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Light-induced retinal degeneration causes a transient downregulation of melanopsin in the rat retina

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ABSTRACT

In this work we study the effects of an acute light-induced retinal degeneration on the population of melanopsin positive retinal ganglion cells (m^+RGCs) and the expression of the melanopsin protein in the retina. The m^+RGCs may be more resistant than other RGCs to lesion, but the effects of an acute light exposure in this population are unknown. Albino rats were exposed to white light (3000 lux) continuously for 48 h and processed 0, 3, 7 or 30 days after light exposure (ALE). Whole-mounted retinas were immunodetected with antibodies against melanopsin, Brn3a, and rhodopsin to study the populations of m^+RGC, Brn3a$^+$RGC and rods (which are the most abundant photoreceptors in the rat retina). Three days ALE there was substantial rod loss in an arciform area of the superior retina and with time this loss expanded in the form of rings all throughout the retina. Light exposure did not affect the number of Brn3a$^+$RGCs but diminished the numbers of m^+RGCs. Immediately ALE there was a significant decrease in the mean number of immunodetected m^+RGCs that was more marked in the superior retina. Later, the number of m^+RGCs increased progressively and reached normal values one month ALE. Western blot analysis showed that melanopsin expression down-regulates shortly ALE and recovers thereafter, in accordance with the anatomical data. This study demonstrates that there is a transient downregulation of melanopsin expression in the RGCs during the first month ALE. Further studies would be needed to clarify the long-term effect of light exposure on the m^+RGC population.

Intrinsically photosensitive retinal ganglion cells (ipRGCs) represent a special subtype of RGC that expresses the photopigment melanopsin and thus responds directly to light (Pickard and Sollars, 2012; Provencio et al., 2000). These neurons have changed the traditional conception that classical photoreceptors (cones and rods) were the only cells able to sense light in the retina. We will refer to the ipRGCs as melanopsin positive RGCs (m^+RGCs), as most works on these cells rely on melanopsin immunodetection.

m^+RGCs have important roles related to non–image forming visual functions (Berson, 2003; Chen et al., 2011; Hattar et al., 2003; LeGates et al., 2014; Sand et al., 2012; Panda et al., 2003; Schmidt et al., 2011; Semo et al., 2014; Vugler et al., 2015) such as the regulation of circadian rhythms, part of the pupillary light reflex, melatonin secretion, regulation of sleep, or masking behaviour. Moreover, recent studies have suggested that these neurons could also have a role in image-forming visual function (Estevez et al., 2012; Schmidt et al., 2011) and that they may also influence vision through the transmission of luminance signals to the outer retina (Prigge et al., 2016).

Our group has demonstrated that the transcription factor Brn3a is expressed by most RGCs (~96%) except those that express melanopsin (~2.6% of the total RGC population; Galindo-Romero et al., 2013; García-Ayuso et al., 2015) and half of their ipsilateral projection (~1.3% of the total RGC population; Nadal-Nicolas et al., 2012; 2014). While melanopsin is expressed by photosensitive RGCs that mainly carry non-image forming visual information, Brn3a is expressed by the population of RGCs that convey image-forming visual information but do not respond to light

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(henceforward the general RGC population or Brn3a$^+$RGCs). Thus, double immunodetection of melanopsin and Brn3a is an effective tool to study these two functionally different types of RGCs in parallel but independently (Agudo-Barriuso et al., 2016; Vidal-Sanz et al., 2015a). This approach has allowed us to document the total number and spatial distribution of m$^+$RGCs in the rat retina in relation to the general RGC population (Galindo-Romero et al., 2013; Nadal-Nicolas et al., 2012; 2014, 2015a,b), their co-expression of Brn3a (Galindo-Romero et al., 2013; Nadal-Nicolas et al., 2012) and that this co-expression changes as photoreceptors die in a model of retinal dystrophy (García-Ayuso et al., 2015).

m$^+$RGCs may be more resistant to different injuries than the general RGC population (Cui et al., 2015; DeParis et al., 2012; Moura et al., 2013; Nadal-Nicolás et al., 2015b; Pérez de Sevilla Müller et al., 2014). However, the higher resilience of m$^+$RGCs to some degenerations is not clear and they may die at the same rates as the rest of the RGCs in some diseases such as experimental glaucoma (Rovere et al., 2016; Valiente-Soriano et al., 2015a,b) or retinitis pigmentosa (Esquiva et al., 2013; García-Ayuso et al., 2015).

