Identification of rhodopsin in the pigeon deep brain

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Abstract We detected rhodopsin gene expression in the pigeon lateral septum, a photosensitive deep brain region that is responsible for the photoperiodic gonadal response. The nucleotide sequence of the deep brain rhodopsin cDNA clone exactly matched that of the retinal one, indicating that a single rhodopsin gene is transcribed in the two tissues. Immunohistochemical analysis localized rhodopsin in the cerebrospinal fluid-contacting neurons, which have been assumed to be photoreceptive cells in the deep brain. Pigeon rhodopsin seems to play dual important roles in the visual and non-visual systems, the latter of which contributes to the photoperiodic response.

Key words: Deep brain photoreceptor; Rhodopsin; Photoperiodic response; Lateral septum; Cerebrospinal fluid-contacting neuron; Pigeon

1. Introduction

A variety of vertebrates have photoreceptor cells not only in the retina but also in the pineal complex, deep brain, and skin [1–4]. These extraretinal photoreceptor cells play important roles in diverse physiological responses, i.e. photo-entrainment of circadian rhythms, detection of seasonal changes in photoperiod (photoperiodicity), color changes of the skin [1–5], etc. In some animal species, the pineal and parapineal photoreceptive molecules have been sequenced, and they are named pinopsin [6] and parapinopsin [7], respectively. The deep brain photoreceptor cells responsible for the photoperiodic response of birds have been localized in the lateral septum and the infundibular region [8]. Except for the presence of opsin-like immunoreactivities [9–15], however, little information is available about the deep brain photoreceptive molecules. This is probably due to the limited distribution of the photoreceptor cells in the deep brain and/or the low level expression of the protein(s).

The present study undertook the molecular identification of the pigeon deep brain opsin as an initial step for elucidation of the molecular mechanism underlying animal photoperiodic responses.

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Abbreviations: CSF, cerebrospinal fluid; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends

2. Materials and methods

2.1. Genomic PCR

Two degenerated oligonucleotide primers, 5′-ATGAA(C/T)GGIACIGA(A/G)GG-3′ and 5′-GTIGC(A/G)AA(A/G)AAICC(C/T)TC-3′, were designed for amplification of exon I of vertebrate opsin genes (see Section 3). The amplification reaction was performed using 2.5 units of Ampli-Taq polymerase (PE Applied Biosystems) with 1 μg of the pigeon genomic DNA under the following conditions: an initial denaturation step of 1 min at 94°C, and 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The final cycle was followed by an extension step of 7 min at 72°C.

2.2. RNA isolation

Freshly isolated pigeon brains were cut into slices (3 mm in thickness) with Rodent Brain Matrix (ASI instruments) on ice, and then the tissue blocks containing the lateral septum were punched out bilaterally with a flat-ended needle (3 mm in diameter). These tissue blocks or the pigeon retinas were homogenized in guanidine thiocyanate, and the RNA fraction was isolated by a density gradient ultracentrifugation using CsTFA (Amersham Pharmacia Biotech). Poly(A)$^+$ RNA was purified from the RNA fraction by an oligo(dT)-cellulose (type 3, Collaborative Biomedical Products) column chromatography.

2.3. RT-PCR analysis

RT-PCR was performed using the GeneAmp XL RNA PCR kit (PE Applied Biosystems) with a pair of the following primers: 5′-TCTCGGCTCTGGCTGCCTACATG-3′ and 5′-TGTAGCAGCCCGTTACTCCAAAGACAAAG-3′ for amplification of the Po-2 sequence (see Section 3), or 5′-GTGTGCTGCTACATCTTCTTCCTCATCTCC-3′ and 5′-GCCCGAAGACGAAGTAACCGTTCC-3′ for amplification of the Po-39 sequence (see Section 3). The amplification reaction was performed under the following conditions: an initial denaturation step of 1 min at 94°C, and 40 cycles of 15 s at 94°C and 225 s (with extension of 15 s per cycle during cycles 21–40) at 70°C. The final cycle was followed by an extension step of 7 min at 60°C.

2.4. Cloning of deep brain opsin cDNA

The 5′ and 3′ flanking regions of the pigeon rhodopsin cDNA were obtained by rapid amplification of cDNA ends (RACE) using retinal total RNA as a template. Then a pair of primers was designed from the 5′/3′ flanking regions for RT-PCR to amplify the entire protein-coding region of the deep brain opsin cDNA. Reverse transcription was performed for 50 min at 50°C in the presence of SUPERSCRIPT II polymerase (Gibco BRL) and template poly(A)$^+$ RNA obtained from the pigeon lateral septum. After that, the cDNA was amplified by PCR with LA Taq polymerase (Takara) under the following conditions: an initial denaturation step of 1 min at 94°C, and 40 cycles of 30 s at 94°C and 90 s at 65°C. The final cycle was followed by an extension step of 7 min at 72°C. Six independent clones obtained were sequenced on both strands by the cycle sequencing method with fluorescence-labeled sequencing terminators.

