Contributions of Second- and Third-Order Retinal Neurons to Cone Electroretinograms After Loss of Rod Function in Rhodopsin P347L Transgenic Rabbits

Taro Kominami,1 Shinji Ueno,1 Satoshi Okado,1 Ayami Nakanishi,1 Mineo Kondo,2 and Hiroko Terasaki1

1Department of Ophthalmology, Nagoya University Graduate School of Medicine, Nagoya, Japan
2Department of Ophthalmology, Mie University Graduate School of Medicine, Tsu, Japan

Correspondence: Shinji Ueno, 65 Tsuruma-Chuo, Showa-ku, Nagoya 466-8550, Japan; ueno@med.nagoya-u.ac.jp.
Submitted: July 17, 2016
Accepted: January 31, 2017
Citation: Kominami T, Ueno S, Okado S, Nakanishi A, Kondo M, Terasaki H. Contributions of second- and third-order retinal neurons to cone electroretinograms after loss of rod function in rhodopsin P347L transgenic rabbits. Invest Ophthalmol Vis Sci. 2017;58:1417–1424. DOI:10.1167/iovs.16-20344

Purpose. To determine the contribution of second- and third-order retinal neurons to the photopic electroretinograms (ERGs) after the degeneration of the rods in rhodopsin P347L transgenic rabbits (Tg).

Methods. Four wild-type (WT) rabbits and four Tg rabbits were studied at 18 months of age. The photopic ERGs elicited at stimulus onset and offset were analyzed. To block different retinal pathways, 2-amino-4-phosphonobutyric acid (APB), 6-cyano-7-nitroquinazolinole-2, 3(1H,4H)-dione (CNQX), tetrodotoxin (TTX), and N-methyl-DL-aspartic acid (NMDA) were injected intravitreally. Digital subtraction of the postdrug ERGs from the predrug ERGs was used to determine the contributions of the ON-components blocked by APB, the OFF-components blocked by CNQX, and the third-order neurons blocked by TTX+NMDA.

Results. Contribution of the cone photoreceptors to the photopic ERGs in Tg rabbits was approximately 10% of that in WT rabbits. The amplitudes of the positive waves of the ON-components at stimulus onset in Tg rabbits were approximately one-half as large as those in WT. On the other hand, the amplitudes of the positive waves of the OFF-components at stimulus offset in Tg rabbits were approximately 1.4 to 2.3 times larger than those in WT. Transgenic rabbits had a positive wave at stimulus offset, which was reduced after the TTX+NMDA injection.

Conclusions. A reduced ON-component and an augmented OFF-component with abnormal responses of the third-order neurons contributed to the cone ERGs after the loss of rod function in Tg rabbits. Our results suggest a complex synaptic remodeling of the residual retinal cells in the advanced stage in Tg rabbits.

Keywords: retina, remodeling, amacrine cells, OFF bipolar cells

Retinitis pigmentosa (RP) is a hereditary retinal disease that is associated with severe visual impairments. The genetic abnormality in patients with RP causes degeneration of the rods followed by the degeneration of cones. However, the genetic abnormality leads to not only direct photoreceptor degeneration but also indirect alterations of the second- and third-order neurons of the retina.1,2 The events following photoreceptor degeneration lead to a gradual deconstruction and functional reprogramming of the middle and inner retina, which is known as remodeling.3 Usually, remodeling in RP eyes has features that take place earlier and more severely among cells directly connected to the photoreceptors, that is, bipolar cells, and less severely on the other inner retinal neurons.

Photoreceptors release glutamate transmitter onto the postsynaptic depolarizing ON- and hyperpolarizing OFF-bipolar cells, which respond with opposite effects due to the different postsynaptic glutamate receptors. Rod bipolar cells and ON-cone bipolar cells express the metabotropic glutamate receptor, mGluR6,4 which closes nonselective cation channels and hyperpolarizes the cell.5,6 On the other hand, glutamate activates the depolarizing kainate/AMPA-type ionotropic receptors (iGluR) on OFF-cone bipolar cells.7

