Editorial

Combined treatment promotes the long-range axon regeneration to right brain targets

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Axons in the peripheral nervous system (PNS) can regenerate after injury. However, the adult mammalian central nervous system (CNS) loses the intrinsic regrowth ability. No robust axon regeneration occurs spontaneously after nerve injury, which was clearly observed by Ramon y Cajal in the early 20th century (1,2). Due to lack of regenerative potentials, the injured axons permanently lose their connections from their targets, e.g. in the optic nerve damage and spinal cord injury. Later, pioneer studies found peripheral nerve segments can bridge the injured axons in the rodent retina (3), medulla and spinal cord (4). The transected axons are able to regrow along the peripheral nerve "bridge". In 2005, Dong Feng Chen Lab at Harvard Medical School found the elevation of calcium signaling by Bcl-2 overexpression triggered the axon regeneration of the retina (5). In addition, Zhigang He's lab at Harvard Medical School demonstrated modulating the PTEN/mTOR signaling pathway promoted axon regeneration in the adult CNS (6). Therefore, manipulating the cellular signaling pathways overcomes intrinsic barriers that limit the regeneration capacity of the CNS. Besides, researchers found transcorneal electrical stimulation could enhance axon regeneration (7) and accelerate the speed of axon growth (8). It implies enhancing neuronal activity of RGCs would promote regrowth of axons *in vivo*. By these treatments, the regenerated axons went through the injury site for several millimeters, which shed lights on rebuilding the injured neural connection. However, there are at least three obstacles in the way to clinical interventions. (I) The regenerated axons can only regenerate for millimeters, far from reaching the brain; (II) how to guide confine regenerated axons to find the right targets; (III) how do regenerated axons re-establish functional connections with downstream neurons. Therefore, there is still a giant gap to the effective therapeutic interventions. In a recent milestone study, researchers from Stanford combined mTOR signaling modulation and visual stimulation to achieve the synergic effects on axon regeneration. The combined treatment promoted injured axons to regenerate along the retinofugal pathway, reinnervate the right targets and partially restore the visual function (*Figure 1*) (9).

Lim *et al*. first examined the axon regeneration after optic nerve crush (ONC), a well-established model of the CNS axon regeneration. The retinal ganglion cell (RGC) axons undergo retrograde degeneration from the lesion site, and then RGCs eventually die (10). Three weeks after ONC, fluorophore conjugated cholera toxin subunit-β (CTB) was intravitreally introduced to label the RGC axons. In the absence of any treatment, few CTB labeled axons could past the lesion site (*Figure 1A*), in line with the limited intrinsic

