Transient Downregulation of Melanopsin Expression After Retrograde Tracing or Optic Nerve Injury in Adult Rats

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Purpose. To investigate the effect of retrograde tracing or axotomy on melanopsin mRNA expression and immunodetection in albino and pigmented rat retinas.

Methods. Groups were (1) intact-naïve retinas; (2) optic nerve crush (ONC) analyzed at 7 days (7d) or 2 months (2m); (3) Fluorogold (FG) tracing from the superior colliculi (SC) analyzed at 7d or 2m; (4) tracing from the intact optic nerve (ON) with FG or hydroxystilbamidine methanesulfonate (OHSt), analyzed 3d later; and (5) sham tracing from the ON or sham surgery. Brn3a and melanopsin were double stained in whole mounts to quantify and assess the distribution of orthotopic and displaced Brn3a+ retinal ganglion cells (Brn3a+RGCs) and melanopsin+RGCs (m+RGCs). Freshly dissected retinas were used for melanopsin mRNA quantitative PCR.

Results. Tracing from the SCi did not affect the number of Brn3a+RGCs or m+RGCs counted in pigmented rats. However, only 55% of m+RGCs were immunodetected in albinos at 7d, although by 2m the m+RGCs counts returned to normal. Optic nerve tracing had a more dramatic effect (38% or 77% of m+RGCs were immunodetected in albino or pigmented rats) that occurred irrespectively of the tracer (OHSt or FG). This effect was not observed in the sham groups. After ONC, Brn3a+RGCs decreased to 37% and 8% by 7d and 2m, respectively. Melanopsin+RGC counts diminished to 30% at 7d, but recovered to 49% of controls by 2m. Melanopsin mRNA was downregulated after ON tracing or 7d after ONC, but did not differ from intact values 2m after ONC.

Conclusions. Following ON injury or retrograde tracing there is a transient melanopsin downregulation that should be taken into account when assessing m+RGC survival.

Keywords: intrinsically photosensitive retinal ganglion cells, superior colliculi, optic nerve, neighbor maps, traumatic axonal injury

Intrinsically photosensitive retinal ganglion cells (ipRGCs) constitute a new type of RGC that responds directly to light due to the expression of the pigment melanopsin.1,2 They send information about ambient light intensity to the brain to control non-image-forming visual functions such as circadian photocentrainment, melatonin secretion cycle, sleep, masking behavior, and the pupillary light reflex.5–8 Moreover, recent reports document their involvement in image-forming functions.9–12 In rats and mice there are five subtypes of ipRGCs (M1–M5)8,13 and a recently described melanopsin-expressing retinal interneuron.14 M1 and M2 ipRGCs are identified by standard melanopsin immunodetection, while M3 to M5 are not, as they express very low quantities of melanopsin.9,13,15 Because all reports in rat and most of the work on mice studying ipRGCs rely on melanopsin immunodetection, we will refer to them as melanopsin+RGCs (m+RGCs).

The vast majority of m+RGCs and the rest of the RGCs are found in the ganglion cell layer (orthotopic), but a small proportion have their soma in the inner nuclear layer; these are known as Dogiel’s or displaced RGCs.16,17

Much work is devoted to deciphering the function, resilience to injury, and regenerative capacities of m+RGCs.15,18 In fact, studies in different animal models indicate that m+RGCs respond differently than the general RGC population to certain types of injury such as axotomy,18,19 N-Methyl-D-aspartate–induced excitotoxicity,20 retinal degeneration,21,22 or elevated intraocular pressure,23–25 although for the latter type of injury the reported results are not homogenous.26–30

Tracing techniques from either the superior colliculi (SCi) or the optic nerve (ON) are commonly used to identify and quantify rodent RGCs.31–36 Fluorogold (FG) or its analogue hydroxystilbamidine methanesulfonate are presently the tracers of choice for many laboratories.37–40

We have recently reported the total number and retinal distribution of m+RGCs, and found that they represent between 2% and 3% of all RGCs in adult albino and pigmented rats37,38,41 and mice.14 In these studies we noticed that FG tracing affected
the mRGC counts in albino rats. Such a decreased melanopsin immunoreactivity appeared greater when the tracer was applied around the ON than when it was applied over the SGI, something we thought could be related to the different interval time after tracing (3 days for ON and 7 days for SGI). Importantly, tracing from the SGI did not seem to affect melanopsin immunodetection in pigmented rats, and neither albino nor pigmented mice showed altered melanopsin immunostaining after tracing—a difference that we thought could also be explained by the retrograde tracer employed, OHS instead of FG.