Previous studies using rodent models of RP have shown that the earliest morphologic signs of remodeling appear in the rod bipolar cells with a reduction and retraction of the dendrites.8,9 Then, these cells postsynaptic to photoreceptors sprout dendrites and seek functional photoreceptors.10 In the progression of photoreceptor degeneration, the ON- and OFF-bipolar cells undergo transsynaptic cell death, which causes a loss of expression of the mGluR6 glutamate– and iGluR glutamate–activated currents.11–13 However, this process is accompanied by new synaptic connections and temporary neuronal networks. For example, there is a functional shifting of the ON-bipolar cells to the OFF-bipolar cells by an expression of iGluR, and a spontaneous iGluR-mediated signaling by amacrine and ganglion cells.12,14

We have created a rhodopsin Pro347Leu transgenic rabbit (Tg) as a model of RP in humans.15 This animal model was shown to have a dominant inheritance pattern and a progressive degeneration of the rods. Electroretinogram (ERG) showed that the rod functions were almost completely lost by 1 year, although cone function still remained. Interestingly, several ERG studies using pharmacologic dissection of the retinal circuits in this model revealed an...
enhancement of the function of the second- or third-order retinal neurons.16–18

This rabbit model of RP has at least two benefits in ERG analyses compared with the rodent models of eyes with RP. The first is that rabbits have large eyes, which allowed us to perform multiple intravitreal injections of different agents.13–15 The second advantage is that it is easier to evaluate the OFF-bipolar component in the rabbits than in rodent models,19 which have only a weak OFF-bipolar cell contribution to the cone ERGs.20

Two studies on Tg rabbits reported enhanced ON-bipolar cell function,17,21 and two other studies showed an abnormal enhancement of the oscillatory potentials (OPs) that arise from activity of retinal ganglion cells and/or amacrine cells in the Tg rabbits at 24 weeks of age.16,22 In addition, Jones et al.14 performed excitation mapping with organic cations and computational molecular phenotyping in this rabbit model. They reported that the rod bipolar cells switched their phenotype to OFF-bipolar cells by expressing iGluRs at 40 weeks of age.

These studies documented that retinal remodeling occurred at a relatively early stage of retinal degeneration. However, there have also been a limited number of reports describing functional remodeling in the late stage of retinal degeneration when cones are the only surviving photoreceptors in animal RP models.23 And it has been undetermined whether the late functional remodeling affects the responses of second- and third-order neurons in Tg rabbits.

Thus, the aim of this study was to determine the functional alterations of the second- and third-order retinal neurons in 18-month-old Tg rabbits when the rods have degenerated. We compared the waveforms of the cone ERGs of Tg rabbits to those of WT rabbits and evaluated the functional remodeling at an advanced stage of retinal degeneration. We used pharmacologic agents to dissect the retinal circuits to investigate how the different types of retinal neurons contribute to the cone ERGs at this late stage.

**MATERIALS AND METHODS**

**Animals**

All experimental procedures adhered to the ARVO Statement for 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).

Four wild-type (WT) and four Tg rabbits whose background was the New Zealand White (NZW) strain were studied at 18 months of age. The generation of this Tg rabbit has been described in detail.15 The rabbits were maintained in a 12-hour light (<40 lux) and 12-hour dark cycle. Before beginning the experiments, we confirmed that scotopic ERGs were not elicited by 0.01 log cd/m² from the Tg rabbits.

**ERG Recordings**

The rabbits were anesthetized with an intramuscular injection of 25 mg/kg ketamine and 2 mg/kg xylazine with topical oxybuprocaine for the cornea. Electroretinograms were recorded with a bipolar contact lens electrode (Gold Lens; Doran Instruments, Littleton, MA, USA). The pupils were fully dilated with topical 0.5% tropicamide and 0.5% phenylephrine HCl. Hydroxyethyl cellulose was used to keep the cornea and conjunctiva hydrated and to ensure good electrical contact between the electrodes and the cornea and conjunctiva. The ground electrode was placed on the ear.

Signals were amplified and band pass filtered between 0.3 and 1000 Hz and digitized at 2000 Hz. A 60-Hz notch filter was used to reduce the contamination from stray line noise. The notch filter was a second-order filter and provided approximately 52 dB attenuation at 60 Hz, thereby reducing the effect of stray 60-Hz signals. Twenty 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 homogenous 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).

