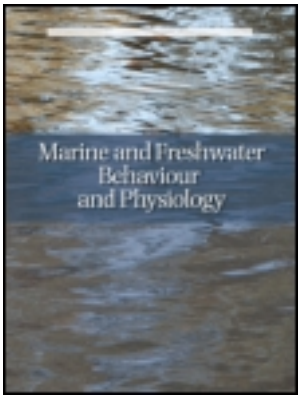

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Diurnal and circadian regulations by three melatonin receptors in the brain and retina of olive flounder *Paralichthys olivaceus*: profiles following exogenous melatonin

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To establish the molecular basis of circadian rhythm control by melatonin receptors (MTs), we investigated the mitochondrial ribonucleic acid (mRNA) expressions of three types of MTs in different tissues of the olive flounder (*Paralichthys olivaceus*). All three types of MT mRNAs were expressed in the neural tissues, while MT1 mRNA was expressed in the peripheral tissues and MT2 and MT3 mRNAs were weakly expressed or undetected in these tissues. We observed increased MT mRNA expression in the neural tissues at night under both light–dark (LD) and constant dark (DD) conditions. Although the melatonin-treated cultured pineal gland samples showed similar diurnal variations with high-MT mRNA expression levels at night compared to those of untreated cultured pineal gland samples, the expression levels were considerably higher in the melatonin-treated samples. The plasma melatonin level also significantly increased at night. Under DD conditions, the expression patterns of MT mRNAs were similar to those under the LD photocycle, but the peak was lower and the circadian change patterns were less clear. These findings reinforce the hypothesis that MTs are active in processing light information, and that these genes are regulated by the circadian clock and light, thus suggesting that MTs play an important role in daily and circadian variations in the brain and retina of olive flounders.

Keywords: circadian rhythm; melatonin receptor; olive flounder; optic tectum; *Paralichthys olivaceus*; pineal gland; retina

Introduction

Organisms exhibit many activities that vary according to periodic changes in environmental factors. The photoperiod, the most potent environmental stimulus, exerts an endogenous effect by causing a rhythmic change in melatonin levels (Bromage et al. 2001). Melatonin is produced mainly in the pineal gland and retina, and its plasma content is higher at night, between zeitgeber times (ZTs) 16 and 20, than during the day. It acts as a neuroendocrine messenger in the regulation of

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circadian and seasonal biological rhythms (Reiter 1991; Falcón et al. 2007). These actions are mediated *via* melatonin receptors (MTs), which belong to the G-protein-coupled receptor superfamily (Iigo et al. 1994a; Reppert et al. 1996).

Vertebrates have three subtypes of MTs: MT1, MT2, and MT3 (Ebisawa et al. 1994). MTs are distributed in the central nervous system and peripheral tissues of vertebrate species (Dubocovich 1995; Reppert et al. 1996) and are believed to be expressed to mediate various physiological functions of melatonin in these tissues (Ikegami et al. 2009). A variety of vertebrates reportedly express MT1 and MT2 (Reppert et al. 1995b; Roca et al. 1996). High MT1 mitochondrial ribonucleic acid (mRNA) expression has been detected in the suprachiasmatic nucleus (SCN; Von Gall et al. 2002; Dubocovich et al. 2003), where the master circadian clock system is located in mammals (Gauer et al. 1993). Therefore, MTs are thought to convey photoperiod information to control the circadian rhythm in the SCN (Masana et al. 2000). The pineal gland is also a candidate for the master circadian clock in teleost fish (Cahill 1996) because this photoreceptive gland functions as both an endocrine organ and a circadian oscillator (Collin et al. 1989). The light signals captured by this gland play important roles in a variety of physiological functions (Simonneaux and Ribelayga 2003).

The mammalian MT2 is expressed in the retina and may mediate the actions of melatonin in terms of retinal physiology (Reppert et al. 1995a). The MT1 and MT2 are expressed in the brain regions involved in processing light information, such as the thalamic region and optic tectum, in rainbow trout (Mazurais et al. 1999), and MT2 expression has been reported in the retina of sea bass (Sauzet et al. 2008). In contrast, MT3 expression has been identified in the brain and retinas of nonmammalian species such as zebrafish (*Danio rerio*), golden rabbitfish (*Siganus guttatus*), African clawed frog (*Xenopus laevis*), and chicks, suggesting that MT3 plays functional roles in neural tissues, and that it corresponds to 'quinone reductase-2,' a cytosolic enzyme that might be involved in detoxification processes (Ebisawa et al. 1994; Reppert et al. 1995b; Wiechmann and Smith 2001; Mailliet et al. 2005; Park et al. 2007b).

Melatonin activity exhibits a circadian rhythm in fish, and control of the circadian rhythm is reportedly associated with a high density of melatonin-binding sites in the brain (Yuan et al. 1990). The brain of goldfish (*Carassius aurata*) reportedly exhibits a regional distribution of melatonin-binding sites, with the highest number occurring in the optic tectum–thalamus region (Iigo et al. 1994b).

MTs were recently cloned in sea bass, and MT1 and MT2 expression levels were investigated by *in situ* hybridization in the retina (Sauzet et al. 2008). In addition, Confente et al. (2010) investigated the expression of three types of MT genes, comparing day–night and seasonal variations. Ikegami et al. (2009) investigated the expression levels of four subtypes of MT genes in the diencephalons of puffer fish (*Takifugu niphobles*) with lunar-related spawning cycles. Previous studies have shown that MTs perform the important role of regulating melatonin activity according to the circadian rhythm, but knowledge of the relationship between the expression of MTs and the control of circadian rhythm is limited, i.e. no previous studies have examined the relationship between melatonin treatment and circadian rhythm. An analysis of the expression patterns of MTs would provide a better understanding of the molecular basis of melatonin activity.

