Divergent Phenotypes of Vision and Accessory Visual Function in Mice with Visual Cycle Dysfunction (Rpe65<sup>rd12</sup>) or Retinal Degeneration (rd/rd)

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PURPOSE. Tests of vision for mice remain limited and the visual phenotype of some retinal disorders in mice remain poorly understood. A novel assay of vision was used to determine how the form and extent of retinal disease affects visual phenotype in mice.

METHODS. Retinal histology, the suppression of locomotion by light and visual guidance of locomotion, were assessed in mice with progressive photoreceptor degeneration (rd/rd) or visual cycle dysfunction (Rpe65<sup>rd12</sup>).

RESULTS. In wild-type mice, there was visual guidance of locomotor activity in dim light and suppression of activity (negative masking) in bright light. In rd/rd mice, vision was sufficient to guide locomotion at postnatal day (P) 54 but was lost from P46 onward. In bright light rd/rd mice had enhanced negative masking. Although Rpe65<sup>rd12</sup> mice had no dim light response, with high illumination, vision was sufficient to guide locomotion at all ages tested.

CONCLUSIONS. A major concern for gene and cell replacement therapies is the development of visual pathways through which restored retinal function can connect to visual centers of the brain. The residual retinal response to high illumination in Rpe65<sup>rd12</sup> mice translates into useful vision, and visual pathways remain functional—a prerequisite for restoring vision in disorders of the retina. Similarly, useful vision in young rd/rd mice shows that there is visual pathway function before photoreceptor degeneration and suggests the potential for early therapy. Together, these findings recommend observation of masking responses in the assessment of gene and cell replacement therapies. (Invest Ophthalmol Vis Sci. 2008;49:2737–2742) DOI:10.1167/iovs.07-1546

The functions of the eye are many. In addition to light detection for vision, the eye provides input to light-regulated adaptations known as accessory visual responses. The contribution and organization of the rods, cones, and melanopsin-expressing photoreceptor retinal ganglion cells (pRGCs) can vary considerably among tasks. For instance, the rods underlie scotopic vision but are not essential for circadian photosensitivity. On the other hand, pRGCs are important in irradiance detection, but not in visual guidance of movement. There is also extensive variation among disorders of the retina, from degeneration to partial loss of function in a discrete cell type or region of the eye. Together, these factors translate into phenotypic diversity in the state of vision and of accessory visual responses.

In human beings, vision can be measured in several ways, and the form and progression of vision loss may be broadly characterized among clinical categories. However, the relationship between the disorders of the eye and accessory visual function is often less well characterized. In mice, the available methods to test or measure vision are inadequate. As a consequence, the relationship between retinal disease and visual phenotype is insufficiently understood. In contrast, some accessory visual systems have been relatively well studied. Nevertheless, the relationship between retinal condition and the phenotype of accessory visual responses is far from simple. For instance, classic photoreceptor loss or dysfunction can also have a secondary effect on the pRGCs, indicating that changes in an accessory visual response cannot be easily attributed to a reduction or increase in classic photoreceptor capacity.

A behavioral assay of both visual guidance of movement and an irradiance-dependent accessory visual response is ideal for the study of these features of visual function. In darkness, the timing and amount of running wheel use by a mouse is predictable. Visual guidance of movement in dim light can result in increased locomotor activity at a given time of day (positive masking) and lesions of the classic photoreceptors or of the lateral geniculate nucleus abolish this response. However, light can also acutely modify locomotor activity in the opposite way: Activity is suppressed (negative masking) under bright light through an accessory visual response. Although a test of an innate behavior in the home cage provides an improvement over existing techniques, studies of the effect of retinal dysfunction on the phenomena of masking of locomotor activity have been limited.

To improve our understanding of how retinal disorders affect visual phenotype and to develop this assay further, we studied masking as a function of age in mice with retinal degeneration (rd/rd) or visual cycle dysfunction (Rpe65<sup>rd12</sup>). In rd/rd mice, loss of cGMP-phosphodiesterase-6b hydrolysis activity results in early degeneration of rod photoreceptors, but some cones persist up to 18 months.

The state of vision and of negative masking in rd/rd mice is unclear early in the degenerative process. In Rpe65<sup>rd12</sup> mice, loss of RPE65 all-trans-retinoid isomerase activity results in chromophore deficits with early degeneration of cone photoreceptors and residual activity in surviving rods. Whether residual rod function in Rpe65<sup>rd12</sup> mice supports useful vision, or how chromophore
deficits affect behavior during negative masking has not been determined.

