Remaining Rod Activity Mediates Visual Behavior in Adult *Rpe65*$^{-/-}$ mice.

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Purpose. C57/Bl6, *Cpfl1*$^{-/-}$ (cone photoreceptors function loss 1; pure rod function), *Gnat1a*$^{-/-}$ (rod α-transducin; pure cone function), and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ double-knockout mice were studied to distinguish the respective contributions of the different photoreceptor (PR) systems that enable light perception and mediate a visual reflex in adult *Rpe65*$^{-/-}$ mice, with a simple behavioral procedure.

Methods. Visual function was estimated using a rotating automated optomotor drum covered with vertical black-and-white stripes at spatial frequencies of 0.025 to 0.5 cycles per degree (cyc/deg) in both photopic and scotopic conditions. Mouse strains with different luminances were tested to evaluate the contribution and the light-intensity threshold of each PR system.

Results. Stripe rotation elicited head movements in the wild-type (WT) animals in photopic and scotopic conditions, depending on the spatial frequency, whereas the *Cpfl1*$^{-/-}$ mice show a reduced activity in the photopic condition and the *Gnat1a*$^{-/-}$ mice an almost absent response in the scotopic condition. A robust visual response was obtained with *Rpe65*$^{-/-}$ knockout mice at 0.075 and 0.1 cyc/deg in the photopic condition. The residual rod function in the *Rpe65*$^{-/-}$ animals was demonstrated by testing *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice that present no response in photopic conditions.

Conclusions. The optomotor test is a simple method of estimating the visual function and evaluating the respective contributions of rod and cone systems. This test was used to demonstrate that in *Rpe65*$^{-/-}$ mice, devoid of functional cones and of detectable 11-*cis*-retinal protein, the rods mimic cone function in part, by mediating vision in photopic conditions. (*Invest Ophthalmol Vis Sci.* 2010;51:6835–6842) DOI:10.1167/iovs.09-3870

Mutations in the *Rpe65* gene are responsible for certain cases of Leber congenital amaurosis (LCA) and retinitis pigmentosa (RP).[1,2] These diseases induce dramatic visual dysfunction starting in early childhood, although photoreceptors remain present in the retina for many years, and, depending on the mutation, develop beyond adolescence.[3–6] The RPE65 protein is expressed inside the retinal pigment epithelial (RPE) cells and is necessary for the regeneration of 11-*cis*-retinal, the ligand of rod and cone opsins. As a regulator of the visual cycle, RPE65 allows the onset of the phototransduction cascade by providing the chromophore that captures the photon energy and thus maintains normal vision.[7] *Rpe65*$^{-/-}$ mice are deprived of rod photopigments and show impaired rod physiology soon after birth.[7] No measurable amount of rhodopsin or 11-*cis*-retinal, both prerequisites for rod function, is detected in *Rpe65*$^{-/-}$ retinas with standard protocols, which explains their lack of detection in earlier studies.[7,8] However, *Rpe65*$^{-/-}$ mice express the opsin apoprotein in the rod outer segments,[7] and there is evidence that rod and cone responses can be recorded in the young *Rpe65*$^{-/-}$ mice. In contrast to cones,[9] rods degenerate very slowly in the absence of the chromophore.[10]

To determine whether the remaining rods can support vision in *Rpe65*$^{-/-}$ mice, we compared these mice with other well-characterized models featuring a selective mutation and dysfunction of rods, or cones, or both. *Gnat1a* $^{-/-}$ mice carry a mutation in the α-subunit of the rod transducin 1 gene, leading to a loss of rod function, with almost no rod cell loss.[11] In contrast, *Cpfl1*$^{-/-}$ mice bear a mutation in the cGMP-phosphodiesterase α-subunit (Pde-6C), a gene specifically expressed in the cone photoreceptors. Although cones still physically exist at the ages of 1 and 2 months, the genetic defect causes failure of the cone phototransduction cascade. However, the rod function remains intact until at least 18 months of age in this strain.[12] In *Rho*$^{-/-}$ mice, the rods do not express opsin protein. In consequence these mice completely lack rod-mediated light responses and develop rod photoreceptors without outer segments.[13–15] In contrast, cones develop normally with the adequate function in the beginning of adulthood (before the cone degeneration induced by the loss of rods).[13–15] In these mice, the cone function is abolished.[16] Furthermore, ERG recordings have demonstrated that the remaining retinal activity in the young adult *Rpe65*$^{-/-}$ retina results from the rod function[17] and persists, even in old animals.[18] Thus, it seems that the lack of RPE65 enables rods to mimic certain cone functions by responding under lighting conditions that normally isolate cones.[17] These results indicate that the rod system generates an electrical activity, but there is no evidence that, in this model, this system can drive a stimulus perceived by the brain, such as an optomotor response (OR). We sought to answer this question by examining double-knockout *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice.

Among various methods that can attest to visual function, the optokinetic reflex reflects the activity of subcortical areas in mice,[19,20] and an independent testing of the two eyes is possible simply by controlling the direction of the drum rotation without having to use occluders or suturing eyelids.[19] The optomotor test allows easy recording of the head movement component of this reflex; this procedure has been shown to be

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efficient in estimating visual acuity in commonly used wild-type mice (C57/BL/6J, 129/SvPas, and CD1) and visually deficient mice: C3HeB/FeJ (*Rd1*) and *Rho*$^{-/-}$;*Cnga3*$^{-/-}$.[19,21–24] Because C57/Bl6 mice showed significant variability between males and females,[22] we decided to use only male animals. In addition, since changes in intensities reflect the transition from rod to cone vision,[25] we tested the visual acuity at different luminances in wild-type and mutant mice lacking rods, cones, or both, to evaluate whether rods allow the formation of a light-sensitive response and are sufficient to mediate visual function in *Rpe65*$^{-/-}$ mice. In the present study, we show that the rod photoreceptor system mediates OR in *Rpe65*$^{-/-}$ mice.

