CHARLES UNIVERSITY

THIRD FACULTY OF MEDICINE

DUAL ROLE OF NEUROTROPHIN BDNF AND VGF IN THE REGULATION OF THE PATHOGENESIS OF RETINAL AUTOIMMUNE INFLAMMATION

Dissertation Summary

Mgr. Miloslav Zloh

Prague, 2024

Postgraduate Doctor degree studies in Biomedical Science

Charles University in Prague

Field of Doctor degree studies: Human physiology and pathophysiology

Chairperson of the Field Board:

Institution: Department of Physiology, Third Faculty of Medicine, Charles University, Prague

Author: Mgr. Miloslav Zloh

Supervisor: PharmDr. Andrea Štofková, Ph.D.

Reviewers: …….

…….

Dissertation summary was sent out on ……

Date of the defense: …….

Place: …….

CONTENT

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP	anteroposterior
APU	autoimmune posterior uveitis
BDNF	brain-derived neurotrophic factor
BrdU	5-bromo-2′-deoxyuridine
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CRALBP	cellular retinaldehyde-binding protein
CXCL	chemokine (C-X-C motif) ligand
DMSO	dimethyl sulfoxide
DV	dorsoventral
EAU	experimental autoimmune uveoretinitis
GFAP	glial fibrillar acidic protein
GS	glutamine synthetase
IL	interleukin
IRBP	interphotoreceptor retinoid binding protein
MCs	Müller cells
ML	mediolateral
NeuN	neuronal nuclear antigen
NGF	nerve growth factor
NF-κB	nuclear factor-κB
SC	superior colliculus
SOX2	SRY (sex determining region Y)-box 2
VC	visual cortex
VGF	neurotrophins-inducible factor VGF (non-acronymic)
VS	visual stimulation

SUMMARY

Autoimmune posterior uveitis (APU) is an inflammatory condition of the choroid, the posterior segment of the uvea. However, given its close proximity to the retina, APU often affects the adjoining retinal tissue, which may ultimately lead to vision impairment. Despite advancements in recent research, current treatments for APU are unable to completely cure the disease or provide strategies for retinal tissue regeneration. Neurotrophins, including brain-derived neurotrophic factor (BDNF), play crucial roles in nervous system development, growth, and regeneration and also regulate the activity of the immune system. Moreover, BDNF increases the expression of the nerve growth factor inducible VGF (non-acronymic), which is also involved in the modulation of neural and immune functions.

This doctoral dissertation aimed to assess the dual effects of BDNF and VGF, specifically their involvement in the neuroprotective and immunomodulatory mechanisms that may control the pathogenesis of APU, using a mouse model of experimental autoimmune uveoretinitis (EAU) and the ocular administration of BDNF and the VGF-derived peptide TLQP-21. The study also explored the neuroprotective and immunomodulatory effects of BDNF and TLQP-21 on Müller cells (MCs) in vitro. Additionally, this study investigated the ability of high-contrast visual stimulation (VS) to naturally induce BDNF and VGF expression in the retina and MCs in healthy and uveitic mice and the possibility of the retrograde axonal transport of BDNF from the visual brain areas to the retina.

Results from this study demonstrated that high-contrast VS in optomotor drum enhanced mRNA and protein expression of BDNF in retinal neurons and MCs but upregulated VGF only on the mRNA level in both healthy and uveitic retinas. Furthermore, high-contrast stimulation with pulsed light as well as, BDNF, promoted the neuroprotective properties of MCs in vitro by suppressing inflammatory reactive gliosis and inducing their dedifferentiation into neural progenitor cells, pointing to the potential therapeutic mechanism for retinal regeneration. When examining in more detail, we revealed that VS increased not only retinal BDNF expression but also induced the expression of BDNF in the neurons and astrocytes of the superior colliculus, from which it was retrogradely transported to the retina.

In addition, ocular administration of the exogenous BDNF to EAU mice in the form of eyedrops produced similar results as in vitro experiments on MCs. BDNF significantly reduced reactive gliosis in the retina and promoted the neurogenic attributes of MCs by stimulating their proliferation and dedifferentiation into neural progenitors. At the same time, we observed generation of the newly formed retinal neurons in the ganglion cell layer and

inner nuclear layer of the retina. These protective functions of BDNF ultimately resulted in improved clinical symptoms of EAU.

TLQP-21 treatment of MCs in vitro also promoted MC neurodifferentiation and inhibited their pro-inflammatory properties like BDNF. Comparably, the topical ocular treatment of EAU mice with TLQP-21 displayed partial protective effects, as evidenced by a moderate improvement of the clinical manifestations of EAU, decreased reactive gliosis, and downregulation of pro-inflammatory mediators in the retina. In addition, TLQP-21 also enhanced dedifferentiation of MCs and supported neuronal proliferation in EAU mice. However, given that BDNF treatment of EAU mice did not influence the expression of VGF in the retina, it can be assumed that the beneficial effects of BDNF were not mediated through TLQP-21, a peptide derived from the VGF precursor molecule.

Our findings suggest that BDNF and VGF play a significant neuroprotective and immunomodulatory role in the retina. Therefore, enhancement of BDNF or VGF activity, whether achieved endogenously by VS or augmented by exogenous administration, holds a promising therapeutic potential in the treatment of neurodegenerative and neuroinflammatory conditions of the retina, such as APU.

SOUHRN

Autoimunitní zadní uveitida (APU) je zánětlivé onemocnění cévnatky, zadního segmentu uvey. Avšak, vzhledem k těsné blízkosti sítnice, často postihuje i přilehlou tkáň sítnice, což může v konečném důsledku vést k poruše zraku. Navzdory pokrokům ve výzkumu nejsou současné léčebné postupy pro APU schopny toto onemocnění zcela vyléčit nebo poskytnout strategie pro regeneraci sítnice. Neurotrofiny, včetně mozkového neurotrofického faktoru (BDNF), hrají klíčovou roli ve vývoji, růstu a regeneraci nervového systému a také regulují činnost imunitního systému. BDNF navíc zvyšuje expresi induktivního nervového růstového faktoru VGF (neakronymický termín), který se rovněž podílí na modulaci nervových a imunitních funkcí.

Cílem této disertační práce bylo prozkoumat duální účinky BDNF a VGF, konkrétně jejich zapojení do neuroprotektivních a imunomodulačních mechanismů, které mohou řídit patogenezi APU, použitím myšího modelu experimentální autoimunitní uveoretinitidy (EAU) a očního podání BDNF a TLQP-21, peptidu odvozeného od VGF. Studie rovněž zkoumala neuroprotektivní a imunomodulační účinky BDNF a TLQP-21 na Müllerovy buňky (MCs) in vitro. Dále tato práce zkoumala schopnost vysoce kontrastní vizuální stimulace (VS) přirozeně indukovat expresi BDNF a VGF v sítnici

a MCs u zdravých a uveitických myší a potenciálně i retrográdní axonální transport BDNF ze zrakových oblastí mozku do sítnice.

Výsledky této studie ukázaly, že vysoce kontrastní VS v optomotorickém bubnu zvyšuje mRNA a proteinovou expresi BDNF v neuronech sítnice a MCs, avšak expresi VGF zvyšuje pouze na úrovni mRNA ve zdravé i uveitické sítnici. Dále vysoce kontrastní stimulace pulzním světlem a také BDNF zlepšily neuroprotektivní vlastnosti MCs in vitro potlačením zánětlivé reaktivní gliózy a indukcí jejich dediferenciace na nervové progenitorové buňky, což poukazuje na potenciální terapeutický mechanismus v regeneraci sítnice. Při podrobnější analýze jsme odhalili, že VS zvýšila nejen expresi BDNF v sítnici, ale také indukovala expresi BDNF v neuronech a astrocytech superior colliculus, odkud byl BDNF retrográdně transportován do sítnice.

Navíc oční podání exogenního BDNF myším s EAU ve formě očních kapek přineslo podobné výsledky jako experimenty in vitro na MCs. BDNF významně snížil reaktivní gliózu v sítnici a podpořil neurogenní vlastnosti MCs tím, že stimuloval jejich proliferaci a dediferenciaci v nervové progenitory. Současně jsme pozorovali proliferaci neuronů sítnice ve vrstvě gangliových buněk a vnitřní jaderné vrstvě sítnice a zlepšení klinických příznaků EAU.

Podávání MC in vitro rovněž podpořilo neurodiferenciaci MCs a inhibovalo jejich prozánětlivé vlastnosti podobně jako BDNF. Podobně topická oční aplikace TLQP-21 u EAU myší vykazovala částečné protektivní účinky, což se projevilo mírným zlepšením klinických projevů EAU, snížením reaktivní gliózy a snížením exprese prozánětlivých mediátorů v sítnici. Kromě toho TLQP-21 také podpořil dediferenciaci MCs a podpořil proliferaci neuronů sítnice u myší s EAU. Avšak vzhledem k tomu, že podávání BDNF u myší s EAU neovlivnilo expresi VGF v sítnici, lze předpokládat, že příznivé účinky BDNF nebyly zprostředkovány TLQP-21, peptidem odvozeným od prekurzorové molekuly VGF.