Exposure to light is often used to induce retinal degeneration (Noell et al., 1966). Our method of phototoxicity has been well characterized in rats (García-Ayuso et al., 2011; Marco-Gomariz et al., 2006) and mice (Montalbán-Soler et al., 2012). We have described in detail how phototoxicity affects first photoreceptors

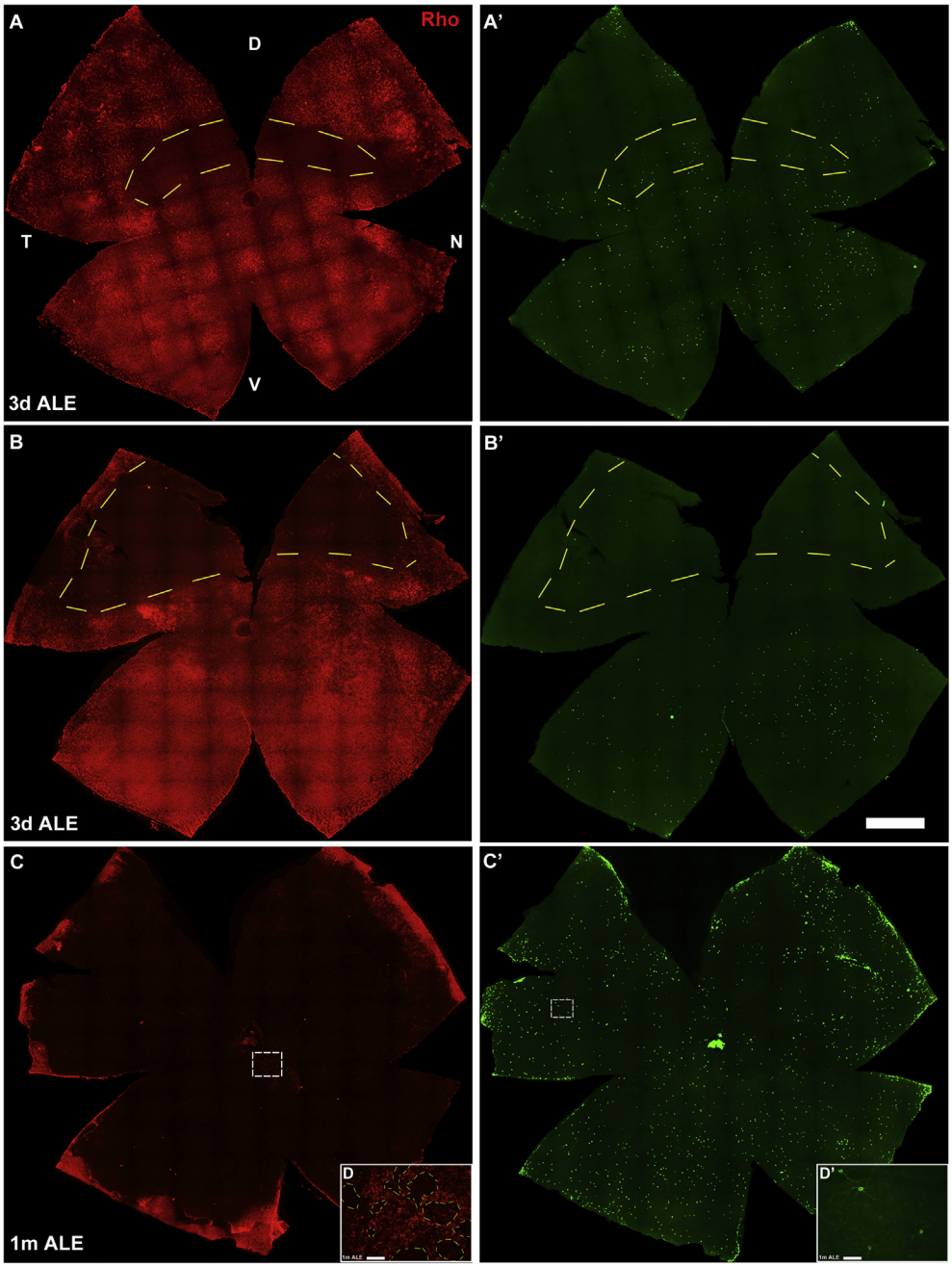
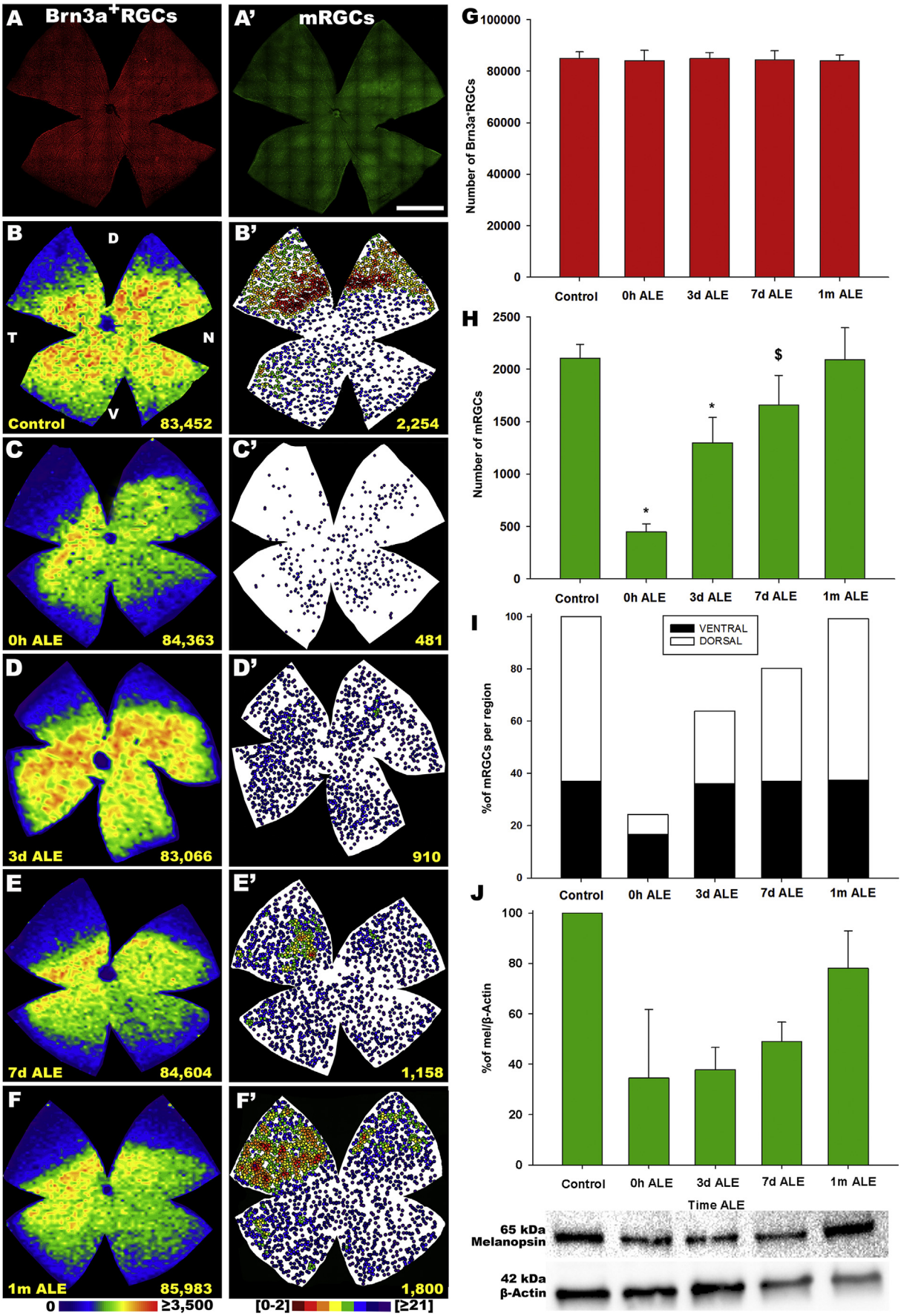


