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Review article

Utilizing extracellular vesicles as a drug delivery system in glaucoma and RGC degeneration

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ABSTRACT

Retinal diseases are the leading cause of blindness, resulting in irreversible degeneration and death of retinal neurons. One such cell type, the retinal ganglion cell (RGC), is responsible for connecting the retina to the rest of the brain through its axons that make up the optic nerve and is the primary cell lost in glaucoma and traumatic optic neuropathy. To date, different therapeutic strategies have been investigated to protect RGCs from death and preserve vision, yet currently available strategies are restricted to treating neuron loss by reducing intraocular pressure. A major barrier identified by these studies is drug delivery to RGCs, which is in large part due to drug stability, short duration time at target, low delivery efficiency, and undesired off-target effects. Therefore, a delivery system to deal with these problems is needed to ensure maximum benefit from the candidate therapeutic material.

Extracellular vesicles (EV), nanocarriers released by all cells, are lipid membranes encapsulating RNAs, proteins, and lipids. As they naturally shuttle these encapsulated compounds between cells for communicative purposes, they may be exploitable and offer opportunities to overcome hurdles in retinal drug delivery, including drug stability, drug molecular weight, barriers in the retina, and drug adverse effects. Here, we summarize the potential of an EV drug delivery system, discussing their superiorities and potential application to target RGCs.

1. Introduction

RGCs process and transmit visual information from the retina to the visual centres of the brain *via* their axons bundled into an optic nerve (ON). With this crucial role, RGCs are prone to degeneration in diseases including glaucoma, and traumatic optic neuropathy. RGC degeneration in glaucoma is a complex, multifactorial process involving neurotrophic factor deprivation, the imbalance between production and elimination of nitric oxide, imbalance in intracellular calcium level, glutamate-induced excitotoxicity, activation of apoptosis cascade induced by cytochrome *c* release, and dysregulation of the immune system [1]. In many instances (but not always), the degeneration is a secondary response to raised intraocular pressure (IOP), and IOP remains the only clinically proven target for therapies. To protect RGCs directly from degeneration, manipulation of the above pathways is the main method to promote RGC neuroprotection, with varying preclinical success. Neurotrophic factors are one of the examples that have been utilized to address the neurotrophic deprivation theory and promote neuroprotection of RGCs [2]. Neurotrophic factors including nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin 3 have roles in neuronal growth, plasticity, and survival [2,3] and have been demonstrated to elicit a potent RGC survival effect, more so when combined [4]. Despite decades of promising results *in vitro/ in vivo,* they still lack clinical use as a neuroprotectant due to drug stability, molecular weight, and low delivery yields to RGCs. Calcium (Ca^{2+}) channel blockers and *N*-methyl-D-aspartate (NMDA) antagonists have also become promising candidates to protect RGCs in glaucoma [5–7]. However, off-target systemic effects of these drugs are the main hurdle to clinical use in glaucoma, exacerbated by the high dosages required to achieve neuroprotection. Another example, memantine, is an NMDA

Abbreviations: NMDA, *N*-methyl-D-aspartate; BDNF, Brain derived neurotrophic factor; AMD, Age related macular degeneration; AAV, Adeno-associated Virus; RGC, Retinal ganglion cell; EV, Extracellular vesicles; sEV, Small extracellular vesicles; IOP, Intraocular pressure; ON, Optic nerve; ONC, Optic nerve crush; BMSC, Bone marrow derived mesenchymal stem cell; UMSC, Umbilical cord derived stem cell; MSC, Mesenchymal stem cell; ES, Embryonic stem cell; SC, Schwann Cell; ILM, Inner limiting membrane; TNF-α, Tumour necrosis factor α.

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receptor antagonist to block the excessive glutamate activation, which showed promise in preclinical studies, but failed in clinical studies [8–10], although this may be due to the study design such as the focus on patients with the advanced form of the disease, or only measuring functional and not structural endpoints. A recent promising example to combat RGC loss is nicotinamide. According to a recent clinical trial, daily 1.5 g nicotinamide intake in the first 6 weeks, and then 3 g/day nicotinamide in the second 6 weeks provide improved visual function [11]. These high doses however may burden the kidney and liver over chronic periods, a problem that can be alleviated by an improved drug delivery system. Nutritional supplements can used for the attenuation of mitochondrial dysfunction in glaucoma. Gingko biloba is the most prominent candidate that can modulate oxidative stress and elicit vasodilation [12]. Despite these and other preclinical studies, these small molecules failed in large part due to the various drug delivery challenges such as long-term durability, and off target effects.

Gene, stem cell, and extracellular vesicle (EV) therapies can be thought of as an extension of the above treatment approaches, aiming to deliver therapeutic compounds through alternative, more efficacious means. For example, neurotrophic factor delivery, particularly as bolus, leads to receptor downregulation [13]. To address this, gene therapy studies have shown that intracellular neurotrophic factor receptor overexpression (in RGC) *via* adeno-associated virus (AAV) resulted in RGC protection in acute and chronic glaucoma models [14]. Additionally, AAV-mediated overexpression of both BDNF and its receptor in RGCs led to an increase in RGC viability in an animal model of glaucoma [15]. Another gene therapy strategy targets ATF4/CHOP (through AAV-mediated CRISPR knockdown), a gene involved in endoplasmic reticulum stress after ON injury and silicone induced hypertension, whose down-regulation prevents RGC degeneration [16]. While viral based gene therapies may evocate an immune response [17], there has been recent success in the application of Luxturna, the first FDA-approved AAV-mediated gene therapy for inherited retinal dystrophy. In contrast to gene therapies, which seek to transduce endogenous cells to overexpress neuroprotective compounds, stem cell therapies seek to transplant exogenous cells into the eye, to provide a source of secreted neuroprotectants [18]. Mesenchymal stem cells from dental pulp or bone marrow have been shown to protect RGCs after ONC [19] and glaucoma [20,21]. However, cell therapies may cause issues related to uncontrolled cell differentiation [22], and immunogenicity [23]. Recently, EVs have gained attention as a possible alternative to stem cells and have shown promise at recapitulating their therapeutic efficacy when delivered into animal models of RGC loss [24,25]. There is still a need to investigate possible off-target and toxicity-related effects, but these results have catapulted EVs as a candidate drug delivery system for the eye.

