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Optic nerve diseases and regeneration: How far are we from the promised land?

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Abstract

The retinal ganglion cells (RGCs) are the sole output neurons that connect information from the retina to the brain. Optic neuropathies such as glaucoma, trauma, inflammation, ischemia and hereditary optic neuropathy can cause RGC loss and axon damage, and lead to partial or total loss of vision, which is an irreversible process in mammals. The accurate diagnoses of optic neuropathies are crucial for timely treatments to prevent irrevocable RGCs loss. After severe ON damage in optic neuropathies, promoting RGC axon regeneration is vital for restoring vision. Clearance of neuronal debris, decreased intrinsic growth capacity, and the presence of inhibitory factors have been shown to contribute to the failure of post-traumatic CNS regeneration. Here, we review the current understanding of manifestations and treatments of various common optic neuropathies. We also summarise the current known mechanisms of RGC survival and axon regeneration in mammals, including specific intrinsic signalling pathways, key transcription factors, reprogramming genes, inflammation-related regeneration factors, stem cell therapy, and combination therapies. Significant differences in RGC subtypes in survival and regenerative capacity after injury have also been found. Finally, we highlight the developmental states and non-mammalian species that are capable of regenerating RGC axons after injury, and cellular state reprogramming for neural repair.

Keywords

axon regeneration; optic nerve injury; optic neuropathies; retinal ganglion cells; survival

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1 | INTRODUCTION

Vision is essential for humans to explore the world. Visual information is focused and projected onto the retina, where it is captured as light impulses by photoreceptors and transmitted to bipolar cells and then to the third order neuron retinal ganglion cells (RGCs). RGCs extend their axons toward the optic disc, forming the nerve fibre layer, which make a 90° turn and leaves the eye at the optic disc. The 1.2 million RGC axons then form the optic nerve (ON) that help transmit signals from the eye to the brain.[1,2] The ON is surrounded by CSF and three layers of meninges. It is set apart from the peripheral nervous system (PNS) partly due to its limited regenerative ability and the presence of oligodendrocytes instead of Schwann cells for myelin formation.[3] Due to their length, trajectory, and spatial constraints, these axons are vulnerable to various damages.[4,5] Understanding the pathophysiology and clinical manifestation of various optic neuropathies is crucial for their timely diagnoses and treatments to prevent massive RGCs death, which will lead to inevitable of visual loss. After sever injury of the ON, promoting the RGC and axon regeneration is a matter of course for visual function recovery.

Similar to other parts of the adult mammalian central nervous system (CNS), damaged axons in the ON do not automatically regenerate after injury. Unlike mammals, however, other vertebrates such as *Dario renio* (zebrafish) and *Xenopus laevis* (African clawed frogs) possess extraordinary intrinsic regenerative abilities.[6] The loss of mammalian CNS regeneration could be the result of evolution and natural selection. With the evolution of species, the CNS has become more and more sophisticated and complex. Unlike the orderly regulation during development, it is extremely difficult to restore the original tissue structure and physiological function after CNS injury for adult mammals. Incorrect nerve regeneration in the CNS might cause serious consequences, such as long-term memory disruption or other mental disorders, which probably lead to the elimination of mammalian individuals with regenerative abilities in the evolutionary process.[7] However, the regrowth ability of PNS is preserved much better than CNS during evolution, since its structure and function are relatively simple and consistent, and its incorrect regeneration may not always cause grievous consequences. As part of the CNS, the regenerative capacity of ON is correspondingly lost, leading to enormous difficulties for visual restoration, which eventually lead to blindness.[8]

The failure of mammalian CNS neurons to regenerate axons is caused by a combination of intrinsic defects and extrinsic factors. Though there are still hurdles to overcome and very few reparative strategies have moved from the bench to the bed, some promising candidates have been reported. In this review, we first discuss the pathophysiology, diagnoses and treatments of common optic diseases, followed by therapeutic advance of RGC survival and axonal regeneration, which could be considered as potential candidates in clinics for vision recovery.

2 | OPTIC NEUROPATHIES

Optic neuropathy refers to compromised function of the optic nerve that may be caused by a variety of pathologic processes, etiologically categorised as inflammatory optic

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neuropathy, glaucomatous optic neuropathy, ischemic optic neuropathy, toxic/nutritional optic neuropathy, traumatic optic neuropathy, and hereditary optic neuropathy. The damage of optic nerve by these various etiologies may result in the loss of RGCs which do not regenerate in higher species animals leading to irreversible visual impairment or complete vision loss. The accurate diagnosis of optic neuropathy is crucial for timely treatments to prevent irrevocable RGCs death, which is made on the basis of history taking and clinical examination. Optic neuropathies result in alteration of the five optic nerve functions, including visual acuity, visual field, colour vision, subjective brightness and a relative afferent pupillary defect (RAPD) when the defect is unilateral or asymmetrical. The optic nerve structure will also undergo changes with initial swelling then disc pallor.[9]

2.1 | Inflammatory optic neuropathy

Inflammatory optic neuropathy is an inflammation of the optic nerve, with classic clinical manifestations of subacute and unilateral vision loss, eye movement pain, decreased contrast and colour sensitivity, and a RAPD. Optic neuritis is an inflammation of the optic nerve and may be due to demyelination, infectious, para-infectious and vasculitic causes. For demyelinating optic neuritis, it is in a continuum with clinically isolated syndrome being on one end and systemic multiple sclerosis being on the other end. The MRI scan is the most powerful predictor to determine the risk of conversion. There is no consensus regarding the classification of optic neuritis, and precise diagnostic criteria are not available. Usually, autoimmune optic neuritis is recrudescent and require long-term immune suppression therapy, and infectious optic neuritis is generally monophasic, although there are some overlaps between them such as Multiple Sclerosis-associated optic neuritis (MS-ON) which is related to autoimmune disorder but remains monophasic.[10] Vision improvement and the relief of symptoms usually occur over the next few weeks, with remyelination and sodium channels redistribution improving conduction but increasing the vulnerability of surviving axons.[11]

