Long-Term Effect of Optic Nerve Axotomy on the Retinal Ganglion Cell Layer

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Purpose. To analyze the long-term effect of optic nerve injury on retinal ganglion cells (RGCs) and melanopsin+ RGCs orthotopic and displaced, and on the rest of the ganglion cell layer (GCL) cells.

Methods. In adult albino rats, the left optic nerve was crushed (ONC) or transected (ONT). Injured and contralateral retinas were analyzed at increasing survival intervals (up to 15 months). To study all GCL cells and RGCs, retinas were immunodetected with Brn3a and melanopsin to identify the general RGC population (Brn3a+) and mRGCs, and counterstained with 4′,6-diamidino-2-phenylindole (DAP). Brn3a+ RGCs and mRGCs displaced to the inner nuclear layer were analyzed as well. In additional retinas, glial cells in the GCL were identified with glial fibrillary acidic protein (GFAP) or Iba1, and in some retinas, Brn3a, calretinin, and γ-synuclein were immunodetected.

Results. Orthotopic and displaced RGCs behave similarly within the RGC and mRGC populations. Both lesions cause an exponential loss of RGCs (4%–1% survival at 6 months after ONC or ONT), but not of mRGCs, whose number remains stable from 1 to 15 months (34%–44% of the initial population). γ-synuclein is expressed by RGCs and displaced amacrine cells (dACs), allowing us to confirm that axotomy does not affect the latter, and to determine that out of the approximately 217,406 cells that compose the GCL (excluding endothelia), 10% are glial cells, 50% dACs, and the remaining 40% are RGCs.

Conclusions. In the GCL, only RGCs are lost after axotomy, and there are important differences in the course of loss and rate of survival between melanopsin+ RGCs and the rest of GCL cells.

Keywords: intrinsically photosensitive retinal ganglion cells, topography, Brn3a, melanopsin, displaced amacrine cells, gamma synuclein, displaced retinal ganglion cells

Retinal ganglion cells (RGCs) are the only afferent neurons of the retina. Their axons form the optic nerve and convey the luminous information from the retina to the retinorecipient areas in the brain. Luminous stimuli elicit image forming responses that are sensed by photoreceptors, cones, and rods, preprocessed by intermediate retinal neurons and sent to the brain by the general RGC population (henceforward RGCs). In addition, luminous stimuli also elicit non-image-forming responses, these are sensed and sent to the brain by the intrinsically photosensitive RGCs (ipRGCs), which express the photopigment melanopsin. Intrinsically photosensitive RGCs have a major role in nonimage-forming responses but also contribute to image-forming responses.1-7

Retinal ganglion cells are preferentially found in the ganglion cell layer (GCL; orthotopic RGCs), but a small population of them, including ipRGCs, are displaced to the inner nuclear layer (displaced RGCs).8-11

In the GCL there are endothelial cells, glial cells (astrocytes and microglia12-14), and displaced amacrine cells (dACs) which are, at least, as abundant as RGCs.15-19 Although the proportion of each of them is not yet fully addressed it is known that neurons, RGCs and dACs, conform most of the GCL.15-19 Thus, to analyze RGCs these must be specifically identified. A good strategy consists of double immunodetection of Brn3a and melanopsin, because Brn3a in the rodent retina is expressed by all RGCs except melanopsin expressing RGCs and half of the RGCs projecting ipsilaterally.20,21 Thus, this approach allows to study, in parallel but independently, RGCs and ipRGCs.10,11,22

Melanopsin immunodetection only identifies two of five subtypes of ipRGCs, M1 and M223, because the M3 to M5 ipRGC subtypes express very low levels of this protein. Nevertheless, melanopsin labeling is, at present, the way to study ipRGCs in rats. Because all reports in rat and most of the works on mice addressing ipRGCs are based on melanopsin immunodetection, we will refer to them as melanopsin+ RGCs (mRGCs).

Optic nerve axotomy induced by crush (ONC) or transection (ONT) causes RGC death. This model has been widely used to study the response of central nervous system neurons to axonal injuries as well as to test neuroprotective therapies. The temporal course of axotomy-induced RGC degeneration has been analyzed mostly at early time points20,24-29 and varies depending on the distance from the cell somata,30 and on the type of insult (ONC versus ONT) both at the molecular11,32 and anatomical level.20,21,53 To our knowledge, few reports address the long term effect of axotomy on rat RGCs,50-53 and these studies were carried out before the discovery of mRGCs.7 This
is important because the reaction of RGCs and m’tRGCs to injury or retinal degeneration appears to be different: m’tRGCs are more resistant to some insults such as axotomy,36,37 excitotoxicity,38 or mitochondrial optic nerve neuropathy39 (reviewed in Ref. 40), but not to ocular hypertension–induced retinal degeneration.41,42 and, on the other hand, they do not respond to neuroprotective treatments that are beneficial for the general RGC population.42

Retinal ganglion cells and dACs are highly interconnected synaptically.43–47 Therefore, it is conceivable that the loss of RGCs affects the survival of dACs. Several studies demonstrate that within the first 2 weeks, approximately 90% of RGCs die all across the retina.20,24,28,34 without any quantifiable effect on other GCL cells.34,35 There are at least 10 types of dACs in rodents,48 and to date there is not a specific marker to label them all. Thus, most works focused on dACs use neurotransmitters to identify dAC subtypes49–52 or classical staining methods to study all the GCL layer, assuming that the GCL is composed mainly by RGCs and dACs.18,19,35,53

Because little is known about the total number and distribution of cells in the GCL and about the long term effect of axotomy on RGCs, m’tRGCs and the rest of GCL cells, we propose to (1) determine the total number and topography of cells in the GCL using automated quantification routines, and calculate the percent of RGCs, dACs, macro, and microglia that compose it; (2) study and compare the temporal course of RGC and m’tRGC loss (orthotopic and displaced) up to 6 or 15 months after ONC or ONT, respectively; (3) assess whether the massive death of RGCs triggered by axotomy induces a protracted secondary death of other GCL cells; and (4) that γ-synuclein and Brn3a immunodetection and 4’,6-diamidino-2-phenylindole (DAPI) staining reveal that γ-synuclein is expressed by all Brn3a’tRGCs and, probably, most of dACs. Thus, taking advantage of the fact that at 15 months after axotomy the majority of RGCs have died, we have quantified the total number of γ-synuclein’dACs and ascertained their distribution.