Fig. 1. Light exposure causes a progressive rod loss and a transitory melanopsin downregulation. Retinal whole-mounts showing rhodopsin (A–C) and melanopsin (A′-C′) immunodetection in three representative retinas from animals processed 3 days (A, A′, B, B′), and 1 month (C, C′) ALE. The arciform area of rod degeneration is clearly observed at 3 days ALE. D and D′, magnifications showing the areas highlighted in C and C′, respectively. In D, rings delineated by rods are observed at 1 month ALE (C). In D′, specific melanopsin immunostaining. Scale bars: 1 mm in (A-C, A′-C′) and 100 μm in (D, D′).


A Brn3a+RGCs
A' mRGCs
B
D
T
N
V
Control
83,452
B'
2,254
C
0h ALE
84,363
C'
481
D
3d ALE
83,066
D'
910
E
7d ALE
84,604
E'
1,158
F
1m ALE
85,983
F'
1,800
0
≥3,500
[0-2]
[≥21]
G
Number of Brn3a+RGCs
100000
80000
60000
40000
20000
0
Control
0h ALE
3d ALE
7d ALE
1m ALE
H
Number of mRGCs
2500
2000
1500
1000
500
0
*
*
$
Control
0h ALE
3d ALE
7d ALE
1m ALE
I
%of mRGCs per region
100
80
60
40
20
0
VENTRAL
DORSAL
Control
0h ALE
3d ALE
7d ALE
1m ALE
J
%of mel/β-Actin
100
80
60
40
20
0
Control
0h ALE
3d ALE
7d ALE
1m ALE
Time ALE
65 kDa
Melanopsin
42 kDa
β-Actin

and, with time, other retinal layers, causing eventually (from 6 months onwards) RGC death (García-Ayuso et al., 2011; Marco-Gomariz et al., 2006), following a similar pattern to that observed in inherited photoreceptor degenerations (García-Ayuso et al., 2010, 2014; Villegas-Pérez et al., 1996, 1998). It is now widely accepted that there is extensive retinal remodelling following photoreceptor degeneration (Marc et al., 2008).

To the best of our knowledge, the effect of an acute light exposurein the population of m^+RGCs is not known and, because m^+RGCs are directly stimulated by light, it is plausible that they will respond to a light insult.

For this study, two months old albino Sprague Dawley (SD) female rats obtained from the University of Murcia breeding colony (n = 28) were housed in temperature- and light-controlled rooms with a 12:12 h light-dark cycle (light from 8 a.m. to 8 p.m.), and had food and water *ad libitum*. Light intensity within the cages ranged from 5 to 30 lux. Animal manipulations were performed following the Spanish and European Union regulations for the use of animals in research and were approved by the Ethical and Animal Studies Committee of the University of Murcia.

Light exposure was always started between 10 and 12 a.m. following our previously reported methods (García-Ayuso et al., 2011). Briefly, the rats were placed individually in standard transparent cages and food and water were placed in Petri dishes at the bottom. A metal grid was placed also at the bottom to avoid the use of litter and thus prevent the animals from burying their heads to hide from light. The animals were then exposed to cold white light (linear bulbs situated 20 cm above the cages) at an intensity of 3000 luxes during 48 h.

Immediately ALE or at 3, 7 days or 1 month ALE, rats (n = 5 per time point) were euthanized with an intraperitoneal lethal dose of sodium pentobarbital (Dolethal Vetoquinol, S.A., Lure, France) and then perfused transcardially. This was carried out always between 10:00 a.m. and 12:00 a.m. to avoid melanopsin diurnal fluctuations (González-Menéndez et al., 2009; Hannibal et al., 2013). Retinas were dissected as whole-mounts keeping record of the orientation (as described in detail in Salinas-Navarro et al., 2009) and the general population of RGC immunodetected (goat anti-Brn3a C20, diluted 1:750; Santa Cruz Biotechnologies, Heidelberg, Germany), m^+RGCs (M1 and M2 subtypes; rabbit anti-melanopsin PAI-780 antibody, diluted 1:500; Thermo Scientific, Madrid, Spain) and rods (mouse anti-rhodopsin, diluted 1:10,000; Sigma-Aldrich, Madrid, Spain) were also immunodetected following previously described methods (García-Ayuso et al., 2015; Nadal-Nicolas et al., 2012). For secondary detection a combination of donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 594 or donkey anti-mouse Alexa 594 antibodies were used (diluted 1:500, Molecular Probes, Thermo Scientific. Madrid, Spain).

