Photopic ERG Negative Response from Amacrine Cell Signaling in RCS Rat Retinal Degeneration

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PURPOSE. The authors investigated photopic electroretinographic changes during degeneration in the Royal College of Surgeons (RCS) and transgenic P23H rhodopsin rat models, including the cellular origins of a large corneal-negative component that persists in the RCS rat.

METHODS. Photopic and scotopic electroretinograms (ERGs) were recorded from dystrophic RCS (RCS-β+/Lav) rats (4–18 weeks old) and transgenic rhodopsin Pro23His line 1 (P23H) rats (4–30 weeks old). Age-matched congenic (RCS-rdy+/Lav) and Sprague-Dawley rats were used as controls. N-methyl-DL-aspartic acid (NMA), dopamine, and γ-aminobutyric acid (GABA) were injected intravitreally, and optic nerve sectioning (ONS) was performed to suppress or remove inner retinal neuron activity. Retinal morphology for cone cell counts and immunohistochemistry for quantification of Kir4.1 channels were performed at various stages of degeneration.

RESULTS. As degeneration progressed, the photopic ERG of RCS dystrophic rats was distinctly different from that of P23H rats, primarily because of the growth of a corneal-negative response (RCS-NPR) after the b-wave in RCS rats. This response had a peak time similar to the photopic negative response (PhNR) in controls but with a more gradual recovery phase, and it was not affected by ONS. The PhNR in P23H rats declined linearly with the b-wave. NMA and GABA eliminated the RCS-NPR and uncovered a larger b-wave in RCS rats at late stages of degeneration, but NMA had little effect on the ERG in P23H rats. The NMA-sensitive negative response in RCS rats declined with age more slowly than did the NMA-isolated b-wave. The density of Kir4.1 channels at the endfeet of Müller cells and in the proximal retina increased significantly between 6 to 10 weeks and 14 weeks of age in the RCS rat retina but not in the P23H rat retina.

CONCLUSIONS. The photopic ERG of the dystrophic RCS rat retina becomes increasingly electronegative because of an aberrant negative response, originating from amacrine cell activity, which declines more slowly than the b-wave with degeneration. The absence of this response in the P23H rat indicates that the inner retinal cone pathway pathology is different in the two models. A relative increase in Kir4.1 channels on Müller cells of RCS retina may contribute to the enhanced negative ERG response in the RCS rat. (Invest Ophthalmol Vis Sci. 2008;49:442–452) DOI:10.1167/iovs.07-0291

Photoreceptor degeneration initially spares the proximal retinal layers but may subsequently lead to transsynaptic degeneration of retinal neurons in later stages of disease.1–3 Assessment of proximal retinal function may thereby help in evaluating the state of the retina and provide insight into disease mechanisms. The electroretinogram (ERG) traditionally is used to monitor photoreceptor4–6 and second-order neuron7,8 function. Some ERG parameters, however, reflect proximal retinal function. The scotopic threshold response (STR) is a rod-driven, predominately negative ERG response that originates in potassium (K+) currents in the proximal retina.9,10 Studies on the human and nonhuman primate photopic full-field corneal ERG have also described a cone-driven negative response, the photopic negative response (PhNR), originating in the proximal retina.11 The PhNR in primates is suppressed in experimental glaucoma, consistent with an origin in ganglion cells or activity of their axons.12 Optic nerve sectioning (ONS) substantially reduced a response identified as the PhNR in rats,13 further implicating ganglion cell activity. However, like the STR, for which the contributing cell types appear to vary across species,14 proximal retina cells other than ganglion cells may contribute to the PhNR in rat.

The Royal College of Surgeons (RCS) rat is a model of progressive photoreceptor degeneration.15 It harbors a mutation in the receptor tyrosine kinase merT gene16 that leads to rod cell loss from defective phagocytosis by the retinal pigment epithelium (RPE) and causes an abnormal accumulation of debris between the RPE and the neural retina. We previously demonstrated in the RCS retina that the STR persists even into very late stages of photoreceptor degeneration, despite severe reduction of the scotopic b-wave. Ultimately, in late stages of RCS degeneration, the negative STR comes to dominate the ERG.17 Cone cells and cone-driven visual function persist into later stages of RCS disease18,19 beyond the loss of recordable rod or cone b-waves.15,20,21 Even when only 1% of the receptor nuclei remain, ganglion cells still retain measurable cone-driven sensitivity.22 Therefore, by analogy to the rod ERG, it may be possible to record cone pathway inner retinal signals in RCS rats at late stages of degeneration.

In this study we examined cone-driven inner retinal electroretinographic function in the dystrophic RCS rat and in a second model of photoreceptor degeneration, the transgenic P23H rhodopsin rat. Both rodent models have genetic counterparts in human retinitis pigmentosa.23,24 Analogous to the STR in the dark-adapted ERG, we found that a negative wave persisted in the light-adapted ERG even during advanced stages of RCS retinal degeneration when no apparent residual photopic b-wave remained. We found that this negative-going photopic response (RCS-NPR) could be eliminated by application of the same neurotransmitters and neurotransmitter agonists that suppressed the STR but that it was not affected by
ONS. These data indicated that cone-signaling to the inner retina persists beyond what might be expected based on recording the ERG photopic b-wave in the RCS rat. Surprisingly, the ERG of the P23H rhodopsin rat showed a very different photopic ERG pattern during degeneration in which the b-wave and the subsequent negative response, presumed to be the PhNR, declined in parallel. Based on work that had implicated K⁺ currents in the proximal retina as generating the STR and photopic inner retinal ERG currents, we conducted a semiquantitative analysis of Kir4.1 channel density in the proximal retina by immunohistochemistry and found a significant increase between 6 to 14 weeks of age in the RCS rat but not the P23H rhodopsin retina.

