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Commentary

Do growth-stimulated retinal ganglion cell axons find their central targets after optic nerve injury? New insights by three-dimensional imaging of the visual pathway

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ABSTRACT

Retinal ganglion cells (RGCs) do not normally regenerate injured axons. However, several strategies to transform RGCs into a potent regenerative state have been developed in recent years. Intravitreal CNTF application combined with conditional PTEN and SOCS3 deletion or zymosan-induced inflammatory stimulation together with cAMP analogue injection and PTEN-deletion in RGCs induce long-distance regeneration into the optic nerve of adult mice. A recent paper by the Benowitz group (de Lima et al.) claimed that the latter treatment enables full-length regeneration, with axons correctly navigating to their central target zones and partial recovery of visual behaviors. To gain a more detailed view of the extent and the trajectories of regenerating axons, Luo et al. applied a tissue clearing method and fluorescent microscopy to allow the tracing of naïve and regenerating RGC axons in whole ON and all the way to their brain targets. Using this approach, the authors found comparable axon regeneration in the optic nerve after both above-mentioned experimental treatments. Regeneration was accompanied by prevalent aberrant axon growth in the optic nerve and significant axonal misguidance at the optic chiasm. Less than 120 axons per animal reached the optic chiasm and only few entered the correct optic tract. Importantly, no axons reached visual targets in the olivary pretectal nucleus, the lateral geniculate nucleus or the superior colliculus, thereby contradicting and challenging previous claims by the Benowitz group. The data provided by Luo et al. rather suggest that potent stimulation of axonal growth per se is insufficient to achieve functional recovery and underscore the need to investigate regeneration-relevant axon guidance mechanisms in the mature visual system.

Retinal ganglion cells (RGCs) do not normally regenerate their axons after optic nerve injury, but rather undergo apoptotic cell death (Berkelaar et al., 1994; Berry et al., 2008). This regenerative failure has manifold reasons, amongst others the growth-inhibitory environments of the injured optic nerve (myelin) and the glial scar as well as the insufficient intrinsic ability of mature RGCs to regrow axons (Berry et al., 2008; Fischer and Leibinger, 2012; Silver and Miller, 2004; Yiu and He, 2006). Research in the past two decades indicates that neutralization of myelin inhibition alone might be insufficient to promote significant RGC regeneration (Fischer et al., 2004a, 2004b; Liu et al., 2011; Sengottuvel et al., 2011). However, several experimental approaches have been discovered to markedly delay RGC apoptosis and/or to promote regeneration of lengthy axons into the inhibitory environment of an injured optic nerve (Berry et al., 1999; Diekmann and Fischer, 2013; Fischer et al., 2001; Heskamp et al., 2013; Leaver et al., 2006; Moore et al., 2009; Park et al., 2008; Pernet et al., 2013; Smith et al., 2009; Sun et al., 2011; Yin et al., 2003).

Stimulation of optic nerve regeneration by inflammatory stimulation

Stimulation of a low-grade inflammatory response in the eye induced either by lens injury (Fischer et al., 2000; Leon et al., 2000; Lorber et al., 2005) or by intravitreal injection of crystallins (Fischer et al., 2008) or toll-like receptor 2 agonists, such as zymosan or Pam_3Cys (Hauk et al., 2010; Yin et al., 2003), transforms axotomized RGCs into an active regenerative state. As a consequence, RGC survival is increased and axons regenerate over considerable distances in the injured optic nerve. This inflammatory stimulation (IS) is characterized by the activation of retinal astrocytes and Müller cells and an influx of activated macrophages into the eye (Leibinger et al., 2009; Leon et al., 2000; Lorber et al., 2009; Muller et al., 2007; Yin et al., 2003). Glial-derived ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) have been identified as key mediators of IS-induced neuroprotection and axon growth stimulation (Leibinger et al., 2009). In addition, IL-6 has been found to mediate disinhibition towards myelin (Leibinger et al., 2013). Consistently, constant CNTF supply via viral expression in retinal cells results in long distance regeneration, with axons reaching the optic chiasm 5 weeks after an intra-orbital optic nerve crush (Hellstrom et al., 2011; Leaver et al., 2006; Pernet et al., 2013). The

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proposed prominent role of macrophage-derived oncomodulin in this context, as claimed by the Benowitz lab, is a matter of debate (Fischer, 2008, 2010; Hauk et al., 2008; Yin et al., 2006, 2009).

Stimulation of axon regeneration by genetic modulation of signaling pathways

In a ground-breaking study, genetic deletion of phosphatase and tensin homolog (PTEN) has been shown to potently promote RGC neuroprotection and axon regeneration to an extent similar to IS (Park et al., 2008). PTEN counteracts the activity of phosphatidylinositide-3 kinase (PI3K) and thereby negatively regulates the PI3K/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway. Consistent with downstream signaling pathways, enhanced axonal regrowth promoted by genetic PTEN deletion is compromised by mTOR inhibition (Park et al., 2008, 2010).

Manipulation of another signaling cascade downstream of IL-6-type cytokines, namely the Janus kinase/signal transducers and activators of transcription-3 (JAK/STAT3) pathway, also results in an improved regenerative ability of RGCs upon axotomy. Conditional knockdown of suppressor of cytokine signaling 3 (SOCS3), which acts as feedback inhibitor of the JAK/STAT3 pathway (Jo et al., 2005; Lehmann et al., 2003; Nicholson et al., 2000; Shouda et al., 2001), enables RGCs to regenerate axons beyond the lesion site of a crushed optic nerve (Smith et al., 2009). These effects are markedly enhanced upon intravitreal CNTF injection, suggesting that SOCS3 at least partially restricts CNTF-induced axonal regeneration (Smith et al., 2009; Sun et al., 2011). Moreover, SOCS3 expression is suppressed by increased intracellular cAMP levels (Park et al., 2009), which might explain why IS-induced axonal regeneration is enhanced by intravitreal injections of cAMP-elevating compounds (Fischer, 2008; Muller et al., 2007, 2009).

