Kole et al., 2020). A recent study that used ONC model in WT and Arginase enzyme arginase 2 (A2) KO mice revealed that $A2^{-/-}$ presented increased axonal sprouting 14 dpi as indicated by CTB staining. In detail, there was a 1.5-fold increase between $A2^{-/-}$ and WT at 200μm from the crush site and a 2.5-fold increase at 300μm from the lesion site compared to WT mice (Xu et al., 2018).

Axon regeneration is a slow phenomenon- it occurs over approximately 1 month (Kurimoto et al., 2010; Luo et al., 2013). Immunohistochemistry (IHC) against various markers (GAP-43, L1) is usually utilized to study axon regeneration across the damaged tissue. Growth Associated Protein-43 (GAP-43) is a cell membrane phosphorylation protein located in the nerve terminal membrane (Grasselli & Strata, 2013). GAP-43 is associated with regeneration, nerve outgrowth and synapse formation (Wang et al., 2017). Kitamura et al., 2019 proved that administration of a cocktail of neuroprotective agents for 2 weeks in ONC rats, lead to a significant increase in axon regeneration, almost 300 μm away from the crush site, compared with the control group.

Another useful marker to study axon regeneration is the Immunoglobulin superfamily member of CAMs (IgSF). L1, a protein involved in axon regrowth and remyelination (Roonprapunt et al., 2003; Barbin et al., 2004; Chen et al., 2007; Becker et al., 2001) is known to mimick the expression pattern of GAP-43 in regenerating axons after a CNS trauma (Savvaki et al., 2021). L1 is a cell-adhesion molecule that is significantly elevated during axon regrowth (Castellani et al., 2002; Xu et al., 2004). Immunolabelling in the proximal ON after ONC in mice revealed L1 immunoreactivity up to the lesion site 1 week post crush. 4 weeks after ONC, L1 staining was maintained, however the intensity of L1

signal was lower, consistent with RGC apoptosis. Spared axons 8 months after injury maintain some L1 immunoreactivity (Becker et al., 2001). Another study examined L1 mRNA expression after ONC in rats and showed that 2 dpi $L1^+$ RGCs are more than control (no lesion) rats (1600 vs 1400 per mm^2) and over the following 28 days after crush the number of $L1^+$ RGCs progressively decreased. Only a small proportion of these cells were $GAP43^+$. Most $GAP43^+$ RGCs appeared on 28dpi. (Jung et al., 1997). Furthermore, 2 weeks post ONC the number of $L1^+$ axons were significantly lower ($p<0.001$) in wild type mice compared to $Cntn2^{-/-}$ close (<1000 μm) to the lesion site (Savvaki et al., 2021). Finally, increased expression of L1.1, L1.2 genes induces CNS axon regeneration in RGCs (Bernhardt et al., 1996).

Assays of Demyelination and Remyelination

Demyelination process starts the first days following an ONI, as inflammatory cells (microglia) remove the myelin debris *(Fig. 1-4)*. Demyelination is usually studied by evaluating axon and myelin integrity. Grinblat and his group, proved that 5dpi ONC there is a 15- fold demyelination in ONC mice compared to control, after Luxol Fast Blue staining for the axon and Myelin Associated Glycoprotein (MAG) staining for the myelin (Grinblat et al., 2018). Furthermore, Bei et al., 2016, designed an Optic Tract Injury mouse model that received intravitreal injection with the construct AAV-ChR2-mCherry and sacrificed 13 weeks after injury. This proved that in injured mice ChR2 (which labeled the axons) was not colocalized with MAG (which labeled the myelin) in and across the lesion site and implied that regenerated axons were poorly remyelinated. The quantification of the result, showed that in control group 80% of $ChR2^+$ axons were colocalized with MAG, whereas in the crush group no of the $ChR2^+$ axons was colocalized with MAG.

The reconstitution of vision demands not only axonal regeneration, but also remyelination of injured axons so as to propagate successful the electrical signals from the retina to the brain. As a result, poor myelination leads to weak synaptic activation in the SC (Bei et al., 2016). Remyelination is carried out by OPCs and can be evaluated immunohistochemically by counting A2B5 immunoreactivity. A2B5 is a marker localized in OPCs, and is presented significantly elevated (approximately 2- fold) 12 weeks after ONC in $PTEN^{-/-}$ mice that received intravitreal injections with cyclic AMP and zymosan at 3- and 6-weeks post injury (Regenerating group) compared to ONC group. O4 is another useful marker for remyelination studies. O4 antigen is expressed by late OPCs/preoligodendrocytes and is found almost 5- fold increased at 2 weeks post crush and 2-fold increased at 12 weeks pi in the Regenerating group (Mendoca et al., 2020).

1.3 Neurotrophins in Traumatic Optic Neuropathy

1.3.1 Neurotrophins, Neurotrophin Receptors & their Expression in ON

Neurotrophins and Neurotrophic Receptors

Neurotrophins (NTs) are proteins that mediate neuronal survival and regeneration throughout the CNS. The neurotrophic family includes: NGF, BDNF, NT-3 and NT-4/5. Mature neurotrophins arrive from pro-neurotrophin precursor forms. Pro-neurotrophins are cleaved either intracellularly by furin or proconvertases or extracellularly by metalloproteases and plasmin (Houlton et al., 2019).

Mature neurotrophins bind to Trk receptors, whereas pro-neurotorphins have a high affinity to $p75^{NTR}$ receptor which induces pro-apoptotic effects (Nykjaer et al., 2004; Teng et al., 2005). $p75^{NTR}$ belongs to the tumor necrosis factor receptor (TNFR) superfamily and binds to all neurotrophins with similar nanomolar affinity (Bothwell, 1995). Regarding Trk receptors, TrkA binds selectively NGF, TrkB binds BDNF and NT-4/5 and TrkC binds NT-3. The binding of the neurotrophin ligand to a specific type of Trk receptor induces receptor dimerization and subsequent transphosphorylation at specific intracellular tyrosine kinase residues, which recruit adaptor proteins and activate specific intracellular signaling pathways (Houlton et al., 2019). Most neuronal populations co-express both $p75^{NTR}$ and Trk receptors that can also interact. This interaction positively regulates Trk function following the binding of a mature neurotrophin (Reichardt, 2006).

The binding of a neurotrophin to its Trk receptor induces an intracellular cascade of molecular events. Trks can induce the phosphorylation of PLC-γ which activates IP3 / DAG and increases intracellular Ca^{2+}. The increased Ca^{2+} triggers enhanced neuronal and synaptic plasticity (Chao, 2003; Yoshii & Constantine-Paton, 2010). Furthermore, Trk receptors can activate molecular pathways that activate Ras GTP-binding protein and the MAPK pathway, which induces ERK phosphorylation and CREB activation. CREB is responsible for transcriptional changes to genes associated with neuronal differentiation and neurite outgrowth (Kaplan & Miller, 2000). Trk receptors also activate the PI3K pathway, which leads to AKT activation and subsequently the activation of the transcriptor factor NF-κB. NF-κB is associated with neuronal survival (Reichardt, 2006), cell growth, proliferation (Cantley, 2002) and also axonal sprouting in hippocampal neurons (Atwal et al., 2000; Sanchez et al., 2001; Jaworski et al., 2005).

The pan-neurotrophin receptor $p75^{NTR}$ forms a complex with sortilin that promotes apoptotic signaling (Teng et al., 2005; Nykjaer et al., 2004). The complex of a pro-neurotrophin with $p75^{NTR}$ and sortilin induces activation of the JNK pathway, which leads to apoptotic cell death (Reichardt, 2006; Harrington et al., 2002). The complex of $p75^{NTR}$ with NRIF1/NRIF2 or NADE also activates the pro-

apoptotic Rac pathway (Mukai et al., 2003; Dechant & Barde, 2002; Yeiser et al., 2004), whereas the complex of $p75^{NTR}$ with Lingo-1 or Nogo-66 (NgR1) induces RhoA activation, inhibits axonal sprouting and neural plasticity (Scwab, 2010). Furthermore, RhoA activation inhibits OL differentiation and myelination (Tan et al., 2012).

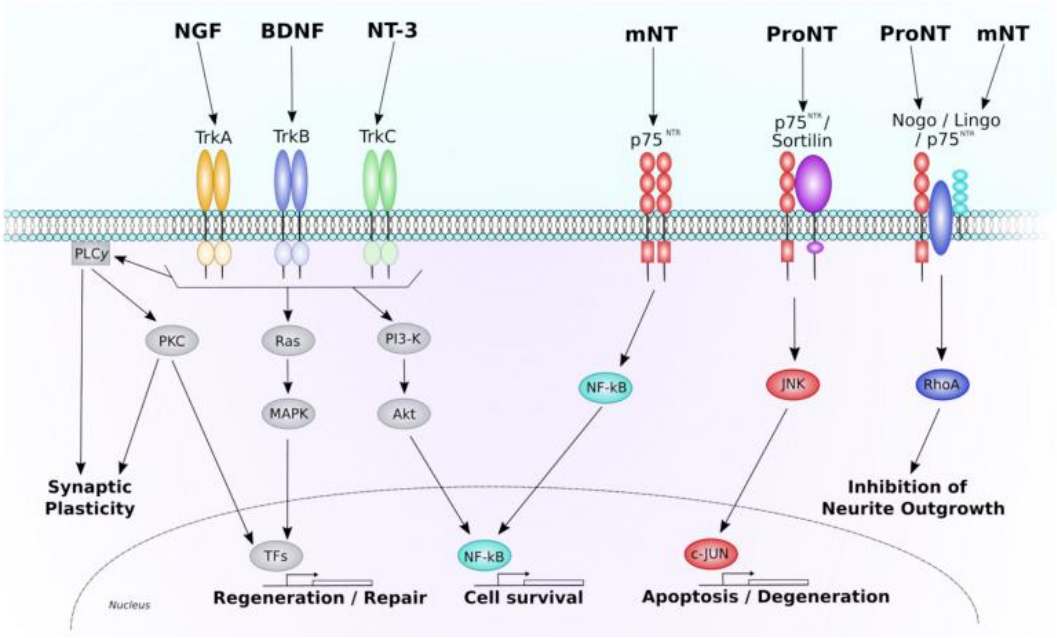


Figure 1-6: Depiction of neurotrophins and their receptors. Mature neurotrophins bind to Trk receptors and activate intracellular pathways that regulate gene transcription, survival and regeneration. The $p75^{NTR}$ receptor can bind either mature neurotrophins or pro-neurotrophins and is implicated in pathways that control cell survival, apoptosis/degeneration and neurite outgrowth inhibition. NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; Trk, tropomyosin receptor kinase; mNT, mature neurotrophin; ProNT, proneurotrophin; p75NTR, pan neurotrophin receptor 75; PLCγ1, phospholipase C gamma one; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TFs, transcription factors; PI3-K, phosphoinositide 3-kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinase (Houlton et al., 2019).

Neurotrophic expression in the mammalian retina and ON

Cells in retina and in ON require secretion of neurotrophins and expression of neurotrophic receptors so as to ensure their survival and proliferation. TrkA mRNA can be found in cells in INL and GCL in rat retina (Ernfors et al., 1992). NGF but not TrkA is found in Muller cell processes (Chakrabarti et al., 1990; Vecino et al., 1998a, b). In rat retina NGF is also localized to the RPE, photoreceptor outer segments, ONL and IPL (Chakrabarti et al., 1990; Vecino et al., 1998; Bronzetti et al., 2007; Sun et al., 2008; Garcia et al., 2014). In the INL and in the GCL, NGF is encountered in cell bodies and in RGC somata respectively (Garcia et al., 2014). Processes of positive glutamine

synthetase (GS)-Muller cells that cross the ONL present NGF immunoreactivity, whereas $GFAP^+$ astrocytes do not (Garcia et al., 2014). However, it has been shown that during development NGF is weakly expressed by $GFAP^+$ astrocytes (Liu et al., 2010). proNGF is expressed in Muller cells (Ali et al., 2011), RGCs (Wei et al., 2012) and microglia (Garcia et al., 2016) in rats. Bipolar cells, express TrkA (Garcia et al., 2014), whereas the expression of TrkA in photoreceptors is still controversial. Kokona et al., 2012 support the presence of TrkA in rat photoreceptors, whereas Di Polo et al., 2000 support that no TrkA protein was detected in rat retina.

TrkB is expressed in the visual system of most vertebrates as its ligand BDNF is important for RGC differentiation and maturation (Frost et al., 2001; Garcia et al., 2003). BDNF expression in adult porcine retina is prominent in RGCs and INL (Garcia et al., 2003). TrkB expression is quite complex, as 6 distinct TrkB transcripts are found the mouse brain (Klein et al., 1990) and 8 transcripts in the rat brain (Middlemas et al., 1991). TrkB is not detected in photoreceptors in normal rat retina (Richman & Brecha, 1995; Jelsma et al., 1993), whereas TrkB is found in most GCL neurons (Perez & Caminos, 1995; Hallbook et al., 1996; Vecino et al., 1998) and also in some amacrine, Muller (Seki et al., 2003) and bipolar cells in the INL (Garner et al., 1996). TrkB mRNA is located in INL and also in GCL in the adult rat retina (Jelsma et al., 1993; Perez & Caminos, 1995).

TrkC shows weak expression in normal rat retina (Cui, 2003) or even no expression (Rickman & Brecha, 1995). On the other hand, some contradictory results suggest the presence of TrkC mRNA in the INL and GCL of the adult rat retina (Ernfors et al., 1992).

The pan-receptor $p75^{NTR}$ is present in mammals in INL and RPE (Schatteman et al., 1988; Vecino et al., 1998a, b), mainly in Muller cells that extend through the entire retina (Vecino et al., 1998b). In photoreceptors $p75^{NTR}$ seems to be present in low levels (Srinivasan et al., 2004; Santos et al., 2012), whereas in bipolar cells of adult rats $p75^{NTR}$ is not expressed (Garcia et al., 2016). Studies on $p75^{NTR}$ expression in RGCs provide contradictory results, as some studies agree that $p75^{NTR}$ is expressed in RGCs of adult rats (Coassin et al., 2008; Colafransesco et al., 2011), whereas others believe that there is no $p75^{NTR}$ expression in RGCs of adult rats and mice (Lebrun – Julien et al., 2010; Hu et al., 1998; Xu et al., 2009; Ding et al., 2001). Astrocytes in adult mice retinas are characterized by faint $p75^{NTR}$ labeling whereas there is no positive labeling in astrocytes of adult rat retinas (Hu et al., 1999; Wei et al., 2008).

NT-3 is present in most retinal cell types in different species, which implies that maybe NT-3 function is important for retinal development and cell differentiation (De La Rosa et al., 1994; Barolenta et al., 1996; Vecino et al., 1998). Finally, NT-4/5 is present in neonatal rat retina and in the superficial layers of the superior colliculus (Spalding et al., 2004).

Trafficking of Neurotrophins and their Receptors

Neurotrophic factors in the retina are either transported to RGC somata from the superior colliculus retrogradely through the ON or are locally produced by neurons and glial cells located in the retina (Von Bartheld, 1998). According to the classical neurotrophic theory, neurons receive trophic support from their targets (Levi-Montalcini, 1987; von-Bartheld et al., 1996b) and this process can be achieved by four distinct ways: anterogradely from afferent neurons, retrogradely from innervated neurons, autocrine and paracrine (Korsching, 1993; Curtis & Distefano, 1994; Von Bartheld et al., 2001; Cohen-Cory & Lom, 2004). Retrograde axonal transport of neurotrophins provides neurons with useful information about their surrounding microenvironment and neurons can respond by surviving, by undergoing apoptosis or by altering the synaptic connections with their targets (Hendry, 1980). NGF was the first NT that showed to undergo retrograde transport. Injection in the anterior eye chamber with ^{125}I- labeled NGF revealed that NGF was transported retrogradely in neural crest-derived sensory neurons (Henry et al., 1980; Johnson et al., 1978). This result comes into agreement with endogenous NGF which is also retrogradely transported (Palmetier et al., 1984), whereas BDNF, NT-3 and NT-4/5 presented retrograde transport only in certain neural cell populations (Di Stefano et al., 1992; Curtis et al., 1995).

