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Postsynaptic neuronal activity promotes regeneration of retinal axons

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SUMMARY

The wiring of visual circuits requires that retinal neurons functionally connect to specific brain targets — a process that involves activity-dependent signaling between retinal axons and their postsynaptic targets. Vision loss in various ophthalmological and neurological diseases damages these connections from the eye to the brain. How postsynaptic brain targets influence retinal ganglion cell (RGC) axon regeneration and functional reconnection with the brain targets remains poorly understood. Here, we established a paradigm in which the enhancement of neural activity in the distal optic pathway, where the postsynaptic visual target neurons reside, promotes RGC axon regeneration, target reinnervation, and leads to the rescue of optomotor function. Furthermore, selective activation of retinorecipient neuron subsets is sufficient to promote RGC axon regeneration. Our findings reveal a key role for postsynaptic neuronal activity in the repair of neural circuits and highlight the potential to restore damaged sensory inputs via proper brain stimulation.

INTRODUCTION

Vision is the primary sense humans use to navigate the world and survive. Retinal ganglion cells (RGC) are the output neurons of the eye and the sole conduit for visual information

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Author contributions: SGV and ADH conceived and designed the experiments and wrote the manuscript; SGV performed experiments, histology, image acquisition, injections, and data analysis and assembled the figures and manuscript. SGV and FW performed behavioral assays with XD's supervision. FW performed electrophysiological recordings under XD's supervision. OSD performed histology; PL performed injections.

Declaration of interests: Authors declare that they have no competing interests.

to reach the brain, where it undergoes perceptual, sensory-motor, and autonomic processing. RGCs and their axons thus represent a key bottleneck to restoring lost vision following degenerative diseases and injuries that deplete neurons. One example is glaucoma- a progressive condition in which RGCs die - and the second leading cause of blindness worldwide. To restore vision after injury or disease RGC axons must re-grow back into the brain and connect with specific synaptic targets, as they did in development. However, RGC axons, like all axons of the central nervous system, do not spontaneously regenerate their axons following injury.

During development, RGC axons rely on a wide range of signals from within the retina and their central targets in the brain to wire up correctly, a process known as axon-target matching [1]. Factors inherent to RGC-axons and target-derived signals, such as axon guidance cues, trophic factors, and neural activity, direct the processes of topographic mapping, eye-specific axonal segregation, synaptic choice, synapse formation, and refinement [2-5]. Many studies have demonstrated the importance of target-derived signals arising from postsynaptic partners. For example, when trophic factors are injected into the superior colliculus, a major central target of most RGC axons, RGC death is reduced during development [6]. Conversely, an absence of trophic support from target cells increased RGC degeneration during development [7-10]. Divorcing RGC axons from their central targets by lesioning retinorecipient targets during development leads to RGC death [11,12]. Target cells thus play a critical role in developing and maintaining visual circuits. However, whether target cells can be leveraged to promote the regeneration of RGC axons remains unclear.

Recent studies have identified strategies to increase the growth potential of RGC axons in a diseased or injured environment [13,14]. Most of these studies focus on molecular and cellular events in the retina and/or optic nerves. Although target neurons play an important role in the development of visual circuits, far less is known about the role of retinorecipient target cells in the regeneration process. To address this, we used a chemogenetic approach to stimulate neural activity in specific visual target nuclei and neurons following injury and evaluated how this impacts RGC axon regeneration. Our findings reveal that increased neural activity in target neurons promotes RGC axon regeneration and rescues deficits in optomotor responses typically observed following injury to the distal optic pathway. The potential to leverage postsynaptic partners of injured retinal axons to promote their regeneration and thereby restore visual system function represents an underappreciated and potentially effective strategy for repairing neural circuits.

RESULTS

Increasing neural activity in the distal optic pathway promotes RGC axon regeneration

Enhancing RGC neural activity in the retina can promote regeneration of RGC axons in an optic nerve crush model [15,16]. Here we tested the hypothesis that increasing neural activity in cells distal to the lesion site (within the brain proper) would promote regeneration of injured RGC axons. We used a distal injury model, in which RGC axons were unilaterally severed at a location rostral to the pretectum, resulting in a partial injury to the optic tract (see Methods, Figure 1A). There are two advantages to using this distal-injury model for probing the role of target cells in axon regeneration: (i) it requires a shorter distance for

regenerating axons to reach their targets; (ii) RGC axon collaterals innervating visual targets rostral to the lesion site would retain connectivity and trophic support and thereby minimize RGC death in the retina, which is a major confounding issue of optic nerve crush models [17-19].

We first set out to determine if the distal injury model indeed minimizes RGC death. RGC axons were visualized by labeling them with cholera toxin subunit b (Ctβ), an anterograde tracer conjugated to Alexa-Fluor-488, injected intravitreally into the left eye of 9-week-old wildtype mice (Figures 1A-B). Two days later, the distal lesion was performed to partially sever the contralateral optic tract (Figures 1C-I). To assess RGC death induced by the lesion, we sacrificed mice two weeks after the injury and processed the retinas for immunohistochemistry using the RGC-specific marker RBPMS [20,21] (Figures 1J-M). We observed no significant reduction in the number of RGCs compared to sham (uninjured) retinas ($p = 0.5857$ from one-way ANOVA with post-hoc Tukey's multiple comparisons, n = 5 animals/group) (Figures 1M) indicating that the distal injury model minimizes RGC death.

To test the hypothesis that stimulating postsynaptic neurons could promote RGC axon regeneration, we used a previously established chemogenetic approach to increase neural activity in pretectal neurons caudal to the lesion (Figures 2A-F) [15,20]. We overexpressed an engineered G-protein-coupled receptor hM3Dq [modified human M3 muscarinic designer receptor exclusively activated by designer drug (DREADD)] [22] into the pretectum of 8-week-old mice using adeno-associated virus (AAV) expressing hM3Dq behind the human synapsin promoter to restrict expression to neurons (AAV2-hSyn-hM3Dq-mCherry) (Figure 2G). Two weeks later, the distal optic tract was lesioned, and mice were given intraperitoneal injections of either saline (control group) or the synthetic ligand clozapine-N-oxide (CNO) twice daily to activate hM3Dq (neural activity group) and thereby increasing the neuronal activity of the infected cells [15] (Figures 2A-F). We first confirmed hM3D activation by immunostaining for cFos, an immediate early gene induced by neural activity [23]. Double-labeled $mCherry^+$ $cFos^+$ cells were observed 24 hours following CNO injection (Figures S1D-F) and following a 2-week period (Figures S1J-L) but not after injecting saline (Figures S1A-C and S1G-I). No $mCherry^+$ $cFos^+$ cells were observed in the dLGN or SC of CNO-injected mice (Figures S1M-N). Recordings from $mCherry^+$ cells showed a significant increase in the firing rate ($p = 0.019$ after adding CNO, $p = 0.0019$ after washout, from paired t-test, n= 5 cells from 3 mice) and resting potential ($p = 0.0097$ from unpaired t-test, n = 5 cells from 3 mice) following CNO administration (Figures S1O-S). A subsequent CNO washout significantly decreased the firing rate ($p = 0.0019$ from paired t-test) (Figure S1Q). CNO administration was not found to exert any independent effects on regeneration and was not statistically significant compared to saline injected controls ($p = 0.5565$ from unpaired t-test, n=2 animals/group) (Figure S1T). To unambiguously identify regenerating axons, mice received intravitreal injections of Ctβ conjugated to Alexa-Fluor 488 (Ctβ-488) and Alexa-Fluor 647 (Ctβ-647) two days before the injury and twelve days after injury, respectively (Figures 2G, P, S2A-F). Whole-mount retinas from uninjured and injured mice injected with Ctβ-488 and Ctβ-647 2 weeks apart were collected and immunostained for RBPMS to measure the number of RGCs that were double-labelled with both tracers. Quantification showed that the number of RGCs labeled with the first (91%), second (93%), or both (88%) tracers were not statistically significant ($p = 0.61$ for uninjured and $p = 0.9077$

with a two-way ANOVA followed by post-hoc Tukey's multiple comparisons, n = 4 animals for control and 5 animals for activity) (Figures S2G-M).

