REVIEW   **Open Access**

Retinal ganglion cell repopulation for vision restoration in optic neuropathy: a roadmap from the RReSTORe Consortium

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Abstract

Retinal ganglion cell (RGC) death in glaucoma and other optic neuropathies results in irreversible vision loss due to the mammalian central nervous system's limited regenerative capacity. RGC repopulation is a promising therapeutic approach to reverse vision loss from optic neuropathies if the newly introduced neurons can reestablish functional retinal and thalamic circuits. In theory, RGCs might be repopulated through the transplantation of stem cell-derived neurons or via the induction of endogenous transdifferentiation. The RGC Repopulation, Stem Cell Transplantation, and Optic Nerve Regeneration (RReSTORe) Consortium was established to address the challenges associated with the therapeutic repair of the visual pathway in optic neuropathy. In 2022, the RReSTORe Consortium initiated ongoing international collaborative discussions to advance the RGC repopulation field and has identified five critical areas of focus: (1) RGC development and differentiation, (2) Transplantation methods and models, (3) RGC survival, maturation, and host interactions, (4) Inner retinal wiring, and (5) Eye-to-brain connectivity. Here, we discuss the most pertinent questions and challenges that exist on the path to clinical translation and suggest experimental directions to propel this work going forward. Using these five subtopic discussion groups (SDGs) as a framework, we suggest multidisciplinary approaches to restore the diseased visual pathway by leveraging groundbreaking insights from developmental neuroscience, stem cell biology, molecular biology, optical imaging, animal models of optic neuropathy, immunology & immunotolerance, neuropathology & neuroprotection, materials science & biomedical engineering, and regenerative neuroscience. While significant hurdles remain, the RReSTORe Consortium's efforts provide a comprehensive roadmap for advancing the RGC repopulation field and hold potential for transformative progress in restoring vision in patients suffering from optic neuropathies.

Keywords Retinal ganglion cells, Transplantation, Neuroprotection, Organoids, Stem cells, Regenerative medicine, Ophthalmology, Glaucoma, Optic neuropathy

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Introduction

Retinal ganglion cell (RGC) dysfunction is the pathological feature of all optic neuropathies and, in the case of RGC death, results in irreversible vision loss [1, 2]. RGC axons transmit visual information from the eye to the brain, and progressive vision loss occurs when RGCs and their axons degenerate. Blindness caused by optic neuropathies is irreversible due to the limited regenerative capacity of the mammalian central nervous system (CNS) [3]. Therefore, developing innovative regenerative medicine approaches to restore vision loss to optic neuropathies would be transformative.

Ophthalmology is at the frontier of regenerative cell therapy as applied to chronic neurodegenerative diseases and has had several success stories. Transplantation of retinal pigment epithelium is under active investigation in patients as a part of an ongoing clinical trial, and transplantation of photoreceptors derived from human pluripotent stem cells is poised to begin phase I clinical trials soon [4]. These interventions are pioneering the replacement of dysfunctional retinal cells to restore vision and support the premise that RGC transplantation may be capable of reversing vision loss from optic neuropathies.

There are several reasons why the field of RGC repopulation is relatively immature compared to repopulation efforts for other types of retinal cells, but there is reason for optimism. RGCs are inherently more diverse than retinal pigment epithelium and retinal photoreceptors, comprising more than 40 subtypes in mice and more than 15 in primates [5–7]. Moreover, their wiring properties are more intricate than photoreceptors, receiving synaptic input from a variable number (up to dozens) of bipolar and amacrine cells and extending a long axon that must navigate through the optic nerve and optic chiasm into one of several subcortical visual nuclei in the brain [8]. Although RGC repopulation has long been considered [9], recent scientific advances (reviewed here) suggest that this audacious goal may be feasible [10, 11].

To help propel the field forward, the RGC Repopulation, Stem Cell Transplantation, and Optic Nerve Regeneration (RReSTORe) Consortium was established to bring together a diverse group of investigators (more than 200 worldwide) from complementary fields and with broad expertise (http://rrestore.info) [12]. From January through April 2022, the RReSTORe Consortium engaged in a virtual consensus-building process to identify the most pressing challenges and questions that need to be addressed to bring RGC repopulation towards clinical translation. On April 29th, 2022, consortium members met in Denver, Colorado, to engage in a daylong workshop designed to delineate these challenges, review the current state of the field, and brainstorm experimental frameworks to advance goals of vision restoration in optic neuropathies. Workshop discussions were held by five *subtopic discussion groups (SDGs)* running concurrently, which addressed a comprehensive set of goals that need to be obtained to restore functional vision in patients suffering from severe optic neuropathy. The SDGs included: (1) RGC Development and Differentiation, (2) Transplantation Methods and Models, (3) RGC Survival, Maturation, and Host Interactions, (4) Inner Retinal Wiring, and (5) Eye-to-Brain connectivity. Subsequently, the RReSTORe Consortium has continued to foster international collaboration through a series of discussions. Herein, pertinent aspects of those discussions are summarized, briefly recapitulating the state of the field and, more importantly, focusing on the most critical outstanding questions and obstacles that must be overcome within the next several years to enable the clinical translation of RGC replacement to prevent and reverse blindness. Notably, some of the obstacles/goals identified transcend RGC replacement. Focused work in these areas may also be applicable to neuroprotective or regenerative paradigms that seek to preserve or enhance function in optic neuropathies short of overt neuronal replacement.

SDG #1. RGC development and differentiation

A fundamental challenge to functional RGC repopulation is the derivation of *bona fide* RGCs from de novo sources. Advances in stem cell biology have yielded multiple approaches for differentiating RGCs from pluripotent cells [13–15]. Indeed, developing techniques to reliably produce RGC-like cells from pluripotent stem cells at the scale needed for transplantation is a key achievement from the past decade and supports the current feasibility of RGC transplantation approaches. A second approach, induced transdifferentiation of RGCs from endogenous retinal cells, as exhibited by several non-mammalian species [16, 17], might obviate the need for transplantation, especially if such an approach can be harnessed efficiently and safely. Because the molecular mechanisms underlying RGC differentiation from pluripotent cells and endogenous retinal cells overlap, collaborative efforts focusing on RGC specification and maturation from stem cell differentiation and endogenous transdifferentiation provide an opportunity to advance the field (Fig. 1).

Although they do not yet recapitulate the mature retina, retinal organoids derived from pluripotent stem cells are a promising tool for broadening our understanding of RGC development in humans, as they mimic the development, structure, and function of the retina. However, RGCs that develop within organoids are often immature compared to their in vivo counterparts, tend to degenerate as the organoids age, and lack their postsynaptic target, limiting their usefulness for studying long-term RGC survival and maturation
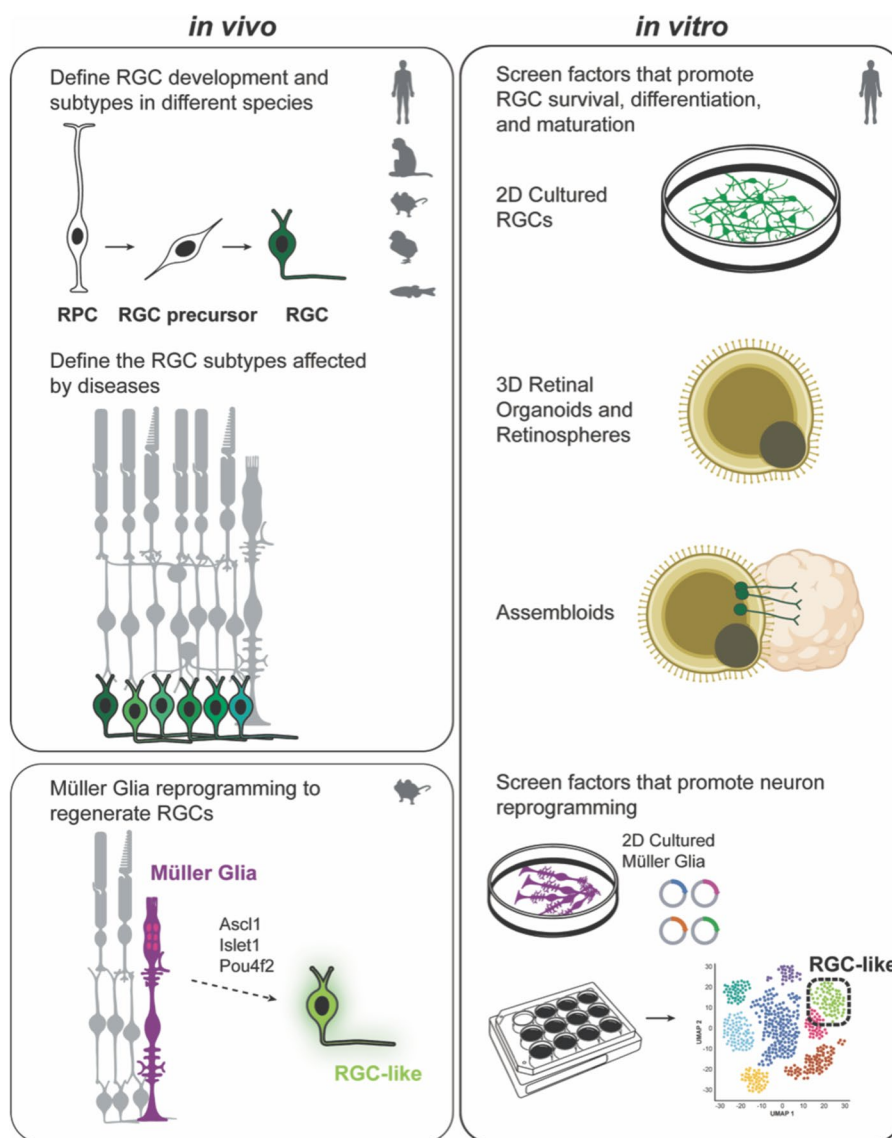
Fig. 1 RGC development, subtype specification, differentiation, and regeneration. Retinal progenitor cells (RPC), RGC precursors, and mature RGCs can be defined and isolated from various species. Neuronal replacement therapies will be advanced by defining the RGC subtypes affected by various optic neuropathies and developing methods to target donor RGC maturation into specific subtypes. In vitro systems serve as a source of RGCs for cell replacement therapies and are useful for screening factors that promote RGC differentiation, maturation, and survival. RGCs can be cultured in monolayers, 3D retinal organoids, retinospheres, and assembloids. Through direct reprogramming in vivo and in vitro, Müller glia can also be a source for newborn mammalian RGCs, and factors that promote neuron reprogramming can be identified. While RGCs can be isolated from various species, RGC repopulation through reprogramming is currently only studied in mice, but in vitro studies can be performed using human samples

[18–20]. As we will discuss, the field would benefit from further identifying the mechanisms underlying RGC development and inevitable degeneration in retinal organoids. Knowledge gained will help researchers identify strategies to promote donor neuron survival and maturation for cell repopulation and will also lead to the development of new neuroprotective therapies for diseases affecting the retina.

Müller glia transdifferentiation for RGC replacement

Retinal regeneration occurs naturally following damage or injury in some lower vertebrates, and recent advances have suggested genes important in this process [21, 22]. For instance, in teleost fish, this process is driven by Müller glia (MG), support cells that dedifferentiate into progenitor cells, proliferate, and replace damaged retinal neurons [23–26]. In mammals, this regenerative process

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is absent, and MG instead respond to injury by generating an inflammatory gliotic state [27]. Over the past decades, considerable advancements have been made in our understanding of the molecular mechanisms underlying MG transdifferentiation in regenerative species and neuroinflammatory gliotic responses in mammals, which may help translate endogenous regeneration strategies to humans. While MG transdifferentiation can repopulate the outer retina, RGCs do not regenerate de novo after optic nerve injuries in zebrafish; rather they are highly resilient to primary degeneration [28], Therefore, this model may not be the most informative for RGC repopulation. Nevertheless, comparative species studies identified upregulation of a pro-neural transcription factor, *Ascl1*, in MG after injury in fish and birds but not mammals [29]. Overexpressing *Ascl1* in MG can stimulate the regeneration of functional neurons in adult mice after injury [30]. These cells express markers of bipolar neurons and wire into pre-existing retinal circuits. Conditional deletion of NFI$_{a/b/x}$ factors also induces MG to generate cells expressing bipolar neuron markers, though it is unknown whether these cells are functional [31]. The addition of *Atoh1* in combination with *Ascl1* increases the number of new neurons generated through MG transdifferentiation [21]. Importantly, this is currently the only combination of transcription factors not requiring retinal damage to induce regeneration in the adult mouse retina [21].

Most regeneration models for RGCs are injury-based, where damage prompts a regenerative response. However, following an injury, RGCs exhibit signs of active cell death, which raises questions about the applicability of the findings for the repopulation of healthy RGCs. Studying teleost models, like zebrafish, in which RGCs are preserved and then shift into a regenerative state, may provide insights for improved regeneration in other organisms. Specifically, additional combinations of transcription factors have demonstrated enhanced potential for regenerating disease-relevant neurons in mammals. Induced expression of *Pou4f2*, *Isl1*, and *Ascl1* in MG was recently shown to produce RGC-like cells in adult mice after injury [22]. These newborn RGC-like cells transcriptionally resembled immature RGCs sequenced from the developing mouse retina and formed synapses within the inner retinal circuitry, but axon regeneration into the optic nerve and towards their cerebral targets has yet to be demonstrated. Though further work remains to demonstrate RGC subtype specification or long-distance axonal extension from endogenously regenerated neurons, these data suggest that RGC reprogramming from MG is achievable in mammals.

A critical goal in the study of RGC repopulation is to establish a definitive demonstration of inducing newly generated RGCs from endogenous neurons, which may have survived a neurodegenerative insult [32]. Typically, neural differentiation is defined based on the expression of reporter constructs meant to label only newly derived neurons. However, multiple factors can lead to artifactual labeling of endogenous host neurons with such reporters, which can prompt erroneous conclusions about transdifferentiation approaches to retinal neuronal repopulation. Such artifacts have arisen in studies based on AAV-mediated expression of transgenes, which are a common approach to inducing endogenous transdifferentiation of MG. A clear drawback of AAV-mediated retinal transduction from MG is that glial-specific promoters lose their cell type specificity when certain transcription factors are expressed, resulting in leaky expression within endogenous neurons [33–35]. This surprising result has led to dubious claims of MG reprogramming that remain to be validated with rigorous controls [36]. To avoid similar issues from impeding progress, the research community must be aware of these pitfalls and provide strong validations for RGC repopulation. Induction of MG transdifferentiation, using transgenic mouse lines rather than AAV, partially mitigates this concern [29], but using transgenic mice is not directly translatable to primate models. Other methods of verifying newborn cell origin involve lineage tracing using EdU and/or BrdU to label actively proliferating cells and track their differentiated progeny. However, these methods require that transdifferentiation occurs through a proliferative progenitor intermediary and would not label new neurons derived from direct transdifferentiation. Importantly, MG-derived neurons are rarely identical to normal retinal neurons in morphology, gene expression, or physiology, and these cells often retain a glial-specific transcriptional signature. Therefore, rigorous transcriptional, microscopic, and electrophysiological assays that document evolution over time can typically parse newborn neurons from endogenous retinal cells.

Although induction of endogenous regeneration in the rodent retina is possible, several questions must be answered before the translational potential of this approach can be appreciated. These include whether MG reprogramming is feasible in primates, including humans, whether the transcription factors capable of driving functional reprogramming of MG into RGC phenotypes are conserved among species, and whether there are any negative long-term effects of MG depletion after reprogramming. Designing a reprogramming system that is narrowly targeted or with less than 100% efficiency might be used to leave a percentage of MG unmodified to support retinal function and ameliorate the effects of MG depletion. Unfortunately, data describing the mechanisms underlying RGC specification in humans

remain sparse due to limited tissue availability and ethical restrictions on studying human embryos. This limitation is further complicated because RGCs are the first neurons to appear in the retina, and early time points are particularly challenging to study [37]. Therefore, developmental studies of human retinal organoids will be important because they can provide answers to at least some of these questions.

Sources of RGCs and models to study RGC development

Translational approaches to therapeutic RGC repopulation will require the study of human RGCs, but access to human and primate tissue is limited. Therefore, there is a need for relevant, in vitro methods of studying RGC specification, maturation, physiology, and survival. Stem cell-derived RGCs, 3D retinal organoids, and brain-retinal assembloids can support developmental research when human tissue cannot be readily accessed [19, 38, 39]. Direct differentiation of plated stem cells produces relatively pure RGC populations by converting pluripotent cells to neurons through activation of relevant transcription factors, such as *Neurog2, Atoh7, Isl1, Ascl1*, and *Pou4f2*, using small molecule signals or transfection [40–42]. In contrast, 3D organoids drive RGC differentiation by pushing stem cells through retinal development stages and yielding complex tissues with multiple interacting cell types. However, differences in RGC phenotype and maturation states have been identified when cells are derived by monolayer as compared to 3D culture approaches. For this reason, comparative studies are needed to determine which approach might be best suited for specific goals.

One potential source of RGCs for transplantation is to collect MG from donor retinas, as MG can transdifferentiate into RGCs [43]. However, the current limitations in maintaining MG in vitro for an extended period and the constraint of obtaining these cells from patients render them a less viable source for generating RGCs in vitro.

An alternative and more promising source of RGCs is pluripotent stem cells, which include both embryonic stem cells and induced pluripotent stem cells (iPSCs). These cells have the potential to differentiate into all cell types in the human body, and their ability to be maintained indefinitely in vitro makes them a potentially unlimited source for generating specific cell types, including RGCs [44]. Notably, patient-derived iPSCs, which can be reprogrammed from adult somatic cells, offer a unique advantage as they could be genetically and immunologically matched to the patient, increasing the potential for disease- or injury-based cellular therapies. Multiple studies have demonstrated the successful generation of RGC-like cells from differentiated rodent and human stem cells through direct and organoid-based differentiation [45–53].

