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

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

Jung-Hwan Albert Lim

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2016

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Chair

University of California, San Diego

2016

DEDICATION

For my mother and grandmother who have supported me throughout my academic career across the Pacific Ocean. Thank you both for giving me the opportunity to excel academically and pursue my passion.

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ALM	anterior lateral microtubule neuron
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CEBP-1	CCAAT/enhancer-binding proteins 1
ChABC	chondroitinase ABC
CNO	clozapine- <i>N</i> -oxide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CoCH-GFP	cochlin-GFP
CT β	cholera toxin subunit- β
CT β -488	CT β conjugated to Alexa-Fluor-488
CT β -594	CT β conjugated to Alexa-Fluor-594
CSPGs	chondroitin sulfate proteoglycans
dLGN	dorsal lateral geniculate nucleus
DLK-1	dual-leucine zipper kinase MAPKKK 1
DREADD	designer receptors exclusively activated by designer drugs
DRGs	dorsal root ganglion cells
GABA	γ -aminobutyric acid
GAG	glycosaminoglycan
GFP	green fluorescent protein
hM3Dq	Gq-coupled human muscarinic M3 DREADD

hM4Di	Gi-coupled human muscarinic M4 DREADD
IGF	insulin growth factor
IGL	intergeniculate leaflet
ipRGCs	intrinsically photosensitive RGCs
JAK/STAT	janus kinase/the signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KLFs	kruppel-like factors
KO	knockout
MAG	myelin-associated glycoprotein
MAK-2	MAP kinase activated protein kinase 2
MLP	MARCKS-like protein
MTN	medial terminal nucleus
mTOR	mammalian target of rapamycin
NgR	nogo receptor
NOT	nucleus of the optic tract
OKR	optokinetic reflex
OMgp	oligodendrocyte myelin glycoprotein
ONC	optic nerve crush
OPN	olivary pretectal nucleus
OPN	osteopontin
PAX	paxillin
p-S6	phosphorylated S6 ribosomal protein
PLM	posterior lateral microtubule neuron

PLR	pupillary reflex
PNS	peripheral nervous system
PTEN	phosphatase tensin homolog
Rheb1	ras homolog enriched in brain 1
RGCs	retinal ganglion cells
SC	superior colliculus
SCN	suprachiasmatic nucleus
SGS	stratum griseum superficiale
SO	stratum opticum
SOCS3	suppressor of cytokine 3
SVH-1	suppressor of vhp-1
TTX	tetrodotoxin
V1	primary visual cortex
vLGN	ventral lateral geniculate nucleus
YFP	yello fluorescent protein

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FIELDS OF STUDY

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Studies in Axon Regeneration

Studies in Circuit Repair

ABSTRACT OF THE DISSERTATION

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

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2016

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Axons in the mammalian central nervous system (CNS) fail to regenerate after injury. A major goal of neuroscience is to develop strategies to promote axon regeneration and to re-establish accurate circuit connections. Using the mouse visual system as a model I show that neural activity of adult retinal ganglion cells (RGCs) induced by either visual experience or by chemogenetic activation, promotes axon regeneration after optic nerve crush. When combined with activation of the cell growth-promoting protein, mammalian target of rapamycin (mTOR), visual stimulation-driven RGC activity triggers axons to regenerate all the way back into the brain. Analysis of

genetically-labeled RGC types revealed that remarkably, regrowth is target specific: RGC axons navigated back to their correct visual targets and avoided incorrect visual targets suggesting the guidance cues that direct RGC axons are either still present or are re-expressed in response to regeneration. Moreover, the target-specific regrowth of adult RGC axons resulted in partial recovery of several different visual functions. These findings indicate that visually-evoked and artificially-induced sensory activity can be powerful tools for enhancing axon regeneration and they highlight the remarkable capacity of mammalian CNS neurons to re-establish accurate circuit connections in the adult brain.

INTRODUCTION

Neurons in the adult mammalian central nervous system (CNS) fail to regenerate their axons after injury. This is in contrast to neurons in the peripheral nervous system (PNS), which regenerate their axons at rates up to 4.4mm/day (Gutmann et al., 1941). The failure of CNS neurons to regenerate establishes a major roadblock to recovery of sensory, cognitive and motor defects that occur in response to traumatic injuries and neurodegenerative diseases (reviewed in: Goldberg and Barres, 2000; De Vos et al., 2008; Liu et al., 2011; He and Jin, 2016). In the last four decades, the regeneration field has advanced considerably in its efforts to understand the discrepancy between axon regrowth capacity in the PNS versus the CNS, and has used this distinction to develop strategies to promote injured CNS neurons to regenerate the axons.

While regenerative failure is generally an issue faced by mammalian CNS neurons, researchers have used invertebrate models such as nematodes and fruit flies to overcome some of the technical limitations inherent in the mammalian CNS. Cold-blooded vertebrates such as fish and amphibians - in which CNS neurons readily regenerate - have also been used to investigate cellular and molecular mechanisms of axon regeneration. Before introducing background on mammalian CNS regeneration, I will highlight a subset of the invertebrate and cold-blooded vertebrate studies which stand as particularly important contributions to the field. In terms of mammalian CNS regeneration, two major sets of factors have been shown to prevent regeneration of injured axons: extrinsic and intrinsic factors. I will highlight the different strategies neurobiologists have implemented to either down-regulate extrinsic inhibitory factors and/or up-regulate intrinsic growth factors to promote axon regeneration.

Invertebrates and cold-blooded vertebrates as models for regeneration

The use of invertebrate genetic model organisms such as nematodes and fruit flies has provided significant contributions to parsing out the molecular mechanisms of axon regeneration. For the invertebrate organisms that are either transparent or semi-transparent, laser ablations to selectively sever axons while causing minimal damage to surrounding tissues present unique single-axon resolution to examine detailed axonal responses (Ghosh-Roy et al., 2010; Soares et al., 2014). In general, invertebrate animals have the capacity to regenerate their nerves after injury, and researchers have used forward and reverse genetics to identify a set of genes that are either favorable or disruptive to axon regeneration. Recently, multiple groups have used *C. elegans* and *D. melanogaster* to discover that dual-leucine zipper kinase MAPKKK 1 (DLK-1), a molecule known to be involved in facilitating synaptogenesis and axon outgrowth during development and in regulating neurodegeneration after injury, is also critical in promoting axon regeneration after nerve injury in these organisms (Hammarlund et al., 2009; Yan et al., 2009; Ghosh-Roy et al., 2010; Xiong et al., 2010). Bastiani and colleagues identified DLK-1 as required for promoting axon regeneration of γ -aminobutyric acid (GABA)-releasing motor neurons in *C. elegans* (Hammarlund et al., 2009). Furthermore, Jin and coworkers identified the downstream effectors of DLK-1 - MAP kinase Activated protein Kinase 2 (MAK-2) and CCAAT/enhancer-binding proteins 1 (CEBP-1) - as critical for promoting axon regeneration of PLM and ALM neurons in *C. elegans* (Yan et al., 2009). In *D. melanogaster*, Wallenda, a conserved MAPKKK homologous to DLK-1 in *C. elegans*, was essential for activating an injury signaling cascade including the downstream effectors - c-Jun NH2-terminal kinase

MAPK (JNK) and the transcription factor Fos - and was required for promoting axon regeneration of motor neurons and sensory neurons of the *D. melanogaster* in the larval stage (Xiong et al., 2010).

Testing the novel function of key molecular pathways in higher mammals is essential for potentially identifying therapeutic targets to treat traumatic brain injuries and neurodegenerative diseases in humans. Building on results in worms and flies, investigators studied the role of DLK-1 in mammalian models. Using DLK gene-trap mice and DLK knockout (KO) mice, they showed that dorsal root ganglion neurons (DRGs) without functional DLK protein are impaired in axon regeneration after crush (Itoh et al., 2009; Shin et al., 2012). The critical role of DLK in regulating axon regeneration in mammals illustrates the conserved function of molecular signaling pathways from invertebrate organisms to mammalian organisms. Because neurons in invertebrate organisms readily regenerate their axons, testing powerful manipulations in those animals, which are not feasible in the higher mammalian organisms, can further elucidate the mechanism of axon regeneration.

The other model organisms that have been widely used to aid our understanding of axon regeneration are cold-blooded vertebrates, such as fish and amphibians. Cold-blooded vertebrates can naturally regenerate some (but not all) of their CNS axons after nerve injury (reviewed in: Gaze, 1970). One way these species promote axon regeneration is by continuously adding new neurons after development - a phenomenon called neurogenesis. Meyer (1978) showed that in adult goldfish new retinal cells that were labeled with tritiated thymidine migrated to the entire retinal circumference (Meyer, 1978). Another way they promote axon regeneration is by providing an environment that

is favorable for axon regeneration. Studies in the 1980s and 90s characterized the CNS environment of both cold-blooded vertebrates (that can regenerate their CNS axons) and warm-blooded vertebrates (that fail to regenerate their CNS axons). Different groups observed that rat DRGs could regenerate their axons through optic nerve sheath explants from goldfish but not through optic nerve explants from rats (Carbonetto et al., 1987; Caroni and Schwab, 1988). A series of biochemical studies revealed that the fish CNS environment harbors oligodendrocytes which are molecularly distinct from mammalian CNS oligodendrocytes by virtue of their myelin protein expression and by their ability to promote, rather than inhibit, axon regeneration (Caroni and Schwab, 1988; Jeserich and Rauen, 1990; Bastmeyer et al., 1991). These important findings suggest that the CNS environment of cold-blooded vertebrates lacks axon growth inhibiting molecules present in the mammalian CNS environment. Furthermore, axotomy of CNS neurons in cold-blooded vertebrates triggers up-regulation of growth-associated proteins including GAP-43 and cell adhesion molecules including E587-Ag and neurolin in fish and amphibian CNS neurons (Mizobuchi et al., 1990; Bisby and Tetzlaff, 1992; Stuermer et al., 1992; Petrausch et al., 2000).

