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Retino-retinal projection in juvenile and young adult rats and mice

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ABSTRACT

Identification of retino-retinal projecting RGCs (ret-ret RGCs) has been accomplished by tracing RGCs in one retina after intravitreal injection of different tracers in the other eye. In mammals, rabbit and rat, ret-ret RGCs are scarce and more abundant in newborn than in adult animals. To our knowledge, ret-ret RGCs have not been studied in mice. Here we purpose to revisit the presence of ret-ret RGCs in juvenile and young adult rats and mice by using retrograde tracers applied to the contralateral optic nerve instead of intravitreally. In P20 (juvenile) and P60 (young adult) animals, the left optic nerve was intraorbitally transected and Fluorogold (rats) or its analogue OHSt (mice) were applied onto its distal stump. P20 animals were sacrificed 3 (mice) or 5 (rats) days later and adult animals at 5 (mice) or 7 (rats) days. Right retinas were dissected as flat-mounts and double immunodetected for Brn3a and melanopsin. Ret-ret RGCs were those with tracer accumulation in their somas. Out of them some expressed Brn3a and/or melanopsin, while other were negative for both markers. In young adult rats, we found 2 ret-ret RGCs displaced to the inner nuclear layer. In both species, ret-ret RGCs are quite scarce and found predominantly in the nasal retina. In juvenile animals there are significantly more ret-ret RGCs (9 ± 3, rats, 13 ± 3 mice) than in young adult ones (5 ± 6 rats, 7 ± 3 mice). Finally, juvenile and young adult mice have more ret-ret RGCs than rats.

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Visual information, sensed by photoreceptors and conveyed to retinal ganglion cells (RGCs), and circadian information, directly perceived by the intrinsically photosensitive type of RGCs (ipRGCs), travel from the retina through the optic nerve, formed by the RGC axons, to the image forming and non-image forming target territories in the brain.

In rats and mice, RGCs project massively to the contralateral superior colliculi, (Dräger and Olsen, 1980; Lund, 1965; Lund et al., 1980; Nadal-Nicolás et al., 2014; Salinas-Navarro et al., 2009) but also send collaterals to the lateral geniculate nucleus, intergeniculate nucleus, the dorsal, lateral and medial terminal nuclei, the olivary pretectal nuclei and the supraquiasmatic nuclei (reviewed in Sefton et al., 2004).

RGCs projecting from one retina to the other have been described in anuran (Bohn and Stelzner, 1979, 1981a, 1981b, 1981c; Tennant et al., 1993; Toth and Straznicky, 1989), chicken (Halfter, 1987; Thanos, 1999) and mammals (Bunt and Lund, 1981; Lam et al., 1982; Müller and Holländer, 1988). To date, retino-retinal projecting RGCs (ret-ret RGCs) have been identified by intravitreal injection of different tracers and formulations in one eye and analysis of traced RGCs in the contralateral retina, with the exception of a very recent report in pigmented rats where they apply the tracer onto the contralateral optic nerve (ON) (Avellaneda-Chevrier et al., 2015).

In mammals, rabbit and rat, ret-ret RGCs are few and mainly located in the nasal retina. They are, however, more abundant in newborn than in adult animals. In fact, Müller and Holländer (1988) identified ret-ret RGCs in all newborn rat retinas, but not in all adult retinas. To our knowledge, ret-ret RGCs have not been studied in mice.

Thus, we have revisited the retino-retinal projection in juvenile and young adult rats and mice. We have applied the tracer onto the optic nerve instead of intravitreally and studied whether ret-ret RGCs express the RGC markers Brn3a and/or melanopsin. Brn3a is a transcription factor expressed by all RGCs except ipRGCs and half of the ipsilateral projection (Nadal-Nicolás et al., 2012), and

