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Photochemical Properties of Mammalian Melanopsin

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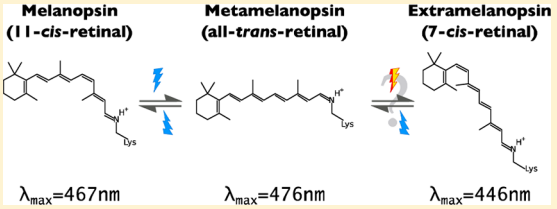
Supporting Information

ABSTRACT: Melanopsin is the photoreceptor molecule of intrinsically photosensitive retinal ganglion cells, which serve as the input for various nonvisual behavior and physiological functions fundamental to organisms. The retina, therefore, possess a melanopsin-based nonvisual system in addition to the visual system based on the classical visual photoreceptor molecules. To elucidate the molecular properties of melanopsin, we have exogenously expressed mouse melanopsin in cultured cells. We were able to obtain large amounts of purified mouse melanopsin and conducted a comprehensive spectroscopic study of its photochemical properties. Melanopsin has an absorption maximum at 467 nm, and it converts to a meta intermediate having an absorption maximum at 476 nm. The melanopsin photoreaction is similar to that of squid rhodopsin, exhibiting bistability that results in a photosteady mixture of a resting state (melanopsin containing 11-*cis*-retinal) and an excited state (metamelanopsin containing all-*trans*-retinal) upon sustained irradiation. The absorption coefficient of melanopsin is 33000 ± 1000 M^{-1} cm^{-1}, and its quantum yield of isomerization is 0.52; these values are also typical of invertebrate bistable pigments. Thus, the nonvisual system in the retina relies on a type of photoreceptor molecule different from that of the visual system. Additionally, we found a new state of melanopsin, containing 7-*cis*-retinal (extramelanopsin), which forms readily upon long-wavelength irradiation (yellow to red light) and photoconverts to metamelanopsin with short-wavelength (blue light) irradiation. Although it is unclear whether extramelanopsin would have any physiological role, it could potentially allow wavelength-dependent regulation of melanopsin functions.



Melanopsin is an opsin molecule initially isolated from dermal melanophores of *Xenopus laevis*.[1] It later became clear that this photoreceptor molecule was primarily expressed in the retina, in a subset of the retinal ganglion cells now known as ipRGCs (intrinsically photosensitive retinal ganglion cells), which serve as the input for various nonvisual behavioral and physiological systems of fundamental importance, such as PLR (pupillary light response), photoentrainment of circadian rhythm, and negative masking by light in mammals.[2−6] Although ipRGCs can relay light signals from the canonical photoreceptor cells and also respond to light themselves, it is clear that the light response evoked from melanopsin is essential for normal function.[6−9] Additionally, it was recently shown that some vertebrates can elicit a PLR directly from the iris, through the input of melanopsin.[10] Moreover, light input from melanopsin is an important regulator of the circadian clock, which has been implicated in a range of disorders, including behavioral disorders (circadian rhythm sleep disorders and seasonal disorders),[11] metabolic disorders (cardiovascular disease, obesity, and diabetes),[12−14] migraines,[15] and even cancer.[16] A study of melanopsin will therefore yield valuable insights into its potential therapeutic use.

The discovery of ipRGC and melanopsin has prompted interest from evolutionary biology as well. Phylogenetically, melanopsins belong to the group of opsins that includes visual pigments of cephalopods and arthropods (Gq opsins), and ipRGCs have seemingly originated from the same types of cells as these invertebrate visual photoreceptor cells.[1,17,18] Although we possess both types of photoreceptor molecules, there seems to be a division of labor for visual and nonvisual functions. This may reflect the differences in the molecular requirements for their physiological function. However, little is known about Gq opsins in comparison to the well-studied vertebrate visual opsins (Gt opsins). Understanding the functional differences between opsins is crucial for understanding the evolution and functions of visual and nonvisual systems in animals.

In spite of considerable interest, our understanding of melanopsin at the molecular level has largely been hindered by technical difficulties in its heterologous expression and purification. Although several groups have successfully expressed melanopsin in various cells,[18−24] only a few have successfully purified melanopsin,[18,24] yet key photochemical properties of melanopsin remain unknown. In fact, only a handful of opsins belonging to the Gq opsins have been successfully expressed.[24] Here we report on the photochemical properties of mouse melanopsin, expressed in HEK293 cells. We were able to express melanopsin in HEK cells and purify sufficient amounts to conduct a comprehensive characterization of the photochemical properties of melanopsin for the first time.

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EXPERIMENTAL PROCEDURES

Heterologous Expression of Melanopsin. *Mus musculus* melanopsin cDNA (GenBank entry BC139827) was purchased from Open Biosystems, Inc., and inserted into the pCAG expression vector.[25] Melanopsins, in general, contain a relatively large C-terminus compared to vertebrate visual pigments. The long C-terminus of melanopsin was truncated to improve expression yield and purification, and the epitope sequence for anti-bovine rhodopsin monoclonal antibody rho1D4 was added for purification with an immunoaffinity column. The amino acid sequence of the melanopsin construct is provided in the Supporting Information.

