Bistable UV pigment in the lamprey pineal

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Lower vertebrates can detect UV light with the pineal complex independently of eyes. Electrophysiological studies, together with chromophore extraction analysis, have suggested that the underlying pigment in the lamprey pineal exhibits a bistable nature, that is, reversible photoreaction by UV and visible light, which is never achieved by known UV pigments. Here we addressed the molecular identification of the pineal UV receptor. Our results showed that the long-hypothesized pigment is a lamprey homologue of parapinopsin, which exhibits an absorption maximum at 370 nm, in the UV region. UV light causes *cis–trans* isomerization of its retinal$_2$ chromophore, forming a stable photoproduct having an absorption maximum at 515 nm, in the green region. The photoproduct reverts to the original pigment upon visible light absorption, showing photoregeneration of the pigment. *In situ* hybridization showed that parapinopsin is selectively expressed in the cells located in the dorsal region of the pineal organ. We successfully obtained the hyperpolarizing responses with a maximum sensitivity of ≈380 nm from the photoreceptor cells at the dorsal region, in which the outer segment was clearly stained with anti-parapinopsin antibody. These results demonstrated that parapinopsin is the pineal UV pigment having photointerconvertible two stable states. The bistable nature of the parapinopsin can account for the photorecovery of the pineal UV sensitivity by background green light in the lamprey. Furthermore, we isolated the parapinopsin homologues from fish and frog pineal complexes that exhibit UV sensitivity, suggesting that parapinopsin is a common molecular basis for pineal UV reception in the vertebrate.

Pineal and its related organs are known to be photosensitive in lower vertebrates (1, 2). It is widely accepted that the photoreception of the pineal is involved in various light-regulated physiological events such as the biological clock. Although extraocular photoreceptors typically exhibit maximum sensitivities in blue to green light, several electrophysiological studies have demonstrated UV reception in the pineal complex of a variety of lower vertebrates, such as lamprey (3), trout (4), pike (5), and frogs (6, 7). In these animals, UV light suppresses the neuronal firing of a specific kind of ganglion cell in the pineal complex, whereas visible light stimulates the neuronal activity, which is called chromatic responses. The antagonistic chromatic responses allow the pineal complex to detect the ratio of UV light to visible light in environmental light, which achieves the wavelength-discrimination in the pineal complex independently of eyes (8). Electrophysiological studies also showed that the response to UV light is influenced by the background illumination of visible light, providing a possibility that the mechanism, which is different from that of eyes, underlies the pineal wavelength-discrimination (9, 10).

In the river lamprey, whose pineal UV reception is well investigated, reversible photoisomerization of the chromophore retinal in the pineal by UV and visible light has been reported (11, 12). This biochemical evidence suggested that a previously uncharacterized UV pigment is involved in the pineal UV reception, because such photoreversibility has not been found in known vertebrate opsins including the UV cone opsin, which achieves the UV vision in many vertebrates (13, 14). Because the property of the underlying pigment seemed to be closely related to the pineal physiology, we tried to clarify the molecular basis of the pineal UV reception by using the lamprey.

Here we show that the lamprey homologue of parapinopsin, which was first identified in the catfish pineal complex (15), is the UV pigment in the pineal. Furthermore, spectroscopic analysis revealed that the parapinopsin exhibits a bistable nature, that is, reversible photoreaction by UV and visible light. These characteristics of the parapinopsin can account for the electrophysiological characteristic of the pineal UV reception.

