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Source: Zoological Science, 18(3):325-330. 2001.
Published By: Zoological Society of Japan
DOI: http://dx.doi.org/10.2108/zsj.18.325
URL: http://www.bioone.org/doi/full/10.2108/zsj.18.325

ZOOLOGICAL SCIENCE **18**: 325–330 (2001)

Immunohistochemical Localization of Opsins and Alpha-Subunit of Transducin in the Pineal Complex and Deep Brain of the Japanese Grass Lizard, *Takydromus tachydromoides*

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ABSTRACT—Extraretinal photoreceptor cells have been found in the pineal complex and deep brain of a variety of non-mammalian vertebrates. Light signals received by these photoreceptor cells seem to be a potent regulator of diverse physiological responses. Here, the pineal complex and deep brain of the Japanese grass lizard, *Takydromus tachydromoides*, were immunohistochemically analyzed to localize the photoreceptive molecule (opsin) and the light signal-transducing G-protein (transducin). In addition to the pineal organ and parietal eye constituting the pineal complex, we unexpectedly found a parapineal organ, which is located just below the parietal eye and is morphologically similar to the pineal organ. Both organs had photoreceptor-like cells with outer segments immunostained by anti-rhodopsin and anti-pinopsin antibodies. Neither opsin- nor transducin-like immunoreactivities were detected in the parietal eye with all the antibodies tested in this study, although its morphology resembles that of the lateral eyes. In the deep brain region, rhodopsin-like immunoreactivities were observed in the posterior pallial commissure and median eminence. The cerebrospinal fluid-contacting neurons in the paraventricular organ were immunoreactive to an antibody against α-subunit of cone transducin. In lizards, this is the first report showing (1) rhodopsin- and pinopsin-like immunoreactivities in the parapineal organ, (2) rhodopsin-like immunoreactivity in the deep brain, and (3) putative photoreceptive areas in the hypothalamus.

INTRODUCTION

Since the pioneering works on extraretinal photoreceptors by von Frisch (1911), Scharrer (1928) and Benoit (1935), a number of studies have shown the existence of photoreceptors in the vertebrate brain. It is now widely accepted that non-mammalian vertebrates have extraretinal photoreceptor cells in the pineal complex and deep brain (reviewed by Yoshikawa and Oishi, 1998). These photoreceptor cells seem to play important roles in diverse physiological responses such as photo-entrainment of circadian rhythms and detection of seasonal changes of the photoperiod.

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It has been described that the pineal complex of lizards consists of the pineal organ and parietal eye (Oksche, 1965). Circadian rhythms in melatonin synthesis of the isolated pineal organ and parietal eye of lizards are entrained to light-dark cycle (Tosini and Menaker, 1998), and both organs exhibit electrophysiological responses to light stimulation (Dodt, 1973; Solessio and Engbretson, 1993). These results indicate the presence of endogenous photoreceptive molecule(s) in the pineal organ and parietal eye serving as circadian photoreceptors. In addition, the extraocular and extrapineal photoreceptive sites (probably the deep brain) of lizards may be involved in the regulation of seasonal reproduction and circadian physiology (Underwood and Menaker, 1976). However, photoreceptive molecules in the pineal complex and deep brain are less understood in reptilian species, when they are compared with other vertebrate classes (reviewed by Kojima and Fukada, 1999). In birds, pinopsin and rhodopsin are photoreceptive molecules in the pineal gland

