Brain photoreceptors and the pineal organ are considered as extra-retinal photoreceptors in birds. Deep-brain photoreceptors have not yet been identified morphologically, although physiological studies have accumulated evidence for the existence of a photoreceptor in the avian brain for several decades. Several pioneering works concerning an extra-retinal photoreceptor in birds have been published in the last decade. First, in 1988, Silver et al.51) demonstrated that opsin-like immunoreactivity in the avian brain was present in cerebrospinal fluid (CSF)-contacting neurons of the lateral septum and infundibulum. Since then, a CSF–contacting neuron has emerged as a viable candidate for a deep-brain photoreceptor in birds. Then, in 1994, Okano et al.36) isolated a cDNA clone encoding an opsin-like molecule from a chicken pineal cDNA library, and showed that the coded protein with bound 11-cis-retinal exhibits blue–light sensitivity with maximal absorbance at ~470 nm. They named this protein “pinopsin” in reference to pineal opsin. Most recently, Wada et al.63) (1998) detected rhodopsin gene expression in the lateral septum of the pigeon. In this paper, I review especially the immunohisto- and cytochemical issues relevant to the localization of the brain photoreceptors in the avian brain and give an overview of the morphological bases for the avian extra-retinal photoreceptor. Before presenting morphological considerations, I describe briefly the physiological background of brain photoreceptors in birds.

Physiological evidence for existence of brain photoreceptors

Since the concept of extra-retinal photoreception originated with Karl von Frisch (1911), many studies have been done to clarify his concept and to localize the extra-retinal photoreceptor in several nonmammalian species (see reviews of Oliver and Baylé46), Oksche36,40), Kuenzel27). On the basis of physiological responses to photic stimuli, several kinds of experiments have been reported over half a century. Photic stimuli are one of the most important environmental signals regulating reproductive function in birds. At an early step in their research, Benoit and his
colleagues demonstrated that a photoreceptor other than the retina is involved in the photoperiodic response of the testis in the duck (Benoit4)). There is a relationship between circadian rhythms and the photoperiodic control of reproduction. Photic stimuli act as the primary zeitgeber among birds. Entrainment of a light-dark cycle of birds can thus serve as an assay of photic perception. The pioneer work of Menaker31) showed that the circadian activity rhythms of blinded house sparrows entrain to 24-hour cycles of visible light. An injection of India ink under the bird's head skin abolished the entrainment and free run (Menaker34)). He thought that the sparrow must possess an extra-retinal photoreceptor which is coupled to its biological clock, and that the photoreceptor is in the brain. Subsequent experiments showed that removal of the pineal organ does not abolish the entrainment response of blinded birds (Menaker34)). In the field, the testis of birds shows a dramatic annual change in spermatogenic activity reflected by changes in weight. Recrudescence in postrefractory birds can be stimulated by artificially long photoperiods in the laboratory. With regard to the testicular response to the photic period in the house sparrow, Menaker and his colleagues demonstrated that the testes of blinded house sparrows recrudesce in response to artificially long days (Menaker and Keatts32)). Removal of the pineal organ does not affect either the response to light or the process of recrudescence (Menaker et al.33)). Recrudescence was also abolished if India ink was injected under the head skin of the sparrows, and was restored after removal of the ink (Menaker et al.33)). Based on these data, they considered that in the house sparrow, as in the duck, extra-retinal photoreception is involved in the photoperiodic response of testicular growth, and that the extra-retinal photoreceptor mediating testicular growth must be in the brain.

