Supporting Information

Nguyen et al. 10.1073/pnas.1013965108

SI Methods

Sample Preparation for Scanning Block-Face Serial EM. A 9-mo-old female C57BL/6J mouse was anesthetized with pentobarbital and transcardially perfused with Ringer solution followed by 2.5% glutaraldehyde/2% paraformaldehyde in cacodylate buffer (Ted Pella) containing 2 mM $CaCl_2$ (CAC+Ca buffer). An eye was removed and the ONH carefully dissected and postfixed in the same fixative agent for 2 h at 4 °C. The nerve was washed in CAC +Ca buffer for 30 min at 4 °C. The nerve was placed in CAC +Ca buffer containing 2% OsO_4 and 1.5% potassium ferrocyanide for 3 h at room temperature (RT). The nerve was washed with ddH_2O three times for 5 min and then placed in 1% aqueous thiocarbohydrazide (Sigma) for 20 min at RT. The nerve was washed three times for 5 min in ddH_2O and then placed in 2% aqueous OsO_4 for 1 h at RT. The nerve was washed three times for 5 min and placed in 1% aqueous uranyl acetate at 4 °C overnight. The nerve was washed three times for 5 min in ddH_2O and incubated in lead aspartate solution in 60 °C oven for 30 min, as described previously (1). The nerve was washed three times for 5 min in ddH_2O and then dehydrated in a series of ice-cold ethanol solutions (20%, 50%, 70%, 90%, 100%, 100%) followed by ice-cold dry acetone for 10 min. The nerve was placed in acetone at RT for 10 min and then infiltrated with an ascending series of Durcupan:acetone solutions. The nerve was infiltrated with 100% Durcupan and then cured at 60 °C for 2 d. The nerve was trimmed to remove excess plastic and attached to an aluminum pin, grounded with silver paint, and sputter coated with gold–palladium before imaging.

1. Walton J (1979) Lead asparate, an en bloc contrast stain particularly useful for ultrastructural enzymology. *J Histochem Cytochem* 27:1337–1342.

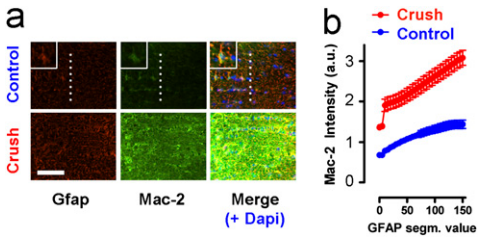


Fig. S1. Optic nerve astrocytes up-regulate Mac-2 after optic nerve crush. (*A*) Optic nerve crush of the myelinated nerve near the MTZ promotes up-regulation of GFAP and Mac-2. Note the GFAP expression within Mac-2–expressing cells at the MTZ in the absence of crush (left of dotted line). Inset shows 2× magnified view of a Mac-2–positive GFAP-positive cell amid doubly negative cells. (*B*) Mac-2 is increased by crush in optic nerve astrocytes. Mac-2 intensity represents the mean intensity value within pixels identified based on GFAP expression, at increasingly stringent GFAP segmentation values. Mac-2 expression is higher after crush; for example, $P < 0.0001$, two-tailed unpaired *t* test, at a segmentation of 100. Values are mean values from six nerves each (an average of six sections per nerve), and error bars represent SEM. (Scale bar: 100 μm.)

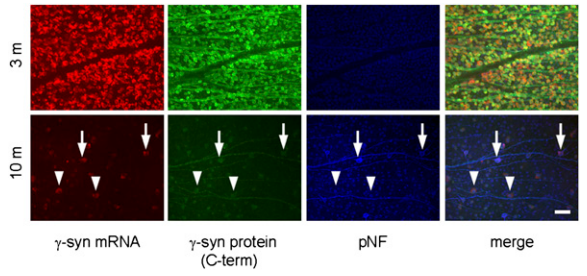


Fig. S2. Loss of RGCs and increase in pNF^+ RGCs in glaucomatous DBA/2J. Midperipheral regions of a 3-mo and a highly degenerated 10-mo DBA/2J retina show that the loss of RGCs and appearance of damaged RGCs in glaucomatous DBA/2J mice can be detected by the RGC-specific gene γ-synuclein and a pNF antibody, respectively. Note the overlap of γ-synuclein mRNA and protein labeling in both healthy and degenerated retinas, the comparable numbers of cells without γ-synuclein expression (showing that amacrine cells do not die in this glaucoma animal model), the loss of nuclei (cross-reacting nuclear epitope of this pNF antibody), and the fact that some (arrows) but not all (arrowheads) remaining RGCs have somatodendritic phosphorylated neurofilament labeling. (Scale bar: 50 μm.)

