Detailed insights into functional retinal neurodegeneration of aged DBA/2J glaucoma mice

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Introduction

Glaucoma is the second leading cause for blindness worldwide and affects approximately 3% of the population with a higher incidence at people aged 40 years and older. In pigment dispersion glaucoma as one important form of glaucoma, pigment particles from the iris get loose and distribute inside the anterior chamber. The DBA/2J mouse is a widely used inbred strain that develops progressive eye abnormalities that closely mimic human pigment dispersion glaucoma.

The incidence and the extent of iris atrophy in DBA/2J mice increases with age, and the iris atrophy is usually severe at the age of 18 months. Therefore, the IOP rises significantly by six to nine months of age. As a consequence of the glaucoma developing in DBA/2J mice, the nerve fibre layer becomes thinner, and the number of RGCs is greatly depleted, accompanied by optic nerve atrophy and disorganisation. Although there is a high phenotypic variability in glaucoma development in the DBA/2J mice, glaucoma develops in nearly all eyes. The disease follows a predictable progressive path, and the majority of mice suffer increase of IOP, loss of RGCs and optic nerve atrophy by 22 months of age.

To determine functional loss due to RGC death, visual evoked potentials (VEP) can be measured in these animals, because VEP give the best idea about the presence, the state and the function of RGCs and their axons in the optic nerve. Surprisingly, decreased Ganzfeld electroretinographic (ERG) amplitudes were also found in DBA/2J mice at ages up to approximately 300 days.

In this study, we examined two-year-old DBA/2J mice that can be considered a kind of "end point" regarding the degeneration of RGCs. Our aim was to evaluate the extent of RGC degeneration in such old DBA/2J mice and to investigate whether visual function on both retinal and cortical levels could still be recorded. For this purpose, we performed ERG and VEP measurements in aged DBA/2J mice and age-matched C57BL/6J mice and labelled RGCs retrogradely to quantify them. To our knowledge, this is the first combined ERG and VEP investigation in two-year-old DBA/2J mice.

Material and Methods

Animals

All mice were handled according to German animal protection laws. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology. DBA/2J and C57BL/6J mice were obtained from the European distributor of Jackson Laboratories mice (Charles River Laboratories). All mice were kept in a 12 h light/12 h dark cycle. For the DBA/2J strain, a special fat chow containing 5.5% fat instead of 4.5% was provided ad libitum. Control eyes were collected from C57BL/6J mice that did not display any ocular abnormalities.

Retrograde labelling and immunohistochemistry

RGCs were labelled retrogradely by injecting a solution of Fluorogold (FG; Fluorochrome, LLC, Colorado) in PBS and 2% DMSO into each of the *superior colliculi.* One week after the injection, retinas were prepared and fixed. Digital images were taken using a fluorescence microscope (Axioplan2, Zeiss, Germany). RGCs were counted in the images.

Neurofilament staining: Briefly, retinas from DBA/2J and C57BL/6J mice were fixed, washed, treated with Proteinase K and 3% H_2O_2, and followed by three washing steps and a blocking step. Then retinas were incubated with an anti-neurofilament antibody (R&D Systems, Germany), washed, blocked again and incubated with a Cy3 donkey anti-goat antibody (Dianova, Germany) and finally embedded in FluorSave (Calbiochem, Germany) and inspected using a fluorescent microscope.

Visual electrophysiology

General procedure:

Eight animals of each strain were used. Mice were dark adapted for a period of at least 24 hours. All manipulations were performed under dim red light. Corneal anaesthesia was performed using Novesine (Novartis Ophthalmics). Body temperature was kept constant by placing the animals on a heating. A gold wire ring electrode placed into the mouth served as a reference electrode. The pupils were dilated with a drop of Tropicamide (Novartis Ophthalmics). Measurement was performed using the commercial RetiPort32 device (Roland Consult Systems, Brandenburg, Germany).

Electroretinography:

Animals were anaesthetised by ketamine and xylazine (120 mg/kg ketamine, 10 mg/kg xylazine). Gold wire ring electrodes placed on the corneas of both eyes served as working electrodes. Standard electroretinographic measurements were performed simultaneously on both eyes, with scotopic flash ERG at eight different light intensities from 0.0003 to 100 cds/m², an additional run for scotopic oscillatory potentials at 100 cds/m², photopic 30 Hz Flicker at 3 cds/m² after 10 minutes of light adaptation, and finally photopic flash ERG and photopic oscillatory potentials at 100 cds/m².