Methods

Animals

The animals were handled in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and approved protocols of the local institutional committee (Service Vétérinaire du Canton de Vaud, Lausanne, Switzerland). The mice were bred and housed in communal cages (five to six animals per cage), maintained in controlled temperature (22°) and light (12-hour light–dark cycle), with free access to food and water. The behavioral study included wild-type C57/Bl6 mice (provided by Charles River, Lyon, France), *Gnat1a*$^{-/-}$ mice (provided by Janis Lem, Tufts University, Boston, MA), *Cpfl1*$^{-/-}$ mice (provided by Bo Chang, The Jackson Laboratory, Bar Harbor, ME), *Rpe65*$^{-/-}$ mice (provided by T. Michael Redmond, National Eye Institute, Bethesda, MD), and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice (provided by Christian Grimm, Laboratory of Retinal Cell Biology, University of Zürich, Zürich, Switzerland; $n = 5$ males per group). Ages of the tested animals ranged from 4 to 8 weeks.

Behavioral Tests

The experimental setup consisted of a central platform (10-cm diameter) surrounded by a vertical drum (46-cm diameter) rotating at a velocity of nine rotations per minute. The wall of the drum was covered with white and black vertical stripes at various spatial frequencies, or with a uniform white and then black setting without stripes, to evaluate the baseline anxiety and the OR threshold of each strain tested (Fig. 1).

The animals were tested with spatial frequencies increasing from 0.025 to 0.5 cycles per degree (cyc/deg): 0.025, 0.05, 0.075, 0.1, 0.2, 0.3, 0.4, and 0.5 cyc/deg. Behavior was recorded for 4 minutes in each test session with a camcorder that enabled infrared recording in scotopic conditions (CDR-HC90E; Sony, Tokyo, Japan). During each session, the drum rotated alternately clockwise and counterclockwise, with the rotation direction inverted at mid session. The drum was lighted by a white LED, placed above the cylinder. Neutral-density filters were superimposed to obtain light intensities ranging from 0.01 to 1750 lux, measured on the platform center, with the following parameters: 0.01, 0.15, 0.6, 3.2, 18.5, 150, and 1750 lux.

Procedure

In the first step, the animals were trained by handling the day before the task, to reduce baseline anxiety and to adapt the mice to the setup. Two successive sessions with background settings were run without recording, and the animals were returned to their communal cages in the animal facility. The tests were first performed in photopic conditions with a light intensity of 650 lux, measured with an illuminance meter (T-10; Konica-Minolta, Osaka, Japan). The mouse was placed on the platform, allowed to move freely, and adapted during 1 minute to the setup before the test session. Then, the sessions were repeated with each spatial frequency. For scotopic conditions, the mice were dark-adapted overnight, and tests were performed under dim illumination (<0.01 lux). The procedure was the same as for photopic conditions. Each animal was videotaped, and head movements were then scored manually. Clockwise movement drives tracking through the left eye, whereas counterclockwise motion activates the right eye.[19] A movement was counted when the animal followed the drum rotation at the same angular speed and in the same direction. We considered a one-movement event to be when the head axis (frontal-occipital) clearly rotated by minimum 15°. We used the same quantification method to evaluate the background. All movements slower or faster than drum speed were not taken into account. We checked first the response obtained with the left and then the right eye and observed no significant differences between eyes. In consequence, the ORs from the clockwise and counterclockwise stimuli were then pooled.

Figure 1. Optomotor drum, designed to estimate visual acuity for the behavioral experiment. To test OR, the mice were placed individually on the platform in the center of the rotating drum, and results were videotaped. The pattern of vertical stripes was made by plastic foil attached to the drum.

In the *Rpe65*$^{-/-}$ mice, the first tested eye (left) did not respond as well as the second (right) eye, thus precluding pooling of the results. This finding could be explained by the fact that individuals with low vision need a longer time to adapt. To abolish these disparities, we performed test sessions of 6 minutes in the mice, changing the direction of the drum every 2 minutes, and we collected data during the last 4 minutes only. In this condition, we observed no significant differences between eyes.

Tissue Processing and Immunolabeling

For immunohistochemical analyses, *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice were killed at 4 weeks of age after optomotor recordings. The eyes were cauterized for orientation indication, enucleated, fixed in 4% paraformaldehyde, rinsed in PBS, cryoprotected in 30% sucrose overnight, embedded in albumin from hen egg whites (Fluka, Buchs, Switzerland), and cut into 14-μm cryostat sections. Immunohistochemistry was performed as described previously.[26] Briefly, the first antibody was incubated overnight at 4°C in PBS containing 0.2% TritonX-100 and 10% normal horse serum. Sections were then washed and incubated with the secondary antibody at 37°C for 1 hour. The following primary antibodies were used: goat polyclonal blue-sensitive opsin (N20; 1/1000) and rabbit polyclonal GNAT2 (1:100; both from Santa Cruz Biochemicals, Santa Cruz, CA). For visualization, fluorescence-labeled secondary antibodies (AlexaFluor 488 and AlexaFluor 633; Molecular Probes, Eugene, OR) were used. Sections were counter-

stained with DAPI (Molecular Probes) and mounted under coverslips (Mowiol 4-88 Reagent; VWR International AG, Lucerne, Switzerland).

Data Analysis and Statistics

We measured the average between the results found in both white and black settings (background) for each mouse strain, and the mean value was carried over in the statistical analysis to determine whether the number of head movements (at spatial frequency settings) was significant compared with the background. The data were analyzed by one-way, repeated-measures ANOVA (Prism 5.0 Software; GraphPad Software Inc., San Diego, CA). The Turkey and Dunnett tests were used for post hoc analysis. A level of $P < 0.05$ (two-tailed) was considered significant.