2.5. Production of antibody

Amino acid sequences of chicken [16] and pigeon rhodopsins (determined in the present study) are identical to each other within the amino-terminal region (Met^1–Leu^{79}; see Fig. 2). Therefore, mice were immunized with chicken retinal rhodopsin purified as described pre-

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viously [17], and the resultant antisera were subjected to affinity purification of the amino-terminal region-specific antibodies. To this end, a fusion protein consisting of maltose-binding protein and the amino-terminal 29 residues (Met^1–Tyr^{29}) of pigeon rhodopsin were produced in *Escherichia coli*, and purified as described previously [18]. The fusion protein was coupled to NHS-activated Sepharose column (Amersham Pharmacia Biotech), to which the anti-rhodopsin antisera were applied for purification of the amino-terminal region-specific antibody termed RhoN.

2.6. Immunohistochemical studies

Pigeons deeply anesthetized with ether were transcardially perfused with solutions in order of 0.7 ml of 1000 U/ml heparin in distilled water, 5–10 ml of 0.75% NaCl, and then 200 ml of freshly prepared 4% paraformaldehyde in 0.75% NaCl. Then the brains and eyes were dissected, immersed in a postfixation solution for 1 h and dehydrated successively in 15%, 20% and 30% sucrose (w/v) solutions in 0.75% NaCl for 2 h each at 4°C. Then the tissues were frozen, sectioned at 5 μm thickness, mounted onto gelatin-coated glass slides, and air-dried. After the sections were incubated for 30 min at room temperature in 10 mM phosphate-buffered saline (PBS, pH7.4) containing 1.5% normal horse serum, they were incubated for 72 h at 4°C with the RhoN antibody diluted 500-fold with PBS containing 0.3% Triton X-100 and 1.5% normal horse serum. Positive signals were detected using a Vectastain Elite ABC kit (Vector Laboratories) and a substrate solution of diaminobenzidine (DAB) in 0.03% H_2O_2.

3. Results

3.1. Identification of a deep brain opsin

We previously reported that the vertebrate retinal opsins are classified into four groups (group L, M1, M2, and S) based on their amino acid sequences. Opsins in the same group show similar wavelength sensitivities to each other when they bind 11-*cis*-retinal [19]. For example, vertebrate rod opsins (rhodopsins) and green-sensitive cone opsins belong to group M2, and their absorption maxima are located at a middle-wavelength region of the visible light (465–511 nm). The action spectra for the photoperiodic responses of the Japanese quail showed maximal sensitivity at wavelengths near 500 nm [20,21]. These facts together with the rhodopsin-like immunoreactivities in avian (quail, ring dove, and duck) brains [9] have led us to hypothesize that a group M2-related opsin(s) is present in the avian deep brain.

For designing a forward degenerate primer to identify a gene encoding the pigeon deep brain opsin, we took advantage of the fact that the amino acid sequence at the amino-terminal region (MNGTEG) is highly conserved among the group M2 opsins. A reverse primer was designed on the basis of the partial sequence of the third transmembrane domain (EGFFAT in chicken rhodopsin) that is highly conserved among all vertebrate opsins. PCR employing these degenerate primers and the pigeon genomic DNA as a template is expected to amplify any gene for group M2 opsin(s). As expected, we obtained only two kinds of cDNA clones, Po-2 and Po-39, of which the deduced amino acid sequences were very similar to chicken rhodopsin [16] (99.1% identical) and chicken green [19] (99.1% identical), respectively. This together with the results of the cDNA cloning (see below) indicates that Po-2 and Po-39 encode pigeon rhodopsin and green, respectively.

As we detected no amplification of any other opsin-like gene, a possible expression of rhodopsin and green in the pigeon deep brain was examined by RT-PCR using primers specific to Po-2 and Po-39. A DNA fragment of 225 bp was amplified by RT-PCR employing the rhodopsin (Po-2)-specific primers and template poly(A)$^+$ RNA purified from the pigeon lateral septum (Fig. 1A) or from the retina (data not shown). The nucleotide sequences of the products amplified from the two tissues exactly matched the genomic sequence of pigeon rhodopsin (Po-2). This suggests that rhodopsin is expressed not only in the retina but also in the lateral septum of the deep brain. On the other hand, the pigeon green (Po-39) sequence was amplified only from retinal poly(A)$^+$ RNA but not from lateral septum poly(A)$^+$ RNA (data not shown).

