Retinal attachment instability is diversified among mammalian melanopsins*.
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Background:
Melanopsin is involved in non-visual photoreception in mammals, but molecular characteristics underlying these non-visual functions are unclear.

Result:
Stability of the bond with the retinal chromophore is weakened and diversified in mammalian melanopsins.

Conclusion:
Mammalian melanopsins have acquired characteristics suited for their non-visual functions.

Significance:
The weaker retinal attachment in melanopsin may contribute to the functional tuning of non-visual photoreception in mammals.

SUMMARY
Melanopsins play a key role in non-visual photoreception in mammals. Their close phylogenetic relationship to the photopigments in invertebrate visual cells suggests they have evolved to acquire molecular characteristics that are more suited for their non-visual functions. Here we set out to identify such characteristics, by comparing the molecular properties of mammalian melanopsin to those of invertebrate melanopsin and visual pigment. Our data show that the Schiff base linking the chromophore retinal to the protein is more susceptible to spontaneous cleavage in mammalian melanopsins. We also find this stability is highly diversified between mammalian species, being particularly unstable for human melanopsin. Through mutagenesis analyses, we find that this diversified stability is mainly due to parallel amino acid substitutions in extra-cellular regions. We propose that the different stability of the retinal attachment in
Molecular characteristics of mammalian melanopsins

Introduction

Mammals use external light signals for both visual and non-visual (or non-image forming visual) processes, such as circadian clock regulation and pupil constriction. Non-visual photoreception in mammals is mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs)\(^1\) as well as rod and cone visual photoreceptor cells in the retina (1-3). Electrophysiology studies show ipRGCs have unique and different properties compared to visual photoreceptor cells. Their light-induced depolarization response occurs via Gq activation (2,4), and they exhibit a maximum spectral sensitivity of \(~480\text{-nm} \) (2,5). Moreover, ipRGCs are more than \(\sim10,000\)-fold less sensitive to light than visual cells (6). This extremely low photosensitivity is thought to be important for the ability of these cells to detect ambient light intensity without saturation, and the wavelength sensitivity might be relevant to effects of blue light on sleep disorder.

Some of the ipRGC characteristics are closely tied to the molecular characteristics of melanopsin (or Opn4) (7,8), which acts as a photopigment in the cells (1). Melanopsin is a member of the opsin family, and is more closely related to Gq-coupled invertebrate visual pigments [or rhabdomeric opsins (r-opsins)] than to Gt-coupled vertebrate visual pigments [or ciliary opsins (c-opsins)] (7,9-11) (Fig. 1A). As with all opsins, melanopsin possesses seven transmembrane \(\alpha\)-helices and covalently binds the chromophore retinal via a Schiff base linkage with a highly conserved Lys residue (position 296 in bovine rhodopsin numbering system, Fig. 1, B and C) (12-15). Previous studies have found the absorption maxima of purified melanopsins and the maximal spectral sensitivity of melanopsin-expressing cultured cells are both around 480-nm (13,14,16-21). Although some Gq-independent signaling by melanopsin has been suggested (22), a variety of melanopsins are reported to show light-dependent Gq activation \textit{in vivo} and \textit{in vitro} (14,17,18,23-26), consistent with their high sequence similarity with the invertebrate visual pigments, which also use Gq-dependent signaling (7,9) (Fig. 1A).

What is the cause of the extremely low photosensitivity of ipRGCs, and could it be related to molecular characteristics of melanopsin? Previously, the low photosensitivity of mouse ipRGCs was attributed to low (photoreceptive) melanopsin density, based on electrophysiology analyses (6). Whether this low density is due to low melanopsin-promoter activity and/or suppression of melanopsin folding and trafficking in ipRGCs was not determined.

However, reducing the amount of melanopsin protein may not be the only way to regulate ipRGC photosensitivity, as the chromophore retinal is required for photoreception. Recent studies showed that addition of exogenous retinal to mouse ipRGCs causes a \(\sim3\)-fold increase in the photosensitivity, indicating that a significant fraction of melanopsin molecules exists in an apo-protein (retinal-unbound and photo-insensitive) form (6,12). This pool of the retinal-free melanopsin molecules would effectively decrease the number of photoreceptive molecules in ipRGCs.

What could cause the presence of the retinal-free melanopsin? One possibility is a shortage of retinal supply from retinal-producing enzymes (27-29). Alternatively, they could be due to constant retinal release from the protein via...
Molecular characteristics of mammalian melanopsins

cleavage of covalent bond (Schiff base linkage) with retinal. The latter possibility would be similar to what occurs in cone photoreceptor cells, where retinal is known to spontaneously dissociate from visual pigments, resulting in reduced overall cellular photosensitivity (30,31).

We thus speculated that analogous to cone visual cells, an unstable retinal bond could contribute to the low photosensitivity of ipRGCs, a possibility that has not been previously explored in detail. In addition, not much is known about the characteristics of melanopsin from diurnal mammals (such as humans) that live primarily in much brighter environments. Although some diurnal mammals have also been investigated (32,33), the majority of melanopsin studies have been carried out on nocturnal mice and rats that live primarily in low-light environments.

Thus, in this study, we set out to identify molecular characteristics of mammalian melanopsins that are relevant to their non-visual functions. We also investigated how the characteristics of melanopsin are diversified between mammalian species living in different light environment, focusing primarily on the molecular characteristics of human and mouse melanopsins.

Our data show that in these mammalian melanopsins, the covalent bond between the retinal chromophore and protein is unstable and hydrolyzes spontaneously. Notably, in human melanopsin, the retinal attachment to the protein is much less stable than in mouse melanopsin. Even among primate melanopsins, we find the stability of the bond with retinal is highly diversified. We also identify specific differences in the extra-cellular region that appear responsible for the varied thermal stability among mammalian melanopsins. Based on these results, we discuss how the molecular characteristics of mammalian melanopsins contribute to their non-visual functions in each species.

Experimental Procedures

Ethics statement- All animal experiments in this study complied with the guidelines of the Animal Care Committee of the National Institute for Physiological Sciences (Okazaki, Japan) and were performed with an approval of the committee.

cDNAs- cDNA of human melanopsin was kindly provided by Dr. Ignacio Provencio (University of Virginia, USA). Melanopsin cDNAs from squirrel monkey (XM_003929949), olive baboon (XM_003903672) and small-eared galago (XM_003803012) were synthesized by Eurofins Genomics, Tokyo, Japan.

