Anatomical and functional damage in experimental glaucoma
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Glaucoma is a progressive neurodegenerative disease caused by retinal ganglion cell (RGC) loss. One important risk factor for glaucoma is elevated intraocular pressure and thus many animal models are based on spontaneous or induced ocular hypertension (OHT). Using these models it has been shown that RGCs initially suffer an impairment of the active axonal transport that progresses to a lack of passive diffusion along the axon. This axonal damage eventually causes the death of the parent RGCs in pie-shaped sectors of the retina, but there is also diffuse RGC loss, without involving displaced amacrine cells. Recent data show that OHT results in a protracted insult to the inner and outer retina that causes functional alterations and ultimately, degeneration and death of cones.

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Introduction
While the end point of glaucoma is well recognized as the loss of RGCs and their axons, which causes vision impairment and eventually, blindness [1–3], the exact mechanism causing retinal ganglion cell (RGC) death is not known. Most of the working hypotheses identify the optic nerve (ON) head as the site of injury, and some of the currently proposed mechanisms are crush-like injury to axonal bundles, ischemia, loss of trophic support by astrocytes, and activation of phagocytic astrocytes (reviewed in [4]).

One of the main risk factors in glaucoma is an elevated intra-ocular pressure (IOP) [5] and thus the majority of the animal models developed to study the anatomical [6,7], molecular [8,9,10,11,12], and functional [13] changes associated with this disease, and to test neuroprotective therapies [14,15–19], are based on experimentally induced or genetically acquired elevation of the IOP (for review see [20,21]).

In our studies in the laboratory we have induced ocular hypertension (OHT) by laser photocoagulation (LP) of the periferal and episcleral vessels of adult albino Sprague-Dawley rats and Swiss mice [6,13,22] which resulted in an increased IOP very early after LP (12 hours) that reached 35 mm of Hg in both species (basal IOP values were 10.2 and 15.7 mm of Hg in rat and mouse, respectively). Significant elevation of the IOP was maintained for 1 week in mouse and nearly 4 weeks in rat, but the damage produced in the retina was found to be similar in both models. In this review we summarize the main anatomical and functional changes in the mouse and rat retina subjected to LP (Figure 1).

Identification of retinal neuronal populations
In rodents, RGCs share their location in the innermost layer of the retina, the ganglion cell layer (GCL), with the equally numerous population of displaced amacrine cells [23–25]. Hence, to study RGCs it is necessary to univocally identify them. Because the vast majority of rodent RGCs project to the superior colliculi, these neurons can be traced by applying neuronal tracers to these brain areas. Alternatively, tracers can be applied to the ON stump, though this procedure involves an axonal damage and thus retinas need to be analyzed before RGC death commences [26,27]. Fluorogold (FG) or its analog hydroxystilbamidine methanesulfonate (OHSt) are tracers that are transported retrogradely and actively from the axonal terminals to the neuronal soma and have been shown to be effective in the rodent visual system [28,29]. The dextan of tetramethylrhodamine (DTMR) on the other hand, diffuses passively through the axon, filling the cell bodies, proximal dendrites and axons [30]. Thus, when tracing RGCs, it is worth having in mind that if a lesion impairs the axonal flow, only the RGCs with a competent axonal transport will be detected, while those that are alive but functionally impaired may be missed. Identification of RGCs may also be achieved by in situ hybridization of RGC-specific mRNAs (such as γ-synuclein [7,31]) or by immunodetection of RGC-specific proteins (such as Brn3a; brain-specific homeobox/POU domain protein 3A [32,33]). Brn3a is a transcription factor expressed by majority of RGCs in rats [33] and mice [32] whose expression is maintained after injury and neuroprotective strategies [34].
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The intraretinal RGC axons express the highly-phosphorylated high molecular isoform of the neurofilament triplet (pNFH), a protein that changes its expression pattern upon RGC injury [35,36]. Thus, immunodetection of this protein is a good tool to assess RGC axonal integrity.