Materials and Methods

Isolation and Sequencing of Opsin cDNAs. Total RNAs extracted from the pineal complexes of river lamprey (*Lethenteron japonica*), clawed frog (*Xenopus tropicalis*), and rainbow trout (*Oncorhynchus mykiss*) were reverse transcribed to cDNAs by using an oligo(dT) primer, and the cDNAs were used as templates for PCR amplification. The sense and antisense degenerate primers that were used for obtaining the cDNA fragments of opsin from the lamprey and the rainbow trout were as follows: 5′-GCGGATCCICCIITNNTNGGNTGG-3′, corresponding to the amino acid sequence PP(F/L/V/I/M)(F/L/V/I/M)GW for the sense primer, and 5′-GCGAATTCIIIGC(A/G)TANGGN(C/A/G)NCCA-3′, corresponding to WXPYAX for the antisense primer. PCR amplifications were carried out with annealing temperatures of 40 or 46°C. The partial cDNA of the clawed frog parapinopsin was obtained with the aid of the sequence in the *X. tropicalis* Genome Project at the Joint Genome Institute (www.jgi.doe.gov). The full-length cDNAs of these opsins were obtained by using the 3′ RACE and 5′ RACE systems (Invitrogen). The cDNA encoding goldfish UV cone pigment was obtained from the lateral eye of goldfish (*Carassius auratus*) by RT-PCR with gene-specific primers, designed according to the sequence in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases.

Phylogenetic Tree Inference. Multiple alignment of the amino acid sequences of opsin and inference of the molecular phylogenetic tree were carried out with the aid of XCED software (16). On the basis of the alignment, the evolutionary distance was calculated by a simple Poisson correction (17). The evolutionary distance was then applied to phylogenetic inference by the neighbor joining method (18). Bootstrap analysis was carried out by the method of Felsenstein (19).

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Data deposition: The sequences reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. AB116380–AB116382, AB159672, and AB159673).

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Expression and Purification of Pigments. The coding region of the lamprey parapinopsin and the goldfish UV cone pigment cDNAs were isolated by PCR, being tagged by the monoclonal antibody Rho 1D4 epitope-sequence (ETSQVAPA) (20). The tagged cDNA was inserted into a plasmid vector SRα (21). One hundred micrograms of the plasmid DNA were used for the transient expression in 10 dishes (100 mm in diameter) of HEK293s cells. The vector was cotransfected with the pRSV-Tag into HEK293s cells by the calcium-phosphate method according to the previous report (22, 23). The transfected cells were harvested for 2 days and collected by centrifugation. To reconstitute the pigment, the expressed proteins were incubated with excess 11-*cis* 3-dehydro-retinal (in the case of lamprey parapinopsin) or 11-*cis* retinal (in the case of goldfish UV cone pigment) overnight. 11-*cis* 3-dehydro-retinal was a generous gift from Liu R. S. H. (University of Hawaii, Manoa). The pigments were then extracted with 1% dodecyl β-D-maltoside (DM) in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (pH 6.5) containing 140 mM NaCl (buffer A), bound to 1D4-agarose, washed with 0.1% DM in buffer A (buffer B), and eluted with buffer B containing the C terminus peptide of bovine rhodopsin. L-α-phosphatidylcholine (0.1 mg/ml) was added to buffer B through the wash and elution steps to keep its photoreaction that is observed in the membrane preparation according to the previous report (24).

Spectrophotometry and HPLC Analysis. Absorption spectra of purified samples were recorded at 0°C with a Shimadzu UV2400 spectrophotometer (25). A 1-kW halogen lamp (Philips) was used for the sample irradiation. UV, orange, and white light were supplied by the light source with a UV-D36C glass filter, with an O-56 glass cutoff filter (Toshiba) and without a filter, respectively. The chromophore configurations of each samples were analyzed by HPLC with a Shimadzu LC-7A interfaced with a CR-5A according to the method described previously (23, 26).

***In Situ* Hybridization.** Digoxigenin-labeled antisense RNA probes for the lamprey parapinopsin and rhodopsin were synthesized by using the DIG RNA labeling kit (Roche Applied Science). The pineal organs were immersion-fixed in 4% paraformaldehyde, cryoprotected in 0.1 M phosphate buffer containing 15% sucrose, frozen with OCT Compound (Sakura), and sectioned at 12 μm. The pineal sections were pretreated with proteinase K and hybridized with the antisense RNA probe. The probe on the sections was detected by using alkaline phosphatase-conjugated anti-digoxigenin (Roche Applied Science) by a blue 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium color reaction.