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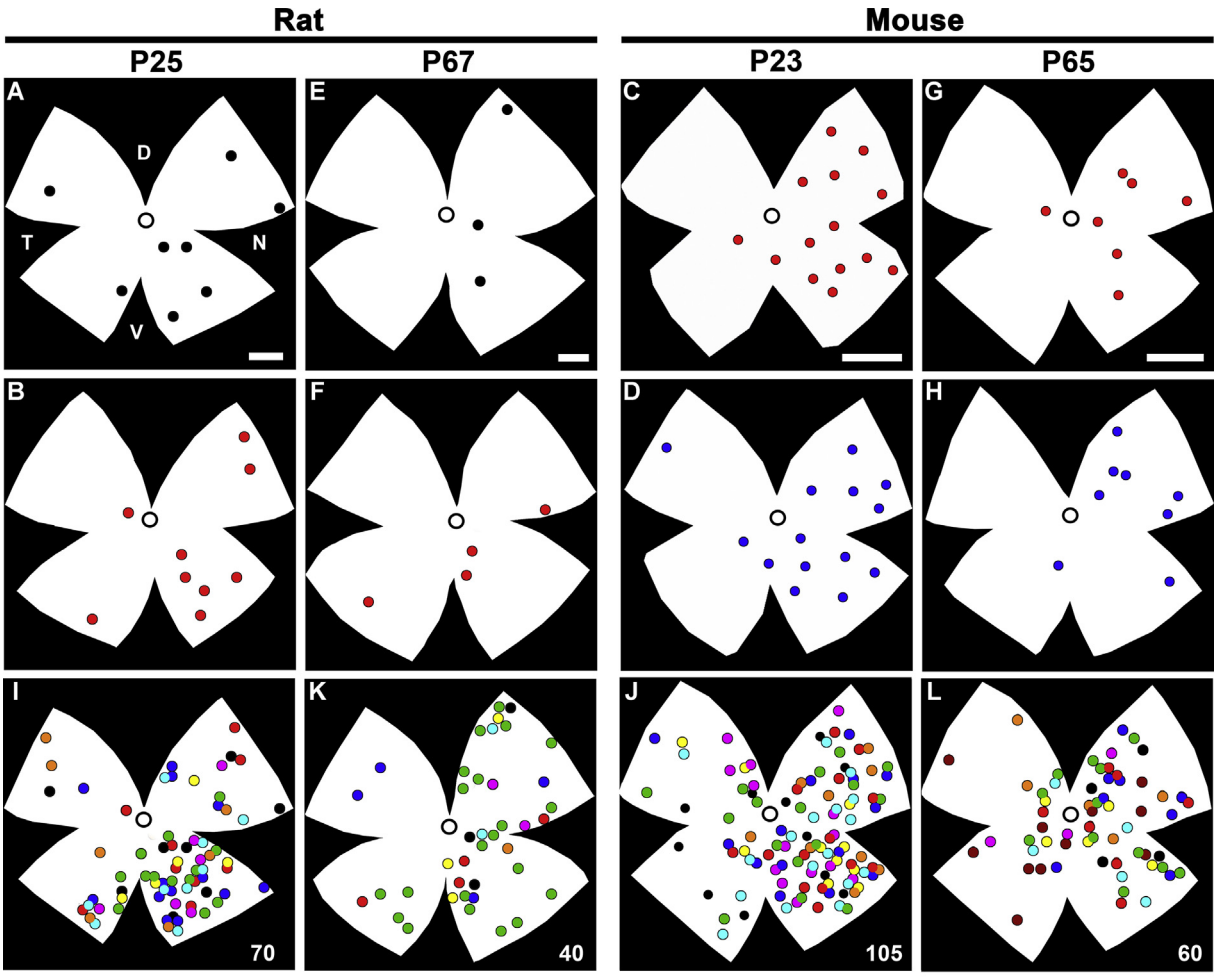

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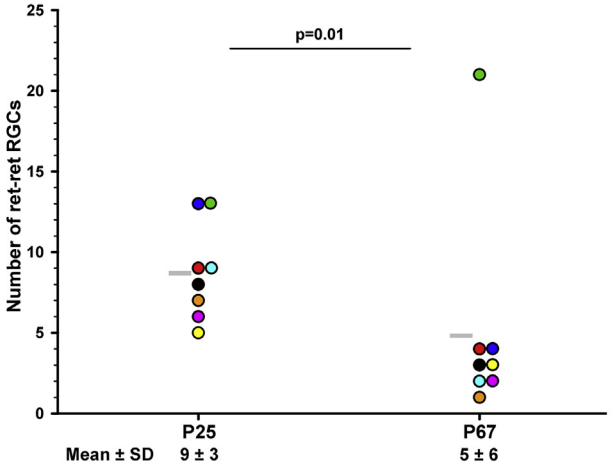