(Okano *et al.*, 1994) and deep brain (Wada *et al.*, 1998), respectively. In frogs, the majority of pineal photoreceptor cells are rhodopsin-immunopositive (Masuda *et al.*, 1994; Yoshikawa *et al.*, 1994), whereas both pinopsin and rhodopsin seem to work as deep brain photoreceptive molecules (Yoshikawa *et al.*, 1994; Yoshikawa *et al.*, 1998; Okano *et al.*, 2000). These results suggest roles of rhodopsin and/or pinopsin as non-visual photoreceptive molecules in birds and frogs, and it is possible that lizards have rhodopsin and/or pinopsin-like photoreceptive molecules in their pineal complex and deep brain. To date, no rhodopsin-like immunoreactivity has been detected in the lizard deep brain (Foster *et al.*, 1993, 1994; Grace *et al.*, 1996), whereas pinopsin-like immunoreactivity was observed in the pineal organ (Vigh *et al.*, 1998). From the evolutionary point of view, it is interesting to know whether lizards have either or both of rhodopsin and pinopsin as non-visual photoreceptive molecule.

In the course of immunohistochemical analyses of the pineal complex of the Japanese grass lizard, *Takydromus tachydromoides*, we found an organ, termed parapineal organ, which is morphologically quite similar to the pineal organ. In this study, we examined the immunohistochemical localization of rhodopsin, pinopsin and transducin in the pineal complex including the parapineal organ and in the deep brain.

MATERIALS AND METHODS

Five male and five female Japanese grass lizards (*Takydromus tachydromoides*) were collected in the field in August, 1999 and November, 2000. Animals were treated in accordance with the guidelines of the University of Tokyo.

Lizards were sacrificed at 16:00 (light adapted) or 23:00 (dark adapted), and no significant differences in immunorectivities were observed between tissues from light- and dark-adapted animals. For immunohistochemical analyses, the eye balls, brains (with the pineal organ) and parietal eyes (with the parapineal organ) were fixed with 4% paraformaldehyde in PB (100 mM Na-phosphate; pH7.4) for 9 hr at 4°C or with Bouin's solution for 9 hr at room temperature (23°C). For histological analyses, the heads were fixed with 4% paraformaldehyde in PB for 2 hr at 4°C, then decalcificated with 5% nitric acid and 5% sodium sulfate. The tissues were cryoprotected with increasing concentrations of sucrose (10, 20 and 30% [w/v]) in PB, and embedded in OCT compound (Sakura, Tokyo, Japan). Ten-μm-thick sections were cut out from the embedded tissues, mounted on gelatin-coated glass slides, and air-dried. The sections were pretreated with a blocking solution (1.5% horse or goat normal serum, 0.1% Triton X-100, 10 mM Na-phosphate, 140 mM NaCl; pH 7.4), and then incubated with a primary antibody diluted in the blocking solution for 3 days at 4°C. They were rinsed with PBS (10 mM Na-phosphate, 140 mM NaCl; pH7.4), treated with a secondary antibody for 2 days at 4°C, and washed again with PBS. Then, the sections were coverslipped with Vectashield Mounting Medium (Vector Laboratories, Burlingame, USA). The primary antibodies used were: RhoN-Ab (Wada *et al.*, 1998; mouse antiserum raised against amino-terminal region of chicken rhodopsin, diluted 1:500), anti-dg4 (Y. Taniguchi, O. Hisatomi, M. Yoshida, F. Tokunaga, to be published elsewhere; mouse antiserum raised against carboxyl-terminal region of gecko pinopsin, diluted 1:1000), P9 (Okano *et al.*, 1997; mouse antiserum raised against carboxyl-terminal region of chicken pinopsin, diluted 1:250), PinC-Ab (Yoshikawa *et al.*, 1998; mouse antiserum raised against carboxyl-terminal region of toad pinopsin, diluted 1:540), R2 (Shichida *et al.*, 1989; mouse antibody raised against chicken iodopsin, diluted 1:400), and anti-pTrα and anti-pTcα (Kokame *et al.*, 1993; rabbit antisera raised against the internal sequences of rod and cone transducin α-subunits, respectively, diluted 1:500). For control staining, a primary antibody was replaced by mouse or rabbit preimmune serum. The secondary antibodies used were the horse anti-mouse IgG conjugated with FITC (diluted to 2.5 μg/ml) and the goat anti-rabbit IgG conjugated with FITC (diluted to 2.5 μg/ml), both of which were purchased from Vector Laboratories (Burlingame, USA).