Increasing neural activity in pretectal neurons post-injury for two weeks led to significantly greater RGC axon regeneration than was observed in control mice ($p < 0.0001$ with Mann Whitney test, n = 12-15 sections/mice from 10 mice in the control group, and 14 mice in the activity group) (Figures 2H-N). We sought to determine if a relationship exists between the proportion of RGC axons spared from injury and the degree of regeneration observed. The degree of axon sparing was determined by measuring the pixel density of axons labeled with both Ctβ fluorophores (Figure 2P). A comparison of spared axons between groups revealed no significant differences ($p = 0.8408$ with Mann Whitney test) (Figure 2O). We quantified cell death by immunostaining retinas from each group with RBPMS. We found no significant difference in the number of RGCs between the control and activity groups (Figure 1M). These results suggest that the increase in regeneration is due to the enhancement of neural activity in the chemogenetically manipulated pretectal neurons.

We sought to determine the extent to which increasing neural activity in the pretectum could promote regeneration. HM3D-mCherry^{+} neurons were observed throughout the pretectum but not in the dLGN or SC (Figure S3A). We measured the observed regeneration in the distal optic pathway as a function of distance. We found that while control animals exhibited the highest degree of regeneration at only 200 μm from the lesion site, increasing neural activity promoted regeneration at all distances up to 2000 μm from the lesion site, with the highest degree of regeneration observed at 1400 μm from the lesion site ($p < 0.0001$ from t-test with post-hoc multiple non-parametric t-tests; 12-15 sections/mice from 10 mice in the control group and 14 mice in the activity group) (Figure 2Q). Although the total pixel density of regenerating axons varied between animals, regeneration was observed in all 10 mice of the neural activity group (Figures S3B-S).

Regenerating axons reach target nuclei

To assess whether regenerating axons also re-innervated visual targets, we quantified the density of regenerating axons within each subcortical visual target. We focused on the pretectal targets near the hM3Dq injection site, such as the olivary pretectal nucleus (OPN; responsible for pupillary light reflex), the nucleus of the optic tract (NOT; horizontal image stabilization), the medial division of the posterior pretectal nucleus (mdPPN; function unknown) and the superior colliculus (SC; head and eye movements) [24]. Because the lesion site was medial to the dorsal lateral geniculate nucleus (dLGN), RGC collaterals innervating the dLGN are proximal to the lesion and thus not injured; nevertheless, we expected injured axons to degenerate a few hundred microns from the injury site before regenerating [14,25]. Therefore, we also evaluated regeneration within the dLGN. Whereas regenerating axons were observed in all target nuclei in both groups (Figures S4A-T), we observed significantly more regenerating axons within all target nuclei in the neural activity group ($p < 0.001$) (Figures S4U-Y). We examined whether regenerating axons navigated more favorably towards one or more targets within the neural activity group. Since each visual target varies by size, we normalized the degree of regeneration within each target by the target area. We found no significant difference in regeneration between the targets

(Figure S4Z) ($p = 0.185$ from ordinary one-way ANOVA with post-hoc Tukey's multiple comparisons). These findings suggest that injured axons died back as far as the dLGN (~at least 500 μm) and that regenerating axons navigated towards all regions that are highly electrically active.

A genetic driver to manipulate retinorecipient cells in the NOT

To dissect the role of neural activity more specifically in postsynaptic cells that receive RGC inputs in regeneration, we focused on the NOT, a subcortical visual target nucleus. RGC inputs to the NOT, a component of the accessory optic system (AOS) critical for image stabilization, are required to control horizontal eye movements [26-28]. We sought mouse lines to target and manipulate the cells within the NOT selectively. We screened a library of BAC-transgenic Cre recombinase driver lines (GENSAT) and identified the Synaptotagmin 17 Cre line (*Syt17::Cre*) as a possible candidate. To characterize the Cre-labeled cells in the *Syt17::Cre* line, we crossed the mice with a reporter expressing tdTomato and examined the brains of their offspring: *Syt17::cre; Ai9::tdTomato* (Figures S5A-E, S5I). We injected Ctβ-488 into the eyes of these mice to visualize the axons of all RGCs and retinorecipient innervation of the NOT (Figure S5D). We then analyzed the subcortical visual targets using Ctβ-labeled projections and quantitated which regions contained $tdTomato^+$ cells.

We observed $tdTomato^+$ cells within the NOT ($p = 0.0012$ from t-test, n = a total of 32 sections from 4 animals) (Figures S5C-E), but none within the dLGN, mdPPN, or SC (Figures S6A-C, G-L). We also occasionally observed a few $tdTomato^+$ cells within the OPN (Figure S5E, Figures S6 D-F); however, $tdTomato^+$ OPN cells were absent in all mice. We also crossed the *Syt17::cre;Ai9* mouse line with the *Hoxd10::GFP* mouse line that labels RGCs projecting to retinorecipient targets of the accessory optic system (AOS), which includes the NOT, DTN, and MTN [28]. As expected from previous reports, we confirmed the presence of Ai9-$tdTomato^+$ cells in the vicinity of GFP^+ RGC axons within the NOT (Figures S5F-H). Additionally, previous reports have confirmed that RGCs are not labeled in the *Syt17::Cre* line [29]. These findings confirm that the *Syt17::Cre* mouse line is useful for labeling and manipulating NOT neurons restrictively.

Anatomically, the NOT is a target containing RGC afferents and excitatory and inhibitory neurons from other brainstem nuclei [27,30]. To confirm whether the Cre^+ cells in the NOT receive RGC inputs, we used pseudotyped rabies viral tracing to label pre-synaptic inputs to Cre^+ cells in the NOT in the *Syt17::Cre* mouse line (Figures S5J-V). In this approach, the rabies virus can only jump one synapse, and pre-synaptic inputs can be identified with a mCherry tag [31,32] (Figure S5T). We analyzed sections of the brain and the retina and found $mCherry^+$ axons in the optic tract and $mCherry^+$ cells in the retina (Figures S5N-S). Cross-sections of the retina immunostained with choline acetyltransferase (ChAT) showed that the majority of $mCherry^+$ RGCs were bistratified in the inner plexiform layer, where the dendritic arbors co-label with the ChAT layer, suggesting these RGCs may be on-off direction-selective ganglion cells or other bistratified RGC subtypes (Figures S5K-M)[24]. Since RGCs are the only cells that extend projections out of the retina, these data confirm that Cre^+ cells in the *Syt17::Cre* mouse line receive monosynaptic inputs from RGCs and validate Cre^+ cells in the NOT as *bona fide* retinorecipient cells.