Because the majority of the above-mentioned studies rely on the immunodetection of mRGCs and many of them employ retrogradely transported neuronal tracers to compare mRGCs with the rest of the RGC population, we have analyzed the short- and long-term effect of tracing or traumatic injury to the ON on melanopsin immunodetection and mRGC counts, as well as melanopsin mRNA expression in albino and pigmented rats. Our results indicate that both situations induce a transient downregulation of melanopsin that should be taken into account in assessment of mRGC survival.

METHODS

Animal Handling and Ethics Statement

Adult albino (Sprague-Dawley) and pigmented (Piebald Virol Glaxo) rats were obtained from the University of Murcia (Spain) breeding colony. Animal care and experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and European Union guidelines for the use of animals in research and were approved by the Ethical and Animal Studies Committee of the University of Murcia (Spain). For anesthesia, a mixture of xylazine (10 mg/kg body weight; Rompun; Bayer, Kiel, Germany) and ketamine (60 mg/kg body weight; Ketolar; Pfizer, Alcobendas, Madrid, Spain) was used intraperitoneally (i.p.). During recovery from anesthesia, an oculair ointment (Tobrex; Alcon Cusí, S.A., Barcelona, Spain) was applied on the cornea to prevent corneal desiccation.

Surgery

Tracing From the Optic Nerve. The meninges of both ONs were opened and a strip of gelatine sponge soaked in FG, vehicle, or saline was applied surrounding the nervous tract. Four groups were formed: (1) 6% FG (Fluorochrome, LLC, Denver, CO, USA) diluted in 10% dimethylsulfoxide (DMSO)-saline; (2) 10% hydroxyethylamidine methanesulfonate (OHSt; Molecular Probes, Leiden, The Netherlands) diluted in 10% DMSO-saline; (3) 10% DMSO-saline (sham tracing group); and (4) saline (sham surgery group). Animals were processed 5 days later.

Tracing From the Superior Colliculus. Fluoroceil DNA (3% in 10% DMSO-saline) was applied to both SGI as previously reported, and animals were processed 7 days or 2 months later.

Intraorbital Optic Nerve Crush. The left ON was crushed at 2 mm from the optic disc using previously reported methods. Animals were processed 7 days or 2 months later.

Experimental design is detailed in Table 1.

Retinal Dissection and Immunodetection

Rats were euthanized with an i.p. overdose of pentobarbital (Dolethal; Vetoquinol Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain) and perfused with saline followed by paraformaldehyde 4%. Then, retinas were dissected as flat mounts and processed in parallel. Immunofluorescence was carried out as previously reported.

The general population of RGCs was detected using goat anti-BrdU (1:500, C20; Santa Cruz Biotecnologies, Heidelberg, Germany). Melanopsin+RGCs (M1 and M2 subtypes) were detected using rabbit anti-melanopsin PAI-780 antibody (1:500; Thermo Scientific, Madrid, Spain) that binds to the NH₂ terminal of the melanopsin protein and thus identifies both melanosin isoforms.

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

Image Acquisition, Automated Quantification, and Topographical Maps

Retinal whole mounts were photographed under an epifluorescence microscope (Axioskop 2 Plus; Zeiss Mikroskopie, Jena, Germany) using for each marker the same acquisition settings. In the case of melanopsin, these were chosen based on intact retinas to get the maximum signal with the minimum background. Individual frames (154/retina) were later reconstructed as retinal montages as previously reported. Displaced mRGCs were identified as reported previously.

The number of traced and/or Brn3a+RGCs was automatically quantified (image analysis software: Image-Pro Plus, IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD, USA) using routines established by our group. Their topography was visualized using isodensity maps as previously described.

Melanopsin+RGCs were manually dotted on the retinal photomontage, and the dots were automatically quantified (IPP software). Then their distribution was assessed by the fixed-radius (0.276 mm) near neighbor algorithm, which allowed as well extraction of the number of them in each retinal quadrant as previously described.

All maps were performed using SigmaPlot (SigmaPlot 9.0 for Windows; Systat Software, Inc., Richmond, CA, USA).