Melanopsin was heterologously expressed in HEK293 cells as previously described.[26] HEK cells were transfected by calcium phosphate coprecipitation, with the melanopsin-expressing plasmid. Traditionally, heterologously expressed opsins are regenerated with retinal after they have been harvested; however, we found that incubation with retinal during culture increased the final yield of the pigment. Therefore, 11-*cis*-retinal (final concentration of 2.7 μM) was added to the culture medium 24 h after transfection and cultured in the dark. Cells were cultured for an additional 24 h and then harvested. All the following procedures were conducted on ice in the dark under dim red light.

Purification. HEK cells were harvested and solubilized with 1% *n*-dodecyl β-D-maltoside (DM) in buffer PM [50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH adjusted to 7.0)]. The DM extract was incubated with rho1D4-conjugated agarose overnight and washed with 1% digitonin and 25% glycerol in buffer PM. Finally, the pigment was eluted with the same buffer containing the synthetic nonapeptide corresponding to the rho1D4 epitope sequence.

Preparation of Membrane Fractions. Membrane fractions of HEK cells containing mouse melanopsin were prepared by sucrose floatation. Harvested HEK cells were homogenized, and membrane fractions were separated by 50% sucrose floatation as previously described.[27] The supernatant, containing membrane fractions, was then collected and precipitated by diluting the sucrose to 25% and centrifuging. Membrane fractions were then washed and suspended in buffer PM. Spectroscopic measurements were taken by resuspending these membrane fractions in 50% sucrose in buffer PM after a brief sonication (typically, cells from a single 150 mm dish were suspended in 500 μL).

Spectroscopy. Spectroscopic measurements were taken using a Shimadzu UV-2400 spectrophotometer.[28] Samples were loaded on a black masked quartz cell with an inside width of 2 mm and an optical path length of 10 mm. The temperature of the cell holder was regulated within 0.1 °C with a thermostat, and nitrogen gas was blown against the cell to prevent the condensation of dew. All spectra were recorded at 0 °C. When light irradiation is indicated, the light source was a 1 kW tungsten−halogen lamp (Rikagaku-Seiki) beam, passed through a 5 cm water layer to remove infrared radiation. The wavelength of irradiation light was controlled by combining band-pass filters and glass cutoff filters. The tungsten−halogen lamp provided a steady flux of photons, and the amount of light was regulated by neutral density filters and by manually controlling irradiation time. The photon flux of a particular wavelength was measured with a NEOARK PM-245 laser power meter.

Estimation of the Absorption Coefficient. The absorption coefficient, or molar extinction coefficient, of melanopsin was estimated by acid denaturation of purified melanopsin.[29−31] Acid-denatured pigments produce an absorbance peak at 440 nm that is independent of the apoprotein, because of the acid trapping of the protonated retinal Schiff base. The absorption coefficient was estimated relative to the absorption coefficient of bovine rhodopsin, 40600 M^{-1} cm^{-1}.[32] After the pH had been measured and the dark state spectrum of purified pigments recorded, a small amount of 2 N HCl was added and the mixture incubated until the spectral change ceased and denaturation was completed. Samples were collected immediately after spectroscopic measurements, and their pH was checked (pH <2.0). The denatured spectrum was corrected for dilution, and denatured spectra of melanopsin and rhodopsin were normalized.

Chromophore Extraction and HPLC Analysis. Extraction of the chromophore from samples was performed according to the method previously described.[28,33] Retinal oxime was generated from purified pigment samples (kept in the dark or irradiated with light) by incubating a 250 μL aliquot with hydroxylamine (final concentration of 100 mM) and denaturing pigments with 250 μL of methanol and 250 μL of dichloromethane. After thorough mixing, the mixture was supplemented with 1 mL of hexane and mixed again to transfer retinal oximes to the hexane layer. The mixture was then centrifuged to separate the hexane layer, and the hexane extraction was repeated once more. The collected hexane layer containing retinal oximes was dried over anhydrous Na_2SO_4 and then evaporated under a N_2 stream. All procedures were conducted under dim red light, and the sample was stored at −80 °C until it was used.

Extracted retinal oximes were analyzed by HPLC to determine their isomeric composition, as previously described,[33−35] using a Shimadzu LC-10AT apparatus equipped with a silica column (150 mm × 6.0 mm, A-012-3, YMC). The solvent consisted of 98.8% (v/v) benzene, 1.0% (v/v) diethyl ether, and 0.2% (v/v) 2-propanol. Dried retinal oxime samples were dissolved in 20 μL of hexane, and 10 μL was used for HPLC analysis. The HPLC patterns were obtained by monitoring the absorbance at 360 nm. A sample containing authentic syn and anti isomers of 11-*cis*-, 9-*cis*-, 13-*cis*-, and all-*trans*-retinal oximes was used as a standard to assign retention times. The isomeric composition of the sample was calculated from the areas of the peaks and the molar extinction coefficients at 360 nm:[36] 13s, 49000; 13a, 52100; 11s, 35000; 11a, 29600; 9s, 39300; 9a, 30600; 7s, 47000; 7a, 46200; Ts, 54900; Ta, 51600.