Sections were analyzed on a microscope equipped for epifluorescence (model BX60; Olympus Suisse SA, Aigle, Switzerland) and coupled to software (Cell-P; Soft Imaging System, Olympus, Hamburg, Germany). To quantify the number of cells positive for GNAT2 and S-opsin, we counted the number of outer segments that stained positive throughout the retina of the most transverse section containing the optic nerve. Histologic counting of cone markers was analyzed by one-way ANOVA (Prism 5.0; GraphPad Software, Inc.) to determine the statistical significance between *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice.

Results

Background Noise

We first investigated whether head movements can be elicited without the presence of a contrasting decor, resulting in a background threshold that allows discrimination between spontaneous activity (background noise) and visual acuity of mice induced by striped patterns. In photopic conditions, the C57/Bl6, *Cpfl1*$^{-/-}$, and *Rpe65*$^{-/-}$ mice showed similar background noise. In contrast, activities of the *Gnat1a*$^{-/-}$ (1.2 ± 0.2 mov/min) and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ (1.3 ± 0.27 mov/min) mice were significantly higher than those in the C57/Bl6 ($P < 0.01$) animals, indicating that spontaneous activity was increased in these strains (Fig. 2). Under scotopic conditions, we showed similar results. The background thresholds of the

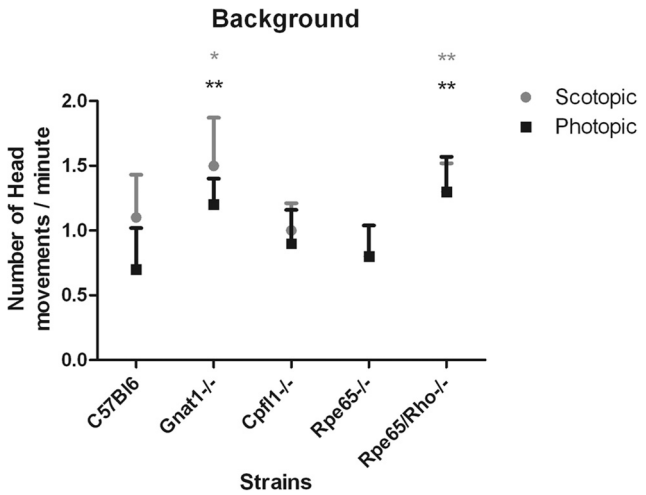

Figure 2. Background quantification: the number of head movements per minute (means ± SE; $n = 10$ per group) of C57/Bl6, *Gnat1a*$^{-/-}$, *Cpfl1*$^{-/-}$, *Rpe65*$^{-/-}$;*Rho*$^{-/-}$, and *Rpe65*$^{-/-}$ mice without a stripe pattern in photopic and scotopic conditions. Bars represent the mean ± SEM ($n = 10$ per group). Wild-type C57/Bl6 mouse was used as reference and statistical analysis compared mutants versus C57/Bl6 mice. Strain-related differences in the background are observed for *Gnat1a*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice in both conditions. One-way ANOVA and Turkey post hoc tests: $^{*}P < 0.05$, $^{**}P < 0.01$.

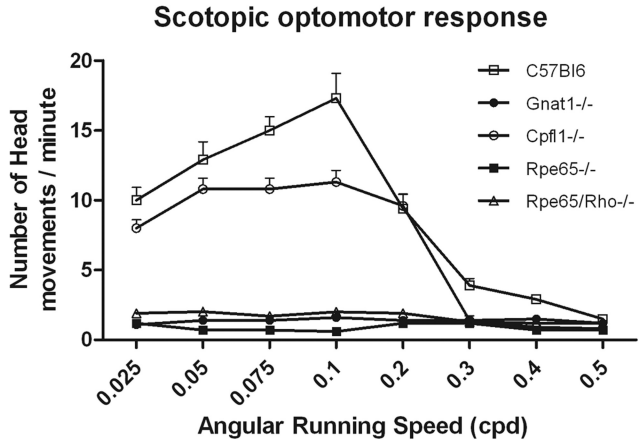

Figure 3. ORs in scotopic conditions: Results are expressed as the number of head movements per minute for C57/Bl6, *Gnat1a*$^{-/-}$, *Cpfl1*$^{-/-}$, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice. The results are expressed as mean ± SEM ($n = 10$ per group).

Gnat1a$^{-/-}$ (1.5 ± 0.37 mov/min) and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ (1.3 ± 0.22 mov/min) mice were significantly higher than those observed in the C57/Bl6, *Cpfl1*$^{-/-}$, and *Rpe65*$^{-/-}$ mice (Fig. 2). These discrepancies between strains could be explained by a different level of baseline anxiety between these strains, or by a change in behavior due to severe rod function loss. The latter hypothesis suggests that rods are necessary to establish a normal neuronal network with the vestibular system. Such elevation of the head movement number could thus be related to the nystagmus phenomenon observed in patients showing early and severe vision loss.

