Editorial

Comment on retinal ganglion cell electrical activity, mammalian target of rapamycin signaling and optic nerve regeneration

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Lim and coworkers have recently described impressive results on optic nerve regeneration in mice. In their paper published on *Nature Neuroscience* (1), the authors found that increasing electrical activity of adult retinal ganglion cells (RGCs) could stimulate their axons to grow through the injured optic nerve. Furthermore, when in addition to visual stimulation, the contralateral eye was removed, the magnitude of the results was such that regenerated axons reconnected to specific targets in the brain and the visual function was partially restored. It is remarkable that the authors have performed several experimental groups and controls to confirm their observations, in a study carefully designed to bring a complete proof of concept.

Like other central neurons, RGCs are unable to efficiently grow injured axons, which ultimately leads to neuronal death and irreversible visual loss (2,3). Harboring a simply organized neuronal stack in the retina and RGCs as long-distance projecting neurons, the eye and the optic nerve have anatomical advantages that favor their use to model visual and non-visual neurodegenerative disorders. Notably, seminal observations on the optic nerve regeneration were made by Ramon y Cajal and later confirmed by Aguayo, demonstrating that the absence of regeneration in the central nervous system (CNS) is not necessarily due to an intrinsic inability of neurons, but rather to the presence of an inhibitory environment (4). These findings led to a general effort to overcome CNS inhibitory environment, and more recently to the use of multiple approaches to bring RGC back to a growth state.

The intrinsic ability of neurons to regenerate can be markedly stimulated upon increasing ocular inflammation through lens injury or intraocular injection of zymosan (5,6). Moreover, in the last decade, new possibilities brought by transgenic technologies led to a deeper study of the mechanisms of neuronal survival and regeneration (7-11). Meanwhile, cell therapies performed to exert neuroprotection and support regeneration of RGCs have also emerged (12-14), taking advantage of a possibly shorter road to the clinic (15) but so far showing less robust results than gene manipulation approaches. Indeed, cell growth-related molecules such as the mammalian target of rapamycin (mTOR), Kruppel-like family (KLF) transcription factors and the suppressor of cytokine signaling 3 (SOCS3) are some of the key players that controls regeneration in the visual system (16).

Nevertheless, gene targeting alone has not yet produced stable RGC axon regeneration and functional recovery, suggesting the need of synergistic therapies. Goldberg and coworkers showed that RGC axonal growth in response to trophic factors is dramatically increased by electrical activity (17). Based on these evidences, Lim and coworkers used visual stimulation to increase RGC electrical activity and induce axon regeneration. Visual stimulation was performed using an Optometry apparatus, which was designed to measure the optokinetic nystagmus in rodents and, for that purpose is equipped with monitors that display

high-contrast moving bars surrounding the animal to create a virtual cylinder. The authors performed an elegant and careful set of chemogenetic experiments to assure that visual stimulation effects were dependent on the electrical activity of RGCs. Briefly, adeno-associated virus AAV2 were used to overexpress designer receptors exclusively activated by designer drugs (DREADD) to induce either the suppression or the increase of RGC spiking. By reducing RGC activity, visual stimulation effects on RGC axon regeneration were abolished and the number of axons anterior to the crush site was reduced, indicating an impact both in regeneration and survival of RGCs. In contrast, elevating RGC spikes increased axon regeneration even without visual stimulation, confirming the initial hypothesis on the regenerative effect of electrical activity.

As multi-targeted therapies have been shown to be the most likely way to achieve long-distance regeneration and functional recovery (10), the authors have performed an all-embracing comparative study considering genetic tools combined to additional approaches. Based on previous studies that showed that mTOR activation boosts RGC axon regeneration (8), they used AAV2 to overexpress ras homolog enriched in brain 1 (Rheb1) protein, which is a positive regulator of that pathway. Although axonal numbers were lower when compared to mTOR activation by conditional deletion of *Pten*, in both cases there was increased regeneration through the optic nerve. By combining mTOR signaling to visual stimulation, the authors had observed axonal regeneration in the proximal nerve, but still a failure to extend the completeness of the nerve and reach RGC targets.

