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Ocular hypertension impairs optic nerve axonal transport leading to progressive retinal ganglion cell degeneration

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ABSTRACT

Ocular hypertension (OHT) is the main risk factor of glaucoma, a neuropathy leading to blindness. Here we have investigated the effects of laser photocoagulation (LP)-induced OHT, on the survival and retrograde axonal transport (RAT) of adult rat retinal ganglion cells (RGC) from 1 to 12 wks. Active RAT was examined with fluorogold (FG) applied to both superior colliculi (SCi) 1 wk before processing and passive axonal diffusion with dextran tetramethylrhodamine (DTMR) applied to the optic nerve (ON) 2 d prior to sacrifice. Surviving RGCs were identified with FG applied 1 wk pre-LP or by Brn3a immunodetection. The ON and retinal nerve fiber layer were examined by RT97-neurofibrillar staining. RGCs were counted automatically and color-coded density maps were generated. OHT retinas showed absence of FG^+ or $DTMR^+$RGCs in focal, pie-shaped and diffuse regions of the retina which, by two weeks, amounted to, approximately, an 80% of RGC loss without further increase. At this time, there was a discrepancy between the total number of surviving FG-prelabelled RGCs and of $DMTR^+$RGCs, suggesting that a large proportion of RGCs had their RAT impaired. This was further confirmed identifying surviving RGCs by their Brn3a expression. From 3 weeks onwards, there was a close correspondence of $DTMR^+$RGCs and FG^+RGCs in the same retinal regions, suggesting axonal constriction at the ON head. Neurofibrillar staining revealed, in ONs, focal degeneration of axonal bundles and, in the retinal areas lacking backlabeled RGCs, aberrant staining of RT97 characteristic of axotomy. LP-induced OHT results in a crush-like injury to ON axons leading to the anterograde and protracted retrograde degeneration of the intraocular axons and RGCs.

1. Introduction

Retinal ganglion cells (RGCs) are the innermost layer of neurons in the retina whose axons converge radially towards the optic disc where they form the optic nerve (ON) which conveys visual information gathered in the retina to the subcortical retinorecipient target regions in the brain. In rats the superior colliculus (SC) receives axons from 98% of the RGC population (Salinas-Navarro et al., 2009a, b). RGC axons leaving the retina are organized retinotopically with respect to their retinal quadrant and centroperipheral position (Guillery et al., 1995; Fitzgibbon and Taylor, 1996; Jeffery, 2001), so that fibers carrying the information from different eccentricities are mixed within a wedge, extending from the periphery to the centre of the nerve (Fitzgibbon and Taylor, 1996), but this topographic organization may not be completely maintained as they travel towards the chiasm (Guillery et al., 1995; Jeffery, 2001; Jeffery et al., 2008). One human disease that characteristically affects this neuronal population is glaucoma, a chronic neurodegenerative disease that affects RGCs and their axons, provokes optic disc changes and leads to visual field losses that may progress to complete blindness. Elevation of the intraocular pressure (IOP) is one of the most important risk factors associated with glaucoma in humans (Nouri-Mahdavi et al., 2004), and yet the only variable for which a number of medical and surgical treatments are available to control or slow down disease progression (Morrison et al., 2005, 2008).

To further study elevated IOP-induced retinal damage we have used one model of ocular hypertension (OHT), based on laser photocoagulation (LP) of the trabecular meshwork and perilimbal and episcleral veins, originally described to induce OHT in monkey (Quigley and Hohman, 1983) and later modified for the rat (WoldeMussie et al., 2001, 2004; Levkovitch-Verbin et al., 2002). These models do not fully mimic the human glaucomatous optic neuropathy, but are useful to understand the pathophysiology

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involved in OHT-induced retinal damage (for review see; Morrison et al., 2005, 2008), as well as to test for neuroprotective substances (WoldeMussie et al., 2001; Pang et al., 2005; Zhou et al., 2005) and can be used to investigate RGC responses to OHT within a relative short period of time in the albino rat, an available laboratory animal for which there are few transgenic animals in which to study neurodegenerative diseases. Injured RGCs are known to undergo early functional deficits (Schlamp et al., 2001; Alarcón-Martínez et al., in press), including alterations in their axoplasmic flow properties (McKerracher et al., 1990a, 1990b; Quigley and Anderson, 1976, 1977; Lafuente López-Herrera et al., 2002), in several metabolic functions (Nash and Osborne, 1999; Schlamp et al., 2001; Chidlow et al., 2005; Parrilla-Reverter et al., 2009) and in the regulation of a substantial number of genes (Agudo et al., 2008; 2009), including the downregulation of Brn3a (Nadal-Nicolás et al., 2009) shortly before they die.

