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Immunoelectron-microscopic investigation of the subcellular localization of pinopsin in the pineal organ of the chicken

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Abstract. Pinopsin is a photoreceptive molecule cloned from the chicken pineal organ. An antibody highly specific for pinopsin was applied in light- and electron-microscopic immunocytochemical studies of the pineal organ of 1 to 2-month-old chickens. Intense immunoreactivity was found in the follicular lumen at the light-microscopic level. In addition, small immunoreactive spherical or fibrous structures were diffusely distributed at the parafollicular aspect of the pineal organ. To identify immunoreactive elements precisely, we used pre-embedding immunoelectron microscopy. These studies revealed immunoreactive outer segments of pinealocytes arranged closely side by side in the follicular lumina. The thin initial portion of the outer segment arose from a basal body located in the inner segment. Immunoreactive pear-shaped outer segments occupied small lumina. Follicular lumina displayed immunonegative arrays of whorl-like lamellar membranes. Occasionally, these immunonegative structures were surrounded by immunoreactive concentric lamellar complexes. In the parafollicular pineal parenchyma, long slender cilium-like structures or enlarged cilia and concentric lamellar arrays showed intense immunoreactivity. All immunoreactive structures observed in this study were considered to represent outer segments of pinealocytes of the chicken pineal organ.

Key words: Pineal organ – Pinealocytes – Pineal photoreceptors – Sensory structures – Photopigment – Pinopsin – Immunocytochemistry – Chicken

Introduction

In light- and electron-microscopic immunocytochemical investigations with anti-opsin antibodies, a positive immunoreaction has been found in the outer segment-like structure of the modified photoreceptor cells of the avian pineal organ (Vigh and Vigh-Teichmann 1981, 1988; Vigh et al. 1982; Araki et al. 1992). The modified photoreceptor cell of the avian pineal organ is generally considered to be an element having the phylogenetic capacity of light sensitivity (for reviews, see Oksche 1971, 1983; Menaker and Oksche 1974; Vollrath 1981; Vigh and Vigh-Teichmann 1988; Korf 1994). The photoreceptive molecule of the chicken pineal organ has been implicated in the photic entrainment of the circadian pacemaker in the gland (Deguchi 1979, 1981). Recently, Okano et al. (1994) have isolated a cDNA clone, encoding an opsin-like molecule, from a chicken pineal cDNA library and have shown that the coded protein with bound 11-cis-retinal exhibits blue-light sensitivity with maximal absorbance at ~470 nm. We have named this protein pinopsin in reference to pineal opsin. By means of light-microscopic immunohistochemstry with a pinopsin-specific antibody, we have detected positive cellular elements in the pineal organ of the chicken and pigeon (unpublished data), without being able to define the subcellular structures corresponding to these elements. In the present study, we have applied the same pinopsin-specific antibody in pre-embedding immunoelectron microscopy for the subcellular localization of pinopsin in the chicken pineal organ.

Materials and methods

Pinopsin-specific antibody P7 was raised in mice against the MBP-p7 fusion protein, which consists of maltose-binding protein (MBP) and carboxyl-terminal 75 amino-acid residues of pinopsin (Pro 247-Val 351: p7 peptide). The anti-MBP antibodies were eliminated from the antiserum by passing it through an MBP-immobilized Sepharose column. The carboxyl-terminal region (p7 peptide) was selected as an antigen, because the amino-acid se-