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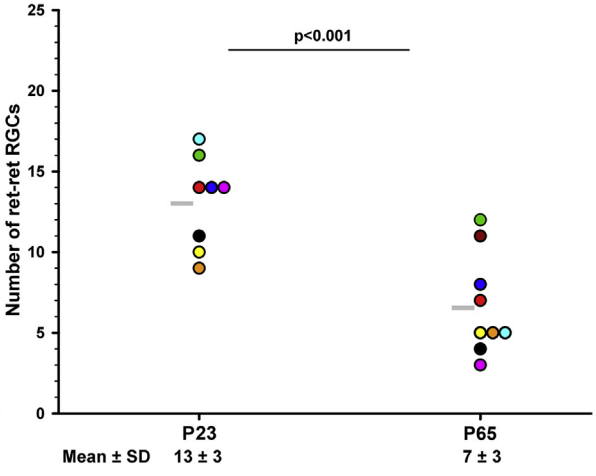

O

P25		P67
50% (35)	Brn3a+ Mel-	85% (34)
41% (29)	Brn3a- Mel-	5% (2)
9% (6)	Brn3a- Mel+	10% (4)
0% (0)	Brn3a+ Mel+	0% (0)
0% (0)	Displaced	5% (2)

P

P23		P65
84% (88)	Brn3a+ Mel-	88% (53)
10% (10)	Brn3a- Mel-	8% (5)
6% (6)	Brn3a- Mel+	2% (1)
1% (1)	Brn3a+ Mel+	2% (1)
0% (0)	Displaced	0% (0)

melanopsin is the pigment that confers photosensitivity to ipRGCs. Melanopsin immunodetection detects the M1, M2 and M3 subtypes of ipRGCs, since the M4 expresses very low levels of this protein (Estevez et al., 2012) and the M5 subtype is not stained with anti-melanopsin antibodies (reviewed in Schmidt et al., 2011).

Juvenile (P20, 40–44 g body weight, n = 8) and young adult (P60, 180 g, n = 8) albino Sprague Dawley rats, and juvenile (P20, 15 g, n = 8) and young adult (P60, 25–30 g, n = 9) pigmented C57/Bl6 mice were obtained from the University of Murcia (Spain) breeding colony. Animal care and experimental procedures were performed in accordance with the 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). As 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.

The left ON was intraorbitally transected at approximately 2 mm from the optic disk following previously reported methods (Galindo-Romero et al., 2011, 2013b; Lafuente López-Herrera et al., 2002; Nadal-Nicolás et al., 2009, 2012; Sánchez-Migallón et al., 2011). A pledge of gelatine sponge (Spongostan Film, Ferrosan A/S, Denmark) soaked in the tracer was applied to the distal end of the ON stump. For rats, the tracer was Fluorogold (FG, Fluorochrome, LLC, USA) diluted at 6% in 10% dimethylsulfoxide(DMSO)-saline, and for mice the FG analogous hydroxystilbamidine methanesulfonate (OHSt, Molecular Probes, Leiden, The Netherlands) diluted at 10% in 10%DMSO-saline. Animals were sacrificed 3 days (P20 mice), 5 days (P20 rats and P60 mice) and 7 days (P60 rats) after the tracing. Thus, ret-ret RGCs were analysed at P23, P25, P65 and P67, respectively.

Animals were euthanized with an i.p. overdose of pentobarbital (Dolethal, Vetoquinol®, Especialidades Veterinarias, S.A. Madrid, Spain), and perfused with saline followed by paraformaldehyde 4%. Right retinas were dissected as flat mounts and double immunodetected for Brn3a (goat anti-Brn3a C20, diluted 1: 750 (rat) or 1:500 (mouse), Santa Cruz Biotechnologies. Heidelberg, Germany) and melanopsin (rabbit anti-melanopsin UF006 diluted 1:5000 for mice retinas-Advance Targeting Systems, The Netherlands-, and rabbit anti-melanopsin PA1-780 diluted 1:500-Pierce, Thermo Scientific. Madrid, Spain-for rat retinas). Secondary detection was done with a combination of donkey anti-rabbit Alexa 488 and donkey anti-goat Alexa 594 (each diluted 1:500, Molecular Probes, Thermo Scientific. Madrid, Spain).

