

# Restoration of optic neuropathy

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**Abstract:** Optic neuropathy refers to disorders involving the optic nerve (ON). Any damage to ON or ON-deriving neurons, the retinal ganglion cells (RGCs), may lead to the breakdown of the optical signal transmission from the eye to the brain, thus resulting in a partial or complete vision loss. The causes of optic neuropathy include trauma, ischemia, inflammation, compression, infiltration, and mitochondrial damages. ON injuries include primary and secondary injuries. During these injury phases, various factors orchestrate injured axons to die back and become unable to regenerate, and these factors could be divided into two categories: extrinsic and intrinsic. Extrinsic inhibitory factors refer to the environmental conditions that influence the regeneration of injured axons. The presence of myelin inhibitors and glial scar, lack of neurotrophic factors, and inflammation mediated by injury are regarded as these extrinsic factors. Extrinsic factors need to trigger the intracellular signals to exert inhibitory effect. Proper regulation of these intracellular signals has been shown to be beneficial to ON regeneration. Intrinsic factors of RGCs are the pivotal reasons that inhibit ON regeneration and are closely linked with extrinsic factors. Intracellular cyclic adenosine monophosphate (cAMP) and calcium levels affect axon guidance and growth cone response to guidance molecules. Many genes, such as *Bcl-2*, *PTEN*, and *mTOR*, are crucial in cell proliferation, axon guidance, and growth during development, and play important roles in the regeneration and extension of RGC axons. With transgenic mice and related gene regulations, robust regeneration of RGC axons has been observed after ON injury in laboratories. Although various means of experimental treatments such as cell transplantation and gene therapy have achieved significant progress in neuronal survival, axonal regeneration, and restoration of the visual function after ON injury, many unresolved scientific problems still exist for their clinical applications. Therefore, we still need to overcome hurdles before developing effective therapy to treat optic neuropathy diseases in patients.

**Keywords:** retinal ganglion cells, optic nerve injury, neuronal survival, axonal regeneration, vision restoration

Optic neuropathy refers to disorders involving the optic nerve (ON). Because ON transmits optical signals from the eye to the brain, any damage to ON or ON-deriving neurons, the retinal ganglion cells (RGCs), may lead to the breakdown of this transmission, thus resulting in loss of visual field and eventually partial or complete vision loss. The causes of optic neuropathy include trauma, ischemia, inflammation, compression, infiltration, and mitochondrial damages (nutritional, toxic, and hereditary).

Primary ON injuries, such as mechanical perturbation, ischemia, hypoxia, and inflammation, lead to direct axonal damage, while secondary injury may exacerbate the damage by complicated mechanisms including immune reaction, oxidative free radicals, and excitatory cellular toxicity. During these injury phases, various factors

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orchestrate injured axons to die back and become unable to regenerate, and these factors could be divided into two categories: extrinsic and intrinsic.

Extrinsic inhibitory factors refer to all conditions in the ON but outside RGCs, namely the environmental conditions that influence the regeneration of injured axons. Lack of neurotrophic factors, the presence of myelin inhibitors, and glial scar composed of chondroitin sulfate proteoglycans (CSPGs) are being investigated. Inflammation mediated by injury including immune cell infiltrations is also included as an extrinsic factor, but is more complicated. Intrinsic factors of RGCs are the pivotal reasons that inhibit ON regeneration and are closely linked with extrinsic factors. Intracellular cyclic adenosine monophosphate (cAMP) and calcium levels affect axon guidance and growth cone response to guidance molecules. Extrinsic factors need to trigger the intracellular signals to exert inhibitory effect. Guanosine triphosphatase (GTPase) and Ras homolog gene family member A (RhoA) – a small GTPase, involved signals are associated with myelin-associated glycoprotein (MAG) and other myelin-derived inhibitory factors in axonal regeneration. Proper regulation of these intracellular signals has been shown to be beneficial to ON regeneration. Recent research works found that many genes, usually playing roles in cell proliferation, axon guidance or growth during development, were down-regulated after development and played important roles in the regeneration and extension of RGC axons. These genes include B-cell lymphoma 2 (*Bcl-2*), phosphatase and tensin homolog (*PTEN*), and the mammalian target of rapamycin (*mTOR*). With transgenic mice and related gene regulations, robust regeneration of RGC axons has been observed after ON injury in laboratories.<sup>1–3</sup>

Although various means of experimental treatments such as cell transplantation and gene therapy have achieved significant progress in neuronal survival, axonal regeneration, and restoration of the visual function after ON injury, many unresolved scientific problems still exist for their clinical applications. Therefore, more time is needed before the aim of clinical applications of these effective therapeutic methods is achieved.

## Experimental neurorestorative studies

### Factors and medicine

#### Anti-myelin-derived inhibitors

The use of anti-myelin-derived inhibitors is an important strategy for ON regeneration. Techniques include antibodies, antisense oligonucleotide, gene knockout, interruption

of ligand-receptor combination, and blockage of associated signal pathway.

Nogo-A is one of the most potent oligodendrocyte-derived inhibitors for axonal regrowth in injured adult cerebral nervous system (CNS). Using an anti-Nogo-A antibody or by expressing a dominant-negative form of the Nogo receptor (NgR) in RGCs can block the activity of Nogo. However, Cui et al found that application of IN-1, an antibody against Nogo, alone failed to enhance regeneration of transected RGC axons in a peripheral nerve (PN) graft. IN-1 hybridoma cells also failed to significantly promote crushed ON axons to reenter the distal part of the ON. However, a combined application of IN-1 and ciliary neurotrophic factor (CNTF) had a synergistic effect in ON crush paradigms, suggesting that the action of IN-1 antibodies in promoting axonal regeneration in the CNS could be more effective when coupled with other appropriate factors.<sup>4</sup> Su et al demonstrated that Nogo-A-deficient mice showed significantly longer axon regrowth than control, according to growth-associated protein-43 immunohistochemistry and Nogo-A/B/C knockout resulted in more active regeneration of RGC axons, and suggested Nogo knockout could be an effective way to eliminate this inhibition and accelerate axonal regeneration.<sup>5</sup> Knockout of the Nogo-66 receptor enhanced regeneration after ON crush.<sup>6</sup>