EVs are a new opportunity for drug delivery systems. They are naturally encapsulated, nano sized, and lipid structured particles released by all cells. With their lipid and protein content, EVs are able to overcome barriers and transport their cargo to target cells [26,27]. Due to their endogenous origin, EVs are more biocompatible with the immune system and less likely to evoke the immune response [28]. This is likely due to their immunosuppressive and anti-inflammatory cargo including human leukocyte antigen G, interleuikin-10 and transforming growth factor β, and it is reported that even allogenic sources of EVs show no side effects in human clinical trials [141]. Despite all these advantages, EVs still require optimization for clinical usage such as manufacturing scale-up, optimal (and cheap) storage, GMP manufacturing guidelines, improved pharmacokinetics for retinal diseases, and an optimized loading system for EVs [29].

In this review, we will discuss the potential for EVs as a drug delivery system to RGCs. Moreover, we will explore EVs characteristics that mediate EV cargo transport and could affect EV pharmacokinetics. We will elaborate on EVs in glaucoma/RGC degeneration and possible barriers for EV drug delivery. We will also discuss possible engineering solutions to optimize drug delivery.

1.1. Glaucoma/RGC degeneration

Glaucoma is an eye condition that affects >57.5 million people worldwide, recorded as the leading cause of irreversible blindness [30]. It is characterised by RGC degeneration and chronic progressive optic nerve damage, often caused by increased IOP. IOP is determined by the balance between aqueous humor production and the rate of outflow from the eye. The ciliary body, a tissue placed posterior to the peripheral iris, produces aqueous humor, which nourishes the cornea before draining through the trabecular meshwork located at the iridocorneal angle as a sponge-like tissue. In open angle glaucoma, the trabecular meshwork (and in particular the juxtacanalicular tissue) becomes fibrosed (angle still remains open), leading to blocked drainage, whereas in closed angle glaucoma, the iris adheres to the inside of the cornea, effectively closing the angle. This angle becomes narrow and obstructed in glaucoma for aqueous humor outflow [31,32]. Asymptomatic progression of glaucoma in early disease is a common condition that considerably impacts RGC degeneration. The mechanisms of pathophysiological conditions that trigger this degeneration are still enigmatic, but several theories have been proposed (reviewed in [33]). One theory suggests that glaucomatous alterations are due to elevated IOP acting mechanically to compress the optic nerve head [34]. Another theory is that inadequate oxygen supply to the optic nerve head is the principle cause of sight loss, and is due to systemic hypotension, vasospasm, atherosclerosis, or compression of the vasculature secondary to elevated IOP [35]. Other mechanisms include those previously mentioned, such as the inhibition of retrograde (brain-to-retina) transport of the neurotrophic factors that regulate cell function, survival, and plasticity in the nervous system [36]. Paradoxically, despite being the main modifiable risk factor, lowering IOP is not always therapeutic, and glaucoma can occur without evidence of elevated IOP (normal-tension glaucoma) [37]. The involvement of IOP is not just to kill the RGCs however, and studies suggest RGCs may be in a dysfunctional stage in early glaucoma, which is called the comatose state and these dysfunctional RGCs may be recoverable with IOP lowering [139]. Morphological alterations in the optic nerve head are generally observed in glaucoma, which shows nerve atrophy as biomechanical remodeling of the lamina cribrosa with cupping or excavation of the optic disc. The changes are related to optic nerve degeneration; however, it is possible that the modifications indicate a collection of pathophysiological factors of the posterior segment in glaucoma [38]. A variety of other risk factors outside IOP considered to influence glaucoma have been explored, including age, several gene variants, race, central corneal thickness, and severe myopia [39], with this diversity in risk factors showing the complexity of the disease. A more recent theory suggests RGC degeneration may be a consequence of the impairment of balance between neuroprotective and damaging mechanisms of RGCs [140]. These mechanisms are classified as neuroprotective pathways, processes regulating the redox status, factors/pathways regulating the cell death, survival and neuroinflammation and they may be considered as a therapeutic target.

1.2. Extracellular vesicles

As a biological material, the term of EV was first used by Aaronson et al. in 1971 [40] reviewed in [41]. In the early 1980s, these vesicles were considered waste disposal systems [42], shuttling unwanted molecules out of a cell. With the advancements in this area, it is revealed that EVs mediate intracellular communication through their contents, such as RNAs [43,44]. In one of the most ground-breaking studies in the EV field, Valadi et al. demonstrated that the recipient human mast cells would translate endogenous mRNA into proteins, after receiving mRNA from EVs [45]. After this discovery, EV studies were directed to investigate them as mediators for intercellular signalling, biomarkers for diseases, drug delivery vehicles, and therapeutic agents to modulate injured cells [46–50]. Moreover, their ability to deliver the cargo to

specific cells further enhances their drug delivery potential and studies suggest this delivery may even be targeted based on specific receptors driving uptake by specific cells, which may even be exploited through engineering [51,52].

Debates have persisted on the correct nomenclature [41] to be used, with "exosomes" being more favourable outside of the EV community (and particularly within industry), whereas the minimal information for studies of extracellular vesicles (MISEV) guidelines, a consensus published by the International Society for Extracellular Vesicles states that "EVs" should be used [54]. This review follows the MISEV guidelines, that EVs are "particles that are released from cells, are delimited by a lipid bilayer, and cannot replicate on their own". Terms such as "exosomes", "microvesicles", and "apoptotic bodies" are EVs of a specific biogenesis pathway, of which, few studies (rightfully) seek to identify as it is in many cases unnecessary for investigations into their therapeutic purposes. While historically they have been classified based on size (apoptotic bodies (100–5000 nm), exosomes (50–100) and microvesicles (100−1000)), this is discouraged. In this review, we use EVs based on MISEV guidance 2023 [53] and thus it should be noted that this may conflict with the original authors nomenclature for many of the referenced studies.