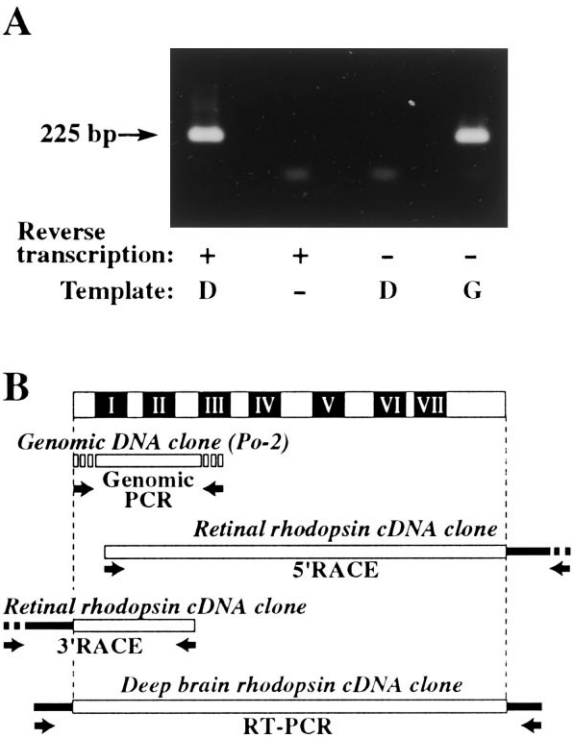

Fig. 1. RT-PCR analysis and cloning strategy. A: RT-PCR analysis of Po-2 gene expression in the pigeon deep brain. RT-PCR was performed in the presence of pigeon brain RNA template (lanes 1, 3), pigeon genomic DNA template (lane 4), or in the absence of template (lane 2). The reverse transcription step was omitted in lanes 3 and 4. D, deep brain poly(A)$^+$ RNA; G, genome DNA. B: A schematic drawing of the cloning strategy of deep brain rhodopsin cDNA. The entire protein-coding region (1053 bp; top bar) of deep brain opsin cDNA was obtained by a combination of genomic PCR and 5′/3′ RACE with retinal total RNA and by RT-PCR with poly(A)$^+$ RNA extracted from the lateral septum. Arrows represent the positions of the oligonucleotide primers.

3.2. Complementary DNA cloning of rhodopsin from deep brain and retina

Prior to the cDNA cloning of the entire protein-coding region of the pigeon deep brain opsin, we employed 5′ RACE and 3′ RACE with a template of pigeon retinal total RNA to isolate two partial cDNAs overlapping with the coding region of rhodopsin (Fig. 1B). This provided the nucleotide sequences of the 5′ and 3′ flanking regions of pigeon rhodopsin cDNA without amplifying a cDNA covering the entire coding sequence of retinal rhodopsin. This strategy enabled us to eliminate a possible contamination by retinal rhodopsin cDNA in the course of the following PCR.

Finally, we performed RT-PCR using a template of lateral septum poly(A)$^+$ RNA and the specific primers corresponding to the 5′ and 3′ flanking sequences of pigeon rhodopsin

