Color opponency with a single kind of bistable opsin in the zebrafish pineal organ

Seiji Wada*, Baoguo Shen*, Emi Kawano-Yamashita*, Takashi Nagata*, Masahiko Hibi,b,c, Satoshi Tamotsu,d, Mitsumasa Koyanagi,a,e, and Akihisa Terakita*a,e,1

*Department of Biology and Geosciences, Graduate School of Science, Osaka City University, 558-8585 Osaka, Japan; 1Division of Biological Science, Graduate School of Science, Nagoya University, 464-8602 Nagoya, Japan; *Laboratory of Organogenesis and Organ Function, Bioscience and Biotechnology Center, Nagoya University, 464-8601 Nagoya, Japan; 2Department of Chemistry, Biology, and Environmental Science, Faculty of Science, Nara Women’s University, 630-8506 Nara, Japan; and *The Osaka City University Advanced Research Institute for Natural Science and Technology, Osaka City University, 558-8585 Osaka, Japan

Edited by King-Wai Yau, Johns Hopkins University School of Medicine, Baltimore, MD, and approved September 12, 2018 (received for review February 12, 2018)

Lower vertebrate pineal organs discriminate UV and visible light. Such color discrimination is typically considered to arise from antagonism between two or more spectrally distinct opsins, as, e.g., human cone-based color vision relies on antagonistic relationships between signals produced by red-, green-, and blue-cone opsins. Photosensitive pineal organs contain a bistable opsin (parapinopsin) that forms a signaling-active photoprotein upon UV exposure that may itself be returned to the signaling-inactive “dark” state by longer-wavelength light. Here we show the spectrally distinct parapinopsin states (with antagonistic impacts on signaling) allow this opsin alone to provide the color sensitivity of this organ. By using calcium imaging, we show that single zebrafish pineal photoreceptors held under a background light show responses of opposite signs to UV and visible light. Both such responses are deficient in zebrafish lacking parapinopsin. Expressing a UV-sensitive cone opsin in place of parapinopsin recovers UV responses but not color opponency. Changes in the spectral composition of white light toward enhanced UV or visible wavelengths respectively increased vs. decreased calcium signal in parapinopsin-sufficient but not parapinopsin-deficient photoreceptors. These data reveal color opponency from a single kind of bistable opsin establishing an equilibrium-like mixture of the two states with different signaling abilities whose fractional concentrations are defined by the spectral composition of incident light. As vertebrate visual color opsins evolved from a bistable opsin, these findings suggest that color opponency involving a single kind of bistable opsin might have been a prototype of vertebrate color opponency.

bistable opsin | pineal organ | color opponency | UV-sensitive opsin | molecular evolution

Color discrimination is achieved through antagonistic responses to different wavelengths of light, known as color opponency. Such color opponency has been described in many animal species and has previously been considered to require antagonistic interaction between signals produced by two or more opsins each sensitive to a different portion of the spectrum. The best-known instance of color opponency originates in the retina and is well known as color vision. Thus, in humans, trichromatic vision arises from red-, green-, and blue-sensitive cone opsins, which are exclusively present in different photoreceptor cells (1–4). These photoreceptor cells transmit light information to the brain via a complex neural network involving neurons that subtract outputs from different photoreceptor cells to generate color opponency. Such operations enable humans to recognize an enormous number of colors. Many vertebrates also have extracellular photoreception, and color opponency in these systems has also been reported in the context of sensing dawn/dusk (5, 6). Mechanisms for such extracellular color opponency can be quite different from those of color vision. In lizard parietal eyes, blue vs. green discrimination is achieved at the level of single cells by comparing the activity of two opsins, blue-sensitive pinopsin and green-sensitive parietopsin, expressed in a single photoreceptor (5, 7). Interestingly, color opponency is generated through two distinct intracellular signal transduction cascades driven by pinopsin and parietopsin, respectively. Thus, in the lizard parietal eye, color opponency is a property of single cells rather than neural networks, as is the case for color vision.