In all retinas multi-frame acquisitions were taken for Brn3a signal in a raster scan pattern under an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany). The individual frames were reconstructed as retinal photomontages and Brn3a$^+$RGCs were automatically quantified as reported (Galindo-Romero et al., 2011; Nadal-Nicolás et al., 2009). Next FG or OHSt signal was examined to identify ret-ret RGCs and their position was then manually dotted on the retinal photomontages. All ret-ret RGCs were individually photographed. Displaced ret-ret RGCs were identified by changing the focus from the ganglion cell layer to the inner nuclear layer as previously described (Nadal-Nicolás et al., 2014; Valiente-Soriano et al., 2014). By changing the microscope filter, Brn3a and melanopsin signals were acquired in the same frame and focus. Individual frames were merged using Adobe Photoshop CS3 v10.0 (Adobe System Incorporated, USA) to assess the proportion of ret-ret RGCs expressing Brn3a, melanopsin, both or neither. Data were analysed with SigmaStat® for Windows Version 3.11 program; (Systat Software, Inc., Richmond, CA). Differences were considered significant when $p \leq 0.05$.

Ret-ret RGCs were identified in all animals (n = 8-9 per age and species). Müller and Holländer (1988) did not detect ret-ret RGCs in 8 out of 13 adult rat retinas, this discrepancy with our data may be due to the tracing approach: they injected the tracer intravitreally while here the tracer was applied to the distal stump of the intraorbitally transected ON.

As shown in Fig. 1A–L, ret–ret RGCs are found predominantly in the nasal retina. This is in agreement with an study in rabbits and rats (Müller and Holländer, 1988), but is in contrast with a recent article (Avellaneda-Chevrier et al., 2015) reporting that ret-ret RGCs are homogenously distributed in pigmented rats. Such discrepancy may be due to the different rat strain, as it has been shown that the topography of retinal cells such as cone photoreceptors, melanopsin$^+$RGCs, displaced RGCs or those RGCs that project ipsilaterally, varies between albino and pigmented animals (Nadal-Nicolás et al., 2012, 2014; Ortin-Martinez et al., 2014). In P25 rats, out of the 70 ret-ret RGCs identified in the 8 analysed retinas, 54 were found in the nasal hemiretina (77%); in P67 rats this proportion increases to 82% (33 ret-ret RGCs out of 40). In young mice the percent is 75%(79 of 105) but, contrary to rat, it decreases in adult mice (66%, 40 ret-ret RGCs of 60).

The number of ret-ret RGCs is highly variable between animals (Fig. 1M and N), which seems to be a general feature of this projection in mammals (Müller and Holländer, 1988; Avellaneda-Chevrier et al., 2015). In fact, the number of ret-ret RGCs ranges between 5 and 13 in P25 rats, 9 and 17 in P23 mice, and 3 and 12 in adult mice. Between 1 and 4 ret-ret RGCs were found in 7 out of the 8 young adult rat retinas analysed. In one P67 rat we found 21 ret-ret RGCs. So, the mean number of ret-ret RGCs in young adult rats results in 5 ± 6. However this is biased by the outlier animal. Without it, the mean number of ret-ret RGCs in young adult rats drops to 2.7 ± 1.1.

The mean number ± standard deviation of Brn3a$^+$RGCs in these same retinas was 81,678 ± 2810 and 79,534 ± 1919 n P25 and P67 rats, and 38,211 ± 2642 and 37,471 ± 1336 in P23 and P65 mice. Thus, ret-ret RGCs represent between 0.006% and 0.03% of the RGC population.

Ret-ret RGCs are significantly more abundant in juvenile than in young adult animals (Fig. 1), an observation in accordance with Müller and Holländer's (1988) results in rats and in rabbits, and with the developmental analysis carried out by Bunt and Lund (1981) in rats. Additionally, mice have, at both ages, significantly more ret-ret RGCs than rats (t-test: p = 0.014 between juvenile animals, and p = 0.029 between young adult animals).

Next, we addressed whether ret-ret RGCs express Brn3a and/or melanopsin (Fig. 1O and P, Fig. 2). Most of the ret-ret RGCs in both species express Brn3a, such finding is not surprising since most RGCs express this transcription factor (Galindo-Romero et al., 2011; Nadal-Nicolás et al., 2009, 2012, 2014). Brn3a is expressed by a

