Most retinal ganglion cells receive input from rods and cones and most of them project to the brain regions involved in image-forming vision. A small subset of ganglion cells are also intrinsically photosensitive (1–5). They express a light-sensitive bacterial protein, channelrhodopsin-2, which can be used to activate retinal ganglion cells. Channelrhodopsin-2 expressed in ON bipolar cells confers a form of visual function that is distinctively different from that of photoreceptors for image-forming vision. They transmit information by means of a chain of intermediate cells to the retinal ganglion cells, which in turn send signals to the brain. Loss of photoreceptor cells, as happens in a number of human diseases, leads to irreversible blindness. In a mouse model (rd/rd) of photoreceptor degeneration, we used a viral vector to express in a large number of retinal ganglion cells the light-sensitive protein melanopsin, normally present in only a specialized subset of the cells. Whole-cell patch-clamp recording showed that these cells even after degeneration of the photoreceptors and additional pharmacological or Ca²⁺ block of synaptic function. Interestingly, similar responses were observed across a wide variety of diverse types of ganglion cell of the retina. The newly melanopsin-expressing ganglion cells provided an enhancement of visual function in rd/rd mice: the pupillary light reflex (PLR) returned almost to normal; the mice showed behavioral avoidance of light in an open-field test, and they could discriminate a light stimulus from a dark one in a two-choice visual discrimination alley. Recovery of the PLR was stable for at least 11 months. It has recently been shown that ectopic retinal expression of a light sensitive bacterial protein, channelrhodopsin-2, can restore neuronal responsiveness and simple visual abilities in rd/rd mice. For therapy in human photodegenerations, channelrhodopsin-2 and melanopsin have different advantages and disadvantages; both proteins (or modifications of them) should be candidates.

Results

We used adeno-associated virus (AAV) to ectopically express mouse melanopsin in the retina of rd mice homozygous for the Pde6brd1 mutation (12, 13). This gene codes for a specific cGMP phosphodiesterase present exclusively in rod photoreceptors. These cells in the retina of rd/rd mice begin to degenerate soon after their terminal differentiation and are essentially absent by postnatal day 30 (P30). Cone photoreceptors subsequently degenerate, and all but a very small residual subset in the peripheral retina are lost by P90 (14–16). At ~P80, we injected intravitreally one of three viral constructs: AAV-Opn4, coding for the melanopsin protein; AAV-Opn4-RES-EGFP, coding for melanopsin protein and EGFP; and AAV-GFP, coding for the green fluorescent protein alone. Because we planned whole-

Author contributions: B.L., A.K., S.P., and R.H.M. designed research; B.L., A.K., and N.T. performed research; N.T. and S.P. contributed new reagents/analytic tools; B.L., A.K., N.T., and R.H.M. analyzed data; and B.L., S.P., and R.H.M. wrote the paper.

Conflict of interest statement: A patent application (U.S. no. 60/397,088; July 18, 2002) has been filed by R.H.M. and assigned to the Massachusetts General Hospital.

This article is a PNAS Direct Submission.

1Present address: National Institute for Physiological Sciences, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Aichi, Japan.

2Present address: Faculty of Medicine, Kyorin University, 6-20-2 Shinkawa, Mitaka-shi, Tokyo 181-8611 Japan.

3To whom correspondence should be addressed. E-mail: richard.masland@hms.harvard.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0806111410/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA
animal behavioral testing of the animals, both eyes of a mouse were injected with the same construct.