**METHODS**

**Animals**

Dystrophic RCS (RCS-p<sup>-/−</sup>/Lav), congenic control RCS (RCS-rdy<sup>−/−</sup> p<sup>+</sup> /Lav), Long Evans (LE), heterozygous transgenic P23H rhodopsin line 1 (P23H), and albino Sprague-Dawley rats were born and reared at the University of Michigan, Kellogg Eye Center animal facility (non-SPF), and National Eye Institute (NEI) animal facility (SPF). All animals were kept on a cycle of 12 hours on/12 hours off fluorescent white light, 5 lux for the albino P23H rats and 50 lux for the pigmented RCS rats at cage level. Breeding pairs of each were kindly provided by Matthew LaVail (University of California at San Francisco School of Medicine, Beckman Vision Center). All rats were fed high-fat breeding chow (Formulab; PMI Feed, Richmond, IN) ad libitum. These studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the Committee on the Use Committee at NEI.

**Electroretinographic Recordings**

ERGs were recorded from 56 RCS (age 4–18 weeks), 10 congenic RCS, 30 P23H-1 rats (age 4–30 weeks), and 9 Sprague-Dawley rats to identify waveform change as a function of age. Animals were kept in total darkness for 12 hours and then were prepared under dim red light for ERG recordings. Rats were anesthetized with a loading dose of xylazine (15 mg/kg, intramuscularly) and ketamine (86 mg/kg, intramuscularly) and then were maintained by a slow subcutaneous infusion of the same mixture by pump (Razel Instruments, Stamford, CT). The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine HCl. The animals were held steady for recordings with a bite bar and a nose clamp. A heating pad maintained body temperature at 37°C.

ERGs were recorded using a gold or chloride-silver wire loop positioned on the center of the cornea after topical anesthesia with 1% tetracaine. One drop of methylcellulose was added to maintain corneal hydration and electrical contact. Chloride-silver or gold reference electrodes were positioned on both eyes on the temporal sclera approximately 1 mm behind the corneal limbus. A clip attached to the left ear lobe served as the ground electrode. Responses were amplified at 10,000 gain from 0.1 to 1000 Hz, filtered to remove 60-Hz noise, and digitized at 10 kHz. Responses were computer averaged, with flash intervals from 3 to 60 seconds for scotopic recording. For photopic recording, 20 to 40 responses were averaged with a stimulus interval of 1 to 2 seconds. ERGs were elicited using a Ganzfeld bowl and a white (2800 K) 50-ms shuttered stimulus with a maximum intensity of 2.2 log cd/m² or white xenon photostrobe 10-μs flashes (PS33; Grass Instruments, Warwick, RI) with a maximum intensity of 0.6 log cd ⋅ s/m². Intensities were attenuated with neutral-density filters and were measured with a research radiometer and photopic probe (IL 1700; International Light, Inc., Newburyport, MA). Photopic ERGs were recorded on a white rod-suppressing background of 34 cd/m² after light-adapting for at least 10 minutes. Recording began at intensities below ERG threshold and progressed upward in 0.3 or 0.4 log unit steps. The criterion threshold amplitude was 8 μV. Amplitudes of the b-wave and the negative response after the b-wave in the photopic ERG were measured from prestimulus baseline, and amplitudes elicited by maximum intensity stimuli were designated as the photopic V<sub>bmax</sub> and PhNR V<sub>bmax</sub> (control and P23H) or RCS-NPR V<sub>bmax</sub> (RCS dystrophic, respectively).

Rats have both short-wavelength (S-cone, 359 nm maximum) and long-wavelength (M-cone, 509 nm maximum) photoreceptors; 11% to 12% of the total are S-cones. The S-cone ERG in rats is approximately 0.3 to 0.4 log unit less sensitive than the M-cone ERG at the respective wavelengths of maximum sensitivity. In addition, because our ERG light source was greater than 1 log unit more intense at the M-cone maximum than at 400 nm, the shortest wavelength we measured, it can be assumed that M-cone responses dominated the photopic ERG in our experiments. As in mouse, rat M-cones have a spectral sensitivity very close to that of rhodopsin. Although we report photopic intensity in this study, it is reasonable to multiply our photopic measurements by a factor to convert to scotopic units to provide a more precise measure of stimulus intensity for the rat M-cone response. This factor for our 2800 K source is approximately 1.4.