While retinal organoids contain several RGC subtypes, including J-RGCs, alpha RGCs, intrinsically photosensitive RGCs (ipRGCs), and direction-selective ganglion cells [54, 55], RGC specification and maturation remain to be exhaustively characterized through multiple developmental states. Indeed, clarifying in greater detail the RGC subtypes unique to primates, including humans, is an important goal. Studies in rodents have identified RGC subtypes with particularly high resilience, susceptibility to optic nerve injury, and/or propensity for axon regeneration [6, 56]. Identifying correlates in primates may yield translational insights that could augment regenerative and/or neuroprotective paradigms. Critically, retinal organoids lack a fovea (which contains the highest density of midget RGCs), do not recapitulate topographic (i.e., dorsal–ventral, nasal-temporal) differences, and fail to form a retinal pigment epithelium layer as the retina does in vivo. Developing specific methodologies to specify RGC subtypes overrepresented in the macula and exhibiting regional heterogeneity may be necessary to restore high-acuity central and lower-acuity peripheral vision, respectively. Retinal pigment epithelium may be required to support retinal organoid maturation in vitro [57, 58], and it is conceivable that promoting outer retinal organoid development may drive secondary maturation of inner retinal circuits and RGCs. Supplementing 2D retinal cultures and 3D retinal organoids with other cell types, such as microglia, astrocytes, or pericytes, would likely also augment the development and maturation of retinal neurons.

Retinospheres are an alternative approach for the long-term in vitro culture of fetal retinal tissue to differentiate and study human RGCs [39, 59]. Retinospheres are generated directly from retinal fetal tissues rather than stem cells and maintain better lamination than retinal organoids [59], potentially providing a more physiologically relevant environment for RGC development. While single-cell sequencing has uncovered differences in gene expression between the fetal retina and retinal organoids, the signaling pathways that regulate RGC development in retinospheres remain largely undefined. However, despite their potential advantages, both retinospheres and retinal organoids suffer from the limitation that RGCs, more so than other retinal neurons, eventually die in culture [60], potentially due to the lack of retrograde transport of survival cues, such as brain-derived neurotrophic factor (BDNF), from central subcortical targets. To overcome this challenge, novel approaches, such as assembloids, which fuse 3D retinal and cerebral organoids, might be employed to increase the survival of RGCs in vitro [19]. Interestingly, RGCs can survive for lengthy periods in 2D

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culture; therefore, the lack of postsynaptic target innervation in retinal organoids may not independently explain the improved RGC survival documented in brain-retinal assembloids. Further investigations into the factors that support RGC survival and differentiation across in vitro culture models will be essential to developing effective cell replacement products for optic neuropathy.

RGC specification

Various signaling pathways tightly control RGC specification, patterning, and differentiation during development. Several key transcription factors are involved in RGC fate determination, including *Atoh7*, *Isl1*, and *Pou4f2* [7, 61–65]. *Atoh7* is expressed early in RGC development and is essential for RGC differentiation and survival [61]. *Isl1* is a critical transcription factor that regulates RGC fate, while *Pou4f2* controls RGC survival, maintenance, and fate [66]. Whereas transcription factors that maintain RGC fate are well described, much less is known about factors repressing RGC specification. While the Sonic Hedgehog pathway regulates RGC numbers during development by shifting progenitor cell differentiation towards other retinal cell types [67], a more comprehensive understanding of the gene regulatory networks that positively and negatively influence RGC specification would improve our ability to generate this cell type efficiently.

Extrinsic mechanisms, including signals from neighboring cells and extracellular matrix components, also play a crucial role in RGC differentiation and survival. For example, neurotrophins, such as BDNF, promote RGC survival and neurite outgrowth [68, 69]. Laminin at high concentrations in basement membranes, including the internal limiting membrane (ILM), promotes RGC differentiation, polarization, and axon guidance [70–72]. These findings suggest that a complex interplay between intrinsic and extrinsic factors regulates RGC specification and differentiation, which might indicate that incorporating a more complex signaling milieu could enhance RGC generation from pluripotent sources.

Epigenetic modifications are essential for regulating gene expression and cellular differentiation, including RGC development, plasticity, and survival. For example, histone deacetylase inhibitors drive RGC differentiation and promote neurite outgrowth [73, 74]. Furthermore, resetting the epigenetic modifications associated with aging in RGCs induces significant endogenous RGC regeneration in injury models [75]. Moreover, following *Pten* knockout, ipRGCs downregulate several genes, including those involved in subtype specification, indicating that subtype specification is not a determinant of intrinsic regeneration capacity but rather a function of proximity to a particular transcriptomic state [76]. In fact,

ipRGCs are more similar to the embryonic RGC transcriptome state compared to other subtypes, and *Pten* knockout causes ipRGCs to become even more transcriptomically similar to embryonic RGCs and have a greater regenerative capacity as a result [76]. Such approaches to "reset" or dedifferentiate RGCs may help identify molecular targets to prime RGCs for repopulation.

In addition to expressing RGC-specific genes, it may be relevant whether donor cells "match" the recipient's age through similar epigenetic modifications. RGCs derived directly from fibroblasts retain the age of the original cell [77], whereas RGCs derived from iPSCs are epigenetically "younger." It remains unclear how relevant epigenetic state or biological clock "age" is to the ability of newborn RGCs to integrate into retinal circuitries, and this represents an interesting question for further research.

In sum, a more comprehensive understanding of the positive and negative genetic and epigenetic regulators of RGC specification and maturation will likely improve protocols aimed at generating RGCs that are highly efficient at integrating into recipient retinas and functioning within the visual pathway.

RGC subtypes and vulnerability (susceptibility) to injury

RGC diversity is necessary to process the various features of visual information (i.e., motion, color, direction, contrast, and non-vision forming inputs to subnuclei entraining circadian signals) that comprise our perception of the world. Multiple RGC subtypes have been identified in the retina of various species using physiological, morphological, and molecular criteria [8, 78]. RGC subtypes also form distinct postsynaptic connections with different retinorecipient brain targets. However, it remains undetermined whether all these RGC subtypes need to be re-established to restore visual function after optic neuropathy or if some rudimentary vision restoration is possible by regenerating only the most prevalent or functionally critical subtypes. RGC subtype heterogeneity has been shown to underlie visual functions in mice and zebrafish [79, 80], but these relationships have yet to be rigorously explored in other species, including humans and non-human primates.

The numbers of RGC subtypes vary dramatically across species, and it remains unclear which populations of cells should be regenerated/transplanted to reverse blindness. For instance, 30–60 RGC subtypes have been identified in mice compared to only 18 in macaques and humans [8]. Moreover, the distribution and frequency of these subtypes vary across species [81]. In humans, more than 86% of RGCs are midget types (with roughly equal distributions of ON and OFF subtypes) [82], while in mice, the most frequent RGC subtypes (W3 RGCs) comprise only ~7.5% of all RGCs [81]. From an evolutionary

perspective, animals with a high-acuity visual system may have whittled away such vast RGC subtype diversity over time in favor of greater central processing of visual stimuli [83]. Moreover, unique retinal characteristics, such as foveation in primates, may underlie this RGC subtype variation among different species. While single-cell transcriptomic studies have identified key species differences in subclasses of RGCs, some studies may have underestimated the number of RGC subtypes due to limited RGC capture, as RGCs typically represent less than 1% of cells in the adult retina [84, 85]. Therefore, further evolution in single-cell transcriptomics will likely increase sensitivity and identify greater complexity among RGC subtypes, especially during development or disease.

One issue underlying apparent discrepancies in reported distributions of RGC subtypes relates to the multiple methods by which subtypes have been defined. Recent efforts have been made to unify the classification of RGC subtypes across morphologic, electrophysiologic, and transcriptomic modalities [79]. Advancements in techniques like Patch-seq enable simultaneous transcriptomic and physiological profiling of cells [86]. Spatial transcriptomics at cellular resolution can also be leveraged to capture subtype differences on both the morphological and transcriptomic levels [87], using platforms such as 10×Xenium (https://10xgenomics.com), MERFISH [88], and NanoString GeoMx (https://nanostring.com). For example, MERFISH has been applied to the retina as part of the Cell Atlas Eye [89]. Additional questions, such as whether specific RGC subtypes are preferentially lost with age and whether RGC subtypes differ based on retinal location and sex, may also be answered through these technologies.

A crucial but unresolved question in glaucoma and other optic neuropathies is whether and why distinct subtypes of RGCs are vulnerable to damage and, therefore, preferentially lost in these conditions. Topographical patterns of RGC loss corresponding to visual field defects characteristic of specific optic neuropathies have been appreciated for decades. The earliest RGCs to exhibit degeneration in glaucoma tend to be localized to the temporal retina and correspond to a "nasal step" defect [90]. In contrast, toxic and nutritional optic neuropathies are classically defined by cecocentral defects arising from the loss of macular RGCs. While there are some morphological and functional differences in RGCs based on their topographical location, specific transcription factors have also been associated with ipsilateral/contralateral targeting segregation [59], retinal eccentricity, and axonal location within the optic nerve [91], which may also underlie potential differences in vulnerability.

Whether RGC subtypes exhibit differential vulnerability to optic nerve insult independent of topographic location remains to be determined. Selective vulnerability of human RGC subtypes in glaucoma was first reported in the 1980s and described as a preferential loss of RGCs with larger somas and axon diameters [92, 93]. Subsequently, numerous other studies evaluated the selective loss of human RGCs in glaucoma [94–108], of which more than half failed to identify such RGC type-specific loss [101–108]. Interestingly, only one of the contradictory reports evaluated the loss of RGCs in glaucomatous tissues using histological approaches [108], suggesting that methodological differences may have contributed to conflicting conclusions. Indeed, both anatomical and functional studies of non-human primates on the selective vulnerability of RGCs in glaucoma have remained inconclusive. Specifically, RGCs with larger somas and axon diameters may be preferentially vulnerable in glaucomatous monkeys [109–116] and pigs [117]. On the other hand, results from multiple functional and anatomical studies showed the opposite, suggesting a universal exposure of RGC subtypes in nonhuman primate models of glaucoma [118–124].

Subsequently, an overwhelming number of studies in cat [125–130], rat [131–138], and mouse [81, 139–156] glaucoma or other RGC degeneration models—spanning from 1989 to 2020—reported significant variation among RGC subtypes in their response to experimental perturbations including elevated IOP, optic nerve trauma, and excitotoxicity. Few mouse studies have failed to identify a selective vulnerability of RGC subtypes in glaucoma [157, 158]. The vulnerability of RGC subtypes in mouse and other animal models of RGC degeneration (e.g., hypertensive microbead injection glaucoma model, optic nerve crush model, and DBA/2 J mice) appears well established. For instance, direction selective-RGCs and alpha-RGCs are more vulnerable after an injury in mice, while ipRGCs are more likely to survive than other subtypes [138]. Spatially, the mid-peripheral and peripheral RGCs are more vulnerable in experimental glaucoma in pigs and rats [117, 159, 160]. Interestingly, there is also an increase in the RGC soma size in experimental glaucoma [117, 159, 161, 162]. The propensity for selective loss of RGC subtypes in humans (and non-human primates) requires further clarification. Although new technologies, like single-cell RNA sequencing technologies, have greatly facilitated the identification and quantification of molecularly distinct subtypes, answering this question in humans will be challenging due to variations in the severity (stage) of various optic neuropathies and availability of tissues, which enable only cross-sectional evaluations.

For several reasons, it is necessary to understand whether human RGC subtypes differ in their vulnerability to damage. First, the molecular and cellular features of susceptible and/or resistant RGCs could be identified and

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leveraged to develop molecularly targeted neuroprotective treatments. Second, RGC subtype specificity could influence the design of therapeutics to repopulate specific RGC subtypes in the eye [163, 164]. Finally, understanding the molecular mechanisms underlying RGC subtype susceptibility to optic nerve damage would significantly advance our understanding of disease pathophysiology [81]. Indeed, care must be taken not to promote the regeneration of one RGC subtype at the expense of a more vulnerable, but functionally necessary, population.

Several experimental approaches have been proposed to identify which RGC subtypes are most relevant to target from a therapeutic standpoint. These include individually depleting all but one RGC subtype in the retina through transgenic approaches to better understand isolated subtype function, prioritizing the study of rare diseases preferentially affecting specific RGC subtypes, using transgenics to more comprehensively characterize RGC subtype development and functions [165], and performing highly specific visual modality testing in patients with optic neuropathy to identify potential subtype-specific deficits [166]. In addition to commonly used automated perimetry, other assessments such as contrast sensitivity, direction sensitivity, color perception, and specialized electroretinography (ERG) metrics might complement visual fields in measuring RGC subtype-specific functions. Specifically, contrast and color vision tests directly measure specific attributes of RGC function and subtype specification [166]. However, their added value and potential for predicting disease progression earlier than visual fields alone require further study, and any value in integrating these tests with conventional perimetry in routine glaucoma practice rather than for research purposes remains to be determined.

Future directions for RGC development and differentiation
Over the past ten years, methodologies have significantly expanded for driving RGC differentiation from stem cells. These protocols hold great promise for developing cell-based therapies for retinal degenerative diseases, such as glaucoma. However, which protocols are best suited to generate RGCs for experimental or therapeutic goals remains to be determined. We propose that direct comparisons (based on morphology, transcriptomics, and electrophysiology) of human RGCs generated through independent approaches (e.g., gradual differentiation in monolayers, directed differentiation in monolayers, and within retinal organoids) would be highly informative. Furthermore, comparisons of RGC engraftment following transplantation into the eye using these various sources could help elucidate the best source for donor RGCs.

One key question is whether it is necessary to replicate progression through all stages of RGC development, as occurs in retinal organoids, or if it is possible to bypass specific developmental steps and directly induce RGC differentiation using vectors that drive the expression of specific transcription factors. It is unknown if doing so would enable subtype specificity, but recent data suggest that identifying and manipulating the molecular and cellular factors that regulate RGC subtype development in vivo may offer a solution [167]. This approach might involve single-cell transcriptomics to identify subtype-specific gene expression profiles and manipulate signaling pathways involved in RGC subtype specification in vitro and in vivo. If achieved, directed differentiation of RGCs into specific subtypes would provide a valuable tool for shedding light on the selective vulnerability of RGC subtypes in humans and could also be critical to repopulating the most visually relevant RGC subtypes in patients. Therefore, developing novel methods for controlled subtype specification of stem cell-derived RGCs is a crucial area for future research.

Establishing protocols to generate RGC subtypes may yield targeted interventions to restore specific aspects of vision. For example, the fovea subserves high acuity central vision and contains a high density of midget RGCs, for which no specific differentiation protocol yet exists. Developing a fovea in organoids is under investigation in several labs and may help address this challenge. Although increasing our knowledge of RGCs subtypes is crucial for transplantation-based RGC repopulation, an alternative might involve engineering a 'generic' RGC that would be robust and resilient once integrated into the host injured environment. However, the ultimate level of visual function that such an RGC could support remains unclear. Ongoing work in optogenetics and implantable visual prostheses may partially answer this question since current technologies activate RGCs indiscriminately, without regard for their subtype.

Access to primary retinal tissue from human postmortem donors would help validate whether these findings are relevant to patients. According to recent work, primary neurons from human postmortem donors can survive in specially designed culture conditions for up to 6 weeks [168]. Using this tissue, human-derived-MG cultures could be used to screen a large set of transcription factors and focus on the most competent ones for RGC reprogramming before performing more complex experimental tests in vivo. For in vitro screens, relative transcription factor expression levels must be considered. The ability to study human cells in culture could help answer whether gene signaling networks underlying regeneration are evolutionarily conserved. However,

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studying species close to humans, like non-human primates, represents an experimental alternative.

In summary, various models exist for studying RGC development and generating this class of neurons for experimental and therapeutic purposes, each with advantages and limitations. Stem cell-derived RGCs, 3D retinal organoids, retinospheres, brain-retinal assembloids, and dissociated primary cultures are helping investigators to better understand RGC biology and develop cell-based therapies for retinal degeneration. However, further research is necessary to improve these models and ensure their relevance to human vision disorders (Table 1).

SDG #2. Transplantation methods and models

Once exogenously derived RGCs are introduced into a diseased eye, many significant obstacles must be overcome to yield survival and engraftment of large numbers of donor neurons. Lessons learned from the retinal pigment epithelium and photoreceptor transplantation fields inform some approaches to achieving these goals. Animal models remain essential to studying and developing treatments for human diseases (Fig. 2). These preclinical models must recapitulate human anatomy, physiology, and genetics to the greatest extent possible as they relate to optic neuropathy pathophysiology and cell transplantation methodology. Each model has unique advantages and disadvantages for answering experimental questions in a clinically translatable manner, and some models are better suited than others for studying aspects of RGC transplantation. Rigorous efforts utilizing multiple independent disease models and species have the highest potential for avoiding disappointing results in human clinical trials.

Preclinical animal models

Given that the recipient microenvironment influences the survival and behavior of transplanted neurons, consideration of specific optic neuropathy models for evaluating RGC transplantation is critical. Many distinct inherited, traumatic, excitotoxic, and ocular hypertension-induced RGC loss and damage models have been developed [169, 170]. RGC cytotoxicity induced by NMDA, glutamate, or kainic acid is rapid, severe, and exhibits low variability, but their clinical relevance is limited by the mechanism of action and concomitant loss of inner retinal neurons beyond RGCs [171, 172]. Traumatic injury to the optic nerve, including transection, crush, and ocular blast injury, is more clinically relevant but leads to profound and sometimes variable RGC loss.

Optic neuritis and ischemic optic neuropathy models represent other clinically relevant strategies to cause the death of RGCs [173–175]. Optic neuritis occurs in the murine model of multiple sclerosis, wherein myelin oligodendrocyte glycoprotein is administered to induce experimental autoimmune encephalomyelitis [176]. Ischemic optic neuropathy can be induced in rabbits using a sustained endothelin-1 release via minipump [177]. Alternatively, selective capillary occlusion at the optic nerve head can be accomplished in both rodents and non-human primates by the intravenous injection of a photosensitive dye (e.g., Rose Bengal) in conjunction with low-intensity laser light to generate dye-induced superoxide radicals that circulate within the optic nerve capillaries [175, 178].