Four weeks later, we studied the retinas morphologically and electrophysiologically and evaluated the visual behavior of the mice. The testing occupied approximately two weeks. All of the constructs were expressed in retinal neurons (Fig. 1). The most effective, as judged by the number of cells transduced, brightness of GFP, and/or intensity of melanopsin immunostaining, was AAV-GFP (18,906 ± 1,184 GFP-expressing cells per retina), followed by AAV-Opn4 (4,437 ± 1,222 Opn4-expressing cells per retina) (Fig. 1J). Retinas injected with AAV-Opn4-IRES-EGFP were used primarily to identify the transduced cells for recording; the number of cells was not counted. Most of the transduced cells were retinal ganglion cells, although a scattering of amacrine and bipolar cells were also transduced. This selectivity may occur in part because ganglion cells are the first cells encountered by the virus particles after intravitreal injection, but there is a viral tropism as well (17). In untreated or sham-injected (AAV-GFP) retinas, the Opn4-expressing ganglion cells made up 572 ± 10.7 and 577 ± 23 cells per retina. Thus, the total

Fig. 1. Ectopic expression of melanopsin protein in retinal ganglion cells of different morphological types in the rd/rd mouse. (A–C) Spatial distributions of native melanopsin-expressing ganglion cells in uninjected (A) and sham-injected (C) rd/rd mouse retinas, and of total melanopsin-expressing ganglion cells in an AAV-Opn4 injected rd/rd mouse retina (B). Orientation of the retina: T, temporal; N, nasal; D, dorsal; and V, ventral. (D–G) Different types of ganglion cells were targeted in the melanopsin-treated mouse retina. Two monostratified ganglion cells (D and E) and one bistratified ganglion cell (F) are indicated here. (G) Their dendritic arbors are denser and smaller than those of the native melanopsin cell (G). (H and I) Sections of mouse retina stained with DAPI. In the rd/rd mice there appeared to be total loss of rod photoreceptors. Rare cones, which lacked inner and outer segments, were present but limited to the retinal periphery. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bars, 500 μm in A, B, and C; 100 μm in D–G; and 20 μm in H and I.) (J) Quantitation of melanopsin-expressing ganglion cells in rd/rd mice. For comparison, numbers of GFP-expressing ganglion cells in retinas injected with AAV-GFP are also indicated. Values are mean ± SD, with n indicating the number of retinas counted.
number of Opn4-expressing ganglion cells increased by almost an order of magnitude in the treated animals.

The transduced cells had various different dendritic morphologies, stratifications, and sizes (Fig. 1 D–F; see also Fig. S1). They were distinctly different from the unique and stereotyped native melanopsin cells (1, 18), which have sparse, crooked dendrites that spread far across the retinal surface (Fig. 1 J). For six retinas, we mapped the position of every melanopsin-expressing ganglion cell in the transduced retinas (Fig. 1 A–C). They were distributed across entire surface of the retina, with occasional concentrations that may have been near the injection sites. We cannot be certain that representatives of all ~12 types of ganglion cells in the mouse (19) were transduced, but it is clear that many of them were.

The responses of the ganglion cells to light were studied by whole-cell patch–clamp recording (Fig. 2). Cells were targeted for recording by their expression of GFP in eyes injected with AAV-Opn4-IRES-EGFP. GFP was identified in a ganglion cell soma during brief fluorescence illumination, after which the electrode was advanced while visualized by infrared differential interference contrast (DIC) microscopy optics. The recording pipettes were filled with Lucifer yellow CH, allowing visualization of the dendritic arbors. Although there was considerable variability from cell to cell (presumably due to varying amounts of melanopsin expression and/or bleaching during exploration of the retina) all of the cells that expressed Opn4 and GFP (n = 18) responded to light. The responses had the characteristics expected for melanopsin: long latency of onset (several hundreds of milliseconds to several seconds), and persistence for seconds after termination of the stimulus (1, 3, 18, 20, 21).

