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Axotomy-induced retinal ganglion cell death in adult mice: Quantitative and topographic time course analyses

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ABSTRACT

The fate of retinal ganglion cells after optic nerve injury has been thoroughly described in rat, but not in mice, despite the fact that this species is amply used as a model to study different experimental paradigms that affect retinal ganglion cell population. Here we have analyzed, quantitatively and topographically, the course of mice retinal ganglion cells loss induced by intraorbital nerve transection. To do this, we have doubly identified retinal ganglion cells in all retinas by tracing them from their main retinorecipient area, the superior colliculi, and by their expression of BRN3A (product of *Pou4f1* gene). In rat, this transcription factor is expressed by a majority of retinal ganglion cells; however in mice it is not known how many out of the whole population of these neurons express it. Thus, in this work we have assessed, as well, the total population of BRN3A positive retinal ganglion cells. These were automatically quantified in all whole-mounted retinas using a newly developed routine. In control retinas, traced-retinal ganglion cells were automatically quantified, using the previously reported method (Salinas-Navarro et al., 2009b). After optic nerve injury, though, traced-retinal ganglion cells had to be manually quantified by retinal sampling and their total population was afterwards inferred. In naïve whole-mounts, the mean (±standard deviation) total number of traced-retinal ganglion cells was 40,437 (±3196) and of BRN3A positive ones was 34,697(±1821). Retinal ganglion cell loss was first significant for both markers 5 days post-axotomy and by day 21, the last time point analyzed, only 15% or 12% of traced or BRN3A positive retinal ganglion cells respectively, survived. Isodensity maps showed that, in control retinas, BRN3A and traced-retinal ganglion cells were distributed similarly, being densest in the dorsal retina along the naso-temporal axis. After axotomy the progressive loss of BRN3A positive retinal ganglion cells was diffuse and affected the entire retina. In conclusion, this is the first study assessing the values, in terms of total number and density, of the retinal ganglion cells surviving axotomy from 2 till 21 days post-lesion. Besides, we have demonstrated that BRN3A is expressed by 85.6% of the total retinal ganglion cell population, and because BRN3A positive retinal ganglion cells show the same spatial distribution and temporal course of degeneration than traced ones, BRN3A is a reliable marker to identify, quantify and assess, ex-vivo, retinal ganglion cell loss in this species.

1. Introduction

Rodent retinal ganglion cells (RGCs) are widely used to understand the degenerative events underlying injury-induced neuronal death in the central nervous system (CNS) (Salvador-Silva et al., 2000; Lafuente et al., 2002a; Mayor-Torroglosa et al., 2005; Agudo et al., 2008, 2009; Nadal-Nicolas et al., 2009; Parrilla-Reverter et al., 2009b). RGCs are located in the innermost layer of the retina where they share location with a similarly numerous population of displaced amacrine cells (Perry, 1981). Classic morphological criteria do not distinguish reliably RGCs from the many displaced amacrine cells within the RGC layer (Drager and Olsen, 1981; Perry, 1981; Perry et al., 1983; Jeon et al., 1998). Indeed, displaced amacrine cells and small RGCs overlap in size, making it difficult to discriminate between them (Villegas-Perez et al., 1988, 1993). Thus, to study RGCs it is necessary to label them specifically (Thanos et al., 1987; Vidal-Sanz et al., 1988). A well established method to label RGCs consists on the use of neuronal tracers applied to the optic nerves,

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tracts or their main target regions in the brain which, in rats and mice, are the superior colliculi (SCi) (Lund, 1965; Perry, 1981; Linden and Perry, 1983). Fluorogold, or its analogue hydroxystilbamidine methanesulfonate (OHSt) are the tracers of choice for many laboratories (Peinado-Ramon et al., 1996; Selles-Navarro et al., 1996; Villegas-Perez et al., 1996; Wang et al., 2000, 2003; Salvador-Silva et al., 2000; Danias et al., 2006; Reichstein et al., 2007; Murphy et al., 2007; Sobrado-Calvo et al., 2007; Lebrun-Julien et al., 2009). In fact, application of these tracers on both SCi results one week later in the labelling of 98.4% or 96.5% of the RGC population in rats (Salinas-Navarro et al., 2009c) or mice (Salinas-Navarro et al., 2009b), respectively. This tracing, using automated methods accounts, in pigmented mice, for 41,192 ± 3395 RGCs (Salinas-Navarro et al., 2009b). An alternative to trace RGCs is to detect proteins or transcripts specifically expressed by them (Barnstable and Drager, 1984; Casson et al., 2004; Chidlow et al., 2005; Bernstein et al., 2006; Soto et al., 2008; Surgucheva et al., 2008; Nadal-Nicolas et al., 2009; Nguyen et al., 2011). Recently, our group reported that BRN3A is a specific and reliable marker to detect and quantify the whole population of adult rat RGCs (Nadal-Nicolas et al., 2009), and that BRN3A is a useful tool to quantify the RGC population in various models of retinal injury, including axotomy (Nadal-Nicolas et al., 2009; Sanchez-Migallón et al., 2011), ocular hypertension (Salinas-Navarro et al., 2009a, 2010; Cuenca et al., 2010) and photoreceptor degeneration (García-Ayuso et al., 2010).