OR in Scotopic Conditions

To estimate the visual acuity in scotopic conditions (<0.01 lux), we tested stripes with spatial frequencies ranging from 0.025 to 0.5 cyc/deg (Fig. 3, Table 1). As previously demonstrated,[22–24] the C57/Bl6 mice showed clear head movements in scotopic conditions between 0.025 and 0.2 cyc/deg. ORs in the C57/Bl6 mice reached maximum at 0.1 cyc/deg (17.3 ± 1.78 mov/min), and the visual reflex was limited to 0.3 cyc/deg ($P < 0.05$). Although the number of head movements per minute was reduced in the *Cpfl1*$^{-/-}$ mice, the response range was comparable, being at its maximum at 0.1 cyc/deg (11.3 ± 0.83 mov/min), but the responses were significantly lower than those observed in the C57/Bl6 animals at 0.075 and 0.1 cyc/deg ($P < 0.001$). When spatial frequencies were higher than 0.2 cyc/deg, responses were equivalent to background values ($P > 0.05$). In contrast, no OR was scored in the *Gnat1a*$^{-/-}$ mice, which show cone function only. The values were similar to those obtained with background settings ($P > 0.05$), with the maximum number of head movements never exceeding 1.6 ± 0.10 mov/min at 0.1 cyc/deg. These results indicate that rods are essential in mediating ORs in scotopic conditions.

Optomotor Response in Photopic Conditions

A similar approach was undertaken in photopic conditions (650 lux), to reveal the respective contributions of rods and cones to the OR (Fig. 4, Table 2). The C57/Bl6 mice showed head movements between 0.025 and 0.3 cyc/deg, with the maximum response at 0.1 cyc/deg (12.5 ± 0.68 mov/min). At 0.4 and 0.5 cyc/deg, the OR decreased and became identical with that observed at background settings ($P > 0.05$). The *Gnat1a*$^{-/-}$ mice behaved similarly to the C57/Bl6 control animals when they were placed in photopic conditions, with a

TABLE 1. Relative Responses in Wild-Type and Knockout Mice in Scotopic Conditions

Strain	Cycles per Degree								Background Value
	0.025	0.05	0.075	0.1	0.2	0.3	0.4	0.5	
C57/B16	10.0 ± 0.91***	12.9 ± 1.29***	15.0 ± 0.97***	17.3 ± 1.78***	9.4 ± 1.03***	3.9 ± 0.50*	2.9 ± 0.28	1.5 ± 0.15	1.1 ± 0.33
Gnat1α$^{-/-}$	1.1 ± 0.10	1.4 ± 0.14	1.4 ± 0.11	1.6 ± 0.10	1.4 ± 0.13	1.4 ± 0.20	1.5 ± 0.17	1.2 ± 0.15	1.5 ± 0.37
Cpfl1$^{-/-}$	8 ± 0.59***	10.8 ± 0.78***	10.8 ± 0.77***	11.3 ± 0.83***	9.6 ± 0.84***	1.4 ± 0.32	0.9 ± 0.05	0.8 ± 0.11	1.0 ± 0.21
Rpe65$^{-/-}$	1.2 ± 0.21	0.7 ± 0.17	0.7 ± 0.13	0.6 ± 0.10	1.2 ± 0.22	1.2 ± 0.21	0.7 ± 0.17	0.7 ± 0.13	0.8 ± 0.24
Rpe65$^{-/-}$; *Rho*$^{-/-}$	1.9 ± 0.19	2.02 ± 0.15	1.7 ± 0.15	2.01 ± 0.17	1.9 ± 0.17	1.3 ± 0.07	1.2 ± 0.08	1.2 ± 0.08	1.3 ± 0.22

Number of head movements per minute (mean ± SEM; $n = 10$ per group) at different spatial frequencies was measured for in-type (C57/B16) and mutant mice lacking functional rods, cones, or both (*Gnat1α*$^{-/-}$, *Cpfl1*$^{-/-}$, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$; *Rho*$^{-/-}$). Strain background was used as the reference to determine the OR for each strain.

* Differs significantly from the values of the corresponding strain background. Strain-related differences were observed: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, by one-way, repeated-measures ANOVA and Dunnett post hoc tests.

maximum response observed at 0.2 cyc/deg (13 ± 0.84 mov/min). There was no major strain-related difference in the ORs compared with the C57/Bl6 control animals between 0.025 and 0.2 cyc/deg ($P > 0.05$), except at 0.3 cyc/deg ($P < 0.01$), which induced a more pronounced activity in the *Gnat1a*$^{-/-}$ mutants. With frequencies higher than 0.3 cyc/deg, no response was detected, and the number of head movements was not significant in regard to the baseline threshold. Compared with the C57/Bl6 and *Gnat1a*$^{-/-}$ mice, the *Cpfl1*$^{-/-}$ cone-deficient mice were significantly less sensitive and less stimulated in photopic conditions between 0.025 and 0.3 cyc/deg ($P < 0.001$). However, the *Cpfl1*$^{-/-}$ mice showed an OR significantly higher than background values ($P < 0.01$), and their OR was limited to 0.2 cyc/deg with a maximum OR obtained for 0.05 cyc/deg (5.4 ± 0.49 mov/min). These results suggest that cones are essential to properly induce OR with bright-light stimuli.

OR in *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ Mice

We measured ORs in *Rpe65*$^{-/-}$ mice at 8 weeks of age, when almost no more cones remained.[9] Contrary to the C57/Bl6 and *Cpfl1*$^{-/-}$ mice, the *Rpe65*$^{-/-}$ animals did not display a visual response under scotopic conditions. From 0.025 to 0.5 cyc/deg, we did not observe any significant difference in the *Rpe65*$^{-/-}$ responses compared with those at background settings or the *Gnat1a*$^{-/-}$ responses (Fig. 3, Table 1), indicating that *Rpe65*$^{-/-}$ mice are not responsive to dim stimuli in comparison to pure rod *Cpfl1*$^{-/-}$ or C57/Bl6 animals. Similarly, no OR was detected in the *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice under scotopic conditions (Fig. 3, Table 1). Scores obtained between 0.025 and 0.5 cyc/deg were analogous and did not significantly differ from background data. This absence of OR is consistent with the lack of rod function in *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mutants.

