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Immunohistochemical characterization of a parapinopsin-containing photoreceptor cell involved in the ultraviolet/green discrimination in the pineal organ of the river lamprey *Lethenteron japonicum*

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Summary

In the pineal organ, two types of ganglion cell exhibit antagonistic chromatic responses to UV and green light, and achromatic responses to visible light. In this study, we histologically characterized UV-sensitive photoreceptor cells that contain a unique non-visual UV pigment, lamprey parapinopsin, in order to elucidate the neural network that is associated with antagonistic chromatic responses. These characteristics were compared with those of lamprey rhodopsin-containing cells, most of which are involved in achromatic responses. RT-PCR analysis revealed that lamprey parapinopsin was expressed in the pineal organ but not in the retina, unlike lamprey rhodopsin, which was expressed in both. Lamprey parapinopsin and lamprey rhodopsin were immunohistochemically localized in the dorsal and ventral regions of the pineal organ, respectively. The two pigments were localized in distinct photoreceptor cells throughout the pineal organ, namely the dorsal and ventral regions as well as the peripheral region, which corresponds to the dorso-ventral border region. The ratio of the number of lamprey parapinopsin-containing cells to lamprey rhodopsin-containing cells around the peripheral region was higher than in the central region. Electron-microscopic analysis revealed that lamprey parapinopsin-containing dorsal cells have outer segments and synaptic ribbons similar to those of ventral photoreceptor cells. However, unlike lamprey rhodopsin-containing cells, lamprey parapinopsin-containing cells connected with each other in a wide area of dorsal and peripheral portions and made direct contact with ganglion cells, mainly in the peripheral portion. These results suggest that UV light information captured by lamprey parapinopsin-containing photoreceptor cells is converged and directly transmitted to chromatic-type ganglion cells in the peripheral region to generate antagonistic chromatic responses.

Key words: pineal organ, parapinopsin, rhodopsin, ganglion cell, antagonistic chromatic response.

Introduction

Lower vertebrates have a directly photosensory pineal organ that is involved in the photoregulation of various physiological functions such as circadian rhythm (Falcon, 1999; Gaston and Menaker, 1968; Morita et al., 1992; Reiter, 1993). The pineal organ is widely accepted as a synthesizer and secretor of melatonin (Falcon et al., 1989; Gern and Greenhouse, 1988; Samejima et al., 2000; Samejima et al., 1997; Underwood, 1985). In addition to the secretory response, the pineal photoreceptor transduces a light signal to an electrical response, which is transmitted to the brain via pineal ganglion cells (Dodt, 1973; Dodt and Heerd, 1962; Morita, 1969). Physiologically, the pineal organ has two types of ganglion cell, chromatic and achromatic. Several electrophysiological studies have demonstrated that the neural activity of chromatic-type ganglion cells is inhibited by UV/short wavelength light and excited by green/long wavelength light, whereas that of achromatic-type ganglion cells is inhibited by visible light in the pineal organ of the lamprey (Morita and Dodt, 1973), teleost (Morita, 1966) and frog (Dodt and Heerd, 1962). Therefore, chromatic-type ganglion cells detect the ratio of short wavelength light to long wavelength light in environmental light (Dodt, 1973), suggesting that the pineal organ is involved in the wavelength discrimination of the photic component in incident light.

The river lamprey *Lethenteron japonicum* also has a well-developed photosensory pineal organ, in which some ganglion cells exhibit antagonistic chromatic responses, in addition to a large number of achromatic-type ganglion cells (Morita et al., 1989; Uchida and Morita, 1994). Electrophysiological studies have identified two types of photoreceptor cell in the pineal

organ, UV- and green-sensitive photoreceptor cells (hereafter referred to as UV and green photoreceptor cells, respectively), both of which hyperpolarize in response to light stimuli (Tamotsu and Morita, 1986; Uchida and Morita, 1990). The sensitivity curve of green photoreceptor cells fits that of achromatic-type ganglion cells with maximum sensitivity at 525 nm (Uchida et al., 1992) but does not fit that of chromatic-type ganglion cells with maximum sensitivity at 540 nm (Uchida and Morita, 1994). We found that the lamprey rhodopsin with an absorption maximum at ~520 nm (Hisatomi et al., 1997) (M. Koyanagi, E. Kawano-Yamashita, Y. Kinugawa, T. Oishi, Y. Shichida, S. Tamotsu and A. Terakita, unpublished observations) is expressed in a large number of pineal photoreceptor cells (Koyanagi et al., 2004). Taken together, these results suggest that green photoreceptor cells containing lamprey rhodopsin achieve achromatic responses, whereas green/long wavelength photoreceptor cells responsible for the antagonistic chromatic response remain to be clarified. On the other hand, it has been suggested that UV photoreceptor cells are responsible for the antagonistic chromatic response but not for the achromatic response, based on the sensitivity curves of the two types of ganglion cell.

Recently, we found the UV pigment lamprey parapinopsin in the lamprey pineal organ (Koyanagi et al., 2004). Lamprey parapinopsin is distinct from vertebrate cone and rod opsins, and has a bistable nature as it exhibits photointerconversion by UV and green light (Koyanagi et al., 2004). Lamprey parapinopsin underlies a unique characteristic of photoreceptor cells that differs from that of lamprey rhodopsin-containing photoreceptor cells.

To understand the neural connections that generate the antagonistic chromatic response, an effective strategy is to investigate how UV light signals are transmitted to chromatic-type ganglion cells, because the green/long wavelength photoreceptor cells responsible for the antagonistic chromatic response have not been identified. In this study, we carried out histological characterization of the lamprey parapinopsin-containing cells involved in generating the antagonistic chromatic response and compared it with that of the lamprey rhodopsin-containing cells, most of which generate the achromatic response. Lamprey parapinopsin is expressed in the dorsal region of the pineal organ, whereas lamprey rhodopsin is expressed in the ventral region as revealed by *in situ* hybridization analysis (Koyanagi et al., 2004), which showed that UV photoreceptor cells and green photoreceptor cells are distributed in the dorsal and ventral layer of the pineal organ, respectively. We elucidated the structural characteristics and localization of UV photoreceptor cells containing lamprey parapinopsin in detail by using an antibody against lamprey parapinopsin, and compared them with those of lamprey rhodopsin-containing cells. Furthermore, we histologically showed that the light information from UV photoreceptor cells is directly transmitted to ganglion cells.