Axon regeneration in the fish and frog visual system is a widely studied regeneration model. After optic nerve transection in fish, retinal ganglion cells (RGCs) - a type of CNS neuron - spontaneously regenerate their axons to re-innervate and occasionally even synapse in the optic tectum - their distal-most visual target (Sperry, 1963; Jacobson and Gaze, 1965). Moreover, they re-innervate the tectum with retinotopic precision (Gaze, 1970). Cline and colleagues observed that removing the optic tectum in one side of the brain induced RGC axons to regenerate to the opposite tectum in a

retinotopically precise manner in developing tadpoles (Ruthazer et al., 2003). Others have discovered that the regenerating RGC axons are more likely to misroute to the wrong side of the brain compared to the projection patterns of normal, uninjured RGCs (Stelzner et al., 1981; Bohn and Stelzner, 1981). Notably, some studies directly attributed cold-blooded vertebrates' capacity to regenerate their CNS axons to their neurogenesis of some CNS neurons over their lifetime (reviewed in Holder and Clarke, 1988). RGCs of frog species *R. pipiens* that undergo neurogenesis over lifetime of the organism can re-innervate their axons to optic tectum after optic nerve transection (Gaze, 1970). On the contrary, tectal efferents in *R. pipiens*, which stop proliferating after metamorphosis, fail to regenerate their axons after brainstem hemisection (Lyon and Stelzner, 1987). This observation suggests that CNS neurons' ability or inability to undergo neurogenesis may be the central distinction between their capacity and failure, respectively, to regenerate consistently after injury.

In addition to goldfish and frogs, researchers have used the salamander 'axolotl' to identify the types of neural progenitors and their migration patterns that take place during spinal cord regeneration after injury. Tanaka and colleagues grafted green fluorescent protein (GFP)-labeled spinal cord to a host with a transected spinal cord and found that a small region next to the amputation plane generates PAX7- and PAX6-expressing neural progenitors. Those progenitors in turn produced daughter cells to expand along the anterior/posterior axis but also stay close to the dorsal/ventral location of the parent for regeneration, indicating that the factors establishing the domains of the regenerating spinal cord comes from the mature spinal cord (McHedlishvili et al., 2007). More recently, using an expression cloning strategy followed by *in vivo* gain- and loss-of-

function assays, Tanaka's group has identified axolotl MARCKS-like protein (MLP) that activates the initial cell cycle response during appendage regeneration (Sugiura et al., 2016). Together, cold-blooded vertebrates' capacity to spontaneously regenerate CNS axons provide important molecular distinctions from mammalian organisms that can be used to further elucidate the molecular pathways that regulate mammalian CNS axon regeneration.

Extrinsic limits on regeneration of mammalian CNS axons

Many decades of work indicate that factors inherent to the mature CNS environment provide an unfavorable milieu for axon regeneration (reviewed in: Yiu and He, 2006). By contrast, neurons in the PNS readily regenerate. During 1970s and 80s, Aguayo and colleagues investigated whether the CNS environment constitutes an unfavorable barrier for axon regeneration. They transected either optic nerve or spinal cord and bridged the severed ends with sciatic nerve grafts - a PNS nerve - and then assessed regeneration of CNS axons through the graft. Remarkably, CNS neurons that do not normally regenerate in their native CNS environment regenerated long distances through the sciatic nerve graft (Richardson et al., 1980; David and Aguayo, 1981; Vidal-Sanz et al., 1987). When the group repeated the experiments using CNS nerve grafts, there was no axon regeneration (personal communication with A. Aguayo). This indicated that factors inherent to the mature CNS environment provide an unfavorable milieu for axon regeneration.

Myelin-associated inhibitors

After this significant discovery, many neurobiologists focused on identifying the extrinsic factors that inhibit mammalian CNS axon regeneration. Schwab and colleagues identified myelin as a potent inhibitor of CNS regeneration and biochemically purified the myelin-associated protein Nogo as one source of this inhibition. When they added antibodies against Nogo, the central branch of axons from dissociated DRGs could regenerate through the rat spinal cord and the optic nerve explants (Schnell and Schwab, 1990; Chen et al., 2000). Other groups discovered additional myelin-associated inhibitors such as myelin-associated glycoprotein (MAG) (Mukhopadhyay et al., 1994; McKerracher et al., 1994) and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002) that also inhibit axon regeneration of dissociated developing and adult CNS neurons in MAG- and OMgp-expressing substrates, respectively. It was later discovered that Nogo receptor (NgR) binds with high affinity to MAG and OMgp, suggesting that neutralizing all three myelin-associated inhibitors are functionally redundant in disinhibiting axon regeneration (Wang et al., 2002; Domeniconi et al., 2002; Liu et al., 2002).

In vivo studies of Nogo knockout mice suggest that deleting genes for myelin inhibitors alone may not be sufficient to promote substantial amount of CNS axon regeneration (Cafferty and Strittmatter, 2006; Lee et al., 2010a; Lee et al., 2010b; Cafferty et al., 2010). Zheng and colleagues have assessed the regenerative capacity of injured corticospinal and raphespinal serotonergic axons by knocking out all three major myelin inhibitors, Nogo, MAG, and OMgp, in mice and discovered that these triple-mutant mice failed to show enhanced regeneration (Lee et al., 2010). Their results

indicate that while myelin-based factors play an inhibitory role in CNS neurite outgrowth, removing them alone is insufficient to promote axon regeneration *in vivo*.

Scarring as a barrier

In addition to myelin, reactive glial scarring is another factor that acts as a barrier of CNS axon regeneration. CNS injury triggers mass recruitment of microglia, oligodendrocyte precursors, meningeal cells, and reactive astrocytes to the lesion site (Davies et al., 1997). Silver and others reported that reactive astrocytes upregulate extracellular molecules called chondroitin sulfate proteoglycans (CSPGs) at the core of the lesion and that such extracellular molecules inhibit neurite extension (Gallo et al., 1987; Snow et al., 1990; McKeon et al., 1991). The Fawcett and McMahon labs investigated whether neutralizing CSPGs in the reactive glial scar could promote regeneration of CNS axons. They administered chondroitinase ABC (ChABC) to the lesioned dorsal columns of adult rats, in order to remove CSPG glycosaminoglycan (GAG) in the area of reactive glial scarring. This promoted some regeneration of descending cortico-spinal axons and ascending sensory projections and restored some locomotor and proprioceptive behaviors (Bradbury et al., 2002). Recently, Giger and colleagues observed that CSPGs bind to NgR 1 and 3 and discovered shared molecular mechanisms of myelin-associated and CSPG-specific inhibition of axon regeneration (Dickendesher et al., 2012). Furthermore, Sofroniew and colleagues revealed that astrocytic glial scars are in fact aiding, rather than inhibiting, axon regeneration after spinal cord lesions. Genetically removing or attenuating the astrocytic scars *reduced* the extent of regeneration of transected corticospinal, sensory, and serotonergic axons

(Anderson et al., 2016). The extent of the inhibitory role of glial scar in *in vivo* axon regeneration models remains to be further investigated.

Repellent guidance cues

Other major extrinsic factors are repellent guidance cues that remain expressed in the adult CNS after development and regulate axon regeneration after injury. Semaphorins are classic short-range inhibitory guidance cues that repel growing axons from innervating incorrect targets during development (Behar et al., 1996). In the adult CNS, one of their roles is to stabilize the established circuitry by limiting axonal growth as adult DRGs undergo growth cone collapse in the presence of Sema3A *in vitro* (Reza et al., 1999). The inhibitory role of Sema3A was evident when preconditioned adult DRG axons failed to grow through the PNS injury lesion sites that express Sema3A (Pasterkamp et al., 2001). On the contrary, the Sema3 expression was down-regulated in the PNS nerves while the spinal and facial motor neurons spontaneously regenerated their axons through the lesion site, suggesting one potential mechanism of why axons fail to regenerate in the CNS (Pasterkamp et al., 1998). In addition to the semaphorins, ephrins predominantly repel migrating axons away from the site of Eph/ephrin activation (Marquardt et al., 2005). In response to CNS injury, ephrin expression is upregulated in myelin and reactive astrocytes (Wilson et al., 2002; Bundesen et al., 2003; Benson et al., 2005). EphA4-deficient mice enhanced axon regeneration of corticospinal and rubrospinal tracts and reduced the size of gliosis and expression of CSPGs at the lesion site after spinal cord hemisection (Goldshmit et al., 2004). This suggests potential shared mechanism between glial scar and repellent cues in preventing CNS axon regeneration.

Studies that targeted extrinsic factors to trigger CNS axon regeneration revealed that removing the extrinsic factors promote regeneration of some CNS axons in certain contexts but is not sufficient to trigger robust, long-distance regeneration of CNS axons, due to repressed intrinsic growth capacity of mature CNS axons.

Intrinsic limits on regeneration of mammalian CNS axons

Another barrier to CNS regeneration is the dramatic reduction of neuronal growth capacity as they age. As neurons transition from embryonic to postnatal stages, they downregulate their expression of cell growth-related pathways and proteins (reviewed in: He and Jin, 2016). A number of groups have shown that isolated early embryonic CNS neurons can extend their neurites through both neonatal and adult CNS substrates but that their growth capacity declined sharply after neonatal development (Shewan et al., 1995; Chen et al., 1995; Goldberg et al., 2002a). Numerous potential factors include steady decline of classic secondary messenger cyclic adenosine monophosphate (cAMP) levels during neuronal development (Cai et al., 2001). Gene profiling analyses further revealed that the expression of Kruppel-like factors (KLFs) in RGCs changes around the neonatal period (Moore et al., 2009). Functional studies further confirmed that KLF6 and KLF7 could promote axon regeneration while KLF4 could inhibit axon regeneration (Moore et al., 2009; Blackmore et al., 2012). Other groups revealed that in many types of CNS neurons, the expression of mammalian target of rapamycin (mTOR) - a master regulator of protein synthesis - becomes downregulated gradually over the course of neuronal development (Park et al., 2008; Liu et al., 2010; Belin et al., 2015; Geoffroy et al., 2016). He and colleagues discovered that activating the mTOR signaling cascade in mature CNS

neurons by removing the negative regulator of mTOR - phosphatase tensin homolog (PTEN) - triggered robust axon regeneration of RGCs and cortico-spinal tract after optic nerve crush and spinal cord pyramidotomy, respectively (Park et al., 2008; Liu et al., 2010; Zukor et al., 2013). Identifying the intrinsic factors that are downregulated in the mature CNS neurons has led to key discoveries of molecular pathways that could be manipulated to promote robust CNS axon regeneration.

A variety of cellular and molecular responses at the lesion site after injury can be potentially targeted to promote axon regeneration. Some of the direct molecular responses to CNS insult include local activation and retrograde transport of several MAP kinases - the Erk (Hanz et al., 2003; Perlson et al., 2005), JNK (Cavalli et al., 2005; Lindwall et al., 2005), and the protein kinase G (Sung et al., 2006). Matsumoto and coworkers identified a novel growth factor - suppressor of vhp-1 (SVH-1) - and its receptor tyrosine kinase SVH-2 that regulate axon regeneration of motor neurons via JNK-MAPK pathway in *C. elegans* (Li et al., 2012). Moreover, glial cells at the lesion site release cytokines to activate Janus kinase/the signal transducer and activator of transcription (JAK/STAT) signaling pathway in response to injury (Schobitz et al., 1992). The activation of JAK/STAT pathway subsequently triggers secondary messengers to activate axon growth, as numerous studies demonstrated that overexpression of ciliary neurotrophic factor (CNTF) - a cytokine that directly activates the JAK/STAT pathway - triggered CNS axon regeneration after injury (Leaver et al., 2006; Pernet et al., 2013; Jin et al., 2015).

