Neural activity promotes long-distance, target-specific regeneration of adult retinal axons

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Axons in the mammalian CNS fail to regenerate after injury. Here we show that if the activity of mouse retinal ganglion cells (RGCs) is increased by visual stimulation or using chemogenetics, their axons regenerate. We also show that if enhancement of neural activity is combined with elevation of the cell-growth-promoting pathway involving mammalian target of rapamycin (mTOR), RGC axons regenerate long distances and re-innervate the brain. Analysis of genetically labeled RGCs revealed that this regrowth can be target specific: RGC axons navigated back to their correct visual targets and avoided targets incorrect for their function. Moreover, these regenerated connections were successful in partially rescuing a subset of visual behaviors. Our findings indicate that combining neural activity with activation of mTOR can serve as a powerful tool for enhancing axon regeneration, and they highlight the remarkable capacity of CNS neurons to re-establish accurate circuit connections in adulthood.

Neurons in the adult mammalian CNS fail to regenerate after injury, thereby preventing recovery of numerous CNS functions. A major goal of neuroscience is to identify the factors that limit CNS regeneration and devise therapeutic strategies to overcome them. Previous work has illustrated that factors inherent to the mature CNS environment are unfavorable for axon growth but that damaged CNS axons are capable of regenerating through peripheral nerve grafts. Subsequent work identified some of the CNS factors responsible for inhibiting axon regrowth, including myelin-associated proteins and reactive glial scarring, upregulation of extracellular matrix factors and repellant guidance cues. Factors intrinsic to CNS neurons also prevent axon regeneration. For example, during development, CNS neurons downregulate expression of growth-promoting molecules. Some CNS axon regeneration can be achieved by augmenting intrinsic cell-growth-promoting factors such as mammalian target of rapamycin (mTOR), cyclic adenosine monophosphate (cAMP) and ciliary neurotrophic factor (CNTF). However, neither Pten deletion nor combined Pten and Socs3 deletion stimulates regeneration of RGC axons back into the brain. Instead, the regenerating RGC axons stall at the optic chiasm or steer away from the brain and travel down the opposite optic nerve, leaving RGCs divorced from their targets.

The purpose of this study was twofold. First, we sought to identify strategies that alone or in combination allow adult RGC axons to regenerate through the optic chiasm and back to the brain. We found that, if neural activity was enhanced along with levels of mTOR, RGC axons re-innervated their targets, including the most distal subcortical visual nuclei. That discovery, in turn, allowed us to address a second major question: whether regenerating RGC axons have the ability to reconnect with their correct targets and restore visual function. Together, our data support the combined use of neural activity and molecular programs for intrinsic growth as strategies to regenerate visual circuits. Our results reveal the remarkable ability of adult CNS neurons to re-establish correct patterns of connectivity following injury.

RESULTS

Enhancement of RGC axon regeneration by visual stimulation

We lesioned RGC axons by crushing the optic nerve just posterior to the orbit of the eye using fine forceps (Fig. 1a). Three weeks later we labeled RGCs and their axons with intravitreal injections of cholera toxin subunit-β (CTβ) conjugated to Alexa-Fluor-594 (CTβ-594) (Fig. 1a,c,d). In the absence of any therapeutic intervention (group 1), very few RGC axons extended beyond the crush site (Fig. 1e) (control...
group details in Supplementary Fig. 1) and the majority of RGCs died (Supplementary Fig. 2). This regenerative failure is consistent with hundreds of previous reports spanning many decades15. An earlier study showed that electrically stimulating developing RGCs in vitro accelerated outgrowth of their axons20. This inspired us to investigate whether enhancing the electrical activity of adult RGCs would promote regeneration of their axons in vivo. Vision is a potent stimulus to drive RGC firing (Fig. 1b), an effect that, while relatively limited in distance, was statistically significant compared to controls (Fig. 1g).

To further explore the influence of neural activity on axon regeneration, we employed chemogenetic technology21. First we tested whether reducing RGC activity would block the enhancement of RGC axon growth caused by visual stimulation. We overexpressed the engineered G-protein-coupled receptor hM4Di [Gi/o-coupled human muscarinic M4] receptor receptor exclusively activated by a designer drug (DREADD)21 in RGCs by injecting mice with adeno-associated-virus AAV2-Cre into the optic nerve, waited 2 weeks for expression, and then crushed the same eye's optic nerve and systemically administered the mice CNO twice a day for 3 weeks following ONC would trigger regeneration of RGC axons (Fig. 2c,d) (see Online Methods). This protocol (group 2) was effective in causing some RGC axons to regenerate a short distance past the lesion site (Fig. 1f), an effect that, while relatively limited in distance, was statistically significant compared to controls (Fig. 1g).

Next we applied the hM4Di strategy to test whether suppression of RGC activity would affect the axon regeneration caused by daily visual stimulation. We injected AAV-hM4Di-mCitrine into one eye, waited 2 weeks for expression, and then crushed the same eye's optic nerve and systemically administered the mice CNO twice a day for 3 weeks, while also exposing the animals to high-contrast visual stimulation to drive RGC firing (Fig. 2d). Reducing RGC activity with hM4Di and CNO abolished the effect of visual stimulation on RGC axon regeneration and also reduced the total number of CTβ-labeled RGC axons anterior to the lesion site (compare Fig. 2e with Fig. 1f) (Fig. 2k). These findings suggest that high-contrast visual stimulation promotes regeneration of RGC axons by increasing their overall levels of electrical activation. The reduced number of CTβ-labeled
RGC axons anterior to the lesion site observed in mice that received hM4Di and CNO treatments also suggests that neural activity may affect the number of RGCs that survive optic nerve damage.

Next we tested whether chemogenetically increasing levels of RGC activity can promote their axons to regenerate. We overexpressed the CNO-sensitive synthetic receptor hM3Dq (Gq-coupled human muscarinic M1 DREADD)21 in RGCs by intravitreal injections of AAV2-hM3Dq-mCitrine. (Fig. 2f,g). Two weeks after injection, expression of hM3Dq-mCitrine was observed in all four quadrants of the RGC layer ( Supplementary Fig. 3e–h) and co-labeling of these retinas with RBPM522 showed that >90% of RGCs expressed the hM3Dq ( Supplementary Fig. 3i–l). In whole-mount retinas from these mice, CNO-driven activation of the hM3Dq receptor caused a large increase in the number of spikes elicited from infected RGCs by current injection or by high-contrast visual stimulation (Fig. 2h and Supplementary Fig. 4e–h).