RESULTS

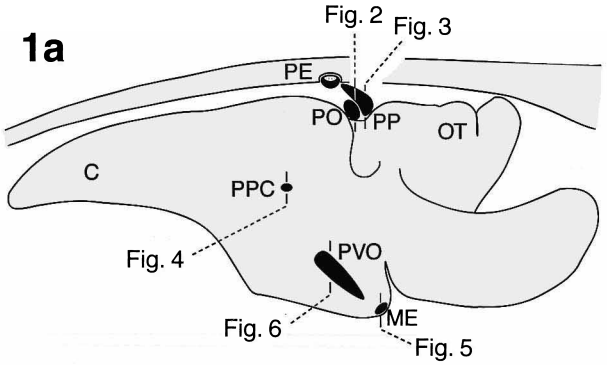
Pineal complex

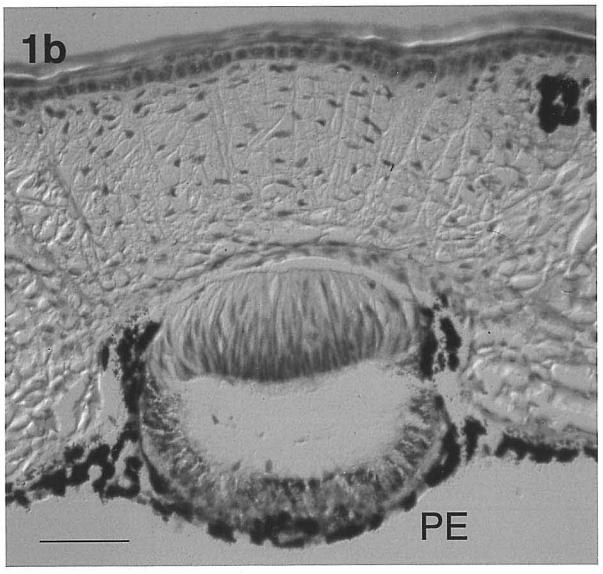
Immunohistochemical localization of rhodopsin and pinopsin was investigated first in the pineal complex located at the parietal region of the Japanese grass lizard (Fig. 1). The follicular cells of the pineal organ protruded their outer segments into the lumen, and some of the outer segments were immunopositive to RhoN-Ab and anti-dg4 (Fig. 2). Although the signals to the former were stronger than those to the latter, we observed almost the same number of immunoreactive outer segments to each antibody. The parietal eye was located within the parietal foramen (Figs. 1a, 1b). It partly resembled the lateral eyes in structure, but no immunoreactivities to RhoN-Ab, anti-dg4, P9 nor PinC-Ab were observed in this organ. In microscopic examination of the nearby region, we unexpectedly found an organ, located just below the parietal eye, with immunoreactivities to RhoN-Ab and anti-dg4 (Fig. 3). The shape and population of the immunoreactive membranes in the newly found organ were quite similar to those in the pineal organ (Figs. 1c, 2, 3). Therefore, we provisionally term it "parapineal organ" in this study (PP in Figs. 1a, 1c). We observed no remarkable differences in morphology between the parapineal organ and pineal organ at the light-microscopic level (Figs. 1c, 2, 3), but these two organs were obviously separated from each other. We observed the parapineal organ in at least 11 individuals out of 13 animals examined.