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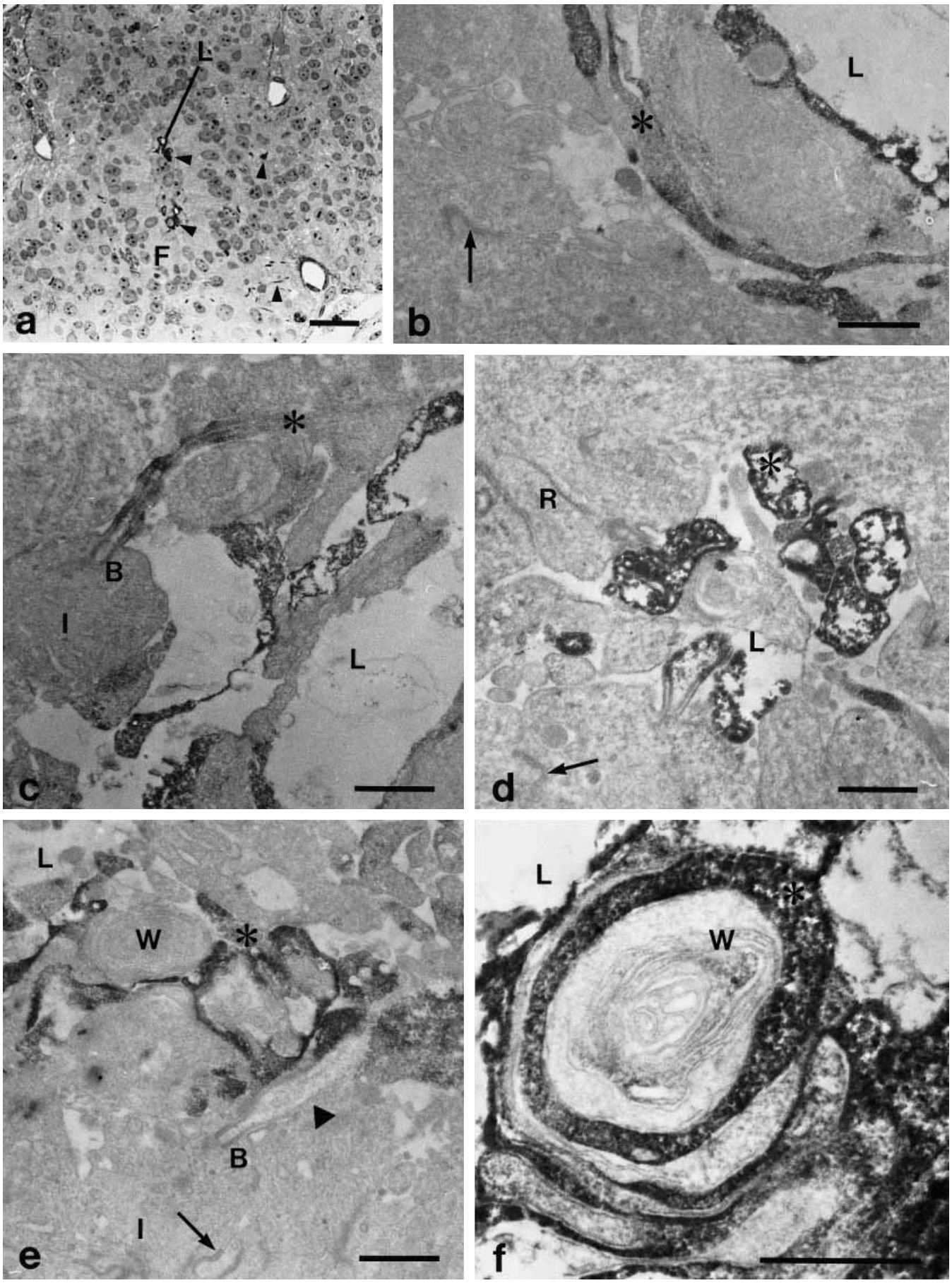
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quence of pinopsin at this region is highly divergent from that of retinal photoreceptive molecules. Indeed, the resultant antibody P7 is highly specific for pinopsin and cross-reacts with neither rhodopsin nor cone pigments purified from chicken retinas.

Four 1 to 2-month-old male chickens were used for immunocytochemical studies. Anesthetized animals were perfused through the heart with a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 100 mM phosphate buffer (pH 7.4). After perfusion, pineal glands were removed from the brain and cut into 50-μm-thick sections with a Vibratome (Technical Products International, USA). These sections were processed for immunocytochemical staining by the P7-antibody against pinopsin. Sections were incubated for 30 min at room temperature in 100 mM phosphate-buffered saline (PBS) containing 10% normal rabbit serum. They were subsequently incubated for 3 days at 4°C in mouse polyclonal antibody directed against pinopsin (P7) diluted 1:50 in 100 mM PBS containing 1% bovine serum albumin. Following rinses in three changes of 100 mM PBS for 30 min, sections were incubated for 90 min in biotinylated rabbit anti-mouse IgG (1:50 dilution; DAKO, Denmark) and then rinsed in 100 mM PBS. They were incubated for 90 min in 100 mM PBS containing peroxidase-conjugated streptavidin (1:80 dilution; DAKO, Denmark), rinsed in two changes of 100 mM PBS and TRIS-HCl buffer (pH 7.6) for 30 min, and incubated for 5 min in TRIS-HCl buffer containing 0.05% diaminobenzidine and 0.01%H_2O_2 for visualization.