Construction and expression of mutants of melanopsins and jumping spider Rh1- The cDNAs of mutants of human, mouse, amphioxus, squirrel monkey, baboon, galago melanopsins were inserted into a mammalian expression vector pMT. The cDNA of jumping spider Rh1 was also inserted into the pMT vector. On each C-terminus, the coding sequence of the 1D4 tag (ETSQVAPA), a recognition sequence of the antibody 1D4, was added to enable purification using 1D4-antibody columns. For FSEC (fluorescence detected size-exclusion chromatography) analyses, melanopsin mutant cDNA was inserted into the EGFP-pMT vector, which is designed to code the melanopsin mutants fused with EGFP on the C-terminus. The mutations in this study (swapping of N-terminus and deletion of C-terminus as well as point mutations at positions 96, 102 and 192) were introduced by conventional PCR reactions. The exact locations of the introduced mutations are indicated in Fig. 1C. Note that the residue
numbering in this paper is based on the amino acid sequence of bovine rhodopsin.

Protein expression and purification- The melanopsin mutants and jumping spider Rh1 were transiently expressed in COS-1 cells (typically 10 to 20 plates), and the cells were harvested 48 h after transfection as described previously (34). The collected cells were incubated with 11-cis-retinal overnight, and membrane proteins were solubilized with 1.25 % DDM (Dojindo, Kumamoto, Japan), 20 mM HEPES, 140 mM NaCl, 0.25 % cholesterol hemisuccinate (Sigma-Aldrich, St. Louis, MO) 25 mM Tris, 10 % glycerol, pH 7.0. The solubilized materials were mixed with 1D4-agarose overnight, and the mixture was transferred into Bio-Spin columns (Bio-rad, Hercules, CA). The columns were washed with 0.05 % DDM, 2 mM ATP, 1 M NaCl, 3 mM MgCl₂ 0.01 % cholesterol hemisuccinate, 1 mM Tris, 10 % glycerol in PBS, and subsequently washed with 0.05 % DDM, 140 mM NaCl, 20 mM HEPES, 0.01 % cholesterol hemisuccinate, 1 mM Tris, 10 % glycerol, pH 7.4 (buffer A). The 1D4 tagged pigments were eluted with buffer A containing 0.45 mg/mL 1D4 peptide (TETSQVAPA) (TOYOBO, Osaka, Japan).

FSEC analysis- FSEC analysis was conducted as described in Kawate and Gouaux (35). Each melanopsin construct (containing a GFP-tag on the C-terminus) was transfected into COS-1 cells in one 100-mm diameter plate as described previously (34). After 48 hours, the transfected cells were collected and solubilized with 1 % DDM, 140 mM NaCl, 20 mM HEPES, pH 7.0. The supernatant after centrifugation was directly injected into a Superdex 200 10/300 GL column (GE Healthcare, Pittsburgh, PA) equilibrated with 0.05 % DDM, 140 mM NaCl, 20 mM HEPES, pH 7.0. Flow rate was at 0.5 mL/min and the EGFP fluorescence was detected using a RF-20A fluorescence detector (Shimadzu, Kyoto, Japan). The excitation wavelength was set to 480-nm and the emission wavelength was set to 512-nm.

Spectrophotometry- Absorption spectra of purified photopigments were recorded with a Shimadzu UV-2450 spectrophotometer (Shimadzu). For thermal decay measurement (Figs. 3, 4 and 5), the samples were kept at 37 °C, and for photoreaction measurement (see Fig. 2, C and D), the samples were kept at 10 °C. The thermal decay measurement recorded the absorption spectra for 160 min while incubating at 37 °C, and residual pigments were bleached by illumination with >520-nm light. For bleaching of the amphioxus melanopsin and jumping spider Rh1, hydroxylamine was added (final concentration of 100 mM), as the photoproducts of these pigments are thermally stable. After bleaching, the control absorbance at 490-nm (for melanopsins) or 560-nm (for jumping spider Rh1) after loss of pigments was obtained. For the acid denaturation experiments on human and mouse Opn4 (Fig. 3, E and F), 6.6 µL of 1N HCl was added to 140 µL of purified samples (final pH values were 1.8 – 2.0).

HPLC analysis to determine retinal isomer content- The time-dependent changes in retinal configurations of melanopsin samples (Fig. 3, G and H) were analyzed as previously described (36-38). Briefly, this analysis involved mixing 0.2 mL of each purified melanopsin sample after incubation at 37 °C was mixed with 50 µL of 1 M hydroxylamine and 400 µL of cold 90 % methanol to convert retinal chromophore into retinal oxime, followed by extraction of the retinal oxime with 0.7 mL n-hexane. The extract (150 µL) was then injected into a YMC-Pack SIL column (particle
size 3 μm, diameter 150 x 6.0 mm, YMC Co-Ltd., Kyoto, Japan) and eluted with n-hexane containing 15 % ethyl acetate and 0.15 % ethanol at a flow rate of 1 mL / min. The absorbance at 360 nm was monitored using an SPD-20A detector (Shimadzu).

Preparation of Xenopus oocytes- Isolated Xenopus oocytes were prepared from frogs as previously described (39-41). Briefly, Xenopus oocytes were surgically collected from frogs anesthetized in water containing 0.15 % tricaine. 5'-capped cRNA was prepared from the pGEMHE vector containing cDNA of human or mouse Opn4-Cdel mutants using an in vitro transcription kit (mMESSAGE mACHINE Kit, Life Technologies, Carlsbad, CA). Oocytes were injected with prepared cRNA (~200 pg in 50 nL water) and incubated in MBSH, a standard frog ringer solution (88 mM NaCl, 1 mM KCl, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.8 mM MgSO₄, 2.4 mM NaHCO₃, 15 mM HEPES, pH 7.6), for 1 day in the dark at 17 °C. For measurement of M1 ACh-R current, ~50 ng of cRNA was injected.