Materials and methods

Animals

River lampreys *Lethenteron japonicum* (Martens) were commercially obtained from Ishikari River in Hokkaido, Japan, and kept in aquaria with aerated and filtered water at 4–10°C under light–dark cycle conditions (12 h:12 h L:D). Our investigations were conducted in accordance with the Animal Care Committee of Nara Women's University.

Reverse transcription (RT)-PCR

Total RNA from the pineal organ, retina and brain was reverse transcribed to cDNA using an oligo(dT) primer and Superscript III (Invitrogen, Carlsbad, CA, USA), and the cDNAs were used as templates for PCR amplification. To obtain an internal standard for normalizing expression levels of the lamprey parapinopsin (AB116380; all accession nos are for GenBank/EMBL/DDBJ) and the lamprey rhodopsin (AB116382), we cloned glyceraldehyde 3-phosphate dehydrogenase (GAPDH, AB300852), a housekeeping gene, from lamprey with degenerate primers; forward: 5′-CCIISIGCIGAYGCNCCNATGTT-3′, reverse: 5′-GTAICC-RHAYTCRTTRTCRTACCA-3′. Gene-specific PCR amplifications were performed with the following primer pairs: 5′-ACGTGTCGTACATTACGAGC-3′ and 5′-TCACC-ACGATCATGGCGAAG-3′ for lamprey parapinopsin; 5′-ACGAGTCGTACGTAGTCTAC-3′ and 5′-GTGAAGA-TGTAGAAGGCCAC-3′ for lamprey rhodopsin; 5′-ACG-ACAACTTCGTGATCCTG-3′ and 5′-CTTCCTTCACCT-TAGCCTTG-3′ for GAPDH. The optimal annealing temperature was 60°C for the lamprey parapinopsin and GAPDH primer pairs, and 65°C for the lamprey rhodopsin primer pairs.

Generation of anti-lamprey parapinopsin and anti-lamprey rhodopsin antibodies

The anti-lamprey parapinopsin and anti-lamprey rhodopsin antibodies were generated as reported previously (Koyanagi et al., 2005), with the following modifications. The 50 amino acids of the C-terminal region of the lamprey parapinopsin (Met324–Ser373) and the 47 amino acids of the C-terminal region of the lamprey rhodopsin (Ile307–Ala353) were fused to maltose-binding protein in the expression vector pMAL-c2X (New England Biolabs, Ipswich, MA, USA). The fusion proteins were expressed in *Escherichia coli* and purified by amylose affinity column chromatography (New England Biolabs). JW/CSK female rabbits or BALB/c female mice were immunized four to six times with the purified fusion proteins of lamprey parapinopsin and lamprey rhodopsin, respectively.

Immunohistochemistry

Animals were quickly decapitated and their pineal organs were isolated with a small piece of adjacent tissue. The pineal organs were fixed in Zamboni's fixative [4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (PB) containing 0.2% picric acid, pH 7.4] overnight at 4°C. Each organ was cryoprotected by immersion in 0.1 mol l^{-1} PB containing increasing concentrations of sucrose (15 and 30%), and embedded in OCT compound (Sakura, Tokyo, Japan). Frozen sections (thickness: 15–30 μm) were prepared using a cryostat (Bright Instrument Co. Ltd, Huntingdon, Cambridgeshire, UK), and mounted on 0.5% gelatin-coated slides.

Immunohistochemical analyses were conducted as reported previously (Kawano et al., 2006). In brief, the sections were incubated overnight at 4°C with primary rabbit antiserum

against lamprey parapinopsin [diluted 1:1000 in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4) containing 0.3% Triton X-100 (PBS-T) and 1% bovine serum albumin], and subsequently incubated with Alexa 488-conjugated anti-rabbit IgG (diluted 1:500 in PBS-T; Molecular Probes, Eugene, OR, USA) for 5 h at room temperature. In the double-immunostaining experiment, sections were incubated overnight at 4°C with the first primary rabbit antiserum against lamprey parapinopsin (diluted 1:1000 in PBS-T containing 1% bovine serum albumin) and subsequently incubated with Alexa 488-conjugated anti-rabbit IgG (diluted 1:500 in PBS-T) for 5 h at room temperature. The same sections were then incubated with the second primary mouse antiserum against the lamprey rhodopsin (diluted 1:1000 in PBS-T containing 1% bovine serum albumin) overnight at 4°C, and subsequently incubated with Alexa 594-conjugated anti-mouse IgG (diluted 1:500 in PBS-T; Molecular Probes) for 5 h at room temperature.

For the whole-mount analysis, the dorsal region was dissected from the fixed pineal organs. The specimens were incubated with the primary rabbit antiserum against lamprey parapinopsin (diluted 1:1000 in PBS-T containing 1% bovine serum albumin and sodium azide) for 5 days at 4°C, and then with Alexa 488-conjugated anti-rabbit IgG (diluted 1:500 in PBS-T containing sodium azide) for 5 days at 4°C.

To confirm the specificity of the antisera against lamprey parapinopsin and lamprey rhodopsin, sections were incubated in rabbit or mouse normal serum (diluted 1:250 in PBS-T containing 1% bovine serum albumin) instead of the primary antisera. For control staining of lamprey parapinopsin, both the antigen (lamprey parapinopsin peptide) and the antiserum against lamprey parapinopsin were first mixed and incubated overnight at 4°C. Control sections were then incubated in the primary antiserum absorbed by lamprey parapinopsin. In all control preparations, no immunoreactivity was observed.

Intracellular injection of the dye into the UV photoreceptor cells

The removed pineal organ with a piece of adjacent tissue was placed in a recording chamber and perfused with oxygenated Ringer solution under dark adaptation for at least 30 min. Intracellular recording was carried out with a glass microelectrode filled with 2% neurobiotin (Vector Laboratories, Burlingame, CA, USA) in 1 mol l^{-1} KCl, with a resistance range from 120 to 200 MΩ. Responses were amplified by a high-input impedance amplifier (IR-183, Cygnus Technology, Delaware, PA, USA). After the UV-sensitive photoresponse had been confirmed, intracellular injections of neurobiotin were performed by passing 2–5 nA depolarizing rectangular pulses of 200 ms duration at 2 Hz for about 3 min. The pineal organ was fixed with 4% paraformaldehyde in 0.1 mol l^{-1} PB, and frozen sections (thickness: 30 μm) were obtained using a cryostat. Visualization of the injected cells was achieved with Alexa 568-conjugated streptavidin (Molecular Probes).

