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Diurnal gene expression of *Period2*, *Cryptochrome1*, and arylalkylamine *N*-acetyltransferase-2 in olive flounder, *Paralichthys olivaceus*

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The suprachiasmatic nucleus (SCN) of the teleost hypothalamus contains a central circadian pacemaker, which adjusts circadian rhythms within the body to environmental light-dark cycles. It has been shown that exposure to darkness during the day causes phase shifts in circadian rhythms. In this study, we examined the effect of exposure to darkness on the mRNA expression levels of two circadian clock genes, namely, *Period2* (*Per2*) and *Cryptochrome1* (*Cry1*), and the rate-limiting enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase-2 (*Aanat2*), in the pineal gland of olive flounder, *Paralichthys olivaceus*. The expression of these genes showed circadian variations and was significantly higher during the dark phase. These changes may be involved in the mechanism of dark-induced phase shifts. Furthermore, this study suggests that olive flounder may be a teleost model to investigate the localization and function of circadian oscillators.

Keywords: *Period2*; *Cryptochrome1*; arylalkylamine *N*-acetyltransferase-2; melatonin; circadian clock

Introduction

All organisms have daily rhythms that are driven by endogenous oscillators called circadian clocks, which regulate various physiological, biochemical, and behavioral functions (King and Takahashi 2000). In natural conditions, circadian rhythms are entrained to a 24 h cycle by environmental time cues, such as light (Pierce et al. 2008).

The suprachiasmatic nucleus (SCN) of the hypothalamus is the central coordinator or pacemaker of circadian rhythms, and SCN lesions of these nuclei result in arrhythmia, which causes a loss of behavioral and neuroendocrine circadian rhythms (Meijer and Rietveld 1989). It is believed that the circadian rhythm is maintained by the electrical activity of neurons in the SCN during the day and a lack of activity at night. In addition, these neurons play a crucial role in maintaining seasonal rhythms since they detect changes in light/dark cycle (Meijer and Rietveld 1989). The SCN transmits this information to the pineal gland, which is a photoreceptive organ that uses this information to produce hormones that modulate various physiological functions, such as growth and reproduction (Simonneaux and Ribelayga 2003).

The molecular mechanism of the circadian clock involves autoregulatory transcriptional/translational feedback loops (Klein et al. 1997), which include transcription factors that act as positive and negative regulators. For example, the positive clock proteins BMAL and CLOCK heterodimerize and activate the transcription of the negative clock proteins period (Per) and cryptochrome (Cry), which suppress the activity of the positive regulators (Cheng et al. 2002). Therefore light information is transduced to clock genes, such as *Per* or *Cry*, in the pineal gland, which acts as the circadian pacemaker (Cahill 2002).

Per, which was first characterized in *Drosophila* (Reddy et al. 1984), is a key protein in the circadian system of animals. In vertebrates, three *Per* homologs (*Per1*, *Per2*, and *Per3*) have been identified, and their roles as circadian oscillators have been studied extensively (Albrecht et al. 1997). In particular, *Per2* is a circadian oscillator that is rapidly induced by light information from the SCN of the hypothalamus and appears to be involved in light-dependent clock resetting (Vallone et al. 2004).

Cry (*Cry1* and *Cry2*) is an important contributor to circadian photoreception in *Drosophila melanogaster* (Okano et al. 1999), and negatively regulates its own expression, thereby setting up the rhythmic oscillations of gene expression that drive the circadian clock in mammalian (van der Horst et al. 1999). In particular, *Cry1* exhibits a circadian oscillation in cultured zebrafish cells exposed to a LD cycle; this oscillation dampens quickly after the transfer of the cells to a dark condition (Cermakian et al. 2002). However, light-dependent clock genes have only been studied in

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a few non-mammalian vertebrates, such as Japanese quail, *Coturnix coturnix* (Fu et al. 2002), African clawed frog, *Xenopus laevis* (Kubo et al. 2010), and zebrafish (Vallone et al. 2004).

The pineal organ of teleosts functions to directly perceive and transduce light-dark signals in aquatic environments (Klein et al. 1997), so fish may be good model organisms to elucidate the molecular mechanisms of the biological clock.