MATERIALS AND METHODS

Animal Handling and Ethics Statement

Adult albino (Sprague Dawley) rats (∼180 g body weight, 2 months old) were obtained from the University of Murcia (Murcia, Spain) breeding colony at the Animal Housing Facilities. Animal care and experimental procedures were performed in accordance to the Association for Research in Vision and Ophthalmology, European Union guidelines for the use of animals in research and were approved by the Ethical and Animal Studies Committee of the University of Murcia. Animals were anesthetized with a mixture of xylazine (10 mg/kg body weight; Rompun; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight; Ketolar, Pfizer, Alcobendas, Madrid, Spain) administered intraperitoneally (i.p.). During surgery and while the animals were recovering from the anesthesia, an oculovar ointment (Tobrex; Alcon Cuséi, S.A., Barcelona, Spain) was applied on the cornea to prevent corneal desiccation. All efforts were made to minimize animal suffering, and rats were given oral analgesia (Buprex, Buprenorphine 0.3 mg/mL; Schering-Plough, Madrid, Spain) at 0.5 mg/kg (prepared in strawberry-flavored gelatin) the day of the surgery and during the next 3 days.

Surgery and Animal Groups

Intraorbital Optic Nerve Crush (ONC). The left optic nerve was crushed at 3 mm from the optic disk using previously reported methods.20,26,31–35 Animals were processed at 7 days, 1, 2, 3, or 6 months later (n = 6/time point).

Intraorbital optic nerve transaction (ONT) the left optic nerve was transected at 0.5 mm from the optic disk using previously reported methods.14,20,27,28,30 For quantitative studies, animals were processed at 7 days, 1, 2, 3, 6, or 15 months later (n = 6/time point). To assess γ-synuclein and calretinin coexpression after axotomy, two additional retinas processed 3 months after ONT were used. For Western blotting animals were euthanized at 7 or 14 days, or 2 months (n = 4 retinas/time point).

As control, 2-month-old intact (naïve) animals (n = 6 retinas) were used for quantification. The right retinas, contralateral to the injured ones, were analyzed in parallel. Twelve additional intact retinas were used to assess the proportion of glial cells and γ-synuclein cells in the GCL (n = 10) or to perform cross-sections (n = 2). Finally, four intact retinas were used for Western blotting.

Western Blotting

After an overdose of sodium pentobarbital, retinas were fresh dissected and immediately frozen in dry ice. Protein extraction was carried out as previously described.31,32 Forty micrograms of protein from a pool of four retinas per experimental condition were loaded and resolved in 4% to 20% polyacrylamide gels (Amersham GE Healthcare Europe GmBH, Barcelona, Spain). Primary antibodies were goat-anti Brn3a (1:1500, G-20; Santa Cruz Biotechnologies, Heidelberg, Germany), rabbit anti–γ-synuclein (1:100, ab55424; Abcam Plc, Cambridge, UK), and mouse anti-calretinin (1:200, 34; Santa Cruz Biotechnologies) diluted in blocking buffer (Western Blocker Solution; Sigma Aldrich, Alcobendas, Madrid, Spain), Secondary detection was carried out with donkey anti-mouse, donkey anti-rabbit, or donkey anti-goat antibodies conjugated to horseradish peroxidase antibodies (1:5000; Santa Cruz Biotechnologies) and visualized by chemiluminescence (Enhanced Chemi Luminiscence [ECL]; Amersham GE Healthcare Europe GmbH). The signal was acquired with an Image LAS 500 (Amersham GE Healthcare Europe GmbH). Westerns were replicated three times. As loading control, actin detection was carried out using rabbit anti-actin (1:500; Sigma Aldrich).

Retinal Dissection and Immunodetection

Rats were euthanized with an i.p. overdose of pentobarbital (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain), and perfused with saline followed by paraformaldehyde 4%. Retinas were dissected as flat-mounts and subjected to double immunofluorescence as previously reported.20–22

For cryostate sectioning (15 μm) the two eyes from an intact animal were cryoprotected in increasing gradients of sucrose (Sigma-Aldrich Quimica SL, Madrid, Spain) before embedding them in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). The general population of RGCs was detected using goat antibrain-specific homeobox/POU domain protein 3A (Brn3a, 1:750, C-20; Santa Cruz Biotechnologies). Melanopsin’tRGCs (M1 and M2 subtypes) were detected using rabbit anti-melanopsin PAI-780 antibody (1:500; Thermo Scientific, Madrid, Spain) that binds to the NH3 terminal of the melanopsin protein and thus detects both melanopsin isoforms.54 γ-synuclein’cells were detected with rabbit anti–γ-synuclein (1:100, ab55424; Abcam Plc) and amacrine cells with mouse anti-calretinin (1:200, 34; Santa Cruz Biotechnologies). Astrocytes were detected with rabbit anti–glial fibrillary acidic protein (GFAP; 1:500; Sigma Aldrich Quimica SL), and microglial cells with rabbit anti-ionized calcium-binding adapt-
er molecule 1 (Iba1, 1:1000; Wako Chemicals GmbH, Neuss, Germany).

Secondary detection was carried out with donkey anti-goat IgG(H+L) Alexa Fluor 594, donkey anti-rabbit IgG(H+L) Alexa Fluor 488, donkey anti-mouse IgG(H+L) Alexa Fluor 488, or 594 (1:500; Molecular Probes, ThermoFisher, Madrid, Spain).