Naše výsledky naznačují, že BDNF a VGF hrají v sítnici významnou neuroprotektivní a imunomodulační roli. Zvýšení aktivity BDNF nebo VGF, ať už endogenně pomocí VS nebo exogenním podáním, má slibný terapeutický potenciál v léčbě neurodegenerativních a neuroinflamačních stavů sítnice, jako je APU.

1. INTRODUCTION

Neurotrophins are a family of regulatory proteins that play a crucial role in the regulation of neuronal growth, proliferation, and survival (Chao, 2003). One of the most studied neurotrophins is brain-derived neurotrophic factor (BDNF), which is known for its neuroprotective and immunomodulatory functions (Bayas et al., 2003; Nagahara et al., 2009; Razgado-Hernandez et al., 2015). Moreover, BDNF was found to display potent therapeutic properties in the models of several ocular conditions, such as optic nerve injury, glaucoma, or photoreceptor damage (Chen et al., 2001; Domenici et al., 2014; Cerri et al., 2015).

One of the mechanisms by which BDNF might exert its effects could be through the protein VGF, given that BDNF and VGF are known to mutually enhance each other's activity (Alder et al., 2003; Jiang et al., 2019). Furthermore, VGF also serves as a precursor molecule to several biologically active peptides, including TLQP-21 (Levi et al., 2004), which is known to have an important role in the regulation of nervous and immune system activity (Severini et al., 2008; Hannedouche et al., 2013; Fairbanks et al., 2014; Sahu et al., 2021).

Considering the neuroprotective and immunomodulatory effects of BDNF and VGF, these factors may hold great potential in the treatment of neuroinflammatory and degenerative diseases of the retina. One such condition could be autoimmune posterior uveitis (APU), a condition mediated predominantly by $CD4^+$ T cells that are autoreactive to several retinal antigens, including interphotoreceptor retinoid binding protein (IRBP) or S-antigen (Luger et al., 2008; Oh et al., 2011). APU is marked by substantial retinal degeneration and inflammation, which leads to the disruption of the functional and structural stability of the retina, ultimately resulting in vision loss (Caspi et al., 2010). Antigen-induced experimental autoimmune uveoretinitis (EAU) is the most widely used animal model of APU, thanks to its close resemblance to human uveitis in terms of basic immunological mechanisms, such as mediation of immune response through autoreactive Th1 and Th17 cells (Luger et al., 2008; Bansal et al., 2015). As such, EAU offers great possibilities for investigating pathophysiologic mechanisms and potential treatments for APU.

1.1. Therapeutic potential of BDNF in the treatment of neurodegenerative and neuroinflammatory diseases of the retina

A growing body of evidence has demonstrated that BDNF has great therapeutic potential in conditions of retinal neurodegeneration. For example, Yin et al. (2020) found that intravitreal therapy of BDNF in a mouse model of retinitis pigmentosa significantly reduced the expression of the pro-apoptotic protein BCL-2 and increased the anti-apoptotic protein Bax, thus reducing photoreceptor degeneration. BDNF has also been shown to be an

effective therapeutic agent in experimental models of glaucoma. Domenici et al. (2014) demonstrated in mice that intravitreal and local administration of BDNF significantly improved the function of retinal neurons damaged by intraocular pressure. Additionally, Liu et al. (2013) showed that BDNF treatment significantly enhanced the survival of rat neurons exposed to a hyperglycemic environment in an in vitro model of diabetic retinopathy.

Another therapeutic approach to treating the retina affected by neurodegenerative diseases could involve supporting the endogenous neurotrophic system in the retina. One such approach could be harnessing the neurotrophic potential of glial Müller cells (MCs), since these cells represent a dominant source of endogenous BDNF, indicating their potential in neurotrophic therapy (Seki et al., 2005). One of the ways in which MC neurotrophic properties could be evoked is by exposing them to visual stimuli. Several authors have already studied the effect of visual stimulation (VS) in mice and its potential for retinal protection. Lim et al. (2016) found that VS improved the regeneration of damaged retinal ganglion cell axons in a mouse model of an optic nerve crush injury. Another study by Mui et al. (2018) discovered that daily exposure of mice to visual stimuli in an optomotor drum significantly improved the visual functions of these animals. In addition, improved visual functions were mediated by pathways associated with BDNF. Considering that MCs are the main source of BDNF in the retina and also express several members of the opsin family (Morshedian et al., 2019; Rios et al., 2019), enabling them to respond to light stimuli, it is likely that VS could be a promising therapeutic approach supporting the neuroprotective functions of MCs.

Giving these findings into perspective, this thesis focuses on the investigation of the dual neuroprotective and immunomodulatory effects of BDNF and VGF on the onset and progression of autoimmune inflammation in the retina. In this work, we explored the effects of VS on BDNF and VGF expression in mice with and without EAU with a particular focus on the role of MCs in these processes. Moreover, we examined the effects of exogenous BDNF therapy for treating EAU, and explored the possibility that the VGF-derived peptide TLQP-21 could mediate its actions.

2. AIMS AND HYPOTHESES

The aims of our study were:

1) To determine whether our models of (a) in vivo VS of control and EAU mice with high-contrast moving patterns may induce changes in retinal BDNF and VGF expression.
2) To identify the cellular origin of BDNF in the retina after VS.
3) (a) To determine whether in vitro stimulation of MCs with pulsed light with the same frequency of pulses as the movement of high-contrast moving patterns in vivo may induce changes in BDNF and VGF expression and, (b) to investigate the neurotrophic and immunomodulatory roles of BDNF and VGF-derived peptide TLQP-21 in vitro in MCs and in a mouse EAU.

In our experimental work, we had three major hypotheses:

1) *We hypothesized that VS of control and EAU mice induces protein and gene expression of BDNF and VGF in the retina.*
2) *We assumed that the main source of BDNF in the retina of visually stimulated mice could be MCs and retinal neurons, or glia and neurons of brain structures activated during VS, due to the possible retrograde transport of BDNF into the retina.*
3) *We expected (a) that stimulation of MCs in vitro with pulsed light induces protein and gene expression of BDNF and VGF and (b) that BDNF and TLQP-21 administered to MCs in vitro or to mice will activate neuroprotective and anti-inflammatory mechanisms, which in EAU mice could alleviate the clinical manifestations of the disease.*

3. MATERIALS AND METHODS

- ***Animals***: Both male and female C57BL/6J mice, 7–10 weeks old were used in the experiments (Charles River Laboratories (Sulzfeld, Germany). Mice were housed under standard conditions with a 12 h/12 h light/dark cycle, regulated temperature (24 ± 1 °C) and humidity (50 ± 10%), and free access to food and drinking water. At the end of experiments, mice were euthanized by an anesthetic overdose with ketamine (100 mg/kg) and xylazine (10 mg/kg), after which transcardial perfusion was performed with 0.1 M PBS.
- ***Cells***: In vitro experiments were carried out using immortalized rat MCs (rMC-1; Applied Biological Materials, Richmond, BC, Canada). The cells were seeded into 24-well plates with collagen coating at a density of 50,000 cells/well in Prigrow III medium, supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin/streptomycin solution.
- ***Induction of EAU***: EAU was induced by subcutaneous administration of an emulsion of retinal antigen $IRBP_{1-20}$ (GPTHLFQPSLVLDMAKVLLD) in an emulsion of complete Freund's adjuvant (*Mycobacterium tuberculosis* + incomplete Freud's adjuvant) on day 0, followed by intravenous administration of pertussis toxin into the tail vein on days 0 and 2.

- ***Eye fundus imaging and EAU clinical score grading***: Clinical manifestations of EAU were investigated using topical endoscopic fundus imaging (TEFI), according to Paques et al. (2007). The examination took place under general anesthesia (ketamine and xylaxine), with the use of mydriatic atropine sulfate to induce pupil dilatation. The fundus images were obtained using an endoscope attached to an Olympus Pen E-PL8 camera (Evident, Tokyo, Japan) and analyzed according to the scoring system developed by Xu et al. (2008).
- ***Visual stimulation of mice***: VS of mice was carried out in the optokinetic drums with a rotating high-contrast grated pattern (black and white vertical stripes), according to the previously published studies (Schmucker et al., 2005; Stofkova et al., 2021). VS was carried out for 14 days, 12 hours daily, during the light phase of the photoperiod. The black and white vertical stripes revolved around the mouse with alterations of 91 ms/stimulus. The light intensity in the optokinetic drums was 30-35 lux.
- ***Stimulation of Müller cells with pulsed light***: Stimulation of MCs was conducted in a humid incubator at 37 °C with 5% CO_2 for 48 hours. The VS was ensured by a custom-made device that emitted pulsed light, i.e., repeated alteration of high-illuminance (230 lux) and low-illuminance (2 lux) light. The frequency of pulses was equivalent to that of the in vivo high-contrast VS (1 pulse per 91 ms). The VS device was installed to uniformly light up the 24-well plate containing the cells. The unstimulated cells were treated under the same conditions, however, in the dark.
- ***Stereotaxic surgery and BDNF administration***: The stereotaxic surgery was performed using a stereotaxic drill and injection robot (Neurostar, Tubingen, Germany), which was operated via the Neurostar StereoDrive software. During the entire procedure, mice were anesthetized with 1.5% isoflurane and had their heads were fixed with ear and incisor bars. After determining the precise coordinates of the superior colliculus (SC) (ML = -0.80 mm, AP = -3.52 mm, DV = +2 mm), both visually stimulated and unstimulated healthy and EAU mice were administered with BDNF-FITC (1 µg/µl). After the procedure, mice underwent VS or were in stationary conditions for another 2 hours.
- ***Topical treatment of EAU mice with BDNF or TLQP-21***: Daily topical treatment of EAU mice with either BDNF (1 µg/day) or TLQP-21 (1.5 µg/µl) eyedrops was performed from the 8^{th} until the 14^{th} day after immunization. Mice were administered with vehicle eyedrops (moisturizer 0.5% carboxymethyl cellulose and 1% dimethyl sulfoxide (DMSO), with or without 1 mg/ml 5-bromo-2′-deoxyuridine (BrdU)) into the ipsilateral eye. BDNF or TLQP-21, diluted in vehicle, were administered into the contralateral eye.
- ***Protein isolation and Western blot***: Both retinal and MC samples were homogenized and treated with ultrasonic waves. The MC samples were then