The retinas of the rd/rd mice appeared to lack rod photoreceptors (Fig. 1B), and the few residual cones were truncated (they lacked inner and outer segments) and restricted to the retinal periphery. To eliminate any possibility that the responses were driven by these cones (14–16, 22), the experiment was repeated in a series of rd/rd retinas (nine retinas, 15 cells) incubated in a mixture of NMDA-receptor antagonist [2-amino-5-phosphonovaleric acid (APV)], AMPA-receptor antagonist [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)], and metabotropic glutamate receptor agonist 2-amino-4-phosphonobutyrate (APB), to block the glutamergic synapses that would transmit potential signals from these photoreceptors to the ganglion cells. Responses to light persisted under these conditions, indicating that the ganglion cells had become intrinsically photosensitive. In four cases, Cd²⁺ at 200 μM was used to block synaptic transmission instead of the glutamate receptor drugs. The cells continued to respond to light, albeit at a higher intensity than in untreated retinas or retinas treated with glutamate antagonists. The higher threshold is likely due to the direct effect of Cd²⁺ on the ganglion cells themselves, because Ca²⁺ ions permeate the channel opened by melanopsin in response to light (23, 24). The dendritic arbors of the cells expressing melanopsin and GFP had many morphologies (Fig. 2), corresponding to many types of ganglion cells. There was no noticeable correlation between the morphology of the cell and the characteristics of the light-induced response. We conclude...
mediate a preference of mice for nesting in the dark under contains a dark refuge. The fraction of time spent by the mice
the test is to place mice in an illuminated open field that also
physiologically in the transduced ganglion cells (Fig. 2).

cell, a functional pathway that can couple activation of melan-
These results show that the signaling system that couples mela-
nopsin to membrane depolarization is ubiquitous, or at least very
widespread, in retinal ganglion cells; it is clearly not restricted to
the native melanopsin-expressing cells. Although there is no
certainty that the signaling pathway is identical in every ganglion
cell, a functional pathway that can couple activation of melano-
pin to a membrane cation channel appears to be present in
most types of retinal ganglion cell, as well as in many other neural
and nonneuronal cells (1, 3, 6–9).

In the absence of light-driven inputs, the responses of the
different morphological types of ganglion cells were similar to
each other. This finding suggests that ganglion cells, which
normally send diverse kinds of functional signals to the brain
(ON responses, OFF responses, sustained, transient, etc.), be-
electrophysiologically more uniform when driven by mela-
In three different behavioral measures indicated that visual
function, at least of a simple sort, was restored in nominally blind
rd/md mice by ectopic expression of melanopsin. The PLR is the
rd/md mouse. (A and B) Representative infrared images of pupil area taken in
dark (A) and light (B). White dots indicate the pupil areas. For a time series of
images, see Fig. S2. Pupil area was measured from such images by using
ImageJ. (C) Intensity-response curves for pupillary constriction. The stimulus
was exposure to 20 s of white light. The threshold for response is dramatically
reduced in melanopsin treated eyes (red curve) compared with sham-injected eyes (blue curve). Data from uninjected C57BL mice are shown for comparison.
The data are fitted with a sigmoidal function. (D and E) Time course of pupil
constriction over the first few seconds of dim (D) and bright (E) light exposure
are shown for melanopsin-treated (red) and sham-injected (blue) rd/md mice,
and uninjected C57BL mice (black). The area of the pupil is depicted as a
percentage of its size immediately preceding the onset of light. Values are
mean ± SEM, with n indicating the number of eyes examined.

that ectopic expression of the melanopsin protein rendered many
types of retinal ganglion cells intrinsically sensitive to light.

Could the visual information transmitted to the brain by the
transduced ganglion cells restore any of the visual function that
is lost when the photoreceptor cells degenerate in these mice? We
performed several tests of visual function. The first was the
PLR (25–27). A high-threshold PLR is retained in rd/md mice,
mediated by the small complement of native melanopsin cells (4,
26). Our results in the sham-injected (transduced with GFP
alone) mice confirm this finding (Fig. 3C). In these animals, as
in untreated mice with photoreceptor degeneration, the PLR was
~3 log units less sensitive than the PLR in wild-type C57BL
mice and had a long latency. In rd/md mice with ectopic expres-
sion of melanopsin, the PLR was returned to almost the sensi-
tivity observed in mice that had not suffered photoreceptor
degeneration (Fig. 3C). The responses to light did have some-
what longer latencies than normal (Fig. 3 D and E). This delay
is in accord with the long latency of response recorded electro-
physiologically in the transduced ganglion cells (Fig. 2).