Retinas were examined and photographed using an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany). The total number of $Brn3a^+$RGCs was automatically quantified and their distribution assessed by isodensity maps as previously reported (Vidal-Sanz et al., 2015b). m^+RGCs were manually dotted in a blinded masked fashion in the retinal photomontage, and then the dots were automatically quantified and their topography depicted by neighbour maps (Galindo-Romero et al., 2013; García-Ayuso et al., 2015; Nadal-Nicolás et al., 2015a,b). Photomontage reconstruction and cell quantification were carried out using the image analysis software Image-Pro Plus (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA). The distribution maps were performed using SigmaPlot (SigmaPlot 9.0 for Windows; Systat Software, Inc., Richmond, CA, USA).

For western blotting, retinas were fresh dissected and immediately frozen in dry ice (n = 4 retinas/group). Then, retinas were homogenized in lysis buffer (Pro-prep protein extraction solution, Intron Biotechnologies, Eurovet Veterinaria SL, Madrid, Spain) and the amount of protein was determined using a bicinchoninic acid test (BCA, B9643 Sigma-Aldrich, Madrid, Spain). A total of 10–20 μg of protein from pool samples (composed by four individual retinal extracts) were resolved in 4–20% SDS-PAGE gels (Mini-Protean® TGX™ Gels, Cat#45–1095; Bio-Rad, Madrid, Spain), transferred to a nitrocellulose membrane and incubated overnight at 4 °C with rabbit anti-melanopsin (1:500). Secondary detection was carried out with donkey anti-rabbit horseradish peroxidase (1:5000; Santa Cruz Biotechnologies, Heidelberg, Germany). As a loading control, β-actin (rabbit anti-β-Actin-HRP mouse monoclonal antibody, 1:5000; Sigma Aldrich) was identified. Membranes were developed using Enhanced Chemo Luminiscence (GE Healthcare Europe GmBH, Barcelona, Spain). The signal was acquired with ChemidocTM XRS+ (Bio-Rad, Hercules, CA) and the density of the bands was quantified using the software ImageLabTM 5.2.1. Westerns were replicated three times.

Statistical analysis was done by using SigmaStat 3.1 for Windows (SigmaStat for Windows TM version 3.11; Systat Software, Inc.). One way ANOVA was used when comparing more than two groups and the Mann-Whitney or the *t*-test when comparing two groups only. Differences were considered significant when $P < 0.05$.

Three days ALE, the absence of rods was clearly observed in an "arciform area" of degeneration in the superior retina (Fig. 1A and B) that showed some inter-animal variation in size and shape. We have previously described this arciform area as the area in which there is maximal photoreceptor death and vascular leakage of horseradish peroxidase ALE, both in albino and pigmented rats (García-Ayuso et al., 2011; Marco-Gomariz et al., 2006). Here, we document for the first time that rod loss ALE begins in this arciform area. With time ALE (7 days onwards) the loss of rods expands all throughout the retina (Fig. 1C) and rings of rod degeneration can be seen in the areas where some rods are still present (Fig. 1D). These rings of photoreceptor degeneration have been previously described for rods and cones in two animal models of inherited retinal degeneration bearing a rhodopsin mutation: the P23H-1 rat (García-Ayuso et al., 2013) and the 334ter-line-3rat (Ji et al., 2012; Yu et al., 2016), but not in light-induced phototoxicity.