Electrophysiology- Before electrophysiological recording, oocytes injected with cRNA were incubated in MBSH solution containing 5 μM 11-cis-retinal for 30 min at 17 °C, and subsequently AA92593 (Glixx Laboratories, Southborough, MA) in DMSO or DMSO without the antagonist was added to the oocytes in MBSH solution (final AA92593 and DMSO concentrations are 120 μM and 0.6 %, respectively). The oocytes with or without AA92593 were incubated at 37 °C for 90 min. Light-induced electrophysiological responses were recorded in a Ca²⁺-free bath solution (96 mM NaCl, 2 mM KCl, 3 mM MgCl₂, 5 mM HEPES, pH 7.4). The increase in Ca²⁺-Cl⁻ current as a result of Gq activation was monitored with continuous depolarizing pulses of 175 ms to 60 mV every 2 sec from the holding potential of -80 mV (39,40). After 15 times of the depolarizing repetition (~30 sec), melanopsin molecules were activated by illumination with white light, and the recording was subsequently obtained for ~60 sec. As shown in Fig. 7, the maximal current before and after activation of melanopsin was plotted as a light-induced current. For measurement of M1 ACh-R current, 100 μM acetylcholine was added to activate the receptor.

Results
Purification and characterization of human and mouse melanopsins
In order to biochemically and spectroscopically analyze melanopsin molecules, we purified the human and mouse melanopsins using dodecyl maltoside (DDM), a detergent widely used for purification and characterization of various rhodopsin-related photopigments (17,42,43) and G protein-coupled receptors (44). Recent studies have shown that the C-terminus in melanopsin is involved in phosphorylation and arrestin binding similar to other G protein-coupled receptors (45,46). In this study, we tested how the melanopsin C-terminus affects the receptor expression levels. To do so, we compared the yields of purified human melanopsin (human Opn4-full) and mouse melanopsin long (mouse Opn4L) and short (mouse Opn4S) isoforms (47), as well as their C-terminus truncated forms (human/mouse Opn4-Cdel) (see Fig. 1C). The absorption spectra shown in Fig. 2, A and B indicate that these photopigments were successfully purified from the same number of transiently transfected COS-1 plates (ten dishes with 100-mm diameter), albeit with different yields. Human and mouse melanopsins showed
similar absorption maximum ($\lambda_{\text{max}}$) at 468-nm (Fig. 2A) and 470-nm (Fig. 2B), respectively. Note that the $\lambda_{\text{max}}$ value we obtained for mouse melanopsin is very similar to a reported value (467-nm) (13), and the $\lambda_{\text{max}}$ values for human and mouse melanopsin are consistent with reported $\lambda_{\text{max}}$ values of ipRGCs and heterologously melanopsin-expressing cultured cells (around ~480-nm) (5,14,18,20), although some studies have reported quite different values (23,48). Consistent with a previous study on mouse melanopsin (13), we find deletion of the C-terminus resulted in higher purification yields, not only for mouse but also for human melanopsins (Fig. 2, A and B). This result suggests the C-terminal region of both human and mouse melanopsins may negatively affect the expression levels at least in COS-1 cultured cells. Interestingly, the C-terminus seems to have less of an effect on mouse Opn4S than Opn4L (Fig. 2B). In addition, the C-terminal deletion has a much stronger effect on human Opn4 than mouse Opn4S, but the effect is similar to mouse Opn4L (Fig. 2, A and B), whereas the human Opn4 C-terminal sequence corresponds to the short isoform (47) (see Fig. 1C).

We next analyzed the photoreaction of purified human and mouse melanopsins (due to its poor yield, human Opn4-full was not tested further). The data show that upon irradiation with blue light (440-nm) at 10 °C, the absorption spectra of human and mouse melanopsin Cdel mutants are converted to slightly blue-shifted photoproducts (Fig. 2, C and D), which are subsequently converted to an ~380-nm species in a time-dependent manner (Fig. 2, C and D). Mouse Opn4S and Opn4L showed very similar photoreactions to mouse Opn4-Cdel (data not shown). For mouse melanopsin, the photoreaction behavior we observed is similar to that seen for ipRGCs extracts from transgenic mice (15), but differs from the slightly red-shifted and stable photoproduct observed in a spectroscopic study using purified mouse melanopsin expressed in HEK cells (13) and an electrophysiological study using isolated mouse ipRGCs (5). The behavior of melanopsin photoproduct is also controversial at the level of cellular responses (49,50). The origin of these differences in melanopsin photoreaction was not further pursued in our present work.

To test if the different purification yields for the different melanopsins reflected differences in their expression levels, or instead other factors such as purification efficiency, we next probed how the C-terminal regions affect absolute expression levels using fluorescence detected size-exclusion chromatography (FSEC) analysis (35) on the melanopsin constructs. FSEC analysis provides a rapid and quantifiable way to assess expression levels and molecular conditions of membrane proteins prior to purification, and works by monitoring the fluorescence from a GFP that is fused to the protein of interest (35).

For FSEC analyses, we expressed melanopsin mutants containing EGFP fused onto the C-terminus in COS-1 cells, and then injected an aliquot of total solubilized membrane proteins directly onto a size-exclusion column connected with a fluorescence detector (see "Experimental Procedures"). The results are shown in Fig. 2, E – I. The first elution peak in the FSEC chromatograph (at or near the void volume) reflects aggregated species. The subsequent elutions reflect the various folded states of the protein, and the final peaks reflect free-GFP species (see Fig. 2E) (35). For our melanopsin samples, the fluorescence intensities of the folded species are consistent with difference in the yields after purification (Fig. 2, A and B).
human melanopsin mutants, the order of fluorescence intensity of folded species was Opn4-Cdel > Opn4-full (Fig. 2, E and F), and in the case of mouse melanopsin mutants, the order was Opn4-Cdel > Opn4S > Opn4L (Fig. 2, G, H and I). These FSEC data confirm that the different yields after purification are mainly due to different expression levels of folded proteins.

Spontaneous hydrolysis of the Schiff base linkage between retinal and mammalian melanopsins

We next assessed the spontaneous cleavage of the covalent bond between the retinal chromophore and melanopsin, since this process could contribute to the characteristics observed for melanopsin in ipRGCs (see "Introduction"). We assessed the spontaneous hydrolysis for human and mouse melanopsins as well as for an invertebrate melanopsin [amphioxus melanopsin (16,17)] and an invertebrate visual pigment [jumping spider Rh1 (51)], since these photopigments are closely related and classified into the same group (Gq-coupled opsin or r-opsin) in the opsin family (10) (Fig. 1A). The amphioxus melanopsin and jumping spider Rh1 were purified from transfected COS-1 cells under the same conditions (Fig. 3, A - D). In these experiments, human and mouse Opn4-Cdel forms were used as a "wild-type (WT)" because of their high yields. A C-terminally truncated amphioxus melanopsin mutant was used as this deletion is necessary for purification (17) (see Fig. 1C).