In the pathophysiology of the of MS-ON which is very similar to MS lesions in the brain, the occurrence of the immune system dysregulation and inflammatory demyelination leads to pro-inflammatory cytokines releasing and conduction block and thus visual impairment, which is associated with the activation of T-cell principally, B-cell and microglial cell probably.[12] Neuromyelitis optica associated optic neuritis (NMO-ON) is another subtype of inflammatory optic neuropathy with more severe loss of visual function, less chance of spontaneous recovery and a high risk of recrudescence. This disease results in CNS demyelination lilkely due to IgG autoantibody–mediated loss of aquaporin-4 (AQP4) water channel protein on astrocytes. The pathophysiology of NMO–ON seems to involve the interaction between astrocytes and microglia and its C3a receptor signal pathway critically associated activation of cytolytic complement in NMO lesions.[13]

According to patient's characteristics of suspected inflammatory optic neuropathy, assessment of OCT, brain MRI and various serum biomarkers should be taken into consideration as paraclinical tests.[10] For management of inflammatory optic neuropathy, finding the cause of the inflammatory optic neuritis is highly important and would be governed by patient demographics. For instance, in MS-ON in Caucasian population,

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intravenous steroid followed by oral treatment hasten the rate of vison recovery but does not change the final vision. In NMO-ON, the patient may require higher dose and longer immunosuppression. In acute attack phase, plasmapheresis can also be considered in severe refractory situations.[10,12] Though these timely treatments can alleviate inflammation to prevent further damage of RGCs and axons, they are incapable of promoting regeneration of RGCs and axons.

2.2 | Glaucoma

Glaucoma can be classified into open-angle and angle-closure anatomically. Each of these is further divided into primary or secondary, denoting the absence or presence of underlying disorders responsible for the development of glaucoma.[14] Primary open-angle glaucoma (POAG) is the most common optic neuropathy worldwide with the characteristic of slow, progressive, and irreversible RGC degeneration, clinically presenting absence of symptoms in early stage, damaged visual field, progressive optic nerve head (ONH) cupping with classically vertical elongation, with or without (normal tension glaucoma) increased intraocular pressure.[15,16] The pathophysiology of glaucoma is characterised as degeneration of RGC related to increased intraocular pressure which causes compression and deformation of ONH and impediment to axonal transport,[17] and dysfunction of RGC mitochondria mediated by oxidative phosphorylation stress.[16] Other factors such as low ocular perfusion pressure, and gene mutations may also play a role.[15] It is significant for ophthalmologist to refer patients with family history of glaucoma or suspicious ONH findings for further ophthalmologic examination. The generally accepted therapeutic strategies for glaucoma are topical ocular hypotensive drops, laser therapy, and surgical intervention to reduce the risk of further progression.[8] Investigations indicated that erythropoietin[18] and blockage of purinergic P2X7 receptor, an inflammation mediator, are beneficial on neuroprotection in rat glaucoma models.[19]

2.3 | Ischemic optic neuropathy

Ischemic optic neuropathy (ION), the most common acute optic neuropathy in patients of age over 50, results from infarction of ON. It is classified clinically into anterior ischemic optic neuropathy (AION) with the presence of the optic nerve head swelling, and posterior ischemic optic neuropathy (PION) which is much rarer than AION with the posterior part of the ON affected.[20] Both AION and PION can be further classified into arteritic when caused by vasculitis such as giant cell arteritis, and nonarteritic when secondary to other entities such as thromboembolism and systemic hypotension.[20]

The pathophysiology of nonarteritic AION, the most common type of ION, is not fully established, but probably associated with ischemic event from small vessel ischemia or hypoperfusion. This results in optic nerve swelling and a vicious circle of compartment syndrome. The swelling of ON is mechanically compressed within a tight scleral canal, leading to axonal flow and capillary perfusion impairments, with subsequently cytotoxic factors released and ischemia worsened, and this cycle continues, inducing axonal degeneration and RGCs loss through apoptosis.[20,21] The arteritic AION cases are usually caused by giant cell arteritis (GCA) in population over 65 years of age, which is a systemic granulomatous vasculitis mediated by immunity with histological evidence

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affecting medium and large arteries, accompanied with dreaded complication of visual loss and second eye involvement.[22]

The diagnosis of ION principally relies on clinical manifestation of painless, acute and commonly unilateral visual loss, visual field defects and RAPD. Arteritic AION has waxy disc pallor oedema due to disc infarction and NAION has hyperemic disc oedema in the acute stage, following optic disc pallor 4–6 weeks in both forms after the beginning of visual loss.[20] Although there is still no proven treatment for ION, it is essential for ophthalmologist to rule out GCA when assessing patients with suspected ION especially those older than 50 years old, through blood tests of complete blood count (CBC), platelet count, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP).[20,22] There are some empirical therapies for NAION in practice such as steroids, levodopa and erythropoietin (EPO), however, a recent meta-analysis concluded that no effective therapy for NAION is recommended and further investigations are needed.[23]