In fish and other vertebrates, melatonin, which is a hormone that controls the circadian clock and conveys rhythmic information, is synthesized mainly in the pineal organ from serotonin by two enzymatic reactions. First, arylalkylamine *N*-acetyltransferase (AANAT) catalyzes the conversion of serotonin to *N*-acetylserotonin, which is then catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT) to form melatonin (Klein et al. 1997). Although serotonin levels are high during the day and low at night, melatonin levels increase during the night and decrease during the day (Falcón 1999; Kim et al. 2002). The nocturnal rise in melatonin production is due to an increase in the activity of AANAT, which is the rate-limiting enzyme of melatonin synthesis (Klein et al. 1997), whereas HIOMT activity remains relatively constant throughout the light-dark cycle. In addition, BMAL/CLOCK heterodimers drive the rhythmic expression of a number of genes, including *Aanat2*, which is the main circadian clock gene, in a light-dependent manner (Klein et al. 1997). Unlike other vertebrates, teleosts prosses two *Aanat* genes (Coon et al. 1999); *Aanat1* is expressed in the retina and brain, whereas *Aanat2* is expressed in the pineal organ. In pineal cells, melatonin is synthesized by AANAT, and then secreted into the bloodstream, where it conveys photic information to various central and peripheral organs, such as the liver, gonads, and intestine (Falcón et al. 2010).

In this study, we elucidated the underlying mechanism of the circadian rhythm in the olive flounder, *Paralichthys olivaceus*, a marine flatfish that lives in benthic environments and is cultured in the coastal areas of Korea. Specifically, we investigated the diurnal changes in the mRNA expression of *Per2*, *Cry1*, and *Aanat2* in the pineal gland (in vivo, in vitro). Also, cultured pineal gland was treated with melatonin, the pattern of melatonin synthesis and secretion by the pineal organ may be directly regulated by the light-dark cycle in this marine species.

Materials and methods
Experimental fish and conditions
Olive flounder ($n = 40$, 15 ± 1.2 cm, 25 ± 3.3 g) were obtained from a commercial fish farm and were allowed to acclimate to the experimental conditions for 2 weeks in three 300-l circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at $20 \pm 1°C$ and 12-h light:12-h dark (light-on 7:00, dark-off 19:00, 200 lx during photophase and 0 lx during the scotophase at the water surface) respectively. The fish were fed a commercial feed twice daily (09:00 and 17:00). For the diurnal gene expression study fish were sacrificed at 4-h intervals. Pineal glands are removed immediately placed in RNA Trizol for tissue preservation for zeitgeber time (ZT) 4, 8, 12, 16, 20, 24, and 28 (11:00) of the following day.

In vitro culture of the pineal gland and melatonin treatment
After anesthetizing the fish, the pineal gland was dissected from the fish and placed in an ice-cold medium (pH 7.5), which was composed of 150 mM NaCl, 3.5 mM KCl, 1.0 mM $CaCl_2$ H_2O, 1.0 mM $MgCl_2$ $6H_2O$, 0.7 mM NaH_2PO_4 $2H_2O$, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 1.0 mM $MgCl_2$, 10 mM Hepes, and 0.88 g l^{-1} Eagle's MEM (Sigma, USA) containing antibiotics (0.06 g l^{-1} penicillin and 0.1 g l^{-1} streptomycin; Penicillin-Streptomycin, Gibco, USA). The pineal gland was transferred into a 24-well culture plate with 1 ml of the medium and incubated at $20 \pm 1°C$ in an incubator under LD conditions (LD 12:12, light switched on at 7:00). The light intensity at the surface of the culture plate was approximately 700 lx during the light phase.

The pineal gland was sampled at 4-h intervals ($n = 5$) from ZT4 to ZT28; each sample was centrifuged (20°C, 10,000 × g, 15 sec), and the supernatant was removed and stored at $-80°C$ until RNA extraction.

Melatonin (Sigma, USA) was dissolved in ethanol and was then diluted in physiological saline at the appropriate doses, which were added to the culture medium in the ratio 1/1000e (v/v) and indicated concentrations of melatonin (10 μM) were added at ZT0. Cultured pineal glands were treated from ZT4 to 28 without renewing the medium. Each sample was centrifuged (4°C, 10,000 × g, 15 sec), and supernatant was removed and stored at $-80°C$ until RNA extraction.