Pineal organs of lower vertebrates such as lamprey and most teleosts also show color opponency, discriminating UV and visible light. This property has been recorded at the level of pineal ganglion cells (that receive light information from several pineal photoreceptor cells), with firing of these neurons suppressed by UV and enhanced by visible light (6, 8). We previously found that parapinopsin, an opsin first identified in catfish pineal and parapineal organs (9), is a UV-sensitive opsin and that it supports the UV reception involved in color opponency of the lamprey pineal organ (10). Interestingly, parapinopsin is a bistable opsin: exposure of dark-adapted parapinopsin to UV light produces a stable photoprotein that is itself maximally sensitive to visible light and, on light absorption, can regenerate the original dark state, showing interconvertibility between the dark state and its photoprotein. Therefore, parapinopsin has two stable “color states” with their absorption maxima at largely separated wavelengths, unlike other vertebrate cone opsins whose photoprotein is unstable (10, 11). However, it remains

Significance

Color discrimination in animals is considered to require opponent processing of signals from two or more opsins sensitive to different parts of the spectrum. We previously reported that lower vertebrate pineal organs, which discriminate between UV and visible light, employ a bistable opsin called parapinopsin that has two stable photointerconvertible states, a signaling-inactive state maximally sensitive to UV and a visible light-sensitive signaling-active photoprotein. Here, we present evidence that the photoequilibrium between these two states in the zebrafish pineal organ is dependent on the spectral composition of incident light, setting the opsin’s signaling activity according to color to allow parapinopsin alone to generate color opponency between UV and visible light at the level of single pineal photoreceptor cells.
unclear how the stable photoproduct of parapinopsin is involved in the pineal color opponency.

Bistable opsins are also employed in visual cells of many invertebrates (12). Interestingly, under specific light conditions, the bistability can give rise to a form of “color opponent” behavior. Thus, for example, Drosophila visual cells contain a blue-sensitive rhodopsin whose photoproduction show green/yellow sensitivity. The blue-sensitive opsin is not signaling-active, but its photoproduct is, driving depolarization of the photoreceptor cell. In white-eyed Drosophila, a bright blue light stimulus generates prolonged depolarization after light-off. This is known as prolonged depolarizing afterpotential (PDA), which occurs because the photoproduction (signaling active metarhodopsin) out-titrates the arrestin required to inactivate it. A subsequent long-wavelength (yellow/orange) light stimulus photoisomerizes signaling-active metarhodopsin to signaling-inactive rhodopsin and results in suppression of the PDA, thereby generating an effective “hyperpolarization” (13). The opposite effects of blue and green/yellow light on photoreceptor polarization in this paradigm reveal the potential for bistable opsins to support a sort of color opponency but this does not translate to more physiological lighting conditions.

Recently, we found that parapinopsin in the zebrafish pineal organ exhibits a bistable nature identical to that observed for lamprey parapinopsin (SI Appendix, Fig. S1). We also obtained the promoters sequence of zebrafish parapinopsin (14). Here, we investigated the light response of parapinopsin-expressing cells (PP cells) by using genetic procedures. We found that parapinopsin alone can support color opponency at the single photoreceptor level. Color opponency in the zebrafish pineal organ has been considered to have little relevance to light regulation of pineal melatonin secretion (14, 15), and there is no evidence directly demonstrating its involvement in light entrainment of the circadian rhythms in zebrafish. Here, we also discuss physiological relevance of the zebrafish pineal wavelength discrimination on the basis of light conditions that enable parapinopsin-based color opponency.