2.4 | Toxic/nutritional optic neuropathy

Toxic optic neuropathy (TON) refers to the damage of ON caused by exogenous agent exposure, including medications, metals, organic solvents and methanol; while nutritional optic neuropathy is thought to arise from nutritional deficits such as B vitamins, folic acid and copper. The increasing incidence of nutritional optic neuropathy may be related to the widespread of gastric bypass surgeries and vegan diets.[9,24] However, the causal connection remains unproven and very few unequivocally confirmed cases of optic neuropathy are described to be caused by a identified single toxin or nutrient deficiency.[25]

The pathophysiology in both forms involve in biochemical pathways of cell energy metabolism, oxidative stress and free radicals, triggering dysfunction of the ON.[26,27] Toxic and nutritional optic neuropathies have similar clinical manifestations: progressively bilateral and symmetrical central visual impairment, declined colour and contrast sensitivity, central or cecocentral scotomas, and pale optic disc in the late stage due to the loss of papillomacular bundle where the parvocellular RGCs have more limited mitochondrial energy reserves and a lower threshold to apoptosis.[9,28]

The diagnosis of TON can be supported by clinical suspicion of symptoms and history of toxin exposures, any degree of recover after removal of toxins, and exclusion of alternate causes.[29] For nutritional optic neuropathy, laboratory tests such as serum vitamin, serum protein, micronutrient (cobalamin, folic acid, thiamine, and copper), blood count and metabolic panel are recommended for diagnosis.[30] The management of toxic and nutritional optic neuropathies involves suspension of the offending toxins and supplementation of deficient nutrients. Timely identification and intervention can prevent further damage to RGCs and axons, and result in better visual prognosis.[9,30] Lost neurons and their axons, however, cannot be replaced and regenerated automatically.

2.5 | Hereditary optic neuropathy

Hereditary optic neuropathy (HON) refers to the dysfunction of optic nerve determined genetically, among which the most common two are Leber hereditary optic neuropathy (LHON) and autosomal-dominant optic atrophy (DOA), with the underlying mechanisms of

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selective loss of RGCs and papillomacular bundle involvement.[3,31] Disturbed mitochondrial function and complex I respiratory chain defect likely contributes to the RGC loss in LHON and DOA. The underlying genetic abnormality in LHON is a point mutation of mitochondrial DNA (mtDNA) gene encoding complex I, most commonly 11778G > A (MT-ND4), 14 484T > C (MT-ND6), or 3460G > A (MT-ND1). On the other hand, the mutation of DOA is primarily due to OPA1 gene mutation which encodes a mitochondrial inner membrane protein GTPase related to transmembrane dynamic.[31] However, the trigger for selective RGC loss presents great complexity in mitochondrial disturbances, other mechanisms including disruption of mitochondrial network dynamics, impairment of mtDNA maintenance, instability of mitochondrial respiratory chain complexes, and defect in mitochondrial gradient maintenance.[31,32] Clinical manifestation of hereditary optic neuropathy includes symmetric and bilateral central visual loss without pain, cecocentral scotomas and temporal optic disc pallor due to papillomacular bundle involvement.[3,31] The primary therapeutic strategy for LHON is use of antioxidants agents, particularly oral idebenone 900 mg/day for 24 weeks to help prevent further vision impairment. Idebenone is a coenzyme Q10 analogue and approved by the Food and Drug Administration (FDA) for the treatment of LHON. Other strategies under consideration include drugs that target the key pathogenic mechanisms, as well as gene therapy and gene editing.[33]

2.6 | Traumatic optic neuropathy

The optic nerve is vulnerable to direct trauma and more commonly, indirect trauma. Direct trauma arises from penetrating injury especially orbital and mid-facial fractures presenting various ON injury such as avulsion, transection, and ON sheath haemorrhage. Indirect trauma is caused by the force of orbital or cranial impact results in traction, increased intraorbital pressure, ON compression or axonal shearing.[34]

Since the dura of ON tethers the periosteum within the canal, the transmitted indirect compression forces may create a shearing force, leading to either ON or the pial vascular compression. The pathophysiology of traumatic optic neuropathy may involve lipid peroxidation of cell membranes caused by oxygen-free radicals, intracellular calcium and arachidonic acid releasing, as well as cell mediated inflammation.[35,36] In the injured optic nerve, the poor phagocytic capacity of resident microglia leads to ineffective clearance of growth-inhibitory myelin debris, contributing to the inhibition of RGC regeneration and poor ON recovery.[37]

The clinical presentation is diverse, depending on the type and severity of injuries. The history of head trauma is distinctive, occurrence of visual impairment of one eye with a RAPD and visual field defects, and atrophy of ON 4–6 weeks later. However, sometimes it may be difficult to detect in acute stage of trauma if there are other multitrauma and co-morbidity.[3] The diagnosis of location and nature of the injury depends on clinical examination and neuroimaging examination especially Orbital CT. The management of traumatic optic neuropathy should be individualised for each patient, which is still short of consensus and high-quality evidence, therapeutic strategies including observation, corticosteroids and surgical decompression.[38]

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3 | NEURAL REPAIR AFTER OPTIC NEUROPATHY

For various types of optic neuropathy, the corresponding treatment strategies mentioned above aim to halt the progression of the diseases and prevent further RGC loss. In addition to the etiological treatments, further understanding the molecular mechanisms of RGC loss after ON injury may help to achieve maximum RGC rescue, which is discussed in more detail below.[39] Promoting RGC survival, however, does not meant to granted vision recovery, especially in situations with massive axonal degeneration and neural connection loss. Therefore, promoting ON axon regeneration is crucial to re-establish neural connections to the brain for visual signal transduction. Unlike the PNS, however, injured axons in the ON lack of the ability to spontaneously regenerate. Illustrating contributing factors and their underlying molecular mechanisms will benefit the discovery of novel treatment strategies for promoting ON regeneration and to achieve vision recovery.