separated into cytosolic and nuclear fractions. Proteins underwent denaturation, were separated according to their molecular weight by SDS-PAGE, and were transferred to a PDVF membrane. Then, incubation with primary and secondary antibodies followed. Protein bands were visualized with Radiance Plus Chemiluminescence Substrate and Radiance Peroxide, and membranes were imaged with the Azure Biosystems C300 Digital Imager.

- ***RNA isolation and RT-qPCR***: Isolation of RNA from both retinal and MC homogenates was performed using the RNeasy Mini Kit (Qiagen, Hiden, Germany) according to the manufacturer's instructions. Using a High-Capacity cDNA Reverse Transcription Kit, we next performed reverse transcription, after which cDNA was used for RT-qPCR. The reaction took place in QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems).
- ***Immunohistochemistry and preparation of retinal wholemounts***: Experiments were carried out according to Kawamoto (2003). Eyes and brains were placed in the super cryoembedding medium and frozen to -80 °C in hexane. Next, 10 μm-thick eye and brain sections were made and were dehydrated in 99% ethanol, fixed in 4% paraformaldehyde, and blocked with 2% bovine serum albumin. Sections that were incubated with mouse primary antibodies were also treated with F(ab) anti-mouse IgG (H&L). Sections were then treated overnight at 4 °C with primary antibodies and a rat monoclonal IgG2b anti-mouse CD16/CD32 antibody, Clone 2.4G2, after which incubation with secondary antibodies followed. When preparing retinal wholemounts, whole eyes were fixed in 4% PFA overnight, after which the retinas were isolated and incubated in blocking buffer. Retinas were placed on a glass slide, and ProLong™ Glass Antifade Mountant was applied on the tissue. Both tissue sections and retinal wholemounts were visualized using the IX83 inverted microscope and analyzed in Olympus CellSens Dimension software (Evident).
- ***Flow cytometry***: Flow cytometry analysis was conducted on MCs exposed to pulsed light and/or BDNF or TLQP-21 in vitro and on the retinas excised from mice treated with BDNF or TLQP-21 eyedrops. In vitro experiments involved determining cellular proliferation and staining each group with Fixable Viability Dye eFluor 780 to determine dead cells. Cells were fixed with Fixation/Permeabilization Diluent, after which they were incubated with antibodies. In vivo experiments involved euthanizing mice, enucleating their eyes, and excising retinas. Retinas were disrupted into single cells, incubated with collagenase D, filtered, and lysed. Retinal cells were then washed with RPMI-1640 and incubated with IgG2b anti-mouse CD16/CD32 antibody, Clone 2.4G2, followed by incubation with anti-mouse monoclonal antibodies. Data were obtained using the Attune NxT Flow Cytometer and Attune NxT version 3.1.2 software.

- ***Statistical analysis:*** A paired or unpaired t-test was used to determine statistical significance between two groups. For more than two analyzed groups, one-way analysis of variance (ANOVA) was used to analyze changes in one parameter and two-way ANOVA to examine differences in multiple parameters. The statistical difference was significant when $p < 0.05$.

4. RESTULTS

4.1. VS enhanced retinal BDNF but not VGF protein expression in control and EAU mice

In vivo VS of mice was shown to increase both gene and protein expression of BDNF in the retina of control and EAU mice (Fig. 1A-C). More specifically, increased BDNF levels were observed in retinal MCs (labeled with cellular retinaldehyde binding protein, CRALBP, Fig. 1B) and neurons (labeled with neuronal nuclear antigen, NeuN, Fig. 1C). However, significant changes in VGF were observed only at mRNA levels (Fig. 1D) but not at protein levels (not shown).

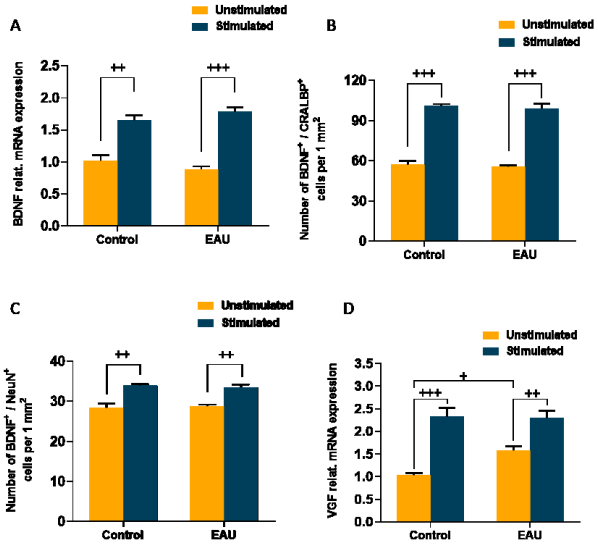

Fig. 1: Effects of in vivo VS on BDNF expression in control and EAU mice: BDNF gene expression (**A**), numbers of $BDNF^+/CRALBP^+$ cells (**B**), and $BDNF^+/NeuN^+$ cells (**C**). Effects of VS on VGF gene expression (**D**). Significant differences: Stim. vs Unstim. with and without EAU: + $p<0.05$, ++$p<0.01$, +++$p<0.001$.

4.2. VS upregulated BDNF expression in the superior colliculus and increased retrograde transport of BDNF from the superior colliculus to the retina in control and EAU mice

Augmentation of the BDNF fluorescent signal was observed only in the SC, specifically in neurons (labeled with NeuN) (Fig. 2A, B) and astrocytes (labeled with glutamine synthetase, GS) (Fig. 2C, D). No substantial differences in BDNF protein levels were observed in the dorsolateral geniculate nucleus (DLG) or visual cortex (VC) (not shown). To further elucidate whether BDNF produced in SC could be retrogradely transported from the brain to the retina, we analyzed retinal wholemounts for the presence of a BDNF fluorescent signal. Although all groups exhibited BDNF fluorescent signal in the retina, visually stimulated control and EAU mice exhibited higher BDNF levels compared to unstimulated control (2E-G). Stimulated EAU mice showed lower amounts of BDNF, when compared to stimulated control mice, indicating that uveitis decreased retrograde transport of BDNF. Retrogradely transported BDNF was observed in neurons and MCs (not shown).

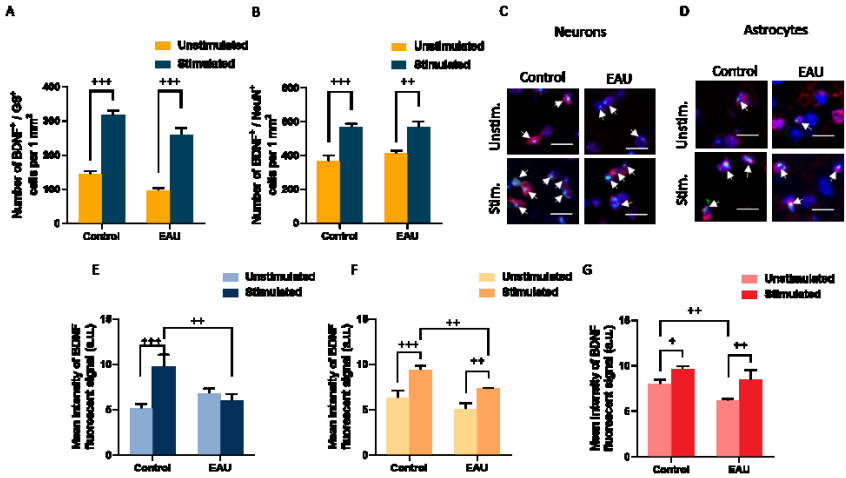

Fig. 2. Number of neurons ($BDNF^+/NeuN^+$ cells) (A, B) and astrocytes ($BNDF^+/GS^+$ cells) in the SC (C, D). Mean fluorescence intensity of exogenous BDNF in the central (E), intermediate (F), and peripheral (G) segment of retinal wholemount. Significant differences: Stim. vs Unstim. with and without EAU: + $p<0.05$, ++$p<0.01$, +++$p<0.001$

4.3. High-contrast stimulation upregulated BDNF but not VGF expression in MCs in vitro

By conducting in vitro stimulation of MCs with pulsed light, we observed significantly higher protein levels of BDNF (Fig. 3A), but not VGF (Fig. 3B) after high-contrast stimulation. However, combination treatment of MCs with pulsed light and exogenous BDNF (0.1 nM, 1 nM, 10 nM) resulted in a dose-dependent increase of VGF synthesis (Fig. 3B). Combination treatment of MCs with BDNF and pulsed light managed to enhance MC proliferation (Fig. 3C), increase the numbers of MCs positive for SOX2 and nestin, markers of neurodifferentiation (Fig. 3D), and decrease numbers of $SOX^+/GFAP^+$ MCs, indicating suppressed MC gliosis (Fig. 3E). Moreover, BDNF reduced nuclear translocation of the p65 subunit of the NF-κB (Fig. 3F), which reflected on the gene expression of downstream pro-inflammatory mediators (not shown).