Intracellular Recordings. Intracellular recordings were carried out as described (27) with the following modifications. Before recording, tissues were kept in darkness for at least 30 min. Electrodes, whose resistances ranged from 120 to 200 MΩ, were used for recording. Intracellular responses were amplified by a high-input impedance amplifier, IR-183 (Cygnus Technology, Delaware Water Gap, PA), and recorded on a data acquisition system, PowerLab (AD Instruments). A stimulating light was delivered from a 500-W Xenon arc lamp. Monochromatic light of equalized quantum flux was obtained by using interference filters (half bandwidth, 20 nm) and neutral density filters. The duration of the stimulus was 300 ms in all experiments. After physiological characterization of the cells, intracellular injections of neurobiotin (Vector Laboratories) were performed. Neurobiotin-injected pineal organs were fixed as described above and sectioned at 20–30 μm. To visualize the neurobiotin, the sections were incubated with Alexa Fluor 568 streptavidin (Molecular Probes).

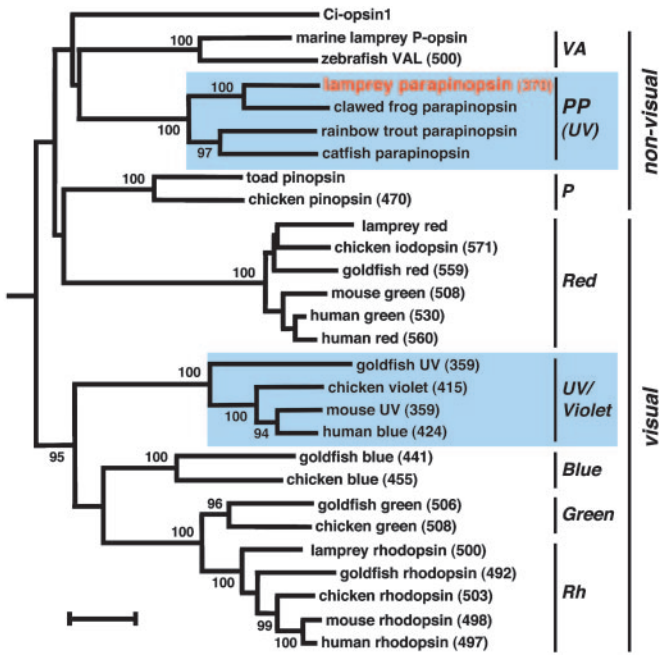

Fig. 1. Phylogenetic tree of the vertebrate opsin subtype. From a comparison of the alignment of conserved regions, the tree was inferred by the neighbor joining method, using other opsin subtypes including invertebrate rhodopsins as an outgroup (data not shown). The bootstrap probabilities >90% are indicated at each branch node. The names of evolutionarily and functionally classified groups are shown on the right side of each cluster. Two groups that contain UV-sensitive opsins are shaded. (Scale bar, 0.1 substitutions per site.) Absorption maximum values (nm) of pigments, which have been reported (14), are indicated in the parentheses. The value of the retinal$_2$-based lamprey parapinopsin was revealed in this work. Accession numbers of the sequence data from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database are as follows: Ci-opsin1, AB058682; marine lamprey P-opsin, U90671; zebrafish VAL, AB035277; lamprey parapinopsin, AB116380 (this work); clawed frog parapinopsin, AB159672 (this work); rainbow trout parapinopsin, AB159673 (this work); catfish parapinopsin, AF028014; toad pinopsin, AF200433; chicken pinopsin, U15762; lamprey red, AB116381 (this work); chicken iodopsin, X57490; goldfish red, L11867; mouse green, AF011389; human green, AH005296; human red, AH005298; goldfish UV, D85863; chicken violet, M92039; mouse UV, U92562; human blue, AH003620; goldfish blue, L11864; chicken blue, M92037; goldfish green, L11866; chicken green, M88178; lamprey rhodopsin, AB116382 (this work); goldfish rhodopsin, L11863; chicken rhodopsin, D00702; mouse rhodopsin, M55171; human rhodopsin, U49742.

Generation of Antibody and Immunohistochemistry. Anti-parapinopsin antibody was generated against a synthetic peptide corresponding to 18 residues (C308–S325) near the C terminus of the lamprey parapinopsin. Pineal sections, prepared as described above, were incubated with the 1:500 diluted anti-parapinopsin antiserum, followed by incubation with Alexa Fluor 488 anti-rabbit IgG (Molecular Probes) for the immunofluorescent detection of parapinopsin.