Retinorecipient target cell activity promotes RGC axon regeneration

Non-specific activation of neurons in the pretectum included cells that receive visual information and other diverse types of neurons that do not receive visual input. Thus, we next asked if any cells distal to the lesion could be manipulated to promote regeneration or if only target activity from synaptic partners was required to promote regeneration of injured axons. We overexpressed Cre-dependent hM3Dq (AAV2-hSyn-DIO-hM3Dq-mCherry) in the NOT of *Syt17::Cre* mice to allow for selective increase neural activity in retinorecipient cells of the NOT (Figures 3A- F). Two weeks later we performed a distal-injury and administered CNO twice daily for two weeks; Cre^- mice that received cre-dependent hM3Dq injections and CNO were used as controls (Figure 3B). We identified regenerating axons unambiguously using Ctβ-488 and Ctβ-647, injected before and after injury, respectively (Figures 3G-H). Quantitation of regenerating axon pixel density in the distal optic pathway showed that increasing neural activity selectively in Cre^+ cells in the NOT significantly increased RGC axon regeneration as compared to controls ($p = 0.033$ from Mann Whitney test, n = 12-15 sections/mice from 7 animals in the activity group and 3 animals in the control group) (Figure 3I). Measurement of the pixel density of spared axons showed no significant difference between the groups ($p > 0.99$ from Mann Whitney test) (Figure 3J). Binning the pixel density of regenerating axons as a function of distance from the lesion site revealed that maximum regeneration caudal to the lesion was observed closest to the injection site ($p = 0.0079$ from paired t-test with multiple post-hoc t-tests) (Figure 3K), indicating that regenerating axons navigate preferentially towards activity-induced signals. To determine if injured RGC axons were regrowing or if spared axons were sprouting collaterals in response to increasing neural activity, we used the rabies tracer to retrogradely label RGCs in the retina. Nine days after injury, mice in both groups were injected with pseudotyped rabies with a GFP tag (ΔG-Rabies-GFP) into the NOT. Whole-mount retinas collected five days later (two weeks post-injury) showed four-fold more GFP^+ RGCs in the neural activity group compared to control group (average 80 cells in the activity group and 20 cells in the control group, $p = 0.02$ using an unpaired t-test, n= 4 animals for control and 3 for activity group) (Figures 3L-N). These results suggest that retinorecipient target-cell activity is sufficient to promote RGC axon regeneration following a distal injury.

We also find that selectively stimulating retinorecipient neurons within the NOT is sufficient to promote regeneration within that target and to neighboring visual targets, albeit to a lesser degree than with non-specific pretectal activation. Quantification of regenerating axons within each target revealed significantly greater regeneration in all targets in the activity group compared to controls (Figures S7A-E). The average pixel density was highest within the NOT compared to other pretectal targets in the activity group (Figure S7F). However, a comparison of the pixel density of regenerating axons (normalized to the area of the corresponding targets) showed no statistically significant difference among the targets ($p=0.0551$ from two-way ANOVA with post-hoc Tukey's multiple comparisons) (Figure S7G). These results indicate that stimulating neurons within the NOT directed regenerating axons to the NOT and other visual targets.

Increasing neural activity rescues deficits in optomotor response induced by injury

To understand if the degree of regeneration observed was sufficient to restore function following injury, we compared the optomotor reflex of animals before and after injury. This assay has previously been established as a reliable way to gauge the functional recovery of RGC projections to subcortical targets in rodent models [15,33,34]. All animals generate reflexive head and eye movements in response to moving stimuli. These compensatory movements stabilize visual images and are called optomotor responses; their generation requires multiple subcortical nuclei [27,28,35]. In this behavioral assay, otherwise unrestrained mice are placed on a raised platform and presented with drifting gratings of varying spatial frequency; mice reflexively move their head to track the stimuli and thereby offset image slip on the retina (Figures 4A-B) [36]. The visual performance or acuity of mice is determined by fully automated software (OptoDrum, Striatech) that identifies the spatial frequency threshold that mice can track. To test for functional recovery, we increased neural activity non-specifically in the pretectum of wild-type mice. We assayed their optomotor response two days before and two weeks after the distal injury (Figure 4C). Whereas control mice showed a significant deficit in tracking after injury ($p=0.0081$ from two-way ANOVA with posthoc Sidak's multiple comparisons tests, n= 5 mice), mice that had increased neural activity to stimulate RGC regeneration showed no significant deficit in the threshold of spatial frequency detected before and after injury ($p=0.5696$, n= 6 mice; $p =0.1543$ for control vs activity post-injury response) (Figure 4D). A similar trend was observed for the length of time an animal tracked the drifting gratings at the lowest spatial frequency (0.056 cycles/degree), ($p=0.036$ for control pre- vs post-injury, $p=0.5429$ for activity pre- vs post-injury, $p=0.81$ control vs activity post-injury response from two-way ANOVA) (Figure 4F). As each animal had a slightly different threshold for tracking before the injury, we normalized each animal's post-injury response as a percentage of its corresponding pre-injury response. Normalization of post-injury responses showed a significant difference between animals from the control versus activity groups in terms of threshold ($p=0.0107$ from two-way ANOVA) and tracking duration ($p=0.0415$ from two-way ANOVA) (Figures 4E and 4G). Taken together, these results suggest that while a distal injury to the optic tract leads to a significant functional deficit in the optomotor response, increasing neural activity in the pretectum rescues the function of the visual circuits for reflexive image stabilization.

DISCUSSION

During development, both the retina and the postsynaptic RGC-targets in the brain play crucial roles in specifying functional visual circuit connectivity. Previous studies have shown that ablating cellular targets in the brain or target-derived signal leads to RGC death, indicating the importance of postsynaptic RGC-targets during development [6,11]. Here we demonstrate that activity-induced target-derived signals are essential to regenerate RGC axons in the mature brain following injury. We observe that increasing the activity of neurons in the optic pathway promotes regeneration of RGC axons up to 2 mm past the injury site, a considerable distance for mature central nervous system (CNS) axons to grow within two weeks. Moreover, we observe robust regeneration of RGC axons that re-connect to subcortical visual targets and rescue visual acuity. In this study, we assayed the optomotor response, a well-established behavioral assay for assessing the presence and

precision of mouse vision, including in the context of regeneration [15,33,34]. We show that increasing neural activity rescues the deficit in optomotor response produced by distal injury to the optic tract. Further, we demonstrate that stimulating specific subsets of retinorecipient neurons in the NOT is sufficient to promote the regeneration of RGC axons. Thus, our findings identify two key strategies to achieve CNS circuit regeneration: (1) stimulating large collections of neurons caudal to a lesion site as a therapeutic strategy to broadly encourage regeneration of CNS axons and, (2) stimulating specific postsynaptic target neurons to promote axon regeneration in defined circuit pathways.

Attempts to repair most optic neuropathies often suffer from two major barriers – promoting regeneration of sufficient numbers of RGC axons and ensuring those extend sufficiently long distances down the optic pathway to re-interface with their normal targets. The distal injury model we described here—by producing a lesion to the optic tract and without damaging retinorecipient target nuclei, provides a model in which the role of RGC axon-target re-innervation can be examined. The distal injury model also mimics certain aspects of clinically-relevant CNS diseases that manifest as axonopathy whereby some axons are spared, and others are lesioned and/or degenerate, such as in glaucoma, multiple sclerosis, amyotrophic lateral sclerosis [37-39]. In optic nerve crush models, there is profound cell death (~80%) between 1-3 weeks post-injury [18]. It is notable that RGC cell death does not appear to be affected following distal injury. This could presumably be due to the increased distance between the retina and the lesion site compared to optic nerve crush injuries. However, we cannot exclude the possibility that RGC cell death may be stalled or occurring at a slower rate in the distal injury model.

Our observation that activity-induced signals promote RGC axon regeneration is unlikely to be the mere consequence of spared axons since there was no significant difference in the density of spared axons between control and activity groups. Further, our retrograde tracing data show more RGCs labeled in retinas from the activity group suggesting that injured RGC axons are truly regenerating. We do not, however, rule out the potential role of spared axons sprouting new collaterals, or providing a structural scaffold for the regeneration of activity-stimulated axons. Pioneering axons have long been known to provide cues to guide follower axons during development [40-44]. Similarly, axon-axon fasciculation and isotypic interactions between axons are well-established mechanisms that determine how axons are arranged and navigate in a tract [45-47]. Thus, the partial-injury model provides a tractable solution to examine multiple aspects of axon regeneration and axon-target pathfinding following injury not made possible by standard optic crush or similar injury models where entire pathways are severed.