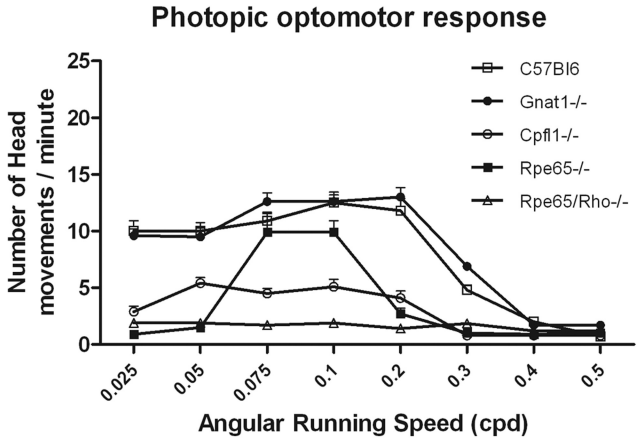

FIGURE 4. ORs in photopic conditions: Results are expressed as the number of head movements per minute for C57/Bl6, *Gnat1a*$^{-/-}$, *Cpfl1*$^{-/-}$, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice. Results are expressed as mean ± SEM ($n = 10$ per group).

Under photopic conditions, the *Rpe65*$^{-/-}$ strain showed a significant OR for 0.075, 0.1, and 0.2 cyc/deg (respectively, 9.9 ± 1.62, 9.9 ± 1.01, and 2.7 ± 0.50 mov/min; Fig. 4, Table 2). At 0.075 and 0.1 cyc/deg, no obvious difference in response was observed compared with those of the C57/Bl6 and *Gnat1a*$^{-/-}$ mice. At 0.2 cyc/deg, the OR decreased significantly compared with that of C57/Bl6 or *Gnat1a*$^{-/-}$ animals, but was still significant compared with the baseline level ($P < 0.05$). Nevertheless, when spatial frequencies were lower than 0.075 cyc/deg and greater than 0.2 cyc/deg, scores of the *Rpe65*$^{-/-}$ mice were analogous to the baseline threshold. These results indicate that, under photopic conditions, the OR of the *Rpe65*$^{-/-}$ mice was limited to a range of spatial frequencies between 0.075 and 0.2 cyc/deg.

To assess whether the photopic OR recorded in the *Rpe65*$^{-/-}$ mice was driven by rods, we investigated the ORs in 4- and 8-week-old double-knockout *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice. At 4 and 8 weeks after birth under photopic conditions, there were no differences in the ORs between the *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ age-matched animals and background values (Figs. 4, 5; Table 2). We next analyzed the expression of cone-specific markers in 4-week-old *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ animals, an age at which the mouse retina can be considered mature, to verify that the absence of OR in the double-knockout was not due to a difference in the number of cones compared with those of the *Rpe65*$^{-/-}$ mice. We compared the retinal morphology and the expression of SWL-opsin in 4-week-old *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice by immunostaining (Fig. 6) and did not observe significant differences in the expression pattern of SWL-opsin. In both strains, SWL-opsin expression was mislocalized to the axon and cone pedicle, indicating that there is no major difference in SWL-opsin expression and localization (Fig. 6). In addition, we quantified the number of cells positive for the cone opsin short wavelength (SWL-opsin) and the cone-specific transducin α-subunit (GNAT2), and it appeared that it was similar in both strains (Fig. 7). These results indicate that even if some cones were present in the *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ animals, they did not mediate visual behavior.

Rod–Cone System Responses

To specify the ranges of rod and cone sensitivity and their contributions to visual acuity under various light intensities, we used a spatial frequency of 0.1 cyc/deg corresponding to

TABLE 2. Relative Responses in Wild-Type and Knockout Mice in Photopic Conditions

Strain	Cycles per Degree								Background Value
	0.025	0.05	0.075	0.1	0.2	0.3	0.4	0.5	
C57/B16	10.0 ± 0.91***	10.0 ± 0.75***	10.9 ± 0.75***	12.5 ± 0.68***	11.8 ± 1.06***	4.8 ± 0.44***	2.0 ± 0.26	0.7 ± 0.11	0.7 ± 0.32
Gnat1α$^{-/-}$	9.6 ± 0.59***	9.5 ± 0.78***	12.6 ± 0.77***	12.6 ± 0.83***	13.0 ± 0.84***	6.9 ± 0.32**	1.7 ± 0.05	1.7 ± 0.11	1.2 ± 0.20
Cpfl1$^{-/-}$	2.9 ± 0.47*	5.4 ± 0.49**	4.5 ± 0.44**	5.1 ± 0.65**	4.1 ± 0.61*	0.8 ± 0.11	0.8 ± 0.10	0.8 ± 0.08	0.9 ± 0.26
Rpe65$^{-/-}$	0.9 ± 0.17	1.5 ± 0.38	9.9 ± 1.62***	9.9 ± 1.01***	2.7 ± 0.50*	1.0 ± 0.11	0.9 ± 0.10	1.0 ± 0.10	0.8 ± 0.24
Rpe65$^{-/-}$; *Rho*$^{-/-}$	1.9 ± 0.19	1.9 ± 0.06	1.7 ± 0.15	1.9 ± 0.08	1.4 ± 0.08	1.85 ± 0.07	1.2 ± 0.08	1.2 ± 0.08	1.3 ± 0.27

Number of head movements per minute (mean ± SEM; $n = 10$ per group) at different spatial frequencies was measured in wild-type (C57/B16) and mutant mice lacking functional rods, cones, or both (*Gnat1α*$^{-/-}$, *Cpfl1*$^{-/-}$, *Rpe65*$^{-/-}$, and *Rpe65*$^{-/-}$; *Rho*$^{-/-}$). Strain background was used as the reference to determine the OR for each strain.