The ectopic melanopsin cells could also guide more complex
behaviors. Normal mice avoid open, brightly lit spaces, and this
innate tendency is the basis of a simple test of their ability to see.
The test is to place mice in an illuminated open field that also
contains a dark refuge. The fraction of time spent by the mice
in the open space is measured. Mice were placed in the apparatus
shown in Fig. 4A for a total of 300 s. The distorted cones
persisting in the dorsal retinas of rd/md mice could conceivably
mediate a preference of mice for nesting in the dark under
chronic living conditions (16). However, these cones were not
sufficient to mediate light avoidance by rd/md mice under our
conditions: mice with normal retinas spent 244 ± 14.2 s (mean ±
SEM, n = 5) in the dark field, whereas untreated rd/md mice and
mice injected with AAV-GFP spent 136 ± 4.8 s (n = 10) and
150 ± 14.5 s (n = 9) in the dark area, respectively. Injection of
AAV-Opn4 returned the mice lacking rods and cones almost to
normal behavior (221 ± 7.7 s, n = 18). The difference between
untreated and untreated or AAV-transduced rd/md animals was
statistically significant at P < 0.01 (Fig. 4B).

Last, we sought a test that had a cognitive component (i.e., one
in which the mice were required to make a decision based on
visual information) (28). For this purpose, we used a two-choice
visual discrimination. Mice were taught that a bright target
represented safety in the form of a submerged platform. The
mice swam down an alley and had to choose between a bright
target (safe platform) or a dim target (no platform) (Fig. 4C).
For normal mice, this task is easy; they learned it to >90% accuracy
in only a few days (Fig. 4D, open squares). Rd/md mice injected
with the AAV-GFP construct did not reach above-chance performance after 8 days of training. Rd/md mice injected
with the AAV-Opn4 construct showed a steady improvement,
reaching a level of ~80% correct after the 8-day sequence (Fig.
4D). The difference between the latter two groups was signifi-
cant at P < 0.001 (two-way ANOVA).

The PLR was retested in one series of mice 11 months after
injection of AAV vectors. The sensitivity of the PLR in the
melanopsin-treated rd/md animals remained near the sensitivity
of the PLR in the wild-type animals (Fig. S3).

Discussion

These results show that the signaling system that couples mela-
opsin to membrane depolarization is ubiquitous, or at least very
widespread, in retinal ganglion cells; it is clearly not restricted to
the native melanopsin-expressing cells. Although there is no
certainty that the signaling pathway is identical in every ganglion
cell, a functional pathway that can couple activation of melano-
pin to a membrane cation channel appears to be present in
most types of retinal ganglion cell, as well as in many other neural
and nonneuronal cells (1, 3, 6–9).

In the absence of light-driven inputs, the responses of the
different morphological types of ganglion cells were similar to
each other. This finding suggests that ganglion cells, which
normally send diverse kinds of functional signals to the brain
(ON responses, OFF responses, sustained, transient, etc.), be-
electrophysiologically more uniform when driven by mela-
opsin. Presumably, the melanopsin system bypasses the normal
interplay of excitatory and inhibitory inputs to the cells. Subtle
differences, for example, those due to differing expression of ion
channel proteins or amounts of melanopsin, may well exist (20),
because their effects could have been obscured by the large and
long-lasting depolarization initiated by the photoactivation of
melanopsin.

