Ocular Melatonin Rhythms in a Cyprinid Teleost, Oikawa Zacco platypus, Are Driven by Light-Dark Cycles

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[Short Communication]

Ocular Melatonin Rhythms in a Cyprinid Teleost, Oikawa *Zacco platypus*, Are Driven by Light-Dark Cycles

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ABSTRACT—Ocular melatonin contents in a cyprinid teleost, oikawa *Zacco platypus* were determined by newly-developed radioimmunoassay. Under light-dark (LD) cycles of 12:12 hr, melatonin levels in the oikawa eye exhibited daily rhythms with higher levels during the dark phase than those during the light phase. However, the rhythms disappeared under either constant light (LL) or constant darkness (DD). Ocular melatonin contents under LL remained at low levels while those under DD kept high titers. These results indicate ocular melatonin rhythms in oikawa are driven not by a circadian clock but by LD cycles.

INTRODUCTION

The pineal organ and the retina of vertebrates synthesize melatonin (*N*-acetyl-5-methoxytryptamine). It has been demonstrated in a number of species that melatonin levels in the pineal organ, lateral eye and blood exhibit marked daily rhythms, with high levels during the dark phase under light-dark (LD) cycles (Binkley, 1987; Underwood and Goldman, 1987; Yu and Reiter, 1993). Two enzymes, serotonin *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), are involved in melatonin biosynthesis from serotonin in the pineal organ and retina (Binkley, 1987; Underwood and Goldman, 1987; Yu and Reiter, 1993). The activity of NAT, the rate-limiting enzyme, oscillates and controls melatonin production with large amount produced during the dark phase. In nonmammalian vertebrates, both the pineal organ and the retina have photosensitivity, and lines of evidence suggest the involvement of photoreceptor cells in indole metabolism such as melatonin biosynthesis (McNulty, 1986; Wiechmann *et al.*, 1988; Cahill *et al.*, 1991; Falcón *et al.*, 1992, 1994).

Recent studies have revealed several characteristics of the regulatory mechanisms of melatonin synthesis in the teleostean pineal organ. Both *in vivo* and *in vitro* studies have demonstrated that melatonin secretion from the pineal organ of pike *Esox lucius*, goldfish *Carassius auratus*, white sucker *Catostomus commersoni*, and zebrafish *Danio rerio* are regulated by a circadian clock located in the pineal organ itself (Falcón *et al.*, 1992; Zachmann *et al.*, 1992a; Iigo *et al.*, 1994; Cahill, 1996). A recent *in vitro* comparative study has also demonstrated the involvement of a circadian clock in the regulation of melatonin secretion in the pineal organ of 8 fresh water and 6 marine teleosts (Bolliet *et al.*, 1996). However, in the pineal organ of rainbow trout *Oncorhynchus mykiss* and masu salmon *O. masou*, the circadian regulation is lacking (Gern *et al.*, 1992; Iigo *et al.*, 1997b). Light, day length and temperature are also involved in the regulation of pineal melatonin rhythms (Falcón *et al.*, 1992; Max and Menaker, 1992; Zachmann *et al.*, 1992a,b; Iigo *et al.*, 1994; Iigo and Aida, 1995). These results indicate that the teleostean pineal organ is playing important roles as a photoneuroendocrine and thermoendocrine transducer.

Although existence of melatonin synthesizing enzymes, NAT and HIOMT, has been reported in the retina of teleosts (Quay, 1963; Wiechmann and Hollyfield, 1988; Nowak *et al.*, 1989; Falcón and Collin, 1991), relatively little is known on ocular melatonin rhythms in fishes and only a few data is available. Ocular melatonin exhibited daily rhythms under LD cycles with higher levels during the dark phase, and circadian rhythms under constant darkness (DD) in pike, zebrafish and goldfish (Falcón and Collin, 1991; Cahill, 1996; Iigo *et al.*, 1997a). Whereas in salmonids such as rainbow trout and brook trout *Salverinus fontinalis*, reversed melatonin profiles in the retina with higher levels during the light phase under LD cycles have been reported (Gern *et al.*, 1978; Zachmann *et al.*,

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1992b). Thus, no consensus has been obtained on ocular melatonin rhythms in fishes.