Ocular hypertension-induced optic neuropathy is most relevant to human glaucoma. This form of optic neuropathy can be achieved in multiple species through inherited or virally transduced gene mutations (e.g.,

Table 1 Future directions for RGC development and differentiation (SDG1)

| Research Area	Future Goals
RGC subtype identification	Identify the human RGC subtypes most affected by diseases like glaucoma and study the underlying mechanisms of their vulnerability. Prioritize research into diseases that might give insights into RGC subtypes most relevant from a therapeutic standpoint
RGC differentiation protocols	Determine how bypass of developmental differentiation stages with directed induction of RGC specification may influence or enable subtype specification
Subtype-specific gene expression profiles	Use cutting-edge transcriptomic technologies to identify subtype-specific gene expression profiles across species and manipulate relevant signaling pathways to better understand RGC subtype specification in vitro and in vivo
Subtype-specific transplantation protocols	Develop protocols for RGC subtype-specific differentiation to advance the study of RGC subtype biology and pathophysiology, and to enable subtype specific transplantation protocols
Fovea in organoids	Develop a methodology for generating foveated human retinal organoids, which would be scientifically valuable and may also provide an efficient way to generate midget RGCs, which could support high acuity vision if transplanted
Glial reprogramming	Use human stem cells or postmortem tissue to derive MG for screening a large set of transcription factors for RGC reprogramming, followed by further evaluation in human retinal organoids and/or nonhuman primates

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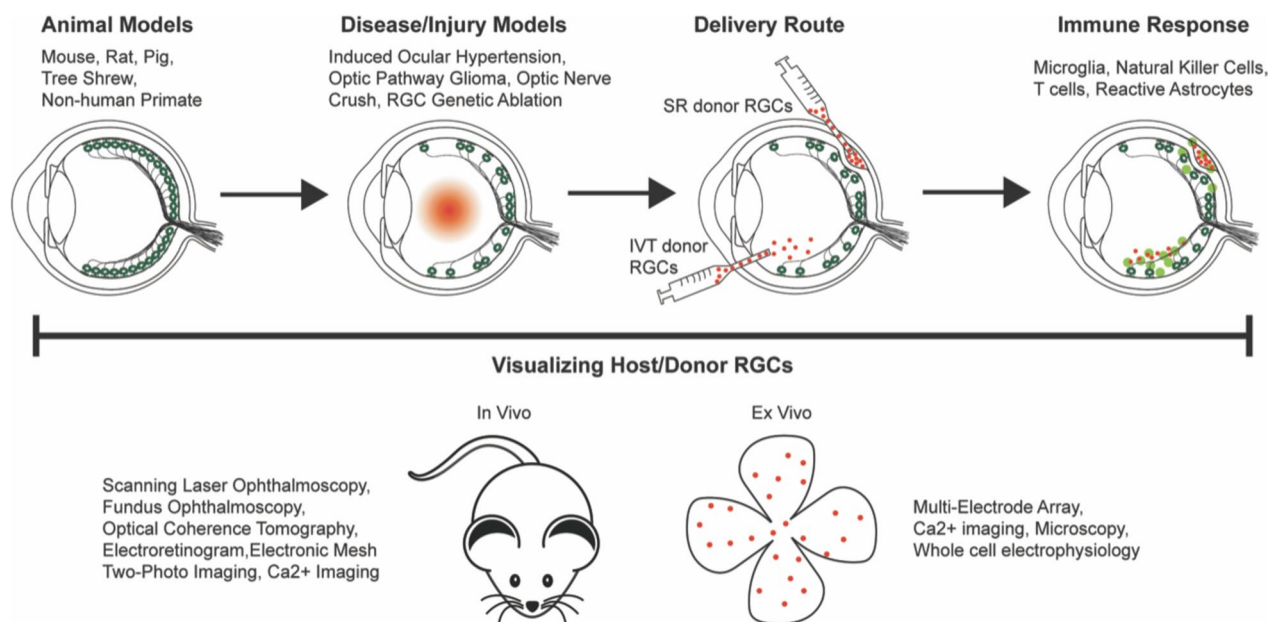
Fig. 2 RGC transplantation models, methods, and assessment. Each animal and disease/injury model possesses advantages and disadvantages for studying essential aspects of RGC replacement and mimicking different characteristics of optic neuropathies. Donor RGCs can be delivered to the intravitreal (IVT) or subretinal (SR) space, but each route has unique barriers to overcome to achieve structural integration. In addition to integrating within the host retina, donor RGCs must avoid being targeted by the adaptive and innate immune systems. Visualizing donor and host RGCs is essential to translate cell replacement therapies to the clinic, and a combination of techniques is required to properly assess the structural and functional integration of the transplanted cells

DBA/2 J, Myocilin, Angiopoietin, TGFβ, etc.) [179, 180], intracameral microbead or silicon oil injection, laser photocoagulation of the trabecular meshwork, and episcleral vein cauterization or sclerosis via hypertonic saline injection [181–183]. Importantly, more than 30% of glaucoma patients experience progressive optic neuropathy despite intraocular pressure (IOP) in the normal range (≤ 20 mmHg). Corresponding models of "normal tension" glaucoma have been developed [184, 185], such as through mutation of the *Optn* gene, which encodes an adaptive protein involved in vesicle trafficking and autophagy [186]. These models differ in the timing and extent of IOP elevation and the degree of endogenous RGC loss, enabling evaluation of neuronal transplantation in various glaucoma-like pathological contexts.

Rather than inducing active neurodegeneration, mouse strains exhibiting developmental defects in RGC specification also offer an opportunity to evaluate the influence of endogenous RGCs on donor engraftment. Knockout of *Brn3b (Pou4f2)*, *Brn3a (Pou4f1)*, and *Math5 (Atoh7)* leads to a developmental depletion of RGCs [187, 188]. These models provide an opportunity to test the hypothesis that endogenous RGCs impact donor RGC integration without the confounding effects of an actively neurodegenerative retinal environment, which is inherently accompanied by chronic neuroinflammation and may be a hostile environment for donor cells.

Additionally, murine models of optic glioma provide opportunities to study chronic axonal injury, RGC degeneration, and vision loss. Leveraging conditional transgenesis approaches, *Neurofibromatosis type 1* (*Nf1*)-mutant mice have been genetically engineered to develop optic nerve gliomas, which subsequently result in RGC death and impaired performance on virtual optokinetic system testing [189, 190], mimicking the clinical progression of optic pathway gliomas (OPGs) and associated vision loss in NF1 [191]. Understanding the cell-intrinsic [192] and cell-extrinsic [193] molecular and cellular events that culminate in RGC loss may yield new insights into therapeutic approaches for this and other chronic optic neuropathies [194].

With their genetic and anatomical similarities to humans, zebrafish are another valuable model for studying vision loss [195] and various therapeutic interventions [196]. Specific zebrafish mutants, such as brass and bugeye, exhibit chronic elevated IOP, resulting in symptoms akin to glaucoma, including retinal stretching, RGC loss, and progressive optic nerve damage [197]. Detailed investigations into these models have promoted ou understanding of the genetic underpinnings of glaucoma, such as the involvement of the low-density lipoprotein

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receptor-related protein 2 gene in the bugeye mutant [197, 198]. Understanding the behavior of RGCs under elevated IOP conditions and the mechanisms of RGC death in these models can provide insights into therapeutic strategies to prevent RGC loss or promote their recovery. Additionally, due to the transparency of zebrafish embryos, the process of RGC development, death, and replacement can be directly visualized, offering an opportunity to assess multiple aspects of RGC biology in vivo, including function and subtypes [80].

Each model and species present advantages and disadvantages for studying various aspects of RGC replacement, including cellular derivation, formulation, and dose; surgical methodologies; immunogenicity and tumorigenesis; and neural circuit integration. Ex vivo tissue culture models and retinal organoids enable high throughput experiments with significant microenvironmental control at the expense of enabling long-term studies or incorporating the peripheral immune or vascular systems. Nonetheless, postmortem human retinal tissue and human retinal organoids enable studies of donor RGC integration into clinically relevant host tissue.

Mice are advantageous as transplant recipients because they are broadly accessible, inexpensive, and have been the subject of many inducible and developmental optic neuropathy models [199]. However, several fundamental differences between human and murine ocular anatomy represent drawbacks to their translatability. Rodent retinas do not contain a macula [200], and their RGC subtypes and inner retinal wiring patterns differ substantially from primates, including humans [8, 82]. Furthermore, rodents possess a glial, rather than collagenous, lamina cribrosa, which is thought to be a critically important site for glaucomatous injury of RGCs in human and non-human primate eyes. As donor RGCs will need to extend axons through the optic nerve head to reach subcortical visual targets, it may be necessary to study axonal pathfinding and efferent synaptogenesis in species with a collagenous lamina cribrosa. While remodeling of the lamina cribrosa is a hallmark of glaucoma in humans [201], it is unknown if there are differences in the ability of RGC axons to regenerate through a collagenous vs. a non-collagenous structure before or after remodeling. It may also be important to understand differential effects on axonal regeneration and pathfinding of varying stages of connective tissue remodeling and reactive gliosis within the optic nerve head, just as the microenvironment of the retina will be important for survival and integration of replacement RGCs.

Ocular size is another vital consideration for cell replacement experimentation and eventual translation to human eyes. Though cell suspensions can be injected into the vitreous cavity of rodents, larger animal models are amenable to clinically relevant techniques such as pars plana vitrectomy, ILM peeling, and intravitreal (IVT) implantation of semi-rigid scaffolds.

Cell replacement in non-human primates may be the best predictor of vision restoration in humans and represent the best model to investigate translatable cell delivery strategies, but they are often cost- and resource-prohibitive at the early stages of research. The tree shrew is a para-primate and an alternative animal model with distinct advantages over the mouse, non-human primate, and ex vivo models [202]. Tree shrews have been bred in captivity, have a collagenous lamina cribrosa, exhibit excellent ocular optics enabling high-resolution noninvasive ophthalmoscopy, and have been subject to well-characterized models of optic neuropathies [203]. However, tree shrews have complex colony management requirements, can be challenging to work with, given their temperament and small eyes, and their collagenous lamina cribrosa is structured differently than in primates, including humans. Further, molecular and biochemical reagents to study tree shrews have not been well characterized.

In addition to non-human primates and tree shrews, domestic animals such as cats, dogs, and pigs exhibit anatomical and physiologic ocular parameters relevant to human glaucoma and other optic neuropathies and RGC replacement therapies. For example, both beagles and cats can be affected by glaucoma spontaneously in some lines [204, 205]. Experimentally induced optic neuropathy models in cats closely recapitulate the optic nerve pathology of human glaucoma [206–208]. Furthermore, pigs have similar eye anatomy, vasculature, and photoreceptor distribution to humans [209], and RGCs in the pig retina have been well described [210–212]. Moreover, there are several well-characterized models of experimental glaucoma in pigs [117, 160] that exhibit changes in the retinal microvasculature [213] and trabecular meshwork that are similar to humans [214, 215].

Besides differences in ocular anatomy between models, differences in the retinal microenvironment may be relevant to donor RGC growth, maturation, axon extension, and metabolic demands. Each model used to study cell replacement therapies will provide insights into controlling the retinal microenvironment in optic neuropathies to support successful donor RGC grafting. Studying various animal species, specifically non-human primates, can provide important information on immune compatibility and potential rejection issues, which must be addressed before conducting human trials. Due to their genetic versatility, rodents offer fundamental insights into basic mechanisms. Pigs are valuable for disease models due to their similar ocular anatomy and larger eye size, making it easier to perform surgical techniques and transplantation

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methods. However, larger animals are more difficult and expensive to house.

To establish safety, the first patients to be enrolled in RGC transplantation trials will likely be those with advanced optic neuropathy and severe to complete vision loss. However, subsequent efficacy studies must consider and target the patient populations most likely to attain functional improvement, and preclinical experimental work would benefit from studying animal models of analogous diseases. To this end, there is a rationale to conduct early RGC replacement clinical trials in patients with an orphan disease, such as NF1-OPG [216]. In this case, younger patients with pathology primarily localized posterior to the optic nerve head may provide a more favorable environment for donor RGC integration into the retina and could exhibit enhanced neural plasticity. While models of NF1-OPG are highly variable in their presentation, improving upon the current models by varying the cell of origin [217] or genetic mutation [218, 219], as well as developing NF1-OPG models in different species, like pigs [220], to study RGC replacement may enable these therapies to enter the clinic with fewer regulatory hurdles if given an orphan disease designation. Alternatively, there are many phenotypes of glaucoma, and patients with greater intrinsic RGC susceptibility (such as those harboring the OPTN-E50K mutation) might be particularly favorable subjects for early RGC replacement therapies based on the hypothesis that cell-extrinsic pathogenic drivers may be less active in such patients and wild type RGCs transplanted into this environment may exhibit greater survival.

Lastly, acute ischemic optic neuropathies may represent favorable substrates for RGC replacement since the insult is typically acute and often occurs in relatively younger patients. In zebrafish, signaling between retinal glia and the endothelial cells that comprise the blood vessels in the retina is necessary for retinal regeneration [221]. Therefore, perhaps patients with significant neurodegeneration yet good blood flow, slow or remote (rather acute and active) neurodegeneration, low levels of chronic inflammation, and well-controlled IOP at the time of transplant will yield the greatest graft success. Standardizing the "ideal" optic neuropathy patient to mirror in an experimental model is of great significance going forward.

In addition to selecting the "ideal" experimental model to study RGC repopulation, it is essential to investigate the long-term effects of various interventions before they can be used in patients. While some studies suggest that donor RGCs survive for > 1 year in rodents [54], most transplantation studies have focused on shorter time points (up to 3 months) [222]. More studies need to be designed to include later-stage time points after treatment. These studies may be included as a part of the principal research or as flow-up reports to better facilitate the rapid dissemination of promising results.

Graft specifications

Previous studies show that the survival rate of intraocular primary RGC transplants is around 1% [10, 223]. Although the survival rate is relatively low, transplanting more neurons may not necessarily increase donor cell survival. Indeed, more donor RGCs survived in one study when 40,000 cells were injected intravitreally in rats compared to 60,000 cells [10]. There are several possible reasons for this outcome. First, transplanting a greater number of cells into the volume-limited rodent eye requires increasing the cell density of the payload, which may lead to cell aggregation and can increase mechanical shear stress on the neurons as they pass through the needle and/or result in clumping rather than dispersion on the host retina. Second, the metabolic burden from donor cells may worsen the existing metabolic deficiency in pathologic conditions. Transplanting too many donor cells may further deprive the host retina of nutrients, harming the donor cells and remaining host RGCs [224]. Finally, more donor cells may induce a greater local and systemic immune response. Therefore, further investigation is required to determine the optimal number of donor cells that should be transplanted across the range of model species.

Most prior studies transplanting RGCs have injected cells purified using RGC-specific markers [222]. However, there is precedent for non-RGC cells contained within a graft as beneficial to support the health and integration of the target RGC population [225]. Depending on the various differentiation protocols or development stages, it might be advantageous to transplant the donor cells as a mixture of RGCs and other cells, such as retinal microglia, astrocytes, MG, and/or retinal progenitor cells and semi-differentiated retinal ganglion neuroblasts, rather than pure RGC populations. RGC-microglia and RGC-astrocyte cocultures have led to more robust functional responses and complex morphology in RGCs [226, 227]. Hence, whether it is necessary or optimal to purify the donor cells before transplantation remains to be determined. Furthermore, donor cells must have limited proliferation ability to obviate the risk of tumorigenesis regardless of the preparation protocol.

Transplantation timing

Cell replacement therapy requires careful consideration of the timing, both in terms of the recipient's disease stage and the developmental stage of donor cells. Some optic neuropathies, such as glaucoma, pose a particular challenge in terms of treatment timing as the disease

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is often chronic and disproportionately affects elderly adults. The aging retina undergoes para-inflammatory dysregulation, and alterations in microglial phenotype contribute to neurodegeneration [228]. Furthermore, aging-related oxidative stress accumulation promotes neuroinflammatory dysregulation of the nervous system, possibly contributing to neurodegeneration [228, 229]. An inflammatory microenvironment might be necessary for regeneration [230], but it also might negatively affect the survival and integration of donor neurons.

Moreover, RGC death is heterogeneous in glaucoma. Advanced glaucoma is associated with reactive gliosis, scarring, and biomechanical remodeling of the optic nerve head, possibly hindering engraftment. Therefore, it appears critical to rigorously explore the influence of recipient age and disease stage in clinically relevant animal models of optic neuropathy on the efficacy of RGC transplantation. Most rodent models commonly used in RGC transplantation studies are 2–6 months old [231], roughly equivalent to a 20- to 30-year-old human. In mice, the incorporation of donor brain progenitor cells seems to decrease as host age increases [232]. In addition, neural progenitor cell transplantation in the Brazilian opossum retina demonstrates more extensive survival and morphological integration in the developing retina (postnatal 15 and younger) than in older retinas (postnatal 35 and older) [233]. Interestingly, a study of photoreceptor transplantation showed greater cell integration in older mice [234]. However, older mice with retinal degeneration exhibit greater photoreceptor loss, which confounds the association with age alone. Thus, it remains to be determined whether greater photoreceptor integration is due to age or degenerative state, and it is critical to explore this association for RGC replacement in aged animals.

Many human patients blind from optic neuropathy seen as potential RGC repopulation candidates are elderly. Moreover, the first-in-human RGC transplantation procedures will likely be in individuals with late-stage vision loss and no light perception. Consequently, exploring the anatomical and physiological changes in models with severe optic neuropathy and how later disease stages relate to engraftment will be necessary.

Some models, like optic nerve crush, induce rapid and robust RGC death, while others, such as microbead-occlusion and silicon oil-occlusion models, cause sub-acute RGC injury, ultimately resulting in the loss of only a minority of RGCs. However, none of these models fully mimic the natural disease progression or fully recapitulate the role of immune reaction and gliosis in human pathology. A combination of multiple disease models is likely required to comprehensively understand how transplanted RGCs respond to the heterogeneity of human disease.

In preclinical models, optimizing the timeline is crucial for successful engraftment. As optic neuropathy advances, the retina may become less receptive to engraftment due to reactive gliosis, scarring, and optic nerve head changes. While achieving RGC engraftment in the early stages of optic neuropathy may be more practical, studying engraftment in end-stage disease on aged animals is vital for translational purposes. For instance, treatments could significantly differ in terms of efficacy and off-target effects as a function of recipient age. Moreover, topographically heterogenous RGC loss occurs in many optic neuropathies and requires validating that attempts to repopulate new RGCs do not disrupt the survival or function of surviving, endogenous RGCs. Understanding the role that host cells and environmental changes play in engraftment will enable RGC transplantation optimization, thereby enhancing its efficacy in both experimental preclinical models and clinical translation to human patients.

