Supporting Information

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SI Methods

Constructs and Viral Vectors. Experiments were performed on adult C57BL and C3H/HeJ (*rd*/*rd*) mice, which were maintained at the Massachusetts General Hospital. All experimental procedures were in accordance with institutional guidelines. After anesthesia with a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg), mice with photoreceptor degeneration (*rd*/*rd*, strain C3H/HeJ, The Jackson Laboratory) were injected intravitreally with 1 μl of AAV2 vector, under visual control, in both eyes. They showed no sign of distress of following the injections, nor did the visual function of the control (AAV2-GFP injected) animals fall below that of normal mice. A total of 20 *rd*/*rd* mice were injected with AAV2-GFP, 50 with AAV2-Opn4, and 30 with AAV2-Opn4 IRES-EGFP.

Immunocytochemistry and Cell Counting. At four weeks postinjection, animals were anesthetized with the same mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). Eyes were quickly enucleated after a reference point was taken to label the superior pole and the retina was dissected free of the vitreous and sclera in carboxygenated Ames medium (Sigma). The retina was fixed in 4% paraformaldehyde (PFA) for 1 h and then blocked for 1 h in a solution containing 10% normal goat serum (NGS), 1% BSA, and 0.5% Triton X-100 in PBS (pH 7.4). 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 applied overnight. After washes in PBS, secondary antibody conjugated either to Alexa 488 (1:500; Molecular Probes) or Alexa 594 (1:500; Molecular Probes) were applied for 2 h. The tissue was mounted in Vectashield (Vector Laboratories).

Retinal whole mounts were used to estimate the number of retinal ganglion cells. We counted total native melanopsin ganglion cells in retinas of uninjected and GFP-injected *rd*/*rd* mice, GFP positive ganglion cells in AAV-GFP-injected *rd*/*rd* mouse, and all melanopsin expressing ganglion cells in AAV-Opn4-injected *rd*/*rd* mice.

Confocal micrographs of fluorescent specimens were taken from retinal flat-mounted preparations with a Bio-Rad Radiance confocal microscope equipped with a krypton-argon laser at a resolution of 1024 $\times$ 1024 pixels. Zeiss Plan Apochromat 25-/0.8 and C-Apochromat 40-/1.2 W lenses were used. Images were adjusted in brightness and contrast by using Photoshop 8 (Adobe Systems).

Electrophysiology. Patch pipettes (10–15 MΩ resistance) were pulled from Pyrex tubing on a micropipette puller (P-97; Sutter Instrument). The pipette solution consisted as following (in mM): 125 K-gluconate, 5 KCl, 10 Hepes, 1 CaCl2, 1 MgCl2, and 11 EGTA (pH adjusted to 7.2 with KOH). The piece of retina was placed in a recording chamber, ganglion cell layer up, and continuously perfused at a rate of 2 ml/min with oxygenated Ames solution at 32–35°C. The recording pipette was connected to the input stage of a patch-clamp amplifier Axopatch 200B (Axon Instruments), and signals were sampled at 10 kHz with DigiData 1322A interface-type and pCLAMP8 software (Axon Instruments). Liquid junction potentials were corrected after recordings ($V_m = V_{record} - 11$ mV).

The intensity of the light was measured by using a photometer (LS-100, Minolta) and/or a photodiode calibrated by a Gossens Luna Pro illuminance meter. For some of the behavioral experiments (see below) wide fields of illumination were required; it was thus impractical to use monochromatic light. Several different light sources were used, all of them spectrally broad. Because a major goal of these experiments was to assess the visibility of the stimuli by a mammalian retina, we measured illuminance in photometric units (lux), i.e., units adjusted for the sensitivity of a mammalian (human) retina. For the purpose of these experiments, the human and mouse eyes do not differ greatly in their spectral sensitivities. To compare our results with others, a rough conversion to units of irradiance may be made by assuming all of the light to be at 550 nm, under which conditions 1 lux $\approx 4.1 \times 10^{11}$ photons $\times$ cm$^{-2} \times$ s^{-1}. This is an approximation accurate to approximately a factor of 2, which is well within the requirements of experiments of this type.

The retinas studied electrophysiologically were less sensitive than those in the pupillary light responses (PLR) or behavioral testing. This is partly because they were transduced with AAV-OPN4-IRES-EGFP, which yielded less expression of melanopsin than simple AAV-Opn4. In addition, the GFP-expressing cells were targeted for recording by using the bright epifluorescence illumination of the microscope, which isomerizes some of their melanopsin and may desensitize the signal transduction pathway as well.

