Enhancement of ON-Bipolar Cell Responses of Cone Electroretinograms in Rabbits with the Pro347Leu Rhodopsin Mutation

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PURPOSE. To determine how the different stages of retinal processing change after photoreceptor degeneration in rabbits carrying the Pro347Leu rhodopsin mutation (Tg rabbits).

METHODS. Cone electroretinograms (ERGs) were elicited by 150-ms duration stimuli from 15 Tg rabbits at 12 and 24 weeks of age. The ERG recordings were made before and after an intravitreal injection of tetrodotoxin citrate (TTX) plus N-methyl-D-aspartic acid (NMDA), with the addition of 2-amino-4-phosphonobutyric acid (APB) and then cis-2,3-piperidine-dicarboxylic acid (PDA) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Digital subtraction of the ERG after the injection from the ERG before the injection was used to extract the components that were blocked by these drugs. Thirteen age-matched, wild-type (WT) rabbits were studied with the same protocol.

RESULTS. In Tg rabbits, the cone ERGs elicited by intermediate intensities had a depolarizing pattern. At 12 weeks of age, the photoreceptor and OFF-bipolar/horizontal cell responses reflected in the ERG in the Tg rabbits did not differ significantly from those in the WT rabbits. The ON-bipolar cells and the third-order neuronal responses recorded after pharmacologic blockade were significantly enhanced in the Tg rabbits compared with those recorded in the WT rabbits. At 24 weeks of age, the ERG waveforms representing the photoreceptors and OFF-bipolar/horizontal cell responses were significantly decreased, but those representing the ON-bipolar cell and third-order neuronal responses were still preserved in the Tg rabbits.

CONCLUSIONS. A depolarizing pattern of the cone ERG responses was seen in Pro347Leu Tg rabbits. The enhancement or preservation of the ON-bipolar cell response in the ERGs contributed to shaping the waveform in the Tg rabbits. In this model, the functional alterations in the ON-pathway took place before the deterioration of cone photoreceptor function. (Invest Ophthalmol Vis Sci. 2011;52:7610–7617) DOI:10.1167/iovs.11-7611

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rabbits have a depolarizing pattern. We also used pharmacologic agents to dissect the cone ERGs elicited by long-duration stimuli to investigate how the different types of retinal neurons contribute to the depolarizing pattern of the ERGs in Tg rabbits.

**METHODS**

**Animals**

We used heterozygous Pro347Leu rhodopsin Tg rabbits, line 7, which have the highest level of transgene expression and the most severe photoreceptor degeneration.21 Thirteen 10-week-old Tg New Zealand albino rabbits were purchased from Kitayama Laboratories Co., Ltd. (Nagano, Japan) and kept in the animal colony until the experiments. Thirteen wild-type (WT) New Zealand albino rabbits were used as controls. The experiments were performed on different sets of rabbits at 12 and 24 weeks of age.

All animals were housed and handled under the authorization and supervision of the Institutional Animal Care and Use Committee of the Iwate Medical University. All the procedures involving the rabbits conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**ERG Recordings**

The rabbits were anesthetized with a loading dose of intramuscular xylazine (2 mg/kg) and ketamine (25 mg/kg), and anesthesia was maintained by hourly injection of a mixture of xylazine (1 mg/kg) and ketamine (15 mg/kg). The pupils were maximally dilated with a mixture of 0.5% tropicamide and 0.5% phenylephrine HCl applied topically.

After the cornea was anesthetized by topical oxybuprocaine, a contact lens electrode containing light-emitting diodes (LEDs; EW-102; Mayo, Nagoya, Japan) was inserted. The LEDs provided homogenous white (color temperature, 4000–9000 K) stimuli and background illumination. The luminance was measured at the corneal surface with a photometer (model LS-100; Minolta, Tokyo, Japan). The characteristics of the contact lens electrode have been described in detail.22 A needle reference electrode was inserted subcutaneously into the forehead, and the ground electrode was placed on the right ear lobe.