After immunostaining, sections were treated with 1% OsO_4 in 100 mM phosphate buffer for 90 min and dehydrated in a graded ethanol series. They were flat-embedded in Araldite on glass slides by means of a silicon rubber cover. After polymerization, embedded sections were examined and small areas of interest were cut out and re-embedded in Araldite for semi- or ultra-thin sectioning. Semithin sections (1 μm thick) stained with toluidine blue or unstained were observed and photographed with a light microscope. The ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Jeol, JEM-1210).

Two types of immunohistochemical control were employed: (1) Vibratome sections of the pineal organ were incubated with P7 antiserum (1:50) that had been pre-adsorbed with MBP-p7 fusion protein (60 μg/ml), and (2) sections were processed without the primary antibody. No immunopositive staining was observed in these control sections.

Results

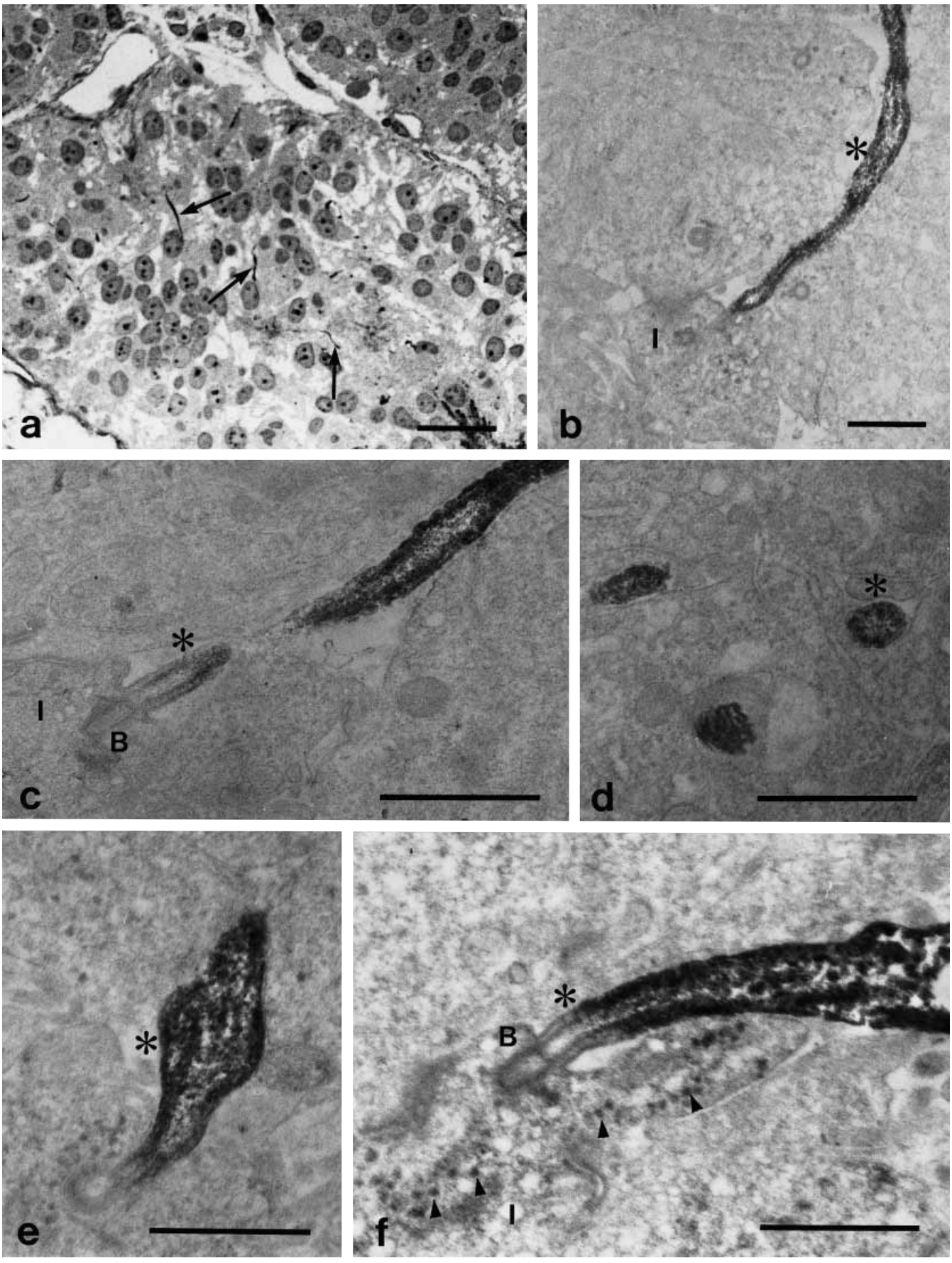
The parenchyma of the pineal organ of the 1 to 2-month-old chicken consisted of many follicles. Light-microscopic examination of semithin sections showed that the follicular lumina were lined by tall columnar cells; these lumina had a relatively small caliber (Fig. 1a). Parafollicular formations of the parenchyma were visible outside the follicles. Immunocytochemical investigations of Vibratome or semithin sections of plastic-embedded pineal organs revealed the regular occurrence of pinopsin-positive cellular elements throughout specific tissue. Pinopsin immunoreactivity was observed exclusively in the follicular lumina and in the parafollicular parenchyma (Figs. 1a, 2a, 3a). In some follicles, the entire lumen seemed to be occupied by immunoreactive material at the light-microscopic level. Randomly distributed immunoreactive cellular elements in the parafollicular layers were bulb- or string-shaped (Figs. 2a, 3a). In order to identify immunoreactive structures more precisely, the chicken pineal organ was subjected to pre-embedding immunoelectron microscopy.

