Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2011 | 117 | 904–914 doi: 10.1111/j.1471-4159.2011.07260.x

Effect of experimental glaucoma on the non-image forming visual system

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Abstract

Glaucoma is a leading cause of blindness worldwide, characterized by retinal ganglion cell degeneration and damage to the optic nerve. We investigated the non-image forming visual system in an experimental model of glaucoma in rats induced by weekly injections of chondroitin sulphate (CS) in the eye anterior chamber. Animals were unilaterally or bilaterally injected with CS or vehicle for 6 or 10 weeks. In the retinas from eyes injected with CS, a similar decrease in melanopsin and Thy-1 levels was observed. CS injections induced a similar decrease in the number of melanopsin-containing cells and superior collicular retinal ganglion cells. Experimental glaucoma induced a significant decrease in the afferent pupil light reflex. White light significantly decreased nocturnal pineal melatonin content in control and glaucomatous animals, whereas blue light decreased this parameter in vehicle- but not in CS-injected animals. A significant decrease in light-induced c-*Fos* expression in the suprachiasmatic nuclei was observed in glaucomatous animals. General rhythmicity and gross entrainment appear to be conserved, but glaucomatous animals exhibited a delayed phase angle with respect to lights off and a significant increase in the percentage of diurnal activity. These results indicate the glaucoma induced significant alterations in the non-image forming visual system.

Keywords: glaucoma, melanopsin, melatonin, pupil light reflex, suprachiasmatic nuclei.

J. Neurochem. (2011) **117**, 904–914.

Glaucoma is a leading cause of blindness worldwide, characterized by specific visual field defects because of the degeneration of retinal ganglion cells (RGCs) and damage to the optic nerve head. Elevated intraocular pressure (IOP) is one of the most important risk factors for the development of glaucoma. The underlying mechanisms that link elevated IOP to RGC death are still not fully understood. An experimental model of pressure-induced optic nerve damage would facilitate the understanding of the cellular events leading to RGC death, and how they are influenced by IOP and other risk factors. Recently, we have developed a new experimental model of glaucoma in rats through weekly intracameral injections of chondroitin sulphate (CS), which mimics central features of human glaucoma (Belforte *et al.* 2010).

Although the eye has been largely recognized as 'The organ of sight (in man and animals)', over the past decade, a second role for the eye has been uncovered: even in the absence of 'cognitive' vision, the eye can serve as a sensor

Received November 23, 2010; revised manuscript received March 16, 2011; accepted March 22, 2011.

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Abbreviations used: CS, chondroitin sulphate; GCL, ganglion cell layer; IOP, intraocular pressure; mRGC, melanopsin-expressing retinal ganglion cell; PBS, phosphate-buffered saline; PLR, pupil light reflex; RGC, retinal ganglion cell; SC, superior colliculus; SCN, suprachiasmatic nuclei.

for ambient lighting, akin to the light meter in a camera. Several light-regulated functions, including entrainment of circadian clocks, suppression of activity by light, photic suppression of pineal melatonin synthesis, and pupillary light responses are retained in animals that are blind as a result of mutations causing complete or near-complete degeneration of the classical photoreceptors, the rods and cones (Freedman *et al.* 1999; Lucas *et al.* 1999, 2001; Mrosovsky *et al.* 1999; Van Gelder *et al.* 2003). These light-responsive functions are controlled by a retinal photoreceptor because animals lacking RGCs lose circadian photoresponses, behavioral masking, and pupillary light responses (Wee *et al.* 2002; Van Gelder *et al.* 2003). The discovery of intrinsically photosensitive RGCs has given non-visual phototransduction an anatomical basis (Berson *et al.* 2002; Hattar *et al.* 2002). A subtype of RGCs [melanopsin-expressing retinal ganglion cells (mRGCs)] containing a novel photopigment, melanopsin, respond to light independently of the input from rods and cones, and project their axons through the retinohypothalamic tract to the suprachiasmatic nuclei (SCN), where the principal pacemaker center for circadian rhythms is located. A strong body of evidence supports that mRGCs are involved in modulating circadian rhythms (Provencio *et al.* 2000; Hattar *et al.* 2002; Berson 2003). Moreover, mRGCs send monosynaptic projections to the intergeniculate leaflet, responsible for circadian photoentrainment, and the olivary pretectal nucleus, responsible for the pupil light reflex (PLR) (Hattar *et al.* 2006).

Although most studies on glaucoma have focused on RGCs involved in conscious visual functions, clinical studies show that glaucoma patients have afferent pupillary defects during the early stages of the disease (Kaback *et al.* 1976; Kohn *et al.* 1979), and sleep disorders during later stages (Onen *et al.* 2000). These clues imply that there could be some damage to the non-image-forming visual system, and that mRGCs, as well as other RGCs, can be damaged in glaucoma patients. Presently, however, there is no clear support for the impact of glaucoma on circadian and/or sleep disturbances, and the degenerative loss of mRGCs in glaucoma is still controversial. One study shows that mRGC degeneration is proportional to that of the entire RGC population (Jakobs *et al.* 2005), while another report claimed selective sparing of melanopsin-containing neurons (Li *et al.* 2006). In view of these contradictory results, we considered it worthwhile to analyze the non-image-forming visual system in an experimental model of glaucoma induced by weekly injections of CS in the eye anterior chamber.

Materials and methods

Animals and tissues

Male *Wistar* rats (average weight, 200 ± 40 g) were housed in a standard animal room with food and water *ad libitum* under controlled conditions of humidity and temperature (21 ± 2°C), under a 12 h light : 12 h dark lighting schedule (lights on at 07.00 h). Rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (0.5 mg/kg) administered intraperitoneally. With a Hamilton syringe and a 30-gauge needle, under a surgical microscope with coaxial light, 20 μL of CS (Sigma Chemical Co., St. Louis, MO, USA, catalog # C9819, 0.4 g/mL in saline solution), were injected into one eye of anesthetized rats, and an equal volume of vehicle (saline solution) was injected in the fellow eye, as previously described (Belforte *et al.* 2010). In some experiments, animals were bilaterally injected with vehicle or CS. For the assessment of the PLR, animals were weekly injected in one eye with vehicle or CS, while the contralateral eyes remained intact.

Intraocular pressure assessment

A tonometer (TonoPen XL; Mentor, Norwell, MA, USA) was used to assess IOP in conscious, unsedated rats, as previously described (Benozzi *et al.* 2002). All IOP determinations were assessed by operators who were blind to the treatment applied to each eye. Briefly, five IOP readings were obtained from each eye by using firm contact with the cornea and omitting readings obtained as the instrument was removed from the eye. The mean of these readings was recorded as the IOP for that eye on that day. IOP was assessed between 11.00 and 12.00 h to correct for diurnal variations in IOP (Benozzi *et al.* 2002).

