Editorial

Central nervous system: re-establishing lost connections

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In the July issue of *Nature Neuroscience*, Lim *et al*. published their study on regeneration of target-specific retinal ganglion cell (RGC) axons by enhancing visual activity and intrinsic signaling pathways after optic nerve crush (ONC) injury (1,2). This study provides promising data to support the notion that adult neurons of the central nervous system (CNS) can regenerate and reconnect after axonal damage.

Neurodegeneration is marked by progressive loss of structure and function of neurons, culminating in cell death. Neurons of the peripheral nervous system (PNS) have the ability to repair and regenerate themselves after injury as well as recover lost functions. The PNS also has favorable environmental factors and intrinsic factors for axonal regrowth, as well as the ability to up-regulate growth-promoting genes upon injury (3). However, neurons of the CNS lose their regenerative ability after maturation (2). Unlike the growth promoting environmental and intrinsic factors of the PNS, the CNS of the adult mammal has been shown to have an inhibitory environment for axonal growth (2,3). These inhibitors of regeneration include CNS myelin (4,5), proteoglycans, and other molecules associated with glial scars that occur after axonal damage (5-7). Slower debris clearance in the CNS may also be a reason for inhibited axonal regrowth (3,8). When CNS axons mature, growth factor receptors necessary for axon growth are lost from the axons. These neurons also lose their ability to induce transcription of genes required for growth after injury (9). Recently, new evidence has suggested that neurons of the CNS may have the ability to regenerate under specific conditions. Watanabe *et al*. demonstrated the restoration of the regenerative potential of CNS neurons by permissive peripheral neural graft, which has opened new prospects to induce axonal regrowth in CNS neurons after injury (10).

The retina is an anatomical extension of the CNS. The axons of RGCs in the retina form the optic nerve, which then connects to different target centers in the brain. Damage to the optic nerve (optic neuropathy) causes axonal degeneration, neuronal cell death, and irreversible vision loss. Thus, ONC is a common method used to study the effect of axonal injury on the CNS (11). Recent studies have shown that manipulation of cellular signaling pathways can induce axonal regeneration in experimental animal models (12-14). One such regenerative signaling pathway is the mTOR pathway. Activation of mTOR pathway helps cell survival and proliferation. However, mTOR signaling is greatly downregulated after maturation of RGCs and is further reduced during CNS injury. Activating mTOR signaling in RGCs by deleting the gene for one of its inhibitors, phosphatase and tensin homolog gene (PTEN), initiates RGC survival and axonal regeneration after axonal injury (13). Similar results of RGC survival and axonal regeneration have been shown by overexpressing growth promoting cytokines such as ciliary neurotrophic factor (CNTF) and inhibiting suppressor cytokines such as SOCS3 (15,16). Some recent findings also demonstrate the importance of neural activity in regeneration (17,18). *In vitro* as well as *in vivo* studies have shown that electrical stimulation accelerates motor and sensory axon outgrowth (19,20). However, none of these interventions successfully regenerated axons through the

entire length of the optic nerve, preventing re-established axonal connections to the visual centers of the brain, and restored visual functions.

To summarize, CNS recovery after damage requires axons to regenerate and reconnect to their targets in the brain, as well as regain their neuronal functions. In an effort to discover an effective strategy for the reversal of CNS damage, Lim *et al*. in their study, employed a combination of extrinsic and intrinsic stimulations after optic nerve injury. The authors induced ONC injury in C57BL/6 mice and showed axonal degeneration and failure of axons to extend beyond the crush site via CTβ-594 anterograde labeling 3 weeks post ONC. These data confirm previously published studies. As discussed earlier, increased electrical activity in the neurons have been shown to promote axonal growth. To test this theory in the retina, the authors used high contrast visual stimulation daily for 3 weeks post ONC as a means to increase the electrical activity of RGCs. Through this experiment, the authors were able to demonstrate some degree of axonal growth past the crush site as seen by CTβ-594 anterograde labeling. To test the effects of amplified electrical activities on axonal regeneration, they carried out targeted overexpression of synthetic G-protein coupled receptors hM4Di or hM3Dq via AAV2 vectors in the RGCs of C57BL/6 mice. These receptors are exclusively activated by clozapine-*N*-oxide (CNO), which was administered intraperitoneally, and upon activation increases (hM4Di) or decreases (hM3Dq) electrical activity. Data from these experiments show that RGCs with diminished electrical activity have no axonal regeneration upon visual stimulation and the numbers of CTβ-594 labeled axons prior to the lesion site are also reduced. This led the authors to consider the involvement of increased electrical activity in promoting cell survival. Over stimulation of electrical activity through synthetic receptors alone was sufficient to drive axonal regeneration past the lesion site with some axons extending half way through the optic nerve. Through these sets of data, the authors conclude that visual stimulation causes the axons of the RGCs to regenerate by increasing their electrical activity.