Sampling and Measurement of Melanopsin+RGC Soma Diameter

In four retinas per strain and group, 12 samples of 0.1575 mm² were acquired, 3 per quadrant. The first sample was taken at 0.875 mm from the optic disc and the other two at 1 mm from each other. For each sample two images were acquired, one for orthotopic and the other for displaced mRGCs. The soma of each mRGC was outlined and its diameter calculated as the average length of diameters measured at 2° intervals and passing through the cell body centroid (IPP software). The number of measured mRGCs is shown in Results. Data representation was carried out with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

Real-Time Quantitative PCR

Each sample was composed of one left or right retina of a given animal. Retinas were collected immediately after sacrifice and stored in RNAlater stabilization reagent (Qiagen, Venlo, The Netherlands). Total RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA, USA) and the RNA samples were dissolved in 16 μL Milli-Q water (Merck Milipore, Billerica, MA, USA). Total RNA concentration was determined using NanoDrop ND1000 (Thermo Scientific,
Waltham, MA, USA). Complementary DNA amplification was performed according to the instructions provided by the manufacturer (NZYTech, Lisbon, Portugal), using 1 µg total RNA. The resultant cDNA was treated with RNase-H for 20 minutes at 37°C, and a 1:2 dilution was prepared for quantitative PCR (qPCR) analysis. All samples were stored at −20°C until analysis. Genomic DNA contamination was assessed with a conventional PCR for β-actin using intron-spanning primers (5′-GCTCCTCTGAGCGCAAG-3′; 3′-CATCTGCTGGAAGGTGGACA-5′), as previously described.48

SYBR Green-based real-time qPCR was performed using Step On ePlus (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions were as follows: iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) 200 nM primers (Opsin4; 5′-TTGCTGGTGATCTCGCACT-3′; 3′-CCCTGTACCTGGGTAGTG-5′) and 2 µL 1:2 dilution cDNA, in a total volume of 20 µL. Cycling conditions were a melting step at 95°C for 15 seconds and annealing-elongation at 60°C for 45 seconds, and extension at 72°C, with 40 cycles. A dissociation curve at the end of the PCR run was performed by ramping the temperature from 60°C to 95°C while continuously collecting fluorescence data. The Ct values were converted to relative quantification using the 2^−ΔΔCt method previously described.49 Three candidate housekeeping genes (Tbp, Hprt, and Ytebaz) were evaluated using NormFinder.50 Thermo stable gene was Hprt (5′-ATGGGAGGCCATCA CATTGT-3′; 3′-ATGAATCCAGCAGTGCTAGCAA-5′) and was therefore used as housekeeping gene.

Statistics

Comparison of two groups (t-test or Mann-Whitney test) and more than two groups (pairwise multiple comparison procedures, ANOVA, or Kruskal-Wallis ANOVA, and post hoc tests: Tukey or Dunn) was done with GraphPad Prism v. 6 software (GraphPad). Differences were considered significant when P < 0.05.

RESULTS

This work focused on analysis of the response of mRGs, orthotopic (m'oRGs) and displaced (m'dRGs), to retrograde tracing and ON injury. Immunodetected mRGs were counted in all retinas and to monitor the response of the general RGC population, Brn3a'RGs were quantified as well, since melanopsin and Brn3a immunodetection by double staining has been shown to be an excellent tool to study in parallel but independently the general population of RGCs (Brn3a') and m'RGs.14,28,30,31 To verify that the tracing was successful, traced RGCs were also quantified.

In all figures, neighbor maps of m'oRGs and m'dRGs are from the same retina, and so are Brn3a'+RGCs isodensity maps from m'oRGs and m'dRGs when shown (labeled with the same letter). Magnifications from whole-mounted retinas were always taken from the same areas: dorso-temporal, temporal, and ventral (shown in the retinal drawings in the corresponding figures).

Intact Retinas

In intact retinas, immunodetection of melanopsin gave a neat and clear signal in m'RG somas and dendritic processes (Figs. 1A–F). In both strains m'oRGs are more abundant in the dorsal than in the ventral retina (Figs. 1G–J', see below for quantitative data) and their topography is similar, although in the albino rat the highest densities were shifted to the temporal quadrant, a shift that is not so clear for the pigmented strain.17,37

The two strains had a similar population of m'oRGs,17,37 but the number of m'dRGs was significantly higher in the pigmented rat (Table 2).17 Finally, the mean soma diameter of m'oRGs was bigger than that of m'dRGs (see data below).

The total number of Brn3a'RGs (Table 2) in intact, traced, or sham groups was comparable among them within each strain and slightly bigger in pigmented than in albino rats, in agreement with previously published studies.17,36,41

Traced Retinas

The total number of traced RGCs is shown in Table 2, and these results are in agreement with previous reports.34,36 As previously reported,17,34,36 the number of RGCs was slightly higher in the retinas traced from the ON than from both SCI. In addition, there were no differences in the number and topography of Brn3a'RGs among all traced groups within each strain, indicating that neither tracing method affected RGC survival at these time points.

