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Research article

The challenge of regenerative therapies for the optic nerve in glaucoma☆

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ABSTRACT

This review arose from a discussion of regenerative therapies to treat optic nerve degeneration in glaucoma at the 2015 Lasker/IRRF Initiative on Astrocytes and Glaucomatous Neurodegeneration. In addition to the authors, participants included Jonathan Crowston, Andrew Huberman, Elaine Johnson, Richard Lu, Hemai Phatnami, Rebecca Sappington, and Don Zack. Glaucoma is a neurodegenerative disease of the optic nerve, and is the leading cause of irreversible blindness worldwide. The disease progresses as sensitivity to intraocular pressure (IOP) is conveyed through the optic nerve head to distal retinal ganglion cell (RGC) projections. Because the nerve and retina are components of the central nervous system (CNS), their intrinsic regenerative capacity is limited. However, recent research in regenerative therapies has resulted in multiple breakthroughs that may unlock the optic nerve's regenerative potential. Increasing levels of Schwann-cell derived trophic factors and reducing potent cell-intrinsic suppressors of regeneration have resulted in axonal regeneration even beyond the optic chiasm. Despite this success, many challenges remain. RGC axons must be able to form new connections with their appropriate targets in central brain regions and these connections must be retinotopically correct. Furthermore, for new axons penetrating the optic projection, oligodendrocyte glia must provide myelination. Additionally, reactive gliosis and inflammation that increase the regenerative capacity must be outweigh pro-apoptotic processes to create an environment within which maximal regeneration can occur.

1. Introduction

Glaucoma causes blindness through degeneration of the retinal ganglion cell (RGC) projection to the brain, which effectively separates the visual cortex from its sensory input. Degeneration arises in most forms of the disease from chronic (e.g., progressive) stress due to sensitivity to intraocular pressure (IOP). This stress is conveyed or transduced to the unmyelinated RGC axon segment as it passes through the optic nerve head in complex ways that include mechanical, inflammatory, and bioenergetic components. In exiting the nerve head, the axon becomes myelinated by oligodendrocytes in forming the remainder of the optic nerve. Both the pressure-dependent nature of this stress and its origin at the nerve head are considered defining features of glaucoma in its many forms. Even so, signs of RGC degeneration in glaucoma can be found early in progression at distal sites in the projection. This observation underscores that glaucoma is like other neurodegenerative disorders in that neuronal stress at one site can be manifest pathogenically at quite another.

The distal RGC projection is important in the context of regeneration or repair for a number of reasons. One of the hallmarks of glaucoma is RGC axonal dysfunction early in progression (Libby et al., 2005; Nickells, 2007; Calkins, 2012; Howell et al., 2013).

☆ This article summarizes the results of a targeted session on this topic at the March 2015 conference Astrocytes and Glaucomatous Neurodegeneration. This meeting was a follow-up to the 2010 meeting on the same topic, both of which were conducted as part of The Lasker/IRRF Initiative for Innovation in Vision Science. For more information about this conference, its participants and other review articles that originated from it see Tamm and Dowling, 2016.

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One index of this dysfunction is depletion of anterograde intra-axonal transport, that is, transport from the retina to central projection targets in the brain (Crish et al., 2010). Degradation of anterograde axonal transport to the brain marks the beginning of an interventional window, defined by the period between the onset of axon dysfunction and actual degeneration of RGC axons in the optic projection and RGC bodies in the retina, which occur later. Neuroprotective interventions that rescue axon transport to the brain also prevent axon degeneration in the optic nerve and subsequent RGC body loss in the retina. These include systemic delivery of brimonidine tartrate, an alpha-adrenergic receptor agonist (Lambert et al., 2011), and topical delivery of a selective p38 MAP kinase inhibitor (Dapper et al., 2013). Conversely, conditions that accelerate axon dysfunction also accelerate degeneration in the nerve and RGC loss in the retina (Ward et al., 2014). The bottom line is this: if axon function can be repaired early enough, either by reducing IOP-related stress on the axon or by ameliorating the influence of this stress, loss of tissue in glaucoma can be avoided – at least in experimental models. That the same might hold true for human glaucoma is gleaned from results showing reversal of physiological deficits with early IOP-lowering interventions (Sehi et al., 2010).