3.1 | Retinal ganglion cell loss after optic nerve injury

Optic nerve damage leads to retrograde signalling to RGC somas, and subsequently cause devastating neuron death. The apoptosis mechanism in RGCs may be related to the direct destruction of membrane integrity after the mechanical injury to the axons, the lack of trophic factors caused by the interruption of axonal transport, and the loss of nutrient support after ischemia. Many endogenous genes are involved in the regulation of apoptosis and the survival of RGCs after optic nerve injury (ONI) including *Bcl-2/Bax, DLK/LZK, c-Jun, p53, Caspases*, and *FasL*. The proteins encoded by these genes form a complex network and are regulatory switches that determine cell survival and apoptosis.

The Bcl-2 gene family includes anti-apoptotic genes (Bcl-2, Bcl-xl), pro-apoptotic genes (such as Bax gene), and BH3-only genes. In the retina, interaction of these Bcl-2 family members modulates intrinsic apoptotic programs induced by death signals.[40] Up-regulation of Bax protein was found in degenerating RGCs 3–5 days after ONI.[41] Overexpression of Bcl-2 or deletion of Bax gene shows RGC neuroprotection following optic nerve damage.[42,43] A series of stress response signals following Bax activation occurs early after ONI, including dual leucine zipper kinase/leucine zipper kinase (DLK/LZK) signalling and c-Jun N-terminal kinase (JNK) signalling pathway. DLK may be responsible for regulating the initial stress response signal after injury. The expression of DLK is increased in axons immediately after ONI and then in the cell soma.[44] Downstream targets of DLK include activation of MKK4/7 and JNK, which can participate in feedback loops and stabilise DLK activity. Double Jnk2/3 knockout protected RGCs from chromosomal death after optic nerve crush (ONC) in mice.[45] Endogenous and exogenous apoptosis pathway involves activation of different caspases, which are a family of protease enzymes playing essential roles in programmed cell death. Active caspase-2,[46] −3, and −9 are found to be involved in RGC death after ONI.[47] Intraocular injection of caspase-3 or capase-9 inhibitors promotes modest protection of RGCs against optic nerve transection-induced apoptosis.[47,48] Intravitreal injection of siRNA for caspase-2 enhances RGC survival rate.[46] Apoptotic protease activating factor-1 (APAF-1) is a key mediator of caspase-induced cell death. siRNA-based knockdown of APAF-1 also improved RGC survival.[49] In addition, mitochondria are the core sites of interaction between anti- and pro-apoptotic Bcl-2 family

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members. When the balance tilts to apoptosis, the permeability of mitochondrial membrane increases and a variety of apoptotic mediators are released, such as cytochrome c, which leads to eventual RGC death.[50]

3.2 | Intrinsic pathways and transcriptional regulation for axon regeneration

Similar to other parts of CNS in the mammals, RGCs also exhibit different regenerative capacities in different species and developmental stages. These observations hint the possibility of manipulating intrinsic factors to achieve RGC axon regeneration in the adulthood. Embryonic RGCs showed significantly greater axon regeneration than those harvested after birth,[51] which indicates the switch of intrinsic states for axon regeneration. Therefore, these innate programming molecules have been considered important for understanding RGC survival and regeneration. With the advances in gene manipulating techniques and viral vectors, several critical pathways for intrinsic regenerative ability in RGCs have been found over the past decade. These include the phosphatase and tensin homologue (PTEN)/PI3K/Akt/mTOR pathway[52] and the Janus kinase (JAK)/Signal transducer and activator or transcription 3 (STAT3)/Suppressor of cytokine signaling 3 (SOCS3) signalling pathways.[53] Beside the direct manipulation of the regenerative signalling pathways, changing the expression of target genes for axon regeneration by modulating transcription factors (TFs) is another efficient approach. TFs are deoxyribonucleic acid binding proteins that can activate or repress gene expression. Their effects are amplified by their ability to bind to multiple sites in the genome, resulting in a large number of genes being turned on or off.[54] Several promising TFs have been discovered and investigated intensively for their roles in RGC axon regeneration including the Klf family members, Sex-determining region Y (SRY)-box-containing gene 11 (Sox11), c-Myc, and RE1-Silencing Transcription Factor (REST).[55] Studies on intrinsic pathways and transcriptional factors have been reviewed and discussed comprehensively previously,[56-58] which will not be elaborated again in this review.

3.3 | Subtype-specific RGC survival and regeneration

With the advances of single-cell RNA sequencing, RGCs in the retina can be divided into at least 45 subtypes by transcriptional analysis. RGCs are essentially similar in their developmental origin, general morphology and overall synaptic structure. However, distinct subtypes of RGCs differ in their ability to recover from axonal injury.[59] Moreover, axonal regeneration is consistently limited to <10% of RGCs after ONI, regardless of the number of surviving RGCs. Twelve RGC subtypes have been found to survive better than the rest. These neurons are mostly alpha-RGCs (α-RGCs), the largest RGC subtypes, and intrinsically photosensitive RGCs (ipRGCs), a type with the ability to respond directly to light due to the presence of melanopsin (Opn4) protein.[59,60] Higher levels of mTOR were detected in αRGCs compared to other RGC subtypes. Alpha RGCs and M1-ipRGCs preferentially survived and extended axons after ONI in the background of Pten-deficiency.[60] When overexpressed, Sox11 promoted the regeneration of non-α-RGCs but killed α-RGCs.[61] RNA-seq analysis comparing ipRGCs and other subtypes revealed that thrombospondin-1 (Thbs1) is highly expressed in ipRGCs, and ectopic overexpression of Thbs1 promoted regeneration for both ipRGCs and non-ipRGCs. Taken together, these studies have demonstrated that different subtypes of RGCs have different cell survival

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rate and intrinsic axonal regeneration capacity after injury due to different transcriptome patterns.