Previous work in rats (WoldeMussie et al., 2001, 2004; Levkovitch-Verbin et al., 2002; Danias et al., 2006) and pigmented mice (Danias et al., 2003; Jakobs et al., 2005; Filippopoulos et al., 2006; Schlamp et al., 2006; Howell et al., 2007; Buckingham et al., 2008; Soto et al., 2008) has provided important information regarding the effects of OHT in the retina of these animals. Using retrogradely transported and diffusing neuronal tracers and molecular markers to identify RGCs and their axons, as well as a recently developed technology to image their distribution within the retina and to count the total population of RGCs (Salinas-Navarro et al., 2009a,b; Nadal-Nicolás et al., 2009; Parrilla-Reverter et al., in press) we have further investigated OHT-induced damage in the Sprague Dawley rat retina and addressed the following questions: i) Does increased IOP result in retinal damage; ii) Is OHT-induced retinal damage a progressive event; iii) Does the lack of labeled RGCs represent an impairment of retrograde axonal transport (RAT) and/or an actual loss of RGCs; iv) Is the lack of retrograde labeling due to a functional deficit and/or to a mechanical compression of the RGCs axons at the level of the ON head, and; v) Do the degenerative events in the RGC layer mimic an axotomy-like type of insult. Our first group of experiments shows that increased IOP results in large areas of the retina lacking backlabeled RGCs from the SCi and in anterograde axonal degeneration of sectors within their optic nerves. A second group established that lack of RAT progresses between 1 and 2, but not between 2 and 12 weeks post-LP. While lack of labelled RGCs is mainly due to actual RGC degeneration, at early time periods there is also a substantial proportion of surviving RGCs that have their RAT impaired, as shown by Brn3a as well as by RT97 immunofluorescence. The lack of RAT may appear functional at the beginning, within the first two weeks but is shortly followed by an impairment of passive retrograde dye diffusion, implying optic axon compression somewhere near the ON head. This is further evidenced by the neurofibrillar staining of the RGC fiber layer showing characteristic features of crush injury-induced to ON axons (Parrilla-Reverter et al., in press). Short accounts of this work have been published in abstract form (Mayor-Torroglosa et al., 2004; Villegas-Pérez et al., 2005; Vidal-Sanz et al., 2005; Salinas-Navarro et al., 2006).

2. Methods

2.1. Animals and anesthetics

Female adult (180–200 g) albino Sprague–Dawley (SD) rats from the breeding colony of the University of Murcia (Murcia, Spain) 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. All surgical manipulations were carried out under general anesthesia; intraperitoneal (i.p.) ketamine (70 mg/kg, Ketolar®, Parke-Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, Rompún®, Bayer, S.A., Barcelona, Spain)]. A drop of topical anesthetics (Colircusí anestésico doble®, Alcón Cusí, S.A., Barcelona, Spain) was instilled on both eyes prior to IOP measurements. During recovery from anesthesia a topical ointment (Tobrex®, Alcón Cusí, S.A., Barcelona, Spain) was applied to prevent corneal desiccation. Additional measures were taken to minimize discomfort and pain after surgery. For sacrifice an i.p. overdose of pentobarbital (Dolethal Vetoquinol®, Especialidades Veterinarias, S.A., Alcobendas, Madrid, Spain) was used.

2.2. Induction of OHT

The left eyes were treated on a single session with diode laser burns (Viridis Ophthalmic Photocoagulator-532 nm laser, Quantel Medical, Clermont-Ferrand, France). Laser beam was directly delivered without any lenses and aimed to the trabecular meshwork, the perilimbar and episcleral veins with modifications of previous methods (WoldeMussie et al., 2001; Levkovitch-Verbin et al., 2002). The spot size, duration and power used were 50–100 μm, 0.5 s and 0.4 W, respectively. A number of ocular complications such as hyphema and corneal opacities were observed shortly after lasering in a small number of animals and, as described (Levkovitch-Verbin et al., 2002), these resolved within several days in most cases and in those instances in which these did not resolve the animals were discarded.

2.3. Retrograde tracing of RGCs: FG and DTMR

Application of 3% FG (Fluorochrome Inc., Engelwood, CO, USA) to the SCi followed described methods (Vidal-Sanz et al., 1988; 1993; 2000; 2001, 2007; Salinas-Navarro et al., 2009a, b). The fluorescent tracer dextran tetramethylrhodamine (DTMR; 3000 MW; Molecular Probes, Inc. Eugene, OR, USA) was applied intraorbitally 2 days prior to sacrifice as previously described (Sellés-Navarro et al., 1996; Lafuente López-Herrera et al., 2002; Salinas-Navarro et al., 2009a,b). DTMR diffuses passively through the axon towards the cell soma producing an intense labeling in normal (WoldeMussie et al., 2001, 2004; Salinas-Navarro et al., 2009a, b) and OHT eyes (WoldeMussie et al., 2001). Although we do not have total counts for $DTMR^{+}$RGCs, the labeling is efficient enough as to depict the spatial distribution of RGCs along a visual streak in the superior retina, something only observed when practically all RGCs are labeled (see Fig. 3A of Salinas-Navarro et al., 2009a). A summary of the different experimental manipulations, survival intervals and number of animals used to address the main questions posed in this study is provided in Table 1.

2.4. Measurement of IOP

The intraocular pressure (IOP) was measured under anesthesia in both eyes with a tonometer (Tono-Pen® XL Reichert® Ophthalmic Instruments Depew, NY, USA) (Moore et al., 1993, 1996) prior to, and 24, 48, 72 h and 1, 2 or 4 weeks after LP for groups 2–3 (Table 1), except for animals surviving 12 weeks in which IOP was measured prior to, and at 6, 12, 24, 48 and 72 h, and 1, 2, 3, 4, and 12 weeks after LP (Table 1). At each time point 8–12 consecutive readings from each eye were averaged. IOP was tested at the same time in the morning and right after deep anesthesia to avoid IOP fluctuations due to circadian rhythm (Moore et al., 1996; Krishna et al., 1995; Jia et al., 2000) or to elevation of the IOP itself (Drouyer et al., 2008).