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Number and spatial distribution of intrinsically photosensitive retinal ganglion cells in the adult albino rat

C. Galindo-Romero [a,1], M. Jiménez-López [a,1], D. García-Ayuso [a], M. Salinas-Navarro [a], F.M. Nadal-Nicolás [a,b], M. Agudo-Barriuso [a,b], M.P. Villegas-Pérez [a], M. Avilés-Trigueros [a,**,2], M. Vidal-Sanz [a,*,2]

[a] Departamento de Oftalmología, Facultad de Medicina, Regional Campus of International Excellence "Campus Mare Nostrum", Instituto Murciano de Investigaciones Biosanitarias, Campus de Espinardo Universidad de Murcia, E-30100 Espinardo, Murcia, Spain
[b] Unidad de Investigación, Hospital Universitario Virgen de la Arrixaca, Fundación para la Formación e Investigación Sanitarias de la Región de Murcia, Instituto Murciano de Investigaciones Biosanitarias, Murcia, Spain

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ABSTRACT

Intrinsically photosensitive retinal ganglion cells (ipRGCs) respond directly to light and are responsible of the synchronization of the circadian rhythm with the photic stimulus and for the pupillary light reflex. To quantify the total population of rat-ipRGCs and to assess their spatial distribution we have developed an automated routine and used neighbour maps. Moreover, in all analysed retinas we have studied the general population of RGCs – identified by their Brn3a expression – and the population of ipRGCs – identified by melanopsin immunodetection – thus allowing the co-analysis of their topography. Our results show that the total mean number ± standard deviation of ipRGCs in the albino rat is 2047 ± 309. Their distribution in the retina seems to be complementary to that of Brn3a$^+$RGCs, being denser in the periphery, especially in the superior retina where their highest densities are found in the temporal quadrant, above the visual streak. In addition, by tracing the retinas from both superior colliculi, we have also determined that 90.62% of the ipRGC project to these central targets.

1. Introduction

Intrinsically photosensitive retinal ganglion cells (ipRGC) are a subtype of retinal ganglion cells (RGC) that express melanopsin, an opsin that responds directly to light (Berson et al., 2002; Kumbalasiri and Provencio, 2005; Provencio et al., 2000, 2002). ipRGCs are responsible for non-image forming visual functions such as the regulation of the circadian rhythm and the pupillary light reflex (Berson et al., 2002; Hankins et al., 2008; Hattar et al., 2002, 2003; Lucas et al., 2003; Panda et al., 2003). Recently, however, it has been suggested that they also play a role in image forming vision (Schmidt et al., 2011) and may contribute to signal brightness discrimination (Brown et al., 2012; Semo et al., 2010).

ipRGCs that drive the circadian rhythm project mainly to the suprachiasmatic nucleus (Berson et al., 2002; Gooley et al., 2001), while those that are responsible for the pupillary reflex project to the intergeniculate leaflet and pretectal olivary nucleus (reviewed in Berson, 2003). In mice, it has been shown that ipRGCs project as well to the superior colliculi (SCi) (reviewed in Hattar et al., 2006).

In the adult rat, ipRGCs represent around 1–3% of the population of RGCs and are distributed throughout the entire retina, being denser in the supero-temporal pole (Hannibal and Fahrenkrug, 2002; Hattar et al., 2002). In rats and mice, RGCs project mainly to the SCi (Lund, 1965; Perry, 1981), reviewed in Sefton et al. (2005), thus, by applying retrogradely transported neuronal tracers such as fluorogold (FG) to these target regions, approximately 98.4% of the RGC can be identified (Salinas-Navarro et al., 2009a, 2009b). To identify the entire retinofugal population retrogradely transported neuronal tracers may be applied to the optic nerve stump and this method also allows identification of the small percentage of rodent RGCs that do not project to the SCi (Salinas-Navarro et al., 2009a, 2009b). RGCs may also be identified by their expression of specific proteins, such as Brn3a (Nadal-Nicolas et al., 2009) or mRNAs, such as γ-synuclein (Nguyen et al., 2011; Soto et al., 2008; Surgucheva et al., 2008). In rat, Brn3a is expressed by approximately 92.2% of the RGC population (Nadal-Nicolas et al., 2009), leaving aside half of the RGCs that project ipsilaterally and most of the ipRGCs (Nadal-Nicolas et al., 2012).

* Corresponding author. Tel.: +34 868 883961; fax: +34 868 883962.
** Corresponding author. Tel.: +34 868 887572; fax: +34 868 883962.
E-mail addresses: marcelin@um.es (M. Avilés-Trigueros), manuel.vidal@um.es (M. Vidal-Sanz).
[1] Joint first authors.
[2] Joint last authors.

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In our laboratory we have developed several automated methods to quantify different retinal populations, such as photoreceptors (Ortin-Martinez et al., 2010) and RGCs, either traced (Galindo-Romero et al., 2011; Salinas-Navarro et al., 2009a, 2009b) or immunodetected by their expression of Brn3a (Galindo-Romero et al., 2011; Nadal-Nicolas et al., 2009). Automated quantification in whole-mounted retinas allows the analysis of their spatial distribution using isodensity maps and to assess objectively their survival after different injuries, pathologies or neuroprotective strategies (Garcia-Ayuso et al., 2010, 2011; Nadal-Nicolas et al., 2009; Salinas-Navarro et al., 2010; Sanchez-Migallon et al., 2011).