**Bleaching and Light Adaptation**

To investigate the difference in the sensitivity of the photopic and scotopic ERG responses to a strong bleach, four dystrophic RCS rats at age 12 weeks with fully dilated pupils were exposed to a Ganzfeld 500 lux light for 10 minutes. The light source was the same as that used to produce the adaptation background for photopic ERG recordings with neutral-density filters removed. This intensity gives a near complete bleach of rhodopsin out to at least 40° eccentricity in pigmented rat eyes. Scotopic ERGs were then recorded at 5-minute intervals using −0.8 log cd/m² stimuli. The rats were then exposed again to the bright bleaching light for 10 minutes, after which photopic ERGs were recorded using 2.2 log cd/m² stimuli and 34 cd/m² background.

The change in amplitude of the RCS-NPR during light adaptation was examined in four 10-week-old RCS dystrophic rats. After overnight dark adaptation, photopic ERGs to a 50-ms stimulus of 2.2 log cd/m² intensity were just recorded after a background light of 34 cd/m² was turned on and again 15 minutes after starting light adaptation. RCS-NPR amplitudes obtained at these two time points were compared.

**Drug Administration**

N-methyl-DL-aspartic acid (NMA; Sigma, St. Louis, MO), γ-aminobutyric acid (GABA; TOCRIS, Ballwin, MO), and 3,4-hydroxyphenethylamine (dopamine; Sigma) were applied by vitreal intraocular injection to RCS dystrophic rats (n = 3–5 per drug) at an advanced stage of degeneration (age 10–12 weeks). NMA was also injected into P23H rats (n = 2) at a comparable stage of degeneration (16 weeks). Drugs were dissolved in PBS, and the pH was adjusted to 6.8 to 7.2 with 1 N NaOH and was passed through a 0.2-μm filter (Corning Glass Works, Corning, NY). Ascorbic acid (1 mg/mL) was added to the dopamine solution to prevent oxidation. Intravitreal injections of 1 μL were given through a 30-gauge needle inserted approximately 1 mm behind the corneal limbus using a Hamilton syringe (#80001; Hamilton, Reno, NV). Approximate intravitreal drug concentrations of 5 mM NMA, 0.25 mM dopamine, and 10 mM GABA were estimated by assuming full mixing in 38 μL vitreous volume (calculated from the schematic rat eye<sup>38</sup>). Dose effects were predicted based on our previous experience in cat, primate, and rat electroretinographic recordings.<sup>17</sup> ERGs were recorded in both eyes simultaneously 30 to 90 minutes after drug injection, and the drug-injected eye was compared with the uninjected eye. Previous reports using saline in control eyes did not find an effect of intraocular injection alone on the photopic ERG,<sup>35</sup> though elevated intraocular pressure may reduce the PhNR in primate<sup>34</sup> and the STR in rat.<sup>35</sup> Based on the latter study, the effects of a small, transient elevation in pressure caused by injection of a quantity less than 5% of the vitreous volume in our study would be unlikely to cause significant and consistent reduction in the ERG when we recorded it 30 to 60 minutes after injection.

In addition, we used NMA injections to isolate the full bipolar and inner retinal responses in RCS dystrophic rats and to monitor their...
progression with age. NMA suppresses synaptic transmission between second-order bipolar cells and third-order retinal neurons through its action at NMDA glutamate receptors. It has been used extensively to remove the inner retinal contributions and to isolate the bipolar PII contribution to the ERG. 8,33-37,40 RCS dystrophic rats at six different ages from 8 to 18 weeks were injected with NMA (n = 3-5 per time point).

Optic Nerve Sectioning

The right optic nerve was severed intracranially in three RCS rats at age 12 weeks using a ventral approach through the roof of the mouth by removing a section of the nerve just anterior to the optic chiasm, as previously described. This approach avoided damage to the ocular blood supply and other ocular tissues and was performed after baseline ERG. All three rats recovered and were behaving normally within 24 hours of surgery. A second ERG was performed 2 weeks after surgery, and the eyes then were removed for ganglion cell counts. ONS by this method and an intraorbital approach have previously been shown to cause maximal loss of cells in the ganglion cell layer by 2 weeks in the rat.

Histology

Cone Cell Counts. One day after ERG recording, rats were killed with an overdose of sodium pentobarbital or by CO2 asphyxiation, and the eyes were removed for histology after marking the temporal margin for later orientation. Eyes were placed overnight at 4°C in Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer), trimmed, and postfixed in 1% osmium for 1 hour. Epon-embedded tissue was cut into 1-μm sections along the vertical meridian of the eye passing through the optic nerve and stained with toluidine blue. The number of cone nuclei in 100-μm lengths was counted at 400-μm intervals beginning 200 μm from the optic nerve head using the 100× objective. Identification of cone nuclei was based on morphologic criteria previously reported for mouse retina. Numbers in each retinal half were averaged.