Numerous groups have observed that the downstream effector of JAK/STAT signaling pathway, STAT3, is exclusively activated in response to PNS nerve injury but

not CNS nerve injury, suggesting its important role in axon regeneration (Schweiger et al., 2000; Qiu et al., 2005). Kerschensteiner's group elegantly demonstrated that conditional deletion of STAT3 in DRGs abolished its axon regeneration of peripheral branch while overexpressing STAT3 in DRGs enhanced its axon regeneration of central branch (Bareyre et al., 2010). Consistently, deleting the negative regulator of the JAK/STAT pathway, suppressor of cytokine 3 (SOCS3), promoted RGC axon regeneration after optic nerve crush (Smith et al., 2009). Studies that manipulate injury signaling factors in order to disinhibit axon growth after injury continue to elucidate the molecular mechanism of the injury response in the PNS and CNS systems and provide valuable insight to the mechanism of CNS axon regeneration.

One important factor that is now starting to be more heavily explored in the context of axon regeneration is neural activity. During development, neural activity promotes cell survival, dendritic and axon growth, and axon refinement (Mao et al., 1999; McAllister et al., 1996; Meyer-Franke et al., 1995; Sretavan et al., 1988; Goldberg et al., 2002b). After development is completed, activity-dependent plasticity continues throughout the brain. For instance, exercise-conditioned animals showed enhanced regrowth of DRG axons compared to sedentary animals after nerve crush injury (Molteni et al., 2004). To enhance or suppress neural activity, neurobiologists have used electrical stimulation or tetrodotoxin (TTX), respectively, and more recently, designer receptors exclusively activated by designer drugs (DREADD) (Urban and Roth, 2015). Goldberg et al. (2002b) demonstrated that electrically stimulating purified RGCs coupled with brain-derived neurotrophic factor (BDNF) treatment enhanced axon growth *in vitro*. Others discovered that in the PNS, periodic electrical stimulation increased the speed and

accuracy of motor neuron axon regeneration back to their correct targets after nerve transection, whereas TTX treatment abolished this electrical stimulation-induced axon regeneration (Al-Majed et al., 2000). In mouse models, trans-corneal stimulation of the eye enhanced RGC axon regeneration after optic nerve crush (Tagami et al., 2009). Furthermore, elevating melanopsin expression in RGCs enhanced axon regeneration after optic nerve crush (Li et al., 2015). These studies highlight the potential therapeutic benefits of neuronal activity to treat mammalian CNS degeneration.

Probing specificity of axon regeneration

In addition to triggering long distance regeneration of CNS axons, ensuring that the regenerated axons find and reconnect to the correct nuclei in the brain is critical for functional recovery. The innervation of CNS axons to correct targets during development relies on a host of guidance expressions including ephrins, slits, Wnts, and cadherins (e.g., Dickson, 2002; Schmitt et al., 2006; Harada et al., 2007; Osterhout et al., 2011). Axon guidance/repellent cues that are expressed during development undergo changes in their expression as the animal ages (reviewed in: Harel and Strittmatter, 2006). In addition, injury itself may alter the expression of these cues, which in turn could present a major challenge for regenerating axons to steer toward correct targets (reviewed in: Guan and Rao, 2003; Koeberle and Bahr, 2003; Harel and Strittmatter, 2006; Giger et al., 2011).

.Regeneration literature presents evidence that axons that regenerate long distances possess the capacity to re-innervate correct targets. Studies in invertebrates or cold-blooded vertebrates demonstrate that some neurons are capable of regenerating back

to the correct target region. For example, in gold fish, retinal axons can regenerate back to and re-innervate the correct layer of the optic tectum after optic nerve crush (Murray and Edwards, 1982). In the larval sea lamprey, the majority of the spinal axons regenerate beyond the transection site in a proper direction with a similar projection pattern of uninjured spinal axons (Mackler et al., 1986). In salamander, DRGs fully regenerate their axons back to proper targets for recovery of limb movement (McHedlishvili et al., 2012).

In the mammalian visual system, Aguayo and coworkers observed that long-distance regenerating RGC axons through the PNS graft preferentially regenerate to the stratum opticum (SO) and stratum griseum superficiale (SGS), the main layers in superior colliculus (SC) that normally receive RGC axonal innervation (Vidal-Sanz et al., 1987). This target-specific re-innervation suggests that regenerating RGCs can recognize which SC layers are visual and correctly re-innervate. Moreover, Bahr and colleagues reported that the RGC axons from the retinal explants preferentially regenerated towards the deafferented SC (e.g. optic nerve lesioned) (Wizenmann et al., 1993) and they grew towards the correct region of the SC (Wizenmann and Bahr, 1998). More recently, Benowitz's group showed that triple manipulations that included enhancement of mTOR signaling and cAMP as well as chronic injections of zymosan to increase oncomodulin could trigger long-distance regeneration of RGC axons and furthermore, guide regenerating RGC axons to visual targets in the brain for partial recovery of function (de Lima et al., 2012).

In the spinal cord, numerous studies showed target specificity of regenerating DRG axons in both central and peripheral branches. For example, investigating the regeneration of DRG axons showed that systemic induction of the GDNF family

member, artemin, could trigger re-innervation of regenerating DRGs to a correct layer of the dorsal horn (Harvey et al., 2010; Smith et al., 2012). Furthermore, Brushart and coworkers showed that electrical stimulation allowed normally regenerating peripheral axons of DRGs to correct targets in the periphery (Brushart et al., 2005). In all the studies, either the regenerating neurons are intrinsically capable and/or their environment presents correct guidance cues for target re- innervation.

The eye-to-brain ‘retinofugal’ pathway as a model for CNS axon regeneration

The optic pathway consisting of RGC projections down the optic nerve and into the brain is a classic and still highly utilized model for studying CNS regeneration (Vidal-Sanz et al., 1987; Park et al., 2008; Benowitz and Yin, 2010; de Lima et al., 2012). Normally, following the unilateral optic nerve crush, RGC axons fail to regenerate through the lesion sites and eventually die (Chierzi et al., 1999). Recent work showed that increasing the mTOR signaling pathway can promote a substantial number of RGCs to survive and regenerate their axons down the optic nerve after lesions (Park et al., 2008), an effect that is further increased by the co-deletion of the SOCS3 (Smith et al., 2009; Sun et al., 2011).

A critical step for the re-establishment of functional visual circuits after optic nerve injury is the regeneration of RGC axons past the optic chiasm and into the brain. The retinofugal pathway includes several dozen target nuclei distributed from the hypothalamic forebrain to the caudal midbrain (reviewed in: Morin and Studholm, 2014). The precise connectivity between RGCs and their targets is essential for proper visual perception. For RGC axons to regenerate long distances in a target-specific manner, they

must 1) be triggered to regenerate (i.e. by enhancing cell growth signaling pathways and attenuating regulatory pathways), 2) overcome inhibitory factors (i.e. by removing myelin-associated inhibitors and glial scars), and 3) be able to pathfind to and re-innervate correct visual targets and select a correct layer in a topographically precise manner (i.e. by re-expressing appropriate guidance cues and enhancing neural activity). Strategies that provide sustained, long-distance RGC axon regeneration back into the brain, however, have remained scarce. RGC axons that regenerate long distances remain stalled at the optic chiasm and thus, remain divorced from their targets in the brain (Park et al., 2008; Sun et al., 2011). Benowitz and coworkers revealed that RGCs could be stimulated to regenerate long distances and re-innervate into the various visual targets in the brain for partial recovery of visual functions (de Lima et al., 2012). However, they observed reduced number of axon regeneration over time, suggesting that the regenerated RGCs eventually die, and thereby prevent enduring functional recovery.

Mammals have ~30 types of RGCs (reviewed in: Dhande et al., 2015); each type responds best to a particular feature in the visual world (i.e. direction of motion, color or edges) and sends its axons to specific retinorecipient target(s) in the brain. The Huberman lab and others previously discovered and characterized various transgenic mouse lines that harbor GFP in specific RGC types (reviewed in: Dhande et al., 2015) and others have shown that regeneration is biased mainly toward α -like RGC types (Duan et al., 2015). These transgenic mouse lines can be useful to probe cell-type specific regeneration of visual circuitry, especially at the level of target re-innervation. The questions posed and addressed here in the context of the mammalian CNS regeneration are as follows:

1. What are the factors that can promote long-distance CNS axon regeneration?
2. What are the patterns of regenerated CNS projections and connections?
3. What are the behavioral capacity of regenerated visual circuits?

Rationale

I investigated the above questions using the mouse eye-to-brain retinofugal pathway. The main objective of my thesis was to develop strategies for promoting RGC axon regeneration long distances and replenishing functionally precise connections in the brain after optic nerve injury. The first necessary step toward repairing the damaged neural circuitry in the visual system was promoting long-distance axon regeneration of RGCs. The next step was to probe whether RGC axons that regenerate long distances have the capacity to re-innervate to correct targets using the transgenic mouse lines that genetically label specific RGC type. One way to assess the function of the RGC axons that regenerated to their correct targets was to run visual behavior tests that would require proper connection of the RGCs to their target nuclei. Ultimately, my goal is to uncover the biology of neurological defects through understanding the cellular and molecular mechanisms of how traumatic brain injuries and neurodegenerative diseases progress over time. All of this groundwork could lead to the development of better treatments for patients suffering from these diseases.

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CHAPTER 1: Neural activity promotes long-distance, target-specific regeneration of
adult retinal axons

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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 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 functions¹. 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 grafts². Subsequent work identified some of the CNS factors responsible for inhibiting axon regrowth, including myelin-associated proteins^{3,4}, reactive glial scarring⁵, upregulation of extracellular matrix factors⁶ and repellent guidance cues^{7,8}. Factors intrinsic to CNS neurons also prevent axon regeneration. For example, during development, CNS neurons downregulate expression of growth-promoting molecules⁹. 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) or ciliary neurotrophic factor (CNTF)^{10–12}, or by downregulating transcriptional inhibitors of axon growth such as Kruppel-like factor 4 (KLF4)¹³ or suppressor of cytokine signaling 3 (SOCS3)¹⁴. In general, it is thought that both extrinsic and intrinsic factors limit CNS axon regeneration¹⁵.