What is the impact of chemogenetically enhancing neural activity on RGC axon regeneration? To test this, we injected a group of mice with AAV2-hM3Dq-mCitrine, crushed the optic nerve 2 weeks later and administered CNO once a day for 3 weeks (Fig. 2i). To isolate the effects of hM3Dq-driven RGC activity, these mice were housed on normal light-dark cycles, with no additional visual stimulation. CTβ labeling of RGC axons at the end of the 3-week chemogenetic stimulation period revealed that hM3Dq and CNO treatments led to a greater degree of RGC axon regeneration (Fig. 2j,k) than occurred in control mice (group 1) or to mice that underwent daily visual stimulation alone (group 2) (Fig. 2k). A large number of hM3Dq-treated RGCs regrew their axons through the lesion site in the proximal nerve, and indeed, some even extended into mid-optic nerve (Fig. 2). These results indicate that elevating RGC spiking levels is sufficient to promote axon regeneration and support the idea that visual stimulation exerts its influence on RGC axon regeneration by increasing levels of activity.

Synergistic effects of visual stimulation and mTOR elevation

Previous studies showed that increasing mTOR signaling by deletion of one of its repressors, PTEN, can trigger RGC axon regeneration.10,16,18,19 Here we tested whether expressing a positive regulator of mTOR signaling, ras homolog enriched in brain 1 (Rheb1) protein, also could promote regeneration. We injected AAV overexpressing constitutively active Rheb1 (AAV2-cRheb1)23 into one eye of adult mice, waited 2 weeks for cRheb1 overexpression, and then crushed the optic nerve of the AAV-injected eye. Three weeks later, we labeled RGC axons by intravitreal injection of CTβ–594 and assessed their regeneration (Fig. 3a) (see Online Methods). Injections of AAV2-cRheb1 significantly increased the number of cells in the adult RGC layer that express phosphorylated S6 ribosomal protein (p-S6), a downstream marker of phosphorylated mTOR activity (Fig. 3b–e and Supplementary Fig. 5).

Overexpression of cRheb1 enhanced the ability of adult RGCs to regenerate their axons after injury. Three weeks after ONC, we observed
RGC axons extending through the lesion site and into the proximal optic nerve (Fig. 3f), an effect that was statistically significant compared to control mice receiving either (i) no treatment, (ii) intravitreal injections of saline or (iii) injections of a control virus (AAV2-Cre) (collectively, group 1) (Fig. 3h and Supplementary Fig. 1). The effects of AAV2-cRheb1 on RGC axon regeneration were attenuated by chronic administration of the mTOR inhibitor rapamycin (Supplementary Fig. 6), supporting the idea that the effect of AAV2-cRheb1 stemmed from elevation of mTOR signaling. Although there were quantitative differences in the overall number of axons that regenerated in response to cRheb1 overexpression versus visual stimulation versus Pten deletion (ref. 10 and Supplementary Fig. 7), the general patterns of regeneration observed in these three groups were similar: RGC axons regenerated past the lesion site but failed to grow the full distance of the optic nerve to reach the optic chiasm or brain (Fig. 3f,h and Supplementary Fig. 7).

Next we tested whether combining visual stimulation with enhanced mTOR signaling would increase the distance that RGC axons regenerate beyond that observed with either treatment alone. We injected one eye with AAV2-cRheb1 then allowed 2 weeks for elevation of mTOR signaling in RGCs. We then crushed the optic nerve and exposed the mice to high-contrast visual stimulation daily for 3 weeks (group 5; Supplementary Fig. 8a). In these mice, RGC axons regenerated past the lesion site but failed to extend beyond the mid-optic nerve and optic chiasm (Supplementary Figs. 8a and 9a).

In the motor system, forced use of an impaired limb promotes sprouting of corticospinal axons24. We therefore tested whether, after treatment with AAV2-cRheb1 and crushing one optic nerve, suturing shut the eye corresponding to the non-lesioned optic nerve could further enhance RGC axon regeneration of the lesioned eye pathway (group 6). Biased use of the lesioned eye pathway in this manner enhanced RGC axon regeneration compared to group 3 mice treated with AAV2-cRheb1 alone (Supplementary Figs. 8b and 9a). However, if these mice were also exposed to high-contrast visual stimulation every day for 3 weeks (group 4; Fig. 3g and Supplementary Figs. 10–12), this combination of treatments (hereafter referred to as ‘biased visual stimulation/AAV2-cRheb1’) triggered long-distance regeneration of RGC axons. In 7 of 10 mice treated in this manner, RGC axons regenerated through the ONC site, down the full length of the optic nerve and into the optic chiasm (Fig. 3g and Supplementary Figs. 10–12), an effect that was highly robust compared to control mice (group 1), to mice that received AAV2-cRheb1 treatment (group 3) (Fig. 3h) or to mice that received visual stimulation/AAV2-cRheb1 but that had both eyes open (group 5). Also, when we sutured shut...
Combining biased visual stimulation and enhancement of mTOR signaling with cRheb1 overexpression allows RGCs to regenerate their axons back to their targets. (a) Schematic of experimental group (group 4). Protocol for the combined treatments: inject cRheb1 virus, perform optic nerve crush and suture opposite eye, provide mice daily visual stimulation, inject CTβ neurotracer, and collect optic nerve and brain. (b) Photomicrograph and schematic of a sagittal mouse brain section showing the major visual targets labeled with CTβ. (c–i) Coronal brain sections from one animal showing CTβ-labeled regenerating RGC axons in the SCN, vLGN, dLGN, OPN, MTN (d, dorsal; v, ventral) and SC (SGS, stratum griseum superficiale; SO, stratum opticum). Dashed lines indicate the boundary of each visual target. Arrows in c point to regenerating axons in the SCN and arrows in g point to regenerating axons in pretectum. Arrowhead in g indicates highly specific axon regeneration of posterior limitans. (j–o) Coronal sections of another animal’s brain, showing CTβ-labeled regenerating RGC axons in SCN (j), vLGN (k), dLGN (l), OPN (m), MTN (n) and SC (o). Dotted outlines indicate the approximate area of each boxed region. High-magnification images of boxed regions are shown in c1–e1, k1, l1 and o1. Scale bars: c 100 μm (left) and 20 μm (c1); d, 100 μm (left) and 50 μm (d1); e, 200 μm (left) and 50 μm (e1); f, 100 μm; g, 50 μm; h, 100 μm; i, 200 μm; j, 200 μm; k, 100 μm (left) and 50 μm (k1); l, 200 μm (left) and 100 μm (l1); m, 100 μm; n, 100 μm; o, 250 μm (left) and 50 μm (o1).

The number of RGCs surviving ONC did indeed more than double in mature RGCs9–12. This high-throughput of regeneration required critically on cRheb1 overexpression of the RGCs. When the daily visual stimulation was biased toward the lesioned eye pathway but we did not overexpress cRheb1 (group 8), the number of RGC axons that regenerated was markedly reduced (Supplementary Figs. 8d and 9b). Further, in the absence of cRheb1 overexpression and visual stimulation (group 9), the number of regenerated RGC axons was significantly reduced (Supplementary Figs. 8e and 9b). This highlights the need for elevating intrinsic growth programs missing in mature RGCs9–12. Together, the different combinations of treatments we designed to promote regeneration highlight the importance of providing visual stimulation to the AVV2-cRheb1 treated eye and they argue against indirect effects of the biased visual stimulation/AVV2-cRheb1 protocol on nonvisual factors such as enhanced locomotion (Fig. 3 and Supplementary Figs. 8–12). We considered whether the enhanced axon regeneration we observed in mice given biased visual stimulation/AVV2-cRheb1 reflected an increase in RGC survival. The number of RGCs surviving ONC did indeed more than double in the biased visual stimulation/AVV2-cRheb1 group (Supplementary Fig. 13). The most parsimonious conclusion from all of these experiments is that it is the combination of simultaneously enhancing activity and enhancing mTOR signaling that allows a set of RGC axons to regenerate through lesion sites and extend long distances down the optic nerve.