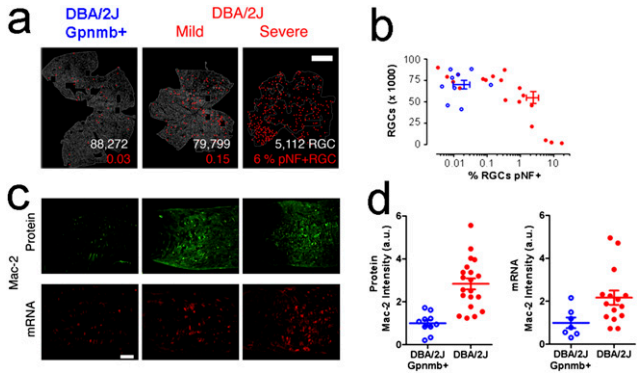


Fig. S3. DBA/2J have increased Mac-2 expression at the MTZ relative to age-matched DBA/2J Gpnmb$^+$ mice. (*A*) Loss of RGCs, detected by γ-synuclein mRNA (gray), and increase in pNF$^+$ RGCs (red dots) in 9- to 10-m DBA/2J mice relative to age-matched DBA/2J Gpnmb$^+$ mice. (*B*) DBA/2J mice have reduced number of RGCs, based on counts of cells expressing Sncg mRNA, and increased numbers of pNF$^+$ RGCs. (*C*) DBA/2J mice also have higher levels of Mac-2 protein and mRNA at the MTZ. (*D*) Quantification of fluorescence intensity (using a single segmentation value) shows significant increases in Mac-2 mRNA and protein (*$P < 0.0001$, **$P = 0.02$, Mann–Whitney two-tailed *t* test). Values are the mean of 10 DBA/2J Gpnmb$^+$ and 15 to 19 DBA/2J nerves. Error bars represent SEM. (Scale bars: *A*, 500 μm; *C*, 50 μm.)

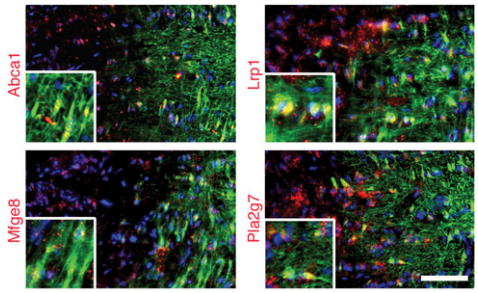


Fig. S4. MTZ astrocytes express phagocytosis-related genes. Some of the phagocytosis-related genes expressed by MTZ astrocytes include Abca1, Lrp1, Mfge8, and Pla2g7. Mac-2 labeling is shown in green and mRNAs for the respective genes are shown in red. Nuclei labeled by Dapi are shown in blue. Boxes are 2× magnified view of select MTZ astrocyte somata. (Scale bar: 50 μm.)