In order to obtain more information on melatonin rhythms in the teleostean eye, in the present study, we developed a new sensitive radioimmunoassay (RIA) for melatonin and examined ocular melatonin rhythms in a cyprinid teleost, oikawa *Zacco platypus*, under LD 12:12, constant light (LL) and DD.

MATERIALS AND METHODS

Experimental fish

The oikawa (wild fish) were caught by fishing in Akiyama River, Kanagawa, Japan. They were reared in stock tanks under LD 12:12 (light on 06.00-18.00) at 22 ± 1°C for at least three weeks until use. Illumination (500 lx at the water surface) was supplied with a white fluorescent bulb (10 W) for the light phase. Fish were fed commercial trout pellets.

Ocular melatonin rhythms in the oikawa under LD 12:12, LL and DD

Thirty oikawa (7.6 ± 0.4 g in body weight) were used for melatonin measurements under LD 12:12. Samples of the eyes were taken from the oikawa every 4 hr from 16.00 to 12.00 under LD 12:12 for 6 times.

Thirty-five oikawa (9.6 ± 0.5 g in body weight) were used for melatonin determinations under LL. Light conditions were changed from LD 12:12 to LL at the normal light offset (18.00). Samples of the eyes were taken at 16.00 under LD 12:12 and every 4 hr from 20.00 to 16.00 under LL for 6 times.

Sixty-five oikawa (6.8 ± 0.3 g in body weight) were used for melatonin analysis under DD. Light conditions were changed from LD 12:12 to DD at the normal light offset (18.00). Samples of the eyes were taken at 16.00 under LD 12:12 and every 4 hr under DD for 12 times.

Sampling procedure

The oikawa were anesthetized with 0.06% 2-phenoxyethanol. After decapitation, the eyes were dissected out and immediately frozen on dry ice. For the sampling during the dark phase, fish were caught and anesthetized in the dark. Then, a dim-red light was turned on during the eye removal. Samples were stored at -80°C until further processing. The eyes were thawed and individually homogenized with a teflon headed-glass homogenizer (10 strokes) in 1 ml of ice-cold assay buffer (10 mM phosphate buffer containing 140 mM NaCl and 0.1% sodium azide, pH 7.5; PBS) and centrifuged at 30,000 × g for 20 min at 4°C. Supernatants were subjected to the RIA.

RIA procedure

A new sensitive melatonin RIA was developed and used for determination of ocular melatonin contents. The rabbit anti-melatonin serum (HAC-AA92-03RBP86) and anti-rabbit γ-globulin goat serum (HAC-RBA2-05GTP91) were supplied from Prof. K. Wakabayashi, Gunma University (Maebashi, Japan). Bovine serum albumin (BSA), authentic melatonin and related compounds were purchased from Sigma (St Louis, MO, USA). [*O*-methyl-³H]Melatonin (85 Ci/mmol) was obtained from Amersham Japan Limited (Tokyo, Japan).

A standard curve (1.6-204.8 pg/tube) was constructed by using serial two-fold dilutions of authentic melatonin dissolved in PBS containing 1% BSA (BSA-PBS). For the initiation of RIA, anti-melatonin serum (1:120,000 dilution with PBS containing 50 mM EDTA and 1% normal rabbit serum, 100 μl) and [*O*-methyl-³H]melatonin (approximately 10,000 dpm in BSA-PBS, 100 μl) were added to test tubes (Milli-3PP HD-polypropylene LSC vial, Lumac Lsc., Groningen, The Netherland) containing BSA-PBS (100 μl) and the standard or samples (100 μl) in duplicate or triplicate. After incubation for 24 hr at 4°C, anti-rabbit γ-globulin goat serum (1:100 dilution with PBS

containing 50 mM EDTA, 100 μl) was added to each vial. After incubation for additional 24 hr at 4°C, vials were centrifuged (2,000 × g, 30 min, 4°C) and the supernatant was aspirated. The precipitate was dissolved in 0.1N NaOH (100 μl) followed by the addition of Aquasol-2 (2 ml; Dupont NEN, Boston, MA, USA). Radioacivity was then determined with a liquid scintillation counter (Aloka, Tokyo, Japan).