The total number and distribution of $Brn3a^+$RGCs remained however unaltered and similar to those observed in control retinas at all time points ALE (Mean ± Standard Deviation: 84,035 ± 2295; n = 5 retinas; Fig. 2A–E,F), in agreement with previous studies from

Fig. 2. Effect of light exposure on the topography and numbers of $Brn3a^+$RGCs and m^+RGCs, and on melanopsin expression. Representative control retina showing Brn3a immunostaining (A) and melanopsin immunostaining (A′) and their corresponding isodensity maps (B, B′). Isodensity maps (B–F) showing the distribution of $Brn3a^+$RGCs and neighbour maps (B′-F′) showing the distribution of m^+RGCs in the same retinas in control (non-exposed) animals (B, B′), and light exposed animals at 0 h (C, C′), 3 (D, D′) or 7 days ALE (E, E′) and one month ALE (F, F′). The number of cells per retina is represented at the bottom of each map. Color scale bars indicate from 0 (purple) to ≥3500 (red) Brn3a + RGCs/mm2 in the isodensity maps and from 0 to 2 (purple) to ≥21 (dark brown) neighbors in the m^+RGC neighbour maps. Mel, melanopsin; I, inferior; N, nasal; S, superior; T, temporal. In G and H, histograms showing the mean number ± SD of immunodetected $Brn3a^+$RGCs (F) and m^+RGCs (G) in control and light-exposed retinas. * and $ indicate significant differences, $p < 0.001$ and $p = 0.001$, respectively. In I, histogram showing the mean percentage of m^+RGCs per group and in the dorsal or ventral hemiretina (white and black, respectively). These percentages were calculated by considering 100% the number of m^+RGCs in P30 SD control rats. In J, western blot showing the expression level of melanopsin in retinal extracts from control and light exposed animals. In the bar graph above it, is shown the quantification of melanopsin signal intensity in arbitrary units taking intact retinas as 100% and normalized vs. β-actin signal. Error bars show the SEM for each time-point. Scale bar: 1 mm.

our laboratory (García-Ayuso et al., 2011).

In control non-exposed animals, the mean total number and distribution of m$^+$RGCs (2106 ± 132; n = 10 retinas; Fig. 2A', B' and H) was similar to those previously published (Galindo-Romero et al., 2013; García-Ayuso et al., 2015; Hattar et al., 2002). However, in light exposed animals immediately ALE (0 h) there was a substantial reduction in the total number of immunodetected m$^+$RGCs (449 ± 75; n = 10 retinas; Fig. 2) that was significant when compared to control animals ($p < 0.001$) Surprisingly, 3 days ALE there was a significant recovery (1298 ± 24; n = 10 retinas; $p < 0.001$), although their number was still significantly lower than in control animals ($p < 0.001$). One week ALE, the number of m$^+$RGCs increased significantly once more (1658 ± 284; n = 10 retinas; $p = 0.001$), but it was at 1 month when the total number of m$^+$RGCs reached values comparable to those of control animals (2092 ± 303; n = 10 retinas; $p = 0.91$). These results suggest that the decrease in the number of immunoidentified m$^+$RGCs might be due to a transient downregulation of melanopsin caused by phototoxicity.

We studied also the m$^+$RGCs topography, which in control animals was similar to that described previously (Fig. 2B'; Galindo-Romero et al., 2013; García-Ayuso et al., 2015). However, immediately ALE there was a diminution of m$^+$RGCs densities (warm colors) in the superotemporal retina (Fig. 2C'). This region is coincident with the arciform area where light exposure causes, shortly ALE maximal rod loss (Fig. 1A–B′). Interestingly, this area has been described also as the "photosensitive area" in normal rats (Marc et al., 2008; Tanito et al., 2007), and co-localizes with the rat visual streak, an area with the highest densities of RGCs and L-cones (Ortín-Martínez et al., 2010; Salinas-Navarro et al., 2009). This visual streak is most probably the preferential fixation area in rats and therefore an area more exposed to light (García-Ayuso et al., 2011; Ortín-Martínez et al., 2010; Salinas-Navarro et al., 2009). Thus impact of phototoxicity in RGCs and m$^+$RGCs would be higher in the cells in this area (Nadal-Nicolás et al., 2015a; Osborne et al., 2014). With time ALE the normal topography of m$^+$RGCs gradually recovers (Fig. 2D′,E′) and by one month ALE is similar to that observed in control animals (Fig. 1C', 2 F'). To verify the loss of warm colors in the superior retina, we quantified the mean numbers of m$^+$RGCs in the dorsal and in the ventral retina and we observed a higher loss of immunodetected m$^+$RGCs in the superior retina. Indeed, by 3 days ALE almost all m$^+$RGCs in the ventral retina have recovered their melanopsin expression, while in the dorsal retina this expression is not totally recovered until 30 days ALE (Fig. 2I).