Photopic ERGs were elicited by stimulus intensities of 1.5, 2.0, and 2.5 log cd/m² after 10 minutes of light adaptation. Three types of stimuli were used: a 150-ms-duration stimulus (long duration), a 2-Hz sawtooth rapid-ON (rapid-ON) stimulus, and a 2-Hz sawtooth rapid-OFF (rapid-OFF) stimulus. The stimuli were presented on a constant white background of 40 cd/m². The ERGs elicited by the three types of stimuli were recorded prior to the drug injection and 60 to 90 minutes after the drug injections.

**Drug Injections**

The drugs that were injected intravitreally were 2-amino-4-phosphonobutyric acid (APB; Sigma-Aldrich Japan, Tokyo, Japan), 6-cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione (CNQX; Sigma-Aldrich Japan), tetrodotoxin (TTX; Wako, Osaka, Japan), and N-methyl-DL-aspartic acid (NMDA; Wako). APB was injected into the vitreous cavity of the right eye followed by an injection of CNQX. A mixture of TTX and NMDA was injected into the left eye of the same rabbit within 1 week after the intravitreal injection of the right eyes. The intravitreal concentrations were 4 mM for APB, 0.4 mM for CNQX, 4 mM for TTX, and 5 mM for NMDA assuming that the vitreous volume of the NZW rabbit is 1.5 mL.18 The drugs were dissolved in phosphate-buffered solution, and 0.05 mL was injected into the midvitreous. Four right eyes of four WT and Tg rabbits were analyzed after the injections of APB followed by CNQX. Three left eyes of three WT and Tg rabbits were analyzed following the TTX+CNQX injection. We recorded ERGs before the TTX+CNQX injection and confirmed that the previously injected APB and CNQX into the right eyes did not affect the left eyes.

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

We digitally subtracted each type of postdrug ERG from the predrug ERGs as reported previously.24–26 We defined the ON-component as the waveform obtained by subtracting the post-APB ERGs from the baseline ERGs. The OFF-component was defined as the waveform obtained by subtracting the post-CNQX ERGs from the post-APB ERGs. The post-APB+CNQX ERGs were subtracted from the baseline ERGs to isolate the response of the third-order neurons.
Contribution of Inner Retina to Cone ERGs in Advanced RP

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.

Statistical Analyses

Mann-Whitney U tests were used to determine the significance of differences in the amplitudes of the ERG components between the WT and Tg rabbits. $P < 0.05$ was considered statistically significant.

RESULTS

Predrug ERGs

The ERGs elicited from the right eyes of four WT and four Tg rabbits by the three types of stimuli, namely, long duration, sawtooth rapid-ON, and sawtooth rapid-OFF, are shown in Figure 1. The ERGs were elicited by stimulus intensities of 1.5, 2.0, and 2.5 log cd/m$^2$. The amplitudes of the ERGs of both the WT and Tg rabbits increased as the intensity of the stimulus increased. The ERGs elicited by weaker stimuli had similar changes as those elicited by the stronger intensities.

The ERGs elicited by long-duration stimuli are shown in Figure 1A. The ERG of the WT rabbits was composed of small a-waves and large b-waves at light onset and small OPs at light offset. On the other hand, the Tg rabbits had similar-sized a-waves but smaller b-waves and had positive responses at light offset (Fig. 1, arrow). The size of these positive responses at light offset differed among the Tg rabbits, but distinctive positive waves were found in all Tg rabbits. We also noted that the slow negative response after the b-wave, that is, the photopic negative response (PhNR), was smaller in the Tg rabbits after the APB injection as was seen in the ERGs elicited by long-duration and rapid-ON stimuli. The deep wide negative wave was more visible in WT as shown in the ERGs elicited by long-duration and rapid-ON stimuli.