Long-range RGC axon regeneration to targets in the brain
A critical milestone for the re-establishment of functional eye-to-brain circuits is long-distance regeneration of RGC axons back to the brain. The retinofugal pathway includes several dozen target nuclei located in the forebrain and midbrain (Fig. 4b and Supplementary Fig. 14)32. In mice receiving biased visual stimulation/AVV2-cRheb1 treatments (group 4) (Fig. 4a,b), regenerated CTβ-labeled RGC axons were observed in multiple subcortical visual targets (Fig. 4c–o). Seven of ten mice that received the biased visual stimulation/AVV2-cRheb1 treatments had RGC axons that regenerated past the ONC site, through the optic chiasm and optic tract, and back to visual targets in the brain (Fig. 4c–o, Table 1 and Supplementary Fig. 15). Three weeks after nerve crush, CTβ-labeled RGC axons were observed in the most proximal visual target, the hypothalamic suprachiasmatic nucleus (SCN) (Fig. 4c,j), and in the thalamic ventral lateral geniculate nucleus (Fig. 4d,k) and dorsal lateral geniculate nucleus (Fig. 4e,l) (vLGN and dLGN, respectively). Regenerated RGC axons were also observed in midbrain pretectal nuclei such as the olivary pretectal nucleus (OPN) (Fig. 4f,m) and posterior limitans (Fig. 4g) and in accessory optic targets of the brainstem, such as the medial terminal nucleus (MTN).
Remarkably, RGC axons were also detected in the subcortical visual target located furthest from the eye, the superior colliculus (SC) (Fig. 4h,n). Notably, we did not observe any CTβ-labeled RGC axons in nonvisual subcortical regions such as the somatosensory or auditory thalamus (data not shown), indicating that regenerating RGC axons appropriately confined their trajectories to the retinofugal pathway. The extent of RGC axon regeneration along the retinofugal pathway and within each target varied from one mouse to the next (Table 1). Nevertheless, in every animal receiving biased visual stimulation/AAV2-cRheb1 treatment, the extent of regeneration was marked compared to mice treated only with AAV2-cRheb1 (group 3) or that received only visual stimulation but not AAV2-cRheb1 (group 2) (Figs. 1 and 3 and Table 1).

To ensure that the CTβ-labeled axons we observed in the optic nerve and brain were regenerated axons and not spared RGC axons, we carried out several control experiments. For the first set of controls, we labeled RGC axons by intravitreal injections of CTβ-594, and 2 d later we crushed the optic nerve. Then we waited 1 week, at which time we relabeled all RGC axons by intravitreal injection of CTβ-488 (Supplementary Fig. 16b). In every mouse examined, CTβ-594-labeled axons were observed in the vicinity of the lesion site but never at the distal nerve or optic chiasm, contrary to abundantly labeled RGC axons down the entire length of the non-lesioned nerve (Supplementary Fig. 16a,c), indicating that the ONC indeed caused RGC axons to degenerate. Additionally, RGC axons labeled after the ONC with CTβ-488 were observed posterior to the eye and in the vicinity of the lesion site, but never in the mid- or distal optic nerve, optic chiasm or brain (Supplementary Fig. 16c and data not shown). These results support the conclusion that the ONC procedure did not spare RGC axons.

As a second set of controls, we tested whether the biased visual stimulation/AAV2-cRheb protocol induced regenerating RGC axons to regenerate down the optic nerve in a time-dependent manner, which would not occur if the crush procedure had spared RGC axons. For these experiments, we allowed the mice a period of either 1 or 2 weeks for RGC axons to regenerate before assessing the distance of axon growth (Supplementary Fig. 17a). In mice allowed to survive 1 week after crush for regeneration to occur, RGC axons extended through and beyond the lesion site (Supplementary Fig. 17b), but were never observed in the distal nerve or in central visual targets (Supplementary Fig. 18a–d). In mice allowed 2 weeks for regeneration to occur, there were an even greater number of RGC axons growing through the lesion site (Supplementary Fig. 17c,d); some RGC axons were observed in the mid- and distal optic nerve but no axons were observed in the chiasm or the brain (Supplementary Fig. 17c,d). Only in mice treated with biased visual stimulation/AAV2-cRheb1 and allowed a minimum of 3 weeks for regeneration did we observe RGC axons regenerating through the optic chiasm and back into the brain (Figs. 3 and 4).

Together, these two sets of control experiments support the conclusion that our crush procedure did not spare RGC axons. Rather, the CTβ-labeled profiles observed in the optic nerve, chiasm and brain represent RGC axons that regrew in a time-dependent manner through and beyond the crush site.

### Table 1 Targets innervated by regenerated RGC axons in each of the treatment conditions

| Group | Animals | Rostral | Caudal |
|-------|---------|---------|--------|
| 1     | n = 16  | 0/16    | 0/16   |
| 2     | n = 5   | 0/5     | 0/5    |
| 3     | n = 13  | 0/13    | 0/13   |
| 4     | n = 10  | 7/10    | 6/10   |
| 5     | n = 6   | 0/6     | 0/6    |
| 6     | n = 5   | 1/5     | 0/5    |
| 7     | n = 5   | 0/5     | 0/5    |
| 8     | n = 5   | 0/5     | 0/5    |
| 9     | n = 5   | 0/5     | 0/5    |
| 10    | n = 9   | 6/9     | 4/9    |

Table summarizes the number of animals in each group with regenerated RGC axons to different visual nuclei. Control group is group 1. The two groups with combined biased visual stimulation with AAV2-cRheb1 are group 4 and group 10. Only these two groups showed regenerated RGC axons that re-innervated visual nuclei in the brain. Group 1, no treatment/saline/AAV2-Cre; group 2, visual stimulation; group 3, AAV2-cRheb1; group 4, AAV2-cRheb1/suture opposite eye/visual stimulation; group 5, AAV2-cRheb1/visual stimulation; group 6, AAV2-cRheb1/suture opposite eye; group 7, AAV2-cRheb1/suture same eye/visual stimulation; group 8, suture opposite eye/visual stimulation; group 9, suture opposite eye; group 10, AAV2-cRheb1/remove opposite eye/visual stimulation.