Finally, all retinas were mounted vitreal side up with antifading medium containing DAPI (VectaShield mounting medium with DAPI; Vector Laboratories, Burlingame, CA, USA).

Image Acquisition and Quantification

Retinal whole-mounts were photographed under an epifluorescence microscope. DAPI nuclei in the GCL, mRGCs or γ-synuclein+ cells, and Brn3a+ RGCs were photographed in the same retinas. Individual frames (15/retina) were later reconstructed as retinal photomontages as previously reported.41,55,56 The total number of DAPI+ nuclei, γ-synuclein+ cells, and Brn3a+ RGCs was automatically quantified (image analysis software: Image-Pro Plus, IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA) using established routines by our group.20,27,55–57 Of note, the automated routine was able to quantify separately round and fusiform DAPI+ nuclei (see Results).

Displaced RGCs (Brn3a or melanopsin-positive) were identified and quantified as reported.10,11 mRGCs were manually dotted on the retinal photomontage, and the dots automatically quantified using the IPP software.10,11,22

γ-synuclein+ Cells, Astrocytes, and Microglial Cells in the GCL of Intact Retinas

To assess the proportion of GCL cells that were γ-synuclein+ but not Brn3a+ RGCs, both proteins were double immunodetected. Magnifications (0.1575 mm²) were acquired from 12 samples, 3 per quadrant, n = 4 retinas. To quantify astrocytes or microglial cells, GFAP or Iba1 were single immunodetected. Magnifications (0.1575 mm²) were acquired from 12 samples, 3 per quadrant (central, medial peripheral, n = 3 retina/iris). All retinas were counterstained with DAPI, DAPI, γ-synuclein, and Brn3a, or DAPI and GFAP, or DAPI and Iba1 signals were acquired from each frame and their numbers manually quantified. Data are given as densities (number of cells/mm²).

Topographic Maps

The topography of round DAPI+ nuclei and γ-synuclein+ cells was carried out with isodensity maps as previously described.41,55,56 The distribution of mRGCs and displaced Brn3a+ or mRGCs was assessed by the fixed-radius near neighbor algorithm.10,11,22 In these maps each dot represents a single cell, and its color indicates the number of neighbors around it: the warmer the color, the higher the number of neighbor cells in a given radius.

Up to date, we have represented the topography of orthotopic Brn3a+RGCs using isodensity maps. However, because after axotomy the number of surviving RGCs is very small, neighbor maps are more suitable to visualize the distribution of the remaining ones. The radius within which the neighbors are counted was smaller for orthotopic Brn3a+RGCs (0.0552 mm) than for mRGCs or displaced RGCs (0.276 mm). In addition, the color scale was set to an increase of four neighbors for orthotopic Brn3a+RGCs and of three neighbors for the rest. These conditions were chosen because axotomized retinas were compared with intact retinas in which the number of orthotopic Brn3a+RGCs is around 80,000.

Finally, data from the neighbor maps, that give back the X,Y location of each cell using as 0,0 point the optic nerve, were translated as well to plots depicting the number of RGCs at a given distance from the optic nerve (data shown in these plots are based on data from 3–4 retinas per group).11,22 All maps were performed using Sigmaplot (SigmaPlot 9.0 for Windows; Systat Software, Inc., Richmond, CA, USA).

Statistics

Statistical analysis was done with GraphPad Prism v. 6 software (GraphPad, San Diego, CA, USA). Data are given as mean ± SD and differences were considered significant when P was less than 0.05.

Each cell type was analyzed independently along time within each lesion (e.g., [1] loss of Brn3a+RGCs after ONC, [2] loss of mRGCs after ONC, [3] Brn3a+RGCs after ONT, and so on). When data passed the normality test, comparisons were performed with 1-way ANOVA (post hoc Tukey’s test), and when data were not normal comparisons were done with Kruskall Wallis and post hoc Dunn’s test.

The loss of RGCs between ONC and ONT along time was compared using 2-way ANOVA analysis (variables: time and lesion) post hoc Bonferroni.

The temporal loss of Brn3a+RGCs (orthotopic and displaced) fitted a curve of exponential decay (exponential lineal combination) with an R2 (goodness of the fit) greater than or equal to 0.94. These data were modeled using Sigmaplot (SigmaPlot 9.0 for Windows; Systat Software, Inc.).

Results

We have quantified and assessed the distribution of the total number of (1) DAPI+ nuclei in the GCL, (2) orthotopic and displaced Brn3a+RGCs, and (3) orthotopic and displaced melanopsin+RGCs (Figs. 1A–C). DAPI+ nuclei were divided into two classes: fusiform, which most probably belong to endothelial cells, and round that correspond to glia, and neurons (RGCs and dACs; Fig. 1A, arrow and arrowhead, respectively). Of these, only round nuclei were plotted on isodensity maps. In addition, in retinas analyzed 15 months after ONT, γ-synuclein+ cells were quantified and mapped (see Fig. 7). All magnifications shown here were taken from the same retinal area: the centrotemporal quadrant, except in Figure 2 where they were taken from the dorso medial temporal retina.

