Sir, retinal ganglion cells (RGCs) are normally unable to regenerate axons. However, they can be transformed into a robust regenerative state by lens injury (LI) or by intravitreal injections of either lens-derived β-/γ-crystallins or zymosan (Fischer et al., 2000, 2001, 2004, 2008; Leon et al., 2000). Based on evidence provided in Müller et al. (2007) and the literature, we propose that astrocyte-derived CNTF and activation of its major downstream signalling pathway in RGCs, the JAK/STAT3 pathway, are both essentially involved in switching these neurons to an active regenerative state after LI. By arguing against a role of CNTF and JAK/STAT3 signalling in this context, Benowitz and colleagues have ignored important evidence in the literature and also presented data from our own and other groups inaccurately, as described later.

The literature contains compelling evidence that CNTF directly stimulates axon regeneration of RGCs. For instance, using purified (immunopanned) RGCs, Lingor et al. (2008) have recently demonstrated that CNTF stimulates axon outgrowth of primary RGCs without requiring the elevation of cAMP. Benowitz and colleagues ignore this important piece of evidence, instead only mentioning the significant, but less-pronounced effects of CNTF on RGC-5 cells shown in the same paper (Lingor et al., 2008). However, RGC-5 cells are not primary neurons but rather a transformed RGC cell line derived from animals at postnatal day 1 (Krishnamoorthy et al., 2001). For this reason, the reported stronger effects on primary RGCs are much more relevant for assessing the neuritogenic potency of CNTF. Other papers provide further evidence that CNTF stimulates axon outgrowth of postnatal and adult RGCs in culture without requiring cAMP elevation (Lorber et al., 2002, 2007). Consistent with these, we have also found that CNTF highly significantly stimulates axon regeneration of adult RGCs in dissociated cell cultures without cAMP-elevation (Müller et al., in preparation). Under these conditions the effects of CNTF were maximal at a concentration of 200 ng/ml (~9 nM), whereas concentrations of 10 ng/ml (~0.4 nM) as used in Yin et al. (2006) remained ineffective (Müller et al., in preparation). Nevertheless, in the presence of forskolin, (which causes cAMP elevation), even CNTF at low concentrations (0.4 nM) significantly stimulated axon outgrowth of postnatal and adult RGCs in culture (Jo et al., 1999; Yin et al., 2003, 2006), whereas oncomodulin (another protein that has been proposed as mediating the effects of intraocular inflammation) remained ineffective at concentrations as high as 1 nM in culture (Yin et al., 2006).

Importantly, without cAMP elevation even high concentrations of oncomodulin (>10 nM) failed to stimulate axon regeneration of RGCs in vitro and in vivo (Yin et al., 2006). Thus, the effects of oncomodulin in vivo reportedly remain heavily dependent on the co-administration of drugs elevating intracellular cAMP, although intravitreal application of beads that continuously released oncomodulin did induce inflammation by itself, indicated by an influx of macrophages into the eye (Yin et al., 2006). Therefore, oncomodulin’s dependence on cAMP-analogs appears not to be overcome in the presence of macrophage-derived or other physiological factors in vivo. In contrast, both CNTF and LI do not require cAMP elevation to stimulate axon regeneration, but their effects are potentiated by cAMP elevation as shown in Müller et al. (2007).

Similar to an LI or intravitreal zymosan injection, the continuous release of CNTF from virally transfected retinal cells strongly protects axotomized RGCs from cell death and stimulates axon regeneration over long distances into the injured optic nerve (Leaver et al., 2006). This demonstrates that the continuous release of CNTF from retinal cells (which does not require overcoming the inner limiting membrane barrier of the retina) is much more potent than intravitreally applied recombinant CNTF. Consistent with this, an LI continuously increases the expression and release of CNTF from activated retinal glial cells, which is temporally closely correlated with the activation of CNTF’s major downstream pathway (JAK/STAT3) in RGCs (Müller et al., 2007). These data suggest that CNTF is one of the major mediators of the effects of intraocular inflammation. Further evidence is provided by
the intravitreal application of an anti-CNTF antibody in vivo diminishing LI-induced axon outgrowth by 32%, and by that of the JAK/STAT3 pathway inhibitor by 43 and 75%, respectively (Müller et al., 2007). We consider these reductions to be strong and functionally relevant, since the antibody probably cannot quench all the CNTF in vivo due to the close anatomical association of astrocytes and RGCs and the continuous expression and release of CNTF. Another possible reason is the short half-life time of reagents in the vitreous body. As discussed in Müller et al. (2007), these are also possible reasons why Leon et al. (2008) found no reduction of LI effects by one single intravitreal injection of CNTF antibodies and evaluating axon regeneration 3 weeks afterwards. In this context we would also like to emphasize that Lorber et al. (2002) have not shown that an antibody against the CNTF receptor failed to diminish LI-induced axon regeneration in vivo. Instead, they investigated the axon-growth-promoting effects of lens-conditioned medium in culture (Lorber et al., 2002).