Successful retrograde transport of a NT requires its release from the presynaptic membrane. Experiments in rat hippocampal slices and primary cultured hippocampal neurons revealed that NGF release depends upon the activity, extracellular Na^+ and intracellular Ca^{2+} (Blochl & Thoenen, 1995). It is hypothesized that NT themselves regulate the release of NTs from the presynaptic cells (Kruttgen et al., 1998; Canossa et al., 1997). The release of a NT is followed by its binding to a suitable Trk receptor or to $p75^{NTR}$ in the presynaptic plasma membrane (Rodriguez-Tebar et al., 1992; Thoenen, 1995).

The binding of a NT to its receptor induces receptor clustering at the membrane (van 't Hof et al., 1989) or dimerization, forming either homodimers or heterodimers (Jungbluth et al., 1994). As soon as receptors dimerize, they become phosphorylated and activated.

The binding of the ligand to its receptor induces receptor internalization. In PC-12 cells NGF induces the internalization of TrkA mainly in a clathrin-mediated manner. During internalization, the complex of NT and Trk remains bounded, activating intracellular cascades (Grimes et al., 1996). However, except from clathrin-dependent endocytosis, there is also a clathrin-independent means of internalization, known as the caveolae-based system (Galbiati et al., 1998). $p75^{NTR}$ and TrkA are known to interact with caveolin-1 in PC12 cells, implying that maybe caveolin-1 is associated with receptor internalization (Bilderback et al., 1999).

Once the receptor is internalized, it must be decided if it will be recycled to the plasma membrane or targeted for retrograde transport. NTs and phosphorylated Trks are transported mainly retrogradely (Ehlers et al., 1995; Tsui-Pierchala et al., 1999). PI-3 kinase is a potent regulator of retrograde transport of NGF *in vivo* and this process is inhibited by its antagonist wortmannin in sympathetic and sensory neurons in adult mice (Barlett et al., 1998). It is hypothesized that different isoforms of PI-3 kinase are involved in mediating NTs' retrograde axonal transport in different cell populations (Reynolds et al., 2000). Experiments conducted using ^{125}I- labeled NGF proved that is transported at a rate of 1-5 mm/h to the cell bodies of sympathetic neurons, and the transport process is highly dependent on the microtubular system (Hendry et al., 1974).

Once the receptor - ligand complex reaches the cell body, signaling to the nucleus takes place. Intracellular cascades activate specific transcription factors such as CREB (Riccio et al., 1997) or other effects are mediated independently of the nucleus as a result of mRNA stabilization (Raynaud et al., 1988). As soon as the NT together with its receptor reach the cell body, the degradation process begins. It has been proved that NGF conjugated with HRP is incorporated in secondary lysosomes and is finally broken down (Schwab et al., 1977). This destruction of the NT in the lysosomes implies ''switching-off'' its signal (Reynodls et al., 2000).

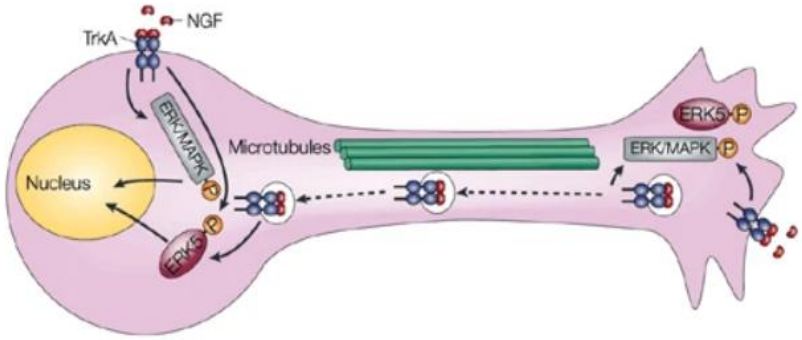


Fig. 1-7: Retrograde transport of NGF and its receptor TrkA from the axon to the cell body. NGF binding to TrkA causes ERK5 and ERK/MAPK phosphorylation and activation and subsequent gene expression (Sorkin & von Zstrow, 2002).

1.3.2 Alterations of NTs and NTRs Expression after ONI

Axotomized neurons that survive ONC are more likely to die by apoptosis as injury takes place closely to their soma. Apoptotic signals are triggered by the deprivation of trophic factors, as retrograde transport of NTs by their distant targets and also by glia is severely impaired (Mesentier-Louro et al., 2017). As a consequence, neurons respond to acute injury by downregulating their neurotrophic

receptors, as also reported in SCI (Kobayashi et al., 1997) and TBI animal models (Venero et al., 1994).

NGF protein levels do not alter significantly during the first 1 to 3 days post ONC/ONT compared to controls (Lebrun-Julien et al., 2009) while they are increased at 7 and 14 days after ONC in rat retinas (Mesentier-Louro et al., 2017). Pro-NGF follows a similar expression pattern in the retina at the same timepoints after injury (Mesentier-Louro et al., 2017, 2019; Lebrun-Julien et al., 2009) or not at all alteration at 3dpi and 10dpi (Harada et al., 2015).

Quantification of TrkA in the in retina provided also contradictory results. Lebrun-Julien et al., 2010 showed that TrkA in retina is significantly increased 48 hours after axotomy. Another study suggests that TrkA protein levels are increased in retina during the first 5 days post crush and then decline during the following 3 weeks as most RGCs are lost (Cui et al., 2003). Furthermore, TrkA mRNA levels in retina are elevated after ONT (Cui et al., 2003). Finally, some rat retinal studies show that there is no significant decrease in the first and the second week after injury (Mesentier-Louro et al., 2019). Another ONC study conducted in a Tench fish model revealed that TrkA mRNA in retina is elevated 7-30 dpi (Caminos et al., 1999).

BDNF plays a significant role in the neural response after injury (Struebing et al., 2017) as it mediates neuroprotective processes (Gao et al., 1997). Gao proved that there are no alterations in BDNF expression within the first 6 hours after injury. 1-day post-crush there is an increase in BDNF expression and the fraction of $BDNF^+$ cells in GCL reaches 10% (in controls this fraction is approximately 5- 6%). 2 days post-ONC BDNF expression levels reach a peak and the fraction of $BDNF^+$ cells in the GCL is 28%. During the next days until 1-week post-crush, BDNF levels are elevated compared to control but BDNF signal declines progressively (Gao et al., 1997). A recent study revealed that BDNF expression level is decreased by 50% 4 days after ONC in the optic tectum of zebrafish, a structure analogous to the superior colliculus in mammals, while 3 weeks post-crush, BDNF expression is increased about 1.5 times compared to control animals (Sato et al., 2010). In rats BDNF levels in retina remained normal 2 weeks after crush when most RGCs died (Chen &Weber, 2004). Furthermore, 2 weeks post-crush BDNF expression levels in retina returned to basal and during the following weeks there is no obvious signal to be detected (Gao et al., 1997). Duprey-Diaz et al., 2002 found that BDNF immunoreactivity is increased significantly 1 and 3 months after axotomy in the INL of Rana papiens retinas, however 4 months post injury BDNF levels in retina returned to slightly above control levels (Zhang et al., 2012). Regarding alterations in gene expression, it has been presented a decrease in the expression of *Bdnf-4 and Bdnf-1* genes in mice retinas survived after ONC (Struebing et al., 2017). On the contrary, in Rana papiens there are no significant alterations in BDNF mRNA expression, in retinal tissue 1, 4 and 12 weeks post ON injury (Duprey-Diaz et al., 2002).

The effects caused by ONC on TrkB receptor expression depend on the type and severity of the injury (Canals et al., 1999; Han & Holzman, 2000). It is reported that a mild damage at the ON leads to an increase in TrkB mRNA expression in retina, which is linked to neuroprotection (Chen & Weber, 2004). Cheng et al., 2002 revealed a steady decrease of RGCs' TrkB mRNA during the first 2 weeks following injury in rats. Specifically, 3 days post axotomy TrkB mRNA levels in RGCs decline by 40%, while 1 week post axotomy, TrkB mRNA signal in the GCL was 45-60% of uninjured controls. On the other hand, TrkB protein expression in retina is reported to be near normal 1 week after ONC and then is decreased to about 54% over the following 1 week. Regarding trkB mRNA, peaks 3 days post-injury, returns to basal levels 7 days following ONC and then it decreases to 40% of normal within the following week. (Chen & Weber, 2004). A different study in Rana papiens showed that there are no changes in the number of $TrkB^{+}$ RGCs between 6- and 12-weeks post ONC (Duprey-Diaz et al., 2002). However, TrkB mRNA levels in amacrine cells were unaltered after axotomy, because amacrine cells do not project to the ON (Chen et al., 2002).

The expression of the $p75^{NTR}$ receptor is significantly increased already 1 day after injury, in retina and reaches its peak 14 days in a rat ONC model (Mesentier- Louro et al., 2017). Contrary to this result, other studies showed that retinal expression of $p75^{NTR}$ is unaltered from 1dpi- 60dpi after ONT (Hu et al., 1999; Lebrun-Julien et al., 2009)

The expression of neurotrophin NT-3 was measured by *in situ* hybridization but no significant changes were detected (Gao et al., 1997). Finally, neurotrophic receptor TrkC in the normal retina is not abundant, whereas TrkC expression is increased between the first 3-5 days upon injury and is maintained until 3 weeks following injury (Cui et al., 2003).

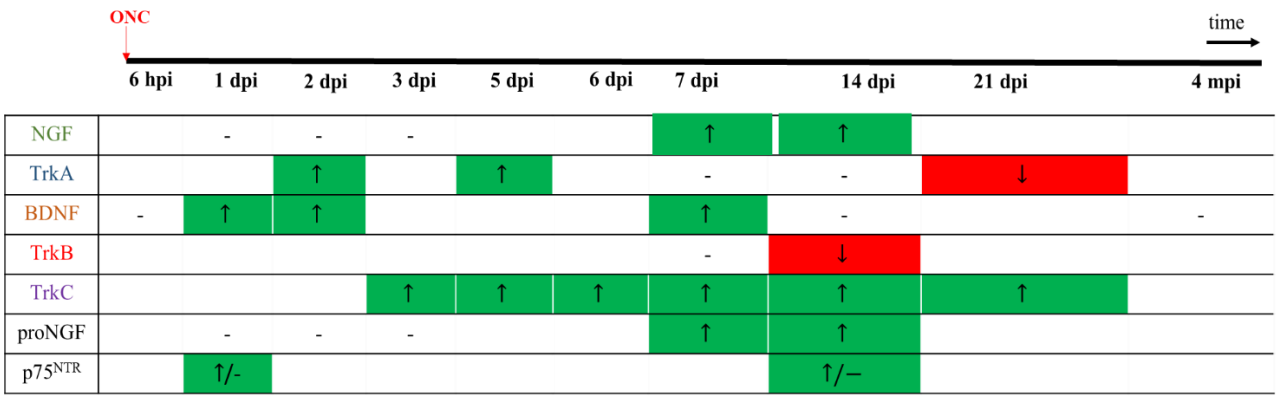


Fig. 1-8: Timeline regarding the alterations of NTs and NT-receptors following ONC. Data obtained only from rodent studies.

1.3.3 Neurotrophins as Optic Nerve Injury Treatments

RGC axon terminals that form the ON are responsible for the uptake and trafficking of NTs across the axon to the cell body (Raff et al., 1993). However, injured axons are susceptible to apoptotic signals which lead to RGC death (Almasieh et al., 2012; Calkins, 2012). Nowadays, there are different types and combinations of neurotrophin-based therapeutic treatments that are administered at different times after trauma and aim the protection of RGCs (Thanos et al.,, 1997; Gates et al., 2000; Chierzi & Fawcett, 2001; Harvey et al., 2006). NT administration increases the survival, proliferation and growth of axons in the CNS. However, the efficacy of the treatment depends on the disease state, drug properties and also the successful delivery to the target sites (Subrizi et al., 2019). The most common delivery treatment strategies that have been applied after TON are: eye drops, injections, cell transplantation, PNS grafts and delivery via biomaterials.

Delivery via Eye Drops

The most common ocular drug delivery method is via eye drops. Eye drops are delivered locally to the eye, applied to the ocular surface where the drug is mixed with the lacrimal fluid. It is considered an easy procedure, as patients can apply the treatment to their eyes by their own. Yet, it is quite effective for anterior segment diseases that influence the ocular surface such as dry eye disease and infections, or for the tissues surrounding the anterior chamber which can lead to elevated IOP, inflammation etc. Eye drops treatment is not appropriate for posterior segment diseases as the drug cannot reach in effective concentrations in the posterior segment of the eye (Urtti et al., 1990; Subrizi et al., 2019). Furthermore, eye drops demand frequent administration (1-8 times daily) due to the short duration of the drug action (Subrizi et al., 2019). The short duration of eye drops is caused by the leakage of the drug from the ocular surface to the nasal cavity in a few minutes without the use of viscosity enhancers on the ocular surface (Chrai et al., 1973). Another serious drawback that limits drug delivery via eye drops is that macromolecules such as proteins cannot pass through the corneal epithelium surface due to the tight cellular junctions. Therefore, drops are appropriate only for the topical delivery of small-molecule compounds (Ahmed & Patton, 1987; Toropainen et al., 2007).

Mesentier-Louro et al. studied the daily administration of rhNGF via eye drops (180 or 540 μg/ml) in rats after ONC. 7 days after ONC, rhNGF treatment led to significant increase in RGC survival in the peripheral retina. However, no significant alterations in axon regeneration were observed despite the presence of GAP-43 positive fibers beyond the crush site. Administration of rhNGF for 2 weeks post injury increased RGC survival in both central and peripheral retina, while injured axons achieved to regenerate 0.50 mm beyond the crush site (Mesentier-Louro et al., 2019). In

contrast, NT-4 administration via eye drops (twice per day) in a rat ONC model did not increase RGC survival (Kitamura et al., 2019). Furthermore, 2 eye drops/day of rhNGF for 3 consecutive weeks in ONT rats leads to increase in RGC survival and reduced glial activation (Guo et al., 2020).

Delivery via Injection

Eye injections can be performed intravitreally, subconjuctivally or suprachoroidally. They are suitable therapeutic methods for posterior eye segment diseases. Injections in the eye are applied mainly to the vitreous body (intravitreal injections, ivt). Bioavailability of the drug is complete after an intravitreal injection, as long as there is an adequate drug dose of the drug so as to enable prolonged duration of action (Subrizi et al., 2019). Experimental studies in rats proved that ivt injections can target RGCs and by extension ON (Chiha et al., 2020). Furthermore, intraocular injections of recombinant growth factors such as BDNF lead to increased RGC survival and axon regeneration of lesioned RGCs (Mey & Thanos, 1993). A recent study, examined the effect of ONC on RGCs in mice, and found that RGC loss was about 65% in the first 7 days and a single injection of BDNF on the day of the surgery delays RGC death and caspase-3 activation by 1 day. (Sanchez-Migallon et al., 2016).

However, NTs transferred by a bolus injection are characterized by physiochemical instability, rapid diffusion and short half-life (Flachsbarth et al., 2014). The short half-life of recombinant BDNF leads to only temporary neuroprotection (Poduslo-Curran, 1996). This can be overcome by repeated number of injections (Di Polo et al., 1998), but also induces many side-effects such as cytotoxicity and inflammation (Harvey et al., 2006; Isenmann et al., 2004; Bertram et al., 2010).

Because intraocular injections of NTs (BDNF, CNTNF, NT4/5) lead to transient RGC neuroprotection even with repeated injections (Harvey et al., 2006; Isenmann et al., 2003), gene therapy is considered a more effective therapeutic tool in ONI. Gene therapy utilizes non-enveloped replication-defective non-immunogenic ssDNA viruses (adeno-associated serotype 2 vectors, AAV2) that are injected intraocularly (Surace & Auricchio, 2003; Dinculescu et al., 2005). Gene therapy using NTs targets the transduction of neurons with neuroprotective genes (Harvey et al., 2002), providing a long-term supply of trophic factors, leading to an increase in RGC survival (Harvey et al., 2006; Martin & Quigley, 2004). Studies using AAV2 encoding the BDNF gene lead to long-term expression of the peptide promoting RGC viability of injured RGCs for at least a year (LeVaillant et al., 2016; Osborne et al., 2018; Nafissi & Foldvari, 2016). Leaver and his colleagues revealed that the AAV-BDNF-GFP construct increased RGC survival in rats, however it did not enhance axonal regeneration distally to the ON even at 7 weeks post injury. Another important finding is that non-transduced RGCs also

presented increased survival rates, which means that these cells possibly received paracrine trophic support from cells transduced-with NTs (Leaver et al., 2006).