3.4 | Epigenetic regulations for axon regeneration

During differentiation and development of the retina and optic nerve, the entire gene expression profile of RGCs changes dramatically, with “youth” genes switched off and genes related to mature RGCs expressed. The regulation of gene expression is achieved primarily through epigenetic modifications, including DNA methylation, histone modifications and non-coding RNA. Theoretically, RGCs can be reprogrammed to regenerative “youth” stage by overexpressing several reprogramming factors resulting in global epigenetic remodeling.[62] Studies have found that Ascl1a, Apobec2a and Apobec2b were involved in optic nerve regeneration, implying that DNA demethylation may underlie the regeneration program.[63] In fact, forced expression of Oct4, Sox2 and Klf4 in RGCs induced global DNA demethylation, which promotes axonal regeneration in the ONC model.[64] Furthermore, mRNA methylation-dependent maintenance of protein translation has been shown to be required for Pten deletion-induced optic nerve regeneration.[65]

In addition, epigenetic regulation through microRNAs and altering genomic accessibility is a major mechanism for the formation of certain transcriptomes. The Lin28 protein normally acts by inhibiting the biogenesis of let-7 microRNAs. Lin28a also has DNA binding capacity and can drive gene transcription by recruiting the epigenetic modifier Tet1.[66] Overexpression of Lin28 in RGCs with adeno-associated virus 2 (AAV2) significantly promoted optic nerve regeneration. This may be attributed to activated Akt and mTOR pathways with concomitant inhibition of the GSK3β pathway shown in sensory neurons.[67] Lin28b overexpression also led to increased transcription of c-Myc and Tet3, linking Lin28 signalling to c-Myc and DNA methylation.[68] Tet enzymes involved in DNA demethylation have been shown to play a key role in cellular reprogramming. Thus, in addition to acting as a reprogramming factor, association with c-Myc and Tet3 further implies that Lin28 may enhance axonal regeneration through epigenetic regulation.[69]

3.5 | Inflammatory stimulation for optic nerve regeneration

Inflammation in immune diseases is often assumed to be harmful. However, studies have shown that inflammation induced by zymosan or lens injury is beneficial to neural repair after optic nerve injury. Immune cells such as macrophages, neutrophils and microglia, together with their secreted factors and neurotrophic factors contribute to the inflammation-promoted neural repair after ONI.

Lens injury can occur unintentionally during intravitreal injection procedure when delivering repair-promoting factors experimentally in rats and mice, and cause inflammation in the eye. This inflammatory reaction strongly promotes RGC survival and regeneration.[70] Intravitreal injection of inflammation inducer, zymosan but not lipopolysaccharide (LPS), was shown to stimulate macrophage/neutrophils infiltration and neural repair after ONI.[71] Although LPS can similarly recruit macrophages/neutrophils, it does not promote axon regeneration.[72] This is because zymosan binds to the receptors dectin-1 expressed in both resident retinal microglia and invading blood-borne macrophages, and uniquely stimulates a signalling

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cascade that can induce axon regeneration.[72] Ciliary neurotrophic factor (CNTF), a cytokine that belongs to the interleukin 6 (IL-6) family, plays key roles in immune homeostasis and inflammation. When exogenously delivered, CNTF has been shown to attract macrophages to the eye and promote RGC survival and axon regeneration.[73] Oncomodulin from macrophages and neutrophils was shown by the Benowitz group to be an critical mediator of inflammation-mediated axon regeneration in the background of cAMP elevation.[74,75] Furthermore, a chemokine known as C-C motif chemokine ligand 5 (CCL5), was recently discovered to be expressed in several immune cells including macrophages, and retinal glia. Importantly, this chemokine mediated the CNTF induced RGC axon regeneration. Inversely, CRISPR-mediated knockdown of the cognate receptor (CCR5) in RGCs suppressed the effects of CNTF gene therapy.[76]

Another chemokine stromal cell–derived factor 1 (SDF1 or CXCL12) was found highly expressed in the infiltrative macrophages and played a central role in inflammation-induced RGC survival and axon regeneration. Selective deletion of *Cxcl12* in myeloid cells ($CXCL12^{flx/flx}$LysM-$Cre^{-/+}$ mice) or selective deletion of the complementary receptor CXCR4 (intraocular AAV2-Cre in the $CXCR4^{flx/flx}$ mice) in RGCs greatly suppressed inflammation-induced axon regeneration and RGC survival.[77] Inversely, recombinant SDF1 complements the effects of oncomodulin/cAMP and promoted RGC survival and axon regeneration, similar to those induced by intraocular inflammation. In contrast to the deletion of Pten, which promotes regeneration selectively from αRGCs, SDF1 promoted regeneration from non-αRGCs and enabled the latter cells to respond robustly to Pten deletion.[77] In addition, chemokine CXCL5 was later discovered to be a mediator capable of attracting immune cells into the eye for neural repair after ONI. Delivery of recombinant CXCL5 promoted RGC survival and axon regeneration both in vivo and in retinal explants, with increase in the number of activated microglia. This effect was inhibited by the CXCL5 receptor CXCR2 antagonist SB225002. CXCL5 further promoted lens injury-induced RGC protection with increase in the number of activated $CD68^{+}$ cells. SB225002 inhibited lens injury-induced cell infiltration and activation, and attenuated the survival and regeneration promoting effects on RGCs through reduction of lens injury-induced Akt activation.[78] Although the role of activated microglia in the CXCL5–induced RGC survival and axon regeneration is unclear, it has been shown to be required for RGC axon regeneration with complement proteins C1q and C3. Importantly, genetic deletion of C1q, C3, or CR3 attenuates RGC axon regeneration induced by lens injury. Collectively, these results indicate that the complement-myeloid phagocytic signalling is required for RGC axon regrowth.