The specificity of the RIA was evaluated by determining the relative potency of the melatonin related compounds listed in Table 1. We first tested the inhibition of the [*O*-methyl-³H]melatonin binding to the antibody by melatonin related compounds at 10 and 100 ng/tube levels. When significant inhibition was obtained, inhibition curves for serial two-fold dilution of the respective compounds were drawn and 50% inhibition doses (IC_{50}) were calculated by third-order regression analysis after log-logit transformation of the data. The cross-reactivity was calculated by deviding the IC_{50} of melatonin by that of a compound tested.

Cross-reactivities of the anti-melatonin serum with melatonin-related compounds are shown in Table 1. The antiserum slightly cross-reacted with 2-iodomelatonin (14.9%), 6-hydroxymelatonin (0.65%) and *N*-acetylserotonin (0.098%). Other compounds did not show any appreciable cross-reactivities (< 0.025%). These results indicate that the melatonin RIA is highly specific for melatonin.

Validation of RIA

For the validation of the RIA, parallelism and quantitative recovery studies have been performed. Parallelism of the inhibition curves for serial two-fold dilution of the pooled eye extract and the melatonin standard were tested by parallel line assay (2 × 3 points). As shown in Fig. 1, the inhibition curve for serial two-fold dilution of the oikawa eye extract was parallel to the curve for the standard melatonin.

The relationship between the quantity of melatonin added (0 – 102.4 pg/tube) to the pooled eye extract (containing 10.8 pg melatonin/ tube) and the recovered amount was analyzed using linear regression analysis. A significant correlation was obtained between the two [Y =

Table 1. Cross-reactivities of the anti-melatonin serum (HAC-AA92-03RBP86) with selected melatonin-related compounds

Compound	IC_{50} (ng/tube)	Cross-reactivity (%)
Melatonin	0.0251	100
2-Iodomelatonin	0.169	14.9
6-Hydroxymelatonin	3.87	0.65
N-Acetylserotonin	25.7	0.098
5-Methoxytryptamine	a	< 0.025
5-Methoxytryptophol	a	< 0.025
5-Methoxyindole-3-acetic acid	b	< 0.025
Serotonin	b	< 0.025
5-Hydroxytryptophol	b	< 0.025
5-Hydroxyindole-3-acetic acid	b	< 0.025
Tryptamine	b	< 0.025
Tryptophan	b	< 0.025
5-Hydroxytryptophan	b	< 0.025
N-Acetyltryptophan	b	< 0.025
5-Methoxytryptophan	b	< 0.025
Indole-3-acetic acid	b	< 0.025
5-Hydroxyindole	b	< 0.025
5-Methoxyindole	b	< 0.025
3-Methylindole	b	< 0.025
*N*ω-Methyl-5-hydroxytryptamine	b	< 0.025
5-Methoxy-*N*, *N*-d-tryptamine	b	< 0.025

a: Less than 50% inhibition at 100 ng/tube.
b: No inhibition at 100 ng/tube.

1.029X − 0.44 (r = 0.996, n = 8, P < 0.001); X: melatonin added (pg/tube); Y: melatonin recovered (pg/tube)] (Fig. 2).

Intra- and interassay coefficients of variation of the RIA were 4.9% (n = 7) and 7.4% (n = 5) at the 25.1 pg/tube level, respectively. The minimum detectable level defined as 2 SD from the buffer controls was 1.0 pg/tube.

Statistics

Ocular melatonin rhythms under LD 12:12, LL or DD were analyzed by one-way analysis of variance followed by Duncan's multiple range test.

RESULTS

Ocular melatonin rhythms under LD 12:12

Ocular melatonin rhythms in the oikawa under LD 12:12 are shown in Fig. 3. Melatonin contents in the eye exhibited clear daily variations: The melatonin levels were low at 16.00. After the onset of darkness, the levels increased and kept high titers during the dark phase, and returned to the basal level after the light onset. Melatonin levels during the dark phase (20.00, 00.00 and 04.00) were significantly higher than those during the light phase (16.00, 08.00 and 12.00) (P < 0.01).