### Target-specific axon regeneration in the brain

A critical unresolved issue in the field of CNS regeneration is whether regrowing axons can find and reconnect to their correct targets. The growth of RGC axons back into the brain we observed in mice receiving biased visual stimulation/AAV2-cRheb1 treatment (Figs. 3 and 4), provided us the opportunity to address this issue. Mammals, including mice and humans, have ~30 types of RGCs, each of which responds to a particular feature in the visual world and connects to a small subset of the 40-plus retinorecipient targets. Our laboratory and others have created and/or characterized various transgenic mouse lines, each harboring green fluorescent protein (GFP) in specific RGC types25,26. When combined with the axon-regrowth protocol described above, these mice offer a powerful opportunity to explore the specificity of RGC axon regeneration. Others have shown that mTOR-induced regeneration is biased toward α-RGC types.27 We made use of a new mouse line: cochlin-GFP (CoCH-GFP). In these mice many of the GFP-expressing RGCs are α-RGCs (Supplementary Fig. 19) and we tested whether GFP+ RGCs regenerated their axons back to their correct targets in the brain.

In normal non-lesioned mice, CoCH-GFP+ RGC axons densely innervate the vLGN, dLGN, OPN, and MTN and interrupt the visual pathway from the eye to the brain. The vLGN, dLGN, OPN, and MTN are retinorecipient targets that are normally failed to innervate: the SCN, the nucleus of the optic tract (NOT), and the MTN. In optic nerve-lesioned CoCH-GFP mice treated with biased visual stimulation and AAV2-cRheb1 (Fig. 5a), a subset of the CTβ-594+ axons in the optic nerve also expressed CoCH-GFP (Supplementary Fig. 20) indicating they indeed are part of the regenerating cohort. CTβ-594-labeled RGC axons were observed in various retinorecipient targets in the brain, including the SCN, vLGN, dLGN, OPN, and MTN (Fig. 5c–l,o–u). Remarkably, the only targets that contained axons that were double-labeled with both CTβ-594 and CoCH-GFP (i.e., regenerated CoCH-GFP+ RGC axons) were those targets that normally receive input from CoCH-GFP+ RGCs, namely the vLGN, dLGN, and MTN (Fig. 5f–h), the SCN, and the nucleus of the optic tract (NOT) and the MTN. Although these targets contained CTβ-594 labeled RGC axons (Fig. 5c,0,r), indicating they are capable of accepting regenerating RGC axons, none of the regenerated axons expressed GFP (Fig. 5d,e,0,9,q,s,t), indicating they arose from other, non-CoCH-GFP+ RGC types.
To investigate whether other types of RGCs reconnect to appropriate targets, we examined regeneration in OPN4-GFP mice. OPN4 is expressed by a subpopulation of RGCs—so-called intrinsically photosensitive RGCs (ipRGCs)—that are thought to be more amenable to regeneration.27,28 In normal non-lesioned mice, the axons of OPN4-GFP+ RGCs heavily target the SCN, vLGN, IGL and OPN, while minimally targeting the dLGN and SC. OPN4-GFP+ RGC axons avoid the MTN entirely (ref. 28 and Supplementary Fig. 21). The biased visual stimulation/AAV2-cRheb1 protocol induced a subset of OPN4-GFP+ RGC axons to regenerate back to the brain, where they reinnervated several correct targets such as the IGL (Supplementary Fig. 22j–l) and avoided incorrect targets such as the NOT and MTN (Supplementary Fig. 22p–r).

In the brains of both CoCH-GFP mice and OPN4-GFP mice, we observed GFP+ axons that were not labeled with CTβ-594. To test the assumption that the GFP+ CTβ- axons arose from the retina that was treated with AAV-Rheb1 and not from any other source (Fig. 5h,k,n,w), we injected AAV2-cRheb1 into one eye of CoCH-GFP mice, nerve crushed the AAV2-cRheb1 eye and then enucleated the opposite eye, forcing its RGC axons to degenerate. We then provided biased visual stimulation through the remaining eye every day for 3 weeks to trigger regrowth of RGC axons (Fig. 6a,b). In this experiment, any CTβ+GFP+ axons observed in the brain must have originated from the lesioned and treated eye. Six of nine mice in this group (group 10) exhibited regenerated CoCH-GFP+ axons in the optic nerve and chiasm (Fig. 6c), as well as the vLGN (Fig. 6d–f), dLGN (Fig. 6g–i) and SC (Fig. 6j–l and Table 1). Notably, all the regenerat ed CTβ-RGCs were labeled with CTβ-594, indicating they regenerated from the lesioned eye. Together, our experiments on regrowth and steering of axons from GFP-labeled RGCs reveal the remarkable capacity of adult CNS axons to navigate back to and re-innervate their correct targets in the brain when provided with the appropriate combination of regeneration-inducing stimuli.

Functional restoration of visual behaviors

To test whether the regenerated RGC connections described above can support visual function, we assayed behavioral performance in four different tests of visual function25. The optokinetic reflex (OKR) probes the function of the AOS connections to the oculomotor brainstem29, the pupillary light reflex (PLR) probes retino-pretectal connection to the OPN shell30, the visual cliff test probes the retinogeniculo-cortical pathway31, and the looming avoidance response probes the retino-collicular pathway32–34. We tested three groups of mice: a pure-control non-lesioned group, a unilaterally optic-nerve-lesioned group that received no regeneration-enhancing treatment (‘lesioned and untreated’) and a lesioned and treated group (identical to group 4 above) that received the biased visual stimulation/AAV2-cRheb1 treatments capable of inducing long-range axon regrowth into the brain (see Online Methods) (Fig. 7a–c). To ensure that any observed functional recovery was mediated by regenerated RGC connections originating from the lesioned eye and not by RGCs from the non-lesioned eye, we sutured shut the non-lesioned eye. The only exception to this was during testing of the consensual PLR, where, by requirement, both eyes had to be kept open (see below).

First we assessed the OKR, in which animals generate slip-compensating head movements in response to drifting gratings moving along the horizontal axis (Fig. 7d). As described previously35,36 we quantified the percentage of 15-s trials in which the animals successfully tracked the stimulus (see Online Methods). The non-lesioned group tracked ~33% of the stimulus trials (n = 5 animals)—a value lower than is typical in binocularly sighted mice but still far greater than observed in the lesioned and untreated group, which failed to track any stimulus trials (n = 5 animals) (Fig. 7e). The lesioned and treated mice, by contrast, tracked ~23% of the stimulus

Figure 5 Specificity of axon regeneration from distinct RGC types to their visual targets. (a) Schematic of experimental group (group 4). Protocol for the combined treatments: inject cRheb1 virus, perform optic nerve crush and suture opposite eye, provide mice daily visual stimulation, inject CTβ neurotracer, and collect optic nerve and brain. (b) Photomicrograph of sagittal mouse brain with retinofugal projections from CoCH-GFP+ RGCs shown in green and pan-RGCs shown in magenta. (c–w) Images of CTβ-labeled regenerated axons in GFP-immunostained coronal sections of brain from CoCH-GFP animal showing RGC axons in the SCN (c–e), vLGN (f–h), dLGN (i–k), OPN (l–n), MTN (o–q), NOT (r–t) and SC (u–w). Dashed outlines indicates the approximate boundary of each visual target. Boxed region in d is magnified in inset. Boxed regions in f–n and u–w are magnified in f1–n1 and u1–w1, respectively. Scale bars: c–e, 200 µm; f–h, 50 µm (left) and 25 µm (f1, g1, h1); i–k, 100 µm (left) and 25 µm (i1, j1, k1); l–n, 100 µm (left) and 25 µm (l1, m1, n1); o–q, 100 µm; r–t, 250 µm; u–w, 500 µm (left) and 50 µm (u1, v1, w1).