Displaced amacrine cells cannot be immunodetected because of the lack of specific markers. However, it is possible to counter-stain all the GCL nuclei with DAPI (4',6-diamidino-2-phenylindole) after Brn3a immunodetection and assume that the majority of non Brn3a+ nuclei would be displaced amacrine cells and the rest endothelial, astrocytic and microglial cells [22].

In the outer retina are the photoreceptors, rods and cones. In mouse and rat there are two types of cones: those responding to short wave length light or S-cones, and those responding to medium-long wave length light or L-cones. Each type of photoreceptor, rod, S-cone, and L-cone, can be specifically immunodetected because each one expresses a type of opsin: rhodopsin, S-opsin and L-opsin, respectively [37].

Automated routines: quantification and distribution of retinal neurons
In our group we have developed computerized routines that allow the automated quantification of the whole population of RGCs, either traced or immunodetected, DAPI+ nuclei in the GCL, and S-cones and L-cones [22,28,29,37]. Thus, once processed, the flat mounted retinas are photographed and the desired cell population quantified. With these data it is possible to generate isodensity maps of the retina that show the normal distribution of a given cell and/or its topographical loss after an insult.

Active retrograde axonal transport impairment precedes obstruction of passive diffusion along RGC axons
By combining active retrograde transport, passive diffusion along the axon, and Brn3a immunodetection, it is possible to monitor the course of OHT-induced degeneration and death of RGCs [6*,13*]. The loss of traced-RGCs, mainly in pie-shaped sectors of the retina, was observed as early as 8 days after OHT and did not progress beyond 1 week in mice, and 2 weeks in rat. However, in the retinal areas lacking traced-RGCs there were still Brn3a+ RGCs, and quantitative data showed that at those early time points the number of surviving RGCs was significantly higher than the number of traced-RGCs. Because FG and OHSt are actively transported, it was possible to conclude that OHT impairs RGC retrograde axonal transport within the first week. But, is the passive diffusion along the axon also altered? In other words, is OHT causing a mechanical obstruction that blocks axonal transport? To solve this question FG or OHSt and DTMR were used in combination. Between 1 and 2 weeks after OHT, the areas of the retina containing...
DTMR+ RGCs were larger than those containing FG+ or OHSt+ RGCs. However, by 21 days in rat and 17 days in mouse there was a very close match between the areas with FG+ or OHSt+ RGCs and the areas with DTMR+ RGCs. Thus, it appears that in both species OHT initially damages active axonal transport, which is followed by a mechanical insult that blocks the passive diffusion of the dextran, and this is in agreement with several other studies in rats [38,39] and mice [7,40,41].

Selective retinal ganglion cell loss
The lack of back-labeled RGCs appeared as early as one week after OHT, amounted to approximately 80% of the RGC population, and did not progress further in mice or rats whose retinas were analyzed up to 9 or 12 weeks after LP, respectively. This lack of progressive RGC loss was surprising because in previous studies of axotomy-induced or ischemia-induced RGC loss, this process was shown to be progressive [42,43]. However, when Brn3a was used to determine RGC survival, it was observed that between one and five weeks after LP there was progressive RGC loss, indicating that the apparent loss identified by back-labeled RGCs reflected an alteration of the retrograde axonal transport rather than RGC loss. By three weeks after LP in rats and four weeks in mice, the numbers and distribution of back-labeled and Brn3a+ RGCs coincided, indicating that in the retinal areas where at 1 week there were no traced RGCs, these surviving, dysfunctional RGCs died progressively [6*,13*].

Topographically, RGC loss was mainly sectorial and occurred in the dorsal retina, coinciding with the retinal areas devoid of tracing. Consequently, surviving RGCs were located, in general, in pie-shaped sectors of the ventral retina, with their apex pointing to the optic nerve. In addition, a scattered damage was observed either throughout the retina or in the sectors with surviving RGCs.

