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Anatomical and Molecular Responses Triggered in the Retina by Axonal Injury

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1. Introduction

After receiving their visual input from photoreceptors via the intermediate neurons (bipolar, horizontal and amacrine cells), retinal ganglion cells (RGCs) relay this intraretinally pre-processed visual information to the centres in the brain for further processing. Thus, RGCs are the only projecting neurons in the retina, and their axons form the optic nerve. These central nerve axons originate at the RGC bodies located in the neural retina, travel along the nerve fibre layer converging to the optic disk and finally exit the eye through the optic nerve head (ONH).

Glaucomatous optic neuropathy is a complex, multifactorial and heterogeneous human chronic neurodegenerative disease that characteristically affects the RGC population and their axons. It is a slowly progressive form of optic nerve damage and blindness that begins with loss of peripheral vision and is followed by gradual narrowing of the remaining central vision.

Glaucoma currently affects over 70 million people (Quigley, 1996). Second only to cataract is the leading cause of irreparable blindness. This disease affects all age groups, but is more frequent in the elderly (Quigley & Vitale, 1997). Glaucoma is broadly classified into three main groups, i) primary open glaucoma (POAG); ii) primary acute closed angle glaucoma; and iii) primary congenital glaucoma. Among these, the most frequent is POAG (Shields et al., 1996). In this type of glaucoma the main risk factors are age and an elevated intraocular pressure (IOP). In most cases, lowering the IOP has a beneficial effect on RGC survival. However, some individuals with low or even normal IOP develop this disease with associated RGC loss, yet others with high IOP do not develop glaucoma or lose these neurons. Because of this, it has been proposed that there are genetic variants in humans that affect the relative susceptibility or resilience of RGCs to the same insult. This hypothesis is based on recent works showing that RGCs from different mice strains have an intrinsically different resistance to optic nerve injury. Li et al (2007) analyzed the survival of RGCs in 15 mice strains after optic nerve crush, and found that RGCs from the DAB/2J strain were the most resistant, while the ones from BALB/cByJ were the most susceptible. Interestingly, this

rank of relative vulnerability to optic nerve crush does not translate to other lesions or regions of the CNS, which means that the genetic background has a complex effect on the resistance to injury of specific neurons from specific areas. In addition to this putative implication of the different human genotypes on developing glaucoma, there are 25 loci that have been found linked to POAG. However, only 3 genes are known to cause glaucoma if mutated (*Myocilin*, *WDR36* and *Optineurin*), though at least 30 more genes have been reported to be associated with this neuropathy (for reviews see Ray & Mookherjee, 2009; Fuse, 2010). This is further complicated by the fact that environmental factors and systemic disorders play a role in glaucoma, making it a multifactorial disease. Because, to date, there is not a cure for this neurodegenerative disease many efforts are being devoted to understand its molecular, cellular and etiological causes.

Two of the more popular hypotheses to explain the initial glaucomatous damage to the ONH are the mechanical and the vascular theory of glaucoma. The mechanical theory states that the raised IOP obstructs RGC axons, thus causing their degeneration and death. With respect to the vascular theory, there is evidence of vascular dysregulation in some forms of glaucoma (Flammer & Mozaffarieh, 2007) that may endanger ONH blood flow. Thus, this might induce an ischemia-reperfusion nerve injury. Additionally, the role of glial cells at the ONH has received increasing attention (Ramirez et al., 2010; Nguyen et al., 2011).

2. Animal models of glaucoma

The high incidence of glaucoma, and the lack of non-invasive approaches to study in humans the subjacent neuronal responses, make necessary the use of animal models to understand the pathogenesis of this neuropathy (for a more detailed account of these models see Morrison et al., 2010).

2.1 Spontaneous, transgenic or deficient mice

Some mice strains have spontaneously developed mutations that produce a chronic elevation of the IOP, and result in a phenotype similar to chronic age-related glaucoma. The best studied is the DAB/2J inbred line (John et al., 1997, 1998). This strain is homozygous for two mutations related to melanosomes accumulation. Thus, it has been suggested that the initial pathology of the anterior chamber is due to an abnormal iris pigmentation and melanosome structure (John, 1998) though it is not the only cause as there is also an immune component involved (Chang et al., 1999). These mice start to exhibit increased IOP by 8-13 months old. The course of the disease, however, varies on individual colonies and environmental factors.

Mice can be genetically manipulated, and so transgenic mice have been generated targeting proteins implicated in the aqueous outflow or those for which human mutations related to glaucoma have been described. Examples of such transgenic mice are the ones with the targeted mutation in the α1 subunit of collagen type 1 (Aihara et al., 2003) or the ones expressing the Tyr437His mutation in myocilin (Zhou et al., 2008), associated with human patients developing early onset glaucoma (Alward et al., 1998). Recently, it has been reported that mice deficient of Vav 2 and/or Vav 3 proteins (guanine nucleotides exchange factors for Rho guanosine triphosphatases) show early onset of iridocorneal angle changes and elevated intraocular pressure (Fujikawa et al., 2010). Interestingly, *VAV2* and *VAV3* genes seem to be candidates for associated genes in Japanese open-angle glaucoma patients.

2.2 Induced

The majority of the induced animal models of glaucoma are based on experimental elevation the IOP. Most of these models are carried out in rats and in mice. There are some advantages of these experimental models over the spontaneous models. First, the onset of the elevated IOP is predictable, making possible to determine the temporal course of the degenerative events in retina and optic nerve. Second, unilateral IOP elevation leaves the fellow eye as internal control, hence accounting for inter-individual variability. Third, IOP increase occurs soon after the treatment, and thus it is not necessary to wait long periods for the animal to develop the symptoms. However, these models have in common a high variability, in terms of the IOP reached and the damage induced.

The goal of any experimental procedure to elevate the IOP is to inhibit aqueous humour outflow without interfering with the eye's ability to produce this humour. There are four main approaches, scarring the anterior angle chamber by hypertonic saline injection (rats, Johnson et al., 1996; Morrison et al., 1997; mice, Kipfer-Kauer et al., 2010) laser photocoagulation (rats and mice, Salinas-Navarro et al., 2009a, 2010, Cuenca et al., 2010), venous cautery (Shareef et al., 1995) and injection of microbeads in the anterior chamber (mice, Cone et al., 2010; Chen et al., 2011; rats and mice, Sappington et al., 2010).