There have been morpho-physiological attempts to localize the photoreceptive sites of the brain in photoperiodic birds. Homma and Sakakibara22) demonstrated that the implantation of small discs or spheres coated with radioluminescent paint (RLP) along the fissura longitudinalis cerebri in the hypothalamic vicinity induced a rapid testicular development in the Japanese quail. Similar experiments were done by Oliver and his colleagues. After RLP was implanted in the tuberal or the dorsal area of the infundibular complex of non-photostimulated quails, testes were markedly developed (Oliver and Baylé42()). This confirmed that neuronal populations located in the infundibular complex are directly photosensitive. By similar radioluminous implantation and hypothalamic deafferentation, Oliver et al.42) showed that (1) neuronal populations of the basal hypothalamus were directly stimulated by radioluminous material (RLM) implants without any participation of retinal photoreceptors, and (2) that basal hypothalamic structures exhibited intrinsic photosensitivity and gonadostimulating ability. Direct selective photic stimulation of RLM in the infundibular complex increased the testicular weight as well as the plasma testosterone and luteinizing hormone levels in quail (Oliver et al.42()). Gonadal growth of the white-crowned sparrow can be induced by direct encephalic photostimulation. Direct illumination of light-conducting fibers within and near the ventromedial hypothalamus resulted in testicular growth and increased plasma levels of luteinizing hormone and testosterone (Yokoyama et al.65()).

In addition to the major photoreceptor in the basal hypothalamus (the infundibular complex), there seem to be other light-sensitive sites in the brain of the bird. Direct implantation of RLP into the olfactory lobe of the quail induced testicular growth in more than 50% of the birds (Homma and Sakakibara22)). Furthermore, the 1979 work of Homma et al.23) described at least two photosensitive sites in
the brain of the quail. According to their results (shown in Fig. 1 of Homma et al.23), deep intrahypothalamic implants of radioluminous beads induced testicular growth. In addition, surface implants in which beads were implanted along the fissura longitudinalis cerebri also induced testicular growth (birds No. 5-9 in Fig. 1 of Homma et al.23)). In that work, the authors seemed to consider the later photosensitive site as "a vicinity of the hypothalamic area." Direct photic stimulation of the paroolfactory lobe by RLM resulted in significant testicular growth and an increased plasma testosterone level (Oliver et al.45)). In an electrophysiological study using multiunit activity, Sicard et al.50) suggested that some paraolfactory cells are endowed with an intrinsic photosensitivity. From these studies, photosensitive areas of the brain were located in or close to the hypothalamus, and in the telencephalic structures. However, "nothing is known about the mechanisms by which deep rhinencephalic or infundibular neurons are able to pick up and transduce photic energy, allowing for gonadotrophic activation in birds" (Oliver et al.45)). Recently, Perera and Follett48) have used hypothalamic explants from quail and measured the dynamics of GnRH release induced by photoperiodism. It was thought that the hypothalamic block included the GnRH neurons as well as the anterior and tuberal hypothalamus, which are essential for the transduction of photoperiodism. A physiological study of an action spectrum for photoperiodic response suggests that a photopigment contained in the brain photoreceptor very closely resembles a rhodopsin in the quail (Foster and Follett8, Foster et al.9)).

Search for brain photoreceptor with conventional light and electron microscopy

Compared with the physiological approach, morphological approaches to a search for deep encephalic photoreceptor cells have not been as fruitful, probably because of methodological limitations. Even using a conventional electron microscope, a very thorough search for a photoreceptor-like structure such as the outer segment membrane specialization in the avian hypothalamus has failed (Oksche40)). No specialized cells with structures and pigments characteristic of typical photoreceptive units have been demonstrated in the avian hypothalamus (Hartwig, 1975, from Yokoyama et al.65)). However, the CSF-contacting neurons of the avian hypothalamus have long been

Fig. 1. Cerebrospinal fluid-contacting neuron revealed by VIP (vasoactive intestinal peptide)-immunocytochemistry in the infundibulum of the pigeon. VIP-immunoreactivity is observed throughout the neuron. Arrow indicates a dendritic process running toward the third ventricle (3 V). A ventricular terminal (VT) of the neuron protrudes into the third ventricle. The shape of the ventricular terminal closely resembles the inner segment of a pinealocyte. NU: nucleus of CSF-contacting neuron, EP: ependymal cell. Bar: 500 nm (from A. Okamura, graduate thesis of Nagoya Univ. 1996).
considered as a candidate for the deep-brain photoreceptor (Yokoyama et al.). This special kind of neuron was named cerebrospinal (CSF)-contacting neuron by Vigh, a Hungarian neuroanatomist, in 1969 (Vigh and Vigh-Teichmann) (Fig. 1). Such neurons send one of their processes into the ventricle. These ventricular terminals are ciliated. The peculiar location of these neurons such as neuronal elements of the paraventricular organ (PVO) suggests that their activity may be connected to some receptive function. The PVO is well developed in reptiles and birds. It has two deeper layers of ganglion-like arrangements of nerve cells. On the basis of neuroanatomy of the PVO, it resembles somewhat the organization of a retina. Furthermore, conventional electron microscopy demonstrated that they have some basic fine structural features in common with modified pineal photoreceptors and the photoreceptor cells of the developing retina. The common ultrastructural features of the CSF-contacting neurons are the inner segment-like bulbous structures which project to the third ventricle. These structures are sometimes endowed with a cilium (see also Vigh-Teichmann and Vigh).