Clearly, inflammatory cells and mediators play important roles in neural repair after ONI. Though some mediators have been discovered in the recent years, the full picture of inflammation-induced RGC survival and axon regeneration is complex in terms of spatial and temporal aspects, involving multiple factors, and inflammatory cells. In-depth studies on mechanisms of inflammation-stimulated neural repair after ON injury are required.

3.6 | Extrinsic factors regulating optic nerve regeneration

The failure of axon regeneration following ON injury is not only due to the low intrinsic regenerative capability, but also to the lack of neurotrophic factors and inhospitable

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surrounding environment for neuron survival and axon regeneration, such as glial scar and growth inhibiting cues.[79] Lengthy regrowth of cut axons from RGCs after peripheral nerve transplantation into the retina of adult rats suggested that RGCs have the intrinsic ability to regenerate axons if the external environment is favourable.[80] A balance between growth promoting cues and growth inhibiting cues may regulate the structural and functional changes of the injured mature CNS. Promotion of axon regeneration after ON injury can be achieved by decreasing the extrinsic growth inhibitory factors and increasing extrinsic growth promoting factors.[81]

3.6.1 | Neurotrophic factors—Neurotrophic factors (NFs) are secreted proteins that play an important role in regulating neuronal survival, axon growth, synapse formation and cell migration.[82,83] Together with the corresponding receptor, NFs can be transported retrogradely to the neuronal cell body through the axon plasma, and promote neuronal survival and axon growth.[84] These NFs include neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and neurokines such as CNTF.[85] Neurotrophin family members bind to tyrosine receptor kinase (Trk) and trigger intracellular signalling pathways, such as the classical PI3K/ AKT pathway and the Ras/Raf/ERK kinase cascade. These pathways have been studied extensively for their roles in axon growth.[86] Intravitreal administration of BDNF or AAV2-mediated overexpression enhances RGC survival, but does not promote significant axon regeneration after ONC injury or after PN grafts transplantation.[87,88] CNTF is a major candidate for the treatment of various eye diseases. It has been shown that CNTF, no other trophic factors, promotes axonal regeneration of axotomized RGCs in adult hamsters.[89] Recombinant CNTF protein or AAV2-CNTF can promote both RGC survival and axon regeneration.[73,90]

3.6.2 | Glial scar—Although glial scar contains and separates damage in space as a repair process, it can be a mechanical barrier to growth and create an inhospitable environment for CNS regeneration. Other studies have indicated that reactive astrocytes in fact aid axon regeneration. CNS scar consists of two components; a lesion core with fibroblasts and inflammatory cells, and an astrocyte lesion border which is primarily composed of reactive astrocytes.[79,91] Historically, the term glial scar refers to the astrocytic component.[92] In the optic nerve crush model, inhibition of microRNA-21 (miR-21) moderates excessive reactive astrocytes and glial scar progression and thus promotes axon regeneration.[93] In addition, up-regulation of matrix metalloproteases (MMP) and down-regulation of tissue inhibitors of MMP (TIMP) may weaken the inhibitory effect of the glial or collagen scar after ONI.[94] Moreover, pharmacologically blocking the interaction between reactive astrocytes and collagen type I (i.e. a process which promotes glial scar formation via integrin and N-cadherin) attenuated the inhibitory glial scar formation and promoted axon regeneration.[95]

3.6.3 | Inhibitory molecules—Apart from glial scar, axon guidance molecules also account for the limited axon regeneration. Ironically, these axon guidance molecules are important for CNS development and homeostasis. Axon guidance molecules can be divided into three families. One, classic axon guidance molecules which work as inhibitory guidance cues after CNS injury including semaphorins, ephrins, netrin and slits.[81] Enhancement

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of ON axon regeneration has been shown in mice with ephrinB3 gene deletion.[96] Two, myelin-associated inhibitors , which originate from oligodendrocytes after injury, and bind to receptors of multiple components that are localised to the membrane of neuronal cells including Nogo receptor 1 (NgR1), Lingo-1, p75NTR, and TROY. Activation of these receptors induces the activation of RhoA/ROCK.[97] Paired immunoglobulin-like receptor B (PirB) is a high-affinity receptor for the myelin-derived inhibitory molecules l.[97] Selective ablation of Nogo-A in ON oligodendrocytes and myelin exerts greater effects on axon regeneration than the prior global Nogo-A knockout model.[98] Three, chondroitin sulphaate proteoglycans (CSPGs), which are secreted by the reactive astrocytes was shown to limit axon sprouting and extension near the injured lesion site.[99] CSPGs also facilitate inhibition effects through binding to two receptors of myelin-associated inhibitors, NgR1 and NgR3, which indicates shared mechanisms of myelin-associated inhibitors and CSPG inhibition.[100]

Multiple growth inhibitory molecules facilitate their inhibitory effects through altering the RhoA/ROCK signaling pathway, which is an important regulator of actin cytoskeleton. Activation of this pathway leads to rapid growth cone collapse and axon regeneration inhibition.[101] Others have shown that Sema3A inhibits ON regeneration through ROCK2.[102] The Rho/ROCK signalling pathway also mediates the inhibitory effects of myelin-associated inhibitors and CSPGs.[97,103] Therefore, numerous growth inhibitory molecules converge on RhoA/ROCK, and inhibition of RhoA/ROCK could be a potential therapeutic strategy to promote ON regeneration.[90,101,102]

3.7 | Stem cell therapy for ON regeneration

Stem cells are widely found in human and animal tissues and organs, which have the potential for infinite proliferation and multi-directional differentiation.[104] These two characteristics pace them an important source of cell therapy materials. There are at least two ways where stem cells could be beneficial after ON injury. One, transplanted exogenous stem cells can produce neurotrophic factors, which in turn promote RGC survival and axon regeneration. Alternatively, stem cells can be programmed to become neurons and replace the damaged RGCs.