Electron microscopy

The pineal organs were fixed by immersion in 2.5% glutaraldehyde prepared with 0.1 mol l^{-1} PB. After the specimens had been washed in PBS, they were postfixed with 1% OsO_4 in 0.1 mol l^{-1} PB for 1 h, dehydrated in a graded series of alcohol, and embedded in epoxy resin (Quetol 812, Nisshin EM, Tokyo, Japan). Semi-thin and ultrathin sections were made using an ultramicrotome (EM-UC6, Lieca, Heidelberg, Germany). Semi-thin sections were stained with a mixture of azur-II and toluidine blue for light-microscopic observation. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (JEM 1220, JEOL, Tokyo, Japan).

For immunoelectron microscopy, the pineal organs were fixed in Zamboni's fixative including 0.05% glutaraldehyde for 2 h, and kept in Zamboni's fixative. After washing in PBS, the specimens were incubated for 1 week at 4°C with anti-lamprey parapinopsin antiserum (diluted 1:1000 in PBS) and rinsed with PBS. The specimens were incubated with biotin-labelled anti-rabbit IgG (diluted 1:1000 in PBS; Biosource, Camarillo, CA, USA) and rinsed with PBS, then incubated with horseradish peroxidase-labelled streptavidin (diluted 1:250 in PBS; ICN, Aurora, OH, USA) and rinsed with PBS. Alternatively, the specimens were incubated with horseradish peroxidase-labelled anti-rabbit IgG (diluted 1:1000 in PBS; Biosource) and rinsed with PBS. To visualize the immunoreaction, the specimens were incubated with 0.025% diaminobenzidine (DAB; Sigma, St Louis, MO, USA) in 0.05 mol l^{-1} Tris-HCl (pH 7.4) with 0.03% H_2O_2 for 10–20 min. The specimens were postfixed with 1% OsO_4 in 0.1 mol l^{-1} PB for 1 h at room temperature, dehydrated in a graded series of ethanol, and embedded in epoxy resin (Quetol 812). Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined with an electron microscope.

Retrograde labelling

Retrograde labelling was carried out as described previously (Samejima et al., 1989) with the following modifications. After decapitation of the lamprey, the intact brains were carefully removed and transferred into oxygenated lamprey Ringer solution (138.6 mmol l^{-1} NaCl, 2.82 mmol l^{-1} KCl, 0.24 mmol l^{-1} $NaHCO_3$, 2.07 mmol l^{-1} $CaCl_2$ in distilled water). The pineal tract was transected 2–3 mm away from the pineal organ using a pair of microscissors, and crystals of a neural tracer (neurobiotin) were applied to the cut region. After 30 min, excess tracer was rinsed away with Ringer solution, and the brain was incubated overnight at 4°C in oxygenated Ringer solution. After incubation, the brains were fixed in Zamboni's fixative overnight at 4°C and sectioned with a cryostat. To visualize the neural tracer, the sections were incubated with Alexa 594-conjugated streptavidin for 5 h at room temperature. Additionally, immunohistochemistry was performed as described above for the immunofluorescent detection of lamprey parapinopsin.

For the control experiment, neural tracer crystals were applied to the surface of the pineal stalk in the intact brain without cutting. In the control sections, ganglion cells were not labelled, confirming that the ganglion cells were retrogradely labelled from the pineal stalk (data not shown).

We examined the stained sections under a fluorescence microscope (BX51, Olympus, Tokyo, Japan) and a confocal laser-scanning microscope (TCS-NT, Leica, Bansheim, Germany).

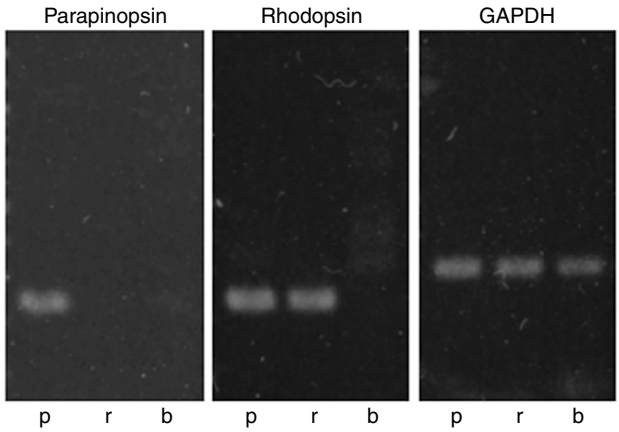

Fig. 1. RT-PCR analysis of lamprey parapinopsin and lamprey rhodopsin gene expression in the pineal organ (p), retina (r) and brain (b) of the lamprey. The lamprey parapinopsin gene is expressed only in the pineal organ, whereas the lamprey rhodopsin gene is expressed in both the pineal organ and the retina. GAPDH serves as an internal standard.

Three-dimensional reconstruction

Three-dimensional reconstruction methods were conducted as described previously (Tamotsu et al., 1997). In brief, serial confocal images were taken by serial optical sectioning using a confocal laser-scanning microscope. Three-dimensional images were reconstructed from these serial confocal images by means of a volume-rendering method with reconstruction software (VoxelView; Vital Image, Minneapolis, MN, USA).

Results

In order to elucidate whether lamprey parapinopsin functions only in the antagonistic chromatic response of the pineal organ, we examined lamprey parapinopsin gene expression by RT-PCR analysis in the pineal organ, retina and brain, each of which contains photoreceptor cells, and compared it with the lamprey rhodopsin gene expression pattern. The lamprey parapinopsin gene was expressed only in the pineal organ, not in the retina or in the brain (Fig. 1). In comparison, the lamprey rhodopsin gene was expressed in both the pineal organ and retina but not in the brain (Fig. 1).

