Temporal Properties of Flicker ERGs in Rabbit Model of Retinitis Pigmentosa

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PURPOSE. We determined the effects of a remodeled inner retina on the flicker electroretinograms (ERGs) in a rabbit eye at an advanced stage of inherited retinal degeneration.

METHODS. Six wild-type (WT) and four rhodopsin P347L transgenic (Tg) rabbits were studied at 18 months of age. Flicker ERGs were elicited by sinusoidal stimuli at frequencies of 3.906 to 50.781 Hz. To block the ON and OFF retinal pathways, 2-amino-4-phosphonobutyric acid (APB), and 6-cyano-7-nitroquinoxaline-2,3(1H, 4H)-dione (CNQX), respectively, were injected intravitreally. The amplitudes and phases of the fundamental components of the pre- and postdrug ERGs were analyzed. The postsynaptic APB (ON-) and CNQX (OFF-) sensitive components were determined by examining the phases and amplitude vectors.

RESULTS. The temporal properties of the Tg rabbits were different from those of the WT rabbits and had unique features; at 3.906 Hz, the amplitude was depressed but it increased by more than 3.5-fold at 15.625 Hz. The reduction of the amplitude at 3.906 Hz in Tg rabbits was caused by a cancelation of the ON and OFF components by a phase difference of 180°. On the other hand, an increase in the amplitude at 15.625 Hz in Tg rabbits was caused by the summation of the ON and OFF components, which had an approximate 120° phase difference.

CONCLUSIONS. The temporal properties of the flicker ERGs of Tg rabbits were affected markedly by the remodeling of the retinal neurons. Evaluations of the flicker ERGs in RP eyes must be done with careful considerations of the current findings.

Keywords: flicker ERG, remodeling, retinal ON bipolar cell, retinitis pigmentosa, retinal OFF bipolar cell

Retinitis pigmentosa (RP) is a hereditary retinal disease that causes severe visual impairments. The genetic mutations in patients with RP cause degeneration of the rod photoreceptors followed by degeneration of the cones. The death of the photoreceptors leads to a gradual deconstruction of the morphology of the retina and functional reprogramming of the middle and inner retina, which have been reported as a remodeling of the retina in humans and animal models of RP.

Flicker electroretinograms (ERGs) have been used to evaluate the residual cone function in patients with RP, and the results showed a reduction in the amplitudes and a prolongation of the intrinsic times. Flicker ERGs have been reported to have larger contributions from the photoreceptor components, viz., the depolarizing ON bipolar cell pathway (ON pathway) and the hyperpolarizing OFF bipolar cell pathway (OFF pathway), with a small contribution from the photoreceptors in primates.

Thus, the amplitudes and implicit times of the flicker ERGs in RP patients are believed to be due not only to the degeneration of the photoreceptors but also to signals from the inner retina which has been remodeled in eyes with RP.

Several studies have used harmonic analyses of the flicker ERGs to determine the temporal properties of the ERGs. In addition, a “vector analyses” method was developed by Kondo and Sieving, which modified the harmonic analyses method. In the vector analyses, flicker ERGs elicited before and after pharmacologic blockades of the ON and OFF pathways of the retina were analyzed by Fourier transformation. The obtained fundamental components of the ERGs are shown in the phase and amplitude vector. These analyses revealed the effects of a subtraction and summation of the ON and OFF pathways, and how they contribute to the amplitudes of the flicker ERGs.

We determined how the remodeled inner retina in transgenic (Tg) rabbit eyes altered the contribution of the photopic ON and OFF pathways to the flicker ERGs. To accomplish this, we used rhodopsin Pro347Leu Tg rabbits, which were generated by BAC transgenesis, as a model of RP in humans. This animal model was shown to have a progressive degeneration of the rods followed by that of the cones. Full-field ERGs showed that the rod components of the ERGs were reduced to only 5% by 48 weeks, while the cone components remained at 35% of the wild-type (WT) at the same age. At 18 months of age, the Tg rabbits had very few nuclei in the outer nuclear layer and there is a remodeling of the inner retina accompanied by an augmented of the OFF component and abnormal responses of the third-order neurons. We showed...
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that the pattern of summation and subtraction of the ON and OFF pathways of the flicker ERGs in Tg rabbits was different from that in WT rabbits.