Results

Antagonistic Chromatic Responses in a Single Pineal Photoreceptor Cell. To investigate responses to light in PP cells, we performed calcium imaging in transgenic zebrafish PP cells expressing the calcium indicator GCaMP6s (16) by using a two-photon excitation microscope at 930 nm (Fig. 1 A and B). The excitation light for GCaMP6s is expected itself to activate photoreceptors and can therefore be considered equivalent to a continuous blue light stimulation (~465 nm) throughout the experiment. After approx- imately 150 s of imaging, fluorescence reached a plateau (SI Ap-pendix, Fig. S2), allowing us to investigate the calcium level changes in PP cells upon additional stimulation with different wavelengths of light. In each region of interest (ROI; ROIs 1–6 in Fig. 1B) containing part of a single PP cell, stimulation with 405-nm light caused a decrease in fluorescence intensity. This response is consistent with parapinopsins’ known ability to show light-dependent interaction with transducin, which is expected to lead to hyperpolarization of the PP cell and closure of voltage-sensitive calcium channels (17). In contrast, stimulation with 588-nm light elevated the fluorescence intensity, demonstrating the antagonistic effect of UV and visible light on the calcium in individual PP cells to UV and visible light (Fig. 1C). The fluorescence changes between the average of ROIs 1–6 and ROI 7 encompassing the whole pineal organ were almost identical, suggesting that the color opponency observed in the whole pineal organ was based on the antagonistic chromatic response of a single PP cell. Such antagonistic responses were reproducibly observed by repeated stimulation with UV and visible light (Fig. 1D). This represents color opponency in a single photoreceptor cell of the teleost pineal organ.

Color Opponency Depending on Molecular Property of Parapinopsin. Color opponency in a single photoreceptor cell has previously been reported in the parietal eyes of the side-blotched lizard (5). In that photoreceptor cell, two different opsins, blue-sensitive pinopsin and green-sensitive parietopsin, drive gustducin (Gt-
but did not significantly respond to 588-nm light (Fig. 2H), suggesting that the bistable nature of parapinopsin is responsible for the visible light-dependent calcium increase.

**Color Opponency Based on Photoequilibrium of Parapinopsin Two States**
How the bistable nature underlies the antagonistic chromatic response in PP cells under imaging light conditions can be speculated as follows. As 930-nm light in two-photon excitation is considered to serve as blue light (~465 nm) in one-photon excitation, the imaging conditions were considered to form a "photoequilibrium" or "semiphotoequilibrium" (i.e., photoequilibrium-like mixture) of the dark state and photoproduct of parapinopsin (Fig. 34). For 10–20 s after starting imaging, fluorescence intensity decreased because of hyperpolarization of the PP cells, which demonstrates accumulation of the parapinopsin photoproduct under excitation light (SI Appendix, Fig. S2). The 405-nm light stimulus increases the short-wavelength component of incident light and thus increases the fractional concentration of the signaling active photoproduct from that produced by the imaging light alone, leading to hyperpolarization (Fig. 3B and D, calcium decrease). Conversely, because the photoproduct is more sensitive to longer wavelengths, the 588-nm light stimulus decreases its fractional concentration, reducing the pool of signaling-active opsin, resulting in depolarization and increased calcium levels (Fig. 3C and D, calcium increase). After the stimuli are turned off, the photoproduct amount is considered to revert to the original photoequilibrium level under "blue light" conditions (Fig. 3D). Therefore, the 405-nm and 588-nm light stimuli may transiently decrease and increase calcium levels by changing the fractional concentration of photoproduct from the photoequilibrium level.

To test this hypothesis, we analyzed calcium level changes under 860-, 930- and 1,000-nm two-photon excitations, which are considered equivalent to violet, blue, and green light (~430, ~465, and ~500 nm) excitations, respectively, and are therefore expected to generate a different "basal photoequilibrium." When imaging was performed with two-photon excitation with 860-nm light (i.e., "violet"), the 588-nm light stimulus increased the calcium levels, whereas the 405-nm light stimulus did not significantly decrease calcium levels (Fig. 3E). In contrast, in two-photon excitation with 1,000-nm light (i.e., "green"), the 405-nm light stimulus decreased the calcium levels, whereas the 588-nm light stimulus did not evoke an obvious increase (Fig. 3F). These results, together with the response profile of two-photon excitation at 930 nm (Fig. 3D), strongly support that the photoequilibrium of parapinopsin is fundamentally important to generate color opponency to light stimuli.