We then generated an antibody against UV-sensitive lamprey parapinopsin and investigated its localization in the pineal organ. Strong lamprey parapinopsin immunoreactivity was observed in the outer segments of the photoreceptor cells in the dorsal region as well as the peripheral region, which corresponds to the dorso-ventral border region in the pineal section (Fig. 2A). Lamprey parapinopsin immunoreactivity was also observed in the pineal stalk (Fig. 2B) and in the parapineal organ (Fig. 2C). Interestingly, by using a high concentration of the antiserum, weak lamprey parapinopsin immunoreactivity was observed in the inner segments and basal processes of the photoreceptor cells, showing that the whole shape of a photoreceptor cell can be traced with this antiserum. As mentioned in detail later, the weak immunoreactivity demonstrated that these basal processes connected with each other (Fig. 2A, Fig. 3A). For control staining of lamprey parapinopsin, no immunoreactivity was observed (Fig. 2D).

We then compared the distribution patterns of UV-sensitive lamprey parapinopsin with those of green-sensitive lamprey rhodopsin, using a double-immunostaining technique. Lamprey parapinopsin and lamprey rhodopsin were localized in the dorsal and ventral region of the pineal organ, respectively (Fig. 3A,B). Strong lamprey parapinopsin or lamprey rhodopsin immunoreactivity was detected in the outer segment of each photoreceptor cell. In the peripheral region, confocal images clearly revealed that each photoreceptor cell showed either lamprey parapinopsin or lamprey rhodopsin immunoreactivity (Fig. 3C,D), demonstrating that each photoreceptor cell even in the peripheral, dorso-ventral border region, contains either lamprey parapinopsin or lamprey rhodopsin.

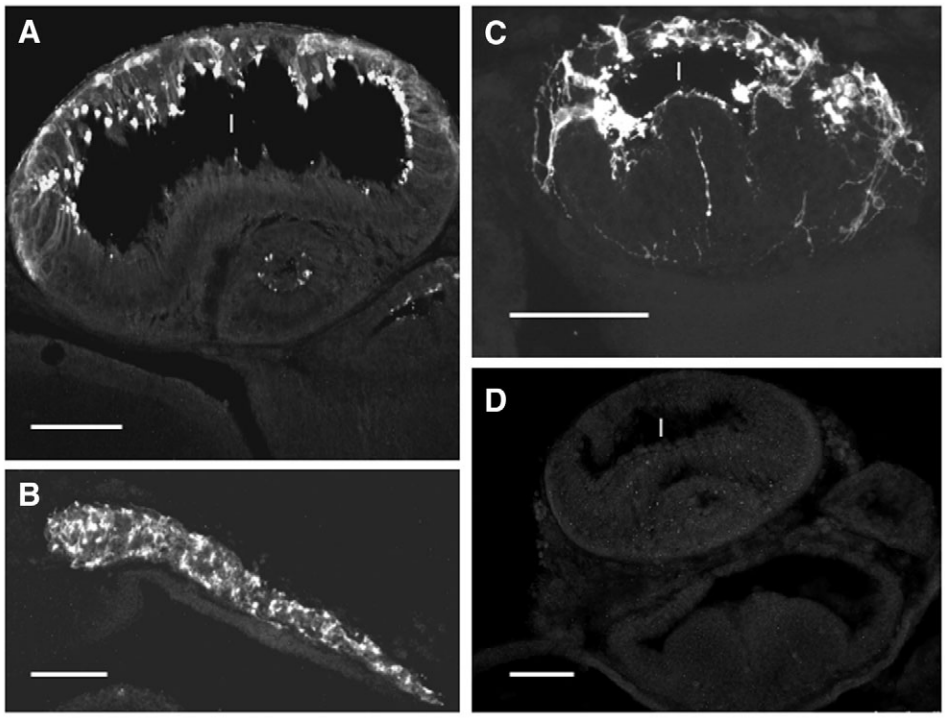

Fig. 2. Immunohistochemical localization of lamprey parapinopsin in the pineal organ. (A) Lamprey parapinopsin immunoreactivity is localized in the outer segment of the dorsal and peripheral photoreceptor cells of the pineal organ. A weak immunoreaction is observed in the cell bodies and the basal processes. (B) The lamprey parapinopsin immunoreactivity is localized in the pineal stalk. (C) In the parapineal organ, lamprey parapinopsin immunoreactivity is mainly localized in the dorsal region. (D) In control staining for lamprey parapinosin, no immunoreactivity was observed. l, lumen. Scale bars, 100 μm.