PLR. The PLR was measured by standard techniques (1, 2). Unanesthetized mice 120 to 190 days old were dark adapted for at least 1 h after the light phase of their 12/12 h light/dark cycle. A quartz halogen lamp provided light stimuli, which were transmitted along a 60-cm fiber optic bundle to illuminate the whole eye. An intervening shutter (Uniblitz) controlled stimulus timing. The intensity of the light was controlled by neutral density filters. The effective intensities of each exposure were calculated by measuring illuminance at the position of the cornea. Background illumination was provided by dim red light throughout the experiment. Mice were grasped manually by the scruff of the neck and loosely supported upon a simple platform. A single trial lasted $<$30 s. Animals were subjected to white light exposures in an ascending intensity series, ranging from 0.1 to 1500 lux. Individual trials were separated by at least 2 min (2). An infrared sensitive video recorder (Sony model SR-42) fitted with a 10x macro lens was focused upon the eye. Pupillary responses were quantified from the video images, by using Sony motion picture software and ImageJ software (NIH). To correct for individual variance in the area of the dark adapted pupil, the data were normalized to pupil area immediately preceding light onset. The PLR curves were fitted by a sigmoidal relationship.

Open Field Test. The open field test was conducted as described previously (3). The light/dark box (45 $\times$ 27 $\times$ 25 cm) was made of Plexiglas and consisted of two chambers connected by an opening (4 $\times$ 5 cm) located at floor level in the center of the dividing wall (Fig. 4*A*). The test field was diffusely illuminated at 2,800 lux by a tungsten filament bulb positioned over the apparatus.

Mice were carried into the testing room in their home cage. A trial began when the mouse was placed inside the dark shelter for a 2-min habituation period, with the opening from dark to light spaces closed. The mouse was then allowed to leave the shelter and explore the illuminated field for 5 min. For each mouse the length of time the animal spent in the light side of the box was recorded. A video camcorder located above the center of the box provided a permanent record of the behavior of the mouse. Mice were then removed from the box and returned to the home cage.

The box was then cleaned with a solution of 70% ethanol/water and permitted to dry between tests.

Two-Choice Visual Discrimination. Mice were pretrained to swim to a hidden platform (37 × 13 × 14 cm, *l* x *w* x *h*) located below a translucent panel with a luminance of 32 cd/m^2. The other side of the alley held a dark panel (0.005 cd/m^2) and no platform. The two sides were separated by a divider (Fig. 4*B*). Mice were pretrained in the overall task in several steps. First, they were placed onto the hidden platform for 1 min. They were then released at increasing distances from the platform. When they swam confidently, they were released from the start of the alley. Discrimination testing consisted of eight trials a day for 8 days (64 trials). The positive stimulus (S+) was alternated between the left and right side of the divider in the sequence LRLLR-LRR for the first, second, fifth, and sixth days of testing and reversed (RRLRLLRL) for the other 4 days of testing. A correct trial was scored when a mouse swam from the release chute to the S+ platform without entering the S− arm. If the animal crossed a line perpendicular to the end of the divider on the S− side of the tank, the trial was recorded as an error. The mouse was then required to run another trial. If the mouse failed to find the platform after 1 min, it was guided to the platform and allowed to stay on the platform for a few seconds and the trial was recorded as incorrect. The number of correct trials and their latency were recorded. A video camcorder located above the box made a permanent record of the performance of the mouse.

Discussion

In principle, the PLR observed here could be mediated by overexpression of melanopsin in the native melanopsin cells, as a recent report shows that the melanopsin cells are a major central pathway of the PLR (1, 2, 4). While this is imaginable, it does not seem likely. The AAV vector transduced only a small fraction of the total population of retinal ganglion cells: assuming a total ganglion cell population of 45,000 cells (5), the Opn4-expressing ganglion cells were 9.9% of the total population. On a statistical basis, we would then expect that only ≈60 of the ≈600 native melanopsin cells would be candidates for the postulated overexpression. In fact, we saw no evidence that any native melanopsin cells (which are easily recognized from their dendritic morphology) expressed unusually high levels of melanopsin in the AA-treated retinas. This could be directly studied in the retinas transduced with both Opn4 and GFP. The retinas were double immunostained for melanopsin and GFP. The native melanopsin cells were identified by their dendritic morphology, and those that had been transduced were identified by their expression of GFP (Fig. S1). In 16 retinas, a total of 88 out of 947 melanopsin-expressing cells sampled (9.2 ± 2.4%) expressed both melanopsin and GFP. There was no suggestion that these cells contained more melanopsin protein than the nontransduced cells. While there is no doubt that the melanopsin cells make a major contribution to the normal PLR (6, 7), other retinal ganglion cells do project to the olivary pretectal nucleus (the brainstem nucleus of the PLR) and the present results suggest that they can contribute to the pupillary response as well. It may be important in this context that the responses to light triggered by ectopic expression of melanopsin are larger and last much longer than those of normal nonmelanopsin ganglion cells.