Melatonin analysis
To determine melatonin concentration in plasma, the immunoenzymoassay method was used, with a commercial enzyme-linked immunoabsorbent assay (ELISA) kit (IBL, Germany). Plasma samples were purified immediately after defrosting in the centrifuge.

They were then purified with the extraction columns. After that, 50 µl of each sample were added to different wells of an ELISA plate precoated with capture anti-body. They were incubated with the melatonin-biotin and antiserum solutions for 15 h at 4°C. The wells were then washed with the assay buffer (phosphate buffer with Tween and stabilizer) and the plate was incubated with the enzyme-labelled solution (antibiotin-alkaline phosphatase in TRIS buffer with stabilizers) for 2 h at room temperature and constant shaking. After washing the plate again, it was incubated with the *p*-nitro-phenyl phosphate solution for 30 min, before adding 50 µl of the stop solution (1 N NaOH with 0.25 M ethylenediamine tetracetic acid (EDTA)). Absorbance was read at 405 nm.

Results

Identification of Per2, Cry1, and Aanat2

The sequences of olive flounder *Per2* (GenBank Accession No. HM107817), Cry1 (HM107816), and AANAT2 (HQ883478) were obtained with RT-PCR using mRNA extracted from pineal gland. A PCR-based cloning strategy (RT-PCR followed by 3′- and 5′-RACE) was used to clone the full-length cDNA encoding AANAT2 (Figure 1).

Diurnal changes in the mRNA expression of Per2, Cry1, and Aanat2 in pineal gland

The diurnal variations in the mRNA expression of *Per2*, *Cry1*, and *Aanat2* mRNA in the pineal gland are shown in Figure 2. *Per2* and *Cry1* mRNA expression levels oscillated in olive flounder kept in light/dark conditions, with peaks of expression at ZT4, 24, 28, which corresponds to the time of light onset. *Per2* mRNA expression levels were 5.3 ± 1.2 times higher during the dawn phase (ZT24) and the beginning of the light phase (ZT4, 28) than during the light phase (ZT8) (Figure 2(a)). Similarly, *Cry1* mRNA expression levels were 2.2 ± 0.8 times higher at ZT24 than in the light phase (Figure 2(b)). *Aanat2* mRNA expression levels were 6.4 ± 1.3 times higher during the scotophase (ZT20) than the photophase (ZT8) (Figure 2(c)). Its expression peaked at ZT20, decreased significantly at
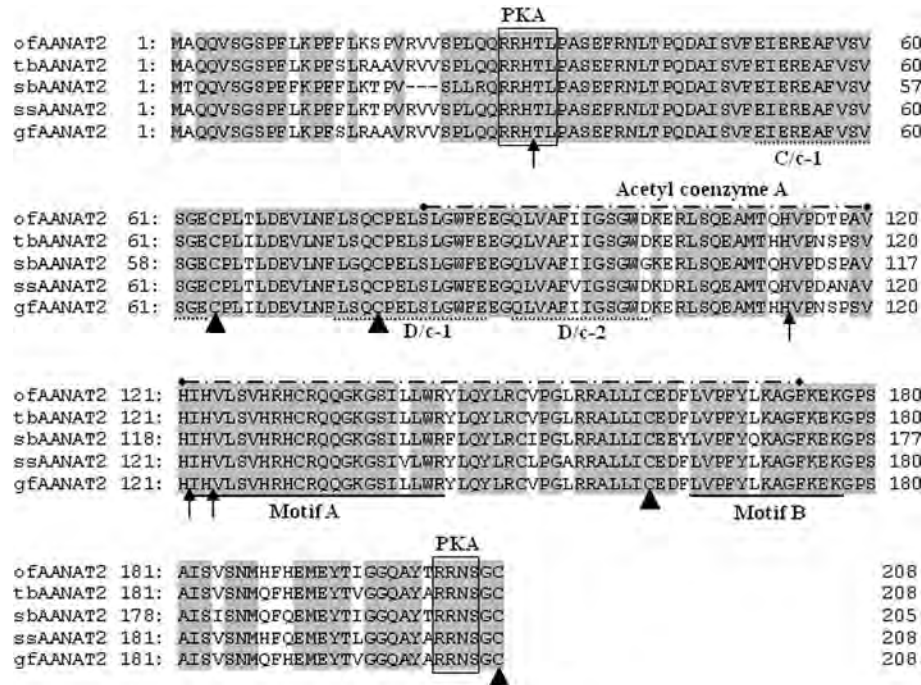