Neurotrophic factors promote RGC survival and axon regeneration after ON injury. Mead et al.[105] have demonstrated that intravitreal injection of dental pulp stem cell (DPSCs) promotes neurotrophin-mediated RGC survival and axonal regeneration after ON injury. Subsequently, mesenchymal stem cells (MSCs),[106] periodontal ligament stem cells (PDLSCs)[107] and neural stem cells[108] were shown to exert protective effects on RGCs and promote axonal regeneration by upregulating the expression of neurotrophic factors. It was shown recently that human PDLSCs after intravitreal transplantation can survive and migrate to the RGC layer and to the ON. The cell–cell interaction is a critical condition to protect RGCs from degeneration since RGC survival is increased in the contact fashion of human PDLSC-retinal explant co-culture. Notably, we discovered a novel mechanism that the injured retina enhances BDNF secretion from human PDLSCs.[107]

Compared to delivering neurotrophic factors, RGC replacement therapy is more challenging. The basis of cell replacement therapy is that new RGCs could be regenerated from stem cells to substitute the damaged RGCs in optic neuropathies. Pluripotent stem cells, including

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embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been studied for their ability to differentiate into various retinal lineage cells. Embryonic stem cell-derived retinal progenitor cells could differentiate into RGCs, integrate into the retinal nerve fibre layer of the damaged retina and express genes associated with RGC differentiation, including Pou4f1, Pou4f2, NFL, Map2, and syntaxin.[109] Human ESCs-retinal neurons have been injected into the eye of squirrel monkeys, and the axons of some transplanted cells extended along the host nerve fibre layer toward the optic papilla and then project to the brain.[110] Pelaez et al. found that human PDLSCs, the neural crest stem cells residing in neural crest-derived adult tissues, can form teratomas in the immunodeficient mice and can be induced into neuronal lineage. This finding indicated that pluripotent adult stem cells can be isolated and enriched from human adult tissues without the necessity of reprogramming.

Another possible strategy for RGC regeneration is the endogenous regeneration by retinal stem cells. Retinal Müller glia can be transiently reprogrammed into retinal progenitor stage through de-differentiation and re-differentiation. However, such reprogramming occurs with extremely low efficiency under normal circumstances. Müller glia reprogramming can be enhanced by retinal damage and induction of Wnt/β-catenin signalling. Further work is required to test the treatment efficacy in other ON injury models, and to refine the differentiation signals for specific RGC regeneration from the de-differentiated Müller glia.

3.8 | Combined approaches for ON regeneration and target innervation

To date, the degree of ON regeneration induced by monotherapy has been limited. Combination therapy using two or more factors has proven to be more effective. Common strategies include a combination of methods that enhance the intrinsic growth state of RGCs or a combination of methods that enhance the intrinsic state and reduce the inhibitors of axonal growth.[111] Depending on the number of combined approaches, it can be divided into double, triple and quadruple therapies.

Strategies that produce strong effects on axonal regeneration include Pten knockout or silencing, accompanied by ocular inflammation, and elevated cAMP or CNTF. Among them, Pten deletion+Zymosan+cAMP[112] augmented regeneration 10-fold over a single treatment. SDF1 treatment could further enhance this regeneration by 2.7 folds.[77] Other studies showed more than 10-folds increase in RGC axon regenerating achieved by additional Pten deletion compared to the merely combined approached such as Soc3 knockout+AAV CNTF[113] or Ocm + cAMP.[114] Other strategies include BCL2 + CNTF [115] and Zn^{2+} chelator+ KLF9 gene knock down.[116] These combined treatments resulted in about 3 to 7 folds increase in regeneration compared to monotherapy. In adult mice, the length of regenerated axons induced by these approaches ranged 2.5–6.25 mm with 20%–50% of the regenerating axons reaching the optic chiasm.[113,114] Some axons extended to the contralateral optic nerve,[117] and a few of them entered the brain.[114] Combined strategies that produce moderate levels of axonal regeneration include CNTF combined with RhoA inhibition,[90] cAMP,[117] Nogo receptor blocking.[118] Among them, CNTF+RhoA inhibitor+cAMP was more effective than that of dual or single therapy.[119] The length of regenerated axons was about 0.25–3 mm[53,117] with some of them extending through the full length of the optic nerve. Methods which enhance the RGC survival include Pten gene knockout combined with ocular

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inflammation,[112] or Soc3 gene knockout.[113] Other strategies include BCL2 + CNTF [115] and FGF + NT3 + BDNF.[120] These combined treatments were able to increase RGC survival about 1.5–2 times higher than that of a single treatment. The combinatorial strategies with diverse efficacies for promoting RGC survival and their axon regeneration are listed in Table 1 for references.