Counteracting the NgR enhances ON regeneration if RGCs are in an active growth state, but when the growth program of RGCs is not activated, the negative form of the NgR would not have any beneficial effect.<sup>7</sup> Chen inhibited NgR by RNA interference and recruited exogenous trophic support by the zymosan intravitreal injection and found that either approach can stimulate ON axonal regrowth but regenerating axons can grow longer and extend further when both methods were combined.<sup>8</sup> When associated with NgR, the transmembrane receptor p75NTR signals the collapse of growth cones. Ahmed et al showed that the *in vivo* ON regeneration correlates with Schwann cell-derived factor-induced cleavage of NgR and Nogo-A, and inactivation of p75NTR signaling by the induction of regulated intramembranous proteolysis and the release of both extracellular (p75ECD) and intracellular (p75ICD) domains. Schwann cell-derived factor induced attenuation of NgR/p75NTR signaling which in turn suppressed epidermal growth factor receptor (EGFR) activation, thereby potentiating the disinhibition of axonal growth.<sup>9</sup>

Interfering with RhoA is a more comprehensive way to overcome inhibitory signals. RhoA is a small GTPase that is part of the intracellular signaling pathway through which multiple inhibitors cause the collapse of growth cone and

arrest axonal growth. Inhibition of RhoA results in modest levels of axonal regeneration of injured ON, while blockade of Rho-associated coil-forming protein kinase (ROCK) promotes the neurite outgrowth of adult RGCs only in the presence of growth-promoting factors.<sup>10</sup> Bcl-2-associated athanogene-1 (BAG1) is an Hsp70/Hsc70-binding protein which can suppress apoptosis and enhance neuronal differentiation. Planchamp et al demonstrated that BAG1 induced an intracellular translocation of Raf-1 (an enzyme, a member of Raf family which belongs to MAP kinase kinase) and ROCK2 and ROCK activity was decreased in a Raf-1-dependent manner by BAG1's overexpression. Therefore, BAG1 promotes axonal outgrowth and regeneration in vivo via Raf-1 and reduction of ROCK activity.<sup>11</sup>

MAG is involved both in the initiation of myelination in the CNS<sup>12</sup> and in the events of cell adhesion during ON regeneration.<sup>13</sup> MAG is necessary for the maintenance of the cytoplasmic collar and periaxonal space of myelinated fibers.<sup>14</sup> Axonal regeneration in the CNS of adult MAG-deficient mice was not improved when compared to wild-type animals,<sup>15</sup> but inactivation of MAG enhances ON regeneration. Wong et al used the retina-ON explants of embryonic chicks and eliminated MAG function acutely along ON using chromophore-assisted laser inactivation (CALI). They found that CALI of MAG permitted significant regrowth of retinal axons passing the lesion site of the CNS.<sup>16</sup>

CSPG, a matrix protein that occurs naturally in the CNS, is considered to be a major inhibitor of axonal regeneration and is known to participate in the activation of the inflammatory response. The isolated CSPG has a molecular mass of 500–600 kDa and carries the HNK-1 (a glycan that could be found in a number of neural cell adhesion molecules) carbohydrate epitope.<sup>17</sup> CSPG is expressed in the glial scar<sup>18</sup> and significantly contributes to the nonpermissive properties of the CNS environment during axonal regeneration. Chondroitin sulfate moieties of the proteoglycans were shown to be involved in patterning the early phase of axonal growth across the midline and at a later stage controlling the axon divergence at the chiasm.<sup>19</sup> A study on zebrafish also showed that CSPGs contribute to repellent axon guidance during regeneration of the optic projection.<sup>20</sup> In adult rats, the microcrush lesion site of the ON was CSPG-immunoreactive and an adjacent GFAP-negative zone developed early after the lesion. Axons regrew towards the lesion, but most of them stopped abruptly at the glial scar. CSPG-positive scar and the myelin-derived growth inhibitory proteins contribute to the failure of in vivo RGC regeneration after injury.<sup>21</sup>

The Rho/ROCK pathway mediates the inhibitory activity of neurite growth associated with CSPGs of the CNS glial scar. Concomitant application of chondroitinase ABC and ROCK inhibitors may make optic fibers pass over barriers in the severely injured ON. Inhibition exerted by CSPGs in vitro can be blocked by application of either C3 transferase, a specific inhibitor of the Rho GTPase, or Y27632, a specific inhibitor of the Rho kinase. RGC axon growth on glial scar tissue was enhanced in the presence of C3 transferase and Y27632, respectively.<sup>22</sup> Suppressing the kinase function of the EGFR blocks the activities of both myelin inhibitors and CSPGs in inhibiting neurite outgrowth. In addition, regeneration inhibitors trigger the phosphorylation of EGFR in a calcium-dependent manner. Local administration of EGFR inhibitors promotes significant regeneration of injured ON fibers, pointing to a promising therapeutic avenue for enhancing axonal regeneration after CNS injury (for a summary of main inhibitors of RGC axon regeneration, Table 1).