Laser photocoagulation, a method first described in monkeys (Gaasterland & Kupfer, 1974) and later in rats (Ueda et al., 1998), is based on the photocoagulation of the trabecular mesh alone or in combination with episcleral veins and/or perilimbar vessels (WoldeMussie et al., 2001; Levkovitch-Verbin et al., 2007; Salinas-Navarro et al., 2009a, 2010, Cuenca et al., 2010). The IOP increase occurs because the drainage of the aqueous humour is obstructed by the closure of the intratrabecular spaces and of the majority of drainage channels. In this model, RGC loss occurs mainly in pie-shaped sectors located in the dorsal retina, with their apex pointing to the optic nerve disk. It has been described that there is first an impairment of the anterograde active axonal transport, followed by a retrograde degeneration of RGCs (Salinas-Navarro et al., 2009a, 2010).

3. Intraorbital optic nerve crush (IONC)

Another good approach is to study separately the RGC response to axotomy. Intraorbital optic nerve crush (IONC) is based on crushing the entire retinofugal pathway at the exit of the eye. This model specifically injures all the RGC axons, avoiding any ischemic insult. Thus, as a model to study the underlying causes of RGC death by axonal injury, IONC is clean and predictable.

Traumatic axonal injury to the optic nerve, either by complete transection or crush, is a well established model (Bray et al., 1987) that can be evaluated morphologically (Nadal-Nicolas et al., 2009; Parrilla-Reverter et al., 2009a, 2009b), molecularly (Agudo et al., 2008, 2009) and functionally, and has been thoroughly analyzed by our group, both in rats and mice (Alarcon-Martinez et al., 2009, 2010). These lesions induce a quick and massive death of RGCs (Peinado-Ramon et al., 1996; Chidlow et al., 2005; Sobrado-Calvo et al., 2007; Agudo et al., 2008, 2009; Galindo-Romero et al., 2011; Sanchez-Migallon et al., 2011). After IONC, the whole RGC population is injured and degenerates within a short period of time. In addition, as long as the lesion is inflicted at the same distance from the optic disk (Villegas-Perez et al., 1993), there is a small variability among different animals.

IONC has several advantages: i) the insult affects the whole RGC population. This is important because the population of RGCs only represents approximately 1% of the retinal

cells, thus, there is a dilution effect that may hinder the study of protein or transcript regulation in the affected RGCs. This dilution effect is higher if only part of the RGC population is affected by the lesion, as it happens in the increased IOP models (Guo et al., 2010); ii) the temporal course of RGC degeneration is well established; iii) there is a body of reports in which the molecular correlates associated to this injury have been studied (Agudo et al., 2008, 2009). Importantly, this lesion mimics the crush-like injury observed in the optic nerve after IOP increase (Salinas-Navarro et al., 2009a, 2010) but leaves aside the possible ischemic insult, thus easing the understanding of the obtained data.

3.1 Early signs of retinal ganglion cell degeneration

Degeneration of CNS axons after a traumatic insult was first described by Ramón y Cajal (Ramón y Cajal 1914) and his co-workers (Tello 1907; Leoz y Arcuate 1914). They observed many degenerative changes such as axons that broke up rapidly and axons ending in club-like shapes known as terminal swellings, ganglioform swellings or cytoid bodies. Later, in 1967, Reimer and Wolter (Reimer & Wolter, 1967) after analyzing the retinas of 3 patients with ocular or systemic diseases and the cordotomized spinal cord of a fourth patient, concluded that these degenerative events were the common reaction of CNS axons to damage. These authors stained the axons with the silver-carbonate or silver nitrate methods. Nowadays axons are identified by immunodetecting proteins specifically expressed by them. Furthermore, because specific proteins (or their isoforms) are expressed in specific neuronal compartments, it is possible to study them separately. Neurofilaments (NF) are the main cytoskeletal proteins in mature axons. They are assembled from three subunits, high (H), medium (M) or low (L). NF suffer post-translational modifications wherein the most relevant is phosphorylation. Phosphorylation of NF, particularly of NFH (pNFH) is considered to decrease their transport rate (for review see Perrot et al., 2008). Thus, highly phosphorylated isoforms of pNFH are found in the mature axons while dephosphorylated isoforms are expressed in the soma and dendrites (Perrot et al., 2008). Degenerating neurons insulted by either disease or trauma show an altered organisation and/or metabolism of pNFH that is associated with several human neurodegenerative diseases or experimental paradigms (for review see Al Chalabi & Miller, 2003). The levels of NF transcripts decrease after different retinal injuries, such as ischemia, excitotoxicity or optic nerve injury (McKerracher et al., 1990a, 1990b; Chidlow et al., 2005b; Agudo et al., 2008). This regulation correlates, after optic nerve transection, with an impairment of the axonal transport if the RGCs are committed to death, but not if RGCs are allowed to regenerate along peripheral nerve grafts (McKerracher et al., 1990a, 1990b; Vidal-Sanz et al., 1991, 2000). In healthy retinas pNFH expression is circumscribed to the mature portion of the intrarretinal axons, i.e. in the central-medial retina, while in the periphery few, thin pNFH positive axons ($pNFH^+$) are observed. In fact, quantitative analyses show that $pNFH^+$ axons occupy 61%, 14% and 0.37% of the central, medial and peripheral retinal surface, respectively (Parrilla-Reverter et al., 2009a). In addition, few or none RGC somas express this isoform. After optic nerve injury this pattern changes dramatically (Drager & Olsen, 1981; Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988, Parrilla-Reverter et al., 2009). Three days after IONC the expression pattern of pNFH resembles that of a control retina when analyzed at low magnification. However, at high magnification two aberrant patterns emerge. Firstly, pNFH signal in the medial and peripheral retina increases significantly, occupying 24% and 9% of the middle and peripheral retinal surface, respectively. Secondly, some RGCs somas, preferentially in these retinal areas, become pNFH positive ($pNFH^+$RGCs) (Figure 1A). With

time, these abnormalities progress further (Figure 1B-D). The distribution of pNFH+RGCs widens, their number increases, peaking at 14 dpl, and the intensity of their staining augments. pNFH+RGCs appear first in the medial and peripheral retina, then they spread centripetally throughout the whole retinal surface. In general, pNFH+somas are weakly stained (Figure 1A arrow), but from day 14 onwards strongly stained ones appear, mainly in the temporal retina (Figures 1B-C arrows).

