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Authors: Masuda, Tomohiro, Iigo, Masayuki, Mizusawa, Kanta, and Aida, Katsumi

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Effects of Macromolecule Synthesis Inhibitors on Light-Induced Phase Shift of the Circadian Rhythm in Melatonin Release from the Cultured Pineal Organ of a Teleost, Ayu (*Plecoglossus altivelis*)

Tomohiro Masuda[1], Masayuki Iigo[2,3*], Kanta Mizusawa[1,4], Katsumi Aida[1]

[1]*Laboratory of Aquatic Animal Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan*
[2]*Department of Anatomy, St. Marianna University School of Medicine, Miyamae, Kawasaki 216-8511, Japan*
[3]*Department of Applied Biological Chemistry, Faculty of Agriculture, Utsunomiya University, 350 Mine-Machi, Utsunomiya, Tochigi 321-8505, Japan*
[4]*Department of Animal Sciences, Teikyo University of Science & Technology, 2525 Yatsusawa, Uenohara, Yamanashi 409-0193, Japan*

ABSTRACT—Effects of macromolecule synthesis inhibitors on the light-induced phase shift of the circadian clock in the photoreceptive pineal organ of a teleost, ayu (*Plecoglosus altivelis*) were investigated using melatonin release as an indicator. A single light pulse during the early- and late-subjective night delayed and advanced the phase of the circadian rhythm in melatonin release, respectively. During the late subjective-night, protein synthesis inhibitor cycloheximide (CHX) delayed the rhythm while RNA synthesis inhibitor 5,6-dichlorobenzimidazole riboside (DRB) had little effect. Light-induced phase advance was diminished by the treatment of CHX but not by DRB. During the early subjective-night, DRB, CHX, light and combination of these (DRB+light, CHX+light) all phase-delayed the rhythm. There were no additive effects of light and DRB or CHX. These results indicate that macromolecule synthesis is somehow involved in generation of circadian oscillation, and that *de novo* protein synthesis is required for light-induced phase shift of the circadian clock in the ayu pineal organ.

Key words: pineal organ, circadian rhythm, melatonin, phase shift, macromolecule synthesis

INTRODUCTION

Physiological, behavioral and biochemical activities in most organisms exhibit a daily variation that is regulated by an internal circadian clock (Aschoff, 1981). In vertebrates, the circadian clocks are localized in the central structures such as the suprachiasmatic nucleus (SCN) of the hypothalamus, the retina, and the pineal organ (Takahashi *et al*., 1987; Klein *et al*., 1991; Cahill and Besharse, 1995; Falcón, 1999). Recent identification of circadian clock genes in mammals advances our knowledge on the molecular basis for the circadian clocks in the mammalian SCN (Dunlap, 1999; Young and Kay, 2001). In addition, molecular analyses have demonstrated that peripheral tissues also harbor circadian clocks (Schibler and Sassone-Corsi, 2002). Circadian clock genes have also been cloned from nonmammalian vertebrates such as Japanese quail, chicken, *Xenopus* and zebrafish (Yoshimura *et al*., 2000; Cahill, 2002; Fukada and Okano, 2002; Green, 2003) but the roles of these clock genes remains to be elucidated.

The teleostean pineal organ provides a useful model to analyze molecular mechanisms of the pineal circadian clock, especially that of photic entrainment, because a single pineal organ or a single pineal photoreceptor cell contains all the three essential components of a circadian system, i.e. the circadian oscillator, the photoreceptor responsible for photic entrainment, and melatonin synthesizing system as the output pathway (Falcón, 1999). Recently, we found that the pineal organ of a teleost, ayu (*Plecoglossus altivelis*) maintained in the superfusion culture exhibited a robust rhythm in melatonin release under constant darkness (DD) and that the melatonin secretion rhythm is entrainable to a given light-dark (LD) cycle (Iigo *et al*.,

* Corresponding author: Tel: +81-28-649-5474;
Fax: +81-28-649-5401.
E-mail: iigo@cc.utsunomiya-u.ac.jp

2003a). In addition, the rhythm could be phase shifted by a 6-hr light pulse according to a typical light-type phase response curve: the light pulses starting during the early and late subjective-night respectively induced phase–delay and phase-advance while the light pulse during the subjective-day was ineffective (Iigo *et al.*, 2003b).