The adoptive transfer of T cells specific to proteins associated with CNS myelin, such as myelin basic protein, can reduce the posttraumatic secondary degeneration of the rat ON. The synthetic peptide copolymer 1 (Cop-1), known to suppress experimental autoimmune encephalomyelitis, can be safely substituted for the natural myelin antigen in both passive and active immunizations for neuroprotection of the injured ON.<sup>23</sup> Protection from glutamate toxicity requires reinforcement of the immune system by antigens that are different from those associated with myelin. The use of Cop-1 apparently circumvents this antigen specificity barrier. In the rat ocular hypertension model, immunization with Cop-1 significantly reduced the RGC loss without affecting the intraocular pressure.<sup>24</sup> Vaccination was tried to enhance ON regeneration because myelin-derived inhibitors

**Table 1** A summary of main inhibitors of axonal regeneration of RGCs and their blockers

Inhibitors	Strategies that block the inhibitors
<b>Myelin-derived inhibitors</b>	
Nogo-A	Anti-Nogo-A antibody or expression of a dominant-negative form of the Nogo receptor
Nogo receptor	RNA interference, interfering with the intracellular signaling pathway such as Rho/ROCK and SCH vaccination
MAG	Chromophore-assisted laser inactivation of MAG
<b>A matrix-derived inhibitor</b>	
CSPG	Chondroitinase ABC

**Abbreviations:** CSPG, chondroitin sulfate proteoglycan; MAG, myelin-associated glycoprotein; RGCs, retinal ganglion cells; ROCK, rho-associated coil-forming protein kinase; SCH, spinal cord homogenate.

were discovered. Spinal cord homogenate (SCH) contains abundant myelins. Enhanced axonal regeneration of RGCs was found after ON microcrush in SCH-vaccinated animals. In culture, serum from SCH-vaccinated animals promoted the neurite growth of RGCs on myelin but not on CSPG.<sup>25</sup>

### Neurotrophic factors

Lack of neurotrophic factors is also a crucial reason for RGC death and axonal regeneration in ON injury or diseases. In support of RGC survival, neurotrophic supplement is a traditional and effective strategy.

Several neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-4 or 5 (NT-4/5) are found to be effective not only for rescuing RGCs from retrograde cell death, but also for promoting growth of intraretinal axons.<sup>26</sup> A single injection of BDNF or the prolonged administration of NT-4/5 by mini-pump increases the median lengths of axon branches after ON transection in adult rats, but neurotrophin-3 (NT-3) does not significantly influence axonal regrowth.<sup>27</sup> Negishi et al compared the effect of grafts combining with trophic factors on ON regeneration in adult rats and found that the degree of regeneration was significantly increased by BDNF and NT-4 in the grafts and in the vitreous. They showed that neurotrophin receptors (tropomyosin receptor kinase [Trk]-A and Trk-B) were detected in nerve fibers in both retina and graft.<sup>28</sup> On the axonal regeneration by PN transplantation in cats, a combined injection of BDNF, CNTF, and forskolin resulted in a 3.4-fold increase of beta-RGCs with regenerating axons. Watanabe et al suggested that the increase of regenerating beta-RGCs was mainly due to the enhanced effect of neurotrophic factors on their survival, and possibly to a change of RGC properties by cAMP to facilitate their axonal regeneration.<sup>29</sup>

Inhibition of CNS axon growth is mediated in part by calcium-dependent phosphorylation of axonal EGFR. A local administration of EGFR kinase inhibitors AG1478 and PD168393 to an ON lesion site can promote axonal regeneration of adult RGCs. However, Douglas et al showed that EGFR was neither constitutively expressed, nor activated in the ON axons in non-regenerating and regenerating ON injury models. Neurotrophins released by AG1478 stimulated disinhibited axonal growth of RGCs in CNS myelin-treated cultures by the induction of regulated intramembranous proteolysis of p75(NTR) and Rho inactivation. They attributed AG1478-stimulated neuritegenesis to the induced release of neurotrophins together with raised cAMP levels in treated cultures, leading to axon growth and disinhibition by neurotrophin-induced regulated intramembranous proteolysis

of p75(NTR). These off-target effects of EGFR kinase inhibition suggest a novel therapeutic approach for the regeneration of CNS including RGC axons.<sup>30</sup>

BDNF released from RGCs acts as a survival factor for postsynaptic neurons in retinal target fields.<sup>31</sup> BDNF protects RGCs from apoptosis mediated by transient ischemia through inhibition of c-Jun and caspase-2 activities.<sup>32</sup> BDNF concentration dependently improved the survival of cultured RGCs, particularly of those small RGCs.<sup>33</sup> The number of BDNF-positive RGCs increased 2-fold 2 days after axotomy, and the percentage of RGCs expressing TrkB was elevated by 50%, while the number of TrkA-positive RGCs doubled after axotomy in comparison to the controls.<sup>34</sup> Insulin-like growth factor-I, an important factor in retinal development, prevents secondary death of RGCs after axotomy via phosphoinositide 3-kinase (PI3K)-dependent protein kinase B (AKT) phosphorylation and by inhibition of caspase-3.<sup>35</sup> In the mature retina, CNTF treatment, via Janus kinase/signal transducers and activators of transcription signaling, enhances survival of RGCs and photoreceptor cells exposed to otherwise lethal perturbation.<sup>36</sup> Adenovirus (ADV)-mediated CNTF expression in rat RGCs enhances RGC survival after ON transection.<sup>37</sup> The protective effect of CNTF may result from activation of its high-affinity receptor expressed in RGCs, that switches the state of RGCs into more neuroprotective phenotype. CNTF also modulates glial cells in the retina and may buffer the high concentration of glutamate.<sup>38</sup>

The ways to supply neurotrophic factors in vivo include systemic application, intravitreal injection, and virus-mediated gene transfection. For instance, systemic application of glial cell-line-derived neurotrophic factor (GDNF) was reported to rescue some RGCs from axotomy. Using electroporation, GDNF gene transferred into RGCs can increase RGC survival 2–4 weeks after ON transection.<sup>39</sup> Administration of adeno-associated virus (AAV) vector expressing CNTF into the eyes protects RGCs undergoing focal injury and local ischemia in rats.<sup>40</sup> AAV containing basic fibroblast growth factor (bFGF) or BDNF is neuroprotective to RGCs from excitatory injury caused by *N*-methyl-D-aspartic acid (NMDA) or nerve crush.<sup>41</sup>

Besides injection of neurotrophic factors and increase of gene expression mediated by virus vectors, olfactory ensheathing cells (OECs) transplanted into the injured ON also increase beneficial neurotrophic factors like BDNF to promote RGC survival in rats,<sup>42</sup> and an in vitro study demonstrated that it is BDNF produced by OECs that can enhance the survival of injured RGCs.<sup>43</sup>

Cui et al compared several neurotrophins including BDNF, NT-4/5, FGF, and GDNF, and concluded that CNTF is the only effective factor that promotes a long-distance axonal regeneration of RGCs, whereas BDNF and NT-4/5 can only enhance neurite sprouting within the retina.<sup>44,45</sup> To enhance the ON regeneration, neurotrophin synergy is required. Logan et al demonstrated that a combination of basic FGF, NT-3, and BDNF delivered to the somata of RGCs promoted greater neuronal survival and axonal growth than each neurotrophic factor alone. They showed that the triple, but not single, neurotrophin treatment may decrease 30% RhoA activation, and thus increase axonal regeneration.<sup>46</sup> Many approaches promoting ON regrowth are combination of neurotrophic factors and other ways such as PN transplantation, lens injury, and chondroitinase application (for a summary of main neurotrophic factors, see Table 2).