We next confirmed whether color opponency based on a single parapinopsin is generated under environmental light conditions. In vitro spectroscopic analysis revealed that, under white light with spectral distribution similar to that of sunlight in the early afternoon, zebrafish parapinopsin formed a photoequilibrium similar to that under the sunlight conditions wherein the ratio of the dark state to the photoproduct was ~4:1 (SI Appendix, Fig. S1, curve 4). The establishment of such a photoequilibrium allows the possibility that PP cells exhibit color opponency based on parapinopsin alone under sunlight. We investigated the calcium level changes

---

*Fig. 2.* Contribution of opsins to color opponency in PP cells under two-photon imaging. (A) RT-PCR analyses of parapinopsin and parapinopsin expression in the eye, brain, and pineal organ of zebrafish. (B) In situ hybridization analyses of two opsins in the zebrafish pineal organ using the double-fluorescence method. Yellow arrowheads in B indicate coexpression of two opsins in the zebrafish pineal organ. Orientations marked with “d” and “r” indicate the dorsal and rostral sides, respectively. White dotted traces indicate the landmarks of the pineal organ. (Scale bars: 100 μm.) (C) Relative difference absorbance spectrum of zebrafish parapinopsin before minus after light irradiation. The spectrum is shown as an average of three measurements. (D–H) Calcium level changes upon 405- or 588-nm light stimuli in PP cells of WT (D; n = 41), parapinopsin-KO (E; PT−/−, n = 28), parapinopsin-KO (F; PP−/−, n = 40), double-KO (G; PP−/−/PT−/−, n = 27), and SW51 opsin-expressing/parapinopsin-KO fish (H; SW51/PP−/−, n = 27). Error bars indicate SE. The light intensities of 405- and 588-nm light stimuli in D–H were ~3.2 × 10^{10} and ~5.4 × 10^{10} photons per cm^{2}s, respectively. The durations of both stimuli were ~450 ms. A/F values are change rates of normalized fluorescence intensity with the averaged intensity of 10 points before initial light stimuli. Statistical evaluation of the differences in amplitudes of calcium level changes among the WT and mutants are shown in SI Appendix, Fig. S5 A and B.