To elucidate the mechanism of photic entrainment of the circadian clock located in the photoreceptive pineal organ in fish, in the present study, we examined whether macromolecule synthesis is involved in light-induced phase shift of the circadian clock in the ayu pineal organ.

MATERIALS AND METHODS

Experimental fish, flow-through culture and radioimmunoassay

Ayu (1.6–23.0 g in body weight) were obtained from Chiba Prefectural Fish Farming Center (Katsuura, Chiba, Japan) or Teganuma Fishing Center (Shonan, Chiba, Japan). They were kept in freshwater tanks under natural light conditions at 20°C. The pineal organ was dissected and individually superfused as previously described (Iigo *et al.*, 1998, 2003a, b). All the pineal glands were maintained at 20°C under LD 12:12 (lights on 06:00–18:00 hr, approximately 1,500 lx) for the first day and then kept in DD for additional 5 days except for the light pulse-treated groups (see below). The flow rate of the medium was set at 0.5 ml/hr. Melatonin contents in perfusates collected at 3-hr intervals were determined by the radioimmunoassay using 2-[^{125}I]iodomelatonin (2200 Ci/mmol, New England Nuclear, Boston, MA) and the rabbit anti-melatonin serum (HAC-AA92-03RBP86, kindly supplied by Prof. K. Wakabayashi, Gunma University) as described (Iigo *et al.*, 1998, 2003a). Although the body weight of ayu used differed significantly, this did not affect melatonin secretory profiles after normalization of the data (see Iigo *et al.*, 2003a).

Treatment with a light pulse and RNA and protein synthesis inhibitors

During the late subjective-night in the first cycle under DD, 5,6-dichlorobenzimidazole riboside (DRB, an RNA synthesis inhibitor; 100 μM, Sigma, St. Louis, MO), cycloheximide (CHX, a protein synthesis inhibitor; 142 μM, Sigma) or vehicle (dimethylsulfoxide, DMSO; 1% in final concentration in the medium) was perfused for 6-hr (00:00–06:00 hr; Zeitgeber Time [ZT] 18-24; ZT0=light on; ZT12=light off) with or without a light pulse (01:00–06:00 hr; ZT19-24; 1500 lx at the surface of the culture chamber). Similarly, during the early subjective-night in the second cycle under DD, DRB, CHX or vehicle was perfused for 6-hr (18:00–00:00 hr; ZT12-18) with or without a light pulse (19:00–24:00 hr; ZT13-18). A dim red light was used to exchange the medium containing drugs. The concentrations of the drugs were determined according to the results of the preliminary experiments using different doses of DRB or CHX and to the concentrations used in previous studies (Ohi and Takahashi, 1991; Mizusawa *et al.*, 2001).

Data analysis

In order to facilitate comparison of rhythms from pineals producing different overall levels of melatonin, melatonin release was normalized relative to the average release during the first day under LD. For measurement of phase shift, the rhythms were smoothed by a three-point running average and the interpolated times of half-rise and half-fall of melatonin rhythm peak were determined (the data depicted in Figs. 1 and 2 are not smoothed). Then midpoint phase reference was calculated by averaging the times for the half-rise and half-fall (Robertson and Takahashi, 1988; Cahill and Besharse, 1991). In this study, the 4th midpoint corresponding to the third cycle in DD was used as the phase reference point to calculate phase shift compared with the vehicle-treated control kept in DD. Immediate phase shift is known to be qualitatively similar to the steady state phase shift (Pittendrigh, 1981). The amplitude of phase