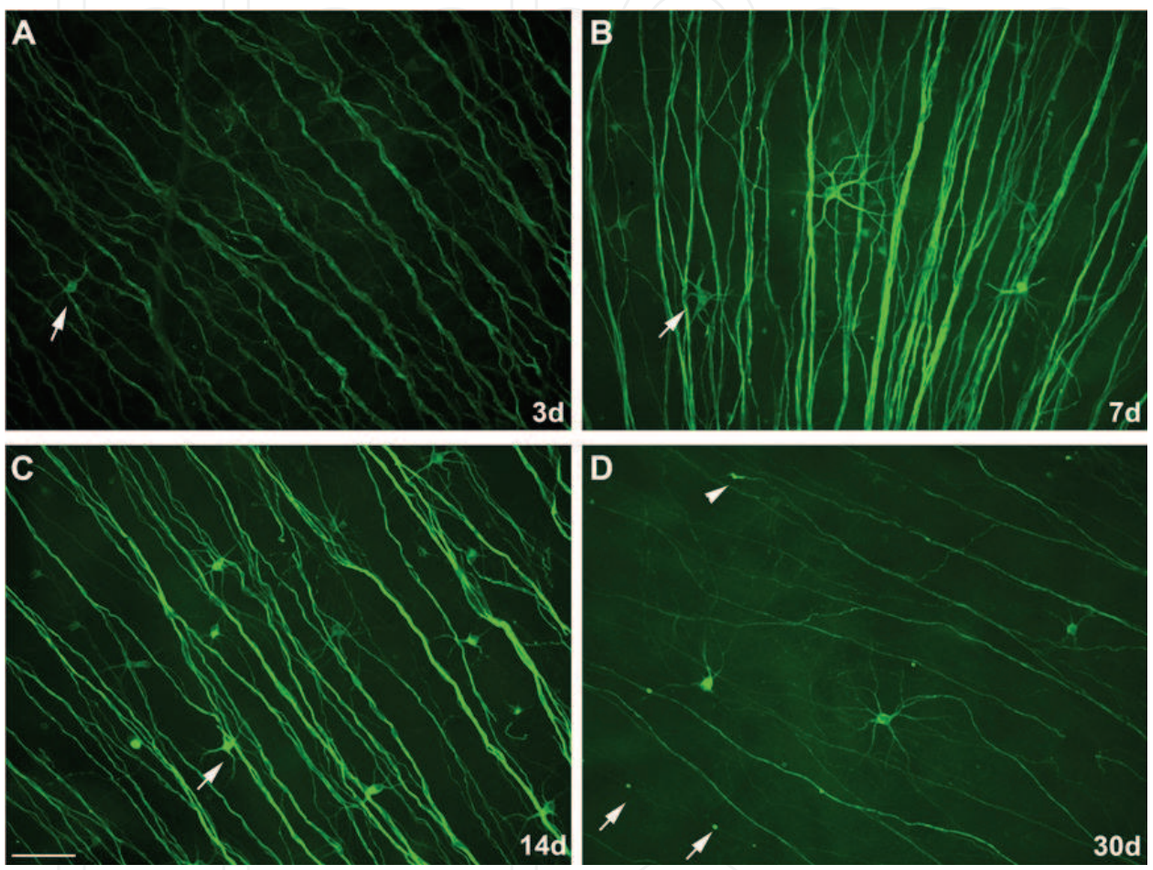

Fig. 1. Aberrant expression of pNFH after IONC. Microphotographs acquired from the retinal periphery of IONC-injured retinas at increasing days post-lesion (dpl). A: As soon as 3 dpl pNFH signal is observed in the retinal periphery and in some RGCs' bodies that have become pNFH positive (arrow). This abnormal expression progresses (B, C), and at 7 and 14dpl there are more pNFH+RGCs somas with variable signal intensity (weak as arrow in A, medium as arrow in B, or strong as arrow in C). D. 30 dpl the general intensity of pNFH signal has decreased, there are fewer axons but still scattered pNFH+somas are observed. In addition, club-like degenerating axonal tips (arrow-head) and axons with rosary-like pNFH accumulations are observed (arrows). Bar: 100μm.

With respect to the axonal expression of pNFH, in the medial and peripheral retina is maintained above the control levels at least till 30dpl. In the central retina, where the RGC axonal bundles converge to exit the eye, pNFH expression decreases gradually. At late times post-lesion axonal degenerative events similar to those described by Ramón y Cajal are

observed, such as rosary-like intra-axonal accumulations and club-like axonal ends (Figure 1D, arrows and arrowhead).

The aberrant expression of pNFH in the ganglion cell layer is common to other insults that affect the RGC either primarily, like optic nerve transection or IOP increase (Salinas-Navarro et al., 2009a, 2010; Parrilla-Reverter et al., 2009a; Nguyen et al., 2011), or secondarily, like phototoxicity (Villegas-Pérez et al., 1996, 1998; Marco-Gomariz et al., 2006; Garcia-Ayuso et al., 2010). It is worth noting than even though all these insults cause the same aberrant patterns, their quantity and time course differs. For example after intraorbital nerve transection there are fewer pNFH$^+$somas but in turn, early after the injury, there are more axons showing rosary-like accumulations of pNFH. These differences allow the correlation of different retinal diseases with a crush or transection-like temporal course of degeneration. In relation to this, our group has shown that after increasing the IOP in mice and rats, the RGCs located in the sectors of RGC loss express pNFH in their soma in a pattern closer to that observed after optic nerve crush than after optic nerve transection (Salinas-Navarro et al., 2009a; 2010). In conclusion, the pathological expression of pNFH is an early event that marks RGC degeneration and it is observed as early as 3 days following the insult.

3.2 Retinal ganglion cell loss after IONC

3.2.1 RGC identification and quantification

RGCs share their location in the ganglion cell layer with the equally numerous population of displaced amacrine cells (Drager & Olsen, 1981). In order to study the RGC population is then, necessary to distinguish them from the amacrine neurons. There are several techniques that specifically label RGCs. These are based either on tracing them from their projection areas, which in rodents are mainly the superior colliculi (SCi) (Linden & Perry, 1983; Thanos et al., 1987) or by detecting proteins or transcripts specifically expressed by these neurons (Barnstable & Drager, 1984; Chidlow et al., 2005). Retrograde tracing is based on applying onto the retinorecipient areas in the brain a fluorescent tracer, among which fluorogold is the most utilized. This tracer is taken up by the RGC terminals and transported actively through their axons till their somas in the retina (Figure 2A). In mice or rats, fluorogold applied onto the SCi detects 97.5% or 98.4% of the total RGC population, respectively (Salinas-Navarro et al., 2009b, 2009c).