Photosensitivity. Photosensitivity of purified pigments was determined by UV−vis spectrophotometry as previously described.[28,37] The pigment's responses to incremental stimulation were recorded, and the amplitude of the light-elicited response was plotted versus photon flux and fit with an exponential function. Pigments were irradiated for 20, 20, 40, 80, 160, and 320 s (additionally, bovine rhodopsin was irradiated for 640 s) so that the accumulated irradiation times were 20, 40, 80, 160, 320, and 640 s (and 1280 s for bovine rhodopsin), with light passing through a band-pass filter (BP 450) and a cutoff filter (L40) to selectively irradiate pigments at 448 nm. Additionally, we used a neutral density filter (ND20) to attenuate the light intensity. The photon flux monitored before and after experiments remained constant at 1.365×10^{14} photons cm^{-2} s^{-1}. Bovine rhodopsin photosensitivity was

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measured in the presence of 50 mM hydroxylamine, assuming a single-exponential reaction, as previously described.[37−39] The obtained exponential rate constant is relative to the photosensitivity. We used bovine rhodopsin to calibrate our measurements and therefore estimated photosensitivity of melanopsin relative to that of bovine rhodopsin. For melanopsin, we assumed a reversible reaction scheme between melanopsin and metamelanopsin, and therefore, the exponential constant of melanopsin represented the combined photosensitivities of melanopsin and metamelanopsin. The individual exponential constants were estimated by using the relative amounts of melanopsin and metamelanopsin in the equilibrium state mixture, obtained from HPLC analyses of retinal composition.

RESULTS

Melanopsin Spectrum. Expression and purification of melanopsin are critical for furthering our understanding of the molecular properties of melanopsin. We have successfully expressed mouse melanopsin in HEK293 cells and were able to characterize its photochemical properties. Typically, we obtained 0.005 absorbance milliliter of regenerated melanopsin per 150 mm dish extract, which is roughly $^{1}/_{10}$ of the yield we obtained for bovine rhodopsin. Purified melanopsin exhibited an absorption spectrum with a maximum (λ_{max}) at 467 nm, as shown in Figure 1. The purity A_{280}/A_{max} was not very good,

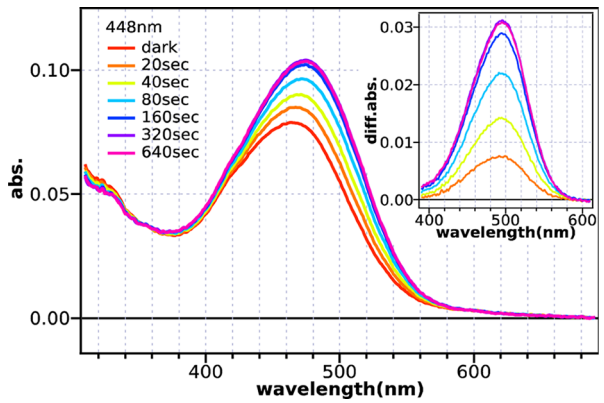

Figure 1. Absorption spectrum and photoreaction of purified melanopsin. Melanopsin (red trace) had an absorption maximum (λ_{max}) of 467 nm. Melanopsin was irradiated incrementally with 448 nm light, which caused the absorbance to increase between 400 and 600 nm, shown in the difference spectra in the inset. After exposure to light for 320 s, melanopsin photoreaction was saturated, and further irradiation did not elicit any change (640 s).

with values typically varying between 8 and 10 (see Figure S1 of the Supporting Information); however, the α-band of the pigment was clearly defined, and the large absorbance at 280 nm is probably due to misfolded and/or denatured melanopsin. Light irradiation of melanopsin results in an increase in absorbance between 400 and 560 nm (see the inset of Figure 1), resulting in a spectrum similar to that of the dark state but with a higher amplitude. This photoreaction is indicative of the formation of the protonated acid-meta state, characteristic of Gq-coupled opsins such as squid rhodopsin. We therefore assigned this photoproduct as acid metamelanopsin (hereafter simply termed "meta" as a contribution from alkali meta was minimal if any, judging by the absorption around 380 nm). Although we were able to partially purify melanopsin with other detergents [0.02% DM and 0.75% CHAPS with 1 mg/mL phosphatidylcholine (CHAPS/PC)], the meta state was stable only in preparations with 1% digitonin (metamelanopsin was stable with or without glycerol). Purified melanopsin was relatively stable to hydroxylamine, showing a modest 3−4% bleach after incubation for 30 min with 15 mM hydroxylamine [the rate constant of hydroxylamine bleach (k) was approximately 0.0015 min^{-1}], while metamelanopsin was more reactive and quickly formed retinal oxime (k = 0.1 min^{-1}).

Melanopsin Bistability between all-*trans*- and 11-*cis*-Retinal-Bound States. Several authors have suggested that melanopsin functions as a bistable pigment, as melanopsin-expressing cells do not have well-known access to 11-*cis*-retinal.[40] Moreover, several spectroscopic studies have shown that Gq opsins function as bistable pigments.[18,24] Bistable pigments, as opposed to bleaching pigments (also termed monostable pigments) such as the vertebrate visual opsins (Gt opsins), have a stable active state capable of reabsorbing a photon resulting in the reisomerization of retinal back to 11-*cis*-retinal, reverting the activated receptor to its resting state. However, as shown in the inset of Figure 1, it is clear from the difference spectrum of melanopsin and metamelanopsin, that meta state (all-*trans*-retinal-bound state) has an absorption spectrum closely resembling that of the dark state (11-*cis*-retinal-bound state), similar to those of metarhodopsin and rhodopsin in the squid.[41] It was therefore impossible to control the formation of the meta state in a wavelength-dependent manner, and irradiation at any wavelength in the absorption range of melanopsin resulted in the formation of metamelanopsin. Figure 2 shows the retinal composition of the resting state and after light exposure. The retinal composition of the photoproduct after saturating light exposure shows that 11-*cis*-retinal is still present in significant amounts. This indicates that light excitation produces a photosteady state mixture consisting of melanopsin (11-*cis*-retinal-bound state) and metamelanopsin