Pou4f1, *2* and *3* genes, members of the Brn3 family of POU-domain transcription factors, were cloned in the early nineties and it was shown that, in the retina, their products (BRN3A, B and C, respectively) were specifically expressed by RGCs (Xiang et al., 1993, 1995). These transcription factors play important roles in the differentiation, survival, and axonal re-growth of mice RGCs (Theil et al., 1993; Latchman, 1998; Gan et al., 1999; Budram-Mahadeo et al., 2002; Hudson et al., 2005). In adult mice, BRN3A is expressed by RGCs that project mainly to the contralateral SCi and, to a minor extent, to the dorsal lateral geniculate nucleus (dLGN) (Quina et al., 2005). However, it is not known which proportion of the entire RGC population expresses this transcription factor, neither their spatial distribution within the adult mice retina nor whether its expression is affected by axonal injury.

In adult rats the temporal loss of RGCs that follows optic nerve injury has been studied by many groups (Berkelaar et al., 1994; Mansour-Robaey et al., 1994) including our own laboratory (Villegas-Perez et al., 1988, 1993; Peinado-Ramon et al., 1996; Vidal-Sanz et al., 2000; Lindqvist et al., 2004) where using modern automated routines we have quantified the whole population of rat RGCs, fluorogold and BRN3A positive, that survive at different times post-lesion after two types of axonal injury, transection or crush (Nadal-Nicolas et al., 2009). In addition, the quantitative data thus obtained allowed for the generation of detailed maps showing RGC distribution within the retina in control as well as after such insults (Nadal-Nicolas et al., 2009).

Transgenic and knockout mice have become an important tool to study a number of relevant questions in the adult mammalian central nervous system. Moreover, albino or pigmented mice are usually animals of choice for many experimental paradigms that affect the RGC population (Grozdanic et al., 2003; Holcombe et al., 2008; Salinas-Navarro et al., 2009a, 2010; Alarcón-Martínez et al., 2010; Cuenca et al., 2010; Fu and Sretavan, 2010).

Axotomy-induced RGC death in mice is a model used to test the role of different molecules or genes in RGC degeneration. In these studies, RGCs are generally identified by retrograde tracing and, because of the appearance of transcellularly traced microglial cells, the quantification analyses are carried out by sampling and manual-counting (Murphy et al., 2007; Lebrun-Julien et al., 2009). Thus, in the present work we propose to investigate whether immunodetection of BRNA in mice retinas is a good tool to identify and quantify RGCs. In addition, we propose to study in detail the temporal loss of mice RGCs after intraorbital optic nerve transection, and specifically to: i) assess whether BRN3A is a reliable tool to identify mice RGCs, naïve and injured, using as baseline criterion OHSt-tracing; ii) develop an automated routine to quantify the whole population of BRN3A$^+$RGCs, iii) compare the population of BRN3A$^+$RGCs with the population of OHSt$^+$RGCs which will be quantified, in the same retinas, using the automated routine previously described by our group (Salinas-Navarro et al., 2009b); iv) analyze and compare the temporal loss of IONT-injured RGCs identified by OHSt-tracing and BRN3A expression in the same retinas, and; v) characterize the spatial distribution of surviving RGCs after axotomy. Finally the data will be compared with that obtained for the adult rat, and the resemblances and differences will be discussed.

2. Material and methods

2.1. Animal handling and surgery

Experiments were performed on adult female pigmented C57BL/6N mice, (25 g of body weight) obtained from the breeding colony of the University of Murcia (Murcia, Spain). Mice were housed in temperature and light controlled rooms with a 12 h light/dark cycle and had food and water ad libitum. When animal manipulations were performed, the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985), the OPRR Public Health Service Policy of the Human Care and the Use of Laboratory Animals (revised 1986) and the US Animal Welfare Act, as amended, were followed, as well as our institutional guidelines, European Union regulations for the use of animals in research and the ARVO statement for the use of animals in ophthalmic and vision research. In addition, additional measures were taken to minimize pain or discomfort. All surgical manipulations were done under general anaesthesia induced with an intraperitoneal (i.p.) injection of ketamine (70 mg/kg, Ketolar®; Parke-Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, Rompún®; Bayer, S.A., Barcelona, Spain). While recovering from anaesthesia, mice were placed in their cages, and an ocular ointment containing antibiotic (Tobrex; Alcon S.A., Barcelona, Spain) was applied.

To identify the RGCs, these were retrogradelly traced by applying hydroxystilbamidine methanesulfonate (OHSt) to both superior colliculi (SCi) one week before surgery or processing, as previously described (Wang et al., 2000; Salinas-Navarro et al., 2009b; Alarcón-Martínez et al., 2010). In brief, after exposing the midbrain, a small pledget of gel foam (Espongostan Film; Ferrosan A/S, Denmark) soaked in saline containing 10% OHSt in 0.9% NaCl and 10% dimethylsulfoxide (DMSO), was applied over the entire surface of both SCi.

The left ON was intraorbitally transected (IONT) at 0.5 mm from the optic disc sparing the blood supply, according to procedures that are standard in our laboratory (Wang et al., 2000; Salinas-Navarro et al., 2009b; Alarcón-Martínez et al., 2010). After surgery, the eye fundus of each animal was checked to verify that the retinal vessels were intact.

Retinas traced with OHSt from de SCi were divided into two groups one control naïve ($n = 14$ retinas) and one experimental undergoing IONT. IONT-injured animals were analyzed at 2 ($n = 10$), 5 ($n = 7$), 7 ($n = 11$), 9 ($n = 7$), 14 ($n = 11$) or 21 ($n = 8$) days post-lesion (dpl). These groups were analyzed to determine the percentage of BRN3A$^+$RGCs that project to the SCi, to quantify the whole population of RGCs BRN3A$^+$ and OHSt$^+$ and to assess their temporal loss after IONT.