Of note, FG is not a long-term persistence tracer38,51 and thus a long time after its application the number of FG-traced RGCs decreases.52

Immunodetection of m'RGs (orthotopic and displaced) was diminished 7 days after tracing from the SCI in the albino but not in the pigmented strain (Table 2, Figs. 2A, 3A–D'). Not

Table 1. Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Quantification and Topography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orthotopic</td>
</tr>
<tr>
<td>Intact, albino and pigmented</td>
<td>6</td>
</tr>
<tr>
<td>Sham ON, albino and pigmented</td>
<td>4</td>
</tr>
<tr>
<td>Vehicle ON, albino and pigmented</td>
<td>4</td>
</tr>
<tr>
<td>FG-SCI 7 d, albino and pigmented</td>
<td>6</td>
</tr>
<tr>
<td>FG-SCI 2 mo, albino</td>
<td>6</td>
</tr>
<tr>
<td>FG-ON, albino and pigmented</td>
<td>6</td>
</tr>
<tr>
<td>OHSt-ON, albino</td>
<td>6</td>
</tr>
<tr>
<td>ONC 7 d, albino</td>
<td>6 LR</td>
</tr>
<tr>
<td>ONC 2 mo, albino</td>
<td>5 LR</td>
</tr>
</tbody>
</table>

Number of retinas used in this study for each of the rat strains, experimental groups, and analysis. In the ganglion cell layer, orthotopic Brn3a' and melanopsin'+RGCs were quantified, and in the traced groups, also FG+ or OHSt+ RGCs. In the inner nuclear layer only melanopsin-displaced RGCs were studied. In the ONC groups prepared for qPCR, the right (RR), contralateral to the lesion, and the left (LR), injured, retinas were analyzed separately.
FIGURE 1. Orthotopic and displaced melanopsin+RGCs in intact albino and pigmented retinas. Top: Magnifications from flat-mounted retinas showing melanopsin+RGCs (m+RGCs) in pigmented (A–C) or albino (D–F) rats. Magnifications in each column were taken from the areas framed in the drawings (top row). In the subsequent figures, all magnifications are from the same retinal locations. Bottom: (G, H) Neighbor maps showing the topographical distribution of orthotopic m+RGCs (G–J) in one right (RR) and one left (LR) retina from a pigmented (G, H) or an albino (I, J) rat. (G’–J’) topography of displaced m+RGCs in the same retinas as in (G–J). In these maps, each dot represents a single m+RGC, and the warmer its color, the more neighbors that m+RGC has in a radius of 0.276 mm. Color scale is shown in (G); each color represents an increase of 3 neighbors, from 0 to 3 (purple) to 44 to 47 or more (bright green). At the bottom left of each map is the number of m+RGCs quantified in the corresponding retina. D, dorsal; T, temporal; V, ventral; N, nasal; RR, right retina; LR, left retina. Scale bars in (A, H).
Melanopsin immunodetection after tracing. Magnifications from flat-mounted retinas showing melanopsin immunodetection 7 days (A) or 2 months (B) after FG tracing from the SCi in albino rats, and 3 days after FG tracing from the optic nerve in albino (C) and pigmented (D) animals. D, dorsal; T, temporal; V, ventral; N, nasal. Scale bar in (A).
only fewer m'RGs were identified (44% of m'oRGs and 64% of m'dRGs were not immunodetected) when compared to intact control, but melanopsin immunoreactivity was restricted to the soma (Fig. 2A). This alteration was transitory because 2 months after the tracing, the melanopsin signal was back to normal in number, cell and dendrite appearance, and retinal topography (Table 2; Figs. 2B, 3E–F').

We have previously reported in albino rats that 91% of the m'oRGs project to the SCi, 37 but in view of this transient diminution in melanopsin expression the reported projection could be an overestimation. Thus, we determined the percentage of m'oRGs traced from both SCi in the pigmented strain. Out of 770 counted m'oRGs, 706 were FG+. Hence in the pigmented strain, 92% of the m'oRGs project to the SCi.