If free drug or gene therapy cannot provide long-term effects by their own, drug incorporation in gels, particles or even implants can be advantageous as they provide sustained drug release, while they are injected intravitreally. The injected material must be endotoxin free, sterile and unable to cause alterations in pH, osmotic pressure and transparency of the vitreous body. Gels soaked with the appropriate drug are injected through a small needle and provide a constant release of the drug (Subrizi et al., 2019). Drug delivery duration can be extended by drug incorporation inside particles (microparticles, nanoparticles) within the gel (Famili et al., 2014). However, at the moment no studies have utilized nanoparticles with NTs for ONI treatment.

Delivery via Peripheral Nervous System Grafts

More targeted drug delivery can be achieved by transplantation of a segment of peripheral nervous system (PNS) into the injured CNS (Thanos et al., 1997; Harvey et al., 2006). PNS grafts contain viable Schwann cells that provide a permissive microenvironment to the lesioned ON and enhance growth cone motility (Thanos et al., 1997). These grafts can be modified by tissue engineering approaches and lead to reconstruction of acellular PNS sheaths with purified Schwann cells (Cui et al., 2003). Studies using genetically modified Schwann cells that overexpress NTs such as BDNF or CNTF lead to an increase in RGC survival and regrowth of RGC axons (Hu et al., 2005). Injections of NTs intravitreally lead only to temporary effects (Parrilla-Reverter et al., 2009). PNS grafts are considered promising tools for the cure of a CNS injury (Cen et al., 2012), yet some studies support that they cannot lead to RGC axon regeneration (Harvey et al., 2006).

Berry et al., 1999 showed that intravitreal transplantation of sciatic nerve segments in a rat ONC model, led to the regrowth of axons in the lesioned optic nerve due to the presence of Schwann cells in the graft that secrete NTs (Heumann et al., 1987; Assouline et al., 1987). Secreted NTs bind to receptors expressed by RGCs (Jelsma et al., 1993; Perez & Caminos, 1995; Koide et al., 1995). Grafts were implanted on the injury day, 14 days post injury or both. Axon regeneration was assessed 30 days post injury. Grafted animals presented more regenerating axons across the ON ipsilaterally to grafted eyes. Axons regenerated through fragmented myelin, axonal debris and inflammatory astrocytes. The cessation of axon growth implied that Schwann cells in the grafts stopped secreting NTs (Berry et al., 1999). Another study that used cellular and acellular PNS grafts in a rat ONC model, proved that cellular grafts lead to higher levels (58%) of neuroprotection 21 dpi when they implanted both intravitreally (like pellets) and also at the ON to induce anastomosis (Ahmed et al., 2020). Leaver and

his colleagues proved that gene therapy using intraocular injections of AAV-CNTF-GFP and application of PN grafts induced survival of rat RGCs by 25% and partial RGC axon regeneration into the graft, when rats sacrificed 7 days pi (Leaver et al., 2006).

Delivery via Biomaterials

Laughter and his colleagues evaluated RGC survival in a rat ONC model, where two types of CNTF-loaded scaffolds, reverse thermal gel (RTG) and sulfonated reverse thermal gel (SRTG), were delivered by intravitreal injections at the day of surgery. Each scaffold included 0.5 μg CNTF. Rats were sacrificed and RGC viability was evaluated 1 week post injury. There was a slight increase in the RTG-CNTF treated group compared to saline. SRTG-CNTF treatment induced significant increase in RGC survival and also lead to significant axon regeneration (Laughter et al., 2018).

Delivery via Cell Transplantation

Cell transplantation strategies after TON are considered very efficient as they focus on both neuroprotection and axon regeneration (Bei et al., 2016; Chen et al., 2013; Mesentier-Louro et al., 2014; Yang et al., 2015; Zaverucha-do-Valle et al., 2014). Transplanted cells are injected ivt, acquire the appropriate phenotype and integrate into the pre-existing neural circuitry (Chen et al., 2013).

Injection of C17.2 cells 24h after ONC induced β-III tubulin expression which is expressed normally in RGCs, whereas no expression of other RGC markers such as Brn3b and Pax6 was detected (Harvey et al., 2007). Another study revealed that Oldfactory Ensheathing Cells (OEC) promote axon regeneration (Yang et al., 2015) as they secrete a variety of NTs (Boyd et al., 2005). These cells can be transplanted either alone or in combination with NTs or other cell types (Liu et al., 2010; Zhang et al., 2017; Torres-Espin et al., 2014). The drawback of this therapeutic strategy is the time of transplantation. Treatment must be applied as soon as possible after injury so as to prevent RGC death. However, the use of autologous cells is not immediately possible, as these cells need to be cultured for 4-6 weeks (Feron et al., 2005). Heterologous transplantation on the other hand elucidates the danger for immune rejection. A recent study in rats transplanted with OECs showed that RGC death started 28 days after injury because of reduction of NTs secreted by OECs (Gong et al., 2018).

Bone marrow-derived cells (MSCs) are a useful tool for cell transplantation, as they secrete trophic factors (Crigler et al., 2006), have immunomodulatory properties (Uccelli et al., 2007) and can be transfected so as to induce the production of specific factors (Heile et al., 2009). Rats that received ivt injection of MSCs immediately after ONC presented significantly increased levels of RGC survival compared to the vehicle group. Specifically, the $Brn3a^{+}$ cells increased 3.5-fold at 1.00 mm and 1.8-

fold at 3.5 mm from the optic disc compared to control group 16 days pi. Regarding axon regeneration, MSC treatment caused significant extension of the axons (3.2- fold compared to vehicle group) up to 0.75mm from the lesion site 16 days pi (Mesentier-Louro et al., 2014).

1.3.4 Microneurotrophins as Therapeutic Molecules for Ocular Diseases

Microneurotrophins (MNTs) are small molecules, synthetic derivatives of dehydroepiandrosterone (DHEA). DHEA is an endogenous steroid precursor which activates sex hormone receptors (Charalampopoulos et al., 2006, 2008; Calogeropoulou et al., 2009; Gravanis et al., 2012; Pediaditakis et al., 2015). DHEA is produced by neurons, glia and also by the adrenal cortex (Charalampompoulos et al., 2008). DHEA is intermediate in the biosynthesis of estrogens and androgens that affect the endocrine system. However, it has been proposed that DHEA increases the risk for developing estrogen- and androgen- dependent tumors. DHEA is a multifaceted agent that exerts its properties by binding at nM affinity to neurotrophin receptors TrkA and $p75^{NTR}$ (Lazaridis et al., 2011) and also to steroid receptors (Charalampopoulos et al., 2008). Neurosteroids such as DHEA provide neuroprotection. Kokona et al., 2012 showed that DHEA reversed the AMPA-induced excitotoxicity in the rat retina as indicated by TUNEL assay.

Since MNTs are derived from DHEA, they bind to NT-receptors as well, presenting neurotrophic, inti-inflammatory and inti-apoptotic activities in multiple cell targets, as demonstrated both *in vitro* and *in vivo* (Charalampopoulos et al., 2006, 2008; Pediaditakis et al., 2015). MNTs are believed to induce neuroprotective effects, as there are DHEA synthetic analogs. BNN27, a DHEA derivative, activates NGF receptors TrkA and $p75^{NTR}$, inducing neuron survival (Pediaditakis et al., 2016a, 2016b), but is unable to bind and activate hormone receptors (Calogeropoulou et al., 2009). In PC-12 cells and in DRG neurons there was a significant increase of axonal length after treatment with a combination of NGF and BNN27, compared to NGF alone (Pediaditakis et al., 2016a). In the cuprizone induced demyelination model, BNN27 treatment leads to successful rescue of demyelination by reducing OL death and microglia and astrocyte activation, although it cannot affect directly OPC proliferation (Bonneto et al., 2016).

Regarding BNN27 treatments for retinal diseases, daily administration of BNN27 eye drops for 4 weeks reversed diabetes effects in a dose-dependent manner on RGC axons as quantified by neurofilament (NFL) immunoreactivity, in a diabetic retinopathy model (Iban-Arias et al., 2019). Daily intraperitoneal injections of BNN27 (10 or 50 mg) for 7 days beginning 4 weeks after diabetic retinopathy introduction, reversed diabetes-induced decrease in NFL, brain Nitric Oxide Synthetase (bNOS) and Tyrosine Hydroxylase (TH) immunoreactivity. Furthermore, BNN27 treatment decreased

$p75^{NTR}$ levels compared to non-treated diabetic mice, decreased significantly cleaved caspase-3 immunoreactivity and prevented glial activation (Iban-Arias et al., 2017). Intraperitoneal BNN27 administration in a mice retinal detachment (RD) model leads to reduced photoreceptor cell death, as indicated by TUNEL assay. However, this study presents contradictory results compared with other studies and propose that BNN27 treatments increase the recruitment of macrophages and microglia to the lesion site (Tsoka et al., 2018).

1.4. Thesis Scope

This study focuses on quantifying the effect of microneurotrophin BNN27 in a murine model of Optic Nerve Crush. BNN27 was delivered by two ways: via eye drops and via a biomaterial graft. The first approach refers to intraocular administration of BNN27 drops daily. In the second approach BNN27 was entrapped in a gel inside a collagen-GAG scaffold graft, which was implanted around the injury site of the ON.

The first objective of this study is to confirm that the ONC procedure was carried out successfully. However, the main objective of this study was to evaluate the effects of BNN27 in the response of key wound healing processes initiated by ONC. First, quantifying BNN27 effects in RGC viability and astrogliosis in the retina. Then, quantifying BNN27 effects in axonal regeneration and demyelination progress in the optic nerve following ONC.

This study aims to shed light to the process of tissue regeneration after CNS injury. Effects of BNN27 can provide valuable information about the underlying molecular mechanisms that control axon regeneration upon CNS injury and subsequently facilitate the development and production of novel therapeutic compounds for TON.

Chapter 2. Materials and Methods

2.1 Animal Model

TON is an irreversible condition that leads to partial or complete blindness and affects the quality of life of millions of people worldwide. In order to study TON and seek appropriate therapies, it is necessary to utilize a suitable animal model, which can imitate the TON response in humans. Primates have the highest homology with humans and are the ideal models for studying disease mechanisms, for example axon degeneration and regeneration after injury. However, primate animal models are expensive and demand special housing facilities. On the other hand, rodents (mice and rats) have high conservation with human genome, are lower-cost, easy to handle and have similar anatomical features with humans, therefore rodents are most common animal models utilized in TON studies.

This study used C57BL6/SV129 mice kept at the specific-pathogen-free (SPF) animal unit of the Institute of Molecular Biology and Biotechnology (IMBB). The temperature was controlled in the facility and there was a 12hr light/dark cycle. Animals were fed by standard chow and water ad libitum.

2.1.1 Optic Nerve Crush Mouse Model

This study presents an established murine unilateral ONC model in order to evaluate the effects of BNN27 after injury. 2-month-old C57BL/6 mice (20-30g) received anesthesia via an intraperitoneal injection of ketamine/xylazine. ONC was induced as follows: the conjunctiva of the left eye was incised and the ON was exposed at its exit from the eye globe by putting aside the orbital muscles. ONC was induced by compressing the ON for 6 sec approximately 1 mm away from the eyeball using a pair of Dumont #5 forceps. Great care was taken so as not to injure the ophthalmic artery. The intact eye group refers to the right eye of each animal that received no ONC.

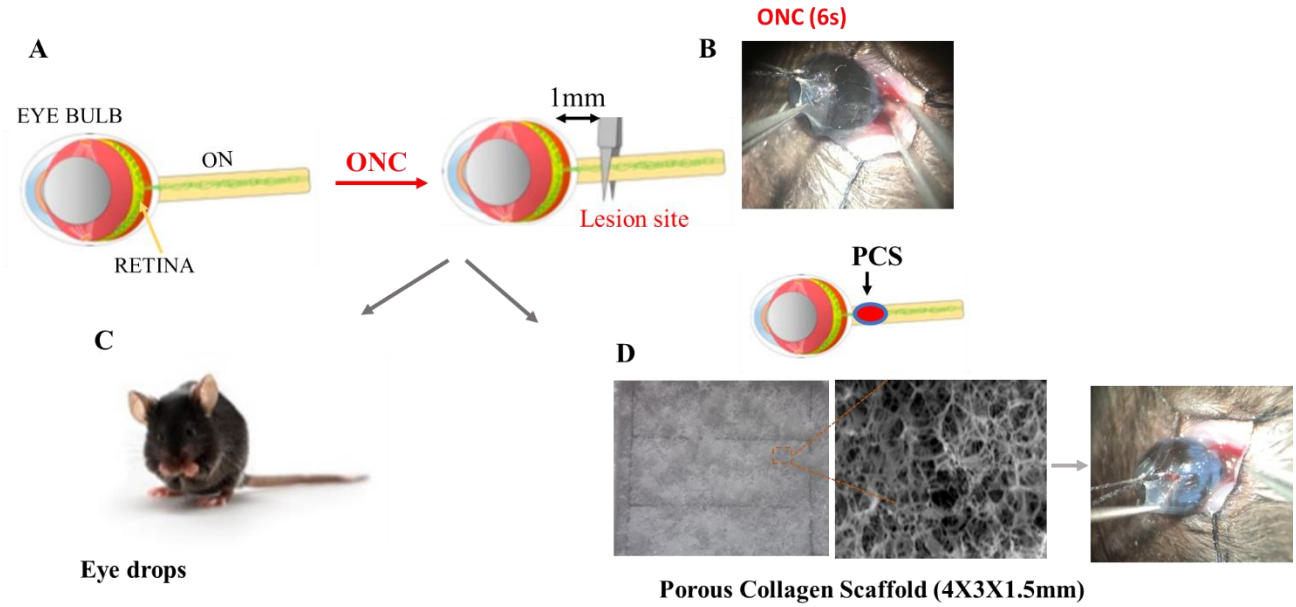


Fig 2.1: Description of ONC model and therapeutic interventions followed. A. schematic of ONC procedure. B. ONC induction 1 mm away from optic disc. C. eye drops administration D. implantation of a porous scaffold-based graft around the crushed optic nerve site. Images prepared by Constantina Georgelou. PCS: porous collagen scaffold, ONC: Optic Nerve Crush.

2.1.2 Therapeutic Treatments & Experimental Design

The 2-week study included the following experimental groups: the "intact" eye group (control), two "BNN27 eye drops" groups, the "scaffold/peptide" group and the "scaffold/peptide/BNN27" group. In all animal groups the crush was unilateral. While only the left ON was crushed, both eyes received the same treatment *(Table 2-1)*.

GROUPS	TREATMENTS		
	LEFT EYE (CRUSHED)	RIGHT EYE (INTACT)	CONCENTRATION
CRUSH	DMSO	DMSO	
CRUSH (SCAFFOLD)	SCAFFOLD/PEPTIDE	-	30mM
CRUSH + BNN27 (1 EYE DROP/ DAY)	BNN27 in DMSO	BNN27 in DMSO	50mM
CRUSH + BNN27 (2 EYE DROPS/DAY)	BNN27 in DMSO	BNN27 in DMSO	50mM
CRUSH + BNN27 (SCAFFOLD)	SCAFFOLD/PEPTIDE/BNN27	-	30mM

Table 2-1: Treatments applied to the four different groups included in this study.