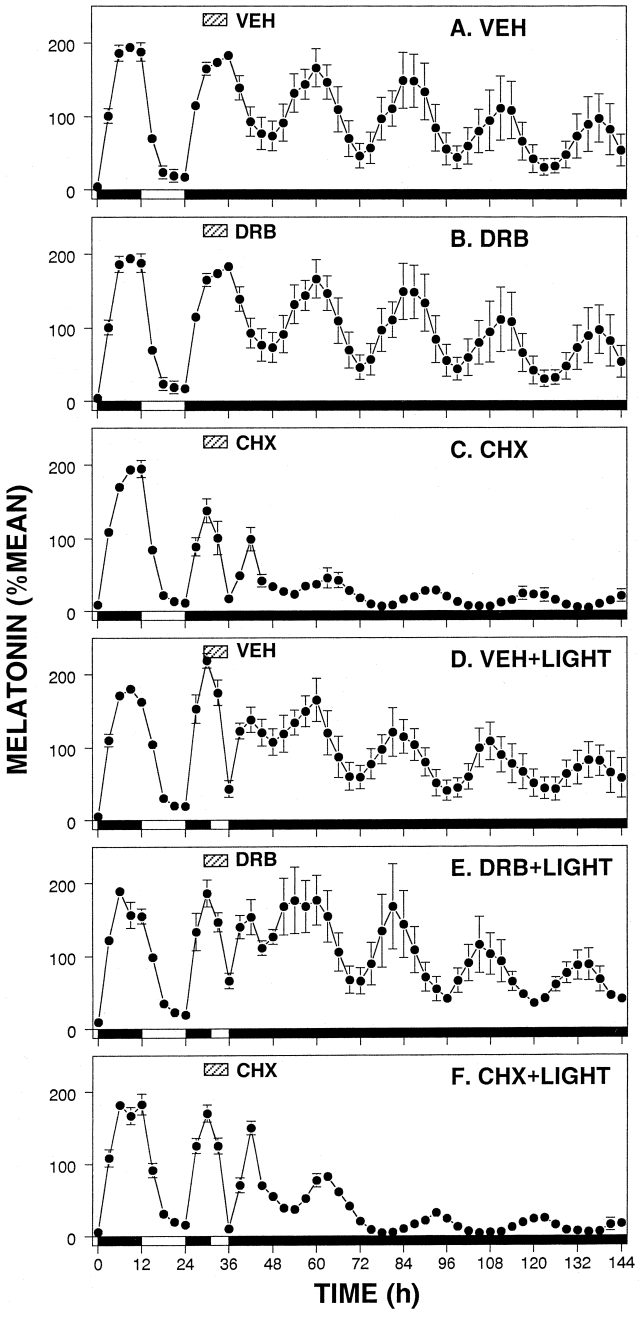

Fig. 1. Effects of a DRB or CHX pulse during the late subjective-night on the circadian rhythm in melatonin release and on its light-induced phase shift in the ayu pineal organ maintained in the superfusion culture. The pineal organs cultured under LD were exposed to DD. Then a DRB, CHX or vehicle (VEH) pulse (ZT18-24, hatched area) was applied with or without a 5-hr light pulse (ZT19-24). The normalized amount of melatonin secreted into perfusates (%; Y-axis) was plotted at the end of collection interval against incubation time (X-axis). Dark and open bars along the X-axis represent the dark phase and the light phase, respectively. Values are means± SEM (n=3–5). Phase shifts calculated from these data are depicted in Fig. 3A.