Pre-embedding immunoelectron microscopy with the pinopsin-specific antibody revealed the subcellular localization of pinopsin in the chicken pineal organ. As described above, immunoreactive elements were observed in the follicular lumina. Slender lamellar structures (Fig. 1b,c), pear-shaped processes (Fig. 1d) and concentric lamellar arrays (Fig. 1f) were immunopositive. These elements were closely arranged and appeared to occupy the entire follicular lumen (Fig. 1e); they are regarded as outer segments of pinealocytes. In the immunonegative inner segments of follicular cells, basal bodies (Fig. 1c,d) and ciliary rootlets (Fig. 1d) were observed. Junctional complexes were found between the columnar cells (Fig. 1b–e). Some immunoreactive lamellar structures were continuous with the ciliary basal body in the inner segment (Fig. 1c). In the follicular lumina, whorl-like lamellar membranes were observed. This type of membranous structure, however, was immunonegative (Fig. 1e,f). Occasionally, the whorl-like complexes were surrounded or apposed by immunopositive lamellar structures (Fig. 1e,f). In addition to the immunoreactive cilium-like structures, immunonegative cilia were occasionally found in the follicular lumen (Fig. 1e).

At least two kinds of immunoreactive cellular elements were distinguished in the parafollicular formations. First, cilium-like structures were immunopositive (arrows in Fig. 2a; asterisks in Fig. 2b–f). String-shaped processes projected from the inner segment (Fig. 2b,c). Some processes arose from the ciliary basal body in the inner segment (Fig. 2c). Immunoreactive cilium-like processes sectioned transversely were also observed in the parafollicular layer (Fig. 2d). Some cilium-like processes had an enlarged distal portion (Fig. 2e). Immunoreactive vesicles occurred near the basal body in the cytoplasm of the inner segment (Fig. 2f).

Another type of immunopositive element was bulb-like or comma-shaped at the light-microscope level (arrows in Fig. 3a). Immunoelectron microscopy revealed that these bodies consisted of immunoreactive concentric lamellae embedded in small cavities (Fig. 3b,c). Immunoreactive lamellar structures encircled whorl-like immunonegative lamellar complexes (Fig. 3b). Ciliary basal bodies and ciliary rootlets were observed in the cy-