Enhanced axon regeneration upon combinatorial approaches

The extent of regeneration observed in the above-mentioned studies is further increased by combining several of these approaches. Genetic co-deletion of PTEN and SOCS3 in combination with intravitreal CNTF injection significantly increases axonal growth in comparison to the respective single knockouts (Sun et al., 2011). Similarly, PTEN deletion in combination with zymosan + cAMP analogue injection enables some RGC axons to regenerate through the whole optic nerve (Kurimoto et al., 2011). These results raised the question whether sufficient numbers of these regenerating axons could enter the brain, find their proper targets and restore functional circuits. Using the same experimental approach, the Benowitz group later claimed that regenerating axons correctly navigate and re-innervate all major visual targets, associated with partial restoration of basic visual functions (de Lima et al., 2012).

Three-dimensional evaluation of axon regeneration

To gain a more detailed view of the extent and the trajectories of regenerating axons in the two experimental conditions described above, Luo et al. (2013) now examined axonal regeneration in whole optic nerve preparations using tetrahydrofuran-based tissue clearing and laser scanning ultramicroscopy, as previously been described for the brain and the spinal cord (Becker et al., 2012; Erturk et al., 2012). This method allows imaging of un-sectioned tissues and thereby the three-dimensional visualization of single axonal trajectories along the visual pathway (Luo et al., 2013). Even small numbers of axons known to mis-project into the contralateral optic nerve in naïve wild-type animals were clearly detectable, underscoring the superiority of this method over the evaluation of histological sections. Moreover, this method enables the accurate determination of regenerating fiber numbers in the whole optic nerve and optic chiasm rather than extrapolation from histological sections. Comparing the regenerative outcome of intravitreal CNTF application in PTEN/SOCS3 deleted RGCs side by side with zymosan-induced IS + cAMP-analogue injection in PTEN knockout RGCs, Luo et al. (2013) found very similar axon numbers at long distances in the optic nerve and at the optic chiasm for both treatments. These data suggest that the impact of CNTF injection + SOCS3 deletion and the effects of IS (which induces endogenous CNTF and LIF expression) + cAMP (which suppresses endogenous SOCS3 expression) in PTEN-depleted RGCs are expectably comparable. Although the stimulation of lengthy axon regeneration is at first glance impressive, less than 120 axons per animal reached the optic chiasm 10–12 weeks after optic nerve injury (Luo et al., 2013). This number represents only ~0.2% of all axons in the uninjured optic nerve (assuming a total of 60,000 RGCs) and is, based on the high number of re-growing axons close to the lesion site, rather disappointing (Luo et al., 2013). The comprehensive analysis of three-dimensional axon trajectories by Luo et al. (2013) may provide a potential explanation for this observation. The authors found that regenerating axons followed circuitous, aberrant paths in the optic nerve, with some extending back to the eye (Luo et al., 2013). A comparable finding was recently described upon retinal CNTF overexpression (Pernet et al., 2013). Therefore, these studies collectively indicate that aberrant axonal growth within the optic nerve might be a general, so far underestimated parameter limiting long distance regeneration (Luo et al., 2013; Pernet et al., 2013). It is currently unclear whether growth inhibitory cues in the extracellular environment contribute to the observed aberrant axon trajectories and will have to be investigated in the future.

Target innervation by regenerating axons

A major proportion of the axons that reached the optic chiasm in CNTF injected PTEN/SOCS3-knockout and zymosan/cAMP treated PTEN deleted mice was found by Luo et al. (2013) to falsely grow into the contralateral optic nerve or the ipsilateral optic tract whereas only few axons projected correctly towards the contralateral optic tract. Some axons extended into hypothalamic brain regions, including the suprachiasmatic nucleus (SCN) and the medial pre-optic area. However, no axons were detected in more distant targets, such as the olivary pretectal nucleus (OPN), the lateral geniculate nucleus (LGN) or the superior colliculus (SC) in both experimental conditions (Luo et al., 2013). These findings therefore challenge data recently published by de Lima et al. (2012), remarkably claiming correct projection of axons into all major visual targets under very similar experimental conditions and also using CTB-labeling. No potential reasons for the contrasting results of the two studies were given, so that one can only speculate. Untranssectioned (spared) axons could potentially be present in individual animals in the de Lima et al. (2012) paper, as the reported functional data seem very variable within the 'regenerating' treatment group. Furthermore, some of the applied behavioral tests are unlikely to absolutely reflect axonal regeneration as their outcome may be influenced by non-retinal input (Xue et al., 2011). These issues will have to be addressed in more detail in the future.

Despite this obvious controversy, we would like to point out that restoration of visual functions, such as optomotor response, pupillary light reflex and depth perception as claimed by de Lima et al. (2012) would likely require substantial innervation of the distant visual targets and proper remyelination of axons. According to de Lima et al. (2012), only 131 axons were quantified at the distal end of the optic nerve (5 mm), of which only 42.5% grew into the contralateral optic tract. These axons still have to navigate into the various brain targets. Even if we ignored the important finding of misguidance at the chiasm as reported by Luo et al. (2013), it is questionable whether such a low number of axons can really measurably restore complex visual functions such as depth perception. Similarly, it is surprising that circadian entrainment was partially restored according to de Lima et al. (2012), as only 1–2% of RGCs are expected to be intrinsically photosensitive due to endogenous melanopsin expression (Robinson and