Results and Discussions

We have tried to isolate opsin cDNAs from the pineal organ of adult river lamprey (*L. japonica*) to identify the UV receptor protein in the lamprey pineal, and have obtained five kinds of opsin cDNAs. Two of the five opsins showed the highest similarity in amino acid sequence to RGR and peropsin, both of which are considered to act as a retinal photoisomerase (23, 28). Because the remaining three opsins show high similarity to the members of vertebrate opsin subtype that contains vertebrate visual pigments, we inferred the molecular phylogenetic tree of the vertebrate opsin subtype (Fig. 1). The phylogenetic tree

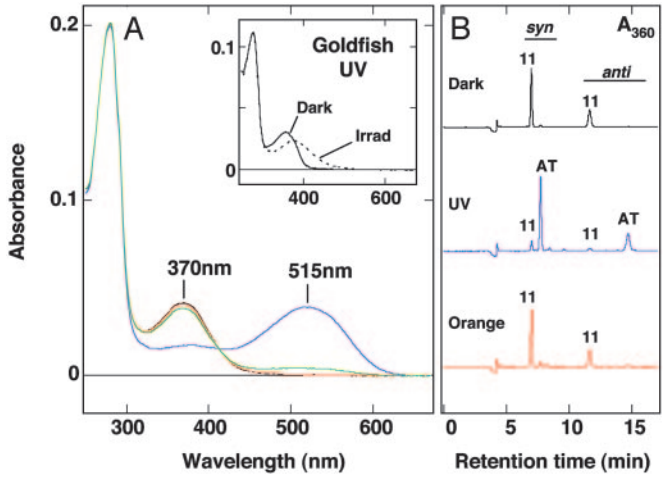

Fig. 2. Absorption spectra and chromophore configurations of the lamprey parapinopsin. (*A*) Absorption spectra of the lamprey parapinopsin in the dark state (black), after irradiation with UV light (blue), after subsequent irradiation with orange light (red), and after irradiation with white light (green). (*Inset*) The absorption spectra of the goldfish UV cone visual pigment before (Dark) and after UV-light irradiation (Irrad). The ratio of the protein absorbance at 280 nm to the pigment absorbance, namely the purity of the pigment, was 4.9 and 3.8 in the parapinopsin and the UV cone pigment, respectively. (*B*) The chromophore configurations of the lamprey parapinopsin were analyzed with HPLC after extraction of the chromophore as retinal oximes (*syn* and *anti*-forms of 11-*cis* and all-*trans* retinal oximes). The color of each trace corresponds to that of each spectrum in *A*. Note that ≈90% of the 11-*cis* chromophore was photoisomerized to all-*trans* form by the UV irradiation.

clearly shows that these opsins belong to the Rh group [lamprey rhodopsin (29)], the Red group (lamprey red), and the PP group (lamprey parapinopsin) (Fig. 1). No opsin belonging to the UV/Violet group (Fig. 1), whose members achieve UV vision, has been isolated from the pineal organ or eye of the lamprey.

The absorption maximum of the lamprey rhodopsin is ≈500 nm (30). The phylogenetic tree (Fig. 1) strongly suggests that the lamprey red serves as a longer wavelength-sensitive opsin. We therefore focused our attention on the lamprey parapinopsin as a candidate for a UV-sensitive pigment in the pineal. The lamprey parapinopsin was expressed in cultured cells and was successfully reconstituted with 11-*cis* 3-dehydro-retinal (retinal$_2$), which is a major component of the chromophore in the pineal organ of the adult lamprey (11). The reconstituted pigment exhibits an absorption maximum at 370 nm, in the UV region (Fig. 2*A*), which is 10 nm longer in wavelength than that of retinal$_1$-based lamprey parapinopsin (31). The absorption spectrum clearly indicates that parapinopsin is a UV-sensitive opsin. Interestingly, the phylogenetic tree shows that two lines of UV pigments, UV cone opsin (UV/Violet group) and parapinopsin (PP group), have evolved independently in the vertebrate lineage (Fig. 1), providing a striking example of convergent evolution.