The total population of ipRGCs in rat and their detailed distribution, and relationship with the distribution of the entire RGC population has not been yet fully characterized, nor has it been assessed whether they project to the SCi and if so, in what proportion. Thus, in the present study we: i) develop an automated method to quantify the total population of rat ipRGCs; ii) analyse their spatial distribution using neighbour maps, and iii) compare it with the distribution of Brn3a$^+$RGCs.

2. Material and methods

2.1. Animal handling and anaesthesia

Two month old female (180–200 g) albino Sprague–Dawley (SD) rats from the breeding colony of Janvier (Le Genest-St-Isle, France) were housed in temperature and light controlled rooms with a 12 h light/dark cycle and food and water ad libitum. Animal manipulations followed institutional guidelines, Spanish and European Union regulations for the use of animals in research and the ARVO statement for the use of animals in ophthalmic and vision research.

Animals groups subjected to surgery: for anaesthesia 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.). After surgery, an ointment containing tobramicin (Tobrex; Alcon S. A., Barcelona, Spain) was applied on the cornea to prevent its desiccation. All animals were sacrificed with an i.p. injection of an overdose of pentobarbital (Dolethal, Vetoquinol®, Especialidades Veterinarias, S. A., Alcobendas, Madrid, Spain).

2.2. Experimental design

Animals were divided in three experimental groups: i) Tracing from the ocular stump of the intraorbitally transacted optic nerve (ON). This group was used to investigate whether all retinal neurons inmunodetected with melanopsin antibodies send their axons through the ON ($n = 8$ retinas); ii) Tracing from both superior colliculi (SCi), to assess the proportion of ipRGCs that project their axons to the SCi ($n = 10$ retinas); iii) Brn3a immunodetection, this latter group was used to quantify and compare the spatial distribution of the total population of ipRGCs and Brn3a$^+$RGCs in the same retinas ($n = 10$, 5 right and 5 left retinas); iv) untouched retinas processed for vertical sections ($n = 2$).

2.3. Retinal ganglion cell tracing

2.3.1. Fluorogold tracing from the optic nerve stump

Three days before processing, a small piece of gelatine sponge (Espongostan Film, Ferrosan A/S, Denmark) soaked in 6% Fluorogold (Fluorochrome Inc., Engelwood, CO, USA) diluted in 10% dimethyl sulphoxide–saline, was applied to the ocular stump of the left transected optic nerve, approximately at 3 mm from the optic disc, following previously described methods (Salinas-Navarro et al., 2009b).

2.3.2. Fluorogold tracing from both superior colliculi

One week before processing, FG was applied onto both SCi following previously described methods (Salinas-Navarro et al., 2009a, 2009b; Vidal-Sanz et al., 1988; Villegas-Perez et al., 1996; Wang et al., 2003).

2.4. Tissue processing

After euthanasia, animals were perfused transcardially first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Eyes were enucleated and the retinas dissected as whole-mounts as previously reported (Salinas-Navarro et al., 2009b). To maintain eye orientation, a suture was placed on the dorsal (superior) pole of each eye and when dissecting the retina four radial cuts were done: nasal, temporal, ventral (inferior), and dorsal (superior); this was the deepest one.

For cryostate sectioning, 2 dissected eyes were cryoprotected in 30% sucrose (Sigma, Alcobendas, Madrid, Spain) before embedding them in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA).

2.5. Immunohistofluorescence

Flat mounted retinas were permeated in phosphate buffer saline (PBS) with 0.5% Triton X-100 by freezing them during 15 min at −70 °C, rinsed in new PBS-0.5% Triton X-100 and incubated overnight at 4 °C with the primary antibody diluted in blocking buffer (PBS, 2% normal donkey serum, 2% Triton X-100). Then, retinas were washed three times in PBS and incubated 2 h at room temperature with the secondary antibody diluted in PBS-2% Triton X-100. Finally, retinas were thoroughly washed in PBS and mounted vitreal side up on subbed slides and covered with anti-fading solution.

Vertical sections obtained by cryostat sectioning (15 μm) were washed 3 times with PBS to eliminate the OCT; then, sections were incubated overnight at 4 °C with the appropriate antibodies diluted in blocking buffer (PBS, 2% donkey normal serum, 0.1% Triton X-100). Next day, sections were washed with PBS-0.1% Triton and incubated 1 h at room temperature with fluorescence conjugated-secondary antibodies diluted in the same blocking buffer. Finally, after thorough washing in PBS-0.1% Triton, sections were rinsed in PBS and mounted with anti-fading Vecta-Shield Mounting Medium with DAPI (Vector Laboratories, Alicante, Spain) to counterstain all retinal nuclei.

2.6. Antibodies and working dilutions

2.6.1. Primary antibodies

Rabbit anti-melanopsin (1:500, PAI-780 Cultek, Spain) to detect M1–M3 ipRGCs, since M4 and M5 subtypes are not stained with melanopsin antibodies (reviewed in Schmidt et al., 2011). Goat anti-Brn3a (1:500, C-20, Santa Cruz Biotechnologies Heidelberg, Germany) to detect the RGC population (Nadal-Nicolas et al., 2009, 2012). Mouse anti-rhodopsin (1:10,000, Sigma–Aldrich, Alcobendas, Spain) to detect rod photoreceptors.

2.6.2. Secondary antibodies

Donkey anti-goat Alexa-594, donkey anti-rabbit Alexa-488, donkey anti-mouse Alexa 594, and donkey anti-rabbit Alexa-594 (Molecular Probes, Invitrogen, Barcelona, Spain) diluted each 1:500.