Different Inner Retinal Pathways Mediate Rod-Cone Input in Irradiance Detection for the Pupillary Light Reflex and Regulation of Behavioral State in Mice

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PURPOSE. Detection of light in the eye contributes both to spatial awareness (form vision) and to responses that acclimate an animal to gross changes in light (irradiance detection). This dual role means that eye disease that disrupts form vision can also adversely affect physiology and behavioral state. The purpose of this study was to investigate how inner retinal circuitry mediating rod-cone photoreceptor input contributes to functionally distinct irradiance responses and whether that might account for phenotypic diversity in retinal disease.

METHODS. The sensitivity of the pupillary light reflex and negative masking (activity suppression by light) was measured in wild-type mice with intact inner retinal circuitry, *Nob4* mice that lack ON-bipolar cell function, and *rd1* mice that lack rods and cones and, therefore, have no input to ON or OFF bipolar cells.

RESULTS. An expected increase in sensitivity to negative masking with loss of photoreceptor input in *rd1* was duplicated in *Nob4* mice. In contrast, sensitivity of the pupillary light reflex was more severely reduced in *rd1* than in *Nob4* mice.

CONCLUSIONS. Absence of ON-bipolar cell-mediated rod-cone input can fully explain the phenotype of outer retina degeneration for negative masking but not for the pupillary light reflex. Therefore, inner retinal pathways mediating rod-cone input are different for negative masking and the pupillary light reflex. (*Invest Ophthalmol Vis Sci.* 2011;52:618–623) DOI:10.1167/iops.10-6146

Detection of light serves many tasks. Independent of form vision, the detection of brightness (irradiance) is used to regulate pupil size,¹ entrain circadian rhythms to the day-night cycle,² and modulate endocrine function.³ Irradiance information is transmitted to central nuclei by a specialized subset of intrinsically photosensitive retinal ganglion cells (ipRGCs).^{4,5}

The response of ipRGCs to light is a composite of their intrinsic melanopsin-generated activity and rod-cone photoreceptor-dependent activation mediated by interneurons of the inner retina.⁶ However, morphologic and functional specialization among ipRGCs has been identified, and these “ipRGC types” show variation in their projections to central nuclei.^{7–10}

How these differences in ipRGC form, function, and central projections relate to a given irradiance response is not well understood. For instance, the pupillary light reflex and negative masking (a suppression of activity by light) both respond to “lights on” in an irradiance-dependent manner.^{1,11} However, the effect of outer retina degeneration differs dramatically between these irradiance responses, with a paradoxical enhancement of irradiance sensitivity in negative masking¹² and a severe loss of sensitivity in the pupillary light reflex.^{1,13,14} At the level of the retina, such phenotypic variability could arise from secondary effects of outer retina disease on ipRGCs or from differences in rod-cone input to the different ipRGC types. Although inner retinal circuitry is diverse, all vertical transmission of rod and cone signals to ganglion cells is, at some point, mediated by bipolar cells (BCs).¹⁵ Therefore, differences between responses could arise from specificity in bipolar cells mediating rod-cone input.

The aim of this study was to determine whether there are differences in inner retinal pathways mediating rod-cone input to functionally distinct irradiance responses and whether such differences could contribute to the divergent effects of outer retinal disease on the different irradiance responses. Negative masking and pupillary light reflex were compared between mice with intact inner-retinal circuitry (wild-type), mice selectively lacking the ON-BC pathway of rod-cone input (*Nob4*), and mice with degenerate rods and cones (*rd1*), effectively lacking input to both ON- and OFF-BCs (Fig. 1).

METHODS

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Iowa Animal Care and Use Review. Male wild-type control (C57BL/6J), *Nob4* (C57BL/6J *Grm6^{Nob4/Nob4}*), and *rd1* mice (B6.C3-Pde6b^{rd1} *Hps4le/J*) were raised and maintained in a repeating 12-hour light/12-hour dark cycle of fluorescent white light at ~20 $\mu\text{W}/\text{cm}^2/\text{s}$, except when undergoing experiments. Food and water were available ad libitum. Light measurements were made using a power meter (PM103; Macam Photometrics Ltd., Livingston, UK). Bonferroni corrections were applied when assessing statistical significance.

Comparison between mice of the same age and background under the same conditions was intended to constrain variables to the genetic dysfunction of the retina. The rod cGMP phosphodiesterase β sub-

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Supported by the Howard Hughes Medical Institute, National Institutes of Health/National Eye Institute grant (EMS), and the Foundation Fighting Blindness.

Submitted for publication June 30, 2010; revised August 17, 2010; accepted August 19, 2010.