**METHODS**

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Animals and Housing**

*Rpe65<sup>rd12</sup>* (B6(A)−Rpe65<sup>rd12</sup>), rd/rd (B6.C3−Pde6<sup>brd1</sup> Hps4<sup>le</sup>), and their common wild-type control (C57BL/6) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were raised in a 16:8-hour light/dark (LD) cycle (fluorescent white light at ~26 μWcm<sup>2</sup>). Light measurements were made with a power meter (model PM103; Macam Photometrics Ltd, Livingston, UK) and a luxmeter (E2x; B. Hagner AB, Solna, Sweden). Irradiances are the average of all cage positions. After they were weaned, mice were either kept for histology (male and female mice) in the same conditions or underwent behavioral phenotyping (only males). Food and water were available ad libitum.

**The Dynamic Range of Masking**

Wild-type mice (*n* = 9) were individually housed in rectangular cages (44 × 23 × 20 cm) fitted with a running wheel (17.5 cm diameter). Temperature was maintained between 18°C and 21°C, and a 16:8-hour LD entraining cycle was scheduled (120 μWcm<sup>2</sup>, ~500 lux). The facility also allowed application of light pulses to mice in their home cages, separate from entraining lights. After 14 days of acclimatization to cages with wheels, mice then 238 ± 43 days old (mean ± SEM), were treated in a 3-day repeating experimental cycle: prepulse baseline day, pulse day, and maintenance day. On pulse days, 3-hour pulses of light were applied starting 1 hour after daily onset of darkness. Irradiance was controlled with gel neutral density filters (Cinegel; Rosco Laboratories, Stamford, CT) at 12 irradiance levels, scheduled to avoid order effects.

Activity was recorded as wheel revolutions in sequential 10-minute bins (DataQuest 3; Minimitter Respirometers, Bend, OR). If activity recording failed in an animal, arbitrary exclusion criteria were applied: If activity was below 20 counts (10% of mean baseline for wild-type mice) in 4 consecutive bins or in 5 of the total 18 bins of the baseline period, that light treatment data set would be excluded; and, if an animal repeatedly failed to meet the inclusion criteria, the entire data set for that animal would be excluded. In the present case, no records were excluded.

Responses were calculated for each treatment as percentage of activity relative to the corresponding time on the preceding baseline day. Two suitable irradiances were derived from the dynamic range: an irradiance that produced an approximately half-maximal negative masking response (16.9 ± 3.0 μWcm<sup>2</sup> or ~33 lux; subsequently called bright), and an irradiance at the brighter end of the positive masking range (6.0 × 10<sup>−3</sup> μWcm<sup>2</sup> or ~0.006 lux; subsequently called dim).

**Responses to Light in Retinal Dysfunction**

After weaning, rd/rd (*n* = 7), *Rpe65<sup>rd12</sup> (*n* = 7), and wild-type (*n* = 8) mice were housed in cages with wheels, as described earlier. After 3 days of acclimatization to the cages, the mice were treated alternately with bright or dim pulses of light in the repeating 3-day protocol. Behavioral responses were recorded between postnatal day (P)28 and P135 and were segregated into defined age groups (P34, 28–39 days after birth; P46, 40–51; P58, 52–63; P76, 64–87; P100, 88–111; and P135), and were segregated into defined age groups (P34, 28–39 days after birth; P46, 40–51; P58, 52–63; P76, 64–87; P100, 88–111; and P135). Age groupings reflect the expected rate of degeneration after birth; P46, 40–51; P58, 52–63; P76, 64–87; P100, 88–111; and P135 and were segregated into defined age groups (P34, 28–39 days after birth; P46, 40–51; P58, 52–63; P76, 64–87; P100, 88–111; and P135). Age groupings reflect the expected rate of degeneration. Typically more than one test for an individual fell within an age group. In this case, 2.3% of treatments were excluded (wild-type, 1 of 273 tests; rd/rd, 6 of 227; *Rpe65<sup>rd12</sup>, 10/227).

Comparison of underlying levels of wheel running during the 3-hour baseline periods revealed differences in activity with age. Therefore, both percentage of baseline and difference from baseline calculations were used to describe response magnitude. For the difference method the number of revolutions in the baseline was subtracted from the corresponding treatment period. For each light condition, age group, and genotype, the mean of responses for each animal was used.