To further enhance axonal regeneration, Lim *et al*. used a positive regulator of the mTOR pathway, ras homolog enriched in brain (Rheb-1). Rheb-1 was constitutively expressed (cRheb-1) via AAV2 vectors to enhance mTOR signaling in the RGCs. In cRheb-1 overexpressing mice, axons regenerated beyond the crush site and through the optic nerve. These data were observed by CTβ-594 labeling of axons 3 weeks post ONC. The effect of cRheb-1 was abolished by administration of rapamycin, which is the inhibitor of mTOR. Thus, the authors demonstrate that the axonal regeneration in this experiment was dependent on the mTOR pathway. However, the authors did not find these independent interventions to be effective in regenerating axons all the way to their target sites in the brain, which also has been a limiting factor in many other regeneration studies. Therefore, the authors explored the effects of combined strategies to increase axonal survival and regrowth.

As reported in studies of motor nerve damage, forced use of the damaged limb promotes axonal regeneration. Analogous to this, the authors sutured shut the non-lesioned eye, exposing only the lesioned eye to visual stimulations. The authors designed different combinations of treatments involving visual stimulations, mTOR pathway enhancement, and biased exposure of lesioned eye to stimulation. The authors report that only when mTOR signaling is enhanced and vision is stimulated while the non-lesioned eye sutured, do the axons grow past the lesion site after ONC through the entire length of the optic nerve. This combination of treatment, referenced by the authors as the "biased visual stimulation/AAV2-cRheb1 treatment", was further used to investigate whether the axons are capable of long distance regeneration by crossing the optic chiasm and connecting to their correct targets in the brain.

Three weeks after biased visual stimulation/AAV2-cRheb1 treatment post ONC, 7 out of 10 animals were reported to have varied degrees of CTβ-594 labeled axonal innervations throughout the retinofugal pathway. Such innervations were not observed in the non-target regions of the brain. To further show that the axons were regenerating and were not residual axons spared during ONC injury, the authors performed anterograde labeling with CTβ-594 2 days before ONC followed by 1 week of biased visual stimulation/AAV2-cRheb1 treatment. After 1 week, anterograde labeling of the axons with CTβ-488 was performed. The authors observed that the only axons that crossed the lesion site were CTβ-488 labeled, whereas CTβ-594 labeled axons were found in the proximal vicinity of the lesion site and never into the distal nerve. These data demonstrate that the axons were indeed regenerating and were not the residual axons spared during ONC injury. The authors also observed that regeneration of axons was time dependent, with maximum regrowth occurring after 3 weeks of treatment compared to 1 and 2 weeks of treatment after injury. Through these experiments the authors concluded

that ONC did not spare any axons and the regeneration of axons was time-dependent when subjected to biased visual stimulation/AAV2-cRheb1 treatment.