The eye-to-brain pathway consisting of retinal ganglion cell (RGC) connections to subcortical targets is a widely used model for studying vertebrate CNS regeneration^{2,10,13,16}. After optic nerve crush (ONC), RGC axons fail to regenerate beyond the crush site, and eventually the RGCs die altogether¹⁷. Recent work showed that increasing mTOR signaling in RGCs by deletion of the gene encoding one of its inhibitors, phosphatase and tensin homolog (PTEN), allows a substantial number of RGCs to regenerate their axons through lesions in the

optic nerve¹⁰, an effect that is further enhanced by deletion of the gene encoding SOCS3 (ref. 18). 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 chiasm¹⁰ or steer away from the brain and travel down the opposite optic nerve^{18,19}, 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

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Figure 1 Visual stimulation triggers regeneration of RGC axons. (a) Experimental design of intravitreal injection and optic nerve crush. (b) Diagram of high-contrast visual stimulation. (c) Experimental timeline. (d) Schematics of group 1 and group 2. (e,f) CT β -labeled optic nerves from the control group (group 1, $n = 16$ mice) (e) and visual stimulation group (group 2, $n = 5$ mice) (f). High-magnification images of boxed regions are shown in e1–e3 and f1–f3. Arrows point to regenerating axons. Asterisks indicate the lesion site. Vertical dashed lines indicate the 500- μ m mark beyond the lesion site for axon quantification. (g) Number of regenerating axons as a function of distance from the lesion site (data presented as mean $\pm$ s.e.m.). Asterisks indicate significance in unpaired one-tailed Student's t -test; $*P < 0.05$ (the following sets of P values are from comparison of group 1 and group 2 for data points from left to right along the y axis: $P = 0.032$, $t = 2.393$, d.f. = 4.83; $P = 0.0658$, $t = 1.873$, d.f. = 4.167; $P = 0.1465$, $t = 1.208$, d.f. = 4.048; $P = 0.1793$, $t = 1.035$, d.f. = 4.027; $P = 0.213$, $t = 0.8829$, d.f. = 4.1). Scale bars in e,f, 500 μ m (top) and 250 μ m (e1–e3 and f1–f3). OC, optic chiasm. Group 1 includes control animals that received intravitreal injections of either AAV2-Cre ($n = 5$ mice) or saline vehicle ($n = 6$ mice) or control animals that received no injections ($n = 5$ mice) before the optic nerve crush. We observed no difference in the number of RGC axons past the lesions among the three control groups (Supplementary Fig. 1).

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 for driving the electrical activity of RGCs. Thus, we examined whether exposing adult mice to high-contrast visual stimulation daily for 3 weeks following ONC would trigger regeneration of RGC axons (**Fig. 1b,c**) (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**).

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 [$G_{i/o}$ -coupled human muscarinic M_4 designer receptor exclusively activated by a designer drug (DREADD)]²¹ in RGCs by injecting mice with adeno-associated-virus AAV2-hM4Di-mCitrine in one eye *in vivo* (**Fig. 2a,b**). Two weeks later, hM4Di-mCitrine-expressing cells were observed throughout the RGC layer (**Supplementary Fig. 3a–d**). They also expressed the marker RBPMS²², confirming them as RGCs (data not shown). When exposed to the synthetic ligand clozapine-*N*-oxide (CNO), hM4Di causes membrane hyperpolarization and silencing of CNS neurons²¹. We confirmed this in RGCs by targeting the AAV2-hM4Di-mCitrine-infected RGCs for whole-cell current-clamp recordings of their responses to current injections and high-contrast visual

stimulation (**Fig. 2c** and **Supplementary Fig. 4a–d**). In the absence of CNO, expression of AAV2-hM4Di-mCitrine had no impact on RGC activity. Application of CNO, however, led to strong hyperpolarization and suppression of RGC spiking in response to current injection and visual stimulation (**Fig. 2c** and **Supplementary Fig. 4a–d**).

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 CT β -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 CT β -labeled

Figure 2 Neural activity regulates regeneration of RGC axons. (a) Diagram of intravitreal injections. (b) Diagram of hM4Di receptor. (c) Representative example of spike train before and after CNO administration during whole cell recording of a RGC infected with AAV2-hM4Di-mCitrine. (d) Experimental timeline. I.p., intraperitoneal. (e) CTβ-labeled optic nerves from the AAV2-hM4Di group ($n = 5$ mice). High-magnification images of boxed regions are shown in e1–e3. (f) Diagram of intravitreal injection. (g) Diagram of hM3Dq receptor. (h) Representative example of spike train before and after CNO administration during whole-cell recording of a RGC infected with AAV2-hM3Dq-mCitrine. (i) Experimental timeline. (j) CTβ-labeled optic nerves from the AAV2-hM3Dq group ($n = 5$ mice). High-magnification images of boxed regions are shown in j1–j3; a higher magnification image of boxed region in j2 is shown in j2'. Asterisks in e and j indicate the lesion site. Vertical dashed lines indicate the 500-μm mark beyond the lesion site for axon quantification. (k) Number of regenerating axons as a function of distance from the lesion site (data presented as mean $\pm$ s.e.m.). Asterisks indicate significance in unpaired one-tailed Student's *t*-test; $*P < 0.05$ (statistics in **Supplementary Table 1**). Scale bars in e and j, 500 μm (top), 250 μm (e1–e3 and j1–j3) and 50 μm (j2').

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 (G_q -coupled human muscarinic M_3 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. 3c–h**) and co-labeling of these retinas with RBPMS²² 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 twice a day for 3 weeks (**Fig. 2f,i**). 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. 2j**). 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

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Figure 3 Combining biased visual stimulation and enhancement of mTOR signaling with cRheb1 overexpression triggers long-distance regeneration of RGC axons. **(a)** Experimental time line. **(b,c)** Images of flat mount retina immunostained with p-S6 for baseline levels **(b)** and cRheb1-induced levels of p-S6 expression **(c)**, with high-magnification images of boxed regions shown in insets. **(d)** Quantification of p-S6-expressing cells ($n = 5$ retinas per group) (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's *t*-test; $***P < 0.0005$ ($P = 0.0003$, $t = 1.553$, d.f. = 19.67). **(e)** Schematics of group 3 and group 4. **(f,g)** CT β -labeled optic nerve in AAV2-cRheb1 group (group 3, $n = 13$ mice) **(f)** and AAV2-cRheb1 plus biased visual stimulation group (group 4, $n = 10$ mice) **(g)**. High-magnification images of boxed regions are shown in f1–f3 and g1–g4. Arrows point to regenerating fibers. Asterisks indicate lesion site. Vertical dashed lines indicate the 500- μ m mark beyond the lesion site for axon quantification. **(h)** Number of regenerating axons as a function of distance from the lesion site (data presented as mean $\pm$ s.e.m.). O.c., optic chiasm. Asterisks indicate statistical significance in unpaired one-tailed Student's *t*-test; $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$ (statistics in **Supplementary Table 2**). Scale bars: **b,c**, 250 μ m; **f,g**, 500 μ m (top) and 250 μ m (f1–f3 and g1–g4).

RGC axons extending through the lesion site and into the proximal optic nerve (**Fig. 3f**) (group 3), 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

Figure 4 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 CT β neurotracer, and collect optic nerve and brain. (b) Photomicrograph and schematic of a sagittal mouse brain section showing the major visual targets labeled with CT β . (c–i) Coronal brain sections from one animal showing CT β -labeled regenerating RGC axons in the SCN (c), vLGN (d), dLGN (e), OPN (f), pretectum (g), MTN (d, dorsal; v, ventral) (h) and SC (SGS, stratum griseum superficiale; SO, stratum opticum) (i). 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 CT β -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 visual target. Arrowheads in j show axons in optic chiasm that do not re-innervate to visual targets. High-magnification images of boxed regions are shown in c1–e1, k1, l1 and o1. Scale bars: c 100 μ m (left) and 20 μ m (c1); d, 100 μ m (left) and 50 μ m (d1); e, 200 μ m (left) and 50 μ m (e1); f, 100 μ m; g, 50 μ m; h, 100 μ m; i, 200 μ m; j, 200 μ m; k, 100 μ m (left) and 50 μ m (k1); l, 200 μ m (left) and 100 μ m (l1); m, 100 μ m; n, 100 μ m; o, 250 μ m (left) and 50 μ m (o1).

the lesioned and AAV2-cRheb1 treated eye (group 7), the number of RGC axons that regenerated was similar to that of the group 3 animals that received AAV2-cRheb1 treatment and, notably, no axons reached the optic chiasm (Supplementary Figs. 8c and 9a).

We found that the effect of the combined treatments also relied 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 RGCs^{9–12}.

Together, the different combinations of treatments we designed to promote regeneration highlight the importance of providing visual stimulation to the AAV2-cRheb1 treated eye and they argue against indirect effects of the biased visual stimulation/AAV2-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/AAV2-cRheb1 reflected an increase in RGC survival. The number of RGCs surviving ONC did indeed more than double in the biased visual stimulation/AAV2-cRheb1 group (Supplementary Fig. 13). The most parsimonious conclusion from all 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)²⁵. In mice receiving biased visual stimulation/AAV2-cRheb1 treatments (group 4) (Fig. 4a,b), regenerated CT β -labeled RGC axons were observed in multiple subcortical visual targets (Fig. 4c–o). Seven of ten mice that received the biased visual stimulation/AAV2-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, CT β -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)

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(Fig. 4h,n). Remarkably, RGC axons were also detected in the subcortical visual target located furthest from the eye, the superior colliculus (SC) (Fig. 4i,o). Notably, we did not observe any CT β -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 CT β -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 CT β -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 CT β -488 (Supplementary Fig. 16b). In every mouse examined, CT β -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 CT β -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-cRheb1 protocol induced 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 nerve crush procedure did not spare RGC axons. Rather, the CT β -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							
		OC	SCN	vLGN	dLGN	OPN	MTN	NOT	SC
1	<i>n</i> = 16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16
2	<i>n</i> = 5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
3	<i>n</i> = 13	0/13	0/13	0/13	0/13	0/13	0/13	0/13	0/13
4	<i>n</i> = 10	7/10	5/10	6/10	6/10	6/10	6/10	5/10	6/10
5	<i>n</i> = 6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
6	<i>n</i> = 5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
7	<i>n</i> = 5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
8	<i>n</i> = 5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
9	<i>n</i> = 5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
10	<i>n</i> = 9	6/9	4/9	5/9	5/9	4/9	4/9	4/9	6/9

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 of 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 RGCs²⁵, 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 types^{25,26}. 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 types²⁷. 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 SC, and they avoid the SCN, MTN and intergeniculate leaflet (IGL) (Fig. 5b and Supplementary Fig. 19). In optic-nerve-lesioned CoCH-GFP mice treated with biased visual stimulation and AAV2-cRheb1 (Fig. 5a), a subset of the CT β -594⁺ axons in the optic nerve also expressed CoCH-GFP (Supplementary Fig. 20) indicating they indeed are part of the regenerating cohort. CT β -594-labeled RGC axons were observed in various retinorecipient targets in the brain, including the SCN, vLGN, dLGN, MTN and SC (Fig. 5c–l,o–u). Remarkably, the only targets that contained axons that were double-labeled with both CT β -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 (Fig. 5f–h), the dLGN (Fig. 5i–k), the OPN (Fig. 5l–n) and the SC (Fig. 5u–w). Thus, regeneration of CoCH-GFP⁺ RGC axons was target-specific. Moreover, the regenerated CoCH-GFP⁺ RGC axons avoided visual targets they normally fail to innervate: the SCN, the nucleus of the optic tract (NOT) and the MTN. Although these targets contained CT β -594 labeled RGC axons (Fig. 5c,o,r), indicating they are capable of accepting regenerated RGC axons, none of the regenerated axons expressed GFP (Fig. 5d,e,p,q,s,t), indicating they arose from other, non-CoCH-GFP⁺ RGC types.