Interestingly, by combining DAPI nuclear counterstaining and Brn3a immunodetection, it was observed that in the areas lacking Brn3a+ RGCs, that is, in the areas of RGC loss, there was no loss of DAPI+ nuclei [22]. In fact, after automated quantification of the total number of DAPI+ nuclei and Brn3a+ RGCs two important observations were made: firstly, in control retinas the number of the DAPI+ nuclei was approximately double the number of RGCs, in agreement with the fact that in the GCL half of the neurons are RGCs and the other half displaced amacrine cells [24,25] and, secondly, in the OHT-injured retinas the decrease in DAPI+ nuclei correlated with the decrease in RGCs. These data indicate that in the GCL, OHT specifically damages RGCs but not non-RGC neurons (i.e. presumed displaced amacrine cells) [22,44].

The sectorial and selective death of RGCs suggests that OHT may be causing a compression-like injury somewhere in the optic nerve head that affects axonal bundles exiting the retina, with a highly retinotopic arrangement. It has been reported that there is an OHT-dependent ATP decrease in the optic nerve axons that produces a decrease in their metabolic capacity, which causes their dysfunction and eventually their loss [45]. Metabolic impairment has been observed, as well, in the fortified astrocytes of the optic nerve head after OHT [46*] and additional evidence indicating a major role of astrocytes in the pathology of ONH axons has been provided [31**].

The nerve fiber layer shows signs of a crush-like injury
Degenerating neurons insulted by either disease or trauma show an altered organization and/or metabolism of cytoskeleton proteins [47]. For instance, in the healthy retina, pNFH expression is circumscribed to the mature portion of the intraretinal axons, that is, in the central-medial retina, while in the periphery, few, thin pNFH positive (pNFH*) axons are observed. Upon axonal injury, three aberrant patterns emerge: firstly, pNFH signal in the medial and peripheral retina increases significantly, secondly, axons show highly pNFH* bead-like accumulations along their length, and thirdly, some RGCs somas become pNFH* [36]. The temporal pattern of these abnormalities as well as the frequency of each abnormal expression depends on the axonal lesion. For example, after intraorbital nerve crush few axons show bead-like accumulations of pNFH, but there are plenty of pNFH+ somas, however after intraorbital nerve transection the observation is the opposite [36]. These differences allow the correlation of different retinal diseases with a crush or transection-like temporal course of degeneration. The distribution of pNFH* RGCs was compared to the topological distribution of FG+ RGCs or OHSt+ RGCs, in mice or rats retinas. It was observed that the RGCs that still survive in sectors lacking back-labeled RGCs express pNFH in their soma, in a pattern closer to that observed after ON crush than after ON transection [6*]. Moreover, the aberrant expression of pNFH in the RGC somas was observed preferentially within the retinal sectors devoid of traced-RGCs, while none of the traced-RGCs showed this aberrant pNFH staining. These observations suggest that pNFH was expressed in the somas of those RGCs whose retrograde transport was impaired and that in the retinal sectors with maintained retrograde transport the axons and RGC somas had not been injured.

Glial cells and inflammatory response in the retina and optic nerve head
While the glaucomatous optic neuropathy affects mainly RGCs, glaucoma is a disease that also affects the brain [48,49,50,51,52*].

The glial and immune response in glaucomatous eyes and their optic nerves is a rising area of interest.
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[53–55,56,57,58]. In the OHT-retina there is an activation of Müller cells and astrocytes [59] that precedes RGC loss. The role of the activated astrocytes in the retina is not clear, but recent reports suggest that they play an important part in the immune response and activation of the complement [53,57] that follows OHT. In the ONH the activated astrocytes become phagocytic [31**] and internalize degenerating RGC axonal evulsions. It is worth highlighting that this glial and immune response is not circumscribed to the injured retina. In fact it has been reported that in the contralateral, non-OHT eye, there is an increase of astrocytes, microglial cells and MHC-II molecules [60*,61*], while in the dorsal lateral geniculate nucleus and superior colliculi, retinoreceptin areas in the brain, astrocytes are also activated [59].