**Opsin-immunohistochemistry of brain photoreceptors in avian and non-avian species**

Immunohistochemistry is of great use in localizing the retinal and extra-retinal photoreceptor cells. By the use of antibodies against photoreceptor-specific proteins such as opsin, transducin, and S-antigen, several authors conducted an immunocytochemical search for the deep-brain photoreceptor of the avian brain. As described above, the CSF-contacting neurons of the avian hypothalamus (especially in the paraventricular organ) have been considered as a candidate for deep-brain photoreceptors. Using antibodies against opsin, transducin, and retinal S-antigen (arrestin), Foster et al. failed to identify the photoreceptor-specific molecules in the hypothalamus of Japanese quail, although opsin immunoreactivity was demonstrated within many retinal photoreceptor cells and in a limited number of pinealocytes of the quail. On the other hand, Silver et al. demonstrated opsin-like immunoreactivity in the avian brain in CSF-contacting neurons of the septal and infundibular areas using a monoclonal antibody (RET-P1) raised against a membrane fraction of rat retina, and which is known to bind the amino-terminal of rhodopsin (Barnstable et al., Feket and Barnstable, Hargrave et al.). They used also Cos-1, a monoclonal antibody, raised against a chicken cone visual pigment (Silver, Saldanha et al.). According to their studies, this antibody recognizes CSF-contacting neurons of identical morphology as RET-P1 in the lateral septum of the ring dove, although the number of immunoreactive cells is less than that of RET-P1 immunoreactive cells. Concerning another type of photoreceptor-specific protein, anti-arrestin-labelled cells were reported in the medial preoptic area, and in the periventricular area of the paraventricular nucleus in the dove. However, no immunoreactive cells were reported in the septal or infundibular regions. The antibody used seems to cross-react with neurophysin in the dove brain (Saldanha et al.). The recent work of García-Fernández and Foster showed opsin-like immunoreactive hypothalamic CSF-contacting neurons in the larval lamprey with the antibodies CERN-JS 858, a polyclonal anti-bovine rod-opsin, and Cos-1. However, using the same methodologies, they failed to identify any opsin-immunoreactive cells in the brain of the Japanese quail or chick. Most recently, rhodopsin cDNA was cloned from the pigeon lateral septum RNA fraction (Wada et al.). An antibody (RhoN) which recognizes the amino-terminal region of pigeon rhodopsin was prepared for use in an immunohistochemical study to identify photoreceptor cells in the lateral septum of the pigeon. Immunoreactive CSF-contacting neurons were detected.
Immunoreactivities were found throughout neurons including ventricular terminals, cell bodies and basal processes (Wada et al. 63). These findings are in agreement with the findings of RET-P1 immunoreactive CSF-contacting neurons described by Silver et al. 51)

Taking these immunocytochemical studies into account, although the chemical nature and localization of deep-brain photoreceptors of avian species is still controversial among researchers, the CSF-contacting neurons of the lateral septum were detected immunohistochemically by the use of two types of amino-terminal specific rhodopin antibodies (see Table 1 and Foster et al. 12). About three decades ago, they probably did not focus their attention on the lateral septum of birds as a location of deep-brain photoreceptors. However, an experiment in brain surface implants of radioluminous beads showed induced testicular growth in mail quail (Homma et al. 23). In that experiment, radioluminous beads were implanted along the fissura longitudinalis cerebri of the quail. The sites of implantation were anatomically very close to the lateral septum of the quail. Photo-stimuli of radioluminous beads may penetrate into the lateral septum from the brain surface and stimulate the CSF-contacting neurons of the lateral septum. To date, no electrophysiological evidence for the photoreception of the CSF-contacting neuron of the avian lateral septum has been reported. Further studies are needed to characterize the photoreceptor-specific proteins of the avian deep brain in the infundibular region and to define species differences in the brain photoreceptive systems in birds.