The aim of this study was to establish the molecular basis of circadian rhythm control by MTs in the olive flounder (*Paralichthys olivaceus*). We observed the

changes in mRNA expression of three types of MTs in the retina, pineal gland, and optic tectum over a 28-h time period under both 12 h light:12 h dark (LD, light–dark) and constant dark (DD) photocycles. In addition, to investigate the effect of melatonin on MT mRNA expression, we treated cultured pineal gland samples with exogenous melatonin and examined the changes in MT mRNA expression.

Materials and methods

Experimental fish and conditions

Olive flounder ($n = 50$; length 15 ± 0.5 cm; weight 27.5 ± 2.5 g) were obtained from a commercial fish farm and allowed to acclimate in three 300-L circulation filter tanks in the laboratory. During the experiments, the water temperature and photoperiod were maintained at $20 \pm 1^{\circ}$C and 12 h light:12 h dark (lights on 07:00–19:00 h, 200 lux during photophase and 0 lux during the dark phase at the water phase) for 2 weeks. For the DD experiment, the flounders were kept for 1 day and provided commercial feed twice daily (09:00 and 17:00 h). The fish were anesthetized with tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO, USA) before blood collection. Blood was collected from the caudal vein using a 1-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4°C, 10,000× *g*, 5 min) and stored at −80°C until the analysis, then killed at 4-h intervals to collect the retina, pineal gland, and optic tectum.

Tissue distribution of three subtypes of MT mRNAs

To examine tissue distribution of three subtypes of MT mRNAs, total RNA was extracted from the nervous and peripheral tissues. The brain, retina, olfactory bulb, pituitary, gill, liver, kidney, and skin were removed and immediately stored at −80°C. Total RNA was extracted from the whole brain using a TRIzol kit (Gibco-BRL, Grand Island, NY, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed with specific primer sets (Table 1) and using a 2× Taq Premix I (Solgent, Daejeon, Korea). PCR was carried out as follows: initial denaturation at 95°C for 2 min, 33 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min, followed by 7 min at 72°C for the final extension. Amplification of β-actin mRNA was also conducted to verify the quality of the RT products using a primer set for olive flounder β-actin complementary deoxyribonucleic acid (cDNA) (Table 1). The amplified PCR products were electrophoresed on 1% agarose gels, detected by staining with ethidium bromide, and visualized by illumination with UV light.

Quantitative PCR

Total RNA was extracted from the whole brain using a TRIzol kit (Gibco-BRL). RT was performed using M-MLV reverse transcriptase (Bioneer) according to the manufacturer's instructions. Quantitative PCR (QPCR), performed using cDNA from the protocol above, was conducted to determine the relative expression levels of

Table 1. Primers used.

Primer	5′-sequence-3′
cDNA amplification from different tissues	
MT1-F	CCTCATCTTCACCATCG
MT1-R	GTGGTTTGATTGCTACAGCC
MT2-F	ACTGCTACATCTGTCACTCG
MT2-R	AGTAGGCCATGAAGTAGCTG
MT3-F	TGTACAGTCTGAGGAACACC
MT3-R	TGAGGCAGCTGTTGAAGTAC
β-actin-F	TCGAGCACGGTATTGTGACC
β-actin-R	ACGGAACCTCTCATTGCCGA
Real-time PCR	
MT1-F	CCTCACCTCCATCTTCCA
MT1-R	ATGTAGCAGTAGCGGTTAATG
MT2-F	CAGAATGTCAGCACTTCCT
MT2-R	CCTCCTCGGTCTTCACTT
MT3-F	TGCTGGTGGTGTCTTACT
MT3-R	GTCGCTCGGTTTCAGTTT
β-actin-F	GCAAGAGAGGTATCCTGACC
β-actin-R	CTCAGCTCGTTGTAGAAGG

MT1 (GenBank accession no. ADI59670), MT2 (HQ844737), and MT3 (HQ844738) mRNAs using total RNA extracted from the whole brain.

Primers for QPCR were designed with reference to the known sequences of the olive flounder (Table 1). PCR amplification was conducted using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ^{TM} SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 20 s, and annealing at 55°C for 20 s. As an internal control, experiments were duplicated with β-actin. The efficiencies were found to be as follows: β-actin = 94.7%, MT1 = 95.1%, MT2 = 95.0%, and MT3 = 95.3%. As an internal control, experiments were duplicated with β-actin; all data were expressed as change with respect to the corresponding β-actin calculated cycle threshold (ΔCt) levels. The calibrated ΔCt value ($\Delta\Delta Ct$) for each sample and internal control (β-actin) was calculated [$\Delta\Delta Ct = 2^{\wedge} - (\Delta Ct_{\text{sample}} - \Delta Ct_{\text{internal control}})$]. The data were analyzed using the delta–delta method (Livak and Schmittgen 2001).