**MATERIALS AND METHODS**

**Animals**

All experimental procedures adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the Guidelines for the Use of Animals of the Nagoya University Graduate School of Medicine. The Nagoya University Animal Experiment Committee approved this project (Approval Number, 28405).

Six WT (WT1-6) and four TG (Tg1-4) rabbits whose background was the New Zealand White (NZW) strain were studied at 18 months of age. The generation of this Tg rabbit was described in detail.22 Before beginning the experiments, we confirmed that scotopic ERGs were not elicited by 0.01 log cd/s/m² from the Tg rabbits, which indicated that the rod function essentially was absent.

**ERG Recordings**

The procedures of recording ERGs have been reported in detail.23 Briefly, the rabbits were anesthetized with an intramuscular injection of ketamine and xylazine. ERGs were recorded with a bipolar contact lens electrode (Gold Lens; Doran Instruments, Littleton, MA, USA) after the pupils were fully dilated. The signals were amplified and band-pass-filtered between 0.3 and 1000 Hz and digitized at 2000 Hz. A total of 20 to 30 ERGs were averaged with a computer-assisted signal averaging system (Power Lab; AD Instruments, Castle Hill, Australia).

**Visual Stimulation**

Rabbits were placed in a Ganzfeld bowl (Model 2503SH; LKC Technologies, Gaithersburg, MD, USA) and stimulated with light-emitting diodes (LEDs) providing white stimuli (modified version of LS200; Mayo Corporation, Aichi, Japan). The luminance of the LED light was measured with an integrating radiometer (40X-Spotmeter; United Detector Technology, Hawthorne, CA, USA). The LEDs were controlled by a digital function generator (WF1945, NF Corporation, Tokyo, Japan) which controlled the intensity and frequency of the stimulating light.

Flicker ERGs were elicited by sinusoidally modulated stimuli whose maximal intensity was 2.40 log cd/m² and minimal intensity was 0.00 cd/m². The stimuli were presented on a constant white background of 40 cd/m². We chose these intensity was 0.00 cd/m². The stimuli were presented on a white stimulus light-emitting diodes (LEDs) providing white stimuli (modified version of LS200; Mayo Corporation, Aichi, Japan) and stimulated with light-emitting diodes (LEDs) providing white stimuli (modified version of LS200; Mayo Corporation, Aichi, Japan) which controlled the intensity and frequency of the stimulating light.

**Statistical Analyses**

Two-factor factorial ANOVA followed by the Tukey-Kramer test was used to determine the significance of the differences in the amplitudes and phases of the ERGs components between WT and Tg rabbits. P < 0.05 was considered statistically significant.

**Retinal Histology**

Two WT and two Tg rabbits were euthanized after the ERG recordings. Eyes were enucleated and fixed in Davidson's fixative for 6 hours and then transferred to 10% neutral buffered formalin. The tissues were trimmed and embedded in paraffin, sectioned vertically through the optic nerve, and stained with hematoxylin and eosin.

**RESULTS**

**Representative ERGs**

Representative ERGs of the WT and Tg rabbits before and after APB and APB+ CNQX at four frequencies ranging from 3.906 to 46.875 Hz are shown in Figure 1. The predrug ERGs were composed of several components with different shapes at the lower stimulus intensities, but became simpler and more sinusoidal at 46.875 Hz. The amplitudes of the ERGs of the Tg rabbit were smaller than that of the WT rabbit at 3.906 Hz; however, it became comparable to that of the WT rabbit at other frequencies (Supplementary Fig. S2A).

After the APB injection, the ERGs were composed of the signals of the photoreceptor potentials and the OFF pathway. The amplitudes of the post-APB ERGs of the Tg rabbit at 3.906 and 15.625 Hz were larger than that of the WT, and the
amplitude of the ERGs at 31.250 Hz was comparable to that of the WT rabbits (Supplementary Fig. S2B).

After the APB+CNQX injection to isolate the photoreceptor component, the amplitude of the photoreceptor component was smaller in the Tg than in the WT rabbits at all frequencies (Supplementary Fig. S2C).

These results indicated that the temporal properties of Tg and WT rabbits were different before and after injection of the drugs. The vectors were analyzed to determine the mechanisms for these differences.