To assess spontaneous cleavage of the retinal-Schiff base linkage, we monitored changes in chromophore absorbance as a function of time. We used this approach because retinal absorbance decreases after Schiff base hydrolysis (52), and this type of analysis is widely used to assess retinal-protein stability in purified rod and cone visual pigments (30,53,54).

During incubation at 37 °C in the dark, human and mouse melanopsins showed a gradual decrease in absorbance at ~470-nm, with a corresponding increase at ~380-nm absorbance (Fig. 3, A and B), indicative of free retinal production due to Schiff base linkage hydrolysis. In contrast, under the same conditions, amphioxus melanopsin and jumping spider Rh1 showed little spectral changes (Fig. 3, C and D). Together, these results indicate the retinal bond is spontaneously hydrolyzed in human and mouse melanopsins but not in amphioxus melanopsin and jumping spider Rh1.

We also measured loss of the Schiff base linkage by acid protonation (55), before and after incubation at 37 °C. This acid denaturation experiment showed that human and mouse melanopsins produced a product with λ max at ~440-nm before incubation at 37 °C, indicating the Schiff linkage in these melanopsins is present (Fig. 3, E and F). In contrast, after incubation for 160 min at 37°C, the amount of ~440-nm λ max species reduced after the acid treatment. Instead, an ~380-nm λ max species was observed (Fig. 3, E and F). These data clearly indicate that the retinal-Schiff base attachment to the protein had hydrolyzed during incubation.

We next determined which isomer (11-cis or all-trans) was being released from the human and mouse melanopsins incubated at 37 °C using HPLC. This analysis shows that before incubation, both melanopsins possess an 11-cis isomer (Fig. 3, G and H). After incubation for 40 min, although the human melanopsin retained only ~20 % absorbance in the visible region (Fig. 3A), ~50 % of the retinal was still in the 11-cis form (Fig. 3G). Similarly, after incubation for 160 min, mouse melanopsin retained ~60 % of visible absorbance (Fig 3B), but ~80 % of retinal was in the 11-cis form (Fig. 3H).
Molecular characteristics of mammalian melanopsins

These results suggest that at least some part of the melanopsin molecules released the retinal chromophore as the 11-cis form and the released retinal is thermally isomerized to the all-trans form (and then to the 13-cis form) in solution (see Fig. 3G).

Interestingly, these studies indicate that the lifetime (stability) of the retinal Schiff base is quite different between human and mouse melanopsins (Fig. 3I). The human melanopsin showed an apparent lifetime (τ) at 37 °C of 21 min compared to 170 min for the mouse melanopsin, in spite of a high amino acid sequence identity (~80 %) between them.

We next assessed which regions in melanopsin contribute to these stability differences. Our preliminary experiments with chimeric melanopsin mutants found that introducing the N-terminal and the second transmembrane regions of mouse melanopsin into human melanopsin increased the apparent retinal-Schiff base stability (data not shown). Encouraged by these results and previous studies showing the extracellular region of visual pigments regulates retinal-linkage stability (52, 56), we noted two significant differences in the extracellular end of the second transmembrane helix at positions 96 and 102 (see Fig. 1, B and C), where the two melanopsins have amino acid residues with different side chains polarity (Fig. 1A).

We thus tested if these differences could contribute to the observed differences in retinal-Schiff base stability, by swapping the N-terminus and amino acid residues at positions 96 and 102 between human and mouse melanopsins. As seen in Fig. 4, introducing the N-terminus of mouse melanopsin (Nm), T96A or Q102K substitution to human melanopsin decelerated cleavage of the bond with retinal (Fig. 4, A and C). Similarly, the reverse effect occurred upon introduction of the N-terminus of human melanopsin (Nh), A96T or K102Q to mouse melanopsin (Fig. 4, B and D). The combined introduction of Nm/T96A/Q102K to human melanopsin caused further deceleration (Fig. 4A), and Nh/A96T/K102Q substitution in mouse melanopsin resulted in further acceleration (Fig. 4B). These results clearly showed that the N-terminal region and residues at positions 96 and 102 are primarily responsible for the different stability of the bond with retinal between human and mouse melanopsins.

We next looked for differences in retinal-Schiff base bond stability among primate melanopsins. Many primate melanopsins have the same length (478 amino acid residues) as human melanopsin, and exhibit more than 85 % amino acid sequence identity with human melanopsin (see Fig. 1C). Using synthesized melanopsin cDNAs of squirrel monkey (Bolivian squirrel monkey), baboon (olive baboon) and galago [small-eared galago (bush baby)], we expressed and purified these melanopsin in their C-terminal truncated forms, as with human Opn4-Cdel (Fig. 1C). Purified squirrel monkey, baboon and galago melanopsins showed λ_{max} values at 469-nm (Fig. 5A), 469-nm (Fig. 5B) and 477-nm (Fig. 5C), respectively, indicating that they are blue-sensitive photopigments.

Interestingly, these primate melanopsins also showed markedly different retinal-Schiff base stabilities. As seen in Fig. 5, A – C, upon incubation at 37 °C in the dark, the Schiff base in these melanopsins decayed with very different rates, yielding apparent lifetimes for 10 min of galago Opn4, 49 min of squirrel monkey Opn4 and 112 min of baboon Opn4 (Fig. 5, D and G). Of note, even though squirrel monkey and baboon melanopsins have ~95 % of amino acid sequence identity, the apparent lifetime of their bond with
Molecular characteristics of mammalian melanopsins

retinal differs by ~2-fold. Galago and squirrel monkey melanopsins have a Gln at position 102, and baboon melanopsin has a His at position 102 (Fig. 1, A and C), similar to the Gln and Lys that human and mouse melanopsins have at the site, respectively. Thus, we next tested if these residues could be responsible for the different lifetime among primate melanopsins.

The Q102H substitution in squirrel Opn4 increased the lifetime ($\tau = 110$ min) (Fig. 5, E and G) up to almost the same level as baboon Opn4. Correspondingly, the H102Q substitution in baboon Opn4 decreased the stability ($\tau = 56$ min) (Fig. 5, E and G) to almost the same degree as seen for squirrel monkey Opn4. Together, these results indicate that ~2-fold difference in the decay rate between squirrel monkey and baboon melanopsins can be explained by the single amino acid substitution at position 102.

