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Retinal ganglion cell numbers and delayed retinal ganglion cell death in the P23H rat retina

Diego García-Ayuso [a], Manuel Salinas-Navarro [a], Marta Agudo [b], Nicolás Cuenca [c], Isabel Pinilla [d], Manuel Vidal-Sanz [a], María P. Villegas-Pérez [a,*]

[a] Laboratorio de Oftalmología Experimental, Facultad de Medicina, Universidad de Murcia, Campus de Espinardo, 30100 Espinardo, Murcia, Spain
[b] Unidad de Investigación, Hospital Universitario Virgen de la Arrixaca, Servicio Murciano de Salud, Fundación para la Formación e Investigación Sanitarias de la Región de Murcia, 30120 El Palmar, Murcia, Spain
[c] Departamento de Biotecnología, Universidad de Alicante, San Vicente del Raspeig, 03080 Alicante, Spain
[d] Servicio de Oftalmología, Hospital Clínico Universitario Lozano Blesa, Avenida de San Juan Bosco 15, 50009 Zaragoza, Spain

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ABSTRACT

The P23H-1 rat strain carries a rhodopsin mutation frequently found in retinitis pigmentosa patients. We investigated the progressive degeneration of the inner retina in this strain, focussing on retinal ganglion cells (RGCs) fate. Our data show that photoreceptor death commences in the ventral retina, spreading to the whole retina as the rat ages. Quantification of the total number of RGCs identified by Fluorogold tracing and Brn3a expression, disclosed that the population of RGCs in young P23H rats is significantly smaller than in its homologous SD strain. In the mutant strain, there is also RGC loss with age: RGCs show their first symptoms of degeneration at P180, as revealed by an abnormal expression of cytoskeletal proteins which, at P365, translates into a significant loss of RGCs, that may ultimately be caused by displaced inner retinal vessels that drag and strangulate their axons. RGC axonal compression begins also in the ventral retina and spreads from there causing RGC loss through the whole retinal surface. These decaying processes are common to several models of photoreceptor loss, but show some differences between inherited and light-induced photoreceptor degeneration and should therefore be studied to a better understanding of photoreceptor degeneration and when developing therapies for these diseases.

1. Introduction

Inherited photoreceptor degenerations are a group of genetic diseases, or retinal dystrophies, with multiple genotypic variations. All these diseases have in common mutations on genes coding for proteins implicated in phototransduction, visual pigment cycle, vesicular transport, structural proteins and transcription factors, although some of those mutations have yet to be identified (Hartong et al., 2006; Swaroop et al., 2007; Den Hollander et al., 2008; Fritsche et al., 2008; Baehr and Frederick, 2009).

One of the commonest forms of inherited retinal degeneration in humans is retinitis pigmentosa. This disease comprises a heterogeneous group of retinal degenerations that lead first to night blindness and afterwards to progressive loss of peripheral and central vision. Retinitis pigmentosa represents a major cause of irreversible blindness and is characterized by a progressive apoptotic death (Yu et al., 2004; Wenzel et al., 2005; Pennesi et al., 2008) of photoreceptors, first rods and secondarily cones (Mohand-Said et al., 2000; Shen et al., 2005; Komeima et al., 2006).

In humans and animals, inherited photoreceptor degeneration causes, with time, alterations in all retinal layers (Villegas-Pérez et al., 1996, 1998; Milam et al., 1998; Fariss et al., 2000; Wang et al., 2000, 2003, 2005; Jones et al., 2003; Cuenca et al., 2004; Jones and Marc., 2005; Aleman et al., 2007, 2008; Walia et al., 2007; Walia and Fishman, 2008). Patients with retinitis pigmentosa usually show optic disc pallor and, while some investigators have documented retinal ganglion cell (RGC) loss and thinning of the retinal nerve fiber layer in patients with retinitis pigmentosa (Stone et al., 1992; Santos et al., 1997; Walia et al., 2007; Lim et al., 2008; Walia and Fishman, 2008), other have failed to do so (Hood et al., 2009; Oishi et al., 2009). However, because many proposed treatments for retinitis pigmentosa such as photoreceptor/retinal pigmented epithelium transplantation or visual prosthesis rely on the integrity of the inner retina, it is important if the disease causes damage to the

