The distribution of proliferating cell nuclear antigen-immunoreactive cells in the pineal organ of the rainbow trout *Oncorhynchus mykiss**

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Summary. Cell proliferation in the pineal organ of the immature rainbow trout *Oncorhynchus mykiss* was investigated by immunocytochemical demonstration of the proliferating cell nuclear antigen (PCNA) together with photoreceptor-specific opsin. Numerous PCNA-immunoreactive cells were found throughout the pineal end-vesicle and stalk. Two types of PCNA-immunoreactive cells were distinguished: intensely stained, large ovoid and round cells, and mildly stained, slender fusiform cells. The ovoid type of the former cell was found often in the apical region and the round type in the basal region of the epithelium, while the latter fusiform cells were scattered through the apical and middle regions. Occasionally, close approaches were found between the opsin-immunoreactive photoreceptor outer segments and the PCNA-immunoreactive cells, which expressed mildly stained, nuclear and cytoplasmic signals. In addition, overlaps of the opsin-immunoreactive outer segments with the BrdU-labelled cells were occasionally found within the pineal epithelium. These findings suggest that the proliferation and neurogenesis of the pineal photoreceptor cells might persist also in the adult rainbow trout, thus

maintaining highly sensitive, photo-signal transduction mechanisms for melatonin synthesis.

Introduction

Although new neurons are continuously generated with enormous potential in the adult brain and sensory organs of many fish (Johns and Easter 1975, 1977; Zupanc and Horschke 1995; Zikopoulos *et al.*, 2000; Byrd and Brunjes 2001; Ekström *et al.*, 2001; Zupanc 2001; Otteson *et al.*, 2001; Otteson and Hitchcock 2003; Zupanc *et al.*, 2005), there is still a lack of information whether the fish pineal organ continues to produce new photoreceptor cells. Using proliferating cell nuclear antigen (PCNA)-immunocytochemistry, we previously demonstrated that retinal rod cells continuously proliferate in two metamorphic nocturnal fish, *Anguilla japonica* and *Paralichthys olivaceus*, throughout life (Omura *et al.*, 2003, 2004). In the pineal organ of fish, however, PCNA-immunoreactive, proliferative cells were demonstrated only in the developmental stage in the sea lamprey (Meléndez-Ferro *et al.*, 2002; Villar-Cheda *et al.*, 2002) and at an early stage of neurogenesis in the cichlid fish *Aequidens pulcher* (Negishi and Wagner, 1995).

The capacity of the trout pineal organ to synthesize melatonin has been repeatedly demonstrated (Oguri *et al.*, 1968; Hafeez and Quay, 1969; Oguri and Omura, 1973; Gern and Greenhouse, 1988), and it is now well established that, in most species of fish, the pineal organ is involved in photoreception, melatonin-synthesis, and circadian rhythm generation (cf. Falcón *et al.*, 1992, 2003; Ekström and Meissl, 1997). In the rainbow trout *Oncorhynchus mykiss*, however, melatonin biosynthesis is exclusively controlled by light and darkness, and a circadian rhythm generator is lacking in its pineal organ

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(Gern and Greenhouse, 1988; Gern *et al.*, 1992; Bégay *et al.*, 1998). For at least up to two years after hatching, proliferative cells or photoreceptor precursors exist in the mature retina of the rainbow trout (Julian *et al.*, 1998), which commonly has a long life span and spawning activity (approximately 5–7 years). More than 100 years ago, Hill (1894) described how the pineal organ of the rainbow trout gradually increases in size at least two years after hatching, though the parapineal organ — the second component of the pineal complex in teleost fish — remains almost at the original site and size (Rüdeberg, 1969; Korf, 1974; Omura *et al.*, 1990).

In the present study, numerous PCNA-immunoreactive cells were demonstrated in the pineal organ of the immature rainbow trout, together with opsin-immunoreactive photoreceptor outer segments. The findings were discussed especially with reference to the continuous proliferation and neurogenesis of the pineal photoreceptor cells in the adult rainbow trout.

Materials and Methods

Experiments were conducted in accordance with the guidelines of the Committee for Animal Experimentation of Nagoya University. Immature rainbow trout *Oncorhynchus mykiss* (19.7 to 25.2 cm in body length) were obtained from the Shiga Prefectural Samegai Trout Farm. Before the experiments, the animals were kept in an aquarium at 15 to 18°C under a 12 h: 12 h light/dark cycle. Twelve fish were deeply anaesthetized with 1/1000 FA100 (TANABE, Osaka) and killed during the light phase. Their pineal organs with or without brains were fixed by immersion with Zamboni's solution for paraffin sections. Ten additional immature fish (containing 2 controls) were injected intraperitoneally with BrdU (0.3 mg/g body weight, SIGMA B9285, Saint Louis, MO, USA) in phosphate-buffered saline (PBS), killed and fixed by perfusion with 4% paraformaldehyde-0.1M phosphate at 1, 3, 10, and 16 days after the injection. The pineal organs were then incubated in 30% sucrose and cut into 20–30 µm thick frontal or sagittal frozen sections.