Introduction of Q102H to galago Opn4 increased the lifetime ($\tau = 20$ min), as did introduction of N-terminus of baboon melanopsin (Nbab) ($\tau = 14$ min) (Fig. 5, F and G). Although significant, these effects on retinal Schiff base stability are not by themselves sufficient to explain the ~10-fold difference in stability between galago and baboon melanopsins. Moreover, at position 96, both melanopsins possess an Ala residue, indicating that some other residues are involved in the accelerated spontaneous cleavage of the bond in galago melanopsin.

We noted one possible candidate for this residue could be position 192 in the second extracellular loop region (see Fig. 1, B and C). Galago melanopsin possesses an uncommon Thr residue at this position, whereas most other mammalian melanopsins, including human, mouse, squirrel monkey and baboon ones, have Met (Fig. 1, A and C, and Fig. 6). In addition, a previous mutational study on a visual pigment in bovine rod photoreceptor cells (bovine rhodopsin) found that amino acid substitution at position 192 increases the rate of spontaneous retinal Schiff base hydrolysis (56). We thus substituted this residue, and found introducing a T192M into galago Opn4 increased the lifetime ($\tau = 33$ min), and the combined substitution of Nbab/Q102H/T192M caused further increase of the lifetime ($\tau = 88$ min), which is a value near that of baboon Opn4 ($\tau = 112$ min) (Fig. 5, F and G). In addition, introduction of the N-terminus of galago melanopsin (Ngal) with H102Q/M192T substitutions to baboon Opn4 caused a dramatic decrease in stability of the bond ($\tau = 23$ min) (Fig. 5, E and G).

Taken together, these results suggest that most of ~10-fold difference in the retinal Schiff base stability between galago and baboon melanopsins can be explained by different amino acid sequences in the N-terminus and at positions 102 and 192. The results also indicate that even among primate melanopsins, the stability of the bond with retinal is highly varied presumably due to amino acid substitutions in the N-terminus and at positions 96/102/192. A comparison of amino acid residues at positions 96, 102 and 192 (Figs. 1A and 6) suggests that human and other apes' melanopsins achieve a low stability of the bond with retinal by the acquisition and retention of destabilizing residues Thr-96 and Gln-102 (see "Discussion").

Retinal release from melanopsin molecules in cell membranes of Xenopus oocytes assessed electrophysiologically

Our analysis using purified proteins in a detergent solution showed that the bond between the retinal chromophore and protein is unstable in mammalian melanopsins, particularly so for human melanopsin (Fig. 3). In cells, the cleavage
of this bond would result in retinal release from a protein moiety (opsin) and loss of light-dependent Gq activation ability. In order to assess such a reaction in cells, we utilized light-induced current in *Xenopus* oocytes heterologously expressing human or mouse melanopsin. Since melanopsins are coupled with Gq-type G proteins, light-dependent Gq activation by melanopsin can be detected as an increase in the Ca\(^{2+}\)-activated Cl\(^-\) current (14,39,40). To detect the difference in the retinal release rate from melanopsin, melanopsin-expressing oocytes were treated with retinal and subsequently incubated at 37 °C in the dark for 90 min in the presence or absence of a melanopsin-specific antagonist AA92593 (24). A previous study has shown that the antagonist can bind to the retinal-free form of melanopsin and block retinal binding to the protein, leading to reduction of light-dependent current by melanopsin (24).

Thus, we expected that if retinal release from melanopsin molecules is frequent, the current due to melanopsin photo-activation should be reduced by exchange of the chromophore retinal with the melanopsin-specific antagonist. To test this idea, we measured the light-induced Cl\(^-\) current after incubation of oocytes expressing retinal-bound human or mouse Opn4-Cdel mutants at 37 °C in the presence or absence of the antagonist. As expected, significant light-dependent current was observed in oocytes expressing either melanopsins, indicating these melanopsins can activate Gq in a light-dependent manner (Fig. 7, A and C). Interestingly, incubation with the antagonist significantly decreased the light-dependent current of oocytes expressing human melanopsin, but had little effect on oocytes expressing mouse one (Fig. 7, B, D and E). As a control, we tested and confirmed that the antagonist treatment does not show significant effect on the current caused by activation of the M1 muscarinic acetylcholine receptor (M1 ACh-R) that is a photo-insensitive Gq-coupled receptor (Fig. 7E).

These results showed that in oocytes, human melanopsin frequently releases the chromophore retinal and instead binds the antagonist, whereas mouse melanopsin does so less frequently. Incubation with the antagonist reduced photocurrent for human melanopsin by ~40 %, but did not significantly affect photocurrent triggered by mouse melanopsin (Fig. 7E), although after 90 min incubation at 37 °C, purified human melanopsin molecules almost completely lose their absorbance in the visible region, and only ~40 % of purified mouse melanopsin decays (Fig. 3I). These differences are likely due to these melanopsins releasing the retinal chromophore less frequently in cells (lipid membranes) than in purified conditions (detergent micelles). Taken together, our data indicate that human melanopsin releases retinal chromophore more frequently than mouse melanopsin in cell membranes as well as in detergent solutions.

**Discussion**

Below, we discuss how mammalian melanopsins have been fine-tuned to facilitate their non-visual functions.

*The effect of the C-terminus on expression levels of melanopsin*

Melanopsin possesses a relatively long C-terminal region compared with other opsins. Our analysis here indicates that the extended C-terminal region in human and mouse melanopsins decreases their expression levels, at least in cultured COS-1 cells. An increase in expression levels of mouse melanopsin after C-terminal truncation has been previously noted
Molecular characteristics of mammalian melanopsins

(13). Here, we find the C-termini of mouse Opn4L and Opn4S negatively affect the expression levels to different degrees (Fig. 2, A, B, E, F, G, H and I). Specifically, we find a modestly reduced expression level for mouse Opn4S in comparison with the Opn4L. Interestingly, the C-terminus of human Opn4 severely affects the expression levels like that of mouse Opn4L, whereas the C-terminal sequence of human Opn4 is more similar to mouse Opn4S rather than Opn4L (47). In other words, the negative effect of C-terminus on the expression level appears to be enhanced in human melanopsin.

Of course, since these analyses are based on data using cultured cells, they do not prove that the C-terminal regions of mammalian melanopsins negatively affect their expression levels in native ipRGCs. However they do raise the interesting possibility that the different effects of the C-termini contribute to the low melanopsin density and the low photosensitivity in ipRGCs. Furthermore, the more severe C-terminal effect of mouse Opn4L may be relevant to the lower photosensitivity of ipRGC subg roup M2 cells compared to M1 cells (57,58), since M2 cells express only Opn4L whereas M1 cells express both Opn4S and Opn4L (47). In a similar way, the expression levels of melanopsin might be strongly suppressed in human ipRGCs (see below).