Spontaneous neural activity, including retinal waves, occurs during development; these waves drive particular patterns of correlated neuron firing more centrally throughout the subcortical visual shell [48-50]. Spontaneous neural activity in the dorsal lateral geniculate nucleus (dLGN) during development is required for the proper wiring of thalamocortical axons to the visual cortex [48,51-53]. These studies indicate that neural activity in the retina, subcortical visual targets and visual cortex are interlinked and influence visual connectivity. It is unclear if activity-dependent regeneration observed in the present study results from cell-autonomous effects of neuronal stimulation. However, we show that specific

stimulation of retinorecipient neurons in the NOT promotes regeneration of RGC axons to the OPN, mdPPN, and the SC, suggesting non-cell-autonomous effects of increasing activity. Dissociating direct and indirect regeneration-promoting signals is important for understanding the mechanism underlying activity-induced repair and its potential application to other CNS injuries outside the visual system.

How might neural activity of target neurons in the brain promote RGC axon regeneration? Disruption of neural activity in RGCs leads to aberrant terminal arborizations of RGC axons during development [54]. Neural activity also plays a key role in regulating gene expression, transcription, and trophic factor responsiveness in RGCs—not just during development but throughout the lifespan [23,51,55-58]. Furthermore, both loss-of-function and gain-of-function studies have shown that trophic factors expressed in retinorecipient targets regulate RGC survival in the retina during development [6,7,9,10]. Our data indicate that increasing neural activity in the NOT leads to regeneration of RGC axons to the NOT itself, and other pretectal targets. This suggests that activity-induced regeneration signals extend through and beyond the local neuropil; such activity patterns are observed in development, particularly in the subcortical visual pathway [48,50]. Activity-dependent, target-derived events, including up or down-regulation of transcription factors, guidance cues, or neurotrophins thus, may be recruited when target neurons are activated or hyperactivated following injury.

Chronic stimulation of target neurons promoted significant regeneration within two weeks (this study), a relatively short period of time after a CNS injury. Understanding whether stimulating neurons for longer periods might control other aspects of RGC growth, morphology and connectivity, is important for understanding therapeutic potential. Chronic stimulation of neurons in the SC with optogenetic approaches has proven moderately neuroprotective to RGCs in a mouse glaucoma model [59]. Establishing a therapeutic window is vital to determine if stimulation of specific postsynaptic retinorecipient neurons (as done here; Figure 3) would slow disease progression in neurodegenerative disease models that cause progressive loss of function.

Work in humans has identified various approaches to modulate neural activity via deep brain stimulation or brain-responsive neurostimulator (RNS system) to treat movement disorders, depression, Parkinson’s disease, and epilepsy [60-62]. Non-invasive cuff electrodes have become an alternative approach and can restore some aspects of vision, but whether that is due to axon regeneration remains unknown [63-65]. More recently, stimulating RGCs in humans using optogenetic approaches resulted in partial functional recovery in neurodegenerative diseases such as retinitis pigmentosa [66]. With these discoveries, the clinical application of stimulating postsynaptic target neurons in specific neural circuits is gaining favor. More generally, reapplying developmental mechanisms to restore human visual pathways and function have become fathomable.

Limitations of the Study

Further studies are needed to understand the functional contribution of regenerating axons fully. Since a partial injury allows some spared axons to remain connected, tonic depolarization due to increased activity may allow spared axons to drive functional rescue. Further, retrograde tracing from the NOT to label truly regenerating RGCs only labeled

a relatively small number of neurons. This is likely due to the small number of RGCs projecting to the NOT compared to major targets such as the dLGN or SC. Though this indicates that injured RGCs are regenerating in this approach, further studies are needed to identify the total number of regenerating RGCs. Although a partial injury mimics many clinically relevant injuries and is useful from a therapeutic standpoint to identify approaches that promote repair, a complete injury would provide further information regarding the potential of postsynaptic neuronal activity to promote regeneration and functional recovery in the absence of spared axons.

STAR METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrew Huberman (adh1@stanford.edu).

Materials availability—This study did not generate any unique reagents.

Data and code availability—Data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals—Mice of either sex were used and ranged from 7-8 weeks old. C57Bl/6J wildtype mice and Ai9-tdTomato lines were obtained from Jackson Laboratories (stock no. #000664, stock no.#007909), and *Syt17::Cre* line from (GENSAT # RRID:MMRRC_034355-UCD. Animals were housed on a 12-hour light/dark cycle with unrestricted access to food and water. All animal care and experimental procedures were conducted in accordance with NIH guidelines and as approved by the Institutional Animal Care and Use Committee at Stanford University School of Medicine and University of California, San Francisco.

METHOD DETAILS

Intravitreal injections of tracers—The following anterograde tracers were injected into the vitreal chamber of the left eye of anesthetized mice using a glass micropipette (Drummond #5-000-1001-X10):1-2 μl cholera toxin subunit-β (Ctβ) conjugated to Alexa-Fluor 488 (Ctβ-488; Invitrogen #C22841) and Alexa-Fluor 647 (Ctβ-647; Invitrogen #C34778) to label RGC axons. Ctβ-injected mice were given 2 days to allow the tracer to travel into the brain to label RGC axons.

Intraperitoneal injection of Clozapine-N-oxide—Clozapine-N-oxide (CNO) (Tocris Bioscience #4936) was dissolved at 1mg/ml in DMSO (0.5% saline) and administered at 1.5 mg/kg via intraperitoneal injections twice a day, 8hrs apart, for two weeks.

Stereotaxic brain injections—Mice were anesthetized with 1.5-3% isoflurane. A midline scalp incision was made to expose the skull and perform a craniotomy above the injection site. Stereotaxic injections of the virus (~0.4 μl) AAV2-hSyn-hM3Dq-mCherry (Addgene #50474) were injected into the pretectum (bregma: −2.7 mm, midline: 0.8 mm, dorsal surface: 2.25mm) of 7-8 week old wildtype mice using a Nanoject II (Drummond) injector. We used the human synapsin promoter (hSyn) to restrict expression to neurons. Control mice were also injected with the same AAV-hM3Dq virus but administered saline instead of CNO. One cohort of mice was used to evaluate the independent effects of CNO, where one group of mice received AAV-hM3Dq injections followed by i.p. injections of saline (control). In contrast, another group of mice received AAV-GFP injections followed by i.p. injections of CNO (CNO) twice a day for two weeks. *Syt17::Cre* mice were injected with a Cre-dependent virus (~0.4 μl) of AAV2-hSyn-DIO-hM3Dq-mCherry (Addgene #44361) into the NOT (bregma: −2.8 mm, midline: 1.0mm, dorsal surface: 2.25mm). Cre-negative mice injected with the same Cre-dependent AAV-hM3Dq virus and receiving CNO injections were used as controls for the cre experiments in Figure 3 and Figure S7. To prevent backflow, the needles were left in place for ten minutes following injections before slowly retracting to the surface.

To determine inputs to Cre-labeled cells in the NOT of *Syt17::Cre* mice, stereotaxic injections of the helper virus AAV8-hSyn-FLEX-TVA-P2A-GFP-2A-oG (0.4 μl Salk Institute #85225) was injected into the NOT of 9-week old mice. Injected mice were given three weeks for adequate expression of Cre-dependent TVA and G protein in starter cells and then injected with EnVA-ΔG-rabies-mCherry (0.35 μl Salk Institute #32636). EnVA can only bind TVA-expressing starter cells, ensuring expression only in Cre-labeled cells; the rabies virus acquires the G-glycoprotein expressed in starter cells to spread trans-synaptically, thus also preventing the spread of rabies virus beyond one synapse[67]. For retrograde tracing to identify regenerating versus spared axons, ΔG-rabies-GFP (0.35 μl Salk Institute #32635) was injected into the NOT nine days after injury. Rabies-injected mice were housed in the bio-safety cabinet for 6 days to allow adequate trans-synaptic spread before analysis.