1.3. EV therapeutics in glaucomatous RGC degeneration

Despite various studies in therapeutics for cancer and cardiovascular diseases, there are few studies on EVs in retinal diseases, particularly for glaucoma [54] (see Table 1). For the first study to investigate the therapeutic potential of EVs for RGC degeneration, we tested their efficacy at promoting RGC survival and functional preservation *in vitro* and *in vivo* in both optic nerve crush [55] and glaucoma models [24,25]. Bone marrow stem cell (BMSC)-derived EV particles were provided to primary rodent retinal cells at different doses, and 3×10^9 EV particles were determined as the optimal dose for RGC protection *in vitro*. Subsequently, an *in vivo* investigation was conducted after rat optic nerve crush [57], rat microbead or laser-induced glaucoma [24], as well as a mouse DBA/2 J glaucoma model [25]. Intravitreal injection of BMSC-derived EVs demonstrated significantly improved RGC protection with preserved function (as determined by electroretinography), and retinal nerve fiber layer (RNFL) thickness. When investigating the mechanism behind the BMSC neuroprotection on RGCs, the study showed the ablation of miRNA in EVs reduced their subsequent neuroprotective effect on RGCs [24]. Efficacy was retained even when the delivery was restricted to monthly, as opposed to weekly injections, and in the case of the DBA/2 J model, were effective for over 6 months in aged mice [25]. More recently, these findings have been corroborated by other groups, demonstrating that MSC derived EVs were RGC neuroprotective and promoted PIK3/AKT activation that abrogated the RGC damage caused by ONC and consequently, improved RGC survival [56]. Similarly, umbilical cord mesenchymal stem cells (UMSC) enhanced RGC survival after optic nerve crush and promoted the glial activity around optic nerve injury [57]. Also, UMSCs were neuroprotectant in a chronic optic nerve injury model [58]. Human embryonic stem cell (ES) derived MSCs have also been trialled as an EV source for treatment after ONC [59]. Interestingly, EVs were systemically delivered (15 μg) *via* the tail vein and elicited $Brn3a^+$ RGC neuroprotection at days 21 and 60 alongside some visual behavioural improvements and RNFL preservation. Finally, Schwann cell derived EVs were shown to protect RGC at Day 7 and Day 14 after ONC, possibly through CREB activity modulation in RGCs [60].

In summary of the above studies, RGC neuroprotection exceeded the preserved functional activity [24] suggesting improvements are still needed perhaps for the EVs to be engineered for sustained and long-term effect. Since fibroblast-derived EVs did not demonstrate a therapeutic effect *in vitro*, and differing effects were seen with different MSC EVs, the source of the EV is critical. This is further emphasised by the miRNA content [55]. When considering the differing cargo of EVs, more investigation is needed to unveil the potentially multifactorial mechanisms, as the different therapeutic actions might be caused by different cellular uptake kinetics of EVs or different EV contents such as proteins and lipids. With regards to biodistribution/pharmacokinetic studies in BMSC-derived EVs, a recent study investigated RGC uptake kinetics, showing that EVs are distributed around the RGC cell body [60]. EVs injected intravitreally will naturally cluster in the vitreous, and this vitreal distribution of EVs peaked on Day 1 and was detectable in the vitreous after 14 days. This drug duration in the eye is likely an important challenge for intravitreal delivery of EV-therapies and the possibility of extending it should be explored. Consequently, this emphasises that EV engineering is necessary for sustained delivery, particularly when considering that intravitreal injection in the human eye is tolerated up to once a month in age related macular degeneration (AMD) patients, a sight threatening condition affecting the retina. While systemic delivery looks promising [61], more studies are needed to corroborate this finding, as well as report on any toxicity. Intravitreal delivery was investigated for possible EV toxicity in the retina and demonstrated that EVs did not change retinal histology [60]. Although this data gives important information about possible EV toxicity in the retina, it only considers gross changes to the retinal architecture, and EV distribution after retina delivery is required to investigate more subtle, potentially toxic effects on the eye. For example, a study observed severe fibrosis after cell transplantation in human and mouse eyes despite improved function [62], and similar effects may occur with their EVs.

Table 1
List of studies that investigated EV therapies in RGC degeneration.

EV Source	EV Dose	Survival/Functional Investigation	Model	Species	Targeted Mechanisms	References
BMSC	3×10^9 particles	RGC survival ON preservation ERG Retinal structure	ONC, DBA2J Microbead induced glaucoma Laser induced glaucoma model	Rat (female) Mouse (female)	miRNA mechanism	Mead&Tomarev (2017) [55] Mead et al. (2018) [24] Mead et al. (2018) [25]
MSC	N/A	RGC survival	ONC		PI3K/AKT	Cui et al. (2021) [56]
HESC-MSC	15 μg	RGC survival Visual behavioural improvement Retinal structure	ONC	C57BL/J6 mice (male)	N/A	Seyedrazizade et al. (2020) [59]
Schwan Cell	4.5 μl (1 μg/ μl	Survival	ONC	SD rats (male)	CREB	Zhu et al. (2023) [46]
UMSC	1×10^9 5 μl (200 μg/ml, ~5×10^4)	RGC survival ON preservation	ONC Conjunctival fibroblast injection	Wistar rats (male) SD rats (male)	-Caspase 3 inhibition	Pan et al. (2019) [57] Yu et al. (2023) [58]

EV source, EV dose, RGC degeneration model, species, targeted mechanisms are presented. EV: Extracellular vesicles, ON: optic nerve, SD: Sprague Dawley, ONC: Optic Nerve Crush, CREB: cAMP-responsive element binding protein, RGC: Retinal Ganglion Cell, N/A: Not Available.

While the above highlights the therapeutic application of EVs, it is worth highlighting that EVs have been implicated as pathological mediators of glaucoma, as well as other retinal diseases. EVs isolated from naive microglia during elevated hydrostatic pressure induce pro-inflammatory cytokine formation, retinal microglial motility, phagocytic efficiency, and proliferation [63]. In addition, they were shown to lead to an increase in cell death and reactive oxygen species, resulting in retinal degeneration in glaucoma.

Currently, 592 studies exist on https://www.clinicaltrials.gov (last search dated in June 2024), which include exosomes or EVs. Most of these studies are related to cancer, and cardiovascular diseases. In contrast, only seven of them are associated with retinal diseases, while there are no glaucoma related studies. Three of these studies are investigating the therapeutic effect of MSC derived EVs for retinal pigmentosa (NCT05413148, NCT06242379) and macular hole healing (NCT03437759). The other four explore blood/intraocular fluid EVs in diabetic retinopathy (NCT03264976, NCT06198543, and NCT06188013) and retinoblastoma (NCT04164134) for their potential as biomarkers.