**Retinal Condition**

Histology was described for each genotype/age group (*n* ≥ 3 per group). Mice were killed by cervical dislocation. After confirming death, the eyes were marked on the superior surface with a heated inoculating loop for orientation. Eyes were then fixed by immersion in 4% paraformaldehyde-10 mM phosphate-buffered saline (pH 7.4; PBS) for 4 hours, then transferred to PBS. After removal of the lens, the eyes were infiltrated and embedded in acrylamide solution, followed by freezing in optimal cutting temperature solution (Ted Pella, Redding, CA). Sections were collected along the inferior–superior axis with a cryostat and stained with hematoxylin-eosin. Photographs were taken with a microscope (model BX41; Olympus, Lake Success, NY) equipped with a digital camera (SPOT-RT; Diagnostic Instruments, Burlington, CA) calibrated by using a stage micrometer. The thicknesses of the inner nuclear layer (INL) and outer nuclear layer (ONL) were obtained at a minimum of five positions.

**RESULTS**

Masking of Locomotor Activity by Light: Dynamic Range

Wild-type mice showed a dose-dependent transition between negative and positive masking (Fig. 1). There was negative masking of locomotor activity between 124.4 and 2.3 μWcm<sup>2</sup> (~250 and 4 lux, respectively) with response magnitude correlated to irradiance. The derived irradiance for a half-maximum suppression of activity was ~15 μWcm<sup>2</sup>. Positive masking was apparent between 4.3 × 10<sup>−2</sup> and 2.0 × 10<sup>−6</sup> μWcm<sup>2</sup> (below ~0.1 lux), a ~4-log-unit irradiance range of relatively dim light. Activity was not different from baseline during a sham (dark) pulse.

Age-Dependent Changes in Activity and Responses

At each age group all three genotypes exhibited similar levels of underlying activity on baseline days (two-way ANOVA *P* =
0.704; Bonferroni corrected $P = 0.025$; Fig. 2). However, in all three genotypes, there was an increase in use of the wheel from P34 to P46 (paired two-tailed $t$-test $P < 0.0001$). There appeared to be a gradual decline in activity at P100 and P124.

**Masking of Locomotor Activity by Light: Effects of Genotype and Age**

Analyses of responses to light were made as a percentage of baseline activity and as a change in activity.

In wild-type mice, there was partial negative masking under fixed irradiance bright-light pulses (mean ± SEM: % baseline method [%] $71.5 ± 9.2$; difference from baseline method $[\Delta] -89.3 ± 9.8$), and positive masking under dim light pulses (% $129.8 ± 8.4$; $\Delta +62.2 ± 4.2$; Fig. 3A). Positive masking in wild-type mice appeared to be higher at P34 than at P46 but this was not significant (paired $t$-test: % $P = 0.016$; $\Delta P = 0.15$; Bonferroni $P = 0.0125$). However, when compared with responses at P46 and older, the bright-light response at P34 was significantly reduced irrespective of the lower activity at that age (paired $t$-test: % $P = 0.003$; $\Delta P = 0.003$). This therefore suggests that there is reduced sensitivity or magnitude in negative masking responses at P34 in wild-type mice.

In rd/rd mice, negative masking under bright light was significantly enhanced compared with the wild-type (% $22.1 ± 9.8$; $\Delta -222.7 ± 14.9$; equal variance $t$-test $P = 5.04 \times 10^{-6}$; Bonferroni $P = 0.025$; Fig. 3B). This was also true at P34, although in terms of the change in running, the amount of underlying activity that was suppressed was less. Although there was no overall change in activity under dim light (% $101.5 ± 5.5$; $\Delta +1.1 ± 3.6$), at P34 rd/rd mice showed positive...
masking compared with baseline (% 114.5 ± 3.8; Δ +26.2 ± 5.3; paired t-test \( P < 0.003 \)).

\( Rpe65^{rd12} \) mice exhibited a surprising phenotype (Fig. 3C). There was no clear effect of age (one-way ANOVA: dim light \( P = 0.19 \); bright light \( P = 0.06 \); Bonferroni \( P = 0.0166 \)) and no change in activity under dim light (grouped ages: % 100.0 ± 3.8; Δ +0.1 ± 3.8) but there was positive masking under bright light (grouped ages: % 124.9 ± 7.5; Δ +52.8 ± 5.3). The positive masking response to bright light in \( Rpe65^{rd12} \) mice was indistinguishable from the response in wild-type mice under dim light (compare the filled diamonds in Figure 3A with the open circles in Figure 3C; grouped ages: equal variance t-test \( P = 0.94 \)).

