

Opn5 is a UV-sensitive bistable pigment that couples with Gi subtype of G protein

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Edited by Rosalie K. Crouch, Medical University of South Carolina, Charleston, SC, and accepted by the Editorial Board November 1, 2010 (received for review August 23, 2010)

Opn5 (neuropsin) belongs to an independent group separated from the other six groups in the phylogenetic tree of opsins, for which little information of absorption characteristics and molecular properties of the members is available. Here we show that the chicken Opn5 (cOpn5m) is a UV-sensitive bistable pigment that couples with Gi subtype of G protein. The recombinant expression of cOpn5m in HEK 293s cells followed by the addition of 11-cis- and all-trans-retinal produced UV light-absorbing and visible light-absorbing forms, respectively. These forms were interconvertible by UV and visible light irradiations, respectively, indicating that cOpn5m is a bistable pigment. The absorption maxima of these forms were estimated to be 360 and 474 nm, respectively. The GTPγS binding assay clearly showed that the visible light-absorbing form having all-trans-retinal activates Gi type of G protein, whereas no Gt or Gq activation ability was observed. Immunohistochemical studies using an antibody against cOpn5m clearly showed that this pigment is localized within some types of amacrine cells and some cells in the ganglion cell layer of the retinas, the vast majority of cells in the pineal gland and serotonin-positive cells in the paraventricular organ. Because cOpn5m is the only UV-sensitive opsin among the opsins found so far in chicken, this study provides the molecular basis for UV reception in chicken.

G protein-coupled receptor | nonvisual photoreception | phototransduction

Opsins are the universal photoreceptive molecules that underlie the molecular basis of visual and nonvisual photoreceptions in animals. Recent progresses in cloning technology and genome sequencing have revealed seven distinct groups of opsins based on their amino acid sequences (1, 2). In vertebrates, more than 1,000 opsin genes belonging to five groups have been identified (Fig. S1). Despite the diversity of opsins, all the members are thought to have seven α -helical transmembrane domains and a lysine residue in helix VII to which a retinal chromophore binds via a Schiff base linkage. In contrast to these conserved structural features, the phylogenetic classification of opsins correlates well with their functional differences, i.e., G protein-coupled receptors (GPCRs) and retinal photoisomerases. In most groups, opsins act as GPCRs containing 11-cis-retinal as a light-absorbing chromophore (3). Light isomerizes the retinal chromophore from 11-cis to all-trans form, leading to the formation of the active states of opsins. These can couple with trimeric G proteins, resulting

in the triggering of the intracellular signal transduction cascades. These types of vertebrate opsins such as visual opsins in rod and cone photoreceptor cells as well as several nonvisual opsins can be categorized by their coupling to either Gi-type [including transducin (Gt)] or Gq-type G protein (1, 2, 4). On the other hand, opsins such as retinal G protein-coupled receptor (RGR) and peropsin bind to all-trans-retinal as chromophores (5, 6). Irradiation of these opsins results in photoisomerization of all-trans chromophore to 11-cis form. Thus these opsins are thought to work as retinal photoisomerases, which supply 11-cis-retinal to the other opsins. Characterizations of opsins have been achieved by the advance of the techniques to produce and purify the recombinant proteins in mammalian cultured cells.

The Opn5 (neuropsin) group was first identified in mouse and human genomes and found to be expressed in neural tissues (7), but its function had remained uncharacterized. An interesting character of Opn5 is that Opn5 is phylogenetically closely related with retinal photoisomerases, RGR, and peropsin. In fact, Opn5 shares intron positions with peropsins and, in some phylogenetic trees, the pigment is considered to be diverged from peropsin and retinochrome/RGR after diversification from other opsin groups such as the Go-coupled rhodopsin group (1). However, there have been no reports about the molecular properties of purified Opn5 protein. Thus it remains unknown whether Opn5 can function as a retinal photoisomerase or a GPCR. In this study, we successfully expressed the active protein of chicken homolog of mammalian Opn5 (cOpn5m) in cultured cells. The pigment having 11-cis-retinal chromophore is sensitive to UV light, and it converts to the active state having all-trans-retinal chromophore to efficiently activate Gi-type G protein. Therefore, cOpn5m is not a retinal photoisomerase but a UV-sensitive GPCR that couples with Gi-type G protein. Immunohistochemical analyses showed that cOpn5m-positive cells are localized in chicken retina, pineal

Author contributions: T.Y., H.O., and Y.S. designed research; T.Y., H.O., S.T., K.I., and K.S. performed research; T.Y., H.O., S.T., K.I., K.S., and Y.S. analyzed data; and T.Y., H.O., and Y.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. R.K.C. is a guest editor invited by the Editorial Board.