Parameters of photoreceptor response were computed by using the well-known relationship by Hood and Birch (1994), based on the theory by Lamb and Pugh (1992):

$$P3_{(i,t)} = Rm_{P3} \cdot \left(1 - e^{-i \cdot S \cdot (t - t_d)^2}\right) \quad \text{for } t \geq t_d \qquad (1)$$

with i – stimulus intensity, t - time after flash onset, P3 - sum of individual photoreceptor responses, Rm_{P3} - saturated response, t_d - delay that allows for biochemical and other recording latencies, S - sensitivity parameter.

We approximated measured a- and b-wave amplitudes to the parameters $R_{max,1}$ and $R_{max,2}$ (maximal response amplitudes), $i_{50,1}$ and $i_{50,2}$ (half-saturating light intensities) and n,1 and n,2 - exponents describing the slope of the Hill function using the biphasic equation

$$R = R_{max,1} \frac{i^{n,1}}{i^{n,1} + i_{50,1}^{n,1}} + R_{max,2} \frac{i^{n,2}}{i^{n,2} + i_{50,2}^{n,2}} \qquad (2)$$

firstly introduced without the exponent n in the monophasic shape by Naka and Rushton (1966), with R - response amplitude and i – intensity of light stimulus.

Visual evoked potentials:

Animals were anaesthetised with an intraperitoneal injection of a solution of chloral hydrate in physiologic saline (0.42 mg chloral hydrate/kg). A self-tapping stainless-steel screw with a shaft diameter of 1.12 mm and a length of 3.18 mm (Y-TX00-2, Small Parts, Inc., Miami Lakes, Florida) served as a measuring electrode and was inserted more than one week before the measurement through the skull into the left visual cortex (1.5 mm laterally to the midline, 1.5 mm anterior to the lambda), penetrating the cortex to approximately 1 mm. For the actual measurement, the skin was opened; the screw was cleaned of connecting tissue and blood and connected to the amplifier. Both eyes were stimulated for the measurement. Visual evoked potentials were recorded, with scotopic flash VEP at eight different light intensities ranging from 0.0003 to 100 cds/m², and photopic flash VEP at 100 cds/m² after 10 minutes of light adaptation. After finishing the measurements, the wound was stitched and antibiotic ointment applied.

The averaged signals of DBA/2J mice and wild-type mice were compared with respect to both latency and amplitude.

Statistics

The single ERG and VEP parameters as well as numbers of RGCs obtained in DBA/2J mice and wild-type C57BL/6J mice were compared using the Student's t-test. The significance of parameter differences between wild-type mice and DBA/2J mice was indicated as follows: * p<0.05, ** p<0.01, *** p<0.001.

Results

Figure 1: A clear impairment of the inner retinal function in DBA/2J mice is obvious: all waveforms of DBA/2J mice show considerably smaller amplitudes

Representative appearance of electroretinographic waveforms obtained in wild-type C57BL/6J mice (black curves) and DBA/2J mice (red curves). The intensities of light stimuli in scotopic ERG are indicated nearby the curves. Please note the different scales.

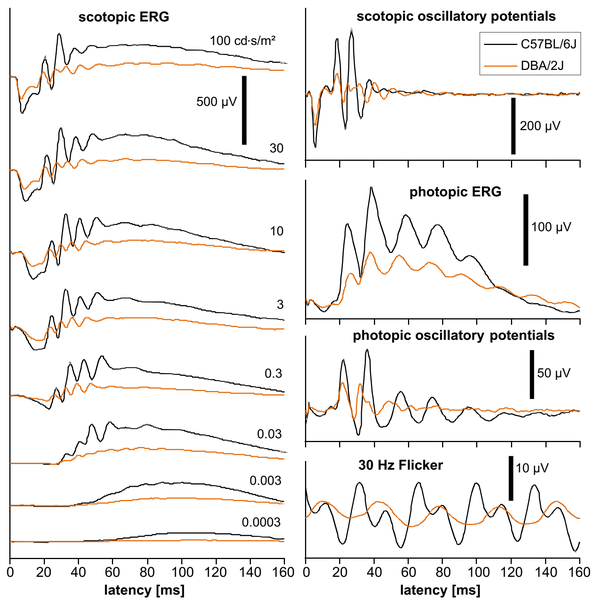


Figure 2: a-wave and b-wave amplitudes but not the latencies are significantly reduced in DBA/2J-mice