Further spectroscopic analysis discovered the definite difference in the photoreaction between the two kinds of UV pigments. UV light irradiation converted the parapinopsin to the photoproduct with an absorption maximum at 515 nm, in the green region (Fig. 2*A*). The photoproduct was stable at 0°C and exhibited no spectral change in the dark. It was then reverted by subsequent orange light irradiation to a pigment having an absorption spectrum almost identical to the dark state, showing photoregeneration of the pigment (Fig. 2*A*). These photoreactions can be repetitively achieved by irradiation with UV and orange light (data not shown). These results clearly demonstrate

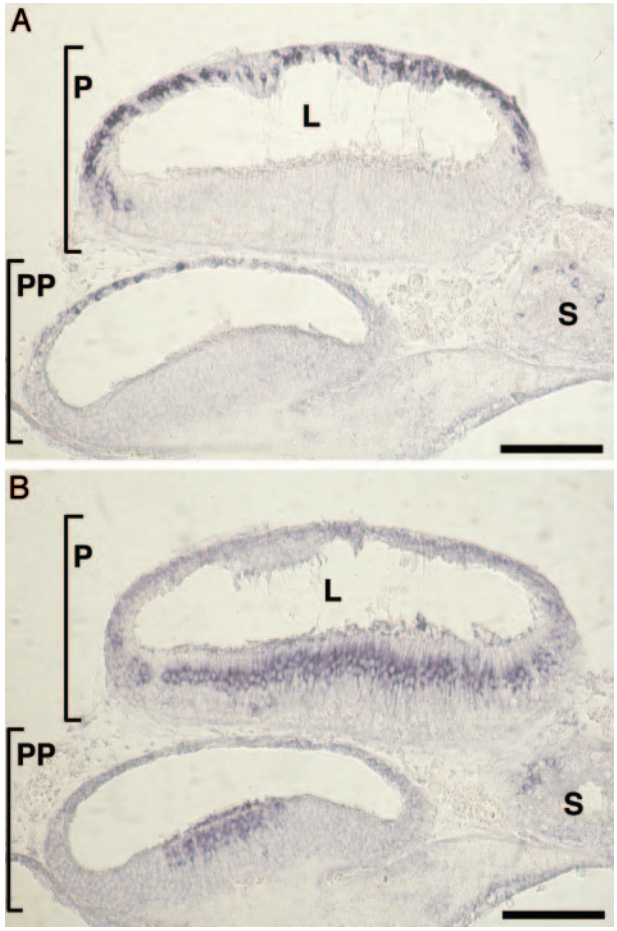

Fig. 3. Localization of parapinopsin and rhodopsin in the pineal complex. *In situ* hybridization with the antisense probes of parapinopsin (*A*) and rhodopsin (*B*) show that they are expressed in the photoreceptor cells in the dorsal region (*Upper*) and the ventral region (*Lower*) in both pineal and parapineal organs. P, pineal organ; PP, parapineal organ; L, pineal lumen; S, pineal stalk. (Scale bar, 100 μm.)

that the parapinopsin has photointerconvertible two stable states, the UV-sensitive dark state and the green-sensitive photoproduct, which is called a bistable nature. We also observed the photointerconvertible reaction of the parapinopsin in the detergent-free preparation, namely in the HEK293s membranes, at 15°C, which could be closer to physiological conditions (data not shown). The photoisomerization of the chromophore retinal between the 11-*cis* and all-*trans* forms underlies the reversible spectral change (Fig. 2*B*), which can account for the previous observation in the lamprey pineal, a reversible photoisomerization of the chromophore retinal (11, 12). The formation of a stable photoproduct having an absorption maximum at visible region is quite different from the photoreaction of cone UV pigment, such as goldfish UV (Fig. 2*A Inset*). Like all known vertebrate cone pigments, the goldfish UV cone pigment shifts to an absorption maximum at ≈380 nm upon irradiation because of the dissociation of the all-*trans* retinal from the opsin (32–34).