Disclosure: S. Thompson, None; S.F. Stasheff, None; J. Hernandez, None; E. Nylén, None; J.S. East, None; R.H. Kardon, None; L.H. Pinto, None; R.F. Mullins, None; E.M. Stone, None

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FIGURE 1. Vertical transmission of rod-cone input to ganglion cells. *Green*: ON pathways. *Blue*: OFF pathways. Loss of function is indicated by *gray shading* or cell absence. (A) When rods and cones respond to light, metabotropic or ON-BCs depolarize, and ionotropic or OFF-BCs hyperpolarize. Rod BCs are metabotropic (ON-BCs) and, acting through AII amacrine cells, can depolarize cone ON-BCs through gap junctions (resistor symbol) and can hyperpolarize cone OFF-BCs through glycinergic synapses (*bar, filled circle*). Gap junctions also propagate signals between rods and cones and between rods and cone BCs. Although inner retinal pathways for transmission of rod-cone input to ipRGCs are specialized, they also almost certainly depend on the same principal components, with vertical signal transmission by way of ON-BCs or OFF-BCs, or both. (B) In *rd1*, the extent of rod cone loss at P90 results in effective absence of rod-cone input to bipolar cells. (C) In *Nob4*, rods, cones, and OFF-BCs function normally, but ON-BCs are not activated by rod-cone synaptic input.

unit (Pde6b) has rod photoreceptor-specific expression, and the *rd1* allele of this gene results in the degeneration of rods and cones.¹⁶ Similarly, the metabotropic glutamate receptor 6 (mGluR6) is specifically expressed in ON-bipolar cells, mediating the ON-BC response to glutamate at the synapse from rods and cones, and the mutation in *Nob4* mice abolishes expression of the protein.¹⁷

As in all such models, an influence of retinal remodeling cannot be entirely excluded from the interpretation of our results. Stratification and distribution of ipRGCs is normal in *rd1* mice on a different background (CBA/J), though ipRGC number is increased.¹⁸ Although there is likely remodeling of the inner retina with rod-cone loss, multielectrode array recordings of *rd1* retinas show that rod-cone-generated light-evoked responses are undetectable by postnatal day (P) 28¹⁹ (and see Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6146/-DCSupplemental>). Therefore, at the mouse age at which these studies were conducted (P90), negligible light would be evoked input from the outer retina, and responses to light would be reasonably attributed to the intrinsic photosensitivity of ipRGCs. For *Nob4* mice, previous studies did not observe any examples of retinal remodeling, and phenotypic changes in *Nob4* mice are likely to primarily reflect absent ON-BC function²⁰ (and see Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6146/-DCSupplemental>).

Effects of Light on Running Wheel Activity

The dynamic range of negative masking and the presence or absence of useful vision were assessed according to previously reported methods.²¹ Briefly, the effect of light on running wheel activity was measured across a wide range of irradiances in wild-type ($n = 9$; age at test, $P97 \pm 32$ days [mean $\pm$ SD]), *Nob4* ($n = 9$; age at test, $P121 \pm 16$ days), and *rd1* ($n = 12$; age at test, $P118 \pm 23$ days) mice.

Mice were individually housed in wheel cages (Thoren Caging Inc., Hazleton, PA) mounted in environmental control cabinets (University

of Iowa Medical Instruments, Iowa City, IA). Wheel running was continuously recorded using a customized data acquisition system (ClockLab; Actimetrics, Inc., Evanston, IL). A 12-hour light/12-hour dark cycle of fluorescent white light at $\sim 19 \mu\text{W}/\text{cm}^2/\text{s}$ was applied throughout. Animals were allowed to acclimatize to wheel cages for 14 days before testing. A 3-day repeating experimental cycle of pre-pulse baseline day, pulse day, and maintenance day was applied. On pulse days, a remotely controlled 1-hour pulse was applied to mice in their home cage starting 1 hour after daily dark onset. Under this protocol, circadian activity remains entrained throughout testing, but acute changes in activity during the dark phase are induced. Neutral-density film (Cinegel; Rosco, Glendale, CA) was used to regulate the irradiance of the applied light. Nine light levels between 3.3×10^{-7} and $15.4 \mu\text{W}/\text{cm}^2/\text{s}$ were applied in a sequence that distributed bright and dim pulses over the course of testing. Changes in activity over the 1-hour light treatment were calculated as percentage of baseline activity at the corresponding time on the preceding day for each animal.