Unfortunately, loss of vision in glaucoma is difficult to detect early on (Crabb et al., 2013), so by the time patients are identified they often have substantial visual field deficits, corresponding to loss of RGC axons in the optic nerve. Since the nerve and retina are integral components of the central nervous system (CNS), their intrinsic (or spontaneous) capacity for regeneration in adult tissue is severely limited by the same multitude of factors that limit regeneration in other CNS tissues, such as the spinal cord. Several factors compound the problem. RGC axon degeneration in the optic nerve is not confined to a discrete locus, nor does it follow a single mechanistic process. Rather, evidence combined from chronic and inducible models suggests that axon degeneration in glaucoma involves components of both early and progressive distal axonopathy and more rapid Wallerian degeneration, involving disassembly of the axon at multiple points along the nerve (Calkins, 2012). Thus, regenerative strategies either have to replace entire lengths of axon or repair axons at multiple break points. In either case, the task is daunting, as the optic nerve in the human brain stretches some 5 cm in total length.

2. A brief history of optic nerve regeneration

As recently as 20 years ago, most observers considered the possibility of optic nerve regeneration to be remote. Although rodent RGCs show robust axon outgrowth through the late-embryonic or early postnatal period when placed in culture and *in vivo*, this growth capacity is lost shortly after birth due to cell-cell contacts that occur during the formation of retinal circuitry (Chen et al., 1995; Goldberg et al., 2002a). Nonetheless, in the early twentieth century, Cajal's student Tello found that a few RGCs could regenerate axons into a peripheral nerve graft sutured to the cut end of the optic nerve, and several decades later, Aguayo and his colleagues investigated this phenomenon in considerable depth (Ramon y Cajal, 1991; Aguayo et al., 1991).

Axon regeneration through the optic nerve itself was thought to be impossible until 1996, when Berry et al. discovered that implanting a peripheral nerve graft into the back of the eye – with the intention of providing Schwann cell-derived trophic factors – enabled many RGCs to regenerate axons well into the optic nerve itself. While testing whether this latter phenomenon might be mediated by a glial cell-derived factor that they were studying at the time, the Benowitz lab discovered that several manipulations that induce intraocular inflammation, including lens injury or injection of Zymosan, a yeast cell wall preparation, was a sufficient stimulus to induce regeneration (Leon et al., 2000; Yin et al., 2003). The primary mediator of this phenomenon was identified as Oncomodulin (Ocm), an atypical growth factor that is heavily expressed by both neutrophils and macrophages and that binds to a high-affinity receptor on RGCs in a cAMP-dependent manner (Yin et al., 2006; Yin et al., 2009; Kurimoto et al., 2010; Kurimoto et al., 2013). The effects of Zymosan are strongly enhanced when it is combined with manipulations that counteract cell-extrinsic or cell-intrinsic suppressors of axon growth. Whereas counteracting cell-extrinsic suppressors of axon growth by itself results in only modest levels of regeneration, combining any of these treatments with Zymosan increases the effects of the latter several-fold (Lehmann et al., 1999; Fischer et al., 2004a,b; Stiles et al., 2013).

Some of the most potent cell-intrinsic suppressors of regeneration increase during the course of development and account in part for the decline in regenerative capacity in the early postnatal period noted earlier (Wang et al., 2007; Park et al., 2009). These include the transcription factor Klf-4 (Moore et al., 2009), PTEN, a suppressor of cell signaling through the PI3 kinase-Akt pathway (Park et al., 2008), and SOCS3, which suppresses signaling through the Jak-STAT pathway and prevents agents such as CNTF from having a major effect (Smith et al., 2009). Other Klf family transcription factors that promote regeneration decline during development (Moore et al., 2009). Combining Zymosan, a cAMP analog, and *pten* gene deletion results in approximately 10 times more regeneration than any of these treatments in isolation and enables some RGCs to regenerate axons into central target areas (Fig. 1), with a partial return of simple visual responses (Kurimoto et al., 2010; De Lima et al., 2012). Combining double-deletion of *pten* and *socs3* with CNTF also has strongly synergistic effects, though not many axons extend past the optic chiasm unless surgery is done in the distal optic nerve, in which case there is an eventual innervation of the suprachiasmatic nucleus (Sun et al., 2011; Li et al., 2014a,b). Another major factor that suppresses optic nerve regeneration is the massive elevation of ionic zinc that occurs in synaptic contacts between amacrine cells and RGCs within an hour after nerve injury (Li et al., 2014a,b). Finally, at least one strain of mice has been identified that shows considerably greater optic nerve regeneration in response to fzymosan than the highly inbred strains that are more commonly used for this type of research (Omura et al., 2015).