Intact Retinas

Orthotopic and Displaced Retinal Ganglion Cells and All Nuclei in the GCL. The central and medial regions of the rat retina are more densely populated than the periphery (DAPI+ nuclei, Fig. 1E), and the dorsal central retina, that contains the rat visual streak58,59 has the highest densities of orthotopic RGCs (Figs. 1F–F’). The highest densities of mRGCs are located in a complementary fashion to Brn3a+RGCs, in the dorsal periphery, (Fig. 1, compare 1F’ with 1G). In agreement with our previous report, displaced Brn3a+RGCs are denser in the mediotemporal retina, and homogenously distributed in the nasal retina without following the distribution of their orthotopic counterpart (Fig. 1H), while displaced mRGCs, however, are located in the same areas as orthotopic mRGCs (Fig. 1I).10

Within the GCL, the total number of RGCs (the addition of orthotopic melanopsin+ and Brn3a+RGCs) amounts to 83,707
FIGURE 1. Topography of DAPI+ nuclei, RGCs, and m+RGCs in intact retinas. In all retinas, DAPI+ nuclei in the ganglion cell layer (A), Brn3a+RGCs (B, B’), and melanopsin+RGCs (C, C’) were identified in parallel. (B, C) Orthotopic, (B’, C’) displaced. All these magnifications were taken from the same frame, but the focus was changed to the INL in (B’, C, D) retinal cross-sections where a displaced Brn3a+RGC (left) and a displaced m+RGC (right) are observed in the INL. The arrow in (A) points to a round nucleus (neuron or glia), and the arrowhead to a fusiform nucleus (endothelial cell). Both were counted separately and only round nuclei were used for topographic analysis. The topography of each of these cells is shown in (E-I). (E) Isodensity map of round DAPI+ nuclei in the ganglion cell layer. (F-F’) Distribution of orthotopic Brn3a+RGCs using either an isodensity map (F) or a neighbor map (F’). (G-I) Neighbor maps of orthotopic m+RGCs (G), displaced Brn3a+RGCs (H), and displaced m+RGCs (I). The square in these maps is the area where the corresponding magnifications in (A-C’) were taken from (centrotemporal retina). At the bottom of each map is shown the number of cells quantified. Isodensity map color scale (E, F) goes from 0 cells/mm² (purple) to greater than or equal to 5200 nuclei/mm² or greater than or equal to 3200 Brn3a+RGCs/mm² (red). Neighbor maps color scale: (F’) Each color represents an increase in four neighbors in a radius of 0.0552 mm from purple (0–4 neighbors) to red (≥35–39 neighbors). (G-I) Each color represents an increase in three neighbors in a radius of 0.276 mm from purple (0–3 neighbors) to bright green (12–15 neighbors for displaced RGCs, or 44–47 neighbors for orthotopic m+RGCs). oRGC, orthotopic RGCs; dRGCs, displaced RGCs; D, dorsal; T, temporal; V, ventral; N, nasal; RR, right retina. Scale bars in (A, D, F’).
features of astrocytes or microglial cells, nor did they seem to be endothelial cells as they had round nuclei (Figs. 3A–A’). Hence, we interpret them as dACs (γ-synuclein⁺/dACs).

**Density of γ-synuclein⁺ Cells in the GCL.** All Brn3a⁺/RGCs expressed γ-synuclein but not vice versa, as there were many Brn3a⁻ cells that were γ-synuclein⁺ (Fig. 3A’; Table 2). Based on the previous experiment these cells were dACs. Quantification of the total number of γ-synuclein⁺ cells in intact retinas was not feasible because the axonal signal masked the cell bodies in the central retina. For this reason, the density of γ-synuclein⁺ cells was calculated in magnifications taken from the medial and peripheral retina. In the same frames, DAPI⁺ nuclei (round) and Brn3a⁺/RGCs were quantified as well to deduce the proportion of cells in the GCL. As shown in Table 2, approximately 61% of the GCL cells are Brn3a⁺ RGCs. 4% γ-synuclein⁺/dACs, and 10% are glial cells.

**Density of Glial Cells in the GCL.** The polymorphism of microglial cells and the overlapped processes of astrocytes (Figs. 3B, 3C) in the GCL did not allow automated quantification. Thus, we assessed their densities by sampling and manual quantification of round DAPI⁺ nuclei and astrocytes in some retinas and DAPI⁺ nuclei and microglial cells in another retinas (Table 2; Figs. 3B, 3C). This analysis indicates that approximately 9% of cells in the GCL are glial cells (4.9% astrocytes, 4.3% microglial cells) in agreement with the previous analysis.

**Table 2.** Density of γ-synuclein⁺ cells, RGCs, dACs, and Glial Cells in the GCL of Intact Retinas

<table>
<thead>
<tr>
<th></th>
<th>Cells/mm²/Retina</th>
<th>% in the GCL, DAPI⁺ Nuclei 100%</th>
<th>% vs. γ-synuclein⁺ cells</th>
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<tbody>
<tr>
<td><strong>Density of RGCs and γ-synuclein⁺ cells</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DAPI⁺ nuclei, round</td>
<td>7095 ± 148</td>
<td>90 ± 4</td>
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<tr>
<td>Brn3a⁺ RGCs</td>
<td>6381 ± 77</td>
<td>41 ± 0.8</td>
<td>45 ± 0.8</td>
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<tr>
<td><strong>Density of γ-synuclein⁺/dACs and glial cells</strong></td>
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<td></td>
<td></td>
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<tr>
<td>γ-synuclein⁺ dACs</td>
<td>3492 ± 33</td>
<td>49 ± 1.4</td>
<td>55 ± 0.8</td>
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<tr>
<td>(γ-syncells – Brn3a⁺/RGCs)</td>
<td></td>
<td></td>
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<tr>
<td>γ-synuclein⁻ cells, glia</td>
<td>713 ± 140</td>
<td>10 ± 1.7</td>
<td></td>
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<tr>
<td>(DAPI⁺ nuclei – γ-syncells)</td>
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<tr>
<td><strong>Astrocyte density</strong></td>
<td></td>
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<tr>
<td>DAPI⁺ nuclei, round</td>
<td>6769 ± 80</td>
<td>4.9 ± 0.3</td>
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<tr>
<td>Astrocytes, GFAP⁺</td>
<td>355 ± 21</td>
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<tr>
<td><strong>Microglial cell density</strong></td>
<td></td>
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<td></td>
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<tr>
<td>DAPI⁺ nuclei, round</td>
<td>7017 ± 101</td>
<td>4.3 ± 0.2</td>
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<td>Microglial cells, lba⁺</td>
<td>303 ± 8</td>
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From top to bottom: mean number ± SD of DAPI⁺ nuclei, γ-synuclein⁺ cells, and Brn3a⁺RGCs per mm² (8 samples/retina, n = 4 intact retinas). In each frame, the number of γ-synuclein⁺ dACs was calculated subtracting the number of Brn3a⁺ RGCs from the number of γ-synuclein⁺ cells and the number of glial cells subtracting γ-synuclein⁻ cells from the number of DAPI⁺ nuclei. Note that the 10% of nuclei that are not γ-synuclein⁺ corresponds to glial cells. Mean number ± SD of DAPI⁺ nuclei and astrocytes, DAPI⁺ nuclei and microglial cells per mm² (8 samples/retina, n = 3 intact retinas/marker). All these data were collected from frames acquired from the medial and peripheral retina. Percent of cells in the GCL were calculated using as 100% the number of DAPI⁺ nuclei.