Fig. 1. Number and distribution of retino-retinal projecting RGCs (ret-ret RGCs) in juvenile and young adult rats and mice. A–H: Outline from individual right retinas showing the distribution of ret-ret RGCs in two juvenile (P25/P23, A–D) and two young adult (P67/65, E–H) rats (left columns) and mice (right columns). Each dot represents a single ret-ret RGC. In I–L is shown the retinal position of all the ret-ret RGCs identified in this study at both post-natal times and in both species. Each colour indicates a single animal. The total number of ret-ret RGCs represented is shown at the bottom of each panel. M–N: Plot showing the number of ret-ret RGCs in each rat (M) or mouse (N) at P25/P23 and P67/65. Colours are the same as in I–J. The horizontal grey line marks the mean value. Below each graph is shown the mean ± standard deviation (SD) of ret-ret-RGCs within each group. In both species the number of ret-ret RGCs was significantly higher at P25/P23 than at P67/65 (t-test p = 0.01 rat, p < 0.001 mouse). O–P: number and percent of ret-ret RGCs that express Brn3a but no melanopsin (Brn3a + Mel−), negative for both markers (Brn3a − Mel−), Brn3a and melanopsin positive (Brn3a + Mel+) or displaced to the inner nuclear layer (displaced). The total number of ret-ret RGCs identified within each group was considered 100%. D: dorsal, N: nasal, V: ventral, T: temporal. Scale bar: 1 mm.