Variable slope sigmoid dose-response curves were fitted to the data (Prism; GraphPad Software Inc., La Jolla, CA) with a fixed constant for the minimum set at 0%. The irradiance producing a half-maximal response (EC_{50}) and HillSlope were calculated (Prism; GraphPad Software Inc.) from fitted curves. Features of fitted curves were then compared by an *F*-test of a two-fit comparison. Curves were fitted to the data sets for two genotypes independently and then to the combined data set for both genotypes; the effect of combining data sets on the quality of fit to a given parameter was then used to calculate whether there was a difference.

Useful vision was assessed by running wheel activity at dim light levels below irradiances that generate a negative masking response (2.2×10^{-3} to $3.1 \times 10^{-6} \mu\text{W}/\text{cm}^2/\text{s}$). If useful vision is present, it results in running wheel activity in dim light, greater than baseline activity in complete darkness. Significance was determined by a two-

FIGURE 2. Activity records for wheel running. Representative activity records (actograms) for wheel running are shown for each genotype. *Horizontal axis:* Time of day. *Vertical axis:* Sequential days. The black bars and white bars at the top show the timing of the daily cycle of light and dark. With normal entrainment to the light cycle, activity is restricted to the dark phase. *Arrows:* On a 3-day protocol, pulses of light are applied during the dark or active phase of the daily cycle. Activity is acutely suppressed during a bright pulse of light on day 4 ($15.4 \mu\text{W}/\text{cm}^2/\text{s}$), but not during a dim light pulse of light on day 7 ($3.2 \times 10^{-5} \mu\text{W}/\text{cm}^2/\text{s}$).

tailed *t*-test of data at these irradiances compared with a hypothetical mean at 100%, the baseline to which data are normalized.

Pupillary Light Reflexes

Pupillary responses were assessed using a light ketamine-xylazine (46 mg/kg; 4.6 mg/kg) sedation method validated in other studies^{22–24} and in our own control experiments (see Supplementary Fig. S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6146/-/DCSupplemental>). Critically, this avoids the variable effect on pupillary responses of anxiety with restraint,^{25,26} particularly because anxiety behaviors in mice are heightened in retinal degeneration.²⁷ Applied light was initially at a lower irradiance range to assess responses primarily generated by inner retina-mediated rod-cone input rather than the intrinsic melanopsin-generated sensitivity of ipRGCs.^{1,14} To further concentrate on the contribution of rod-cone input, between-strain analysis was restricted to the initial constriction of the pupillary light reflex. Because it is within 2 seconds of stimulus onset, particularly at lower irradiances, this aspect of the pupillary light reflex is dominated by rod-cone input.^{28,29}

The pupillary light reflex with both eyes illuminated was measured at five irradiance levels (0.014, 0.12, 1.4, 12.2, and $133 \mu\text{W}/\text{cm}^2/\text{s}$) in wild-type ($n = 11$; age at test, $P114 \pm 29$ days), *Nob4* ($n = 11$; age at test, $P102 \pm 7$ days), and *rd1* ($n = 8$; age at test, $P121 \pm 12$ days). However, in our protocol, loss of sensitivity in *rd1* was marked.

Therefore, to allow comparison of fitted curves, a second test was made at nine irradiance levels at log unit intervals up to $1980 \mu\text{W}/\text{cm}^2/\text{s}$ in wild-type ($n = 5$; age at test, $P106 \pm 4$ days), and *rd1* ($n = 8$; age at test, $P98 \pm 6$ days).

Animals were tested during the 6 hours corresponding to the mid-light cycle. Mice were dark adapted for a minimum of 2 hours and then sedated using 46 mg/kg ketamine with 4.6 mg/kg xylazine. Under dim far red light ($>700 \text{ nm}$) animals were positioned manually onto a platform in a Ganzfeld bowl illuminator. A brilliant white light halogen bulb (Osram Sylvania Inc., Danvers, MA) was mounted in a light tight chamber onto the Ganzfeld bowl. The timing of applied light was controlled with a manually operated shutter, and irradiance was regulated by neutral density filters (Andover Corp., Salem, NH). Pupil measurements were made from infrared (IR source 850 nm) digital video equipment fitted to the Ganzfeld bowl, and pupil size over time was determined (ViewPoint Eye Tracker; Arrington Research Inc., Scottsdale, AZ). Under a standard protocol, recording was initiated once animals were situated and pupil capture was stable. After a baseline period of 60 seconds, the response to white light was recorded at progressively increasing irradiance, with 10-second exposures separated by 50 seconds of dark adaptation. There were no instances in which mice showed any sign of poor sedation (twitching, blinking).