Although a limited number of immunohistochemical studies on the avian deep-brain photoreceptor is available, the study of opsin-like protein-expressing cells has recently been undertaken on brains of non-avian species, because the phylogenetic point of view is important in precisely understanding such photoreceptors. Early in the 1980s, immunocytochemical studies using an antibody raised against bovine opsin failed to demonstrate any immunoreactive cells within the hypothalamus of lower vertebrates. They could not find any opsin immunoreactivity in CSF-contacting neurons of the paraventricular organ, the infundibular nucleus or the magnocellular and parvocellular preoptic nuclei of the newt at the light microscopic level (Vigh-Teichmann et al. 61). Furthermore, they failed to demonstrate opsin immunoreactivity in either the hypothalamic CSF-contacting neurons in the magnocellular preoptic nucleus or in the anterior periventricular nucleus of the European minnow, nor in the periventricular gray of the mammillary recess of the goldfish or the paraventricular organ of the frog (Vigh et al. 60). Ultrastructurally, the ciliary membrane of the intraventricular terminals of the CSF-contacting neurons studied were negative (Vigh et al. 60). However, opsin immunoreactive cells were recently reported in the brain of lower vertebrates, e.g., within the hypothalamus of the larval lamprey (García-Fernández and Foster 13) and of three species of adult lamprey (García-Fernández et al. 14). These cells were labelled with opsin antibodies CERN-JS 858 and Cos-1. Immunoreactive cells are CSF-contacting neurons, and most possess a bipolar cell body. Many cells had a long thin process that extended to the ventricular surface and terminated in a single bulb-like protrusion, contacting the cerebrospinal fluid of the third ventricle.

Concerning the deep-brain photoreceptors of amphibians, using three antisera against bovine rhodopsin, α-subunits of bovine rod and cone transducine, all three antisera–immunoreactive cells were found in areas corresponding to the suprachiasmatic nucleus and preoptic nucleus of the hypothalamus in the bullfrog (Yoshikawa et al. 65). They are
Table 1. Summary of published and unpublished results of opsin- and VIP (vasoactive intestinal peptide)-like immunostaining within the avian brain

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<th>Antiserum</th>
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<td>Ring dove</td>
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<td>Hypothalamus</td>
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<td>Junco</td>
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<td>Septum</td>
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<td>Hypothalamus</td>
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Distributed along the third ventricular wall and have a round or spindle-like cell body with one or two neuronal processes. This feature characterized some immunoreactive cells as CSF-contacting neurons. In 1998, Yoshikawa et al. have cloned a cDNA encoding a deep brain photoreceptive molecule from the hypothalamus of Bufo japonicus. The deduced amino acid sequence closely resembles that of pinopsin (toad pinopsin). Using antibodies against toad pinopsin, they demonstrated immunoreactive CSF-contacting neurons in the anterior preoptic nucleus. This hypothalamic region is considered to play an important role on reproductive behaviour of toad. They suggested that toad pinopsin acts as a photoreceptive molecule for the photoperiodic gonadal response.