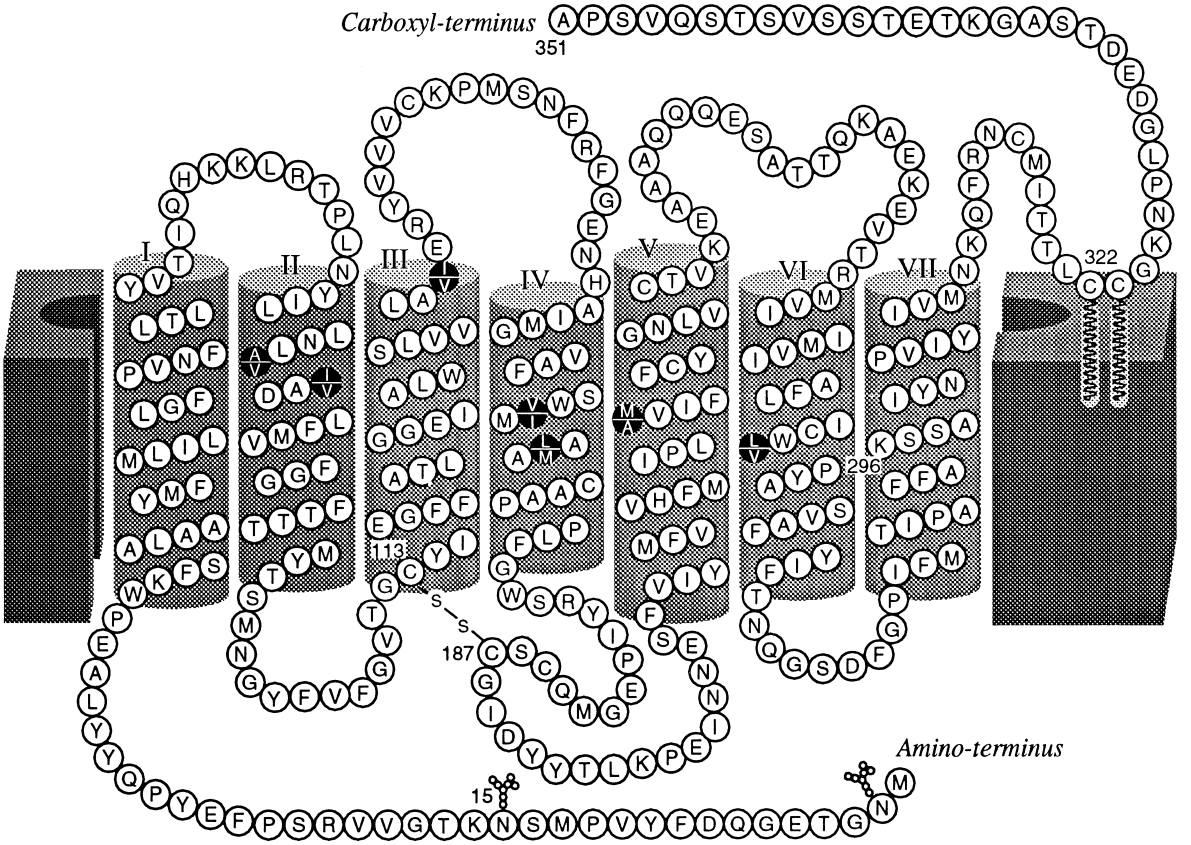

Fig. 2. The deduced amino acid sequence of pigeon rhodopsin. On the basis of the deduced amino acid sequence of pigeon rhodopsin, a model is constructed for a heptahelical membrane-spanning structure composed of helices I–VII. Amino acid residues different from chicken rhodopsin are shown as white characters on a black background (upper: pigeon, lower: chicken).

cDNA (Fig. 1B). Then we isolated a single PCR product spanning 1210 bp, whose nucleotide sequence was completely identical to that of retinal rhodopsin. These results indicate that a single rhodopsin gene is expressed in the two tissues, the retina and lateral septum of the pigeon. The amino acid sequence of pigeon rhodopsin was 98.0% identical to chicken rhodopsin (Fig. 2).

3.3. Detection of rhodopsin protein in the brain

To localize rhodopsin in the pigeon brain at a protein level, we prepared an antibody (RhoN) which recognizes the amino-terminal region of pigeon rhodopsin. The RhoN antibody immunostained a number of subependymal neurons in a highly circumscribed region of the lateral ventricular wall of the lateral septum in the pigeon brain (Fig. 3C), as well as the rod outer segments of the pigeon retina (Fig. 3E). The majority of the RhoN-immunopositive subependymal neurons emitted an immunopositive process with a bulb-like ending which projected into the lateral ventricle (Fig. 3D). In addition to the bulb-like dendritic terminals and perikarya, basal processes were also immunostained strongly (Fig. 3D).

4. Discussion

In the present study, we cloned rhodopsin cDNA from the pigeon lateral septum RNA fraction. The immunohistochemical analysis revealed RhoN antibody-positive subependymal neurons in the lateral septum of the pigeon brain (Fig. 3C,D). These neurons correspond to cerebrospinal fluid (CSF)-contacting neurons which have been assumed to be photoreceptor cells due to their sensory cell-like morphology [22] and opsin-like immunoreactivities [9–15]. Curiously, it was reported that some of the rhodopsin-specific antibodies did not stain the deep brain cells [9,12,23]. For example, Rho-1D4, a monoclonal antibody specific for the carboxyl-terminal region of rhodopsin [24], detected no positive signal in the deep brain [9]. Consistent with this, we observed no immunoreactivities in the pigeon lateral septum when we used a polyclonal antibody raised against the carboxyl-terminal region of the chicken rhodopsin (data not shown), though this antibody heavily stained the rod outer segments of the pigeon retina. This may represent a difference in intracellular localization or structure of the carboxyl-terminal region of the rhodopsin molecule between the retina and the deep brain. It may also correlate with the membrane structure of the CSF-contacting neurons lacking photoreceptor-like lamellar membranes [22]. Alternatively, the deep brain rhodopsin might be differently processed by post-translational modifications such as phosphorylation and/or proteolysis, which prevented the recognition by the carboxyl-terminal region-specific antibodies.