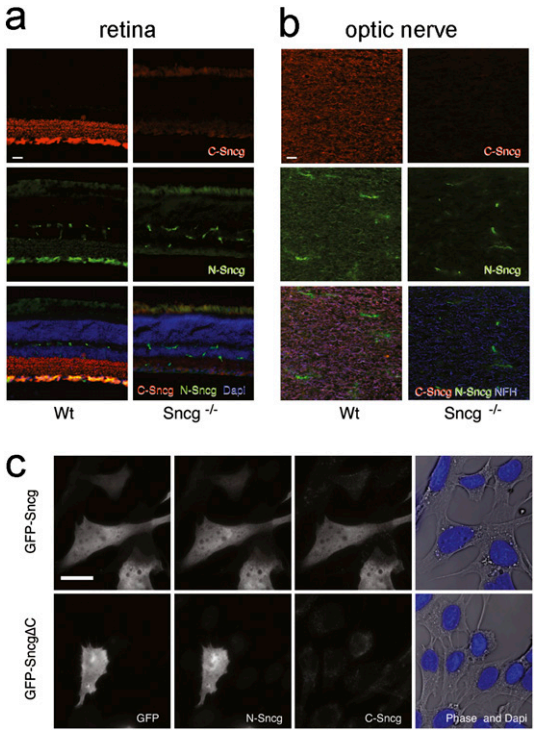


Fig. S5. Two γ-synuclein antibodies show specific labeling and recognize different parts of γ-synuclein. (*A*) γ-Synuclein antibodies label approximately half of somata in the ganglion cell layer, as well as axon bundles and IPL only in mice with γ-synuclein. (*B*) γ-Synuclein antibodies label optic nerve axons only in mice with γ-synuclein. Some axons are visualized by their expression of heavy neurofilament (NFH). Residual labeling for the mouse N-Sncg antibody in Sncg$^{-/-}$ mice is caused by labeling of blood vessels by the secondary antibody. (*C*) Cells transfected with GFP fusion constructs containing the full-length mouse γ-synuclein (GFP-Sncg) or mouse γ-synuclein lacking 15 C-terminal aa (GFP-SncgΔC), and labeled with antibodies to GFP, to the C-terminal 16 aa of mouse γ-synuclein (C-Sncg), or a commercial mouse monoclonal antibody generated against human γ-synuclein (N-Sncg). Note that the N-Sncg antibody equally labels cells transfected by γ-synuclein with or without the C terminus; that is, this antibody recognizes an epitope outside the C terminus. (Scale bars: 20 μm.)

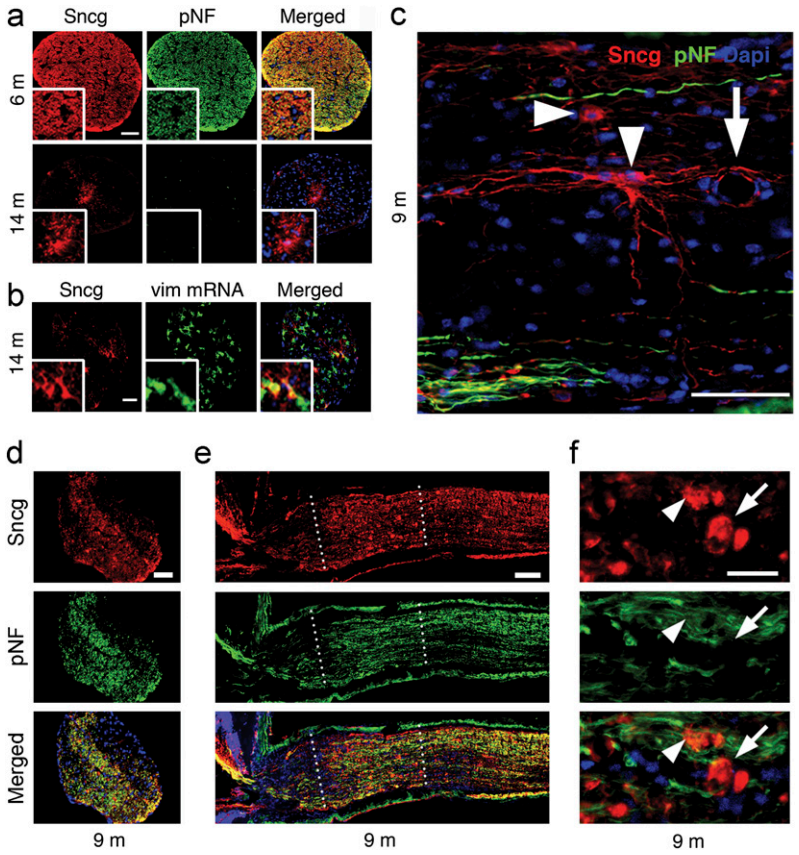


Fig. S6. γ-Synuclein is found within astrocytes and axonal spheroids in glaucomatous DBA/2J mice. (*A*) Nerve cross sections showing that γ-synuclein is found in axons in a healthy nerve from a 6-mo DBA/2J mouse but also within cells in a highly degenerate nerve from a 14-mo DBA/2J mouse. (*B*) Nerve cross sections showing that γ-synuclein–positive cells express Vim mRNA, an astrocyte marker. (*C*) Longitudinal section shows two cells labeled by γ-synuclein (arrowheads), which are astrocytes based on their morphology and wrapping of a blood vessel (arrow). (*D*) Cross-section in the orbital portion of the optic nerve shows sectorial axon loss. (*E*) Longitudinal section through the same optic nerve has numerous γ-synuclein–containing axonal spheroids concentrated at the MTZ (flanked by dotted lines). (*F*) Longitudinal section of the MTZ in another 9-mo DBA/2J optic nerve shows that some axonal spheroids are contiguous with axons labeled by pNF (arrowhead) but others are not (arrow). Nuclei labeled by Dapi are shown in blue. *Insets*: *A*, magnification of 2×; *B*, magnification of 3×. (Scale bars: *A*–*D*, 50 μm; *E*, 100 μm; *F*, 20 μm.)

