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Assessment of inner retina dysfunction and progressive ganglion cell loss in a mouse model of glaucoma

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ABSTRACT

The DBA/2J mouse is a model of ocular hypertension and retinal ganglion cell (RGC) degeneration, the main features of which are iris pigment dispersion (IPD) and iris stromal atrophy (ISA). These animals also experience glaucomatous changes, including an increase in intraocular pressure (IOP) beginning at about 9–12 months of age and sectorial RGC death in the retina. The aim of this study was to determine the onset of functional changes exhibited by DBA/2J mice in the inner retina. This was performed by means of electroretinographic recordings (scotopic threshold response, STR) and their correlation with morphological changes (loss of RGCs). To this end, we recorded the scotopic threshold response in control C57BL/6J and in DBA/2J mice at different ages. The RGCs, in both DBA/2J and C57BL/6J animals, were identified at 15 months of age by retrograde tracing with an analogue of fluorogold, hydroxystilbamidine methanesulfonate (OHSt), applied on the superior colliculi. Whole mount retinas were processed to quantify the population of RGCs identified by fluorogold tracing and Brn3a immunodetection, and were counted using image analysis software; an isodensity contour plot was generated for each retina. DBA/2J mice showed a significant reduction in the positive STR (pSTR) amplitudes at 12 months of age, as compared to control C57BL/6J mice of the same age. The pSTR mean amplitude decreased to approximately 27.82% of the values recorded in control mice ($p = 0.0058$). STR responses decreased in both strains as a result of the natural process of aging, but the decrease was more pronounced in DBA/2J mice. Furthermore, quantification of the total number of RGCs identified by OHSt and Brn3a expression showed a reduced population of RGCs in DBA/2J mice as compared to control mice. Regression analysis revealed significant correlations between the decrease in pSTR and a non-homogeneous reduction in the number of RGCs throughout the retina. Our results indicate the existence of a correlation between retinal function impairment and RGC loss. This functional and morphological analysis allows a reliable assessment of the progression of the disease.

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1. Introduction

Glaucoma is a significant cause of human blindness, characterized by RGC degeneration and the loss of axons (Quigley and Broman, 2006). So far, the mechanisms triggering the development of glaucoma have remained elusive. Despite the lack of information on the origin of this disease, glaucoma is very often associated with an abnormal elevation of intraocular pressure (IOP). On this basis, the current treatment for glaucoma is essentially aimed at lowering intraocular pressure in an attempt to protect RGCs (Schuettauf et al., 2002).

There are a number of animal models of glaucoma in which RGC degeneration and visual loss can be induced by methods such as

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limbal laser photocoagulation of episcleral veins (Mabuchi et al., 2003) or sclerosis of episcleral veins by injection of hypertonic saline (Morrison et al., 1997). Intraocular injections of hyaluronic acid, which can induce elevated IOP, have also been used to produce a model for the study of glaucoma (Moreno et al., 2005). Spontaneous genetic mutations have generated strains of animals suffering from glaucoma, such as the DBA/2J mouse, in which the development of the disease occurs spontaneously and subsequent to anterior chamber changes. These mice develop glaucomatous degeneration caused by iris pigment dispersion (IPD) and iris stromal atrophy (ISA) as the result of mutations in the Gpnmb and Tyrp genes (John et al., 1998; Libby et al., 2005; Howell et al., 2007). The accumulation of pigments in the trabecular meshwork of DBA mice may occlude trabecular spaces and cause a decrease in aqueous humor outflow, which leads to increased IOP. IOP values increase moderately between 2 and 6 months of age and more significantly between the ages of 8–12 months (Saleh et al., 2007; Harazny et al., 2009), showing a maximum value once the animal reaches 11 months of age (Scholz et al., 2008). The variability in the temporal course of IOP elevation is an important characteristic of this mouse model, as it provides information about the progression of the pathology and the course of RGC loss (McKinnon et al., 2009).

The changes in IOP have been correlated with a deficit in RGC axonal transport that leads to RGC loss (Crish et al., 2010; Danias et al., 2003; Schuettauf et al., 2004; Libby et al., 2005; Schlamp et al., 2006; Buckingham et al., 2008; Salinas–Navarro et al., 2009c, 2010; Cuenca et al., 2010; Nguyen et al., 2011). Metabolic vulnerability due to mitochondrial damage has also been reported (Baltan et al., 2010), which is related to the dysfunction in the inner retina (Ju et al., 2009; Salinas–Navarro et al., 2009c; Cuenca et al., 2010). The death of RGC during the development of this pigmentary glaucoma is the product of different mechanisms. Controversial results have pointed to cell death by apoptosis (Jakobs et al., 2005; Zhou et al., 2005) or necrosis (Schuettauf et al., 2004). Furthermore, the loss of RGC in the retina has been described as sectorial (Danias et al., 2003; Schlamp et al., 2006; Salinas–Navarro et al., 2009c; Vidal-Sanz et al., 2012).