In the “BNN27 eye drops” groups animals received 5 μl BNN27 drops (50 mM BNN27 diluted in DMSO) once or twice per day, beginning the following day after the surgery. Eye drop treatments lasted for 14 days post injury. In the “scaffold/peptide” group, a 4x3x1.5 mm porous collagen-GAG scaffold (CGS; 0.5% mass fraction, 95 μm mean pore diameter) was implanted around the lesion site of the ON. Prior to grafting, grafts were soaked in 20 μg/ml (Fmoc-FF) peptide solution. In the ‘’CRUSH (SCAFFOLD)’’ group CGS were soaked in 6μl Peptide (Fmoc-FF) peptide solution. In the ‘’CRUSH + BNN27 (SCAFFOLD)’’ group, CGS were soaked in 6μl Peptide Fmoc-FF peptide solution containing 30mM BNN27. The Fmoc-FF peptide was dissolved in ethanol, heated at 50ºC for 9 minutes and then was sonicated for 5 seconds. Peptide solution either alone or with BNN27 was diluted in ethanol and then was mixed with H_2O. The resulting Fmoc-FF peptide solution (with or without BNN27) was mixed with H_2O (1 volume of peptide/BNN27 and 3 volumes of water) in order to initiate the spontaneous gelation of the peptide. The resulting mix was pipetted quickly 3 times and then was polymerized within 30-40 sec. In order to polymerize the peptide inside the scaffold, 6 μl peptide/BNN27 solution (mixed with water) was put on a coverslip and the scaffold was quickly placed on the top of the drop. The scaffold absorbed the peptide solution and remained for up to 7 minutes until placed around the lesion site.

The 10 week-long study included almost the same animal groups as the 2-week study. Here BNN27 eye drops were applied only twice per day. In this case, 10 weeks post-injury mice were also injected intravitreally with Cholera Toxin B (CTB), an anterograde neuroanatomical tracer which labels the axons that constitute the ON (Savvaki et al., 2021). Prior to CTB injection, an insulin syringe was placed 0.5mm inside the vitreous of the eye 2 mm away from the superior limbus. Then a capillary was used in order to remove 2μl of vitreous humor within 40 sec. Afterwards, 2μl CTB solution (1mg/ml CTB diluted in PBS) was injected in the vitreous within 30 sec. Sacrifice and tissue collection (eyes, ONs and brain) took place 3 days after CTB injection.

ONC induction, scaffold placement around the injury site, preparation of BNN27 for eye drop treatments, animal sacrifices and tissue harvesting were conducted by Constantina Georgelou (IMBB Neural Tissue Engineering Lab). Administration of eye drops was performed in cooperation with Constantina Georgelou, whereas grafts preparation was conducted by Constantina Georgelou in collaboration with the Mitraki Lab (Department of Materials Science and Engineering, University of Crete).

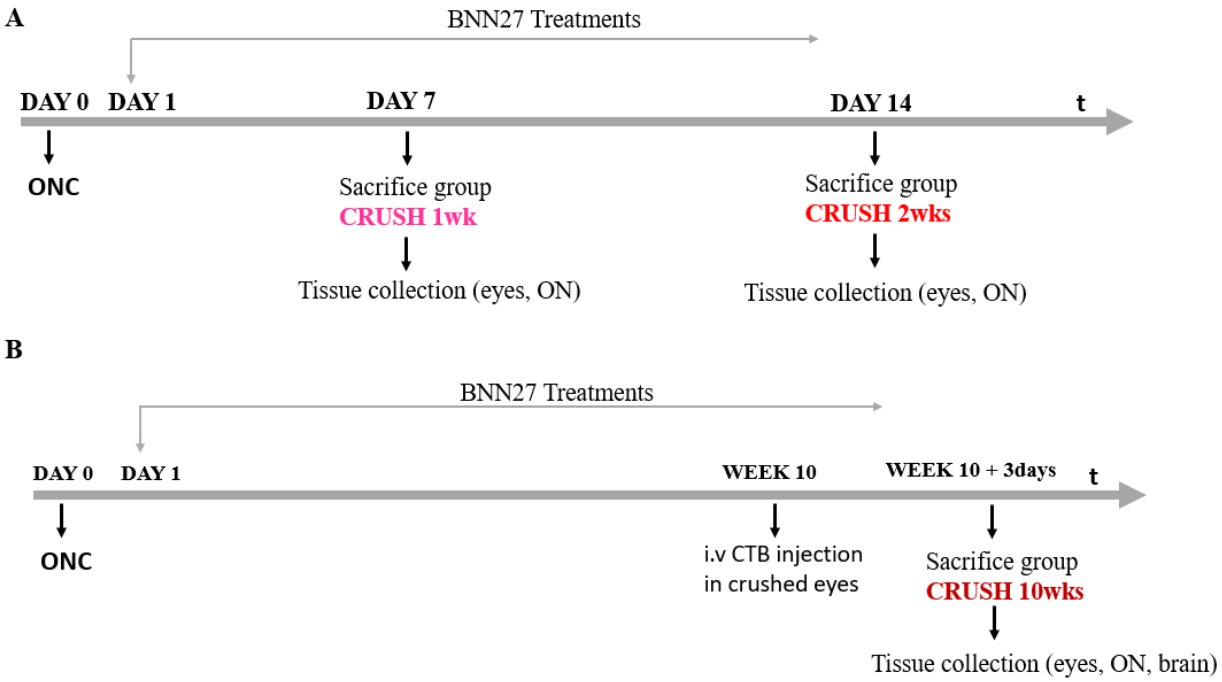


Fig 2-2: Timelines of BNN27 administration via eye-drops after ONC. A. In the 2-week study, ONC was carried on day 0 and animals were sacrificed 7 or 14 days later. B. In the 10-week study, ONC was conducted on day 0 and CTB was injected intravitreally at week 10. Three days later animals were sacrificed and tissue was collected. BNN27 treatments began the next day of the surgery and ended on the day prior to the sacrifice.

2.2 Tissue Processing

In the 2-week study, mice were sacrificed via cervical dislocation. Eyes were carefully removed using a pair of forceps. In detail, the two parts of the forceps were maintained in a small distance, trying not to damage the ON, pushing the eyeball and pulling out the eye together with its ON from the eye socket. Harvested eyes were placed in 4% paraformaldehyde (PFA) solution at 4°C for 24 h, briefly washed twice with PBS (phosphate buffered saline) at RT, and placed in 30% sucrose solution in PB (phosphate buffer) at 4°C for at least 24 h. In the 10-week study, mice were transcardially perfused 3 days after the intravitreal injection of CTB. Retinas, ONs and brains were dissected and post-fixed in 4% PFA diluted in PBS for 1h, rinsed with PBS, and kept in 30% sucrose until freezing. In both studies, ON was carefully separated from the retina using scissors and immediately cut into cryosections. Retinas were immersed in handmade molds filled with OCT (Optimal Cutting Temperature) compound (VWR Chemicals, 361603E) and then snap frozen at -70°C using methylbutane placed on dry ice. Cryo-sectioning was performed at -25°C: ON was placed directly on frozen OCT and sectioned longitudinally in 10 µm thick sections. Frozen retinas were sectioned

vertically in 20-μm thick sections at -25°C. For each ON sample, serial sections were placed on 5 slides (4-6 sections/slide). For each retina sample, sections were placed on 6 slides (6-8 sections/slide). Cryo-sectioned samples were stored at -80°C.

2.3 Immunohistochemistry (IHC)

All manipulations were conducted at room temperature (RT) except from primary antibody overnight incubation, which took place at 4°C. Sections were immersed in cold acetone at -20°C for 5 min, air-dried in laminar flow for 10 min, washed twice in PBS for 10 min, washed in 0.1% PBST (Triton X-100, 0.1% in PBS) for 15 min, washed in 0.3% PBST (Triton X-100, 0.3% in PBS) for 30 min, blocked in 0.1% PBST (0.1% Triton X-100 in PBS) supplemented with 10% horse/goat serum (depending on the host of the secondary antibody) and 0.1% Bovine Serum Albumin (BSA) for 1 h. Then, samples were incubated in the desired primary antibodies diluted in the aforementioned blocking solution at 4°C overnight. The next day, samples were washed 3 times in 0.1% PBST for 15 min, incubated in fluorophore-conjugated secondary antibodies diluted 1:1000 in 0.1% PBST for 1h, washed in 0.1% PBST for 15 min, washed in PBS for 15 min, counterstained with Hoechst 1:10000 (10mg/ml) in PBS for 15 min, washed in PBS for 15 min, washed in PB for 15 min, mounted and stored at 4°C. Stained sections were imaged in a Leica TCS SP8 inverted confocal microscope.

Antibody	**Vendor, Catalogue #**	**Dilution/Concentration**
Rec. Cholera Toxin B conjugated to Alexa Fluor™ 647	Thermo C34778	1 mg/ml in PBS
NeuN	Millipore MAB 377	1:200 in 0.1% BSA, 10 % goat serum, 0.1 % PBS-Triton and PBS
GFAP	Millipore AB5541	1:2000 in 0.1% BSA, 10 % goat serum, 0.1 % PBS-Triton and PBS
L1	Prof. F. Rathjen (Max-Delbrück Centrum Molek. Med, Germany), Prof. D. Karagogeos (U. Crete)	1:1000 in 0.1 % BSA, 0.1 % PBS-Triton, 10 % horse Serum and PBS
MAG	Cell Signaling 9043	1:200 in 0.1 % BSA, 0.1 % PBS-Triton, 10 % horse Serum and PBS
NF	Biolegend 837904	1:200 in 0.1% BSA, 01% PBS-Triton, 10% goat serum and PBS

Table 2.2: Primary antibodies and stains utilized in immunohistochemical analysis.

2.4 Imaging and Image Processing

Fluorescently labeled retinas and ONs were imaged in a Leica TCS SP8 inverted confocal microscope using a 40× oil-immersion objective lens (Leica Microsystems, Wetzlar, Germany) by Constantina Georgelou. All images were acquired either from the peripheral retina next to the iris or from the ON as close as to lesion site. 2 to 8 confocal z-stacks (1.5 μm z step) were acquired per sample.

Image analysis was performed using ImageJ (Fiji) software. Initially, each image was converted into an RGB image and each z-stack was converted to a maximum intensity z-projection image that included the appropriate channels for each analysis. The scale of each image was set using the "Set Scale" command of ImageJ. RGC viability in mouse retina was evaluated by manually counting $Hoechst^+$ $NeuN^+$ cells in the GCL and calculating the number of $Hoechst^+$ $NeuN^+$ cells per 100 μm GCL length. The level of astrogliosis in mouse retina was evaluated by manually counting $GFAP^+$ Müller cell processes next to the RGL, and then normalizing the number of processes to a standard length of 100 μm. $L1^+$ axons were manually counted in ON sections along transverse lines (normal to the ON axis) located approximately 250 μm, 500 μm and 750 μm away from the lesion site. The number of axons that cross these lines and also the thickness of each ON at each site is counted. The presence of MAG was described qualitatively.

Quantification of RGC survival

This thesis follows the procedure for quantifying RGC survival developed by Xenofon Mallios (Mallios, 2020). Some minor modifications will be highlighted below.

Images were acquired by a Leica TCS SP8 inverted confocal microscope and were processed using ImageJ software *(Import options: view stack with Hyperstack; color mode set to 'composite')*. The scale of each image was obtained from the LasX software (*Properties* and *X, Y dimensions*). The scale of the image was set from the *Set Scale* option in ImageJ. Channels containing NeuN (green) and Hoechst (blue) were selected and then the image was converted into RGB. The max intensity z-projection was shown in grayscale. The brightness and contrast of each channel was adapted, channels were merged and the image converted into RGB. The *color balance* tool was used to improve image contrast. $NeuN^+$ cells were counted manually via the *Cell counter* tool of ImageJ. Then, the *Segmented line* tool was used to count the length of the GCL. RGC viability was quantified as $NeuN^+$ nuclei /100μm GCL. Images were saved as PNG files.

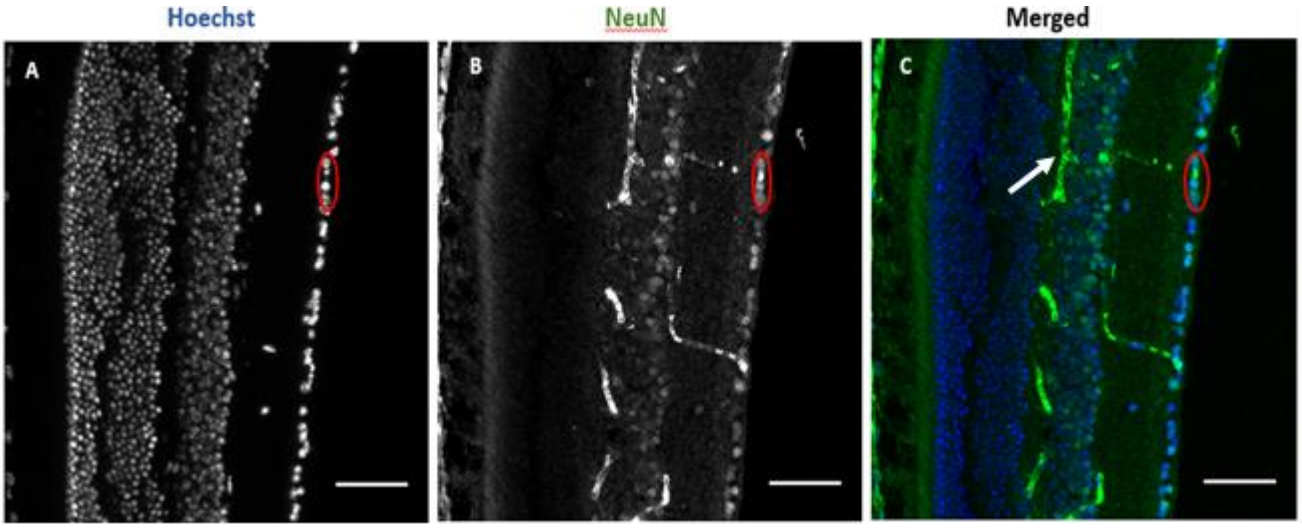


Fig 2.3: Image analysis procedure for quantifying RGC survival in immune-stained mouse retinal sections. (A) Representative image of Hoechst emission showing cell nuclei. (B) Representative image of NeuN emission. (C) Maximum z-projection of a confocal image stack. Red circles illustrate representative Hoechst$^+$ NeuN$^+$ nuclei in the GCL. Yellow arrow indicates the presence of vessels that were stained for NeuN but did not count. Scale Bars: 50 μm

Exclusion criteria for RGC survival evaluation

Although 2-week and 10-week experiments provided a large number of retina samples, not all of them were included in the statistical analysis. In order to include samples from a particular mouse in subsequent analysis specific prerequisites were taken into consideration:

- analysis considered only animals where both retinal tissue sections and ON tissue sections were available in order to assure that ONC was conducted correctly and completely.
- ON sections were examined so as to validate that crush was complete. This was achieved by evaluating the L1 or CTB immunostaining in the lesion site.
- samples that had no obvious lesion site were immediately excluded.
- samples were considered for RGC survival quantification as long as RGCs were quantified in at least 3 different retina locations (positions) in the peripheral retina.
- retinal slices where RGL did not have definite borders were not included in the subsequent analysis as they did not provide credible qualification.
- retina positions where the RGL was not a monolayer were excluded because multilayer RGL does not represent the peripheral retina.

The abovementioned criteria eventually turned out to be considered too strict as they lead to the exclusion of too many animal samples. Therefore, several animal samples that did not satisfy all the

above conditions were included as long as their inclusion did not improve the RGC survival rate of the corresponding animal group. For instance, samples that did not have a visible ONC site but presented a mean of RGC/100μm that was estimated below of the highest mean of an acceptable-in-the-analysis sample of the same group, were added in the statistical analysis.

Quantification of astrogliosis & inflammation

This thesis follows the procedure for quantifying astrogliosis developed by Xenofon Mallios (Mallios, 2020). Some minor modifications will be highlighted below. Astrogliosis/inflammation was quantified based on the number of $GFAP^+$ processes extended by activated Muller cells. Although astrogliosis concerns also astrocytes, quantification was based on Muller cells. Images were acquired by Leica TCS SP8 confocal microscope *and* processed using ImageJ software *(Import options: view stack with Hyperstack; color mode set to 'composite')*. The scale of each image was provided by the LasX software (*Properties* and then *X, Y dimensions)* and was set from the *Set Scale* option in ImageJ. The channel depicting GFAP (red) was selected from the *Channels Tool* and a maximum z-projection image was created. A line up to 100 μm was designed longitudinally and the number of processes that crossed the line was counted manually.