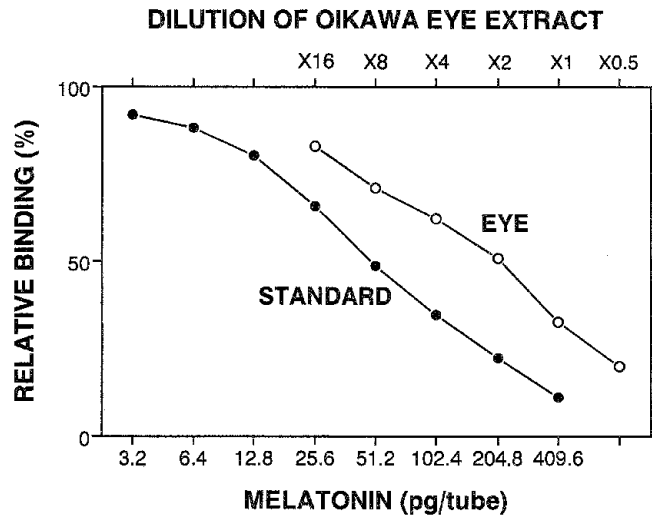**DILUTION OF OIKAWA EYE EXTRACT**

Fig. 1.   Parallel inhibition curves of the melatonin standard (closed circles) and the oikawa eye extract (open circles). Each point represents the mean of tripicate determination.
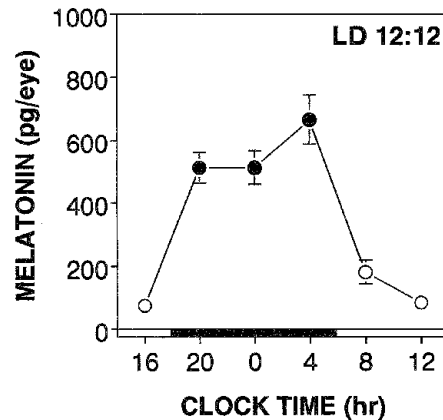

Fig. 3.   Ocular melatonin rhythms in the oikawa under LD 12:12. Each point represents mean ± SEM of melatonin contents in the eye (n = 10 from 5 individuals). Each time point comes from different animals. Solid and open bars along the X-axis represent the dark phase and the light phase, respectively. Ocular melatonin contents during the dark phase were significantly higher than those during the light phase.
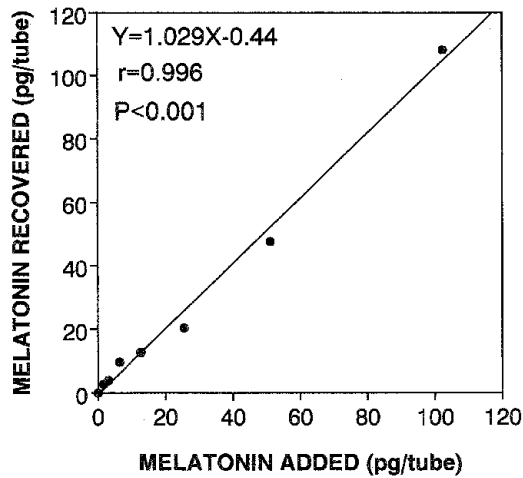

Fig. 2.   Recoveries of melatonin added to the oikawa eye extract. Each point represents the mean of triplicate determination. The results obtained from linear regression analysis indicate there was significant correlation between the amount of melatonin added (X-axis) and that which was recovered (Y-axis).