### Factors related to lens injury

Mansour-Robaey et al discovered that an injury on the lens secretes unidentified factors which promote neuronal survival and axonal regeneration of RGCs.<sup>47</sup> Further investigations showed that such a lens injury also resulted in re-innervation of the visual center<sup>48</sup> and recovery of visual evoked potentials.<sup>49</sup>

Macrophage activation by lens injury appeared to play a key role, because intraocular injections of zymosan, a yeast cell wall preparation, stimulated monocytes in the absence of the lens injury and induced RGCs to regenerate their axons into the distal ON or a PN graft.<sup>50</sup> The axonal growth of RGCs is also enhanced by delaying macrophage activation for a few days after ON transection.<sup>51</sup> Oncomodulin is a calcium-binding protein secreted intravitreally in combination with cAMP by macrophages, and accumulates rapidly in the eye after intravitreal inflammation and exhibits cAMP-dependent, high-affinity binding to a cell surface receptor on RGCs. Oncomodulin promotes axonal regeneration of RGCs possibly regulated

through mammalian sterile 20-like kinase-3b, a homolog of a yeast kinase<sup>52,53</sup> and is potentiated by both the sugarman-nose found in vitreous humor and forskolin-stimulated cAMP elevation.<sup>51,54,55</sup> However, one group observed that neuroprotective and axon growth-promoting effects of intraocular inflammation did not depend on oncomodulin or the presence of large numbers of activated macrophages.<sup>56</sup>

Astrocyte-derived CNTF and leukemia inhibitory factor are also the major contributors to the neuroprotective and axonal growth-promoting effects on mature RGCs caused by lens injury and zymosan.<sup>57,58</sup> Muller reported that astrocyte-derived CNTF activated the STAT/JAK pathway and promoted RGC survival and axonal regeneration after lens injury.<sup>59</sup> Alpha-crystallins of the beta/gamma superfamily are the lens-derived activators of cascades and mimic the effects of lens injury, which led to axonal regeneration after ON crush in rats.<sup>60</sup>

### Cyclic AMP

The level of cAMP plays a role in modulating outgrowth of nervous system. Urbina et al found that cAMP level was crucial for retinal outgrowth and regeneration. Forskolin, a potential activator of adenylate cyclase system and biosynthesis of cAMP, decreased the outgrowth of post-crushed goldfish retina in a dose-dependent manner.<sup>61</sup> In the guidance of growth cones of RGCs, cAMP was found involved in conversion of netrin-1-induced attraction of growth cones to repulsion. Höpker et al reported in *Xenopus* laminin or fragment of laminin that YIGSR converted netrin-1 attraction to repulsion of RGC growth cones, and low levels of cAMP in growth cones also led to such conversion. Moreover, the amount of cAMP decreased in the presence of laminin or YIGSR.<sup>62</sup> Years later, the same group demonstrated that a low level of cAMP developmentally and intrinsically regulated the aged axon responsiveness to netrin.<sup>63</sup> Another negative regulator of RGC growth cone movement, Sonic hedgehog, may decrease the level of cAMP in growth cones.<sup>64</sup>

In the PN-grafted ON stump, a combinational intravitreal injection of neurotrophins such as BDNF, CNTF, and forskolin may increase neuronal survival and axonal regeneration of RGCs, and the cAMP elevation by forskolin may contribute to the promotion of the RGC regenerative profile.<sup>29</sup> Some small molecules including mannose and glucose were proved to have the effect of promoting axonal regeneration of RGCs in a cAMP-dependent manner in the higher or lower vertebrates.<sup>54</sup> High level of cAMP abrogates the ability of CNS molecules to inhibit axonal elongation. Using the ON crushing model in adult rats, Monsul et al showed that intra-

**Table 2** A summary of main neurotrophic factors that enhance the neuronal survival and axonal regeneration of RGCs

Neurotrophic factors	Effects of the factors
BDNF and NT-4/5	Rescuing RGCs from retrograde cell death and promoting growth of intra-retinal axons
CNTF	Enhancing survival of RGCs. The only effective factor that promotes a long-distance axonal regeneration of RGCs
GDNF	Rescuing some RGCs
bFGF	Protecting RGCs

**Abbreviations:** BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; GDNF, glial cell-line-derived neurotrophic factor; NT-4/5, neurotrophin-4 or 5; RGCs, retinal ganglion cells.

ocular injection of cAMP analog dibutyryl-cAMP promoted ON regeneration without increasing RGC survival. Their study also proved that cAMP did not enable axons to undergo rapid, sustained, and lengthy elongation.<sup>65</sup> In the cat ON crush model, elevation of intracellular level of cAMP by forskolin also promoted axonal regeneration beyond the crush site to some extent.<sup>66</sup> Another cAMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) was unable to promote RGC axons to regenerate into the PN graft, but significantly potentiated CNTF-induced axonal regeneration. Blockade of cAMP pathway did not affect spontaneous RGC survival and regeneration but effectively blocked CNTF/CPT-cAMP induced survival and regeneration. The CNTF/CPT-cAMP effect was proved to be a result of multiple pathways actions, with protein kinase A (PKA) as an essential component.<sup>67</sup> CPT-cAMP and PKA inhibitor accelerated and decelerated the rates of ON regeneration, respectively, and the inhibitor application altered the RGC axon guidance *in vitro*, suggesting that the therapeutic manipulation of cAMP should ensure topography and consequently functions.<sup>68</sup>