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 CT β 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 CT β -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 μ m; f–h, 50 μ m (left) and 25 μ m (f1, g1, h1); i–k, 100 μ m (left) and 25 μ m (i1, j1, k1); l–n, 100 μ m (left) and 25 μ m (l1, m1, n1); o–q, 100 μ m; r–t, 250 μ m; u–w, 500 μ m (left) and 50 μ m (u1, v1, w1).

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 CT β -594. To test the assumption that the GFP⁺CT β [−] 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 CT β +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 regenerated CoCH-GFP⁺ axons were labeled with CT β -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 retino-geniculate-cortical pathway³¹, and the looming avoidance response probes the retino-collicular pathway^{32–34}. 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 previously^{35,36} 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

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Figure 6 Removing one eye and enhancement of mTOR signaling with cRheb1 overexpression trigger RGC regeneration to correct visual targets in the brain. **(a)** Schematic of the experimental group (group 10, $n = 9$ mice). Protocol for the combined treatments: inject cRheb1 virus, perform optic nerve crush and suture opposite eye, provide mice daily visual stimulation, inject CT β neurotracer, and collect optic nerve and brain. **(b)** Photomicrograph and schematic of sagittal mouse brain with projections from CoCH GFP $^{+}$ RGCs in green and pan-RGCs shown in magenta. **(c)** CT β -labeling of regenerated RGC axons in the optic nerve. High-magnification images of boxed regions are shown in c1–c4. Arrows in c3 point to long-distance regenerating axons. Arrows in c4 point to regenerating axons in the optic chiasm. Asterisks indicate lesion site. **(d–l)** Images of CT β -labeled, GFP-immunostained coronal sections of brain from CoCH GFP $^{+}$ animal showing vLGN (**d–f**), dLGN (**g–i**) and SC (**j–l**). Boxed regions in the left panels are magnified in g1, h1 and i1; boxed region in i1 is magnified in i1'. Scale bars: **c**, 500 μ m (top left), 250 μ m (c1–c3) and 100 μ m (c4); **d,e**, 100 μ m; **g**, 100 μ m (top) and 20 μ m (f1); **g–i**, 100 μ m (left), 50 μ m (g1, h1, i1) and 20 μ m (i1'); **j,k**, 250 μ m; **l**, 250 μ m (top) and 50 μ m (l1).

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 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 pathway³¹. 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 overhead 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)³². Upon

Figure 7 Combined daily visual stimulation and enhancement of mTOR signaling with cRheb1 overexpression partially rescues visually guided behaviors. (a–c) Schematics of the non-lesioned group ($n = 5$ mice) (a), lesioned and untreated group ($n = 5$ mice) (b), lesioned and treated group (group 4, $n = 3$ mice) (c). Protocol for the combined treatments: inject cRheb1 virus, perform optic nerve crush and suture opposite eye, provide mice daily visual stimulation, inject CT β neurotracer, and collect optic nerve and brain. (d) Schematic of the optokinetic reflex behavior. (e) Mean percentage of the trials with optokinetic reflex (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's t -test; $*P < 0.05$, $**P < 0.005$ (non-lesioned group versus lesioned and untreated group: $P = 0.0008$, $t = 7.67$, d.f. = 4; lesioned and untreated group versus lesioned and treated group: $P = 0.022$, $t = 4.613$, d.f. = 2; non-lesioned group versus lesioned and treated group: $P = 0.0857$, $t = 1.605$, d.f. = 4.845). (f) Schematic of the direct pupillary reflex response. (g) Schematic of the consensual pupillary reflex response. (h) Mean percentage of the direct pupil constriction (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's t -test; $***P < 0.0005$ (non-lesioned group versus lesioned and untreated group: $P < 0.0001$, $t = 13.03$, d.f. = 5.862; lesioned and untreated group versus lesioned and treated group: $P = 0.1712$, $t = 1.085$, d.f. = 3.779; non-lesioned group versus lesioned and treated group: $P = 0.0042$, $t = 7.927$, d.f. = 2.443). (i) Mean percentage of the consensual pupil constriction (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's t -test; $***P < 0.0005$ (non-lesioned group versus lesioned and untreated group: $P < 0.0001$, $t = 14.55$, d.f. = 6.207; lesioned and untreated group versus lesioned and treated group: $P = 0.4799$, $t = 0.05594$, d.f. = 2.327; non-lesioned group versus lesioned and treated group: $P = 0.0193$, $t = 4.709$, d.f. = 2.097). (j) Schematic of the visual cliff behavior. (k) Mean percentage of the shallow side-selected trials (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's t -test; $*P < 0.05$, $***P < 0.0005$ (non-lesioned group versus lesioned and untreated group: $P = 0.0002$, $t = 5.89$, d.f. = 7.748; lesioned and untreated group versus lesioned and treated group: $P = 0.4682$, $t = 0.08575$, d.f. = 3.474; non-lesioned group versus lesioned and treated group: $P = 0.0135$, $t = 4.025$, d.f. = 3.03). (l) Schematic of the looming response. (m) Mean percentage of the trials responded to looming stimulus (data presented as mean $\pm$ s.e.m.). Asterisks indicate statistical significance in unpaired one-tailed Student's t -test; ($P = 0.0918$, $t = 2$, d.f. = 2; $P = 0.2113$, $t = 1$, d.f. = 2); n.s., not significant statistically.

presentation of the looming stimulus, normal mice either immediately froze or ran under the shelter (Fig. 7m)³². 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

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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 degeneration 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 been shown to prolong RGC both survival and function in various models of ophthalmic disease⁴⁰. Thus, there is growing evidence that electrical activity is a key parameter adjusting the regrowth potential of CNS neurons.

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 chiasm, 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 sciatic 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 CT β ⁺ 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 is 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 cervical 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 development^{29,35} 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 retino-SC connections (the looming avoidance response)³⁴ while the assays where we failed to observe any recovery were the pupil response (retino-OPN connection)³⁰ and visual cliff test. The first three behaviors involve retino-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 retino-geniculate-cortical pathway may therefore be higher than that of the other retinofugal 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 restoration⁴⁵. Only by enhancing neural activity in regenerating RGCs did their axons recruit myelinating glia—just as RGCs do during development⁴⁶—and allow functional transmission between RGCs and their targets⁴⁵. 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 demonstrating 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. C.W. prepared AAV-cRheb1 viruses. K.Z. provided technical assistance for optic nerve crush surgery and AAV-cRheb1. Z.H. provided AAV-cRheb1 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-EGFP) (from MMRRC), Opsin 4 (melanopsin)-EGFP mice (OPN4-EGFP) (from MMRRC) and *Pten*^{fl/fl} 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^{10,14,18}. 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 IACCUC 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 previously²⁶; the anterograde tracer cholera toxin subunit-β (CTβ) conjugated to Alexa Fluor 594 (CTβ-594; Invitrogen) or to Alexa Fluor 488 (CTβ-488; Invitrogen) to label RGC axons; adeno-associated virus serotype 2/1 with constitutively active Rheb1 (AAV2/1-cRheb1, 6×10^{10} ifu/ml) to overexpress cRheb1; adeno-associated virus serotype 2 with hM4Di (rAAV2/hSyn-HA-hM4D(Gi)-IRES-mCitrine, 5.6×10^{12} vg/ml) and hM3Dq (rAAV2/hSyn-HA-hM3D(Gq)-IRES-mCitrine, 5.1×10^{12} vg/ml) (Gene Therapy Vector Core, UNC, NC) to overexpress hM4Di and hM3Dq, respectively; adeno-associated virus serotype 2 with Cre (AAV2-iCre, 1×10^{13} GC/ml) (Vector Biolabs) to knock out *Pten* gene in *Pten*^{fl/fl} 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. CTβ-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-cRheb1 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 previously¹⁰. 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-cRheb1 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 previously²¹. 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 eye¹⁰. 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-RBPMS (PhosphoSolutions, 1:1,000, Cat. No. 1830-RBPMS), 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 (Sternberger 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-cRheb1-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 RBPMS²².

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^{10,16}, we quantified the total number of regenerating axons, Σad , by using the following formula: $\Sigma ad = \pi r^2 \times [\text{average axons/mm}] / t$, 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 previously³⁵. Briefly, retinas were collected and dissected in gassed (95% O₂ and 5% CO₂) Ames medium (Sigma) under infrared illumination and cut along the dorsal-ventral axis. Only ventral pieces were used. A piece of retina was placed in a chamber on an upright microscope and superfused (~5 ml/min) with gassed 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-Mg²⁺ 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 2.6×10^5 R*/S-cone s⁻¹. 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⁻¹, the optimal stimulus for driving the OKR^{35,36}. 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 unanesthetized 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^{30,38} 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^{32,34}. 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 cRheb1 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.

CHAPTER 1 SUPPLEMENTAL DATA

Supplementary Figure 1. RGC axons do not regenerate after optic nerve crush.