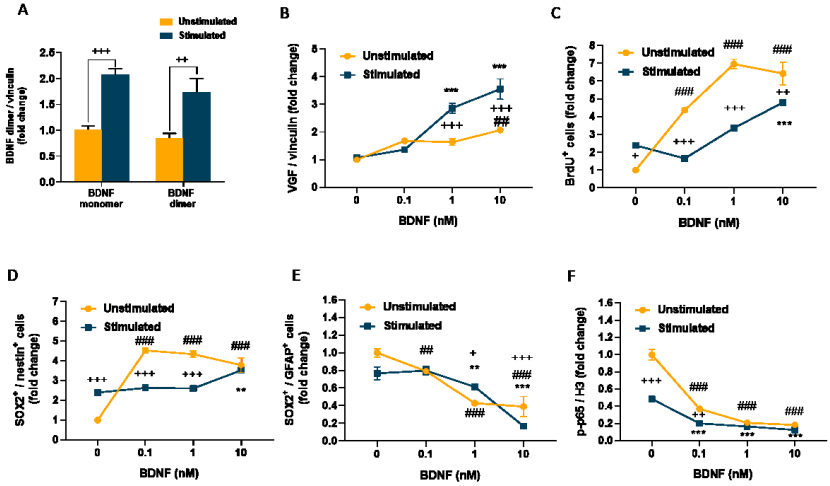

Fig. 3. Effects of VS on BDNF monomer and dimer protein levels (**A**). Effect of VS and BDNF (0.1 nM, 1 nM, 10 nM) on the VGF protein levels in MCs (**B**), MC proliferation (**C**), number of $SOX2^+/nestin^+$ cells (**D**) and $SOX2^+/GFAP^+$ cells (**E**). Levels of p-p65 in the MC nuclear extracts (**F**). Difference between Stim. and Unstim. MCs: $+p<0.05$ $++p<0.01$ $+++p<0.001$. Difference between Stim. cells with and without BDNF treatment: $**p<0.01$ $***p<0.001$. Difference between visually unstimulated cells with and without BDNF treatment $\#\#p<0.01$, $\#\#\#p<0.001$.

4.4. In vitro treatment of MCs with TLQP-21 promoted their anti-inflammatory and neurogenic properties

Treatment with the VGF-derived peptide TLQP-21 appeared to promote MC proliferation (Fig. 4A) and neurodifferentiation (Fig. 4B), as well as suppress MC differentiation to reactive glia (Fig. 4C). TLQP-21 also decreased NF-κB translocation to the nucleus (Fig. 4D), and downregulated gene expression of pro-inflammatory chemokine CCL2, but not other pro-inflammatory mediators (not shown).

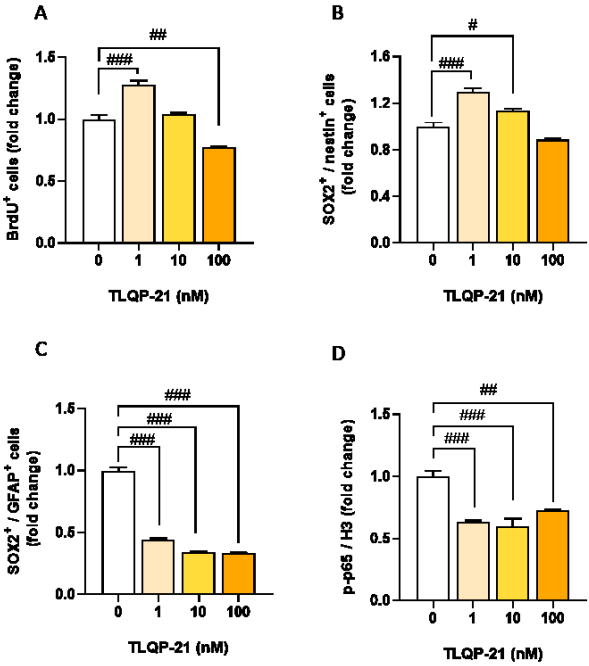

Fig. 4. Effect of TLQP-21 treatment on MC proliferation (**A**), number of $SOX2^+$/$nestin^+$ cells (**B**) and $SOX2^+$/$GFAP^+$ cells (**C**). Protein levels of p-p65 protein in nuclear extracts (**E**) of MCs. Difference between TLQP-21-stimulated and unstimulated cells: #p<0.05, ##p<0.01, ###p<0.001.

4.5. BDNF topical ocular treatment attenuated EAU development and supported retinal regeneration

Treatment with BDNF resulted in a significant amelioration of the disease, as evidenced by the improved clinical score of EAU: optic disc edema, vasculitis, and inflammatory infiltrates in the retina (Fig. 5A). These findings were confirmed by significantly downregulated expression of proinflammatory chemokine CCL2 and cytokine IL-6 (Fig. 5B), as well as decreased numbers of $CD4^+$ and $CD8^+$ T cells and $CD11b^+$ myeloid cells (Fig. 5C) and suppressed reactive gliosis in the retina (not shown). BDNF also supported retinal regeneration, by promoting neurodifferentiation of retinal MCs (Fig. 5D, E), proliferation of MCs (Fig. 5F), and retinal neurons (Fig. 5G). Topical administration of BDNF did not cause any differences in VGF protein levels, which suggests that these neuroprotective and anti-inflammatory functions of BDNF were not mediated via VGF-dependent mechanisms. (Fig. 5H).

4.6. TLQP-21 topical ocular treatment improved EAU and promoted neurodifferentiation of MCs

In our experiments, we observed that TLQP-21 significantly alleviated the EAU severity, as evidenced by the subsided optic disc edema, decreased presence of scars in the retinal tissue, and vasculitis of retinal blood vessels (Fig. 6A). TLQP-21 also downregulated the gene expression of several pro-inflammatory mediators, including IL-6, IL-1R1, as well as the chemokines CXCL1 and CXCL10 (Fig. 6B), indicating that TLQP-21 displayed anti-inflammatory effects. Nevertheless, flow cytometry analysis showed there were no significant changes in the numbers of $CD4^+$ and $CD8^+$ T cells and $CD11b^+$ myeloid cells, although a non-significant decreasing trend could be observed (not shown). Furthermore, we found that treatment with TLQP-21 significantly promoted retinal regeneration by increasing neurodifferentiation of MCs (Fig. 6C, D) and proliferation of MCs (Fig. 6E) and retinal neurons (Fig. 6F).

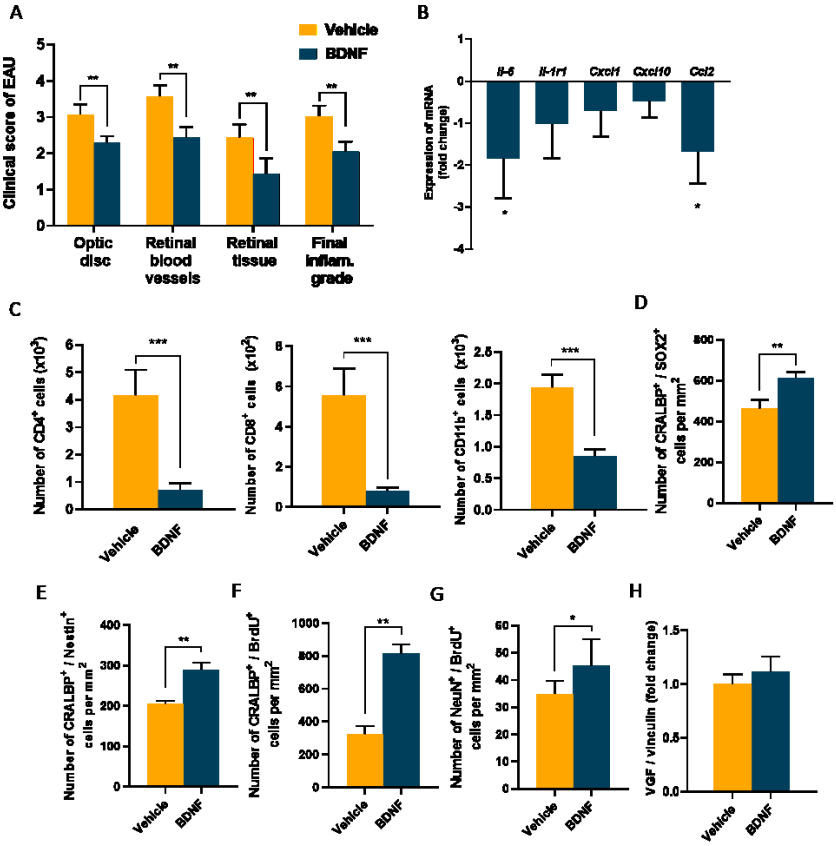

Fig. 5. Effects of BDNF topical therapy on the clinical score of EAU: optic disc edema, vasculitis of retinal blood vessels, and inflammatory infiltrates in the retina (**A**), gene expression of pro-inflammatory mediators (**B**), and numbers of $CD4^+$, $CD8^+$ T cells and $CD11b^+$ myeloid cells in the retina (**C**). Numbers of $CRALBP^+/SOX2^+$ cells (**D**), $CRALBP^+/nestin^+$ cells (**E**), $CRALBP^+/BrdU^+$ cells (**F**), and $NeuN^+/BrdU^+$ cells (**G**). Levels of VGF in the vehicle- and BDNF treated retina (**H**). Statistical difference between vehicle- and BDNF-treated group: *p<0.05, ***p*<0.01 ****p*<0.001

