A deep brain photoreceptive molecule in the toad hypothalamus

Tomoko Yoshikawa[a,b,c], Toshiyuki Okano[b,c], Tadashi Oishi[a], Yoshitaka Fukada[b,c,*]

[a] *Department of Biology, Faculty of Science, Nara Women's University, Kitauoyanishi-machi, Nara 630-8263, Japan*
[b] *Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Tokyo 113-0032, Japan*
[c] *CREST, JST, Tokyo, Japan*

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Abstract We have isolated a cDNA clone encoding a deep brain photoreceptive molecule from the hypothalamic cDNA library of the toad, *Bufo japonicus*. The deduced amino acid sequence showed the highest similarity to that of pinopsin (75–76%) among vertebrate retinal opsins, indicating the expression of toad pinopsin in the deep brain. Antibodies raised against the C-terminal tail of toad pinopsin stained cell bodies and the knob-like structures of the cerebrospinal fluid-contacting neurons in the anterior preoptic nucleus. This region is known to play an important role in breeding behavior, suggesting that toad pinopsin acts as a photosensor for the photoperiodic gonadal response.

Key words: Pinopsin; Hypothalamus; Photoperiodic response; Cerebrospinal fluid-contacting neuron; Anterior preoptic nucleus; Toad

1. Introduction

Animals show various seasonal behaviors such as breeding, molting and migration. Photoperiod is the predominant zeitgeber for the endogenous annual timing systems regulating animal seasonalities (photoperiodicity). In many vertebrates that might be restricted to non-mammalian species, photoreceptive molecules responsible for the photoperiodicity are located in the deep brain [1] to which light reaches [2]. The photoreceptive molecule has been called 'deep brain (encephalic) photoreceptor' since the discovery of encephalic photosensitivity early in this century [3]. More recently, rhodopsin-like photosensitivities regulating photoperiodic gonadal response have been detected in the hypothalamus of the Japanese quail [4,5]. Such a relationship between the deep brain photoreceptor and the photoperiodic gonadal response has also been suggested in amphibians [6]. Consistently, light-induced electrophysiological response was recorded from the frog diencephalon even after removal of the eyes and the pineal complex [7], and opsin-like immunoreactivities were found in the frog hypothalamus [8]. In addition, a chromophore of vertebrate opsin, 11-*cis*-retinal, was detected in the ventral part of the frog diencephalon including the hypothalamus [9]. These results suggest that frogs have opsin-like deep brain photoreceptive molecule(s) in their hypothalamus, though the molecular identity remains to be elucidated.

Here we report the primary structure of the toad deep brain photoreceptive molecule and its immunohistochemical localization in the brain.

*Corresponding author. Fax: (81) (3) 5802-8871.
E-mail: sfukada@hongo.ecc.u-tokyo.ac.jp

Abbreviations: APON, anterior preoptic nucleus; CSF, cerebrospinal fluid

2. Materials and methods

2.1. cDNA cloning and phylogenetic analysis

A cDNA library was constructed in λgt11 phage using poly(A)$^+$ RNA purified from hypothalamus of the toad (*Bufo japonicus*) collected in Tokyo, Japan. DNAs of the cDNA library were used as a template of PCR. For PCR amplification, four kinds of degenerate forward primers (Op-Fw) and a reverse degenerate primer (Op-Rv) were designed on the basis of the amino acid sequences of vertebrate opsins (Fig. 1). The λgt11-specific primers (λFw; 5′-GGTGGCGACGACTCCTGGAGCCCG-3′ and λRv; 5′-TTGACACCAGACCAACTGGTAATG-3′) were also used for the initial PCR under the following cycling conditions: 94°C for 1 min, 55°C for 1 min and 74°C for 1 min for 35 cycles in the presence of AmpliTaq Gold polymerase (PE Applied Biosystems). Genomic PCR was performed with the aid of the LA PCR in vitro cloning kit (Takara) in the presence of a template of toad genomic DNA partially digested by *Sau*3AI under the following cycling conditions: 98°C for 20 s and 68°C for 15 min for 30 cycles. A cDNA clone encoding the toad deep brain opsin was obtained by reverse transcription (RT)-PCR as follows. First, a pair of primers corresponding to the 5′- and 3′-flanking regions of the coding sequence was synthesized (NC-Fw: 5′-CTCTGGCCTATGCCTCAAACC-3′ and NC-Rv: 5′-GGTTCTGTGCTGATGACAAGC-3′). Then, total RNA isolated from the toad hypothalamic region was reverse transcribed by SuperScript II (Gibco BRL) priming with NC-Rv, and the cDNA covering the entire coding sequence was amplified by PCR in the presence of NC-Fw, NC-Rv and LA Taq polymerase (Takara) under the following cycling conditions: 94°C for 1 min, 60°C for 1 min and 74°C for 1 min for 30 cycles. Twelve independent clones were sequenced on both DNA strands.