Distal injury—9-10 week old mice were anesthetized with 1.5-3% isoflurane. The midline incision made during virus injections was reopened, and scar tissue was cleared. Craniotomy was performed by drilling 3-4 burr holes using a 500 μm drill-bit (Fine Science Tools, #19007-05) and joining the individual drill sites to make a contiguous hole. A horizontal line was drawn using a surgical pen on a sterile surgical blade (11, Feather #2976#11) to mark the maximum depth of insertion, and then attached to a scalpel and inserted (bregma: −2.0 mm, midline: 0.5-2.5 mm, dorsal surface: 2.7 mm) severing the optic tract from lateral to medial. Bleeding was controlled using sterile surgical spears (Sugi #30601), and the craniotomy was covered with Bone wax (Ethicon #W31G). Mice were administered with slow-release buprenorphine and/or carprofen post-surgery as needed.

Immunohistochemistry—Mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA), and brains were harvested and postfixed in 4% PFA (24 hours at 4°C). Postfixed brains were sectioned using a microtome in the sagittal or coronal plane to yield 45μm thick sections following cryoprotection with 30% sucrose in PBS. Eyes

were removed, postfixed in 4% PFA (2 hours at 4°C), and dissected to remove the retina was dissected, and relieving cuts were made to allow the retina to lay flat. Samples were incubated in blocking buffer (5% normal donkey serum, 0.5% Triton X-100 in PBS) for 1-2 hours at room temperature. Sections were incubated overnight at 4°C in primary antibodies, while whole-mount retinas were incubated for 2 days at 4°C. The samples were washed with PBS 3x and incubated for 2 hours at room temperature with secondary antibodies. Samples were mounted with Prolong Gold Antifade Medium or Vectashield. Primary antibodies were guinea pig anti-RBPMS (Phosphosolutions #1832; 1:500), rabbit anti-DsRed to enhance tdTomato (Clontech #632496, 1:2000), goat anti-GFP (Abcam #ab6673, 1:2000), goat anti-ChAT (Millipore #AB144P, 1:100), chicken anti-GFAP (Aves #ab_2313547, 1:1000), goat anti-IBA1 (Abcam #ab5076, 1:500), rabbit anti-cFos (Millipore #abe457, 1:1000), rabbit anti-GFP (Invitrogen #A-6455, 1:1000). Species-specific secondary antibodies conjugated to Alexa-Fluor 594 or 647 (1:1000, Invitrogen and Jackson Laboratories) were used.

c-Fos analysis—AAV-hSyn-DIO-hM3Dq-mCherry was injected into the NOT of *Syt17::Cre*$^{+}$ mice. Two weeks later, one cohort of mice (24-hour timepoint) received i.p. injections of CNO (1.5mg/ml, activity group) or saline (control) twice a day, while a second cohort of mice (2-week timepoint) received i.p. injections of CNO or saline twice a day for two weeks. At the end of the respective timepoints, mice were housed in the dark overnight, injected with CNO, kept in the dark, and transcardially perfused 60 minutes after receiving CNO.

Electrophysiology—For confirming hM3Dq activation by CNO, pretectal neurons labeled with mCherry were targeted for whole-cell recording [5]. Briefly, mice were anesthetized with ketamine and xylazine (100 mg kg−1, 12.5 mg kg−1) and transcardially perfused with ice-cold cutting solution (78.3 mM NaCl, 2.3 mM KCl, 33.8 mM Choline-Cl, 0.45 mM CaCl2, 6.4 mM MgCl2, 1.1 mM NaH2PO4, 23 mM NaHCO3, 20 mM D-glucose, 0.5 mM L-glutamine, pH 7.4). The brains were dissected, and coronal sections of 250 μm thickness were prepared using a vibratome (VT1200S, Leica). Sections containing labeling in the pretectum were transferred to a chamber filled with cutting solution and incubated for 30 minutes at 32.5°C. After incubation, slices were transferred to artificial cerebrospinal fluid (ACSF) (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 1 mM MgCl2.6H2O, 2 mM CaCl2, 20 mM D-glucose, pH 7.4) and continuously bubbled with 5% CO_2/95%O_2. The current clamp mode was used to record the action potentials and resting membrane potentials of mCherry^{+} neurons in the pretectum. Spontaneous firing and resting membrane potential were recorded for two minutes before adding CNO. After bath perfusion with CNO (10μM) in ACSF for 3 minutes, the CNO was washed out, and the recording continued for another several minutes to observe the washout effects.

Optomotor response—The OptoDrum (Striatech Inc, Germany) was used to assay the visual behavioral response. The OptoDrum comprises a closed box with four digital displays to simulate drifting gratings. A camera attached to the top of the box records the animals' movements. At the same time, the fully-automated software was used to present the gratings, determine the spatial-frequency threshold and score the tracking performance [36]. Mice were first acclimated to the room in their cages for 30 minutes. Following the acclimation

period, freely behaving mice were placed on a platform and allowed to acclimate to the chamber for five minutes. Briefly, drifting gratings (12 °/s) in the clockwise direction, to gauge left-eye movements contralateral to the injury site, were presented from low spatial frequencies (0.056 cyc/deg), and the mouse was allowed to track the stimulus. To determine the threshold an animal could track, the software alternated between stimuli with high spatial frequencies (0.3-0.5 cyc/deg) and low spatial frequencies (0.06-0.1 cyc/deg) until the animal could no longer track beyond a particular frequency. Two "tracked" scores for a particular frequency and three "not-tracked" scores for the next higher frequency were determined as the threshold the animal could track. Stimuli were only presented for 3-5 seconds at a time and only if the animals' position was in the center of the stage/circle to avoid adaptation. Each animal was tested for 5-10 minutes. The stimulus was paused if an animal engaged in grooming behavior and resumed when the animal stopped grooming. False positives were identified manually and marked as invalid immediately, allowing the software to retest the same frequency or the next appropriate frequency. The assay was performed double-blind.

Imaging—All images were obtained with a Zeiss LSM 880 Airyscan confocal microscope or a Zeiss AxioScan microscope. Tissue collected separately for imaging purposes was cleared using the F-DISCO protocol [68], and imaged using the LaVision Light Sheet Microscope from the Stanford Neuroscience Microscopy Service.

QUANTIFICATION AND STATISTICAL ANALYSIS

Cell Number Quantification—For RBPMS counts, four images were acquired from each quadrant of flat-mount retinas, and cells were counted manually for Figure 1. An automated plugin for FIJI (Simple RGC counter and Batch [69] was used to count $RBPMS^+$ cells for images analyzed in Supp. Fig. 2. For $tdTomato^+$ cells, all sections containing the NOT and OPN were imaged for each animal (n=5 mice), and $tdTomato^+$ cells lying within the NOT/OPN were manually counted. The NOT/OPN was identified using landmark structures and confirmed by densely populated Ctβ-labeled RGC axons [20].

Quantification of double-labeled RGCs—To measure the number of RGCs labeled with both Ctβ tracers whole-mount retinas from uninjured and injured mice injected with Ctβ-488 and Ctβ-647 two weeks apart were collected and immunostained for RBPMS. The $RBPMS^+$ cells were counted as having one, or both tracers.

Quantification of Regenerating and Spared Axons—Ctβ conjugated to Alexa-Fluor 647 was injected intravitreally into the left eye two days prior to the distal injury to label the intact visual pathway, i.e., all RGC axons. Ctβ conjugated to Alexa-Fluor 488 was injected intravitreally into the same eye twelve days after distal injury to label RGC axons that are connected to the retina: axons spared from injury would take up both Ctβ tracers and be visible at both wavelengths, whereas injured axons that are regenerating would only be labeled with the post-injury Ctβ-488 label. All images have been pseudocolored with appropriate colors for clarity.

To quantify regenerating and spared axons, confocal images of brain sections were processed through an ImageJ Macro written for this purpose (Distal cut macro), available upon request. The macro was used to split the channels in the image, perform thresholding and despeckling, and then multiply the resulting red and green channels to generate a 'yellow' image that only displays pixels in both the red and green channels, indicating these are spared axons. The 'yellow' image was then subtracted from the red channel to generate a 'red-only' image that displays uniquely-red pixels, i.e., "regenerating axons." The raw integrated pixel density was measured in ImageJ from each newly generated image for each section, summed together for each animal, and then averaged. To measure regeneration within targets, a polygon outline was first drawn around each target on the merged image as regions of interest (ROI), processed through the same macro, and the raw integrated density for each ROI was measured.