Effects of OHT on the inner and outer retina

In glaucomatous human eyes and in OHT-animal models, effects on the outer nuclear layer (ONL) have been reported [62,63*,64,65]. To further investigate this issue we analyzed the electroretinographic recordings (ERG) from OHT-injured mice retinas [15*]. This approach allows simultaneous functional assessment of the innermost, inner and outer layers of the retina. It was observed that as soon as 24 hours after the OHT, the amplitude of the pSTR (positive component of the scotopic threshold response) that is associated with the RGC activity, was significantly diminished compared with values before LP. The same effect was seen for the major ERG waves that reflect the functionality of photoreceptors (a-wave) and the bipolar and Müller cells (b-wave). Furthermore, this decrease lasted up to 12 weeks, the longest time analyzed, suggesting that the damage caused to retinal functionality by OHT is persistent and affects both the inner and the outer retina. Moreover, quantitative analysis of morphological parameters in the inner and outer retinal layers, showed a progressive disorganization and anatomical changes of the inner and outer retinal circuitries [63*] that validate the permanent functional alterations of the ERG recordings.

These functional and anatomical data are additionally supported by the observation that in rat retinas analyzed 1 month after LP, there is a significant loss of S-cones and L-cones that progresses further by 6 months. This loss of cones does not parallel the sectorial death of RGCs but is diffuse throughout the retina. To determine whether the cone death was a consequence of the loss of RGCs or a secondary effect of the OHT, we analyzed the cone population at the same time points, but after ON transection, a lesion that specifically injures RGCs [33,34,66]. In these retinas with RGC loss without OHT there was no significant loss of cones [67]. Overall, these experiments indicate that OHT is also inflicting a protracted damage to the inner and outer retina, perhaps of ischemic nature to the choroidal vasculature, which is likely to be independent of that caused to the innermost retina.

Concluding remarks

Glaucoma is a heterogeneous and complex disease whose causes and progression will be better understood by extensive research in animal models, and when possible, in human samples. To date we have learned from animal models that raising the IOP above normal levels induces the death of RGCs that occurs even if the IOP returns to basal values one week later. In addition, OHT causes a protracted damage to both inner and outer retinal layer neurons, affecting their functionality and morphology. It is therefore important that the neuroprotective therapies designed to fight glaucoma are directed not only to safeguard RGCs and their axons, but also to preserve the inner and outer retina.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


*This review summarizes the current concepts of glaucomatous disease of the optic nerve, their clinical variabilities as well as their present treatments.*


*This article shows the effects in adult albino rats of laser-induced ocular hypertension on the population of retinal ganglion cells (alterations in the retrograde axonal transport, alteration in the nerve fiber layer of the retina, and geographical loss of these neurons).*
This review summarizes the current knowledge of the different models used up to date to investigate the effects of ocular hypertension in adult rats.


In this article the authors show that at the optic nerve head there is a population of phagocytic astrocytes that constitutively internalize large axonal evolutions. These astrocytes show upregulation of phagocytosis markers (Mac-2) upon glaucoma induction and their phagocytic capacity depends on γ-synuclein. Furthermore, mice lacking γ-synuclein fail to upregulate Mac-2 and develop more axonal loss after glaucoma.


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58. Tezel G: The immune response in glaucoma: a perspective on the roles of oxidative stress. Exp Eye Res 2011, 93:178-186. This review proposes that alterations in the immune system regulation due to accumulating risk factors may shift the physiological equilibrium, from a protective immunity into a neuroinflammatory degenerative process. The review provides a perspective on the complex interplay of cellular events during glaucomatous neurodegeneration.


This paper shows in a mouse model of ocular hypertension (OHT), that there are widespread and persistent changes in GFAP (astrocytes) and MHC-II (antigen-presenting cells) expression in the retina contralateral to the OHT-retina. Thus, the use of the contralateral eye as an internal control in experimental induction of unilateral IOP should be reconsidered.

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This article shows the deleterious long-term effects in adult albino mice of laser-induced ocular hypertension on the innermost, inner and outer retinal layers.