Although the data presented in Müller et al. (2007) suggest that astrocyte-derived CNTF directly stimulates axonal regeneration, we do not rule out the possibility that additional indirect effects also mediated by this cytokine might contribute. However, there is no evidence in the literature that pure CNTF or CNTF released from retinal glia or from virally transfected cells is a chemotractant for blood-borne macrophages, or that it mediates its neuroprotective and axon-growth-promoting effects via these cells. Cen et al. (2007) exclusively used recombinant CNTF containing up to 2% impurities including endotoxins that are highly potent activators of monocytes and microglia. Direct CNTF effects on monocytes were not shown (Cen et al., 2007). Furthermore, that study did not distinguish between microglia and blood-borne macrophages and did not include essential control groups in macrophage-depletion experiments (Cen et al., 2007). In contrast, systemic and local macrophage depletion has recently been shown to not significantly reduce the effects of LI (Hauk et al., 2007), suggesting that alternative, macrophage-independent mechanisms are mainly involved in this process. Consistently, two independent studies recently demonstrated that axon regeneration after LI is closely correlated with the activation of retinal glia rather than macrophages (Hauk et al., 2007; Lorber et al., 2007). Müller et al. (2007) also showed that co-culturing retinal explants with lens proteins increased CNTF expression in the absence of macrophages and Fischer et al. (2008) that this elevation was followed by STAT3 activation and a highly significant increase in axon outgrowth in these cultures (Fischer et al., 2008).

We also consider that the statement by Benowitz and colleagues that ‘Lingor et al. (2008) report a much weaker effect of CNTF’ (in terms of the number of regenerated axons in the crushed optic nerve after intravitreal CNTF injections) is not justified for at least three reasons: (i) Lingor et al. (2008) presented average numbers of axons per single optic nerve section, whereas we presented the numbers of regenerating axons in the whole optic nerve, (ii) Lingor et al. evaluated the numbers of axons 4 weeks after optic nerve crush, whereas we evaluated them after 2 weeks, a time point when more RGCs are still alive and (iii) Lingor et al. used Wister rats, whereas we used Sprague-Dawley rats. Nevertheless, although the numbers of regenerating axons in the optic nerve are difficult to compare between these two studies, both consistently and clearly demonstrate that repeated intravitreal injections of CNTF sufficiently stimulate RGCs to regenerate axons into the injured optic nerve.

Müller et al. (2007) demonstrated that intravitreal injections of a polyclonal anti-oncomodulin antiserum, which has previously been shown to efficiently bind native oncomodulin (Yin et al., 2006), does not reduce the effects of LI. Since this antiserum was made against the total protein, we consider it very likely that it prevents oncomodulin from binding to its putative receptor, and therefore blocks its neuritogenic effects provided that it was the principal mediator of these effects. However, Hauk et al. (2007) used several positive and negative controls and different methods to show, that oncomodulin levels are not significantly elevated in the inner eye after LI and that the antibody used in Yin et al. (2006) strongly cross reacts with lens proteins of a similar size as dimeric oncomodulin making it unsuitable for measuring oncomodulin at the protein level after LI (Hauk et al., 2007). These data together with the effects of oncomodulin on RGCs being heavily dependent on cAMP elevation (as discussed earlier), oncomodulin not being neuroprotective and lens proteins stimulating axon outgrowth of RGCs in the absence of macrophages (Lorber et al., 2002; Fischer et al., 2008) suggest that oncomodulin is not the principal factor mediating the effects of intraocular inflammation, although it might have neuritogenic potential together with drugs elevating cAMP.

References