In vitro *cultures of the pineal gland and melatonin treatments*

Cultures of the pineal gland were measured using the modified methods of Park et al. (2007b). After anesthetizing the fish, the pineal gland was dissected (06:00 h) 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 $CaCl_2 \cdot H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 0.7 mM $NaH_2PO_4 \cdot 2H_2O$, 7.0 mM $NaHCO_3$, 2.8 mM glucose, 1.0 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.88 g L^{-1} Eagle's minimum essential medium (Sigma) containing antibiotics (0.06 g L^{-1} penicillin and 0.1 g L^{-1} streptomycin and penicillin–streptomycin; Gibco-BRL). The pineal gland was transferred into a 24-well culture plate with 1 mL of the medium and placed in an

incubator at 20 ± 1°C under LD and DD conditions (LD12:12, light on at 7:00 h). The light intensity at the surface of the culture plate was approximately 700 lux during the light phase. The pineal gland culturing was started at 07:00 h and sampling conducted at 4-h intervals ($n = 5$–6) from ZT4 to ZT28. Each sample was centrifuged (20°C, 10,000× *g*, 15 s), and the supernatant was removed and stored at −80°C until RNA extraction.

Melatonin (Sigma) dissolved in ethanol was added to the culture medium in the ratio 1:1000e (v/v), and the indicated concentrations of melatonin (10 μM) were added. Melatonin treatments were given at 07:00 h (ZT0). Cells were treated for 4 or 28 h without renewing the medium. Each sample was centrifuged (20°C, 10,000× *g*, 15 s), and the supernatant was removed and stored at −80°C until RNA extraction.

Melatonin determination by enzyme-linked immunosorbent assay

To determine melatonin concentration in the plasma, the immunoenzymoassay method was used with a commercial enzyme-linked immunosorbent assay (ELISA) kit (IBL, Hamburg, Germany). Plasma samples were purified in the centrifuge using extraction columns immediately after defrosting. Next, 50 μL of each sample was added to different wells of an ELISA plate pre-coated with capture antibody. The samples were then incubated with the melatonin–biotin and antiserum solutions for 15 h at 4°C, after which the wells were washed with the assay buffer (phosphate buffer with Tween and stabilizer), and the plate was incubated with the enzyme-labeled solution (antibiotin–alkaline phosphatase in TRIS buffer with stabilizers) for 2 h at room temperature and constant shaking. Following a second washing of the plate, 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). The absorbance was read at 405 nm.

Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test was conducted to detect statistically significant differences among the different time points of diurnal variations. If necessary, values were transformed (logarithmic or square root) to fulfill the conditions of the parametric ANOVA (normal distribution and homogeneity of variances). A value of $P < 0.05$ was considered statistically significant.

Results

Tissue distribution of MT mRNAs

Figure 1 shows the tissue distribution of the MT mRNAs. MT1 and MT3 mRNAs were strongly expressed in the neural and peripheral tissues. The mRNAs of all MT subtypes were expressed in the pineal gland, pituitary, optic tectum, diencephalon, cerebellum, telencephalon, and retina. MT1 mRNA was expressed in almost all peripheral tissues except the kidney, whereas the other mRNAs were weakly expressed or undetected.

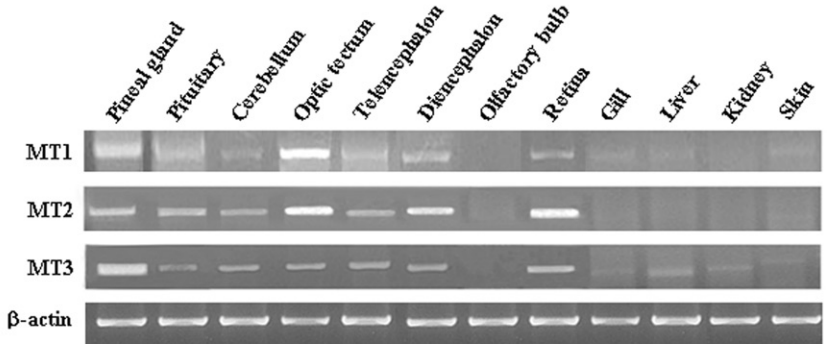

Figure 1. MT mRNAs blots showing the tissue distribution of the three subtypes of MT mRNAs in olive flounder. β-actin mRNA was amplified to verify the integrity of each mRNA sample.

Diurnal and circadian variations in MT mRNA expression in the retina, pineal gland, and optic tectum

To examine the circadian rhythm in the flounder, we examined the diurnal variation in MT mRNA expression in the retina, pineal gland, and optic tectum by QPCR (Figures 2–4). All tissues examined showed significant differences ($P < 0.05$) in mRNA expression with time under the LD conditions. The mRNA expression levels of all three subtypes peaked at night and decreased at the start of daytime, but the time to peak and expression levels varied among the subtypes. Statistically significant results were as follows: the MT1 mRNA expression levels were approximately 2-fold lower than those of the other subtypes in the retina, whereas the MT1 and MT3 mRNA expression levels were approximately 3-fold higher in the pineal gland. In addition, the MT1 and MT2 mRNA expression levels were approximately 1.3-fold higher than those of MT3 in the optic tectum, which is in agreement with the tissue distribution results (Figure 1). Finally, under DD conditions, the MT mRNA expression levels were as rhythmic, but significantly lower with lower amplitude, compared to those under LD conditions.

Diurnal and circadian variations in MT mRNA expression in untreated and melatonin-treated cultured pineal gland samples

As an *in vitro* experiment, we also examined the diurnal variations in the MT mRNA expression in untreated and melatonin-treated cultured pineal gland samples (Figures 5 and 6). In the untreated samples, the mRNA expression of all three subtypes peaked at nighttime and decreased at the start of daytime. Specifically, the peak amounts of MT1 and MT3 mRNAs were three to four times higher than that of MT2 mRNA at night. Similarly, in the melatonin-treated samples, the mRNA expression peaked at night and decreased at the start of daytime, but these samples showed approximately 5–40-fold higher peak amounts than the untreated samples. Under DD conditions, the MT mRNA expressions were as rhythmic as those under LD, but the expression levels and amplitude were significantly lower.