*Fig. 3.* Color opponency involving shift of photoequilibrium between the dark state and photoproduct of parapinopsin. (A–C) Schematic models of photoequilibrium between the dark state and photoproduct of parapinopsin and changes in the amount of its photoproduct upon different light stimuli under two-photon imaging. Circles filled with purple and green indicate molecules of the dark state and photoproduct of parapinopsin, respectively. Photoequilibrium of parapinopsin under two-photon excitation with 930-nm light (i.e., blue light) is shown with blue arrows (A). The shifts in photoequilibrium caused by stimuli of 405- and 588-nm light are shown as purple (B) and orange (C) arrows, respectively. Red arrows indicate the amount of cone transducin (GT2) activated by the photoproducts, which is equivalent to the hyperpolarization level. Note that the circles and the arrows schematically illustrated in A–D are not quantitatively drawn. The recovery of photoequilibrium to the original state after stimuli turn off under "blue light" condition could account for the antagonistic calcium response under two-photon imaging conditions. (D–F) Calcium level changes upon 405- and 588-nm light stimuli in WT PP cells under two-photon excitation with 930-nm light (D; n = 26), 860-nm (E; n = 13), and 1,000-nm light (F; n = 13) lights (i.e., blue, violet, and green lights, respectively). Schematic models for shifts in photoequilibrium by light stimuli are also shown in D. Error bars indicate SE. The light intensities of 405- and 588-nm light stimuli in D–F were ~3.2 × 10^{10} and ~5.4 × 10^{10} photons per cm^{2}s, respectively. The durations of both stimuli were ~450 ms. A/F values are change rates of normalized fluorescence intensity with the averaged intensity of 10 points before initial light stimuli.
in photoreceptor cells at 10-s intervals to reduce the effect of the two-photon imaging light, under continuous irradiation by white light with intensity ~1% of that found in a sunny location in the early afternoon and with a similar spectral distribution (Fig. 4A). In WT and PT−/−, a decrease followed by a steady state of calcium level was observed when the white light was turned on (SI Appendix, Fig. S6), similar to the case under imaging “blue” lights (SI Appendix, Fig. S2). In contrast, PP−/− and PP−/−/PT−/− did not show large decreases in calcium levels in response to white light (SI Appendix, Fig. S6). After the calcium level reached a plateau (~10 min), PP cells were exposed to “mixed white light” with UV or visible LED light, which has a broad spectral distribution (UV light- or visible light-mixed white light; Fig. 4A), to investigate whether color opponency based on parapinopsin is observed under natural light-like conditions. We found decreased and increased calcium levels following exposure to UV and visible light-mixed white light, respectively, in WT and PT−/− but not in PP−/− and PP−/−/PT−/− (Fig. 4B). Changes in calcium level were also observed in PP cells following exposure to different intensities and durations of UV and visible light-mixed white lights (SI Appendix, Fig. S7 A–C). These observations suggest that parapinopsin alone can allow pinel photoreceptors to respond to changes in the spectral distribution of incident light (i.e., color opponency) under physiological sunlight conditions.

**Discussion**

In this study, we describe a chromatically antagonistic response of calcium in a single zebrafish pineal cell produced by a single kind of bistable opsin, parapinopsin. The mechanism relies upon parapinopsin’s ability to form two photoconvertible opsin states with different spectral sensitivity and signaling capacity. Under extended light exposure, an equilibrium between these two states is formed in which the fractional concentration of the signaling active state is dependent on the spectral composition of incident light, which is biased towards UV for greater hyperpolarization than that biased toward visible light. A previous study revealed a correlation of photoresponses between calcium dynamics and electrophysiological outputs in photoreceptor cells (i.e., cones) of the zebrafish retina (21). Therefore, the antagonistic chromatic changes in calcium levels measured in this study qualitatively suggest electrophysiological outputs of color opponency in the PP cells of zebrafish. Parapinopsin is found in a wide variety of lower vertebrates (9, 10, 14, 18), and its bistable nature might contribute to a single kind of opponent-based color opponency in pinel-related organs other than the teleost pineal organ.

Photoreceptor cells in many invertebrates such as insects, crustaceans, and cephalopods employ bistable opsins (12). Photoreceptor cells of white-eyed *Drosophila* show “opponent” responses to blue and orange light under specific artificial experimental conditions (13). Following intense blue light irradiation, fly photoreceptors generate a depolarizing PDA that persists in the dark as a result of insufficient shut-off of light-activated rhodopsin; orange light then causes “opponent” responses (i.e., hyperpolarizations) because of the reverse photo-reaction of the accumulated “signaling-active metarhodopsin” (arrestin-free) to the inactive dark-state rhodopsin (13). Because, similarly to the mechanism of PDA, one might speculate that PP cells require abundant “active” or “not-inactivated” photoproducts to generate the reverse responses of photoreceptor cells (i.e., depolarization), it is important to consider the activity of biochemical deactivation mechanisms in this system. We therefore investigated the recovery kinetics of light response via shut-off of light-activated parapinopsin after turning off the white lights (i.e., in the dark). Interestingly, calcium levels recovered to that of the dark level by ~20 s after turning off the white light (SI Appendix, Fig. S8), showing no prolongation of the light response, as in a typical PDA, which continues for hours in the dark. Therefore, it is of interest to discuss how the active photoproduct accumulates under white light.