The cone ERGs were elicited by stimuli of 150-ms duration presented on a diffuse white background of 40 cd/m². The animals were light-adapted by the white background light of 40 cd/m² for at least 10 minutes before the cone ERG recordings. The ERGs were amplified 50000× and band-pass filtered from 0.5 to 1000 Hz (Neuropack MED 5210; Nihon Kohden, Tokyo, Japan). The stimulus intensity ranged from 1.5 to 3.5 log cd/m², with increments of 0.2, 0.5, or 0.5-log unit steps. The intensity and duration of the stimuli were controlled by an electronic function generator (model WLS-20; Mayo) connected to the LEDs.

**Drug Injections**

TTX (Latoxan, Valence, France), N-methyl-D-aspartic acid (NMDA), 2-amino-4-phosphonobutyric acid (APB), and cis-2, 3-piperidine-dicarboxylic acid (PDA) were purchased from Sigma-Aldrich (St. Louis, MO) and were injected into the vitreous cavity of the Tg and WT rabbits. Each of these drugs blocked the activity of a specific type of retinal neuron, which then allowed us to determine the neuron’s contribution to the ERGs.

First, TTX+NMDA were injected into the vitreous. TTX blocks voltage-gated sodium channels and thus blocks action potentials produced by RGCs and spiking amacrine cells.13–15 NMDA depolarizes the different types of amacrine cells and most RGCs,20 rendering them unresponsive to light stimuli. Therefore, the third-order neuronal responses should have been largely eliminated after an intravitreal injection of TTX+NMDA.21

Second, APB was injected intravitreally. APB is an agonist of metabotropic glutamate receptor type 6 (mGlur6) and blocks the synaptic transmission between the photoreceptors and ON-bipolar cells.22 Finally, we injected PDA, which is an antagonist of AMPA/Kainate class ionotropic glutamate receptors (gGlur) and blocks the light responses of OFF-bipolar cells, horizontal cells, and many amacrine and RGCs.23 Because PDA is not commercially available, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which has effects similar to those of PDA on retinal neural cells and ERGs, was used in some of the experiments.24,25

Each drug was dissolved in normal saline, and the pH was adjusted to 6.8 to 7.2 with 1 N NaOH. Then, a 30-gauge needle attached to a 1-mL syringe was inserted 3.5 mm posterior to the corneal limbus into the midvitreous, and 0.04 mL of the solution was injected. The estimated concentration of each solution was 4 μM for TTX; 5 mM for NMDA, APB, and PDA; and 200 μM for CNQX, assuming a complete mixing in the 1.2-mL vitreous of rabbits.26 These estimated concentrations of the drugs were determined according to earlier studies in rabbits and primates.21,18,21,27,28 The ERG recordings were made between 60 and 90 minutes after the intravitreal injection of each pharmacologic agent or agent combination.

**Statistic Analyses**

A two-way repeated-measures ANOVA was used to compare the intensity–response curves of the WT rabbits to those of the Tg rabbits. In addition, two-tailed Student’s t tests were used to determine the statistical significance of differences in the amplitudes at each stimulus intensity between the WT and the Tg rabbits. The level of statistical significance was set at P < 0.05 (Farris 5.1; GraphPad Software Inc., San Diego, CA).

**RESULTS**

**Cone ERGs of Tg and WT Rabbits Elicited by Long-Duration Stimuli**

All ERG waveforms in this article represent the averaged ERGs recorded from three or five animals. For example, the ERG shown in Figure 1A was recorded at a stimulus intensity of 3.0 log cd/m² in five WT rabbits and then averaged (represented by the thick line).

The cone ERGs elicited by long-duration stimuli from the 12- and 24-week-old Tg (red lines) and WT (black lines) rabbits are shown in Figure 1B. The ERGs shown are the averages of five animals. At 12 weeks of age, there was no difference between the Tg and WT rabbits in the amplitudes and implicit times of the b-wave for all stimulus intensities. However, in the intermediate stimulus range between 2.0 and 3.0 log cd/m², the potential level after the b-wave peak remained elevated and did not return to the baseline until the stimulus was turned off in the Tg rabbits. The waveform in the Tg rabbits seen in this intermediate stimulus range had a depolarizing pattern. On the other hand, the potential level after the b-wave rapidly returned to baseline level in the WT rabbits. The waveforms of the Tg rabbits at the lowest and highest intensities did not differ noticeably from those of the WT rabbits. In the Tg rabbits 24 weeks of age, a depolarizing pattern was also seen at the intermediate stimulus intensities.