Figure 1. Multiple sequence alignment of the amino acid sequences of arylalkylamine *N*-acetyltransferase 2 (AANAT2) from olive flounder (ofAANAT2, HQ883478), turbot *Scophthalmus maximus* (tbAANAT2, EF033250), gilthead seabream *Sparus aurata* (sbAANAT2, AY533403), Senegalese sole *Solea senegalensis* (ssAANAT2, GQ340973), and goldfish *Carassius auratus* (gfAANAT2, NM1032699). The shaded amino acids indicate residues that are identical to those in the olive flounder sequence. Dashes indicate gaps that were inserted to enhance the sequence similarity. The acetyltransferase domain is underlined, while the putative acetyl coenzyme A binding motifs (Motif A and Motif B) and the two protein kinase A (PKA) phosphorylation sites are boxed. Three highly conserved regions (C/c-1, D/c-1, and D/c-2) that are found in vertebrate AANATs are indicated by dotted underlines. In addition, four highly conserved cysteine residues that are important for disulfide bond formation are indicated by solid triangles. Four conserved histidine residues also are indicated by black arrowheads.
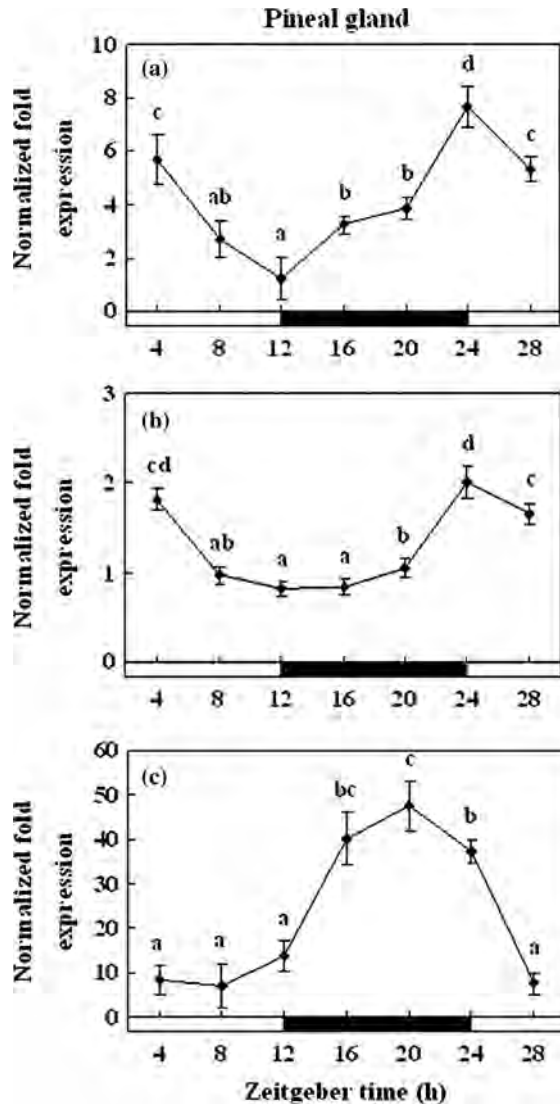
Figure 2. The mRNA expression levels of *Per2* (a), *Cry1* (b), and *Aanat2* (c) in pineal gland of olive flounder during the daily light-dark cycle, as measured by quantitative real-time PCR. Results are expressed as normalized fold expression relative to β-actin in the same sample. The white bar represents the photophase and the black bar represents the scotophase. Data points with different letters indicate statistically significant differences ($P < 0.05$). All values are expressed as mean $\pm$ SD ($n = 5$).

the beginning of the light phase, and reached its minimum during the mid-day (ZT8).