Based on a study that showed that forced use of an impaired limb after corticospinal tract injury induced behavioral recovery in an activity-dependent manner (18), the authors have tested whether suturing the fellow eye could have a regenerative outcome to the crushed-nerve eye. This approach could simply stimulate the animal to keep the nerve-lesion eye opened to receive more visual stimuli or even influence binocular interactions that could accelerate regeneration in the injured eye. Indeed, axon outgrowth was increased, similar to what was observed with Rheb1 overexpression. However, when Rheb1 overexpression, visual stimulation and suturing shut the fellow eye were all combined, 7 of 10 mice regenerated axons through the whole optic nerve, entering the optic chiasm. In addition, RGC survival was increased from approximately 10% to 40% from untreated to treated mice. Importantly, robust axonal regeneration was only achieved with all of these approaches together (biased visual stimulation/mTOR activation).

The authors also describe multiple target reinnervation in mice receiving biased visual stimulation/mTOR activation. Anterogradely labeled axons reached the optic chiasm, navigated trough the optic tract and followed specifically the retinofugal pathway to several nuclei: suprachiasmatic, ventral and dorsal lateral geniculate, pretectal, accessory optic targets in the brainstem and, importantly, the furthest and predominant nuclei for rodent RGCs: the superior colliculus.

Long distance axon regeneration with partial restoration of the visual function has been demonstrated previously in rodents. Opening the precedent to a pioneer line of investigation, Fischer and coworkers observed regeneration of RGCs to the rat superior colliculus by injuring the lens after optic nerve axotomy, by 1 month after surgery (19). The regenerative effects of lens injury were linked to the recruitment of macrophages and most importantly neutrophils that release oncomodulin and requires cAMP elevation (5,20), while other studies attribute it to the activation of STAT3 in RGCs and to glial-derived CNTF and LIF (21). Cell therapy with bone marrow-derived cells has also achieved long-distance regeneration of RGCs and the formation of functional synapses in the superior colliculus, with trophic effects attributed to a paracrine activity (22).

Park and coworkers first described gene deletion experiments that achieved robust axon regeneration (8), although the effects of single gene deletion were observed only during the first 2 weeks after injury. Co-deletion of *Pten* and *Socs3* promoted sustained axon regeneration seen up to 4 weeks, with approximately 20% of RGC axons reaching the optic chiasm. They also observed a minority of axons that crossed the chiasm and projected to the suprachiasmatic nucleus, but the authors did not report innervation to the thalamus and superior colliculus (7). A recent study from the same group showed that overexpression of *C-myc* enhanced regenerative effects of co-deletion of *Socs3* and *Pten*, with several axons crossing the optic chiasm 4 weeks after crush, although a part of them grew ectopically on the contralateral optic nerve. However, after 8 weeks, the number of regenerated axons was reduced, possibly due to the elimination of axons that have not formed synaptic connections (23).

Prior to the study described by Lim and coworkers, the combination of gene deletion experiments with inflammatory stimulation had accounted for the most

vigorous effects on regeneration observed after optic nerve crush and anterograde labeling of RGC axons. Kurimoto and coworkers showed in 2010 that combining zymosan injection, elevation of cAMP and *Pten* gene deletion resulted in approximately 10-fold more regeneration than *Pten* deletion or zymosan injection alone, and approximately 1% of the regenerating axons entered the thalamus, as observed 6 weeks after injury (11). De Lima and coworkers repeated Kurimoto's experiments in 2012, allowing a longer survival (10–12 weeks) of the animals following crush (10). Eleven out of 13 animals had regenerating fibers in the contralateral dorsal lateral geniculate nucleus, in association to pre- and post-synaptic markers. The authors describe that animals showed variable degrees of innervation of the contralateral superior colliculus, and little innervation of the ipsilateral superior colliculus. Treated animals had improved depth perception, circadian activity and 28% of them showed optokinetic reflex in a frequency of approximately 10% of normal mice threshold (maximum frequency responded).