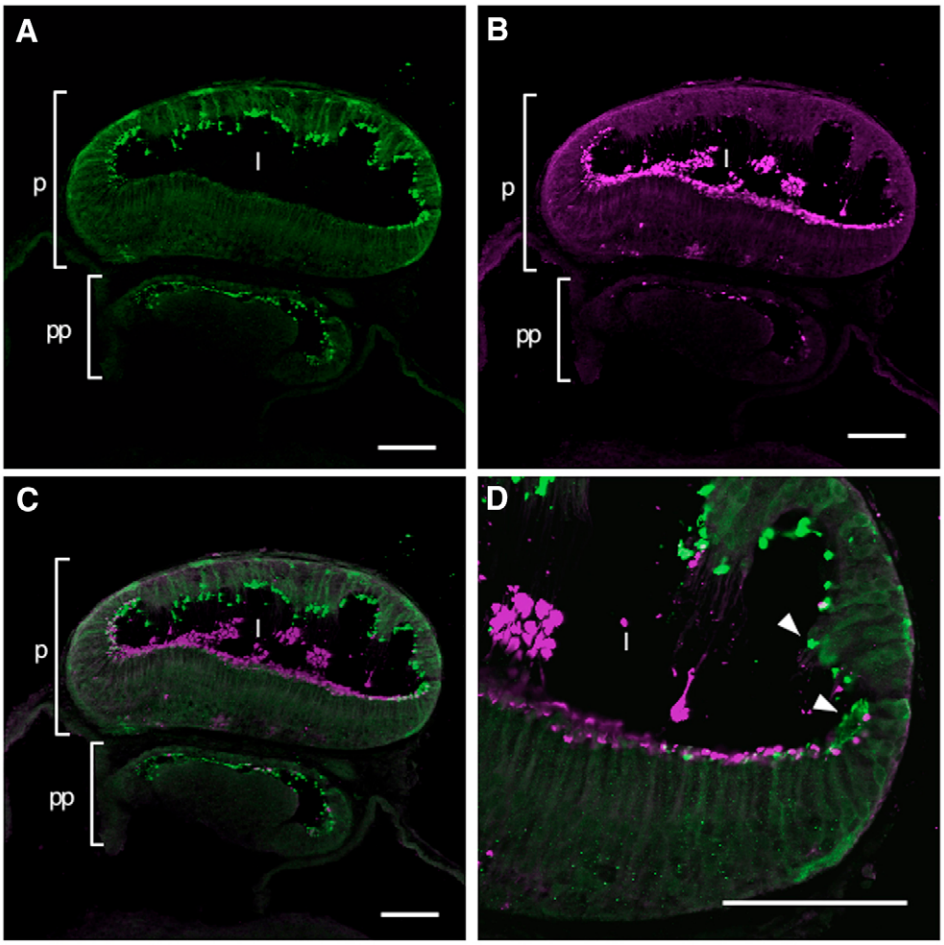

Fig. 3. Immunohistochemical localization of lamprey parapinopsin and lamprey rhodopsin in the pineal organ by confocal imaging. (A) Lamprey parapinopsin immunoreactivity (green) is localized in the dorsal and peripheral regions. (B) Lamprey rhodopsin immunoreactivity (magenta) is localized in the ventral and peripheral regions. (C) In the peripheral region, the photoreceptor cells contain either lamprey parapinopsin or lamprey rhodopsin (arrowheads). (D) Panel C at higher magnification. l, lumen; p, pineal organ; pp, parapineal organ. Scale bars, 100 μm.

We then prepared serial sections across the rostro-caudal axis and immunohistochemically investigated the distribution of photoreceptor cells. Typical results are shown in Fig. 4. In the cross-section around the peripheral portion, a larger number of lamprey parapinopsin-containing cells than lamprey rhodopsin-containing cells were observed (Fig. 4B). On the other hand, a larger number of lamprey rhodopsin-containing cells were observed in the cross-section around the central portion (Fig. 4C). Immunohistochemical observations of the pineal organ through the rostro-caudal axis revealed that lamprey parapinopsin-containing cells were more abundant in the peripheral portion than in the central portion.

As mentioned above, immunohistochemical analyses with a higher concentration of the antibody against lamprey parapinopsin revealed that the basal processes of lamprey parapinopsin-containing cells connect with each other (Fig. 2A, Fig. 3A), and the dye coupling in the basal processes of the UV photoreceptor cells (Fig. 5A, inset) was corroborated as shown in the previous study (Koyanagi et al., 2004). We therefore investigated the details of the connections between lamprey parapinopsin-containing cells in a wide area, by using the anti-parapinopsin antibody. The confocal images revealed that some lamprey parapinopsin-containing cells were connected with each other in the basal processes and formed a neuropile that spread across a wide area, at least about 250 μm × 100 μm (indicated in Fig. 5B), in the whole-mount specimens of the dorsal and peripheral portion (Fig. 5A,B).

The structure of dorsal photoreceptor cells was then compared with that of ventral photoreceptor cells by light- and electron-microscopic analysis. Dorsal and ventral photoreceptor cells were observed in the light micrograph (Fig. 6A). The outer segment of the lamprey parapinopsin-containing photoreceptor cells in the dorsal region (Fig. 6B) was similar in shape to that of the cone-type photoreceptor cells, like the pineal photoreceptor cells in the ventral region (Fig. 6C). The basal processes of the dorsal photoreceptor cells contained synaptic ribbons that were similar to those of ventral photoreceptor cells (Fig. 6D,E). These electron-microscopic similarities suggest that functionally important parts of the photoreceptor cells, the outer segment for capturing light and the synaptic ribbon for transmitting light information to a neural cell, are similar for lamprey parapinopsin-containing dorsal photoreceptor cells and lamprey rhodopsin-containing ventral photoreceptor cells.

In order to understand the neural connection that transmits UV light information to ganglion cells, we investigated the structural connection of lamprey parapinopsin-containing cells and ganglion cells. The retrograde labelling method demonstrated that most ganglion cells were localized in the basal layer of the ventral and peripheral regions, and a few ganglion cells were localized in the dorsal region (Fig. 7). Both the lamprey parapinopsin-containing cells and ganglion cells were then labelled with the double-staining method. According to the confocal images, the basal processes from the lamprey parapinopsin-containing cells extended to and made contact with the ganglion cells in the peripheral (Fig. 8A,B) and the dorsal (Fig. 8C,D) regions. The single optical section, which was taken by confocal laser-scanning microscopy, revealed that the varicosity-like structure and spherules in the basal processes of the lamprey parapinopsin-containing cells contact the dendrites and the cell body of the ganglion cells (Fig. 8A, inset). This result suggests that

lamprey parapinopsin-containing cells directly connect to ganglion cells.

Discussion

The spectral sensitivities of ganglion cells in the lamprey pineal organ demonstrate that UV reception is responsible for the antagonistic chromatic response (Uchida and Morita, 1994) but not its achromatic response (Uchida et al., 1992). In this study, we therefore histologically characterized lamprey parapinopsin-containing cells and their connection to ganglion cells in order to find out the details of the antagonistic chromatic response.

We investigated the expression pattern of lamprey parapinopsin in the pineal organ, retina and brain with RT-PCR analysis. This demonstrated that lamprey parapinopsin functions only in the pineal organ (Fig. 1), suggesting that lamprey parapinopsin is responsible for only the pineal UV reception. In contrast, our results and previous reports (Tamotsu et al., 1990; Tamotsu et al., 1994) demonstrated that lamprey rhodopsin functions in both the pineal organ and the retina (Fig. 1), showing that lamprey rhodopsin is responsible for both visual and non-visual photoreception. In the brain, immunoreactivity against the rod and cone opsins has been shown in three species of lamprey, *Petromyzon marinus*, *Lampetra fluviatilis* and *Ichthyomyzon unicuspis* (Garcia-Fernandez et al., 1997). Since neither the lamprey parapinopsin nor the lamprey rhodopsin gene was expressed in the brain in this study (Fig. 1), photopigments other than lamprey parapinopsin and lamprey rhodopsin would be expressed in the brain of *Lethenteron japonicum*.