Few proteins are known to be specifically expressed by RGCs, among them are γ-synuclein, Bex1/2, Thy1, NeuN or Brn3a (Barnstable & Drager, 1984; Quina et al., 2005; Bernstein et al., 2006; Soto et al., 2008; Buckingham et al., 2008; Nadal-Nicolas et al., 2009). Detection of γ-synuclein mRNA (Soto et al., 2008; Nguyen et al., 2011) has been used to investigate the fate of mice RGCs after ocular hypertension. Immunodetection of the transcription factor Brn3b has been used to estimate RGCs in a mice model of ocular hypertension (Fu & Sretavan, 2010). Detection of another member of this family of transcriptions factors, Brn3a, is a reliable method to identify the whole population of rats and mice RGCs in control retinas, after insults such as optic nerve axotomy, ocular hypertension or photoreceptor degeneration (Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009a, 2010; Garcia-Ayuso et al., 2010; Galindo-Romero et al., 2011) and importantly, to test the efficacy of neuroprotective therapies (Sanchez-Migallon et al., 2011).

Quantification of RGCs can be done by sampling and manual counting, or by automated routines. Automated quantification is an objective routine that allows counting the whole population of a given cell (Soto et al., 2008; Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009b, 2009c; Ortin-Martinez et al., 2010) while avoiding the tiresome and time consuming

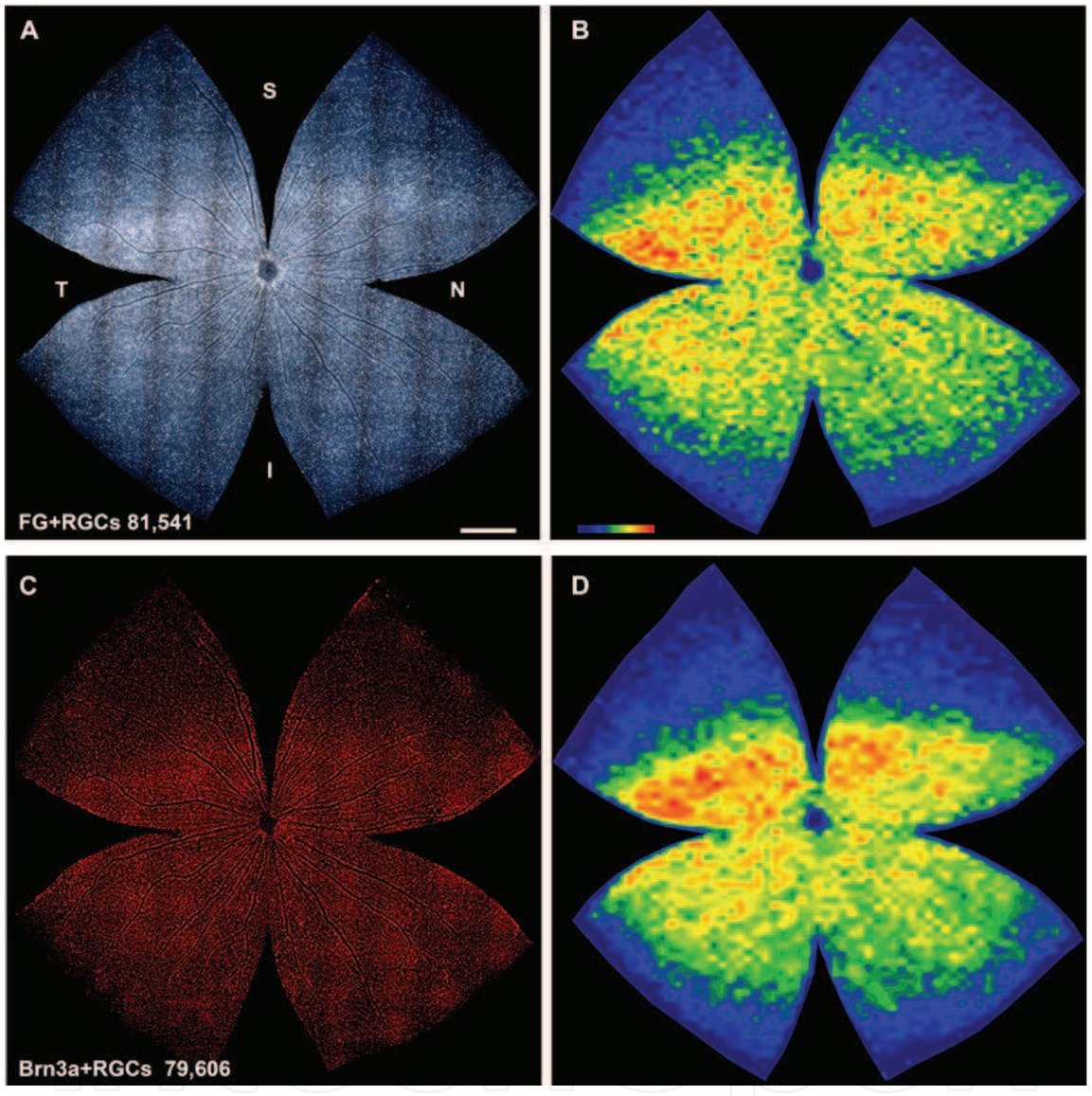

Fig. 2. Retinal ganglion cell distribution in the adult rat. In A and C is shown the same whole-mounted control rat retina where RGCs have been identified by retrograde tracing with fluorogold (A) or immunodetection of Brn3a (C). In this retina, the number of FG or Brn3a positive RGCs has been automatically quantified (A and C bottom left). In B and D are shown the isodensity maps generated from these quantitative data showing the spatial distribution of FG^{+}RGCs (B) and of $Brn3a^{+}$RGCs (D). The density colour-scale goes from 0 (purple) to 3,500 or higher (red) RGCs/mm^2 (B, bottom left). S: superior I: inferior, N: nasal, T: temporal. Scale bar: 1mm.

effort of sampling and manual-counting. These routines have been shown to be successful to quantify RGCs identified by their Brn3a (Figure 2C) or γ-synuclein expression and can be applied to control or injured retinas (Soto et al., 2008; Nadal-Nicolas et al., 2009; Nguyen et al., 2011). In addition, automated routines allow the generation of detailed isodensity maps (Figure 2 B,D). These maps are useful to assess the distribution and densities of the studied cells in control retinas as well as to understand their topographical loss after an insult (Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009a, 2009b, 2009c, 2010 Garcia-Ayuso et al., 2010; Ortin-Martinez et al., 2010; Galindo-Romero et al., 2011; Sanchez-Migallon et al., 2011).

By using this approach our group has reported that RGCs are not homogenously distributed in the retina (Figure 2 B,D). In fact, their lower densities are located in the periphery (blue colours) while their higher ones are found in the superior retina (red-oranges), along the nasotemporal axis, wherein the highest densities are located in the superotemporal quadrant. Furthermore, because in this retinal area L-cones reach their maximum densities, paralleling the RGC distribution, and the S cones reach their lowest, this area of high RGC density has been proposed to be the visual streak of rats (Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2009b, 2009c; Ortin-Martinez et al., 2010).