The duration of light exposure for maximum changes in increase of calcium level in PP cells (10–20 s; SI Appendix, Fig. S7), which is considered to be correlated to accumulation of the photoproduct, is shorter than the duration (more than 100 s) estimated to be required for ~99% level of the photoequilibrium formation (SI Appendix, Supplementary Information Text). To address this inconsistency, we roughly estimated a time evolution of the signaling active photoproduct after exposure to the white light, based on the very simplified reaction scheme of the dark state and photoproduct of parapinopsin according to that of bistable *Drosophila* rhodopsin and metarhodopsin (13) (SI Appendix, Supplementary Information Text and Fig. S9). The estimated profile suggests that the change in signaling active photoproduct amount in PP cells reaches maximum at approximately 20 s under exposure of PP cells to different white light (SI Appendix, Fig. S9C). The duration is similar to that for maximum changes in calcium level in PP cells under exposure to white light (SI Appendix, Fig. S7C). The profiles estimated with different values for reaction parameters suggest that the dark-inactivation of active photoproducts of parapinopsin under white light might contribute to accumulation of active photoproducts (SI Appendix, Fig. S9 C–E). Because the estimation is based on a very simplified model, investigation of a detailed mechanism(s)
involved in the dark inactivation of the signaling-active photoproducts, such as phosphorylation of and arrestin binding to parapinopsin photoproducts and their kinetics is important to understand how a certain amount of the active photoproduct is kept in PP cells under white light (SI Appendix, Supplementary Information Text). In addition, this issue is also important to investigate if activities of proteins, such as arrestin (22), are regulated when the photoproducts revert to the dark state upon absorption of visible light.

We also observed slow elevation of calcium levels after these levels were greatly decreased under continuous blue light irradiation (i.e., two-photon excitation) or xenon white light (SI Appendix, Figs. S2 and S6). Such slow elevation of calcium may be related to the kinetics of signaling-active photoproduct (SI Appendix, Fig. S9C), which could constitute a mechanism of light adaptation. As calcium itself affects electrophysiological responses by regulating several signal transduction proteins (23, 24), it is important to investigate such parapinopsin-based light adaptation by comparing WT and SWS1/PP−/− zebrafish.

We found coexpression of parapinopsin and green-sensitive parietopsin, which underlies depolarization in the lizard parietal eye, in the photoreceptor cells of the zebrafish pineal organ. Although parapinopsin-deficient PP cells exhibited a slight residual increase in calcium levels, suggesting depolarization (Fig. 2), the residual calcium increase in PP cells under white light (SI Appendix, Fig. S5B), suggesting that the residual increase in the calcium level did not involve a large contribution by parietopsin. Additionally, following exposure to white light, no significant difference in calcium changes was observed between PP+/− and PP−/−/PT−/− (SI Appendix, Fig. S6). Taken together, these observations suggest a limited contribution of parietopsin in regulating calcium levels under white light conditions used in this study. Such little involvement of parietopsin under these light conditions may be explained by its molecular polarity as a bleaching opsin (19). Under bright light conditions such as those in early to late afternoon (Fig. 4A and SI Appendix, Fig. S10A), the amount of functional parietopsin decreases because the photoproduct releases its retinal chromophore and loses its function after light absorption. In this circumstance, it remains possible that parietopsin contributes to color opponency by activating Go under dim light conditions (e.g., dusk and dawn), as suggested for the lizard parietal eye (3, 7).