Ganglion Cell Counts. Eyes were marked and removed as described, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin for immunohistochemistry with antibody to neuron-specific nuclear protein (NeuN; Chemicon, Temecula, CA) to label neurons in the ganglion cell layer. NeuN primarily labels the nucleus with lighter staining of the cytoplasm and is useful in distinguishing surviving neurons from glia in the ganglion cell layer after an optic nerve lesion. NeuN-positive cells in the ganglion cell layer were counted along the entire length of 4-μm-thick vertical retinal sections through the optic nerve of ONS eyes and fellow eyes with intact optic nerves. Counts included the total number of NeuN-positive cells and NeuN-positive cells greater than 10 μm in diameter. Approximately 50% of the cells in the ganglion cell layer survive ONS in the rat, even with survival times out to 5 months. Approximately the same number cannot be retrogradely labeled with horseradish peroxidase from the brain in rats with intact optic nerves. Most of these have cell soma diameters less than 10 μm and dendritic structures similar to those of cells at the margin of the inner nuclear layer (INL) and the inner plexiform layer (IPL). Thus, these surviving cells are considered to be displaced amacrine cells. Two weeks after ONS, RCS rats in our study had a 94% ± 6% loss of cells larger than 10 μm, which was a 65% ± 2% reduction when all cells in the ganglion cell layer were counted.

Confocal Immunohistochemistry. Eyes from RCS and Long-Evans (LE) rats at 6, 10, and 14 weeks (n = 3-11 at each age), and from P23H and Sprague-Dawley rats at 4, 8, and 15 weeks (n = 3 at each age) were enucleated as described and fixed in 4% paraformaldehyde in PBS for 6 hours, washed, and embedded in OCT for labeling with antibody to Kir4.1 channel. Frozen sections (10 μm) were collected and immunolabeled with an anti-Kir4.1 polyclonal antibody developed against the peptide corresponding to residues 356 to 375 of rat Kir1.1 (Alomone Laboratories, Jerusalem, Israel) followed by goat anti-rabbit Alexa 568 secondary antibody. Imaging and analysis were performed on a confocal system (Leica SP2; Leica Microsystems, Exton, PA). Ideally, RCS congenic controls would have been used rather than LE rats, but they were not available at the time this portion of the study was conducted. Based on the use of LE as controls for RCS dystrophic rats in at least 13 retinal studies since 1981 (when congenics became available) and as a source of cells for transplantation into RCS dystrophic animals in other studies, the LE strain is considered a reliable genetic and phenotypic control for RCS.

Staining intensity was analyzed blindly with regard to the strain and age of the rat. Mean pixel intensity (MPI) was measured in regions of interest (ROIs) that were defined manually to include the ganglion cell layer (GCL) and the IPL, where much of the Kir4.1 channel immunolabeling in the rat has been predominately localized. Unexpectedly, the RPE layer in P23H rats labeled intensely with the Kir4.1 antibody. We therefore quantified the MPI in this layer for the P23H and Sprague-Dawley rats. ROIs in three equally spaced areas in a vertical section through the optic nerve were measured and averaged for each eye. For each eye, the MPI was also measured from a control slide in which the primary Kir4.1 antibody was omitted and samples were labeled with only the secondary antibody. This control value was subtracted from the averaged MPI value obtained from the anti-Kir4.1 secondary antibody-labeled slide. Data were collected in several sessions, and rats of different strains and ages were examined in every session, making comparison between sessions possible. Confocal system parameters were preserved as much as possible, but small variations inherent in the system might have been exhibited between sessions. Values in a session were shifted (all higher or all lower) relative to previous measurements. Therefore, a correction was applied as follows: For each individual retinal measurement, the percentage difference from the average of all previous measurements of retinas from the same strain and age was calculated. The average of these percentage differences for a session was the correction factor. Then all measurements of that session were corrected by this factor. Statistical significance for MPI values and for ERG measurements was determined with Student’s two-tailed t test unless otherwise specified.

RESULTS

Photopic ERG Waveform and Amplitude Changes with Age

Photopic ERG waveforms of 4-week-old RCS congenic and Sprague-Dawley control rats (Fig. 1) exhibited a prominent b-wave and a negative-going response after the b-wave, which in primates and normal rats was identified as the PhNR. Unlike the photopic ERG of primate and other species, the photopic ERG of rat has little or no leading a-wave. Both the b-wave and the PhNR gradually diminished with age, particularly in Sprague-Dawley rats. In 4-week-old RCS dystrophic and P23H rats, both the b-wave and the PhNR were smaller than in controls. In RCS rats in particular, the post-b-wave negative wave was shallower and broader and lacked the rapid initial recovery phase typical of the PhNR. The photopic b-wave became progressively smaller with age in dystrophic RCS rats until it could no longer be recorded by 12 to 14 weeks of age, whereas the trailing negative response in the region of the PhNR grew until it dominated the photopic waveform. This unusual negative photopic response in RCS rats (RCS-NPR) maintained a time-to-peak similar to the PhNR in congenic controls at all stages of degeneration (Fig. 1, dashed lines) but had a more gradual recovery. By contrast, the relative amplitudes of the b-wave and trailing PhNR appeared not to change in the heterozygous P23H rhodopsin rat, which retains approximately 60% of cells in the ONL at 4 weeks of age and has a more gradual loss of photoreceptors over the next 30 weeks.

For the RCS dystrophic rat, plotting the RCS-NPR Vbmax and Vbmax against age (Fig. 2A) showed that, though the b-wave decreased dramatically between 6 and 10 weeks of age, the RCS-NPR amplitude actually increased with age up to 18 weeks (one-way ANOVA; P < 0.001; n = 4/time point). In P23H, the
photopic ERG overall declined rapidly between 4 and 6 weeks of age and then at a slower rate out to 30 weeks of age, as previously described.49 Unlike the RCS rat, there was no enhanced response after the b-wave in P23H rat, and the PhNR declined steadily (Fig. 2A).