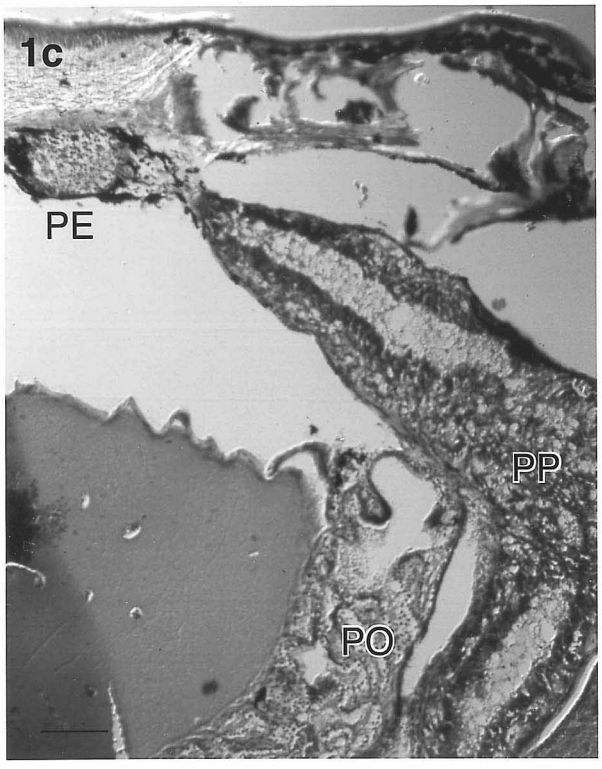
Deep brain

All the sections of whole brain regions dissected out from animals were subjected to immunohistochemical analyses using RhoN-Ab and anti-dg4. RhoN-positive fibers were observed in two regions, the posterior pallial commissure (PPC in Fig. 1, Fig. 4) and median eminence (ME in Fig. 1, Fig. 5) of the brain. The RhoN-positive fibers were immunonegative to anti-pTrα. No immunoreactivity to anti-dg4 was detected in the brain.

pTcα-positive cells were observed in the paraventricular organ (PVO in Fig. 1, Fig. 6a). Most of them were bipolar in shape with bulb-like processes protruding into the third ventricle (arrows in Fig. 6a) and with distal thin processes arborizing into numerous fine fibers (small arrowheads in Fig. 6a). These pTcα-positive cells display characteristics of cerebrospinal fluid (CSF)-contacting neurons, which have been postulated as photoreceptor cells in the brain of various classes of vertebrates (Vigh and Vigh-Teichmann, 1973, 1998). To







investigate whether the pTcα-positive cells have any opsin-like photoreceptive molecules, PVO sections were subjected to extensive immunohistochemical analyses using RhoN-Ab, anti-dg4, P9, PinC-Ab and R2. None of these antibodies, however, immunostained the pTcα-positive cells in PVO, suggesting the absence of rhodopsin-, pinopsin- or iodopsin-like photoreceptive molecules in these pTcα-positive cells.