Change in pupil size was expressed as a percentage of baseline pupil area defined by the 5-second period immediately preceding light exposure. Initial responses were defined as the minimum pupil area during the first 2 seconds of the response. Sigmoid response curves were fitted (Prism; GraphPad Software Inc.) with fixed minimum set at

FIGURE 3. Dose-response for positive and negative masking. The relationship between irradiance and change in wheel running is shown for (top) wild-type and *rd1* mice and (bottom) wild-type and *Nob4* mice. Mean $\pm$ SEM activity at each irradiance shown as a percentage of baseline (activity in complete darkness, line at 100%). Variable slope sigmoid dose-response curves are fitted to data. Wild-type responses are shown in both panels for comparison.

0% and maximum at 52.75% (the mean maximum in wild-type mice). Comparison of the irradiance generating a half-maximal response was made by an *F*-test assessment of the fitted curves.

RESULTS

Effects of Light on Running Wheel Activity

Mice remained entrained to the light-dark cycle, with acute deviations from normal circadian activity (masking) only during the application of light during the active (normally dark) phase of the daily light cycle (Fig. 2). In wild-type mice, negative masking—the suppression of wheel running by light—was characterized by a progressive suppression of activity with increasing irradiance (Fig. 3). The irradiance producing a half-maximal response (EC_{50}) was $2.06 \mu\text{W}/\text{cm}^2/\text{s}$, with a slope of 0.169. In both *rd1* and *Nob4* mice, responses were induced at lower irradiances, with EC_{50} significantly reduced for both *rd1* ($0.097 \mu\text{W}/\text{cm}^2/\text{s}$; *F*-test $P < 0.0001$; $F = 82.8$) and *Nob4* ($EC_{50} = 0.104 \mu\text{W}/\text{cm}^2/\text{s}$; *F*-test $P < 0.0001$; $F = 115.8$) mice. The slope of the response was also significantly increased in *rd1* (0.499; *F*-test $P < 0.0001$; $F = 26.8$) and *Nob4* (0.551; *F*-test $P < 0.0001$; $F = 28.6$) mice. However, there was no identifiable difference in the fitted curves between *rd1* and *Nob4* mice (simultaneous *F*-test of EC_{50} and slope, $P = 0.68$, $F = 0.393$).

These data also allow an assessment of performance in a gross visual task, the contribution of form vision to wheel running.²¹ Baseline activity levels are based on activity in complete darkness; therefore, if form vision enhances task performance, wheel running is greater than baseline. This effect is seen only at light levels below irradiances that generate a negative masking response. In wild-type mice, activity was significantly greater than baseline between 2.2×10^{-3} and $3.1 \times 10^{-6} \mu\text{W}/\text{cm}^2/\text{s}$ ($P = 0.005$) but was not different from baseline in very dim light. As expected,

there was no increase in activity over baseline with dim light in *rd1* mice lacking rods and cones ($P = 0.19$). However, there was also no increase in activity over baseline with dim light in *Nob4* mice ($P = 0.16$).

Pupillary Light Reflex

In wild-type mice, the pupillary light reflex was characterized by an expected rapid initial constriction followed by an escape to a relatively sustained level of constriction (Fig. 4). Except at high irradiances ($\geq 133 \mu\text{W}/\text{cm}^2/\text{s}$), redilation after the end of the stimulus was rapid. The initial responses of *Nob4* and *rd1* mice were reduced compared with those of wild-type mice but less so at higher irradiances. Comparison of the irradiance generating a half-maximal initial response shows a significant reduction in sensitivity of both *Nob4* (*F*-test, $P < 0.0001$) and *rd1* (*F*-test, $P < 0.0001$). Further comparison shows that pupillary light reflex sensitivity was more severely reduced in *rd1* than in *Nob4* (*F*-test, $P < 0.0002$) mice.

DISCUSSION

In the present study, we investigated inner retinal pathways mediating rod-cone input to two different irradiance responses, the pupillary light reflex and negative masking. This was intended to extend our very limited understanding of the neural basis of irradiance detection for functionally distinct irradiance responses.

For negative masking, the striking similarity between *Nob4* and *rd1* mice shows that an enhanced negative-masking sensitivity phenotype can be generated by the discrete absence of ON-BC function. This supports the prediction that ON-BCs, but not OFF-BCs, would mediate rod-cone input in negative masking. However, it is still not clear how altered rod-cone input limits the dynamic range of negative masking. Form vision does not appear to determine negative-masking sensitivity. In aging

FIGURE 4. Pupillary light reflex. (A) The dynamic response of the pupil to a pulse of light at $133 \mu\text{W}/\text{cm}^2/\text{s}$ is shown for (top to bottom) wild-type, *Nob4*, and *rd1* mice. The response is plotted as pupil size relative to the dark-adapted baseline, with stimulus timing illustrated by the central nonshaded column. (B) Mean $\pm$ SEM initial constriction (percentage of constriction during the first second of stimulus) for the range of applied irradiances is shown fitted with a variable slope sigmoid dose-response curve.