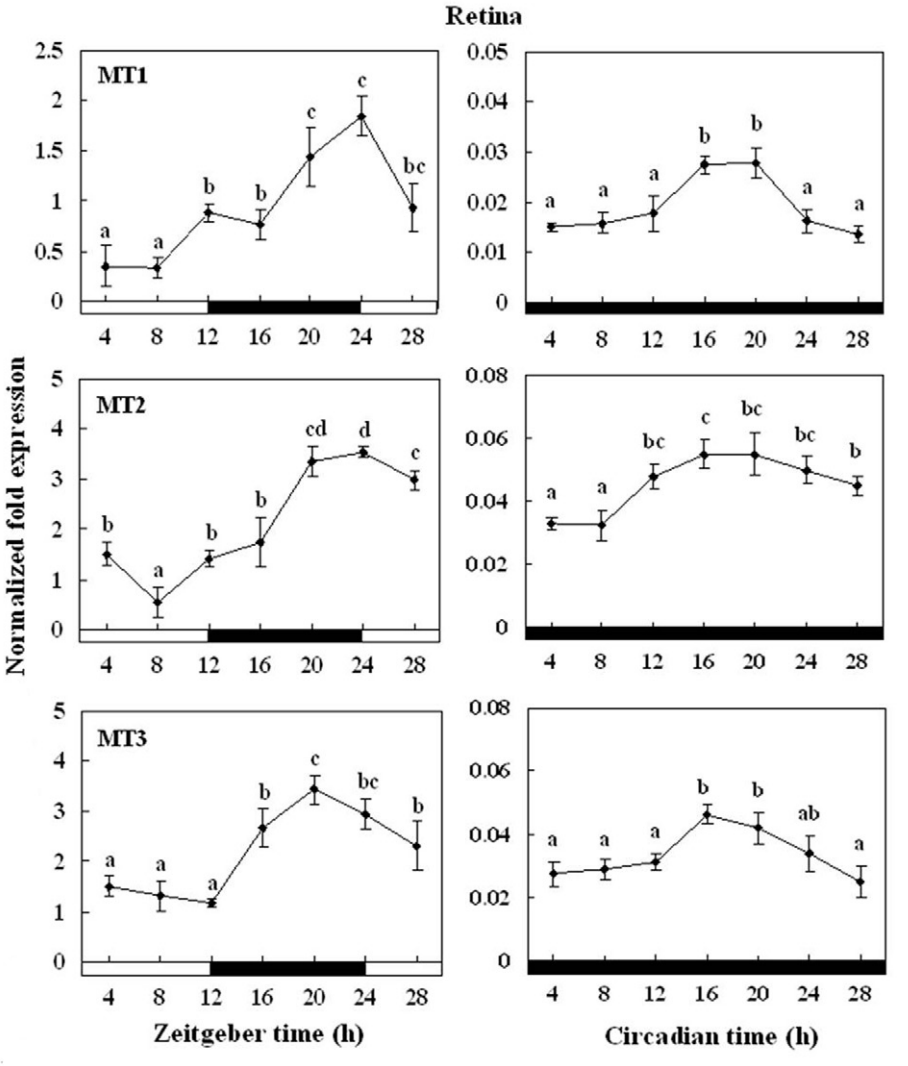

Figure 2. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by quantitative real-time PCR analysis, in the retina of olive flounder. The fish were reared under 12:12 LD and DD conditions. Total retina RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold expression with respect to β-actin in the same sample. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and circadian time (CT) ($P < 0.05$). All values are the mean ± SD ($n = 5$).

Plasma melatonin concentrations

Figure 7 shows the plasma melatonin levels in olive flounder exposed to a normal 24-h LD cycle. The melatonin levels were low during the light phase, increased by approximately 3-fold during the dark phase, and then decreased to low daytime values before lights-on (ZT24). The melatonin concentrations were significantly