Quantification of optic tract regeneration over distance—To quantify regeneration as a function of distance from the lesion ('0 mm'), the same images were binned by 200 μm blocks, measuring up to 400 μm proximal to the lesion and up to 2000 μm distal the lesion site. Images from the non-specific activation experiments are in the sagittal plane. In contrast, images from the Cre-specific activation are in the coronal plane and were accordingly measured to reflect distance from the lesion. The images were processed through an ImageJ plugin written for this purpose (Distal Cut Processor), and raw integrated density was measured within each bin. All analyses were performed blind.

Statistics—To determine statistical significance, we used the Mann-Whitney t-test to compare two groups of mice. Due to the variation in sample sizes, a non-parametric test was used. Two-way ANOVA and One-Way ANOVA were used for distance and normalized area quantification, respectively, followed by Student's t-test on individual pairs. Two-way repeated-measures ANOVA was used with posthoc multiple comparisons for the optomotor response analysis. All statistical analysis was performed with Prism v9 (GraphPad). All data in the graphs represent mean + SEM. Significance levels are indicated: * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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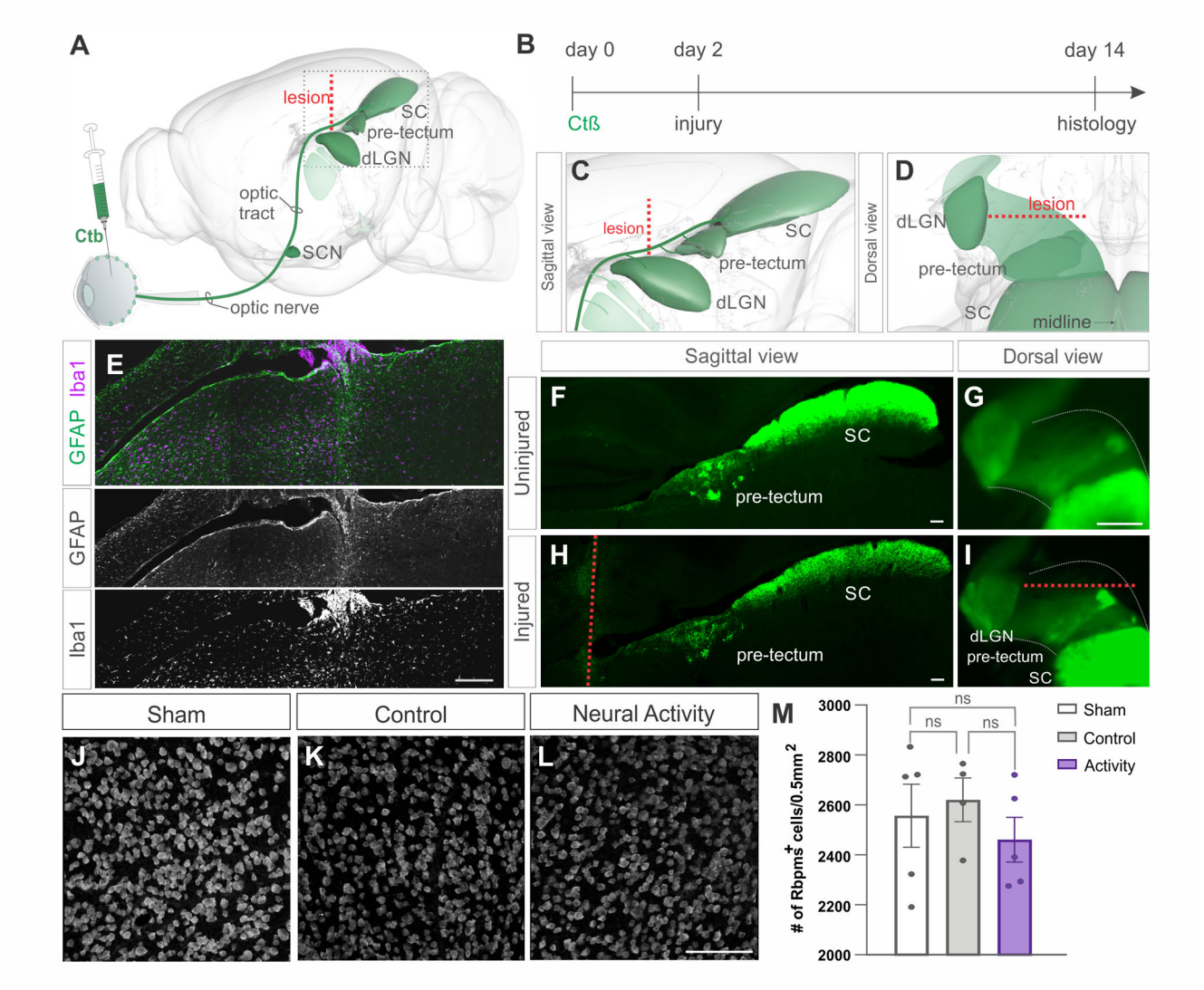

Figure 1: Injury to the distal optic tract of adult mice

(A) Schematic of distal injury (red dotted line) to the optic tract showing an anterograde tracer cholera toxin subunit b (Ctβ)-conjugated to Alexa-Fluor-488 injected into the eye to label RGCs and their axonal projections to central visual targets: dorsal lateral geniculate nucleus (dLGN); pretectum; superior colliculus (SC). The grey dotted rectangle shows the magnified regions shown in C and D.

(B) Experimental timeline to assess the distal injury and its effect on RGC death.

(C, D) Schematic of sagittal (C) and dorsal (D) views of the optic tract and RGC projections into central visual targets.

(E) Sagittal sections demarcating the lesion area immunostained for astrocytes (GFAP, green) and microglia (IBA1, magenta).

(F-I) Sagittal (F) and dorsal (G) views of normal uninjured RGC axon projections into the pretectum and SC. Sagittal (H) and dorsal (I) views of RGC axon projections 2 weeks after distal injury. The red dotted line indicates the lesion site.

(J-M) Whole-mount retinas from sham-uninjured (J) and injured-saline-control (K), injured-CNO-activity (L) mice labeled with an RGC marker (RBPMS). Quantification of RGCs two weeks after distal injury from the contralateral eyes (M). Ordinary one-way ANOVA: $p = 0.5857$; Tukey's multiple comparisons test: $p = 0.9102$, $p = 0.7882$, $p = 0.5675$. $N = 5$ animals/group for sham and activity; $N = 4$ animals for control. Error bars indicate SEM. Scale bars: 100 μm.