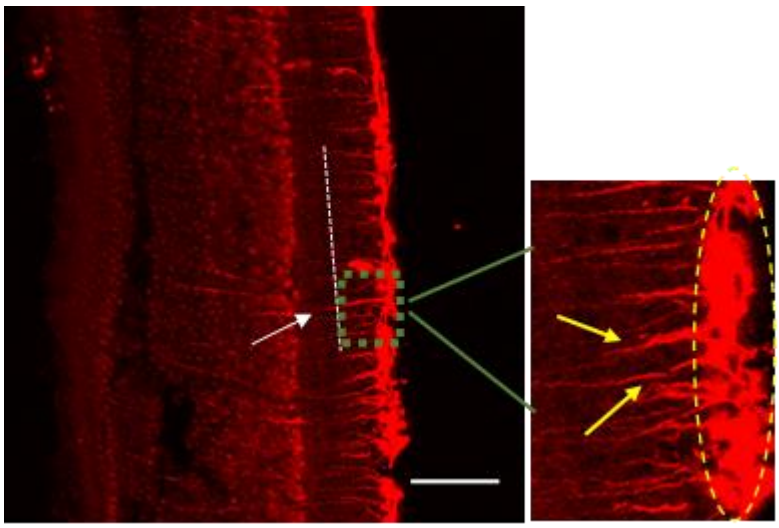


Fig. 2.4: Image of $GFAP^+$ processes that extended from Muller cells through the entire thickness of the retina, upon injury. The white line represents the line (up to 100μm) that is used to count GFAP processes that cross the line. Yellow arrows show 2 Muller cell processes. The yellow dotted ellipse shows $GFAP^+$ astroglia. Scale bar= 50μm.

Quantification of Axon Regeneration

Quantification of axonal regeneration was based on a protocol developed by PhD candidate Constantina Georgelou (IMBB, Neural Tissue Engineering Lab).

Axonal regeneration was evaluated by manually counting the number of $L1^+$ axons at certain distances from the ONC lesion site. Images acquired by Leica TCS SP8 confocal microscope *(. lif extension file)* and processed using the ImageJ software *(Import options: view stack with Hyperstack; color mode set to 'composite')*. The scale of each image was provided by the LasX software (*Properties* and then *X, Y dimensions)* and was set from the *Set Scale* option in ImageJ. The appropriate channels were selected: L1 (green), CTB (red), Hoechst (blue), and a maximum z- projection image was created and was converted into an RGB image. Channels were then split using the *Split channels* command in ImageJ. Afterwards, *Roi manager* tool from ImageJ was selected and lines of 250 μm, 500 μm and 750 μm length from the lesion site were drawn. Points (+) were saved in *Roi manager* and the image was saved in tiff format. Most background signal was removed using the *Subtract background* tool (the pixels parameter was chosen as 20), a horizontal line was drawn from each of the aforementioned points and the number of $L1^+$ axons that cross each line was counted manually. Axons were also checked in parallel in every z-stack so as to confirm the presence of the same axons as in grayscale. The length of each horizontal line (thickness of the ON at the particular point) was calculated using the *Measure* tool. Eventually, axonal regeneration was evaluated using the number of $L1^+$ axons per 100 μm at each distance (250 μm, 500 μm, 750 μm) from the lesion site.

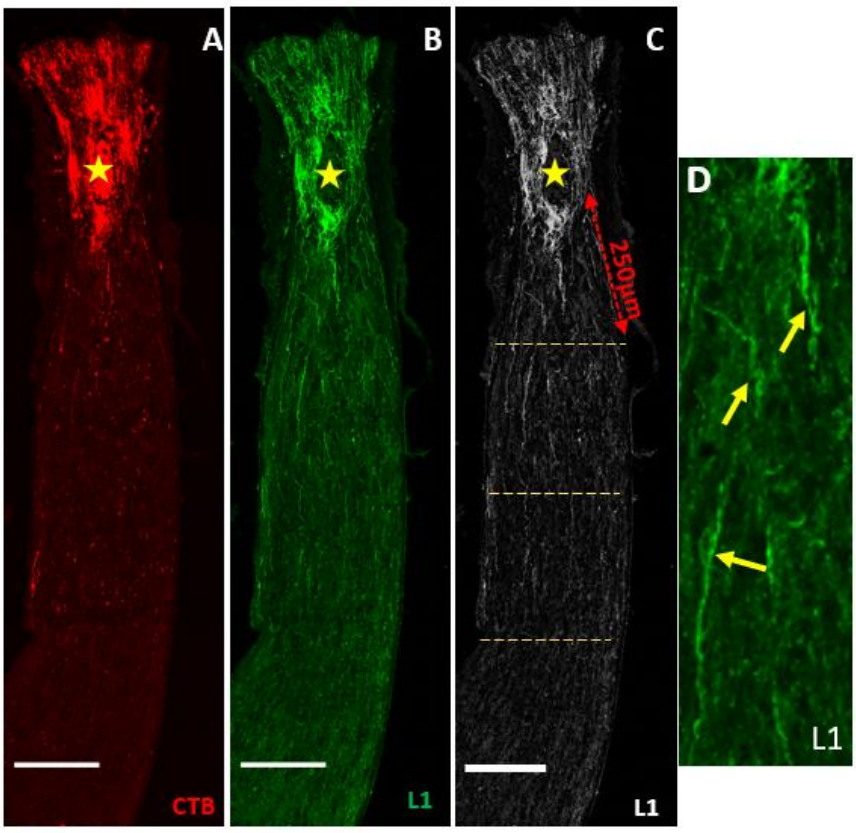


Fig. 2-5*: Quantification of axon regeneration. A. CTB staining of RGC axons in the injury site. B. L1 marker for the assessment of axon regeneration. C. The number of L1 axons was measured 250μm, 500 μm, 750*

μm and 1000 μm (not depicted) away from the lesion site (yellow asterisk). All images are depicted in Maximum Projection. D. Zoom of image B. Yellow asterisks represent three regenerating axons (intense green).

Evaluation of demyelination

Remyelination was evaluated qualitatively by observing neurofilament (pan-NF) and Myelin Associated Glycoprotein (MAG) immunoreactivity. In normal optic nerve tissue, myelin sheaths surround the axon. Upon injury, this structure is disrupted and myelin (MAG) is distinct from axons (NF), leaving the axon bare. All images were acquired by a Leica TCS SP8 inverted confocal microscope *(. lif extension file)* and were processed via the ImageJ software *(Import options: view stack with Hyperstack; color mode set to 'composite')*. The appropriate channels were selected: NF (green) and MAG (red), and a maximum z- projection was acquired.

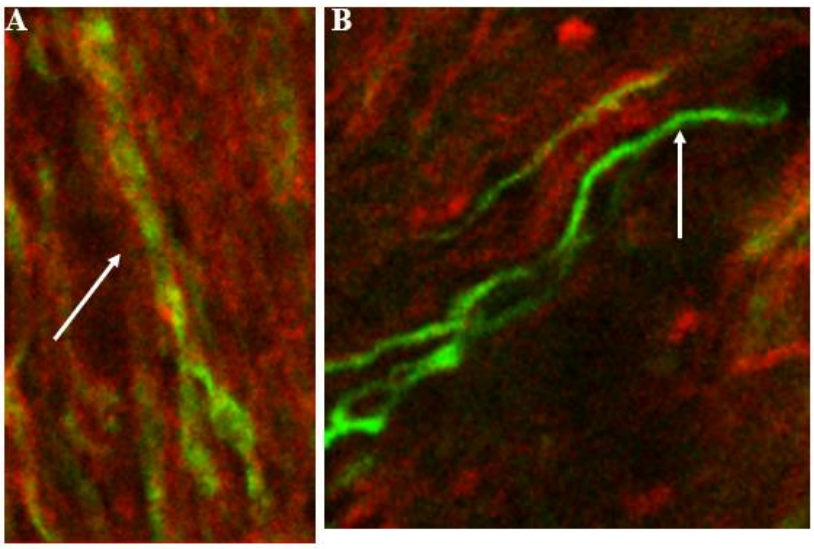


Fig. 2-6: Representation of coherence of NF (green) and MAG (red) in one z-stack. A. INTACT eye. Myelin sheath surrounds the axon very closely, B. CRUSH (SCAFFOLD) eye. Myelin structure is disrupted and axon is bare. White arrows indicate the axons in each case.

2.5 Statistics

Statistical analysis and graphical representation were performed using the Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) software. Statistical significance analysis regarding the mean values of multiple groups was evaluated using one-way Anova followed by Turkey post-hoc analysis. Statistically significant differences were considered when $p < 0.05$. Data were represented as mean ± SEM.

Chapter 3. Results

3.1 RGC survival after Optic Nerve Crush

3.1.1 RGC survival 2 weeks after Optic Nerve Crush

In order to characterize if ONC was conducted successfully, RGC survival was evaluated after ONC induction. For this purpose, 5 intact (right) eyes were collected together with 2 left eyes from the "CRUSH 1 week" group and 7 left eyes from the "CRUSH 2 weeks" group. The samples shown on the graph fulfilled the criteria referred above (see the *exclusion criteria* in section 2.4).

RGC survival quantification was carried out using NeuN as a marker of RGCs, since RGCs located in the RGL are $NeuN^+$ cells. The number of $NeuN^+$ cells was measured for each retinal sample and the resulting number was expressed as RGC number/100µm of RGL length.

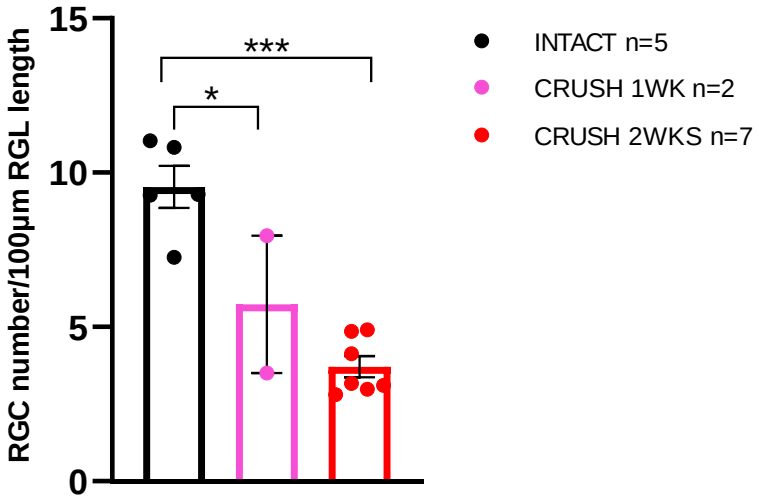
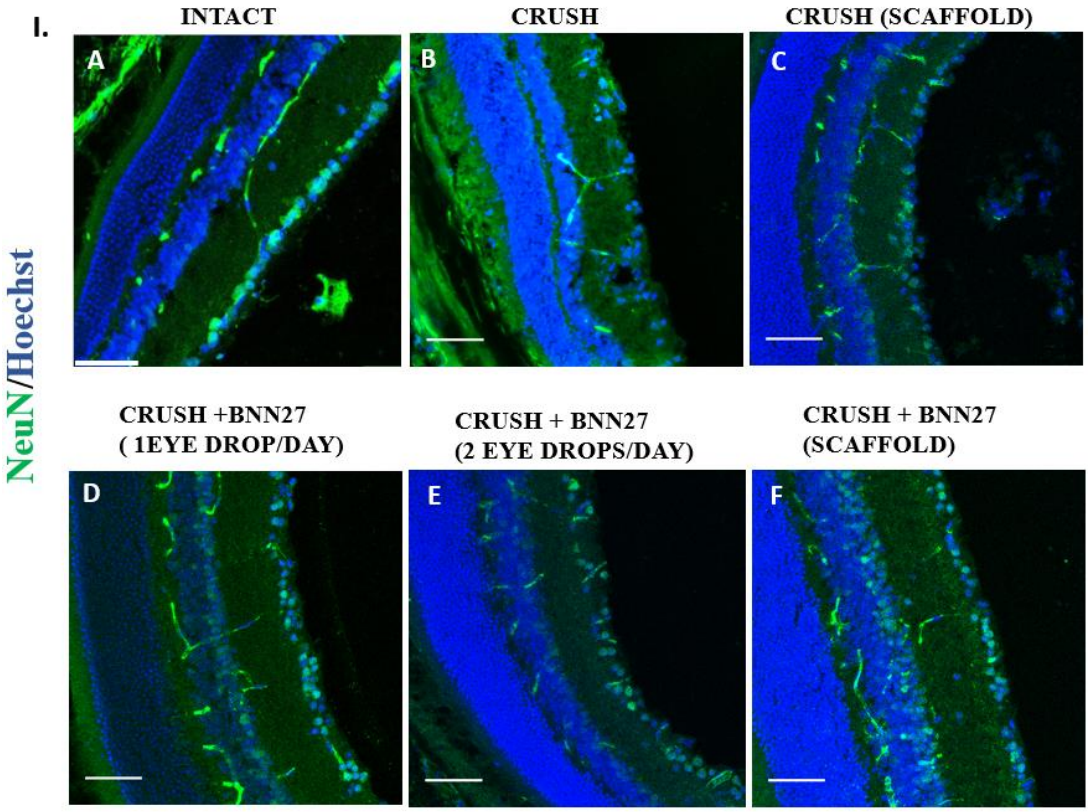


*Fig. 3-1: RGC viability in retinal sections from intact eyes and eyes that did not receive any treatment, 1- and 2-weeks post-ONC. Data are represented as mean ± SEM. *p< 0.05; **p<0.01, ***p<0.001. Turkey's post-hoc pairwise test assuming* $P_{1\text{-way-Anova}} < 0.05$.

RGC viability was reduced significantly after ONC compared with intact eyes. Specifically, 1 week after crush (CRUSH 1WK group) about 43% of RGCs did not survive ($p<0.05$), whereas 2 weeks after ONC (CRUSH 2WKS group) about 63% of RGCs were lost ($p<0.001$). Both CRUSH groups are statistically different compared to the intact eyes. The progressive loss of RGCs suggests that ONC induction was successful and reproducible. However, in order to acquire a better estimation regarding

RGC survival 1-week post-crush it is necessary to increase the number of samples (n) in this group as they present high variability *(Fig 3-1)*.

RGC viability was then studied across all groups 2 weeks after ONC either untreated or after receiving various BNN27 treatment, beginning from the next day of the surgery until one day prior to the sacrifice. This study included the following groups: INTACT, CRUSH 2WKS, CRUSH (SCAFFOLD), CRUSH + BNN27 (1DROP/DAY), CRUSH + BNN27 (2 DROPS/DAY) and CRUSH+ BNN27 (SCAFFOLD) *(Fig.3-2)*.