We investigated the photoreaction of the parapinopsin under white light, which is similar to the environmental light condition for the lamprey. As shown in Fig. 2, ≈90% of the pigments were converted to the photoproduct by UV light irradiation. In contrast, white light irradiation only converted <10% of the pigments to the photoproduct (Fig. 2*A*), although the white light has a UV light intensity much higher than that of the UV light used for the experiments. This photoreaction is due to the

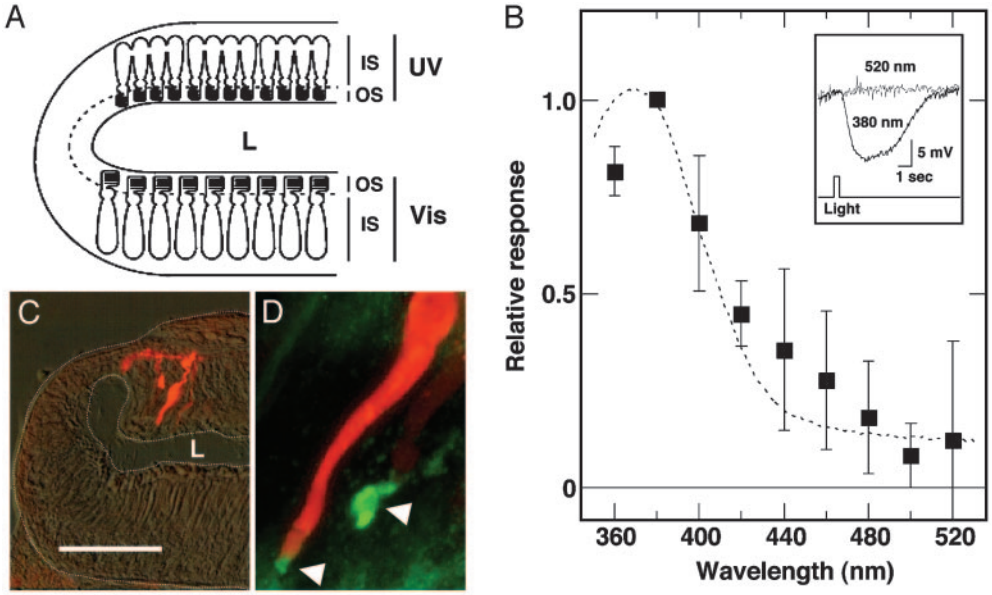

Fig. 4. UV sensitivity from the photoreceptor cells containing the parapinopsin. (*A*) Schematic drawing of the photoreceptor cells in the lamprey pineal. OS, photoreceptor outer segment; IS, photoreceptor inner segment; L, pineal lumen. (*B*) Relative response curve of UV-sensitive cells. The response amplitude to each wavelength-light stimulus was normalized by the maximum amplitude. The individual relative response curves were averaged ($n = 3$). Vertical bars indicate the SD. The absorption spectrum of the parapinopsin in Fig. 2 is superimposed (broken curve). (*Inset*) The electrical recording traces to 380- and 520-nm light stimuli. (*C*) UV-sensitive cells labeled with intracellularly injected neurobiotin at the dorsal region of the pineal. The fluorescent image and Nomarski image are merged. The white dotted traces indicated the landmarks of the pineal organ. L, pineal lumen. (Scale bar, 100 μm.) (*D*) High-magnification confocal image around the outer segment of the cell that exhibits the strongest red-fluorescence in *C*. The outer segments of the UV-sensitive cells are labeled with anti-parapinopsin antibodies (arrowheads).

photoregeneration of pigment by visible light contained in the white light. A similar photochemical change was observed by sunlight exposure (data not shown), suggesting that, under strong environmental light, the bistable nature of the parapinopsin is important in maintaining the high amount of UV pigment in the photoreceptor cells.

We then investigated the localization of parapinopsin in the pineal complex. *In situ* hybridization clearly showed that parapinopsin is selectively expressed in the photoreceptor cells located in the dorsal region of the pineal and parapineal organs (Fig. 3*A*). This finding is in contrast to the localization of rhodopsin in the ventral region (Fig. 3*B*), as reported (35). In the pineal stalk, there are a few cells where parapinopsin or rhodopsin is expressed (Fig. 3). These results demonstrate that pineal and parapineal organs are functionally divided into at least two parts, a UV-light-sensitive region and a visible-light-sensitive region (Figs. 3 and 4*A*).