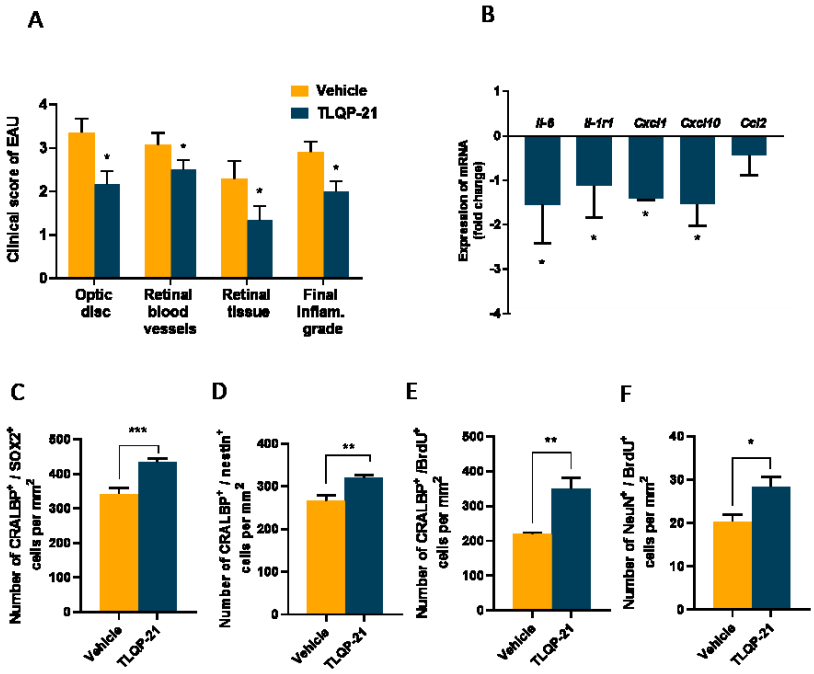

Fig. 6. Effects of TLQP-21 on the clinical manifestation of EAU: optic disc edema, vasculitis of retinal blood vessels, and inflammatory infiltrates in the retina (**A, B**) and gene expression of pro-inflammatory mediators (**C**). The numbers of CRALBP$^+$/SOX2$^+$ cells (**C**), CRALBP$^+$/nestin$^+$ cells (**D**), CRALBP$^+$/BrdU$^+$ cells (**E**), and NeuN$^+$/BrdU$^+$ cells (**F**). Statistical difference between vehicle- and TLQP-21-treated group: *p<0.05, **p<0.01 ***p<0.001.

5.DISCUSSION

5.1. VS increases gene and protein expression of BDNF, but induces only gene expression of VGF in the control and EAU retina

VS was previously found to have a beneficial effect on neuronal growth and regeneration in a mouse model of optic nerve crush (Lim et al., 2016) or during a critical period of mouse development, with BDNF being suggested as a key player in these mechanisms (Mui et al., 2018). Moreover, VS showed therapeutic potential in patients with glaucoma or dry age-related macular degeneration (Gudlin et al., 2008; Sabel and Gudlin, 2014; Li et al., 2020; Horantner et al., 2021). Thus, we explored the possibility of high-contrast VS as a potential approach to promote the expression of retinal endogenous BDNF, which may influence EAU. Our results demonstrated that the VS significantly upregulated both gene expression and BDNF protein levels in control and EAU retinas. More specifically, elevated BDNF expression was observed in the retinal neurons and glial MCs. Since BDNF is known to be involved in the activation of VGF and vice versa (Jiang et al., 2019; Lin et al., 2021), we investigated the effect of VS on VGF expression. Our results showed that VGF exhibited significant upregulation only at the mRNA level, both in healthy and EAU mice. This could be explained by the posttranscriptional/posttranslational modifications of VGF, given that the 3′-untranslated region (3′UTR) of the Vgf mRNA represses VGF translation as reported previously (Lin et al., 2021).

5.2. VS promotes retrograde transport of BDNF from the SC to the retina

In addition to being produced locally in the retina, BDNF may also originate in the brain, from which it could be retrogradely transported of BDNF (DiStefano et al., 1992; Quigley et al., 2000; Lambuk et al., 2022). Thus, we studied if VS may also promote expression of BDNF in the brain structures of the visual pathway (DGN, SC, and VC) in both healthy and EAU mice and promote retrograde transport of BDNF from these structures to the retina. Our results revealed that VS promoted BDNF expression only in neurons and astrocytes of SC but not DLG or VC in control and EAU mice. SC was previously shown to have access to input from most of the RGCs that innervate the DLG but a number of RGC types appear to innervate the SC but not the DLG (Ellis et al., 2016). This robust innervation of the SC by RGCs could explain why significant upregulation of BDNF expression was observed mainly in the SC. Additionally, SC plays a crucial role in processing visual stimuli, orienting attention, and eye and head movement coordination (Basso et al., 2021), which are processes active in mice during the VS, suggesting increased activity of SC neurons may have led to the elevated expression of BDNF. In addition to the SC neurons, a significant

augmentation in BDNF levels was also observed in the SC astrocytes. This elevation may be attributed to the astrocytes' ability to uptake BDNF from the extracellular environment as well as neighboring neurons (Bergami et al., 2008; Crish et al., 2013; Stahlberg et al., 2018), which was found to be beneficial in an experimental model of glaucoma as a protective mechanism of potential re-release in focal sites of neurodegeneration and synapse preservation (Crish et al., 2013). Using stereotaxic delivery of fluorescently labeled BDNF to SC, we revealed that both visually stimulated and unstimulated mice, with and without EAU, had exogenous BDNF in the retina, showing that retrograde transport of BDNF took place in all groups. However, both control and EAU mice exposed to VS had significantly greater amounts of BDNF in the retina, in contrast to the unstimulated animals. Unstimulated mice displayed an exogenous BDNF fluorescent signal in their retinas presumably due to still being kept in the lightened conditions. Even though retrograde transport of BDNF was observed in both healthy and EAU mice, it is clear that EAU mice had significantly reduced intensity of the BDNF fluorescent signal in the central and intermediate segments of the retina after VS when compared to the healthy group, which is in line with our observation of the reactive gliosis in the retina in stimulated and unstimulated mice. The reason of decreased quantities of BDNF in EAU mice might also be due to the inflammation of the optic nerve head (Chu et al., 2013; Selmi et al., 2014) or RGC death (Schwartz and Bakalash, 2007).

5.3. Stimulation of MCs with pulsed light, BDNF and TLQP-21 has anti-inflammatory and neurogenic effects

Considering that MCs express several opsin molecules (Morshedian et al., 2019; Rios et al., 2019) and could respond to light stimuli, we next exposed MCs to pulsed light in vitro. Our results confirmed that MCs exhibit significantly higher protein levels of BDNF after stimulation with pulsed light, which was in line with our in vivo findings and with previously observed effects of electrical stimulation on MCs in vitro (Sato et al., 2008) or enriched environment in mice with non-exudative age-related macular degeneration in vivo (Dieguez et al., 2021). Based on these data, we next evaluated the effect of the stimulation of MCs with either pulsed light or by adding exogenous BDNF in three different concentrations (0.1 nM, 1 nM, and 10 nM), or both, on the neurogenic characteristics of MCs, expressed as the numbers of the cells double positive to markers of neurodifferentiation, SOX2 and nestin (Lendahl et al., 1990; Ellis et al., 2004). We found that the stimulation with pulsed light significantly elevated the numbers of $SOX2^{+}$/$nestin^{+}$ cells when compared to unstimulated group. However, after adding exogenous BDNF, we observed notable increase in the numbers of unstimulated cells positive to SOX2 and nestin in all three concentrations of BDNF. On the other hand, it appeared that only highest concentration of

BDNF significantly augmented the numbers of $SOX2^+$/$nestin^+$ cells stimulated with pulsed light in relation to the MCs without BDNF treatment. Similar trend for high-contrast-stimulated and unstimulated cells was observed in their proliferative capacity. It should be stated that the neurotrophic activity of BDNF is mediated primarily via the TrkB receptor, which is known to be downregulated with increasing BDNF concentrations by the negative feedback loop (Frank et al., 1996). Considering that the unstimulated cells were treated only with BDNF, it is plausible that this negative feedback loop was a mechanism by which MCs did not exhibit further increases in proliferation and neurodifferentiation after the 10 nM concentration. It is also likely that in MCs exposed to both high contrast and exogenous BDNF, not only BDNF but also other mechanisms triggered by high-contrast stimulation may have influenced the regulation of TrkB receptor sensitivity and/or MC proliferation and neuronal differentiation.