Fig. 1a–f. Immunoreactive structures in the follicular lumen of the chicken pineal organ. **a** Light micrograph of immunoreactive structures in a semithin section counterstained with toluidine blue. *Arrowheads* indicate immunoreactive structures in the pineal parenchyma. **b–f** Electron micrographs of immunoreactive structures in the lumen of pineal follicles. **b** Lamella-shaped structure (*asterisk*) in the lumen. **c** An immunoreactive process (*asterisk*) arises from the basal body (*B*) in the inner segment (*I*). **d** Pear-shaped outer segment (*asterisk*) located near similar reactive structures in the lumen. **e** Immunoreactive structure (*asterisk*) occupying the lumen. A whorl-like structure (*W*) and a cilium-like process (*thick arrowhead*) extending from a basal body (*B*) are immunonegative. **f** Immunoreactive lamellar complex encircles a whorl-like structure (*W*). *F* Pineal follicle, *L* lumen of follicle, *R* ciliary rootlet, *arrows* cell junctions of pinealocytes. *Bars:* 20 μm in **a**, 1 μm in **b–f**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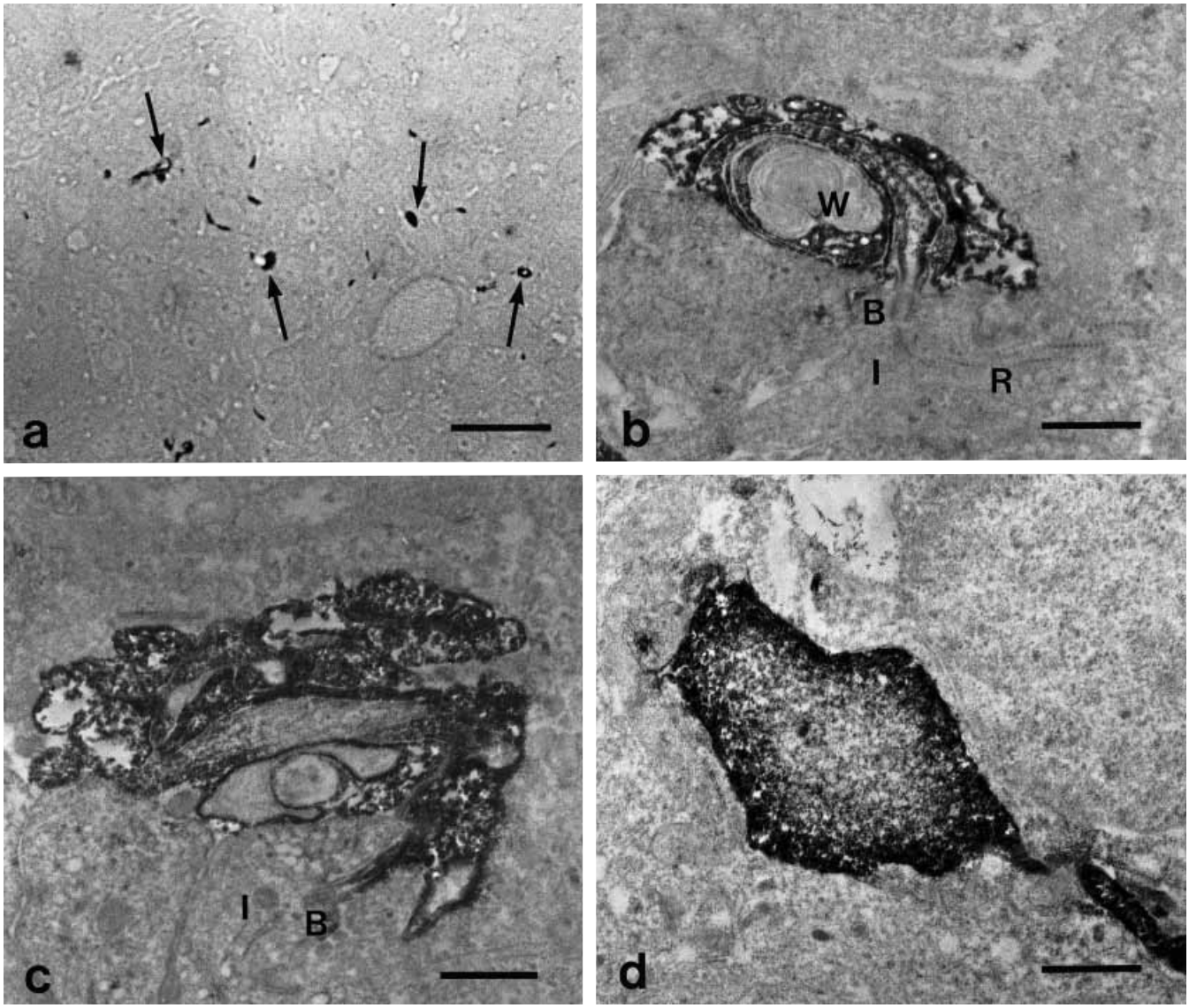