A phylogenetic tree was constructed by the neighbor-joining method [10] using several invertebrate opsins as outgroups [11]. Amino acid sequences of known opsins were obtained from the GenBank database.

2.2. Preparation of antibodies against toad pinopsin

Two kinds of fusion proteins were prepared: MBP/PinC and GST/PinC are composed of the C-terminal region of the toad pinopsin (PinC; Gly^{314}–Ala^{346}, Fig. 2) and maltose-binding protein (MBP) or glutathione *S*-transferase (GST), respectively. The fusion proteins were expressed in *Escherichia coli* BL21 strain, and purified by affinity column chromatography. Two mice were immunized with purified MBP/PinC, and the antisera obtained were purified by a GST/PinC-immobilized HiTrap Sepharose column (1 ml, Amersham Pharmacia Biotech).

2.3. Immunohistochemistry

Toads (*B. japonicus*) were killed by decapitation, and the dissected brains were fixed with Bouin's solution. After the tissues were dehydrated through graded alcohols, they were embedded in paraffin and sectioned at 4–6 μm. The sections were subjected to immunostaining as described [8].

3. Results and discussion

The initial PCR was performed using combinations of the primers (Op-Fw or Op-Rv; Fig. 1, and λFw or λRv: see Section 2) and a template of the cDNA library-derived DNAs. This PCR amplified a cDNA fragment possibly encoding a deep brain photoreceptive molecule with the 3′-flanking region. Two internal PCR primers were additionally

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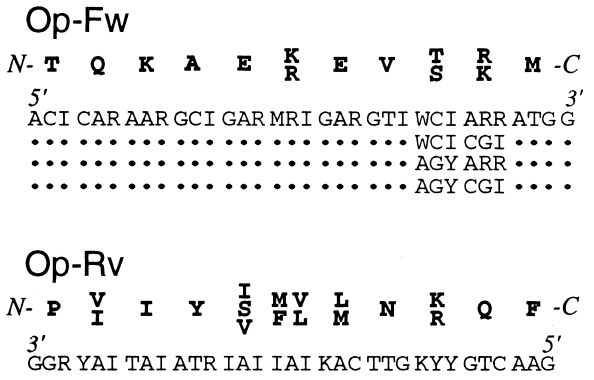

Fig. 1. Nucleotide sequences of the degenerate primers for PCR. The forward primers (Op-Fw) correspond to the loop region between the fifth and sixth transmembrane segments of opsins, and the reverse primer (Op-Rv) corresponds to the seventh transmembrane segment. To reduce combinations of nucleotide sequences, inosine was used instead of three or four nucleotide mixtures. Dots denote nucleotides identical to those in the first line. R, A+G; Y, C+T; M, A+C; W, A+T; K, G+T; and I, inosine.

designed from the newly acquired sequence data to obtain nearly the entire coding sequence. In order to precisely determine the initiation site of translation, we employed genomic PCR which amplified a DNA fragment containing the longer 5′-flanking region (120 bp) of the molecule. A cDNA (1147 bp) covering the entire coding sequence was obtained from a template of toad hypothalamic RNA by RT-PCR in the presence of primers corresponding to the 5′- and 3′-flanking regions (NC-Fw and NC-Rv, respectively).