Diurnal changes in the mRNA expression of Per2, Cry1, and Aanat2 *in cultured pineal gland and melatonin treatment*

The diurnal variations in the mRNA expression of *Per2*, *Cry1*, and *Aanat2* mRNA in cultured pineal gland are shown in Figure 3. Results showed high levels of both *Per2* and *Cry1* mRNA in ZT4, 24, 28 in cultured pineal gland, followed by a marked decline from ZT8 to 20. *Per2* mRNA expression levels were 3.7 ± 0.6 times higher during the night-day transition phase (ZT24) than the light phase (ZT12) (Figure 3(a)). Similarly, *Cry1* mRNA was 15.5 ± 2.4 times higher during the night-day transition phase (ZT4, 24, 28) than the photophase (Figure 3(b)). Likewise, *Aanat2* mRNA expression levels were 11.0 ± 2.5 times higher during the dark phase (ZT20) than the light phase (ZT8) (Figure 3(c)).

The diurnal variations in the mRNA expression of *Per2*, *Cry1*, and *Aanat2* mRNA in cultured pineal gland after treatment with 10 μM melatonin are shown in Figure 3. *Per2* and *Cry1* mRNA expression levels were approximately each 3.2 ± 1.4 times and 4.8 ± 0.8 by melatonin treatment (Figure 3(d,e)). Their expression peaked at ZT24 and decreased significantly at the beginning of the light phase (ZT28). Expression of *Aanat2* mRNA was increased approximately 6.2 ± 0.4 times by treatment with melatonin (Figure 3(f)). Its expression peaked at ZT20 and decreased significantly at the beginning of the light phase (ZT28).

Plasma melatonin levels

The plasma melatonin levels in olive flounder during exposure to a normal 24 h light-dark cycle are shown in Figure 4. The plasma melatonin concentrations during the dark phase (16.0 ± 1.9 pg ml^{-1}) were 5.2 ± 2.6 times higher than those during the light phase (3.2 ± 2.1 pg ml^{-1}).

Discussion

In this study, we examined the diurnal changes in the expression of three genes, namely *Per2*, *Cry1*, and *Aanat2*, in olive flounder pineal gland to elucidate the molecular mechanism of the circadian rhythm. We found higher expression of *Per2* during the dawn under the LD conditions, whereas expression of *Per2* mRNA disappeared at dawn under the day condition in the pineal gland of olive flounder (Figure 2(a)). This is consistent with the results of several previous studies of *Per2* expression in pineal gland of reef fish (Park et al. 2007), zebrafish (Delaunay et al. 2003), chicken (Chong et al. 2003), and fly (*Sarcophaga crassipalpis*). At night, it was apparent that an endogenous component participates in the oscillations, and spontaneous oscillations were found to continue upon transfer to continuous darkness (Košotál et al. 2009).