**DISCUSSION**

In this study, we describe the visual phenotype of two retinal disorders with divergent genetic cause and mechanism. Positive masking was used as a measure of visual guidance of movement and negative masking, to describe irradiance-dependent accessory visual function. Whereas minor variations were observed with age, phenotype was dramatically different between the three genotypes.

**Age-Dependent Changes in Activity**

The approximately twofold increase in wheel use from P34 to P46 in all genotypes suggests an effect of familiarization with use of the wheel.\(^ {14} \) The reduced activity in older mice most obviously reflects physical senescence. Concordance of the direction of changes with age and of the differences among genotypes bolsters confidence that interpretations do not depend on the method of scoring.

**Responses to Dim Light**

Wild-type mice displayed positive masking across a wide range of irradiances and at all ages, consistent with the normal condition of the retina. The increased percentage of response to dim light at P34 was exaggerated by the low underlying activity. However, this effect may not entirely account for the observed response in younger animals. Useful vision leads to more running on a wheel, presumably because the animal can judge foot placement and observe absence of obstructions. It is therefore plausible that there would be a greater performance-improving effect of visual guidance on a task when it is relatively unfamiliar.

In \( rd/rd \) mice, retinal degeneration alters anxiety-related behavior on the visual cliff,\(^ {24} \) casting doubt on the visual cliff as a way to show visual guidance of movement in \( rd/rd \) mice.\(^ {25} \) The present findings with positive masking demonstrate that \( rd/rd \) mice have vision useful to this task in dim light, although this was only true early in the degenerative process (at P34). In view of the extent of photoreceptor degeneration even at P34, it is surprising that there was useful vision at all.

In \( Rpe65^{rd12} \) mice, despite a relatively intact outer retina, the absence of a dim light response is consistent with observations of residual retinal electrical activity only at elevated illumination.\(^ {20} \)

**Responses to Bright Light**

In the wild-type mice, the reduced negative masking response at P34 was unexpected and has no immediate explanation. In \( rd/rd \) mice, although enhanced negative masking has been reported at ages over 110 days,\(^ {15} \) we found that negative masking was enhanced even at P34. We did not observe neg-
ative masking in Rpe65<sup>rd12</sup> mice at 16.9 μW/cm<sup>2</sup>. In fact, positive masking was apparent at all ages at this irradiance, one that was ~10,000 times more intense than the dim light pulses, allowing positive masking in the wild-type.

### Explaining the Unexpected Bright-Light Response in Rpe65<sup>rd12</sup> Mice

The increased irradiance required for positive masking in Rpe65<sup>rd12</sup> mice most obviously reflects the increase in photons necessary for sufficient light capture events. Despite some thinning in the photoreceptor layer, only the reported deficits in rod-opsin<sup>26</sup> can fully account for the degree of reduced sensitivity. Our observation shows that residual rod function can provide useful vision and demonstrates that visual pathways develop and remain functional in these mice.

The absence of negative masking at the bright irradiance is not explained as simply. pRGCs can maintain negative masking in the absence of classic photoreception. Thus, the present finding demonstrates that, contrary to previous reports, the pRGCs are affected by dysfunction in the visual cycle. This result suggests that, in Rpe65<sup>rd12</sup> mice, sensitivity is reduced in both the rods and pRGCs. It is possible that some unidentified change in the classic photoreceptors downregulates the sensitivity of the pRGCs. Alternatively, deficits in chromophore could directly limit the responsiveness of the pRGCs, as initial generation of active melanopsin would require vitamin-A in the appropriate form and amount.

### Interactions between Negative and Positive Masking

Negative and positive masking are different responses to light. Positive masking depends on rod function, whereas negative masking proceeds in the absence of rods. Positive masking is thought to enable more locomotor activity to occur by providing vision that helps the mouse move about more freely at times when it is motivated to do so. Negative masking takes away the motivation to move around when the light is bright. Despite this distinction, negative and positive masking may interact. For example, the enhancement of negative masking that often occurs when rods are dysfunctional could be interpreted as resulting from decreased positive masking. However, the nature of such interactions remain to be elucidated. We mention a few possibilities, none of which is entirely satisfactory.