Re-innervation of the brain by CTβ-594-labeled, CoCH-GFP+ RGC axons in the brains of both CoCH-GFP mice and OPN4-GFP mice.
trials \((n = 3\) animals\), which was significantly more than the lesioned and untreated group and approached the percentage tracked by the non-lesioned group (Fig. 7e). This indicates that regenerated RGC axons can partially restore OKR behavior.

Next we measured the PLR, a behavior driven by ipRGC projections to the OPN shell\(^{30,37,38}\). The PLR has both a direct component and a consensual component. The direct PLR represents constriction of the pupil in the illuminated eye. The consensual response is the constriction of the pupil in the opposite eye, mediated by interhemispheric connections (Supplementary Fig. 23). We recorded both the direct and consensual PLR to ipRGC-optimized blue light stimulation of the lesioned eye at \(25\ \text{lx}\) for \(30\ s\) (refs. 30,38) (Fig. 7f,g). Measuring the consensual PLR required opening the previously sutured eye at the time of testing. Both the direct and consensual response was markedly reduced by ONC (Fig. 7h,i). Restoration of the direct constriction response in the lesioned eye was increased compared to no treatment, but this was not statistically significant (Fig. 7h). There was no difference in direct (Fig. 7h) or consensual pupil constriction (Fig. 7i) between the treated and untreated groups, indicating that the regenerated RGC axons in the treated group failed to rescue the consensual pupil constriction.

Next we performed the visual cliff test to assess depth perception and the functional integrity of the retino-geniculo-cortical pathway\(^{31}\). We placed each mouse on a platform, below which the floor on one side was painted with a low spatial frequency pattern of large black squares while the other was painted with a high-spatial-frequency pattern of small black squares. This creates an illusion of a shallow versus deep drop from the platform, respectively (Fig. 7j; see Online Methods). Normal non-lesioned mice chose to step down on the perceived shallow side of the chamber in \(~70\%\) of trials, an effect that disappeared in mice with lesions to their optic nerves regardless of whether they received a treatment to induce regeneration or not (Fig. 7k). This indicates that the regeneration resulting from biased visual stimulation/AAV2-cRheb1 treatment failed to restore the connections that mediate visual cliff avoidance behavior. This could reflect defects in synapse formation and/or insufficient numbers of axons regenerating to the dLGN (discussed below).

Finally, we assayed the visual fear response of animals to an over-head looming stimulus. In these experiments, animals are placed into a chamber equipped with a shelter in which to hide and then a rapidly expanding black circle (the looming stimulus) is presented from the top of the chamber (Fig. 7l; see Online Methods).
presentation of the looming stimulus, normal mice either immediately froze or ran under the shelter (Fig. 7m). By contrast, none of the lesioned and untreated animals responded to the looming stimulus; they simply continued exploring the chamber (Fig. 7m). In the lesioned and treated group, two of three mice responded to the looming stimulus appropriately by running under the shelter to hide (Fig. 7m) and the third mouse responded to looming stimulus by orienting its head and eyes upward each time the looming stimulus was presented, but never by freezing or hiding, indicating it perceived the stimulus despite its failure to engage the appropriate behavioral response. Overall, these results indicate that biased visual stimulation/cRheb1 treatments lead to regrowth of RGC axons that, in turn, can sustain partial recovery of some visual functions and vision-driven behaviors.

DISCUSSION

By enhancing neural activity and mTOR signaling in RGCs, we observed long-distance, target-specific RGC axon regeneration in
adult mice. These results indicate that, under the appropriate conditions, mature RGCs are capable of regrowing axons into the brain and forming connections with appropriate target neurons. This regeneration leads to partial recovery of several visual functions, suggesting that some degree of functional synapse re-formation can take place in the adult visual pathway.

Biased visual activity as a trigger for RGC axon growth

The greatest degree of regeneration was observed in mice that received enhanced RGC activity and unilateral lid suture (or eye removal) to eliminate vision through the non-lesioned eye pathway. Why might this be so? Complete optic nerve lesions, as used here, eliminate the opportunity for binocular interactions among RGC axons located within central visual targets. Thus, the observed effects of unilateral visual bias may arise from one or several other sources. One possibility is that the bias effect is purely behavioral; that is, suturing shut the non-lesioned eye encouraged animals to keep the opposite, lesioned eye open, which in turn promoted more spiking activity in those RGCs. The reduced amount of regeneration observed in control animals where AAV2-cRheb1 and visual stimulation were provided through both eyes (as well as the controls that experienced no visual stimulation) support this idea. Another possibility is that, even though crushing the optic nerve triggers degeneration of RGC axons, their regeneration is not instantaneous but takes place over 1–3 d following the crush. Thus, there may be a short window whereby binocular interactions driven by biased visual activity could boost or accelerate pathways controlling RGC regeneration in the open, lesioned eye. Regardless of the mechanism, the impact of biased visual stimulation on RGC axon regrowth is evident from our data because only in animals where one eye was sutured shut or removed did we observe long-distance regeneration of cRheb1-treated RGC axons from the unsutured, intact eye.

In theory, visual stimulation could influence RGC axon regeneration by non-activity-dependent means, although the downstream mechanisms for that process are not clear at this time. However, our findings that a reduction in RGC activity via hM4Di and CNO prevents visual-stimulation-mediated axon regeneration and that increasing RGC activity with hM3Dq and CNO promotes axon regeneration support the idea that RGC activity levels are a key parameter regulating axon growth after injury. Increasing activity in ipRGCs has recently been shown to enhance RGC regrowth down the optic nerve but not regrowth into the brain39, and electrical stimulation has recently been shown to enhance RGC regrowth down the optic nerve and indeed all the way into the brain39, although the downstream effects of unilateral visual bias may arise from one or several other sources. One possibility is that the bias effect is purely behavioral; that is, suturing shut the non-lesioned eye encouraged animals to keep the opposite, lesioned eye open, which in turn promoted more spiking activity in those RGCs. The reduced amount of regeneration observed in control animals where AAV2-cRheb1 and visual stimulation were provided through both eyes (as well as the controls that experienced no visual stimulation) support this idea. Another possibility is that, even though crushing the optic nerve triggers degeneration of RGC axons, their regeneration is not instantaneous but takes place over 1–3 d following the crush. Thus, there may be a short window whereby binocular interactions driven by biased visual activity could boost or accelerate pathways controlling RGC regeneration in the open, lesioned eye. Regardless of the mechanism, the impact of biased visual stimulation on RGC axon regrowth is evident from our data because only in animals where one eye was sutured shut or removed did we observe long-distance regeneration of cRheb1-treated RGC axons from the unsutured, intact eye.