To obtain direct evidence that the photoreceptor cells exhibiting the UV sensitivity really express parapinopsin, we first identified the UV-sensitive cells by electrical recordings, and then stained those cells with an antibody against parapinopsin. We obtained hyperpolarizing responses with the highest sensitivity at ≈380 nm from the cells in the lamprey pineal (Fig. 4*B*), consistent with the previous report (27). The spectral sensitivity is well matched to the absorption spectrum of the recombinant parapinopsin. An intracellular injection of neurobiotin into UV-sensitive cells showed that these cells were situated at the dorsal region of the pineal organ (Fig. 4*C*). In addition to the neurobiotin-injected cell, which exhibits the strongest fluorescence, a few photoreceptor cells were weakly labeled with neurobiotin, demonstrating gap junction connections (Fig. 4*C*). Immunofluorescence analysis showed that the anti-parapinopsin antibodies clearly stained two outer segments of neurobiotin-labeled cells that exist in this section (Fig. 4*D*, arrowheads). These results demonstrated that the parapinopsin is involved in the UV reception at the dorsal part of the lamprey pineal.

The molecular property of the newly found pineal UV pigment, parapinopsin, accounts for the electrophysiological characteristics in the lamprey pineal. In the lamprey pineal, UV light inhibits the neuronal firing of a specific kind of ganglion cell (3). Interestingly, the inhibitory effect under strong UV light irradiation is diminished within a minute, whereas it is retained in the presence of background green light (10). The parapinopsin photoregeneration due to the bistable nature (Fig. 2) allows the photoreceptor to recover the UV sensitivity from a "light-adapted" state, and to capture UV light efficiently and continuously with a consequent high amount of UV pigments. Therefore, the pigment photoregeneration leads to the recovery of the UV sensitivity of the ganglion cells. This conclusion is strongly supported by the observation that the inhibitory effect on the ganglion cells was recovered not by incubation in the dark but by green-light illumination that promotes photoregeneration of the UV pigment (S.T., K. Uchida, and M. Samejima, unpublished observation). In addition, the slight photochemical change of the parapinopsin under white light (Fig. 2*A*) can also account for the persistent UV reception of the pineal under environmental light.

A similar inhibitory ganglionic response to UV light in the pineal complex has been also found in fish and frogs (4–7). We can speculate that the parapinopsin homolog is also involved in the pineal UV reception in these animals. In support of this idea, we successfully isolated the parapinopsin homologues from the rainbow trout and the clawed frog pineal complexes where the UV sensitivity has been electrophysiologically detected (4, 7). They exhibit 61% and 71% identity to the lamprey parapinopsin and clearly belong to the PP group (Fig. 1). Therefore, parapinopsin is possibly a common molecular basis for pineal UV reception in the vertebrate.

In the pineal complexes of these lower vertebrates, the inhibitory response to UV light, together with the excitatory response to visible light, constitutes chromatic responses (3–7). The antagonistic nature of these two responses has suggested that the

pineal complex detects the ratio of UV light to visible light in environmental light, a kind of "color discrimination" (8). Interestingly, electrophysiological study in the frog proposed a model that the color discrimination in its pineal complex is achieved through a single hypothesized pigment called "bistable pigment," which has photointerconvertible two stable states and therefore behaves like two distinct visual pigments (9). The model is extended to the case of the color discrimination in pineal complexes of other vertebrates including the parietal eye of lizards (8, 36, 37). Taken together with our current findings, parapinopsin possibly fits the "bistable pigment" in this model. We emphasize that this is, to our knowledge, the first report which describes the functioning of the pigment with bistable nature in the vertebrate. Our result gives a typical example that the pigment property directly generates physiological characteristics. Therefore, by using parapinopsin as a starting point, the biological meaning of the pineal UV reception as well as that of the bistable nature of pigment would be elucidated.

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