Transplant location and technique

Donor neurons must be transplanted in a manner that facilitates migration into the retina, elaboration of dendrites within the inner plexiform layer, formation of synapses within specific retinal circuits, and axonal growth through the optic nerve before visual function can be restored. Delivery location is a fundamental consideration for RGC transplantation that will influence the feasibility of meeting these challenges.

The subretinal (SR) delivery route is the standard for photoreceptor transplantation, as it provides direct access to the outer retina and sequesters donor neurons near the retina in a metabolically supported space between the photoreceptors and retinal pigmented epithelium [235]. However, while SR transplantation provides reliable donor-host contact, this procedure results in retinal detachment (at least locally) and causes secondary injury to the native photoreceptors. While this may be acceptable for treating outer retinal degeneration, it is challenging to rationalize induction of photoreceptor injury for treating optic neuropathies wherein the outer retinal is otherwise largely intact. The larger the volume that is injected into the SR space, the greater the extent of photoreceptors that are damaged, which limits the volume and number of donor RGCs that can be delivered via this route. Regardless, donor RGCs transplanted in the SR space must migrate through the host retina to arrive at the ganglion cell layer (GCL), which may decrease integration efficiency. For SR delivery, the RGC payload would also be sequestered topographically to the extent of the SR bleb, necessitating lateral migration if RGCs are

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to be replaced more peripherally within the retina. Methods to address this challenge are under development [236]. Specifically, by establishing an exogenous stromal cell-derived factor-1 gradient within the retina through IVT delivery of the purified protein, donor RGCs can be stimulated to migrate into the inner retina in vivo following SR delivery [236]. However, the efficiency of trans-retinal migration and potentially deleterious effects on host retinal circuits of that intraretinal migration remain unclear.

IVT or epiretinal transplantation can circumvent these obstacles and provide the shortest route for donor cells to migrate and integrate into their ultimate anatomical location [222]. However, donor RGCs may require more direct metabolic support after delivery into the vitreous cavity because of the relative extent of hypoxia in that compartment. Moreover, they must navigate through the highly viscous vitreous known to limit nanoparticle displacement [237], potentially hindering donor RGC migration into the retina. Although the vitreous cavity is a relatively immune-privileged site, it is less immune-privileged than the sub-retinal space, and peripheral immune cell infiltration can and does occur following intraocular surgery [238, 239].

Intravitreally transplanted cells also face the obstacle of the ILM, a basement membrane composed of extracellular matrix proteins such as laminin and collagen IV. The ILM is a physical barrier to transplanted stem cells, viral vectors, therapeutic compounds, and nanoparticles [240, 241]. By impeding donor cell migration, the ILM can block RGCs from engraftment. Indeed, developmental ILM dysgenesis and enzymatic digestion of the ILM are associated with increased donor RGC survival, retinal localization, and dendrite lamination within the inner plexiform layer (IPL) [242, 243]. Though enzymatic ILM degradation has been successful in rodents, there may be a better approach for clinical translation since intravitreal enzymes can elicit an inflammatory response and may cause off-target retinal toxicity. Fortunately, the ILM is routinely surgically peeled in human patients to treat macular holes and vitreomacular traction, providing a translatable approach to enhancing RGC engraftment. Indeed, surgical ILM removal in large animal models is feasible [244]. As an alternative to *en bloc* ILM removal, laser photoporation is being developed to create focal, precisely patterned ILM defects in a manner that obviates underlying neuroretinal damage and elicits minimal immune response [245].

Despite ILM disruption or removal potentially increasing RGC integration following IVT transplantation, IVT injection of a dissociated cell suspension has only been successful in rodent eyes with proportionately larger lenses and smaller vitreous cavities. The narrow vitreous cavity facilitates sufficient spread and contact for donor cells to the host retina. In larger animal models with ocular anatomy more similar to humans, the injected cells are more likely to be trapped in the vitreous far from the retina and aggregate [246]. Thus, in larger animal models, transplantation into the inner retina may benefit from using biocompatible cellular scaffolds, which can sequester the donor neurons near the target engraftment site [247]. Epiretinal transplantation of RGCs on a biocompatible scaffold may also be beneficial to abrogate the mechanical stresses imposed by intravitreally injecting a dissociated cell suspension. Several types of scaffolds have been developed, such as natural tissue scaffolds, alloplastic synthetic scaffolds, and biodegradable electrospun scaffolds for donor RGC transplantation [246–250]. In general, a scaffold should act as a supportive microenvironment to improve the survival of RGCs, direct the growth of axons radially and towards the optic nerve head, be optically transparent, and cause no impediment to the functional electrophysiological properties of transplanted cells. The delivery of a scaffold will likely require a vitrectomy and the development of new surgical methods, including techniques for fixating the scaffold in place.

Lastly, sub-ILM injection has been developed as an attractive alternative to viral vector delivery that bypasses the ILM [251]. While sub-ILM delivery would allow donor RGCs to be sequestered within microns of their final positions in a nutrient-rich tissue, like SR injection, it would require extensive lateral migration of RGCs to subserve the peripheral retina. Moreover, this method has yet to be successful for cell delivery despite numerous attempts in large animals, and it is incompatible with small rodents.

Barriers to graft survival and integration

The success of donor RGC transplantation in the retina is challenging due to several additional factors, including the inherent stress that donor cells experience during preparation and transplantation. RGCs, like most neurons, are inherently fragile cells, and a significant source of stress includes suboptimal nutrient support, oxidative stress, and pH changes in their culture media during short-term storage while waiting for the transplantation procedure. To dissociate cells for transplantation, they must be removed from their extracellular matrix, which results in anoikis [252], leading to decreased donor cell viability. The process of dissociation can also lead to membrane rupture, as neurites are forcibly broken from the cell soma [253]. Additionally, donor cells experience shear stress during transplantation due to the physical forces involved in injecting them into the vitreous cavity. Moreover, donor cells must also face a vitreous cavity

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microenvironment relatively devoid of oxygenation and metabolic support once transplanted until they can migrate into the retina. This lack of support can further increase donor cell stress and decrease survival rates.

One potential solution to address these issues is to embed donor cells in hydrogels, which can provide mechanical support and buffer metabolic stress during transplantation. Hydrogels are water-based materials that mimic the retina's extracellular matrix and provide an environment conducive to cell survival and migration. Several studies have demonstrated the benefits of hydrogels in enhancing donor cell survival and integration in the retina [248, 249, 254]. Utilizing other 3D culture-based strategies, such as microtopographic or Matrigel scaffolds, may help to improve donor neuron viability [255, 256]. Another potential strategy to improve donor cell survival is hypoxic preconditioning which can enhance the survival and integration of donor cells in multiple tissues and improve their functional outcomes [257]. Hypoxic preconditioning involves subjecting donor cells to a low-oxygen environment before transplantation, increasing their resistance to stress and improving their survival rates in the retina.

Glia and immune system responses

The pathogenesis of optic neuropathies often involves the failure to resolve inflammation, either as a primary (e.g., optic neuritis) or secondary feature. Understanding the inflammatory microenvironment of animal optic neuropathy models and how they relate to the immunological responses of human patients is of great importance. Neuroinflammation is driven primarily by the inflammatory reactions of macroglia (e.g., astrocytes and MG) and immune cells (e.g., resident microglia and macrophages) and results in complex alterations in gene expression, morphology, and function in those cells. While numerous heterogeneous populations of 'reactive' astrocytes [258, 259] and microglia [260, 261] are defined at the transcriptomic level, very few are further defined at the functional level. One example that highlights interactions between immune cells and astrocytes, which, once activated, through tumor necrosis factor (TNF), interleukin 1 alpha (IL-1α), and complement component 1q (C1q) signals from myeloid cells, can induce an inflammatory and neurotoxic response in the CNS [262–265]. Genetic deletion of *Tnf*, *Il1a*, and *C1qa* prevents induction of this reactive astrocytic sub-state and ameliorates RGC death in both acute optic nerve crush [264, 266] and chronic glaucoma models [222, 267]. Importantly, blocking these neurotoxic functions preserves RGC numbers and maintains some electrophysiological functions [266, 268]. An update to this model highlights that *Fatty Acid Elongase 1* can be genetically deleted only from astrocytes to preserve RGC numbers while maintaining global reactivity intact [269] – suggesting reactivity itself is not 'bad' per se but that specific reactive astrocyte functions can be detrimental to RGC health. However, astrocyte responses to neurodegeneration may also be protective, as evidenced by their ability to promote neuronal survival and repair by upregulating neurotrophic factor production in murine glaucoma [264] or by providing a scaffold for axon regeneration following spinal cord injury [270].

Although different glia may share immunological functions, there are instances where cell-specific interactions are particularly important. Deciphering the specific microglial and macrophage interactions activated at various stages of the disease is vital when interpreting RGC transplant outcomes in models of optic neuropathy [271]. The formation of reactive astrocytes and microglia is a response to multiple inflammatory signalss, including the TNF, nuclear factor kappa-light-chain-enhancer of activated B cells, tenascin-C, and Toll-like receptor signaling pathways [272]. Despite early adaptive and protective features of astrocyte and microglia responses, such as the modulation of the microenvironment of individual neurons, maintenance of tissue integrity, immunomodulation, ion homeostasis, and uptake of neurotransmitters [273–275], prolonged reactivity of resident glia can also create a toxic microenvironment to RGCs and their axons [276–278]. A contributor to these interactions is the profusion of anti- and pro-inflammatory cytokines, reactive oxygen species, and toxic lipids, which have also been reported in clinical and experimental glaucoma models [279, 280]. The corresponding responses of astrocytes and microglia to glaucoma-related stress or neuron injury are critical in propagating neuroinflammation and neuroprotection; however, these interactions differ depending on the disease stage and genetic predisposition of individuals, culminating in varying chronic neurodegenerative outcomes. Controlling these neuroinflammatory mediators that drive endogenous RGC loss will be essential for cell replacement therapies because they could put repopulated neurons at risk.

Adaptive immunogenicity of donor RGCs may be a critical characteristic that threatens their survival following transplantation, particularly in clinical applications. When the immune system identifies non-autologous cells, alloreactive cytotoxic CD8^{+} T cells are activated through binding to the major histocompatibility complex class I and T cell receptors, especially in the presence of non-matching major histocompatibility complex (human leukocyte antigen (HLA) in humans) [281, 282], which leads to graft rejection. Transplantation efforts in experimental settings will require developing immunosuppression techniques that target these pathways in RGC transplantation efforts. As immune responses are

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complicated and event-dependent, traditional immuno-suppression is often insufficient; therefore, genetic editing of HLA genes in induced pluripotent stem cells has been proposed to minimize T-cell and natural killer cell activity [283, 284]. An alternative to genetic editing is to use stem cells derived from a patient's blood, skin, or urine samples (i.e., iPSCs) for autologous transplantation. Autologous transplantation of iPSC-derived neurons has already been shown to elicit a minimal immune response in the brain [285]. However, an advantage of allogeneic treatments is that many doses can be manufactured simultaneously from a single batch of iPSCs. In contrast, obtaining and validating iPSCs or banked HLA-matched donor cells for each patient would be expensive, time-consuming, burdensome from a regulatory perspective, and challenging to produce at scale [286, 287].

Despite efforts to limit the immune response to donor RGC delivery, it is not possible to put a needle into the eye without causing some inflammation. Therefore, an anti-inflammatory regime will likely be required, irrespective of the RGC repopulation strategy. Nevertheless, inflammation may also promote axonal regrowth [230, 288]. Inflammation stimulates the recruitment of macrophages into the retina to express pro-regenerative secretion factors and promote axon regrowth via paracrine signaling [230]. Consequently, while inflammation may negatively affect donor RGC grafting, macrophage-centered strategies may also be a promising approach to promote donor RGC regeneration.

Visualizing and evaluating donor cells in the host retina

The success of cell replacement therapy relies on both structural and functional integration of the transplanted cells. Relying solely on endpoint assessment after enucleating the eye as a primary criterion to quantify transplantation outcomes will undoubtedly increase the resources needed to develop cell replacement therapies. Furthermore, endpoint evaluation is incompatible with human trials, so implementing non-invasive strategies to assess donor cell integration longitudinally in vivo is necessary. Early development of innovative functional and morphological readouts will allow investigators to avoid dead ends and improve the potential for successful translation into the clinic. It is also crucial to distinguish between host and donor cells to understand mechanisms of action, such as trophic support, material transfer, or *bona fide* cell replacement – this assessment can only be performed at the single-cell level.

The eye provides an advantageous setting to combine cell transplantation, molecular sensors [289, 290], electrophysiology [291–293], and advanced in vivo imaging techniques [290, 294–296] for the evaluation of functional engraftment. The intraretinal position of donor cells in the x-, y-, and z-axis needs to be defined to study donor cell distribution and structural integration following transplantation. In vivo imaging techniques, such as scanning laser ophthalmoscopy (SLO) and fundus ophthalmoscopy, may resolve the topographic positions of donor cells, provided there is sufficient contrast between donor and host cells [297, 298]. In preclinical studies, specificity has been achieved by genetically modifying the donor cells to express fluorescent reporter proteins. These proteins can be imaged with customized fundus cameras or at the cellular scale with SLO to evaluate neurites extending from donor cells [298].

Newer techniques, such as 3D optical coherence tomography (OCT), two-photon microscopy, and SLO with adaptive optics (AO), can provide depth resolution in the z-axis [299]. Volumetric information is difficult to obtain from fluorescence imaging in patients and non-human primates due to the low numerical aperture of the primate eye. Conversely, in rodents, the higher numerical aperture makes some depth discrimination possible [300], but superior depth resolution is provided by OCT, albeit at the expense of lateral resolution. Generating contrast between donor cells and the host environment in OCT is more challenging. To improve this contrast, it may be possible to genetically alter the transplanted cells to overproduce lipids or self-generate air bubbles, or to load cells with lipid-encapsulated gold nanorods or nanowires [301–304]. Pairing adaptive optics with OCT has recently opened the door to both exquisite lateral and axial resolution in normal retinas, with the contrast arising from natural organelle motility inside cells. With any contrast-enhancing approach, special care and consideration must be given to determine the possibility of material transfer that would result in the mischaracterization of host and donor cells [32] and to ensure the risk of inflammation or foreign body response is minimized [305, 306].

Despite potential mistargeted viral-based expression in RGC transplantation [32, 307], to date, no evidence for neuronal mislabeling via intercellular material transfer in RGC transplantation has been identified. However, material transfer has occurred between donor cell reporters and host cells, resulting in the misidentification of cell origin in previous photoreceptor transplantation studies [32, 308]. Material transfer can potentially occur in any cell transplantation study, including RGC transplantation. As a result, researchers must accurately characterize and label their cells to ensure clarity and interpretation of their experimental results [32]. Implementing rigorous quality control measures throughout the transplantation process is critical to ensure that the transplanted cells are accurately labeled and that any mislabeling due to intercellular material transfer is minimized. Additionally,

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investigators should validate their experimental results using multiple complementary methods to confirm the accuracy of their cell origin determination.

Functional integration may be assessed using a combination of in vivo and ex vivo electrophysiology techniques, such as ERGs, whole mount explants on multi-electrode arrays [309, 310], implanted electronic meshes [291], and the use of genetically encoded calcium indicators in combination with advanced imaging techniques [311]. As a non-invasive measurement of retinal electrophysiology, ERG is currently unable to resolve single-cell activity nor distinguish between host and donor RGCs [312]. On the other hand, high-density multi-electrode arrays can quantify single-cell activity, but assigning individual electrodes to specific cells can be challenging, and recording from the retina is limited to a single endpoint [309, 310]. Unlike MEAs, implanted electronic meshes can record from single neurons chronically in the retina of live mice, but such systems have yet to be used to study transplanted RGCs and are limited in scale and number of recording electrodes [291]. Genetically encoded calcium indicators have shown the most utility in cell transplantation experiments and provide scale to study hundreds to thousands of single donor and host RGCs simultaneously [311, 313]. Still, their use has received mixed acceptance. These approaches require expensive and highly customized imaging systems because visible light stimulates photoreceptors and confounds the ability to image the fluorescent signal from the calcium indicators. Consequently, there is a demand for label-free approaches to measure donor RGC function in the retina. A promising label-free strategy to image RGC function in humans has recently been developed based on full-field swept-source OCT technology, but this approach cannot yet provide cellular scale resolution [314].

Irrespective of the approach, the signal-to-noise ratio remains a significant hurdle for parsing the functional integration of donor cells from endogenous neural activity. Implementing a neuronal activity control mechanism, such as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) or optogenetic techniques that allow researchers to control the activity of specific neurons using small molecules and light, respectively, may be useful for distinguishing between donor and host RGCs [8, 315, 316].

Many of the same parameters and instruments used to study the retina in animals (especially large animals) may be used in human trials [317]. However, unlike preclinical research, where transgenic expression of fluorescent or other proteins can facilitate graft visualization, engineering of cell products specifically for optical monitoring is unlikely to be advisable in clinical trials. Recent developments in adaptive optics OCT have enabled label-free visualization of single ganglion cell somas and single nerve fiber bundles [294], and so this technology will likely constitute the primary structural outcome in human patients. While the availability of AO-OCT instruments is currently limited to a few specialized labs, ultimately, this technology should be applied to preclinical RGC transplantation experiments, ideally in conjunction with the detection of fluorescent reporter tags in SLO or AO-SLO to advance our understanding of how to interpret these label-free techniques until OCT contrast agents are further developed. Lastly, in addition to studying transplanted RGCs, in vivo imaging techniques may be applied to analyze and better understand the mechanisms of retinal neuron degeneration in glaucoma and other optic neuropathies. This will enable the development of models that better recapitulate human disease and our understanding of how the host retina is affected at the cellular scale by the various intervention strategies currently under development.