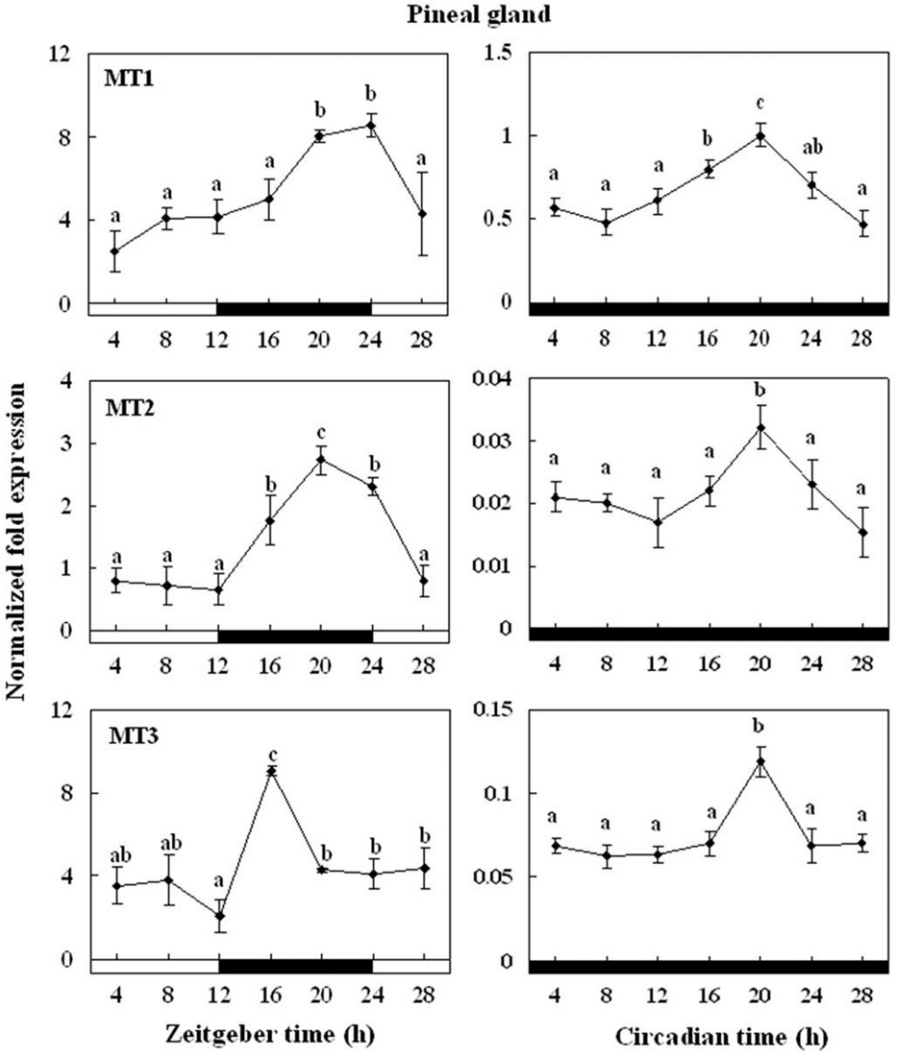

Figure 3. Diurnal and circadian variation in the mRNA expression levels of the three MT subtypes, as measured by quantitative real-time PCR analysis, in the pineal gland of olive flounder. The fish were reared under 12:12 LD and DD conditions. Total retina RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold expression with respect to β-actin in the same sample. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and circadian time (CT) ($P < 0.05$). All values are the mean ± SD ($n = 5$).

higher during the dark phase than during the light phase ($16 \pm 1.9\,\mathrm{pg\,mL^{-1}}$ vs. $5 \pm 2\,\mathrm{pg\,mL^{-1}}$; $P < 0.05$) (Figure 7a).

The plasma melatonin levels under the DD condition were similar to those under the 24-h LD cycle, although they were of lower amplitude; they peaked significantly at ZT20 ($10.0 \pm 1.1\,\mathrm{pg\,mL^{-1}}$) (Figure 7b).

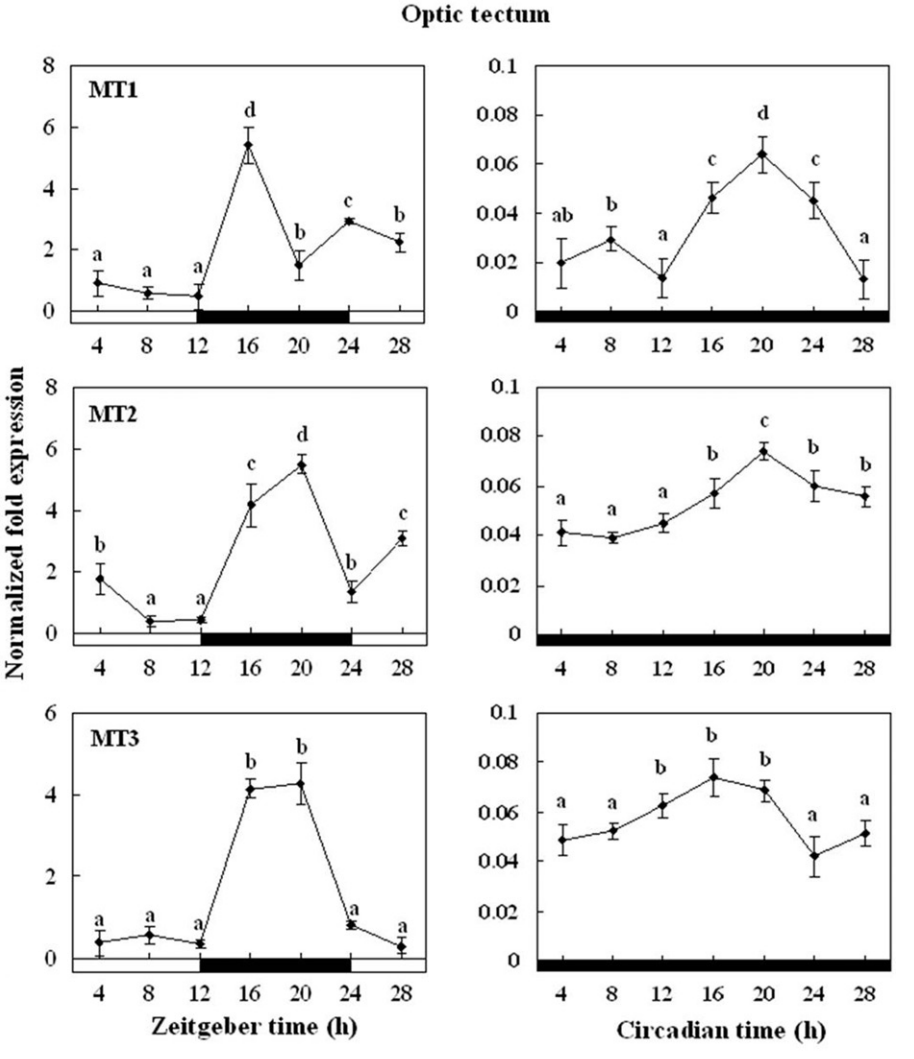

Figure 4. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by quantitative real-time PCR analysis, in the optic tectum of olive flounder. The fish were reared under 12:12 LD and DD conditions. Total retina RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold expression with respect to β-actin in the same sample. The white bars represent the photophase and black ones the scotophase. Different letters to indicate that values are statistically different in ZT and CT ($P < 0.05$). All values are the mean ± SD ($n = 5$).