Both, tracing and immunodetection of RGCs have advantages and disadvantages. For instance, by retrograde tracing is possible to assess the number of RGCs that maintain a functional retrograde axonal transport. However, if the lesion impairs the axonal flow only the RGCs that have a competent axonal transport will be detected and the alive but impaired ones will be missed. On the other hand, while Brn3a or γ-synuclein detect those RGCs that are still alive independently of their axonal transport state, they are not useful to identify transport failures. Combination of tracing and Brn3a immunodetection has served to demonstrate that after IOP increase there is first a loss of the retrograde active transport that is followed by the death of the affected RGCs (Salinas-Navarro et al., 2009a, 2010).

3.2.2 Temporal course of RGC loss after IONC.

RGC loss after axonal injury depends on two main factors. The type of lesion, crush or transection and the distance from the eye where the lesion is inflicted (Villegas-Perez et al., 1993). In albino Sprague Dawley rats, when the optic nerve is completely crushed 3mm away from the eye, the loss of Brn3a$^+$RGCs is first significant 5 days later (Nadal-Nicolas et al., 2009). At this time point the percentage of surviving RGCs is a 48% of the original population. This loss progresses quickly, decreasing to 28 and 14% of the original population at 9 and 14 days, respectively (Figure 3).

If the population of RGCs is identified by tracing, the loss of RGCs is significant later, at 7 dpl, and by day 14pl the percentage of survival is 29% (Nadal-Nicolas et al., 2009; Parrilla-Reverter et al., 2009b). This discrepancy is explained by the different nature of each marker. While Brn3a is an endogenous protein that is expressed as long as the RGC is alive (Sanchez-Migallon et al., 2011), fluorogold is an exogenous molecule that persists in the retina for at least 3-4 weeks after application (Selles-Navarro et al., 1996; Gomez-Ramirez et al., 1999). This means that fluorogold positive but already dead RGCs will be detected and will only disappear from the retina when the phagocytic microglia clears them.

Comparing these results with the time course of RGC loss in our model of IOP increase by laser photocoagulation (LP), it is observed that there is a massive death of these neurons

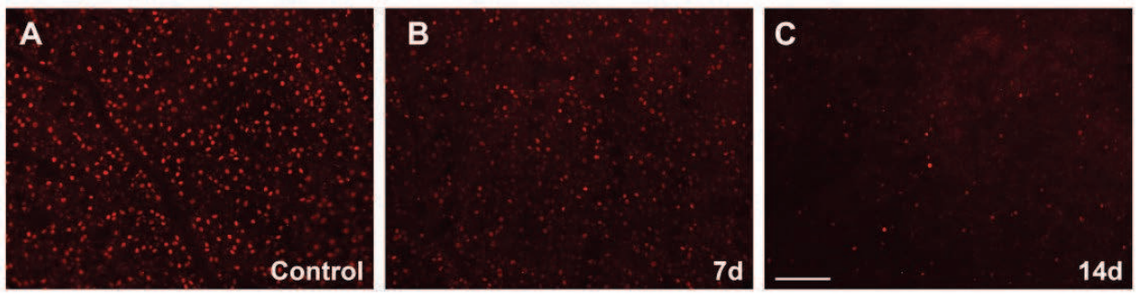

Fig. 3. Temporal loss of Brn3a⁺RGCs after optic nerve crush. Microphotographs from flat mounted retinas in which RGCs have been detected by their expression of Brn3a. A: control; B: 7 days after IONC, C: 14 days after IONC. Bar: 200 μm.

within the first 2 weeks post-LP, which accounts for 53% and 81% of the RGC population at 8 and 14 days post-LP, respectively (Nadal-Nicolas et al., 2009; Parrilla-Reverter et al., 2009b; Salinas-Navarro et al., 2010). Thus, the mean number and course of RGC loss in both models are similar. However there are two important differences. First, in terms of the damage caused IONC induces a consistent injury, while LP insult is highly variable among animals and so, in some retinas the RGC loss accounts for almost 80% of the RGCs, whilst in others only 40% of the original RGC population is affected. Second, after IONC, RGC death is diffuse and affects the whole retina (Figure 4) whereas after LP, RGC death occurs mainly in the dorsal retina in pie-like sectors which are devoid of RGCs, though it has been also observed a diffuse RGC loss in the rest of the retina.

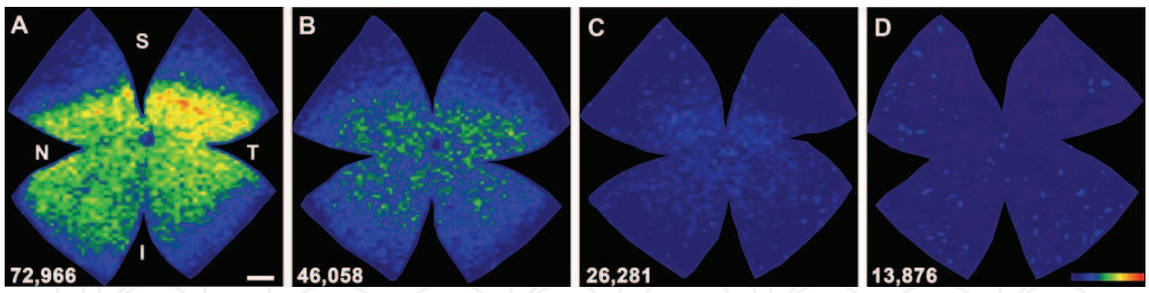

Fig. 4. Temporal loss of RGCs after intraorbital nerve crush. Isodensity maps from IONC-injured retinas analyzed at 2 (A), 5 (B), 9 (C) and 14 (D) days post-lesion. This injury induces a quick and diffuse loss of RGCs that affects the whole retina. At the bottom of each map is shown the number of Brn3a⁺RGCs quantified in the retina wherefrom the map has been generated. Colour-scale goes from 0 (purple) to 3,500 or higher (red) RGCs/mm² (D, bottom right). S: superior I: inferior, N: nasal, T: temporal. Scale bar: 1mm.

3.3 Molecular causes underlying RGC loss after IONC

Why do RGC degenerate upon axonal injury? The main theory is that there is a withdrawal of trophic factors from the retinorecipient areas in the brain once the retina is deafferented. That is the reason why many neuroprotective therapies have been based on trophic factors delivery (see below). However, these therapies are only successful to a point, since the best protection only lasts up to 9 days post-lesion wherefrom the RGCs decay quickly.