Tracing from the intact ON affected m'RG immunodetection in both strains, although it was more severe for the albino (62% of m'oRGs and 75% of m'dRGs were not immunode-

**FIGURE 3.** Effect of tracing from the superior colliculi on m'RG immunodetection. (A–D') Neighbor maps showing the spatial distribution of orthotopic m'RGs (A–D) and displaced m'RGs (A’–D’) 1 week after Fluorogold tracing from the SCI. In the pigmented strain, the number and topography of m'RGs are the same as in intact retinas; however, in the albino rat, fewer m'RGs were immunodetected, mostly in the dorsal retina. (E, F') Two months after FG tracing, the albino retina shows a normal number and topography of m'RGs. At the bottom left of each map is the number of m'RGs quantified in the corresponding retina. Color scale in (B). RR, right retina; LR, left retina; D, dorsal; T, temporal; V, ventral; N, nasal. Scale bar in (A).

only fewer m'RGs were identified (44% of m'oRGs and 64% of m'dRGs were not immunodetected) when compared to intact control, but melanopsin immunoreactivity was restricted to the soma (Fig. 2A). This alteration was transitory because 2 months after the tracing, the melanopsin signal was back to normal in number, cell and dendrite appearance, and retinal topography (Table 2; Figs. 2B, 3E–F').

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Tracing from the intact ON affected m'RG immunodetection in both strains, although it was more severe for the albino (62% of m'oRGs and 75% of m'dRGs were not immunodetected) than for the pigmented rat, since in this strain there were no differences in the counts of m'dRGs, and with respect to m'oRGs, 33% of them were lost (Table 2, Figs. 2C, 2D). Loss of melanopsin immunostaining after ON tracing was stronger than after tracing from the SCi, appeared irrespective of the retrograde tracer employed (OHSt or FG), and was not observed in the sham tracing or sham surgery groups (Table 2, Figs. 2B, 3E–F'). If the reduced melanopsin immunodetection was due to antigen masking, one would expect a homogenous impairment across the retina, but this was not the case. Tracing had a significantly higher impact on m'RGs located in the dorsal retina (Fig. 5). Furthermore, in the pigmented strain the number of m'oRGs in the ventral retina did not change after tracing from the ON; and, importantly, in these same retinas the total number of displaced m'RGs did not differ from that in intact animals.

Soma size measurement further supported that tracing is detrimental to the expression of melanopsin by m'RGs.
Figure 4. Effect of tracing from the intact optic nerve on m\(^+\)RGC immunodetection. (A-D) Isodensity maps of Brn3a\(^+\)RGCs in pigmented (A, B) and albino (C, D) retinas after FG tracing from the intact optic nerve. (A'-D') m\(^+\)RGCs neighbor maps from the same retinas as in (A-D). While the number and topography of Brn3a\(^+\)RGCs are not affected by FG application onto the ON, fewer orthotopic m\(^+\)RGCs (A'-D') and displaced m\(^+\)RGCs (A''-D'') are immunodetected, mostly in the dorsal retina. This effect is stronger in the albino strain. (E-L) In retinas from vehicle (E-G) or sham (I, J) control groups, the number and distribution of m\(^+\)RGCs are normal. At the bottom left of each map is the number of m\(^+\)RGCs quantified in the corresponding retina. Color scales in (B) (isodensity maps) and (C) (neighbor maps): RR, right retina; LR, left retina; D, dorsal; T, temporal; V, ventral; N, nasal. Scale bar in (A).
because their mean soma diameter diminished significantly (Figs. 6B, 6C). Interestingly, in the pigmented strain, m+RGCs were smaller after tracing from the ON but not from the SCI. Furthermore, in the albino retina, m+RGCs recovered their normal size 2 months after tracing from the SCI. All these data demonstrate that the observed decrease in size is not the result of an artifact.

**Axotomized Retinas**

Intraorbital optic injury results in selective death of RGCs. Indeed, 7 days after optic nerve crush (ONC), 63% of Brn3a+RGCs were lost. This loss progressed with time, and by 2 months approximately 8% of the Brn3a+RGC population remained in the retina (Table 2, Fig. 7). In the
Table 2. Number of RGCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Traced RGCs</th>
<th>Brn3a+ RGCs</th>
<th>m+oRGCs</th>
<th>m+dRGCs</th>
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<tr>
<td>Albino</td>
<td></td>
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<tr>
<td>Intact</td>
<td>80,161</td>
<td>2,225</td>
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<tr>
<td>SD</td>
<td>1,000</td>
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<tr>
<td>FG-SCI 7d</td>
<td>81,762</td>
<td>81,480</td>
<td>1,238†</td>
<td>20†§</td>
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<tr>
<td>Mean</td>
<td>748</td>
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<td>192</td>
<td>2</td>
</tr>
<tr>
<td>FG-SCI 2m</td>
<td>59,448*</td>
<td>81,118</td>
<td>2,486</td>
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</tr>
<tr>
<td>Mean</td>
<td>3,892</td>
<td>997</td>
<td>115</td>
<td>11</td>
</tr>
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<td>Vehicle ON</td>
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<td>81,700</td>
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<tr>
<td>SD</td>
<td>2,221</td>
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<td>Mean</td>
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<tr>
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<td>16†‡</td>
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<td>758†</td>
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<td>Mean</td>
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<td>ONC 7d</td>
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<tr>
<td>Mean</td>
<td>29,529†</td>
<td>478¶‡</td>
<td>17†§</td>
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<tr>
<td>SD</td>
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<tr>
<td>ONC 2m</td>
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<td>Pigmented</td>
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<td>Intact</td>
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<td>Vehicle ON</td>
<td>82,910</td>
<td>2,079</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>4,289</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham ON</td>
<td>82,781</td>
<td>2,125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2,790</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FG-ON</td>
<td>87,283</td>
<td>82,739</td>
<td>1,720‡</td>
<td>189</td>
</tr>
<tr>
<td>Mean</td>
<td>1,164</td>
<td>2,123</td>
<td>288</td>
<td>30</td>
</tr>
</tbody>
</table>