Fig. 3a–d. Immunoreactive bulb-shaped structures in the parafollicular zone of the chicken pineal organ. **a** Light micrograph of immunoreactive spherical structures (*arrows*) in a semithin section (no counterstain). **b–d** Electron micrographs of similar structures in a parafollicular position. **b,c** Outer segment-like structures arising from the basal body (*B*) in the inner segment (*I*). Immunoreactive lamellar complex encircles an unstained whorl-like structure (*W* in **b**). Note ciliary rootlets (*R*) in the inner segment. **d** Large bulbous structure with a stalk in the pineal parenchyma. *Bars:* 20 μm in **a**, 1 μm in **b–d**

Fig. 2a–f. Immunoreactive cilium-like structures in the parafollicular zone of the chicken pineal organ. **a** Light micrograph of immunoreactive cilium-like structures (*arrows*) in a semithin section counterstained with toluidine blue. **b–f** Electron micrographs of immunoreactive cilium-like structures in the pineal parenchyma. **b** Long cilium-like immunoreactive structure (*asterisk*) arising from the inner segment (*I*). **c** A cilium-like structure (*asterisk*) originates from a basal body (*B*) in the inner segment (*I*). **d** Immunoreactive cilium-like structure (*asterisk*) sectioned transversely. **e** Enlarged (bulbous) cilium-like structure (*asterisk*) projecting into the parenchyma. **f** Cilium-like structure (*asterisk*) connected to a basal body (*B*). *Arrowheads* indicate immunoreactive vesicle-like structures in the inner segment (*I*). *Bars:* 20 μm in **a**, 1 μm in **b–f**

toplasm of the inner segment of the parafollicular layer (Fig. 3b,c). Basal bodies were continuous with immunoreactive concentric lamellar structures (Fig. 3b,c). Occasionally, enlarged balloon-like structures showed immunoreactivity in the parafollicular layer (Fig. 3d).

Discussion

The pineal parenchyma of the 1 to 2-month-old chicken is multifollicular. Positive pinopsin immunoreaction can be detected in circumscribed areas of the chicken pineal

organ. Immunoreactive structures are present in almost all follicular lumina. It is important to characterize the immunoreactive subcellular equivalent in the follicular lumina at the electron-microscopic level. For this purpose, conventional transmission and scanning electron microscopy has been used (cf. Oksche and Vaupel-von Harnack 1966; Oksche and Kirschstein 1969; Oksche et al. 1969, 1972; Boya and Zamorano 1975; Omura 1977; Boya and Calvo 1978, 1980; Ohshima and Matsuo 1984, 1988; Möller and Möller 1990). In these studies, typical photoreceptor cells with regular comb-like arrangements of outer segment membranes have not been identified, although basic sensory structures such as bulbous cilia with associated lamellar complexes have been observed (Menaker and Oksche 1974; Vollrath 1981). Most of these sensory structures are found in the follicular lumina of the avian pineal organ; they are considered to represent early developmental stages of the photoreceptor outer segment. As shown in Fig. 1, pinopsin immunoreactivity is limited to the follicular lumina of the pineal organ. Immunopositive structures, such as elongated cilium-like elements and lamellar complexes, seem to correspond to sensory structures described in earlier studies with transmission electron microscopy.

Opsin immunohistochemistry has revealed immunoreactive pinealocytes in the avian pineal organ. By using antibodies highly specific to frog opsin, Vigh and Vigh-Teichmann (1981) and Vigh et al. (1982) have shown intense positive reactions in the pineal organ of chickens and pigeons. According to their studies, the immunoreactivity is found in the follicular lumina, especially in the pear-shaped outer segments, including their thin initial portion. Foster et al. (1987) have shown that opsin immunoreactivity is present in all regions of pinealocytes of the Japanese quail. By using a pinopsin-specific antibody, we have detected immunoreactive pear-shaped structures in the pineal lumina of the pigeon (unpublished data). In the present study, we have been able to characterize the immunoreactive elongated cilium-like structures (Fig. 1c) and the pear-shaped outer segments (Fig. 1d) in the pineal lumina of the chicken. Pinopsin is 43%–48% identical in amino-acid sequence to vertebrate retinal opsins (Okano et al. 1994), and thus earlier immunocytochemical studies with antibodies against frog opsin might have detected pinopsin in the pineal organ of birds.