**Table 1.** Number of Cells in Intact Retinas

<table>
<thead>
<tr>
<th></th>
<th>DAPI⁺ Nuclei (Round)</th>
<th>DAPI⁺ Nuclei (Fusiform)</th>
<th>Brn3a⁺ oRGCs</th>
<th>Melanopsin⁺ oRGCs</th>
<th>Brn3a⁺ dRGCs</th>
<th>Melanopsin⁺ dRGCs</th>
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<tr>
<td>Mean</td>
<td>217,406</td>
<td>22,075</td>
<td>81,415</td>
<td>2292</td>
<td>378</td>
<td>61</td>
</tr>
<tr>
<td>SD</td>
<td>11,067</td>
<td>2531</td>
<td>209</td>
<td>210</td>
<td>54</td>
<td>16</td>
</tr>
<tr>
<td>Orthotopic RGCs</td>
<td></td>
<td></td>
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<tr>
<td>Displaced RGCs</td>
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<tr>
<td>Total number of RGCs</td>
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<td></td>
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<tr>
<td>(orthotopic + displaced)</td>
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<tr>
<td>Mean ± SD of the total number of DAPI⁺ nuclei (round or fusiform) and Brn3a or melanopsin-positive RGCs in intact retinas (n = 6). oRGCs, orthotopic RGCs; dRGCs, displaced RGCs.</td>
<td>84,147 ± 2085</td>
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**Figure 2.** γ-synuclein is expressed by RGCs and amacrine cells. (A–A′′) Cross sections from intact retinas showing that γ-synuclein (green) is expressed in the GCL and the inner nuclear layer. In the GCL, γ-synuclein is expressed by RGCs (red), but also by other non-RGC cells (arrows in [A′′]). (B–B′′) Calretinin (green) and Brn3a (red) double immunodetection shows that calretinin is expressed by amacrine cells (inner nuclear layer), RGCs, and dACs (arrows in [B′′]). (C–C′′) γ-synuclein and calretinin immunodetection shows that in the GCL all calretinin+ cells express γ-synuclein ([C′′], arrows); these could be dACs and/or RGCs. (D–D′) In flat mounts analyzed 3 months after ONT there are few RGCs left (red), but many calretinin+dACs (green). (E–E′) All of the calretinin+dACs are γ-synuclein+. Drawings in (C, D) show the retinal areas where these images were acquired. Scale bars in (A, D).
Looking at the densities, the direct quantification of astrocytes and microglial cells (Table 2) resulted in $639 \pm 612$ glial cells/mm² (astrocytes plus microglia) while the inferred density (Table 2) was $713 \pm 130$.

**Different Response of the General RGC Population and m⁺RGCs to Long-Term Axotomy: ONC or ONT**

Seven days after axotomy, Brn3a⁺RGC death is clear and significant (Figs. 4A, 4B, 5A, 6A, 7; Table 3). From 1 month onward, orthotopic and displaced Brn3a⁺RGCs die gradually, with a similar rate, fitting a regression curve of exponential decay and their loss is quicker and more pronounced after ONT than after ONC (Figs. 4C–F, 4H, 5B, 5E, 6B–F, 7; Table 3). Indeed at 6 months 4% of RGCs survive after ONC and 1% after ONT. This different death rate could be due to a different response to either injury20,31–33 and/or to the fact that ONT was performed at 0.5 mm from the optic disk and ONC at 3 mm.30 The loss of displaced and orthotopic Brn3a⁺RGCs is diffuse and affects the whole retina. However, as observed in the topographic maps of orthotopic Brn3a⁺RGCs (Figs. 5, 6) and in the quantification plots (Fig. 8), the areas of the retina with higher densities, such as the central retina and visual streak are more affected. In fact, at the longest time intervals post axotomy analyzed here, surviving Brn3a⁺RGCs are preferentially located in the peripheral retina (Figs. 5E, 6F, 8).

m⁺RGCs behave differently: (1) their response to ONC and ONT is comparable (34% and 35% survival at 6 months, respectively), and (2) while at 7 days after ONC or ONT 52% or 43% of orthotopic Brn3a⁺RGCs were still alive, only 24% or 27% of orthotopic m⁺RGC were detected, which could mean a greater susceptibility to axonal injury for m⁺RGCs than the rest of RGCs.61 However, this is not so because at 1 month there is a significant increase in the number of detected m⁺RGCs (35%) that remains fairly constant up to 6 or 15 months after either axotomy (34%–44%). This behavior is also observed for displaced m⁺RGCs but it does not reach statistical significance. Nevertheless, at 15 months after ONT 35% or 28% of orthotopic or displaced m⁺RGCs survive, while only 0.8% or 1% of orthotopic or displaced Brn3a⁺RGCs are still present in