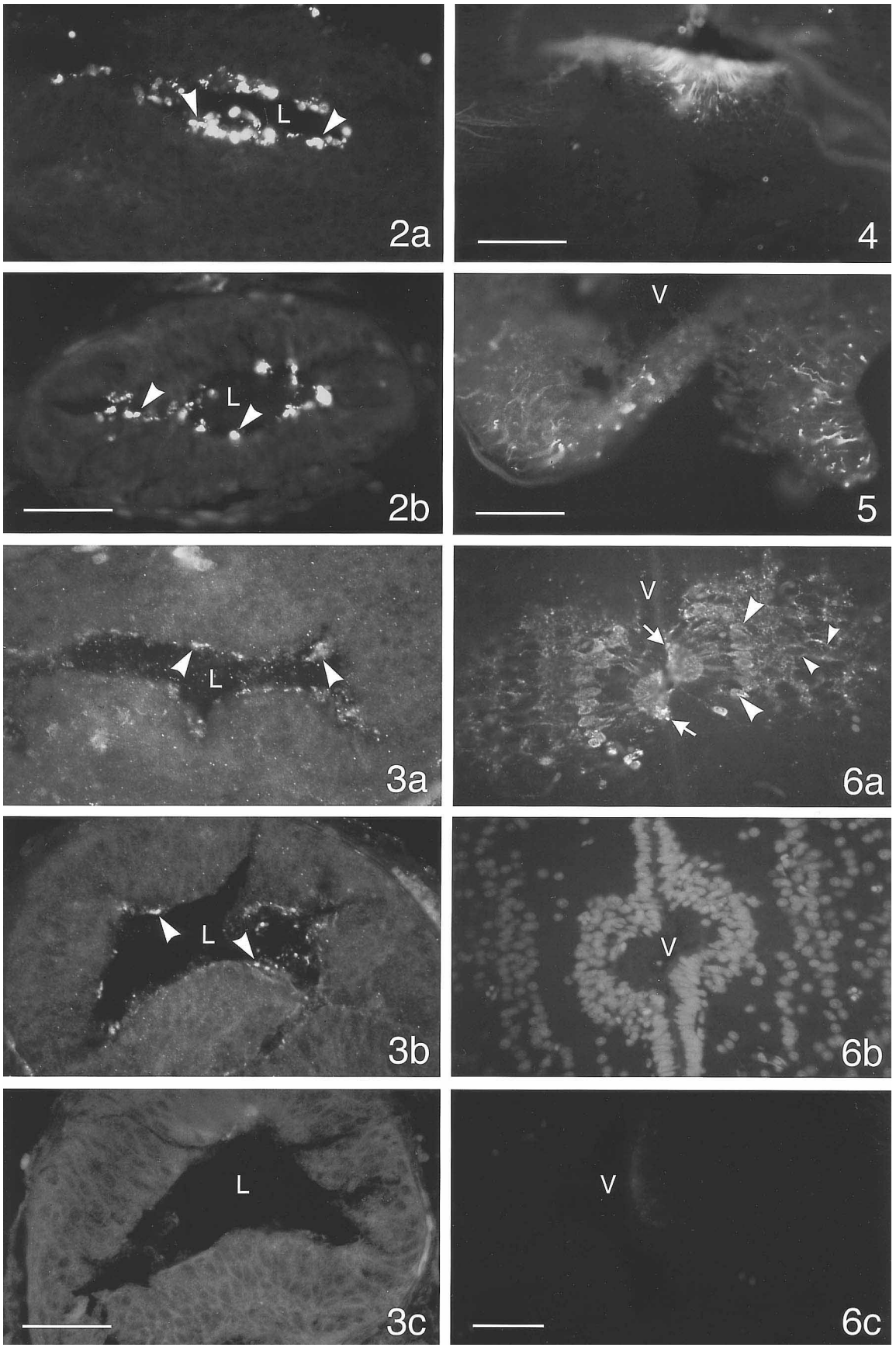
DISCUSSION

The pineal complex of the lizards has been reported to consist of the pineal organ and parietal eye (Oksche, 1965), and the ultrastructure of both organs has recently been described by Ohshima *et al.* (1999). In the course of investigating opsin-like immunoreactivities in the pineal complex, we found an organ immunopositive to RhoN-Ab and anti-dg4, and provisionally termed it “parapineal organ”. To our knowledge, no information is available about the “parapineal organ” of the reptiles, and this is the first report showing the existence of this organ with opsin-like immunoreactivities (Fig. 3).

Lampreys have pineal and parapineal organs, and the two organs are discriminated by their location, morphology and opsin-like immunoreactivities (Tamotsu *et al.*, 1994). On the other hand, the parapineal organ of the lizard observed in this study is similar to the pineal organ in not only its morphological features but also in its opsin-like immunoreactivities (Figs. 1c, 2, 3). The “parapineal organ” might be joined with pineal organ by a thin portion, and correspond to a distal enlargement of the pineal organ (Oksche, 1965). Interestingly, the diagram in the literature (see Fig. 5 in Oksche, 1965) showed the existence of accessory parietal organ just below the parietal eye of lizards. This position is close to the “parapineal organ” found in this study, suggesting another possibility that the “parapineal organ” corresponds to the accessory parietal organ. Alternatively, the lizard “parapineal organ” may be related to one of paired pineal organs which are only occasionally found in quail embryos (Araki and Watanabe,1996). Although we detected no remarkable differences in distribution of opsin-like immunoreactivities between the two organs, further investigations including electrophysiological analyses might reveal the functional significance of this potential photoreceptive site.