To confirm the possibility of melanopsin downregulation, we analysed melanopsin expression in control and light exposed animals using Western blot. Immediately ALE, melanopsin expression down-regulates to 27%, recovering progressively thereafter (Fig. 2J). At 30 days ALE, the expression level of melanopsin reaches 78% of the normal value. However, at this same time point, the numbers of m$^+$RGCs reach normal values and thus we propose that the immunodetected m$^+$RGCs express less melanopsin while they are recovering from phototoxicity. Overall our data shows that up to 1 month, phototoxicity does not induce the death of m$^+$RGCs but alters their homeostasis.

Downregulation of different genes in RGCs following injury has been previously documented (Agudo et al., 2008, 2009; Chidlow et al., 2005; Lonngren et al., 2006). Moreover, transient down-regulation of melanopsin expression by ipRGCs has been recently described by our group following optic nerve crush and acute ocular hypertension (Nadal-Nicolás et al., 2015a,b; Rovere et al., 2016).

Hannibal et al. (2005) demonstrated that melanopsin expression in the retina changes during the daily cycle of light and darkness in albino rats (Hannibal et al., 2005). These authors suggested that this is a regulatory mechanism that allows ipRGCs to control their levels of melanopsin independently of their input from photoreceptors. Other authors however, have proposed that since the response of ipRGC to light is modulated by both, input from classical photoreceptors and melanopsin photosensitivity (Lucas et al., 2012; Wan et al., 2006) if photoreceptors degenerate, ipRGCs will receive less input from photoreceptors and this may cause melanopsin downregulation. Here we have observed that some time ALE the number of immunodetected m$^+$RGCs and the expression levels of melanopsin begin to recover gradually, while photoreceptors continue to die progressively (Fig. 1A–C; García-Ayuso et al., 2011). Thus, our results support the hypothesis of Hannibal et al. (2005), of independent melanopsin regulation by ipRGCs since we observe an early downregulation of melanopsin that is restored with time in spite of the loss of photoreceptors (García-Ayuso et al., 2011).

Why do ipRGCs downregulate their levels of melanopsin shortly ALE? High levels of light could over-stimulate the signalling cascade that commences when light activates melanopsin and this may harm the neurons through for example continuous activation of the cation channels and excitotoxicity. Exposure to light during 48 h could also result in disruption of the circadian rythms and this could also affect the circadian regulation of melatonin expression induced by ipRGCs. Therefore in our model of phototoxic injury, down-regulation of melanopsin expression could be an adaptative response of the cells and in the first instance, an attempt to avoid excitotoxicity.

It is accepted that photoreceptor degeneration leads to secondary RGC loss that commences usually when most photoreceptors have died and is independent of the cause of photoreceptor degeneration (Anastasakis et al., 2012; García-Ayuso et al., 2010, 2011, 2014; Garcia-Martin et al., 2012; Marco-Gomariz et al., 2006; Villegas-Pérez et al., 1996, 1998; Walia & Fishman, 2008). In agreement with this, we have demonstrated in an inherited model of retinal dystrophy, the P23H-1 rat, that, in 1 year old animals there is a significant m$^+$RGCs loss, and the surviving ones show signs of degeneration (García-Ayuso et al., 2015). Therefore we cannot exclude the possibility that at longer times ALE m$^+$RGCs will be secondarily affected as it occurs with the general RGC population (García-Ayuso et al., 2011; Marco-Gomariz et al., 2006).

In summary, this study demonstrates that during the first month ALE there is rod loss in the arciform photosensitive area that expands in the form of rings throughout the whole retina by 1 month and that during this period there is also a transient down-regulation of melanopsin expression. We suggest that melanopsin downregulation could be a protective mechanism to avoid the damaging effects of light. Further studies would be needed to clarify the effect of light exposure on the m$^+$RGC population at longer times.

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