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The retina of the *PCD/PCD* mouse as a model of photoreceptor degeneration. A structural and functional study

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ABSTRACT

In this work, we used the *pcd* (*Purkinje cell degeneration*) mutant mouse with a slow temporal progression of photoreceptor degeneration in order to analyze the structural and functional modifications in the neuronal populations of the outer and inner retina.

Retinal immunocytochemistry and functional electroretinography were performed on the *pcd*/*pcd* mutant mice and control wild type animals of the C57/DBA strain at 45, 90, 180 and 270 post-natal days. Immunohistochemical studies were performed for a series of protein markers: calbindin, calretinin, PKCα, bassoon, synapsin, syntaxin and islet1. Full field electroretinography recordings were performed on control and dystrophic mice. Rod and mixed responses, and oscillatory potentials, were recorded in dark adapted conditions; cone and flicker responses were recorded under light adaptation. Our results show significant structural modifications in the photoreceptor populations and neurons of the inner retina. Changes in cell morphology affect mainly to the bipolar cells, which gradually lose their dendritic tufts. The electroretinography records reveal that in the *pcd* retinas the rod and cone systems show a reduction in the amplitude of the electrical signals. This decrease progresses slowly with the passage of time, although for the most advanced stage of photoreceptor degeneration considered, 270 post-natal days, it is still possible to record light induced responses. We conclude that *pcd* mice experience a loss of retinal function in correlation with the loss of photoreceptors with age, and significant changes in retinal synaptic processes.

1. Introduction

Up to 11% of human blindness is caused by retinal degeneration (http://www.nei.nih.gov/eyedata/pbd_tables.asp) and most is characterized by the loss of photoreceptors, especially the rods (Gaillard and Sauve, 2007). Many gene mutations which result in rod photoreceptor degeneration have been identified (http://www.sph.uth.tmc.edu/retnet).

Diverse animal models with photoreceptor degeneration have been identified, including *rd1* mice (Strettoi et al., 2002, 2003), *rd10* mice (Barhoum et al., 2008; Chang et al., 2002; Gargini et al., 2007), *nr* mice (Chang et al., 2002; LaVail et al., 1993; Ren et al., 2000, 2001), *mnd* mice (Chang et al., 2002; Seigel et al., 2005), *RCS* rats (Dowling and Sidman, 1962; Herron et al., 1974; Villegas-Pérez et al., 1998) and *p23H* rats (Cuenca et al., 2004). The availability of these and other models provide important opportunities to study the disease time course, and to evaluate experimental therapies.

The *pcd* mice (*Purkinje cell degeneration*) (Campbell and Hess, 1996; Chakrabarti et al., 2006; Fernández-González et al., 2002; Harris et al., 2000; Mullen, 1977; Mullen et al., 1976; Wang and Morgan, 2007) undergo a mutation in the *nna1* gene which provokes a progressive degeneration of the photoreceptor cells of the retina (Blanks et al., 1982; Chang et al., 2002; LaVail et al., 1982; Mullen and LaVail, 1975). Even though the progression of death of the rods and cones is well documented in *pcd* mice (LaVail et al., 1982), the effect that the mutation has on the remaining retina is unknown. Moreover, there are no data on the functionality of the

Abbreviations: BSA, bovine serum albumin; CB, calbindin; CR, calretinin; DAB, 3,3′-diaminobenzidine; ERG, electroretinogram; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OP, oscillatory potentials; OPL, outer plexiform layer; PBS-Tx, saline phosphate buffer with triton X-100; *pcd*, *Purkinje cell degeneration*; PKCα, protein kinase C α; RP, retinitis pigmentosa.

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retina of the *pcd* mice at advance stages of degeneration. In this work, we analyze the morphology and functionality of the outer and inner retina of *pcd* mice across age.

2. Material and methods

2.1. Animals

We used the C57BL/DBA mouse strain obtained by crossing between C57BL/6J strain with the pcd^{1J} allele and DBA/2J strain without *pcd* mutation (Jackson Laboratories). As homozygous males are sterile and homozygous females are poor breeders (http://jaxmice.jax.org/strain/000537.html), we crossed *pcd*/+ heterozygous mice to obtain the *pcd/pcd* animals studied here. Although a genotyping method for the mutant *pcd* allele is not available, the use of the DBA/2J mouse strain allowed a clear detection of heterozygous animals by PCR amplification on specific microsatellite sequences (Recio et al., 2007). Since DBA/2J animals are known to develop glaucoma (Libby et al., 2005) we looked for structural differences between wild type (WT) C57BL/DBA and C57BL/6J retinas and noted no significant differences. In this study, 10 *pcd* (*pcd*/*pcd*) mice and 10 WT littermates were used for each age considered: 45, 90, 180 and 270 days. All the experiments were carried out in accordance with the guidelines of the European Community Council (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (RD 1201/2005).

2.2. Immunohistochemistry

The animals, previously anaesthetized with a mixture containing ketamine hydrochloride (95 mg/kg) (Ketolar; Parke-Davis, Barcelona, Spain) and xylazine hydrochloride (5 mg/kg) (Rompun; Bayer, Leverkussen, Germany) were perfused transcardially with a 0.9% NaCl solution followed by 4% paraformaldehyde and 0.2% picric acid in phosphate buffer 0.1 M, pH 7.4 (PB). The eyes were dissected out and post-fixed for 2 h at room temperature (RT) in the same fixative solution. The eyes were cryoprotected in 30% sucrose and 16-μm naso-temporal sections were cut on a cryostat (Leica CM3000, Germany).