In such a very important line of research, identification of the deep-brain photoreceptors of reptilian species is phylogenetically required. Recent progress in the study of deep-brain photoreceptors was made in the reptilian brain. Foster et al. employed three
antibodies which bind retinal cone opsins in the brain of the lizard *Anolis carolinensis*. They have immunolabelled CSF-contacting neurons located at the lateral ventricular border within the nucleus ventromedialis of the septum. The lateral ventricular border of the lizard is anatomically identical to the septal area of the ring dove, quail and duck (Silver et al.51). Grace et al.15 demonstrated fine structures of opsin-immunoreactive CSF-contacting neurons in the brains of *Anolis carolinensis* and *Iguana iguana* by the use of immunohistochemical techniques. Numerous electron-dense vesicles are found in the cytoplasm of opsin-immunoreactive CSF-contacting neurons of both species. These vesicles are large (70–120 nm in diameter) and occur throughout the soma and the dendritic process and within the intraventricular terminal. Similar electron-dense vesicles are found in the VIP-immunoreactive CSF-contacting neurons of the lateral septum of the duck (Hirunagi et al.19). Numerous electron-dense vesicles in the cytoplasm are a common ultrastructural feature of a putative deep-brain photoreceptor of reptiles and birds. However, the functional relationships between electron-dense vesicles and photoreception is still unclear.

**Pinopsin and pineal photoreceptors**

Although a number of experiments suggest that the avian pineal plays no major role in avian photoperiodic response, it certainly appears to contain photoreceptors in the organ. The photoreceptive molecule of the chicken pineal has been implicated in the photic entrainment of the circadian pacemaker which controls the rhythmic production of melatonin in the organ (Deguchi5,43). Recently, Okano et al.36 cloned a blue-light sensitive molecule grouped with a novel class of opsins from a chicken pineal cDNA library. They have named this protein "pinopsin" after pineal opsin. Highly specific antibodies against pinopsin were prepared to uncover the immunocytochemical localization of pinopsin in the pineal organ (Okano et al.37). Using a pinopsin-antibody, Hirunagi et al.20 revealed the subcellular localization of pinopsin in the pineal organ of the chicken. Pinopsin is exclusively expressed in the sensory structures of the modified photoreceptor of the chicken pineal organ. Pinopsin-immunoreactive concentric lamellar complexes and cilium-like structures were frequently observed in the follicular lumen and parafollicular region of the chicken pineal organ (Hirunagi et al.20, Okano et al.37) (Fig. 2). The CSF-contacting neurons in the lateral septum have some basic structural features in common with modified pineal photoreceptors.

Avian pineal photoreceptors and putative brain photoreceptors morphologically resemble each other. Figure 3 shows schematically three different types of modified sensory structures observed in the pineal organ and brain of avian species. First, the concentric lamellar structures connected to the inner segment of pinealocytes with ciliary portion are modified sensory structures (Fig. 3a). These structures are predominantly observed in the pineal organ of the pigeon (Vigh et al.58,59) and *Passer domesticus* (Oksche and Kirschstein38). They are outer segments closely resembling the outer segments of the pinealocytes of lower vertebrates. Second, bulbous cilia protruding from the ciliary basal body are also modified sensory structures (Fig. 3b). Although they do not show lamellae comparable to the comb-like arrangement of membranes in the typical pineal photoreceptor of lower vertebrates, pinopsin-immunohistochemistry shows that this type of modified cilium contains pinopsin as a photopigment in the pineal organ of the chicken (Hirunagi et al.20, Okano et al.37) (Fig. 2). Third, a ventricular bulbous process of CSF-contacting neurons is another modified sensory structure (Fig. 3c). This process usually bears a sensory cilium but lacks the outer segment of a photoreceptor. Opsin – im-
munoreactivity is observed throughout the cytoplasm including the ventricular process of some CSF-contacting neurons in the lateral septum (Silver et al.\cite{51}, Wada et al.\cite{52}) and infundibulum (Silver et al.\cite{51}). This type of modified sensory structure of the avian species is considered as a highly degenerated structure in the reverse evolutionary direction of photoreceptors.