Three different behavioral measures indicated that visual
function, at least of a simple sort, was restored in nominally blind
rd/md mice by ectopic expression of melanopsin. The PLR is the
simplest of the three, because it is a subcortically mediated visual
reflex. At the other extreme, the light–dark discrimination task
would ordinarily be classified as a learned visual discrimination.
However, none of the three tasks studied here require great
temporal or spatial resolution; they can evidently be performed
by using the slow- and long-lasting responses mediated by
melanopsin. The results represent proof of principle that the
visually driven information available from the ectopic expression
of melanopsin in a few thousand ganglion cells can be used to
guide behavior, but do not define the limits of the visual abilities
provided. An obvious next step will be to see whether these mice
Enhancement of visual function in the AAV-Opn4 treated rd/rd mice. (A) The open-field test box consisted of a dark compartment (one third of the floor area) and a larger illuminated compartment (two thirds). A small opening located at floor level in the center of the dividing wall allowed mice to freely move between the lit and dark chambers. (B) Time spent in dark area by four groups of mice. The AAV-Opn4 treated area) and a larger illuminated compartment (two thirds). A small opening located at floor level in the center of the dividing wall allowed mice to freely move between the lit and dark chambers. (C) Time spent in dark area by four groups of mice. AAV-Opn4 treated mice showed behavioral aversion to light: they spent significantly longer time in the dark chamber than their counterparts of either sham-injected or un.injected rd/rd mice (P < 0.01, t test). The exploratory behavior of un.injected C57BL mice is shown as comparison. Dotted line shows the behavior to be expected by chance. (C) Visual discrimination alley. Mice swam down a water-filled alley toward an illuminated or a dark target. The rewarded stimulus indicated the location of a submerged platform. (D) Melanopsin-treated (open circles) mice outperformed sham-injected (triangles) rd/rd mice in visual detection task over an 8-day trial (P < 0.001, two-way ANOVA test). Values represent mean ± SEM, n = number of mice.

Can carry out visual discriminations more demanding than those tested so far.

Could ectopic expression of melanopsin aid vision in humans suffering from photoreceptor cell degenerations (7)? These results suggest that the AAV vector could transduce enough cells in rodents for a crude but useful visual resolution, and that the sensitivity of the cells to light would be within a functional range. However, responses mediated by melanopsin can last for many seconds after the visual stimulus is off, and this persistence would limit the temporal resolution of vision. Modifications of the system to improve temporal resolution [coexpression of arrestin (8) and/or genetic modification of the signal pathway] might reduce this problem.

AAV-mediated expression of channelrhodopsin-2, a light-sensitive microbial protein that contains an intrinsic ion channel, renders ganglion and bipolar cells electrophysiologically responsive to light (11). When expressed in ON bipolar cells of rd/rd mice, it also permitted recovery of certain visual reflexes to high-intensity light: the PLR, an activity test, and the optokinetic response (10). Channelrhodopsin-2 is attractive because it yields responses to light on a millisecond time scale close to that of neurons in normal retinas. However, channelrhodopsin-2 is not a native mammalian protein, raising the possibility of an adverse immune response on long-term expression; this risk would be a significant factor when contemplating its use in humans. Also, it requires stimulation with light at exceedingly high intensities, potentially damaging to the retina when applied chronically. After transduction of ganglion cells with melanopsin, in contrast, the PLR of rd/rd mice returned virtually to its normal sensitivity; and our other behavioral tests were carried out by using stimulus intensities that fall in the range of ordinary indoor lighting. In the future, it might be possible to engineer a fast-acting melanopsin, or a more sensitive channelrhodopsin-2.

Materials and Methods

These experiments were carried out, with the same fundamental results, in two parallel sets of mice, one in San Diego and one in Boston. Because there were many small differences in the protocols, for simplicity this report describes only the Boston experiments, which were the larger series of the two. All methods are described in more detail in SI Methods.

 Constructs and Viral Vectors. Three constructs were used: AAV-GFP, AAV-Opn4, and AAV-Opn4-GFP. ORFs coding for GFP, full-length mouse melanopsin (GenBank accession no. 6693702), or Opn4-ires-GFP were cloned into pAAV-MCS8 vector under the transcriptional control of CMV promoter. These three constructs were packaged into AAV2 serotype virus at the Harvard virus production core. The packaged viruses were concentrated and purified in PBS at titers as follows: AAV-Opn4, 2.1 × 1012; AAV-GFP, 7.8 × 1012; and AAV-Opn4-GFP, 2.9 × 1012 genome copies per milliliter.