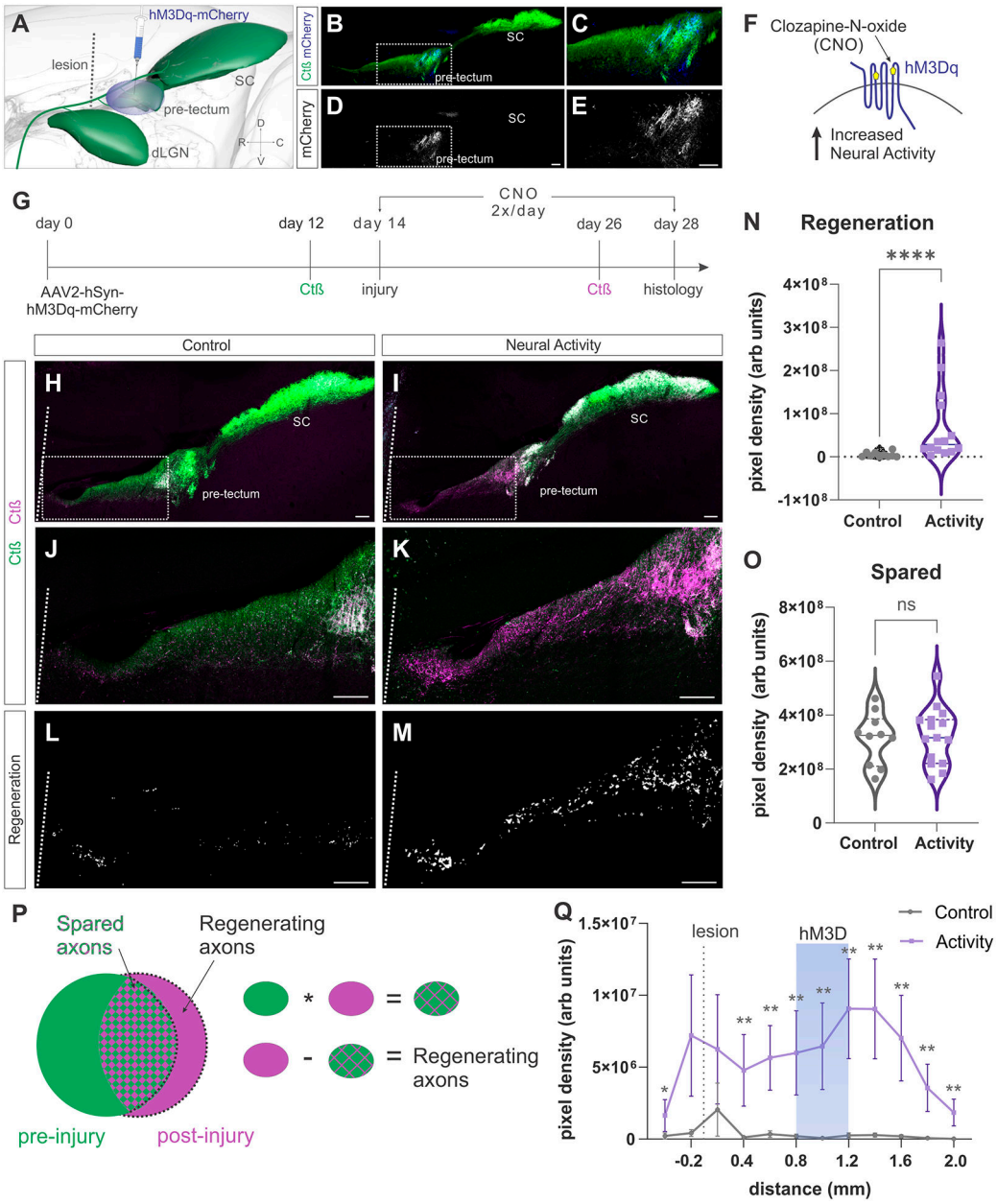

Figure 2: Non-specific stimulation of the distal optic tract promotes RGC axon regeneration

(A) Schematic of chemogenetic stimulation of neurons in the pretectum relative to the lesion site (grey dotted line).

(B-E) Ctβ labeled RGC axon projections (green) in the pretectum, and SC and mCherry labeled neurons in the pretectum expressing hM3Dq-mCherry (blue). White dotted rectangles in B and D show magnified regions in C and E, respectively.

(F) Schematic of chemogenetic stimulation: clozapine-N-oxide (CNO) binds modified hM3Dq to increase neural activity within cells.

(G) Experimental timeline for stimulating neurons in the pretectum following distal injury.

(H-I) Representative images showing Ctβ labeled RGC axon projections in the pretectum and SC. Sagittal sections of the brain with pre-injury Ctβ label (green), post-injury Ctβ label

(magenta) from control (H), and neural activity groups (I). The white dotted line indicates the lesion site.

(J-K) White rectangles in I and J show magnified regions in J and K, respectively.

(L-M) Images in J and K were processed to identify regenerating axons only labeled with post-injury Ctβ.

(N) Quantification of the pixel density of regenerating axons (analyzed as shown in P). Individual data points in each graph represent the sum of pixel density from one animal. Mann-Whitney test: ****$p<0.0001$. N = 10 animals (control), 14 animals (activity).

(O) Quantification of the pixel density of spared axons labeled with both pre-injury and post-injury Ctβ (analyzed as shown in P). Mann-Whitney test: n.s. $p = 0.8408$. N = 10 animals (control), 14 animals (activity).

(P) Pre-injury Ctβ label (green) and post-injury Ctβ label (magenta) injected into the eyes of mice can be processed to distinguish regenerating versus spared axons.

(Q) Quantification of regeneration as a function of distance. The average pixel density of all animals at each point on the x-axis is plotted. The grey dotted line shows the lesion site, the blue bar indicates the hM3Dq-injection site. Paired t-test to compare the group as a whole: ****$p<0.0001$. Multiple Mann-Whitney tests to compare control and activity at each individual distance: $p = 0.022, 0.259, 0.095, 0.0038, 0.00038, 0.0059, 0.0058, 0.0047, 0.0024, 0.0059, 0.0058, 0.0069$. N = 10 animals (control), 14 animals (activity). Error bars indicate SEM. Scale bars: 100 μm.

See also Figure S1-S4.

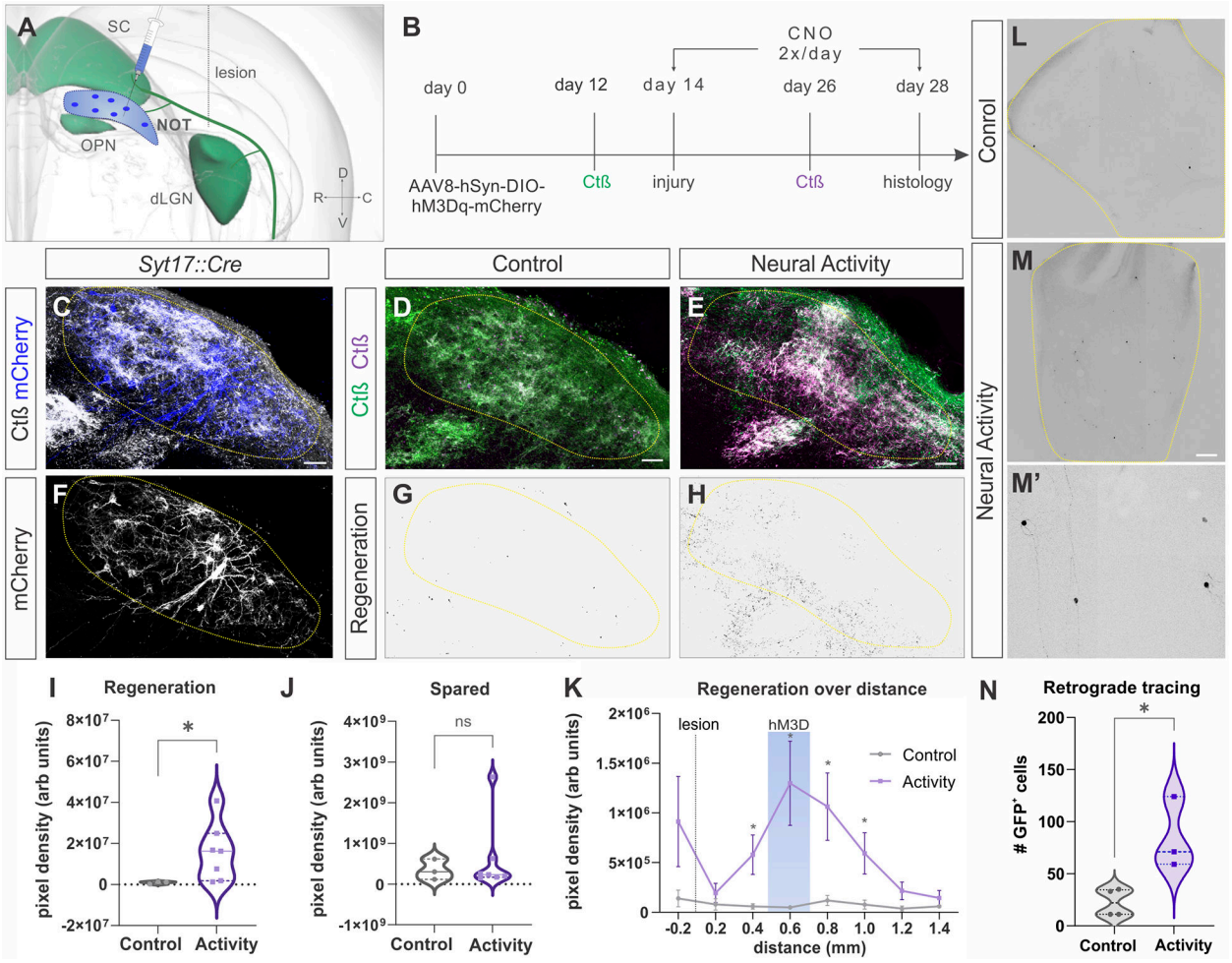

Figure 3: Selective stimulation of retinorecipient cells promotes regeneration

(A) Cre-dependent Flex-hM3Dq injected into the NOT of *Syt17::Cre* mice increases neural activity in Cre^{+} cells postsynaptic to RGCs.