In contrast to the two-opsin system involving parietopsin, the chromatic mechanism involving parapinopsin alone in the zebrafish pineal organ may not be suitable for detecting dawn and dusk. In our previous study at these times (e.g., irradiance of 0.1 W/m2 or less (25), ~3% brightness of the white light used in Fig. 4) is not sufficiently strong to form a photoequilibrium between the two states, which is essential for generating color opponency via this mechanism. The intensity and spectral distribution of natural sunlight varies depending on numerous factors, e.g., the time of day (e.g., early and late afternoon) and local conditions (e.g., sun or shade). Light intensity in the late afternoon is considered to be bright enough for parapinopsin-based color opponency (SI Appendix, Fig. S10A). Interestingly, the spectral distribution of light largely differs between the sunny and shady locations in the late afternoon; late-afternoon sunlight in a sunny location considerably varies depending on different situations. Light intensity in the late afternoon is considered to be bright enough for parapinopsin-based color opponency (SI Appendix, Fig. S10A). Interestingly, the spectral distribution of light largely differs between the sunny and shady locations in the late afternoon; late-afternoon sunlight in a sunny location considerably varies depending on different situations.

Opponency would provide a clue to the physiological function of parapinopsin-based color opponency as well as how putative parapinopsin-based and parapinopsin/parietopsin-based opponency mechanisms are used for the color opponency of PP cells, depending on different situations.

It has been suggested that the ancestral animal opsin had a bistable nature (26). Therefore, a single kind of bistable opsin-based color opponency in a single photoreceptor cell might have been a prototype of color opponency. As shown in Fig. 3, the shift of the quantity ratio of signaling-inactive and -active ("dark" and "photoproduct," respectively) states corresponding to the wavelengths of light is a key to generating color opponency. Bistable opsins in which dark and light states have largely overlapping absorption spectra do not clearly exhibit their photoequilibrium shift depending on the wavelengths of light, and therefore such a bistable opsin is not suitable for a single kind of opsin-based color opponency. In other words, the separation of spectral sensitivities between dark and light states could be critical to detect the color information. Accordingly, the color opponency with a single kind of bistable opsin could have simply developed from the brightness detection system with a single kind of bistable opsin through molecular evolution of the opsin, that is, amino acid mutations resulting in a bistable opsin having two spectroscopically separated states, like parapinopsin. Taken together, such a single kind of bistable opsin-based color opponency could be the first step for developing varied mechanisms of color opponency including color vision with multiple opsins.

Materials and Methods

Animals. Zebrafish (Danio rerio) were obtained from the Zebrafish International Resource Center and National BioResource Project Zebrafish. Zebrafish were maintained on 14-h light/10-h dark cycles at 28.5°C. Embryos (0-7 d postfertilization) were raised in 1 L tanks containing 1 m3 of water. Embryos at 8% and 25%, respectively (SI Appendix, Fig. S10B). Therefore, such changes in wavelength components between sunny and shady locations in the late afternoon may be detected by the mechanism involving parapinopsin alone because late afternoon sunlight in the shade is approximately fivefold brighter than the white light used in this study, in which parapinopsin-based color opponency was generated (Fig. 4A and B and SI Appendix, Fig. S10). Determining the intensity of white light that enables PP cells to generate color variations in amplitude (parietopsin, which underlies depolarization in the lizard parietal eye) may not be suitable for detecting dawn and dusk, as suggested for the lizard parietal eye (3, 7). In contrast to the two-opsin system involving parietopsin, the chromatic mechanism involving parapinopsin alone in the zebrafish pineal organ may not be suitable for detecting dawn and dusk. In our previous study at these times (e.g., irradiance of 0.1 W/m2 or less (25), ~3% brightness of the white light used in Fig. 4) is not sufficiently strong to form a photoequilibrium between the two states, which is essential for generating color opponency via this mechanism. The intensity and spectral distribution of natural sunlight varies depending on numerous factors, e.g., the time of day (e.g., early and late afternoon) and local conditions (e.g., sun or shade). Light intensity in the late afternoon is considered to be bright enough for parapinopsin-based color opponency (SI Appendix, Fig. S10A). Interestingly, the spectral distribution of light largely differs between the sunny and shady locations in the late afternoon; late-afternoon sunlight in a sunny location considerably varies depending on different situations.
RT-PCR. Total RNA from the pineal organ, brain, and eye of zebrafish was purified with an RNAeasy Mini kit (Qiagen) and reverse-transcribed into cDNA by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Purified with an RNeasy Mini kit (Qiagen) and reverse-transcribed into cDNA by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reverse transcription of cDNA was performed with the SuperScript II kit (Life Technologies). The resulting cDNA was amplified by PCR using gene-specific primers. The PCR products were visualized on an agarose gel and purified using a GeneElute PCR Clean-Up Kit (Sigma-Aldrich). The purified DNA was ligated into the pGEM-T Easy vector (Promega) and transformed into E. coli. The positive clones were sequenced by Macrogen Inc. (Seoul, South Korea).