1.4. EVs as a drug delivery system

While the previous section paints a picture that EVs are promising therapies as natural therapeutic compounds, it is perhaps short-sighted to restrict our thinking to only the cargo they come pre-packaged with. The first serious discussions and analyses of EV usage as a delivery system emerged after 2010. The work showed improved curcumin delivery through EVs, which increased curcumin's solubility, stability, and bioavailability [64]. Subsequently, Alvarez-Erviti et al. used targeted EVs for the delivery of siRNA into the brain to demonstrate the therapeutic potential of EV delivery for long-term gene silencing [50]. By engineering dendritic cells to express Lamp2b, GAPDH siRNA was delivered to neurons, microglia, and oligodendrocytes. In the following years, miRNA transport through mesenchymal stem cell-derived EVs was used to overcome the chemo- and radio-resistance of glioblastoma multiforme [65]. This study showed that anti-miR-9 was transferred from MSC derived EVs to glioblastoma cells, and successful miR-9 inhibition was shown, resulting in changing the expression of the multidrug transporter and sensitising the glioblastoma multiforme cells to the anticancer drug.

A recent systematic review [66] of preclinical studies showed that the most popular cargo delivered by EVs was nucleic acids (46.5%) and small molecules (39.5%). Nucleic acid-based therapeutics allow providing long term effects and modulate the genes that guide protein expression. Several candidate nucleic acids have been identified to elicit therapeutic efficacy on RGCs in glaucoma. For example, neuroprotection by miRNA 200a inhibition *via* MAPK signalling pathway [67] as well as miRNA-22-3p by targeting TrkB/BDNF signalling pathway [68] has been explored. However, nucleic acids are unstable, negatively charged molecules and can be easily digested by enzymes. In an effort to capitalize on the potential of miRNA, studies have attempted to develop miRNA mimics to overcome these off-target effects and stability issues. However, some of the chemo-engineering of RNA structure leads to new issues. For example, the modifications for RNA stability can cause alterations in RNA folding [69]. Therefore, designing a drug delivery system might be a better way to maximize the potential of miRNA therapies. As EVs already carry and deliver miRNA with their lipid structure and provide encapsulation and protection of nucleic acids, they are a prime candidate for RNA loading while also avoiding possible cytotoxic effects that may occur using synthetic nanoparticles [70,71].

Despite the long list of candidates amenable to EV drug delivery, targeting retina/RGCs is still very much in its infancy. Currently, there are only a few studies that have investigated exogenous content loaded EVs to target the retina. One of them was investigating UMCS derived EVs as a drug delivery system [83]. In this study, engineered EVs were loaded with an interleukin-β receptor antagonist to reduce microglial activation, which provided significant therapeutic benefit. In another study, EVs were tested for the sustained drug delivery of anti-vascular endothelial growth factor for diabetic neuropathy, with treatments applied monthly and the study demonstrating EV-mediated delivery for up to 3 months [84]. In the following section, we are going to discuss lipid composition in relation to drug delivery.

1.5. Lipid composition of EVs

Lipids are key molecular components of EV composition, playing vital roles in their biogenesis, structure, and function [72]. However, research has predominantly focused on characterising the protein and mRNA profiles of EVs, whilst their molecular lipid composition has received little attention [73].

Studies that investigated lipidome found differences in the lipid composition of EVs compared to their parent cell [74–76]. Llorente et al. showed that EVs were largely enriched in glycosphingolipids, sphingomyelin, cholesterol, and phosphatidylserine (PS) compared to their parent prostate cancer cell line (PC3) [74]. The structure of some of these lipids is shown in Fig. 1 A. Furthermore, studies have found that both vesicle type and source cell could affect the lipid composition of EVs [77]. When Haraszti et al. compared lipid enrichment across U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells, and human BMSC; they found that EVs and microvesicles derived from these cells differed in their lipid contents. Interestingly, the levels of glycolipids and free fatty acids were high in EVs, whereas ceramides and sphingomyelins were enriched in microvesicles. It has also been reported that EVs derived from Huh7 and MSCs are specifically enriched in cardiolipins (a type of diphosphatidylglycerol lipid), whereas U87 EVs are enriched in sphingomyelins. The enrichment of EVs with such lipids, especially cholesterol and PS, which are major components of lipid rafts, suggests that EV membranes contain lipid raft-like domains [77]. In fact, studies showed that proteins with an intrinsic affinity for lipid rafts such as

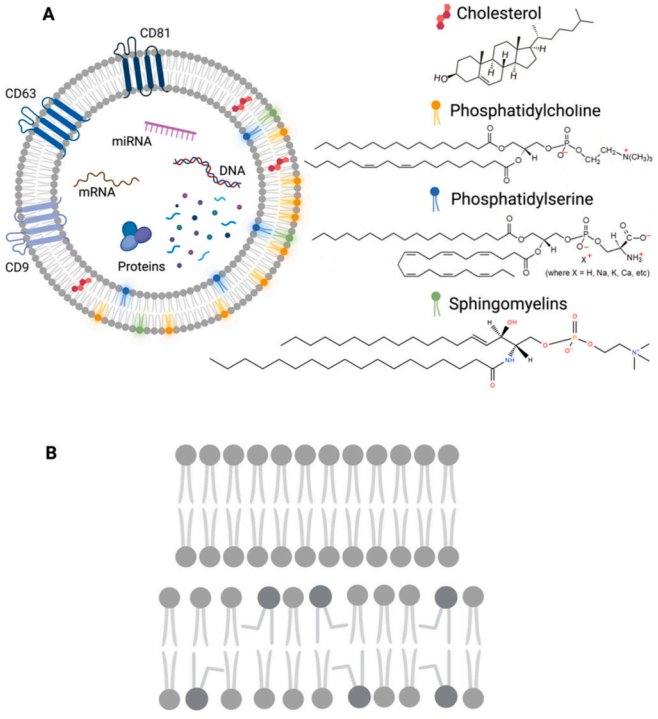

Fig. 1. (A) Schematic of EV and common lipid structures in their bilayer membrane. (B) Schematic of the effect of unsaturation on membrane fluidity. The top membrane has straight saturated hydrocarbon tails, whereas the bottom membrane contains unsaturated fatty acid chains creating kinks in the membrane. Created with BioRender.com.

flotillin-1 and stomatin are sorted into EVs, which indicates the possible involvement of lipid rafts in the selective sorting of proteins into EVs [78].