Mean and standard deviation of the total number of RGCs in each experimental group and rat strain (n = 4-6 retinas per group). ON, optic nerve; SCI, superior colliculi.

* Two months after tracing, some RGCs have lost the labeling.11,12
† Significantly lower number of immunodetected Brn3a+ RGCs or m+oRGCs compared to intact retinas (ANOVA, Tukey test P < 0.001).
‡ Significantly lower number of immunodetected m+oRGCs compared to intact retinas (ANOVA, Tukey test P < 0.01).
§ Significantly lower number of immunodetected m+dRGCs compared to 2-month group (P < 0.05 for m+dRGCs between 7 days and 2 months after ONC; P < 0.01 for the rest).

The mean soma diameter of the remaining m+RGCs was significantly smaller than that found in intact retinas, and did not differ from the diameter measured after ONC or the diameter measured 7 days after tracing from the SCI. Importantly, 2 months after ONC, the mean size of orthotopic, but not displaced, m+RGCs was significantly larger than at 7 days (Figs. 6B, 6C).

Melanopsin mRNA

All the above data rely on the immunodetection of melanopsin protein, which, in the case of traced retinas, could be impaired due to antigen masking. Thus, we investigated as well the regulation of melanopsin mRNA. In this experiment, tracing was performed from the ON because it triggered a stronger response than from the SCI.

Figure 8 shows that indeed, after tracing, melanopsin mRNA is significantly downregulated, thus confirming the anatomical data. Seven days after ONC, melanopsin mRNA was also significantly downregulated, but this was transitory because by 2 months the expression of melanopsin mRNA, although lower than in intact retinas, was not significantly different. In retinas contralateral to the injured retinas, melanopsin mRNA level was comparable to that in intact retinas.

Discussion

The present work using immunodetection and qPCR, we document that melanopsin is downregulated in adult rats by retrograde tracing or by ON axotomy. The effect on m+RGC immunodetection and counts differs between rat strains and tracing methods: Albino are more sensitive than pigmented, and ON tracing elicits a stronger response than tracing from the SCI.

Axotomy and tracing share three features: (1) The response of m+RGCs is stronger in the dorsal retina; (2) their mean soma diameter decreases; and (3) there is a delayed recovery of melanopsin expression and soma size.

Albino and Pigmented Rats

Albinism causes a number of abnormalities in the retina and visual system, such as decreased ipsilateral projection,17,41,54–58 different cone photoreceptor topography,59 and decreased number of displaced RGCs.14,17,56 Our data show that the total number of orthotopic m+RGCs was similar among pigmented and albino animals and that in general terms there are no differences among strains, in agreement with previous reports.14,17 However, there are subtle differences in the topography of these cells: In the albino strain the highest densities of m+RGCs are found in the temporal quadrant, while in the pigmented strain these are observed in the hemidorsal retina.17 Furthermore, in albino rats a “C”-like shape of m+RGCs is observed from the dorsotemporal to the ventrotemporal quadrant that is not found in the pigmented strain.57 With respect to displaced m+RGCs, they are significantly more abundant in the pigmented rat, as has been described for nonmelanopsin+ dRGCs.14,17,56