**Waveform Changes in Cone ERGs after Intravitreal Drug Injections**

The changes in the shape of the ERGs of the 12-week-old WT rabbits after each drug injection at a stimulus intensity of 3.0 log cd/m² are shown in Figure 2A. The ERG waveforms of five animals were averaged. After the intravitreal injections of TTX and NMDA (post-TTX+NMDA), the ERG waveforms became more positive and square shaped with a loss of the oscillatory potentials.

Then, APB was injected intravitreally to block synaptic transmission between the photoreceptors and ON-bipolar cells. After the injection of APB, the b-wave disappeared and the ERG had a negative shape, which was derived from activity...
of cone photoreceptors and OFF-bipolar/horizontal cells (post-TTX/NMDA). Finally, PDA was injected to eliminate responses from OFF-bipolar/horizontal cells, leaving a small, slow, negative response originating from the cone photoreceptors (post-TTX/NMDA/APB).

**Isolation of Responses from Each Type of Retinal Neuron**

Digital subtraction of the ERG waveforms extracted the components that were blocked by the drugs. Therefore, by subtracting the post-TTX+NMDA ERGs from the pre-TTX+NMDA ERGs (baseline waveforms), the response of the third-order neurons could be isolated (Fig. 2, A minus B). By subtracting post-TTX+NMDA+APB from the post-TTX+NMDA waveform, the ON-bipolar cell response was isolated (Fig. 2, B minus C). Finally, we subtracted the post-TTX+NMDA+APB+PDA from the post-TTX+NMDA+APB to isolate the OFF-bipolar/horizontal cell response (Fig. 2, C minus D).

The isolated responses consisted of slow waves, which made it difficult to determine a peak of each wave. Therefore, we measured the amplitude at 75 ms after the light onset (midway between onset and offset). The mean amplitudes with the standard deviations are plotted against the stimulus intensities in Figures 3 to 7.

**Intensity–Response Curves of Pharmacologically Isolated Responses**

The averaged isolated cone photoreceptor responses of the Tg rabbits were very similar to those of the WT rabbits for all stimulus intensities at 12 weeks of age (Fig. 3A). The intensity–response curve of the cone photoreceptors of the Tg rabbits overlapped that of the WT rabbits, indicating that the cone

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**Figure 1.**

(A) Averaged ERG waveforms recorded from five individual rabbits. (B) Averaged waveforms of ERGs recorded from the Pro347Leu Tg (n = 5) and WT (n = 5) rabbits at 12 and 24 weeks of age. Black and red lines: ERGs recorded from the WT and Tg rabbits, respectively.

**Figure 2.** Baseline waveform (A) and waveform changes after intravitreal injection of TTX+NMDA alone (B) or with, APB (C), or APB+PDA (D). Averaged waveforms of ERGs elicited by 3.0 cd/m² from WT rabbits (n = 5) at 12 weeks of age. Digitally subtracted waveforms representing responses from the third-order neurons (A minus B: baseline minus post-TTX+NMDA), ON-bipolar cells (B minus C: post-TTX+NMDA minus post-TTX+NMDA+APB), and OFF-bipolar/horizontal cells (C minus D: post-TTX+NMDA+APB minus post-TTX+NMDA+APB+PDA). The amplitudes of each wave were measured at 75 ms after light onset.
photoreceptor function remained unchanged in the Tg rabbits at this age (Fig. 3B). At 24 weeks of age, the averaged cone photoreceptor responses of Tg rabbits were less negative than those of WT rabbits (Fig. 3A). The curve of the Tg rabbits was significantly lower than that of the WT at all stimulus intensities ($P < 0.0001$), indicating a decrease in cone photoreceptor function (Fig. 3C).