Future directions for transplantation methods and models

Transplantation research has made significant strides in recent years, but many areas still require further development. An important future direction for RGC transplantation methods and models involves further developing and characterizing models that accurately mimic the anatomical features of the human eye, such as the macula and the collagenous lamina cribrosa, and that pathophysiologic changes that occur in these structures in disease. Additionally, more emphasis must be placed on creating models for studying neuronal transplantation in specific pathological contexts, such as normal-tension glaucoma, and models that do not induce active neurodegeneration, such as knockout models of *Brn3b* (*Pou4f2*), *Brn3a* (*Pou4f1*), and *Math5* (*Atoh7*). Exploring autoimmune disease models, including multiple sclerosis, may also improve clinical translatability to various pathological targets. Finally, larger animal models, such as primates and pigs, may be better positioned than rodent models to develop clinically relevant transplantation techniques, including pars plana vitrectomy, ILM peeling, and implantation of semi-rigid scaffolds. Therefore, focused efforts in transplanting RGCs in these models will likely yield significant progress in the field.

Ongoing development of preclinical optic neuropathy models should prioritize those that can better mimic the natural progression of human disease and demonstrate the effect of aging on the survival and integration of donor cells in the retina. While it may be easier experimentally to attain RGC engraftment in early disease stages, clinical translation necessitates success in late-stage disease models. Enhancing engraftment

in advanced optic neuropathy may necessitate immunomodulatory approaches to reduce inflammation and scarring. Additionally, extracellular matrix molecules, hydrogels, or biocompatible scaffolds should be rigorously evaluated for their potential to enhance cell survival and integration in various injury models.

In addition to evaluating the optimal source and developmental stage for donor RGCs, additional focus on cell dosage and composition with non-RGC support cells would be valuable. Such studies might investigate the optimal ratios of different cell types on donor RGC engraftment. Graft rejection and immune responses remain significant challenges for successful transplantation. However, this may represent a more formidable obstacle for experimental xenografts than eventual clinical trials of autologous or allogenic cell sources. Nonetheless, it will be essential to examine the immunogenicity of donor RGCs in clinically relevant models and optimize retinal-specific immunosuppression techniques that target alloreactive T cells or engineer donor RGCs that evade immune surveillance.

Ongoing research must implement rigorous quality control measures, including monitoring for material transfer throughout the transplantation process and validation of results using multiple complementary and standardized methods to facilitate accurate characterization and labeling of transplanted cells. Particular attention should also be given so that dead RGCs inside phagocytosing cells are not counted in successful RGC transplantation results. A combination of in vivo and ex vivo electrophysiology techniques can be used to assess functional integration. For these systems, chemogenetic or optogenetic systems can overcome the high signal-to-noise ratio for discriminating functional integration from endogenous neural activity. Lastly, improving image capabilities in larger eyes for both experimental and translational purposes will be essential, with OCT metrics being the primary structural outcome in human patients (Table 2).

SDG #3. RGC survival, maturation, and host interactions

Challenges in achieving engraftment and long-term survival of donor RGCs within the host mammalian retina are at least partly related to the highly organized tissue structure and interactions among the various resident

Table 2 Future directions for transplantation methods and models (SDG2)

Research Area	Future Goals
Anatomically accurate models	Evaluate RGC transplantation in models that accurately mimic the human eye's anatomical features, including the macula and the collagenous lamina cribrosa, to study neuronal transplantation in various pathological contexts
Disease models	Establish models for studying neuronal transplantation in different pathological contexts such as normal aging, normal-tension glaucoma, autoimmune disease, and developmental models that do not induce active neurodegeneration
Larger animal models	Prioritize larger animal models to develop clinically relevant transplantation techniques, including procedures like pars plana vitrectomy, internal limiting membrane (ILM) peeling, and implantation of rigid scaffolds
First-in-human trials	Define an "ideal" optic neuropathy patient suitable for initial clinical trials and establish an experimental model to mirror this clinical phenotype
Transplantation timing	Investigate the effect of disease progression and aging on the survival and integration of donor cells
Overcoming barriers to engraftment	Evaluate use of immunomodulatory agents and extracellular matrix modulators to promote cell survival and integration
Graft specifications	Investigate the effects of different cell doses on graft survival, integration, and functional outcomes. Explore the potential benefits and optimal ratios of transplanting a mixture of RGCs and non-RGC support cells
Immune responses	Explore methods for promoting immunotolerance of transplanted RGCs, such as immunosuppressive drugs, gene editing techniques, or extracellular matrix modulators that may improve cell survival and integration by inhibiting reactive gliosis and immune cell infiltration
Scaffolds	Explore new techniques for delivering donor RGCs to the retina, such as developing improved scaffolds or designing methods that allow for safe and efficient migration of donor cells from the epiretinal surface
Delivery methods	Evaluate and develop alternative cell delivery methods, such as sub-ILM transplantation, which may offer better donor cell survival and integration outcomes
Preconditioning techniques	Investigate diverse preconditioning methods to improve donor cell resistance to hypoxia, para-inflammation dysregulation, and oxidative stress
Quality control and validation	Implement quality control measures throughout the transplantation process and validate results using multiple complementary and standardized methods to facilitate accurate characterization and labeling of transplanted cells, including the possibility of material transfer
Imaging capabilities	Improve imaging for experimental and translational purposes, benchmarked to OCT metrics as the primary structural outcome in human patients

cell populations. Prior work investigating mechanisms of retinal development, neurodegeneration, and neuroprotection provides numerous potential avenues for promoting long-term survival and maturation of transplanted RGCs, enhancing neurite outgrowth, and supporting appropriate donor neuron function [318], which is necessary for transplanted cells to integrate into the existing neural circuitry and contribute to visual signaling.

RGC survival and neuroprotection
Long-term survival of transplanted RGCs is central to sustained visual improvement and is a significant limitation of most published studies. While some human cells may successfully migrate following SR or IVT injection into a non-human primate retina, the survival rate is typically below 1% [319]. Several methods might be used to improve the survival rate of transplanted cells.

RGCs are particularly vulnerable to metabolic insults and rely on mitochondrial oxidative phosphorylation for their high energy demands [320]. Therefore, ensuring adequate metabolic support for newly transplanted cells is critical for survival in the peri-transplant period when most cell death occurs. Anabolic activity and aerobic glycolysis positively correlate with cell survival in neurogenerative contexts [321]. Nicotinamide, the amide form of vitamin B3, has emerged as a potential neuroprotective agent [322, 323]. Nicotinamide aids in producing NAD^+, a crucial coenzyme in mitochondrial respiration and cellular energy production, prevents axon degeneration (Wallerian degeneration), and improves visual function in existing glaucoma patients [324]. Supplementing the graft with nicotinamide may provide a metabolic boost that helps the cells survive the peri-transplant period.

The vitreous cavity, the site of RGC transplantation, is relatively hypoxic [325], undermining oxidative phosphorylation. This environment might further strain the metabolic machinery of the newly transplanted cells, leading to an increased likelihood of cell death. Therefore, strategies promoting metabolic homeostasis in this challenging environment are essential, and hypoxic preconditioning may be beneficial. Another approach for improving donor RGC survival may be to supplement the vitreous cavity with pyruvate [326]. Pyruvate is critical in cellular metabolism, serving as a key intersection point between anaerobic glycolysis and aerobic oxidative phosphorylation. It serves as a substrate to generate adenosine triphosphate in hypoxic conditions and, in combination with nicotinamide, resulted in significant short-term improvement in visual function in glaucoma patients [327]. Pyruvate is normally supplied to neurons by oligodendrocytes [328], so in addition to providing exogenous pyruvate, promoting metabolic coupling with oligodendrocytes may also be essential for the long-term

survival of donor RGCs and should be investigated. Interventions aimed at improving the antioxidant capacity of transplanted cells may also be beneficial. Since oxidative phosphorylation can generate harmful reactive oxygen species, enhancing the ability of the cell to neutralize these compounds might help prevent cell death due to oxidative stress [329]. Efforts made to better understand the metabolic demands of donor RGCs at all stages of repopulation in pathologic environments will, therefore, provide critical insights for improving cell survival after transplantation.

While several neuroprotective strategies have been explored for promoting host RGC survival in optic neuropathy models, few have been studied in the context of RGC transplantation [330]. Neuroprotection is likely essential for repopulation approaches, as the neurodegenerative environment into which new RGCs are to be introduced is likely to challenge graft survival [331]. While slow-release neurotrophic factors can significantly increase the number of grafted donor RGCs in vivo [236], most donor RGCs do not survive transplantation even with neurotrophic support. While developing neuroprotective therapies will be vital for supporting donor RGC survival, clinical translation of these therapies may also limit the need to replace or regenerate RGCs in patients.

There has been considerable interest in directly suppressing pro-apoptotic signaling for RGC neuroprotection, and many of the same interventions may help promote donor RGC survival following transplantation. For example, Caspase 2 siRNA is currently in clinical trials to treat several optic neuropathies [332, 333]. Other avenues for engineering or blocking pro-apoptotic pathways (e.g., with PARP and RIPK inhibitors [334, 335]) could also be relevant. Targeting the genes and pathways central to RGC apoptosis may also help improve donor RGC survival [336]. Importantly, this could be achieved via pharmacological treatment or genetically engineering the donor RGCs.

BCL2-Associated X protein (BAX) is a pro-apoptotic protein critical in cell apoptosis [337]. Despite a loss of axons following an insult, RGCs that lack BAX exhibit enhanced survival in animal models [338]. Knocking out BAX in RGCs disrupts the usual apoptotic pathway, thereby preventing cell death. This approach, however, does not address the underlying disease pathologies and may not address all sources of cellular stress. Specifically, in the case of BAX knockout, RGCs become quiescent, requiring additional strategies to promote endogenous axonal regeneration [338]. Dual leucine zipper kinase (DLK) and leucine zipper kinase (LKZ) are other important mediators of RGC death [336]. Inhibition of these kinases has improved RGC survival in various injury models, including ocular hypertensive glaucoma and

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traumatic optic nerve injury. Pharmacologic inhibitors of DLK and LZK have the potential to slow or even halt the progression of RGC death [339]. However, while known trophic and pro-regenerative pathways may promote RGC survival and neurite outgrowth in the short term, permanent suppression of such pathways could be counterproductive, given their transient role in neuronal development. For example, while DLK is a negative regulator of RGC survival, it is a positive regulator of neurite outgrowth [340]. Therefore, a combination of therapies will likely be required to promote transplanted RGC survival and integration into the existing retinal neurocircuitry.

In addition to reestablishing lost circuits within the retina and the brain, donor RGCs might benefit the host retina by providing a neuroprotective effect to surviving endogenous neurons. For example, a recent study showed that one week after stem cell-derived RGCs were transplanted, significantly more host RGCs survived an optic nerve crush injury [341]. One possible explanation for how transplanted cells can improve host neuron survival is by transferring their extracellular vesicles that contain diverse, multifactorial cargo to the host neurons [342]. Studying these and other mechanisms by which donor RGCs confer protection to host neurons is an important area of focus, as it could signal a secondary benefit of RGC transplantation.

Taken together, the successful transplantation of RGCs into the retina requires careful consideration of several factors. Genetic enhancement of donor RGCs, optimizing of RGC metabolism early after transplantation, identifying neuroprotective therapeutic targets, and investigating the beneficial effects of donor cells on the host retina are all crucial in optimizing the success of RGC transplantation approaches.

RGC maturation
The optimal stage of maturity for transplanted RGCs remains an important unanswered question. Fully matured donor RGCs may have a reduced ability to migrate and integrate into the retina, as seems to be the case for photoreceptors [343]. Most RGCs differentiated in vitro are relatively immature and typically do not undergo subtype specification [344]. In most RGC differentiation protocols, the maturation state of cells in culture is highly heterogeneous. Though this may be advantageous from a plasticity perspective, clearly defining and controlling the stage of maturity before transplantation will be necessary to rigorously assess this variable in transplantation outcomes. For instance, RGCs derived from day 21 mouse retinal organoids survive better than those from day 16 [54]. Thus, balancing plasticity and maturation by identifying the most suitable

developmental stage for donor cells to achieve optimal outcomes is a key goal.

Interactions between donor cells and host organs are essential in guiding their differentiation and maturation [54], and such interactions may only occur following transplantation. For instance, astrocyte-RGC interactions regulate RGC maturation during development [227]. Investigating whether these interactions are also relevant to RGC transplants will be important. The retinal microenvironment may also promote the differentiation and maturation of transplanted retinal organoids [345]. While molecularly immature (RBPMS negative) RGCs survive following transplantation, there is a propensity for donor RGCs integrated into the GCL to preferentially express the mature RGC cell marker, RBPMS [236]. These findings indicate either that molecular cues in or near the GCL drive RGC maturation in vivo or that RBPMS-expressing RGCs are more likely to integrate [236]. However, the specific molecular signals that promote RGC maturation in the GCL remain unclear and may involve interactions between RGCs and neighboring cells or extracellular matrix proteins within the GCL. Further research is needed to identify these cues and determine their role in RGC maturation.

Lastly, in the early stage of retinal development, electrophysiologic activity in retinal neurons and within their postsynaptic targets, achieved through retinal waves, plays a crucial role in cell differentiation, maturation, and circuit development [346, 347]. While it is unclear the extent to which coordinated retinal activity may promote neuronal maturation or circuit development in adult or diseased retinas, methods to promote electrical activity (e.g., using optogenetic strategies or application of exogenous electrical fields) in donor RGCs may be used to investigate this potential.

Host microenvironment preparation before transplant
In the later stages of optic neuropathies, the retina often exhibits neuroinflammation, peripheral immune cell infiltration, and host glial/immune cell reactivity. In glaucoma, these responses are commonly triggered by chronically sustained high IOP and/or the subsequent death of RGCs, which produces a neurotoxic microenvironment that may impair the acceptance of donor cells by the host retina. Consequently, IOP control will be a prerequisite for any RGC repopulation strategy for glaucoma. Even when IOP returns to homeostatic levels, neuroinflammation and CNS glial reactivity persist [348], making it potentially difficult for transplanted donor cells to survive and integrate into the host retina. To improve the success of RGC transplantation, the host microenvironment may need to be "reset," and host immune cells, particularly phagocytic cells [349, 350], may need to be depleted

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or suppressed, at least temporarily. However, it has become clear that not all phagocytosing cells are harmful. Microglia and astrocytes, for example, play essential roles in developmental processes like vascularization, RGC development, and fine-tuning of neuronal circuit connectivity [351–353], as well as maintaining retinal homeostasis, including immune responses, metabolism, neuronal activities, and phagocytosis [354, 355]. Nevertheless, methods to drive a protective phenotype specifically and reliably in both microglia and astrocytes remain in development.

Studies in mice raised without microflora in a germ-free environment do not exhibit significant RGC death following elevated IOP [356]. This finding indicates that the peripheral immune system and the microbiome may play an important role in glaucoma progression and may have a detrimental effect on donor RGC survival [353]. Moreover, the role of the microbiome in the immune and nervous systems in zebrafish has recently been described to affect regeneration [357]. Therefore, it may be informative to perform RGC transplantation in animals raised in a germ-free environment and assess donor RGC survival.

Lastly, preparing a supportive and nourishing host retinal microenvironment may be necessary to support donor RGC transplantation. A combination of factors may be required to establish this environment, including growth factors, anti-inflammatory agents, antioxidants, and other molecules that promote cell survival and integration. Developing a better understanding of the mechanisms involved in glaucoma and the role of the immune system and glial cells in disease progression will be critical for advancing the field of RGC transplantation and ultimately improving the treatment of glaucoma.

Host microenvironment regulation after transplant

Transplant studies have primarily focused on allogeneic or xenogeneic donor cells, which pose an exceptionally high risk of rejection. In some ways, this represents more of an experimental hurdle than a translational obstacle since allogeneic or autologous transplants into humans are likely subject to greater acceptance than xenografts. Nonetheless, immunosuppressive regimens used for preclinical animal studies are highly variable, and optimal immunosuppression approaches for RGC transplantation remain unclear. Transplantation into immunodeficient animals (Nod/SCID mice or athymic nude rats) may enable researchers to circumvent these experimental hurdles at early stages, but eventual studies in large animals will require optimization of immunosuppressive approaches. Moreover, while immunosuppression in patients receiving autologous transplantation may not be necessary to prevent outright graft rejection, in late-stage glaucoma,

immune cells are already highly active in the retina [358], which will necessitate immunotherapy.

Beyond the adaptive immune system, previous studies suggest that donor cell integration in the retina is enhanced when reactive glia responses in the retina are genetically or pharmacologically suppressed [241, 359], indicating a critical role for reactive astrocytes in neural graft integration [360]. Despite this, there remains an unmet need to develop a reliable protocol for regulating astrocyte and microglia reactions after transplantation to achieve longer-term survival and robust integration. Glial-related disease development can vary between sexes in some disease models. For example, retrospective studies suggest that girls with NF1-associated OPGs restricted to the optic nerves are more likely to lose vision and require treatment than their male counterparts [190, 361]. Similarly, in a genetically engineered mouse model of NF1-associated OPG, a sex-specific effect operates at the level of non-neoplastic glia, where the elaboration of neurotoxic molecules in response to estrogen underlies the observed increase in RGC loss, nerve fiber layer thinning, and visual acuity reduction in female mice [190, 193]. Such variation in glial reactivity highlights the importance of individual and disease-specific immunoregulation in post-transplant management and suggests that clinical translation must carefully consider these factors.

One approach to mitigating innate immune responses to transplanted cells involves the masking of externalized phosphatidylcholine on the plasma membrane, which serves as an "eat-me" signal on donor cells, using Annexin V. This approach reduces the recruitment of microglia to the delivery site [362]. However, this pretreatment has limitations, as it does not provide continuous protection to the donor cells. Therefore, a more comprehensive regimen that addresses post-transplant immune regulation may be necessary. This immunosuppressive regimen could involve a combination of strategies, such as blocking multiple signals or using a mixture of immunosuppressive drugs.

Lastly, by exploring single-cell transcriptome data of the developing human retina, various receptor-ligand candidates have been identified to control donor RGCs in vivo [236]. Establishing an exogenous chemokine gradient across the retina improves the structural integration of donor RGCs through guided migration [236]. Consequently, using transcriptomic data from the adult, diseased, and developing human retina could be a powerful approach for identifying targets to engineer the retinal microenvironment and individual RGCs and control various cellular processes (e.g., synapse formation, phagocytosis, axon growth, etc.).