Melanopsin downregulation after tracing is more severe in the albino than in the pigmented strain. At present we ignore why this is so. However, it has been reported that the melanopsin protein downregulates in conditions of constant light, more strongly in the albino than in the pigmented rat.60,62 The reasons are unknown, but the observation might be due to the presence of melanin in the pigmented epithelium of the pigmented rat, a pigment that absorbs scattered light. Fluorogold has an excitation band between 350 and 395 nm
FIGURE 6. Soma diameter of orthotopic and displaced m°RGCs. Scatter dot plots showing the soma diameter of each m°RGC (open circles) analyzed per group and the mean ± SD values (lines). (A) In intact retinas, the mean diameter of displaced m°RGCs (m°dRGCs) is significantly smaller than that of orthotopic m°RGCs (m°oRGCs). (B) In albino retinas, after ON tracing, at 7 days after tracing from the SCi and after axotomy, the size of m°oRGCs significantly decreases compared to intact retinas. Two months after tracing from the SCi, their soma size returns to intact values. Two months after ONC, surviving m°oRGCs have a significantly bigger diameter than at 7 days; however, they are still smaller than in intact retinas. In the pigmented rat, the decrease of soma size is significant after tracing from the ON but not from the SCi. (C) The soma of displaced m°RGCs in albino animals is significantly smaller after tracing or axotomy. In contrast to m°oRGCs, there is no difference between 7 days and 2 months after ONC. In the pigmented strain, there is not a change in the soma size of displaced m°RGCs. n, number of analyzed m°RGCs.
FIGURE 7. Transient downregulation of melanopsin after optic nerve axotomy. (A–F) Immunodetection of m^+RGCs in flat-mounted retinas analyzed at 7 days (A–C) or 2 months (D–F) after ONC. Seven days after ON, there is a diffuse loss of Brn3a^+RGCs (G, H) and a diffuse, although stronger in the dorsal retina, loss of orthotopic (G', H') and displaced (G'', H'') m^+RGCs. Two months after ONC, the loss of Brn3a^+RGCs has progressed further (I, J), but there is a recovery of m^+RGCs (I', J'). Color scales in (H) (isodensity maps) and (H') (neighbor maps). D, dorsal; T, temporal; V, ventral; N, nasal. Bar scales in (A, G).
and an emission band between 530 and 600 nm; both are within the ranges of the visible spectrum, and thus it is possible that the accumulation of the fluorescent compound in the soma of the m\(^+\)RGCs leads to disruption of their internal clock that regulates melanopsin synthesis.\(^{61}\)

**ON Tracing Versus SCi Tracing**

Optic nerve tracing causes a stronger melanopsin downregulation than tracing from the SCi. This difference could be the result of the different tracer concentrations used in the two methods and/or the time post tracing, although with the present experiments it is not possible to rule out one or the other.

Tracer concentrations were not switched between methods (i.e., 6\% for SCi tracing and 3\% for ON tracing) because the concentrations used here are the standard ones.

We did not perform ON tracing at longer times because 3 days is the minimum time needed to trace the whole retina\(^ {34-36,63,64}\) without quantifiable RGC death.\(^ {34}\) This is important because ON tracing is not an innocuous procedure: The meninges are open, the ON is manipulated,\(^ {34}\) and the tracer is dissolved in DMSO, which has been shown to be toxic for neurons.\(^ {65}\) Thus, ON tracing may be harmful and induce a protracted and retrograde RGC death. By choosing this time point, the effects of ON tracing on melanopsin immunodetection and mRNA expression could be assigned to the tracing itself, without the additional effects of RGC loss.

Retinas traced from the SCi were analyzed at 7 days because this is an optimal time interval of retrograde transport that leads to labeling of the entire RGC population.\(^ {52,66}\) In addition we analyzed the retinas at 2 months because tracing from the SCi does not cause RGC death (present data and Selles-Navarro et al.\(^ {52}\)); thus it was a good model to verify whether melanopsin mRNA expression and immunodetection were to return to basal levels.

**Soma Diameter**

Melanopsin\(^+\)RGC subtypes are distinguished by their soma location, dendritic arborization, and soma diameter (M1<M2<M3<M4\(^ {7,9,13}\)). The observed decrease of the mean soma diameter of m\(^+\)RGCs, both orthotopic and displaced, may be caused by the loss of the bigger m\(^+\)RGCs or by a shrinkage of their cell bodies. Indeed, following ON injury there is a loss of the largest RGCs as well as RGC soma shrinkage.\(^ {71}\) Our analysis does not allow distinguishing between the different m\(^+\)RGC subtypes based on their dendritic arborization; thus with the present data it is not possible to know whether a specific m\(^+\)RGC subtype is the most affected or if all are equally altered but have shrunk. Both explanations are conceivable because the diameter of the surviving m\(^+\)RGCs 2 months after

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**Figure 8.** Melanopsin mRNA downregulation after tracing and ONC. FG tracing from the ON produces a significant decrease of melanopsin mRNA. Seven days after axotomy (ONC), melanopsin mRNA significantly decreases in the injured eye, but at 2 months is recovered to intact values. In the retinas contralateral to the injured retinas, melanopsin mRNA expression does not change.
ONC is significantly larger than at 7 days, and at 2 months after tracing from the SCi their soma size is fully recovered.