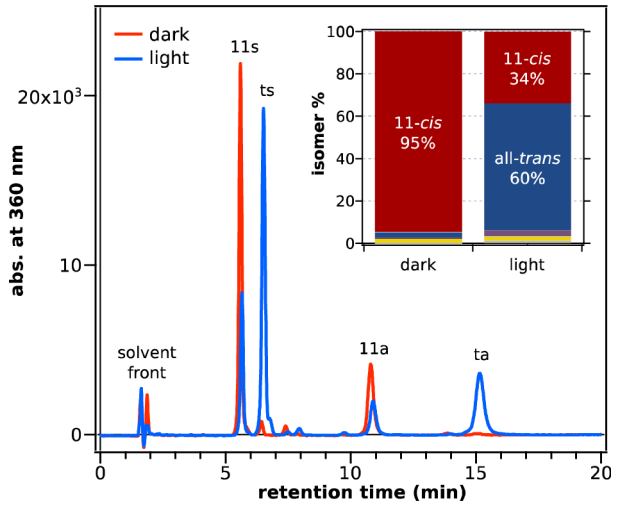

Figure 2. Retinal composition of melanopsin. HPLC pattern of retinal oximes extracted from melanopsin kept in the dark (red) or exposed to light (blue; 448 nm light for 640 s as shown in Figure 1). Positions of the main peaks [syn 11-*cis*-retinal oxime (11s), anti 11-*cis*-retinal oxime (11a), syn all-*trans*-retinal oxime (ts), and anti all-*trans*-retinal oxime (ta)] are indicated. The inset shows the calculated retinal compositions in dark- and light-exposed melanopsin. All calculated values are summarized in Table S1 of the Supporting Information.

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(all-*trans*-retinal-bound state). That is, melanopsin can photoconvert retinal between 11-*cis* and all-*trans* forms. We were not able to regenerate melanopsin directly with all-*trans*-retinal during expression, but this could be due to the inherent instability of metamelanopsin compared to melanopsin.

Model Spectra of Melanopsin and Metamelanopsin. Our purified sample seemed to contain very little nonspecific retinal contamination as shown in Figure 2, and 95% of the retinal of resting state melanopsin was 11-*cis*-retinal (see also Table S1 of the Supporting Information for more detailed information). However, our sample still contained small impurities absorbing in the short-wavelength region of the absorption spectrum. Although these impurities did not significantly affect our analyses, we constructed model spectra of melanopsin to improve our assessments. The model spectrum of the melanopsin α-band was constructed by fitting the difference spectrum of melanopsin before and after light exposure in the presence of hydroxylamine, with the mathematical equation for retinoids proposed by Lamb.[42] As absorption of retinal oxime distorts the spectrum, the curve was fit for wavelengths of >440 nm. In addition to the main α-band peak, we produced a β-band using Govardovskii's criteria.[43]

We used this model melanopsin template to estimate the contribution of impurities in our sample preparations. The linear region between $\lambda_{70\%}$ and $\lambda_{30\%}$ has the most accurate portion of the pigment absorption function, as it is the steepest part of the curve.[44] We adjusted our model melanopsin template so that this nearly linear portion of the right limb of the α-band has the same slope as the melanopsin spectrum. We then subtracted the adjusted model melanopsin spectrum from the melanopsin spectrum, which produced a curve corresponding to impurities and light scattering.

The model spectrum of metamelanopsin was constructed from the photosteady mixture of melanopsin and metamelanopsin. We corrected our data for impurities with the method mentioned above and then calculated the spectrum of metamelanopsin from the spectrum of the photosteady mixture, using the relative amounts of melanopsin and metamelanopsin obtained from the analyses of retinal composition. As most of the nonspecific retinal is expected to isomerize to all-*trans*-retinal and our samples contained only 2.8 ± 0.7% of all-*trans*-retinal (see Table S1 of the Supporting Information), we ignored nonspecific 11-*cis*-retinal and assumed that all 11-*cis*-retinal originated from melanopsin. Our calculations show that λ_{max} = 476 nm for metamelanopsin. Finally, we also fit the metamelanopsin α-band with the retinoid curve, and the remaining β-band with a Gaussian curve. Figure 3 shows the model spectra of melanopsin and metamelanopsin. Note that in our calculations the β-band of metamelanopsin is defined by the β-band of melanopsin, which was arbitrarily assigned; however, the absorbance of the β-band region did not affect our calculations as all of our calculations were based on the α-band region of the spectra. We have provided a complete description of the α-band peak of our model spectra in Figure 3, so others can reproduce and test these model spectra.