Another factor that can affect EV lipid composition is the culture conditions of the parent cells. For example, a study that cultured PC3 cells with a hexadecylglycerol (an ether lipid precursor), found an enrichment of ether lipids in the secreted EVs [79]. Similarly, MSCs supplemented with polyunsaturated fatty acids (PUFAs) resulted in a change of not only the cellular lipidome, but also that of secreted EVs, where phosphatidylcholine had higher levels of the incorporated PUFAs [80]. Haraszti et al., showed a change in the lipid composition of EVs released by serum starved MSCs derived from umbilical cord. They specifically reported enrichment of unsaturated and long-tailed cardiolipins in the EVs from serum starved cells [81]. This observation demonstrates the impact of external stimuli in modifying the lipidome of EVs and offers the potential to achieve increased control of the lipid composition present in EVs.

The level of unsaturation and length of fatty acid chains are known to influence membrane properties as it changes the membrane fluidity and the stability of membrane proteins [82]. Unsaturated fatty acid chains occupy a greater lateral surface area, which reduces the packing between phospholipids and thus increases the fluidity of membranes as shown in Fig. 1 B. In this regard, higher fluidity in the membrane of EVs could facilitate their fusion with target cells during intercellular communication or perhaps encourage the leakage of EV contents slowly over time. This further demonstrates the important role of lipids in the biogenesis and function of EVs. It is noteworthy that there are still many unanswered questions regarding the complete molecular lipid composition of EVs. If revealed, this would improve our knowledge around the mechanisms of EV formation, release, and function and may be utilized in drug delivery and diagnostic applications.

1.6. Drug loading of EVs

The previous section highlights the therapeutic effect of EVs and how this can potentially be improved by loading exogenous cargo into EVs. However, the process of actually loading EVs with candidate compounds is its own challenge. Several methods have been explored all with their own advantages and disadvantages, which will be discussed below.

Loading EVs can be separated into two broad categories: 1), modification of the parent cell to influence the cargo of the subsequently isolated EVs (known as "pre-loading"); and 2), directly loading already isolated EVs referred to in the literature as enrichment, engineering and drug loading.

1.6.1. Preloading

Preloading of EVs involves treating cells with stimulating reagents before isolating the subsequently released EVs on the basis that they will be naturally packed with the drugs of interest [90]. A study investigating interferon-alpha and TNF-alpha priming EVs from gingival MSCs promoted increased CD73 and CD5L expression in EVs, which helped macrophage polarization [86]. More interestingly, pre-treating cells with stimulators such as pro-inflammatory cytokines also led to changes in the RNA profile in EVs [87,88]. Preloading may also be done by treating the parent cell with other compounds, which although are not intended to be directly loaded into EVs, change the cell in some way that leads to a change in their EV cargo. One example is treating cells with different anticancer drugs which was found to promote loading of heat-shock proteins into their EVs [89].

Some studies engineer EVs through increasing their cargo loading. An example is to improve pre-miRNA-mimic loading with the TAT peptide/HIV-1 transactivation response RNA interacting peptide [90]. According to Carolina Villarroya-Beltri et al., the protein heterogeneous nuclear ribonucleoprotein A2B1 binds to specific sequences in miRNAs and sequesters them to multivesicular bodies in T cells [91]. Modifying miRNA to contain this motif could lead to more efficient packaging [92]. Cells expressing Cre recombinase can deliver this to other cells through EVs, inducing the expected switch.

Along with EV testing studies, EV engineering has emerged as a powerful platform for RGC neuroprotection. One of the EV engineering examples is TNF-**α** stimulated gingival MSCs that showed an improved neuroprotective effect on RGCs. They investigated TNF-**α** stimulated enrichment of miRNA 21-5p in gingival MSCs and increased the neuroprotective effect on RGCs in a retinal ischemia perfusion model [93]. We have also demonstrated that TNF-**α** priming of bone marrow MSC EVs increased their therapeutic effects on RGCs [143]. Another example of MSC engineering, hypoxic pre-conditioning to produce miRNA 424 that has been proven as a neuroprotective agent in retinal ischemia [94]. Similarly, pre-conditioning media from human amniotic membrane stem cells resulted in more EVs with higher amounts of neuroprotective proteins.These EVs showed an improved neuroprotective effect in glaucoma [95].

Preloading has several advantages over post-loading. Firstly, it may eliminate the possible negative effects direct loading methods have (*e.g.* electroporation) on EV stability. It is also significantly more efficient as the parent cell can be permanently modified, providing an unlimited source of loaded EVs. The main disadvantage is the reduced control you have over what modifications to the EV are being done, and the stimulation of cells may cause changes in EV content other than the loading of the intended target material.

1.6.2. Post loading

Extensive research has been performed on post loading methods. In the literature, various methods are available including sonication, electroporation, freeze-thawing, calcium mediated drug loading, extrusion, transfection, and saponin-mediated permeabilization [96] (Fig. 2), all with their own advantages and disadvantages. For example, there are therapeutically successful examples of electroporation mediated miRNA loaded EVs, such as the loading of plasma derived EVs *via* incubation and electroporation, which created a more efficacious EV enrichment with antitumor miRNAs to stimulate hepatocellular carcinoma cell apoptosis [97]. However, it has been reported that some of the components of EVs cause siRNA aggregation when exposed to electroporation techniques [98]. Alternatively, rather than using an electrical field to permeabilize the membrane freeze-thawing can be used to elicit the same effect and provide a means by which compounds can be loaded into EVs [99–101]. Unfortunately, such a process has been suggested to damage EVs and cause changes to EV size distribution [102]. Transfection is also another common method for EV loading and relies on a chemical transfection reagent to load EVs [103], often similar to chemicals used to load cells such as lipofectamine. However, these chemical reagents may have harmful effects on the target cells, and it can prove difficult to subsequently purify these engineered EVs from the transfection reagent. Saponin and extrusion-mediated EV drug loading relies on the same principle that creates pores in the membrane with different forces. However, saponin has haemolytic and cytotoxic activity [104,105]. Finally, sonication uses sound waves to agitate and permeabilize the EV membrane to allow compounds to enter but has similar worries regarding possible damage to the EV membrane structure [85].

