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Comparative Biochemistry and Physiology, Part A 147 (2007) 103–111
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Expression of the melatonin receptor Mel_{1c} in neural tissues of the reef fish *Siganus guttatus*

Yong-Ju Park [a], Ji-Gweon Park [a,b], Hyung-Bok Jeong [b], Yuki Takeuchi [a], Se-Jae Kim [b], Young-Don Lee [c], Akihiro Takemura [a,*]

[a] *Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, 3422 Sesoko, Motobu, Okinawa 905-0227, Japan*
[b] *Department of Life Science, Cheju National University, 66 Jejudaehakno, Jeju 690-756, Republic of Korea*
[c] *Marine and Environmental Research Institute, Cheju National University, 3288 Hamdeok, Jocheon, Jeju 690-968, Republic of Korea*

Received 4 July 2006; received in revised form 27 November 2006; accepted 27 November 2006
Available online 5 December 2006

Abstract

The golden rabbitfish, *Siganus guttatus*, is a reef fish exhibiting a restricted lunar-related rhythm in behavior and reproduction. Here, to understand the circadian rhythm of this lunar-synchronized spawner, a melatonin receptor subtype–Mel_{1c}–was cloned. The full-length Mel_{1c} melatonin receptor cDNA comprised 1747 bp with a single open reading frame (1062 bp) that encodes a 353-amino acid protein, which included 7 presumed transmembrane domains. Real-time PCR revealed high Mel_{1c} mRNA expression in the retina and brain but not in the peripheral tissues. When the fish were reared under light/dark (LD 12:12) conditions, Mel_{1c} mRNA in the retina and brain was expressed with daily variations and increased during nighttime. Similar variations were noted under constant conditions, suggesting that Mel_{1c} mRNA expression is regulated by the circadian clock system. Daily variations of Mel_{1c} mRNA expression with a peak at zeitgeber time (ZT) 12 were observed in the cultured pineal gland under LD 12:12. Exposure of the cultured pineal gland to light at ZT17 resulted in a decrease in Mel_{1c} mRNA expression. When light was obstructed at ZT5, the opposite effect was obtained. These results suggest that light exerts certain effects on Mel_{1c} mRNA expression directly or indirectly through melatonin actions.

Keywords: Circadian rhythm; Daily variations; Golden rabbitfish; Mel_{1c}; Melatonin; Melatonin receptor; Pineal gland; Teleost

1. Introduction

Many activities in organisms vary according to periodic changes in environmental factors. The photoperiod is known to be the most potent environmental stimulus, and it exerts an endogenous effect by causing a rhythmic change in melatonin levels, which increases during the dark phase and decreases during the light phase (Bromage et al., 2001). It is well established that melatonin acts as an internal transducer and that melatonin receptors mediate melatonin actions in a variety of neural and peripheral tissues (Dubocovich, 1988; Morgan et al., 1994; Vaneček, 1998). Radioreceptor assays using 2-[^{125}I] iodomelatonin ([^{125}I]Mel) as a radioligand have demonstrated the existence of the melatonin receptors ML1 and ML2 in various vertebrates (Abe et al., 1969; White et al., 1987). ML1 belongs to a superfamily of G protein-coupled receptors and shows high affinity to [^{125}I]Mel, while ML2 (currently known as MT3) with a low affinity to [^{125}I]Mel belongs to the family of quinone reductase 2 (Nosjean et al., 2000). Molecular approaches have shown 3 different melatonin receptor subtypes: Mel_{1a} (currently known as MT1), Mel_{1b} (currently known as MT2), and Mel_{1c}. These subtypes share approximately 60% homology in amino acid sequences (Dubocovich et al., 1997; Ebisawa et al., 1994; Reppert et al., 1995a,b, 1996).