**Figure 3.** γ-synuclein⁺ cells, astrocytes, and microglial cells in the GCL of intact retinas. (A) Magnification from the same frame showing DAPI⁺ nuclei (A), γ-synuclein⁺ cells (green), and Brn3a⁺RGCs (red) (A'), and the coupled image (A''). (B–B’) Magnifications showing DAPI⁺ nuclei, GFAP⁺ astrocytes, and the merged image. (C–C”) Magnifications showing DAPI⁺ nuclei, Iba1⁺ microglial cells, and the merged image. Arrows in (B-B”, C) point to nuclei belonging to astrocytes or microglial cells, respectively.
the retina (Table 3; Fig. 7B). Topographically, mRGCs disappear from all over the retina, but more so in the dorsal region, as observed for Brn3a⁺RGCs (Figs. 5, 6). Curiously, 7 days after axotomy mRGCs have disappeared almost completely from the dorsal retina but from 1 month onward this retinal region recovers some of its mRGCs, indicating that the transitory downregulation of melanopsin occurs preferentially in dorsal mRGCs.61

Axotomy Does Not Cause Secondary Degeneration in the GCL

The number of DAPI⁺ round nuclei decreases numerically in parallel and in similar proportion as the number of RGCs (Table 3; Fig. 7). Topographically, the loss of GCL nuclei follows as well the loss of RGCs (Figs. 5, 6), because densities of DAPI⁺nuclei become lower in the areas of higher RGC loss. Therefore, axotomy results in selective loss of RGCs and does not affect other cells in the GCL, that is, axotomy does not cause a secondary neuronal (dACs), glial (astrocytes and microglial cells), or endothelial (fusiform DAPI⁺nuclei) death in the GCL, at least up to 15 months, the longest time-point studied.

To confirm these anatomical data, we analyzed by Western blot the regulation of Brn3a, calretinin and α-synuclein by ONT (Fig. 9). By 7 days after ONT, the expression level of Brn3a is already diminished, by day 14 almost undetectable and by 2 months no signal is observed. This is in accordance with the quantitative data and expression pattern of Brn3a: it is only expressed by RGCs and at 2 months approximately 2000 RGCs survive, because these are whole retinal extracts, detection of the remaining Brn3a is below the sensitivity of the technique.

Both, calretinin and γ-synuclein levels decrease after ONT, in agreement with the fact that RGCs express them. However, contrary to Brn3a their levels 2 months after ONT are still high, suggesting that amacrine cells have not died. While from retinal extracts we cannot tell whether the source of calretinin or γ-synuclein are the ACs or dACs, these data together with the anatomical analysis indicate that axotomy does not affect non-RGC neurons in the GCL.

Contralateral Retina

The longest time points analyzed were 6 months after ONC and 15 months after ONT. At neither of these time points did we observe RGC or DAPI⁺nuclei loss in the right fellow retinas, contralateral to the injured ones (Figs. 4G, 5F, 6G; Table 3). These data indicate that in adult rats, unilateral axotomy does not cause cell death in the GCL of the contralateral retina.

γ-synuclein 15 Months After ONT: Number and Distribution of Displaced Amacrine Cells

As seen earlier, within the GCL of intact retinas γ-synuclein expression was detected in cell somas and the RGC axons (Fig. 10A). In retinas analyzed at 15 months after ONT γ-synuclein signal was observed in the few remaining RGC axons, that at this time were tortuous and varicose63 (Figs.
in the somas of the few surviving Brn3a⁺RGCs (Figs. 10D'–D'') and in a very large number of dACs (Figs. 10B, 10D–D''). Because at this time point after ONT the axonal γ-synuclein signal was almost absent, it was possible to quantify the total number of γ-synuclein⁺ cells (Table 3) and to assess their distribution (Fig. 10E). Their topography is similar to the distribution of Brn3a⁺RGCs in intact retinas: They are denser in the central-medial retina and sparser in the retinal periphery.

Subtracting from the total number of γ-synuclein⁺ cells (111,354 ± 2236) the few remaining orthotopic RGCs (Brn3a⁺ plus melanopsin⁺), the population of γ-synuclein⁺dACs amounts to 109,874 ± 2234 cells/retina. Because the total number of round DAPI⁺ nuclei and orthotopic RGCs in intact retinas is 217,406 and 83,707, respectively, (Table 1) then, approximately 51% of the GCL cells (excluding endothelial cells) are γ-synuclein⁺dACs, 39% are RGCs and the remaining 10% are glial cells.
Figure 6. Topographic loss of DAPI⁺ nuclei, RGCs, and m⁺RGCs after optic nerve transection. (A–F) Spatial distribution of DAPI⁺ nuclei (isodensity maps), and of orthotopic and displaced Brn3a or melanopsin-positive RGCs (neighbor maps) in injured retinas analyzed from 7 days to 6 months after ONC. (G) Distribution of all these cells in a right retina contralateral to the left injured one analyzed 15 months after ONT. At the bottom of each map is shown the number of cells quantified. Color scales in first row (see Fig. 1 legend for explanation). Scale bars in the right most column; note that as the rat ages, the retina grows.
Axotomy selectively kills RGCs in the GCL: differences between the general RGC population and melanopsin⁺RGCs. (A) Scatterplots showing the total number of RGCs versus time post axotomy. The loss of Brn3a⁺RGCs adjust to an exponential decay curve, while m⁺RGCs after an abrupt downregulation of melanopsin, recover, and their number is maintained stable up to 15 months. (B) Bar graphs showing the percent ± SD of cells with respect to intact retinas (100%) at increasing time points after ONC or ONT. (C) Values of right retinas, contralateral to the injured ones, analyzed at 6 months after ONC or 15 months after ONT. *Significant difference between ONC and ONT (see Table 3 legend for details).
These percents deducted from quantifying the total number of γ-synuclein+ cells at 15 months after ONT (Table 3) and the total number of RGCs and DAPI+ nuclei in intact retinas (Table 1), are comparable to those inferred from sampling intact retinas (Table 2), indicating that γ-synuclein is expressed by all RGCs and most probably by all dACs. Finally, all these data together corroborate the percents of neurons and glia in the RGC layer and show that axotomy does not result in the loss of dACs, at least up to 15 months after the injury.