Western blotting

Rat retinas were homogenized in 100 μL of cold buffer containing 10 mM Tris–HCl, 1 mM $MgCl_2$, 10% Glycerol, 0.5% Triton, 10 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5 μg/mL aprotinin, and 2 μg/mL pepstatin A, pH 7.4. After 15 min at 4°C, retinal homogenates were centrifuged at 900 *g* for 10 min. Samples were mixed 1 : 1 with loading buffer (1.2 mL of 1 M Tris, 2 mL of glycerol, 4 mL of 10% sodium dodecyl sulfate, 2 mL of 1 M dithiothreitol, and 1% bromophenol blue, pH 6.8), boiled for 5 min and electrophoresed (100 μg proteins/lane) on sodium dodecyl sulfate, 10% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes for 60 min at 15 V in a Bio-Rad Trans-Blot SD system (Hercules, CA, USA) in transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 1.3 mM sodium dodecyl sulfate, pH 9.2). Membranes were blocked in 5% non-fat dry milk in Tris-buffer, pH 7.4, containing 0.5% Tween 20 for 60 min at 24°C and then incubated overnight at 4°C in a 1 : 1000 dilution of polyclonal rabbit anti-rhodopsin (obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-melanopsin (obtained from Affinity Bioreagent, Rockford, IL, USA), or anti-Thy-1 (1 : 500 dilution, obtained from Santa Cruz Biotechnology) antibody. Membranes were washed with Tris-buffered saline-T buffer and then incubated for 1 h with a 1 : 2000 dilution of horseradish peroxidase-conjugated secondary antibody. The membranes were washed and the bands were visualized by chemiluminescence (ECL, Western Blotting Analysis System; Amersham Biosciences, Buenos Aires, Argentina) and β-actin was used as an internal load control. Developed membranes were scanned and the intensity of bands was determined by using the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Values were expressed as arbitrary units of rhodopsin, melanopsin or Thy-1/β-actin.

Immunocytochemistry and hematoxylin and eosin staining

Rats were anesthetized and intracardiacally perfused with 150 mL of saline solution followed by 300 mL of a fixative solution containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Then, eyes were carefully enucleated, a slit opening was made in the cornea, and the eyecups were immersed for 24 h in the same fixative. For melanopsin immunohistochemistry, the retinas were isolated and were flat-mounted onto glass slides. Antigen retrieval was performed by heating (90°C) for 30 min unstained retinas immersed in citrate buffer (pH 6.3) and the endogenous peroxidases activity was blocked with 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 20 min. Then, retinas were pre-incubated with 4% normal horse serum in PBS for 1 h. For the detection of melanopsin-positive RGCs, retinas were incubated overnight at 4°C with a 1 : 1000 dilution of a rabbit polyclonal anti-melanopsin antibody (Affinity Bioreagent). The melanopsin positive signal was developed with the labeled streptavidin-biotin (LSAB2® System HRP Dakocytomation, Dako, Carpinteria, CA, USA) reagent kit according to manufacturer's instructions. Each retina was divided into superior, inferior, nasal and temporal quadrants and mRGCs were counted under a light microscope (100×), and a representative drawing of the stained retinas was performed. For hematoxylin and eosin (H&E) staining, the nictitans membrane was maintained in each eye to facilitate orientation. Eyecups were dehydrated in an alcohol series and embedded in paraffin. Sections (4-μm thick) were cut along the horizontal meridian through the optic nerve head and stained with H&E. Retinal morphometry was evaluated as described by Belforte *et al.* (2010). Three sections were randomly selected from each eye. Nine microscopic images at 1 mm from the temporal edge of the optic disc were digitally analyzed. The number of cells in the ganglion cell layer (GCL) was expressed as cells per 100 μm. For each eye, results obtained from three separate sections were averaged and the mean of five eyes was recorded as the representative value for each group. No attempt was made to distinguish cell types in the GCL for enumeration of cell number. Morphometric analysis was performed by observers masked to the protocol used in each eye.

Retrograde labeling of superior colliculus-projecting RGCs

The skin over the cranium was incised and the scalp exposed. Holes approximately 2 mm in diameter were drilled in the skull on both sides of the midline, 6.5 mm posterior to the bregma and 1.5 mm lateral to the midline. These positions correspond to the superior colliculi (SC), as determined from a stereotaxic rat brain atlas. RGCs were labeled retrogradely by injection of 2 μL fluorescent tracer Fluorogold (Fluorochrome, Denver, CO, USA) 2% in saline, into the SC, at each side, using a 10 μL Hamilton syringe (Reno, NV, USA) with a 30-gauge needle. Five days after Fluorogold injection, rats were anesthetized and intracardially perfused with 150 mL of saline solution followed by 300 mL of a fixative solution containing 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Then, eyes were carefully enucleated, a slit opening was made in the cornea, and the eyecups were immersed for 24 h in the same fixative. The retinas were then taken out, washed with PBS (0.01 M PBS, pH 7.4), and flatmounted onto glass slides for cell counting.

Assessment of the pupil light reflex

Animals were dark adapted for 1 h prior to PLR assessment. While one eye received light stimulation from a white light source (1200 lux), the other eye was video-monitored under infrared light with a digital camcorder (Sony DCR-SR60, Tokyo, Japan). Dark adapted pupil diameter prior to light pulse and pupil diameter after 30 s of light were measured. Sampling rate was 30 images per second. The digital video recording was deconstructed to individual frames using OSS Video Decompiler Software (One Stop Soft, New England, USA). The percent pupil constriction was calculated as the percent of pupil area at 30 s after the initiation of the stimulus (steady state) relative to the dilated pupil size. In one experiment, eyes injected with vehicle or CS were stimulated by light, and the PLR was assessed in the contralateral intact eyes, whereas in a second experiment, light stimulated the intact eyes and the PLR was assessed in eyes injected with vehicle or CS.