* Corresponding author. Tel.: +34 868887573; fax: +34 868883962.
E-mail addresses: diegogarcia@um.es (D. García-Ayuso), manuel.salinas@um.es (M. Salinas-Navarro), martabar@um.es (M. Agudo), cuenca@ua.es (N. Cuenca), ipinilla@unizar.es (I. Pinilla), ofmmv01@um.es (M. Vidal-Sanz), mpville@um.es (M.P. Villegas-Pérez).

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inner retinal layers and, more importantly, to the retinal ganglion cells (RGCs), as these are the efferent retinal neurons.

Previous studies from our laboratory have documented, for the first time, that photoreceptor degeneration in dystrophic Royal College of Surgeons (RCSp+) rats (Villegas-Pérez et al., 1996, 1998; Wang et al., 2003), in rd mice (Wang et al., 2000), and after light exposure in non-dystrophic RCS, pigmented Lister-Hooded (Marco-Gomariz et al., 2006a) and albino Sprague–Dawley rats (García-Ayuso et al., 2009, submitted for publication) leads to cell migration and vascular changes that, with time, cause RGC axonal compression and subsequent death. Because the pathophysiology of RGC death in these animals was similar, we concluded that RGC death was secondary to the vascular/axonal changes that follow photoreceptor degeneration and not a consequence of the dystrophy (Marco-Gomariz et al., 2006a; García-Ayuso et al., 2009, 2010, submitted for publication). RCS rats have a defect of the receptor tyrosine kinase gene, Mertk (D'Cruz et al., 2000) and rd mice have a mutation of the gene encoding the beta subunit of the cGMP-PDE (Pittler and Baehr, 1991) and both these mutations have been shown to cause retinitis pigmentosa in humans (Gal et al., 2000; Baehr and Frederick, 2009).

In this article, we have investigated RGC loss in another animal model of retinitis pigmentosa, the P23H rat in order to document whether in this mutant animal, there is also RGC loss and whether it is due to the dystrophy or secondary to vascular/axonal changes as in the animal models that we have investigated before. To document whether there is inner retinal damage in inherited retinal dystrophies, its timing and extent, is important because it implies that therapeutic interventions must then be done early in the course of the disease and before this damage is too extensive.

The P23H rat suffers one of the commonest mutations associated with retinitis pigmentosa: proline23 substitution by histidine in the rhodopsin molecule (Dryja et al., 1990; Steinberg et al., 1996) and was developed to study photoreceptor rescue (Lewin et al., 1998; LaVail et al., 2000). P23H rats undergo a progressive loss of photoreceptors which is faster in the homozygotic than in the heterozygotic strain, in such a way that, at post-natal day (P) 270, only sporadic photoreceptors remain in the retina (Cuenca et al., 2004). Photoreceptor loss correlates with an abnormal electroretinographic (ERG) function as early as 4 weeks of age (Machida et al., 2000; Pinilla et al., 2005; Chrysostomou et al., 2009a). Moreover, beyond the progress of photoreceptor death, changes affecting the outer retina have been described such as neovascularization of the Retinal Pigmented Epithelium (RPE) (Pennesi et al., 2008) and synaptic and morphological neuronal changes (Jones et al., 2003; Cuenca et al., 2004). Two earlier studies have documented that these rats suffer RGC electrical changes and death (Jones et al., 2003; Kolomiets et al., 2010) but have not quantified the amount of cell loss. In this study, we use two different methods to label RGCs and automated counting techniques of whole-mounted retinas developed in our laboratory to determine the RGC numbers in these animals and quantify RGC loss.