**DISCUSSION**

In this work, we have carried out a long-term analysis of the RGC layer after two types of axonal injury: intraorbital optic nerve crush or transection. Our data (summarized in Fig. 11) show that (1) there are approximately 217,406 cells in the rat retina (excluding endothelial cells); of these, 10% are glial cells, 50% dACs, and the remaining 40% are RGCs; (2) RGCs and m’RGCs respond differently to axotomy; (3) there is no protracted death of other GCL cells after axotomy, nor there is cell death in the contralateral uninjured retina (up to 15 months); and (4) γ-synuclein immunodetection identifies all neurons in the GCL (RGCs and dACs), which, incidentally, has allowed us to determine the number and distribution of γ-synuclein+dACs in the rat retina.

**Retinal Ganglion Cells**

The response of RGCs and m’RGCs (orthotopic or displaced) to axotomy differs in three main points: their reaction to ONC or ONT, their course of loss, and their rate of survival. Retinal ganglion cells die quicker after ONT than ONC, although this difference may be related not only to the type of lesion but as well to the site of the injury: farther from the optic head when crushing than when transecting; their loss adjust to an exponential decay curve and has two phases, one acute and very quick (up to 1 month) and one protracted and slow (from 1 month onward) as previously reported^30^, finally RGC loss is extreme, remaining 5% or 1% of them 6 months after ONC or ONT, respectively, in agreement with previous reports.^30,35^ In turn, m’RGCs respond similarly to both lesions (without apparent effect of the site or type of lesion); after an initial transitory downregulation of melanopsin their number remains quite constant and, finally, the percentage of detected m’RGCs at the latest time point (15 months) is much higher (approximately 35%) than that of RGCs (approximately 0.8%). Recovery of melanopsin expression occurs mainly in the dorsal retina by 1 month after the injury and, as shown here, once recovered it is stable up to 15 months after axotomy. The transitory downregulation of melanopsin expression is quite striking and occurs at the protein and mRNA level.83 This means that at 7 days there are at least as many alive m’RGCs as at 1 month. But because upon injury melanopsin is
downregulated, we do not know whether the loss of m\textsuperscript{+}RGCs is gradual or abrupt reaching its peak at 7 days or earlier, neither it is possible to be sure of their death because it may be that the 65% of m\textsuperscript{+}RGCs that are not detected are nevertheless alive but they permanently fail to express melanopsin.

Axotomy triggers RGC death across the whole retina, although their loss is higher in areas of higher density (central–medial retina) and surviving RGCs are found, preferentially, in the periphery. One could argue that peripheral RGCs have a longer axon and thus their retrograde death would be delayed. May as it be, at 7 days there is also loss of peripheral RGCs. m\textsuperscript{+}RGCs loss is also higher in the areas of an initial higher density, the dorsal hemisphere, but surviving ones are found all across the retina. These data are in agreement with recent reports showing the lack of parallelism in the topography of RGC and m\textsuperscript{+}RGC loss in a laser-induced ocular hypertension glaucoma model in mouse\textsuperscript{41} and rat.\textsuperscript{42}

The reasons why some RGC survive, and why those in the dorsal/central retina are more sensitive to injury is, to date, unknown. It could be that RGCs in the central retina are subjected to a higher metabolic stress since they are located in the area of gaze fixation where more light impinges on them, and light has a negative effect on RGC mitochondria.\textsuperscript{62–64} In addition, it might be related to the trophic and homeostatic support that RGCs receive from glial cells. Astrocytes are tightly linked to RGC axons (physically and physiologically);\textsuperscript{55,66} and they are more abundant in the central retina\textsuperscript{12,67} where the nerve fiber layer (RGC axons) is thicker as all RGC axons converge on their way to the optic disk. Thus, it is

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**Figure 8.** Peripheral RGCs are more resistant to axotomy. Line plots depicting the percent (Y-axes) of orthotopic Brn3a\textsuperscript{+}RGCs after ONC and ONT in the dorsal (top) or ventral (bottom) retina at increasing distances from the optic nerve (X-axes, in mm), considering 100% the number of Brn3a\textsuperscript{+}RGCs in intact (2 months, young adults) or right contralateral to the lesion (6–15 months after lesion, i.e., older animals) retinas at the same location. These two controls were used because retinas grow as the animal grows, and old animals have bigger retinas than young animals (see plot from 7 days after axotomy, where there are RGCs up to 5.5 mm, and plots from 3–15 months after axotomy where they reach ≥6 mm). This analysis shows that the percent of surviving RGCs is higher in the peripheral retina (between 4 and 5.5 mm from the ON).

**Figure 9.** Regulation of Brn3a, calretinin, and γ-synuclein after ONT. Western blotting showing the decrease of Brn3a expression (RGCs) already a 7 days after ONT, being almost undetectable at 14 days and absent at 2 months, in accordance with the quantitative data. Calretinin and γ-synuclein (RGCs and ACs) expression also diminishes after ONT, but at 2 months their levels are still high. Actin: loading control.
Figure 10. Topography of γ-synuclein+ cells 15 months after ONT: displaced amacrine cells. In control retinas, γ-synuclein signal is observed in RGC axonal bundles and neuronal somas in the GCL (A). Fifteen months after ONT, when almost all RGCs have died, there are no axonal bundles left and only spared varicose axons are observed. However, there are still many somas γ-synuclein-positive (B). Closer magnifications from intact retinas (C–C’) show that all Brn3a+ RGCs are also γ-synuclein, but no vice versa. This is more clearly observed in the injured retinas ([D–D’], asterisks). Because there are almost no RGCs left, and the morphology of γ-synuclein+ cells does not correspond to astrocytes or microglial cells, these must be dACs ([D–D’], arrows). In addition, note that some round DAPI+ nuclei do not colocalize with γ-synuclein or Brn3a ([D–D’], arrowheads); these must belong to glial populations. (E–G) Topography of DAPI+ nuclei, γ-synuclein+ cells (dACs), and Brn3a+ RGCs at 15 months after ONT. Squares in these maps mark the areas where the magnifications in (B) and (D–D’) were taken from. At the bottom of each map is shown the number of cells quantified. Color scales in last row (see Fig. 1 legend for explanation). Scale bars in (A, C, G).
tempting to think that a peripheral RGC with a longer intraretinal axon will have more astrocytes related to it, helping the RGC to cope better with an injury. Finally, the different survival of RGCs and m’RGCs as well as the fact that some peripheral RGCs die while others do not, indicates that there must be as well an intrinsic component, neuron-type specific.