Absorption Coefficient of Melanopsin. Next we characterized the photochemical properties of melanopsin. We used acid denaturation to estimate the absorption coefficient (molar extinction coefficient) of melanopsin (see Figure 4). The absorption of pigments decayed gradually as a new absorbance peak formed around 440 nm upon acid treatment, indicating denaturation and formation of a protonated retinylidene Schiff base. The spectrum of the

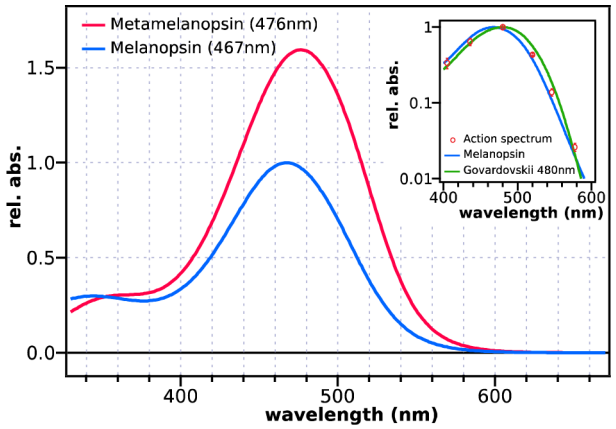

Figure 3. Model spectra of melanopsin (blue) and metamelanopsin (red), calculated from our data using the mathematical description of opsin spectra derived by Lamb.[42] The difference in the amplitude corresponds to the difference in the absorption coefficient of melanopsin and metamelanopsin. The inset shows the model melanopsin (blue) and the universal template curve derived by Govardovskii with a λ_{max} of 480 nm (green), along with the action spectrum of mouse PLR (red) from ref 10. The model spectra are given by the expression $S(x) = 1/\{\exp[A(a - x)] + \exp[B(b - x)] + \exp[C(c - x)] + D\}$, where $x = \lambda_{max}/\lambda$. The coefficients of our model melanopsin spectrum are as follows: λ_{max} = 476.62, A = 41.047, a = 0.91961, B = 12.679, b = 0.91671, C = −15.768, c = 1.1055, and D = 0.45454). Those for metamelanopsin are as follows: λ_{max} = 483.49, A = 44.402, a = 0.90595, B = 11.768, b = 0.87117, C = −14.088, c = 1.1367, and D = 0.2554. Note that "λ_{max}" in Lamb's equation does not necessarily represent the maximum of the curve.

denatured protein indicates the absorption of melanopsin to be approximately 33000 M^{-1} cm^{-1}. Our preparations seemed to contain small impurities, which we calculate to amount to 1000 in our estimate. Also, the spectrum of denatured melanopsin is slightly broader, suggesting that denaturation may not be complete, which may also alter our estimates by −1000. Overall, however, the spectrum of denatured melanopsin resembles that of rhodopsin closely, and we therefore assign the melanopsin absorption coefficient at its λ_{max} as 33000 ± 1000 M^{-1} cm^{-1}. This value is significantly smaller than that of monostable pigments such as bovine rhodopsin (40600 M^{-1} cm^{-1}) but very close to that of cephalopod rhodopsins, which are characterized by their bistability.[32,45] From the absorption coefficient of melanopsin relative to that of metamelanopsin, obtained from the model spectra in Figure 3, the absorption coefficient of metamelanopsin was calculated to be 52600 ± 1600 M^{-1} cm^{-1}.

Photosensitivity and Quantum Yield of Melanopsin. With the absorption coefficient, we can estimate the quantum yield of melanopsin by measuring its photosensitivity. As melanopsin is a bistable pigment, we assumed a reversible reaction scheme: [R] ⇌ [M], where [R] is the concentration of the resting state and [M] the concentration of the meta state. Therefore

$$\frac{d[R]}{dt} = -k_f[R] + k_b[M]$$

where k_f is the rate constant for the reaction from the resting state to the meta state (forward) and k_b is the rate constant for the reaction from the meta state to the resting state (back). We obtained the combined rate constant $k_f + k_b$ by monitoring the

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formation of the meta state (see Figure 5). Additionally, when the mixture is in a photosteady state

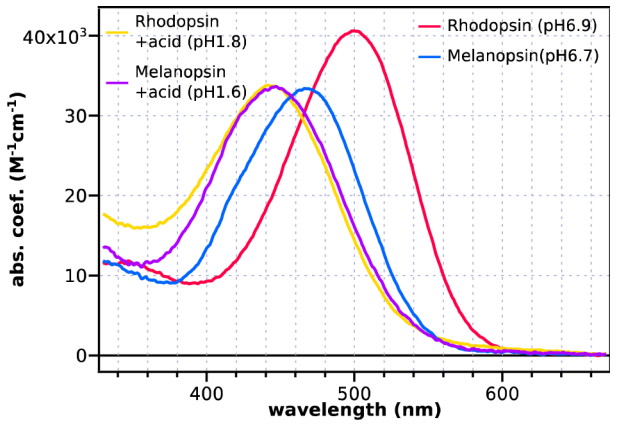

Figure 4. Acid denaturation of melanopsin. Absorption spectra of melanopsin and rhodopsin before and after acid denaturation (pigments were purified with 1% digitonin and 25% glycerol in buffer PM). The pH, indicated in parentheses, was measured before and after spectroscopic measurements. Spectra were normalized at the denatured spectral peak at ~440 nm, and the absorption coefficient of melanopsin (33000 ± 1000 at 467 nm) was calculated relative to that of bovine rhodopsin. Identical results were obtained from duplicate experiments.