4 | LIMITATIONS AND FUTURE DIRECTIONS

Single-cell RNA sequencing has been applied recently to compare the transcriptomics of individual cells including RGCs and microglial cells under different disease states.[121] Many genes and key molecules have been discovered for their abilities to support RGC survival, enhance intrinsic growth ability, reprogram to “youth” stages, and overcome the inhibitory factors including glia scar and myelin-associated inhibitory factor. Key regeneration regulatory mechanisms can be discovered through multi-omics sequencing using artificial intelligence techniques for big data analysis, providing insights into the future treatment of optic nerve injury and diseases.

Despite the progress made in promoting long distance optic nerve regeneration, there are many questions that remain further investigation. Aforementioned, many axons are unable to navigate correctly and often grow tortuously within the optic nerve and in the brain. As such, the number of axons that finally reach LGN and SC is rather small. This likely contribute significantly to the limited or lack of functional recovery. During development, multiple axon guidance molecules are expressed in distinct regions to correctly guide growing axons.[122] It is unclear to what extent these factors contribute to the failed (or successful) target re-innervation in adult animals. Interestingly, a recent study demonstrated that manipulating expression of some of these guidance molecules increases the number of axons that regenerate into the brain.[123] Thus, this study indicated the feasibility of modulating axon guidance of regenerative RGCs to achieve circuit repair in adults. In addition, studies have used a chitosan tube filled with CNTF-chitosan to bridge a 1-mm gap of the transected and excised adult rat ON, which showed RGC axons regenerate over a long distance and re-innervate the visual targets in the brain with partially restored visual functions, including pupillary light reflex, dark light preference, and depth perception.[124]

A large portion of preclinical studies has utilised small rodents (e.g. mice and rats). However, there are many anatomical and cellular differences between rodents and primates. Studies using larger animals (e.g. porcine and primate) is scarce,[125,126] but perhaps they could help identify the limitation (or the feasibility) of translating rodent studies into human. However, it is unclear to what extent the genes and molecular pathways involved in optic nerve regeneration are conserved between rodents and primates. A recent study using human induce pluripotent stem cells-derived RGCs (hRGCs) demonstrated that, much like in rodents, the mTOR pathway plays an important role in the development of hRGCs and regeneration of their axons, indicating that some of the regeneration-regulating molecular pathways might be evolutionarily conserved.[127]

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5 | CONCLUSIONS

RGCs and their axons are responsible for transmitting visual information to the brain, which are particularly vulnerable to damage in various optic neuropathies. The survival of RGCs is crucial for vision maintenance, and is related to the severity of damage and the timing of treatment, which is dependent on accurate diagnoses by neuro-ophthalmologists. After severe ON damage in optic neuropathy, promoting RGC axon regeneration is vital to reconnect the brain for restoring vision. Sustained investigation has been on the march to promote ON regeneration, such as supporting RGC survival, enhancing the intrinsic growth ability, overcoming the extrinsic inhibitory factors and modulating axon guidance. These may address the crucial mechanisms that help establish a model for translating these treatments into pre-clinical trials, and help devise treatments that improve the lives of visually impaired patients in the future.

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TABLE 1

Combined treatments.

Combined treatments	RGC survival	Axon regeneration effects	Length of regeneration	References
Pten knockout+Ocm + cAMP	–	2 or 10 times more effective than single therapy, 1.8 or 1.6 times more effective than dual therapy	0.5–3 mm, 50% reached the optic chiasma, some even reached the optic tracts	[114]
Pten knockout+Soc3 knockout+CNTF	1.5 times more effective than single therapy	10–20 times more effective than single therapy	0.5–2 mm, 20% went beyond the optic chiasma	[113]
Pten gene silencing+AAV−CNTF+cAMP	No synergistic effect	3.8 or 1.9–3 times more effective than single therapy, 1.7, 3.2, or 1.8 times more effective than dual therapy	0.5–2 mm, some reached the optic chiasm, 20% reached the contralateral optic nerve	[117]
shPten+SDF1 + Zymosan+cAMP	–	2.7 times more effective than triple therapy	The optic chiasma	[77]
Pten knockout+Zymosan+cAMP	Twice as effective as Zymosan+cAMP	More effective than dual therapy	The total length of optic nerve	[112]
BCL2 + CNTF	Increased by 50%	3.5 or 7.9 times more effective than single therapy	0.25–2 mm, partially reached the optic chiasma	[115]
Zinc chelation+Klf9 gene silencing	Equivalent to zinc chelation	2.9–7.5 times more effective than single therapy	0.5–3 mm, a few of them reached optic chiasma	[116]
FGF + NT3 + BDNF	Twice as effective as single therapy	Better than single therapy	2–5 mm	[120]
CNTF+Nogo receptor blocking	–	Five times more effective than single therapy	2 mm	[118]
CNTF+shPten	–	Equivalent to CNTF, twice as effective as shPten	0.5–2 mm	[117]
CNTF+cAMP	–	Equivalent to CNTF, twice as effective as Pten	0.5–2 mm	[117]
CNTF+SOCS3 knockout	–	Twice as effective as CNTF	0.25–2 mm	[53]
CNTF+RhoA silencing	Better than RhoA, be equivalent to CNTF	2–5 times more effective than CNTF	0.5–1.5 mm	[90]
Ocm + cAMP	No synergistic effect	2.5–3.5 times more effective than cAMP	0.5–1 mm	[74]
CNTF+RhoA inhibition	–	1.6 times more effective than RhoA inhibition	0.25 mm	[90]
SDF1 + Ocm + cAMP	Equivalent to Zymosan	80% as effective as Zymosan	0.1–0.25 mm	[77]
SDF1 + shPten	Equivalent to shPten	Equivalent to shPten	0.5 mm	[77]
Pten gene silencing+AAV−CNTF+cAMP	No synergistic effect	No synergistic effect	–	[117]

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