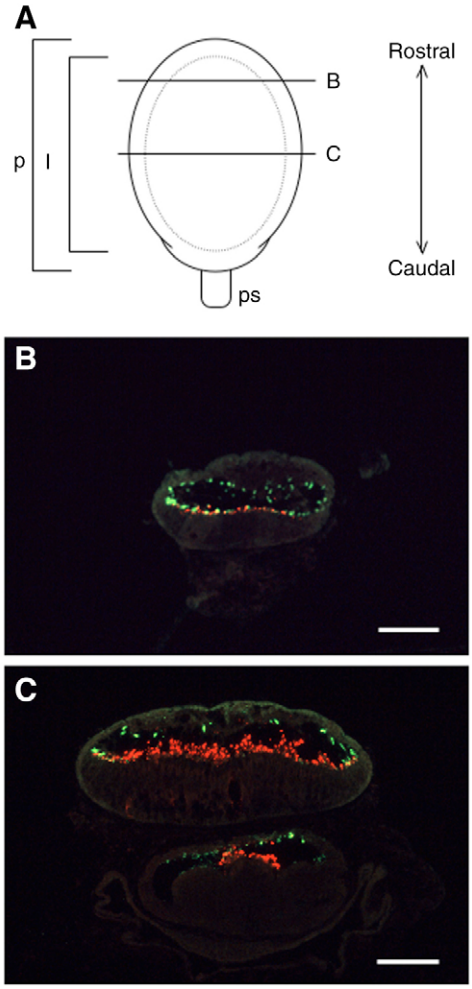

Fig. 4. Comparison of the localization of lamprey parapinopsin-containing cells with that of lamprey rhodopsin-containing cells in the pineal organ. (A) Schematic drawing of the top view of the pineal organ. The horizontal lines show the position of the sections of B and C. (B) In the cross-section around the peripheral portion (about 200 μm rostrally from the centre), there are more lamprey parapinopsin-containing cells (green) than lamprey rhodopsin-containing cells (red). (C) In the cross-section around the central portion, there are fewer lamprey parapinopsin-containing cells (green) than lamprey rhodopsin-containing cells (red). There are more lamprey parapinopsin-containing cells in the peripheral portion than in the central portion. l, lumen; p, pineal organ; ps, pineal stalk. Scale bars, 100 μm.

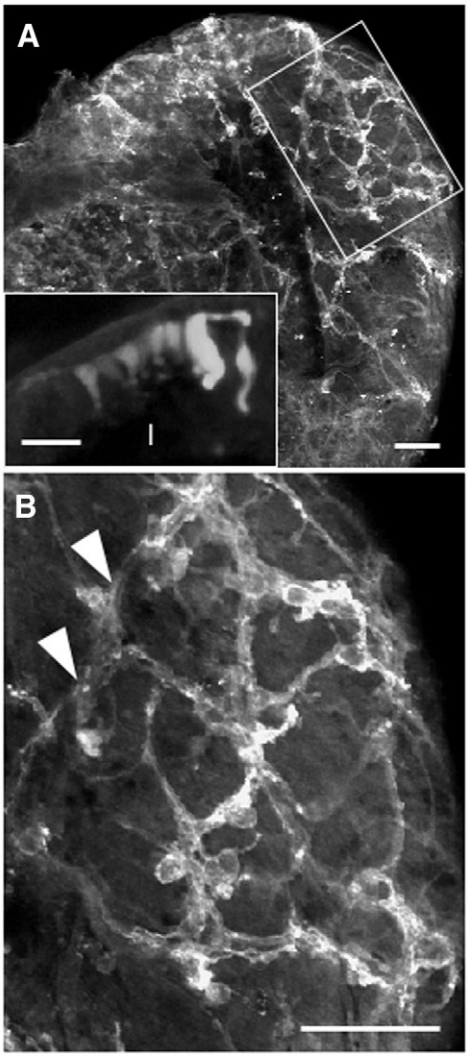

Fig. 5. The basal processes of lamprey parapinopsin-containing cells. Lamprey parapinopsin-containing cells are connected with each other via the basal processes and form a neuropile over a wide area in the whole-mount specimen of the dorsal portion (arrowheads in B). Inset in A shows dye coupling found in the UV-photoreceptor cells. B is a high magnification of the boxed region in A. Scale bars, 50 μm.