The encoded protein was composed of 346 amino acid residues, and it showed all the hallmarks of vertebrate retinal opsins (Fig. 2); cysteines for intramolecular disulfide bond (Cys^{102}, Cys^{179}), a glutamic acid for retinylidene Schiff-base counterion (Glu^{105}) and a lysine for Schiff-base linkage with a chromophore 11-*cis*-retinal (Lys^{286}). The overall amino acid sequence was more similar to the pineal photoreceptive molecule pinopsin (75–76%) than to any retinal photopigments (45–52%). A phylogenetic tree of vertebrate opsins constructed on the basis of amino acid identity [10,11] clearly indicated that the molecule is a member of group P represented by pinopsin [12] (Fig. 3). Thus the molecule was named toad pinopsin though it was cloned from and localized in non-pineal tissue (see below).

Recently, several kinds of non-visual-type opsins (groups P [12], PP [13] and VA [14]) have been cloned from various tissues of vertebrates. A comparison of toad pinopsin with the vertebrate opsins within the second extracellular loop revealed two amino acid insertions into canonical visual-type opsins (groups L, M1, M2 and S [11]) at the position between 188 and 189 in toad pinopsin (Fig. 2, asterisks). Interestingly, all the opsins in groups P, PP and VA can be aligned with invertebrate opsins with no insertion at this position. With respect to this point, the non-visual opsins are more similar to invertebrate opsins than to vertebrate visual opsins, though

Fig. 2. Amino acid sequence alignment of toad pinopsin with several vertebrate photopigments. Gaps (-) were inserted for optimal alignment of the sequences. Putative transmembrane domains (I–VII) are indicated by horizontal lines. Bold face characters represent a potential site for *N*-glycosylation (Asn^{5}), cysteines for an intramolecular disulfide bond (Cys^{102}, Cys^{179}), a glutamic acid for retinylidene Schiff-base counterion (Glu^{105}), a lysine for Schiff-base linkage with a chromophore 11-*cis*-retinal (Lys^{286}), and cysteines for palmitoylation (Cys^{312} and Cys^{313}). Asterisks indicate two amino acid residues to be inserted only into visual opsins for alignment (see text).

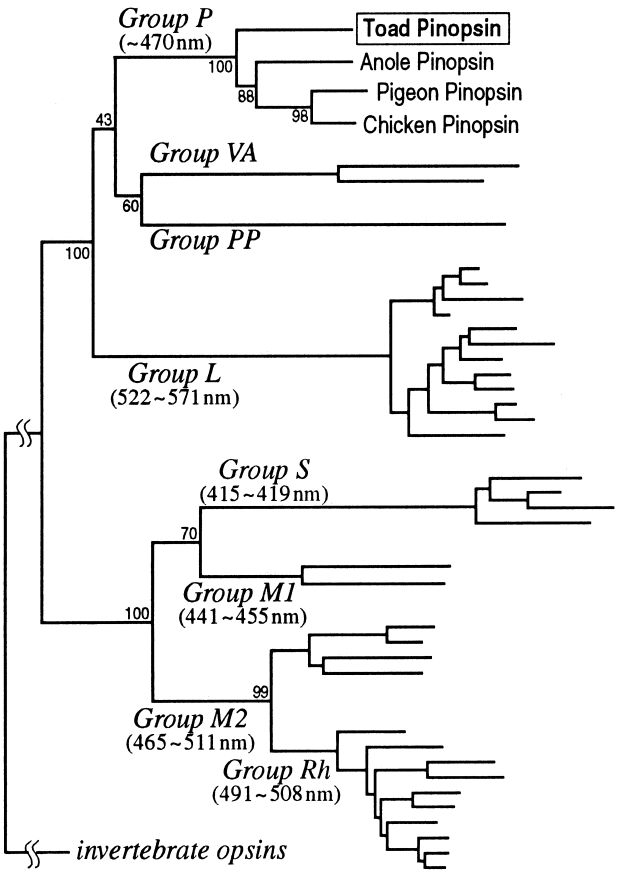

Fig. 3. A phylogenetic tree of vertebrate photoreceptive molecules. The tree was constructed by the neighbor-joining method [10] as described previously [11]. The values on the horizontal lines denote the bootstrap probabilities (%) estimated by 1000 times replications.

this is at variance with the phylogenetic tree constructed on the basis of whole sequences (Fig. 3).