The next step was to determine the contribution of the postsynaptic OFF-components to the photopic ERGs of the Tg retina. We injected CNQX following the APB injection in the same eyes. CNQX is an antagonist of the 3-amino-5-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid (AMPA/KA) class of ionotropc glutamate receptors (iGluRs). It is known to block the light responses of the OFF-bipolar cells, horizontal cells, and many amacrine cells.27

CNQX is assumed to block the OFF-pathway, that is, the OFF-bipolar cells, horizontal cells, and inner retinal neurons driven by the OFF-bipolar cells. The combined APB and CNQX injections should block the entire postreceptoral components, and this allowed us to determine the contribution of the cone photoreceptors to the ERGs.

Effect of Blocking ON-Pathway by APB

To evaluate the contribution of the postsynaptic ON-pathway of the degenerated retina to the photopic ERGs recorded from the Tg rabbits, we injected APB into the vitreous cavity of four eyes of four Tg and four eyes of four WT rabbits. APB is a glutamate analogue that blocks synaptic transmission from the photoreceptors to the ON-bipolar cells, and thus eliminates the neural activities of the ON-pathway, that is, the ON-bipolar cells and inner retinal neurons driven by the ON-bipolar cells.5

In the pharmacologic experiments, the ERGs elicited with a stimulus intensity of 2.5 log cd/m$^2$ are shown because the waveform was large, and the responses had similar changes as those recorded with the two lower stimulus intensities.

The average ERGs of four Tg and four WT rabbits elicited by long-duration, rapid-ON, and rapid-OFF stimuli after APB injection are shown in Figure 2. After the APB injection, the b-wave was not recorded, and a deep negative wave appeared as shown in the ERGs elicited by long-duration and rapid-ON stimuli. The deep wide negative wave was more visible in WT rabbits. On the other hand, the positive OFF response to the stimulus offset emerged in the WT and increased in the Tg rabbits after the APB injection as was seen in the ERGs elicited by the long-duration and rapid-OFF stimuli.

Effect of Blocking OFF-Pathway by CNQX

The next step was to determine the contribution of the postsynaptic OFF-components to the photopic ERGs of the Tg retina. We injected CNQX following the APB injection in the same eyes. CNQX is an antagonist of the 3-amino-5-hydroxy-5-methyl-4-isoxazolepropionic acid/kainic acid (AMPA/KA) class of ionotropic glutamate receptors (iGluRs). It is known to block the light responses of the OFF-bipolar cells, horizontal cells, and many amacrine cells.27

CNQX is assumed to block the OFF-pathway, that is, the OFF-bipolar cells, horizontal cells, and inner retinal neurons driven by the OFF-bipolar cells. The combined APB and CNQX injections should block the entire postreceptoral components, and this allowed us to determine the contribution of the cone photoreceptors to the ERGs.
The average ERG waveforms after the injection of APB+CNQX, which are believed to be the photoreceptor signals, are shown in Figure 2. The ERGs were elicited by a stimulus intensity of 2.5 log cd/m². After APB+CNQX, the ERG amplitudes were consistently smaller than those after APB alone. The amplitudes of negative waves elicited by long-duration and rapid-ON stimuli were much smaller in the Tg rabbits than in the WT rabbits (88% reduction in long-duration and 68% reduction in rapid-ON stimuli). These reductions were most likely due to a degeneration of the cone photoreceptors in the Tg rabbits. The OFF responses elicited by long-duration and rapid-OFF stimuli were still detectable even after the APB+CNQX injection.

**Subtracted ON-Components**

We isolated the ON-component of the photopic ERGs by subtracting the post-APB ERGs from the predrug ERGs (Fig. 2). The ON-component was composed of a positive wave at stimulus onset as seen in long-duration and rapid-ON stimuli, and a negative wave at light offset as seen after long-duration and rapid-OFF stimuli. The amplitude of this positive component was measured from the potential at the stimulus onset to the potential at the maximum peak of the following the positive wave. The average amplitude of the b-wave was 2.0 to 2.2 times larger in the WT rabbits than in the Tg rabbits (Fig. 3). However, the average amplitude of the negative ON-component at stimulus offset was comparable in the Tg and WT rabbits (Fig. 2).