* Differs significantly from the values of the corresponding strain background. Strain-related differences were observed: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, by one-way, repeated-measures ANOVA and Dunnett post hoc tests.

the most efficient stimulus for most strains tested for both photopic and scotopic conditions (Fig. 8). As in the C57/Bl6 mice, the ORs were measured in dim light conditions (0.01 lux) as well as under intense illumination (1750 lux), with a maximum of head movements at 3.2 lux (13.96 ± 1.44 mov/min). In the *Gnat1a*$^{-/-}$ mice, a visual response was detected for light intensities of 3.2 lux (8.1 ± 0.90 mov/min) and above, suggesting that the activation of the cone system starts at this intensity. The maximum response occurred between 18.5 lux (15.7 ± 0.77 mov/min) and 150 lux (15.8 ± 1.20 mov/min), corresponding to the intensity for which most cones were recruited. When the intensity reached 1750 lux, the OR decreased (11.5 ± 0.87 mov/min). Inversely, in *Cpfl1*$^{-/-}$ mutant mice, rod photoreceptors were activated and contributed to constant OR between 0.01 and 18.5 lux, with a maximum OR recorded at 0.15 lux (11.6 ± 1.10 mov/min). At 150 lux, the response decreased (6.4 ± 0.63 mov/min) but was still significant compared with background ($P < 0.001$). Even at 1750 lux, a weak OR was observed (3.7 ± 0.31 mov/min; $P < 0.05$). Concerning the *Rpe65*$^{-/-}$ model, the mice responded significantly only when the light intensity reached 150 lux (6.4 ± 1.61 mov/min). Thus, the ORs of the *Rpe65*$^{-/-}$ mice did not match those of the *Cpfl1*$^{-/-}$ mutants, whose rods functioned between 0.01 and 18.5 lux, indicating that the *Rpe65*$^{-/-}$ rods were less sensitive and unable to respond at intensities lower than 150 lux. Finally, we observed that at 1750 lux, the maximum intensity applied in our experiment, the *Rpe65*$^{-/-}$ ORs were higher but quite similar to those of the C57/Bl6 and *Gnat1a*$^{-/-}$ mice ($P > 0.05$).

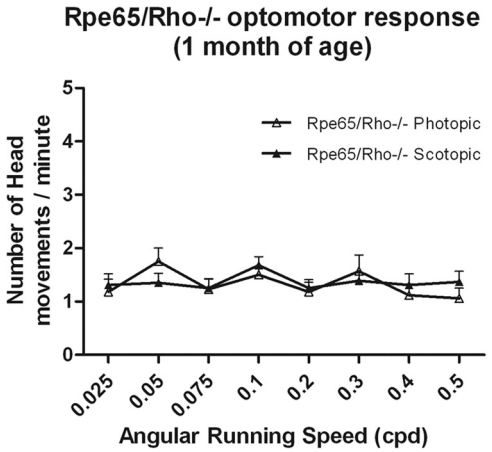

FIGURE 5. *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mouse OR at 1 month of age. ORs were quantified under photopic and scotopic conditions and the results expressed a the number of head movements per minute (mean ± SEM; $n = 10$ per group).

DISCUSSION

Visual Acuity in Wild-Type Mice and in Mice Lacking Rod (*Gnat1a*$^{-/-}$) or Cone (*Cpfl1*$^{-/-}$) Functions

The present findings indicate that the behavioral optomotor test is useful for estimating visual acuity and discriminating rod and cone contributions to vision. Previous studies and data have demonstrated that the optomotor test is a robust and efficient behavioral technique to assess vision in such species as zebrafish,[27,28] chickens,[29] and mice.[19,21–24] Furthermore, the optomotor test provides an easy, noninvasive method of exploring the visual function in mice. In the current work, vision in the C57/Bl6 mice in both photopic and scotopic conditions is consistent with that in the study by Schmucker et al.[23] who estimated the highest sensitivity at 0.3 cyc/deg. Similar to the results found by Umino et al.,[24] the maximum number of responses was detected for spatial frequencies of the same range: from 0.025 to 0.2 cyc/deg (0.03–0.1 cyc/deg in Umino et al.,[24] who used an automated system for speed and size of the rotating bands and for recording).

It is interesting to note that the scotopic OR was higher than the photopic OR. Mice are nocturnal, rod-dominant animals (97% of the photoreceptors),[30] which could explain these variations in visual behavior in bright and dim illumination conditions. The *Gnat1a*$^{-/-}$ pure cone mice behaved similarly to the C57/Bl6 mice under photopic conditions but were unable to elicit head movements in scotopic conditions. A similar sensitivity to spatial frequency under photopic conditions was also described for these two mouse models.[24] These data are in line with the results obtained with ERG recordings and an OR study of other mouse models of pure cone function—the *Rho*$^{-/-}$ and *Cngb1*$^{-/-}$ mice,[17,23,31]—showing that the visual function of these animals is limited to the photopic range. The *Cpfl1*$^{-/-}$ mice (pure rod model) are able to respond to the test in both photopic and scotopic conditions. However, ORs were significantly reduced compared with the C57/Bl6 and *Gnat1a*$^{-/-}$ scores in photopic conditions indicating that cones are essential to respond properly to light stimuli and to provide daylight vision. This result also shows that rods can mediate some visual behavior in photopic conditions. Similarly, Schmucker et al.,[23] using an optomotor test, found that *Cnga3*$^{-/-}$ cone-deficient mice display visual acuity in photopic conditions. These results are in part in contrast to