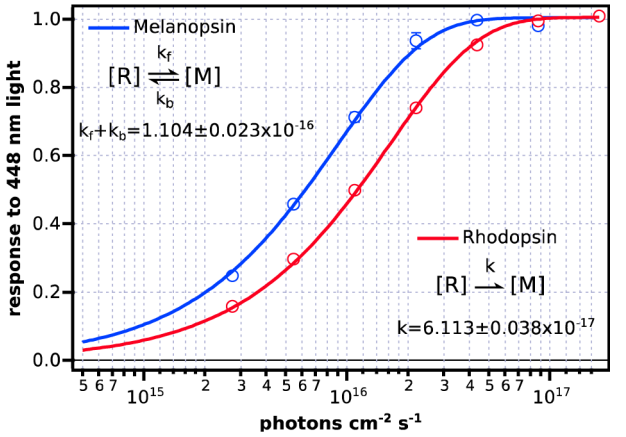

Figure 5. Photosensitivity of melanopsin relative to that of rhodopsin. The photosensitivity of purified melanopsin (blue; melanopsin purified with 1% digitonin and 25% glycerol in buffer PM) was obtained by an exponential fit of the response to 448 nm light, i.e., the peak absorbance change caused by light stimuli (Figure 1, inset), plotted vs the photon number of the excitation light. The light response of bovine rhodopsin was measured in the same experimental setup to calculate the photosensitivity relative to that of rhodopsin. The rhodopsin light response was measured as previously described, with a sample purified with 0.02% DM in buffer PM to facilitate measurements.[28] The obtained rate constant of melanopsin represents the combined photosensitivities of the resting state, [R], and the meta state, [M], because of its bistability, whereas the rate constant of rhodopsin represents the photosensitivity of the resting state, [R], alone. Error bars show the standard deviation from three independent measurements.

$$\frac{[M]_{eq}}{[R]_{eq}} = \frac{k_f}{k_b} = K$$

where $[M]_{eq}$ and $[R]_{eq}$ are the amounts of the respective states at equilibrium. We were able to estimate the equilibrium constant ($K = 1.7 \pm 0.1$) by analyzing the retinal composition of melanopsin in the dark and after formation of the photosteady mixture by light irradiation (see Table S1 of the Supporting Information).

With K and the combined photosensitivity $k_f + k_b$, we determined the individual constants k_f and k_b. We further used bovine rhodopsin to calibrate the photosensitivity in our experimental setup and obtained the photosensitivities of melanopsin and metamelanopsin relative to that of bovine rhodopsin.

Finally, the quantum yield of isomerization of melanopsin was calculated relative to that of bovine rhodopsin using the relationship $S \propto \varepsilon\varphi$ among the photosensitivity (S), the quantum yield of isomerization (φ), and the absorption coefficient (ε).[46] Table 1 shows all the calculated values of the absorption coefficient, photosensitivity, and quantum yield.

Extramelanopsin. In the course of our experiments, we discovered a melanopsin state different from the resting or meta state. When melanopsin is exposed to light, it produces metamelanopsin, manifested as an increase around 500 nm in the absorption spectrum (see the inset of Figure 1). However, when it is exposed to long-wavelength light (orange or red light), a second peak appears around 430 nm as shown in Figure 6. Sustained irradiation increases the magnitude of this peak, while the amplitude of the metamelanopsin peak decreases. Surprisingly, this peak turned out to be a new state containing 7-*cis*-retinal, as revealed by HPLC analysis (see Figure 6c and Table S1 of the Supporting Information). We named this additional state "extramelanopsin". Extramelanopsin (7-*cis*-retinal-bound state) is most probably formed from metamelanopsin (all-*trans*-retinal-bound state), as it is unlikely that a single photon photoisomerizes two double bonds. Interestingly, the extra state was photoconverted to the meta state via short-wavelength irradiation (blue light). That is, melanopsin and extramelanopsin can be freely exchanged by wavelength-dependent irradiation. Extramelanopsin, however, seemed to be unstable in our preparation, and approximately 9−10% of the pigment was lost at each photoconversion cycle. As we were concerned about detergent effects, we prepared membrane fractions from melanopsin-expressing cells, to assess whether this reaction is an artifact of detergent extraction. We obtained very similar difference spectra from membrane fractions as in detergent-solubilized preparations, suggesting that this is not a property that is dependent on the membrane environment; rather, it seems to be an intrinsic propensity of melanopsin to form 7-*cis*-retinal. Note that 7-*cis*-retinal seems to be generated in a stereospecific manner, with insignificant amounts of 13-*cis*- and 9-*cis*-retinal isomers. We calculated the spectrum of extramelanopsin in a manner similar to that used for metamelanopsin, from the retinal composition and spectrum of the steady state mixture of melanopsin, metamelanopsin, and extramelanopsin. Extramelanopsin had an absorption maximum at 446 nm, with an absorption coefficient of 42000 ± 1300.

DISCUSSION

Melanopsin Purification. We showed that melanopsin is readily expressed in HEK293 cells and that it can be purified with the appropriate detergent choice. We have truncated the large C-terminus of melanopsin to facilitate expression and purification. While this deletion may have an effect on some of

Table 1. Summary of Photochemical Properties of Melanopsin, Metamelanopsin, and Extramelanopsin

	λ_{max} (nm)[a]	ε_{max} (M^{-1} cm^{-1})[b]	S_{max}[c]	φ[d]
rhodopsin	499	40600[e]	1	0.65 ± 0.01[f]
melanopsin	467	33000 ± 1000	0.65 ± 0.03	0.52 ± 0.02
metamelanopsin	476	52600 ± 1600	0.44 ± 0.03	0.22 ± 0.01
extramelanopsin	446	42000 ± 1300	not determined	not determined

[a]Wavelength of the absorption maximum. [b]Absorption coefficient at λ_{max}. [c]Photosensitivity at λ_{max} relative to that of bovine rhodopsin, with the standard deviation from three independent experiments. [d]Quantum yield, with the standard deviation from three independent experiments. [e]From ref 32. [f]From ref 39.