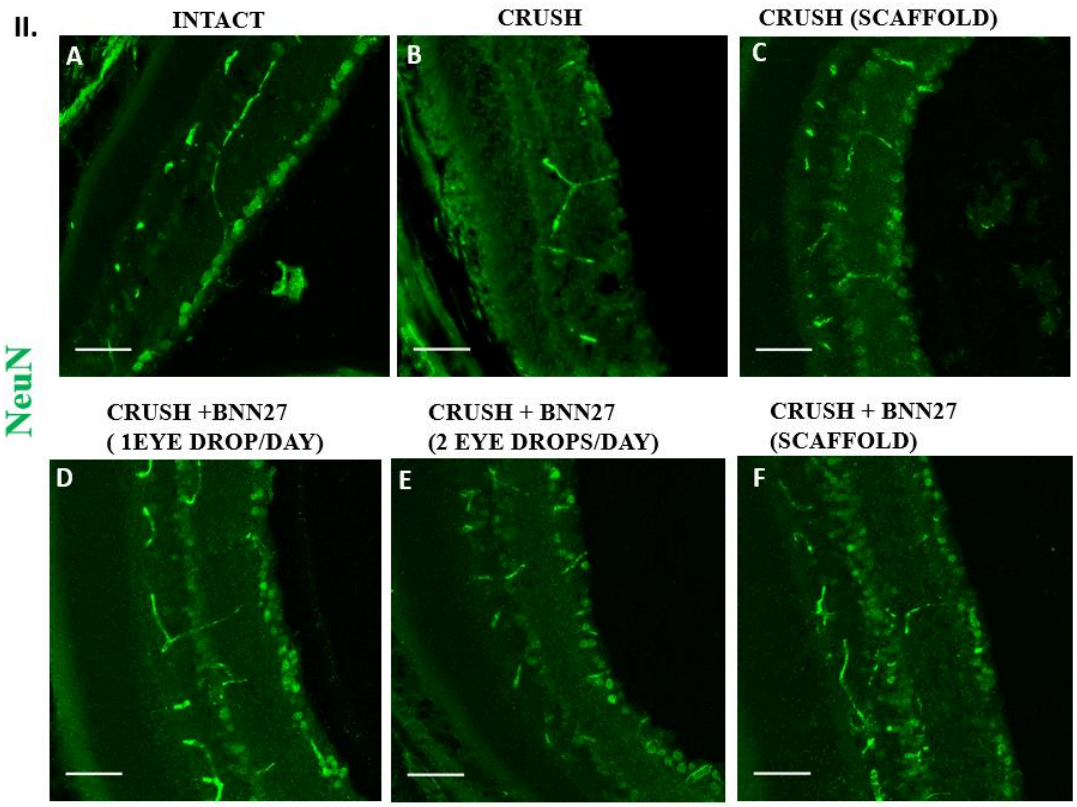


Fig. 3-2: Panels of representative fluorescence images of retina cross sections stained with NeuN from the animal groups of the 2-week study. Images describe RGC survival across the different groups 2 weeks after ONC. I. NeuN (green) stains RGCs in the RGL of the retina. Hoechst (blue) stains all nuclei. II. Same images as panel I., but showing only green channel. NeuN immunoreactivity is mainly nuclear. Scale bars= 50μm

Fig. 3-3 represents the statistical analysis across all different groups 2 weeks post ONC. RGC number was decreased significantly between intact eyes and CRUSH 2WKS group ($p<0.001$) as in *Fig. 3-1*. Furthermore, RGC viability was also significantly decreased between intact eyes groups and CRUSH (SCAFFOLD) group ($p<0.01$) that received no BNN27 treatment. The one eye drops per day BNN27 (CRUSH + BNN27 (1 EYE DROP/DAY)) and two-eye drops per day BNN27 (CRUSH + BNN27 (2 EYE DROPS/DAY)) groups were also statistically different compared to intact eyes group with $p<0.05$ and $p<0.01$ respectively. On the other hand, RGC survival in CRUSH 2WKS group was significantly decreased ($p<0.01$) compared to CRUSH + SCAFFOLD group. BNN27 treatments either via eye drops (1- or 2- eye drops/day) or delivered via a scaffold, present a significantly higher proportion of RGC viability compared to CRUSH 2 WKS group ($p<0.001$). Finally, in the CRUSH + BNN27 (SCAFFOLD) group RGC survival was significantly larger compared to the CRUSH (SCAFFOLD) group ($p<0.05$).

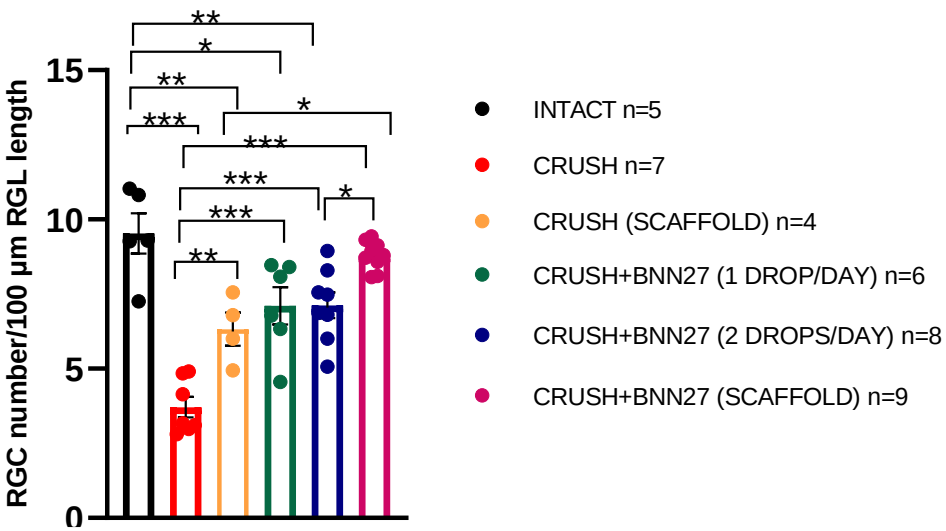


*Fig 3-3: Quantification of RGC survival 2 weeks post-ONC. In this graph are included all the different groups either receiving BNN27 or not. Data are represented as mean ± SEM. *p< 0.05; **p<0.01 and ***p<0.001. Turkey's post-hoc pairwise test assuming $P_{1\text{-way-Anova}} < 0.05$.*

This study used as control eyes the contralateral/intact (right) eyes that received no ONC. Intact eyes received the same treatment as crushed eyes. For this reason, intact eyes were divided in the following subgroups: CONTROL, NO TREATMENT, DMSO, BNN27 (1 DROP/DAY) and BNN27 (2 EYE DROPS/DAY). Graphical representation of RGC survival in the intact eyes' subgroups (*Fig. 3-4)* proves that there are no statistically significant differences (*p> 0.05*).

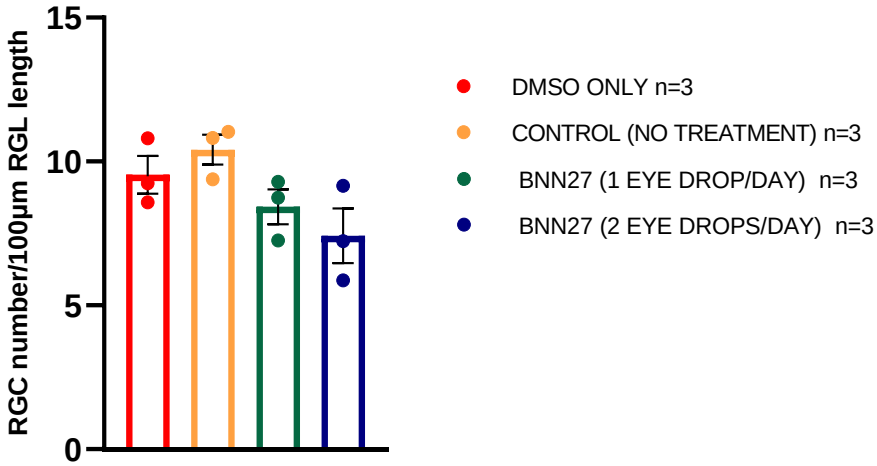


*Fig 3-4: Quantification of RGC survival in subgroups of intact eyes group, 2 weeks post-ONC. Data are represented as mean ± SEM. *p<0.05; **p<0.01, ***p<0.001. Turkey's post-hoc pairwise test assuming $P_{1\text{-way-Anova}} < 0.05$.*

3.1.2 RGC survival 10 weeks after Optic Nerve Crush

A long-term study was designed, in order to evaluate the effects of BNN27 on RGC survival 10 weeks after ONC. This study was comprised of 4 groups: CRUSH, CRUSH (SCAFFOLD), CRUSH + BNN27 (2 EYE DROPS/DAY) and CRUSH + BNN27 (SCAFFOLD).

Panels depicting NeuN immunoreactivity across the different groups prove also that there no significant differences regarding RGC survival *(Fig. 3-5)*. Assessment of RGC survival at 10 weeks after ONC, proved that there were no statistically significant differences across the different groups ($p>0.05$). However, a slight but not statistically significant increase ($p=0.1$) was observed in the CRUSH (SCAFFOLD) group compared to the CRUSH group. Results in the CRUSH + BNN27 (SCAFFOLD) group are characterized by variability, whereas results in the other three groups were more consistent *(Fig. 3-6)*.

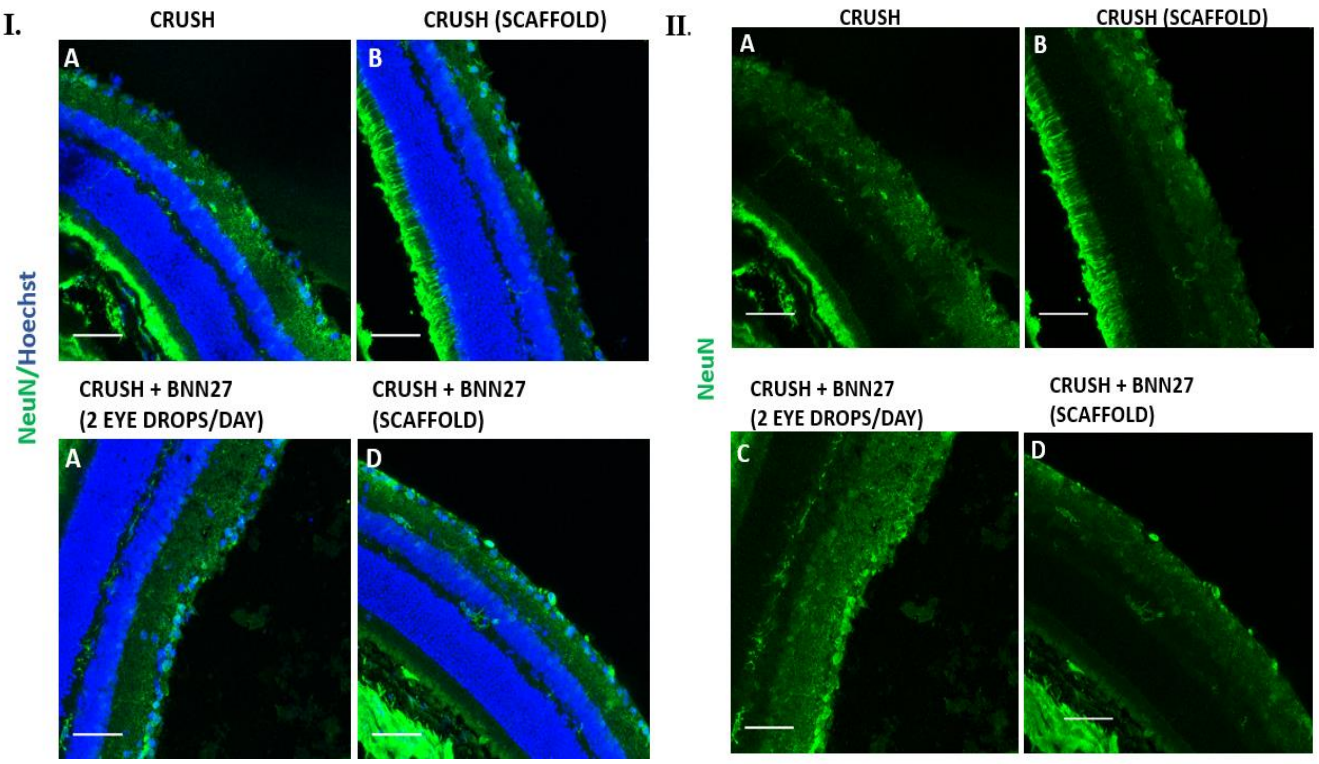


Fig. 3-5: Representative fluorescence images of retina cross sections 10 weeks post-ONC. I. RGCs are stained for NeuN (green) and Hoechst. II. Panel depicting only the NeuN channel, shows that NeuN staining is mainly nuclear in RGCs. Scale bars= 50μm

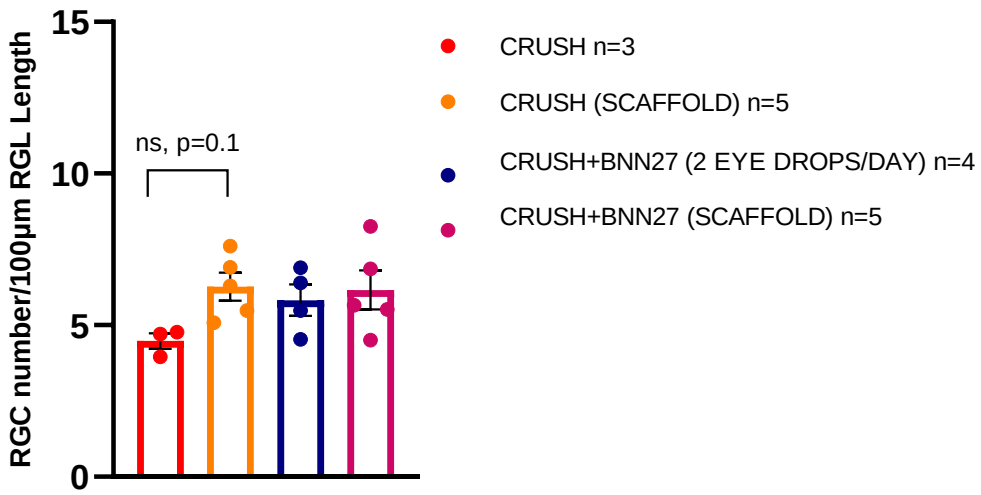


*Fig. 3-6: Quantification of RGC survival 10 weeks post-ONC. No statistically significant differences in RGC survival were observed. There is a slight increase in the CRUSH (SCAFFOLD) group compared to CRUSH group, although not statistically significant (p=0.1). Data are represented as mean±SEM. *p<0.05; **p<0.01 and ***p<0.001. Turkey's post-hoc pairwise test assuming* $P_{1\text{-way-Anova}} < 0.05$.

3.2 Inflammation & Astrogliosis after Optic Nerve Crush

Neuroinflammation is an event that takes place almost immediately after ONC *(Timeline Chapter 1 introduction)*. Therefore, inflammation was studied 2 weeks after ONC induction. Astrogliosis/inflammation concerns two types of cells: astrocytes and Muller cells in the murine retina. In order to quantify the level of inflammation after ONC, the number of processes that extended from Muller cells/100µm was measured.

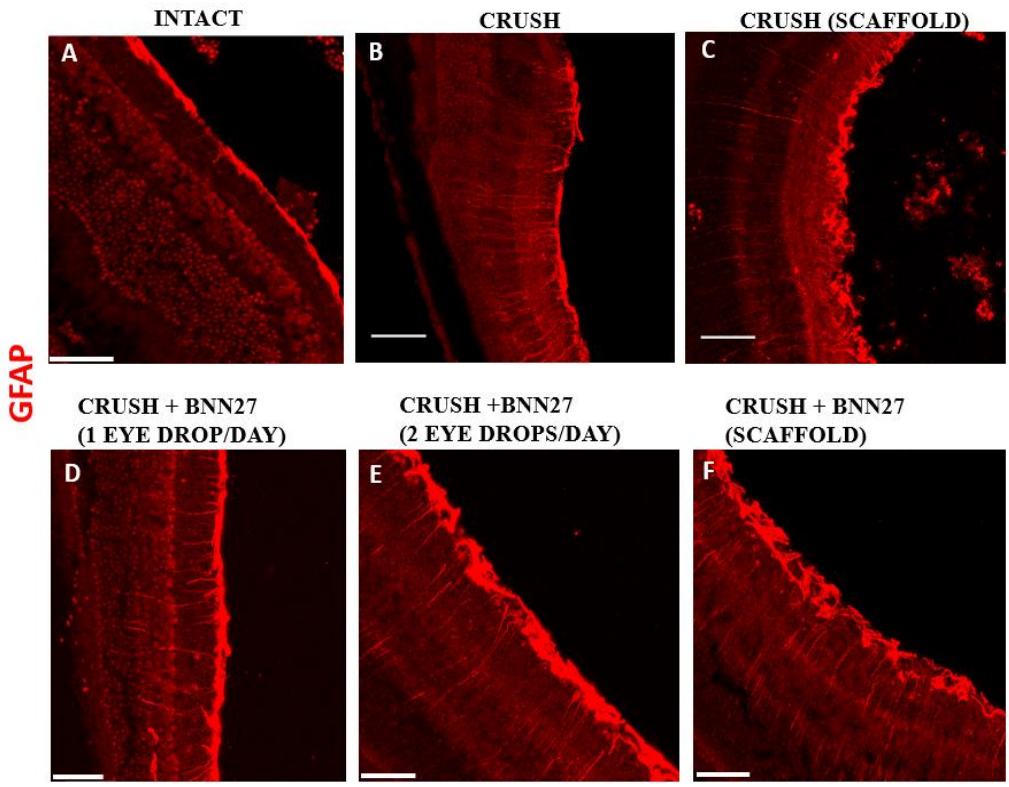


Fig. 3-7: Representative fluorescence images retina cross sections immune-stained for GFAP. Data are indicative of of inflammation/astrogliosis 2 weeks post-ONC. GFAP (red) stains both astrocytes and Muller cell processes. Scale bars = 50 μm