X cells, which are essential for acute vision, suffer from rapid death after ON transection but can be rescued by intravitreal application of neurotrophins accompanied with elevation of cAMP.<sup>69</sup> Astrocyte-derived CNTF and lens injury were proven to switch mature RGCs to a regenerative state following inflammation, and the drug potentiating the effect of lens injury may increase the intracellular cAMP level, thus resulting in strong axonal regeneration *in vivo*.<sup>59</sup> Co-injection of CNTF with CPT-cAMP reduced CNTF-induced upregulation of suppressor of cytokine signal (SOCS) in both mRNA and protein, suggesting that cytokine-induced regenerative responses could be augmented by cyclic nucleotide in another way.<sup>70</sup> SOCS overexpression may result in an overall reduction of axonal regrowth or even complete regeneration failure of RGC axons.<sup>71</sup> The drug that inhibits cyclic nucleotide phosphodiesterase was reported to increase RGC survival possibly through activating PKA signal.<sup>72</sup> Microarray analysis in fish found that a member of cAMP-response element-binding protein family, activating transcription factor 3, increased 8-fold 24 hours after ON injury, indicating the mediator of ON regeneration gene expression in fish.<sup>73</sup>

### PTEN–mTOR signal

Phosphatase and tensin homolog (PTEN) is a negative regulator of the mammalian target of rapamycin (mTOR) pathway. Mechanistic studies of axonal growth during development and axonal regeneration after injury reveal the PTEN-dependent molecular mechanism as a commonality.

Deletion of PTEN in adult RGCs promoted robust axonal regeneration after ON injury. In adult wild-type animals, the mTOR signal was suppressed significantly in axotomized RGCs and the protein synthesis was inhibited, leading to a limited axonal regeneration. By deletion of PTEN or tuberous sclerosis protein 1, one of the protein complexes negatively regulating mTOR, neuronal survival and axonal regeneration of RGCs were significantly enhanced after ON crushed, while administration of rapamycin, the mTOR inhibitor, significantly neutralized the survival and regeneration of RGCs in PTEN deletion. All these findings suggest that mTOR signal is sufficient to promote ON regeneration.<sup>2</sup>

PTEN antagonizes PI3K and catalyzes conversion from phosphatidylinositol 3,4,5-trisphosphate (PIP3) to PIP2, thus inactivation of PTEN results in PIP3 accumulation and activation of AKT which has many downstream effectors and is located in the soma and axon terminals of neurons. Those PI3K/AKT effectors may cooperate with other pathways to promote injured axonal regeneration. Meanwhile, PTEN controls several downstream signals such as 3-phosphorylated phosphoinositides, glycogen synthesis kinase, and mTOR, which are crucial for axonal growth.

Intriguing questions about PTEN relating to CNS regeneration are still open, such as how the pathway is regulated during development and after injury, what other possible signals work in concert to promote axonal regrowth, and the extension of functionality of new connection resulting from PTEN manipulation. Studies in answering these questions will make important steps to understand the factors underlying CNS regeneration.<sup>74</sup>

### Lithium

Lithium was found to have protective effects on the nervous system. Lithium treatment caused Bcl-2 expression in injured RGCs of neonatal mouse retina in a dose-dependent manner, and depleting or forcing Bcl-2 expression deleted the protective effects of lithium.<sup>75</sup> Later on, lithium protection to RGCs and axons was proved also in adult rats with ON crush.<sup>76</sup> Lithium and other drugs have been treated as pharmaceutical approach to promote ON regeneration in animals.<sup>77</sup> To obtain further ON regeneration, those authors suggested to use lithium together with other drugs, citicoline, for instance, that mediate induction of permissive environment in the adult CNS.

Increasing studies documented more mechanisms of lithium protection to the CNS, including the spinal cord and ON. Through Bcl-2 expression, lithium reinforced the promoting effect of chondroitinase ABC that digests CSPG, the main

ingredient of scar.<sup>78</sup> In addition, lithium could activate Wnt or beta-catenin signaling to facilitate healing of bone fracture.<sup>79</sup> While wnt signaling is also crucial for retinal development,<sup>80</sup> activation of Wnt signaling by lithium was proved protective to RGCs under elevated pressure<sup>81</sup> or excitotoxic damage.<sup>82</sup>

## Gene therapy

Besides genes encoding neurotrophins mentioned before, anti-apoptosis genes (such as bcl-2, bcl-xL, p35 and caspase inhibitors) were efficiently upregulated by ADV transduction, human immunodeficiency virus transactivator of transcription containing a protein transduction domain, and AAV vector into the ON stump or vitreous body. Those genes coding apoptotic proteins including BAX, apoptotic protease activating factor, and immediate early gene *c-Jun* can be downregulated by small interference RNA. Increasing studies documented that the regulations of these genes effectively promote the survival and/or viability of injured RGCs. In methodology, non-virus vectors are usually safer than virus vectors, but non-virus approaches are not sufficient because the expression is transient and needs multiple injections. Even the expression of ADV-coding genes is transient and requires modifying. Many groups reported that AVV provided relatively long-term gene transduction of a large proportion of adult RGCs.<sup>1</sup>

When gene-activated matrices (GAM) are placed between the proximal and distal stumps of severed rat ONs, DNA is retained within the GAM, promoting sustained transgene expression in the ON, in the GAM itself, and, more importantly, in axotomized RGCs. Plasmids that encode bFGF, BDNF, and NT-3 promote sustained survival of RGC for over 3 months after the initial injury. These findings suggest that immobilized DNA implanted into a CNS lesion will be delivered by axon terminal uptake and retrograde transport to axotomized neurons. GAM may therefore be a useful agent for promoting the survival and axonal regeneration of sustained neurons. Further optimization of the matrices, plasmids, promoters, and genes present in the GAM will promote even more survival or, alternatively, axonal regeneration remains to be determined.

