Supporting Information

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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$_2$O 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$_2$O 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$_2$O 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$_2$O 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 trimmed to remove excess plastic and attached to an aluminum pin, grounded with silver paint, and sputter coated with gold–palladium before imaging.


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.)
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.)
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 photoagulation 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 nonlased eye, and in both astrocytes and Müller cell endfeet in a lasered eye. (B) Nfl and \(\gamma\)-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 \(\gamma\)-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 nonlased 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 \(\mu\)m; C, 500 \(\mu\)m.)

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

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