Amino acid sequence of the RhoN antigen indicates that RhoN-Ab used in this study will recognize rhodopsin and green-sensitive photopigment in a variety of vertebrates (Wada *et al.*, 1998; Okano and Fukada, 2000). The comparison between the antigen peptides of RhoN-Ab and anti-dg4, along

Fig. 1. Schematic drawing of the brain of the Japanese grass lizard in a sagittal section (**a**). Putative photoreceptive areas are shown in black. The sections of the parietal eye (PE) and pineal organ (PO) with parapineal organ (PP) were stained with hematoxyline-eosin (**b** and **c**, respectively). The parapineal organ seems to conact with the parietal eye. C; cerebrum, ME; median eminence, OT; optic tectum, PE; parietal eye, PO; pineal organ, PP; parapineal organ, PPC; posterior pallial commissure, PVO; paraventricular organ. Bar: 5 μm (**b**), 10 μm (**c**).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with the characterization of these antibodies, supports the notion that the two antibodies recognize opsin-like molecules distinct from each other. In addition, we have previously reported iodopsin-like immunoreactivity in the pineal organ of the same species of the lizard (Masuda *et al*., 1994). Thus, it is likely that the lizard pineal organ has at least three types of opsins (rhodopsin/green, pinopsin and iodopsin) with their populations of positive cells corresponding to each other. This contrasts well with the chicken and frog pineal organs, in which respectively pinopsin- and rhodopsin-immunopositive cells seem to be the predominant photoreceptors (Masuda *et al*., 1994; Yoshikawa *et al*., 1994; Okano *et al*., 1997).

Our efforts to identify opsin(s) in the parietal eye were unsuccessful. The isolated parietal eye shows electrophysiological responses to light stimulation (Solessio and Engbretson, 1993) and also exhibits a melatonin rhythm which is entrainable to the environmental light-dark cycle (Tosini and Menaker, 1998). RT-PCR analysis demonstrated expression of transcripts for four types of opsins, violet, blue, red, and pinopsin, in the parietal eye of *Anolis* (Kawamura and Yokoyama, 1997). This, together with the wide distribution of pinopsin in the non-mammalian vertebrate brains, has let us first to detect pinopsin-like immunoreactivities in the pineal complex of the lizard by using antibodies against chicken pinopsin (P9) and toad pinopsin (PinC-Ab). However, both antibodies gave no positive signals on the lizard brain sections (data not shown). These negative results may be due to amino acid sequence divergence between reptilian and chicken/toad pinopsins in the region used as antigen for raising the antibodies. Then, we used an antibody against gecko pinopsin (anti-dg4). This antibody immunostained a number of follicular cells in the pineal and parapineal organs (Figs. 2b, 3b), but it gave no positive signals in the parietal eye. Our previous immunohistochemical analyses also failed to reveal red- (iodopsin) and rhodopsin-like immunoreactivities in the parietal eye of the Japanese grass lizard (Masuda *et al*., 1994), and others have been unable to detect opsin-like immunoreactivities in the parietal eye of iguanid lizards (Foster *et al*., 1993; Grace *et al*., 1996). One possible explanation for the apparent inconsistency between the results of RT-PCR and immunohistochemical analyses is that some of the photopigment genes (at least red and pinopsin) are transcribed, but not translated to a level detectable by conventional immunostaining procedures. Alternatively, antibodies to violet- and blue-sensitive photopigments may help to investigate opsin molecule(s) obviously expressed in the parietal eye of lizards (cf. Kawamura and Yokoyama, 1997).