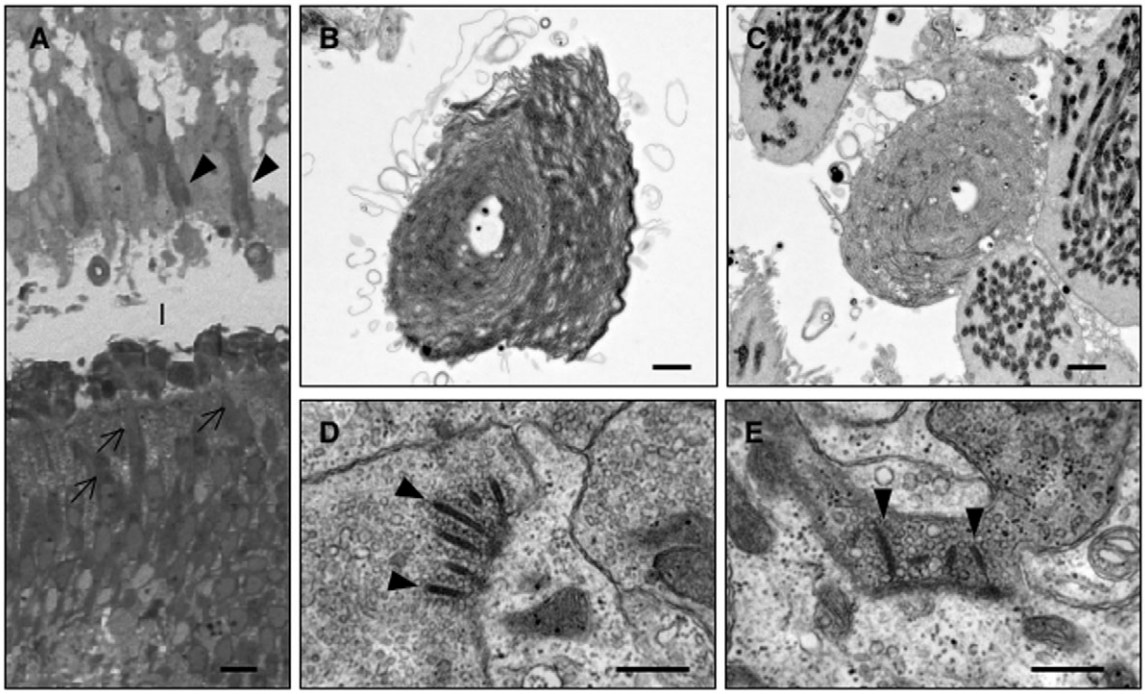

Fig. 6. Light micrograph (A) and electron micrographs (B–E) of pineal photoreceptor cells. (A) Pineal photoreceptor cells exist in the dorsal (arrowheads) and ventral region (arrows). (B) The outer segment of lamprey parapinopsin-containing cells (electron dense) is in the dorsal region. It is similar in shape to that of cone-type photoreceptor cells. (C) The outer segment of pineal photoreceptor cells, which is lamprey parapinopsin immunonegative (electron lucent), is in the ventral region. (D,E) Synaptic ribbons in the basal processes of dorsal (D, arrowheads) and ventral (E, arrowheads) photoreceptor cells. l, lumen. Scale bars, 10 μm in (A), 1 μm in (B,C) and 0.5 μm in (D,E).

In the present study, we immunohistochemically investigated the distribution of lamprey parapinopsin in order to identify a specific UV-receptive region in the pineal organ. Our results showed that lamprey parapinopsin is localized in not only the dorsal region but also the peripheral region, which is the dorso-ventral border region of the pineal organ (Fig. 2A, Fig. 3A). The number of lamprey parapinopsin-containing cells is greater around the peripheral portion than in the central portion (Fig. 4). In a previous study, many responses of chromatic-type ganglion cells were recorded in the peripheral portion of the pineal organ (Uchida and Morita, 1994). These facts indicate the possibility that, in the peripheral portion, light information from lamprey parapinopsin-containing cells is transmitted to chromatic-type ganglion cells. Double immunostaining showed that lamprey parapinopsin and lamprey rhodopsin are distributed mainly in the dorsal and ventral layer of the pineal organ, respectively (Fig. 3A,B), which is consistent with previous *in situ* hybridization results (Koyanagi et al., 2004). Remarkably, our confocal images revealed for the first time that each photoreceptor cell in the peripheral portion contains either lamprey parapinopsin or lamprey rhodopsin; in other words, the two pigments are never colocalized in a photoreceptor cell even in the peripheral region (Fig. 3C,D). These results suggest that UV light information for the inhibitory response and green/long wavelength light information for the excitatory response project to chromatic ganglion cells in the peripheral portion.

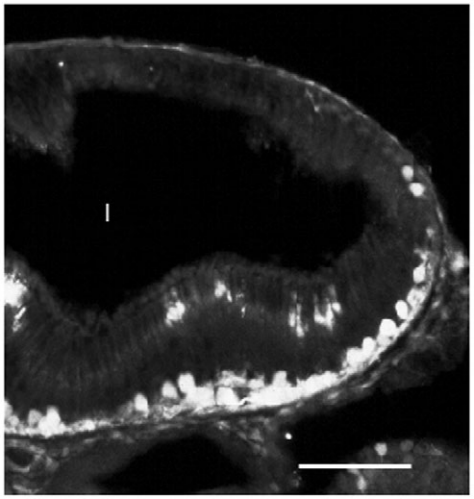

Fig. 7. Ganglion cells in the pineal organ. Most of the ganglion cells are localized in the basal layer of the ventral and peripheral region. A few ganglion cells are localized in the dorsal region. l, lumen. Scale bar, 100 μm.

Our previous studies demonstrated that lamprey parapinopsin has molecular properties quite different from those of vertebrate visual pigments (Koyanagi et al., 2004). It is considered that the molecular properties are possibly related to the functional characteristics of photoreceptor cells, and it is therefore interesting to compare the fine structures of the functional parts, the outer segment and the synaptic ribbon between photoreceptor cells, containing distinct photopigments, lamprey parapinopsin and lamprey rhodopsin. The outer segment of