This prompted us to do an extensive array analysis comparing the transcriptome regulation in naive retinas with retinas extracted at different times post-IONC (12h, 24h, 48h, 3d and 7d) (Agudo et al., 2008). This analysis rendered plenty of data, in fact over a thousand of genes along time showed an altered regulation compared to control retinas. Clustering of the regulated sequences revealed that they were related to several biological processes. Among them, the most significant were: cytoskeleton and associated processes (pEASE: 8,20E-13), primary metabolism (pEASE: 7,80E-14), protein metabolism (pEASE: 1,8E-29), immune response and inflammation (pEASE: 4,80E-11), RNA metabolism (processing and translation pEASE: 3,90E-25), cell cycle (pEASE: 1,20E-05), extracellular matrix remodelling (pEASE: 4,10E-07) and sensory perception of light (pEASE: 1,40E-09). Cell death, as expected, was highly regulated (97 genes, pEASE 2,20E-7). It was surprising to observe a transient down-regulation of genes related to phototransduction such as opsins, rhodopsins and phosducin, since IONC only affects RGCs. The down-regulation of photorreceptor genes may be a reflection of the arrest in transcription observed in the retina soon after injury (Lindqvist et al., 2002; Casson et al., 2004). Thus, while IONC specifically injures RGCs, also induces a retinal response that, even though is not lethal for other retinal neurons, has an effect on them.

Because the more dramatic effect of IONC is the RGC death, we focused our array analyses on the 97 death-related regulated genes. These were further clustered, and the most relevant sub-clusters were inflammation and death receptors, both implicated in triggering the extrinsic pathway of apoptosis and DNA damage, caspases, cell cycle deregulation and stress response, all of which are linked to the induction of the intrinsic pathway of apoptosis (Figure 5). There was a seventh group of death-related genes clustered under lysosomal cell death. A current body of evidence points to a role of lysosomes, and more specifically of cathepsins, in apoptosis. In fact, the "lysosomal pathway of apoptosis" is a phenomenon widely recognized (reviewed in Guicciardi et al., 2004) which would act through caspase activation, and hence activating the intrinsic pathway of apoptosis. However, it has been reported the role of autophagy in RGC death triggered by axotomy (Koch et al., 2010).

Autophagy is a highly regulated pathway that involves the degradation of cytoplasmic organelles or cytosolic components by the lysosomes, thus it is possible that the activation of the lysosomal cell death observed in our arrays analysis is in fact playing a part in autophagy and apoptosis (Figure 5). This is based on the fact that there is a crosstalk between both processes (Zhou et al., 2011) which is complex, and sometimes opposing. Indeed, autophagy can be a cell survival pathway to suppress apoptosis (Yang et al., 2010) or can lead to cell death, either in collaboration with apoptosis or as an alternative mechanism when apoptosis is defective. This crosstalk is mediated, in part, by calpains, which are activated in axotomized RGCs (Paquet-Durand et al., 2007; Agudo et al., 2008, 2009). Calpains cleave and activate Atg5, which is one of the autophagy effectors (Zhou et al., 2011).

Several of these death-effectors have been shown to be expressed by the primarily injured neurons, the RGCs (Cheung et al., 2004; Agudo et al., 2009). Among them, Tnfr1, Caspases 3 and 11, Calpain 1 and Cathepsins B and C are of special interest because each is linked to a different pathway that ends in cell death. The majority of these proteins showed an up-regulation as soon as 12h post-IONC, peaking at 48h, well before the time point when the anatomical RGC loss is first significant (5 days, see above). Thus, these data indicate that axotomized RGCs enter the path to death quickly after the injury, and even though pro-

survival or protective mechanisms are concomitantly activated (Schwartz 2004; Levkovitch-Verbin et al., 2007) they are not enough to overcome the death signals. It is worth highlighting that Tnfr1 is up-regulated in our IONC model and in glaucomatous retinas from human patients (Tezel et al., 2001).

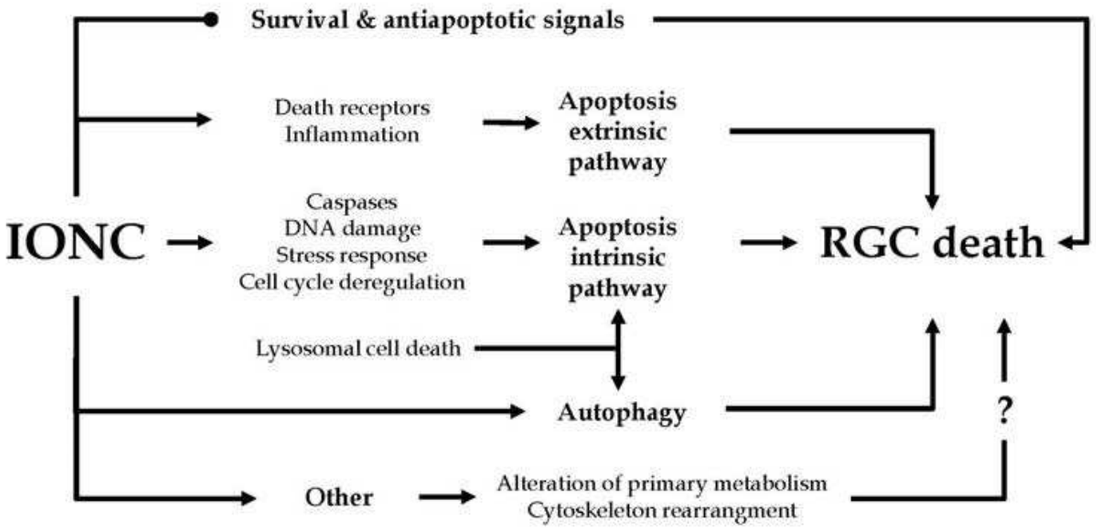

Fig. 5. Scheme summarizing the retinal response to IONC leading to death. After IONC, several pathways ending in cell death are up-regulated. This is accompanied by a down-regulation of survival signals, which, in turn may end in cell death. In addition, there are changes that affect the maintenance of basic cellular functions, such as the cytoskeleton or primary metabolism (catabolism, transcription, biosynthesis), that may tip the scale against survival.