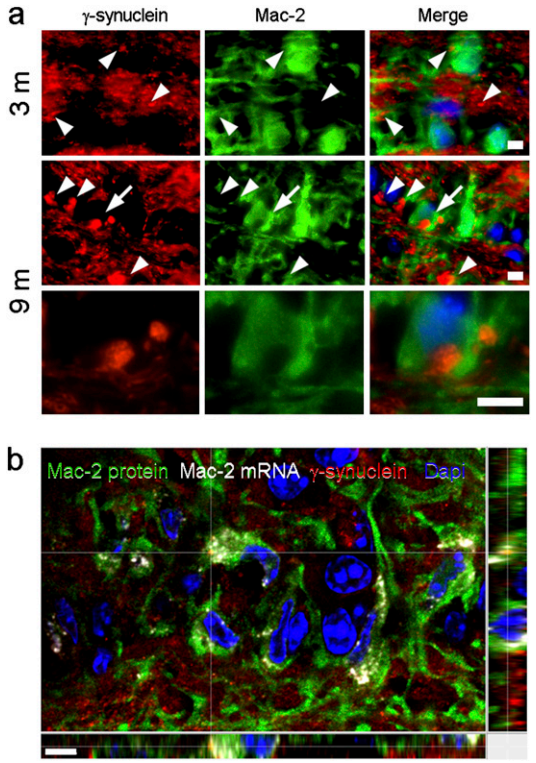


Fig. S7. Axon protrusions and evulsions at the MTZ in preglaucomatous optic nerves contain γ-synuclein. (*A*) Confocal slices show increased size and number of spheroidal structures containing γ-synuclein (arrow and arrowheads) at the MTZ in 9-mo relative to 3-mo DBA/2J mice. Many but not all spheroids are near Mac-2–expressing astrocytes. Arrow points to the spheroids shown in a high-power view (*Lower*). (*B*) Confocal of MTZ 24 h after translimbal laser increase in IOP, showing a γ-synuclein body fully surrounded by Mac-2 mRNA and protein. (Scale bars: 5 μm.)

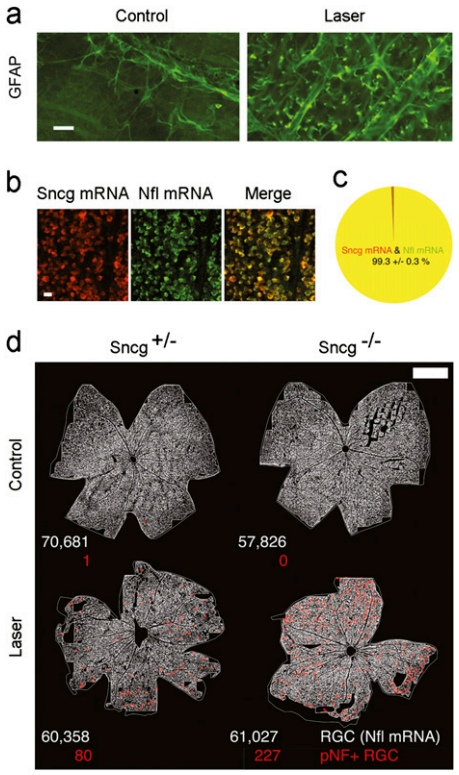
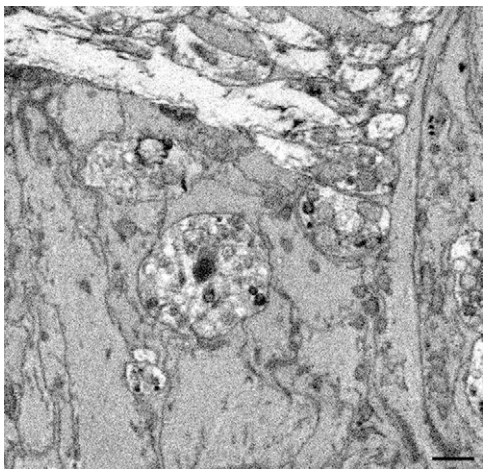
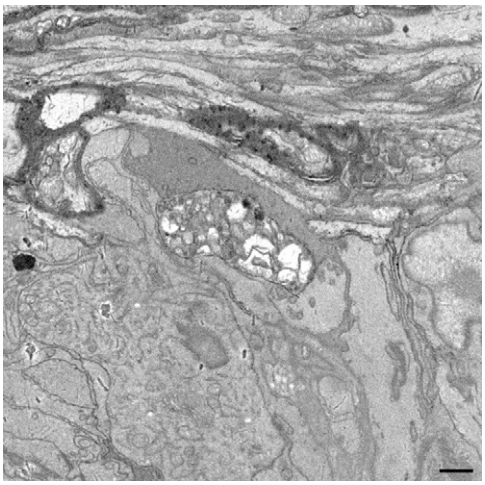