**Subtracted OFF-Components**

The digitally subtracted OFF-components, the CNQX-sensitive components, that were elicited by the three different stimulus patterns are shown in Figure 2. The OFF-component elicited by long-duration and rapid-ON stimuli was composed of a negative wave at stimulus onset and positive waves at stimulus offset as in long-duration and rapid-OFF stimuli. We measured the amplitudes of this positive component, which was measured from the potential at the end of the light stimulus to that at the following largest positive peaks. These amplitudes were 1.4 and 2.3 times larger in Tg rabbits than in WT rabbits (Fig. 3). On the other hand, the average amplitude of the negative wave at stimulus onset was similar after both long-duration and rapid-ON stimuli.

**Effect of TTX+NMDA**

To determine the contribution of the third-order neurons to the cone ERGs of the WT and Tg rabbits at 18 months of age, we injected TTX+NMDA into the vitreous cavity of three eyes of three Tg and three WT rabbits. Tetrodotoxin blocks the voltage-gated sodium channels and prevents the action potentials of the retinal ganglion cells and some types of amacrine cells.28 N-methyl-DL-aspartic acid suppresses the synaptic transmission by its antagonistic action (depolarization) of the NMDA subclass of glutamate receptors located primarily on the third-order neurons.29

It is generally believed that the intravitreal injection of TTX+NMDA can suppress most, if not all, of the electrical activities of the third-order retinal neurons. The average ERG waveforms of predrug and post-PTX+NMDA ERGs elicited by long-duration and rapid-ON and rapid-OFF stimuli are shown in Figure 4. The stimulus intensity was 2.5 log cd/m². After the intravitreal injections of TTX+NMDA (post TTX+NMDA), the ERG waveforms became more positive and slower, with losses of the ONs at the onset of long-duration and rapid-ON stimuli. The amplitudes of these slow positive waves were approximately 1.7 to 2.3 times larger in WT rabbits than in Tg rabbits. On the other hand, the ERGs at stimulus offset became a slow negative wave, and the amplitudes were larger in WT rabbits elicited by long-duration and rapid-OFF stimuli. The positive wave elicited at the offset of the stimulus in Tg rabbits was almost completely absent after the TTX+NMDA injection.

**TTX+NMDA-Sensitive Components**

The digitally subtracted TTX+NMDA components elicited by the three different stimulus patterns are shown in Figure 4. Slow negative potentials following the sharp positive peaks were detected in response to the onset of the long-duration and rapid-ON stimuli in WT and Tg rabbits. These potentials...
FIGURE 3. Comparisons of the amplitudes of ON-component and OFF-component between WT and Tg rabbits at 18 months of age. ON-component was evaluated from the 150-ms long-duration ERGs (A) and sawtooth rapid-ON–elicited ERGs (B). The OFF-component was evaluated from the 150-ms long-duration ERGs (C) and sawtooth rapid-OFF–elicited ERGs (D). The stimulus intensity was 2.5 log cd/m². The amplitudes were measured, shown as gray arrows in the upper row of each column. The amplitudes of each component were measured from the electrical potentials at stimulus onset or offset to the potential at the maximum peaks of the following positive waves. The mean ± standard deviation of the measured amplitudes is shown in lower rows of each part of the figure. *P < 0.05 (Mann-Whitney U tests).

FIGURE 4. Predrug ERGs, post TTX+NMDA, and subtracted components. The waveforms from three Tg and three WT rabbits are averaged. The predrug waveforms (first row), the waveforms after intravitreal injection of TTX+NMDA (second row), and digitally subtracted waveforms (subtracted post-TTX+NMDA ERGs from baseline waveform; third row), which represent the inner retinal components, are shown. ERGs were elicited by long-duration stimuli (A), sawtooth rapid-ON stimuli (B), and sawtooth rapid-OFF stimuli (C). The stimulus intensity was 2.5 log cd/m². The stimulus markers are shown in gray lines beneath each ERG waveform.
were smaller in Tg rabbits. A slow positive component was detected at the offset of the stimuli in WT rabbits, but this was not obvious in the response of Tg rabbits. Instead, a sharp positive wave was present at the stimulus offset in Tg rabbits. This indicated that the sharp positive wave at the stimulus offset in predrug ERG of the Tg rabbit was related to the inner retinal cellular activities.