The presence of $GFAP^+$ processes 2 weeks post-ONC is depicted in *Fig.3-7.* There was a statistically significant increase ($p<0.001$) in the density of $GFAP^+$ processes through all the groups that received ONC compared to intact eyes. BNN27 therapeutic treatments did not affect the process of inflammation as there are no statistically significant differences between treated and untreated groups. Groups ‘’CRUSH’’, ‘’CRUSH (SCAFFOLD)’’ and ‘’CRUSH + BNN27 (2 EYE DROPS/DAY)’’ had larger variability than others. Groups ‘’CRUSH + BNN27 (1 EYE DROP/DAY)’’, ‘’CRUSH + BNN27 (SCAFFOLD)’’ and ‘’INTACT’’ provided more consistent result *(Fig 3.8).*

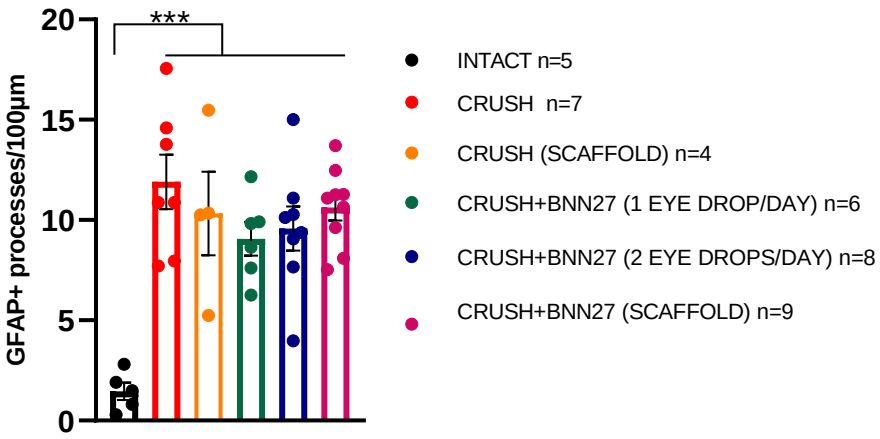


*Fig. 3-8: Quantification of Muller cell GFAP⁺ processes in the murine retina 2 weeks post-ONC. Data are represented as mean ± SEM. *p<0.05; **p<0.01 and ***p<0.001. Turkey's post-hoc pairwise test assuming $P_{1\text{-way-Anova}} < 0.05$.*

3.3 Axonal Regeneration 10 weeks after Optic Nerve Crush

Axonal regeneration occurs after the withdrawal of inflammatory cells, following ONC. This study quantified RGC axon regeneration 10 weeks post-ONC across. In order to assess axon regeneration, the number of L1⁺ axons per 100µm thickness that extended beyond the lesion site and were L1⁺ was measured.

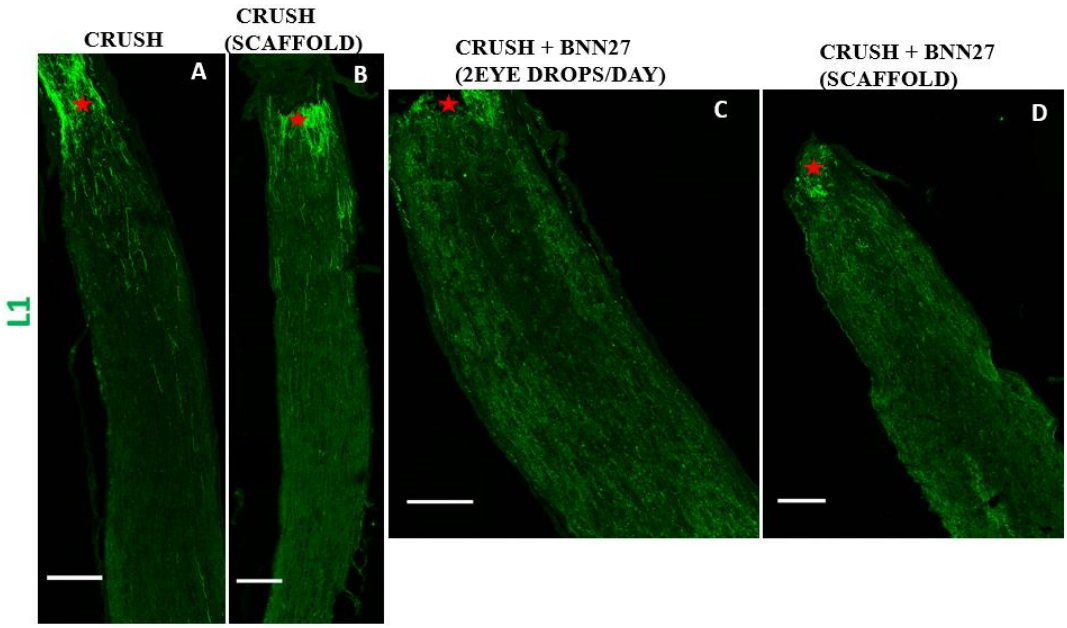


Fig. 3-9: Panel of axon regeneration images evaluated by L1 immunoreactivity. Asterisks denote the lesion site. Scale bars = 150 µm.

The presence of $L1^+$ axons 10 weeks post-ONC is evident in *Fig. 3-9.* There are no significant differences ($p>0.05$) in RGC axon regeneration across the different groups. BNN27 treatment delivered by eye drops or by a scaffold did not improve axon regeneration 10 weeks post injury *(Fig. 3-10).*

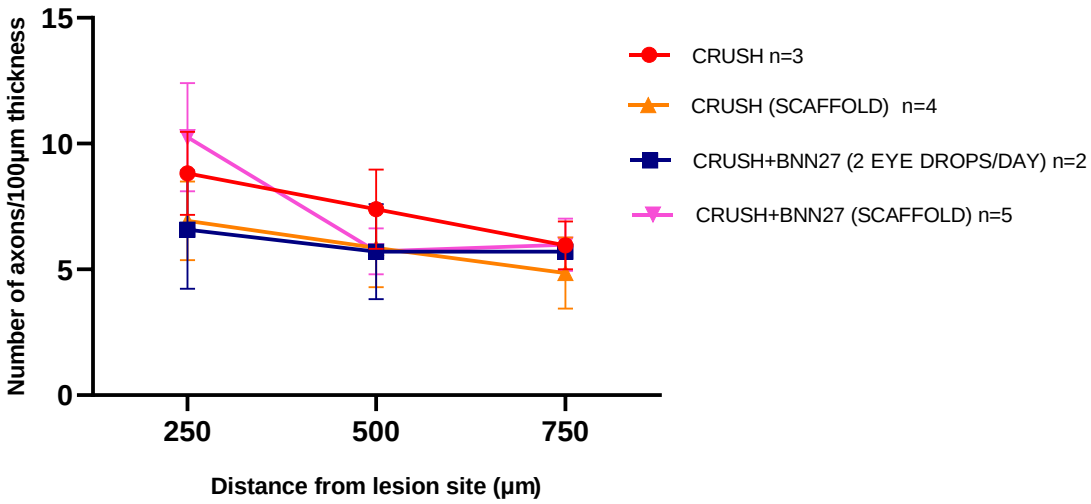


*Fig. 3-10: Quantification of axon regeneration number of $L1^+$ axons per 100 µm of ON thickness 10 weeks following ONC. Data are represented as mean ± SEM. *p<0.05; **p<0.01 and ***p<0.001. Turkey's post-hoc pairwise test assuming $P_{1\text{-way-Anova}} < 0.05$.*

3.4 Axonal Demyelination 2 weeks after Optic Nerve Crush

Demyelination was observed at 2 weeks post-injury, so as to evaluate myelin - axon interactions at this timepoint. Demyelination was evaluated by observing the coherence of NF and MAG staining on ON axons. Assessment of demyelination was performed only qualitatively.

Physiological myelin and axon interactions are represented in *Fig. 3-11 A.* Upon ONC, NF and MAG altered their distribution and myelin does not surround the axons, as occurs in intact eyes. On the contrary, myelin is represented as ''dotted'', which means that axons are bare and myelin structure is disorganized (*Fig. 3-11 B*). BNN27 treatments do not seem to affect the distribution of MAG along the axon. NF remained distinct from MAG, which stained in a ''dotted'' form (*Fig. 3-11 C, D, E*).

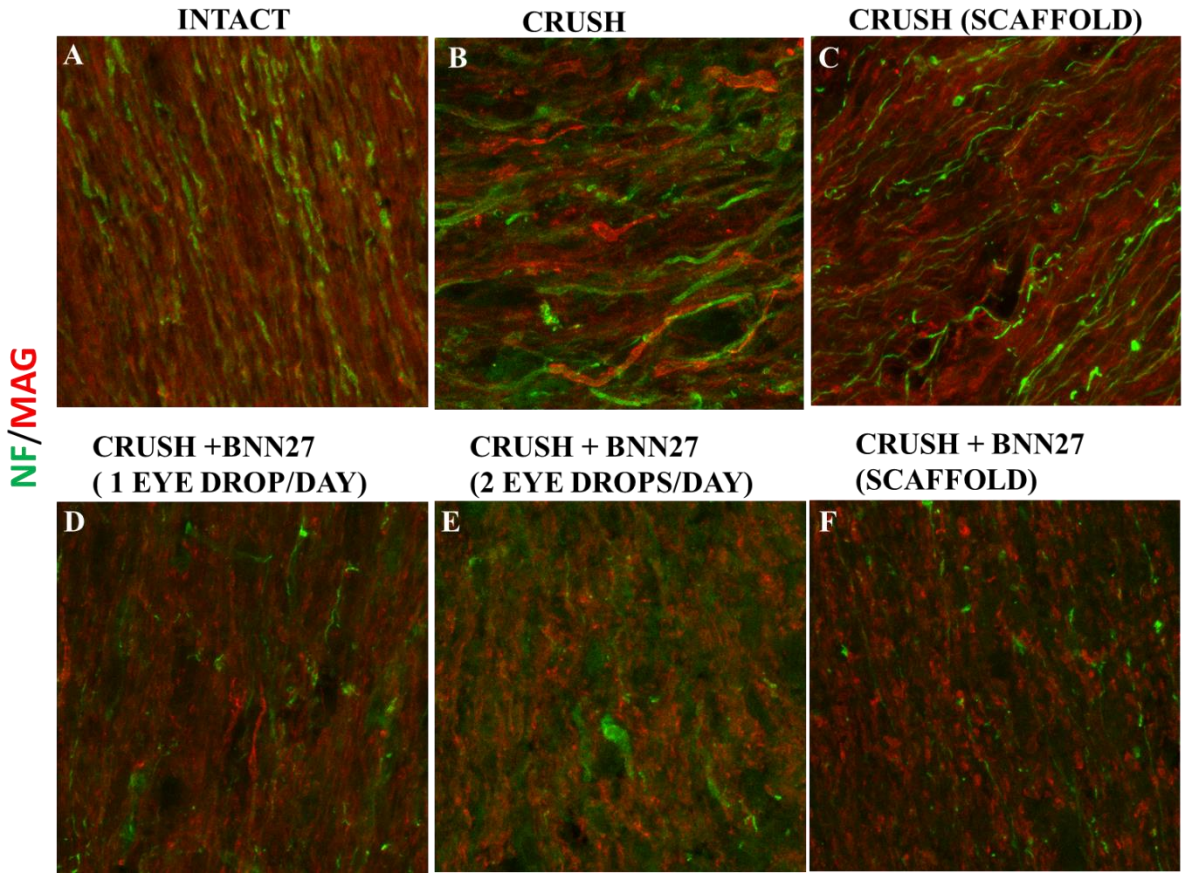


Fig. 3-11: Panel of remyelination assessment 2-weeks post-ONC. Longitudinal mouse ON sections were immune-stained for NF (green) and MAG (red).

Chapter 4: Discussion

The present thesis studies the effects of BNN27 on key processes (RGC apoptosis, inflammation/astrogliosis, RGC axonal regeneration, demyelination) of wound healing induced by optic nerve crush (ONC), a type of TON. TON conditions affect thousands of people wordwide every year, yet the development of appropriate therapies that can ameliorate its consequences (particularly loss of vision) is still an unmet medical need. The pharmacological effects of BNN27 were assessed in an established unilateral ONC murine model. BNN27, a DHEA derivative, mimicks NGF and binds selectively to TrkA neurotrophic receptor. BNN27 has demonstrated neuroprotective effects in various kinds of neural cells. Specifically, BNN27 protected PC-12 cells from serum deprivation-induced apoptosis (Calogeropoulou et al., 2009; Pediaditakis et al., 2016) and reduced apoptosis in superior cervical ganglia following NGF deprivation (Pediaditakis et al., 2016).

BNN27 has already demonstrated beneficial effects in retinal diseases. BNN27 eye drops (1 drop per day for 7 consecutive days) reduced diabetes-induced effects in a dose-dependent manner when administered in a diabetic retinopathy (DR) rat model (Iban – Arias et al., 2017). Intraperitoneal injection of BNN27 decreased retinal neurodegeneration and inflammation in a STZ model of DR (Iban-Arias et al., 2017). A single dose of BNN27 administered within 60 min after injury significantly decreased photoreceptor death at 24h, but not at 7 days after injury in a Retinal Detachment (RD) model (Tsoka et al., 2018). This study evaluated BNN27 in a different mode of retina pathology, Traumatic Optic Neuropathy (TON), which was initiated by optic nerve crush (ONC). Here, BNN27 was administered via two means: 1. via eye drops or 2. via a biomaterial-based graft placed around the optic nerve lesion site.

4.1 RGC survival 2 weeks after Optic Nerve Crush

The study focused initially on evaluating the utilized mouse ONC model. The density of surviving RGCs in the RGL was counted via NeuN immunostaining 1 week or 2 weeks post-ONC. 1-week post-ONC, the density of surviving RGCs was 60% the one in intact eyes, which agrees with a previous study that reported a 50% RGC survival in a model of ischemia one -week post-ischemia induction (Siliprandi et al., 1993). In the present study, 2 weeks post-ONC 40% of RGCs survived. In comparison, a previous study reported that less than 20% of RGCs survived 2 weeks post-ONC model (Mesentier- Louro et al., 2017). RGC survival less than 30% was found by a study of RGC survival in a rodent model of glaucoma (Vidal-Sanz et al., 2017). Small yet significant differences in RGC survival 2 weeks post-injury between the present study and the two abovementioned studies could

originate in differences in animal models utilized. The abovementioned studies refer to different disease models such as ischemia and glaucoma, whereas the present study refers to ONC. Furthemore, induced ONC varies from animal to animal and from user to user due to its manual and elegant nature. Therefore, differences in RGC response could arise due to differences on the force and duration of ONC. Furthermore, partial ONC could induce further experimental noise. Therefore, in this study ON imaging was utilized to validate that ONC was conducted successively. Samples where ONC was induced partially were rejected as described in detail in Section 2.4.

A previous study showed that the administration of BNN27 eye drops (1 drop every 2 days for 2 consecutive weeks) demonstrated a (statistically not significant) trend for RGC neuroprotection. That study used BNN27 eye drops of two concentrations: 10 mM and 50 mM. 50 mM drops lead to the largest yet not statistically significant ($p = 0.11$) improvement in RGC survival. The reason of failing to achieve statistically significant neuroprotection was possibly the long-time interval between drop administration (Mallios, 2020). BNN27 is BBB-permeable and can be detected in mouse brain 30 min after intraperitoneal administration (Bennett et al., 2016). A recent study reported after a single i.p injection of BNN27 (30mg/kg), BNN27 was present in the retina 30 min after the injection, BNN27 concentration in the retina peaked 2 hours post-injection, whereas BNN27 was cleared from the retina 4h following injection (Tsika et al., 2021). Therefore, the present study utilized 2 eye drops per day in order to increase the amount of BNN27 delivered in the retina and the ON. In the 2-week study, 50 mM BNN27 drops were administered once (CRUSH (1 EYE DROP/DAY) group) or twice per day (CRUSH (2 EYE DROPS/DAY) group) for 2 consecutive weeks. These two BNN27 doses did not result in statistically different RGC survival. Yet, providing 2 BNN27 eye drops/day resulted in a more significant result ($p<0.001$) compared to the 1 eye drop/day ($p<0.01$) compared to the CRUSH group. This result agrees with another ONC (15s) study in rats, where the delivery of 2 NGF eye drops (540μl/ml) per day significantly increased RGC survival ($p<0.01$) 2 weeks post injury (Mesentier-Louro et al., 2019).