Our experiments revealed that BDNF treatment of MCs in vitro significantly reduced the numbers of cells expressing SOX2 and GFAP, a commonly used marker of pro-inflammatory reactive gliosis (Yang and Wang, 2016). These data were comparable to the previously published work on a feline model of retinal detachment, where notable attenuation of GFAP expression was observed following BDNF treatment (Lewis et al., 1999). We also observed a substantial decrease in nuclear translocation of the pro-inflammatory transcription factor NF-κB, and a downregulation of pro-inflammatory mediators (CXCL1, CXCL10, CCL2, IL-6, and IL-1R1). Given that NF-κB is one of the most crucial regulators of the expression of these factors, it appears that BDNF with or without additional VS displays anti-inflammatory characteristics, which are mediated via the inhibition of NF-κB-related mechanisms, similarly as previously observed in the in vitro model of diabetic retinopathy (Zhu et al., 2022). BDNF is known to promote expression of VGF (Alder et al., 2003), which could co-act with BDNF to modulate inflammatory responses and promote neuroregeneration. However, we did not observe any significant differences in VGF protein levels after high-contrast stimulation of MCs, meaning that endogenous levels of BDNF increased by VS were not sufficient to potentiate VGF protein synthesis. Thus, apart from the aforementioned possibility of post-translational modifications of VGF (Lin et al., 2021), another reason for unchanged VGF protein levels could also be a relatively low stimulation of VGF expression by endogenously increased BDNF. To examine whether VGF can act like BDNF by promoting neurogenic and anti-inflammatory properties of MCs, we conducted treatment of MCs with the VGF-derived peptide TLQP-21, given its immunomodulatory properties (Severini et al., 2008; Hannedouche et al., 2013; Fairbanks et al., 2014; Sahu et al., 2021). Our experiments revealed that the treatment of MCs with TLQP-21 increased proliferation,

especially when they were treated with 1 nM TLQP-21, which is in line with previous studies on microglia (El Gaamouch et al., 2020) or cerebellar granular cells (Severini et al., 2008). TLQP-21 is known to bind to the C1qBP complement receptor (Elmadany et al., 2020; Sahu et al., 2021), which is also expressed by MCs (Pauly et al., 2019). The C1qBP receptor was shown to support the proliferation and survival of the fibroblasts and cancer cells (McGee et al., 2011). TLQP-21 stimulation also promoted the neural differentiation of MCs, as evidenced by the numbers of $SOX2^+$/$nestin^+$ cells in our study. Similar findings were observed with the VGF-derived peptide TLQP-62 on the neurogenesis of hippocampal neuronal progenitors, where it has been reported to induce the differentiation of type 2a neural progenitors (Thakker-Varia et al., 2014). We also demonstrated that TLQP-21 has anti-inflammatory action by lowering the numbers of $SOX2^+$/$GFAP^+$ cells and decreasing the nuclear translocation of NF-κB in MCs. These findings could be linked to the previously reported anti-inflammatory characteristics of the C1qBP receptor (Waggoner et al., 2005) and its ability to suppress the activity of NF-κB (Fu et al., 2022). Furthermore, given that NF-κB regulates the expression of GFAP, which is linked to reactive gliosis (Angelo et al., 2014), these results may also explain the decreased differentiation of MCs into reactive glia.

5.5. Topical treatment of mice with BDNF attenuates symptoms of EAU

BDNF treatment was already found to have beneficial effects in several models of other ocular conditions, such as optic nerve injury, glaucoma, or photoreceptor damage (Chen et al., 2001; Domenici et al., 2014; Cerri et al., 2015). In our experiments, we found that the topical delivery of BDNF resulted in a significant improvement in the clinical symptoms of EAU, a decrease in the numbers of $CD4^+$, $CD8^+$, and $CD11b^+$ cells, and a downregulation of the pro-inflammatory markers IL-6 and CCL2. Since both IL-6 and CCL2 are well-known mediators of EAU development (Hohki et al., 2010; Zhao et al., 2014), suppression of their expression by BDNF treatment could contribute to EAU amelioration. MCs are recognized to have great regenerative potential in damaged retina in fish (Duprey-Diaz et al., 2002; Mitchell et al., 2018; Mitra et al., 2019), and mammals (Ooto et al., 2004; Karl et al., 2008). In addition, it seems that BDNF plays a major role in these regenerative mechanisms (Duprey-Diaz et al., 2002). Our data showed that BDNF therapy significantly increased the number of $SOX2^+$/$nestin^+$ MCs, suggesting an enhanced neurodifferentiation. Moreover, we found that BDNF increased proliferation capacity of MCs as well as the number of newly generated retinal neurons, signifying the ongoing regeneration of the retinal tissue damaged by EAU. These findings are in line with previously published work, which showed that BDNF potentiated the neurogenesis in the hippocampus (Lian et al., 2016) and supported

regenerative properties of MCs (Ooto et al., 2004; Karl et al., 2008). Altogether, our data indicate the therapeutic effects of BDNF topical ocular treatment on the symptoms of EAU and propose a mechanism for promoting retinal regeneration.

5.6. Topical administration of TLQP-21 ameliorates EAU

VGF and the VGF-derived peptide TLQP-21 are known to regulate neuron regeneration and survival (Severini et al., 2008; Shimazawa et al., 2010; Takeuchi et al., 2018), as well as mediate immune cell activity (El Gaamouch et al., 2020), which points to the potential therapeutic effects in neuroinflammatory conditions. In our study, we revealed that TLQP-21 topical treatment improved the EAU clinical score and downregulated the expression of pro-inflammatory mediators. Although TLQP-21 therapy displayed similar effects to that of BDNF treatment during EAU, we found that BDNF topical administration did not significantly alter the protein levels of VGF in the retina, which suggests that the therapeutic effects of BDNF and TLQP-21 are not interconnected. TLQP-21 treatment decreased gene expression of pro-inflammatory mediators (CXCL1, CXCL10, IL-6, and IL-1R1), which, however, is not comparable with our in vitro experiments on MCs (not shown). This might be due to the presence of other cell types in the retinal tissue, which could influence and alter the overall gene expression profile. In line with these findings, TLQP-21 also appeared to reduce GFAP expression in the retina, suggesting that TLQP-21 treatment may have suppressing effects on retinal reactive gliosis. It has been previously suggested that TLQP-21 has a suppressing effect on microgliosis in the hippocampus and brain cortex in models of Alzheimer's disease (El Gaamouch et al., 2020), which clearly points to the anti-inflammatory effects of TLQP-21 that we also observed during EAU. In addition, we also found that TLQP-21 promoted the expression of SOX2 and nestin in retinal MCs. In addition, both retinal neurons and MCs exhibited increased proliferative capacity after TLQP-21 eyedrop treatment when compared to eyes stimulated with vehicle. These findings demonstrate that TLQP-21 exhibited neurogenic effects on MCs both in vitro and in vivo, which in turn resulted in attenuation of the clinical manifestations of EAU.

6. CONCLUSIONS

This dissertation thesis had three major hypotheses, all of which were shown to be at least partly valid. Based on our results, we have drawn the following conclusions:

1) First hypothesis: *We hypothesized that VS of control and EAU mice induces protein and gene expression of BDNF and VGF in the retina.*

- Our results demonstrate that VS of mice significantly increases gene and protein expression of BDNF in the retinas of healthy and EAU mice. In addition, enhanced BDNF expression after VS is present in both retinal neurons and MCs.
- Our assumption that high-contrast stimulation increases the expression of VGF was only partly confirmed, since mice exposed to stimulation exhibited a significant increase in VGF mRNA level but not protein level in the retina.

2) Second hypothesis: *We assumed that the main source of BDNF in the retina of visually stimulated mice could be MCs and retinal neurons, or glia and neurons of brain structures activated during VS, due to the possible retrograde transport of BDNF into the retina.*
   - Our data have shown that VS upregulates BDNF expression not only locally in the retina but also increases BDNF production in the neurons and astrocytes of the SC, both in healthy and EAU mice.
   - The presented study demonstrates that VS also promotes retrograde transport of BDNF from the SC into the retina via the axons of RGCs. Here, BDNF is transmitted between RGCs and MCs and potentially serves as an important mediator of neuronal-glial interactions.
   - These mechanisms, although significantly reduced, were also shown to be present in mice with an active form of EAU.