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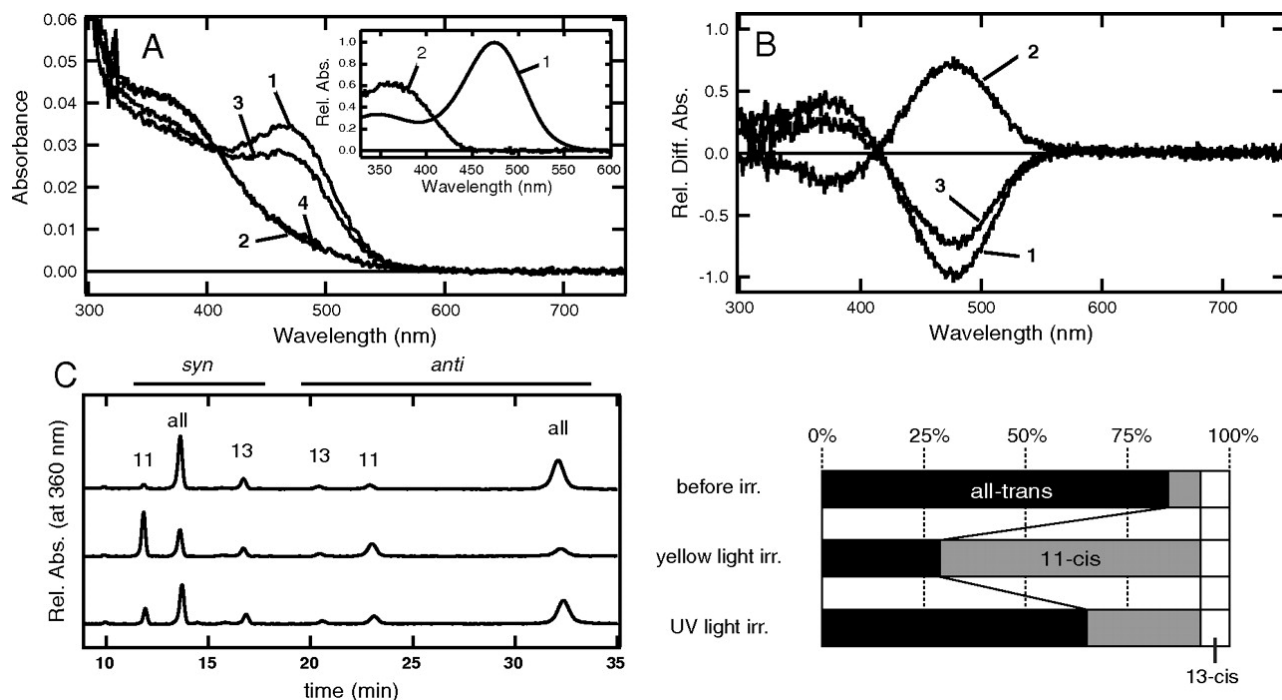


Fig. 1. Absorption spectra and retinal configurations of cOpn5m. (A) Absorption spectra of purified cOpn5m reconstituted with all-trans-retinal. Spectra were measured in the dark (curve 1), after yellow light (> 500 nm) irradiation (curve 2), and after subsequent UV light irradiation (curve 3), and reirradiation with yellow light (curve 4). (Inset) The calculated absorption spectra of cOpn5m in the dark (curve 1) and after yellow light irradiation (curve 2). The calculation procedures are described in the text. (B) Spectral change caused by irradiation with yellow light (curve 1) and subsequent UV light (curve 2) and reirradiation with yellow light (curve 3). (C) Retinal configuration changes by yellow light and subsequent UV light irradiations of cOpn5m. (Left) The retinal configurations were analyzed with HPLC after extraction of the chromophore as retinal oximes (syn and anti forms of 11-cis, 13-cis, and all-trans retinal oximes). (Right) Isomeric compositions of retinal before and after light irradiations of cOpn5m.

gland, and paraventricular organ. Because cOpn5m is the only UV-sensitive opsin among the opsins found so far in chicken, this study reveals the molecular entity of UV reception in chicken.

Results

Molecular Property of cOpn5m. To obtain the information about the molecular properties of cOpn5m, we expressed recombinant protein of cOpn5m in mammalian cultured cells. As already described, Opn5 is phylogenetically closely related with peropsin and retinal photoisomerases that have all-trans-retinal as their chromophores (7). Thus we first tried to reconstitute the pigment by addition of all-trans-retinal in the cells containing expressed cOpn5m proteins. We successfully obtained the active pigment and purified it. Fig. 1A shows the absorption spectrum of cOpn5m and its light-induced spectral changes. The pigment reconstituted with all-trans-retinal has the absorption maximum in the visible region (curve 1 in Fig. 1A). Irradiation of this sample with yellow light (> 500 nm) shifted the spectrum to the blue, indicating the formation of a pigment having absorption maximum in the UV region (curve 2 in Fig. 1A). Subsequent irradiation of this sample with UV light (350 nm) caused a shift of the spectrum to the visible region (curve 3 in Fig. 1A); the spectrum is similar in shape to the original spectrum (curve 1 in Fig. 1A) in the visible region but the absorbance at the maximum is slightly lower than that of the original spectrum. Reirradiation of the sample with yellow light formed a state whose spectrum (curve 4 in Fig. 1A) is identical in shape with that formed by the first yellow light irradiation. The difference spectra calculated before and after the yellow light and UV light

