Supplementary Online Content


**eMethods.** Semiautomated axon counting by targeted sampling

**eFigure.** Intrarater variability of estimated axon counts

**eTable.** Comparison of Brn-3 and NeuN for RGC counting

**Reference**
eMethods. Semiautomated axon counting by targeted sampling

Axon counts of the optic nerve were performed in a masked fashion. First, zones of equal damage were defined on photomicrographs showing the entire nerve cross section. The percentage of the cross section occupied by each zone was then determined using ImageJ (1.42q; http://rsb.info.nih.gov/ij/). Two oil-immersion (original magnification ×100) photomicrographs were then taken from each zone of damage. Four randomly selected photomicrographs were taken of each control optic nerve (ON). The sampled area corresponded on average to 6% of the entire cross section for each ON. Images were contrast and edge enhanced, and each axon with a single, intact myelin sheath was counted using macroroutines written for ImageJ. A weighted average calculation was then used to estimate the number of surviving axons in the total ON. The control group was a “pooled” group (n = 26), consisting of 13 randomly selected contralateral left ONs from each experimental group. The estimated axon count was not different in these 2 control ON subgroups.
Intrarater variability for right optic nerves was assessed. An additional set of images was captured for each animal and optic nerve counts repeated. The data have been represented as dot-plot, with the corresponding correlation coefficient.
eTable. Comparison of Brn-3 and NeuN for RGC counting

<table>
<thead>
<tr>
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<th>Brn-3</th>
<th>NeuN</th>
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<tbody>
<tr>
<td>Controls (n=44)</td>
<td>137.2 ± 3.47</td>
<td>336.2 ± 6.29</td>
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<tr>
<td>NG-OHT (n=24)</td>
<td>95.8 ± 7.84</td>
<td>289.3 ± 11.23</td>
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<tr>
<td>HG-OHT (n=20)</td>
<td>111.0 ± 5.37</td>
<td>320.9 ± 12.11</td>
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</table>

*P* values are given for comparison of retinal ganglion cell (RGC) loss between normoglycemic (NG-OHT) and hyperglycemic (HG-OHT) rats (unpaired 2-tailed *t* test). The numbers represent RGC counts (mean ± SEM) from ciliary body to ciliary body on sections taken at the level of the optic nerve head. The lack of significance using Brn-3 immunostaining may possibly be explained by earlier down-regulation of this transcription factor in functionally compromised, but still viable RGCs. Brn-3 appears to be down-regulated earlier in chronic glaucoma, whereas NeuN expression persists until a later stage of disease (see Buckingham et al1).
REFERENCE