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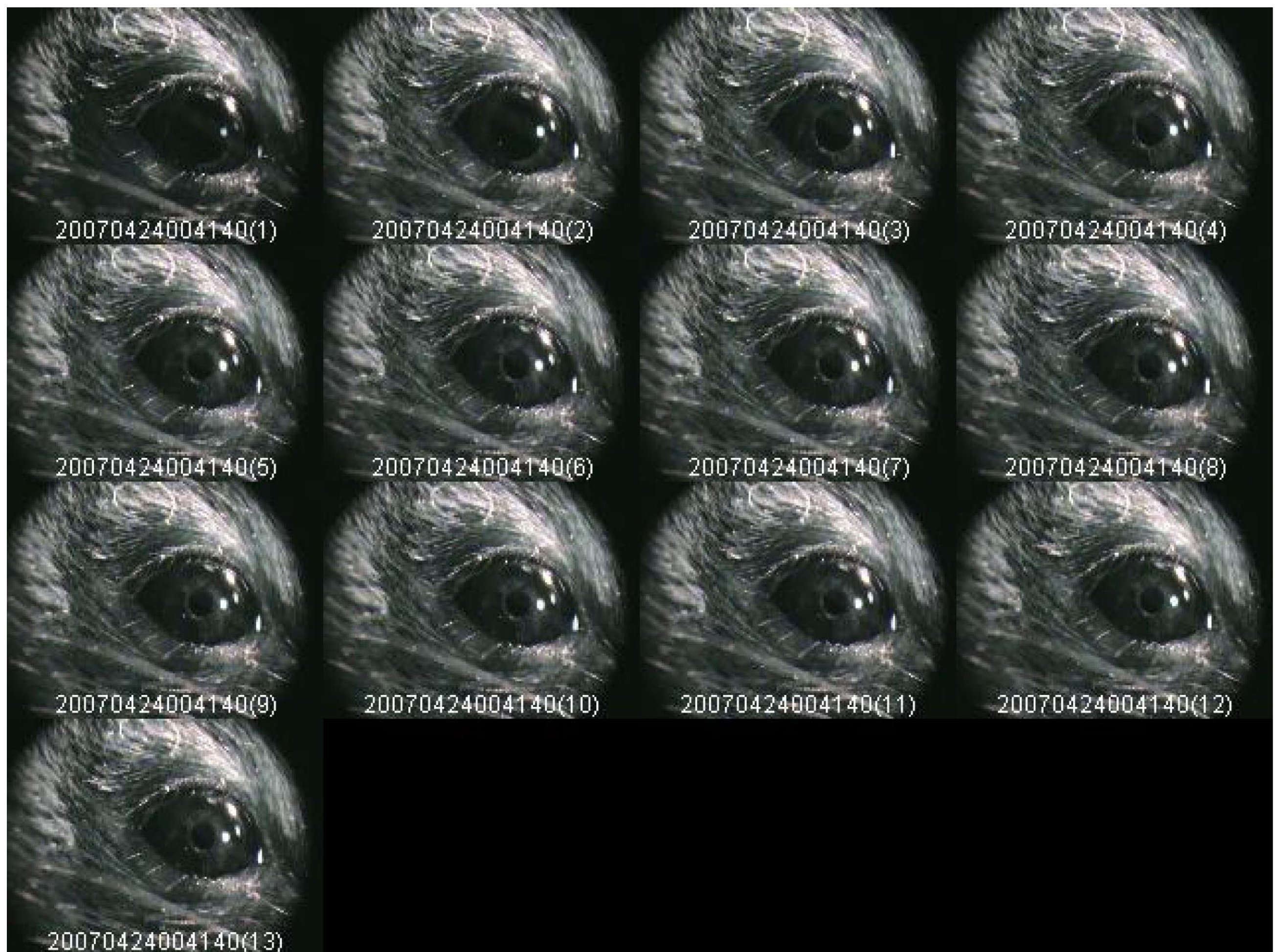

Fig. S1. Few native melanopsin ganglion cells in *rd/rd* mice are transduced by AAV-Opn4-IRES-GFP. Three separate fields are indicated. In each case, *Left* indicates ganglion cells stained for melanopsin. These include both cells transduced by the vector and native melanopsin cells (arrows), recognized at higher power by their depth of stratification and distinctive pattern of dendritic branching. *Center* indicates the expression of GFP. *Right* indicates the merged images. In these fields, none of the native melanopsin cells (arrows) were transduced by the vector, because none of them expressed GFP.