Assessment of the light suppression of nocturnal pineal melatonin content

Animals bilaterally injected with vehicle or CS were either subjected to a 20 min white (1200 lux) or monochromatic blue light which emits around 470 nm (1000 lux) stimulus at 5 h after turning off the lights (i.e. at 12 AM) or kept in darkness until killing. Rats were killed immediately after light stimulus; the pineal gland was removed and freezed at −80°C. The pineal glands were homogenized in 2 mL of 0.1 M HCl, and melatonin was extracted from the whole homogenate (except for 300 μL used for protein assay) with 5 mL of dichloromethane. The organic phase was washed twice with 2% $NaHCO_3$, and distilled water. Aliquots of 1.5 mL of the organic layer were dried under vacuum and stored at −20°C until the radioimmunoassay was performed. The recovery of the extraction procedure was 76 ± 5%. The samples were resuspended in 100 μL of buffer (6 mM NaN_3, 0.1 M KH_2PO_4, and 0.1% gelatin, pH 7.5) and then mixed with 50 μL of [^{3}H]-melatonin (20 000–24 000 dpm; specific activity, 38.8 Ci/mmol) and 50 μL of a rabbit antiserum kindly provided by Dr. Takashi Matozaki (Laboratory of Biosignal Sciences, Institute for Molecular and Cellular Regulation, Gunma University, Japan). The mixture was incubated overnight at 4°C. The bound/free separation was performed by the dextran-charcoal method, and the radioactivity of supernatant was measured by a liquid scintillation counter. Melatonin values were obtained from a melatonin standard curve, with the limit of sensitivity of the assay being 20 pg per tube.

Assessment of light induction of the c-*Fos* in the SCN

Glaucomatous and control rats were either subjected to a 10 min light stimulus (1200 lux), 6 h after turning off the lights (i.e. at 1 AM) or kept under darkness until killing. Rats were anaesthetized 1 h after receiving the light stimulus and perfused intracardially with 100 mL 0.1 M PBS followed by 300 mL 4% paraformaldehyde in 0.1 M PBS. Brains were post-fixed overnight, transferred into 30% sucrose-PBS solution for 48 h and 40 μm coronal sections were collected in 0.1 M PBS and washed with 0.4% Triton X-100 in 0.01 M PBS (PBS-T). Non-specific binding sites were blocked with 0.1% bovine serum albumin and 2% normal horse serum in PBS-T for 1.5 h at 24°C. Sections were incubated for 72 h at 4°C with a rabbit polyclonal antibody anti-c-*Fos* (1 : 5000, Santa Cruz

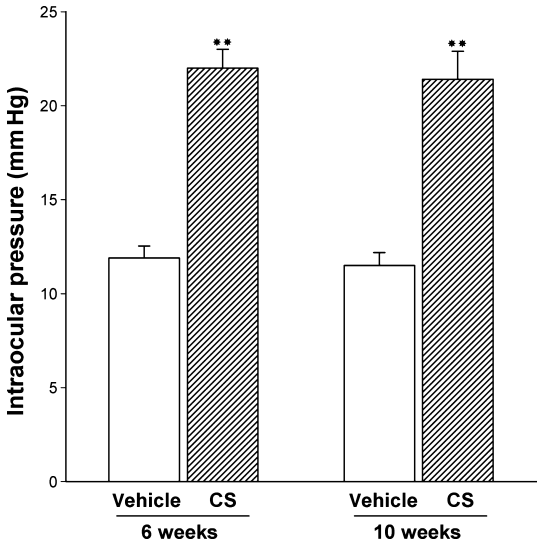

Fig. 1 Effect of weekly injections of CS on rat IOP. Intracameral injections of vehicle or CS were weekly performed for 6 and 10 weeks. Injections of CS for 6 or 10 weeks significantly increased IOP. Data are mean ± SEM ($n = 20$ eyes/group). $^{**}p < 0.01$ versus vehicle-injected eyes, by Student's *t*-test.

Biotechnology). After PBS-T washes, the sections were incubated for 2 h with a biotinylated universal secondary antibody (1 : 200, Vector Labs, Buenos Aires, Argentina). The reaction was visualized with the avidin-biotin complex and the peroxidase chromogen, VIP® (Vectastain Elite ABC kit, Vector Labs).

Locomotor activity rhythm

Circadian rhythms of general locomotor activity were registered in animals bilaterally injected with vehicle or CS for 10 weeks, under 12 h light- 12 h dark (L : D) cycles. Rats were placed in cages equipped with infrared detectors of motion, data were sampled every 5 min and stored for subsequent analysis. Average activity waveforms were constructed for each animal, and the phase angle for activity onset (with respect to time of lights off) was determined. Activity onset was defined as the first of six 5-min activity bins in which locomotor activity was higher than the average value of the diurnal waveform. In addition, the percentage of locomotor activity during the light and dark phases was computed. Re-entrainment rates were calculated after 6-h advance or delay shifts in the L : D cycle, starting by shifting the time for lights off.