**Harmonic Analyses**

The representative waveforms of the fundamental components after FFT filtering are shown in Supplementary Figure S3. The amplitudes (Figs. 2A, 2C, 2E) and phases (Figs. 2B, 2D, 2F) of the amplitude of the photoreceptor component was smaller in the Tg than in the WT rabbits at all frequencies (Supplementary Fig. S2C).

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those for the post-APB ERGs in green, and those of the post-APB+CNQX ERGs in yellow. To estimate the contribution of the ON pathway of the WT and Tg rabbits, we subtracted the vectors of the post-APB from the predrug ERGs (ON component). We also subtracted the vectors of the post-APB+CNQX (photoreceptor component) from those of post-APB ERGs to evaluate the OFF pathway (OFF component). The obtained vectors for the ON and OFF components are shown by red and blue arrows, respectively. The predrug fundamental components are composed of the summation of the vectors of the ON, OFF, and photoreceptor components.

**Vector Analysis at 3.906 Hz**

The results of the phase and amplitude vector analyses of one representative WT rabbit and one Tg rabbit are shown in Figure 3A. For the WT rabbit, the length of the vector for the ON component was longer and approximately 200° out of phase from the OFF component. Consequently, the postsynaptic activity (ON+OFF component) was dominated by the ON component. The photoreceptor component (green arrow) was shifted approximately 90° from the ON component. Thus, the predrug component (black arrow) was slightly smaller than the ON component but larger than the photoreceptor component.
For the Tg rabbit, the ON and OFF components had similar lengths, and the photoreceptor component was shorter than that of these components. The ON component was close to 180° out of phase from the OFF and photoreceptor components. This phase cancellation caused an almost complete elimination of the amplitude of the predrug response.

**Vector Analysis at 15.625 Hz**

At 15.625 Hz, the ON and OFF components were nearly 120° out of phase in both WT and Tg rabbits. They added together to contribute to a large predrug vector. The contribution of photoreceptors was reduced especially for the Tg rabbit, and the lengths of the predrug components of both rabbits were similar. This indicated that the amplitudes of the flicker ERGs were more affected by the postreceptoral than the photoreceptor components.

**Vector Analysis at 31.250 Hz**

The results of the vector analyses of one representative WT rabbit and two Tg rabbits are shown in Figure 3C. The amplitude of the predrug vector (black vector) of WT was similar to those of Tg rabbits but the difference of the phase between the two types of rabbits was approximately 270°. The contributions of photoreceptors were small for both types of rabbits, similar to that at 15.625 Hz. The length of the ON component (red arrow) was relatively longer than that of the
OFF components in both types of rabbits. Thus, the predrug wave tended to be affected by the ON components.

The length of the predrug vector was almost similar in the Tg1 and Tg3 rabbits; however, the amplitude of the ON and OFF components was much larger in Tg1 rabbits. These findings indicated that it is difficult to predict the contribution of the ON and OFF components from the predrug ERGs. The differences in the results of the vector analysis between two Tg rabbits were probably due to differences in the degrees of retinal degeneration and retinal remodeling. These findings indicated that the amplitude of the flicker ERGs in degenerated retinas is affected by the remodeled inner retina and does not always indicate the status of the photoreceptor degeneration.

Amplitudes of ON and OFF Components as Function of Temporal Frequency

We calculated the amplitudes of the ON and OFF components of the WT and Tg rabbits obtained by the vector analysis (Fig. 4). The amplitude of the ON pathway was largest at 13.0 μV in WT and 11.5 μV in Tg rabbits at 3.906 Hz, and it decreased as the stimulus frequency increased. The amplitude of the ON component in the Tg rabbits was smaller than that in the WT rabbit at all frequencies although the differences were not significant. The amplitude of the OFF components in the WT rabbits was smaller than those of ON components at all frequencies in the WT rabbits, but the amplitude of these two components were comparable at most of the frequencies in the Tg rabbits. These findings indicated that there was an increase of the OFF components in the flicker ERGs of Tg rabbits.

To estimate the effect of remodeling of the ON and OFF bipolar cells on flicker ERGs, we analyzed the ratio of the ON bipolar cell/photoreceptor amplitudes and the ratio of the OFF bipolar cell/photoreceptor amplitudes (Supplementary Fig. S4). The results showed the contributions of ON and OFF bipolar cells to the flicker ERGs in Tg rabbits were higher than those of WT rabbits at all frequencies, and the ratio became higher with increasing frequencies.