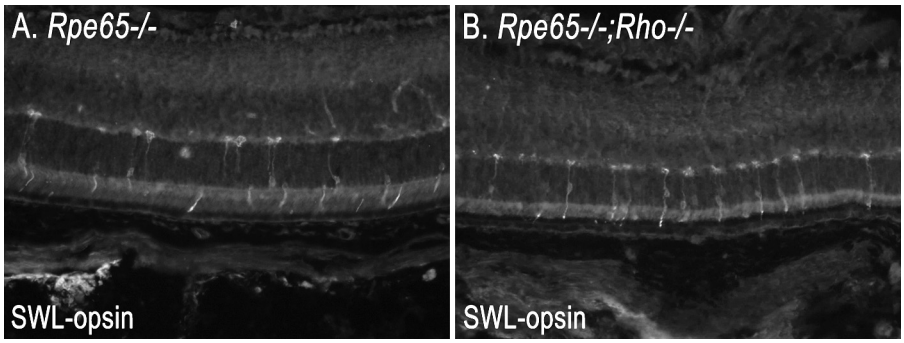

FIGURE 6. Localization of cone SWL-opsin: Retinal sections of 1-month-old mice were stained with antibodies against SWL-opsin in $Rpe65^{-/-}$ and $Rpe65^{-/-}$;$Rho^{-/-}$ retinas. Both strains showed that the S-opsin was mislocalized to the axons and cone pedicles.

those of Umino et al.[24] who showed that $Gnat2^{Cpfl3}$ mice (rod vision) cannot see above -2.0 log cd m^{-2}.

Contributions of Rods and Cones in Different Levels of Light

Our study also specifies the ranges of rod and cone sensitivity and their contributions to visual acuity under various light intensities. In vision-deficient mice, the activation thresholds of the two different photoreceptor systems were determined. According to the results obtained, we stipulate that the threshold of the cone system is at 3.2 lux and reaches the maximum level at 150 lux. $Cpfl1^{-/-}$ mice were able to respond to the test in both photopic and scotopic conditions. However, ORs were significantly reduced at 0.6 lux compared with the C57/Bl6 and $Gnat1a^{-/-}$animals and declined significantly, with higher intensities showing that cones are essential to photopic vision, even though rods can mediate some visual behavior under this condition. Supporting these observations, the $Cpfl1^{-/-}$ ERG recordings indicate that the photopic responses measured are due to rods that are not completely bleached.[12] Thus, we stipulate that particularly high intensities (>1750 lux) are necessary to desensitize the system and to completely abolish rod responses. These results are in agreement with previous works evaluating the retinal activity and rod and cone contributions to OR.[17,18,23,24] Combination of the different results obtained shows that rods mediate an appropriate visual function in mice at luminance levels of less than 18.5 lux (cf., $Cpfl1^{-/-}$ results), defining the scotopic range, whereas cone contribution occurs at luminance levels above 3.2 lux (cf., $Gnat1a^{-/-}$ results), specifying the photopic range. In consequence, a visual reflex can be activated from 0.01 to 1750 lux (at least) and cones and rods work together from 3.2 lux and higher until the rod system is bleached (>1750 lux).

Rod Activity and Visual Behavior in $Rpe65^{-/-}$ Mice

The purpose of this work was to raise the question of rod- and cone-respective contributions to visual behavior in $Rpe65^{-/-}$ mice. The OR was limited from 0.075 to 0.2 cyc/deg only under photopic conditions and the $Rpe65^{-/-}$ mice started to respond significantly at 150 lux. The $Rpe65^{-/-}$ mice had an OR only at high light intensity, suggesting that either cones respond to these stimuli, but with a lower sensitivity, or cone reaction is absent with a strong decrease of rod sensitivity. It is interesting to remember that children affected by the disease are photophilic. Indeed, both patients and $Rpe65^{-/-}$ mice have a minute amount of chromophore[30] that binds to rare opsin. In consequence, only a high flux of photons can stimulate photoreceptors. In accordance with our results, Aleman et al.[32] indicate that $Rpe65^{-/-}$ mice show a severe impairment of the transient pupillary light reflex (TPLR) compared with the $Rpe65^{+/+}$ mice, but responses elicited with much higher-intensity stimuli in the $Rpe65^{-/-}$ mice have properties similar to those evoked by lower intensities in control $Rpe65^{+/+}$ mice. Because it has been shown that no cones are left at this age[9] and the double-knockout $Rpe65^{-/-}$;$Rho^{-/-}$ mice were not

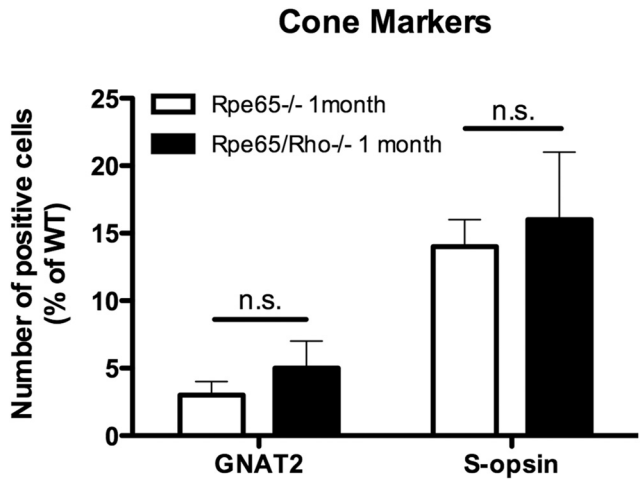

FIGURE 7. Cone marker quantification: quantification of the number of positive cells expressing GNAT2 and SWL-opsin in $Rpe65^{-/-}$ and $Rpe65^{-/-}$;$Rho^{-/-}$ mice at 4 weeks of age (mean ± SEM; $n = 4$ per group).