To block endogenous peroxidase, 7 µm thick paraffin sections were treated with methanol containing 0.3% H₂O₂ for 10 min. After washing with phosphate-buffered saline containing 0.3% Triton X-100 (PBST) and preincubation with 5% normal goat serum for 30 min, the specimens were incubated with 1 µg/ml mouse monoclonal anti-PCNA antibodies (NEOMARKERS, Fremont, CA, USA) diluted with PBST containing 1% bovine serum albumin (BSA). To detect incorporated BrdU, methanol/H₂O₂-

treated frozen sections were incubated in 2N HCl for 30 min at 37°C, washed thoroughly in PBST, blocked in 5% normal goat serum for 30 min, and then incubated with mouse monoclonal anti-BrdU antibodies (SIGMA B2531) diluted 1:500 with PBST containing 1% BSA overnight at room temperature. Binding of the antibodies was detected using biotin-conjugated anti-mouse IgG and HRP-conjugated avidin (VECTASTAIN ABC KIT, VECTOR, Burlingame, CA, USA), with visualizing finally by diaminobenzidine (DAB) or VECTOR VIP. For the control, the primary antibody was omitted.

For identification of the PCNA-immunoreactive cells and BrdU-labelled cells, photoreceptor-specific opsin was demonstrated with the primary antibody (anti-opsin antibody diluted 1:500; Yoshida *et al.* 1991), using conventional ABC procedures mentioned above. Light micrographs were produced with an Olympus System Microscope BX60 and Digital Camera DP70, and the figures were prepared with Adobe Photoshop CS2.

Results

PCNA-immunoreactive cells were exclusively found in the well-convoluted end vesicle and the slender stalk of the pineal organ, the ventricular proliferation zones through the telencephalon and diencephalon, and the monolayer epithelia of the dorsal sac and paraphysis (Fig. 1). In contrast, most of the other brain tissues including the parapineal and subcommissural organs exhibited almost negative reactions for PCNA (Fig. 1C, D).

On closer view, intense immunoreactivity for PCNA was found in ovoid and round cells in the pineal epithelium; the ovoid cells were frequently found in the apical region and the round ones appeared occasionally in the basal region (Fig. 2). Conversely, mildly stained, slender fusiform cells were found through the apical and middle regions of the epithelium. In randomly selected sections (500–600 µm high; 850 µm wide) from the central portion of the pineal end-vesicle, a large number of PCNA-immunoreactive cells were found, with approximately 40–60 PCNA-immunoreactive cells counted in each section (Fig. 3).

In order to reveal the correlation between the PCNA-immunoreactive cells and the pineal photoreceptor cells, double staining of PCNA and opsin was performed (Fig. 4). Strong immunoreaction for photoreceptor-specific opsin was found exclusively on the luminal surface and excavations of the well-convoluted pineal epithelium, so that this immunoreactivity seemed to be exclusively localized in the photoreceptor outer segments.

Fig. 1. Immunocytochemical reactions for PCNA (**A**, **C**, **D**) and a negative control (**B**) found in the pineal region of the immature rainbow trout. PCNA-immunoreactive cells are exclusively found in the well-convoluted end vesicle (**A**) and slender stalk (**C**, **D**) of the pineal organ, the monolayer epithelium of paraphysis (Py) and dorsal sac (Ds) (**A**), and the ventricular proliferation zones of the diencephalon (arrows, **C**, **D**). However, most of the surrounding brain tissues including the parapineal organ (PP) and subcommissural organ (SCO) exhibit almost negative reactions (**C**, **D**). Asterisks indicate the convoluted pineal epithelium. H: habenular nucleus, OT: optic tectum, Pl: pineal lumen, IIIIV: third ventricle. Scale bars: 100 μm (**A**, **B**, **D**), 20 μm (**C**)

Close approaches of the opsin-positive photoreceptor outer segments to the PCNA-immunoreactive cells — expressing mild nuclear and cytoplasmic signals for PCNA — were occasionally found in the pineal stalk part (Fig. 4A, B). On the other hand, the parapineal organ appeared almost free from the immunoreactivity for both PCNA and opsin.