Spontaneous cleavage of the bond with the retinal chromophore in mammalian melanopsins

Our data show that the Schiff base linkage between the retinal chromophore and protein moiety is spontaneously hydrolyzed in mammalian melanopsins (Fig. 3). Although these results were obtained using purified melanopsins in detergent solution, we think they are pertinent for melanopsin properties in the native lipid environment of ipRGCs for the following reasons. First, the $\lambda_{\text{max}}$ values for our purified melanopsins are very consistent with the $\lambda_{\text{max}}$ values obtained in electrophysiological studies of native ipRGCs and cultured cells expressing melanopsins (5,14,18,20). Second, the faster hydrolysis of the linkage with retinal in mammalian melanopsins is consistent with previous studies using native ipRGCs (see below). Third, our electrophysiological analysis utilizing human and mouse melanopsins in membranes of Xenopus oocytes also showed the highly destabilized linkage in human melanopsin (Fig. 7). Finally, faster retinal-Schiff base linkage hydrolysis for cone visual pigments (30) and some mutants of rod visual pigments (53,56) has been determined using purified and detergent solubilized pigments. These molecular properties of the purified photopigments are found to be consistent with cellular responses expressing these pigments (see "Introduction" and below) (31,59). Taken together, we conclude the spontaneous retinal release from melanopsin is relevant and reflects the behavior of melanopsin molecules in cells.

We propose this spontaneous Schiff base hydrolysis and subsequent retinal dissociation could effectively act to decrease the active number of photoreceptive melanopsin molecules, resulting in a lower overall density of photoreceptive melanopsin in ipRGCs. Consistent with this hypothesis, retinal-bound and retinal-unbound melanopsin molecules are reported to co-exist in mouse ipRGCs (6,12). Moreover, spectroscopic and electrophysiological studies of mouse melanopsin recently found that under continuous illumination, a mixture of three states having different retinal isomers and absorption maxima is formed (5,13). In contrast, dark-adapted ipRGCs possess only a single state of melanopsin having
11-cis isomer and the \( \lambda_{\text{max}} \) at \(~480\text{-nm}\) (2,6,15). These facts imply that there are light-independent mechanisms to enrich the 11-cis-retinal bound state (5). Our data here suggest that during the dark adaptation of ipRGCs, melanopsin molecules spontaneously release the chromophore retinal and (re)bind 11-cis-retinal freshly supplied from 11-cis retinal-producing enzymes (27-29).

What is the effect of having a mixture of retinal-bound and retinal-free melanopsin in cells? For vertebrate visual pigments, it is well established that the retinal-free forms can constitutively activate G proteins (30,60-63). In cone visual cells, the light-independent G protein activation by the retinal-free protein acts to desensitize the cells by lowering their overall photosensitivity (30,31). Similarly, in rod visual cells, the constitutive G protein activity accompanying some rhodopsin mutants that exhibit faster retinal-Schiff base hydrolysis can cause night blindness by desensitization of the cells (53,59,64). If retinal-free mammalian melanopsins also inherently possess some level of constitutive G protein activity, the spontaneous Schiff base hydrolysis and retinal release could similarly act to desensitize ipRGCs. We propose that this aspect of mammalian melanopsin behavior has been enhanced in human melanopsin, through further destabilization of the bond with retinal (see below).

Such a relationship between retinal attachment stability in melanopsin and the photosensitivity of melanopsin-expressing cells will not be limited to mammals. For example, unlike mammalian melanopsins (but like invertebrate visual pigment), amphioxus melanopsin possesses a much more stable retinal Schiff-base linkage (Fig. 3D). Consistent with the above hypothesis, amphioxus Hesse and Joseph photoreceptor cells, in which the melanopsin functions as a photopigment (16), show a high photosensitivity comparable to visual photoreceptor cells (65), in contrast to ipRGCs in mammals.

The evolutionary "enhanced" characteristics in human melanopsin

Human and mouse melanopsins share \(~80\%\) amino acid sequence, yet have different stabilities for their retinal linkages (Fig. 3I). Even among primate melanopsins, which have more than \(85\%\) sequence identity, the retinal-Schiff base stability is highly diversified (Fig. 5). Our mutagenesis studies here reveal that most of these differences in stability can be attributed to differences in the N-terminus and positions 96, 102 and 192 (Figs. 4 and 5), regions located in the extracellular side (see Fig. 1B).

Ala/Arg/Met residues are frequently observed at positions 96/102/192 among mammalian melanopsins including Platypus Opn4 (Figs. 1A and 6). Marsupial (wallaby, Tasmanian devil and opossum) melanopsins possess a Thr residue at position 192, but "mammalian-type" melanopsins (Opn4m) in zebrafish and chicken possess Met residue at this site. Taken together, an ancestral type of mammalian melanopsin is presumed to possess A/R/M residues at positions 96/102/192, respectively (Fig. 6). Thus, a hypothetical ancestral mammalian melanopsin would be expected to bind retinal as stably as mouse melanopsin, based on our findings that Ala-96, Met-192 and a positively charged residue at position 102 can decrease the hydrolysis rate for the bond with retinal. In this context, it is interesting to note that parallel substitutions of amino acid residues at positions 96, 102 or 192 have occurred in molecular evolution of mammalian melanopsins. We propose that parallel substitutions have resulted in the
diversification of the thermal stability of the retinal-Schiff base linkage. During this process, it would appear that melanopsins of apes (including human) have acquired and kept destabilizing residues Thr-96 and Gln-102 (Fig. 6).

As discussed above, our data show the C-terminus of human melanopsin acts to decrease receptor expression levels more strongly than it does for mouse melanopsin (Fig. 2, A, B, E, F, G, H and I). We also find that human melanopsin also releases retinal more frequently than mouse one (Figs. 3 and 7). Why these differences have been evolutionally selected is not clear, but they would have acted together to make photoreception through human melanopsin less efficient. In support of this idea, an immunohistochemical study of human retina found that melanopsin-expressing ganglion cells comprise ~0.2 % of total ganglion cells (32), in contrast to mouse retina, where the melanopsin-positive population is significantly greater, ~1 – 2 % (3,66). Together, these results imply that the ability of ipRGCs to detect light is much less efficient in humans than in mice. Such an inefficient light-sensitivity may have some advantages for adaptation to enable tolerating the brighter light environment in which humans live.