Finally, we have compared our array results with the array analyses of Guo et al, 2010 (Table 1). These data show that there is a common response to both types of injuries. In fact all the altered genes follow the same trend (up or down-regulation). These genes are mainly related to inflammation, apoptosis, cytoskeleton and extracellular matrix remodeling. Guo et al, (2010) analyzed the gene expression patterns occurring in the retina in response to elevated IOP induced by hypertonic saline injection. Their big contribution has been that the analysis was performed in extracts from whole retinas and in extracts from cells isolated from the ganglion cell layer. They observed that several genes were regulated in the whole retina and in the isolated cells (both, in the table) while others where only detected in the whole-retinal extracts (retina, in the table) and *vice versa*. The latter will be genes regulated only in the RGC layer, whose regulation might be lost in a whole-retinal extract due to the dilution effect. The main differences found among both sets of experiments were that in the isolated cells there was not regulation of inflammation, which was the leading process observed in whole retinal extracts. In turn, protein biosynthesis and metabolism were the most regulated categories in the isolated cells. This does not mean that inflammation does not take part on RGC death, but rather that cell isolation helps the determination of RGC specific mechanisms.

In conclusion, even though IONC only mimics part of the pathogenesis of glaucoma only by compiling data from different models will be possible to understand the retinal response to injury. Given the complexity of these molecular events, the development of neuroprotective therapies must, probably, be combinatory. This panorama might be further complicated because basic cellular functions, such as primary metabolism and cytoskeleton maintenance are highly altered after IONC and IOP increase, opening up the possibility that the failure to preserve the cellular homeostasis might be the actual trigger of this complex regulation.

Gene name	Gene Symbol	Gene Ontology	IOP increase	IONC
Growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	Activation p38/JNK pathway	↑ (both)	↑
Clusterin	Clu	Apoptosis and cell survival	↑ (retina)	↑
Suppressor of cytokine signaling 3	Socs3	Apoptosis and cell survival	↑ (both)	↑
Tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	Apoptosis and cell survival	↑ (both)	↑
Lysozyme	Lyz	Cell wall catabolism, cytolysis	↑ (both)	↑
Ceruloplasmin	Cp	CNS iron homeostasis and neuroprotection	↑ (retina)	↑
Neurofilament, light polypeptide	Nfl	Cytoskeleton	↓ (both)	↓
Transgelin 2	Tagln2	Development	↑ (retina)	↑
Olfactomedin 1	Olfm1	Development	↓ (both)	↓
Secreted acidic cysteine rich glycoprotein	Sparc	Extracellular matrix	↑ (both)	↑
Alpha-2-macroglobulin	A2m	Immune response and inflammation	↑ (retina)	↑
Beta-2 microglobulin	B2m	Immune response and inflammation	↑ (retina)	↑
Interferon-inducible protein variant 10	Ifitm3	Immune response and inflammation	↑ (retina)	↑
Signal transducer and activator of transcription 3	Stat3	JAK/STAT cascade	↑ (both)	↑
Signal transducer and activator of transcription 1	Stat1	JAK/STAT cascade	↑ (retina)	↑
Cd63 antigen	Cd63	Lysosomal protein	↑ (both)	↑

Gene name	Gene Symbol	Gene Ontology	IOP increase	IONC
Synuclein, gamma	Sncg	Nervous system development	↓ (retina)	↓
Carbonic anhydrase 14	Car14	Primary metabolism	↓ (retina)	↓
Carnitine o-octanoyltransferase	Crot	Primary metabolism	↑ (retina)	↑
Udp glycosyltransferase 1 family, polypeptide a1	Ugt1a	Primary metabolism	↑ (both)	↑
Lipocalin 2	Lcn2	Protection of MMP-9, iron transport	↑ (both)	↑
Arginyl aminopeptidase	Rnpep	Proteolysis	↑ (both)	↑
Corneal wound healing related protein	Mak10	Response to injury	↑ (retina)	↑
Hnrnp-associated with lethal yellow	Raly	RNA metabolism	↓ (retina)	↓
Tax1 binding protein 3	Tax1bp3	Signalling	↑ (both)	↑
Potassium voltage gated channel, member 2	Kcnd2	Synapsis	↓ (both)	↓
Solute carrier family 6, member 6	Slc6a6	Transport	↓ (retina)	↓
ATPAse, NA+/K+ transporting, beta 1 polypetide	Atp1b1	Transport	↓ (both)	↓

Table 1. Genes commonly regulated in glaucoma and after IONC.
↑ gene upregulation, ↓ gene downregulation. See text for further explanations.

4. RGC neuroprotection

Neuroprotection refers to therapies aimed to prevent, stop or at least delay the degeneration that follows CNS trauma. For a neuroprotective agent to be successful it must be directed to a target in the retina, it must be applied before the death of the neurons, it should be safe and it should work on animal models. Once fulfilled these criteria, the next steps are to design the pharmaceutical forms to deliver the compound to the human retina at the appropriate concentrations and to conduct the clinical trials.

After the injury, which cells are the candidates for neuroprotection? Obviously, not the neurons already dead, thus, it is important to know the temporal course of RGC degeneration to narrow the temporal window to intervene. Those cells in the process of degeneration are candidates if their somas are still healthy. In this case, function will only be restored if protection of the cell bodies is followed by regeneration and reconnection. Having this in mind, neuroprotection can be attained by preventing the degeneration of the healthy fibres, or by delaying it in the cell bodies of the damaged fibres.

Neuroprotection can be divided into two main groups: therapies that target specific genes or proteins related to cell death or therapies that increase the resistance of the injured neurons to the trauma. The former approach, though more specific, is very difficult for several reasons: i) it would be necessary to identify all the implicated signals; ii) some of these signals are pleiotropic, playing roles in survival or death depending on the delicate balance of other proteins; iii) some proteins while pathological if over-expressed, are essential for survival; thus, blocking their expression might be deleterious for the neurons that are not yet compromised; iv) signalling pathways are interconnected. Consequently, to tailor an effective therapy all this must be taken into account, and probably should imply the manipulation of several proteins. This requires careful analyses of the pathways and their interactions. Furthermore, the targeted proteins/genes should be head of pathways, provided that the downstream signals do not bifurcate into survival or death. Alternative targets could be the effectors, either proteins (i.e. caspases) or metabolites (i.e. ceramide).

4.1 Targeting specific signals

One way of targeting specific pathways is the use of inhibitors. Rho Kinase (ROCK) is a serine-threonine kinase that regulates the organization of the actin cytoskeleton (Mueller et al., 2005). Its activation is linked to the morphological changes observed during the execution phase of apoptosis (Coleman & Olson, 2002). Tura et al, (2009) showed that after IONC, the intravitreal administration of the ROCK inhibitor H-1152P produced two effects: a significant reduction of apoptosis in the ganglion cell layer and a reduction of the reactive gliosis.