The present result does not always exclude a possible expression of some other photoreceptive molecule(s) in the pigeon deep brain. For example, red-sensitive cone opsin-like immunoreactivity has been detected in the deep brain of ring dove [10]. Cone opsin-like immunoreactivity was also observed in the deep brain of lizards [11,15], gecko, frog,

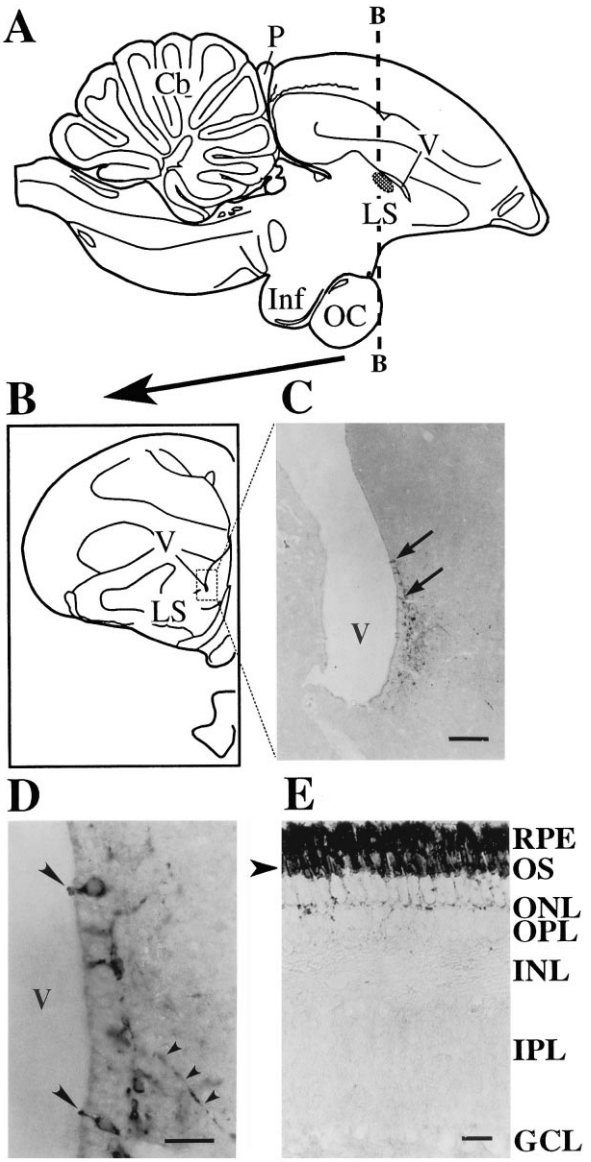

Fig. 3. Immunohistochemical localization of rhodopsin in the pigeon deep brain and retina. Sagittal section (A) and frontal section (B) of the pigeon brain were drawn to depict the location of RhoN-immunoreactive cells. The sections (5 μm thick) of the pigeon brain (C: ×10, D: ×63) and the pigeon retina (E: ×40) were stained with RhoN. Immunoreactivity was observed at the dendritic processes (large arrowheads in D) projected into the lateral ventricle (V), at the perikarya and at the basal processes (small arrowheads in D) of some subependymal neurons (arrows in C) in the lateral septum. In the retinal section, the outer segment layer (OS, arrowhead in E) was heavily stained with RhoN. Dense pigment granules in the RPE layer were also observed in the control section treated without the primary antibody. Cb, cerebellum; Inf, infundibular region; LS, lateral septum; OC, optic chiasma; P, pineal gland; RPE, retinal pigment epithelium; OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 100 μm (C) or 20 μm (D and E).

salamander, fishes [12] and lamprey [14]. Taken together, it is possible to speculate that multiple photoreceptive molecules in the deep brain serve to cover a wide range of the visible wavelengths or even for color discrimination.

The presence of rhodopsin in the deep brain implies that the deep brain photoreceptor cell has a phototransduction cascade common to the retinal cells. This is consistent with the transducin-like immunoreactivities detected in the CSF-contacting neurons of the bullfrog [13] and the lamprey [14]. As well, immunohistochemical studies suggested the presence of phosducin [10] and arrestin [14] in the deep brain of junco and the lamprey, respectively. Identification and characterization of the molecules responsible for intracellular light signal transduction in the rhodopsin-expressing neurons of the deep brain would help to answer the question of how the change in environmental light conditions elicits photoperiodic responses of animals.

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