## Cell and tissue transplantation

Tissue engineering develops scaffolds to serve as a bridge to guide and help injured nerve grow through the injury site. Using a PN graft as a scaffold is also helpful.<sup>83</sup> Moreover, cell transplantation offers not only source of cells substantial to the lost cells caused by injury, but constant sources of neurotrophic factors produced by transplanted cells.

As increasing sorts of cells have been discovered and are available, it becomes feasible to supply RGC pool with transplanted and thereafter differentiated stem cells to replace the lost or dying host RGCs. Embryonic stem cells from eye-like structure differentiated into RGC-like cells and regenerate a new RGC layer after transplanted into the mouse eyes in the presence of NMDA.<sup>84</sup> Unfortunately, more studies showed that stem cells failed to migrate or express the RGC marker after transplanted into the eyes, even some of the grafted stem cells differentiated and formed synapses with host cells. There exist many problems in the replacement of RGCs with transplanted cells, including transplant timing, interaction between intrinsic factors of RGCs, and the microenvironment of retinas.<sup>1</sup> However, many studies indicate that cell transplantation-facilitated ON regeneration or repair is not attributed to cell replacement but via released neurotrophins from grafted cells.<sup>85-87</sup>

Since scientists noticed the difference in the regenerative ability between the central and peripheral nervous systems, studies have been set off to explore the possibility of axonal regeneration of central neurons when the surrounding microenvironments were replaced with the PN grafts.

## PN transplantation

Tello was the pioneer to transplant PN onto the ON stump to induce axonal regeneration in rabbits, though the outcome of the ON regeneration was not fully confirmed at his time.<sup>88</sup> Using modern axonal tracing technique, So and Aguayo finally demonstrated that transected RGC axons can regrow along a PN graft.<sup>83</sup> Interestingly, axotomy seems to be the prerequisite for the regrowth of RGC axons, for intact RGCs would not extend their axon to the PN graft,<sup>89</sup> which is consistent with later research that uninjured RGCs do not respond to protective neurotrophic factors like BDNF.<sup>90</sup>

The regrowth of RGC axons in the PN graft is distance-dependent. The farther the transplantation site of the PN graft onto the ON stump is, the less the regrowth of RGC axons occurs.<sup>91</sup> This phenomenon possibly attributes to the longer time for the diffusible trophic factors produced by Schwann cells in the PN graft to reach the soma of RGCs. Thus, the longer the distance of the remaining ON stump, the more difficult for those factors to reach the soma of RGCs resulting in a decreasing effect of the PN on regeneration of the RGC axons.<sup>92</sup> Pre-degeneration was shown to enhance the axonal regeneration of RGCs in the PN graft, and the number of regenerating RGCs also depends on the time of transplantation after injury. You et al reported that the number of regenerating RGCs decreased with increased delay time,

and pre-degenerated PN grafts overcome this adverse effect to some degree in the rats. To obtain the highest number of regenerating RGCs, pre-degenerated PN should be grafted immediately after ON lesion.<sup>93</sup>

Bray et al documented in rats that replacement of the ON with an autologous PN segment made axotomized RGCs regrow axons into a PN graft, and some of the regenerating axons can even reach the superior colliculus (SC) with terminals forming synaptic contact with SC neurons.<sup>94</sup> PN may serve as a bridge which links axotomized RGC to the brainstem, pretectum, or SC, and guide regenerating axons to these target areas 8–13 months later.<sup>95</sup> Recording from the neurons in SC showed that normal light field can induce postsynaptic potential,<sup>96,97</sup> indicating these synapses are functional.

The underlying mechanisms for the enhancement of axonal regeneration of axotomized RGCs by PN grafts are as follows: 1) the replacement of injured ON with transplanted PN grafts deletes the inhibitory factors that exist in ON as these inhibitory factors are not presented in the PN grafts; 2) a PN graft containing Schwann cells which secrete neurotrophic factors that could diffuse to RGC axons. These factors are also protective to RGC somata and enhance RGC survival; 3) Schwann cells would proliferate and form columns after injury, and the basement membranes of the Schwann cell tubes possess many adhesion molecules such as L1, J1,<sup>98</sup> integrins, and laminins, which are beneficial to axonal regeneration.

Although the axons of injured RGCs can be induced to regenerate along a PN graft, the number of regenerated RGCs is limited. Axons of alpha RGCs regrow faster into the PN graft than those of other types of RGCs.<sup>99</sup> Quantitative studies showed that regenerating axons of RGCs are around 1%–10% of total cell population, according to various reports using rodents and cats,<sup>100</sup> and this number decreased over time. To improve the regeneration of RGC axons, many modified methods of PN transplantation emerged. One of the methods is to reconstruct the PN graft by Schwann cells with lentiviral-mediated transfer of CNTF. This modification significantly enhanced neuronal survival as well as the axonal regrowth of injured RGCs, because the regenerating axons were found myelinated.<sup>101</sup> Using a PN-to-ON graft model, You et al combined gene therapy with pharmacotherapy to promote the long-distance regeneration of injured adult RGCs. Autologous sciatic nerve was sutured onto the transected ON and the distal end immediately inserted into contralateral SC. In experimental groups, three bi-cistronic AAV vectors encoding CNTF were injected into different regions of the grafted eye. Each vector encoded a different fluorescent reporter to assess retinotopic order in the regenerate

projection. To encourage sprouting/synaptogenesis, after 6 weeks some AAV–CNTF injected rats received an intravitreal injection of recombinant BDNF or AAV–BDNF. Four months after surgery, cholera toxin B was used to visualize regenerate RGC axons. RGC viability and axonal regrowth into SC were significantly greater in AAV–CNTF groups. In some cases, near the insertion site, regenerate axonal density resembled retinal terminal densities seen in normal SC. Complex arbors were seen in superficial but not deep SC layers and many terminals were immunopositive for presynaptic proteins vGlut2 and SV2. There was improvement in visual function via the grafted eye with significantly greater pupillary constriction in both AAV–CNTF+BDNF groups. In both control and AAV–CNTF+rBDNF groups, the extent of light avoidance correlated with the maximal distance of axonal penetration into superficial SC. Despite the robust regrowth of RGC axons back into the SC, axons originating from different parts of the retina were intermixed at the PN graft/host SC interface, indicating that there remained a lack of order in this extensive regenerate projection.<sup>102</sup>