3) Third hypothesis: *We expected (a) that stimulation of MCs in vitro with pulsed light induces protein and gene expression of BDNF and VGF and (b) that BDNF and TLQP-21 administered to MCs in vitro or to mice will activate neuroprotective and anti-inflammatory mechanisms, which in EAU mice could alleviate the clinical manifestations of the disease.*
   - High-contrast stimulation of MCs with pulsed light in vitro supports in vivo data and high-contrast stimulation alone or in combination with exogenous BDNF supplementation increases the neurotrophic and anti-inflammatory properties of MCs. Also, BDNF with no additional high-contrast stimulation displays identical effects.
   - The additional supplementation of MCs with exogenous BDNF substantially augmented the protein synthesis of VGF.
   - Treatment of MCs with the neurogenic and immunomodulatory VGF-derived peptide TLQP-21 resulted in similar results to the BDNF treatment, suggesting that TLQP-21 contributed to the neurotrophic and anti-inflammatory activity of BDNF in MC cultures supplemented with higher doses of BDNF.

- The evidence from our in vivo study clearly demonstrates that the anti-inflammatory and neuroprotective properties of BDNF were shown to be beneficial in a mouse model of EAU. Eyes treated with BDNF exhibited significantly attenuated clinical score of EAU when compared to vehicle-treated eyes.
- BDNF also appears to hold strong regenerative potential and aid in the recovery of retinas damaged by inflammation.
- TLQP-21 topical ocular treatment showed to display anti-inflammatory and neurogenic properties, but the inhibitory effect of TLQP-21 on EAU development was weaker compared to BDNF treatment. Therefore, further studies are needed to better understand the mechanism of action of TLQP-21 during EAU.

LIST OF AUTHOR'S PUBLICATIONS WITH IMPACT FACTOR

1. Stofkova A, Zloh M, Andreanska D, Fiserova I, Kubovciak J, Hejda J, Kutilek P, Murakami M. Depletion of Retinal Dopaminergic Activity in a Mouse Model of Rod Dysfunction Exacerbates Experimental Autoimmune Uveoretinitis: A Role for the Gateway Reflex. *Int J Mol Sci*. 23(1):453, 2021. (**IF:5.6**)
2. Zloh M, Kutilek P, Stofkova A. High-Contrast Stimulation Potentiates the Neurotrophic Properties of Müller Cells and Suppresses Their Pro-Inflammatory Phenotype. *Int J Mol Sci*. 23(15):8615, 2022. (**IF:5.6**)
3. Zloh M., Kutilek P., Hejda J., Fiserova I., Kubovciak J., Murakami M., Stofkova A. Visual stimulation and brain derived neurotrophic factor (BDNF) attenuate experimental autoimmune uveoretinitis. Under revision in *Life Sci*. 2024. (**IF: 5.2**)
4. Zloh M., Stofkova A. Involvement of neuroimmune mechanisms in uveitis and clinical relevance of their targets. In preparation of *Mol. Neurobiol.* (**IF:4.6, Q1**)
5. Zloh M., Kutilek P., Stofkova A. Visual stimulation promotes retrograde transport of BDNF from the superior colliculus to the retina during experimental autoimmune uveoretinitis. In preparation for *Acta Ophthalmol.* (**IF: 3.0, Q1**)
6. Topical treatment with VGF-derived peptide TLQP-21 ameliorates experimental autoimmune uveoretinitis and supports retinal regeneration. In preparation to *IOVS* (**IF:5.0, Q1**).

7. REFERENCES

1. Alder J., Thakker-Varia S., et al. Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. J neurosci. 23(34):10800-10808, **2003**.
2. Angelo M.F., Aguirre A., et al. The proinflammatory RAGE/NF-κB pathway is involved in neuronal damage and reactive gliosis in a model of sleep apnea by intermittent hypoxia. *PLoS One*. 9(9): e107901, **2014**.
3. Bansal S., Barathi V.A., et al. Experimental autoimmune uveitis and other animal models of uveitis: An update. Indian J Ophthalmol, 63(3): 211-218, **2015**.
4. Basso M.A., Bickford M.E., et al. Unraveling circuits of visual perception and cognition through the superior colliculus. Neuron. 109(6):918-937, **2021**.

5. Bayas A., Kruse N., et al. Modulation of cytokine mRNA expression by brain-derived neurotrophic factor and nerve growth factor in human immune cells. Neuroscience letters, 335(3): 155–158, 2003.
6. Bergami M., Santi S., et al. Uptake and recycling of pro-BDNF for transmitter-induced secretion by cortical astrocytes. *J Cell Biol*. 183(2):213-221, **2008**.
7. Cerri E., Origlia N., et al. Conjunctivally Applied BDNF Protects Photoreceptors from Light-Induced Damage. Transl Vis Sci Technol. 4(6):1, **2015**.
8. Chu C.J., Herrmann P., et al. Assessment and in vivo scoring of murine experimental autoimmune uveoretinitis using optical coherence tomography. PLoS One. 8(5):e63002, **2013**.
9. Crish S.D., Dapper J.D., et al. Failure of axonal transport induces a spatially coincident increase in astrocyte BDNF prior to synapse loss in a central target. *Neuroscience*. 229:55-70, **2013**.
10. Dieguez H.H., Calanni J.S., et al. Enriched environment and visual stimuli protect the retinal pigment epithelium and photoreceptors in a mouse model of non-exudative age-related macular degeneration. Cell Death Dis. 12(12):1128, **2021**.
11. DiStefano P.S., Friedman B., et al. The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron. 8(5):983-993, **1992**.
12. Caspi R.R. A look at autoimmunity and inflammation in the eye. *J Clin Invest*. 120(9):3073-3083, **2010**.
13. Chen H., Weber A.J. BDNF enhances retinal ganglion cell survival in cats with optic nerve damage. *iOVS*. 42(5):966-974, **2001**.
14. Domenici L., Origlia N., et al. Rescue of retinal function by BDNF in a mouse model of glaucoma. *PloS one*. 9(12): e115579, **2014**.
15. Duprey-Diaz M.V., Soto I., et al. Changes in brain-derived neurotrophic factor and trkB receptor in the adult Rana pipiens retina and optic tectum after optic nerve injury. J Comp Neurol. 454(4):456-469, **2002**.
16. El Gaamouch F., Audrain M., et al. VGF-derived peptide TLQP-21 modulates microglial function through C3aR1 signaling pathways and reduces neuropathology in 5xFAD mice. Mol Neurodegener. 15(1):4, **2020**.
17. Ellis P., Fagan B.M., et al. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Dev Neurosci*. 26(2-4):148-65, **2004**
18. Ellis E.M., Gauvain G., et al. Shared and distinct retinal input to the mouse superior colliculus and dorsal lateral geniculate nucleus. *J Neurophysiol*.116(2):602-610, **2016**.
19. Elmadany N., de Almeida Sassi F., et al. The VGF-derived Peptide TLQP21 Impairs Purinergic Control of Chemotaxis and Phagocytosis in Mouse Microglia. *J Neurosci*. 40(17): 3320–3331, **2020**.
20. Fairbanks C.A., Peterson C.D., et al. The VGF-derived peptide TLQP-21 contributes to inflammatory and nerve injury-induced hypersensitivity. Pain. 155(7): 1229-1237, **2014**.
21. Frank L., Ventimiglia R., et al. BDNF down-regulates neurotrophin responsiveness, TrkB protein and TrkB mRNA levels in cultured rat hippocampal neurons. *Eur J Neurosci*. 8:1220-1230, **1996**.
22. Fu R. H., Tsai C.W., et al. C9-ALS-Associated Proline-Arginine Dipeptide Repeat Protein Induces Activation of NLRP3 Inflammasome of HMC3 Microglia Cells by Binding of Complement Component 1 Q Subcomponent-Binding Protein (C1QBP), and Syringin Prevents This Effect. *Cells*, 11(19):3128, **2022**.