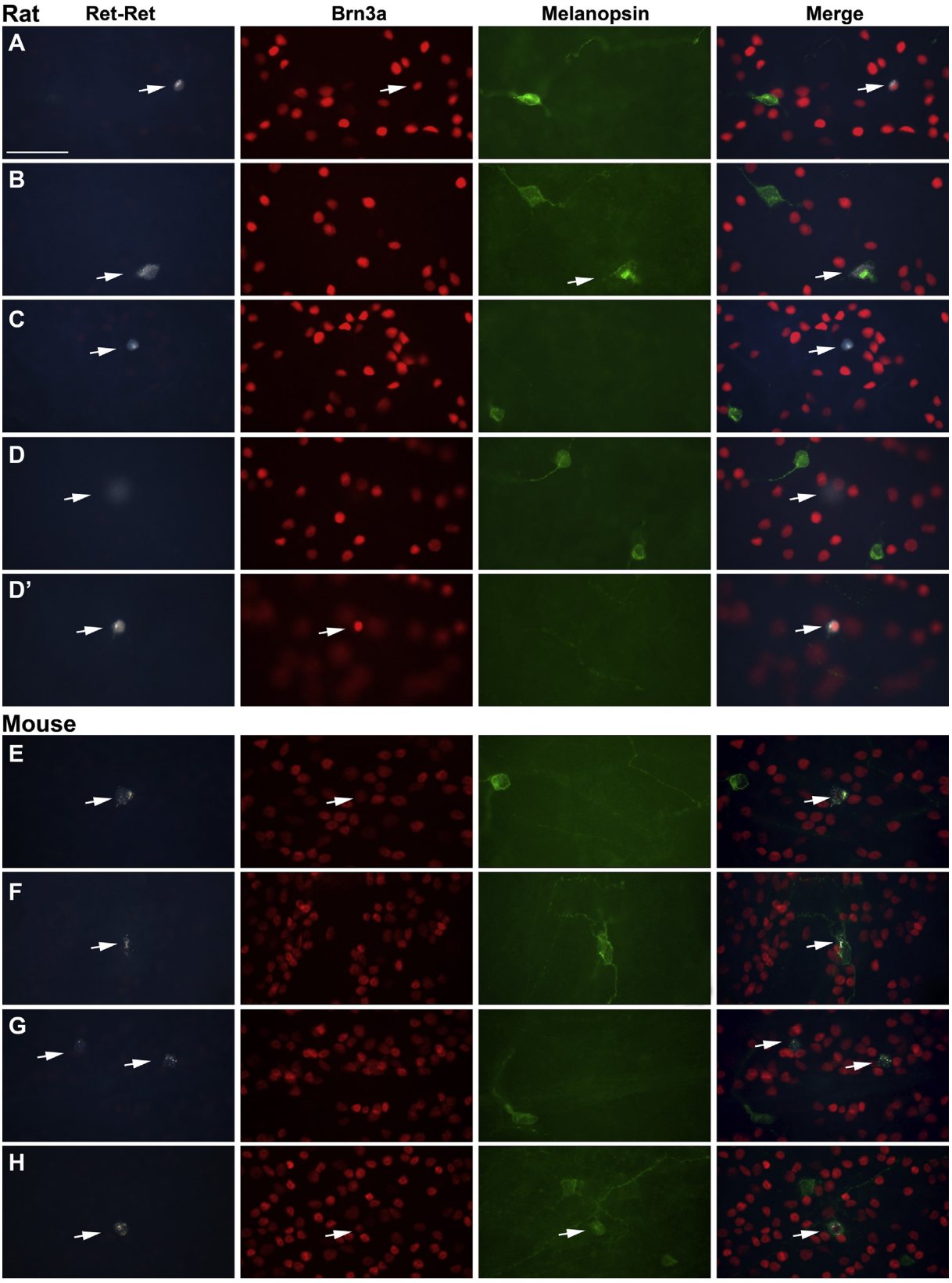

Fig. 2. Diversity of ret-ret RGCs in rats and mice. Photomicrographs from flat mounted right retinas analysed after applying fluorogold (P67 rats, A–D′) or OHSt (P65 mice, E–F) on the left optic nerve stump. In A–H are shown examples of traced ret-ret RGCs. Double immunodetection of Brn3a (red signal) and melanopsin (green signal) shows that some ret-ret RGCs express Brn3a but not melanopsin (A, E rows), some express melanopsin but not Brn3a (B, F rows) and some are negative for both markers (C, G rows). In rats, some ret-ret RGCs were displaced to the inner nuclear layer (D row: ganglion cell layer, D′ row: same frames as in D but focussed on the INL); these were always Brn3a^{+} and were never found in mice. Finally in mice, but not in rat, some ret-ret express both, Brn3a and melanopsin (H row). Arrows point to ret-ret RGCs. Scale bar: 50 μm.

minute proportion of melanopsin$^+$RGCs (Galindo-Romero et al., 2013a; Nadal-Nicolás et al., 2012; Valiente-Soriano et al., 2014, 2015). Our present results indicate that this is also the case for ret-ret RGCs, as we found only 2 cells double labelled in mice and none in rat.

In P25 rats, 41% of ret-ret RGCs were negative for both markers, but in P67 rats this pattern changed and 98% of them expressed Brn3a or melanopsin. Because in adult rats RGCs express either marker, it is tempting to suggest that the decrease of ret-ret RGCs from P25 to P67 is due to the loss of those ret-ret RGCs that do not fully differentiate to adult RGCs. Of note, Brn3a is not expressed by half of the ipsilateral projection (Nadal-Nicolás et al., 2012) but ipsilateral RGCs are located in a temporal crescent (Balkema and Dräger, 1990; Dräger and Olsen, 1980; Lund, 1965; Lund et al., 1980; Nadal-Nicolás et al., 2012, 2014), thus it is likely that ipsilateral RGCs do not contribute to the retino-retinal projection.

Finally, in P67 rats, 2 ret-ret RGCs displaced to the inner nuclear layer (Dräger and Olsen, 1981; Nadal-Nicolás et al., 2014) were found (Fig. 2 D and D′), and both of them were Brn3a$^+$.

It has been shown that unilateral retinal injuries cause a contralateral response (Lönngren et al., 2006) that includes macroglial and microglial activation and proliferation (Bodeutsch et al., 1999; de Hoz et al., 2013; Gallego et al., 2012; Macharadze et al., 2009; Panagis et al., 2005; Ramírez et al., 2010; Rojas et al., 2014) as well as phagocytosis (Galindo-Romero et al., 2013b). What triggers this contralateral reaction is, to date, unknown. It could be due to several mechanisms, which are not mutually exclusive: i/a humoral signal that reaches the contralateral retina but that is independent of the retino-retinal projection; ii/stress signals released from the injured ret-ret RGCs and/or iii/a direct reaction to the death of ret-ret RGCs.

The appearance of phagocytic microglial cells in contralateral mice retinas after unilateral axotomy is a constant response that does not depend on the RGC death rate in the injured retina nor on the time after the lesion (Galindo-Romero et al., 2013b). In fact, around 270 phagocytic microglial cells were found dispersed across the contralateral retina from 3 to 14 days after the injury whether the injured retinas were neuroprotected with BDNF or not. These data suggest that if ret-ret RGCs trigger the contralateral response this may be due to the release of stress or danger signals. This is supported further by the fact that this phagocytic response has not been observed in rat after axotomy (Nadal-Nicolás, unpublished results). Mice have more ret-ret RGCs than rats, and it is thus plausible that they trigger a stronger contralateral response.

In conclusion, we have described here for the first time the number and distribution of ret-ret RGCs in juvenile and young adult mice and juvenile rats. Also we show here that they express RGC markers. Finally our data in adult rat are in agreement with those of Avellaneda-Chevrier et al. (2015) and corroborate the findings of Müller and Holländer (1988), although by tracing from the optic nerve ret-ret RGCs were identified in all retinas, so this approach is more efficient than intravitreal tracing.

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