Additionally, to examine whether the proliferative precursor cells differentiate into the photoreceptor cells, BrdU (a marker of DNA-synthesis) was intraperitoneally injected into the immature rainbow trout. Numerous BrdU-

labeled cells were mainly found in the periphery or basal region of the pineal epithelium (Fig. 5A). Connections or overlaps of the opsin-immunoreactive outer segments with the BrdU-labelled cells were occasionally found within the pineal epithelium at 3 days and 10 days after the BrdU injection (Fig. 5B, C). Because of overly thick sections, however, conspicuous relationships between the two immunoreactive signals were not revealed in the present study.

Fig. 2. PCNA-immunoreactive cells found in the pineal end vesicle of the immature rainbow trout. Intensely stained, ovoid PCNA-immunoreactive cells (arrows) are frequently found in the apical region of the epithelium, with occasional round ones (arrowheads) sometimes in the basal region. Mildly stained, slender fusiform PCNA-immunoreactive cells (asterisks) are scattered through the pineal epithelium. Cp: capillary, Pl: pineal lumen. Scale bars: 20 μ m (A, B)

Discussion

We previously demonstrated by means of PCNA-immunocytochemistry that the retinal rod cells continuously proliferate throughout the life of fish (Omura *et al.*, 2003, 2004). Julian and his collaborators (1998) also revealed that proliferative cells or photoreceptor precursors exist in the mature retina of the rainbow trout for at least up to 2 years after hatching. However,

proliferative progenitor and precursor cells have not yet been investigated in the pineal organ of adult fish, although more than 100 years ago, Hill (1894) described how the pineal organ of the rainbow trout gradually increases in size up to at least 2 years after hatching, and its epithelial wall becomes extremely convoluted and vascularized. Proliferating PCNA-immunoreactive cells were only demonstrated in the early embryonic pineal organ of a cichlid fish (Negishi and Wagner, 1995) and

Fig. 3. Distribution of PCNA-immunoreactive cells in randomly selected sections from the central portion of the pineal end-vesicle of the immature rainbow trout. Note the great number of PCNA-immunoreactive cells distributed throughout the convoluted epithelium. Cp: capillary, Pl: pineal lumen. Scale bars: 50 μ m (A–F)

Fig. 4. Double immunostaining of PCNA (VECTOR VIP) and opsin (DAB) shown in the pineal organ of the immature rainbow trout. Close approaches of the opsin-positive photoreceptor outer segments (arrows) to the PCNA-immunoreactive cells are found (**A**, **B**). Asterisks indicate intensely stained round and ovoid PCNA-immunoreactive cells. Pl: pineal lumen. Scale bars: 10 μm (**A**, **B**)

sea lamprey (Meléndez-Ferro *et al.*, 2002; Villar-Cheda *et al.*, 2002).

PCNA is an essential component for DNA replication and functions as a DNA sliding clamp for DNA polymerase delta, which binds to PCNA on the DNA and accelerates the DNA synthesis (Tsurimoto *et al.*, 1990; Alberts *et al.*, 1998). During cell division cycle, PCNA accumulates in the nucleus from the mid-G₁ phase to the S phase but declines through the G₂ and M phases (Bolton

et al., 1994). The specificity of PCNA for the detection of proliferating cells has been compared to that of BrdU (thymidine analogue) uptake, which is incorporated into the DNA during the S phase, and a mutual correlation between the two methods has been established (Wildemann *et al.*, 2003). Because of advantages in the use of intact experimental animals, we first demonstrated the distribution of PCNA-immunoreactive cells in the pineal organ of the immature rainbow trout.

Fig. 5. Demonstration of BrdU-labelled cells (DAB) and opsin-immunoreactive photoreceptor outer segments (VECTOR VIP) in the pineal organ of the immature rainbow trout. Overlaps (asterisks) of the two immunoreactive signals were occasionally found within the pineal epithelium at 3 days (**A**, **B**) and 10 days (**C**) after the injection of BrdU. Arrows indicate the basal membrane of the pineal epithelium. PI: pineal lumen. Scale bars: 20 μm (**A**), 10 μm (**B**, **C**)

In the present study, we showed the presence of numerous PCNA-immunoreactive, proliferative cells throughout the well-convoluted pineal epithelium of the immature rainbow trout, from the end-vesicle to the proximal stalk. PCNA-immunoreactive cells were also observed in the ventricular proliferation zones through the telencephalon and diencephalon; this distribution pattern of PCNA-immunoreactive cells closely resembled that of ^3H -thymidin-labelled cells in the ventricular proliferation zone demonstrated by means of autoradiography in the trout and other fish (Richter and Kranz, 1981; Ekström *et al.*, 2001).