The present immunohistochemical approach using an antibody against rhodopsin (RhoN-Ab) was successful in labeling fibers in the posterior pallial commisure (PPC; Fig. 4) and median eminence (ME; Fig. 5) of the lizard brain. This is the first demonstration of rhodopsin-like immunoreactivity in the lizard deep brain, although cone opsin-like immunoreactivities have been detected in the septum of the iguanid lizards (Foster *et al*., 1993, 1994; Grace *et al*., 1996). Rhodopsin-like immunoreactivity has been detected also in the deep brain of birds (Silver *et al*., 1988; Wada *et al*., 1998) and frogs (Yoshikawa *et al*., 1994; Okano *et al*., 2000). Taken together, rhodopsin may have been conserved as a deep brain photoreceptive molecule through the evolution of vertebrates from Amphibia to Aves.

In birds, the deep brain photoreceptive areas include median eminence, infundibular region, dorsal inferior hypothalamic region, medial preoptic region and lateral septum (reviewed by Kuenzel, 1993). These areas just correspond to or are located very close to the potential photoreceptive sites of the lizard brain identified in this study: PPC is a part of the septal region which contains the lateral septum (Font *et al*., 1998), and PVO (paraventricular organ) is included in the dorsal inferior hypothalamic region. In the lizard PVO, we were unable to find any rhodopsin-, pinopsin- nor iodopsin-like immunoreactivities, in contrast to our previous observation of rhodopsin-like immunoreactivity in PVO of the Japanese quail (Yoshikawa and Oishi, 1998). Considering recent findings of novel members of the opsin family such as parapinopsin (Blackshaw and Snyder, 1997), melanopsin (Provencio *et al*., 1998), VA/VAL-opsin (Soni *et al*., 1998; Kojima *et al*., 2000), and encephalopsin (Blackshaw and Snyder, 1999), we speculate that the pTcα-positive cells in the lizard PVO may contain such a novel opsin.

Fig. 2. RhoN-Ab (**a**) and anti-dg4 (**b**) immunoreaction of the pineal organ in cross sections. The outer segments of the follicular cells were immunopositive (arrowheads). When the primary antibody was replaced by preimmune serum, no immunoreactivity was observed. L; lumen. Bar: 50 μm.

Fig. 3. RhoN-Ab (**a**) and anti-dg4 (**b**) immunoreaction of the parapineal organ in cross sections. The outer segments of the follicular cells were immunopositive (arrowheads). When the primary antibody was replaced by preimmune serum, no immunoreactivity was observed (**c**). L; lumen. Bar: 50 μm.

Fig. 4. RhoN-Ab immunoreaction of the pallial commissure in a cross section. Dense arrays of immunopositive fibers were observed. When the primary antibody was replaced by preimmune serum, no immunoreactivity was observed. Bar: 100 μm.

Fig. 5. RhoN-Ab immunoreaction of the median eminence in a cross section. Scattered immunopositive fibers occurred in the basal part on the hypothalamus. The fibers were perpendicular to the wall of the third ventricle. When the primary antibody was replaced by preimmune serum, no immunoreactivity was observed. V; third ventricle. Bar: 100 μm.

Fig. 6. Cross sections of the paraventricular organ. (**a**) Cells immunopositive to anti-pTcα (cell bodies, large arrowheads) extend immunopositive processes into the recess of the wall of the third ventricle, where they form bulb-like structures (arrows). The distal immunopositive processes arborize into fine fibers (small arrowheads). (**b**) The same section as in (**a**) stained by DAPI. (**c**) Section adjacent to that in (**a**) treated with preimmune serum (negative control). V; third ventricle. Bar: 100 μm.

ACKNOWLEDGEMENTS

We thank Prof. A. Oksche for critical reading of the manuscript and for helpful comments. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture, and T.Y. was supported by Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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(Received October 14, 2000 / Accepted December 25, 2000)