irradiations are shown as curves 1 to 3 in Fig. 1B, respectively. It should be noted that curves 2 and 3 are a mirror image with each other and curve 1 is identical in shape with inversed curve 2 and curve 3, but absorbance at the maximum (474 nm) is about 1.4 times those of these curves. These results showed that irradiation of the pigment reconstituted with all-trans-retinal converts it to a UV light-absorbing pigment, and these pigments are interconvertible by light irradiations. Smaller absorbance (absolute value) at the maxima of curves 2 and 3 than that of curve 1 in Fig. 1B indicated that UV light irradiation caused the formation of a mixture of two pigments in which the amount of the UV light-absorbing pigment in the mixture is at least 30%. These results demonstrated that cOpn5m is a bistable pigment whose molecular property is in contrast with that of retinal photoisomerase, which does not photoconvert to the original pigment by UV light irradiation (8). These results prompt us to infer that cOpn5m could function as a UV-sensitive GPCR.

To test our hypotheses, we first investigated the retinal configurations of two pigments by means of chromophore extraction from the sample solution followed by HPLC analyses (Fig. 1C). Irradiation with yellow light of the pigment reconstituted with all-trans-retinal increased the amount of 11-cis-retinal, whereas the amount of all-trans-retinal in the sample decreased. Subsequent UV light irradiation changed the retinal configuration from 11-cis to all-trans form. Isomeric compositions before and after light irradiation show that the main reaction in cOpn5m is cis-trans isomerization of the 11-cis double bond (Fig. 1C, Right). The sample irradiated with yellow light to produce the UV-sensitive form seems to contain some all-trans

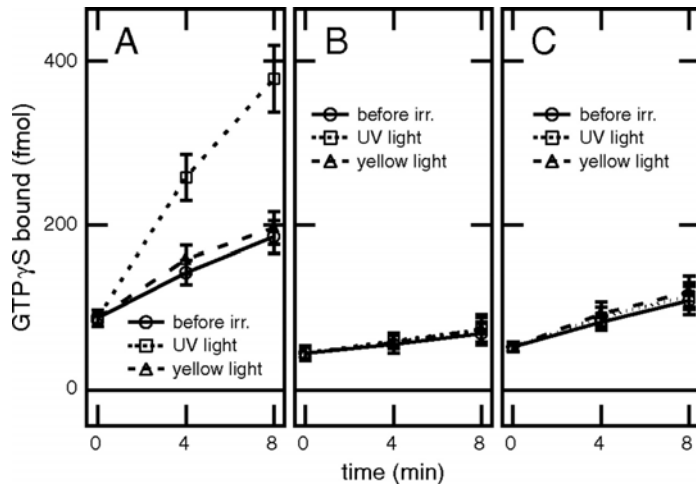


Fig. 2. Time courses of G protein activation ability by cOpn5m. Gi (A), Gt (B), and Gq (C) activation abilities of cOpn5m reconstituted with 11-cis-retinal were measured in the dark (open circle), after yellow light (> 500 nm) irradiation (open square), and after subsequent UV light irradiation (open triangle). Experiments were performed at 0 °C. Data are presented as the means $\pm$ SD of three independent experiments.

isomers, presumably due to the excess all-trans-retinal added to reconstitute the pigment. We also observed the presence of 13-cis isomer in the sample; however, its contribution was small. These results clearly indicated that the two states of the pigment sensitive to UV light and yellow light contain 11-cis- and all-trans-retinals, respectively.

We then reconstituted the pigment by addition of 11-cis-retinal in the cells containing expressed cOpn5m proteins. The formed pigment exhibited absorption maximum in the UV region, and its irradiation with UV light caused the shift of spectrum to the visible region. The difference spectrum calculated before and after the irradiation was identical in shape with curve 2 in Fig. 1B, indicating that the pigment formed with 11-cis-retinal exhibited absorption spectrum and light-induced reaction identical with those produced by the yellow light irradiation of the pigment formed with all-trans-retinal.

Absorption spectra of the UV light-absorbing pigment and the pigment reconstituted with all-trans-retinal (Fig. 1A, Inset) were calculated by the method described in Materials and Methods. The absorption maxima of these pigments were estimated to be 360 and 474 nm, respectively.