We have also demonstrated immunoreactive elements in the parafollicular parenchyma of the chicken pineal organ. From the phylogenetic point of view, it is noteworthy that photopigment-immunoreactive elements are detected outside the follicular lumina, because the avian pineal organ represents a transient form from the tubular or saccular pineal organs of lower vertebrates to the solid pineal gland of mammals. Secretory rudimentary pinealocytes are thought to represent intermediate or transient cell types of this evolutionary process in birds. Interestingly, the presence of outer segment-like cilia has also been reported in the developing ferret pineal gland (Vigh and Vigh-Teichmann 1993). A cytoplasmic cilium-like extension protruding toward the follicular lumen has been demonstrated in the pinealocyte of the adult South American opossum *Didelphis albiventris* (González and Affanni 1995).

In the present study, we have shown two types of pinopsin-immunoreactive elements, viz., cilium-like and bulb-like, in the parafollicular layer. The former is an elongated or bulbous cilium, whereas the latter contains ultrastructually detectable lamellar complexes. Both types have a basal body in the adjacent cytoplasm of the inner segment. These morphological features have led us to the conclusion that pinopsin is also present in modified photoreceptor cells of the parafollicular layer in the avian pineal organ. Menaker and Oksche (1974) have classified this type of cell as “the pinealocyte without connection with the pineal lumen”. Relatively little attention has been directed toward this type of avian pinealocyte (Vollrath 1981). Boya and Calvo (1980) consider that chicken parafollicular cells represent the same type of cells as the follicular cells, although there is no clear-cut structural polarization. Ohshima and Matsuo (1988) have suggested that the parafollicular cell of the chicken pineal organ is a type of pinealocyte, because of the occasional presence of granular vesicles and synaptic ribbons, although they have not found a bulbous protrusion carrying a sensory cilium. Taking into account their morphology, parafollicular cells are probably modified photoreceptor cells.

In this study, we have identified immunocytochemically the outer segment of the parafollicular cell of the chicken pineal organ. Recent immunohistochemical studies have shown that parafollicular cells and follicular cells contain specific molecules possibly involved in pineal phototransduction and/or neuroendocrine processes. For example, hydroxyindole-*O*-methyltransferase (HIOMT), which catalyses the terminal step of melatonin synthesis (Voisin et al. 1988), has been detected immunohistochemically in the parafollicular cells and in the modified follicular photoreceptors in the pineal organ of the chicken after hatching (Bernard et at. 1991). Visinin, a calcium-binding protein identified in chicken cone cells, has also been detected in both follicular and parafollicular cells (Goto et al. 1990). Interestingly, the visinin-positive cells exhibit the same fine-structural characteristics as the follicular and parafollicular cells (Goto et al. 1990). Similarly, neuron-specific enolase, a cytoplasmic marker of neurons and neuroendocrine tissue, is found not only in follicular cells but also in parafollicular elements of the pineal organ of one-month-old chicken (Sato et al. 1995). These results suggest morphological and functional similarities between pinopsin-positive cells in the follicular and parafollicular position.

According to ontogenetic studies based on opsin immunohistochemistry in the chicken pineal organ, the number of opsin-immunoreactive pineal cells decreases considerably during the first two months after hatching (Korf 1994). In the present study, we have noted no remarkable decrease in pinopsin immunoreactivity in the pineal organ of 1 to 2-month-old chicken. Investigations of the follicular lumen and parafollicular region in newly hatched and 1-week-old chickens have revealed identical pinopsin-immunoreative structures to those of 1 to 2-month-old animals (unpublished data).

In conclusion, pinopsin is expressed exclusively in the sensory structures of chicken pinealocytes. In the quail pineal gland, both rod-like and cone-like photoreceptor cells are immunoreactive to highly specific chicken anti-rhodopsin and anti-iodopsin monoclonal antibodies (Araki et al. 1992). Our recent studies have revealed the occurrence of chicken red photopigment in the pineal organ of the chicken (unpublished data). Immunonegative cilium-like outer segments or whorl-like complexes observed adjacent to pinopsin-immunoreactive outer segments may represent this type of photoreceptive element. For a better understanding of the photoreceptive function in pinealocytes of birds, further studies are needed to define how many types of photopigment are involved in this function in the avian pineal organ.

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