Retinal Histology

The retinal histology in the area of the visual streak of WT and Tg 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 (arrows) and five to seven rows of nuclei in the outer nuclear layer (arrowheads). On the other hand, there were very few nuclei in the outer nuclear layer in Tg rabbits (arrows), and the nerve fiber layer to inner nuclear layer was relatively well preserved in Tg rabbits.

DISCUSSION

We have analyzed the photopic ERGs at an advanced stage of degeneration in Tg rabbits and obtained two unexpected findings. The first finding was a positive response to the offset of the light stimuli in Tg rabbits that resembled the d-wave of primate ERGs. Pharmacologic analysis suggested that this wave was related to the neural activities of the third-order neurons. The second observation was that the OFF-components in Tg rabbits were enhanced to nearly twice those in WT rabbits. These results confirmed the findings that the surviving rod bipolar cells assume the phenotype of the OFF-type cone bipolar cells to recover the signaling capacity and ability to activate inner retinal cells through the iGluR-mediated channels after the rods degenerated.12,14

We analyzed not only the ERGs elicited by long-duration stimuli but also ERGs elicited by sawtooth rapid-ON and sawtooth rapid-OFF stimuli.26,50 Rapid-ON and rapid-OFF stimuli enhanced the responses to stimulus onset and offset and made it easier to analyze the contribution of the different retinal neurons to the overall photopic ERGs. Our results indicated that the ERGs elicited by both types of stimuli had neural components similar to those recorded with long-duration stimuli.

Cone Photoreceptor Contribution to Photopic ERGs

We can estimate the degree of residual cone function in Tg rabbits by examining the ERGs after APB and CNQX injection.31 The amplitude of the negative wave at stimulus onset of Tg rabbits for long-duration stimuli was approximately 12% of that in the WT rabbits. These results indicated that the cone function was disrupted to some degree, which corresponded with the histologic data showing a very thin photoreceptor layer.

An earlier report from our laboratory showed that the contribution of the cone photoreceptors to the a-waves of the photopic ERGs was less in Tg rabbits than in WT rabbits.18 Our present results are comparable to those data; the amplitudes of the a-waves were similar in Tg and WT rabbits but had small contributions of cone photoreceptors in Tg rabbits. What is more, the small numbers of cones in Tg rabbits can elicit ERGs with approximately one-half the amplitudes of the ERGs of WT rabbits. This may be caused by not only a buffering but also a gain of function by rewiring and reprogramming of the retinal cells.32

Contributions of ON-Components

We found that the increases of the ON-components were smaller in Tg rabbits. In an earlier study, Nishimura et al.17 reported that the ON-bipolar cells responses of Tg rabbits at 12 weeks of age were significantly enhanced compared with those of WT rabbits, and those of Tg rabbits at 24 weeks of age were still preserved. Our results indicated that the augmented function of the ON-components was found only in the relatively early stage of retinal degeneration but not in the advanced stage. The data of a greater reduction of the ON-components than the OFF-components agree with several previous studies showing that the ON-pathway is more susceptible to the effects of photoreceptor death than the OFF-pathway.12,13,14

The ON-component of the descending potential at stimulus offset in Tg rabbits was not reduced. This might be caused by the differences in the mechanisms of the ON-components elicited by stimulus onset and offset, but the exact reason was not determined.

Contribution of OFF-Components

We found that the OFF-components extracted by CNQX in Tg rabbits were almost double those in WT rabbits. It was reported that the expression of iGluR and function in OFF-bipolar cells tend to remain stable in the course of retinal degeneration in contrast to mGluR6 expression of ON-bipolar cells.13 Previously, a Pro547Leu rhodopsin mutation in porcine eyes showed preserved OFF responses after an APB injection.25 However, the augmentation of the OFF-component in our study might not be explained only by the preservation of healthy OFF-bipolar cell function. In addition, a switching of the ON-bipolar cell phenotypes to OFF-bipolar cell phenotype seemed to be needed.