Protein level assessment

Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

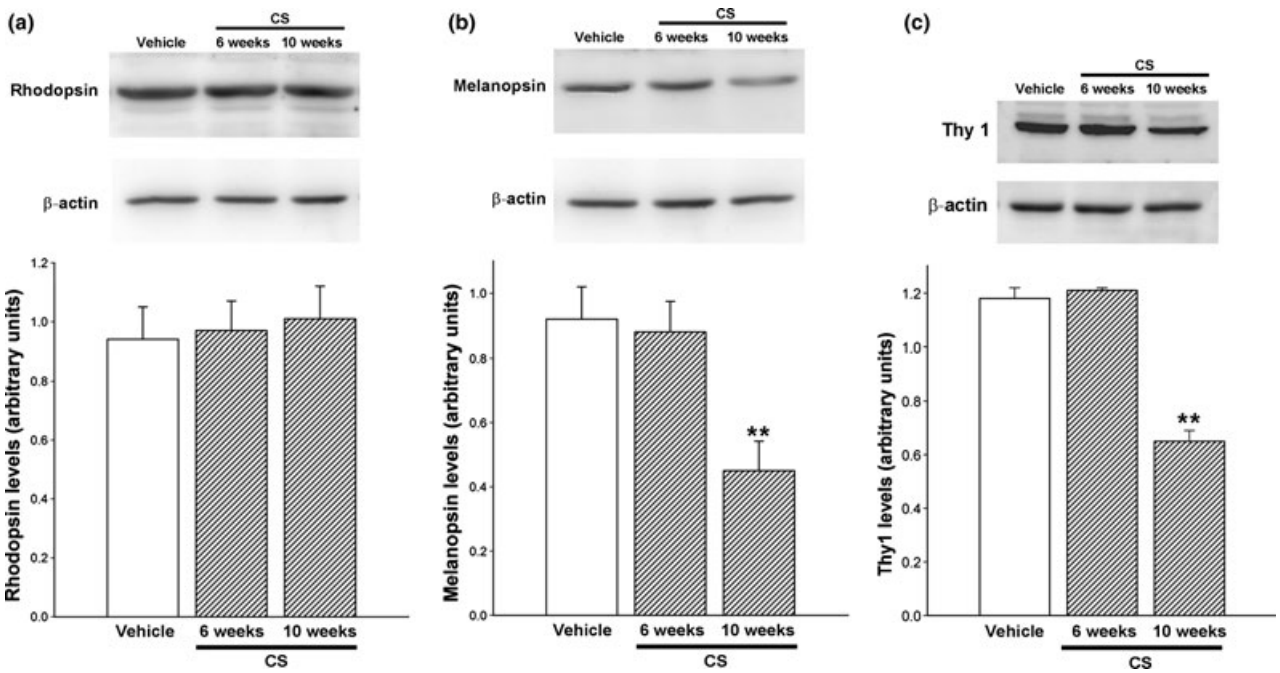

Fig. 2 Rhodopsin, melanopsin, and Thy-1 levels in retinas from eyes injected with vehicle or CS for 6 or 10 weeks. Panel (a) (left): representative gel for rhodopsin in retinas from eyes injected with vehicle or CS. A band of ~ 40 kDa was detected in all the samples. Panel (a) (right): densitometric analysis revealed no significant differences in rhodopsin levels in retinas from eyes injected with vehicle or CS. Panel (b) (left): representative gel for melanopsin in retinas from eyes injected with vehicle or CS. A band of ~ 80 kDa (representing the glycosylated form of melanopsin) was detected in all the samples. Panel (b) (right): densitometric analysis of the samples revealed lower levels of melanopsin in retinas from eyes injected with CS for 10 (but not 6) weeks. Panel (c) (left): representative gel for Thy-1 in retinas from eyes injected with vehicle or CS showing a band of ~ 29 kDa, Panel (c) (right): densitometric analysis of the samples revealed lower levels of Thy-1 in retinas from eyes injected with CS for 10 (but not 6) weeks. Each lane was loaded with 100 μg of proteins. Data are mean ± SEM ($n = 6$ eyes per group). $^{**}p < 0.01$ versus saline-injected eyes, by Dunnett's test.

Statistical analysis

Statistical analysis of results was made by a Student's *t*-test or by a two-way ANOVA followed by a Dunnett's or Tukey's test, as stated.

Results

Figure 1 summarizes the average IOP of rats weekly injected with CS in one eye and vehicle in the other for 6 or 10 weeks. A significant increase of IOP was observed in eyes injected with CS as compared with vehicle-injected eyes. No differences in IOP of vehicle-injected eyes were detected between these time points or between non-injected and vehicle-injected eyes (data not shown).

Rhodopsin, melanopsin, and Thy-1 levels in retinas from eyes injected with vehicle or CS for 6 or 10 weeks were assessed by western blotting. As shown in Fig. 2, bands of ~ 40 kDa, ~ 80 kDa, and ~ 29 kDa were identified in all samples, in agreement with the molecular mass reported for rhodopsin, melanopsin, and Thy-1, respectively. Scanning densitometry of the bands revealed no significant differences in rhodopsin levels between groups, whereas a significant decrease in melanopsin (~ 50%) and Thy-1 (~ 45%) levels was observed in eyes injected with CS for 10 weeks, as compared with control eyes. At 6 weeks of treatment, retinal rhodopsin, melanopsin, and Thy-1 levels did not differ between vehicle- and CS-injected eyes. No differences in these parameters were observed between retinas from eyes injected with vehicle for 6 and 10 weeks (data not shown).

Immunocytochemistry of flatmounted rat retinas with an anti-melanopsin antibody labeled a small percentage of RGCs, including cell bodies, dendrites, and axons, as shown in Fig. 3. Immunopositive-dendrites from adjacent cells overlapped extensively, forming a reticular network. The stained dendrites and proximal axons showed a beaded appearance, with punctuate, and dense labeling. The dendritic fields of labeled cells showed varied sizes and shapes. After 10 weeks of treatment, the total number of melanopsin positive RGCs was 1374 ± 74, and 763 ± 146 cells for vehicle- and CS-treated eyes, respectively (**$p < 0.01$, Student's *t*-test, $n = 4$ eyes/group). A significant loss (~ 40%) in the number of SC RGCs was observed in eyes injected with CS for 10 weeks both in the central and peripheral retina, as shown in Fig. 4. In addition, a significant decrease (~ 45%) in the number of H&E stained cells in the GCL was observed in CS-injected eyes. No differences in the number of Fluorogold labeled and H&E stained RGCs or mRGCs were observed between eyes injected with vehicle or CS for 6 weeks (data not shown).

The PLR was examined in animals in which one eye was injected with vehicle or CS for 6 or 10 weeks, whereas in both cases, the contralateral eye remained intact. When light stimulated eyes injected with CS for 6 or 10 weeks, a significant decrease in the magnitude of pupil contraction of the contralateral intact eye was observed, as shown in Fig. 5.

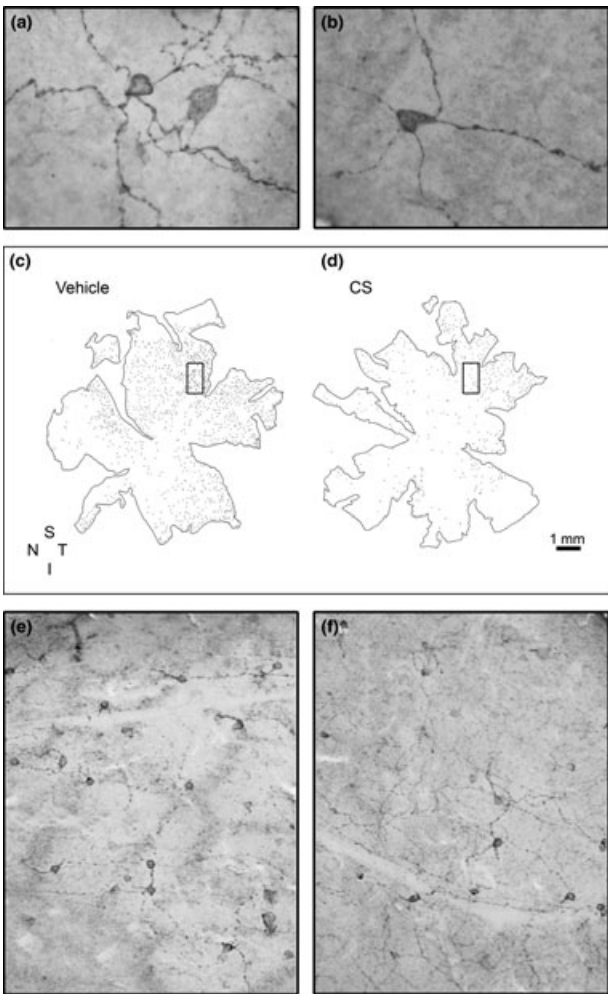

Fig. 3 Immunohistochemical analysis of retinal melanopsin. The presence of melanopsin was assessed in retinas from eyes treated with vehicle (a, b, c and e) or CS for 10 weeks (d and f). Melanopsin was localized in cellular soma, axons, and dendrite ramifications (a and b). Note the beaded appearance of the dendrites. Overall distribution of mRGCs on flatmounted retina from an eye injected with vehicle (c) or CS (d) is shown in the middle panel. Dozens of local dark-field images were taken separately at low magnification, and the montage was assembled with Adobe Photoshop. Each cell body is represented by a dot of about the appropriate size. Details for section (c) and (d) are shown in sections (e) and (f), respectively. Note the high cell density in the superior and temporal quadrants. The density of melanopsin-positive cells was significantly higher in retinas from eyes injected with vehicle than in those injected with CS for 10 weeks. Representative photographs of four retinas/group. S, superior; I, inferior; N, nasal; T, temporal.