(a) Experimental timeline. (b-d) Low magnification images of CT β -labeled crushed optic nerves from the no treatment group (b), saline group (c), and AAV-Cre group (d). (b1-b3), (c1-c3), and (d1-d3) are high magnification images from insets in (b), (c), and (d), respectively. Asterisk in (b), (b1), (c), (c1), (d) and (d1) denotes the lesion site. Vertical dashed line in (b1), (c1), and (d1) indicates 500 μ m mark beyond the lesion site for axon quantification. (e) Quantification of the number of regenerated RGC axons as a function of distance from the lesion site in three separate control groups showing no significant differences between them (unpaired one-tailed Student's *t*-test, +/- SEM). Thus, 'Group 1' (*n* = 16 mice) listed in the **main figures** combines all three into a single control group. Scale bar in (b), (c), (d) = 500 μ m; (b1-b3), (c1-c3), (d1-d3) = 250 μ m.

Supplementary Figure 3. AAV2-hM4Di-mCitrine and AAV2-hM3Dq-mCitrine injections into the vitreous lead to expression of synthetic muscarinic receptors hM4Di and hM3Dq in RGCs, respectively.

(a-d) Images of a flat mount retina from an adult mouse (aged P50) infected with AAV2-hM4Di-mCitrine nasally (a), dorsally (b), ventrally (c), temporally (d). (e-h) Images of a flat mount retina from an adult mouse (aged P50) robustly infected with AAV2-hM3Dq-mCitrine nasally (e), dorsally (f), ventrally (g), temporally (h). (i-k) Images of flat mount retina immunostained with RBPMS (i), with AAV-hM3Dq⁺-labeled cells (j), and merge (k). (l) Bar graph of the percentage of RBPMS⁺ RGCs that are infected with AAV-hM3Dq. Scale bar in (d, h) = 500 μ m; (k) = 50 μ m.

Supplementary Figure 4. Effects of hM4Di and hM3Dq receptor activation on RGC activity.

Whole-cell recording from an AAV2-hM4Di-mCitrine infected RGC as CNO (10 μM) was washed into the recording chamber. Activation of the hM4Di receptor hyperpolarized the cell and inhibited baseline spiking. **(a)** Example whole-cell response of an AAV2-hM4Di-mCitrine infected RGC to a 20 pA current injection under control conditions (left) and in the presence of CNO. CNO reduced the spiking output of the cell. **(b)** Quantification of the effects of CNO on spiking output from a small population of AAV2-hM4Di-mCitrine infected RGCs. The number of spikes (left) and the ratio of the spiking output in CNO relative to control (right) were significantly reduced. **(c)** Example whole-cell response of an AAV2-hM4Di-mCitrine infected RGC to a 300 micron +100% contrast flash under control conditions (left) and in the presence of CNO. CNO reduced the spiking output of the cell. **(d)** Same as in **(c)** for light-evoked responses. **(e-h)** Same as in **(a-d)** for AAV2-hM3Dq-mCitrine infected RGCs.

Supplementary Figure 5. cRheb1 overexpression increases phosphorylated mTOR expression in adult RGCs.

(a-b) Whole mount retinas from adult mice (aged P40 or older) injected either with saline (a) or with AAV2-cRheb1 (b) and immunostained for downstream marker of phosphorylated mTOR, p-S6. (a1) High magnification image from panel (a). (b1), High magnification image from panel (b). Scale bar in (b) = 1 mm; (b1) = 100 μ m; (e) = 25 μ m.

Supplementary Figure 6. Rapamycin prevents AAV2-cRheb1-induced regeneration of RGC axons.

(a) Experimental time line. Rapamycin was administered intraperitoneally every other day for two weeks following AAV2-cRheb1 injections in the eye. (b) Low magnification image of CTβ-traced optic nerve in AAV2-cRheb1 plus rapamycin group. (b1-b3) High magnification images from insets in panel (b). Asterisk in (b) and (b1) denotes the lesion site. Vertical dashed line in (b1) demarcates 500 μm past the lesion site. (c) Line graph showing the number of regenerated axons as a function of distance from the lesion site (+/- SEM). Note: The 'Control group' includes the animals that received intravitreal injections of AAV2-Cre ($n = 5$ mice) and saline vehicle ($n = 6$ mice) and the animals that received no injections ($n = 5$ mice). There were no significant differences between these three control conditions (see **Supplementary Fig. 1**). Asterisks indicate significance in

unpaired one-tailed Student's *t*-test; * $P < 0.05$, ** $P < 0.05$. Scale bar in **(b)** = 500 μm ; and **(b1-b3)** = 250 μm .

Supplementary Figure 7. Conditional deletion of PTEN promotes greater number of RGC axons compared to constitutive overexpression of Rheb1.

(a) Low magnification image of CT β -traced optic nerve in conditional PTEN deletion group. **(a1-a3)** High magnification images from insets in panel **(a)**. Asterisk in **(a)** and **(a1)** denotes the lesion site. Vertical dashed line in **(a1)** demarcates 500 μ m past the lesion site. Arrows in **(a2)** and **(a3)** point to regenerating RGC axons. **(b)** Line graph showing the number of regenerated axons as a function of distance from the lesion site ($\pm$ SEM). Asterisks indicate significance in unpaired one-tailed Student's t -test; $*P < 0.05$. Scale bar in **(a)** = 500 μ m; and **(a1-a3)** = 250 μ m.

Supplementary Figure 8. cRheb1 overexpression and biased visual stimulation of the lesioned eye are critical for triggering long distance regeneration of RGC axons.

(**a-e**) Low magnification images of CT β -labeled optic nerve in animals that received injections of AAV2-cRheb1 plus daily visual stimulation ('Group 5') (**a**), that received injections of AAV2-cRheb1, had opposite eye sutured, and remained in their home cage ('Group 6') (**b**), that received AAV2-cRheb1 in the lesioned eye and received biased visual stimulation of the *non-lesioned* eye ('Group 7') (**c**), that had opposite eye sutured and received daily visual stimulation of the lesioned eye ('Group 8') (**d**) that received AAV2-cRheb1 and had opposite eye sutured and remained in their home cage ('Group 9') (**e**). Panels (**a1-a3**) High magnification images from insets in panel (**a**). Panels (**b1-b3**) High magnification images from insets in panel (**b**). Panels (**c1-c3**) High magnification images from insets in panel (**c**). Panels (**d1-d3**) High magnification images from insets in panel (**d**). Panels (**e1-e3**) High magnification images from insets in panel **e**. Arrows in (**d1**) point to regenerated axons in the proximal optic nerve and those in (**b2**) and (**c2**) point to regenerated axons in mid optic nerve. Arrows in (**b3**) point to regenerated axons in distal optic nerve. Asterisk in (**a**), (**a1**), (**b**), (**b1**), (**c**), (**c1**), (**d**), (**d1**), (**e**), and (**e1**) denotes the lesion site. Vertical dashed lines in (**a1-e1**) demarcate 500 μ m mark beyond the lesion site for axon quantification. Scale bar in (**a-e**) = 500 μ m and (**a1-a3**), (**b1-b3**), (**c1-c3**), (**d1-d3**), (**e1-e3**) = 250 μ m.

Supplementary Figure 9. cRheb1 overexpression and biased visual stimulation of the lesioned eye are critical for triggering long distance regeneration of RGC axons.

(a-b) Line graph of the number of regenerated axons as a function of distance from the lesion site ($\pm$ SEM). Asterisks indicate significance in unpaired one-tailed Student's *t*-test; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. [Note: the line graph of Group 3 in blue is under the line graph of Group 7 in sky blue (**a**).]

Supplementary Figure 10. Examples of optic nerve sections from animals treated with AAV2-cRheb1 and biased visual stimulation.

(a) Schematic of combined biased visual stimulation/AAV2-cRheb1 group ("Group 4"). **(b)**, Summary of the experiments. **(c, d, e)** Low magnification image of CT β -traced optic nerve in "Animal 3" (**c**), "Animal 4" (**d**), and "Animal 5" (**e**) of Group 4. **(c1-c3)**, **(d1-**

d3), (e1-e3) High magnification images from insets in panel **(c)**, **(d)**, and **(e)**. Asterisk in **(c)**, **(c1)**, **(d)**, **(d1)**, **(e)**, and **(e1)** denotes the lesion site. Vertical dashed line in **(c1)**, **(d1)**, and **(e1)** demarcates 500 μm past the lesion site. Arrows in **(c2)**, **(c3)**, **(d3)**, **(e2)**, and **(e3)** point to regenerating RGC axons. Scale bar in **(c)**, **(d)**, **(e)** = 500 μm ; and **(c1-c3)**, **(d1-d3)**, **(e1-e3)** = 250 μm .

Supplementary Figure 11. Examples of optic nerve sections from animals treated with AAV2-cRheb1 and biased visual stimulation.

(a, b, c) Low magnification image of CT β -traced optic nerve in “Animal 6” (a), “Animal 7” (b), “Animal 8” (c) of Group 4. (a1-a3), (b1-b3), (c1-c3) High magnification images from insets in panel (a), (b), and (c). Asterisk in (c), (c1), (d), (d1), (e), and (e1) denotes the lesion site. Vertical dashed line in (a1), (b1), and (c1) demarcates 500 μ m past the lesion site. Arrows in (a2), (a3), (b2), (c2), and (c3) point to regenerating RGC axons. Scale bar in (a), (b), (c) = 500 μ m; and (a1-a3), (b1-b3), (c1-c3) = 250 μ m.

Supplementary Figure 12. Examples of optic nerve sections from animals treated with AAV2-cRheb1 and biased visual stimulation.

(a, b) Low magnification image of CT β -traced optic nerve in “Animal 9” (a) and “Animal 10” of Group 4. **(a1-a3, b1-b3)** High magnification images from insets in panel

(a) and (b). (c) Line graph showing the number of regenerated axons as a function of distance from the lesion site in every animal in Group 4 (+/- SEM). “Animal 1” and “Animal 2” optic nerve images are shown in Main Figure 3e and Supplementary Figure 15, respectively. Asterisk in (a), (a1), (b), (b1), (c) and (c1) denotes the lesion site. Vertical dashed line in (a1-c1) demarcates 500 μm past the lesion site. Arrows in (a2), (b2), and (b3) point to regenerating RGC axons. Scale bar in (a, b) = 500 μm ; and (a1-a3), (b1-b3) = 250 μm .

Supplementary Figure 13. mTOR enhancement combined with biased visual stimulation increases the number of RGCs that survive 3 weeks after optic nerve crush.