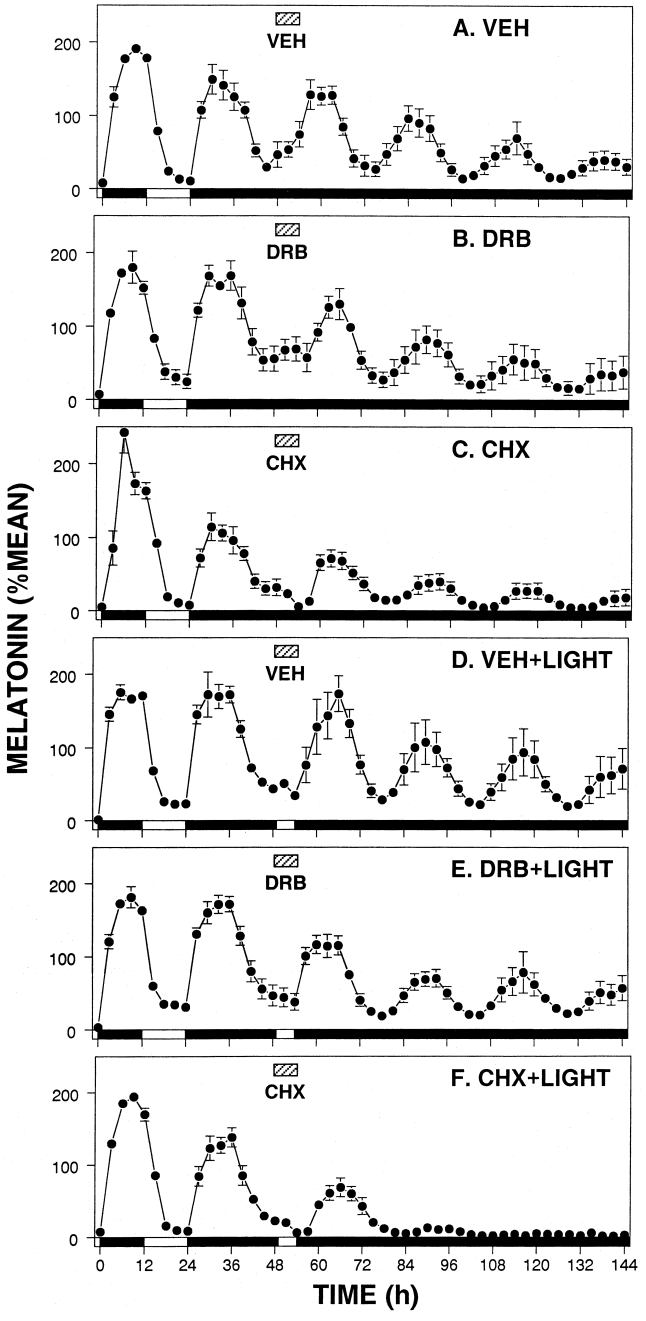

Fig. 2. Effects of a DRB or CHX pulse during the early subjective-night on the circadian rhythm in melatonin release and on its light-induced phase shift in the ayu pineal organ maintained in the superfusion culture. The pineal organs cultured under LD were exposed to DD. Then a DRB, CHX or vehicle (VEH) pulses (ZT12-18, hatched area) was applied with or without a 5-hr light pulse (ZT13-18). Further text the same as for Fig. 1. Phase shifts calculated from these data are depicted in Fig. 3B.

shift was analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test.

Confirmation of inhibition for RNA and protein synthesis

Inhibition of transcription by DRB and inhibition of translation by CHX was confirmed using [5,6-^{3}H]uridine (specific activity 41.0 Ci/mmol, Amersham Pharmacia Biotech, Tokyo, Japan) and [^{35}S]cysteine/methionine (redivue Pro-mix L-[^{35}S]*in vitro* cell labeling mix, specific activity >1000 Ci/mmol, Amersham Pharmacia Biotech), respectively. The pineal organs were dissected and cultured in 24-well culture plates (5 pineals/well for RNA, or 10 pineals/well for protein) filled with 0.5 ml medium/well. The pineal glands were maintained at 20°C under LD 12:12 (lights on 0600–1800 hr) for the first day and then kept in DD as described above. The medium was exchanged at the onset and offset of the light phase. Then, DRB or vehicle with [5,6-^{3}H]uridine (80 μCi/ml), or CHX or vehicle with [^{35}S]cysteine/methionine (100 μCi/ml) was added to the culture medium and incubated for additional 6-hr with or without a 5-hr light pulse at the two circadian phases used for determination of circadian melatonin rhythms as described above. Then, incorporation of radioactive precursors was determined according to the procedure as described (Mizusawa *et al*., 2001). The pineal organs in the same well (5 for RNA and 10 for protein) were pooled and processed simultaneously. Differences in incorporation of [^{3}H]uridine or [^{35}S]cysteine/methionine was analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test.

RESULTS

Effects of RNA and protein synthesis inhibitors on circadian rhythms in melatonin release from the ayu pineal organ

Effects of DRB and CHX on light-induced phase shift of the circadian rhythm in melatonin release from the ayu pineal organ are shown in Figs. 1, 2 and 3. During the late-subjective night, CHX (ZT18-24) and light (ZT19-24) pulses significantly delayed (P<0.001) and advanced (P<0.05) the phase of the melatonin secretion rhythm, respectively. DRB (ZT18-24) had no effects. Light-induced phase advance was diminished by the treatment of CHX but not by DRB (Figs.1 and 3A).