In many animal models examined, extensive remodeling of the bipolar cells has been reported. Some reports referred to molecular changes in the glutamate receptor expression at the bipolar cell dendrites that modified the physiology of bipolar cells with a shift in their functional phenotypes from ON to OFF responses by expressing iGluRs.5,12,33 Jones et al.14 have confirmed a similar phenomenon in Tg rabbits at 40 weeks of age. They reported that approximately 30% to 40% of the bipolar cells were classified as OFF-bipolar cells in the WT retina, but in the Tg rabbit retina at 40 weeks of age, the fraction of bipolar cells with iGluR expression, OFF-bipolar-like cells, increased to over 56%. In addition, the fraction of identifiable rod bipolar cells decreased from 30% to 1%. The increase in the ratio of bipolar cells expressing iGluR was similar to our result of an augmentation of the OFF-component. Our findings might detect these remodelings of retinal bipolar...
cells functionally, although the ages of Tg rabbits were older in our study.

**Contribution of Amacrine and Ganglion Cells**

The most interesting finding was that the sharp positive waves at the stimulus offset in Tg rabbits were not present after the injection of TTX+NMDA. This suggests that the positive wave was caused by a remodeling of the amacrine and/or ganglion cells. Indeed, it has been reported that amacrine cells also remain relatively stable initially but undergo aberrant synaptic connections in the late stage of retinal degeneration. In addition, in vivo excitation mapping revealed that the amacrine cells are extremely active through iGluR-mediated channels. In the study, rabbits. Amacrine cells, which had little contribution to the ERGs, reprogrammed amacrine cells caused a gaining of function or ON- and OFF-bipolar cells. However, we injected APB and then CNQX to the same eye to see the activity of the second- and/or third-order neurons rather than ganglion cells, which had little contribution to the ERGs, caused the sharp positive wave at the stimulus offset in Tg rabbits.

One concern was whether TTX+NMDA really blocked the third-order neuron solely. Several studies reported that TTX reduced the b-wave amplitude in rodents and rabbits, and the authors speculated that TTX might affect the retinal ON-bipolar cells. It is difficult to determine whether TTX has any effect on the retinal ON-bipolar cells. But our result after APB injection revealed an enhancement of the positive wave at stimulus offset, which might indicate that the contribution of the retinal ON-bipolar cells to the sharp positive waves at the stimulus offset in Tg rabbits was not significant. Thus, we suggest that this abnormal wave was likely induced by the third-order neurons rather than the retinal ON-bipolar cells.

**Limitations**

Previously, Nishimura et al. injected TTX+NMDA following APB and then CNQX to the same eye to see the activity of the ON- and OFF-bipolar cells. However, we injected APB+CNQX to the right eye and TTX+NMDA to the left eye to reduce the effects of the long duration of the general anesthesia. We suspect that the long period of general anesthesia might affect the ERG waveform especially for Tg rabbits, which have smaller ERGs. However, our procedure did not allow us to examine the neural activity of the ON- and OFF-bipolar cells directly.

We used Tg rabbits having mutations of rhodopsin Pro347Leu, but the pathophysiology might differ with the genetic mutation underlying the disease. In addition, the retinal structure in rabbits is different from that of humans; rabbits do not have a fovea and the blood supply of the retina is mainly from the choroidal vessels. We have to take into account these differences when we apply our data to RP patients, although most of what is known has been obtained from animal models; the findings in rodents and rabbits are similar and parallel to the limited data collected from humans.

In conclusion, our results indicated that complex functional changes occurred due to synaptic remodeling of residual second- and third-order retinal neurons after a degeneration of the rod photoreceptors in rabbits. Similar changes probably occur in the human retina after the degeneration of the rods in eyes with hereditary degeneration. Synaptic remodeling must be considered when retinal prosthesis, or the implantation of embryonic stem cell-derived or induced pluripotent stem cell-derived retinal tissue, is used in patients with RP because the function of second- and/or third-order neurons is essential for these therapies.

**Acknowledgments**

The authors thank Duco Hamasaki, PhD, of the Bascom Palmer Eye Institute for the discussions and editing the final version of the manuscript.

**Disclosure**

Supported in part by JSPS KAKENHI Grant Number 16K11320.

**References**