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Regrowth distance of RGC axons with various treatments

Our study demonstrates the remarkable capacity of adult CNS neurons to regenerate their axons long distances, if they are treated with the appropriate growth-stimulating conditions. We found that enhanced RGC activity coupled with elevated mTOR signaling allowed axons to regenerate down the full length of the optic nerve and indeed all the way into the brain. We note that other experiments that elevate mTOR signaling, such as deletion of the mTOR inhibitor gene Pten trigger an even greater number of RGC axons to regenerate than we observed here. One possible reason for this is that Cre recombination is all or none whereas cRheb1 expression may vary between infected RGCs and thus induce varying levels of p-S6 expression. However, we note that while Pten deletion causes RGC axons to grow as far as the optic chiasma, it does not allow them to grow into the brain10 unless it is combined with one or more other treatments16 (Figs. 3–6). Together, these data underscore the fact that multiple treatments applied in combination are needed to trigger regeneration of RGC axons back into the brain, and they encourage additional exploration of the molecular pathways activated downstream of neural activity.

Specificity of axon regeneration in the mature CNS

By exploring genetically labeled RGC types—the α-RGCs or ipRGCs—we found that RGCs had a remarkable capacity to reconnect to their correct targets in the brain while simultaneously avoiding incorrect targets. The fact that CoCH-GFP+ RGC axons are among the regenerating cohort is consistent with the previous work that identified cat α-RGCs as the main group undergoing regeneration after sciatric nerve transplants41. These results are also in agreement with those of a recent study showing that α-RGCs account for the majority of regenerating axons following Pten deletion27. We note that we observed many regenerated CTβ+ RGC axons, which were not GFP+ and which targeted brain areas not typically innervated by α-RGCs (Figs. 4–6).

This suggests that elevation of mTOR signaling with enhancement of RGC activity may recruit regeneration of not only α-RGCs, but also other RGC types. The ability of OPN4-GFP+ axons to regenerate supports this idea, and in the future it will be interesting to explore the regeneration capacity of other RGC types as well.

The fact that some RGCs are capable of re-innervating the correct brain targets is remarkable, and yet it not entirely surprising when one considers that others have observed target-specific re-innervation in other systems. Björklund and co-workers observed that when the inhibitory effects of oligodendrocytes and myelin were neutralized, striatal and cortical projection neurons regenerated their axons long distances to re-innervate several of their correct targets in the substantia nigra, pontine nuclei and cerebral spinal cord42. More recently, Frank and co-workers observed that the central branch of lesioned dorsal root ganglion neurons undergo lamina-specific regeneration into the dorsal horn43. Collectively, these studies suggest that, in mammals, ligands and receptors that are expressed to ensure CNS axons arrive at and innervate their proper targets during development29,35 may still be present, or even upregulated, in response to RGC axon injury and/or regeneration in adulthood. Indeed, such upregulation of guidance molecules in response to injury has been observed in the tectum (corresponding to the mammalian superior colliculus) of cold-blooded vertebrates44. It will be important to address this by exploring the molecular pathways that are activated in the RGC types that regenerate in our activity protocol. In addition, it will be interesting to see whether we can bias regrowth of different RGC types and evaluate their targeting by providing activity patterns tailored to their specific receptive field properties.

Functional and clinical implications

There are important functional implications of the anatomical regeneration we observed at the level of visual reflexes and behaviors. Combining elevation of mTOR signaling with enhancement of RGC activity proved effective in partially restoring visual function in two out of the four visual assays we used. Curiously, the behaviors in which we observed recovery were those driven by the accessory optic system (optokinetic reflex)29,35 and retina-SC connections (the looming avoidance response)34 while the assays where we failed to observe any recovery were the pupil response (retina-OPN connection)30 and visual cliff test. The first three behaviors involve retina-subcortical pathways and do not require the cortex25 whereas the visual-cliff depth perception task depends on binocular vision and thus involves the dLGN and primary visual cortex (V1)31. The lack of recovery in
the visual cliff task was somewhat surprising given that we observed regeneration of RGC axons to the dLGn, the nucleus that relays visual information to V1. The threshold for functional recovery of the retina-geniculo-cortical pathway may therefore be higher than that of the other retinafugal parallel pathways at the level of synapse formation and/or precision of within-target wiring. Indeed, retinotopic and spatial precision of connections may not be a prerequisite for pathways driving OKR or looming since they involve large-field illumination, whereas visual cliff tasks require analysis of spatial frequency and thus higher resolution image-formation.

Interestingly, we observed regeneration of RGC axons to the OPN, the nucleus that modulates the PLR, but we did not observe any substantial recovery of direct or consensual pupil constriction. It is, however, worth noting that we intentionally used low light intensities to stimulate the PLR so as not to allow spillover of light to the opposite, non-lesioned eye. Although this was critical to avoid potential confounds, it is possible that higher light intensities could have driven functional activation of the PLR through the regenerated pathway connections. Regardless, our behavioral data support a model in which restoration of neural pathways for visual function and perception may require a large number of RGCs to regenerate.

In addition, a recent study probed the functional recovery of retino-collicular connections after distal nerve cut lesions and found that anatomical regeneration occurred without functional restoration. Only by enhancing neural activity in regenerating RGCs did their axons recruit myelinating glia—just as RGCs do during development—and allow functional transmission between RGCs and their axons recruit myelinating glia—just as RGCs do during development and anatomical regeneration occurred without functional restoration. Thus, additional sources of enhancing neural activity, in particular within the RGC populations that target the dLGn, may prove important for enhancing regeneration of mature central visual pathways and visual perception.

In conclusion, our findings demonstrate long-distance axon regeneration, specificity of connections and partial recovery of visual function may prove informative for devising treatments for the damaged visual system, spinal cord or other CNS regions in patients suffering from neurodegenerative diseases or physical trauma.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.-H.A.L. carried out all experiments, imaging, analysis of the data, and figure preparation. B.K.S. performed recordings from RGCs and the analysis of activity in response to chemical genetic manipulations. P.L.N. assisted with the visual stimulation experiments and tissue collection, and provided technical assistance. B.V.L. contributed to the visual stimulation experiments and tissue collection. C.W. prepared AAV-vRheb1 viruses. K.Z. provided technical assistance for optic nerve crush surgery and AAV-vRheb1. Z.H. provided AAV-vRheb1 virus. A.D.H. supervised the project and data analyses. J.-H.A.L., Z.H., B.K.S. and A.D.H. wrote the paper.