When light stimulated the intact eye, no differences in pupil constriction were observed between both groups at 6 or 10 weeks of treatment (Fig. 5). A similar PLR was observed in eyes injected with vehicle for 6 and 10 weeks (i.e. 75.2 ± 7% and 76.3 ± 9% of pupil constriction at 6 and 10 weeks of treatment with vehicle, respectively).

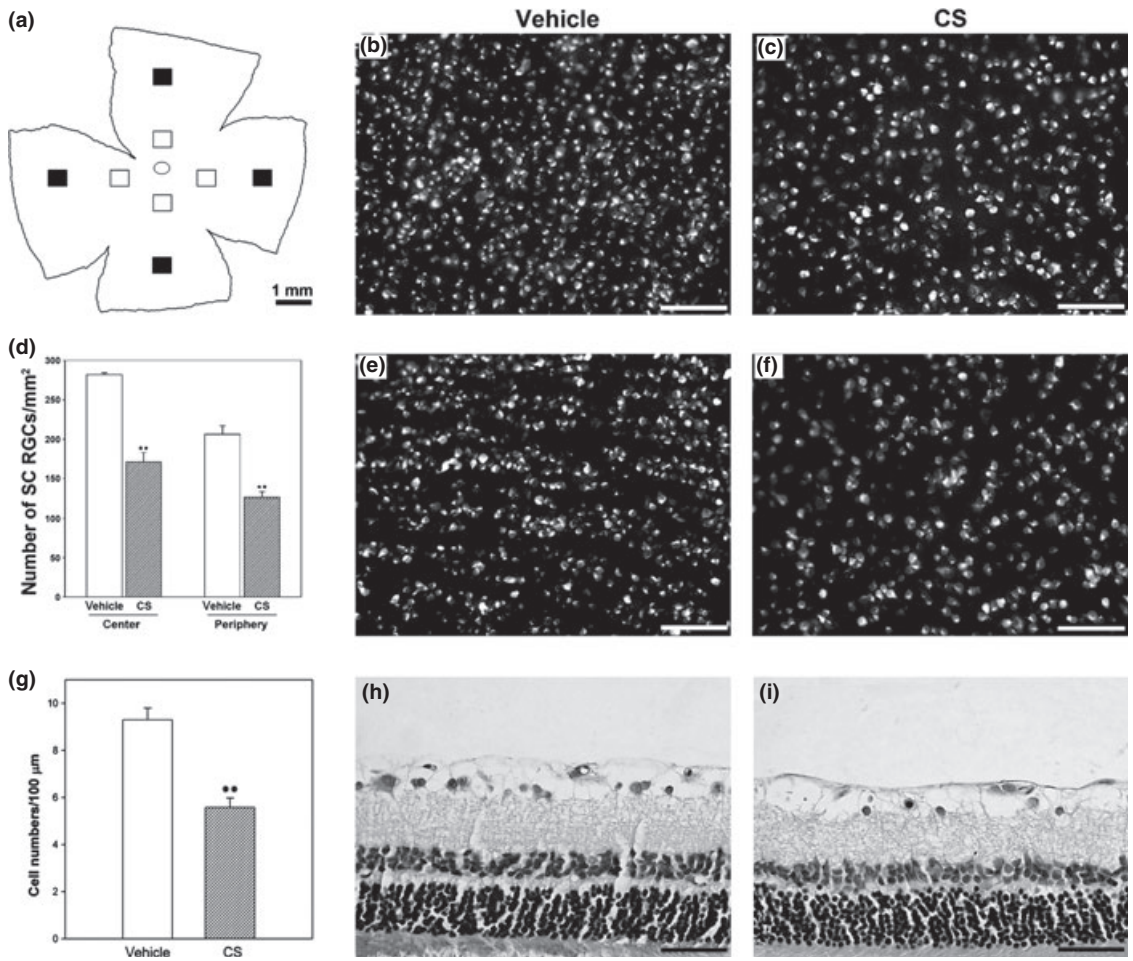

Fig. 4 Analysis of SC RGCs and H&E stained RGCs in retinas from eyes injected with vehicle or CS for 10 weeks. Panel (a): schematic diagram of a flatmounted retina showing the analyzed regions (white squares: center, black squares: periphery). The intensity of Fluorogold labeling in vehicle-injected eyes (b and e, central and peripheral retina, respectively) was significantly reduced in eyes injected with CS, both in the central retina and peripheral retina (c and f, respectively). Panel (d): shown are means ± SEM ($n = 4$ eyes per group), $^{**}p < 0.01$, Student's *t*-test. Panel (h and i): light micrographs of transverse sections of a retina from a rat, in which one eye was injected with vehicle (h) and the contralateral eye was injected with CS for 10 weeks (i). Note the diminution of ganglion cell layer (GCL) cells in the eye injected with CS. The other retinal layers showed a normal appearance. H&E. Panel (g): shown are means ± SEM ($n = 5$ eyes per group), $^{**}p < 0.01$, Student's *t*-test. Scale bar: 50 μm.

In order to analyze the light suppression of nocturnal pineal melatonin content, animals bilaterally injected with vehicle or CS for 10 weeks were exposed to a brief pulse (20 min) of white or blue light at midnight. White light significantly decreased pineal melatonin content in vehicle- and CS-treated eyes. Blue light significantly decreased pineal melatonin content in control animals, whereas in animals bilaterally injected with CS, it was ineffective in reducing pineal melatonin content (Fig. 6). In the absence of photic stimulation, nocturnal pineal melatonin content was significantly higher in control than in glaucomatous animals.

Light induction of the immediate early gene c-*Fos* in the SCN was analyzed in animals whose eyes were bilaterally injected with vehicle or CS. As shown in Fig. 7, a light pulse induced a significant increase of c-*Fos* immunoreactivity in both groups. However, SCN c-*Fos* levels after a light pulse were significantly lower in CS- than in vehicle-injected animals. No differences in c-*Fos* levels in dark conditions were observed between groups.