The RCS-NPR appeared to grow at the expense of the b-wave. Plotting the RCS-NPR $V_{\text{max}}$ against the $V_{b \text{ max}}$ in RCS dystrophic rats from 4 to 18 weeks of age (Fig. 2B) showed a nonlinear relationship between these two ERG potentials as degeneration progressed. The RCS-NPR maintained nearly constant amplitude even as the b-wave became smaller; by the time the b-wave amplitude reached very low levels, the RCS-NPR showed a large relative increase in amplitude following a course similar to an exponential function. In P23H, the PhNR $V_{\text{max}}$ changed linearly with the photopic $V_{b \text{ max}}$ across the entire age range (Fig. 2B; $r^2 = 0.60$; slope = 0.14; $P < 0.0001$). To compare the photopic responses of RCS and P23H rats at matching degrees of degeneration, we selected animals with similar cone numbers (Table 1). Animals from the two groups with similar numbers of remaining cones had similar photopic b-wave $V_{\text{max}}$ but different amplitudes of the negative responses after the b-wave. The dystrophic RCS rat had significantly larger RCS-NPR $V_{\text{max}}$ ($P < 0.0005$) and (RCS-NPR $V_{\text{max}}$/photopic $V_{b \text{ max}}$) ratios ($P < 0.01$) than the transgenic P23H rat PhNR $V_{\text{max}}$ and (PhNR $V_{\text{max}}$/V$_{b \text{ max}}$) ratio.

**Effect of Ganglion Cell Loss on the RCS-NPR**

The RCS-NPR is similar to the PhNR in that it is a transient negative response after the b-wave with approximately the same peak time. To determine whether it contains a significant contribution from ganglion cells, as does the PhNR, we compared ERG responses before and 2 weeks after intracranial ONS performed at 12 weeks of age. Two weeks after ONS, enucle-
and histologic examination showed that 94% ± 6% (mean ± SD, n = 3) of NeuN-positive cells larger than 10 μm in diameter, representing most ganglion cells (see Methods), and 65% ± 2% of all NeuN-positive cells in the ganglion cell layer were gone compared with no change in the untreated contralateral eye. The latter result is essentially identical with the maximum 63% cell loss found by Luikovitch-Verbin et al.43 in the rat superior retina after intraorbital sectioning of the optic nerve fibers. In the group of RCS dystrophic rats used for ONS (see Methods), the RCS-NPR reached peak amplitude by age 12 weeks and then declined as much as 60% between 12 and 14 weeks, even in rats that did not undergo ONS (n = 2). Therefore, ERG responses were lower in both eyes of ONS animals 2 weeks after the procedure but were not different between the ONS and the contralateral untouched eye in any of the three animals (representative example in Fig. 3). These results mean that ganglion cells do not make a major contribution to the RCS-NPR in RCS rats, unlike the PhNR, which is dependent on ganglion cell activity.11,15

### Cone Pathway Origin: Effect of Bleaching and Light Adaptation

The dark-adapted ERG of the RCS rat is increasingly dominated by a negative response as degeneration progresses.12 This response was shown to arise from postreceptorial neurons and was thought to be the STR enhanced by selective loss of the b-wave and with a response range extended to higher intensities because of the loss of photoreceptor output. It has been previously shown that this “STR-like” response in the dark-adapted ERG of RCS can be selectively removed by bleaching using a double flash technique in which a probe flash is presented 1 second after a 1.4 log cd·s/m² conditioning flash to obtain a dark-adapted, cone-driven b-wave.50 To further differentiate the RCS-NPR recorded under light-adapted conditions from the scotopic response, we compared their sensitivity to a continuous strong bleaching light estimated to bleach nearly all rhodopsin (see Methods; Fig. 4). At intensities producing equal amplitudes, these two responses appear similar in waveform, with the photopic response having a slightly faster onset and return to baseline. The dark-adapted response was completely suppressed by a strong bleach and took 15 minutes to recover to 11% ± 7% (n = 4), at which it remained for at least 85 minutes in the dark. By comparison, the bleaching light diminished the RCS-NPR only approximately 25%. It recovered to 84% ± 4% of the prebleach level within 5 minutes (n = 4) and remained at this level for at least 20 minutes. That this rapid partial recovery and sustained plateau of the RCS-NPR after a strong bleach represents a cone-mediated response is supported by a previous study on the RCS rat b-wave at an earlier stage of degeneration, which demonstrated that, under similar conditions of bleaching and recovery, the response recovered along a cone time course, and the remaining sustained response was shown not to be from rods.51

Cone pathway characteristics of the RCS-NPR were explored further by measuring the change in amplitude during light adaptation. One prominent feature of the cone ERG b-wave is rapid partial recovery and sustained plateau of the RCS-NPR and remained at this level for at least 20 minutes. That this rapid partial recovery and sustained plateau of the RCS-NPR after a strong bleach represents a cone-mediated response is supported by a previous study on the RCS rat b-wave at an earlier stage of degeneration, which demonstrated that, under similar conditions of bleaching and recovery, the response recovered along a cone time course, and the remaining sustained response was shown not to be from rods.51