Limited functional recovery following gene deletion experiments could be due to the differential response of RGC subtypes. Indeed, the larger RGC types, alpha-RGCs, accounted for nearly all regeneration following deletion of *Pten* (24). These findings suggest that the modulation of multiple pathways is required to induce a higher number of RGCs to regenerate. In order to access whether re-growing axons can connect to the correct targets, Lim and coworkers used transgenic mouse lines harboring green fluorescent protein (GFP) in specific RGC types. The authors were able to discriminate the innervation from RGC types such as alpha-RGCs and intrinsically photosensitive RGCs, and found that each RGC subset had sent axons to several of their specific targets, avoiding the incorrect ones. The information provided by these experiments revealed a remarkable ability of adult mammals RGCs to re-enter the growth program following the given treatments and find their targets as during the development.

Finally, the authors have tested whether the regeneration of RGC axons could lead to the recovery of visual functions. For that purpose, they used four visual behavioral tests to probe the connection to different RGC targets. When the optokinetic reflex was tested to assess connections to the brainstem, they observed that untreated animals lost the ability to track the visual stimulus, while biased visual stimulation/mTOR activated-treated animals recovered about 70% of the ability observed in non-lesioned controls. Another behavior that was recovered by the treatment was the perception of an over-head looming stimulus, which probes the retinocollicular pathway. On the other hand, pupillary light reflex and depth perception were not recovered, even though re-innervation had been observed in the appropriate targets. Two possible explanations to this failure are the incorrect formation of synapses and insufficient number of regenerating axons to restore the given function. Another issue may be the lack of myelination of regenerated axons. Indeed, the administration of voltage-gated potassium channel blocker 4-aminopyridine improved the conduction of RGC axons and the performance of animals on the optokinetic reflex test after optic tract transection (25).

In summary, from the studies mentioned above, it is clear that combinatorial approaches are needed to promote extensive and functional axonal regeneration. Of importance, de Lima and coworkers used gene deletion to activate mTOR pathway, delivery of cAMP analogue and inflammatory stimulation, eliciting intracellular pathways, enhancing neurotrophins effects and inducing the release of trophic factors, cytokines and other molecules such as oncomodulin by inflammatory cells and/or glial cells. On the other hand, Lim and coworkers used a simpler method to activate mTOR pathway, without the need of transgenic mice, and innovated the field of optic nerve regeneration with the biased visual stimulation approach. In addition, they have shown in a detailed way that adult RGCs conserve or regain the developmental ability to find their correct targets upon regenerative stimulus. Interestingly, the variance in functional recovery obtained in these two studies strengthens the idea that a given treatment may stimulate specific subtypes of RGCs, as previously demonstrated by Duan and coworkers (24).

Challenges to the field remain the development of additional therapies to target even more RGC subtypes and stimulate axonal regeneration in a correct and stable way. The analysis of pre- and post-synaptic markers in the targets is one of the well-accepted ways to assess the formation of new synapses (10). Another possibility is to investigate the activation of post-synaptic neurons. For instance, the immediate early gene *NGFI-A* has its expression driven by light-dependent release of glutamate from RGCs that arrive to the superior colliculus. Thus, using a dark-light cycle, it is possible to assess the expression of this gene as a function of the retinocollicular projections that make functional synapses in the brain (22,26). In the study from Lim and coworkers, the formation of new, functional synapses is clear though, since the animals recovered some visual