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ONLINE METHODS

Subjects. Mice of either sex ranging in age from postnatal day 30 to P80 were used, including seven strains: wild-type mice with no GFP (C57BL/6) (from The Jackson Laboratory); Cochlin-EGFP mice (CoCH-GFP) (from MMRC), Opsin 4 (melanopsin)-EGFP mice (OPN4-GFP) (from MMRC) and Pen1
d mice, all of which were maintained on a C57BL/6 background. Mice were assigned to groups based on genotype (Figs. 5 and 6) or randomly selected for control or experimental groups (Figs. 1–4). Group sample sizes were chosen on the basis of previous studies. Animals were housed on a 12-h light/dark cycle and behavioral analyses were done at consistent afternoon hours during the light cycle. All experiments and procedures were done in accordance with approved animal protocols from APLAC and IACUC committees at the University of California, San Diego.

Intravitreal injections of viruses and tracers. The following dyes and viruses were injected into the vitreal chamber of the eye using a Hamilton syringe as described previously. The anterograde tracer cholera toxin subunit-β (CTb) conjugated to Alexa Fluor 594 (CTb–594; Invitrogen) or to Alexa Fluor 488 (CTb–488; Invitrogen) to label RGC axons; adenov-associated virus serotype 2/1 with constitutively active Rheb1 (AAV2/1-Cre-hRheb1, 6 × 10^{15} ifu/ml) to overexpress Rheb1; adenov-associated virus serotype 2 with hM4Di (AAV2/2-hSyn-HA-hM4Di(Gq)) to label RGC axons; adenov-associated virus serotype 2 with hM4Di and hM3Dq, respectively; adeno-associated virus serotype 2 with Cre (AAV2-2-Cre, 1 × 10^{13} GC/ml) (Vector Biolabs) to knock out Pten gene in Pten^{loxp} mice or to use C57BL/6 mice for AAV control group after optic nerve crush. Injections were done using the following procedure: mice were anesthetized with inhalant isoflurane and a small hole was made near the ora serrata. The injection was made with a 33 gauge Hamilton needle and completeness of the eye fill was confirmed under a fluorescence dissecting microscope. CTb-injected animals were given a 2-d survival period to allow the tracer to travel down and label RGC axons and their terminals. After AAV2-CreRheb1 or AAV2-hM4Di or AAV2-hM3Dq injections, we waited 2 weeks for the virus to express in the RGC population. All virus injections were performed with the experimenter blind to treatment conditions.

Intraperitoneal injection of rapamycin. Rapamycin (LC Laboratories; dissolved at 20 mg/ml in ethanol) was administered as described previously. Before each administration, rapamycin was diluted in 5% Tween 80, 5% polyethylene glycol 400 (0.5–1.5 mg/ml). Rapamycin at 6 mg/kg or the vehicle (5% Tween 80, 5% polyethylene glycol 400 in PBS) was injected intraperitoneally after the AAV2-CreRheb1 injection on the first day and then every 2 d for the duration of the experiment.

Intraperitoneal injection of clozapine-N-oxide. Clozapine-N-oxide (Tocris Bioscience; dissolved at 1 mg/ml in DMSO (0.5%)/saline) was administered as described previously. CNO (1 mg/kg for the hM3Dq group and 5 mg/kg for the hM4Di group) was administered intraperitoneally twice each day for the duration of the experiment.

Optic nerve crush surgery. Animals were sedated by subcutaneous injection of ketamine/xylazine solution (1 ml of ketamine (100 mg/ml) and 0.625 ml of xylazine (20 mg/ml) in 8.375 ml of saline) at 1 µl per gram body weight. Jeweler’s fine forceps (Dumont #5, FST) were used to expose the optic nerve intraorbitally and crushed for 5 s at a distance approximately 2 mm from the posterior pole of the eye. We carefully avoided damaging the ophthalmic artery and monitored the eye for any signs of bleeding in the period immediately after and for several hours and days following the crush. Ointment containing atropine sulfate (Bausch and Lomb, NDC 24208-825-55) was applied pre- and postoperatively to protect the cornea from drying. Any mice with vascular damage in the eye after optic nerve surgery were euthanized immediately after and were not included in the dataset.

Visual stimulation. Mice were placed into a chamber surrounded by four 23-inch widescreen LCD monitors facing each other. The mice were exposed to high-contrast (black and white) vertical lines drifting horizontally, 45- or 270-degree lines moving up and to the right or down and to the left, and horizontal lines moving vertically. The stimulus was delivered for 12–14 h a day for the 21 d after optic nerve crush and was delivered during animals’ wake time. Stimuli were powered by Optomotry VR 1.7.7 (CerebralMechanics Inc., Lethbridge, Alberta, Canada).

Eyelid suture or eye removal. After the animals were sedated, the margins of one eyelid were trimmed slightly and the upper and lower lids were sutured together using nylon monofilament suture. To ensure that sutures remained intact, a drop of ophthalmic surgical bond was applied. To remove an eye, the animals were first sedated and, using curved surgical scissors (ROBOZ, RS-5675), the eye was elevated slightly from the orbit and the optic nerve cut and eye removed. Afterward, the orbit was sutured shut.

Immunohistochemistry. After transcardial perfusion with saline (0.9% NaCl diluted in ddH2O) followed by 4% paraformaldehyde (PFA), the eyes, optic nerves and brain were harvested and postfixed in 4% PFA for 24 h. The eyes were then transferred to phosphate-buffered saline (PBS) and the optic nerve and brain placed in 30% sucrose for cryoprotection. The optic nerve was sectioned using a sledge microtome, cutting longitudinally at 15 µm. The brain was sectioned coronally at 30 µm. All tissue sections were included in the analysis. Retinas, optic nerves and brain tissue were kept at 4 °C overnight with the following antibodies: rabbit-anti-RB1 (PhosphoSolutions, 1:1,000, Cat. No. 1830-RB1PS)), to label RGCs; rabbit or guinea pig anti-GFP (SySy; 1:1,000, Cat. No. 132 003), to enhance GFP signal; rabbit-anti-p-S6 (Cell Signaling Technology; 1:250, Cat. No. 4858S), to label phosphorylated S6 protein; mouse anti-SMI-32 (Sterneberger Monoclonals, 1:2,000, Cat. No. NE1023) to label α-RGCs and other large-soma RGC types; rabbit-anti-melanopsin (Advanced Targeting Systems, 1:1,000, Cat. No. AB-N38). For secondary detection, Alexa Fluor 488 goat anti-rabbit (1:1,000, Invitrogen, Cat. No. A-11012), Alexa Fluor 488 goat anti-guinea pig (1:1,000, Invitrogen, Cat. No. A-11075), or Alexa Fluor 594 goat anti-rabbit (1:1,000; Invitrogen, Cat. No. A-11012) were used. Immunostained tissues were imaged with an epifluorescence microscope (Zeiss Axio imager 2 with HR Zeiss camera, 10× and 20× objectives).