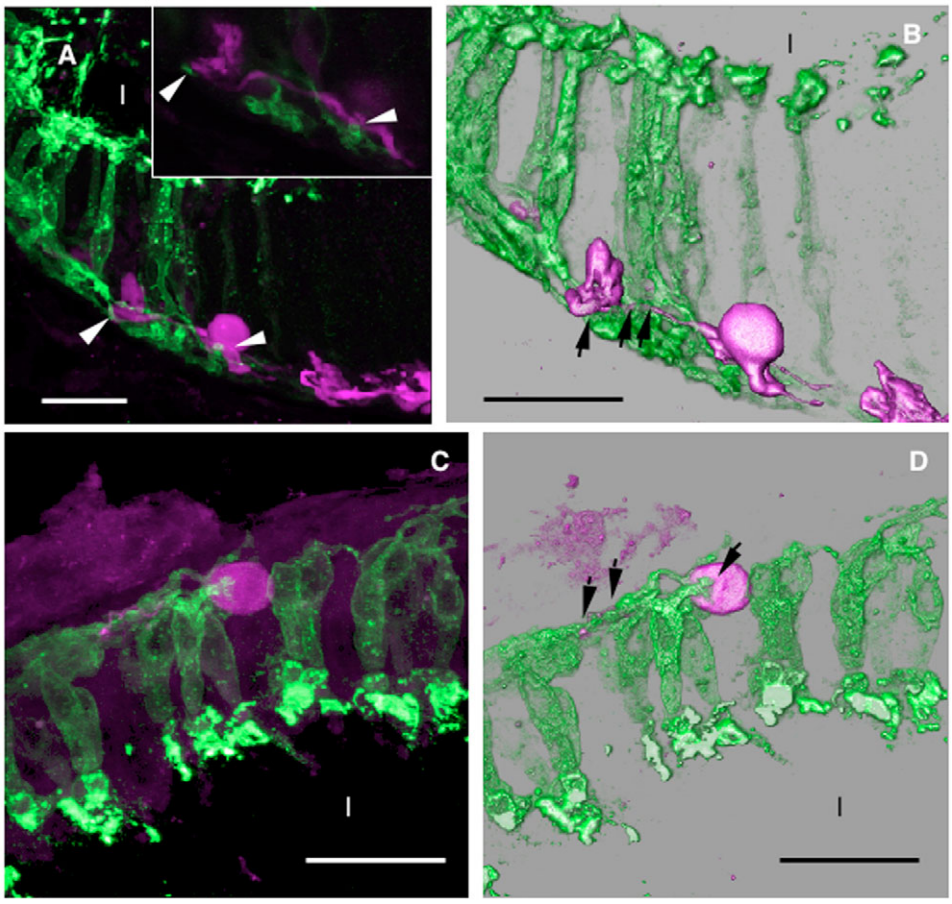

Fig. 8. Neural connection between lamprey parapinopsin-containing cells and ganglion cells by confocal imaging. The basal processes of lamprey parapinopsin-containing cells (green) are extended to and directly connect with ganglion cells (magenta) in the peripheral (A,B) and dorsal (C,D) regions. The single optical section (inset in A), which was taken by confocal laser-scanning microscopy, reveals that the varicosity-like structure and spherules of the lamprey parapinopsin immunoreactive process contact the ganglion cells. The arrows and arrowheads show the contact points. l, lumen. Scale bars, 30 μm.

dorsal photoreceptor cells, which contains lamprey parapinopsin (Fig. 6B), has a cap-like structure, similar to that of pineal photoreceptor cells in the ventral region (Fig. 6C) (Collin, 1971; Pu and Dowling, 1981). We have already found electrophysiologically that dorsal photoreceptor cells containing lamprey parapinopsin are the UV-sensitive cells (Koyanagi et al., 2004). Most of the dorsal photoreceptor cells showed lamprey parapinopsin immunoreactivity in the pineal organ (Fig. 2A, Fig. 3A), and the synaptic ribbon existed in the basal processes of the dorsal photoreceptor cells (Fig. 6D), as in the ventral photoreceptor cells (Fig. 6E). These features suggest that lamprey parapinopsin-containing UV photoreceptor cells have a structure similar to that of lamprey rhodopsin-containing photoreceptor cells in both input and output light information parts, although lamprey parapinopsin has molecular properties distinct from those of the visual pigment lamprey rhodopsin.

We previously found the existence of dye coupling, which indicates cell connection through the gap junction, in the basal processes of UV photoreceptor cells by intracellular injection (Koyanagi et al., 2004). In the present study, the finding was reconfirmed (Fig. 5A, inset) and, furthermore, most of the lamprey parapinopsin-containing cells contacted each other in the dorsal and peripheral portion. These results suggest that UV photoreceptors form a wide neural network, at least 250 μm × 100 μm in area (Fig. 5B). These histological characteristics of lamprey parapinopsin-containing cells are important for speculating on the properties of pineal UV reception and neural projection to chromatic-type ganglion cells. The UV photoreceptor cells are connected with each other, and make a large photoreceptive field. Accordingly, UV photoreceptor cells can average the UV light information reaching the pineal organ by cancelling the effect of shade. On the other hand, in the case of green light photoreceptor cells containing lamprey rhodopsin, the existence of dye coupling like that in UV photoreceptor cells could not be found by intracellular injection into a single photoreceptor cell (E.K.-Y., A.T., M.K., Y.S., T.O. and S.T., unpublished observations).

Most ganglion cells were localized in the ventral and peripheral regions, whilst a few were localized in the dorsal region (Fig. 7). This localization profile of the ganglion cells was different from that of the lamprey parapinopsin-containing cells (Fig. 2A, Fig. 3A), suggesting that they may not be directly connected. However, in this study, we found that lamprey parapinopsin-containing cells form a wide neural network (Fig. 5), and almost all the basal processes from the lamprey parapinopsin-containing cells were in direct contact with the ganglion cells in the peripheral region (Fig. 8). These results suggest that the ganglion cells of the peripheral portion receive UV light information that is converged in the UV photoreceptor layer in the pineal organ, and achieve the antagonistic chromatic response. In the electrophysiological study of the pike pineal, slow potentials that originated in the photoreceptor cells were recorded from all parts of the pineal organ, while ganglion cells were scarce in the distal part and absent in the medial one (Falcon and Meissl, 1981). Moreover, the interconnections between photoreceptor cells were demonstrated in some fish – rainbow trout (Ekström and Meissl, 1988) and ayu (Omura, 1984). Possibly, a similar network to the lamprey UV photoreceptor cells may be present in the pineal organ of the teleost, too.

In chromatic-type ganglion cells, neural firing is inhibited and excited by UV and green/long wavelength light, respectively (Uchida and Morita, 1994). UV photoreceptor cells show

hyperpolarizing responses to light (Koyanagi et al., 2004; Uchida and Morita, 1990). Our results demonstrate that chromatic-type ganglion cells directly receive UV light information from lamprey parapinopsin-containing cells. This suggests that the hyperpolarization of UV photoreceptor cells causes suppression of the release of excitatory transmitters, such as glutamate, and produces the subsequent inhibitory responses of chromatic-type ganglion cells, just as phototransduction does in retinal photoreceptor cells. However, it remains unclear whether the pineal photoreceptor cells that are involved in the excitatory response to green/long wavelength light are depolarized by light stimulation or indirectly connect with chromatic-type ganglion cells via an interneuron. We have not found the neural connection between lamprey rhodopsin-containing cells and chromatic-type ganglion cells. The maximum sensitivities of the excitatory responses were reported to be 540 nm (Uchida and Morita, 1994), which does not fit the absorption maximum of lamprey rhodopsin (M. Koyanagi, E. Kawano-Yamashita, Y. Kinugawa, T. Oishi, Y. Shichida, S. Tamotsu and A. Terakita, unpublished observations) (Hisatomi et al., 1997). Therefore, there is the possibility that an unknown photopigment contained in photoreceptor cells is involved in the excitatory response. It would be of interest to study how the excitatory response to green/long wavelength light is produced in the pineal organ.

The antagonistic chromatic response has also been found in the pineal complexes of lower vertebrates, such as fish and frogs (Dodt and Heerd, 1962; Morita, 1966). We have already isolated the lamprey parapinopsin homologues from rainbow trout and clawed frog pineal complexes (Koyanagi et al., 2004). Parapinopsin possibly generates the antagonistic chromatic response for pineal UV reception in the vertebrate. Therefore, the mechanism of the antagonistic chromatic response could be elucidated by investigating UV photoreceptor cells, using parapinopsin as a marker.

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