1.6.3. Modifications of EVs to improve the delivery efficiency

Another significant aspect of EV engineering is membrane engineering to increase cellular uptake, the crossing of biological barriers, and improve targeted drug delivery (Fig. 2). This is exemplified in the work undertaken by Alvarez-Erviti et al., which used a combined approach: a) Engineered cells to express EVs with Lamp2b-RVG (Rabies Viral Glycoprotein) for better neuron targeting and b) loaded these with β-site amyloid precursor protein cleaving enzyme 1 (BACE1) siRNAs using electroporation. EVs crossed blood brain barrier and knocked down BACE1 with around 60% efficiency in mice [50]. In another example, cells were transfected with plasmid to produce EVs with the ligand targeting the epidermal growth factor receptors [106]. A recent

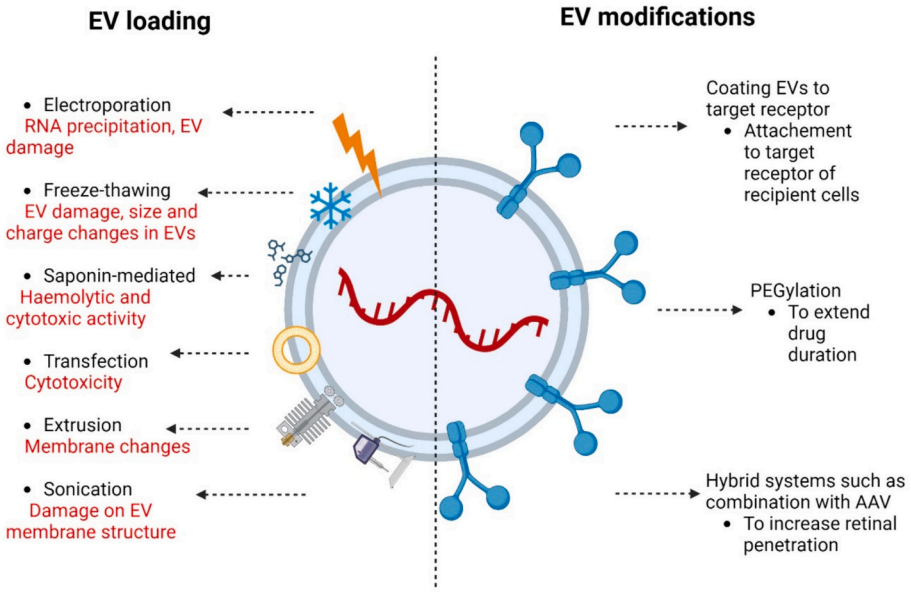

Fig. 2. Schematic representation of EV loading methods and modifications. Red indicates possible disadvantages of the loading methods. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

example of EV engineering was conducted for retinal degeneration and EVs derived from MSCs were coated with cyclic Arg-Gly-Asp peptide to target hyperactivated microglia [88].

Alongside modifications for targeting, some researchers investigated manipulations on EV pharmacokinetics. To improve the EV half-life in the body, Kooijmns et al. investigated EVs with polyethylene glycol (PEG) and they discovered PEG coated EVs had an extended duration in plasma, whereas unmodified EVs were rapidly eliminated from plasma [107]. The second example is the study carried out by Koh et al. (2017) where coating EVs with signal regulatory protein α, masked the CD47 on tumour cells responsible for inhibiting phagocytosis, thereby increasing tumour phagocytosis [108].

Additionally, hybrid systems by intravitreal injection were tested for retinal degeneration, such as the combination of AAV and EV that demonstrated deeper penetration in the retina than conventional AVV gene delivery [109].

1.7. Challenges/clinical translation

Alongside the contextual challenges above, working with EVs has a variety of general challenges that would need to be addressed for their effective use as a drug delivery system. One such challenge limiting the use of EVs is their heterogeneity and the resulting limitations in reproducibility [110]. Biological production systems by nature are hard to standardise, having to rigorously monitor and tightly control the cell lines and culture conditions simultaneously. Furthermore, the population of EVs released by individual cells shows a high degree of variance that is influenced by cell type, developmental stage, cell passage, and external stimuli among other factors, further increasing the need for strict regulations and rigorous evaluations of the finished product. Primary cells likely release EVs that are too heterogenous for clinical translation, so the construction of a cell line is a necessity. Indeed, low levels of purity are a major concern for clinical transition [66,111]. This in turn necessitates more care to be put into safety evaluation and quality control [112]. Considering the scrutiny medical products need to go through, the development of quick and efficient ways for evaluating EV content and purity could greatly benefit the development of these pharmaceuticals.

A further challenge is upscaling production to meet the dosage demand of treating a human eye, a hurdle that involves all stages including production, isolation, and loading of EVs. The efficiency of bioreactors is the most important aspect limiting large-scale EV production, and focusing on the refinement of cultures could offer a great way to increase output while keeping costs low [113]. Even when production is well-optimized, commonly used isolation methods, such as ultracentrifugation, are often tedious or cannot be carried out at large scales without significant infrastructural investment, imposing another hurdle on increasing production rates [114]. Loading similarly suffers from low efficiency. As mentioned above, therapeutic cargo may be pre- or post-loaded into EVs, with preloading being the biggest hindrance to acquiring suitable yields of EVs. In either case, the efficiency of uptake or packaging remains low [98].

Storage represents an additional challenge faced when translating EVs to the clinic. Different conditions may affect the purity and longevity of the EVs stored, with even the generally agreed −80 °C having some time-dependent degradation with significant losses after 6 months [102]. The damage caused by storage and recovery may necessitate the use of cryoprotectants, further increasing the cost of development and production of EV-based therapies [115]. In addition, the requirements for such low temperatures pose serious limitations on both transport and accessibility, and these additional costs may preclude EVs from being used as an ocular treatment, particularly if these are to be self-administered eye drops stored at home.

Finally, the administration brings its own set of challenges and limitations. The most common method for delivering EVs to the retina is intravitreal injection, and it is unclear how well tolerated by glaucoma patients this would be [142]. While AMD patients tolerate this well, it is likely only an option during severe sight loss risk and when only needed for limited time as opposed to indefinitely. Furthermore, the technique may be associated with side effects such as pain, discomfort, and elevation of IOP in the short term, and repeated injections may damage the lens, or cause retinal detachment, and infections in more serious cases. Less commonly observed but more serious complications include retinal toxicity or detachment, corneal abrasions, uveitis, and vitreal or subretinal haemorrhage [116]. As mentioned by two often-cited studies [117,118], liposome formulations, and by extension, the similarly structured EVs, have the capability to cloud the patient's vision as they diffuse in the vitreous. Although visual acuity returned in 2–3 weeks, after complete reabsorption, this temporary distortion not only interfered with the patient's ability to see but also made it difficult for the

ophthalmologist to examine the fundus. The issues arising from this side effect mainly depend on the frequency of injections. As in, if injections needed to be repeated monthly, the patient would spend half or more of the treatment with an even more compromised vision. This in turn could reduce compliance thereby hindering the spread and the application of these therapies. The volume of the human vitreous is approximately 4 ml [119], exponentially higher than the 50 μl of the rat vitreous, suggesting an 80-fold higher dose would be required to elicit similar effects in humans further exacerbating the challenge of upscaling previously mentioned. Below we go into further detail about the different barriers that EVs will encounter when delivered into the eye.