Surprisingly, the stability of galago melanopsin is even lower than that of human melanopsin (Fig. 5, C and G), whereas galago is a typical nocturnal primate. Based on our data and hypothesis, this result would predict that the galago cannot detect light signal efficiently through their melanopsin. A previous study showed that galago has lost blue-sensitive visual pigment, implying that blue light detection is not important for galago (67,68). Since galago melanopsin absorbs in the blue-green region ($\lambda_{\text{max}} = 477$ nm, Fig. 5C), this result suggests that galago does not need to effectively detect blue light, neither for visual or non-visual purposes.

In summary, in the present work we have identified and characterized molecular properties of mammalian melanopsins that are distinct from closely related photopigments. Further understanding of melanopsin properties, as well as understanding of ipRGC properties and ipRGC-driven behaviors, will be needed to fully comprehend non-visual photoreception in mammals.

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Conflict of Interest
The authors declare that they have no conflict of interest regarding this study.

Author Contributions
H. T. designed the research, conducted experiments and analyzed data. Y. K. supervised electrophysiological experiments. D. L. F. supervised protein expression and purification. M. K. provided information to interpret biochemical data and phylogenetic relationship of melanopsins. A. T. supervised protein expression and purification. Y. F. designed and supervised the overall research. All authors edited and wrote the paper.

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**Footnotes**

1 The abbreviations used are: DDM, n-dodecyl-β-D-maltoside; ipRGC, intrinsically photosensitive retinal ganglion cells; FSEC, fluorescence-detected size-exclusion chromatography; λ<sub>max</sub>, absorption maximum; M1 ACh-R, m1 muscarinic acetylcholine receptor; WT, wild-type.
**Figure legends**

**Fig. 1** Sequence and structural properties of mammalian melanopsins

A, Phylogenetic relationships of melanopsins. The phylogenetic tree is constructed by the neighbor-joining method using the software MEGA6 (69). The scale bar represents 0.2 amino acid substitutions per site. Photopigments that are analyzed in this paper are indicated in boldface. Amino acid residues at positions 96, 102 and 192 in mammalian melanopsins are shown. Note that the residue numbering used in this study is based on the amino acid sequence of bovine rhodopsin. In Ballesteros-Weinstein numbering (70), positions 96 and 102 correspond to position 96$^{2.63}$ and 102$^{2.69}$, respectively (position 192 is not located in a transmembrane helix, and thus cannot be described using this nomenclature). B, Location of positions 96, 102 and 192 in the crystal structure of squid rhodopsin (PDB ID: 2Z73) (71), a photopigment closely related to melanopsin. Amino acid residues at positions 96, 102 and 192 are indicated in black. The chromophore retinal is also indicated. C, Amino acid sequence alignment of photopigments used in this study. Amino acid sequences of human, mouse, squirrel monkey, baboon, galago and amphioxus melanopsins and jumping spider Rh1 around mutation-introduced sites are shown. Putative regions corresponding to transmembrane helices are indicated. "N-ter" and "C-ter" (red lines) indicate positions where N-terminal substitutions or C-terminal truncations were introduced in respective mutants. The sequences of mouse Opn4S and Opn4L are indicated in boldface. For amphioxus melanopsin, the last 250 amino acid residues of the very long C-terminus are omitted. Positions 96, 102, 192 and 296 (retinal binding site) are also indicated (red arrowheads). It is not clear if the several cysteine residues in the C-terminal region of melanopsins are palmitoylated.

**Fig. 2** Effect of the C-terminus on expression levels of human and mouse melanopsins.

A, Visible absorption spectra of purified human melanopsin with different C-terminal sequences. Absorption spectra of human Opn4-full (black, with native C-terminus) and Opn4-Cdel (red, with truncated C-terminus) are shown. (Inset) UV-visible absorption spectra of the human melanopsin mutants. B, Visible absorption spectra of purified mouse melanopsin with different C-terminal sequences. Absorption spectra of mouse Opn4L (green, with native Opn4L C-terminus) Opn4S (blue, with native Opn4S C-terminus) and Opn4-Cdel (red, with truncated C-terminus) are shown. (Inset) UV-visible absorption spectra of the mouse melanopsin mutants. C and D, Photoreaction of purified human and mouse melanopsins in DDM micelles. Visible absorption spectra of human Opn4-Cdel (C) and mouse Opn4-Cdel (D) before illumination (black), just after illumination (red), 5 min after illumination (blue) and 15 min after illumination (green). The samples were kept at 10 °C. E – I, FSEC profiles of human and mouse melanopsin mutants. FSEC chromatographs of human Opn4-Cdel (E), Opn4-full (F), and mouse Opn4-Cdel (G), Opn4S (H), Opn4L (I) are shown. The aggregated, folded and free-GFP species are indicated. Note that the peak representing the folded species of human Opn4-Cdel shows a different shape compared to the other constructs, suggesting that deletion of C-terminus in human melanopsin may affect oligomer formation of the protein.
Molecular characteristics of mammalian melanopsins

**Fig. 3** Measurement of spontaneous retinal-Schiff base cleavage in purified melanopsins and an invertebrate visual pigment

A – D, Representative spectra showing time-dependent loss of absorbance in the visible region for human Opn4 (Cdel mutant) (A), mouse Opn4 (Cdel mutant) (B), jumping spider Rh1 (C) and amphioxus melanopsin (C-terminal truncated mutant) (D). Each sample was incubated at 37 °C in the dark, and absorption spectra were recorded at each time point. Incubation time is indicated in panel A. E and F, Representative spectra of acid-denatured human Opn4-Cdel (E) and mouse Opn4-Cdel (F) before (black) and after (red) incubation at 37 °C for 160 min. An intact, protonated retinal Schiff-base appears as a 440-nm absorbing species. For acid denaturation, the pH values of samples were reduced to 1.8 – 2.0 (see "Experimental Procedures"). G and H, HPLC analysis of the retinal configurations present in purified human (G) and mouse (H) Opn4-Cdel mutants after incubation at 37 °C for the indicated times in the dark. Peaks labeled with 11, AT and 13 indicate the 11-cis, all-trans, and 13-cis isomers, respectively. Relative amount of the 11-cis isomer to total retinal isomers in human Opn4 samples are 96, 69, 50 and 11 % after 0, 20, 40 and 160-min incubation, respectively. Relative amount of the 11-cis isomer in mouse Opn4 samples are 99, 98, 96 and 83 % after 0, 20, 40 and 160-min incubation, respectively. It should be noted that the broad peak at ~13 min (black arrow-head) is due to cholesterol hemisuccinate used for purification (see "Experimental Procedures"). I, Plot of relative absorbance in the visible region as a function of incubation time. Time-dependent absorption changes of human Opn4 (black), mouse Opn4 (red), jumping spider Rh1 (purple) and amphioxus melanopsin (green) were indicated. The decay kinetics of human and mouse Opn4 were fitted with single-exponential functions to estimate apparent lifetime values. The jumping spider Rh1 and amphioxus melanopsin were so stable that very little decay occurred, thus these data were fitted with straight lines.