3. Challenges and future directions

3.1. Specificity of connections

In order for regenerated (or replaced) axons to be most relevant for vision, they must satisfy several distinct criteria. One is **target specificity**. The optic projection innervates several subcortical nuclei that serve different aspects of visual processing (Fig. 2). Whereas most RGC axons in the human brain project to the lateral geniculate nucleus (LGN) of the thalamus, other RGCs project to alternative sites, such as the suprachiasmatic nucleus (SNC), olivary pretectal complex, several nuclei concerned with image stabilization, and superior colliculus (SC), the most distal subcortical projection. For now, the goal of regeneration ought to be to restore vision – any vision – where it has been lost, period. However, our expectation is that regenerative strategies eventually will evolve that ensure the right RGCs project to the right neurons in the right target area.

The second issue we must consider is **retinotopic specificity**. During development of the visual system, RGCs of a specific functional type mature to cover the retina such that cells representing adjacent patches of the photoreceptor array maintain the same

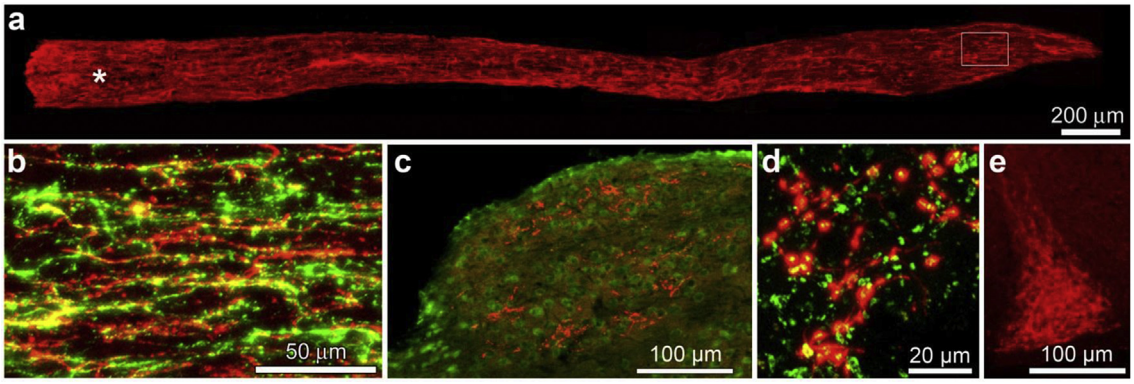

Fig. 1. Optic nerve regeneration and reinnervation of central target areas in mice. **a.** Longitudinal section through a mature mouse optic nerve showing regenerating axons (labeled via intraocular injection of the anterograde tracer cholera toxin B fragment, CTB: *red*) extending the entire length of the optic nerve 10 weeks after nerve damage. *Asterisk:* injury site. **b.** Higher-magnification image of the distal-most optic nerve double-labeled for CTB and the growth associated protein GAP-43 (*green*). Note the presence of many regenerating fibers (GAP-43+) that originate from retinal ganglion cells that did not pick up CTB. **c.** CTB+fibers in the dorsal lateral geniculate nucleus (dLGN) contralateral to the regenerating optic nerve. Section is counterstained for the neuronal protein NeuN. **d.** Evidence of synapse formation: high magnification image of the ventral LGN double-immunostained for CTB and the presynaptic marker VGlut2. **e.** Dense reinnervation of the medial terminal nucleus, a component of the accessory optic system. Modified from De Lima et al., 2012.