Immunocytochemistry and Electrophysiology. The retina was fixed in 4% paraformaldehyde (PFA) for 1 h. Antimelanopsin antibody was applied to reveal melanopsin. The primary antibody was antimelanopsin (1:200; Fisher Scientific), which was diluted in 5% NGS/1% BSA/0.5% Triton X-100 in PBS and

Fig. 4. Enhancement of visual function in the AAV-Opn4 treated rd/rd mice. (A) The open-field test box consisted of a dark compartment (one third of the floor area) and a larger illuminated compartment (two thirds). A small opening located at floor level in the center of the dividing wall allowed mice to freely move between the lit and dark chambers. (B) Time spent in dark area by four groups of mice. The AAV-Opn4 treated rd/rd mice showed behavioral aversion to light: they spent significantly longer time in the dark chamber than their counterparts of either sham-injected or un injected rd/rd mice (P < 0.01, t test). The exploratory behavior of un injected C57BL mice is shown as comparison. Dotted line shows the behavior to be expected by chance. (C) Visual discrimination alley. Mice swam down a water-filled alley toward an illuminated or a dark target. The rewarded stimulus indicated the location of a submerged platform. (D) Melanopsin-treated (open circles) mice outperformed sham-injected (triangles) rd/rd mice in visual detection task over an 8-day trial (P < 0.001, two-way ANOVA test). Values represent mean ± SEM, n = number of mice.
applied overnight. After washes in PBS, secondary antibody conjugated either to Alexa TM 488 (1:500; Molecular Probes) or Alexa TM 594 (1:500; Molecular Probes) were applied for 2 h. Confocal micrographs of fluorescent specimens were taken from retinal flat-mounted preparations with a Bio-Rad Radiance confocal microscope. Images were adjusted in brightness and contrast by using Photoshop 8 (Adobe Systems).

Whole-cell patch–clamp recording was carried out by conventional techniques (24) on intact retinas as whole mounts by using retinas injected with AAV-Opn4-GFP. The expression of GFP was relatively weak in these retinas (Fig. 2) and allowed visualization of only the soma. To prevent photodamage to the retina, after a GFP-expressing soma had been identified by fluorescence microscopy, further manipulation and electrode approach to the cell were carried out under infrared DIC. Photic stimuli were generated by the mercury lamp of the microscope (peak wavelength, 480 nm) attenuated by neutral density filters and were delivered through the epifluorescence pathway of the microscope optics. Note that the process of searching for a transduced cell requires strong short-wavelength illumination, which inevitably isomerizes photopigment and causes an unknown degree of light adaptation (20). For that reason, the unbleached sensitivities cannot be estimated from these electrophysiological experiments. From the results of the behavioral experiments they appear to be near the normal sensitivity of melanopsin, as judged from the sensitivity of the PLR.

Behavioral Tests. The PLR and open-field avoidance tests were carried out by standard techniques. The two-choice visual discrimination closely followed procedures systematically evaluated for various mouse strains by Wong and Brown (28). The apparatus (shown in Fig. 4b) and the testing protocol were close replicas of theirs. It is essentially a classic two-choice alley, with visual stimuli at the end of the alley. The alley was filled to a depth of 15 cm with water, and the reward was access to a safe platform located 1 cm beneath the surface of the water under the positive stimulus.

ACKNOWLEDGMENTS. We thank Aimee Wong and Richard Brown for advice on the testing of mouse visual discriminations and Jeng-Shin Lee and the Harvard vector core for providing the AAV constructs. This work was supported by National Institutes of Health Grants EY 017169 (to R.H.M.) and EY016807 (to S.P.). S.P. was supported by a Pew Scholars award. R.H.M. is a Senior Investigator of Research to Prevent Blindness.