Next, we investigated whether cOpn5m can function as a GPCR. Vertebrate visual opsins can activate Gi-group G proteins including Gt, whereas a vertebrate nonvisual opsin, melanopsin, can couple with Gq-type G protein (1, 2, 4). Thus we measured the activation ability of these G protein subtypes by purified cOpn5m reconstituted with 11-cis-retinal (Fig. 2). UV light irradiation of this pigment caused the elevation of the GTP γ S binding ability of Gi and subsequent yellow light irradiation suppressed the activity (Fig. 2A). In contrast, we did not detect any change of Gt or Gq activation ability in a light-dependent manner (Fig. 2 B and C). Thus cOpn5m can activate Gi-type G protein, and the G protein-activating state is in an all-trans-retinal bound form. The spectral and biochemical analyses of cOpn5m clearly demonstrated that cOpn5m is a UV-sensitive GPCR that couples with Gi-type G protein.

Additionally, we measured the G protein activation ability

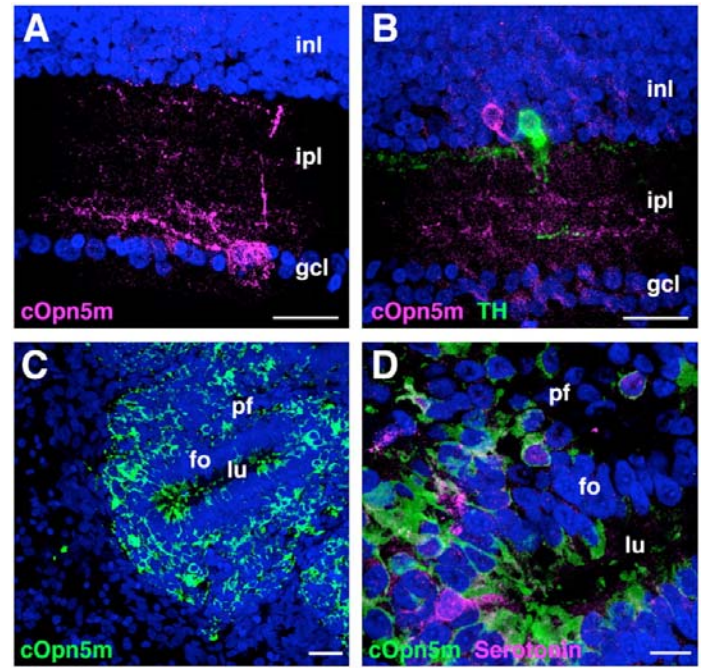


Fig. 3. cOpn5m photoreceptors in the posthatching chick retina (P14) and pineal gland (P11). (A) A cOpn5m-immunoreactive (IR) cell (magenta) in the ganglion cell layer of retina. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer. (B) A cOpn5m-IR cells (magenta) in the vicinity of tyrosine hydroxylase (TH)-IR cells (green). (C) cOpn5m-IR cells (green) are also present in the pineal gland. fo, follicular cells; lu, follicular lumen; pf, parafollicular cells. (D) Enlargement of the pineal gland in C. cOpn5m-immunoreactivity is found in the outer segments of the pinealocytes facing the lumen and perikarya of parafollicular cells. cOpn5m-positive outer segments of pineal photoreceptors are mostly serotonin-positive (magenta). (Scale bars: 10 μ m, D; 25 μ m, A–C.)

of purified cOpn5m reconstituted with all-trans-retinal (Fig. S2). Irradiation of the sample with yellow light caused the decrease of G protein activation efficiency, indicating that cOpn5m reconstituted with all-trans-retinal can really activate G protein and light suppresses the G protein activation ability through trans-cis isomerization of the chromophore. Subsequent irradiation with UV light caused elevation of G protein activation efficiency; however, its magnitude was larger than that of the suppression observed by the first yellow light irradiation. Reirradiation of the sample with yellow light suppressed the G protein activation ability again, with a magnitude identical with the increase caused by the UV light irradiation. The elevation and suppression of the G protein activation abilities with identical magnitudes were repeatedly observed by subsequent UV light and yellow light irradiations. Furthermore, the magnitudes of elevation and suppression are consistent with those observed by UV light and yellow light irradiations of cOpn5m reconstituted with 11-cis-retinal. These results suggest the possibility that the conformation of the cytoplasmic surface responsible for the G protein activation in the pigment reconstituted with all-trans-retinal was not completely identical with that in the pigment produced by the yellow light irradiation of the pigment formed with 11-cis-retinal, even though the spectral properties of these two pigments were identical.