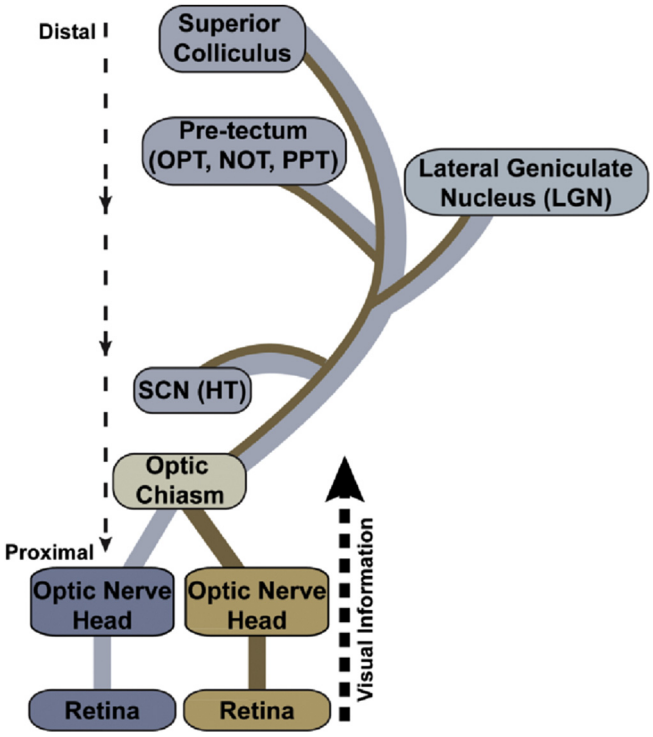

Fig. 2. The Optic Projection. Retinal ganglion cell (RGC) axons exiting the retina through the optic nerve head cross at the optic chiasm to form ipsilateral and contralateral projections to the brain. The contralateral projection dominates in rodents. Central targets for RGC axons are highly conserved across mammals. These include the suprachiasmatic nucleus (SCN) of the hypothalamus (HT) as well as three pre-tectum nuclei in the subcortical midbrain: the olivary pretectal nucleus (OPT), nucleus of the optic tract (NOT), and posterior pretectal (PPT) nucleus. In contrast, for human beings and non-human primates, the lateral geniculate nucleus (LGN) of the thalamus is the primary target for RGC axons. Across mammals, the superior colliculus of the midbrain is the most distal target for ascending RGC axons. In the rodent visual system, nearly all RGCs project to the colliculus with axon collaterals extending to nuclei lying more proximal to the retina. Modified from Crish and Calkins (2015).

spatial order in the ganglion cell layer. That is, neighboring RGCs represent neighboring regions of the photoreceptor array and, by extension, neighboring regions of the visual field. During development, this retinotopic representation is maintained by a multiplicity of axon-guidance cues that enable the formation of a globally accurate map of visual space onto the LGN, SC, and is further refined by correlated activity. Both the LGN and the SC in particular contain highly refined retinotopic maps, necessary for processing detailed, highly organized visual information and coordinating eye movements, respectively. The richness of our spectral, spatial and temporal visual experience depends on accurate maintenance of these maps, and we expect regenerative therapies will eventually need to capture the refinement of retinotopic representations that occurs during development.

3.2. RGC survival and regeneration

In order for regenerating axons to support vision, they must achieve a certain minimum level of physiological stability. For example, newly sprouting axons must re-establish the axon initial segment, which is required for the initiation of an action potential and the selective transport of appropriate components down the axon. Regenerating axons must also become myelinated and establish nodes of Ranvier, both of which are required for efficient signal conduction down the optic nerve. As yet, there is little published data on whether these events occur during axon regeneration. However, for glaucoma, the task may be eased due to the nature of progression.

Degeneration of RGCs in glaucoma can be considered compartmentalized, in that discrete events can be either quantified independently or separated mechanistically. A groundswell of evidence in the last decade or so supports the idea that neurodegenerative events in glaucoma proceed through two primary programs (Calkins, 2012). An early distal program affects RGC axons and their connections to the brain via the optic nerve, whereas a proximal program influences the survival of RGC synapses, dendrites and cell bodies in the retina (Fig. 3). Axonopathy in the distal program occurs early, preceding cell body loss in the retina (Buckingham et al., 2008; Crish et al., 2010). We also know that RGC axonal degeneration is to some extent independent of cell body elimination and therefore warrants attention in its own right (Whitmore et al., 2005). The *Wld*^{S} (*slow Wallerian degeneration*) allele creates a chimeric fusion protein containing 70 N-terminal amino acids of ubiquitination factor *Ube4b* linked to full-length nicotinamide mononucleotide adenylyltransferase 1 (*Nmnat1*) that protects axons from degeneration (Antenor-Dorsey and