(B) Experimental timeline to stimulate NOT cells posts distal-injury.

(C, F) Coronal sections showing Cre+ cells in the NOT that receive Ctβ labeled RGC input expressing hM3Dq (blue, F).

(D-E, G-H) Representative images of coronal sections of the brain labeled with pre-injury Ctβ (green) and post-injury Ctβ (magenta) from control (D, G) and neural activity groups (E, H). Images in D and E are shown processed to identify "regenerating" axons (G, H, respectively).

(I) Quantification of the pixel density of "regenerating" axons. Individual data points in represent the sum of pixel density from one animal. Mann-Whitney test: *p = 0.033. N = 3 animals (control) and 7 animals (activity).

(J) Quantification of the pixel density of spared axons. Individual data points in represent the sum of pixel density from one animal. Mann-Whitney test: n.s. p >0.9999. N = 3 animals (control) and 7 animals (activity).

(K) Quantification of the pixel density of "regenerating" axons as a function of distance.The average pixel density of all animals at each point on the x-axis is plotted. The blue bar in

K represents the hM3Dq injection site. The grey dotted line indicates the lesion site. Paired t-test to compare the group as a whole: **p = 0.0079. Multiple unpaired t-tests to compare control and activity at each individual distance: p = 0.14, 0.34, 0.040, 0.025, 0.031, 0.048, 0.095, 0.318 . N = 3 animals (control) and 7 animals (activity). Error bars indicate SEM. Scale bars: 100 μm.

(L-N) Retinal whole-mounts from control (L) and activity (M) groups show GFP+ RGCs labeled via retrograde tracing from the NOT. (M') The white box in M is magnified in M'. (N) Quantification of GFP+ RGCs from both groups. Unpaired t-test: *p = 0.02. N = 4 animals (control) and 3 animals (activity).

See also Figure S5-S7.

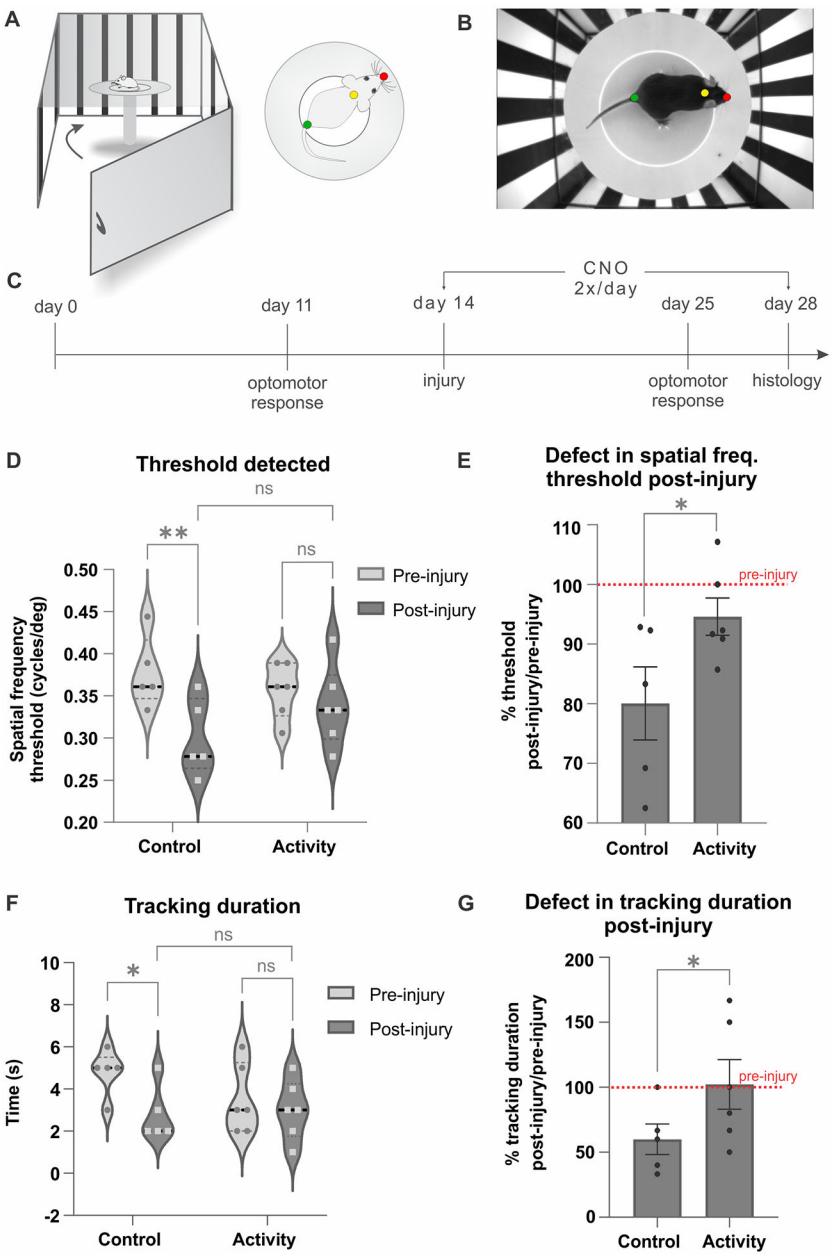

Figure 4: Increasing neural activity rescues deficit in optomotor response caused by distal injury

(A) Schematic of the OptoDrum used to measure optomotor response or OMR (Striatech Inc.). The fully-automated software overlays the red, yellow, and green dots (right) to denote head, body, and tail positions.

(B) Image captured from a video shows a mouse observing low spatial frequency (0.056 cyc/deg) drifting gratings.

(C) Experimental timeline showing two recordings of optomotor response before and after injury.

(D-E) Optomotor response for the threshold of spatial frequency tracked by each animal in control and activity groups before injury (‘pre-injury, light grey) and after injury (‘post-injury, dark grey)

(D). Two-way ANOVA: **p = 0.0081, n.s. p = 0.5696, 0.1543. Quantification of the defect percentage in control and activity groups after injury plotted. Pre-injury responses were scored as 100, and post-injury responses were calculated as a percentage of the pre-injury response for each animal in each group (E). Two-way ANOVA: *p = 0.0107. N = 5 animals/group. Error bars indicate SEM.

(F-G) The length of time each animal tracked the moving stimuli is shown as the tracking duration for both control and activity groups, before injury ("pre-injury," light grey) and after injury ("post-injury," dark grey) (F). Two-way ANOVA: **p = 0.0036, n.s. p = 0.5429, 0.8157. Quantification of the defect percentage in tracking in control and activity groups after injury plotted. Pre-injury responses were scored as 100, and post-injury responses were calculated as a percentage of the pre-injury response for each animal in each group (G). Two-way ANOVA: *p = 0.0415. N = 5 animals/group. Error bars indicate SEM.