Rds/Rds mice, form vision is abolished between 3 and 12 months of age, but negative-masking sensitivity remains unchanged. Further, *rd12* mice retain form vision but have an opposite phenotype in negative masking (loss of sensitivity).^{30,31} Finally, negative-masking sensitivity is enhanced in *Nob4* mice that retain form vision measured by optokinetic responses.³² Therefore, the increase in sensitivity is most obviously explained by removal of a rod/cone inhibitory input to irradiance coding circuits or by compensatory upregulation of melanopsin-generated responses. Electrophysiological evidence suggests that rod-cone input drives rather than inhibits responses of ipRGCs (Schmidt TM, Kofuji P, personal communication, 2010).⁶ Therefore, changes in melanopsin expression offer an appealing mechanism for increased irradiance detection sensitivity. Although some studies show melanopsin mRNA is downregulated in retinal degeneration,^{33–35} the number of ipRGCs is elevated in the CBA strain of *rd1* mice.¹⁸ Therefore, it seems plausible that signal amplification could occur at some other point in the melanopsin photoreponse.

The absence of form vision enhancement of wheel running in *Nob4* mice contrasts with previous findings that *Nob4* mice retain form vision-based optokinetic responses.³² It seems most likely that failure of this visual task in *Nob4* mice reflects a critical role of ON-BC input in some aspect of form vision that contributes to enhanced running wheel use, presumably distinct from, or more demanding than, motion detection measured in the optokinetic response.

With the pupillometry protocol used here, we did observe a greater reduction in sensitivity of *rd1* mice than was observed in previous studies of mice lacking rods and cones.³⁶ Additionally, the maximal constriction achieved appears small compared with that achieved in some previous studies. This may be a consequence of the light sedation protocol we used; however, the most obvious reason for this difference is the comparison of initial and sustained responses. The initial responses compared in this study are largely rod-cone generated and, thus, are severely reduced in *rd1*. By contrast, previous studies in mice lacking rods and cones reported the maximal constriction or sustained response after much longer exposure to the stimulus. This response would then be generated largely by the relatively intact melanopsin input. Irrespective, as a direct comparison under the same conditions and in mice with genetic abnormality restricted to the eye, the intermediate decrease in the pupillary light reflex in *Nob4* mice shows that rod-cone input to the pupillary light reflex is not entirely dependent on mGluR6 of ON-BCs.

Although it is not known how ON-BC independent rod-cone input could generate pupil constriction at stimulus onset, plausible mechanisms have been identified. The major input to ipRGCs appear to be from ON-BCs; OFF input would presumably inhibit the overall response of the pathway to lights-ON.⁶ However, there are ON responses intrinsic to the OFF pathway that are suppressed by the ON pathway and, therefore, disinhibited in mice lacking *Grm6* function.³⁷ It is also possible that OFF-RGC hyperpolarization contributes to pupil constriction. For instance, an inhibition of the dilator pupillae muscle would “enable” pupil constriction driven by residual ipRGC input to the pupillae sphincter muscle.^{38,39}

In conclusion, the absence of rod-cone input by ON-bipolar cells can fully explain the phenotype of outer retina dysfunction in negative masking. By contrast, loss of ON-BC function only partially accounts for the reduction in response sensitivity for pupillary light reflex. Whatever the mechanism, this property does not contribute to negative masking as does the pupillary light reflex. Therefore, the contribution of ON-BCs in mediating rod-cone input to irradiance coding circuits is different for negative masking and the pupillary light reflex. This

identifies one reason outer retina pathology can have such divergent effects on individual irradiance responses.

The role of the different ipRGC types in these responses remains a matter of speculation, but some indications can be drawn from existing evidence. Intriguingly, emerging evidence indicates that rod-cone input induces a more potent response in M2 ipRGCs than M1 ipRGCs (Schmidt TM, Kofuji P, personal communication, 2010). If true in vivo, then absent rod-cone input should result in a pronounced loss of sensitivity in responses or aspects of responses generated by M2 ipRGCs. For negative masking, the nuclei generating responses have not been conclusively identified, and the ipRGC input is unknown. However, the limited effect of absent rod-cone input might argue against an M2 ipRGC input. In contrast, the pupillary light reflex does receive input from M2 ipRGCs,⁷ and rod-cone loss severely reduces pupillary responses to dim light.¹

Acknowledgments

The authors thank Russ Van Gelder and Nicholas Mrosovsky for discussion.

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