Recovery of Melanopsin Expression

Early after tracing or ON injury, fewer m\(^+\)RGCs were identified, and they had a greatly diminished immunoreactivity of melanopsin in their dendrites. Downregulation of a large number of genes in RGCs is a well-known response to retinal injury.43,73–75

Still, the transient downregulation of melanopsin is a striking discovery, as it means that (1) depending on when the retinas are analyzed, more or less survival will be observed (injury) or cells will be identified (tracing); and (2) melanopsin is a rather extraordinary indicator of the well-being of m\(^+\)RGCs but not of their viability.

Neither tracing method nor retrograde tracer had an impact on the number of Brn3a\(^+\)RGCs, nor did we observe melanopsin changes after sham tracing or sham surgery. Two months after tracing from the SCi, melanopsin expression is fully recovered, which indicates that tracing from the SCi itself does not induce m\(^+\)RGC loss. However, because of the transitory downregulation of melanopsin caused by FG or OHSt, care should be taken with use of these fluorescent tracers in quantitative studies of immunostained m\(^+\)RGCs.

In addition to their capacity to sense light, m\(^+\)RGCs differ from the rest of the RGCs in their response to neuroprotection,28 axonal regeneration,18 and injury. They are more resistant to axotomy,18,19,76 excitotoxicity,10 optic neuropathy77 (reviewed in Ref. 15), and ocular hypertension,25–29 though in this paradigm the results are variable24–30 (reviewed in Ref. 78). In hereditary models of retinal degeneration, however, m\(^+\)RGCs die in a proportion similar to the rest of RGCs; but during progression of the disease they remodel anatomically27,28 and molecularly.79 Why m\(^+\)RGCs are more resistant to some insults is yet unknown. It has been proposed that their higher resilience to ON axotomy may be due to undamaged intraretinal axonal collateral that would gather the necessary trophic support from within the retina, although the number of m\(^+\)RGCs sending axonal collaterals does not appear to be large enough to explain the number of surviving m\(^+\)RGCs.5,15,80,81 Moleculary, it has been shown that the PI3 K/Akt pathway is part of their resilience to axotomy.70 Finally, their survival capacity may be related to their constitutive expression of PAP62,83,89 a polypeptide that has been shown to be neuroprotectant for RGCs against excitotoxicity,84,85 axotomy,86 and ischemia.87,88

Our results are in agreement with these reports,18,19,76 because 2 months after intraorbital ONC we have found that ~50% of the m\(^+\)RGCs survive, yet less than 10% of the Brn3a\(^+\)RGCs are present. Had we analyzed the retinas only at 7 days post ONC, we would have concluded that axotomy takes a higher toll on m\(^+\)RGCs than on the general RGC population.

Importantly, although by 2 months approximately half of the m\(^+\)RGCs are lost (or not immunodetected), the levels of melanopsin mRNA, while on average 20% below control levels, do not differ significantly from those of intact retinas. It is possible that the remaining m\(^+\)RGCs express more melanopsin per cell than in an intact retina, perhaps in an attempt to compensate for the loss of m\(^+\)RGCs to regain full functional recovery. Electrophysiological studies have shown that all ipRGC subtypes (M1–M5) have their intrinsic photoresponses with \(\lambda_{\text{max}}\) close to melanopsin’s \(\lambda_{\text{max}}\), thus indicating that all express melanopsin as their photopigment, yet melanopsin antibodies readily identify only the M1 and M2 subtypes of ipRGCs.13 Therefore, an additional explanation for this apparent mismatch between the recovery of melanopsin mRNA expression and the number of detected m\(^+\)RGCs 2 months after ONC is that with use of melanopsin immunodetection, only a proportion of the actual ipRGCs are identified. Nevertheless, early after ON tracing and after ONC, melanopsin mRNA levels decrease in a parallel fashion to the number of detected m\(^+\)RGCs.

In conclusion, caution is warranted when studying the response of m\(^+\)RGCs to injury. Finally, tracing is not an appropriate approach to study in parallel the general RGC population and m\(^+\)RGCs; an alternative and reliable method is to use double staining of melanopsin and Brn3a.

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