Discussion

We observed the changes in MT mRNA expression in the retina, pineal gland, and optic tectum during diurnal and circadian variations to examine the role of MTs in circadian rhythm control in olive flounder. Specifically, we investigated the changes in MT mRNA expression during diurnal and circadian variations by treating cultured pineal gland samples with melatonin.

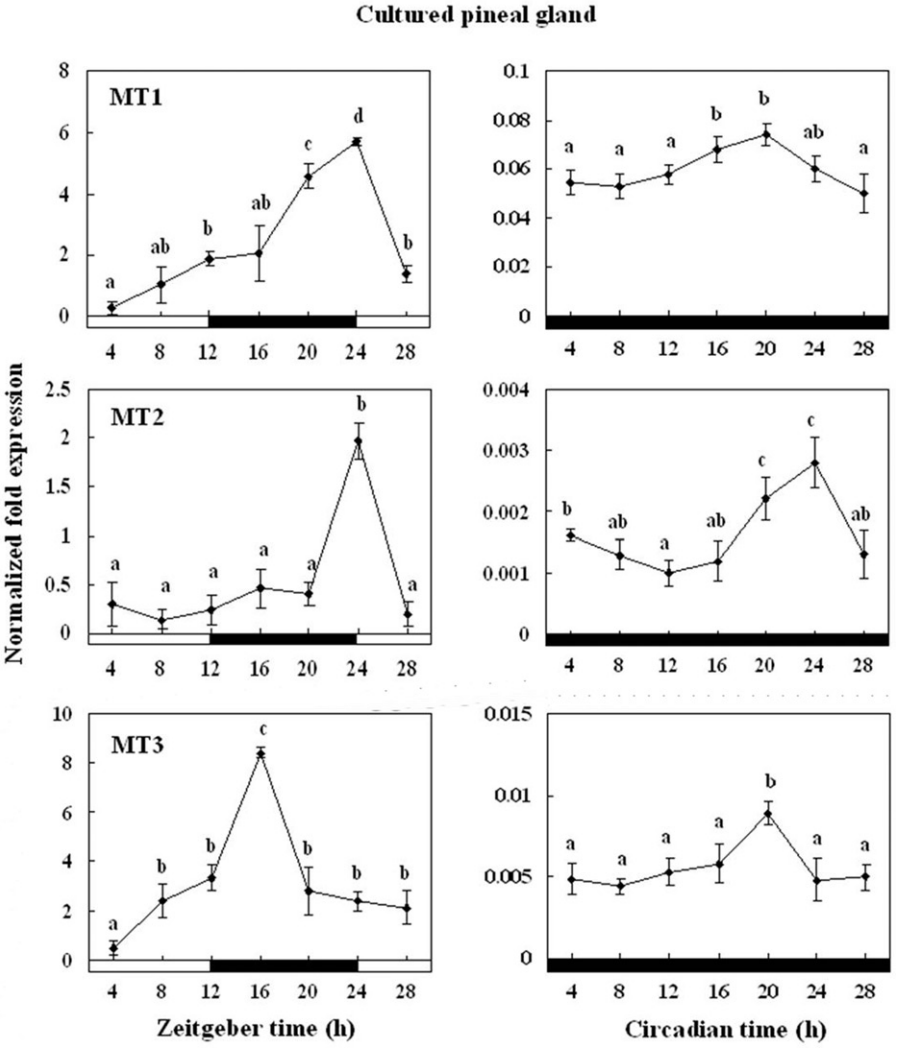

Figure 5. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by quantitative real-time PCR analysis, in the untreated cultured pineal gland samples (*in vitro*) of olive flounder. The fish were reared under 12:12 LD and DD conditions. Total retina RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold expression with respect to β-actin in the same sample. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT ($P < 0.05$). All values are the mean ± SD ($n = 5$).

In terms of their tissue distribution, all three genes were strongly expressed in the pineal gland, pituitary, optic tectum, diencephalon, cerebellum, telencephalon, and retina. The expressions of these genes in parts of the visual system, such as the retina, confirm that they play a role in visual processing (Ikegami et al. 2009). Daily and circadian variations of melatonin-binding sites under LD conditions have been noted in the retina of goldfish (Iigo et al. 1997) and European sea bass (Bayarri et al. 2004) as well as in the whole brain of goldfish (Iigo et al. 1995) and gilthead seabream