Fig. 4.   Ocular melatonin rhythms in the oikawa under LL. Each point represents mean ± SEM of melatonin contents in the eye (n = 10 from 5 individuals). Each time point comes from different animals. Open bar along the X-axis represents the light phase. Ocular melatonin contents failed to show any rhythmicity and remained at low levels.
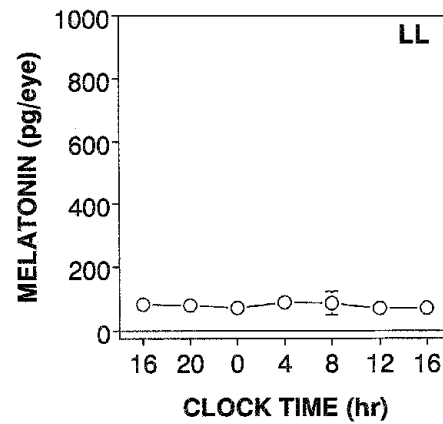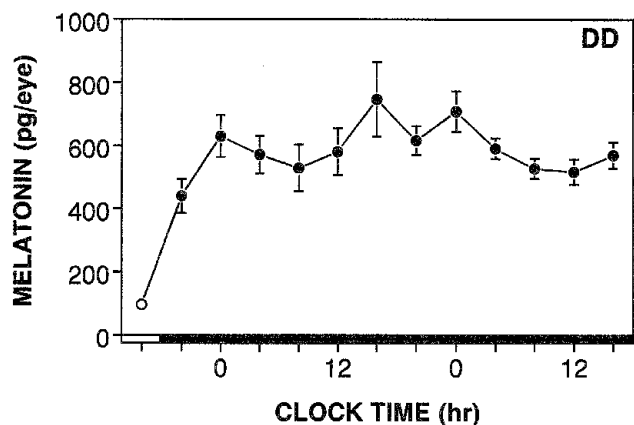
Fig. 5. Ocular melatonin rhythms in the oikawa under DD. Each point represents mean ± SEM of melatonin contents in the eye (n = 10 from 5 individuals). Each time point comes from different animals. Solid and open bars along the X-axis represent the dark phase and the light phase, respectively. Ocular melatonin contents increased after the onset of darkness and kept high titers under DD. However, no endogenous component was evident under DD.

Ocular melatonin rhythms under LL

Ocular melatonin rhythms in the oikawa exposed to LL are exhibited in Fig. 4. Under LL, ocular melatonin remained at low levels just like those during the light phase under LD cycles, with no significant variation.

Ocular melatonin rhythms under DD

Ocular melatonin rhythms in the oikawa exposed to DD are indicated in Fig. 5. Ocular melatonin contents increased after the onset of darkness, and kept high titers both during the subjective day and the subjective night under DD. No circadian variation was observed.

DISCUSSION

In the present study, a new sensitive melatonin RIA was developed using the rabbit anti-melatonin serum (HAC-AA92-03RBP86). The most cross-reactive chemical species with this antiserum is a bioactive melatonin analog, 2-iodomelatonin, with cross-reactivity of 14.9%. The use of 2-[^{125}I]iodomelatonin, with higher specific activity (2,200 Ci/mmol) than [O-methyl-^{3}H]melatonin (specific activity, 85 Ci/mmol) used in the present study may improve the sensitivity of the RIA. Cross-reactivities with 6-hydroxymelatonin (0.65%), N-acetylserotonin (0.098%) and other compounds (< 0.025%) are negligible, indicating the antiserum is highly specific for melatonin. Small intra- and interassay coefficients of variation and the results obtained from parallelism and quantitative recovery studies have demonstrated a reliable and precise RIA.

We have applied this RIA for determination of melatonin contents in the eye of a cyprinid teleost, oikawa *Zacco platypus*, and successfully demonstrated ocular melatonin rhythms. Under LD 12:12, melatonin contents in the oikawa eye during the dark phase were higher than those during the light phase.

Ocular melatonin rhythms in oikawa are similar with those seen in the eye, pineal organ and blood of a number of vertebrate species (Binkley, 1987; Underwood and Goldman, 1987; Cahill *et al.*, 1991; Yu and Reiter, 1993). We speculate that ocular melatonin rhythms may be the result of retinal melatonin synthesis rather than uptake from the circulation, since ocular melatonin synthesis has been reported in other teleost species including cyprinids (Quay, 1965; Nowak *et al.*, 1989; Falcón and Collin, 1991; Iigo, 1996; Iigo *et al.*, 1997a). Measurements of the activities of melatonin synthesizing enzymes, NAT and HIOMT, will be required to confirm melatonin synthesis in the oikawa retina.