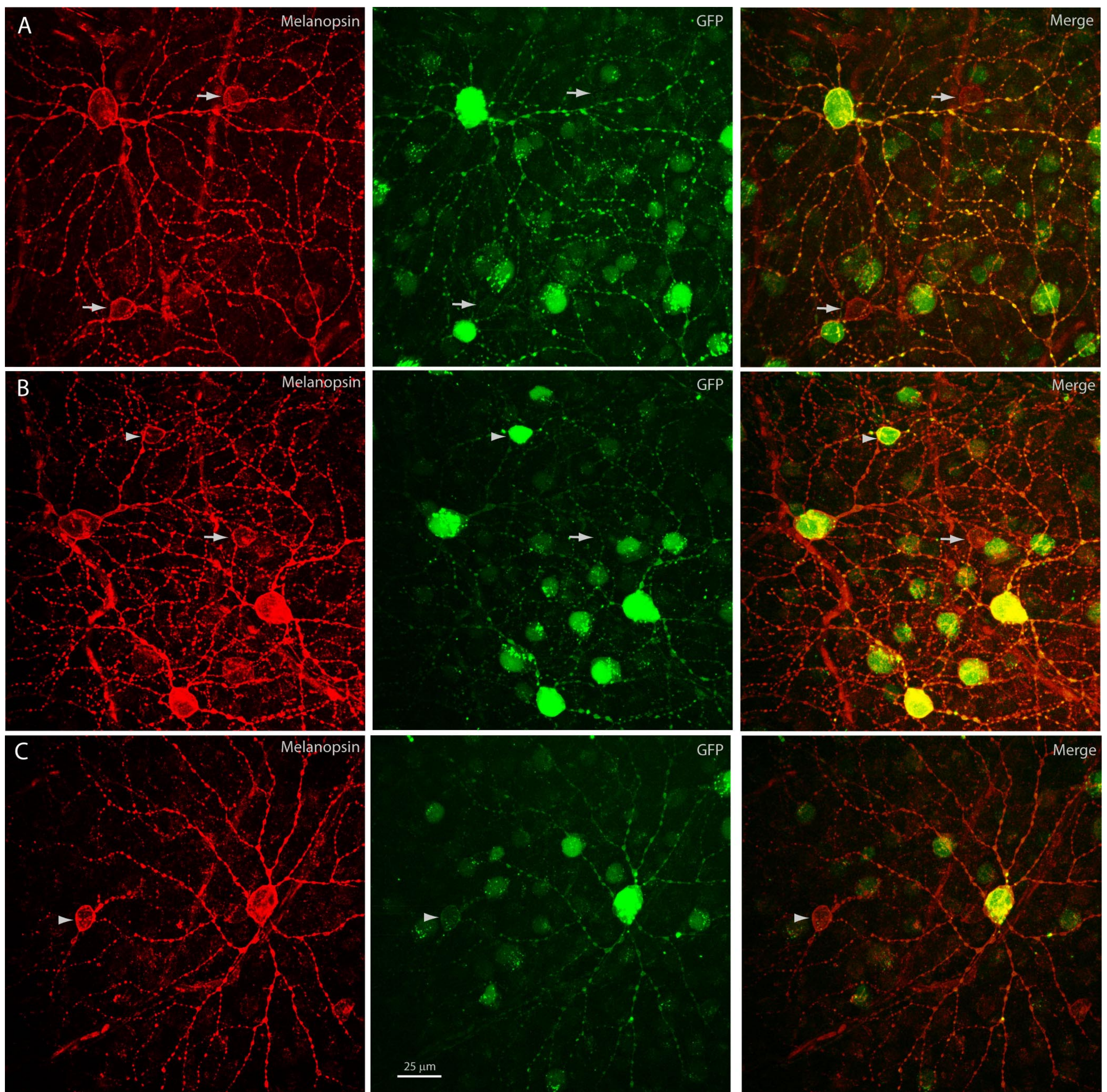

Fig. S2. Pupillary light reflexes in a melanopsin-treated *rd*/*rd* mouse. The montage shows the constriction of pupil area at 333-ms intervals after exposure to halogen (white) illumination at 280 lux.

Fig. S3. Pupillary light reflexes 11 months after injection of AAV vectors. Intensity-response curves for pupillary constriction after exposure 20 s of white light. The area of the pupil is depicted as a percentage of its size immediately preceding the onset of light. The intensity-response function for animals injected with the AAV-Opn4 vector (red curve) remains close to the curve for wild type (uninjected C57BL mice, black curve). Both are more sensitive than the sham-injected *rd/rd* eyes (blue curve). Compared with the measurement at 2 months, there may have been a slight overall loss in sensitivity of both the wild type and the melanopsin-transduced eyes. Since it appeared in the wild type as well as the treated eyes, this may have been due to normal aging (the maximal pupillary constriction was ≈10% less in the older animals), but this effect, if it is real, needs to be studied further. Values are mean ± SEM, with n indicating the number of eyes examined.