Moreover, the diurnal changes in the expression of *Cry1* in the pineal gland were similar to those of *Per2* (Figure 2(b)). The peak expression of *Cry1* occurred at the night-day transition. These results are consistent
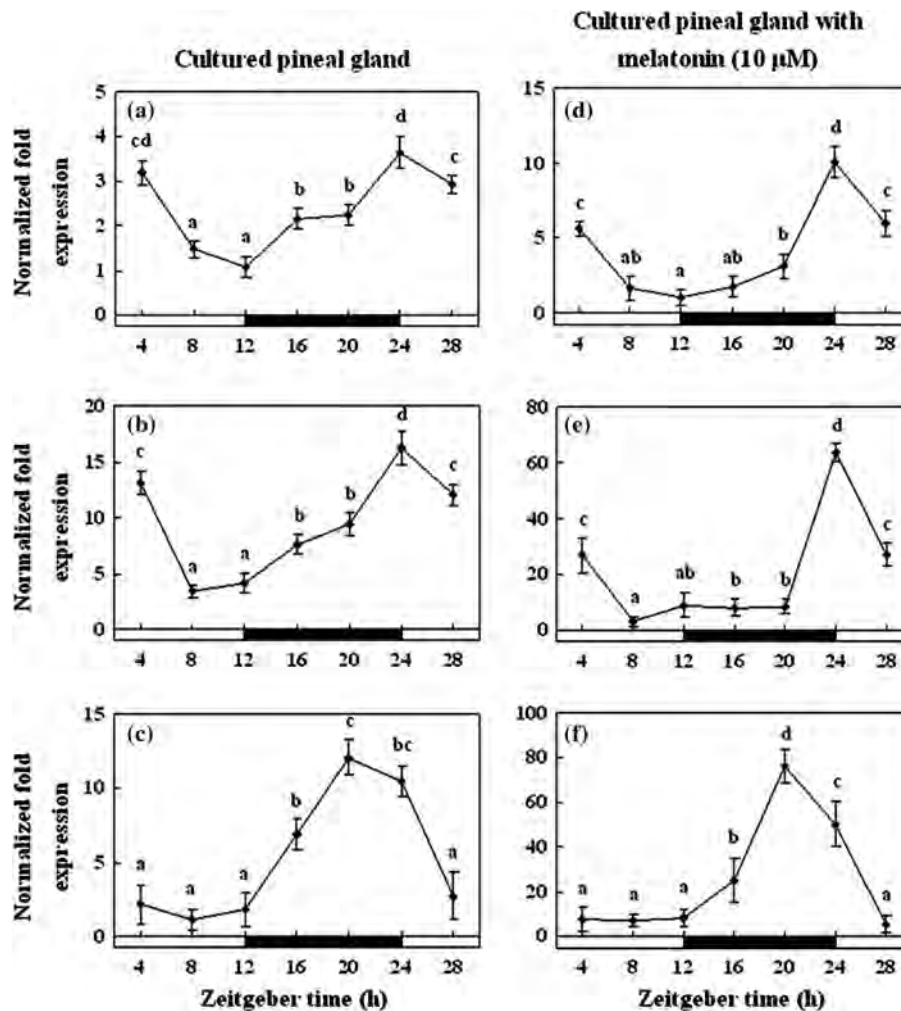
Figure 3. The mRNA expression levels of *Per2* (a, d), *Cry1* (b, e), and *Aanat2* (c, f) in olive flounder pineal gland cultures and treatment with 10 μM melatonin during the daily light-dark cycle, as measured by quantitative real-time PCR. Results are expressed as normalized fold expression relative to β-actin in the same sample. The white bar represents the photophase and theblack bar represents the scotophase. Data points with different letters indicate statistically significant differences ($P < 0.05$). All values are expressed as mean $\pm$ SD ($n = 5$).

with those of Velarde et al. (2009), who reported that *Cry1* mRNA expression increased at night (ZT14) and then decreased at photophase (ZT26) in the goldfish, there were also similar findings in Japanese quail (Fu et al. 2002).

Collectively, these results suggested that the expression of *Per2* and *Cry1* has circadian variations in neural and peripheral tissues (e.g. pineal gland) where circadian genes are considered to play an important role in the regulation of the circadian rhythm of various physiological processes, such as temperature, photoperiod, and the reproductive cycle (Delaunay et al. 2003; Velarde et al. 2009). Specifically, these genes may be involved in the light resetting of the circadian oscillator since gene transcription is rapidly induced in the SCN after exposure to light during the subjective night (Shearman et al. 1997), and *Per2* was