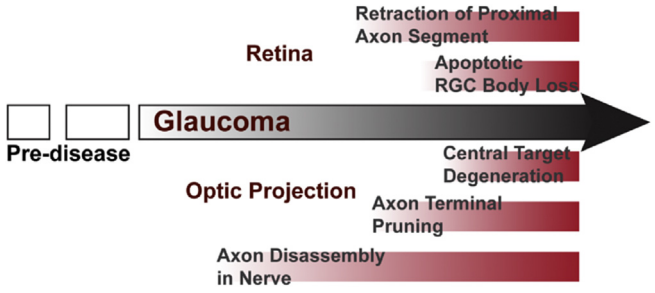

Fig. 3. Progression of Axon Degeneration in Glaucoma. Normal function in the optic projection is interrupted in glaucoma, setting into motion a cascade of degenerative events both in the optic projection and in the retina. Evidence suggests that axon disassembly in the optic nerve may precede loss of RGC axon terminals in central brain targets. In the retina, retraction of the unmyelinated axon segment precedes RGC body loss. Thus, regenerative strategies must address different compartments of tissue loss. Modified from Calkins 2012.

O'Malley, 2012). In two models of glaucoma, *Wld*^{S} preserved RGC axon function as measured by pattern electroretinogram and substantially slowed axon degeneration in the optic projection, with some variability in the degree of rescue (Howell et al., 2007; Howell et al., 2013). In models of glaucoma, RGC body elimination is dependent on the pro-apoptotic gene *Bax*; *Bax*-deficient DBA2J mice undergo axonal degeneration while RGC bodies are spared (Libby et al., 2005). Interestingly, the unmyelinated segment of the RGC axon in the retina also survives with *Bax* knock-out (reviewed in Calkins, 2012). This survival could be a critical variable in regeneration, for the cell body integrates proximal synaptic activity and conveys the net signal to the brain by generation of action potentials in the unmyelinated segment of the axon.

Thus, in glaucoma, the RGC body appears to survive in the retina at least for a while after disassembly of the myelinated segment of the axon in the nerve. Presumably, such survival is necessary for regenerative strategies that would re-grow the axon from the unmyelinated segment. However, cell survival and regeneration appear to be governed to some extent by independent mechanisms, in that factors that promote or increase survival may have little influence or even negative impact in generating new tissue. For example, overexpression of the apoptosis regulator protein Bcl-2 (B-cell lymphoma 2) is sufficient to maintain neonate RGC survival in culture even without glial or trophic factor support, yet Bcl-2+RGCs fail to extend axons or dendrites unless treated with factors such as brain-derived neurotrophic factor (BDNF) (Goldberg et al., 2002b). In mature rats, although BDNF strongly enhances the effect of intraocular inflammation in promoting RGC survival, it eliminates the latter's effects on axon regeneration (Pernet and Di Polo, 2006). Deletion of dual leucine zipper kinase (DLK) in RGCs prevents apoptosis following optic nerve crush, yet axon regeneration associated with *pten* deletion is eliminated (Watkins et al., 2013). Thus, DLK appears to be a switch that is both pro-survival and anti-regenerative.

Other molecular pathways appear to be pro-regenerative - and pro-survival as a direct result. For example, acute injury to axons in the optic nerve induces rapid loss of RGC dendrites, which is accompanied by reduced signaling via mammalian target of rapamycin (mTOR) (Morquette et al., 2015). However, selective inhibition of regulated in development and DNA damage 2 (REDD2), a potent inhibitor of mTOR, prevents dendritic pruning and extends RGC body survival. Thus, while survival of RGC bodies is not sufficient for regeneration, regenerative programs that promote dendrite growth can extend survival. In the context of glaucoma, mTOR is also important for axonal regeneration, in that deletion of PTEN, a negative regulator of mTOR, promotes robust RGC axon regeneration within the injured optic projection (Kurimoto et al., 2010). Pathways such as mTOR that are linked to both dendritic and axonal survival are important, since restoring visual activity via regenerative strategies is likely to involve both.

At the other end of the optic projection, structures important to synaptic transmission from RGC axon terminals to neurons in central brain targets including the LGN and SC persist as well for a period following the onset of axonal dysfunction (Crish et al., 2010; Crish et al., 2013). Like survival of RGC bodies in the retina, such persistence could prove useful for regenerative therapies. One idea is that early in glaucoma progression, RGC axons struggle to maintain connectivity with central targets – in essence, attempting a form of self-repair. Such a process could involve highly focal elevations in BDNF, with the purpose of cementing challenged connections before disassembly of the axon (Crish and Calkins, 2015).