It has been well documented that there are approximately 30 different subtypes of RGCs, each of which innervate different target sites in the brain (12,21). To determine whether the axons that regenerate after treatment innervate their rightful target sites, the authors used transgenic animals expressing green fluorescent protein (GFP) in specific RGC subtypes (22) and subjected them to biased visual stimulation/AAV2-cRheb1 treatment after ONC. Two transgenic mouse strains were used to test the hypothesis; cochlin-GFP (CoCH-GFP) mice that express GFP in most alpha-RGCs subtypes and project to vLGN, dLGN, OPN and SC regions of the brain, and OPN4-GFP mice that express GFP in intrinsically photosensitive RGCs and project to the SCN, vLGN, IGL and OPN regions of the brain. These two particular subtypes were utilized because they have been previously shown to favor regeneration upon mTOR overexpression (12,23). When these transgenic animals were subjected to biased visual stimulation/AAV2-cRheb1 treatment post ONC, some regenerating CTβ-594 labeled axons were co-labeled with GFP indicating that subtype specific axons were also regenerating. Further, even though CTβ-594 labeling was seen in various visual targets of the brain, CTβ-GFP co-labeling was found only in the visual targets of that particular subtype and not in any other non-target regions. The presence of non-CTβ-594 labeled GFP^+ axons was shown to be due to the uninjured contralateral eye. When the uninjured contralateral eye was enucleated there was no evidence non-CTβ-594 labeled GFP^+ axons. Through this experiment, the authors demonstrated the ability of different RGC subtypes to regenerate and reinnervate their specific visual targets in the brain.

The ultimate goal of any neuroprotective and regenerative treatment is to regain lost functions. To ascertain whether the innervations made by the regenerated axons were able to redeem their normal function, the authors conducted 4 different behavioral tests to assess visual functions. The animals were tested in three groups: a non-lesioned (naive) group, a unilateral ONC group with no treatment, and a unilateral ONC group receiving biased visual stimulation/AAV2-cRheb1 treatment. To evaluate gain of function exclusively from the reconnected RGCs, the non-lesioned contralateral eye again was sutured shut. The first test conducted was the optokinetic reflex (OKR) test that evaluates the oculomotor connections. There was significant improvement in OKR responses in mice subjected to treatment as compared to untreated mice. The next test determined the pupillary light reflex (PLR), which evaluates retino-pretectal connections. The contralateral eye was not sutured during this test as direct and indirect responses in both eyes were recorded. There was no significant improvement in light reflexes in the treated animals when compared to the untreated animals. The visual cliff test, which is a test for depth perception attributed to retino-geniculate connectivity, showed that only the naïve mice demonstrated depth perception behavior, while the untreated and ONC treated animals failed to exhibit this behavior. The looming avoidance response test, which tests the ability of the animal to perceive danger and is a characteristic of the retino-collicular pathway, demonstrated that there was an improvement of danger perception in treated mice, whereas the untreated animals had a complete absence of danger avoidance response.

Overall, Lim *et al*. demonstrated that enhancing electrical activity as well as overexpressing key molecular pathways in damaged adult RGC neurons stimulates their ability to regenerate. However, the underlying mechanism is not yet clear, although factors like cAMP and NGF are considered to be involved (19,24,25). This study also demonstrates the effectiveness of combining strategies to develop treatment for neuroprotection and regeneration. The fact that the damaged axons are able to regenerate and travel through the optic nerve to their target centers, suggests the presence of certain guidance cues even after maturation, which are available during RGC development (25). However, based on the four visual function tests, the regenerated neurons were not able to resume all their expected functions. Even though the photosensitive RGCs and alpha RGCs regenerated and reconnected to their visual targets, their corresponding visual functions did not recover. Based on these results, the authors surmise that a higher number of RGCs needed to be regenerated to recover function, or perhaps the regenerated axons did not form proper synapses that are required to relay proper functional cues. Given the authors' supposition of a time-dependent regeneration, one can reason that extending the treatment beyond 3 weeks may yield better functional results. Also, as opposed to the behavioral visual tests, a more objective visual test, like the electrophysiological visual evoked potential test would have been more accurate to evaluate the visual pathway.

Nevertheless, this detailed study has certainly opened

doors to the possibility of complete regeneration and gain of function of damaged adult CNS neurons, where once even the possibility of regrowth was considered highly unlikely. In conclusion, devising a therapeutic strategy by combining interventions that promote axonal survival and regeneration after injury can pave the way for clinical reversal of neurodegeneration in patients suffering from diseases like glaucoma, traumatic brain and spinal cord injury, as well as other CNS related damages.

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Footnote

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

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