**Fig. 4** Effect of substitutions at the N-terminus and positions 96/102 in human and mouse melanopsins

A, Plot of relative absorption of human melanopsin mutant at 490-nm as a function of incubation time at 37 °C. Time-dependent absorption changes of human Opn4 mutants "Nm" (with N-terminus of mouse Opn4) (red), T96A (blue), Q102K (purple) and Nm/T96A/Q102K (green). Each set of data was fitted with a single-exponential function to calculate an apparent lifetime value. The fitted curve of human Opn4 WT (Cdel mutant) is also shown (grey dotted curve). B, Plot of relative absorption of mouse melanopsin mutants at 490-nm as a function of incubation time. Time-dependent absorption changes of mouse Opn4 mutants "Nh" (with N-terminus of human Opn4) (red), A96T (blue), K102Q (purple) and Nh/A96T/K102Q (green). The data were fitted with a single-exponential curve and an apparent lifetime value was calculated. The fitted decay curve of mouse Opn4 WT (Cdel mutant) is also shown (grey dotted line). C and D, Comparison of apparent lifetime values of the bond with retinal in human (C) and mouse (D) melanopsin mutants. The error bars represent the S. D. values (n = 3).

**Fig. 5** Comparison of thermal decay of purified primate melanopsins

A – C, Representative spectra showing the time-dependent loss of absorbance in the visible region for the Opn4 (Cdel mutants) of squirrel monkey (A), baboon (B) and galago (C). Each sample was incubated at
37 °C in the dark, and absorption spectra were recorded in each time point. Incubation time is indicated in panel A. D, Plot of time-dependent absorption changes of Opn4 of squirrel monkey (red), baboon (blue), and galago (green). E, Plot of time-dependent absorption changes for squirrel monkey Opn4 Q102H mutant (red) and baboon Opn4 mutants H102Q (blue), Ngal/H102Q/M192T (purple). "Ngal" mutation means substitution of N-terminal region with galago Opn4 sequence. The fitted decay curves of squirrel (brown dotted line) and baboon (dark blue dotted line) Opn4 WTs (Cdel mutants) are also shown. F, Plot of time-dependent absorption changes for the galago Opn4 mutants "Nbab" (having N-terminal region of baboon Opn4) (red), Q102H (blue), T192M (purple) and Nbab/Q102H/T192M (green). Fitted decay curve of galago Opn4 WT (Cdel mutant) are also shown (grey dotted line). Each set of data in panels D, E and F was fitted with a single-exponential function to calculate an apparent lifetime value. G, Comparison of apparent lifetime values for the retinal-Schiff base linkage in squirrel monkey, baboon and galago Opn4 mutants. The error bars represent the S. D. values (n = 3).

**Fig. 6** Comparison of amino acid residues at positions 96, 102 and 192 among various mammalian melanopsins

Amino acid residues at positions 96, 102 and 192 are shown along with schematic phylogenetic tree of mammalian melanopsins. Amino acid residues that our data predict to destabilize the bond with retinal are highlighted by red. This schematic phylogenetic tree was prepared based on the Ensembl website (http://www.ensembl.org). Amino acid residues at positions 96 and 102 in Mouse Lemur Opn4 are not available in Ensembl or NCBI databases.

**Fig. 7** Assessment of retinal release from human and mouse melanopsins in oocyte cells electrophysiologically.

A - D, Light-induced current recordings of *Xenopus* oocytes expressing human and mouse Opn4 (Cdel mutants) after incubation at 37 °C with or without 120 µM of a melanopsin-specific antagonist AA92593 (24) (see "Experimental Procedures"). Representative current recording data of oocytes expressing human Opn4 (Cdel mutant) in the absence (A) or presence (B) of AA92593 after incubation at 37 °C for 90 min. Representative current recording data of oocytes expressing mouse Opn4 (Cdel mutant) in the absence (C) or presence (D) of the antagonist AA92593 after incubation at 37 °C for 90 min. In panels A – D, the recorded current data in the dark (dotted line) and after illumination (solid line) are shown. E, Comparison of the effect of AA92593 on the amplitude of light-induced current by activation of human and mouse Opn4 as well as acetylcholine-induced current by activation of M1 ACh-R. The error bars represent the S. D. values (n = 7 for human Opn4 –AA92593, n = 9 for human Opn4 +AA92593, n = 6 for mouse Opn4 –AA92593, n = 6 for mouse Opn4 +AA92593, n = 4 M1 ACh-R for –AA92593 and n = 3 M1 ACh-R for +AA92593 conditions). Incubation with the antagonist caused a statistically significant decrease of the current by human Opn4 (p < 0.05), but the effect of the antagonist on the current by mouse Opn4 and M1 ACh-R was not significant (n. s.) by Student's t-test or Wilcoxon test. Similar results were observed in three different batches of oocytes.
Squirrel monkey Opn4
Olive baboon Opn4
Chimpanzee Opn4
Human Opn4
Orangutan Opn4
Galago Opn4
Cattle Opn4
Mouse Opn4
Tasmanian devil Opn4
Zebrafish Opn4.1
Lamprey Opn4
Chicken Opn4
Xenopus Opn4x
Amphioxus melanopsin
Jumping spider Rh1

Molecular characteristics of mammalian melanopsins

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Fig. 3 Tsukamoto et al.
Fig. 4 Tsukamoto et al.
Fig. 5 Tsukamoto et al.
Molecular characteristics of mammalian melanopsins

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Retinal attachment instability is diversified among mammalian melanopsins
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