transiently upregulated in the pineal gland by light (Ziv et al. 2005). In cultured pineal gland, the diurnal changes in the expression of *Per2* and *Cry1* were similar to the changes that were observed in vivo (Figure 3). These results are consistent with the findings of Park et al. (2007), who showed that the mRNA levels of *Per2* in the cultured pineal gland of reef fish are increased prior to the initiation of light phase. As a result, we hypothesized that the pineal gland may act as an independent regulator of the circadian rhythm in teleosts by expressing a dominant-negative transcription factor. In most teleosts, the pineal gland functions as a photoreceptor, circadian oscillator, and central pacemaker (Cahill 2002), and it is tempting to speculate that all the photoreceptive cells of the fish organism possess a molecular clock (Falcón et al. 2010). However, our results do not rule out the
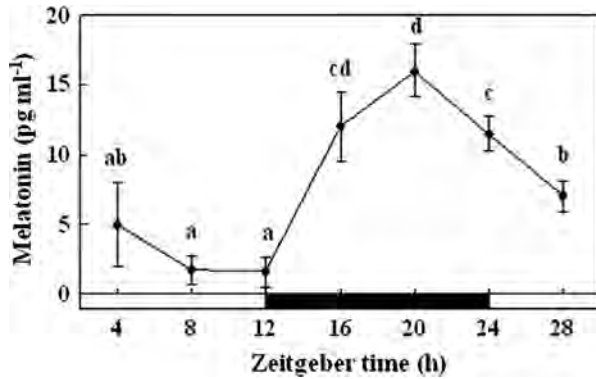
Figure 4. Plasma melatonin levels in olive flounder during the daily light-dark cycle, as shown by an enzyme-linked immunosorbent assay (ELISA). The white bar represents the photophase and the black bar represents the scotophase. Data points with different letters indicate statistically significant differences ($P < 0.05$). All values are expressed as mean $\pm$ SD ($n = 5$).

contribution of other parts of the brain to the circadian oscillation in these fish. Since the melatonin profile persists even in constant darkness (Cahill 2002), it is possible that the pineal organ coordinates environmental cues from other parts of the brain to regulate biological rhythms. Also, melatonin consistent changes, induced expression of clock genes mRNA levels.

The diurnal levels of *Aanat2* in the pineal gland significantly increased at scotophase and then decreased at photophase (Figure 2(c)). These results are consistent with those of previous studies of *Aanat2* expression in zebrafish (Appelbaum et al. 2006), pike (Coon et al. 1999), and rat (Fukuhara et al. 2004).

AANAT2 activity inhibited light and the circadian clock, and melatonin released in vivo and in vitro. In addition, since melatonin synthesis in the pineal gland is stimulated by the SCN, the pattern of melatonin synthesis usually parallels AANAT2 activity (Iuvone et al. 2005) and pineal photoreceptors. In the dark, photoreceptor depolarization allows calcium (Ca^{2+}) entry through voltage-gated Ca^{2+} channels and accumulation of cAMP (Falcón 1999). These effects promote the phosphorylation, and consequently, the activity of AANAT2. This process can be reversed by light, which induces photoreceptor hyperpolarization and dephosphorylation and degradation of AANAT2, which ultimately decreases melatonin synthesis (Falcón et al. 2001). Furthermore, the light-induced decrease in AANAT2 activity and melatonin secretion is a dose-dependent process (Migaud et al. 2006).

Our observation that the plasma melatonin concentration in olive flounder increases at scotophase and decreases at photophase (Figure 4) is consistent with several previous studies in bloch (Renuka and Joshi 2010), gilthead seabream (Falcón et al. 1996), and

goldfish (Iigo et al. 2003). In addition, our results are consistent with those of Vera et al. (2010), who showed that the levels of melatonin in the cultured pineal gland of Atlantic cod and Atlantic salmon are significantly higher during the night than the day. Therefore, we hypothesized that the nocturnal expression of *Aanat2* and synthesis of melatonin regulates the circadian rhythm. Indeed, the pineal gland modulates the secretion of melatonin according to the light intensity, which regulates the expression of genes that are involved in controlling the circadian rhythm (Cahill 2002). Gene expression analysis of *Aanat2*, the rate-limiting enzyme for melatonin synthesis, showed a robust circadian regulation in pineal gland from ZT16 to 24. Also, *Aanat2* rhythmic expression started before the onset of an overt oscillation of *Per2*.

In conclusion, *Per2* and *Cry1* are expressed in olive flounder in a light-dependent manner. We hypothesized that differences in the expression levels of these genes control the circadian rhythm in the pineal gland. The similarity of our results and those of previous studies in other fish suggest that fish may share a common mechanism for circadian rhythms. Also, AANAT may be a key factor for investigating circadian and/or photoperiodic mechanisms. Furthermore, this study suggested that olive flounder may be a teleost model to investigate the localization and function of circadian oscillators. However, further studies are needed to elucidate the mechanisms of the circadian rhythm in more detail.

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