(a) Images of flat mount retina immunostained with RBPMS in a non-lesioned animal (a), lesioned animal (b), and lesioned and treated animal (c). (d) Quantification of RBPMS⁺ RGCs (+/- SEM). *n* = 5 retinas per group. Scale bar in (c) = 50 μ m. Asterisks indicate significance in unpaired one-tailed Student's *t*-test; ****P* < 0.0005.

g Table of major visual targets and their function

Structure	Acronym	Function
Suprachiasmatic nucleus	SCN	Circadian entrainment
Ventral lateral geniculate nucleus	vLGN	Non-photic circadian functions
Intergeniculate leaflet	IGL	Non-photic circadian functions
Dorsal lateral geniculate nucleus	dLGN	Relaying visual information to cortex
Medial terminal nucleus	MTN	Vertical reflexive eye movements
Olivary pretectal nucleus	OPN	Pupillary light reflex
Nucleus of optic tract	NOT	Horizontal reflexive eye movements
Superior colliculus	SC	Visual feature integration

Supplementary Figure 14. RGC projections to subcortical visual targets in a mouse with both eyes intact and that had no optic nerve lesions.

(a) Schematic of a mouse brain in sagittal view with the major visual targets shown. Panels (b-f) Binocular injection of CTβ labels RGC axons in the:

- (b) Suprachiasmatic nucleus (SCN)
- (c) dorsal lateral geniculate nucleus (dLGN) and ventral lateral geniculate nucleus (vLGN).
- (d) medial terminal nucleus (MTN),
- (e) superior colliculus (SC), stratum griseum superficiale (SGS), stratum opticum (SO).
- (e1) The SC is shown at high magnification in the inset of panel (e).
- (f) and olivary pretectal nucleus (OPN).
- (g) Table of major visual targets and their various major functions.
- Scale bar in (b) = 250 μm ; (c) = 250 μm ; (d) = 100 μm ; (e) = 500 μm ; (e1) = 50 μm ; (f) = 100 μm .

Supplementary Figure 15. Combined biased visual stimulation/AAV2-cRheb1 treatments cause long-distance RGC regeneration down the optic nerve and into the chiasm.

(a) Low magnification image of CT β -labeled RGC axons in the optic nerve. This nerve and chiasm correspond to Animal 2 (brain tissue from this animal is shown in main **Fig. 3**).

(a1-a4) High magnification images from insets in (a). Arrows in (a1-a4) point to regenerated axons. Arrowheads in (a1) point to short-distance regenerated axons.

Asterisk in (a) and (a1) denotes the lesion site. Scale bar in (a) = 500 μ m; (a1-a3) = 250 μ m; and (a4) = 100 μ m.

Supplementary Figure 16. Combined pre-optic nerve crush and post-optic nerve crush CT β labeling confirms that the crush procedure does not spare RGC axons.

(a) Low magnification image of CT β labeling of RGC axons in the non-lesioned optic nerve. (a1-a3) High magnification images of insets in panel (a). (b) Experimental time line. (c) Low magnification image of CT β -traced optic nerve section showing pre-optic nerve crush CT β tracing in “magenta” and post-optic nerve crush CT β tracing in “green.” (c1-c3) High magnification images of insets in panel (c). Asterisk in (c) and (c1) indicates the lesion site. Scale bar in (a, c) = 500 μ m and (a3, c3) = 250 μ m.

Supplementary Figure 17. RGC axons regenerate down the optic nerve in a time-dependent manner after combined biased visual stimulation/AAV2-cRheb1 treatments.

(a) Experimental time line. (b) Low magnification images of CT β -labeled RGC axons in optic nerve of a mouse given combined biased visual stimulation/AAV2-cRheb1 treatments sacrificed one week post optic nerve lesion. (b1-b3) High magnification images of insets in panel (b). (c) Low magnification images of CT β -labeled RGC axons in optic nerve from a mouse given combined biased visual stimulation/AAV2-cRheb1 treatments sacrificed two weeks post optic nerve crush. (c1-c3) High magnification images of insets in (c). Arrows in (c2) and (c3) point to regenerating RGC axons. (d) Line graph of the number of regenerated RGC axons as a function of distance from the lesion site ($\pm$ SEM). Asterisks indicate significance in unpaired one-tailed Student's *t*-test; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$. Asterisk in (b), (b1), (c), and (c1) denotes the lesion site. Scale bar in (b) and (c) = 500 μ m; (b1-b3) and (c1-c3) = 250 μ m.

Absence of regenerated RGC axons in the brain one week after nerve crush

Supplementary Figure 18. Lack of labeled RGC axons in the optic nerve post-nerve crush is associated with lack of RGC axons in visual targets.

(a-d) Coronal section images of a one-week post-lesioned ‘Group 4’ animal in SC **(a)**, SCN **(b)**, dLGN and vLGN **(c)**, and MTN **(d)**. Scale bar in **(a)** = 500 μm ; **(b)** = 250 μm **(c)** = 250 μm ; and **(d)** = 100 μm .

Supplementary Figure 19. CoCH-GFP⁺ RGC subtypes project their axons to specific visual nuclei.

[Note: Images in this figure are from a normal, non-optic-nerve lesioned mouse]. **(a)** Schematic of a sagittal mouse brain with projections from CoCH-GFP⁺ RGCs in green.

(b-d) Whole mount retina section immunostained with alpha RGC marker, SMI-32 **(b)**, GFP **(c)** and the merged image **(d)**. **e-i**, Low magnification images of CoCH GFP⁺ axons in SCN **(e)**, MTN **(f)**, LGN **(g)**, OPN **(h)**, and SC **(i)**. Scale bar in **(d)** = 20 μm ; **(e)** = 250 μm ; **(f)** = 100 μm ; **(g)** = 250 μm ; **(h)** = 100 μm ; and **(i)** = 500 μm .

Supplementary Figure 20. CoCH GFP⁺ RGC axons regenerate down the full length of the optic nerve after combined biased visual stimulation/AAV2-cRheb1 treatments.

[These nerves correspond to the animals whose RGC central projections are shown in main **Fig. 4**]. **(a)** Schematic of the experimental group. **(b)** Summary of the experimental paradigm. **(c-e)** Low magnification image of CT β -labeled **(c)**, and GFP⁺ RGC axons **(d)**, and their merge **(d)** of optic nerve sections from CoCH GFP⁺ (same mouse from which brain images in Fig. 4 are shown). **(c1-e3)** High magnification images of insets in **(c-e)**. **(e1'-e3')** High magnification images of insets in **(e1-e3)**. Asterisk in **(c)**, **(c1)**, **(d)**, **(d1)**, **(e)**, and **(e1)** denotes the lesion site. Scale bar in **(c-d)** = 500 μ m and **(c3-e3)** = 250 μ m.

Supplementary Figure 21. Normal, unlesioned OPN4-GFP⁺ RGC subtypes project their axons to specific visual nuclei.

[Projections shown are from a normal, non-lesioned mouse.] **(a)** Schematic of a sagittal mouse brain with projections from OPN4-GFP⁺ RGCs in green. **(b-d)** Whole mount

retina section immunostained for Melanopsin (**b**), OPN4-GFP (**c**) and their merge (**d**). (**e-i**) Low magnification images of normal OPN4-GFP⁺ axon projections in the SCN (**e**), MTN (**f**), dLGN, vLGN, and IGL (**g**), OPN (**h**), and SC (**i**). Scale bar in (**d**) = 20 μm ; (**e**) = 250 μm ; (**f**) = 200 μm ; (**g**) = 250 μm ; (**h**) = 200 μm ; and (**i**) = 250 μm .

Supplementary Figure 22. Biased visual stimulation and enhancement of mTOR signaling regenerate OPN4-GFP⁺ RGC axons to their correct visual targets.

(a) Schematic of experimental group. (b) Summary of the experimental paradigm.

(c) Schematic of sagittal mouse brain with projections from OPN4-GFP⁺ RGCs in green.

(d-u) Images of CTβ-labeled, GFP-immunostained coronal sections of brain from OPN4-GFP⁺ animal showing vLGN (d-f) with inset (f1), dLGN (g-i) with inset (i1), IGL (j-l) with inset (l1), OPN (m-o) with inset (o1), MTN (p-r) with inset (r1), SC (s-u) with inset (u1). Scale bar in (d-f) = 100 μm; (f1) = 25 μm; (g-i) = 250 μm; (i1) = 50 μm; (j-l) = 100 μm; (l1) = 50 μm; (m-o) = 250 μm; (o1) = 50 μm; (p-r) = 100 μm; (r1) = 25 μm; (s-u) = 250 μm; and (u1) = 50 μm.

Supplementary Figure 23. Schematic of the circuitry modulating pupillary reflex behavior.

(a) Schematic of the circuitry modulating pupillary reflex behavior.

Supplementary Table 1. Statistical values for Figure 2k

Group comparison	Value	0.5 mm	1.0 mm	1.5 mm	2.0 mm	2.5 mm
hM4Di / Control	p	0.0846	0.3355	0.278	0.2118	0.1666
	t	1.432	0.4483	0.6144	0.86	1
	dF	18.26	5.535	7.982	5.87	15
hM4Di / Vis stim	p	0.0198	0.082	0.1621	0.1622	0.187
	t	2.974	1.636	1.119	1.123	1
	dF	4.118	4.904	4.106	4	4
hM3Dq / Control	p	0.0117	0.031	0.0315	0.0252	0.0937
	t	3.198	2.393	2.381	2.561	1.527
	dF	5.084	5.008	5.004	5.015	5.007
hM3Dq / Vis stim	p	0.0277	0.0493	0.0471	0.0766	0.1253
	t	2.39	1.996	2.009	1.572	1.283
	dF	5.817	5.363	5.639	8.315	5.548
hM3Dq / hM4Di	p	0.0101	0.0323	0.0324	0.0236	0.0905
	t	3.35	2.358	2.359	2.619	1.553
	dF	5.012	5.042	5.009	5	5

Supplementary Table 2. Statistical values for Figure 3g

Group comparison	Value	0.5 mm	1.0 mm	1.5 mm	2.0 mm	2.5 mm	3.0 mm	3.5 mm	4.0 mm	4.5 mm	5.0 mm
Group 1 / Group 3	p	0.0033	0.0052	0.0204	0.1993	0.0893	0.0887	0.1012	0.1685		
	t	3.209	3.015	2.291	1.357	1.428	1.433	1.348	1		
	dF	13.29	12.33	12.1	12.19	12.05	12	12	12		
Group 3 / Group 4		3.79608									
	p	0.0006	E-05	0.0003	0.0009	0.0015	0.0019	0.002	0.0034	0.0043	0.0447
	t	4.188	6.453	5.176	4.336	4.01	3.867	3.831	3.484	3.346	1.903
	dF	12.72	9.921	9.164	9.041	9.023	9.016	9.007	9.004	9	9
Group 1 / Group 4		8.49036 2.27165									
	p	E-05	E-05	0.0002	0.0009	0.0014	0.0018	0.0019	0.0034	0.0043	0.0447
	t	6.07	7.298	5.419	4.406	4.064	3.911	3.858	3.499	3.346	1.903
	dF	9.2	9.012	9.001	9	9	9	9	9	9	9

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CONCLUSIONS

The work presented here describes a novel strategy to enhance axon regeneration of mature CNS neurons and restore circuitry after severe nerve lesions. Chapter 1 highlighted the importance of visual stimulation-driven neural activity and cell growth-promoting pathway - mTOR - in triggering long-distance regeneration of RGC axons after unilateral optic nerve crush. Chapter 1 further revealed that the regenerating RGC axons mostly stayed on course to re-innervate their correct visual targets in the brain, which induced partial restoration of subset of visual behaviors. These findings can contribute to better understanding of the cellular mechanisms of regenerating CNS neurons after acute injury and can potentially be applied to a clinical setting to treat human patients suffering from traumatic brain injury or neurodegenerative disease.