3.3. The inflammatory environment: a barrier to regeneration?

The unmyelinated segment of the RGC axon is part of a complex microenvironment as it passes through the retina and optic nerve head. This environment includes not only astrocytes, but also a highly mobile population of microglia. In exiting the nerve head, axons become myelinated by oligodendrocyte glia that populate the remainder of the optic projection. These glial groups represent potent sources of interactions with RGCs involving extracellular signals commonly categorized as pro-inflammatory, including cytokines, chemokines, and other soluble signals typically associated with innate immunity (e.g., complement, nitric oxide etc). In response to glaucomatous stress, glia of the retina and optic nerve undergo neurochemical and morphological remodeling that is known broadly as reactive gliosis – a component of what we usually term inflammation. Many of these changes occur over time as part of aging, but are exacerbated by elevations in IOP (Cooper et al., 2015). Inflammation itself is often viewed as pathogenic in glaucoma, as in other diseases. This view is supported by recent results showing that anti-inflammatory p38 MAP kinase inhibitors are protective of RGCs in experimental glaucoma (Dapper et al., 2013). However, under certain circumstances, other immune-derived inflammatory signals can be protective of RGCs (Shechter and Schwartz, 2013). In glaucoma patients with nearly complete RGC axon loss in the optic nerve, we are faced with the prospects of re-sprouting an axon through an optic nerve head complex reshaped by reactive gliosis, which is presumed maladaptive to regeneration. However, during progression, it is more likely that reactive gliosis and inflammation more generally modulate a multidimensional equilibrium that includes a window of opportunity that could promote regeneration.

In neural regeneration, we must distinguish between intrinsic mechanisms governed by neuronal intracellular factors and extrinsic mechanisms contributed by other elements of the neural environment. These include inflammatory interactions with astrocytes, microglia, oligodendrocytes, and the innate immune system. We can infer from transgenic experiments that a subset of inflammatory interactions must hamper regenerative states. For example, when both GFAP and vimentin (important cytoskeletal components of astrocyte gliosis) are removed in mice, both baseline and post-trauma adult neurogenesis increases, neurogenesis from neural stem cells transplanted in the hippocampus improves, and integration and survival of neuronal grafts increases (Pekny and Pekna, 2014). In contrast, in the peripheral nervous system, regeneration requires inflammation at the level of the dorsal root ganglia (DRG) in concert with phagocytosis distal to the injury site. In the so-called conditioning lesion paradigm, cutting the peripheral branch of an axon activates a gene expression program promoting growth and enables some regenerative growth to occur in the central branch within the spinal cord. If inflammation is

eliminated, this phenomenon is lost entirely (Niemi et al., 2013). Conversely, intentional induction of inflammation in the DRG enables the central branch of the axon to regenerate even in the absence of peripheral nerve injury. This research points to the importance of inflammation – if you block inflammation in many repair processes, then you eliminate a crucial partner for regeneration.

4. Summary

To restore vision, regenerative therapies face several difficult problems, which we have outlined here. In glaucoma, one must consider the possibility that experimental therapies that offer neuroprotection by slowing progression could do so by promoting a rudimentary regenerative state. For example, drugs we consider to be anti-inhibitory (such as p38 inhibitors) could be protective of RGCs by reducing glial inflammation within the optic projection. If so, then a key step is to identify the window in progression during which the balance between pro-regenerative and maladaptive inflammation occurs. This requires a far more sophisticated understanding of the extracellular signaling involved in reactive gliosis in the retina and optic nerve head.

The task for regeneration is eased somewhat by the persistence of the RGC body and unmyelinated axon segment during a critical period in progression. Even so, to restore vision requires the growth of axons beyond the nerve head, remyelination via oligodendrocytes, and the correct targeting of axons to multiple central targets with the restoration of retinotopic maps. In the retina, dendrites must be restored with appropriate synaptic connections from interneurons. This stage may represent an even greater challenge than getting injured RGCs that remain alive to regenerate their axons. Cell replacement therapies on the horizon must consider that creating an RGC from a Müller glial cell or astrocyte in the retina involves far more than genetic reprogramming. Critical elements of pre-synaptic circuitry in the retina and post-synaptic targeting in the brain must be recreated as well.

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