2. Materials and methods

P23H-1 homozygous albino female rats at P30, P90, P180, P270 and P365 were used as experimental animals in this study. As age matching control animals we have also analyzed albino female Sprague–Dawley (SD) rats at P60 and P365 that is at a young and at the oldest time points examined in the present study. Transgenic homozygous P23H-1 animals were obtained from Dr. M. LaVail (UCSF School of Medicine; www.ucsfeye.net/mlavailRDratmodels.shtml), and bred in a colony at the University of Murcia; SD animals were obtained from the breeding colony of the University of Murcia (Murcia, Spain). Rats were housed in temperature and light controlled rooms with a 12 h light/dark cycle (light from 8 AM to 8 PM) and had food and water *ad libitum*. Light intensity within the cages ranged from 5 to 30 lux (scotopic to mesopic conditions). Animal manipulations were carried out following the Spanish and European Union regulations for the use of animals in research (Council Directive 86/609/EEC) and the ARVO statement for the use of animals in ophthalmic and vision research. Adequate measures were taken to minimize pain or discomfort.

Surgical manipulations were carried out under general anaesthesia induced with an intraperitoneal (i.p.) injection of a mixture of ketamine (70 mg/kg, Ketolar®, Parke–Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, Rompún®, Bayer, S.A., Barcelona, Spain). For recovery from anaesthesia, rats were placed in their cages and an ointment containing tobramycin (Tobrex® pomada oftálmica, Alcon S.A., Barcelona, Spain) was applied on the cornea to prevent corneal desiccation. Animals were sacrificed by an intraperitoneal injection of an overdose of sodium pentobarbital (Dolethal Vetoquinol®, Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

2.1. Retinal ganglion cells retrograde labeling from the superior colliculi

Fluorogold (FG) was applied to both superior colliculi (SCi), which are the main retino-recipient target regions in the brain, one week before animal processing to retrogradely label the RGCs, following previously described methods (Vidal-Sanz et al., 1988; Villegas-Pérez et al., 1998; Marco-Gomariz et al., 2006a; Salinas-Navarro et al., 2009a,b) that are standard in our laboratory. In brief, after exposing the midbrain, a small pledget of gelatine sponge (Espongostan® Film, Ferrosan A/S, Denmark) soaked in saline containing 3% FG and 10% DMSO, was applied over the entire surface of both SCi. Previous studies in control rats in our laboratory have shown that FG application to both SCi results in the labeling of 98.4% of the RGC population in SD rats (Salinas-Navarro et al., 2009a).

2.2. Tissue processing

Rats were perfused transcardially through the ascending aorta first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Some eyes were used for cross sections and others for retinal whole mounts, as follows:

2.3. Whole-mount preparations

Twenty-one P23H animals that had received FG application in the SCi were processed between P30 ($n = 5$), P90 ($n = 5$), P180 ($n = 6$), P270 ($n = 5$), and P365 ($n = 5$). As controls, nine FG-traced SD rats were processed at P60 ($n = 5$) and at P365 ($n = 4$). After perfusion, the eyes were enucleated and the retinas were dissected as whole-mounts by making four radial cuts in the superior, inferior, nasal, and temporal retinal quadrants. Retinal orientation was maintained by making the deepest radial cut in the superior retina. The retinas were post-fixed, washed, and immunoreacted (see below).

2.4. Cross-sections

After eye enucleation, the superior pole of the eye was marked with china ink. Then, the cornea and lens were removed and the resulting eyecups were post-fixed in the fixative for 1 h.

Microtome sectioning: The eyes of 4 normal SD rats (P60) and of five P23H animals, processed at P30 ($n = 1$), P90 ($n = 1$), P180 ($n = 1$), P270 ($n = 1$) and P365 ($n = 1$), were used. After post-fixation, the eyecups were embedded in paraffin, as previously described (Marco-Gomariz et al., 2006a), for microtome sectioning.