Animals with experimental glaucoma entrained to a normal (T24) light-dark cycle, but with a significantly delayed phase angle of activity onset with respect to the time of lights off [phase angle: controls = 9.7 ± 2.6 min ($n = 10$); glaucoma = −50.0 ± 12.1 min ($n = 15$), $p < 0.01$, Student's *t*-test] (Fig. 8). In addition, the percentage of locomotor activity in the photophase was significantly higher in glaucomatous than in control rats (14.1 ± 1.8% vs. 8.4 ±

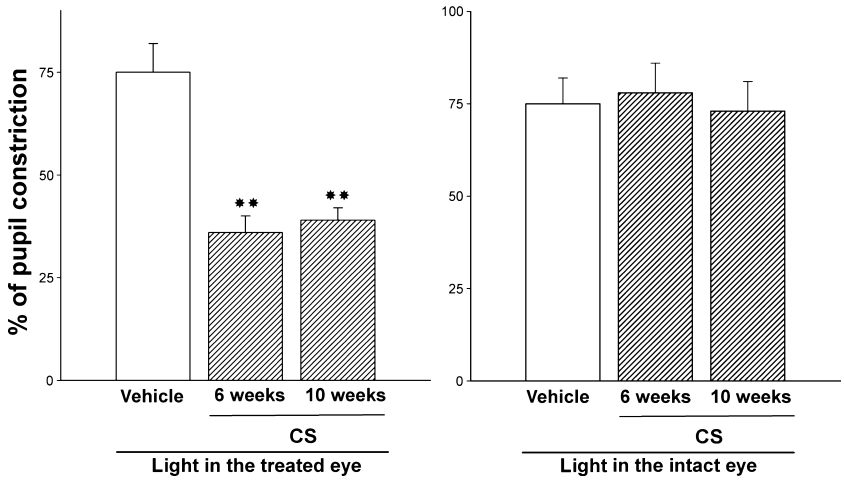

Fig. 5 Assessment of the consensual PLR in animals injected in one eye with vehicle or CS for 6 or 10 weeks. The pupil diameter relative to the limbus diameter was measured before and after a photic stimulus (1200 lux) and the percentage of pupil constriction was calculated as the fraction of the pupil area of the maximum contraction over the pupil area at the maximal dilation (i.e. 2 s before light stimulus). Left panel: THE light pulse was applied to the vehicle (control) or CS-injected eye and the recordings were made in the contralateral intact eye. The maximal contraction of the pupil was significantly lower after 6 or 10 weeks of ocular hypertension. Right panel: the stimulus was applied to the intact eye and the recordings were made in the contralateral eye injected with vehicle or CS. No significant differences were observed among groups. Data are means ± SEM ($n = 6$ eyes per group) $^{**}p < 0.01$ versus vehicle, by Dunnett's test.

1.4%, respectively, $p < 0.05$, Student's *t*-test). Both groups of animals had similar re-entrainment rates after a 6-h delay or advance of the L : D cycle (delays: controls 5.45 ± 0.25 days, glaucoma 5.8 ± 0.49 days, $p > 0.05$; advance: controls = 5 ± 0.30 days, glaucoma = 5.30 ± 0.37 days, $p > 0.05$).

Discussion

The present results indicate that chronic ocular hypertension induced by weekly injections of CS provoked a significant decrease in the number of mRGCs and melanopsin levels. Moreover, these results indicate that experimental glaucoma caused significant alterations in the non-image-forming visual system, as shown by its effect on the PLR, light-induced nocturnal pineal melatonin suppression, light-induced c-*Fos* expression in the SCN, and the locomotor activity rhythm.

Glaucoma is a progressive neurodegenerative disease that results in loss of RGCs. Previous studies demonstrated different degrees of RGC loss, depending on the glaucoma model and animal species (Shareef *et al.* 1995; Chauhan *et al.* 2002; Li *et al.* 2006). In particular, Huang *et al.* (2006) reported that elevated IOP induced by injecting hypertonic saline into aqueous veins of *Brown Norway* rats provokes a 60% decrease in Thy-1 protein, and a 51% decrease in Thy-1 mRNA levels (Huang *et al.* 2006), which agrees with the results reported in this study. A significant decrease in the number of mRGCs was observed after 10 (but not 6) weeks of ocular hypertension. Since the decrease in the number of mRGC (∼ 45%) and in melanopsin levels (∼ 50%) was similar to that observed for Fluorogold labeled RGCs (∼ 40%), and Thy-1 levels (∼ 45%) levels, respectively, it seems that mRGCs and SC RGCs were similarly vulnerable to the deleterious effects of ocular hypertension. In contrast, Li *et al.* (2006) demonstrated that mRGCs are resistant even after 12 weeks of ocular hypertension induced by argon laser photocoagulation of the episcleral and limbal veins in *Sprague-Dawley* rats. However, Wang *et al.* (2008) reported a significant loss of mRGCs in experimental glaucoma induced by cauterization of three episcleral veins in *Wistar* rats. Thus, it seems likely that the experimental model, as well as the rat strain could account for the discrepancy between the report by Li *et al.* (2006) and the present results. One slight issue might be that CS can act as a neuromodulator (Inatani and Tanihara 2002) and so the results from this glaucoma model may differ from other models. Moreover, while previous results were based on immunohistochemical studies, in the present report, the decrease in melanopsin levels was also demonstrated by western blotting.

The large number of projections arising from mRGCs could indicate numerous functions (Gooley *et al.* 2003). In order to identify these functions, several groups created melanopsin-null mice, with the interesting paradox that after years of searching for an elusive circadian photopigment, the race was on to get rid of the cells that produce it (Mrosovsky and Hattar 2003; Panda *et al.* 2003; Güler *et al.* 2008; Hatori *et al.* 2008). Since weekly intracameral injections of CS provoked a decrease in retinal melanopsin levels and in the

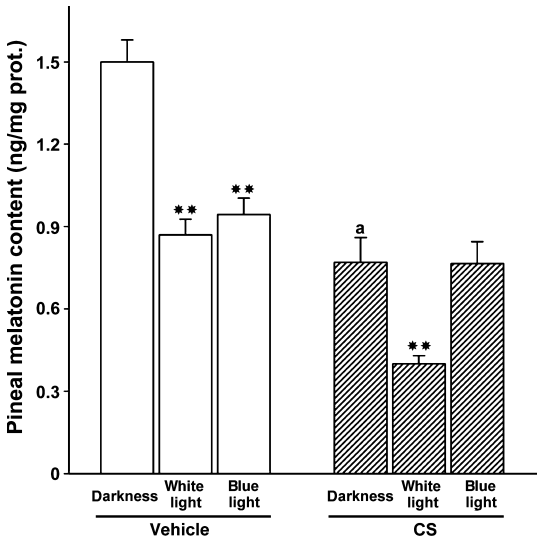

Fig. 6 Effect of light on nocturnal pineal melatonin content in rats injected with vehicle or CS for 10 weeks. Pineal melatonin content was assessed as described in Material and Methods. After a white light pulse (6 h after the beginning of the dark phase), pineal melatonin levels significantly decreased in vehicle- and CS-injected eyes, whereas blue light only decreased this parameter in the pineal gland from vehicle-injected animals. Pineal melatonin levels at night were significantly lower in CS- than in vehicle-injected rats. $^{**}p < 0.01$ versus nocturnal melatonin levels for each group, $^{a}p < 0.01$ versus nocturnal melatonin levels in the control group, by Tukey's test. Data are mean ± SEM (n = 12 animals/group).

number of mRGCs, this model could be a new tool to further study mRGC-related functions.