In situ Hybridization. Preparation of RNA probes and in situ hybridization were carried out as previously described (17). DIGoxigenin (DIG)- and fluorescein-labeled antisense and sense RNA probes for zebrafish parapinopsin and parietoptin mRNAs were synthesized by using the DIG RNA labelling kit and fluorescein RNA labeling kit (Roche), respectively. Sections were pre-treated with protease K and hybridized with each RNA probe in ULTRAhyb UltraSensitive Buffer (Ambion). For in situ hybridization, sections hybridized with DIG-labeled probes were incubated with HRP-conjugated anti-DIG antibody (Roche) and subsequently treated with the TSA plus DNP (HRP) system (Perkin-Elmer), followed by incubation with Alexa 488-conjugated anti-DNP antibody. Fluorescein-labeled probes on the sections were detected by incubation with alkaline phosphatase-conjugated anti-fluorescein antibody (Roche) followed by a color reaction using the HNPP Fluorescent Detection Set (Roche).

Spectroscopic Analyses of Opsin-Based Pigment. Zebrarh parapinopsin and parietoptin were expressed and purified as previously described (30, 31). Expressed proteins were reconstituted by adding an excess of 11-cis-retinal, extracted with 1% n-dodecyl β-d-maltoside (DM) in 50 mM Hepes buffer (pH 6.5) containing 140 mM NaCl (buffer A) and purified by using 1D-agarose with buffer A containing 0.02% DM. Absorption spectra were measured with a UV2450 spectrophotometer (Shimadzu) at 0 °C. Full methods are described in SI Appendix, Materials and Methods.

Measurement of Natural Light Spectra. A CL-500A illuminance spectrophotometer (Konica Minolta) was used for all measurements. Spectrum measurement in the early afternoon was carried out at 2:30 PM on April 16, 2018, in Osaka, Japan. Spectrum measurement of sunny and shady locations in the late afternoon was performed at 6:00 PM on June 16, 2018, in Osaka, Japan. The shade was made by shading direct sunlight with a metallic case (height 30 cm × width 40 cm × depth 3 cm). Shade light, as indirect sunlight from the sky, was measured at a point 100 mm away from the metal case against the direction of the sun. The detector was oriented perpendicularly to the sky and measured at the ground level (∼5 cm).

ACKNOWLEDGMENTS. We thank Robert J. Lucas (University of Manchester) for his valuable comments on this manuscript and Robert S. Molday (Univer- sity of British Columbia) for supplying rho 1D4-producing hybridoma. This work was supported by Japanese Ministry of Education, Culture, Sports, Science and Technology Grants-in-Aid for Scientific Research 15H05777 (to A.T.), 16K00704 and 17H06015 (to M.K.), 14J04099 (to E.K.-Y.), and 13J05782 (to S.W.); Japan Science and Technology Agency (JST) Core Research for Evolutional Science and Technology (CREST) Grant JPMJCR1753 (to A.T.) and JST Precursory Research for Embryonic Science and Technology (PRESTO) Grant JPMJP13A2 (to M.K.); and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (to E.K.-Y. and S.W.).