The ON-bipolar cell response consisted of a slow, positive, trapezoid-shaped wave in both the Tg and WT rabbits. Interestingly, at 12 weeks of age, the averaged ON-bipolar cell responses of the Tg rabbits were more positive than those of the WT rabbits, especially at stimuli from 1.7 to 2.7 log cd/m$^2$ (Fig. 4A). The intensity–response curve of the ON-bipolar cells of the Tg rabbits was always significantly higher than that of WT rabbits, especially in the intermediate intensities from 1.7 to 2.7 log cd/m$^2$ (Fig. 4B; $P < 0.05–0.001$). The intensity–response curve of the Tg rabbits was significantly different from that of the WT rabbits ($P < 0.0001$). The sensitivity of the isolated ON-bipolar cell response was taken to be the stimulus intensity necessary to elicit one half of the maximum amplitude of the ON-bipolar cell response. From the intensity–response curve of each animal, the sensitivity of the ON-bipolar cell response was obtained and then averaged for the Tg and WT rabbits at 12 and 24 weeks of age. The sensitivity of the

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**Figure 3.** (A) Averaged waveforms of the cone photoreceptor responses from Pro347Leu Tg ($n = 5$) and WT ($n = 5$) rabbits at 12 and 24 weeks of age. **Black and red lines:** ERGs recorded from WT and Tg rabbits, respectively. The amplitudes of the cone photoreceptor responses are plotted against stimulus intensities for (B) 12- and (C) 24-week-old Tg and WT rabbits. Error bars, SD.

**Figure 4.** Averaged waveforms of the digitally subtracted response of the ON-bipolar cell recorded from Pro347Leu Tg ($n = 5$) and WT ($n = 5$) rabbits at 12 and 24 weeks of age (A). **Black and red lines:** ERGs recorded from WT and Tg rabbits, respectively. The amplitudes of the ON-bipolar cell responses are plotted against the stimulus intensities for (B) 12- and (C) 24-week-old Tg and WT rabbits. Error bars, SD.
ON-bipolar cell was significantly higher ($P < 0.0000005$) in the Tg rabbits than in the WT rabbits at 12 weeks of age (Table 1). This result indicates a hypersensitivity of the ON-bipolar cell of the Tg rabbits at the early stage of photoreceptor degeneration. At 24 weeks of age, the ON-bipolar cell response was no longer enhanced in the Tg rabbits, and most of the intensity–response curve of the Tg rabbits overlapped that of the WT rabbits (Figs. 4A, 4C). There was no significant difference in the sensitivity between the Tg and WT animals at this age (Table 1).

The feedback from the horizontal cell to the cones could affect the ON-bipolar cell responses after APB was injected and before blocking the horizontal cell activity. After blocking the third-order neuronal activity by the TTX before blocking the horizontal cell activity, we injected CNQX before the APB injections in three Tg and WT rabbits at 12 weeks of age. Then, we isolated ON-bipolar cell responses without the contribution of the horizontal cell feedback by subtracting the post-TTX+NMDA+CNQX+APB from the post-TTX+NMDA+CNQX. The ON-bipolar cell responses of Tg rabbits were more positive than those of WT rabbits (Fig. 5A), and there was a significant difference in the intensity–response curves between Tg and WT rabbits (Fig. 5B; $P < 0.0001$). These findings are identical with those obtained by the APB applications before the PDA injections (Fig. 4), suggesting that the horizontal cell feedback was unlikely to have contributed to the enhancement of the ON-bipolar cell response in the Tg rabbits at 12 weeks of age.

The isolated OFF-bipolar/horizontal cell response consisted of a small, negative wave. At 12 weeks of age, the waveform of the OFF-bipolar/horizontal cell responses of the Tg rabbits was similar to that of the WT rabbits (Fig. 6A). The intensity–response curve of the OFF-bipolar/horizontal cell response of the Tg rabbits overlapped that of the WT rabbits (Fig. 6B). At 24 weeks of age, the OFF-bipolar/horizontal cell responses of the Tg rabbits were significantly lower than those of the WT rabbits ($P < 0.0001$) at all stimulus intensities. The responses of the WT rabbits had a negative shape, with the amplitudes comparable to those at 12 weeks of age (Figs. 6A, 6C).

The third-order neuronal response consisted of a slow negative wave with oscillations corresponding to the onset and offset of the stimulus (Fig. 7A). At 12 weeks of age, the waveform in the Tg rabbits was more negative than that in the WT rabbits for stimuli from 1.5 to 2.5 log cd/m² (Fig. 7A). The amplitudes of the third-order neuronal responses were significantly larger in the Tg than in the WT rabbits from 1.5 to 2.0 log cd/m².
log cd/m² (Fig. 7B, $P < 0.001$). In 24-week-old Tg rabbits, oscillations elicited by ON stimuli were attenuated, but the slow negative response was not decreased (Figs. 7A, 7C).