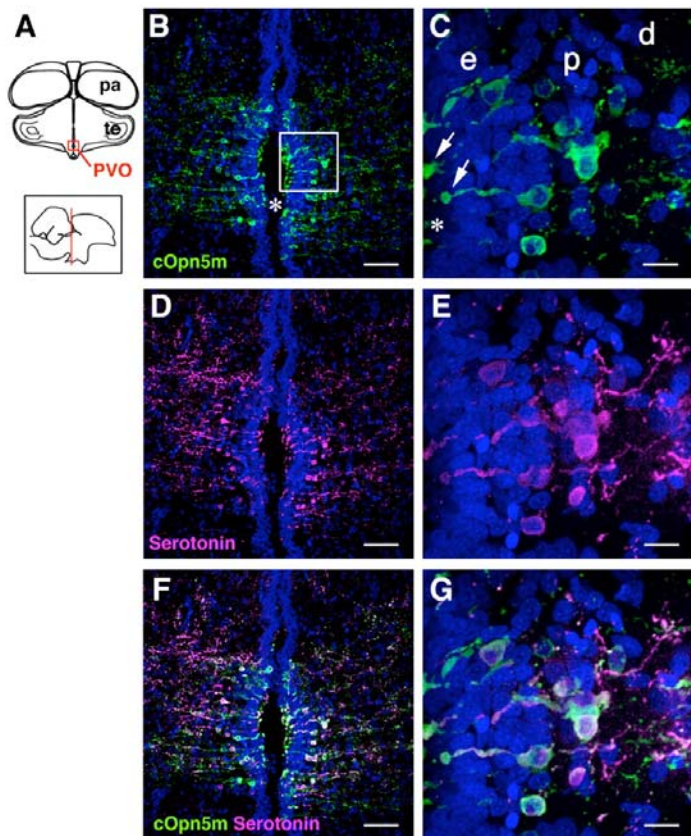


Fig. 4. cOpn5m photoreceptors in the posthatching chick deep brain (P11). (A) Schematic diagram of chick brain. (Upper) Coronal plane; (Lower) sagittal plane. Boxed region is shown in B, D, and F. pa, pallium; PVO, paraventricular organ; te, tectum. (B) cOpn5m-IR cells (green) are predominantly present in the chicken PVO, where serotonin-IR cells (magenta) are mainly located (D) (46). Asterisk, the third ventricle. Nuclei of the cells are stained with DAPI (blue). The boxed region in B is enlarged in C, E, and G. (C) cOpn5m-IR cells are bipolar and their club-like projections (arrows) line the third ventricle. They are present in the ependymal layer (e) and proximal part (p) of the PVO. d, distal part of the nucleus of the PVO (16). (F and G) Most of the Opn5m-IR cells in the PVO are overlapping with serotonin-IR cells. (Scale bars: 10 μ m, C, E, and G; 50 μ m, B, D, and F.)

Localization of cOpn5m Within the Chicken Retina and Pineal Gland.

cOpn5m is the only UV-sensitive pigment identified so far in chicken. Thus we examined which types of cells in chicken can receive UV light through cOpn5m. In the previous report, we showed that mRNA of cOpn5m was predominantly detected within the developing retinas (9). In this study, we prepared the anti-cOpn5m antibody (Fig. S3) and investigated the detailed distribution of cOpn5m within the posthatching chicken retinas. Immunohistochemical analyses clearly showed that cOpn5m is localized in small subsets of cells within the ganglion and amacrine cell layers (Fig. 3 A and B). The amacrine cells have many cell types (10), some of which are characterized by their unique neurotransmitters (11). Although dopaminergic or vasoactive intestinal peptide-positive amacrine cells exhibit no positive signals to cOpn5m (Fig. 3B and Fig. S4A), double stained data showed that several cOpn5m-positive amacrine cells are located in the vicinity of the dopaminergic amacrine cells and dendrites of these two cells are close enough to interact with each other in the inner plexiform layer (Fig. 3B). We

did not yet determine whether the cOpn5m-positive cells in the ganglion cell layer are displaced amacrine cells or true ganglion cells.

We also investigated the expression of cOpn5m within the pineal gland. It should be noted that the vast majority of cells in the pineal gland at posthatching day 11 were immunopositive to anti-cOpn5m antibody (Fig. 3C), indicating that Opn5m is one of the major photopigments present in the pineal gland of young chicken. Serotonin is a precursor of melatonin, which is a neurohormone produced by the pineal gland that regulates pleiotropic circadian functions (12). In the pineal gland of young chicken, serotonin is present in subsets of follicular cells (pineal photoreceptors) and parafollicular cells (13). Although intensities of immunoreactivity for cOpn5m and serotonin seem to vary depending on cell types or cell maturation (Fig. S4 C–E), double stained data showed that the cOpn5m-positive outer segments of pineal photoreceptors are mostly serotonin-positive (Fig. 3D).

Localization of cOpn5m Within the Chicken Deep Brain.

We finally investigated the distribution of cOpn5m within the chicken deep brain. It was previously known that birds have photoreceptors other than in the eye and pineal gland, in the deep brain of the circumventricular region, that is, the lateral septal organ (LSO) in the telencephalon and the paraventricular organ (PVO) in the mediobasal hypothalamus (14, 15) (Fig. 4A and Fig. S5A). We found that cOpn5m-positive cells are clearly observed in the PVO (Fig. 4 B and C), whereas they are absent from the LSO (Fig. S5 B–D). cOpn5m-positive cells are bipolar and have club-like projections toward the third ventricle (Fig. 4C), which is consistent with the morphology of the cerebrospinal fluid-contacting neurons (16). The PVO is a photosensitive organ found in submammalian vertebrates. Although several catecholamines are rich in the PVO depending on the species, serotonin is specifically present in the avian and reptile PVO (17, 18) (Fig. 4 D and E). Notably, the cOpn5m-positive cells in the PVO well overlap with serotonin-positive signals (Fig. 4 F and G), indicating that cOpn5m-positive cells are serotonergic in the PVO.