Fig. S8. Translimbal laser photocoagulation produced large increases in pNF^+ RGCs but no large or sectorial loss of RGCs, as detected by Nfl mRNA. (*A*) GFAP is expressed in astrocytes in a nonlasered eye, and in both astrocytes and Müller cell endfeet in a lasered eye. (*B*) Nfl and γ-synuclein mRNAs label the same cells in retina flat-mounts of C57BL/6J mice. (*C*) Counts in C56BL/6J retinas show that 99.3 ± 0.3% of cells label with both mRNAs, 0.5 ± 0.3% of cells label only with Nfl mRNA, and 0.2 ± 0.2% of cells label only with γ-synuclein mRNA (8,439 cells counted, in five fields in each of five retinas). (*D*) Representative retina whole mounts showing RGCs identified by Nfl mRNA (gray), and pNF^+ RGCs (red dots) in lasered and nonlasered retinas from $Sncg^{+/-}$ and $Sncg^{-/-}$ mice. Number of RGCs and pNF^+ RGCs are shown under each retina in white and red, respectively. (Scale bars: *A*, 10 μm; *C*, 500 μm.)



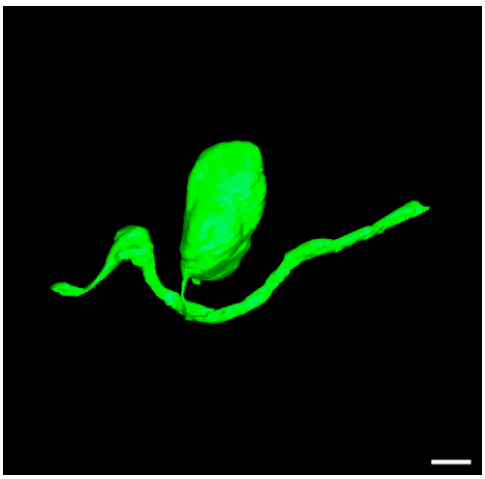
Movie S1. Axonal evulsion at the glial lamina, fully enwrapped by astrocytic processes. Frames are 70 nm apart. (Scale bar: 1 μm.)

Movies S1



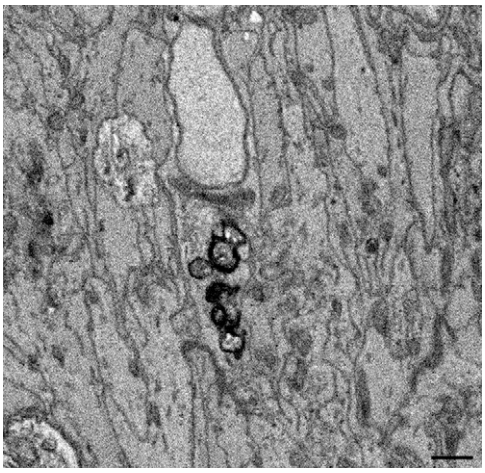
Movie S2. Axonal evulsion at the MTZ, enwrapped by astrocytic processes. Frames are 70 nm apart. (Scale bar: 1 μm.)

Movies S2



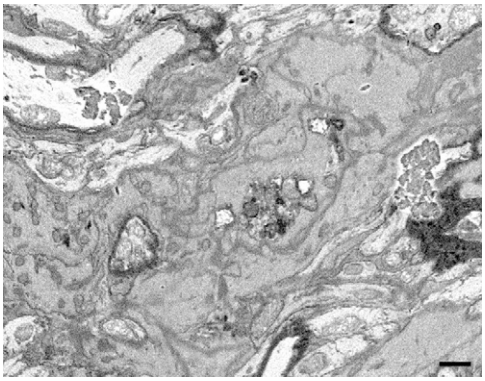
Movie S3. Reconstruction of axonal evulsion at the glial lamina, connected to the axon of origin by a thin stalk. (Scale bar: 5 μm.)

Movies S3



Movie S4. Astrocyte degradation of an axonal evulsion at the glial lamina. Frames are 70 nm apart. (Scale bar: 1 μm.)

Movies S4



Movie S5. Astrocyte degradation of an axonal evulsion at the MTZ. Frames are 70 nm apart. (Scale bar: 1 μm.)

Movies S5