**DISCUSSION**

Our results showed that the ERG component reflecting the ON-bipolar cell responses was enhanced at the early stage of photoreceptor degeneration in Pro347Leu Tg rabbits. In addition, the third-order neuronal responses were enhanced in the Tg rabbits. At a more advanced stage, the ON-bipolar and third-order neuronal responses were preserved despite functional loss of the cone photoreceptors and OFF-bipolar/horizontal cells. These results suggest that the light responses of neurons in the middle or inner retina were altered before the cone photoreceptors showed functional evidence of degeneration.

**Depolarizing Pattern and Photoreceptor Degeneration**

The presence of a depolarizing or a hyperpolarizing pattern of the cone ERG suggests abnormalities of signal transmission between the cone photoreceptor and the bipolar cell. Sieving et al. termed the characteristic waveform of the cone ERGs elicited by long-duration stimuli a “depolarizing pattern,” when the potential level after the b-wave does not return to the baseline level but remains elevated. Experimentally, ERGs with a depolarizing pattern have been recorded after the signals from the cones to the OFF-bipolar/horizontal cells are blocked by glutamate analogs, such as PDA and/or kynurenic acid (KYN).

Although experimental evidence has demonstrated that this unusual waveform is due to abnormalities of the synaptic transmission between the cone photoreceptors and the OFF-bipolar cells, a depolarizing pattern of the cone ERG has also been reported in human photoreceptor diseases. Sieving reported a case of unilateral cone dystrophy with a depolarizing pattern of the cone ERG. Kondo and Miyake reported a case of macular degeneration with a depolarizing pattern of the focal macular ERG. We also reported a case of age-related macular degeneration (AMD) that had a depolarizing pattern of the focal macular ERG after photodynamic therapy. Common to these cases with a depolarizing pattern of the ERG was that the known abnormalities were mainly of the photoreceptors rather than the bipolar cell. The depolarizing pattern of the long-duration cone ERGs was seen in animal models with photoreceptor degeneration in the present study, adding further evidence that eyes with photoreceptor diseases can have a depolarizing pattern of the cone ERGs.

**Enhancement of ON-Bipolar Cell Responses**

We found that the ON-bipolar cell responses, extracted by the use of APB, were enhanced for all the stimulus intensities at the early stage of degeneration when the rod photoreceptor degeneration had already begun. This finding is consistent with our previous result in which the ON-bipolar cell response extracted from the mfERGs was enhanced in the Tg rabbits at the same age. Because the cone photoreceptors and the OFF-bipolar/horizontal cell responses appeared to be nearly functionally normal at this stage, the loss of the rod photoreceptor may be involved in the enhancement of the ON-bipolar cell responses.

Similar findings have been made in rhodopsin-knockout mice without rod outer segments as well as in neural retina leucine zipper (Nrl) knockout mice in which the rods fail to

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**TABLE 1. Sensitivity of ON-Bipolar Cell Response**

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<thead>
<tr>
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<th>12 weeks of age</th>
<th>24 weeks of age</th>
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<td></td>
<td>Tg</td>
<td>WT</td>
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<tr>
<td>$\log cd/m^2$</td>
<td>1.85 ± 0.03</td>
<td>2.19 ± 0.03</td>
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$P < 0.0000005$  NS

Tabulated values give mean ± SD of intensity for 1/2 amplitude max($\log cd/m^2$).
develop and all photoreceptors are replaced by cones. In both mouse models, the photopic cone b-wave amplitudes were larger than normal. Because the contribution of the OFF-bipolar/horizontal cell response to the ERG is very small in mice, the enhancement of the cone b-wave indicates an augmentation of the ON-bipolar cell response of the cone ERGs. These findings support our results and suggest that the functional loss of rods could be a trigger for the enhanced ON-bipolar cell response of the cone ERGs.

**Enhancement of the Third-Order Neuronal Response**

The slow negative wave of the third-order neuronal response was enhanced at 12 weeks of age in the Tg rabbits and persisted at 24 weeks of age. The negative response most likely represents mainly the ON-photopic negative response (PhNR) which is driven by the ON pathway. This finding corresponds to the enhancement of the ON-bipolar cell response, suggesting that enhancement of the ON-bipolar cell response contributes to the alterations in the third-order neuronal response.