**VIP– and opsin–immunohistochemistry of brain photoreceptors**

In the pioneering work of Silver et al.\cite{51}, they demonstrated that opsin–immunoreactive CSF–contacting neurons coexpress VIP (vasoactive intestinal peptide)–immunoreactivity in the lateral septum and infundibulum of birds. Interestingly, a similar observation was made in the lateral septum of the reptile. Using different types of opsin antibodies, Grace et al.\cite{15} demonstrated that opsin–immunoreactive CSF–contacting neurons coexpress VIP immunoreactivity in the brains of Anolis carolinensis and Iguana iguana. VIP–immunoreactive CSF–contacting neurons were reported in the lateral septum of several reptilian species (Hirunagi et al.\cite{17}). According to our unpublished data, opsin–immunoreactive CSF–contacting neurons also coexpress...
VIP-immunoreactivity in the lateral septum of the pigeon. It seems that the opsin- and VIP-immunoreactive cells described here may be photoreceptors mediating light effects on circadian and reproductive rhythms. VIP immunoreactive CSF-contacting neurons are reported by several authors in avian species. Immunohistochemical results from VIP- and opsin-immunohistochemistry of the lateral septum and hypothalamus of birds have been presented in Table 1. In the female rat, recent studies have demonstrated the presence of a monosynaptic projection from the suprachiasmatic nucleus to the GnRH neurons in the preoptic hypothalamus, containing VIP as a putative transmitter (Van der Beek et al.55,56).

**Neural integration of brain photoreceptors**

Very little work has been done on the connectivity of the deep-brain photoreception system in birds. In male Japanese quail at least two hypothalamic regions are responsible for controlling photoperiodically-induced gonadotrophin release and growth of the testes, i.e., the preoptic region and the posterodorsal part of the infundibular nuclear complex. After neural isolation of the basal hypothalamus, testes growth was induced by environmental photostimulation in the quail (Oliver et al.43). Recent immunohistochemical studies have revealed that GnRH-immunoreactive neurons distribute in the septal-preoptic region of birds (Mikami et al.35, Kuenzel and Blähser26, Silver et al.54). The anatomical substrate for the regulation of photostimulation with the hypothalamo-pituitary-gonadal axis is not known. Some information is available on the connectivity of the brain areas, such as the preoptic medial nucleus, but not enough is yet known to construct a hypothetical neural circuit that mediates the processing of environmental photo information in birds (Ball2). Neural input to the hypothalamic GnRH neurons was investigated in male starlings using immuno-electron microscopy. According to that study, there were significantly more axo-somatic terminals and more synaptic modification on the GnRH neurons in the long day refractory birds (Pary and Goldsmith47). VIP immunoreactive processes innervate to a peptidergic neuron of the lateral septum in the pigeon (Hirunagi et al.10). To uncover the brain circuits involved in photoperiodism in birds, several new methods have been employed. A recent study of Meddle and Follett30 demonstrated that photoperiodic stimulation of quail results in the activation of fos-like immunoreactivity within neurons of the basal tuberal hypothalamus. They thought that some neurons of the tubero-infundibular complex are involved in a specific fashion in the avian photoperiodic response. These sorts of double-labelling, tract-tracing studies are useful for the elucidation of neuronal pathways mediating photo information in the brain of birds. Most recently, Kiyoshi et al.40 demonstrated direct anatomical contact between the VIP axon and the GnRH neuron in the septal preoptic area of the pigeon by immunocytochemistry of a confocal scanning- and electron microscope. As shown in Table 1, some VIP neurons of birds show opsin-immunoreactivity in the lateral septum and infundibulum. Although there is no direct evidence of a connection between deep-brain photoreceptor and GnRH neurons in the bird, deep-brain photoreceptors will exist in the neural network involved in the reproductive systems of birds (Silver and Ball20). Further tract-tracing studies will be needed to clarify neuronal connections between avian photoreception and reproductive systems.

In summary, avian deep-brain photoreceptors are not fully understood. However, morphological evidence obtained from opsin-immunohistochemistry suggests the presence of a photoreceptor in the avian brain. CSF-contacting neurons of the lateral septum and infundibulum are the most viable candidates.
for the deep brain photoreceptor, because they contain photoreceptive molecule(s) revealed by biochemical and immunohistochemical studies. Many physiological studies provide abundant evidence in support of the presence of deep-brain photoreceptors in the avian brain, and their functional significance is well defined. However, the anatomical substrate for the correlative functioning of photoreceptive and reproductive systems is still unclear. Further anatomical studies will be needed to elucidate the neuronal circuits involved in these two avian systems.

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