During the early subjective-night, DRB (ZT12-18), CHX (ZT12-18), light pulse (ZT13-18) and combination of these (DRB+light, CHX+light) all phase delayed the rhythm significantly (P<0.001) (Figs. 2 and 3B). There were no additive effects of DRB or CHX and light pulses.

Effects of inhibitors on RNA and protein synthesis in the ayu pineal organ

The effects of DRB on RNA synthesis and those of CHX on protein synthesis were exhibited in Table 1. [^{3}H]Uridine incorporated to the pineal total RNA in the control groups (vehicle-DD) were 14623±1428 and 14260±3091 cpm/pineal (mean±SE, n=3–5) for ZT18-24 and ZT12-18, respectively. [^{35}S]Cysteine/methionine incorporated into the pineal protein in the control groups were 79740±9286 and 73529±9848 cpm/pineal for ZT18-24 and ZT12-18, respectively. There were no significant variations in overall synthesis of RNA and protein at the two circadian phases examined. A light pulse itself did not induce significant changes in RNA and protein synthesis in the ayu pineal organ. Regardless of the presence or absence of a light pulse, DRB inhibited RNA synthesis to ~30% of the control and CHX inhibited protein synthesis to ~20% of the control with no significant inhibition by the vehicle treatment. Thus, RNA and protein synthesis is indeed inhibited by the treatments of inhibitors in the present study.

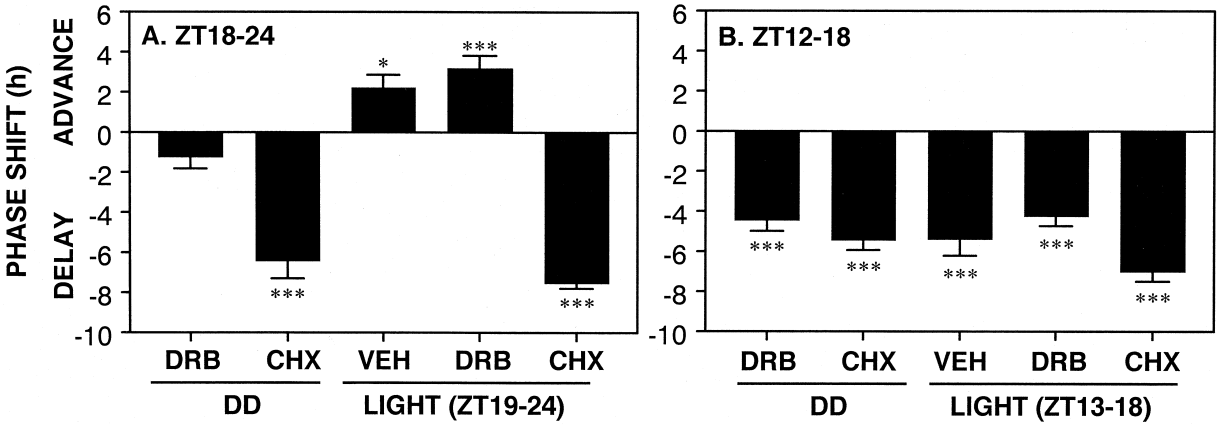

Fig. 3. Comparison of magnitude and direction of phase shift induced by DRB and CHX pulses with or without a light pulse. (A) Late subjective-night (ZT18–24). (B) Early subjective-night (ZT12–18). Phase shift is defined as the phase difference compared with the vehicle (VEH)-treated control kept under DD. Values are means ±SEM (n=3–5). **P*<0.05; ****P*<0.001 vs. the VEH-DD control.

Table 1. Effects of inhibitors for RNA synthesis (DRB) and protein synthesis (CHX) on [^{3}H]uridine and [^{35}S]cysteine/methionine incorporation into the pineal organ *in vitro*.