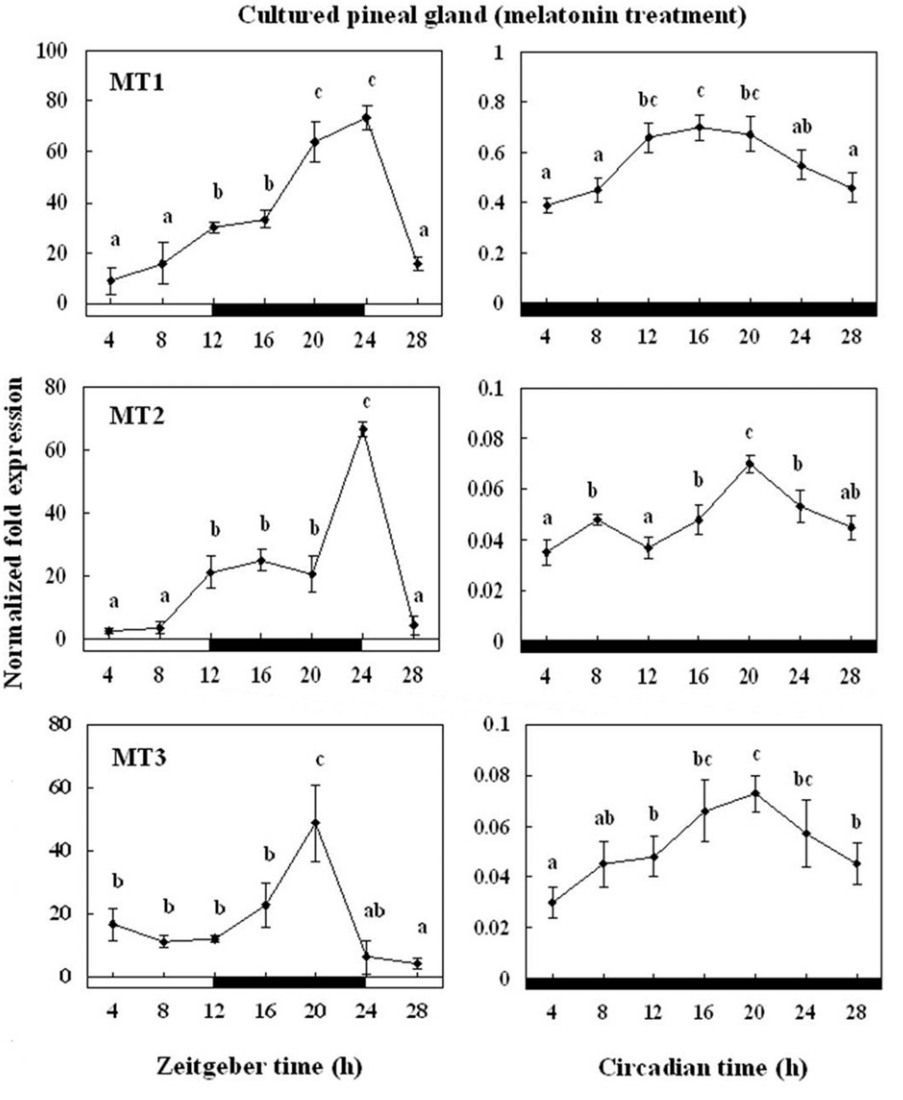

Figure 6. Diurnal and circadian variations in the mRNA expression levels of the three MT subtypes, as measured by quantitative real-time PCR analysis, in the melatonin-treated cultured pineal gland samples (*in vitro*) of olive flounder. The fish were reared under 12:12 LD and DD conditions. Total retina RNA (2.5 μg) was reverse-transcribed and amplified using gene-specific primers. The results are expressed as the normalized fold expression with respect to β-actin in the same sample. The white bars represent the photophase and black ones the scotophase. Different letters indicate that values are statistically different in ZT and CT ($P < 0.05$). All values are the mean ± SD ($n = 5$).

(*Sparus aurata*) (Falcón et al. 1996). MT2 and MT3 were either not expressed or were expressed at very low levels in peripheral tissues such as the gill and liver. Park et al. (2007a) showed that in fish, MT1 plays a role in circadian rhythm control in peripheral tissues as well as the brain, the central control organ of circadian rhythm. All three MTs were also expressed in the central nervous systems, such as the optic

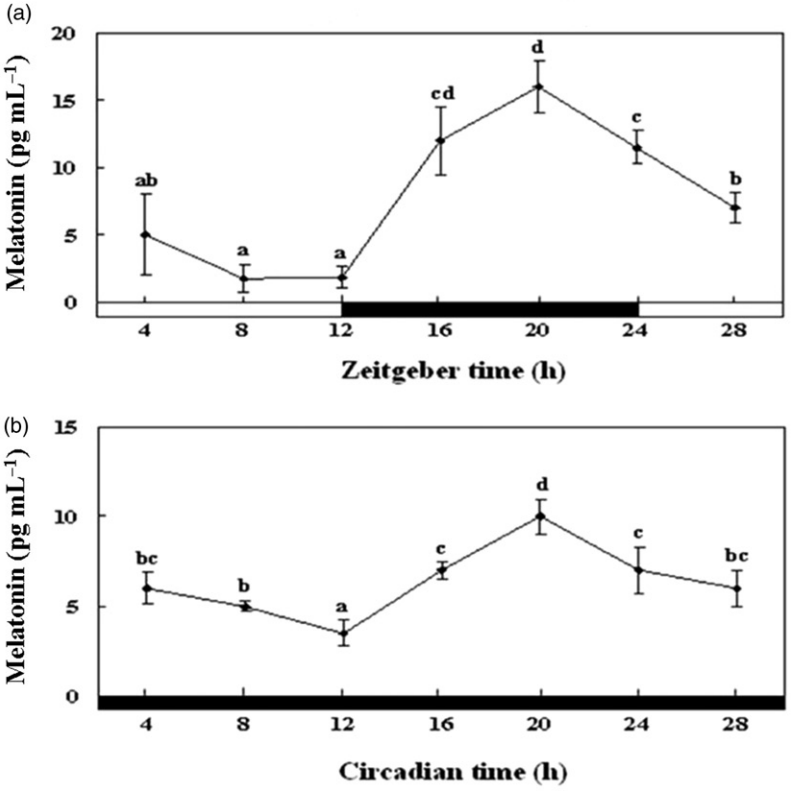

Figure 7. Plasma melatonin measurement by ELISA in olive flounder during the daily LD (A) and DD (B) cycle. The white bars represent the photophase and the black ones the scotophase. Different letters indicate that values are statistically different in ZT and circadian time (CT) ($P < 0.05$). All values are the mean ± SD ($n = 5$).