A drawback of delivering BNN27 via eye drops is that the compound can diffuse across the surrounding tissue, therefore a fraction of the provided BNN27 is eventually absorbed by the retina. In order to improve BNN27 delivery, BNN27 was entrapped in a self-assembled peptide inside a collagen-GAG scaffold (CGS). The resulting CGS soaked in peptide/BNN27 was placed around the injury site immediately after ONC. This method aims to provide a steady release of the compound in the proximity of the lesioned tissue. In the corresponding CRUSH+BNN27(SCAFFOLD) group RGC viability was significantly increased compared to the control CRUSH group ($p<0.001$) and the CRUSH + BNN27 (2 EYE DROPS/DAY) ($p<0.05$). The later suggests that BNN27 administration via the

scaffold graft is more beneficial compared to BNN27 administration via eye drops. Finally, some mice received a scaffold/peptide graft that did not incorporate BNN27. To our suirpise, in the CRUSH (SCAFFOLD) group RGC survival was also significantly improved ($p<0.01$) compared to the untreated CRUSH group, yet this improvement was less significant compared to the CRUSH+BNN27(SCAFFOLD) group. This result was reported in the initial experiments; however, this difference could be attributed to inconsistent ONC and low sample number (Mallios, 2020).

Contralateral (intact) eyes received no ONC, yet were treated with the same type of eye drops as the corresponding injured eye. Results (Figure 3.4) show that there were no significant differences in RGC density among non-treated, DMSO-treated or BNN27-treated intact eyes. This suggests that treatment in the lesioned eyes does not affect the contralateral eyes.

4.2 RGC survival 10 weeks after Optic Nerve Crush

The beneficial effects of BNN27 administration (via eye drops or via a graft implanted around the lesion site) were then evaluated 10 weeks after ONC. This study included 4 experimental groups: CRUSH, CRUSH (SCAFFOLD), CRUSH + BNN27 (2 EYE DROPS/DAY) and CRUSH + BNN27 (SCAFFOLD). Results (Figs. 3-5,6) show that 10 weeks after ONC there was no significant difference in RGC viability across the groups. A slight, yet not significant, increase in RGC survival ($p=0.1$) was presented in CRUSH (SCAFFOLD) group. CRUSH + BNN27 (SCAFFOLD) group was characterized by large variability. This result was not anticipated as several long-term previous studies on local NGF administration in the eye significantly increased RGC survival in rodent models of retina disease (though not ONC). Specifically, NGF administration (200 μg/ml, 4 drops/day for 7 consecutive weeks) lead to significant RGC neuroprotection (1.3-fold compared to untreated eyes) and prevented caspase activation in a glaucoma rat model characterized by elevated intraocular pressure (IOP) (Lambiase et al., 2009). Another study showed that ocular administration of NGF (4 μg NGF, 2 eye drops/day for 7 weeks) caused 30% increase in RGC survival in glaucomatous rats (Colafransesco et al., 2011). Apart from differences in neuropathology induced by ONC and glaucoma rat models (glaucoma was induced by injecting hypertonic saline solution in the superior episcleral vein), a possible explanation for the lack of RGC neuroprotection by BNN27 at 10 weeks post-ONC, is that BNN27 cannot prevent RGC forever but instead delays RGC death.

A few prior studies have already demonstrated the ability of BNN27 to protect RGCs from apoptosis. In a rat model of DR, intravitreal administration of BNN27 mediated pro-survival actions

by binding to TrkA and $p75^{NTR}$, while a TrkA inhibitor reversed BNN27 pro-survival actions. BNN27 administration increased TrkA phosphorylation and attenuated diabetes-induced $p75^{NTR}$ increase (Iban-Arias et al., 2017). Since TrkA is expressed by RGCs, a possible hypothesis is that the observed BNN27 neuroprotective effects in RGCs were mediated by TrkA. Alternatively, it is hypothesized that BNN27 neuroprotective effects could be mediated by other types of receptors expressed in RGCs including acetylcholine receptors and glumate NMDA receptors (Zhang & Diamond, 2006) and AMPA receptors (Iwamoto et al., 2013). Neuroprotective effects of RGCs were observed in cultured RGCs pretreated with acetylcholine or nicotine before glutamate insult, in a rat model of glutamate excitotoxicity in RGCs *in vitro*. Treatment with a nicotinic acetylholine receptor (nAChR) antagonist eliminated the observed neuroprotective effects, implying that RGC neuroprotection was mediated by a7 nicotinic acetylcholine receptors (a7nAChRs) (Iwamoto et al., 2013). Neuroprotection in porcine RGCs treated with acetylcholine was mediated through blockade of apoptosis, as a7nAChRs activated the PI3K/AKT pathway leading to Bcl2 activation and survival (Asomugha et al., 2010). Finally, low Ca^{2+} influx through a7nAChRs resulted in neuroprotective signaling via internalization of NMDA receptors, which eventually lead to RGC survival in a model of excitotoxicity (Schubert & Akopian, 2004; Akopian, 2006). Overall, RGC survival requires not only trophic delivery but also trophic responsiveness achieved by increased electrical activity (Shen et al., 1999), such as increased Ca^{2+} influx. In summary RGC neuroprotection is possibly mediated by not only by Trk receptors, but also by signaling induced by other receptors expressed in RGCs such as nAChRs, AMPA and NMDA.

4.3 Inflammation/Astrogliosis 2 weeks after Optic Nerve Crush

Optic nerve injury induces inflammatory responses both in retina and the ON within the first days after trauma. Regarding inflammation in retina, our results show that BNN27 administration did not manage to decrease the number of $GFAP^{+}$ Muller cell processes 2 weeks post-ONC. This result is contrary to a previous study that reported the ability of BNN27 to prevent diabetic-induced glia activation, leading to a decrease in GFAP expression in Muller cells in a rat model of DR (BNN27 was administered intraperitoneally for 7 days (10mg/kg and 50mg/kg)). In this study, BNN27 increased anti-inflammatory cytokines IL-10 and IL-4 and decreased pro-inflammatory factors TNF-α and IL-1β (Iban-Arias et al., 2017). Similarly, NGF injections (5μg every 4 days for 32 days) in a rat RD model reduced gliosis in retinal cells – as Muller cells express TrkA (Sun et al., 2007). The result of the present study agrees the ability of BNN27 (administrated i.p, 200mg/kg, 1h post RD) to significantly increase GFAP intensity 24h post RD induction in rats (Tsoka et al., 2018). These

conflicting results on the ability of BNN27 to modulate astrogliosis in the retina could be due to differences in BNN27 delivery or differences in the pathophysiology of the animal disease models.

It is still uncertain if Muller cells express both TrkA and $p75^{NTR}$. Some studies report that Muller cells express both receptors, whereas others report that they express only $p75^{NTR}$. Meanwhile, increased GFAP expression in Muller cells does not imply only an inflammatory phenotype, as overactivation of Muller cells can be related to reprogramming and acquisition of a reparative phenotype upon injury (Zhao et al., 2014).

4.4 RGC axonal regeneration 10 weeks after Optic Nerve Crush

Next, we evaluated RGC axonal regeneration 10 weeks post-ONC. BNN27 administration did not affect axon outgrowth beyond the lesion site. All groups, either treated or untreated do not present axon regeneration beyond the lesion site. The result of the present study agrees with previous results from our lab which conclude that BNN27 does not induce axonal outgrowth when administered alone in PC-12 cells or in cultured mouse sensory neurons. BNN27 binds to TrkA and induces fast recycling of the receptor into the membrane. As a result, there is more TrkA available at the membrane. The binding of NGF to its receptor, activates intracellular cascades which lead to axonal outgrowth (Pediaditakis et al., 2016a). Upon ONC, reduction in NGF levels due to disruption of retrograde transport, cannot contribute to BNN27 actions and as a consequence cannot induce axon regeneration. On the contrary, an earlier study showed that NGF administration (180μg/ml and 540μg/ml, 2 eye drops/day for 2 weeks) significantly increased axon outgrowth 0.25 mm (both doses) or 0.5mm (540 μg/ml dose) away from the lesion site (Mesentier-Louro et al., 2019).

Overall, lack of RGC axon regeneration 10 weeks post-ONC was not a surprise. Axonal regeneration requires survival of RGC in the retina. 10 weeks post-ONC, BNN27 had not been able to protect RGC from apoptosis. RGC survival demands not only prolonged NT delivery, but also the ability of neurons to respond to trophic stimuli that is lost after axonal injury. Increased trophic responsiveness demands increased electrical activity by RGCs. The same signals that control RGC survival also control axonal regeneration (Shen et al., 1999). Subsequently, axon regeneration takes place when growth cones of injured axons are stimulated directly by trophic stimuli (Campenot, 1994), and are also characterized by increased electrical activity which assures their trophic responsiveness (Shen et al., 1999). Absence of axon regeneration resulted in lack of functional vision improvement after ONC, since no RGC axons projected to the brain

4.5 Demyelination 2 weeks after Optic Nerve Crush

Demyelination takes place within the first days after ONC and leads to the loss of myelin sheaths that surround RGC axons in the optic nerve. In the present study demyelination was examined by immono-staining for the Myelin Associated Glycoprotein (MAG) and neurofilaments (pan-NF) in RGC axons and qualitatively evaluating the spatial relationship of MAG and pan-NF stainings. MAG is related to the stabilization of contacts between axons and glial cells and also the formation of intact myelin sheaths (Achiron & Miron, 2007).

A recent study demonstrated that BNN27 rescued OLs from apoptosis in a murine model of Multiple Sclerosis (MS) and decreased OPC recruitment, leading to partial recovery of myelin loss. In the same study, microglial population was maintained in a resting state resulting in reduced microgliosis, *in vitro*. The abovementioned actions of BNN27 indicate that it can potentially reduce demyelination (Bonetto et al., 2017). Another study reported that NGF treatment caused partial recovery of MBP immunoreactivity when administrated intravitreally in a rat permanent carotid artery occlusion model (Sivilia et al., 2009). However, in the present study BNN27 did not prevent demyelination in the ON 2 weeks post-ONC. BNN27 did not protect RGC axons from demyelination, although it is not clear how BNN27 influences microglia. The presence of myelin debris 2 weeks post-ONC could indicate that microglia did not exert phagocytotic effects. This agrees with a study that reported that myelin debris following ONC in rats disappeared completely after 5 months (Bignami et al., 1981). On the contrary, axotomy in goldfish revealed a quicker clearance-response by microglia as microglia cleared myelin debris in the lesion site at 2- and 4- weeks post axotomy, creating a permissive-to-axon regeneration microenvironment (Rosenzweig et al., 2010). However, elevated microglial levels do not imply a phagocytotic phenotype. Microglia is presented 4-fold increased 1-week post-ONC compared to uninjured ONs, but there is a delay between initial microglial response and effective myelin clearance; microglia do proliferate upon injury, but do not exert phagocytotic effects immediately (Lawson et al., 1994). Furthermore, another study reported that BNN27 administration kept microglia in a resting state *in vitro* (Bonetto et al., 2017), which could possibly explain the findings of this study. The role of microglia is pivotal. On the one hand, microglia clear the lesion site from myelin debris and other toxic products, contributing to the formation of a more-friendly-to-regeneration microenvironment. On the other hand, microglia initiate immune responses that limit the axons' regenerative capacity upon injury. Overall, BNN27 either induces a persistence of microglia in a quiescent state or it does not affect the microglial component at all; 2 weeks following injury is considered too soon for microglia to have exerted their phagocytotic role.

4.6 Concluding Remarks

This study evaluated the effects of microneurotrophin BNN27, an NGF analog, on a murine ONC model that resembles human TON. BNN27 was delivered via two ways: 1. via eye drops (1 or 2 eye drops/day, 50mM), and 2. entrapped (30mM) in a gel formed by a self-assembled peptide inside a CGS placed once around the ON at the injury site. The biomaterial approach was implemented in order to provide a steady release of BNN27 in the ON.

Our results demonstrate that ONC was carried out in a reproducible manner and lead to 40% RGC loss 1-week post-ONC and 60% RGC loss 2 weeks post-ONC. Unilateral ONC does not influence RGC survival in the contralateral eye. BNN27 increased significantly RGC viability in all three BNN27-treated groups compared to the CRUSH group 2 weeks post-ONC. However, BNN27 did not exert anti-inflammatory properties on Muller cells 2 weeks post-ONC and did not protect RGC axons from demyelination. Contrary to neuroprotective effects of BN227 observed 2-weeks post-ONC, BNN27 administration did not improve RGC survival 10 weeks post-ONC. Furthermore, BNN27 did not enhance RGC axon regeneration through the lesion 10 weeks post-ONC.

The results of this study contribute to the ongoing efforts of developing treatments that can induce tissue regeneration after CNS injury. Optic nerve microenvironment is impotent to support axon regeneration after ONC resulting in loss of functional vision. Therefore, there is need to complement BNN27 effects with other factors that can promote a sustainable axon regeneration, in order to achieve the ultimate goal of restoring the vision of TON patients. Towards this direction, this study contributes novel knowledge on TON pathophysiology and novel data on BNN27 neuroprotective effects on RGCs.

APPENDIX

Immunohistochemical Staining of Retina Sections

Erasmia-Angeliki Saridaki, November 2021

Based on Kanelina Karali, November 2014

Reagents:

1. PBS
2. Triton X-100

Equipment:

1. Aluminum foil
2. 15ml Falcons
3. 1.5- 2ml tubes

Procedure:

DAY 1:

Preparation of PBS1x, PBS-Triton 0.1% and PBS-Triton 0.3%

1. Take slides out of -80^{o} C and immediately immerge them in ice cold acetone, incubate for 5 min in -20^{o} C
2. Air dry in chemical hood for 10 min
3. Wash 2 x 10 min, PBS, RT
4. Wash 1 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT
5. Wash 1 x 30min, PBStx (Triton X-100, 0.3% in PBS) RT
6. Block (10% Normal Serum, 0.1% BSA in PBStx) 1hr –use serum of the species the secondary is raised in

Primary Ab: overnight at 4^{o} C (made in blocking solution). Antibodies used:

Ab	Vendor, Catalogue #	Dilution/Concentration

Rec. Cholera Toxin B conjugated to Alexa Fluor™ 647	Thermo C34778	1 mg/ml in PBS
NeuN	Millipore MAB 377	1:200 in 0.1% BSA, 10 % goat serum, 0.1 % PBS-Triton and PBS
GFAP	Millipore AB5541	1:2000 in 0.1% BSA, 10 % goat serum, 0.1 % PBS-Triton and PBS
L1	Prof. F. Rathjen (Max-Delbrück Centrum Molek. Med, Germany), Prof. D. Karagogeos (U. Crete)	1:1000 in 0.1 % BSA, 0.1 % PBS-Triton, 10 % horse Serum and PBS
MAG	Cell Signaling 9043	1:200 in 0.1 % BSA, 0.1 % PBS-Triton, 10 % horse Serum and PBS
NF	Biolegend 837904	1:200 in 0.1% BSA, 0.1% PBS-Triton, 10% goat serum and PBS

DAY 2:

1. Wash 3 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT
2. **Secondary antibody** (1:1000) made up in PBStx overnight at 4° C cover with aluminum foil:
3. Wash 1 x 15min, PBStx (Triton X-100, 0.1% in PBS) RT
4. Wash 1 x 15min, PBS RT
5. Apply nucleus stain (1:10000 for HOESCHT) in PBS for 15 min at RT
6. Wash 1 x 15min, PBS RT
7. Wash 1x 15min PB and leave for mounting

Coverslip with Vectashield mounting medium or 60% glycerol and fix coverslip with nail polish until imaging in Confocal Microscope.

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