behaviors, although it is not clear whether the non-recovery of other behaviors is associated to malformation of synapses or to other factors. Re-gain of function is a main challenge that was partially overcome by Lim and coworkers and for that reason the achievements made in this study are of wide significance not only to visual diseases but also to the whole field of CNS neurodegenerative disorders.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Lim JH, Stafford BK, Nguyen PL, et al. Neural activity promotes long-distance, target-specific regeneration of adult retinal axons. Nat Neurosci 2016;19:1073-84.
2. Berkelaar M, Clarke DB, Wang YC, et al. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J Neurosci 1994;14:4368-74.
3. Ghaffarieh A, Levin LA. Optic nerve disease and axon pathophysiology. Int Rev Neurobiol 2012;105:1-17.
4. Villegas-Pérez MP, Vidal-Sanz M, Bray GM, et al. Influences of peripheral nerve grafts on the survival and regrowth of axotomized retinal ganglion cells in adult rats. J Neurosci 1988;8:265-80.
5. Kurimoto T, Yin Y, Habboub G, et al. Neutrophils express oncomodulin and promote optic nerve regeneration. J Neurosci 2013;33:14816-24.
6. Leon S, Yin Y, Nguyen J, et al. Lens injury stimulates axon regeneration in the mature rat optic nerve. J Neurosci 2000;20:4615-26.
7. Sun F, Park KK, Belin S, et al. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature 2011;480:372-5.
8. Park KK, Liu K, Hu Y, et al. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 2008;322:963-6.
9. Li S, He Q, Wang H, et al. Injured adult retinal axons with Pten and Socs3 co-deletion reform active synapses with suprachiasmatic neurons. Neurobiol Dis 2015;73:366-76.
10. de Lima S, Koriyama Y, Kurimoto T, et al. Full-length axon regeneration in the adult mouse optic nerve and partial recovery of simple visual behaviors. Proc Natl Acad Sci U S A 2012;109:9149-54.
11. Kurimoto T, Yin Y, Omura K, et al. Long-distance axon regeneration in the mature optic nerve: contributions of oncomodulin, cAMP, and pten gene deletion. J Neurosci 2010;30:15654-63.
12. Mesentier-Louro LA, Zaverucha-do-Valle C, da Silva-Junior AJ, et al. Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. PLoS One 2014;9:e110722.
13. Mead B, Berry M, Logan A, et al. Stem cell treatment of degenerative eye disease. Stem Cell Res 2015;14:243-57.
14. Johnson TV, Martin KR. Cell transplantation approaches to retinal ganglion cell neuroprotection in glaucoma. Curr Opin Pharmacol 2013;13:78-82.
15. Mesentier-Louro LA, Zaverucha-do-Valle C, Rosado-de-Castro PH, et al. Bone Marrow-Derived Cells as a Therapeutic Approach to Optic Nerve Diseases. Stem Cells Int 2016;2016:5078619.
16. Barber A, Farmer K, Martin KR, et al. Retinal regeneration mechanisms linked to multiple cancer molecules: A therapeutic conundrum. Prog Retin Eye Res 2016. [Epub ahead of print].
17. Goldberg JL, Espinosa JS, Xu Y, et al. Retinal ganglion cells do not extend axons by default: promotion by neurotrophic signaling and electrical activity. Neuron 2002;33:689-702.
18. Maier IC, Baumann K, Thallmair M, et al. Constraint-induced movement therapy in the adult rat after unilateral corticospinal tract injury. J Neurosci 2008;28:9386-403.
19. Fischer D, Heiduschka P, Thanos S. Lens-injury-stimulated axonal regeneration throughout the optic pathway of adult rats. Exp Neurol 2001;172:257-72.
20. Yin Y, Henzl MT, Lorber B, et al. Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. Nat Neurosci 2006;9:843-52.
21. Leibinger M, Andreadaki A, Diekmann H, et al. Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. Cell Death Dis 2013;4:e805.

22. Zaverucha-do-Valle C, Gubert F, Bargas-Rega M, et al. Bone marrow mononuclear cells increase retinal ganglion cell survival and axon regeneration in the adult rat. Cell Transplant 2011;20:391-406.
23. Belin S, Nawabi H, Wang C, et al. Injury-induced decline of intrinsic regenerative ability revealed by quantitative proteomics. Neuron 2015;86:1000-14.
24. Duan X, Qiao M, Bei F, et al. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. Neuron 2015;85:1244-56.
25. Bei F, Lee HH, Liu X, et al. Restoration of Visual Function by Enhancing Conduction in Regenerated Axons. Cell 2016;164:219-32.
26. Giraldi-Guimarães A, Mendez-Otero R. Induction of the candidate-plasticity NGFI-A protein in the adult rat superior colliculus after visual stimulation. Brain Res Mol Brain Res 2005;133:242-52.

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