Ganglion Cell Layer

Our data show that the GCL layer is composed of approximately 240,000 cells. Of these approximately 10% are endothelial cells, although there are probably more, because only endothelial cells in the vitreal surface of the blood vessels were photographed and counted. The remaining GCL nuclei belong to neurons (RGCs and dACs) and the glial populations (microglial cells and astrocytes).

Because at all times post lesion we have observed a decrease of round nuclei that could be accounted by the RGC loss, both in numbers and topography, we conclude that axotomy does not cause secondary death of other GCL cells, most of which are dACs (see below), even 15 months after the lesion. These data are in agreement with a long term study in the porcine (9 months 68) and rat (9 months 34) retina after axotomy.

It is worth highlighting here that in a model of anterior ischemic neuropathy in mice, dACs died if the insult was severe and compromised the retinal integrity, but they did not if the insult was moderate and only affected RGCs.49 Similarly, in a rat and mouse model of laser-induced ocular hypertension, RGCs are selectively lost in pie-shaped sectors of the retina without any effect on other GCL cells.41,42,56,69 Thus, RGC death alone does not seem to have an impact on the viability of dACs. Their functionality might, however, be impaired as it has been recently reported in two mouse models of ocular hypertension.70

\(\gamma\)-synuclein and Displaced Amacrine Cells

Our data indicate that \(\gamma\)-synuclein is expressed by all RGCs and dACs. This is quite a controversial result because it has been published that \(\gamma\)-synuclein mRNA is only expressed by RGCs in humans and mice,71,72 and its level decreases concomitantly with RGC death after axotomy or experimental glaucoma in rats and mice.73,74 At present, we do not know why there is this difference between the previous results using \(\gamma\)-synuclein mRNA in situ hybridization and our present studies using \(\gamma\)-synuclein protein immunodetection. It may be that the antibody used here recognizes as well other members of the synuclein family such as alpha-synuclein, since their aminoacid sequence is highly homologous. However, in mice75 around 100,000 \(\gamma\)-synuclein\(^+\) cells were identified by in situ hybridization (i.e., mRNA) in the GCL of control retinas, and this is 2-fold the number of RGCs traced from the optic nerve in this species,76 thus supporting our results.

In addition, several experiments here show that \(\gamma\)-synuclein protein is expressed by all neurons in the GCL, RGCs, and dACs: (1) in controls as well as in axotomized retinas there are many \(\gamma\)-synuclein\(^+\) cells that are not Brn3a\(^+\) but that express calretinin, a marker of amacrine cells,41,42,56,69 (2) \(\gamma\)-synuclein signal is observed in cells located in the inner nuclear layer, where amacrine cells are found, (3) and Brn3a expression decreases below the sensitivity of Western blotting 2 months after axotomy, a downregulation that is caused by the death of RGCs (less than 3% of RGCs survive at 2 months after ONT) and by a lower expression of Brn3a by the surviving ones.21 However, while both calretinin and \(\gamma\)-synuclein levels decrease after axotomy since both are as well expressed by RGCs, at 2

\[\text{FIGURE 11. Summary. The GCL of the rat retina is composed of 10\% glial cells, 50\% displaced amacrine cells, and 40\% RGCs. Within the RGC population, 3\% are melanopsin\(^+\)RGCs. Upon axotomy, Brn3a\(^+\)RGCs die slower after ONC than after ONT and their course of loss is exponential. m’RGCs behave similarly to both injuries, and their number remains quite constant from 1 month onwards. Fifteen months after axotomy, 99\% of Brn3a\(^+\)RGCs and 65\% of m’RGCs are lost. Thus, at this time point, 55\% of the remaining RGCs are melanopsin-positive.} \]
months after ONT their expression level was still high indicating that other retinal cells are expressing them, and (4) quantitative analyses of the GCL composition in control retinas and in 15 months, axotomized retinas concord and support the fact that γ-synuclein is expressed by dACs. According to our results in rat, between 49% and 51% of the GCL cells (excluding endothelial cells) are dACs, 39% and 41% are RGCs, and the remaining 9% and 10% are glial cells, in agreement with a previous report.19 Having into account only RGCs and γ-synuclein+ dACs, the number of neurons in the GCL reaches almost 200,000, being 57% dACs and 43% RGCs. These percentages are in agreement with other reports in mammals where the calculated percent of dACs ranged from 40% (hamster and mouse9,49,53), to 80% (cat 77), with intermediate values found in monkey (47%78), squirrel (50%79), and rat (62%19,54), and indicate that γ-synuclein is probably expressed by all, or most of, dACs. The topography of dACs matches the distribution of RGCs, as shown in monkey79 and human,15 and for catecholaminergic dACs in rat.52 To date there is not a marker to identify all dACs, which are very diverse (up to 10 different subtypes in rats)48 and specific subpopulations are identified using mainly antibodies against neurotransmitters or Ca	extsuperscript{2+} binding proteins.50–52,80,81 Here, we show that double immunodetection of Brn3a and γ-synuclein may be used to differentially study RGCs and dACs.

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