In addition to the morphological changes observed in the inner retina, the functional implications of RGC loss have been investigated in the DBA/2J mouse model of retinal degeneration. Electrical RGC activity has been evaluated using a pattern electroretinogram (PERG), which shows a significant decrease in activity (Porciatti et al., 2007), accompanied by a concomitant increase in IOP (Saleh et al., 2007; Harazny et al., 2009).

Moreover, the analysis of the ERG waves has shown significant reductions in older mice, leading an impairment in the function of photoreceptors (Heiduschka et al., 2010).

The present study focused on the loss of visual function and its correlation with the quantitative and regional analysis of RGC. Retinal ganglion cell dysfunction in DBA/2J mice was studied using ERG measurements as a non-invasive method to monitor the course of the disease. Genetic analysis and IOP measurements were performed on all tested animals in order to confirm the pathological process.

2. Materials and methods

2.1. Animals

Experiments were performed on adult female C57BL/6J ($n = 8$) and DBA/2J ($n = 8$) mice obtained from the European distributor of Jackson Laboratories Mice (Charles Rivers Laboratories). All animal maintenance and experimental procedures followed Spanish and European guidelines for animal care in the laboratory and animal research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed (1-4 mice per cage) in temperature and light-controlled rooms maintained according to a 12-h light/dark cycle; all animals were fed *ad libitum*. DBA/2J and C57BL/6J mice were studied at 3, 6, 9, 12 and 15 months of age.

The experiments were performed on female DBA/2J and C57BL/6J mice, since IOP increased earlier in females and remained elevated longer (Libby et al., 2005). For both IOP and ERG measurements, mice were weighed and anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (95 mg/kg, Imalgene 1000®, Merial, Barcelona, Spain) and xylazine (5 mg/kg, Rompún®, Bayer, S.A., Barcelona, Spain).

The surgery for retrograde labeling was performed as previously described (Salinas–Navarro et al., 2009b, c; Alarcón-Martínez et al., 2010; Cuenca et al., 2010; Galindo-Romero et al., 2011, 2013). General anesthesia was administered by an i.p. injection of ketamine (70 mg/kg, Ketolar®, Parke-Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, Rompún®). While recovering from anesthesia, mice were placed in their cages, and an ocular ointment containing neomycin and prednisone (Oftalmolosa Cusí Prednisona-Neomicina®; Alcon S.A., Barcelona, Spain) was applied to the cornea to prevent corneal desiccation. Animals were euthanized with an i.p. injection of an overdose of pentobarbital (Dolethal Vetoquinol®, Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain).

2.2. Genotyping

DBA/2J mice display two ocular phenotypes: iris stromal atrophy (ISA) and iris pigment dispersion (IPD) (Chang et al., 1999; Anderson et al., 2002, 2006), caused by mutations in the *Tyrp1* and *Gpnmb* genes, respectively. *Tyrp1* (tyrosinase-related protein 1) has two missense mutations, one of which (nt 1151 of the mouse mRNA; GI:13654240) eliminates a restriction site. Thus, to identify this mutation, genomic DNA isolated from tail snips was used to amplify the sequence spanning the restriction site by Polymerase chain reaction (PCR). The PCR product (244 bp) was digested with BbsI (New England BioLabs, Beverly, MA). An undigested product indicates the presence of the mutation.

Gpnmb (glycoprotein transmembrane nmb), the gene responsible for the IPD phenotype, has a point mutation (C →T) at nt 547 of the mouse mRNA (GI:315429906) that creates a premature stop codon and also inserts a PvuII enzyme restriction site. Genomic DNA was used to amplify the 483 bp region surrounding the mutation. The resulting product was digested with PvuII (New England BioLabs). The presence of the mutation is indicated by the existence of two bands of 250 bp and 233 bp.

Genomic DNA was isolated from DBA/2 tail snip tissue using a DNA isolation kit (GentraPuregene, Qiagen, MN), according to the manufacturer's instructions, and 10 ng were used for PCR assays (Invitrogen). Murine *Tyrp1* specific primers (forward 5′-GCATTGCTCAGACCTATAGATATTC-3′ and reverse 5′-CAAAACACCAATTTTGTTTACTTGC-3′) were used to amplify the 244 bp fragment, as previously described (Inman et al., 2006). Two specific primers (forward 5′-CGGATACACTGGAATGCATCA-3′ and reverse 5′-ATGGCGAGCTTAGCCAGGATT-3′) were used to amplify the 483bp fragment of *Gpnmb*. The reactions were performed using TITANUM Taq DNA polymerase (Clontech, CA, USA). The thermal cycling conditions for *Tyrp1* were 40 cycles at 94 °C for 1 min, 55 °C for 30 s and 72 °C for 30 s. *Gpnmb* amplification was performed at 94 °C for 3 min, followed by 40 cycles at 94 °C for 1 min, 68 °C for 1 min and final extension at 72 °C for 7 min. Restriction digest was performed with 10 μL of PCR product, 1 μL of restriction enzyme, 0.5 μL BSA and 2μL of 10× buffer at 37° for 3 h. Digest products were run on a 2% agarose gel, stained with ethidium bromide and photographed using a Kodak GL 200 Imaging system (Kodak, Rochester, NY, USA).

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