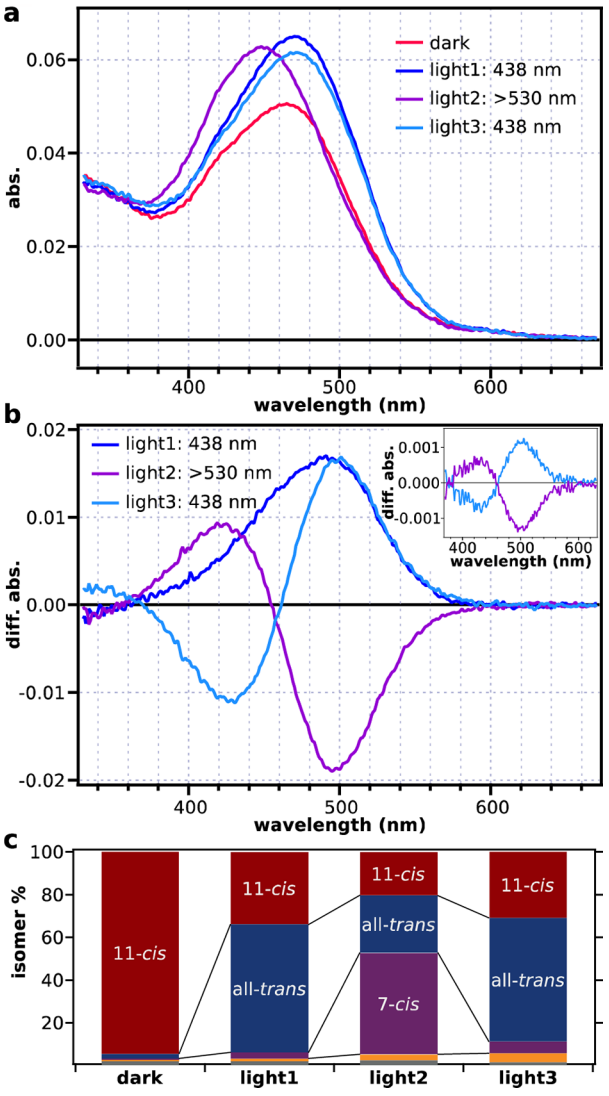

Figure 6. Additional state of melanopsin, extramelanopsin, that forms under sustained irradiation in the long-wavelength region and exhibits bistability with metamelanopsin. (a) Melanopsin (red) was exposed to 438 nm light for 160 s, creating a photosteady state (dark blue, light 1), similar to that shown in Figure 1. The photosteady state mixture was subsequently exposed to >530 nm light for 1280 s (purple, light 2) until the mixture reached a new photosteady state. Finally, the mixture was exposed again to 438 nm light for 1280 s (light blue, light 3). (b) Difference spectra of responses to the respective light irradiations shown in panel a. The inset shows the spectral change from a similar experiment conducted on melanopsin-expressing membrane fractions (average of six cycles of photoconversion). (c) Retinal composition of the respective light-exposed states. All calculated values are summarized in Table S1 of the Supporting Information.

the receptor properties, this region is far from the chromophore, and it has been shown that deletion of this region does not affect photoreaction of cephalopod rhodopsins,[47,48] which are closely related to melanopsin. The λ_{max} of melanopsin we obtained is in apparent disagreement with the action spectrum of ipRGCs[4,49−51] or PLR,[10,52] which has been fit with an A1 visual pigment template (Govardovskii template) with a λ_{max} of 480 nm. Others have reported λ_{max} values between 446 and 477 nm for the action spectrum of melatonin suppression in humans.[53,54] This could be due to the effect of solubilization of melanopsin with digitonin, which is known to shift the absorption maximum by up to 10 nm.[55] In fact, we observed that the formation of meta was slightly different in melanopsin purified in CHAPS/PC and in membrane fractions (see Figure S2 of the Supporting Information). However, as the λ_{max} of the CHAPS/PC sample was also around 470 nm (the λ_{max} of 0.02% DM-purified melanopsin was identical to that of the digitonin sample), it is likely that this difference is due to the shift in the spectrum of metamelanopsin rather than melanopsin. However, these differences may not necessarily reflect inconsistencies or real differences in the absorption spectrum. In fact, our model melanopsin spectrum fits well with electrophysiological data (see the inset of Figure 3). We think differences arise from the shape of our model spectrum and the template spectrum. We obtained the detailed spectral shape by fitting our data with the mathematical description derived by Lamb, which contains eight variables.[42] The Govardovskii template uses the same mathematical expression but fixes seven of the parameters, defining spectral shape solely by λ_{max}, assuming a common shape for all A1 retinal-derived opsins. Our model melanopsin spectrum deviates slightly at the long-wavelength decline from the universal template proposed by Govardovskii (see the inset of Figure 3). These differences are not fundamental, as the shape of this region is temperature-dependent and from our experience also sensitive to detergent conditions. Note also that the retinoid absorption peak is wide; for example, the region between 455 and 480 nm has an amplitude of >95% in our model melanopsin (likewise, the region between 467 and 493 nm has an amplitude of >95% in the 480 nm Govardovskii template). We therefore consider our model spectrum to be in reasonable agreement with electrophysiological experiments.