In oikawa, melatonin levels in the plasma under LD 12:12 also fluctuate as in the eye (Iigo, 1996). It is possible that, as in the Japanese quail (Underwood *et al.*, 1984) the eye functions as an endocrine organ and circulating melatonin may come from the retina, although the contribution of the pineal organ cannot be ruled out. Further studies using pinealectomy and bilateral enucleation will be required to elucidate this subject.

Circadian regulation of melatonin production has been demonstrated in the pineal organ and retina of several teleost species (Falcón and Collin, 1991; Falcon *et al.*, 1992; Zachmann *et al.*, 1992a,b; Iigo *et al.*, 1994,1997a; Bolliet *et al.*, 1996; Cahill, 1996). To test whether or not ocular melatonin rhythms in oikawa are circadian, we determined ocular melatonin contents under constant conditions such as LL and DD. However, melatonin contents in the eye kept low and high titers under LL and DD, respectively. No endogenous component was evident under these lighting conditions. These results indicate that melatonin rhythms in the oikawa eye are regulated not by a circadian clock but by LD cycles. Similar photic regulation of melatonin rhythms has been reported in the pineal organs of rainbow trout and masu salmon (Gern *et al.*, 1992; Iigo *et al.*, 1997b). These imply that the location of a circadian clock regulating melatonin rhythms differs among teleosts, although the pineal organ and/or the retina in some fish are the sites of circadian clocks. It is of great interest to test whether or not pineal melatonin rhythms are regulated by a circadian clock in oikawa in future.

In vertebrates, circadian melatonin rhythms have been reported in the retina and/or the pineal organ of a number of species including mammals, birds, reptiles, amphibia and fishes (Binkley, 1987; Underwood and Goldman, 1987; Cahill *et al.*, 1991; Falcón *et al.*, 1992; Zachmann *et al.*, 1992a; Iigo *et al.*, 1994,1997a; Cahill, 1996; Tosini and Menaker, 1996). However, photic but not circadian regulation is also reported in the retina of frogs *Rana tigrina regulosa* and *Rana pipiens* (Pang *et al.*, 1985; Wiechmann *et al.*, 1986) and in the pineal organ of desert iguana *Dipososaurus dorsalis*, rainbow trout and masu salmon (Gern *et al.*, 1992; Janik and Menaker, 1990; Iigo *et al.*, 1997b). The present study has also demonstrated photic regulation of ocular melatonin rhythms in oikawa. These data support the idea that location of circadian clocks regulating melatonin rhythms is influenced not so much by phylogeny of the animals as by their ecological niches to which they and

their ancestors have been exposed (Janik and Menaker, 1990). Furthermore, comparison of the regulatory mechanisms of melatonin production in the pineal organ and/or retina between circadian and photically regulated species might be a way to elucidate circadian clock mechanisms in vertebrates.

Ocular melatonin in oikawa may have local neuro-modulatory roles in the retina as a signal for darkness. It has been reported that melatonin receptors are present in the retina of vertebrates including fishes (Gern et al., 1980; Dubocovich, 1988; Blazynski and Dubocovich, 1991; Iigo et al., 1994). In addition, melatonin have been demonstrated to be involved in the regulation of retinal physiological processes including outersegment disc shedding, retinomotor movements, dopamine release, horizontal cell sensitivity and electroretinogram in vertebrates (Chèze and Ali, 1976; Wiechmann, 1986; Besharse et al., 1988; Dubocovich, 1988; Wiechmann et al., 1988; Cahill et al., 1991; Kurusu et al., 1993; Lu et al., 1995).

In conclusion, we have developed a new sensitive melatonin RIA and demonstrated ocular melatonin rhythms in a cyprinid fish, oikawa Zacco platypus. The results suggest important roles of melatonin not as a circadian signal but as a chemical expression of darkness in the regulation of retinal physiology. Further investigations will reveal the roles of ocular melatonin in this species.

ACKNOWLEDGMENTS

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