tectum and diencephalon. These results are in good agreement with the high number of melatonin-binding sites in the optic tectum–thalamus region. Iigo et al. (1994b) reported a high number of melatonin-binding sites in the optic tectum–thalamus region of goldfish. The pineal gland receives light information from the retina and acts as the central organ secreting melatonin. We therefore hypothesize that melatonin targets are mainly present in central areas integrating the visual/light information. These findings also suggest that the three types of MTs participate in mediating the melatonin activity involved in processing light information in the brain, and their distribution suggests that they may be involved in diverse functions of melatonin, such as circadian and annual control of behavioral and physiological rhythms, regulation of sleep, and neuronal apoptosis (Iigo et al. 1994a).

The expression of the three genes in the retina, pineal gland, and optic tectum varied diurnally. Specifically, they showed significantly high expression levels in the scotophase, with increasing expression at the start of nighttime (ZT12) and peak expression at ZT16 or ZT20 or ZT24 of approximately 3–18-fold. Under the DD conditions, the mRNA expression for the three genes exhibited similar circadian rhythmicity as under LD conditions. Zawilska et al. (2006) reported that melatonin

synthesis occurs in a light-dependent rhythmic manner controlled by an endogenous circadian clock, with high levels during the dark phase and low levels during the light phase. In retina, the expression levels of the three genes significantly decreased, suggesting that they are regulated by light. Melatonin is an autocrine (neural retina) and paracrine (retinal pigment epithelium) regulator of retinal functions such as cyclic biosynthesis of melatonin and circadian activity of photoreceptor cells (Sauzet et al. 2008). The diurnal expression of the three genes may be important for the circadian regulation of the retinal functions (Ikegami et al. 2009). Despite the low amounts of mRNAs, circadian variations were still visible under DD conditions. Both light and the circadian clock may therefore be responsible for the diurnal expression of the three genes.

Previous studies have shown that melatonin produced by the pineal gland is released into circulation and exerts various biological actions, such as the control of reproduction and prolactin levels in seasonal breeders as well as the regulation of several circadian rhythms when reaching MT-rich target tissues (Zawilska and Nowak 1999). The optic tectum, which is related to the circadian rhythm, also exhibits a high density of melatonin-binding sites, and MTs show significant expression changes following secretion of melatonin (Iigo et al. 1994b; Ikegami et al. 2009). Thus, the major functions of melatonin in the optic tectum are considered to be visual signal transduction. In agreement with previous results (Ebisawa et al. 1994; Reppert et al. 1995b, 1996; Wiechmann and Smith 2001), we found that MT1, MT2, and MT3 mRNA expression levels were slightly higher in the optic tectum than those of the MT genes in the retina. These findings indicate that the MT genes control the circadian rhythm in the brain at the SCN.

Retinal melatonin acts primarily in the eye, where it is involved in the control of circadian rhythm, as well as in the brain and pineal gland (Zawilska and Nowak 1992; Cahill and Besharse 1995). In the retina of golden rabbitfish, the expression of three types of MTs peaked at scotophase, ZT15, and ZT18 during a diurnal rhythm (Park et al. 2006, 2007, 2007b). These previously reported results are similar to ours in terms of the changes in MT mRNA expression in the retina (ZT20 and ZT24). Specifically, the MT2 and MT3 mRNA expression levels were approximately 2-fold higher than those of MT1 in the retina. MT2 and MT3 therefore control the physiological effects of melatonin in the retina.

The expression of the MTs peaked at scotophase during the diurnal rhythm in the cultured pineal gland samples which was similar to the *in vivo* results. Park et al. (2007a) investigated changes in the melatonin concentration and MT1 mRNA expression in cultured pineal gland samples of golden rabbitfish and found that MT1 mRNA was highly expressed in scotophase compared to the photophase. These results indicate that MTs genes are co-expressed in the pineal gland and share regulatory mechanisms of oscillation in the circadian clock. Light may therefore have different influences on the expression of the three genes depending on tissues and regulation by melatonin (Iigo et al. 1995).

In summary, similar to the results reported by Park et al. (2007a), we found that the expressions of the MTs that peaked at scotophase were rhythmic under both DD and LD conditions, but that they were of lower amplitude under the former. Specifically, the expression levels were approximately 10–100-fold higher than in the cultured pineal gland samples not treated with melatonin. Therefore, MT expression varies according to melatonin secretion levels, and melatonin controls the MT expression levels. These results were also supported by another result that the plasma

melatonin concentration peaked at scotophase (ZT20) during the diurnal rhythm. Zawilska et al. (2006) reported that the plasma melatonin concentration in turkey greatly increases in scotophase, indicating that melatonin is a timekeeping hormone that plays a central role in circadian rhythm control. Therefore, melatonin may control the circadian rhythm directly by binding with MTs, and melatonin influences the molecular clock by phasing its circadian activity. Although the SCN of fish is a circadian oscillator is not known, the master clocks could be located in the hypothalamus in addition to the eyes and pineal organ (Falcón et al. 2007).

In conclusion, MT1, MT2, and MT3 show high expression levels in the brain regions receiving light information, particularly the optic tectum–thalamus region, as well as the retina, the organ that primarily handles light information. Our results suggest that MTs play a central role in controlling the diurnal and circadian rhythms in the brain and retina of olive flounder.

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