Time of day	Lighting conditions	[^{3}H]Uridine		[^{35}S]Cysteine/methionine	
		VEH	DRB	VEH	CHX
ZT18-24	DD	100.0±9.8	24.2±1.8**	100.0±11.6	15.1±2.9**
	Light pulse (ZT19-24)	120.6±16.9	35.1±6.6**	100.9±19.4	22.6±2.9**
ZT12-18	DD	100.0±21.7	28.8±6.3*	100.0±10.8	19.2±2.6***
	Light pulse (ZT13-18)	92.9±10.2	34.6±8.2*	95.0±12.2	18.6±2.3***

The pineal organs (5 pineals /well for RNA and 10 pineals /well for protein) maintained in static culture under LD for 1 day were exposed to DD. Then during the late subjective-night in the 1st cycle in DD or the early subjective night in the second cycle in DD, drug and/or light pulses were applied to the culture. [^{3}H]Uridine incorporation to RNA and [^{35}S]cysteine/methionine incorporation into protein were determined and expressed as the relative value (%) to the respective vehicle (VEH)-treated control groups kept under DD. Values shown are the means±SEM (n=3). **P*<0.05; ***P*<0.01; ****P*<0.001 compared with the respective control.

DISCUSSION

The present study demonstrated that *de novo* protein synthesis but not RNA synthesis is required for the phase advance of the ayu pineal circadian clock induced by a light pulse initiated during late subjective-night: treatment with CHX but not DRB diminished the light-induced phase-advance. On the contrary, we could not confirm whether the phase-delay induced by a light pulse initiated during early subjective-night involves *de novo* RNA and protein synthesis because all the treatments (light, DRB, CHX, DRB+light, and CHX+light) phase-delayed the circadian rhythm in melatonin release from the ayu pineal organ. However, it should be noted that effects of light and DRB or CHX were not additive even in the early subjective-night. These results suggest the involvement of *de novo* protein synthesis in the light-induced phase-delay of the circadian clock in the ayu pineal organ as well.

Effects of protein synthesis inhibitors on the light-induced phase shift of the circadian clock were reported in the molluscan eye (Raju *et al.*, 1990) and *Neurospora* (Johnson and Nakashima, 1990). Protein synthesis inhibitors appeared to inhibit or block phase shifts produced by light, indicating that the light pulse phase shifts the circadian clock via activation of translation of a specific protein(s). This is also the case with the ayu pineal organ since general protein synthesis was not affected by a light pulse (see Table 1). Identification of the protein(s) induced by the light pulse will help to elucidate the mechanism by which the light pulse phase-shifts the circadian clock in these circadian systems including the ayu pineal organ.

In the mammalian SCN, light pulses during the subjective-night are known to induce transcription of specific clock genes such as *Per1* and *Per2* (Albrecht *et al.*, 1997; Shigeyoshi *et al.*, 1997) and photic induction of these genes are crucial for the phase shift to occur (Akiyama *et al.*, 1999). The situation in the ayu pineal is slightly different with the mammalian SCN. This might come from the differences in the location of the photoreceptors responsible for the phase shift.

The present study also demonstrated that DRB and CHX induced phase-shift of the circadian rhythm in melato-

nin release from the ayu pineal organ, indicating that macromolecule synthesis is involved in the generation of circadian oscillation of the ayu pineal clock. This is consistent with the results obtained in other circadian systems such as the mammalian SCN (Inouye *et al*., 1988), avian pineal (Takahashi *et al*., 1989; Murakami *et al*., 1995), molluscan eye (Raju *et al*., 1990, 1991), *Neurospora* (Johnson and Nakashima, 1990), and cricket optic lobe (Tomioka, 2000). This is also consistent with the recent molecular data that described circadian expression of circadian clock genes and its products in the SCN (Dunlap, 1999; Field *et al*., 2000; Young and Kay, 2001). From the present limited study, it is too early to conclude that specific mRNA and proteins are produced during the restricted circadian phase in the ayu pineal organ. To resolve this question, we are currently trying to draw phase response curves for DRB and CHX at 8 different circadian phases during a circadian cycle. In addition, we are now trying to characterize circadian clock genes expressed in the ayu pineal organ. This may help to elucidate molecular mechanisms of the circadian clock located in the photosensitive pineal organ in fish.

In conclusion, the present study demonstrated the importance of macromolecule synthesis for photic entrainment of the circadian clock in the ayu pineal organ. Further studies including molecular characterization of clock genes will be required to elucidate the molecular basis of the circadian clock located in the photoreceptive pineal organ of fish.

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