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Future directions for RGC survival, maturation and host interactions

Fortunately, great efforts have been spent identifying the molecular mechanisms underlying RGC death and dysfunction in the context of pathological states, which have yielded numerous neuroprotective approaches to enhancing the survival of endogenous RGCs. The RGC transplantation field is poised to benefit from this rigorous prior research by testing, alone and in combination, many of these pharmacologic, genetic, and microenvironmental interventions, which will hopefully enhance donor RGC survival to a rate needed to achieve functional benefits in optic neuropathy.

Sophisticated 3D retinal tissue culture models have been increasingly important in understanding RGC development and disease pathology. However, further development of these tissue-engineered models to enable longer-term neuronal viability and function and to better mimic the in vivo environment is still necessary. These models might then be used as stand-ins to investigate RGC transplantation without the confounding effects of other systems, such as the immune system. Furthermore, by studying the interactions between RGCs and neighboring cells in these well-defined systems, researchers may be able to identify the specific molecular mechanisms involved in RGC survival and maturation, ultimately improving transplantation outcomes. Separately, developing systems that model the complex interplay between the immune system and RGC degeneration, and developing immune-based therapies to prevent donor RGCs from being collateral damage in a hostile disease environment, are of great interest.

Lastly, recent advances in single-cell sequencing technology have provided new tools for investigating the molecular cues involved in RGC survival and maturation. By analyzing the gene, protein, epigenetic, and metabolic expression profiles of individual RGCs at different stages of development and engraftment following transplantation, investigators may be able to identify the specific molecular pathways involved in promoting the function of successful RGC transplants (Table 3).

SDG #4. Inner retinal wiring

A sound understanding of inner retinal wiring and the ability to manipulate it are central to the anatomical and, perhaps more importantly, the functional success of cellular approaches for retinal regeneration. While our knowledge of this complex process has significantly advanced in recent years (Fig. 3), there are still unmet research needs in this area, summarized as the following: (i) the need to define "success" and intervention endpoints, (ii) how best to maintain dendritic integrity, (iii) the importance of glial and innate immune responses on the inner retinal circuitry, and (iv) when it may be best to intervene during the disease process.

Studying donor RGC polarization, neurite outgrowth and characteristic electrophysiological properties

Research on stem cell-derived RGCs must ensure that the cells can polarize with dendritic and axonal compartments localizing to the correct retinal layers and

Table 3 Future directions for RGC survival, maturation and host interactions (SDG3)

| Research Area	Future Goals
Neuroprotective approaches	Explore the efficacy of neuroprotective approaches, including those initially developed to prevent endogenous RGC death, in the context of RGC transplantation
Epigenetics	Identify specific epigenetic mechanisms that regulate RGC development and survival and develop epigenetic therapies that can be applied to augment donor RGC transplantation
Donor cell maturation	Determine the most appropriate developmental stage and timing for donor RGC harvesting to achieve the best possible transplantation outcomes
Tissue-engineered retina models	Develop more advanced tissue-engineered retina models to provide longer-term neuronal health and better mimic the in vivo environment. These models could then be used to investigate RGC transplantation without the confounding effects of peripheral immunity
Role of the immune system	Better understand the complex interplay between the neuroinflammation and RGC degeneration and develop immune-based therapies to prevent donor RGCs from being collateral damage in a hostile disease environment
Role of CNS resident glial cells	Achieve comprehensive understanding of interactions between astrocytes and Müller glia with immune cells (resident microglia and peripheral macrophages) in maintaining retinal health, preserving RGC viability during disease/trauma, and promoting regeneration and transplant integration
Imaging techniques	Develop new tools and methods for high-resolution imaging and quantifying donor RGC survival and axonal regeneration in vivo to allow for time-course studies
Single-cell sequencing technology	Use the advancements in single-cell sequencing technology to investigate the molecular cues involved in RGC survival, maturation, and functional engraftment. Analyze the gene, protein, and metabolic expression profiles of individual RGCs at different stages of development and engraftment after transplantation

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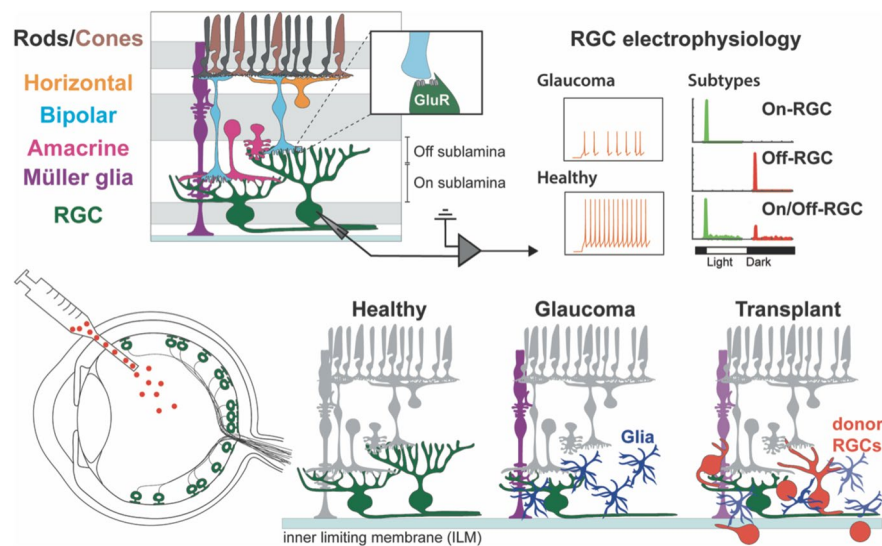
Fig. 3 RGC neurocircuitries in healthy, diseased, and transplanted retinas. Bipolar and amacrine cells establish direct contact with RGCs to relay visual information. Different RGC subtypes extend their dendrites into ON and OFF sublamina in the inner plexiform layer and exhibit different electrophysiological responses. Glaucoma causes dendrite retraction and eventual death of RGCs and the activation of astrocytes, microglia, and Müller glia, while photoreceptor, bipolar, amacrine, and horizontal cells are relatively unaffected. RGC transplantation must replace lost RGCs, return the diseased retina to a homeostatic state, and establish neurocircuitry between host and donor cells. While donor RGCs have been shown to survive in the retina, few are currently able to migrate into the ganglion cell layer, with the inner limiting membrane (ILM) serving as a major barrier for intravitreal (IVT) delivery, and even fewer form de novo neurocircuits in the retina

that cells can form functional synapses while exhibiting characteristic electrophysiological properties. However, the ability to study these processes in vitro is limited, and models must be suitable for high-throughput experimentation.

RGCs in retinal organoids elaborate neurites but do not recapitulate normal retinal circuitries, have no efferent connectivity, and die over time, making RGC polarization, neurite outgrowth, and synaptogenesis challenging to study in these systems [363]. Moreover, organoids lack a fovea and do not faithfully mimic each retinal layer. Nevertheless, these neurons can form de novo synapses after being dissociated [364], providing evidence for synaptic plasticity in stem cell-derived RGCs, which is essential for restoring visual circuits. In addition, in vitro assembloid models have been established to mimic retinofugal projections from RGCs to postsynaptic targets in the brain [19]. Demonstrating the electrophysical properties of RGCs in vitro is essential, as a lack of success in vitro may indicate comparable failure in an in vivo environment, with the caveat that degenerating cultured retinas may be less capable of synaptogenesis. In vitro systems lack functional retinal neurocircuitry, and it is unclear how cell culture conditions impact the cells' electrophysiological response. For example, an array of culture conditions have various pros and cons for electrophysiology experiments in 2D and 3D neuron cultures [365].

Intrinsic and extrinsic factors for successful RGC integration

Recapitulating the RGC connectivity to retinal circuits that occurs developmentally is likely critical to achieving functional therapeutic RGC transplantation. Donor RGCs must extend their dendrites into the IPL with appropriate glutamate receptor expression and specificity for the inner (ON) or outer (OFF) IPL sublamina. However, it remains to be seen whether donor RGCs will do this spontaneously based on environmental cues or will require some additional molecular factors, either inducible cell-intrinsic or extrinsic cues, to be delivered to the IPL.

Several aspects of inner retinal development are pertinent to understanding the signaling factors that may facilitate donor RGC engraftment within the IPL. For instance, semaphorin-plexin interactions contribute to the patterning and stratification of RGC dendrites in different sublayers of the IPL [366]. Insulin and mammalian target of rapamycin (mTOR) signaling pathways can also promote RGC dendrite regeneration and synapse reassembly in the IPL following axonal injury [367].

In addition to molecular cues, retinal cells, including starburst amacrine cells and bipolar cells, themselves play significant roles in pre-patterning the IPL. Starburst amacrine cells help establish the basic structure of the IPL during development [368]. They are among the first cells to stratify within the IPL, effectively acting as

a scaffold for the growth and development of other cells, including RGCs. Starburst amacrine cells also play a crucial role in direction selectivity, a fundamental aspect of visual processing that allows RGCs to respond preferentially to motion in specific directions. Interestingly, in addition to the amacrine cells in the IPL, displaced RGCs can also be found within the IPL, but in orders of magnitude less than in the GCL [294, 369, 370]. Little is currently known about displaced RGCs, but given that they are evolutionarily conserved, they must serve some crucial roles in the retina [371]. Bipolar cells help to convey information from the photoreceptors (rods and cones) to RGCs. In the context of IPL patterning, bipolar cells also contribute to the formation of synaptic connections within distinct IPL sublamina [372, 373]. The stratification of bipolar cell axon terminals within the IPL is thought to influence RGC dendritic development and their ultimate stratification within the IPL. It is fortuitous that afferent inner retinal neurons spontaneously prepattern the IPL during development and that these cell populations are relatively unaffected by optic neuropathies since this suggests that, even in advanced optic neuropathy, there should be an IPL scaffold into which donor RGCs may be able to integrate. Understanding the molecular cues that may be common across all RGC subtypes during development to promote dendritic extension would be very useful.

Cell-intrinsic factors related to RGC survival and axon regeneration have been studied in mouse models following injury. Molecular targets, such as mTOR, phosphatase and tensin homolog (PTEN), BDNF, nerve growth factor, ciliary neurotrophic factor (CNTF), insulin, and Kruppel-like factors (KLF), have been identified as critical for RGC survival and regeneration [374–377]. However, the relevant molecules at each step of retinal circuit restoration remain unclear, and further research is needed to understand the functional role of trophic factors and guidance molecules in IPL regeneration. Therefore, a comprehensive, systematic characterization of developing, degenerating, and transplanted RGCs at multiple levels, including transcriptomic (scRNAseq and spatial transcriptomics), structural (STORM and nanoscopic imaging to visualize the dendritic arbors and synapse arrangements), and functional techniques (electrophysiology) will be required. Using human tissue in addition to animal studies will increase the utility of these investigations.

Extrinsic factors in the ocular microenvironment also influence RGC integration into retinal circuitry. For example, previous work has shown improved donor RGC integration in retinal damage models compared with healthy wild-type retinas [54], consistent with photoreceptor transplantation studies [378]. However, it remains to be determined if donor RGCs can better integrate into these retinas because there is more available physical space or other unknown mechanisms.

As previously mentioned, the ILM is a known physical barrier to transplanted RGCs [242, 243], but its role in donor RGC integration is still being determined. ILM recognition appears essential to proper RGC lamination and polarity during development [379]. Molecular factors, such as integrins expressed by RGCs and laminins associated with the ILM, can affect RGC integration by providing molecular cues to guide neurite outgrowth [236]. Further investigation is needed to determine how the ILM, other retinal cells (e.g., amacrine cells, bipolar cells, and MG), and the IPL extracellular matrix govern donor RGC integration at the molecular level.

Maintaining dendritic integrity

The importance of dendritic integrity in optic neuropathy disease models has been established through the observation that loss of complexity in RGC dendritic trees occurs before axonal loss [147, 150, 154]. This phenomenon is particularly evident in OFF-transient RGCs, which exhibit a rapid decline in both structural and functional organization upon IOP elevation [147, 154]. Indeed, some RGC subtypes undergo significant dendritic rearrangements as early as seven days after induction of elevated IOP [150, 162]. Interestingly, early dendritic remodeling may be linked to axonal regeneration [380]. Furthermore, there is evidence of circuit plasticity ocurring after IOP elevation, with the rewiring of developmental presynaptic bipolar and amacrine cell partners with a resilient RGC type in the inner retina [381, 382].

Given that dendritic resprouting and synaptogenesis can be promoted in injured RGCs, it is plausible that these processes could be induced in newly repopulated RGCs. In fact, changes in spontaneous activity and light-evoked responses in endogenous injured RGCs are noted before any detectable dendritic loss, pointing towards a potential relationship between dendritic remodeling and functional changes in the cells. However, outstanding questions remain regarding the extent and reversibility of dendritic tree remodeling in the context of injury. For instance, while OFF-sustained RGCs show perturbed light-evoked responses following injury, their dendritic structure remains intact [154]. ON-transient and ON-sustained RGCs also demonstrate normal functional receptive field sizes following injury, but their spontaneous and light-evoked firing rates are reduced [154]. How these different responses relate to the extent and reversibility of dendritic remodeling or the propensity of these RGC subtypes to generate new dendrites after introduction into the diseased retina remains to be determined. Further research will be needed to explore these

pathways and the potential for reversing the damage caused by IOP elevation or other insults.

To address these questions, investigations of dendritic and synaptic integrity in various models of optic neuropathy will be necessary. Techniques such as biolistic labeling and rigorous approaches for determining the co-localization of pre- and post-synaptic markers may be useful [381]. Such methods should rely not only on the fluorescent overlap but also on techniques with greater specificity, such as fluorescent protein reconstitution across synaptic partners [383]. Furthermore, much remains to be understood regarding the molecular mechanisms underlying dendritic remodeling, including the role of intracellular signaling pathways and gene expression changes. In particular, much of what is known about dendritic remodeling may be heavily biased towards only a few RGC subtypes because of the availability and use of specific transgenic mice for these studies, such as the Thy1-YFP line (B6.Cg-Tg(Thy1-YFP)HJrs/J), which primarily labels alpha-RGCs [142]. Moreover, RGCs distributed spatially across the retina vary with respect to arborization, irrespective of the RGC subtype [384]. Therefore, it may be necessary to drive dendritic remodeling in repopulated RGCs according to both their specific subtype and spatial distribution across the retina.

Glial factors and innate immunity

Optic neuropathies and RGC death can trigger changes in retinal cells beyond RGCs [292]. MG and innate immune responses are likely crucial factors influencing donor RGC integration. MG are critical in retinal homeostasis and regeneration [385]. However, the re-entry of reactive MG to the cell cycle leads to proliferation and the formation of a glial scar [27, 385]. These scars can act as reservoirs for accumulating extracellular matrix proteins, including chondroitin sulfate proteoglycans [386], which can hinder neurite extension by transplanted cells. Treatment with chondroitinase ABC digests chondroitin sulfate proteoglycans and enhances donor cell migration, neurite outgrowth, and synaptogenesis in the retina [387–390]. Future efforts may explore using chondroitinase ABC or other methods of modulating the extracellular matrix to improve donor RGC integration into the retina.

Innate immune responses, including microglial reactivity and infiltration of peripheral immune cells, can also influence the integration of transplanted cells [391]. While microglia can play a beneficial role in clearing debris, promoting tissue repair, and pruning and revising dendritic arbors and synaptic connections in the retina during development and disease, their chronic activation can lead to neuroinflammation and exacerbate retinal

damage either directly or through promoting additional reactivity response by astrocytes [392, 393].

Modifying the transplanted cells or host environment to avoid or modulate MG, astrocyte, microglial, or other immune responses may be necessary to enhance dendritic integration within the IPL. As discussed, masking the "eat me" signal through preconditioning donor RGCs with annexin V can improve the survival of donor RGCs after xenotransplantation by preventing microglia from phagocytosing donor cells [362] and may also protect immature neurites that would be subject to pruning. Alternatively, changing the environment in which RGCs are transplanted may prevent or modulate these responses. For example, PLX-mediated microglia ablation might improve donor RGC engraftment. However, the potential unforeseen consequences of such modifications must be considered. Microglia ablation could elicit monocyte infiltration to fill the void, but these cells may not function the same way [394]. Glia are essential in regulating the environment around neurons and contribute to synaptic plasticity [354, 395, 396]. Further, depletion has also been related to neurodegenerative changes [397]. Thus, understanding the interactions between glia and retinal neurons could inform methods of enhancing donor RGC integration within the inner retina.

Future directions for inner retinal wiring

Our ability to promote and control RGC integration into the inner retina will be enhanced by a better understanding of the mechanisms by which the inner retina processes visual information. Recent advances in imaging technologies, such as two-photon and light-sheet microscopy, have allowed researchers to visualize the electrophysiological activity of large populations of retinal neurons in real time [398]. These techniques can be used to study the dynamics of retinal circuits and how they respond to changes in visual stimuli. Moreover, it enables the classification of different retinal cell types and allows for high-resolution measurements of calcium entry at synaptic release sites across multiple bipolar cells simultaneously [398]. Applying these functional imaging techniques to RGC transplantation will likely yield important information about their spontaneous functional engraftment capability and provide an essential tool for assessing methods to augment their integration into retinal circuits.

Moreover, optogenetic advancements have uncovered new avenues for studying inner retinal wiring. Optogenetics can selectively activate or inhibit specific cell types in the retina, enabling researchers to probe the function of individual neurons and their interactions with other cells. For example, activating all amacrine cells through optogenetic stimulation promotes the recovery of both

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ON and OFF responses in the retina [399–401]. This method also facilitates studying diverse forms of retinal processing, including sustained and transient responses. Consequently, utilizing optogenetics to modulate specific inner retinal circuits may be useful for studying and/or enhancing donor RGC connectivity within recipient retinas.

Finally, consideration of the goalposts needed to successfully demonstrate the inner retinal wiring of transplanted donor RGCs is warranted. At the minimum, donor RGCs should extend dendrites into the IPL and express functional glutamate receptors. At the other end of the spectrum, we desire synaptic connectivity and function of transplanted RGCs that is indistinguishable from wild-type healthy retina. Goalposts to reach in between these two ends of the spectrum include donor RGCs that exhibit dendritic targeting to the correct sublamina, donor RGCs that show ON vs. OFF responses and generate sustained vs. transient responses, and donor RGCs that have proper connectivity with bipolar cells and amacrine cells. Future research should be directed with these goalposts in mind and with the understanding that translation from mouse to primate circuitry is imperative to make successful RGC transplantation a reality (Table 4).