Alternatively, there is evidence that the third-order neuronal responses are more preserved than the bipolar cell responses. We have shown that the NMDA-sensitive components of the cone ERGs are better preserved than the b-wave in RCS rats despite the progression of photoreceptor degeneration. It has been reported that the TTX-sensitive components of the cone ERG are enhanced in Tg rabbits at the early stage.

**Synaptic Remodeling**

Several immunohistologic studies of animal models with rod degeneration have demonstrated that the rod bipolar cells develop new synaptic connections with the functional cones after they lose their original connections to the rods because of rod degeneration. This ectopic synaptic formation between surviving cones and rod bipolar cells may explain the significant enhancement or preservation of the ON-bipolar cell responses in Tg rabbits.

At 12 weeks of age, the loss of rod function had already started with a decrease in rod photoreceptors, whereas cone photoreceptor function was normally preserved. Our hypothesis of synaptic remodeling can explain the enhancement of the ON-bipolar cell response in the Tg rabbits, although this hypothesis is based solely on functional data without any histopathologic evidence of synaptic remodeling in this model. In the normal state, the cone photoreceptors connect to ON- and OFF-bipolar cells, whereas the rod photoreceptors connect exclusively to ON-bipolar cells. After the rod photoreceptors start to degenerate, the rod bipolar cells receive signals from cones through newly developed synapses, which may contribute to shaping the ON-bipolar cell response of the cone ERGs, possibly leading to the enhancement of the ON-bipolar cell response.

In clinical cases of RP, Marc et al. demonstrated a significant increase in the number of OFF-bipolar cells expressing functional ionotropic glutamate receptors (iGluR) in an RP patient who had surviving cones with shortened outer segments. They suggested that rod bipolar cells switched contacts to cones and expressed iGluR representing the functional characteristics of OFF-bipolar cells, which is not consistent with our ERG data. However, their patients had advanced RPs in contrast to the early stage of rod photoreceptor degeneration in our animals.

**Increased Sensitivity of ON-Bipolar Cell Response**

Although there was no difference in the maximum amplitude of the ON-bipolar cell responses between the Tg and the WT rabbits at 12 weeks, the ON-bipolar response was larger in the Tg than in the WT rabbits in the intermediate stimulus intensity range. As a result, the sensitivity of the ON-bipolar cell response was increased in the Tg rabbits compared with that in the WT.

Although the mechanism of the hypersensitivity of the ON-bipolar cell response remains undetermined, similar findings have been observed in the rod b-wave of rhodopsin transgenic rats carrying the Pro23His mutation. In this rat model, despite progression of rod photoreceptor loss, the sensitivity of the rod b-wave was increased or maintained.

**Limitations of the Study**

The isolated OFF-response consisted of a slow negative wave of low amplitude compared with that of the monkey ERGs. This indicates that the OFF-bipolar cell responses contribute less to the shaping of the cone ERGs in rabbits than in monkeys. These findings also indicate that the ON-bipolar cell responses dominate the OFF-bipolar cell response, even in normal rabbits. The highest intensity of stimuli produce a depolarizing pattern of the long-flash cone ERG in WT rabbits, whereas primate and human cone ERGs with high intensities show a negative waveform because of the photopic hill effect. The relationship between the stimulus intensity and waveform of the rabbit ERG is different from that of human ERGs. Therefore, the results of the present study could not be simply applied to human ERGs in the diseased state.

A search of PubMed did not reveal any studies on RP cases with a depolarizing pattern of the cone ERG. On the other hand, a hyperpolarizing pattern of the cone ERG and dysfunction of the ON-bipolar cell have been reported in RP patients. The relationship between the stimulus intensity and waveform of the rabbit ERG is different from that of human ERGs. Therefore, the results of the present study could not be simply applied to human ERGs in the diseased state.

**Conclusions**

A depolarizing pattern of the cone ERGs elicited by long-duration stimuli was seen in Pro347Leu Tg rabbits. The pharmacologic dissection of the cone ERG demonstrated that the enhancement or preservation of the ON-bipolar cell response contributed to shaping the specific waveforms in Tg rabbits. In this model, functional alterations of the ON-pathway took place before the cone photoreceptor function began to deteriorate.

**References**