ERG parameters obtained in wild-type C57BL/6J mice (n=7)and DBA/2J mice (n=5):

A Amplitudes and latencies of scotopic ERG at different intensities of the light stimuli: Amplitudes of a-waves are significantly smaller in DBA/2J mice at light intensities above 0.01 cds/m² ($p \leq 0.027$), and the same is true for the amplitudes of b-waves over the whole inspected range of light intensities ($p \leq 0.0018$). The curves in the amplitude diagrams were calculated by the equation (2) using the parameters listed in table 1. The curves in the latency diagrams just serve to guide the eye and do not represent a function.

B Indices of amplitudes and latencies of scotopic and photopic oscillatory potentials: The amplitudes of both scotopic and photopic oscillatory potentials are also significantly smaller in DBA/2J mice than in C57BL/6J mice. There was no significant difference in the latencies of a-waves, b-waves and oscillatory potentials between DBA/2J mice and wild-type mice, except for the latency of photopic oscillations that were shorter in DBA/2J mice.

C The amplitudes of photopic b-waves are also significantly smaller in DBA/2J mice than in C57BL/6J mice.

D The ratio of the amplitudes of b-waves and a-waves, the so called b/a ratio, and the ratio of the amplitudes of oscillatory potentials and b-waves (OP/b ratio) were quite similar in both strains, although the values showed a higher variability in DBA/2J mice. There was also no significant difference in the OP/a ratio, although the value was slightly higher in DBA/2J mice, and the variability was much larger in DBA/2J mice.

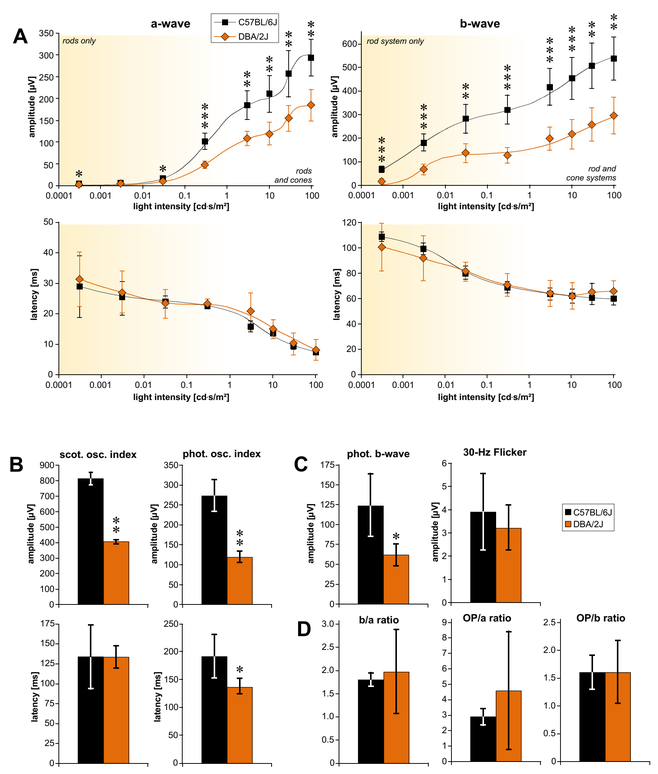


Table 1:

Calculated parameters of the biphasic Naka-Rushton equation (2) with the amplitudes of electroretinographic a-waves and b-waves and VEP amplitudes. R_{max} values given in µV, and i_{50} values in cds/m².