Although PLR defects were described in patients with glaucoma (Prywes 1976; Kohn *et al.* 1979), few reports have previously assessed the pupil response in experimental models of glaucoma in rodents. In that sense, it was shown a significant PLR deficit in *Brown Norway* rats with experimental glaucoma induced by cauterizing three vortex veins and two major episcleral veins (Grozdanic *et al.* 2003). Similarly, our results demonstrated an afferent (but not efferent) pupillary defect in glaucomatous eyes. Historically, it was assumed that the light-evoked neural signals driving the PLR originated exclusively from rods and cones. However, later on, it was shown that mRGCs significantly contribute to the maintenance of pupil constriction in response to light stimuli of 30 s or longer, even at low photopic irradiances, and that cone photoresponses driving pupil constriction adapt considerably and contribute little after 30 s, but rod photoresponses adapt less and contribute significantly to the maintenance of pupil constriction in response to steady-state light stimuli at irradiance levels which are below the threshold of the melanopsin photoresponse (McDougal and Gamlin 2010). In order to avoid cone contribution, a 30-s light stimulus was used herein. Since no changes in rhodopsin levels were observed in CS-treated eyes, the decrease of the PLR in glaucomatous eyes could be attributed to an alteration in the melanopsin system. Notwithstanding, the involvement of blue cones cannot be completely ruled out. The functional and histological alterations induced by glaucoma progress over time, and it was demonstrated that functional disturbance predates morphologic changes (Moreno *et al.* 2005; Belforte *et al.* 2010). The present results suggest that the PLR could be a non-invasive and sensitive indicator of melanopsin system dysfunction, because this measurement revealed injury at a time point in which retinal melanopsin levels appeared relatively normal (i.e. at 6 weeks of ocular hypertension).

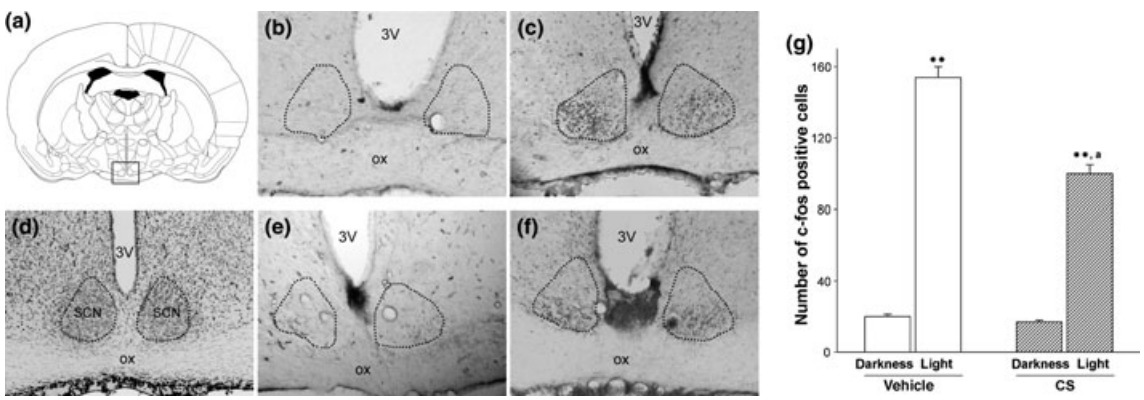

Fig. 7 Light induction of c-*Fos* in the SCN of rats bilaterally injected with vehicle or CS for 10 weeks. Panel (a): representative coronal section at the level of the SCN. The zone of interest is represented in the box, and a Nissl-staining detail is shown in panel (d). The expression levels of c-*Fos* was assessed by immunohistochemistry in the SCN (dotted lines) from vehicle- (b, c) or CS-injected animals (e, f), and the number of c-*Fos* positive cells was counted (g). In darkness, no differences were observed in the level of c-*Fos* in the SCN from vehicle- (b) or CS-injected rats (e). A significant increase in c-*Fos* expression was observed after a light pulse (at 6 h after the beginning of the dark phase) in the SCN from animals whose eyes were injected with vehicle (c) or CS (f). The induction of c-*Fos* by light was significantly lower in glaucomatous than in control rats. Data are mean ± SEM (n = 6 animals/group), $^{**}p < 0.01$ versus darkness; $^{a}p < 0.01$ versus light in animals whose eyes were injected with vehicle, by Tukey's test. 3V, 3rd ventricle; ox, optic chiasm.

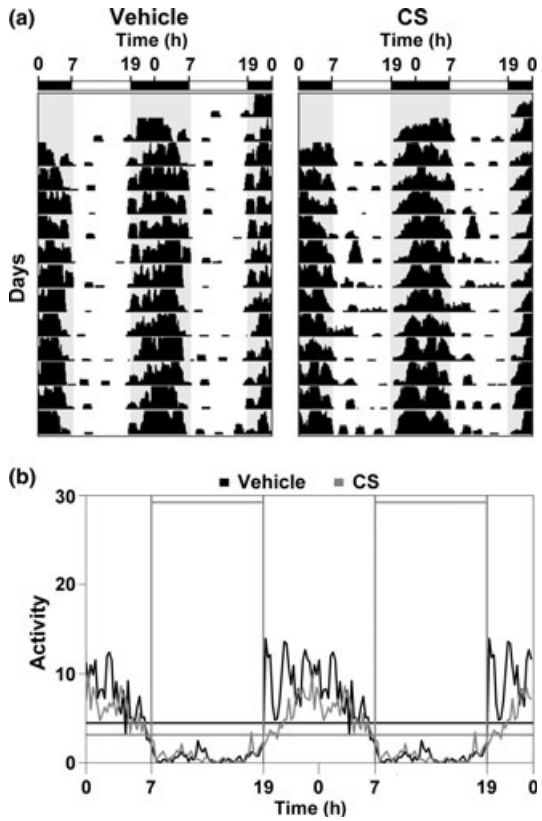

Fig. 8 Locomotor activity rhythm of rats bilaterally injected with vehicle or CS for 10 weeks. Panel (a): double-plotted actogram of vehicle- (left) or CS-injected (right) rats under a L : D cycle. The percentage of locomotor activity in the photophase was significantly higher in CS-injected rats than in vehicle-injected (14.1 ± 1.8% vs. 8.4 ± 1.4%, respectively, $p < 0.05$, Student's *t*-test). Panel (b): average activity waveforms of rats injected with vehicle or CS. A significantly delayed phase angle of activity onset with respect to the time of lights off was observed in animals injected with CS [phase angle: vehicle-injected rats: 9.7 ± 2.6 min ($n = 10$); CS-injected rats: −0.0 ± 12.1 min ($n = 15$), $p < 0.01$, Student's *t*-test].