After washing the tissue with saline PB 0.1 M pH 7.4 with 0.02% of Triton X-100 (PBS-Tx), the endogenous peroxidases were eliminated with methane and hydrogen peroxide at 0.03%; in the case of double labeling, the autofluorescence was eliminated with 5 mg/ml of $NaBH_4$ in PBS. After washing with PBS, the tissue was pre-incubated for 1 h with PBS-Tx and bovine serum albumin (BSA) at 2%. It was later incubated for 12 h at room temperature with diluted primary antibody in the same medium (Table 1).

After the washes, the sections were incubated for 1 h with biotinylated secondary antibody at 1:200 (Vector, Burlingame, USA) in the case of single labeling or with fluorescent secondary antibodies conjugated with Cy2 and Cy3 (Jackson, West Grove, PA, USA) at 1:250 for double labeling. The labeling with biotin was revealed with the avidin–biotin–peroxidase kit (Vector, Burlingame, USA) using 3,3′-diaminobenzidine (DAB) as chromogen. The nuclei were labeled with propidium iodide (Sigma–Aldrich, St Louis, MO, USA) diluted at 1:2000. The negative controls were carried out omitting the primary and secondary antibodies. The sections treated with DAB were dehydrated and mounted with Entellan (Merck, Madrid, Spain) and the fluorescence sections were mounted with a protective mixture of the fluorescence (0.42% of glycine, 0.021% NaOH, 0.51% NaCl, 0,03% sodium azide, 5% of *N*-propylgalate, 70% of glycerine and distilled water).

Microscope images were obtained with an Olympus DP70 digital camera coupled to an Olympus AX-70 photomicroscope (Olympus Optical, Tokyo, Japan) with the appropriate filters. The remainder of the fluorescent images was obtained with a laser confocal microscope Leica TCS SP2 (Leica Instruments, Tokyo, Japan) using simultaneous excitation of the fluorochromes with laser wavelengths of 488 and 543 nm. The original images were later processed with Adobe Photoshop 7.0 software to obtain the optimum contrast within the figure.

Table 1
Primary antibodies.

Antibody	Type	Donor	Concentration	Source
Anti-PKCα	Policlonal	Rabbit	1:5000	Sigma
Anti-islet1	Monoclonal	Mouse	1:500	Hibridoma Bank
Anti-calbindin	Policlonal	Rabbit	1:1000	Swant
Anti-calretinin	Monoclonal	Mouse	1:5000	Swant
	Policlonal	Rabbit	1:5000	Schwaller
Anti-syntaxin	Policlonal	Rabbit	1:400	Santa Cruz
Anti-synapsin	Policlonal	Goat	1:400	Santa Cruz
Anti-bassoon	Monoclonal	Mouse	1:200	Stressgen

2.3. Quantification of cells

Transverse sections of 16 μm from the middle region of the retina were chosen and images were taken using a digital camera (DP70, Olympus, Tokyo, Japan) attached to a microscope (Olympus AX Provis). Afterwards, ImageJ software was used to define rectangular fields longitudinal to the axis of the retina sections. The same software was used to count the number of labeled cells in each field. For each section studied, 3 fields of 200 μm length were defined, and the mean value of the number of labeled cells per field was found. Two-way ANOVA with Bonferroni posttest was used for statistical analysis, using GraphPad Prism version 3.00 for Windows (Graph Pad Software, San Diego California USA, www.graphpad.com); two variables (postnatal times and animal groups) were analyzed for each cell type.

2.4. Electroretinogram recordings

Prior to electroretinogram (ERG) recording, the mice were maintained in darkness overnight and, subsequently, the set up procedures were conducted in dim red light, which was thereafter extinguished for the dark adapted studies. The mice were anaesthetized with an intraperitoneal injection of a solution of ketamine (95 mg/kg) and xylazine (5 mg/kg) and maintained on a heating pad at 37 °C. The pupils were dilated by applying a topical drop of 1% tropicamide (Culircusí Tropicamida, Alcon Cusí, El Masnou, Barcelona, Spain). A topical drop of 2% Methocel (Ciba Vision, Hetlingen, Switzerland) was applied in each eye immediately before situating the corneal electrode. Flash-induced ERG responses were recorded from the right eye in response to light stimuli applied with a Ganzfeld stimulator. The intensity of light stimuli was measured with a photometer (Mavo Monitor USB, Gossen, Nürenberg, Germany) at the level of the eye. At each light intensity, the average of 4–64 consecutive stimuli was obtained; the interflash interval in scotopic conditions ranged from 10 s for dim flashes and up to 60 s for the highest intensity stimuli. Under photopic conditions the interval between light flashes was fixed at 1 s. The ERG signals were amplified and band filtered between 0.3 and 1000 Hz with a Grass amplifier (CP511 AC amplifier, Grass Instruments, Quincy, MA). Electrical signals were digitized at 20 kHz with a Power Lab data acquisition board (AD Instruments, Chalgrove, UK). Bipolar recording was performed between an electrode fixed on a corneal lens (Burian-Allen electrode, Hansen Ophthalmic Development Lab, Coralville, IA) and a reference electrode located in the mouth, with a ground electrode located in the tail. Rod mediated responses were