Retinal Histology

The retinal histology in the area of the visual streak of WT and Tg rabbits at 18 months of age is shown in Figure 5. In WT rabbits, there were two to three rows of nuclei in the inner nuclear layer and five to seven rows of nuclei in the outer nuclear layer. On the other hand, there were very few scattered nuclei in the outer nuclear layer in Tg rabbits, and the nerve fiber layer to inner nuclear layer was relatively well preserved in Tg rabbits.

DISCUSSION

The contributions of the ON and OFF pathways to the temporal responses have been analyzed in mice,19,25 rats,26 rabbits,27 and monkeys.15 However, a search of PubMed showed that no studies have investigated the temporal properties and contributions of the ON and OFF pathways of degenerating retinas.

We analyzed the flicker ERGs elicited by a wide range of frequencies at an advanced stage of degeneration in Tg rabbits. We found a consistent delay of the phases of the fundamental component at all frequencies in the predrug Tg rabbits, and also unique temporal properties of the predrug amplitudes of the fundamental component in Tg rabbits. The amplitude was reduced at 3.906 Hz but it increased more than 3.5 times at 15.625 Hz. To determine the mechanism for this unexpected feature, we analyzed the responses by vector analysis. Using this rabbit model has an advantage in that it was easier to evaluate the OFF-bipolar component in the rabbits than in
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 patrons to those of normal subjects.16 In normal subjects, the OFF pathways in the remodeled inner retina.

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were completely different. Thus, the analyses of the ampli-

previous reports.

An interesting finding determined by the vector model analysis was that the increase in amplitude of the OFF pathway canceled the amplitude of the ON pathway due to a phase difference of 180° at low frequencies. This led to a large reduction in the amplitude of the ERGs at 3.906 Hz in the Tg rabbits.

In addition, the amplitudes of the flicker ERGs of the Tg1, Tg3, and WT2 rabbits were not different at 32.50 Hz but the compositions of the ON, OFF and photoreceptor components were completely different. Thus, the analyses of the amplitudes of the flicker ERGs do not simply reflect the cone photoreceptor function, but they were unexpectedly affected by the summation and subtraction of the signals of the ON and OFF pathways in the remodeled inner retina.

An earlier study compared the temporal properties of RP patients to those of normal subjects.16 In normal subjects, the fundamental component had two main peaks at 3.7 and 41 Hz, while only four of 12 RP patients had a peak at 3.7 Hz. These results are in agreement with our results, and might indicate an unexpected subtraction of the ON and OFF pathways due to a remodeling of the retinal neurons in the inner retina of RP patients.

We evaluated the retinal histology of WT and Tg rabbits at 18 months of age. We found that the WT rabbits had five to seven rows of nuclei in the outer nuclear layer of the retina, but the Tg rabbits had only scattered nuclei in the outer nuclear layer.23 It is assumed that similar histologic changes occur in the retina of RP patients, and these lead to the functional remodeling of the retina.

Flicker ERGs elicited by 30 Hz stimuli are part of the standard ERGs recommended to be recorded by the International Society for Clinical Electrophysiology of Vision (ISCEV).30 The flicker ERGs are used to quantify the functional status of the retinal cone system in patients with retinal diseases.10–14 Our results indicated that recording the flicker ERGs elicited by only one frequency of 30 Hz in RP patients may limit the collection of important information of the physiology of the retina. Thus, analyses of the ERGs for a wide range of frequencies might be more informative about the temporal properties of the cone system. In addition, the implicit times or phases of the major harmonics would be more suitable than the amplitude of fundamental components for evaluating the cone function.

There is a limitation in our study design. Our study was based on the data from four Tg animals because of the shortage of older Tg rabbits, which weakens the conclusions that can be made.

In conclusion, our results indicated that the temporal properties of 18-month-old Tg rabbits are significantly different from those of WT rabbits. Vector model analysis revealed an increase in the amplitude of the OFF component canceled by the ON pathway at low frequencies. Our data indicated that the interpretation of flicker ERGs in eyes with RP must be done cautiously, especially in the evaluation of the amplitudes, because the flicker ERGs are affected by unexpected summation and cancelation of the secondary changes of retinal neurons.

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