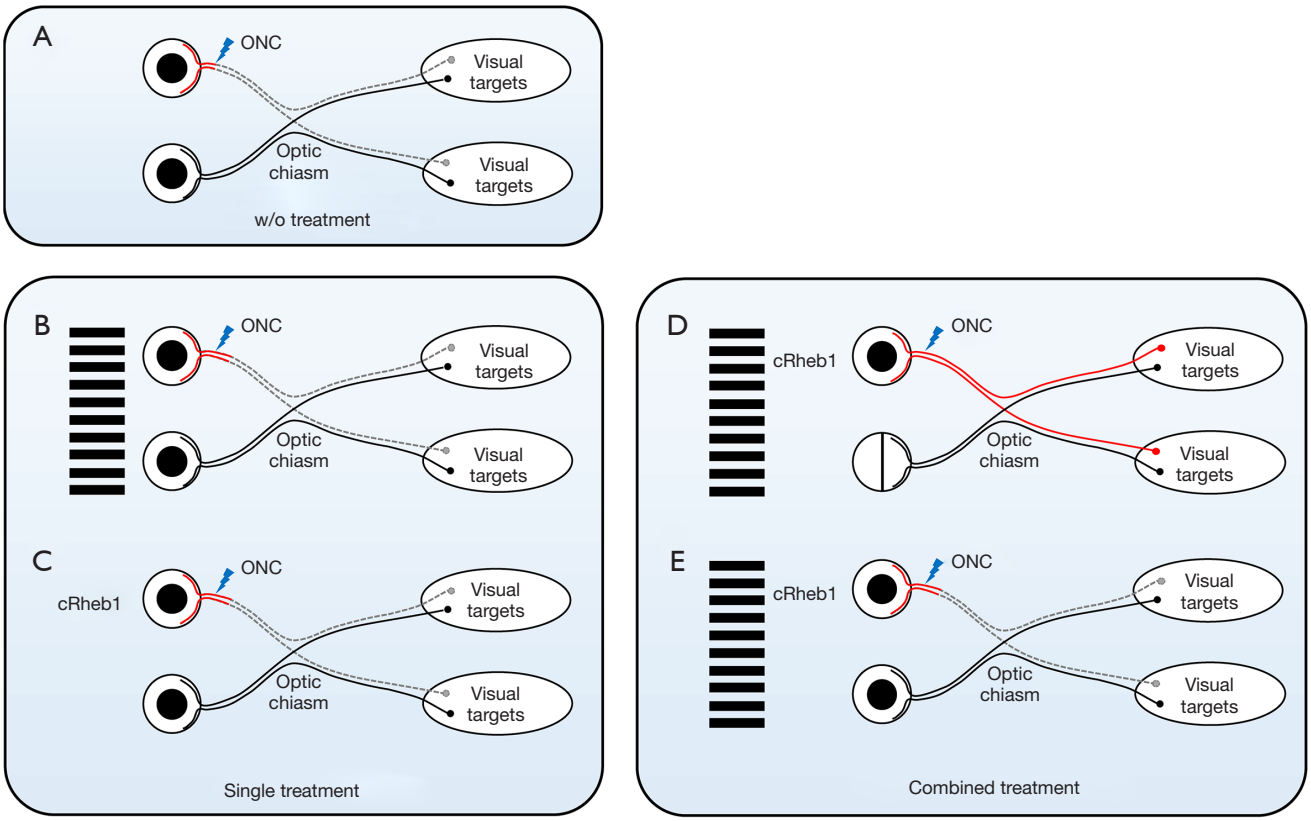

Figure 1 Combined treatment with biased visual stimulation and mTOR signaling elevation (cRheb1) promotes the injured RGC axons to reconnect with their visual targets in the brain. (A) RGC axons do not regenerate spontaneously after ONC; (B) high contrast visual stimulation drives axon regeneration after ONC. But regenerated axons do not reach the middle optic nerve and optic chiasm; (C) the elevation of mTOR signaling by cRheb1 drives axon regeneration after ONC. But regenerated axons do not reach the middle optic nerve and optic chiasm; (D) the combined treatment with the biased visual stimulation and enhancement of mTOR signaling drives long-range axons regeneration and reconnect with their visual targets; (E) the combined treatment with the unbiased visual stimulation and enhancement of mTOR signaling promotes axon regrowth. However, the length of regenerated axons is limited. ONC, optic nerve crush.

regeneration capacity of the adult CNS (11). Then the authors exposed the axon injured mice to a high-contrast visual stimulation. Three weeks after the exposure, there were significantly more CTB labeled axons past the lesion site (*Figure 1B*). It suggests neuronal activity promotes axon regeneration. To further test whether visual stimulation triggered regeneration was induced by the enhanced neuronal activity, the authors utilized designer receptors exclusively activated by designer drugs (DREADDs) to manipulate RGC activities (12). When silenced RGC activities by clozapine-N-oxide (CNO) and hM4Di, reduced RGC activity abolished the visual stimulation triggered regeneration. In contrast, when over-activated RGC activities by CNO and hM3Dq, more regenerated axons were found cross the lesion site. Therefore, the visual stimulation is enhanced by a neuronal activity dependent manner.

Although visual stimulation dramatically enhanced axon regeneration, regenerated axons did not reach the mid-optic nerve and optic chiasm (*Figure 1B*). Previous study demonstrated that the elevation of mTOR signaling promoted axon regeneration (6). The authors reasoned whether the regeneration capacity would be further enhanced by a combination of enhanced mTOR signaling and visual stimulation. Constitutively active ras homolog enriched in brain 1 protein (cRheb1), a positive regulator of mTOR signaling (13), was overexpressed in RGCs by AAV.

Then these mice were situated in the high-contrast visual stimulation environment immediately after ONC. Three weeks after treatment, RGC axons regrew and extended beyond the lesion site; however, they did not reach the mid-optic nerve and optic chiasm (*Figure 1C*). In contrast, when they elevated mTOR signaling and biased stimulated injured eye by suturing shut the contralateral non-lesion eye, the injured optic nerve exhibited remarkable regeneration capacity. The combination of biased visual stimulation plus mTOR signaling enhancement triggered long-distance axon regeneration. The CTB labeled regenerated axons past through the lesion site, along the entire optic nerve and extended to the optic chiasm, where optic nerves from both eyes partially cross (*Figure 1D*). Moreover, the regenerated CTB-positive axons were found in the visual targets of the brain, including the suprachiamatic nucleus (SCN), ventral and dorsal lateral geniculate (LGN), olivary pretectal nucleus (OPN), medial terminal nucleus (MTN), and superior colliculus (SC). In contrast, no CTB-labeled RGC axons were observed in nonvisual brain areas (*Figure 1D*). The results revealed the combined treatment with biased visual stimulation and mTOR signaling elevation was able to drive injured axons find their trajectories of the correct retinofugal pathway. Interestingly, this effect was critically dependent on the biased visual stimulation. When the visual stimulation was delivered to the both injured and uninjured eyes, the RGC axon regenerative capacity was dramatically abolished (*Figure 1E*).