SDG #5. Eye-to-brain connectivity

Axon (re)generation is a complex process that involves overcoming mechanical and inflammatory obstacles, identifying and responding to specific signals in the adult environment, and navigation by diverse RGC subtypes. Axon regeneration is not simply a recapitulation of development, and adult retinas may require unique cues for long-distance reinnervation of central visual targets. Additionally, the role of glia in promoting or inhibiting axon regeneration is complex and varies depending on the type of glial cell, region, and stage of axon regeneration. While glia have traditionally been thought to inhibit axon regeneration, recent studies have demonstrated that

they can also be beneficial. Therefore, a comprehensive understanding of glial cell diversity throughout the visual pathway and their functions at different stages of axon regeneration is necessary to establish effective glial modulation strategies for promoting efferent connectivity to the brain (Fig. 4). This section explores the challenges and potential solutions to promoting axon regeneration over long distances, regulating the immune system and glial cell response, and targeting appropriate regions in the adult brain after potential atrophy. Developing strategies to restore vision in glaucoma and other optic neuropathies requires a comprehensive understanding of RGC diversity, development, the adult healthy and diseased microenvironment, and regeneration.

Axon regeneration over long distances

Axon regeneration remains a significant challenge for optic neuropathy therapy development. The feasibility of RGC axon regeneration over long distances was first shown by anatomical studies demonstrating that RGCs in adult rats can regenerate axons through grafted segments of peripheral nerve tissue [402]. When such grafts are inserted directly between the retina and the brain, RGCs regrow their axons into the graft and the superior colliculus [402–404].

More recently, seminal work over the past 15 years has identified several pathways that can be effectively targeted to promote long-distance axon regeneration of injured, endogenous RGCs in rodents and represents another key advance that makes therapeutic RGC repopulation feasible. These pathways have been reviewed elsewhere and include signaling through thrombospondin-1, Lin28/IGF-1, PTEN/mTOR, suppressor of cytokine signaling 3 (SOCS3)/JAK/STAT3, KLFs, oncomodulin, transcription elongation factors, collapsin-response-mediator proteins, dynein light chains, mitochondrial leucyl-tRNA synthetase, and others [76, 377, 405–411]. It is hoped that by targeting

Table 4 Future directions for inner retinal wiring (SDG4)

| Research Area	Future Goals
Role of non-neuronal cells	Investigate how macroglia (Müller glia and astrocytes) and microglia modulate neural activity and contribute to synaptic plasticity in healthy retinas, disease states, and following RGC transplantation
Pathways underlying circuit development and integration	Identify the cell-intrinsic and extrinsic cues that underlie IPL patterning and circuit development and leverage this information to develop interventions that promote donor RGC integration into these circuits
Visual information processing	Use advanced imaging technologies and optical electrophysiology (e.g., two-photon and light-sheet microscopy) to study the dynamics of retinal circuits and how they respond to changes in visual stimuli. Leverage this information to better understand the mechanisms by which the inner retina processes visual information and how donor RGCs may be contributing to visual processing
Optogenetics	Employ optogenetics to selectively activate or inhibit specific cell types in the retina, enabling the study of individual neurons and their interactions with other cells

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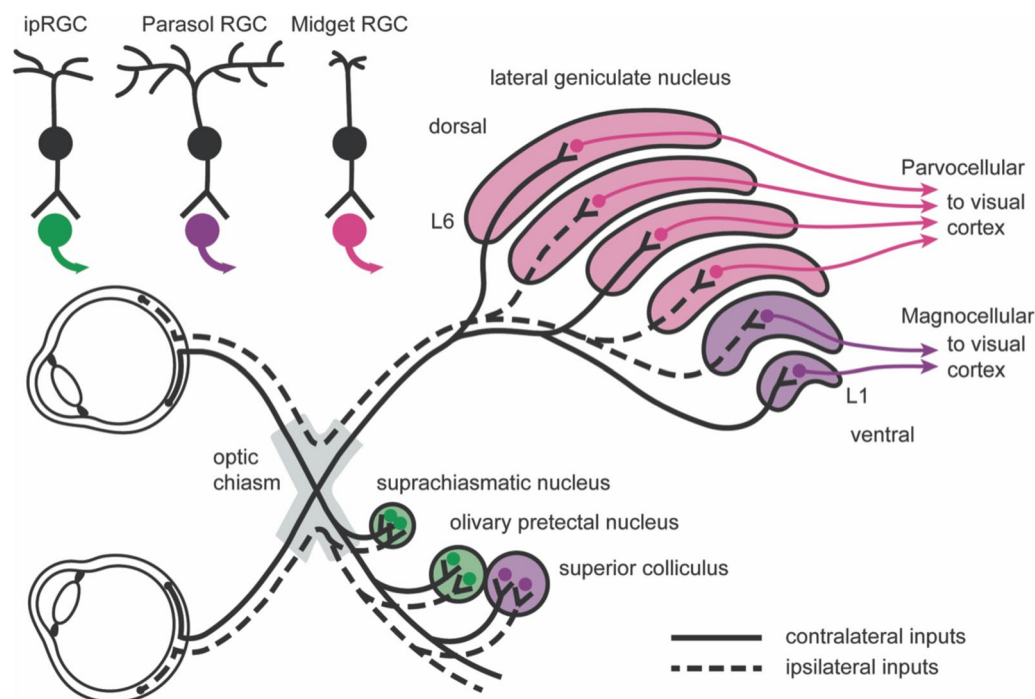
Fig. 4 Retinal ganglion cell (RGC) pathways in the human brain. Visual information travels from each retina through the optic nerve and converges at the optic chiasm. Uncrossed ipsilateral inputs connect to L2, L3, and L5 in the lateral geniculate nucleus, whereas crossed contralateral inputs connect to L1, L4, and L6 in the lateral geniculate nucleus. Both ipsilateral and contralateral inputs connect to the suprachiasmatic nucleus, olivary pretectal nucleus, and superior colliculus. Intrinsically photosensitive RGCs (ipRGCs), among others, connect to the neurons in the suprachiasmatic nucleus and olivary pretectal nucleus (green) to regulate circadian rhythms and the pupillary light reflex, respectively. Parasol RGCs, among others, connect to the neurons in the superior colliculus (purple) to orient head and eye movements and to L1 and L2 in the lateral geniculate nucleus as a part of the magnocellular pathway (purple). Midget RGCs, among others, connect to the neurons in L3, L4, L5, and L6 of the lateral geniculate nucleus as a part of the parvocellular pathway (pink). The magnocellular and parvocellular pathways connect to the visual cortex to primarily process motion and high-contrast information, respectively

similar pathways in transplanted RGCs, long-distance axon growth into the brain can be achieved.

Assuming RGCs can engraft into the retina, their axons will encounter a critical anatomical structure before entering the optic nerve: the lamina cribrosa. Once developmentally formed, this porous, multi-layered collagenous structure may pose a mechanical obstruction that regenerated axons must overcome to exit the eye. Indeed, significant biomechanical changes to the optic nerve head occur in advanced glaucoma. Furthermore, the lamina cribrosa is a site of neuroinflammation in glaucoma and other optic neuropathies, which can stress the axons of donor cells. Given that most work on RGC axonal regeneration has involved lesioning the optic nerve distal to the lamina cribrosa (within the orbit), there is little data that provides insight into how much of a barrier this tissue represents for axonal growth or how to circumvent it. Studies examining how intraretinal RGC axons within the optic nerve fiber layer might traverse the lamina cribrosa and optic nerve head should be a high priority.

Axon regeneration is not simply a recapitulation of developmental axonal genesis. Indeed, the receptor-ligand pairs driving zebrafish axonal regeneration and mammalian peripheral nervous system regeneration are not identical to those driving axonal development [412]. For instance, *Sprr1a* is an essential peripheral nervous system axon regeneration gene. Suppressing *Sprr1a* inhibits axon regeneration of preconditioned sensory neurons [413]. However, Sprr1a is only upregulated during regeneration and not expressed during developmental axon growth. Understanding why factors like Sprr1a are important for regenerative, but not developmental, axon growth may yield insights into the best pathways to target for promoting axon extension from donor RGCs.

Various molecular targets for RGC axon regeneration have been identified, including KLFs, thrombospondin, Oncomodulin, and others [414]. KLF4 and KLF9, part of the KLF family of transcription factors, inhibit RGC axon regeneration, and their deletion has resulted in notable axon growth in experimental models [415, 416]. Thrombospondin-1 and -2, matricellular proteins important for

synapse formation and neuronal plasticity, are potential therapeutic targets for RGC axon regeneration [417, 418]. Oncomodulin binds to RGCs in a cyclic adenosine monophosphate-dependent manner and stimulates optic nerve regeneration [419]. Importantly, these combinatorial approaches promote axon regeneration more than isolated manipulations. *Pten* deletion, *Socs3* deletion, and *Cntf* overexpression have synergistic effects on RGC survival and axon regeneration [374]. To support long-distance axonal regeneration, global changes in metabolism should be a point of focus since lipids are needed to build the considerable volume of plasma membrane needed to traverse the optic nerve [420]. Indeed, *Socs3* knockout and *Pten* knockout-mediated axon regeneration depend on metabolic changes in RGCs to support lipid synthesis [421].

Axon guidance cues are expressed in both spatial and temporal gradients during different stages of development. For example, in the developing *Drosophila* CNS, to decussate, growing axons must exchange cell surface receptor FasII (Fasciclin II) for FasI. Once decussation is complete, neurons must switch back to expressing only FasII [422]. Successful recapitulation of these signaling mechanisms in the adult animal using current molecular techniques has been limited. Although some guidance cues from development persist in adults, it remains unclear whether they function similarly post-development. While the signals that direct RGC axon outgrowth during development have been well studied [423, 424], the expression patterns of these molecules in the post-developmental visual pathway are less well characterized. Fundamental to our ability to leverage intrinsic guidance cues to promote donor RGC axon regeneration will be the development of comprehensive atlases of guidance cue expression throughout the visual pathway as a function of age and disease state. Rather than relying on the maintenance of expression of these cues in the adult, another approach to promoting long-distance axon regeneration is to engineer RGCs to respond to the remaining signals by introducing the appropriate guidance receptors.

Co-culture systems of RGCs with their pre- and post-synaptic targets are one approach to understanding how donor RGCs will interact with the host microenvironment. For example, donor RGCs can be co-cultured with dissociated primary retinal cells to determine whether they will recognize and respond to chemotropic cues in the retina [425]. In this setting, donor RGC neurite complexity and axon length have increased significantly when co-cultured with central versus peripheral retinal cells. This suggests they may respond to a centripetal gradient of intra-retinal guidance cues for orientation towards the optic nerve head. Similarly, by explanting retinorecipient targets from the brain and co-culturing those tissues in vitro, donor RGC axon guidance towards relevant visual targets in the brain can be assessed [19].

In other than the most advanced cases of glaucoma, surviving endogenous RGCs might serve as guidance tracks for new RGCs once transplanted within the retina [426]. Indeed, interactions between donor and host RGC axons within the retinal nerve fiber layer might help guide donor axons to the optic nerve head. Axon guidance during development and in vitro is driven by surface topography or signaling through cell surface receptors and ligands [427, 428]. In particular, molecules such as Sema6D, neuronal cell adhesion molecule (Nr-CAM), and Plexin-A1 that are expressed on midline radial glia and chiasmal neurons play a role in this process [429]. Sema6D typically acts as a repellant for RGC axons, but when combined with Nr-CAM and Plexin-A1, it promotes growth instead. Interestingly, the radial glial marker, slit guidance ligand 1, which is also usually considered to act as an inhibitory guidance cue during development, has been identified in the adult optic chiasm after an optic nerve crush injury, while other markers (radial glial cell marker 2, brain lipid binding protein) remain absent [430]. Lastly, recent research has shown that bone morphogenetic protein 4 signaling interferes with optic chiasm formation and RGC pathfinding [431]. Considering the varying expression of these guidance cues during development, adulthood, and following injury, it remains unclear whether similar axon-specific interactions exist in the retina. Variations in these cues may contribute to the misguidance of regenerating RGC axons through the optic chiasm, or regenerating axons may lack the receptors for those cues.

The optic chiasm is a cross-shaped junction in the brain where the optic nerves meet and a proportion of RGC axons decussate. This structure is crucial for binocular vision by enabling the integration of visual information pertaining to overlapping regions of the visual field from both eyes. RGC axons either cross to the opposite side (contralateral) or remain on the same side (ipsilateral) at the chiasm, depending on specific guidance cues. In many species, the pattern of decussation in the chiasm is influenced by a range of molecular signals. For instance, guidance molecules such as Netrin-1 and Slit proteins, and their respective receptors, play a role in determining which axons cross and which do not [432]. In mice, most RGC axons project contralaterally, with only a small proportion, approximately 3–5%, projecting ipsilaterally [433]. Conversely, in primates, including humans, the proportion of ipsilaterally projecting RGC axons is higher, with estimates suggesting that approximately 45–55% of RGC axons project contralaterally, while the rest remain ipsilateral [433]. While mouse models offer

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numerous advantages for research, these differences in RGC projections between species necessitate caution when selecting animal models for translational applications. Further emphasis is needed to understand the full spectrum of factors influencing RGC axon guidance in the context of retinal transplants across species.

Electrophysiologic activity is another cell-intrinsic factor that mediates axon regeneration. Enhanced neural activity by visual stimulation or chemogenetics increases axon regeneration of injured RGCs [434]. Moreover, increasing neural activity, in combination with the elevation of mTOR, further promotes RGC axon regeneration over the long distances necessary to re-innervate the brain. As such, enhancing the electrophysiologic activity of donor RGCs may augment optic nerve regeneration, though the optimal induction methods and activity patterns remain to be determined.

The diversity of RGC subtypes must also be considered. Different subtypes require different guidance cues for directing axon regeneration, and it is still unclear to what extent such cues may be available endogenously or need to be provided experimentally [435]. Also, some RGC subtypes survive injury better than others or are more likely to regenerate axons following axotomy [6, 149]. Moreover, different RGC subtypes respond differently to gene knockouts. For example, while *Pten* inhibition causes mostly alpha-RGCs to regenerate axon short-distances [374], it is mostly M1 ipRGCs that regenerate axons long-distance [76]. A unique feature of ipRGCs is the expression of photosensitive melanopsin, encoded by the *Opn4* (*melanopsin*) gene, which enables these RGCs to fire action potentials in response to light. Similar to previous work showing improved axon regeneration following electrical stimulation [1], the ability for ipRGCs to be directly activated by light may contribute to their enhanced survivability and axon regeneration capacity [434]. Furthermore, overexpression of *Opn4* itself also promotes axon regeneration [436]. Therefore, combinatorial strategies involving co-targeting different pathways while also stimulating electrical activity will be needed to promote long-distance axon regeneration across multiple RGC subtypes.

Nonetheless, regeneration or replacement of only a specific subset of these RGC subtypes might be sufficient to restore rudimentary forms of vision and drastically improve a patient's quality of life. Vision restoration extends beyond just providing functional vision; it also contributes to regulating the circadian rhythm, which relies on light cues. ipRGCs play a crucial role here, directly responding to light and regulating our internal "clock." Patients with Non-24-Hour Sleep–Wake Disorder, a condition common in people who are blind, suffer from disturbed sleep patterns due to a lack of light perception. Restoring even basic light perception could re-synchronize their sleep–wake cycle and improve their life quality significantly. Current treatments for Non-24-Hour Sleep–Wake Disorder are pharmacological, costly, and have limited effectiveness. A targeted therapy that regenerates specific RGC subtypes could offer a more cost-effective, long-lasting solution. Moreover, ipRGCs project to various vision-processing regions in the brain and regulate other non-image-forming functions, such as the light-evoked constriction of the pupil, and are even involved in contrast sensitivity and visual perception [437]. Hence, treatments aimed at ipRGCs could be promising. However, for research purposes, the aspiration is set above this goal. Short-term goals resulting in clinically significant patient outcomes should be prioritized while striving towards long-term goals of functional vision restoration.

Role of glia in axon regeneration

Glia, in particular reactive astrocytes and microglia, or damaged and dying oligodendrocytes, have traditionally been thought to inhibit axon regeneration due to failure to clear myelin byproducts, including Nogo, MAG, and OMgp, that are released during nerve damage. However, recent studies have revealed that glial cells can also play a beneficial role in axon regeneration. For instance, microglial depletion disrupts the growth of long projecting axons beyond the lesion after spinal cord injury in neonatal mice, suggesting they play an active role in regeneration [438]. Similarly, microglia depletion exacerbates tissue damage and worsens functional recovery after contusion spinal cord injury in adult mice [439]. These findings suggest that the role of glia, at least microglia, in axon regeneration is complex, and it is oversimplistic to classify their behavior as binary: beneficial or inhibitory.

The complexity of glial functions arises from the diverse types of glial cells [440], each of which plays a different role in axon regeneration. Microglia primarily serve immune-related functions and clearance of debris. Astrocytes provide structural and metabolic support and regulate the microenvironment of neurons. MG are the most abundant glia in the retina and have a radial morphology extending from the ILM to the photoreceptor outer segments. Their basal lamina forms the first barrier that must be traversed by RGC transplanted intravitreally. MG also engulf and contact the blood vessels responsible for regulating the extracellular environment and supporting neuronal survival. The interactions and coordination between these different glial cell types are essential for the proper functioning and homeostasis of the retina [354]. For example, MG secret factors are necessary for axon regeneration and RGC survival [441].

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Importantly, there is extreme heterogeneity between glial cells of the same type throughout the CNS. For instance, astrocytes in the retina have unique phenotypes (e.g., transcriptomic and proteomic profiles) compared to those in the optic nerve, and those in the optic nerve head have different functions from those in the myelinated part of the optic nerve or the brain and spinal cord. In the retina alone, there are three at least morphological subclasses of astrocytes: bipolar astrocytes that run along nerve fiber bundles, perivascular astrocytes, and stellate astrocytes that occupy the spaces between blood vessels and nerve fibers [440]. These astrocytes all support retinal neurons and regulate pH and ion levels, but it remains unclear if they have subtype-specific functions that are not easily transferred to astrocytes in other regions (an important factor for consideration in cell transplant therapies). They interact with other glial cells and neurons, influencing synaptic function and participating in vascular development and neurovascular coupling [442]. In the optic nerve, oligodendrocytes wrap axons in myelin, enabling rapid transmission of visual information, maintaining extracellular ion concentrations, and contributing to metabolic substrate delivery. To varying extents across species, astrocytes contribute to the integrity of the lamina cribrosa (or, in rodents, the glial lamina), supporting optic nerve fibers as they exit the eye.