The present study revealed that intensely PCNA-immunoreactive cells were ovoid in the apical region but round in the basal region of the pineal epithelium. On the other hand, mildly stained, slender fusiform PCNA-immunoreactive cells were scattered throughout the apical and middle regions of the pineal epithelium. According to the criteria of the rod photoreceptor lineage in the teleost retina (Otterson *et al.*, 2001; Otterson and Hitchcock, 2003), the ovoid cells located in the apical region might

be considered photoreceptor precursors, and the round ones scattered in the basal region, stem cells. The mildly stained, slender fusiform cells may represent precursor cells in the process of differentiation.

To further characterize the PCNA-immunoreactive cells, a double labelling was performed with an antibody against photoreceptor-specific opsin (Yoshida *et al.*, 1991). In accordance with previous findings (Vigh-Teichmann *et al.*, 1982, 1983), strong immunoreactions for opsin restricted to the photoreceptor outer segments were found along the epithelial surface and excavation. However, close approaches of the two signals of PCNA and opsin were only rarely demonstrated by means of the double immunocytochemistry (Fig. 4). This may be explained by the fact that PCNA-immunoreactive, proliferative cells are precursor and progenitor cells that have not yet differentiated into pineal photoreceptor cells.

To reveal the fate and/or destination of the proliferative cells in the pineal organ of the rainbow trout, an intraperitoneal injection of BrdU was also performed. This investigation demonstrated connections or overlaps of the

opsin-immunoreactive photoreceptor outer segments with the BrdU-labelled cells within the pineal epithelium of the immature rainbow trout at 3 days and 10 days after the injection. However, it did not conclusively display the conspicuous connection because of the thick frozen sections. BrdU-injections in the adult cichlid fish have shown for the retina that rodopsin expression first occurs two days after cell division in the newly formed rod cells (Henderson and Fernald, 2004). Most of the newly divided rod cells migrate to the vitreal side of the outer nuclear layer (Mack *et al.*, 2003; Henderson and Fernald, 2004; Omura *et al.*, 2003, 2004). In the pineal organ of the trout, however, it is not yet known whether the apical ovoid, PCNA-immunoreactive cells divide into the fusiform cells, differentiating to the photoreceptor cells, or if the basal ovoid cells divide and migrate into the middle region. Nevertheless, the present study strongly suggests that photoreceptor cells still be generated in the pineal organ of the adult rainbow trout.

In the early days of our pineal studies, we showed that the photoreceptor outer segments gradually degenerate through the life of a diadromous fish, *Plecoglossus altivelis* (Omura and Oguri, 1971). Although it could be somewhat slowed by raising the animals in diel rhythm, the regression of the outer segments was accelerated in the pineal organ of the blind cave fish *Astyanax mexicanus* kept under continuous light or in continuous darkness (Omura, 1975; Herwig, 1976). The regional rudimentation of the photoreceptor cells, lacking much or all photoreceptor potential, was also indicated in the pineal organ of the pike *Esox lucius* (Falcón, 1979; Falcón and Meissl, 1981). In addition, the pineal organs of the deep-sea fishes were investigated with attention whether the complete or nearly complete absence of ambient light influenced their photoreceptive function (Holmgren, 1959; McNulty, 1976; McNulty and Nafpaktitis, 1977). Many species of deep-sea fish showed a significant increase in size or in the number of lamellar membranes of the photoreceptor outer segments, but some species exhibited signs of regression.

Does the pineal organ functions also in the adult fish? In the present study, a great number of PCNA-immunoreactive, proliferative cells were demonstrated in the pineal epithelium of the sexually immature rainbow trout, suggesting that the proliferative and neurogenetic activities of the pineal photoreceptors might persist also in the adult fish. As is well known, the rainbow trout has a long life span, and regulation of the reproductive cycle is possible by changing of the photoperiod (Bromage *et al.*, 1984). The pineal organ of the rainbow trout is supposed to be a strong candidate for mediating photoperiodic information to brain-pituitary-gonadal axis

through melatonin secretion (see Oguri and Omura 1973; Amano *et al.*, 2000; Falcón *et al.*, 2007). Consequently, continuous neurogenesis of the pineal photoreceptor cells might maintain the highly sensitive photoperiodic-signal transduction mechanism in melatonin secretion necessary for reproductive regulation in the adult rainbow trout.

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