Neural activity regulates axon regrowth after injury

It is possible that chronic application of visual stimulation could enhance axon regeneration by mechanisms other than elevation of neural activity, but the downstream mechanisms for that process need to be further investigated. During development, neural activity regulates axon branching by modifying actin cytoskeleton dynamics (Ohnami et al., 2008). In our study, chronically attenuating RGC activity abolished visual stimulation-mediated axon regeneration while chronically increasing RGC activity alone promoted axon regeneration after optic nerve crush, supporting the role of neural activity in regulating axon growth after injury. It is also worth noting that the amount of RGC axon regeneration seen in mice that received chronic treatment of hM3Dq/CNO was greater than the amount seen in animals that received chronic treatment of the high

contrast visual stimulation, suggesting that greater number of RGCs elevated their activity level in the animals that received hM3Dq/CNO treatment compared to the animals that received visual stimulation treatment. This difference likely stems from the specific type of visual stimulation that our study used - high contrast drifting gratings in fixed orientations - to which all RGCs respond but only certain group of direction-selective RGCs respond most strongly to this stimulus (Dhande et al., 2015). Future studies must address how different RGC types respond to different types of visual stimulation that influences the amount of axon regeneration. This would further elucidate the molecular mechanism of the role of visual stimulation-driven neural activity in axon regeneration.

The distance of axon regeneration was greatest in mice that received combined treatments that included elevation of RGC activity and unilateral lid suture (or eye removal) to eliminate vision through the non-lesioned eye pathway. This was surprising because complete optic nerve lesions would eliminate any potential binocular interactions among RGC axons within the central visual targets. Thus, the enhanced axon regeneration of unilateral visual bias may arise from several other sources. One possibility is that the bias effect observed here is purely behavioral - suturing shut the non-lesioned eye encouraged animals to keep the opposite, lesioned eye open, which would promote additional spiking activity in the RGCs from the lesioned eye. The shift in activity in response to neural defects or injury is well studied in the animal models of the stroke and Parkinson's disease, in which the animals rely more on less compromised motor systems while relying less on the damaged motor systems (Schallert et al., 2000; Whishaw, 2000). Different groups have observed enhanced growth of neuronal processes

during motor training including force use of the impaired limb from the damaged motor systems (Jones and Schallert, 1994; Kleim et al., 1996; Schallert et al., 1997; Kempermann et al., 1998; Coq and Xerri, 1999; Maier et al., 2008; Zhao et al., 2013). In our study, the reduced amount of axon regeneration observed in animals where AAV2-cRheb1 and visual stimulation were provided through both eyes, compared to the long-distance axon regeneration observed in the animals that received combined treatments of AAV2-cRheb1 and biased visual stimulation through the lesioned eye, further support the critical role of biased visual activity in RGC axon regeneration.

Enhanced distance of RGC axon regeneration in multiple treatments

I discovered that the CNS neurons regenerate their axons long distances, if they are treated with the appropriate growth-stimulating conditions. I found that enhanced RGC activity coupled with elevated mTOR signaling promoted axons to anatomically restore damaged visual circuitry. This suggests that activating intrinsic factors such as mTOR signaling and neural activity alone may be sufficient to overcome extrinsic barriers such as myelin-associated inhibitors and glial scars.

Interestingly, PTEN deletion alone could trigger an even greater number of RGC axons to regenerate at proximal distances than the combined treatment paradigm that I implemented in our study. One possibility for the greater number of regenerating axons is that PTEN deletion occurs farther upstream of the mTOR activation and thus likely disinhibit other cell growth signaling pathways. However, while PTEN deletion causes RGC axons to grow as far as the optic chiasm, it does not allow them to grow into the

brain (Park et al., 2008) unless it is combined with one or more other treatments (de Lima et al., 2012).

Together, these data illustrate the complex molecular mechanisms CNS neurons implement during development and further illustrate the fact that multiple treatments applied in combination are required to trigger long-distance axon regeneration of CNS neurons, and they encourage additional exploration of the molecular pathways activated downstream of PTEN and neural activity.

Specificity of mature RGC axon regeneration

Axon regeneration of RGC subtypes

From studying the pattern of axon regeneration of two genetically labeled RGC types, the α -RGCs and ipRGCs, I found that RGCs have the remarkable capacity to reconnect to their correct targets in the brain while simultaneously avoiding incorrect targets. The CoCH-GFP⁺ RGCs, subset of which label classic α -RGCs, are among the group of RGCs that regenerated, consistent with the previous work that identified α -RGCs as the main group of RGCs undergoing axon regeneration after sciatic nerve transplants in cats (Watanabe et al., 1993). These results are also in agreement with a recent study showing that Kcng4-YFP⁺ α -RGCs account for the majority of regenerating axons following either PTEN deletion or combined treatments of osteopontin (OPN) overexpression with insulin growth factor (IGF) administration (Duan et al., 2015). Nearly all α -RGCs expressed elevated basal mTOR and OPN activity levels - conditions that were necessary for axon regeneration of this subset of RGCs. It remains to be further investigated why PTEN deletion that activates mTOR signaling in other RGC subtypes is

insufficient to trigger other RGC subtypes to regenerate. Our study illustrated that other RGC subtypes that are not α -RGCs (i.e. ipRGCs) could regenerate in response to our treatment paradigm. In the future, it will be important to identify other RGC subtypes that can regenerate.

Target-specific re-innervation of CNS neurons

It is remarkable that adult mammalian CNS neurons are capable of re-innervating the correct brain targets, yet it is not entirely surprising since other groups have reported target-specific re-innervation in multiple systems including in the visual system (Vidal-Sanz et al., 1987; Wizenmann and Bahr, 1998; Suave et al., 2001; de Lima et al., 2012) and spinal cord (Brushart et al., 2005; Harvey et al., 2010; Smith et al., 2012). In addition, neutralizing the inhibitory effects of oligodendrocytes and myelin triggered striatal and cortical projection neurons to regenerate their axons long distances and to re-innervate several of their correct targets in the substantia nigra, pontine nuclei and cervical spinal cord in adult rats (Victorin et al., 1990). Ligands and receptors that are expressed in the presynaptic neurons and postsynaptic targets respectively ensure axons arrive at and innervate their proper targets during development in mammals (Sun et al., 2015; Osterhout et al., 2015). The studies demonstrating target specificity of regenerating axons suggest that those cues may still be present, or even upregulated, in response to either nerve injury or regeneration-stimulating treatment. Indeed, Beazley and coworkers observed enhanced expression of ephrin-A2 in the contralateral side of the superior colliculus in an increasing rostro-caudal gradient one month after unilateral optic nerve transection, suggesting that the injury alone can change the expression of guidance

molecules (Roger et al., 2001). Similarly, such upregulation of guidance molecules in response to injury has been reported in the tectum of cold-blooded vertebrates (Roger et al., 2000; King et al., 2003). It will be important to address this hypothesis by exploring the molecular pathways that are activated in the RGC types that regenerate in our combined treatment protocol.

Functional and clinical implications

Restoration of subset of visual behaviors

The ultimate goal of axon regeneration is to restore function in a damaged nervous system for restoration of appropriate behaviors. The combined treatment paradigm reported here partially restored visual function in a subset of visual assays. The behaviors in which I observed partial recovery were optokinetic reflex (driven by the accessory optic system) (Sun et al., 2015; Osterhout et al., 2015) and the looming avoidance response (driven by the retino-SC connections) (Zhao et al., 2014), while the assays where I failed to observe any significant recovery were the pupil response (driven by the retino-OPN connection) (Guler et al., 2008) and visual cliff test (driven by dLGN-primary visual cortex (V1) connection) (Leamy et al., 2007). The first three behaviors require retino-subcortical pathways but not the cortex (Dhande et al., 2015) whereas the visual-cliff depth perception task depends on binocular vision and thus involves the dLGN and V1 (Leamy et al., 2007). Since I observed regeneration of RGC axons to the dLGN - the nucleus that relays visual information to the V1, the lack of recovery in the visual cliff task was surprising. It is possible that the threshold for functional behavioral output of the retino-geniculo-cortical pathway may be higher than that of the other

retinofugal parallel pathways at the level of synapse formation and/or precision of within-target wiring. For partially restored optokinetic reflex and looming avoidance response, retinotopic and spatial precision of connections may not be required for pathways that drive OKR or looming since they involve large-field illumination (Cahill and Nathans, 2008; Yimaz and Meister, 2013). On the contrary, visual cliff tasks require analysis of spatial frequency and thus higher resolution image-formation (Hovda et al., 1989). I observed regeneration of RGC axons to the OPN, the nucleus that regulates the PLR, but failed to observe any substantial recovery of pupil constriction. Notably, I intentionally used low light intensities to stimulate the PLR so as not to allow spillover of light to the opposite, non-lesioned eye. Although the use of low light 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.

My behavioral data support a model in which restoration of neural pathways for visual function and perception may require a greater number of RGC axons to regenerate. He and coworkers probed the functional recovery of retino-collicular connections after distal nerve transection and found that target re-innervation occurred without restoration of functional behavior. The group could restore visual acuity by adding conduction in regenerated RGC axons via administration of voltage-gated potassium channel blockers, 4-AP (Bei et al., 2016). In other models of ophthalmic disease such as retinal dystrophy and retinitis pigmentosa, electrical stimulation has been shown to prolong both RGC survival and function (Fu et al., 2015). Thus, additional sources of enhancing neural activity, in particular within the RGC populations that target the dLGN and SC, may be

critical for enhancing regeneration of mature central visual pathways and visual perception.

In conclusion, during my thesis research, I discovered novel strategy to promote long-distance axon regeneration, specificity of re-connections, and partial restoration of visual function, which may be informative for developing treatment plans for patients suffering from the damaged visual system, spinal cord or other CNS regions as a result of physical trauma or neurodegenerative disease.

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