23. Gudlin J., Mueller I., et al. Computer based vision restoration therapy in glaucoma patients: a small open pilot study. *Restor Neurol Neurosci*. 26(4-5):403-412, **2008**.
24. Hannedouche S., Beck V., et al. Identification of the C3a receptor (C3AR1) as the target of the VGF-derived peptide TLQP-21 in rodent cells. J Biol Chem. 288(38), 27434–27443, **2013**.
25. Hohki S., Ohguro N., et al. Blockade of interleukin-6 signaling suppresses experimental autoimmune uveoretinitis by the inhibition of inflammatory Th17 responses. Experimental eye research, 91(2), 162–170, **2010**.
26. Horantner R., Wolfsgruber J., et al. Effect of repetitive visual training in patients with dry age-related macular degeneration. *Spektrum Augenheilkd*. 35(1):1-15, **2021**.
27. Jiang C., Lin W.J., et al. Role of a VGF/BDNF/TrkB Autoregulatory Feedback Loop in Rapid-Acting Antidepressant Efficacy. J Mol Neurosci. 68(3), 504–509, **2019**.
28. Jiang G., Ke Y., et al. Reactivation of uveitogenic T cells by retinal astrocytes derived from experimental autoimmune uveitis-prone B10RIII mice. Invest Ophthalmol Vis Sci. 49(1):282-289, **2008**.
29. Karl M.O., Hayes S., et al. Stimulation of neural regeneration in the mouse retina. *Proc. Natl. Acad. Sci. USA.*, 105(49), 19508–19513, **2008**.
30. Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. Arch Histol Cytol. 66(2): 123–143, **2003**.
31. Lambuk L., Mohd Lazaldin M.A., et al. Brain-Derived Neurotrophic Factor-Mediated Neuroprotection in Glaucoma: A Review of Current State of the Art. Front Pharmacol. 13:875662, **2022**.
32. Lendahl U., Zimmerman L.B., et al. CNS stem cells express a new class of intermediate filament protein. Cell. 60(4):585-595. **1990**.
33. Levi A., Ferri G.L., et al. Processing, distribution, and function of VGF, a neuronal and endocrine peptide precursor. Cell Mol Neurobiol. 24(4): 517-533, **2004**.
34. Lewis G.P., Linberg K.A., et al. Effects of the neurotrophin brain-derived neurotrophic factor in an experimental model of retinal detachment.*IOVS*. 40(7):1530-1544, **1999**.
35. Li B., Chu H., Yan L., et al. Individualized Visual Reality Training Improves Visual Acuity and Visual Field Defects in Patients with Glaucoma: A Preliminary Study Report. *Cyberpsychol Behav Soc Netw*. 23(3):179-184, **2020**.
36. Li Y.J., Yang C.S., et al. Serum nerve grow factor and brain-derived neurotrophic factor profiles in Sjögren's syndrome concomitant with interstitial lung disease. Clin Rheumatol. 33(8):1161-1164, **2014**.
37. Lian D., He D., et al. Exogenous BDNF increases neurogenesis in the hippocampus in experimental Streptococcus pneumoniae meningitis. *J neuroimmunol.* 294:46–55, **2016**.
38. Lim J.H., Stafford B.K., et al. Neural activity promotes long-distance, target-specific regeneration of adult retinal axons. Nat. Neurosci. 19:1073-1084, **2016**.
39. Lin W.J., Zhao et al. An increase in VGF expression through a rapid, transcription-independent, autofeedback mechanism improves cognitive function. *Transl. psychiatry*. 11(1):383, **2021**.
40. Liu Y., Tao L., et al. BDNF protects retinal neurons from hyperglycemia through the TrkB/ERK/MAPK pathway. Mol Med Rep. 7(6):1773-1778, **2013**.
41. Luger D., Silver P.B., et al. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. J Exp Med. 205(4):799-810, **2008.**

42. McGee A.M., Douglas D.L., et al. The mitochondrial protein C1qbp promotes cell proliferation, migration and resistance to cell death. *Cell Cycle*. 10(23):4119-27, **2011**.
43. Morshedian A., Kaylor J.J., et al. Light-Driven Regeneration of Cone Visual Pigments through a Mechanism Involving RGR Opsin in Müller Glial *Cells. Neuron*. 102(6):1172-1183, **2019**.
44. Mitchell D.M., Lovel A.G., et al. Dynamic changes in microglial and macrophage characteristics during degeneration and regeneration of the zebrafish retina. J Neuroinflammation. 15(1):163, **2018**.
45. Mitra S., Sharma P., et al. Dual regulation of lin28a by Myc is necessary during zebrafish retina regeneration. J Cell Biol. 218(2):489-507, **2019**.
46. Mui A.M., Yang V., et al. Daily visual stimulation in the critical period enhances multiple aspects of vision through BDNF-mediated pathways in the mouse retina. PLoS ONE. 13: e0192435, **2018**.
47. Nagahara A.H., Merrill D.A et al. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. Nat Med. 15(3):331-337, **2009**.
48. Oh H.M., Yu C.R. et al. Autoreactive memory CD4+ T lymphocytes that mediate chronic uveitis reside in the bone marrow through STAT3-dependent mechanisms. J Immunol. 187(6):3338-3346, 2011.
49. Ooto S., Akagi T., et al. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. *Proc. Natl. Acad. Sci. USA*. 101(37):13654–13659, **2004**.
50. Paques M., Guyomard J.L., et al. Panretinal, high-resolution color photography of the mouse fundus. iOVS. 48:2769–2774, **2007**.
51. Pauly D., Agarwal D., et al. Cell-Type-Specific Complement Expression in the Healthy and Diseased Retina. *Cell reports*, 29(9): 2835–2848, **2019**.
52. Pekny M., Pekna M. Reactive gliosis in the pathogenesis of CNS diseases. *Biochim Biophys Acta*. 1862(3):483-491, **2016**.
53. Pozo-Rodrigalvarez A., Ollaranta R., et al. Hyperactive Behavior and Altered Brain Morphology in Adult Complement C3a Receptor Deficient Mice. *Front Immunol*. 12:604812, **2021**.
54. Rios M.N., Marchese N.Aet al. Expression of Non-visual Opsins Opn3 and Opn5 in the Developing Inner Retinal Cells of Birds. Light-Responses in Müller Glial Cells. Front Cell Neurosci. 13:376, **2019**.
55. Razgado-Hernandez L.F., Espadas-Alvarez A.J. et al. The transfection of BDNF to dopamine neurons potentiates the effect of dopamine D3 receptor agonist recovering the striatal innervation, dendritic spines and motor behavior in an aged rat model of Parkinson's disease. *PloS one*. 10(2): e0117391, **2015**
56. Sabel B.A., Gudlin J. Vision restoration training for glaucoma: a randomized clinical trial. JAMA Ophthalmol. 132(4):381-389, **2014**.
57. Sahu B.S., Nguyen M.E., et al. The molecular identity of the TLQP-21 peptide receptor. Cell Mol Life Sci. 78(23):7133-7144, **2021**.
58. Sato T., Fujikado T., et al. Direct effect of electrical stimulation on induction of brain-derived neurotrophic factor from cultured retinal Müller Cells. *IOVS*. Sci. 49:4641-4646, **2008**.
59. Seki M., Tanaka T., et al. Müller Cells as a source of brain-derived neurotrophic factor in the retina: noradrenaline upregulates brain-derived neurotrophic factor levels in cultured rat Müller cells. Neurochem Res. 30(9): 1163-1170, 2005.
60. Selmi C. Diagnosis and classification of autoimmune uveitis. *Autoimmun Rev.* 13(4-5):591-594, **2014**.

61. Severini C., Ciotti M.T., et al. TLQP-21, a neuroendocrine VGF-derived peptide, prevents cerebellar granule cells death induced by serum and potassium deprivation. J Neurochem. 104(2), 534–544, **2008**.
62. Shimazawa M., Tanaka H., et al. An inducer of VGF protects cells against ER stress-induced cell death and prolongs survival in the mutant SOD1 animal models of familial ALS. *PLoS One*. 5(12):e15307., **2010**.
63. Schwartz M., Bakalash S. Autoimmune Uveitis Induces Loss of Retinal Ganglion Cells: Immune Modulation Rather Than Suppression for Neuroprotection. *IOVS*. 48(13): 5213, **2007**.
64. Stahlberg M.A., Kugler S., et al. Visualizing BDNF cell-to-cell transfer reveals astrocytes are the primary recipient of neuronal BDNF. bioRxiv. 255935; 10.1101/255935, **2018**.
65. Takeuchi H., Inagaki Set al. VGF nerve growth factor inducible is involved in retinal ganglion cells death induced by optic nerve crush. Sci Rep. 8(1):16443, 2018.
66. Thakker-Varia S., Jernstedt Krol J., et al. The neuropeptide VGF produces antidepressant-like behavioral effects and enhances proliferation in the hippocampus. *J. Neurosci*. 27, 12156–12167, **2007**.
67. Thakker-Varia S., Behnke, J., et al. VGF (TLQP-62)-induced neurogenesis targets early phase neural progenitor cells in the adult hippocampus and requires glutamate and BDNF signaling. *Stem cell research*. 12(3):762–777, **2014**.
68. Waggoner S.N., Cruise M.W., et al. gC1q receptor ligation selectively down-regulates human IL-12 production through activation of the phosphoinositide 3-kinase pathway. *J Immunol*. 175(7):4706-4714, **2005.**
69. Xu H., Koch P., et al. A clinical grading system for retinal inflammation in the chronic model of experimental autoimmune uveoretinitis using digital fundus images. Exp Eye Res. 87(4):319-326, **2008**.
70. Yang Z., Wang K.K. Glial fibrillary acidic protein: from intermediate filament assembly and gliosis to neurobiomarker. *Trends Neurosci*. 38(6):364-374, **2015**.
71. Yin X., He T., et al. Impact of neurotrophic factors combination therapy on retinitis pigmentosa. *J Int Med Res*. 48(11):0300060520967833, **2020**.
72. Zhao J., Chen M., et al. Experimental autoimmune uveoretinitis (EAU)-related tissue damage and angiogenesis is reduced in $CCL2^{-/-}CX_3CR1$gfp/gfp mice. iOVS. 55(11):7572-7582, **2014**.
73. Zhu M., Li N., Wang Y., et al. Regulation of inflammation by VEGF/BDNF signaling in mouse retinal Muller glial cells exposed to high glucose. *Cell Tissue Res*. 388:521-533, **2022**.