Discussion

In the present study, we have expressed the cOpn5m in cultured cells and succeeded in reconstitution and characterization of the Opn5 pigment. In contrast to the initial expectation from the phylogenetic and exon-intron analyses of cOpn5m, cOpn5m is a UV-sensitive bistable opsin that can activate Gi-type G protein. Immunohistochemical studies clearly showed that this pigment is localized within some types of amacrine cells and some cells in the ganglion cell layer of the retinas, the vast majority of cells in the pineal gland, and serotonin-positive cells in the PVO. Because cOpn5m is the only UV-sensitive opsin found so far in chicken, this study provides the molecular basis for UV reception in chicken.

Molecular Properties of cOpn5m. The spectral analysis of cOpn5m showed that this pigment exhibits the characteristics of a bistable pigment. That is, cOpn5m takes two forms, one is a UV light-absorbing form having 11-cis-retinal and the other is a visible light-absorbing form having all-trans-retinal, and these two forms are

interconvertible by light irradiation. cOpn5m also shows another characteristic of bistable pigment, that is, the ability to directly bind to all-trans-retinal. These two characteristics have never been observed in “monostable” opsin such as vertebrate rod and cone visual pigments (19, 20).

The UV light-absorbing opsins characterized so far are classified into three groups: vertebrate and invertebrate visual opsins, such as mouse and apes UV-sensitive opsins (21, 22), and vertebrate parapinopsin (23). From the viewpoint of phylogenetic relationship, these three opsins and Opn5 have evolved independently to acquire UV light sensitivities. In fact, amino acid residues surrounding the retinal chromophore, which are estimated from the 3D structures of bovine and squid rhodopsins (24, 25), are considerably different among each other (at least 6 different residues out of 16 positions) (Table S1), suggesting that multiple replacements of amino acid residues surrounding the chromophore would have occurred during the divergence of these pigments. The interesting observation is that cOpn5m exhibits UV spectrum with lower absorbance than that of other bistable parapinopsin. In fact, the molecular extinction coefficient of UV light-absorbing form of cOpn5m was estimated to be $24,600 \pm 400$, which is 1.62 times smaller than that of the visible light-absorbing form ($39,900 \pm 650$) (Fig. S6). Thus the comparison of the difference spectrum between UV light- and visible light-absorbing forms in cOpn5m with that in parapinopsin shows that the absorbance at the maximum of cOpn5m in UV region is about half of that of parapinopsin (Fig. S7). These results are consistent with the observations that irradiation of cOpn5m with UV light caused the formation of a photosteady state mixture containing about 30% of UV light-absorbing form, whereas similar experiments using parapinopsin showed the presence of only 10% of UV light-absorbing form in the photosteady state mixture (23). These results indicated that if the visible light-absorbing form of cOpn5m but not parapinopsin is present somewhere in the physiological conditions, irradiation of a steady UV light would cause the conversion of about 30% of visible light-absorbing form to the UV light-absorbing form, resulting in about 30% suppression of the G protein-activating ability. It is evident that cOpn5m is a UV-sensitive receptor that activates Gi-type G protein. However, because of the unique spectral characteristics of cOpn5m, we are unable to neglect the possibility that cOpn5m can function like a Gs-coupled receptor by suppression of the Gi activation ability upon absorbing steady UV light, if it could be present as a visible light-sensitive form under the circumstance that only all-trans-retinal can be supplied.

During the course of the preparation of this manuscript, the action spectrum with the maximum at about 420 nm of quail Opn5 and its coupling with Gq-type G protein were published (26). These properties were estimated by the heterologous expression experiments of quail Opn5 in *Xenopus* oocytes. Sequence comparison of Opn5 in quail and chicken indicates that both pigments show completely identical sequences from positions 18 to 313 (the bovine rhodopsin numbering system), and there are only one and five positions at N and C termini where different amino acid residues are present between them. Thus it should be speculated that both pigments would exhibit identical spectra and similar G protein coupling ability. These

discrepancies will be clarified by future studies.

Immunohistochemistry of cOpn5m in Retinas.