The acute light-induced melatonin suppression response is a broadly used indicator for photic input to the SCN (Brainard *et al.* 1997). Light, phototransduced by mRGCs is conducted through the retinohypothalamic tract to the SCN that connects the retina with the pineal gland through a multisynaptic pathway. Light has two effects on pineal melatonin (Lewy *et al.* 1980): (i) the light-dark cycle synchronizes the circadian rhythm of its secretion, (ii) light of sufficient intensity and duration brusquely inhibits its nocturnal secretion. The action spectrum for light-induced melatonin suppression in the human pineal gland identifies 446–477 nm as the most potent wavelength region providing circadian input for regulating melatonin secretion (Brainard *et al.* 2001; Thapan *et al.* 2001), suggesting that a photopigment distinct from the individual rod and cone photoreceptors may be primarily responsible for melatonin regulation. As shown herein, blue light significantly reduced nocturnal pineal melatonin production in control animals, but it was ineffective in glaucomatous animals, whereas white light significantly decreased this parameter in both groups. Despite abundant evidence supporting that mRGCs provide primary input for neuroendocrine phototransduction (Brainard *et al.* 2001), the fact that white light was significantly effective in suppressing nocturnal melatonin levels in vehicle- and CS-treated animals could suggest that rod and cone photoreceptors still play a role in this response. In fact, polychromatic light is more effective in suppressing human nocturnal melatonin than monochromatic blue light matched for melanopsin stimulation, implying that the melatonin suppression response is not solely driven by melanopsin (Revell and Skene 2007). Notably, nocturnal levels of pineal melatonin were significantly lower in glaucomatous than in control animals. Previously, we showed that chronic ocular hypertension decreases midnight levels of retinal melatonin (Moreno *et al.* 2004). In the retina, melatonin is synthesized through the same pathway as that described in the pineal gland. Thus, is not completely surprising that glaucoma could affect both retinal and pineal melatonin biosynthesis.

As shown herein, the PLR sensitivity to white light was reduced, whereas the melatonin suppression sensitivity to white light was left intact in experimental glaucoma. In that sense, it should be taken into account that projections of mRGCs to the olivary pretectal nucleus are responsible for the PLR, whereas the mRGCs → retinohypothalamic tract → SCN → pineal gland pathway is involved in nocturnal pineal melatonin suppression by light. Thus, it seems possible that experimental glaucoma could differentially affect these pathways. Experiments are planned to examine this issue in the near future.

The proto-oncogene c-*Fos* is a transcription factor which regulates several target genes (Morgan and Curran 1991). In the SCN, the induction of c-*Fos* is mainly regulated by the ambient light. Cells in the SCN expressing c-*Fos* are initially restricted to retino-recipient areas, and neuronal activation later progresses to the dorsomedial portion of the SCN. Moreover, intracerebroventricular injection of antisense oligonucleotides that inhibits *Fos* expression blocks the light-induced phase-shifts of the activity rhythm (Wollnik *et al.* 1995). Therefore, these results suggest that induction of c-*Fos* in the SCN is involved in photic entrainment (Takahashi *et al.* 1984; Wollnik *et al.* 1995) or, at least, it can be used as a marker of phase-dependent photic activation of the SCN. Light induced c-*Fos* expression was significantly lower in CS- than in vehicle-injected animals, suggesting that experimental glaucoma can induce a deficit in the light-induced neuronal activation of the SCN and, presumably, in photic phase shifts of the mammalian circadian clock. In that sense, it was recently demonstrated

that glaucomatous rats show significantly greater variability in activity onsets in comparison with normal rats (Drouyer *et al.* 2008). However, it should be noted that in contrast to our model of glaucoma in which rhodopsin levels did not vary, rod and cone opsin mRNA levels significantly decrease in the model of glaucoma used by Drouyer *et al.* (2008) which could indicate the involvement of classical photoreceptors in the circadian alterations described in the latter report. In the present report, we recorded general activity patterns for both control and glaucomatous animals. Glaucomatous rats exhibited normal circadian rhythms under a light-dark cycle and re-entrained to changes in the photoperiod in both directions – advances and delays. However, subtle changes indicate that entrainment in this group is somewhat impaired. While control rats have an activity onset which coincides with the time of lights off, glaucomatous rats exhibited a significant negative (i.e., delayed) phase angle for the start of their nocturnal locomotion. This could be interpreted either as a deficient fine-tuning for photic entrainment, as a lack of the masking response to lights off, or both. It is important to state that both entrainment and masking to light contribute to normal synchronization of rhythms, and that both mechanisms depend on an intact circadian photoreception pathway (Golombek and Rosenstein 2010). Nocturnal animals under artificial lighting conditions show almost no activity during the light phase of the photoperiod. Here, we show that glaucomatous rats were significantly more active than controls during the photophase, suggesting a decreased mechanism of photic entrainment (or, in this case, negative masking to light). These general differences in entrainment might represent an impaired phototransduction mechanism in the retinohypothalamic tract which would translate into a deficient communication between the retinal and the SCN clocks. Indeed, additional experiments should be able to discern whether these changes affect specific parametric (i.e., photoperiodic), non-parametric (i.e., light pulse-induced phase shifts) or masking (i.e., direct modulation of behavior) effects of light.

This study implies that attention should be paid not only to image-forming visual functions in glaucoma, but also to non-image-forming visual functions. Glaucoma may affect the quality of life in several ways. As shown herein, glaucoma may lead to alterations in non-visual functions, supporting that alterations of the circadian physiology could be another risk to the quality of life of patients with glaucoma. Circadian rhythm disorders may include sleep problems, impaired performance, decrease in cognitive skills, poor psychomotor coordination, and headaches, among many others. There are several therapeutic strategies to restore circadian balance. Thus, by identifying circadian disorders in glaucoma, these results could help to improve the quality of life of patients with this ocular disease.

Acknowledgements

This research was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), the University of Buenos Aires, and CONICET, Argentina.

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