![FIGURE 3. Effect of ganglion cell loss caused by ONS on RCS-NPR amplitude in an RCS dystrophic rat.](A) Light-adapted responses (flash intensity of 0.6 log cd·s/m²) in RCS rats before (12 weeks of age) and 2 weeks after (14 weeks of age) ONS. Responses from the ONS eye are in red, and those from the contralateral eye, with the nerve intact, are in black. There was no effect of ONS on RCS-NPR amplitude. Responses in both eyes were lower at 14 weeks because of the normal course of RCS retinal degeneration. (B) Retinal morphology in ONS and control eyes of the same animal. Ganglion cells and amacrine cells are stained brown (NeuN antibody). GCL, ganglion cell layer; INL, inner nuclear layer; RPE, retinal pigment epithelium. At this advanced stage of degeneration, few photoreceptors remained, so it was difficult to distinguish the photoreceptor cell layer from the INL.

![FIGURE 4. Suppression of the photopic and scotopic responses of a 12-week-old dystrophic RCS rat after a strong bleaching light (500 lux, for 10 minutes).](B)
wave in mice and humans is that the photopic response amplitude increases over the first several minutes of light adaptation.\(^5\) During 15 minutes of light adaptation, the RCS-NPR \(V_{\text{max}}\) of 10-week-old RCS rats increased by 39%, from 30.1\(\pm\)7.4\(\mu\text{V}\) (mean \(\pm\) SD) to 41.8\(\pm\)3.8\(\mu\text{V}\) (\(n\) = 4 each; \(P\) < 0.05), providing additional evidence that the RCS-NPR is cone mediated.

Effect of Blocking Inner Retinal Responses with NMA, GABA, and Dopamine

The post-photoreceptor origins of the RCS-NPR were examined by applying NMA, GABA, and dopamine by intravitreal injections into 10- to 12-week-old dystrophic RCS rats in which the RCS-NPR constituted most of the photopic ERG. Dark-adapted ERGs were recorded for comparison. NMA eliminated the light-adapted RCS-NPR and the dark-adapted STR at all intensities (Fig. 5; \(n\) = 5), consistent with origins in the proximal retina, as is known for the STR in cat\(^8\) and rat.\(^3\) By suppressing the RCS-NPR and the STR, NMA revealed an underlying b-wave in the photopic and scotopic ERG. Apparently the RCS rat photopic b-wave is partially masked by the larger RCS-NPR at this age. The STR before NMA and the uncovered b-wave after NMA of RCS rats were slower and broader than the photopic responses, further evidence for two separate pathways of origin.

NMA Isolated Contributions to the RCS Rat Photopic ERG

Blocking NMDA-mediated light responses has been used in the rat\(^3\) and the cat\(^8\) to remove the STR selectively and thereby uncover the full amplitude of the dim light scotopic b-wave.\(^3\) Similarly, we sought to use NMA to determine the full ampli-
tude of the photopic b-wave in the absence of the inner retinal contribution in RCS rats at different ages as degeneration progressed. We subtracted the waveform after NMA injection from the waveform before treatment to obtain the isolated inner retinal NMA-sensitive response, as shown in the 12-week-old rat in Figure 8A. Before NMA was applied, the PhNR in congenic controls and the RCS-NPR had similar amplitudes (arrows) and peak times, but the RCS-NPR had a slower recovery. After NMA, the NMA-sensitive components had different amplitudes and time courses. The control response was faster and larger, with most underlying the b-wave, and NMA removed only a portion of the PhNR amplitude (arrows). On the other hand, the dystrophic response was slower and broader, with a significant portion occurring later than the b-wave (vertical dashed lines). NMA removed all the RCS-NPR, and the NMA-sensitive component was larger than the original RCS-NPR at its peak (arrows). It was also much larger relative to the post-NMA b-wave than was the NMA-sensitive component in controls. Plotting the log maximum amplitude and log threshold intensity of the NMA-isolated photopic b-wave and the NMA-sensitive response (Figures 8B, 8C) for 8- to 18-week-old dystrophic RCS rats showed that the latter did decline with age because of the loss of photoreceptor output, as expected, but at a slower rate than the NMA-treated photopic b-wave. Over the 10-week period, the b-wave declined 1 log unit of amplitude (10-fold) more, and the threshold increased 0.5 log (3-fold) more than...
the NMA-sensitive response. NMA (Table 1) increased the b-wave amplitude in RCS dystrophic rats almost 5 times (88 μV/H9262V), whereas the NMA-sensitive response was only about twice as large (49 μV/H9262V) as the original RCS-NPR. Thus, the b-wave and the RCS-NPR each reduced the amplitude of the other, and the bipolar cell and inner retinal responses in the ERG were larger than indicated in the untreated response. However, peak amplitudes of the two responses in the untreated waveform were not simply summed because the b-wave increased 30 μV more than the reduction in the RCS-NPR. Interestingly, the full photopic b-wave in RCS rat at a comparable level of degeneration, as measured by cone counts, was much larger than that in P23H rats.