Immunoreaction in the RGC axon environment is an essential condition for ON regeneration. Following ON transection, there is an increase in the numbers of the total and active forms of microglial cells, the phagocytic cells, in the ON stump and retina. PN transplantation onto the freshly-cut ON stump and application of microglia inhibitor minimized the overall regeneration, suggesting an effect of microglial phagocytosis on the ON regeneration.<sup>103</sup> Macrophages, another kind of phagocytic immune cell, also seem to play a beneficial role in axonal regeneration of RGCs. Lens injury leads to activation of macrophages that produce factors to promote the axonal regeneration of RGCs in the PN graft after ON injury. Such effect could also be obtained by application of Zymosan, a yeast cell wall preparation, which stimulates macrophages even better than lens injury. None of the secretion of macrophages is positive to enhance ON regrowth, some negative factors exist as well. The optimal effect of macrophages might be regulated by timing of macrophage activation.<sup>51</sup>

Another problem of PN transplantation is that autologous grafts may exacerbate the PN dysfunction. Cui et al proposed chimerical graft which could be derived from donor or host freeze-thawed PN and repopulated with cultured Schwann cells or OECs. This approach may provide an alternative in clinic.<sup>104</sup> Allograft could be available in the presence of immunosuppressants such as FK506 and cyclosporin-A. In the absence of immunosuppressants, a PN allograft induced little axonal regrowth of RGCs in the grafts.<sup>105</sup>



RGCs could regenerate their injured axons to the PN graft, but those regenerating axons extend only few millimeters in the brain. In order to elevate the effectiveness of RGC axonal regrowth, many treatments and drugs need to be combined in future research.<sup>106</sup>

### Cell transplantation

Cells have been transplanted into the eye to try to replace the dead RGCs or other lost cells after ON injury.<sup>107</sup> Although stem cells were expected to differentiate and migrate to the injured site, there has not been any report to show that transplantation of stem cells could meet this demand up to date. However, plenty of studies found that transplanted stem cells facilitated ON repair and axonal regeneration via secreting trophic factors that play neuroprotective roles.<sup>108</sup>

Umbilical cord mesenchymal stromal cells were neuroprotective and promote axonal regeneration after being transplanted into the lesion site of the optic tract. An *in vitro* analysis proved that these cells secreted several immunomodulatory and neurotrophic factors including TGF beta-1, CNTF, NT-3, and BDNF.<sup>109</sup> Neural stem and precursor cells derived from embryonic rat spinal cord contributed to the survival and axons regrowth of RGCs weeks after being transplanted into the lesion site of the optic tract in the neonatal rat. These cells secreted several neurotrophic factors *in vitro* but did not replace injured neurons as they did not express the marker of neural cells after transplantation.<sup>85</sup> Research with chicken neural tube stem cells indicated that stem cells facilitated ON repair because the transplantation stimulated the expression of matrix metalloproteinase-2 (MMP-2) in the GFAP positive astrocytes in the ON. This process is related to the degradation of inhibitory CSPGs leading to a more favorable microenvironment for axonal regeneration in the distal ON.<sup>86</sup> In experimental glaucoma study, Johnson et al found that mesenchymal stromal cells transplanted into the vitreous could significantly increase the RGC survival, while systemic transplantation of the same cells was not neuroprotective.<sup>110</sup>

Oligodendrocytes precursor cells (OPCs) are multipotential and regarded as stem cells. NG2-expressing OPCs were positioned to inhibit axon growth of both developing and regenerating neurons.<sup>111</sup> However, using the rat glaucoma model, it was found that OPCs were protective to RGCs axons when they interacted with inflammatory cells.<sup>112</sup>

Schwann cells are myelinating cells in the PN and play a key role in Wallerian degeneration and subsequent regeneration. Therefore, the transplantation of Schwann cells has been used to provide a modified environment of ON to enhance regeneration.<sup>113</sup>

Transplants of OECs are able to induce structural regeneration of severed axons from CNS neurons and restore neurological functions in rats. OEC transplantation has become one of the promising strategies in restoring lost functions of injured CNS. Both bulbar and nasal mucosal OECs prolong the survival of the explants. Counts of the surviving RGCs, identified by beta-III-tubulin immunohistochemistry and by their axon trajectory, show that the bulbar cell preparations exhibit around twice the potency of those from the mucosa.<sup>114</sup> Moreno-Flores immortalized primary rat OECs using the SV40 large T antigen expressed from a constitutive cellular promoter, and reported the isolation and characterization of clonal lines. Immortalized OECs have been shown to promote axonal regeneration of RGCs.<sup>115</sup>

An *in vitro* study showed that adult rat OECs were effective promoters of the neurite outgrowth of adult RGCs in coculture. This study also provided evidence for a contribution of an intracellular calcium signaling mechanism, possibly implicating ensheathing cell adhesion molecules in promoting neurite outgrowth.<sup>116</sup>