	wild-type C57BL/6J	DBA/2J	p
ERG a-wave amplitudes			
$R_{max,1}$	207.7 ± 34.6	126.4 ± 19.7	0.0015 **
$n_{,1}$	0.993 ± 0.155	0.926 ± 0.108	0.4639 (n.s.)
$i_{50,1}$	0.361 ± 0.183	0.486 ± 0.070	0.2127 (n.s.)
$R_{max,2}$	95.21 ± 30.65	58.38 ± 20.99	0.0597 (n.s.)
$n_{,2}$	4.191 ± 2.732	4.534 ± 3.093	0.8567 (n.s.)
$i_{50,2}$	29.60 ± 11.29	27.05 ± 3.50	0.6651 (n.s.)
ERG b-wave amplitudes			
$R_{max,1}$	305.3 ± 69.2	129.1 ± 42.0	0.0009 ***
$n_{,1}$	0.729 ± 0.070	1.725 ± 1.156	0.0645 (n.s.)
$i_{50,1}$	0.0018 ± 0.0004	0.0027 ± 0.0006	0.0204 *
$R_{max,2}$	270.1 ± 84.8	205.9 ± 62.3	0.2184 (n.s.)
$n_{,2}$	0.791 ± 0.268	0.709 ± 0.165	0.5936 (n.s.)
$i_{50,2}$	7.98 ± 4.58	17.18 ± 15.87	0.2144 (n.s.)
VEP amplitudes			
$R_{max,1}$	104.7 ± 28.3	10.9 ± 7.2	6.77E-07 ***
$n_{,1}$	0.903 ± 0.275	1.025 ± 0.320	0.4925 (n.s.)
$i_{50,1}$	0.012 ± 0.005	0.009 ± 0.005	0.3540 (n.s.)
$R_{max,2}$	28.56 ± 17.28	3.86 ± 4.48	0.0026 **
$n_{,2}$	3.199 ± 1.915	2.0 ± 0.6	0.1948 (n.s.)
$i_{50,2}$	7.12 ± 4.26	4.75 ± 2.34	0.2769 (n.s.)

Figure 3: Almost no cortical activity can be recorded in DBA/2J mice upon light stimulation

Typical appearance of waveforms of visual evoked potentials obtained in wild-type C57BL/6J mice (black lines) and DBA/2J mice (red lines). The intensities of light stimuli in scotopic VEP are indicated nearby the curves. Please note the different scales.

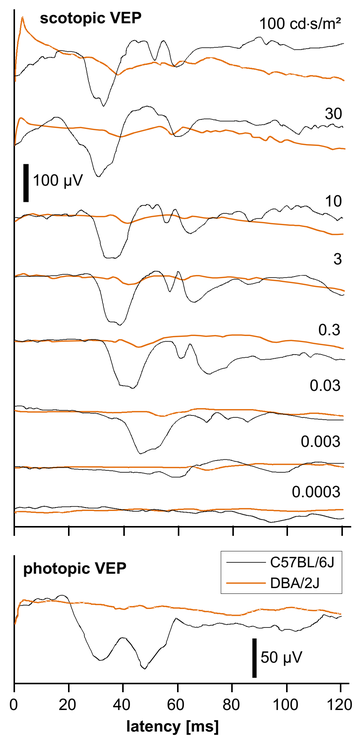


Figure 4: Values of VEP amplitudes are significantly smaller in DBA/2J mice for both scotopic and photopic VEPs

VEP parameters obtained in wild-type C57BL/6J mice and DBA/2J mice (n=8):

A Amplitudes of scotopic VEP at different intensities of the light stimuli and of photopic VEP: In some cases, it was difficult to determine the values of the VEP amplitudes at all due to the flat appearance of the waveforms. Therefore, we used a latency window ranging 30-40 ms around the latency obtained in C57BL/6J mice as a criterion whether a signal could be still interpreted as an evoked potential or if it was just a deflection due to spontaneous cortical activity.

B Latencies of scotopic VEP at different intensities of the light stimuli and of photopic VEP: The latencies measured in DBA/2J mice show a clear tendency of being prolonged compared to C57BL/6J mice, and the difference is significant at higher light intensities. The variability of the latency values was higher in DBA/2J mice than in C57BL/6J mice.

The curves in the amplitude diagrams were calculated by the equation (2) using the parameters listed in table 1. The curves in the latency diagrams just serve to guide the eye and do not represent a function.

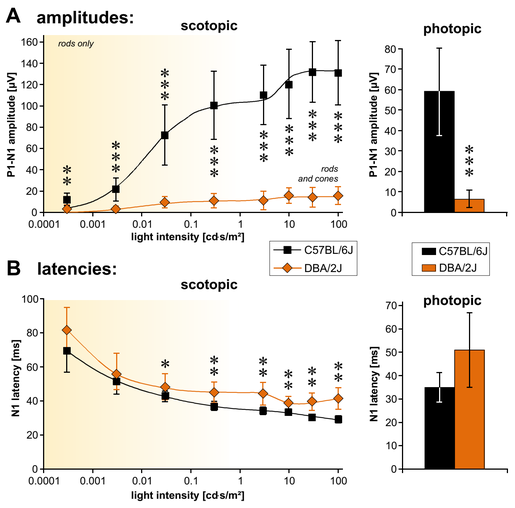


Figure 5: The numbers of retinal ganglion cells were significantly reduced in DBA/2J mice

A Representative fluorescence microscopy images of retinal whole mounts obtained from a wild-type C57BL/6J mouse and an age-matched DBA/2J mouse one week after retrograde staining of the RGCs with Fluorogold. In DBA/2J mice, only a few RGCs were left in the retina compared to age-matched C57BL/6J mice.