1.8. Barriers to retinal delivery and administration routes for the retina

The administration challenges for EV drug delivery mentioned above are in large part due to the various unique biological barriers to drug absorption. EVs must pass through these barriers and deliver sufficient doses to the retina while avoiding off-target effects/damage. There are three main barriers in retinal drug delivery depending on administration routes (Fig. 3).

1.8.1. Tear film and corneal barrier

Topical drug delivery in the form of an eye drop is the most well tolerated by patients and exposes the least number of off-target tissues in its goal of delivering drugs to the retina. To reach the back of the eye, the drug must pass through the cornea although non-corneal routes are also possible. The cornea is composed of the corneal epithelium, stroma, and endothelium, all of which must allow passage of EVs for the drug to reach the anterior chamber (Fig. 3). The negatively charged epithelium has high lipophilicity and thus offers high availability for lipophilic drugs and positively charged molecules. Enzymes present in the epithelium may cause drug degradation, but this is likely avoided entirely by EVs. In contrast to epithelium, the stroma is a barrier for lipophilic drug and provides more permeability for hydrophilic drug transfer [120].

Alongside the cornea, they could pass through the conjunctiva, sclera, and Tenon's tissue to reach the inner eye, although in contrast to the corneal entry, this route must also pass through the choroid which is composed of blood vessels, possibly leading to systemic toxicity and a reduction in the dose that reaches the retina [121].

The corneal surface is covered by a tear film, an additional barrier for drug delivery to the retina. Tear film consists of enzymes and proteins that can change a drug's molecular structure or reduce its activity [122]. Tear film has a high turnover meaning drugs will have a limited retention period on the ocular surface, limiting their effective duration. As the tear film has important roles in lubricating and protecting the eye alongside minor refractive benefits, EVs may protect drugs from these functions and negatively affect vision. Additionally, since the tear film volume and flow change with age [123], it should be considered how EVs-tear film interactions may differ in an elderly patient.

Considerations of precorneal clearance are corneal absorption, eye drop viscosity, pH, and possible toxicities due to systemic absorption or effects on tear production. Small EVs might be a candidate for topical delivery as they are smaller than 200 nm and negatively charged molecules. EVs may need to be trapped within a viscous material such as hydrogel to aid in absorption and reduce clearance.

1.8.2. Vitreous

Vitreous is a gel-like liquid in the eye behind the lens, and its composition is 98–99% water, collagen, hyaluronic acid, glucose, anions, and cations [124] (Fig. 3). Whether drugs are delivered topically or intravitreally, the vitreous is a barrier that EVs must pass through. Due to its structure, negatively charged nanoparticles can easily diffuse in the vitreous, while transport of cationic particles is impeded [125]. Diffusion through the vitreous is also dependent on particle size. A study investigated the distribution of different sized nanoparticles (ranging from 25 to 250 nm) with antibodies of different weights after intravitreal injection [126]. This study showed that larger sized nanoparticles illustrated improved duration in vitreous humor whereas smaller nanoparticles diffused (and were subsequently cleared) faster. Despite these findings on particle size, it has been suggested that particle diffusion in the vitreous is more dependent on particle charge than particle size [127]. This study tested the diffusion behaviour of 36 different lipid-based nanoparticles in 3 different size ranges (<50 nm, 100–200 nm and > 200 nm) and 3 different charges (cationic, neutral, anionic) and showed there are no significant differences between <50

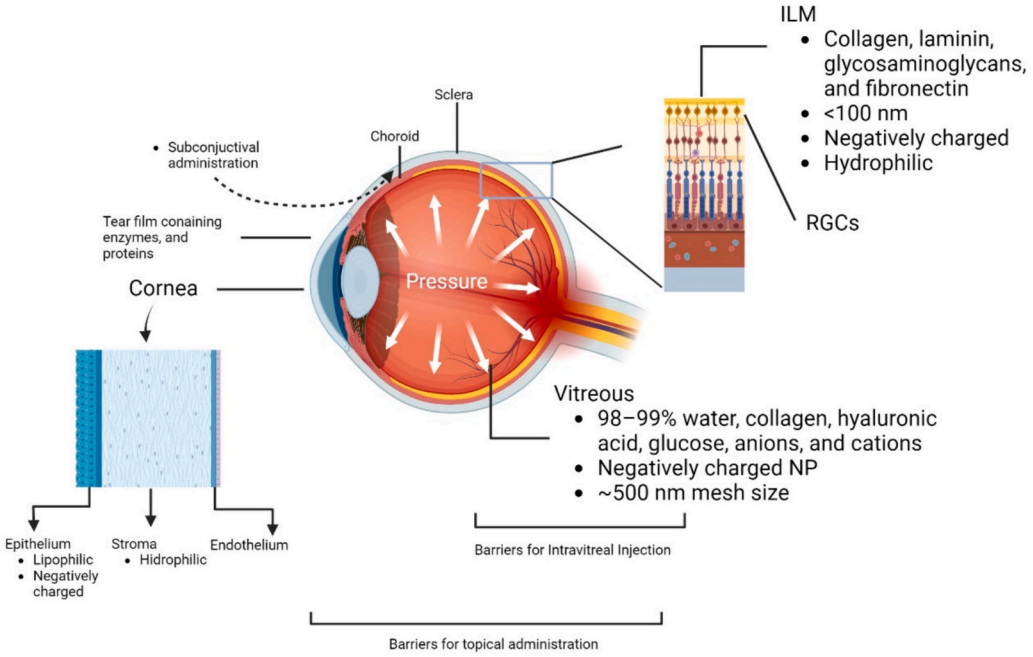

Fig. 3. A representative image of eye anatomy, structural properties for drug delivery system design, and different drug administration methods. Created with BioRender.com.

nm cationic nanoparticles and > 200 nm cationic nanoparticles (D_w/D_v: 126, D_w/D_v: 170, respectively). This study also illustrated that negatively charged and neutral nanoparticles diffuse easily in the vitreous. Interestingly, the study also suggests the corona (outer surface) formation of nanoparticles/EVs may affect cellular uptake and diffusion in the vitreous. PEGylation enhanced the mobility of cationic and larger neutral nanoparticles in the vitreous, due to the steric shielding effect of PEG. Also, hyaluronan can act as an alternative to PEG, as its negative charge promotes diffusion of nanoparticles [128]. Therefore, engineering EVs with these molecules could help improve the transfection efficiency.