Kir4.1 Potassium Channel Immunohistochemistry versus Degeneration

Under dark-adapted conditions, the release of potassium from proximal retinal neurons is known to depolarize Müller cells and contribute to the STR origin.25,26 Given the similarities of the large RCS-NPR in the photopic ERG to the enhanced STR that dominates the scotopic ERG of the dystrophic RCS rat, we explored a possible association between K⁺ channels and the unusual growth of the RCS-NPR with age. Kir4.1 channels are one of the principal potassium channels on Müller cells,57,58 and we compared the distribution and density of Kir4.1 channels in RCS and P23H rhodopsin rat retinas using semiquantitative immunohistochemistry. By 14 weeks of age, the RCS dystrophic rats showed an increase in Kir4.1 staining throughout the IPL (Fig. 9A) compared with earlier ages. Müller cell processes in the proximal retina became more prominent with age and were more heavily labeled with anti-Kir4.1 antibody from the endfeet toward the distal retina, suggesting an increase in the numbers of Kir4.1 channels in this region. Kir4.1 labeling was nearly identical in 6- and 10-week-old RCS dystrophic animals, showing an MPI of 31 ± 3 (mean ± SE, n = 8), and it increased to 45 ± 4 MPI (n = 9; P < 0.05) at 14 weeks (Fig. 9C). Kir4.1 labeling colocalized with glutamine synthetase (GS), a marker for Müller cells (Fig. 9A, inset).
Although we cannot rule out the expression of Kir4.1 channels on other elements in the IPL, the high degree of colocalization with GS and the typical appearance of Müller cell processes suggest that Kir4.1 labeling increased on Müller cells with degeneration in the RCS rats. Pigmented Long-Evans control rats showed a small but not statistically significant increase in MPI over this time (32 ± 2 [mean ± SE], n = 7, at 6 and 10 weeks of age vs. 36 ± 4, n = 11, at 14 weeks; P = 0.41) and were not significantly different from RCS rats at any time point. Staining of Kir4.1 channels in the proximal retina did not change over this time in P23H and Sprague-Dawley rats (Figs. 9B, 9D), and only RCS dystrophic rats showed significantly more staining than P23H rats at age 14 weeks (P < 0.01; 2-way ANOVA; Bonferroni posttest). Immunolabeling in the RPE of the P23H rats increased from 13.6 ± 2.9 (mean ± SE, n = 3) at 4 weeks of age to 21.8 ± 4.5 at 8 weeks of age to 31.5 ± 2 at 15 weeks of age; values were significant between 4 and 15 weeks (P < 0.01 Student’s t-test).

**Discussion**

**Origins of the RCS-NPR**

We have characterized an unusual negative response in the corneal photopic ERG of the dystrophic RCS rat that increases in amplitude during degeneration. This response was seen in the RCS rat but not in the P23H rat model of photoreceptor degeneration. It trails the b-wave at early stages but becomes the sole component seen at late stages, when the b-wave is no longer detectable. Along with its resistance to bleach, its increase in amplitude during light adaptation is characteristic of cone pathway responses in humans and mice. Suppression or elimination of the RCS-NPR by NMA, GABA, and dopamine indicates it originates with neuronal activity in the inner retina, and lack of effect of ONS indicates that amacrine cells rather than ganglion cells are the source. Thus, it appears to reflect a unique pathology in cone pathway inner retinal signaling in the RCS rat. Our data show increased Kir4.1 channel immunohistochemical staining on Müller cells in the inner retina of RCS rat, but not P23H rat, at an advanced stage of degeneration, suggesting that increased K⁺ currents may be associated with its development. This is supported by previous studies on inner retinal slow negative responses, such as the STR and M-wave, showing that they are generated by Müller cell K⁺ currents.

Rewiring during degeneration in the RCS rat could potentially generate the increased electronegative potentials we observed. It is apparent from Figure 1 that the waveform of the negative response in the RCS dystrophic response is different from the control response as early as 4 weeks of age. This suggests that some aspects of cone pathway function in the RCS rat may be compromised during the first several weeks. Indeed early abnormalities in postsynaptic neurochemical function and morphology have been demonstrated. The morphologic changes seen in RCS rat were not observed in P23H. In the RCS rat, the development of the debris zone is associated with increased sprouting of bipolar and horizontal cells into this region, perhaps stimulated by the abnormal products of photoreceptor degeneration found there. It is unclear how this would affect downstream signaling as reflected in the ERG. However, but recent computational molecular phenotyping in rodent and human retinal degenerations suggests rod bipolar cell sprouting and ectopic synapse formation with surviving cones can result in “corruptive” circuits in the cone pathway in models where cone survival is extended well beyond rods, as is true in the RCS rat. The P23H rat, on the other hand, is considered a “cone decimating” model that would only transiently form such connections between the cones and the rod bipolar cells.