B A diagram shows the results of RGC counting in both animal models: counting of RGCs resulted in a significantly lower number of RGCs in DBA/2J mice (3069 ± 350 RGC/mm² in C57BL/6J mice *vs.* 68 ± 11 RGC/mm² in DBA/2J mice, $p=6x10^{-6}$). (n=4)

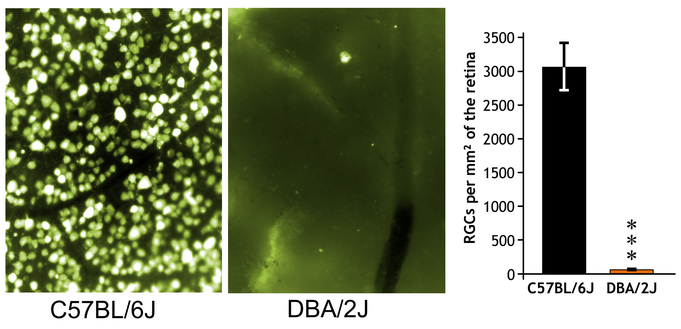
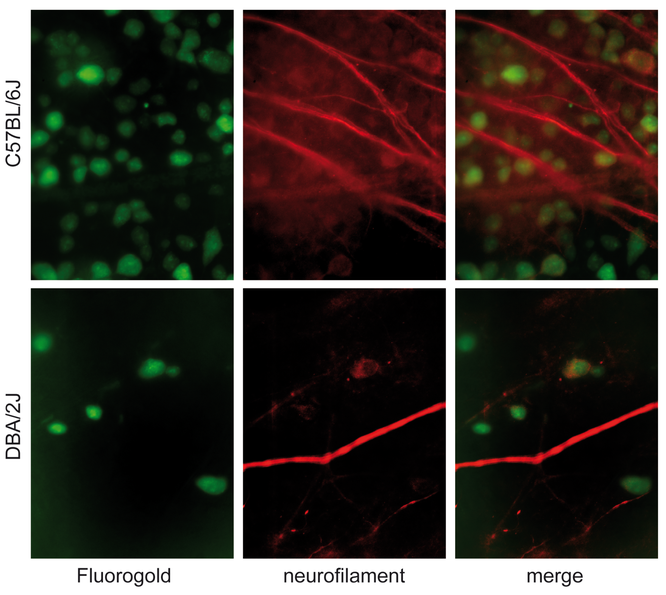


Figure 6: Most of the Fluorogold labelled cells are RGCs

Representative fluorescence microscopy images of retinal whole mounts obtained from a wild-type C57BL/6J mouse and an age-matched DBA/2J mouse one week after retrograde staining of the RGCs with Fluorogold and after immunohistochemical staining using a neurofilament antibody as indicated. Neurofilament immuno-reactivity (IR) was found in the RGC somata and their axons as well as, to a lesser degree, in retinal blood vessels. In rare cases, more RGCs were found to be labelled by Fluorogold than by the anti-neurofilament antibody. There has been almost no case in the C57BL/6J mice that an RGC displayed neurofilament IR without being labelled with Fluorogold, and we did not find such a case in DBA/2J mice. (n=4)



Summary & Conclusion

- **Almost no RGCs were left in the retina of 2 year old DBA/2J mice**
- **VEP in retina of 2 year old DBA/2J mice were hardly recordable**
- **Surprisingly, also ERG amplitudes of scotopic a-waves & b-waves, photopic b-waves & oscillatory potentials were decreased significantly by approximately 40% compared to amplitudes measured in age-matched C57BL/6J mice**
- **The latencies were unchanged in DBA/2J mice compared to controls, and so were the ratios between amplitudes of a-waves, b-waves and oscillatory potentials.**

→ **Our results indicate that, in addition to degeneration of RGCs, also photoreceptors are affected by pathological processes in the eye caused by the mutations present in DBA/2J mice.**

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Conflict of interest: none