Among the best characterized pro-death proteins is BAX. This protein associates with the permeability transition pore of the mitochondrial inner membrane opening it and thus inducing a series of events such as a disruption of the electrochemical membrane gradient, a disruption in ATP production and release of cytochrome C (Marzo et al., 1998). The released cytochrome C in turn, activates the caspase cascade. Li et al. (2000) demonstrated that in Bax deficient mice 2 weeks after optic nerve crush, there was less than a 10% of cell loss in the ganglion cell layer, which was significantly lower than the 41.3% observed in wild type mice. This survival was not due to a slower death-rate in the Bax-/- mice, since 4 weeks after the lesion there was not further cell loss. However, the lack of BAX did not prevent the ganglion cells to undergo early changes in response to optic nerve crush, such as a decrease in the average size of their nuclei. It is worth noting, that the deficiency in this gene protected cells in the ganglion layer after optic nerve crush but not after experimentally induced excitotoxicity. This means that in response to different insults, ganglion cells activate different pathways of cell death.

The discovery of RNA interference (RNAi) has opened new avenues to investigate in neuroscience. RNAi is an endogenous mechanism that silences gene expression after translation. Silencing is highly sequence-specific and ends with the targeted mRNA cleaved into smaller fragments which results in the inhibition of protein synthesis (for review see Sontheimer, 2005). RNAi is mediated by small interference RNA (siRNA). The use of RNAi to knock-down specific pro-death proteins in axotomized RGCs has been only used after complete transection of the optic nerve (Lingor et al., 2005). Lingor et al. showed that in retinas treated with siRNAs against Apaf-1 (component of the apoptosome) or c-Jun (immediate response gene) there was a significant RGC survival compared to control ones.

However, anti-Bax siRNAs did not increase RGC survival; this does not correlate with the experiments carried out on Bax-deficient mice. This discrepancy might be due to the different species (rats *vs* mouse) used and on the lesion, because even though crush and transection are both axonal traumas and there are many commonly regulated genes (Agudo et al., 2008, 2009) there are differences in the temporal course and amount of these regulations.

In glaucoma, RNAi has been directed mainly to lower the IOP (for review see Mediero et al., 2009) rather than to induce a direct RGC survival. Targeting adrenergic receptors, acetylcholinesterase and ATPases, decreases the IOP. This approach has minimal side effects and the reduction of IOP lasts almost 5 days. This is important at the clinical level because the regime is simpler than pharmacological treatments, particularly eye drops that may require several administrations at day. In addition to lower the IOP, RNAi is been tested in vitro to knock-down the gain of function of mutated genes associated to glaucoma, such as myocilin (Li et al., 2009).

4.2 Therapies to increase the resistance of the injured neurons

Administration of neurotrophins to the injured retina is one of the most successful therapies. Our group has shown that a single intravitreal injection of neurotrophin 4 (NT-4), ciliary neurotrophic factor (CNTF), or brain derived neurotrophic factor (BDNF) at the moment of the injury delays IONC-induced RGC death (Parrilla-Reverter et al., 2009b). At 7dpl all of them prevent almost completely the loss of RGCs. However, while at 12 dpl NT-4 and BDNF still protect, CNTF does not. Nevertheless, none of these factors were able to rescue the injured RGCs permanently. It is possible that a single injection is not sufficient. To solve this there are strategies to achieve a sustained expression of neurotrophins: transfection by viral vectors, cell based delivery approaches or microspheres loaded with the neurotrophin of choice (reviewed in Dahlmann-Noor et al., 2010). Another approach consists on the selective stimulation of trophic factor receptors using specific agonists. Thus, it has been shown that a selective agonist of TrkB (BDNF receptor) causes a long term TrKB activation and significantly delays RGC degeneration after IOP increase and after optic nerve transection (Bai et al., 2010).

Brimonidine, an α-2 adrenergic agonist, has been shown to neuroprotect RGCs after retinal ischemia (Lafuente et al., 2001,2002; Lafuente Lopez-Herrera et al., 2002; Vidal-Sanz et al., 2001a,b, 2007; Aviles-Trigueros et al., 2003; Mayor-Torroglosa et al., 2005; Lonngren et al., 2006), after optic nerve crush (Wheeler et al., 1999) and after laser-induced ocular hypertension (Wheeler & WoldeMussie, 2001; Lambert et al., 2011). A randomized trial comparing brimonidine and timolol (β-adrenergic antagonist) in low tension glaucomatous patients, has shown that the loss of visual field is statistically lower in brimonidine treated-patients than in those treated with timolol, thus documenting, for the first time, its neuroprotective effect in human diseases (Krupin et al., 2011).

5. Conclusions

Optic nerve injury induced either by IOP increase or by direct trauma, causes profound structural, functional and molecular alterations in the primarily injured neurons, the RGCs, as well as in the rest of the retina. The consequences of these alterations are permanent as the CNS neurons die upon injury and the surviving ones fail to spontaneously regenerate their axons till their targets (Aguayo et al., 1987). Research is

being focused in understanding the network of changes occurring in the traumatized retina and, more importantly, the effect of one upon another. To date, it is clear that numerous mechanisms are involved; some of them are common to different insults while others are injury-specific, some of them depend on inherited tracts, others implicate the glial and immune response and many reflect the commitment to death of the injured neurons. Only by gathering and unifying all these data we will be able to understand the common responses of the CNS to injury and to decipher the specific ones. With this knowledge it will be possible to design broad-spectrum and tailored therapies to successfully rescue the wounded system.

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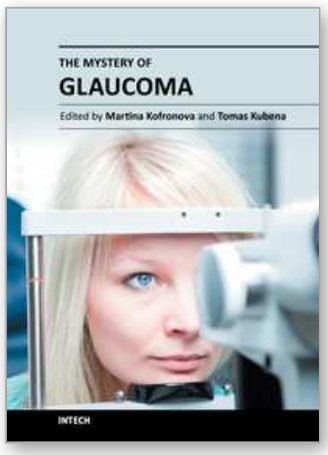

The Mystery of Glaucoma
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Since long ago scientists have been trying hard to show up the core of glaucoma. To its understanding we needed to penetrate gradually to its molecular level. The newest pieces of knowledge about the molecular biology of glaucoma are presented in the first section. The second section deals with the clinical problems of glaucoma. Ophthalmologists and other medical staff may find here more important understandings for doing their work. What would our investigation be for, if not owing to the people's benefit? The third section is full of new perspectives on glaucoma. After all, everybody believes and relies – more or less – on bits of hopes of a better future. Just let us engage in the mystery of glaucoma, to learn how to cure it even to prevent suffering from it. Each information in this book is an item of great importance as a precious stone behind which genuine, through and honest piece of work should be observed.

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