The stage of axon regeneration is a critical factor that influences the overall effect of glial modulation [443]. In acute phases following an injury, microglia, and to a lesser extent astrocytes, clear the myelin debris from degenerating oligodendrocytes and support regeneration. In the context of inflammation and disease, an intriguing division of labor occurs between microglia and astrocytes – with microglia (largely) increasing phagocytic capacity, while some sub-states of reactive astrocytes shutting down phagocytosis almost completely [264]. Modulating glial cells to improve their ability to clear debris may be necessary in cases where excess myelin debris is particularly inhibitory. There is a window of opportunity during which glial cells must be modulated or activated to optimize their clearance functions. If myelin debris persists for an extended period, it can impede regeneration and lead to detrimental effects [444]. Moreover, these clearance and regeneration roles seem to be specialized, with perivascular microglia controlling the entry of materials into the retina from the vasculature and parenchymal microglia being highly motile cells that survey the microenvironment, clear debris, and mediate synapse remodeling [445]. Therefore, promoting or suppressing glial reactivity should be timed appropriately to ensure optimal results depending on the stage of degeneration and regeneration. A comprehensive understanding of glial diversity at different stages of optic neuropathy is

necessary to establish effective glial modulation strategies for promoting axon regeneration while disrupting inhibitory cues. For example, glial cells can be reprogrammed to overcome glial cell inhibition of regeneration and promote structural and functional regeneration after CNS injury by increasing glycolysis [446], while post-injury-born oligodendrocytes incorporate into the glia scar and hinder experimental axon regeneration by presenting myelin-associated inhibitors to the growing axons in an attempt to myelinate them (before they reached respective post-synaptic targets) [447].

It is worth considering that most current knowledge about glial cell contributions to retina/visual system degeneration and regeneration comes from injury models [447, 448], and it is crucial to investigate how this knowledge translates to neurodegenerative diseases that affect human patients. Injury models do not necessarily mimic the chronic nature of neurodegenerative diseases, and the function of glial cells is directly altered in neurodegenerative diseases [449, 450]. Interestingly, unilateral optic nerve damage results in a contralateral glial response [451, 452]. Better understanding the factors that drive contralateral gliosis and development methods to modulate reactive gliosis in each eye (with comparison to bilaterally naïve controls) will be relevant to developing RGC repopulation and protection strategies.

Targeting appropriate visual areas in the brain

Targeting appropriate visual systems in the brain is crucial for successful optic nerve regeneration and vision restoration in optic neuropathies. Co-culture experiments involving RGCs, retinal organoids, or retinal explants with organoids of thalamic tissue (i.e., assembloids) may inform methods for guiding RGC axons to the correct locations in the brain [19]. Leveraging the extensive information gained from studies promoting the regeneration of endogenous RGC axons following injury will also aid the development of strategies to guide donor RGC axons to subcortical visual centers. However, once RGC axons reach their central targets, rebuilding topography will likely be necessary for image-preserving vision. Studies in lower vertebrates, such as zebrafish, may help elucidate how spatial and temporal information is encoded across visual space [453] and can be used to identify mechansims essential for RGC target innervation [454].

Regulating neural activity with exogenous electrical field simulation may help to direct axon regeneration [455–457]. Controlling neural activity may also hold potential in training rudimentary visual functions. One approach for increasing the receptivity of the post-synaptic targets in the brain to innervation is modifying it using chemogenetic tools [458]. For example, by

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enhancing neural activity in postsynaptic neurons in the optic pathway (the pretectal nucleus), endogenous RGC axons can regenerate and reconnect to their brain targets in a distal injury model [458]. In addition, augmentation of axon conduction velocity can help overcome prolonged latency of remyelination after axon reinnervation [459, 460]. This work highlights the potential of targeted brain stimulation to regenerate endogenous RGC connections. However, no studies have examined the retinotopic projections of regenerating *donor* RGC axons within central targets. Careful examination of retinotopic patterning of regenerated RGC axons within the lateral geniculate nucleus, determining whether there is plastic evolution in retinotopic mapping over time, and developing methods to control this process are key areas for future research.

Several experiments have identified atrophy in the visual processing centers of the brain following monocular optic nerve crush across multiple species, and anterograde transsynaptic degeneration appears to be a feature of many human optic neuropathies [461]. Within 90 days of RGC death and an accompanying loss of retinal outputs, the superior colliculus, dorsal lateral geniculate nucleus, and visual cortex experience shrinkage, molecular changes, reduced neural activity, and cell loss [461–467]. Moreover, dark-rearing zebrafish results in reduced brain size [468], indicating the potential importance of visual stimulation for developing and maintaining retinorecipient neurons in the brain. Early intervention after an injury can prevent RGC death, promote optic nerve regeneration, and partially restore vision [75, 434, 469]. Determining the extent to which postsynaptic targets will be receptive to newly established innervation in advanced disease is an important task for the field. Moreover, defining the sequence and timeline of transsynaptic degeneration in various optic neuropathies will help inform the optimal timing for therapeutic innervation. Other subcortical targets, such as the superior colliculus, are also necessary for vision, though the relative contribution of these areas to vision may vary across species. Determining which brain targets are most essential for processing visual information will help guide strategies to target donor RGC axons to the most critical locations.

Functional tests are the most rigorous methods to evaluate the success of optic nerve regeneration and include testing of visual acuity, contrast sensitivity, and electrophysiology function within the brain (e.g., through visually evoked potentials). Unfortunately, results in early transplantation experiments may not yield enough visual pathway regeneration to discern appreciable improvements in behavior. Thus, histology will likely remain the gold standard for assessing optic nerve regeneration in the near term. Future efforts to establish more sensitive approaches to assess donor RGC axon regeneration within the brain in vivo, such as with implantable bioelectronics [470, 471], would be valuable.

Models to study optic nerve regeneration

In vitro models used to study optic nerve regeneration, including retinal explants, purified primary RGCs, patient-derived induced pluripotent stem cell-derived RGCs, and retinal organoids, offer several advantages, such as ease of manipulation of experimental conditions, reproducibility between experiments, and the ability to use human retinal cells. However, these in vitro models also have several limitations. For example, retinal organoids differ in structure and cell composition from the human eye and lack peripheral circulation and immune surveillance, limiting their usefulness in studying visual circuit assembly and disassembly. Moreover, the limited lifespan of RGCs within organoids is a significant drawback to their long-term study in vitro.

Co-culturing retinal organoids with brain tissue may provide postsynaptic contacts for RGCs and overcome some of these limitations, including the limited lifespan and lack of visual circuits in other in vitro systems. Assembloids, created by fusing retinal and thalamic organoids, exhibit decreased RGC apoptosis compared to retinal organoids grown in isolation [19]. In addition to improved survival, some RGC axons extend to and enwrap their postsynaptic targets while others grow into co-cultured cerebral organoids [19]. Co-cultures of the visual centers of the postnatal or embryonic brain with stem cell-derived retinal organoids may represent a powerful approach to studying axonal growth and guidance, particularly if the optic chiasm can be adequately modeled in this system.

To aid in studying axon outgrowth and regeneration, microfluidic chips or nanofibers can direct axons in monolayer and 3D organoids/assembloids [19]. Providing soluble factors in specific compartments of these microfluidic devices is useful as a screening approach to identify cues that drive axonal guidance. Conversely, these tools could identify negative influencers in unhealthy environments that inhibit outgrowth.

Studying regeneration through in vitro experiments will greatly enhance our knowledge, but it is important to also determine how to regenerate retinal connections in vivo. In vivo, assays usually evaluate regeneration after an optic nerve crush or induction of high ocular pressure to mimic glaucoma. Important questions include which animal model to use and how to assess regeneration in a consistent matter. Given the wide availability of transgenic animals, rodents are commonly used as an animal model to study optic nerve regeneration. However, rodents are nocturnal animals relying less on vision

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for day-to-day activities than humans. The absence of a macula and lamina cribrosa also limits their utility as a model system. Pigs are becoming increasingly popular as a model for studying optic nerve regeneration due to the increasing availability of transgenic lines that mimic various optic neuropathies [472, 473]. Feline models have also been used to study optic nerve degeneration, neuroprotection, and RGC transplantation [246, 474].

While other animal models, such as reptiles, amphibians, and fish, can regenerate RGC axons spontaneously, they may still provide valuable insights into this field [475–477]. For example, optic nerve crush in tadpoles and zebrafish [478–480] has been used to study RGC axon regeneration, in this case, from endogenous RGCs. Mammals, including rodents, however, do not regenerate CNS projection neurons spontaneously. Therefore, mice are often used as a model system to study the failure of optic nerve axon regeneration, particularly using optic nerve transection or crush models.

The optic nerve crush injury model is the most widely used in vivo model system. However, there is considerable variability in how optic nerve crush is performed, including the duration of the crush, the type of forceps used, and the extent of injury to the nerve [481]. Partial crushes can lead to extensive variability and reduced reliability compared to total crushes. When studying optic nerve regeneration, a total crush is preferred to prevent surviving RGC axons from being misclassified as regenerating fibers [482]. Several methods, including functional testing with visually evoked potentials, can be used to verify the completeness of the crush [483]. Cholera toxin subunit B is often used to label axons in optic nerve crush, but its effectiveness can be variable depending on the extent of axon damage [484, 485]. Lastly, while optic nerve crush is the standard model used in optic nerve regeneration research, an important limitation of optic nerve crush is its acute nature, making it less suitable for studying chronic and progressive optic neuropathy, such as glaucoma. However, this characteristic also advantageous for studying the mechanisms of optic nerve regeneration without the confounding factors present in more complex optic neuropathies [486].

Ocular hypertension models of glaucoma are also widely used and include the injection of microbeads or silicone oil into the anterior chamber, hypertonic saline injection into the episcleral veins, perilimbal constriction sutures, and genetic models of ocular hypertension. A major challenge with studying axon regeneration in ocular hypertensive glaucoma stems from their chronic progressive nature and difficulties distinguishing regenerated axons from undamaged, surviving axons. Maintaining a state of elevated IOP for prolonged periods of time leading to very severe optic neuropathy may increase the

consistency of experiments utilizing glaucoma models to study optic nerve regeneration. Nevertheless, the choice of a model for studying optic nerve regeneration or glaucoma should be based on specific research questions, and it may be beneficial to study multiple models to obtain a more comprehensive understanding of the processes involved. Indeed, while still in its infancy, whole eye transplantation in rats [487, 488] could represent another model to study optic nerve regeneration outside a disease state.

As discussed, many other animal models have been developed to study other types of optic nerve disease that may help inform RGC axonal regeneration. For example, intraperitoneal injection of myelin-associated glycoprotein or aquaporin-4 protein injection into the subarachnoid space under the optic nerve sheath is employed to study demyelinating optic neuropathies [489]. Frequency double-YAG laser has been used to selectively activate intravascular Rose Bengal dye in the vessels that supply the optic nerve head to induce thrombosis of small capillaries while sparing larger vessels such as the central retinal artery as a model system of ischemic optic neuropathy [490]. Studying RGC axonal regeneration or donor RGC axonal guidance in these models may provide relevant insights into RGC responses to varying optic nerve insults.

Critical period for the regeneration of the visual system

The timeline for therapeutic regeneration is crucial for successful vision restoration in optic neuropathies. In animal models, such as the mouse optic nerve crush injury model, it has been found that almost 90% of RGCs die within the first three weeks after injury [140]. However, injury models do not fully mimic neurodegenerative diseases like glaucoma, and there are inter-species differences between mice and humans. Irrespective of species, long-term denervation, because of RGC death, can cause shrinkage and molecular changes in target regions in the brain [461]. While efforts have been made to prevent superior colliculus and dorsal lateral geniculate nucleus degeneration using neuroprotection, these tissues continue to atrophy [462], seemingly due to a lack of retinal inputs. Prompt initiation of axonal regeneration following peripheral nervous system injuries is associated with reduced degeneration at postsynaptic targets and better functional outcomes [491]. Altogether, these observations suggest that developing early interventions to maintain the postsynaptic targets in the brain after RGC loss may be valuable. Moreover, the initial clinical translation of optic nerve degeneration approaches in humans may be most successful if it targets patients with relatively acute and recent vision loss. While this may not be possible in most cases of patients with glaucoma, future efforts

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may need to focus on developing strategies to regenerate retinorecipient tissue in the brain to maximize the potential for vision restoration.

Future directions for eye-to-brain connectivity

A combinatorial approach to advancing methods of promoting eye-to-brain connectivity must be considered and should combine work investigating signaling pathways necessary for neuronal survival, axon regeneration, and chemotropic guidance of axons. Given the long distance of RGC axonal projections and the complexity of postsynaptic wiring in humans, experimental models that resolve regenerating axons at the single axon fiber level will be needed. This approach may also enable researchers to investigate the role of neural activity and panels of guidance cues in axon regeneration since RGC subtypes are likely to differ in their responsivity to specific signals. A relatively unexplored area of investigation is the development of strategies that aid donor RGCs in connecting with postsynaptic target neurons in the brain. Investigators should also explore mechanisms underlying the retinotopic mapping of synapses and consider ways to manipulate this process so that incoming signals to the lateral geniculate nucleus will be interpretable.

Future studies should undertake multimodal investigations of glial function during development, degeneration, and axon regeneration within the visual pathway. Single-cell sequencing, optogenetics, chemogenetics, and transgenic models might be used to identify specific roles of glial subpopulations in promoting or inhibiting axon regeneration (Table 5).

Conclusions and future directions

RGC death is a major cause of irreversible vision loss, and regenerative approaches for restoring vision lost to optic neuropathies are crucial. Since ophthalmology is at the forefront of regenerative cell therapy, the RReSTORe Consortium was organized to address the challenges associated with RGC repopulation. Through multiple in-depth virtual and face-to-face discussions, members of the RReSTORe consortium have built consensus and identified the most pressing challenges, questions, and suggested approaches that must be addressed to bring RGC repopulation closer to clinical translation. As is often the case for fields on the cusp of transformative progress, discussions within the RReSTORe Consortium have yielded more questions than answers. Through collaborative experimental efforts, the scientists who are a part of this consortium will help to advance the field and bring hope to patients suffering from severe optic neuropathy. While significant obstacles remain, recent scientific advances described here suggest that functional RGC repopulation in humans suffering from optic neuropathy may be feasible, and they provide a roadmap for continued scientific progress.

While consensus building and groupthink can unify a scientific community in its pursuits, we acknowledge that they do not always propel groundbreaking discoveries. Indeed, the visual science field, despite concerted efforts, has yet to develop a definitive cure for optic neurpathic vision loss or even a neuroprotective treatment that substantially slows disease progression. We recognize this criticism and emphasize that the perspectives presented in this manuscript are not exhaustive and

Table 5 Future directions for eye-to-brain connectivity (SDG5)

Research Area	Future Goals
Combinatorial approaches to multimodal reinnervation of the brain	Implement a combinatorial approach to understand different signaling pathways necessary for neuronal survival, axon regeneration, and guidance to direct eye-brain connectivity
Distal injury models and in vitro models	Employ these models to simplify experimentation, study regeneration at the single axon level, and investigate the role of neural activity in axon regeneration. Different RGC subtypes may require different guidance cues
Role of glial cells	Use techniques such as single-cell sequencing, optogenetics, chemogenetics, and transgenic mouse lines to identify specific roles of glial subpopulations in promoting or inhibiting axon regeneration. This may include physiologically 'normal' glial subtypes, or one of many reactive glial sub-states
Overcoming mechanical blockages	Explore the development of strategies that aid donor RGCs in connecting with downstream neurons in the brain, specifically overcoming the mechanical blockage of the lamina cribrosa
Adult retinal and brain microenvironment	Investigate the spatial–temporal expression/induction of guidance signals in the adult environment and engineer RGCs to respond to specific cues present in the adult retina to promote axon regeneration
Neural Activity	Investigate the role of neural activity in axon regeneration among RGC subtypes to develop combinatorial strategies for promoting regeneration broadly
Retinotopic mapping	Evaluate whether regenerating RGC axons synapsing at subcortical visual centers establish a retinotopic map and develop methods for modulating this process
Brain regeneration	Develop strategies to regenerate retinorecipient tissue in the brain in optic neuropathies, which may overcome issues with anterograde transsynaptic degeneration in longstanding optic neuropathy

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should not be viewed as the only path forward. We hope this roadmap can serve as a foundation and a springboard for diverse perspectives and unconventional ideas from the broader research community, including those outside of the consortium, to propel the field forward. Investigators who may wish to join the consortium can find further information at http://rrestore.info, or by contacting the corresponding author.

Appendix
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Abbreviations

AO	Adaptive optics
BAX	BCL2-Associated X protein
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DLK	Dual leucine zipper kinase
ERG	Electroretinography
GCL	Ganglion cell layer
HLA	Human leukocyte antigen
iPSCs	Induced pluripotent stem cells
ILM	Inner limiting membrane
IPL	Inner plexiform layer
IL-1α	Interleukin 1 alpha
IOP	Intraocular pressure
IVT	Intravitreal
ipRGC	Intrinsically photosensitive RGC
KLF	Kruppel-Like Factor
LKZ	Leucine zipper kinase
mTOR	Mammalian target of rapamycin
MG	Müller glia
NF1	Neurofibromatosis type 1
Nr-CAM	Neuronal cell adhesion molecule
OPG	Optic pathway glioma
OCT	Optical coherence tomography
PTEN	Phosphatase and tensin homolog
RGC	Retinal ganglion cell
RPC	Retinal progenitor cells
RReSTORe	RGC Repopulation, Stem cell Transplantation, and Optic nerve Regeneration
SLO	Scanning laser ophthalmoscopy
C1q	Subcomponent q
SR	Subretinal
SDG	Subtopic discussion group
SOCS3	Suppressor of cytokine signaling 3
TNF	Tumor necrosis factor

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Not applicable.

Declarations

Ethics approval and consent to participate
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Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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