Melanopsin, Metamelanopsin, and Bistability. Importantly, we were able to estimate the photochemical properties of melanopsin as well as metamelanopsin, which is likely to be the active state of melanopsin, although the biochemical confirmation is required.[24] Although the formation of extramelanopsin (7-*cis*-retinal-bound state) cannot be ignored during long-wavelength irradiation, we avoided contributions from this reaction by measuring photosensitivity at a short wavelength (448 nm), at which 7-*cis*-retinal formed only marginally (see Table S1 of the Supporting Information) and

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did not affect our estimates of photochemical properties of melanopsin and metamelanopsin. Note also that the difference spectra in the inset of Figure 1 were all identical, and this response to light was fit well with a single-exponential function (Figure 5).

The metamelanopsin absorption coefficient (ε_{max} = 52600 ± 1600 M^{-1} cm^{-1}) is considerably larger than that of melanopsin (ε_{max} = 33000 ± 1000 M^{-1} cm^{-1}). However, in spite of the large absorption coefficient of metamelanopsin, the steady state mixture is dominated by metamelanopsin, because of its low quantum yield (0.22 ± 0.01) relative to that of melanopsin (0.52 ± 0.02). Although these properties are also common among invertebrate visual pigments, melanopsin serves as an irradiance detector and may well form a steady state mixture under physiological conditions. In fact, many studies indicate that melanopsin function is more relevant under strong or sustained irradiation, suggesting that bistability and formation of a photosteady state mixture may play a more prominent role in nonvisual photoreception.[56−59] The metamelanopsin spectrum largely overlaps with the resting state spectrum, and sustained irradiation at any wavelength in the absorption range of melanopsin will result in the formation of a photosteady mixture of melanopsin and metamelanopsin. This may be physiologically relevant, as it allows melanopsin to regenerate its retinal by itself. Moreover, a single melanopsin would "flicker" on and off, allowing an intermittent response to a sustained stimulus. According to our results, approximately one-third of the melanopsin molecules would be inactive at any one time during saturating light exposure. This could allow cells to recover signaling components, preventing depletion or desensitization of signaling molecules. This would allow melanopsin to respond under strong light, while preserving high sensitivity at low light levels.

Extramelanopsin. We found that an additional state, which we named extramelanopsin, formed when melanopsin is exposed to >460 nm light and gradually accumulates under sustained irradiation. The content of 11-*cis*-retinal as well as 7-*cis*-retinal and all-*trans*-retinal changes (see Figure 6c and Table S1 of the Supporting Information) during this reaction. This indicates that 11-*cis* isomerization is also involved, and the protein is simultaneously undergoing two reversible reactions:

$$\text{7-}cis\text{-retinal} \rightleftharpoons \text{all-}trans\text{-retinal} \rightleftharpoons \text{11-}cis\text{-retinal}$$

However, it is unclear whether 7-*cis*-retinal has any physiological role. Robinson et al. extracted retinal from mouse ipRGCs and found melanopsin uses 11-*cis*-retinal as a chromophore, which is photoisomerized to all-*trans*-retinal, with no other significant retinal isomers.[60] However, the mobile phase used in this study for retinal separation does not separate all-*trans*-retinal and 7-*cis*-retinal.[61] Rhodopsin has been shown to regenerate 7-*cis*-retinal and photoisomerize it to all-*trans*-retinal like 11-*cis*-retinal.[62,63] Moreover, 7-*cis*-retinal has also been reported in the photoproduct of squid rhodopsin, bovine rhodopsin, and frog rhodopsin, accounting for more than half of the retinal content in some cases.[45,64−67] These reactions were generally encountered at low temperatures or under specific detergent conditions. However, the interconvertibility by light observed between extra (7-*cis*-retinal-bound) and meta (all-*trans*-retinal-bound) states in melanopsin is not likely a product of the protein environment, as very similar spectral changes were obtained from melanopsin embedded in membrane fractions, which can be considered to be close to the physiological membrane environment. The absorption maximum of extramelanopsin (λ_{max} = 446 nm) is in agreement with the expected hypsochromic shift of a 7-*cis*-retinal pigment compared to those of 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-retinals.[68] Sustained light excitation in the long-wavelength region of the spectrum, therefore, leads to the gradual accumulation of 7-*cis*-retinal. However, we observed in our experiments that extramelanopsin formed readily even with brief exposure, whenever melanopsin was exposed to >460 nm light. Sekharan and Morokuma have recently published a quantum mechanics/molecular mechanics study in which they showed that the structures of 7-*cis*-retinal and all-*trans*-retinal inside squid rhodopsin are very similar. It may be, therefore, that the propensity to form 7-*cis*-retinal may be a reflection of the similar structures of 7-*cis*-retinal and all-*trans*-retinal inside the receptor. The difference in the absorption maximum between meta and extra states opens the possibility to wavelength-dependent regulation of melanopsin. It would therefore be interesting to test whether sustained irradiation at the long-wavelength region has any effect on the response of melanopsin-expressing cells.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Text file containing the amino acid sequence of the mouse melanopsin construct used in this study, values of all the HPLC analyses (Table S1), the purity (A_{280}/A_{max}) of the purified melanopsin (Figure S1), and the photoreaction of melanopsin in various sample preparations (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

HPLC, high-performance liquid chromatography; PLR, pupillary light response; ipRGCs, intrinsically photosensitive retinal ganglion cells.

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