RGCs are not a homogenous population, but differ in their morphology and function. There are at least 20 types of RGCs. Each of them exhibits diverse electrophysiological properties and projection targets (14). So the next question is whether the long-range regenerated axons can retain their heterogeneity of retinofugal projections. To address this question, the authors observed retinofugal pathways from two different types of RGCs. They first utilized the CoCH-GFP mice to genetically label the α-RGCs. They found the regenerated α-RGC axons reinnervated their natural targets including the ventral and dorsal LGN, OPN and SC. In contrast, no regenerated axons from α-RGCs were found in the SCN or MTN, neither of which is the target of normal α-RGCs. Then the authors used OPN4-GFP mice to label the intrinsically photosensitive RGCs (ipRGCs) (15), which are critical for non-image-forming vision (16). In normal mice, ipRGCs mainly innervate SCN, vLGN, intergeniculate leaflet (IGL) and SCN, while they do not innervate MTN (16,17). After the combined treatment, the regenerating axons of ipRGCs reached their natural target including IGL, and avoided the wrong targets such as MTN. Therefore, Lim *et al*. revealed the regenerated RGC axons found the right path and reinnervated their right targets by the combined treatment.

The anatomical reconnection is necessary for the restoration of visual function, but not sufficient. The functional recovery also critically relies on the number of reconnected axons and the electrical signal conduction (18). Therefore, the author reasoned whether the rebuilt retinofugal pathway would support visual functions. To address this question, the authors performed four behavioral tests with four different brain regions of visual function: the optomotor test for the oculomotor brainstem, the pupillary light reflex (PLR) for the OPN shell, the visual cliff test for retino-geniculo-cortical pathway, and the looming avoidance response for the retino-collicular pathway. In optomotor and looming response test, the visual behaviors of ONC mice with combined treatment were significantly improved compared to untreated animals. The results indicated the reinnervation of injured RGC axons could rebuild the impaired visual function. Surprisingly, the combined treated mice showed no significant difference in the PLR and visual cliff tests, despite that the regenerated axons were found in OPN and LGN. Three reasons may explain the failure of PLR and visual cliff function recovery. First, lots of RGCs die after optic nerve injury (10,19). The regenerated axons can only arise from surviving RGCs, while the RGC degeneration largely limits the number of regenerated axons. Second, despite some long-distance regenerated axons were found in brain targets, not all the regenerated axons could regenerate such a long distance to their targets. As a result, the number of synaptic reconnection is not sufficient to reach the functional recovery level. Third, axons propagate action potentials with myelin sheath, whereas regenerated axons are not myelinated (18). Consequently, the visual signal is difficult to be delivered to the brain. In summary, the enhancement of mTOR signaling and biased visual stimulation exhibit remarkable synergic effects on axon regeneration. By the combined treatment, axons regenerated a long distance, confined right trajectories to their natural targets, and partially restored visual functions.

Although the remarkable regeneration capacity was found under the combined treatment, there are still some open questions. First, why visual inputs from the uninjured eye largely inhibited the regeneration capacity of contralateral injured eye (*Figure 1D,E*). Probably, visual signals and/or molecular cues from the intact eye competitively suppress

the axon outgrowth from the contralateral injured eye. Alternatively, the answer is simply behavioral: suturing the uninjured eye can force the animal to use the injured eye more frequently. Second, the regenerated axon number is not comparable to the intact eyes. It may partially due to the RGC degeneration induced by ONC (10). Neuroprotective interventions, e.g., manipulating microglial cells, would be applied to save more RGCs (20). Third, the regenerated axons are not myelinated, which leads the poor conduction of electrical signals (18). Stimulating the remyelination of optic nerve (21) or pharmacologically enhance the conduction (18) would be helpful for functional recovery. In summary, this study sheds light on functional restoration of optic nerve damage and spinal cord injury.

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Footnote

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

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