Immunohistochemical analysis revealed that cOpn5m is expressed in some types of amacrine cells and in some cells in the ganglion cell layer. An interesting observation is that several cOpn5m-positive amacrine cells are located in the vicinity of the dopaminergic amacrine cells and dendrites of these two cells are close enough to interact with each other in the inner plexiform layer. It has been recognized that dopaminergic amacrine cells play roles to modulate the formation of tight junctions among horizontal cells, connectivities among retinal neural cells, and the states of chemical and electrical synapses to adjust the visual stimuli under the current background light conditions (27). Thus it would be an intriguing argument that cOpn5m-positive amacrine cells regulate the dopamine release from dopaminergic amacrine cells in a UV light-dependent manner. In mammals, melanopsin is present in some types of ganglion cells that regulate the activity of dopaminergic amacrine cells by dendrite interactions (28). In chicken retinas, we already identified the melanopsin-containing amacrine cells in the posthatching chicken retinas (29). Therefore, in chicken retinas, these two opsins, UV-sensitive cOpn5m and blue-sensitive melanopsin, may concertedly control the activity of dopaminergic amacrine neurons, resulting in retinal adaptation.

Immunohistochemical Analysis of cOpn5m in Pineal Glands.

As shown in Fig. 3C, cOpn5m is predominantly distributed within subsets of follicle and parafollicle cells of the pineal gland. Although several opsins such as pinopsin, red-sensitive cone pigment, and melanopsin are reported to be present in chicken pineal gland (30, 31), all these opsins exhibit sensitivities to visible light. Thus cOpn5m is unique as it is UV light sensitive. In this context, Rosiak et al. (32) reported that exposure of organ cultured chicken pineal glands to UV light suppressed the tissue cAMP level and declined the activity of serotonin N-acetyltransferase (NAT). Because cOpn5m is a UV-sensitive pigment and couples with Gi to inhibit adenylate cyclase activity, the presence of cOpn5m would account for this UV-dependent change of NAT activity. Thus cOpn5m in the pineal gland possibly functions as a pigment to regulate melatonin production and release.

Immunohistochemical Analysis of cOpn5m in PVOs.

We also detected the expression of cOpn5m in the PVO of the hypothalamus. Our data clearly showed that the cOpn5m-positive cells are serotonergic neurons but not dopaminergic neurons (Fig. 4 F and G and Fig. S5 E and F). Although the recently published studies showed that Opn5-positive cells in the quail PVO are responsible for the photoperiodic induction of testicular growth (26), the photoperiodic response of the domestic chicken is less robust than that of the quail (33). Thus we speculate that serotonergic neurons containing cOpn5m would project the median eminence, resulting in secretory regulation of the adeno-hypophysial hormones. As already described, one of the prominent properties of cOpn5m is the ability to directly bind to all-trans-retinal. Thus cOpn5m can form a pigment in the deep brain by direct binding to available all-trans-retinal without the molecular mechanism of

11-cis-retinal supply. cOpn5m can not only trigger Gi-mediated signal transduction cascade after UV light reception, but it can also quit the cascade after visible light reception. It is possible that cOpn5m in the PVO regulates other physiological functions modulated by UV and visible light in chicken.

Conclusion. We have succeeded in analyzing the molecular properties of Opn5 and the distribution in the chicken retina, pineal gland, and deep brain. Although mammalian Opn5 is not examined yet, it can also be sensitive to UV light, because of complete conservation of 16 amino acid residues that are speculated to surround the chromophore from the 3D structures of bovine and squid rhodopsins (Table S1) (24, 25). In particular, there are no UV-sensitive opsins characterized so far in human. Therefore, although the human lens and cornea contain the UV light-absorbing compounds (34, 35), the human eye and brain have the potential ability to receive UV light through Opn5. UV reception within the mammalian brain and its physiological function will be a major issue to be resolved in the future.

Materials and Methods

Animals. Chicken (*Gallus gallus*) was used in this study, which was approved by the Animal Experiment Committee of University of Tokushima. Fertilized eggs were incubated at 37.5 °C temperature and about 65% humidity under 12 h light/12 h dark cycles. Posthatching chicks were kept in the same light cycle and fixed in the middle of the light and dark phase at desired stages.

Preparation of Purified cOpn5m Pigments. The cDNA encoding cOpn5m (accession no. AB368182) (9) was tagged by the epitope sequence of the anti-bovine rhodopsin monoclonal antibody Rho1D4 (ETSQVAPA) at the C terminus and was inserted into mammalian expression vector pCAGGS (36). The plasmid DNA was transfected into HEK293 cells by calcium-phosphate methods according to the previous report (37). After 1 d incubation of the transfected cells, all-trans- or 11-cis-retinal was added into the medium (final retinal concentration; 5 μ M) to reconstitute the pigments. After additional 1 d incubation in the dark, the cells were collected by centrifugation. The pigments were extracted with 1% dodecylmaltoside (DM) in buffer A (50 mM Hepes (pH 6.5) and 140 mM NaCl) and were applied to the Rho1D4-conjugated agarose. The purified pigments were eluted with 0.02% DM in buffer A containing the synthetic peptide corresponding to the C terminus of bovine rhodopsin.