In spite of many trials, we failed to express recombinant toad pinopsin in mammalian cell lines (293EBNA and COS7), so that its spectral sensitivity curve is unavailable at present. But we expect that toad pinopsin with bound 11-*cis*-retinal would show a blue light sensitivity like chicken pinopsin [12], based on the fact that photoreceptive molecules in the same group show similar spectral sensitivities to each other [11]. 11-*cis*-Retinal is the most common chromophore of vertebrate retinal opsins and, in fact, it is detected in the extract of the ventral area of the frog diencephalon [9].

The cells expressing pinopsin in the toad brain were localized using a toad pinopsin-specific antibody (PinC antibody) raised against its C-terminal region that shows a low amino acid identity to any other opsins including pinopsin (25–45%). The antibody labeled subependymal cells in the anterior preoptic nucleus (APON) of the toad hypothalamus. Most of the immunopositive cells emitted a short process with a knob-like terminal contacting cerebrospinal fluid (CSF) of the third ventricle (Fig. 4). These PinC-immunopositive cells represent characteristics of a CSF-contacting neuron that has been assumed to be a deep brain photoreceptor cell due to its morphological feature of sensory cells [15] and opsin-like immunoreactivities [16,17]. Interestingly, no immunoreactivity was observed at the toad pineal gland, suggesting the presence of an additional opsin(s).

Toad pinopsin is localized in the APON (Fig. 4), which generally plays an important role in the sexual behaviors of a variety of vertebrates [18]. In frogs, the APON is not only a crucial locus for triggering male mate-calling behavior [18] but also one of the components in the hypothalamo-hypophysial neurosecretory system [19]. Furthermore, the volume of the APON changes along with the development of the gonads through the seasons: it is larger in the hibernating period (pre-breeding period) than in the post-breeding period [20]. Thus, the frog APON seems to be closely related to seasonal breeding, and it is an attractive speculation that toad pinopsin acts as a daylength-photosensor molecule in the APON and that it controls photoperiodic gonadal responses through the hypothalamo-hypophysial neurosecretory system.

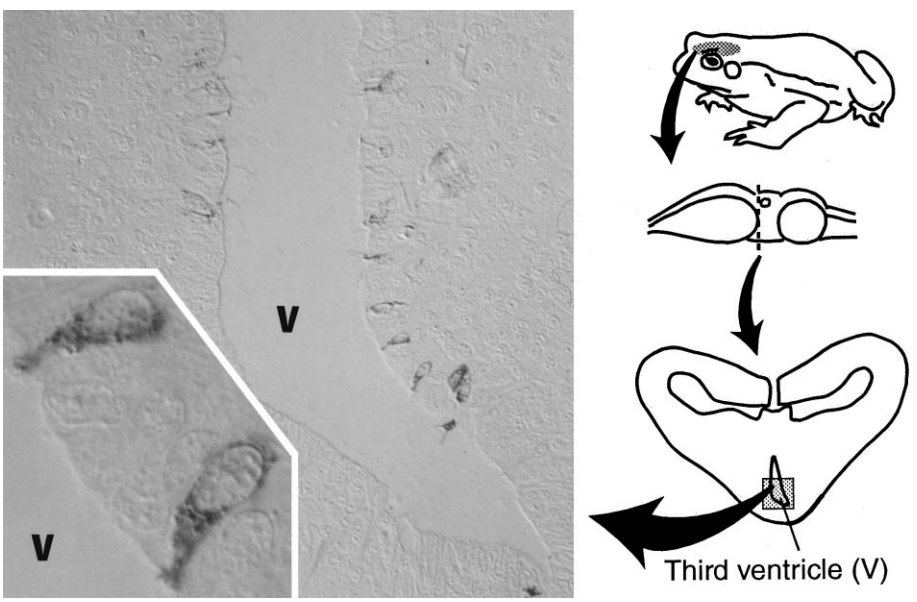

Fig. 4. PinC-immunopositive cells in the toad deep brain. PinC antibody stained CSF-contacting neurons which contact the third ventricle of the hypothalamus (original magnification ×300). Inset, a magnified view of the immunopositive cells (original magnification ×1500).

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