**The RCS-NPR and the PhNR**

The RCS-NPR has some resemblance to the PhNR of primates, in that it appears after the photopic b-wave and has a similar time to peak. However, the PhNR in primates is generated by ganglion cell activity and is eliminated or greatly reduced by optic nerve damage or TTX, which blocks the activity of spiking neurons. In our study, eliminating most of the ganglion cells by optic nerve section did not affect the amplitude of the RCS-NPR. Because some amacrine cells also exhibit spiking activity, and transsynaptic effects of ganglion cell damage cannot be ruled out, it remains possible that amacrine cells also contribute to the PhNR. This is especially true in the rat, in which approximately 66% and 45% of the PhNR remained 2 weeks and 3 months, respectively, after ONS. This would be consistent with differences between primates and rodents in the origin of STR, and it raises the possibility that part of the PhNR in normal rat originates in the same cells as the RCS-NPR.

**Unmasking the Full b-Wave and Inner Retinal Contributions in RCS and P23H with NMA**

Our NMA results and those of Xu et al. indicate a substantial inner retinal negative contribution, probably from amacrine cells, to the normal rat photopic ERG. Its removal by NMA greatly enhances the b-wave. Because the PhNR is only partially blocked by NMA (Fig. 8) and eliminating the PhNR through suppression of ganglion cell responses does not affect b-wave amplitude, this NMA-sensitive response is not identical with the PhNR and is mostly masked by the b-wave under normal conditions. Our data indicate that, in the RCS rat, the b-wave declines more rapidly, allowing the NMA-sensitive negative response to progressively dominate the ERG. In addition, its waveform is altered by the degeneration, becoming slower and broader (Fig. 8), which contributes to producing a larger post-b-wave negative response.

The large increase in b-wave amplitude after NMA indicates that the b-wave in RCS dystrophic animals is substantially masked by this inner retinal response. Comparing the post-NMA b-wave to the untreated b-wave of P23H rats with a similar number of cones remaining (Table 1) indicates that the bipolar cell response is also much larger in RCS than in P23H rats at a similar stage of degeneration. RCS rat cone pathway might thus function better than indicated by the pre-NMA ERG and better than the cone pathway in P23H at the same stage of degeneration. Alternatively, cone pathway signaling might have been altered by ectopic bipolar cell connections or responses enhanced through Müller cell K⁺ currents. Although masking of the b-wave by an enhanced negative response has not yet been demonstrated for human cases of retinitis pigmentosa, the existence of homologous genetic forms of human disease to RCS and P23H rhodopsin rodent degeneration suggested that some caution might well be appropriate in modeling human disease by recording the b-wave.

The finding of masked waveforms in the ERG uncovered by treatment with NMA should be interpreted cautiously. The presence of an amacrine cell negative feedback loop through GABA receptors on bipolar cells means this treatment has the potential to alter light-induced bipolar cell currents that could affect the b-wave and hence other downstream ERG potentials. A recent study in diabetic rats with altered GABA-induced rod bipolar cell currents found no change in the rod a- and b-waves; however, there was a significant change in flicker amplitude and pattern of inner retinal oscillatory potentials. Despite the potential for alterations in ERG signals, NMA has been used extensively to remove the inner retinal contributions and to isolate the bipolar PI contribution to the ERG.
Similiarity of the RCS-NPR to the Enhanced STR in RCS Dystrophic Rats

The behavior of the RCS-NPR parallels that of the STR in dystrophic RCS rat. Both responses show preserved sensitivity and amplitude across nearly 18 weeks of RCS retinal degeneration and are observed over an expanded stimulus range as the b-wave is progressively reduced. Although the amplitude of each does diminish with age and stage of retinal degeneration, the rate of loss is considerably slower than the b-wave, and they eventually become the predominant waveform of the ERG in the RCS rat by 10 to 12 weeks of age. Further, suppression of both the RCS-NPR in RCS rat and the STR in cat by NMA, GABA, and dopamine suggests that the RCS-NPR originates in the same cell types as the STR, though the RCS-NPR is cone driven, whereas the STR is rod driven.

Possible Role of Glial Cell Potassium Channels in Producing the RCS-NPR

Evidence implicates K+ release in the IPL in generating the STR and photopic inner retinal potentials. Furthermore, Mattig et al. showed that in isolated RCS retinas, even a small remaining degree of signaling from the degenerating photoreceptors yields considerable neuronal activity and K+ increase in the proximal retina. Because of the similarities between the RCS-NPR and the STR, we looked for an association between the relative increase in RCS-NPR and changes in K+ current generators. We concentrated on Kir4.1 channels because these have been implicated as principal channels in K+ regulation in the proximal retina.

We found an increase in immunohistochemical staining of Kir4.1 as degeneration progressed to late stages in the RCS rat, possibly because of Muller cell hypertrophy and, hence, to an increase in Muller cell membrane area or to a net increase in Kir4.1 channel density on Muller cell membranes. Kir4.1 channels allow K+ to move from the extracellular space through the Muller cell and to reach the vitreous, which acts as a K+ sink. Increasing the number of K+ channels may enhance current flow. Hence, even in the face of decreased photoreceptor responses in the distal retina, a decrease in K+ current would not be as great as expected. This could explain the prominence of both the STR and the RCS-NPR when other ERG potentials of the outer and mid retina decline with degeneration. The opposite scenario in the P23HR rats, in which PhNR and other inner retinal potentials diminish and the Kir4.1 channel labeling is not increased, lends further support to the concept that Kir4.1 channels are involved in RCS-NPR generation.

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References