Cell number quantification. We compared p-S6 expression (cell numbers) in the retinas of AAV2-CreRheb1-injected animals and control animals injected with saline. After we immunostained the retinas with identical protocols for the downstream marker of phosphorylated mTOR, p-S6, each flat-mount retina was imaged with an epifluorescence microscope. Multiple 500 µm × 500 µm regions of the retina were analyzed for each animal (n = 5 mice per group). Photoshop (Adobe, CS6) was used to convert each image to grayscale and to threshold the image to eliminate background noise (same thresholds applied). Each p-S6 profile was then counted and included in the analysis. Using the same analysis, we also quantified the number of RGCs in different experimental conditions by immunostaining the retinas for the RGC marker RBPMS.

Axon quantification in the optic nerve. After we imaged the serial optic nerve sections, Photoshop (Adobe, CS6) was used to stitch the images into a complete montage. Lines spaced equidistant from each other at 500-μm intervals from the rear of the eye to the optic chiasm were introduced to the montage for bin-by-bin axon quantification. We manually counted the number of individual axons that transected each vertical line. As used by other groups, we quantified the total number of regenerating axons, Σa, by using the following formula: $\Sigma a = \pi r^2 \times (\text{average axons/mm}) / r$, where total number of axons extending distance $d$ in a nerve having a radius of $r$ was estimated by summing over all sections with thickness $t$ (in our case, 15 µm). The axon counts were verified by a blind-to-condition, independent viewer.

Electrophysiology. Procedures were similar to those described previously. Briefly, retinas were collected and dissected in gassed (95% O2 and 5% CO2) Ames medium heated to 33–35 °C. RGCs expressing mCitrine were visualized at 40× by attenuated mercury light passed through a GFP dichroic mirror and then targeted for recording under infrared illumination. Cells were recorded with borosilicate glass pipettes (4–6 MΩ) filled with intracellular solution containing (in mM) 120 potassium methanesulfonate, 10 HEPES, 5 NaCl, 0.1 EGTA,
2 ATP-Mg<sup>2+</sup> and 0.3 GTP-Na, titrated to pH 7.3. Chemicals were purchased from Sigma-Aldrich or Tocris.

Current-evoked spiking responses were recorded in response to a series of ten monotonically increasing 20 pA current steps. The baseline current step used for analysis varied between cells (20–80 pA) but was always the smallest step that produced at least one spike under control conditions. Patterned light stimuli were generated by custom software developed in Psychophysics Toolbox and MATLAB. Stimuli were projected onto the retina using a Dell video projector (M109s DLP) custom fitted with a UV LED (NC4U134A; final emission, 398 nm; Nichia), attenuated by a neutral density 1.0 filter and focused using a 10× objective to the level of rod and cone outer segments. Stimulus intensity produced 2.6 × 10<sup>5</sup> R*/S-cone s<sup>−1</sup>. Stable S-cone mediated responses can be recorded from RGCs in the ventral mouse retina following targeting by epifluorescence under these conditions. The receptive field center was mapped by recording responses to square-wave modulations of a 300-µm-diameter spot at eight positions. In subsequent experiments, stimuli were presented as a contrast pulse (100% Weber contrast), 200–400 microns in diameter. The same size stimulus was always used for both control and CNO recordings for a given cell.

Behavioral analyses. Optokinetic reflex. Mice were placed on an elevated platform surrounded by four 23-inch widescreen LCD monitors. Each trial consisted of vertical drifting-bar stimuli presented at a spatial frequency of 0.16 cycles per degree and temporal frequency of 12° s<sup>−1</sup>; the optimal stimulus for driving the OKR<sup>33,36</sup>. Each trial lasted 15 s; if the head of the mouse moved in concert with the gratings, the trial was scored as “tracked.” Each mouse was presented with 10 trials per day, for 3 consecutive days, at the same time of day. Responses were averaged to generate a mean percentage of trials tracked (n = 3–5 mice per group; see main text).

Pupillary light reflex. Mice were dark-adapted within their home cage for 1 h before the experiment. All mice were unaesthetized and restrained by hand for the duration of the experiment. A single blue (470 nm) LED was placed in front of the stimulated eye. Both the direct (stimulated eye) and consensual (contralateral eye) PLR were recorded with two infrared video cameras placed on either side of the head. The pupils were recorded before (baseline) and during (constriction) the light stimulus (30 s at 25 lx). The light intensity was chosen as 25 lx to prevent light spillover to the other eye. Individual frames of the initial resting pupil size (baseline) and at maximal constriction were extracted from the video recordings and pupil diameters were measured using ImageJ. The percentage of the pupil constriction<sup>30,38</sup> was calculated from the pupil diameter measurements at the initial resting size and maximal constriction (n = 3–5 mice per group; see main text).

Visual cliff. The visual cliff behavior was analyzed in an open-top Plexiglas chamber. Half of the box protruded from the counter to provide a 3-foot depth. The box on the counter displayed a base with a checkerboard pattern and the box off the counter showed the base with the same checkerboard pattern, except for the 3 feet of depth. A 2-foot-high platform was stationed in the middle of the box intersecting both the shallow side and deep side. The mouse was placed on top of the platform and allowed to choose between the two sides. If the mouse stepped down to the shallow side, that trial was scored as “relative depth perceived.” If the mouse either stepped down to the deep side or stayed on top of the platform for 5 min, that trial was scored as “relative depth not perceived.” Each mouse performed this task 5 times per day for 3 d. The visual cliff behavior was averaged to generate mean percentage of trials in which the mouse chose to step down to the shallow side (n = 3–5 mice per group; see main text).

Looming response behavior analysis. The looming response is a well-documented behavior in which mice freeze or flee to an escape area in response to a dark expanding disk overhead<sup>32,34</sup>. This analysis was performed with mice in an open-top Plexiglas chamber. A 24-inch LCD monitor was stationed on top of the chamber facing downward to display the stimulus. One end of the box included a black shelving board to provide a shelter in which to hide. A video camera recorded the mouse’s movements during the trial at 30 fps. The mouse was free to roam inside the box for 5 min before the first presentation of the stimulus. The looming stimulus was presented three times during a 3-s epoch. If the mouse responded to the stimulus by either freezing or hiding in the shelter, that trial was scored as “looming response.” Each animal performed the task once. Looming responses as a percentage of total looming stimulus presentations were averaged for each animal as to generate a mean percentage of trials responded (n = 3–5 mice per group; see main text).

Statistics. Statistical tests indicated in our study were performed using Prism 7 (GraphPad Software, La Jolla, CA). In addressing our hypothesis that visual stimulation alone or together with cRhe1 overexpression and/or other manipulations can enhance axon regeneration, we tested the increases in RGC axon regeneration by one-tailed t-test. Continuous data were tested with parametric tests and data were assumed to be normally distributed, but this was not formally tested.

A Supplementary Methods Checklist is available.