In the present experiment, enhanced negative masking was evident before the loss of positive masking in rd/rd mice, and in Rpe65<sup>rd12</sup> mice reduced sensitivity of positive masking was not accompanied by enhanced negative masking. Also, negative masking changed without any apparent effect on positive masking, as in melanopsin knockout mice. Therefore, downstream competition for control of locomotion does not seem viable as a general explanation of relationships between positive and negative masking.

Another possibility is interaction at the level of the retina. Enhanced negative masking after degeneration of the classic photoreceptors could result from removal of an inhibitory influence from these photoreceptors onto the pRGCs. This could be in the form of an acute inhibitory input to the pRGCs from classic photoreceptors. In terms of our findings, the increase in negative masking in rd/rd mice coincides with the early and severe thinning of the retina, and reduced classic photoreceptor influence might then disinhibit the pRGC input to negative masking. The unusual response in Rpe65<sup>rd12</sup> mice would be understandable if the damaged rods treat bright light as though it were dim light and then inappropriately inhibit the pRGC input to negative masking at higher irradiances. An alternative equally supported by these findings is that classic photoreceptor status regulates the basal sensitivity of the pRGCs and thereby the sensitivity of negative masking. In Rpe65<sup>rd12</sup> mice, fewer pRGCs are detected. In addition, classic photoreceptor degeneration can alter the levels of melanopsin mRNA in the pRGCs.<sup>11,12</sup>

### Vision and Accessory Visual Systems in Circadian Phase Alignment

In some cases, mice with a dysfunction in irradiance detection pathways are diurnal.<sup>28,29</sup> Our observation of positive instead of negative masking in bright light in Rpe65<sup>rd12</sup> mice goes some way toward explaining this phenomenon. In wild-type mice, a relatively bright LD cycle is expected to segregate activity into the dark period through circadian entrainment and negative masking. Loss of irradiance detection sensitivity may mean that there is only weak photic segregation of activity, creating conditions that allow an animal to become diurnal. Animals may then be predisposed to activity in conditions in which they can see (daytime). In addition, activity in the light phase may feed back to provide an alternate or competing entrainment cue.<sup>30</sup> The sum of such changes could therefore account for the diurnality, or advanced activity onset.<sup>29</sup>

### Clinical Relevance

Animal models were key to the development of Rpe65 gene replacement therapy<sup>31-32</sup> which has now entered clinical trials in the United Kingdom and United States. A concern in these therapies is the development of visual pathways, through which restored retinal function can connect to the visual centers of the brain. Successful reversal of congenital blindness depends on visual pathway development before treatment or on treatment at a stage that allows subsequent development of visual pathways.<sup>33-34</sup>

It follows that identifying the state of visual pathways in a mouse is important when they are used as a model for disease and therapy assessment. Although measures of retinal function are useful<sup>13,35,36</sup> the most meaningful way to demonstrate function in visual pathways is to test vision directly.<sup>7</sup> The positive masking assay of an innate visually guided behavior in mice is quantifiable, sensitive to changes in dynamic range and practical on a large scale.<sup>14</sup> The further improvement of this assay and detailed characterization of more phenotypes will benefit future translational research.

The retention of visual guidance of movement in older Rpe65<sup>rd12</sup> mice is comparable to the effect of Rpe65 dysfunction in people<sup>37</sup> and shows the presence of functional visual pathways that would support gene replacement therapy.<sup>32</sup> Similarly, the rd/rd mouse continues to be useful as a general model of severe and early photoreceptor degenerations. In rd/rd mice (P4) treatment with gene replacement therapy can attenuate the loss of rods,<sup>38</sup> but whether this is sufficient to sustain visual capacity is not known. Although visual guidance of movement was lost after P34, the early vision use in rd/rd mice shows that functional visual pathway development does occur, suggesting that gene or cell replacement therapy is warranted.

Finally, accessory visual responses are typically marginalized in assessment of visual disorders. However, they have diagnostic value (Sinclair JD, et al. IOVS 2007;48:ARVO E-Abstract 5535), and can affect quality of life. Irradiance detection has been implicated in endocrine function,<sup>39,40</sup> seasonal depression,<sup>40</sup> alertness,<sup>41</sup> and circadian entrainment. Therefore, although treatments are being developed with the goal of at least partially restoring vision, the benefit of normal function in irradiance detection is less obvious but tangible.

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*The Visual Phenotype of Rpe65<sup>rd12</sup> and rd/rd Mice*
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References