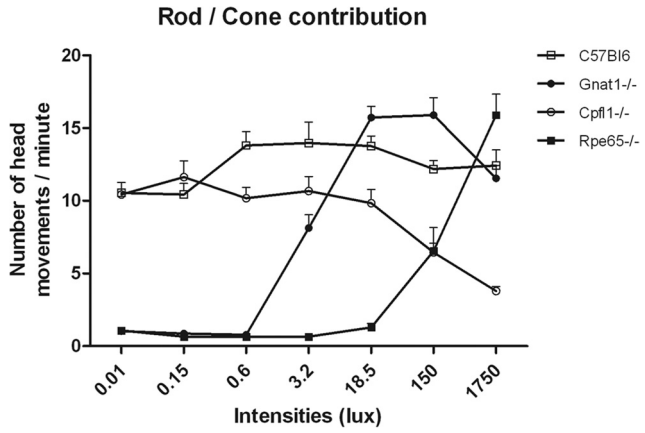

FIGURE 8. Evaluation of rod and cone system contributions: Quantification of the ORs was measured at 0.1 cyc/deg as a function of illumination intensity (from 0.01–1750 lux) and is expressed in the number of head movements per minute in C57/Bl6, $Gnat1a^{-/-}$, $Cpfl1^{-/-}$, and $Rpe65^{-/-}$ mice (mean ± SEM;($n = 10$ per group). Results represent the contribution of rod–cone systems. Statistical analysis: one-way, repeated-measures ANOVA and Dunnett post hoc tests.

able to respond in photopic conditions in the present study, *Rpe65*$^{-/-}$ ORs can be attributed to the rod system, demonstrating that RPE65 deficiency may affect cones more intensely than rods. Furthermore, the loss of function in the *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice was not associated with changes in the retinal morphology, and the number of cells positive for cone markers (GNAT2 and SWL-opsin) remains the same between the age-matched *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice. As previously described,[31,33] we found that the SWL-opsin was mislocalized to the axons and cone pedicles indicating that the cone morphology was unchanged between these models. Even if some cones remain in the *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice (at 4 weeks), in the present study, they did not elicit an OR under photopic conditions. In accordance with previous studies,[17] our results show that rods, and not cones, are the source of visual acuity and are sufficient to induce ORs in *Rpe65*$^{-/-}$ mice.

Although the *Rpe65*$^{-/-}$ and *Cpfl1*$^{-/-}$ models both lack cones and have altered photopic vision, the ORs recorded in both cases were different. The *Rpe65*$^{-/-}$ responses were smaller and occurred at a higher light level than with the *Cpfl1*$^{-/-}$ mice. In contrast, the *Cpfl1*$^{-/-}$ animals were able to respond in a larger range of spatial frequencies, demonstrating that *Rpe65*$^{-/-}$ rod opsins have a markedly reduced capacity to capture light because of the minute amount of chromophore.[34] This finding could be explained by the following hypothesis. Given that rods are able to respond to even a single photoisomerization,[35] small amounts of photopigment could allow them to respond to light stimuli in appropriate conditions in *Rpe65*$^{-/-}$ mice[17] and permit visual guidance of locomotion to bright light in *Rpe65*$^{rd12/rd12}$ mutants, showing that the residual rod function can provide useful vision and visual pathways remain functional.[36]

In fact, the *Rpe65*$^{-/-}$ ORs present a particular pattern and the sensitivity threshold is reduced compared with that in C57/Bl6 or mutant mice. This observation could be partly explained by the fact that photoreceptors with an almost complete deficit of rhodopsin cannot absorb sufficient light to react under all circumstances to transduce the signal to the effectors.[37] In consequence, *Rpe65*$^{-/-}$ rods must be placed in a suitable spatial frequency and appropriate (bright) light intensity to elicit an OR. Other observations involve the localization of the 11- and/or 9-*cis*-retinal used to regenerate rhodopsin after bleaching. Chromophores are stored in a pool inaccessible to light and are stabilized.[38,39] In *Rpe65*$^{-/-}$ animals, no 11-*cis*-retinal is detectable in the retina or RPE using standard protocols,[7,8] but a small rod response leading to visual acuity or ERG recordings can be observed,[17,18] suggesting another source of chromophore. The 9-*cis*-retinal generated from the liver is a candidate for this residual rod activity. Ablation of RPE65 eliminates the cone function, and thus the remnant visual response can be attributed to rod function due to the presence of 9-*cis*-retinal forming the isorhodopsin visual pigment.[17,34] This retinal activity may be responsible for the OR recorded in the present study using *Rpe65*$^{-/-}$. Moreover, as demonstrated recently,[31] in situations of limited amounts of 11-*cis*-retinal in the retina (e.g., R91W mice that possess 5%–10% of normal 11-*cis*-retinal levels produced by a hypomorphic RPE65 protein), there is a competition between rods and cones for limited amounts of chromophore: The chromophore uptake is presumably based on the tyranny of the majority rule, which favors rods over cones. In consequence, in *Rpe65*$^{-/-}$ and *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice, any type of chromophore should be preferentially available for cones (the role of 9-*cis*-retinal in cone function remaining to be determined). Despite this advantageous situation for cones, the lack of an OR in the *Rpe65*$^{-/-}$;*Rho*$^{-/-}$ mice shows that the rods were the unique source of the OR in the *Rpe65*$^{-/-}$ mice.

Summary

Our study showed that cones and rods mediate ORs in response to light stimuli, but with different sensitivities and at different spatial frequencies. Using diverse mouse strains, it appears that, in wild-type mice, both photoreceptor systems (rods and cones) are essential to generate a visual reflex in scotopic and photopic conditions. In addition, this work provides evidence that the rod system is the source of a particular visual function in *Rpe65*$^{-/-}$ mice. Finally, the optomotor test is a simple behavioral method for estimating visual acuity in mice and is efficient to discriminate the relative contribution of different physiologic structures.

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