As the vitreous humor can limit drug diffusion, it can play an important role in prolonging drug delivery. For example, prolonged bevacizumab delivery (4 months) for AMD was achieved with hydrogel rods by controlling the crosslinking degree and swelling ratio of hydrogel and therefore degradation of hydrogel, to control drug release pattern in vitreous humor [129]. Finally, it is important to highlight that species differences in the vitreous may affect EV diffusion [130,131]. Age related structural changes are also important factors that can change drug pharmacokinetics, and as the vitreous of older eyes is substantially less viscous [131], EV retention may be lower than anticipated based on *in vivo* studies of young adult rodents. Any EV therapies should consider their dynamics within the vitreous, their release profile, suitable charge and lipophilicity as well as ensuring there are no undesired effects on the vitreous elasticity and refractive index.

1.8.3. Inner limiting membrane

To pass from the vitreous to the retina, the inner limiting membrane (ILM) is the final barrier to cross (Fig. 3). It includes collagen, laminin, glycosaminoglycans, and fibronectin, and this changes with aging, becoming stiffer, irregular, and thicker due to changes in collagen IV and laminin composition [132]. The ILM as a barrier is the most notable when it comes to cell transplantation, as cells delivered into the vitreous are prevented from moving to the retina due to the ILM. Significant research has gone into modifying the ILM, to allow cells to integrate into the retina [133].

The ILM does not hinder the penetration of negatively charged and < 50 nm molecules, and a study showed 40 nm polystyrene can easily reach the retina, whereas 100 and 200 nm nanoparticles were significantly more hindered in their ability to reach the retina [134]. A separate study showed <100 nm sized nanoparticles can penetrate the retina [135]. Another study investigated 4 different formulations to test different coating, charge, and size effects on ILM pharmacokinetics [136]. The first formulation was 316 nm sized cationic polyethylenimine nanoparticles, which aggregated in the vitreous humor and could not diffuse to any parts of the eye, possibly due to its strongly cationic property. Furthermore, glycol chitosan nanoparticles (229.1 nm, 16.4 mV) pass easily through the vitreous due to their cationic charge, however accumulated at the ILM due to their large size. Modification with hydrophilic shell polymers such as human serum albumin and hyaluronic acid increased penetration through the ILM, despite their larger sizes (326.3 nm and 213.4 nm, respectively). Another study also investigated lipophilic, and lipid conjugated hydrophilic nanoparticles, showing that lipophilic compounds loaded on liposome membrane reached posterior layers [137]. Alongside different nanoparticle designs, more effective drug delivery has been achieved with direct enzymatic digestion of the ILM [138]. Intravitreal delivery of EVs is detectable in the retina, demonstrating that the ILM is not a complete barrier to EV entry [55], but it is difficult to know how much of a hindrance they are until retinal delivery is quantified before and after ILM disruption (Fig. 4).

2. Conclusion and future directions

Several studies exist that show effective EV based therapies for RGC degeneration. Moreover, many studies have investigated EVs as a drug delivery system to deliver different therapeutics, such as miRNAs and proteins. EVs have a lipid bilayer and are naturally coated with specific proteins and lipids which may mediate drug delivery. EVs natural origin provides various advantages such as biocompatibility and reduced side effects as well as the ability to pass through various drug delivery barriers. We have discussed the potential of EVs to be used as a drug delivery system for degenerating RGCs, particularly in glaucoma. Challenges remain in identifying the therapeutic material in EVs, due to their heterogeneity as well as if any of their cargo may yield undesired effects. How this cargo is modified under different conditions, and whether this can be exploited to engineer more efficacious EVs is still in its infancy, and there is equally a limited number of studies about EV biodistribution after their delivery alongside their stability and duration within the different ocular spaces. Despite these challenges, EVs have shown great promise in targeting RGC and engineering them to improve therapeutic efficacy as well as their drug delivery potential might offer new hope for treating retinal diseases that have so far proved resistant in the quest for a clinically translatable neuroprotective therapy.

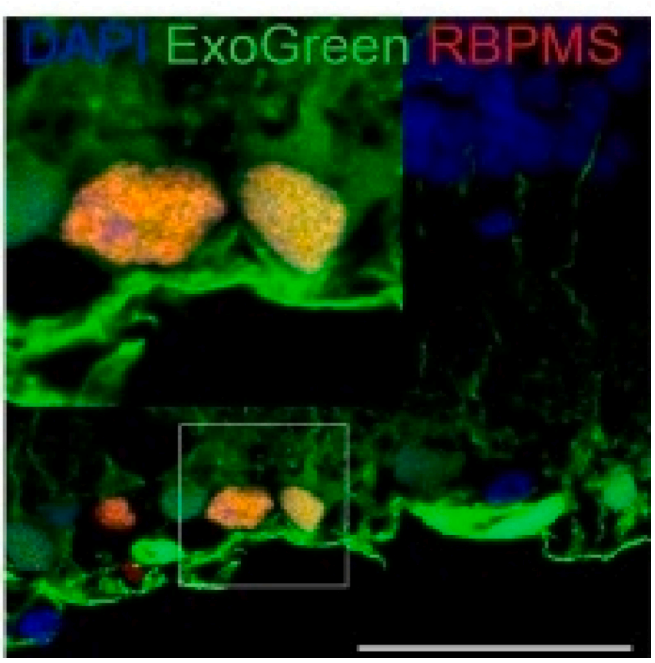

Fig. 4. EV (green) distribution on retinal ganglion cells (red) with nuclear marker DAPI (blue scale bar: 50 μm) [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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CRediT authorship contribution statement

Esmahan Durmaz: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Lujien Dribika:** Writing – original draft, Visualization. **Matyas Kutnyanszky:** Writing – original draft, Visualization. **Ben Mead:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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