Preparation of G Proteins. Purification of Gt from bovine rod outer segments was carried out according to the methods previously described (38). The rat Gi α 1 subunit was expressed in *Escherichia coli* strain BL21 by using Gi α 1 cDNA constructed into pQE6 plasmid vector and was purified as described (39). The purified Gi α 1 was mixed with equal amount of purified Gt $\beta\gamma$. The purified mouse Gq α complexed with β 1 γ 2, which is expressed in Sf9 cells, was kindly provided by T. Doi (Kyoto University, Kyoto, Japan) (40).

Spectrophotometry and HPLC Analysis. Absorption spectra were recorded at 0 °C with a Shimadzu UV-2400

spectrophotometer. The sample was irradiated with UV light through a UV-D35 glass filter (Asahi Technoglass) or with yellow light through Y-52 cutoff filter (Toshiba) from a 1-kW halogen lamp (Master HILUX-HR; Rikagaku).

Absorption spectra of visible light-absorbing and UV light-absorbing forms of cOpn5m were calculated from the methods described previously (41). Briefly, the spectral region at wavelengths longer than the maximum of the main peak of the difference spectrum between the visible light- and UV light-absorbing forms of cOpn5m was best fitted with a template spectrum described by Lamb (42) and Govardovskii et al. (43). The best-fitted spectrum was considered to be the visible light-absorbing form of Opn5m. The absorption spectrum of UV light-absorbing form was then calculated by adding the visible light-absorbing form to the difference spectrum.

The chromophore configurations of each sample were analyzed by HPLC (LC-10AT VP; Shimadzu) equipped with a silica column (150 $\times$ 6 mm, A-012-3; YMC) according to the previous report (44).

G Protein Activation Assay. A radionucleotide filter-binding assay, which measures GDP/GTP γ S exchange by G protein, was performed as described previously (37, 45). All procedures were carried out at 0 °C. The assay mixture consisted of 50 mM Hepes (pH 7.0), 140 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.01% DM, 1 μ M [³⁵S]GTP γ S and 2 μ M GDP. cOpn5m pigments purified after reconstitution with retinal (final concentration: 100 nM) were mixed with G protein solution (final concentration: 600 nM) and were kept in the dark or irradiated with UV light for 1 min or with subsequent yellow light (> 500 nm) for 1 min. After irradiation, the GDP/GTP γ S exchange reaction was initiated by the addition of [³⁵S]GTP γ S solution into the mixture of the pigment and G protein. After incubation for the selected time in the dark, an aliquot (20 μ L) was removed from the sample into 200 μ L of stop solution (20 mM Tris/Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl₂, 1 μ M GTP γ S and 2 μ M GDP), and it was immediately filtered through a nitrocellulose membrane to trap [³⁵S]GTP γ S bound to G proteins. The amount of bound [³⁵S]GTP γ S was quantitated by assaying the membrane with a liquid scintillation counter (Tri-Carb 2910 TR; PerkinElmer).

Preparation of Antibodies Against Opn5m, Tyrosine Hydroxylase, and Serotonin. The anti-chicken Opn5m antibody was generated against the C-terminal region (49 amino acids) of the Opn5m, by using pGEX protein fusion and purification system (GE Healthcare) according to the standard method. The antibody specifically detected the recombinant cOpn5m expressed in HEK293 cells (Fig. S3). The antityrosine hydroxylase (TH) antibody was commercially obtained (MAB318, Chemicon, Millipore). The antiserotonin antibody was kindly provided by David V. Pow (University of Newcastle, Callaghan, Australia).

Microscopic Images. Photomicrographs were taken by using a Leica DM5000B microscope equipped with epifluorescence and a Leica DFC300F digital camera. Confocal microscopy was done by using a Leica TCS-SP5. Images were optimized for color, brightness, and contrast, and overlay images composed by Adobe Photoshop CS3 10.0.

Immunohistochemistry. The whole brain (P11) and eye balls (P14) of the chicken were immersion-fixed in 4% paraformaldehyde, washed in phosphate-buffered saline, and cryoprotected in 30% sucrose. The tissues were embedded, frozen in OCT compound (Sakura), and sectioned at 18 μ m. The sections were incubated with diluted antibody to cOpn5m (1:2,000), TH (1:200), or serotonin (1:2,000) followed by incubation with Alexa Fluor 488/546 anti-guinea pig IgG (Invitrogen), Cy3 anti-rabbit IgG (Jackson ImmunoResearch), or Alexa Fluor 488 anti-mouse IgG for immunofluorescent detections.

Acknowledgments. We thank Dr. Kei Tsutsui, Shinya

Nakata, Shuang Song, Sari Fujita, Take Matsuyama, and Keita Sato for technical support and helpful discussion. We also thank Professor Sumihare Noji at Tokushima University for his helpful discussion. We are grateful to Dr. Tomoko Doi for providing us with recombinant Gq-type G protein and Dr. David V. Pow for antiserotonin antibody. This work was supported in part by Grants-in-Aid for Scientific Research and the Global Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research: from Genome to Ecosystem" of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a grant from Astellas Foundation for Research on Metabolic Disorders (to H.O.).

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