In the first *in vivo* study of transplanted OEC into damaged ON by Li et al, unpurified GFP-positive OECs were obtained, embedded in a matrix of their own production and transplanted into a completely transected rat ON. These transplanted OECs survived within the ON and were able to stimulate and guide the axonal regeneration of cut RGCs for up to 10 mm into the distal stump.<sup>117</sup> Although the regenerating axons were ensheathed by the transplanted cells, none of the regenerating axons became myelinated by either central or peripheral type myelin. They used the same operative procedure but transplanted Schwann cells instead of OECs. As with the OEC transplants, the Schwann cell transplants also induced regeneration of the severed RGC axons into the graft region. In contrast to the situation with the OEC transplants, however, a considerable number of the regenerating axons became myelinated by peripheral type myelin produced by the transplanted Schwann cells.<sup>118</sup> Pastrana et al used microarrays to analyze three OEC populations with different capacities to promote axonal regeneration in cultured rat retinal neurons and identified a number of candidate genes that might play a role in promoting adult axonal regeneration, suggesting that MMP-2 does participate in adult axonal regeneration induced by OECs.<sup>119</sup> Hayat et al demonstrated that pretreatment of OECs with pertussis toxin increased the number of subsequently cocultured adult RGCs which regrew neurites without affecting neuronal survival. The result supports the hypothesis that G-protein-regulated calcium signaling plays a significant role of OECs to support axonal regeneration of

injured CNS.<sup>120</sup> Chimeric PN grafts containing adult OECs do not promote regeneration of RGCs.<sup>104</sup> However, an *in vitro* study showed that adult OECs, but not Schwann cells, promoted the long-distance growth of adult RGC neurites.<sup>121</sup>

A conditioned medium (CM) is the supernatant of culture medium in which cells have been cultured for a short period of time. The OEC-CM protected PC12 (cell line derived from a pheochromocytoma of the rat adrenal medulla) cells from Zn<sup>2+</sup> insult,<sup>122,123</sup> but was unable to protect normal cultured hippocampal neurons and RGCs.<sup>116,124</sup> Wang demonstrated that such OEC-CM protected cultured RGCs from scratch insult, and neutralization of BDNF by a BDNF neutralizing antibody attenuated such neuroprotection of the medium.<sup>43</sup>

OEC in co-grafts had minimal impact on the target recognition of axons. OECs might be an appropriate cell type for direct transplantation into injured neuropil when attempting to stimulate the reconstruction of a specific pathway.<sup>125</sup> Transplanted OECs have a particular potential within CNS neuropil because they can support axonal growth and also allow correct target innervation.

A mixture of OECs and fibroblasts cultured from the adult rat olfactory mucosa was transplanted through a scleral incision into the retina. Transplanted OECs have been shown to be incorporated into the ON head ensheathing RGC axons.<sup>126</sup> OECs transplanted into the ocular stump of transected ON had a transient neuroprotective effect on axotomized RGCs. OEC transplantation induced the expression of BDNF in the ocular ON stump and retina and delayed the death of axotomized RGCs at a certain survival period (for a summary of main tissue and cells used for transplantation, Table 3).<sup>42</sup>

## Clinical neurorestorative strategies Medicine and factors

Vitamin therapy has been suggested for several decades as a possible way to slow down the retinal degenerating process, but to date, there is no approved pharmacological therapy. The

nerve growth factor is potential for the optic neuropathy. A study evaluated the pharmacokinetics of CNTF delivered by an intraocular encapsulated cell technology (ECT) implant in patients with retinitis pigmentosa (RP) and geographic atrophy. Results revealed that the intraocular ECT implant has a favorable pharmacokinetic profile for the treatment of chronic retinal degenerative diseases without systemic exposure. The researchers found that implants produced CNTF consistently over a 2-year period. The calculated half-life of CNTF in the vitreous continuously delivered by ECT implants was 51 months, with CNTF levels statistically equivalent between the 6- and 24-month implant periods. CNTF, anti-CNTF antibodies, and antibodies to the encapsulated cells were not detected in the serum of patients.<sup>127</sup>

## Cell transplantation

Controlled human embryonic stem cell (hESC) differentiation resulted in >99% pure retinal pigment epithelium (RPE). Schwartz et al performed two prospective clinical studies to study the safety and feasibility of subretinal transplantation of hESC-derived RPE in patients with Stargardt's macular dystrophy and dry age-related macular degeneration. Post-implantation, structural evidence demonstrated that the cells had attached and continued to persist for 4 months. There was no hyperproliferation, abnormal growth, or immune-mediated transplant rejection. Best corrected visual acuity improved from hand motions to 20/800 in eye of the patient with Stargardt's macular dystrophy, and vision also improved in the patient with dry age-related macular degeneration.<sup>128</sup>

## Neuromodulation

The Argus II epiretinal prosthesis has been developed to provide partial restoration of vision to subjects blinded from outer retinal degenerative disease. Safety and efficacy data support the development of higher resolution systems that target macular placement from implant design and surgical perspectives.

**Table 3** A summary of main tissue and cells used for transplantation to enhance neuronal survival and axonal regeneration of RGCs

Tissue and cells used for transplantation	Effects
PN graft	Developing scaffolds as a bridge to guide injured ON through the lesion site and secreting neurotrophic factors to enhance neuronal survival and axonal regeneration of axotomized RGCs
Umbilical cord mesenchymal stromal cells	Secreting immunomodulatory and neurotrophic factors, thus can protect injured RGCs and promote axonal regeneration
Neural stem and precursor cells	Secreting neurotrophic factors
Oligodendrocytes precursor cells	Protecting RGC axons when interacting with inflammatory cells
Schwann cells	Providing a modified environment of ON to enhance axonal regeneration
Olfactory ensheathing cells	Secreting neurotrophic and cell adhesion factors, thus can protect injured RGCs and induce structural regeneration of severed RGC axons

**Abbreviations:** ON, optic nerve; PN, peripheral nerve; RGCs, retinal ganglion cells.

Currently, the device has been implanted in multiple subjects with profound RP. Most subjects showed an improvement in tasks assessing orientation and mobility, spatial-motor localization, and ability of discerning the direction of motion of moving stimuli. About one third of subjects experienced measurable improvement in visual acuity with the implant.<sup>129</sup>

In conclusion, from PN transplantation to PTEN-mTOR signals and epiretinal prosthesis, researchers have obtained much more insight of ON restoration and functional recovery. Due to the complexity of the CNS injury, combinational strategies are always the optimal choice, which will facilitate the restoration of the optic neuropathy.

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## Disclosure

The authors report no conflicts of interest in this work.

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