MT1 and MT2 have been widely identified in vertebrates (Dubocovich et al., 2000; Reppert et al., 1996; Slaugenhaupt et al., 1995) and are considered to be involved in the regulation of periodic activities in mammals; this is because daily and circadian variations in the expression of these melatonin receptors were noted in the suprachiasmatic nucleus (SCN),

* Corresponding author. Tel.: +81 980 47 6215; fax: +81 980 47 4919.
E-mail address: tilapia@lab.u-ryukyu.ac.jp (A. Takemura).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2006.11.027

where the master circadian clock in mammals is located (Reppert et al., 1994). On the other hand, the presence of Mel_{1c} has been proven only in the retina or brain of non-mammalian species such as zebrafish, *Xenopus laevis*, and chicken (Ebisawa et al., 1994; Natesan and Cassone, 2002; Reppert et al., 1995b; Wiechmann et al., 1999; Wiechmann and Smith, 2001).

In our previous studies, we cloned and characterized the cDNA of MT1 and MT2 from the brain of the golden rabbitfish, *Siganus guttatus* (Park et al., 2006, in press). With day/night changes, MT1 and MT2 mRNA were expressed in neural and peripheral tissues. Since daily variations in MT1 and MT2 mRNA expression were also found in the pineal gland–the possible oscillator of the central biological clock in teleost fishes (Cahill, 2002)–it was considered that melatonin receptors play an important role in the exertion of rhythmic activities in this fish. The difference in the expression patterns of MT1 and MT2 mRNA was also reported in the retina and brain of the chum salmon, *Oncorhynchus keta* (Shi et al., 2004). However, in contrast to MT1 and MT2, knowledge on the molecular aspects of Mel_{1c} in fishes is limited. The aim of the present study was to clone and characterize the Mel_{1c} cDNA of the golden rabbitfish, which primarily resides in reefs, with lunar-synchronized spawning activity. This study also aimed to examine daily and circadian variations of Mel_{1c} mRNA expression in neural tissues. Moreover, the effect of light on Mel_{1c} mRNA expression in the pineal gland was also assessed using an *in vitro* culture system.

2. Materials and methods

2.1. Experimental fish and sample collection

Juvenile golden rabbitfish (*S. guttatus*, Siganidae, Perciformes) were collected in July 2004 from the brackish creeks of Kijikina River, Okinawa, Japan, using hand nets with a small mesh size during daytime at low tide. They were transferred to the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Japan, and reared in outdoor tanks (6000-L capacity) under conditions of running seawater and ambient aeration for 1 year. The fish were fed commercial pellets (EP1 and EP2, Marubeni Nisshin, Tokyo, Japan) daily at 10:00 h. The body mass of the fish used in the present study ranged from 37.6 to 60.4 g.

The fish were transferred to 200-L tanks under a natural photoperiod (sunrise around 06:30 h and sunset around 18:30 h) and water temperature at 25.0±0.5 °C; and the fish were fed EP2 daily at 10:00 h for 2 weeks. The fish were taken from the tanks at 12:00 h ($n=8$) and 24:00 h ($n=8$), anesthetized with 2-phenoxyethanol (Kanto Chemicals, Tokyo, Japan), and sacrificed by decapitation. The retina, whole brain, liver, spleen, heart, intestine, and kidney were removed from the fish, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Dissection of the tissues at 24:00 h was done under dim red light. All experiments were in compliance with the animal care and use committee guidelines of University of Ryukyus and with the regulations for the care and use of laboratory animals in Japan.

To examine the daily and circadian variations in Mel_{1c} mRNA expression in the retina and whole brain, the fish were introduced into 500-L indoor tanks and acclimated under artificial light–dark cycle (LD 12:12, lights on at 06:30 h) for 6 days with daily feeding of EP2 at 10:00 h. On day 7, the fish were maintained under LD 12:12, constant darkness (DD), or constant light (LL) conditions, respectively. A white fluorescent bulb (40 W) was used for illumination during the light phase. Light intensity near the water surface of the tanks was approximately 800 lx. After 3 days under these conditions without feeding, the fish were anesthetized and sacrificed at 3-h intervals to collect the retina and whole brain samples ($n=6$–8).

2.2. In vitro culture of the pineal gland

After anesthesia, the pineal gland was dissected from the fish and transferred individually to a well in a 24-well microplate (Iwaki glass, Funabashi, Japan) with 1 mL of the ice-cold medium [150 mM NaCl, 10 mM HEPES, 7 mM $NaHCO_3$, 2.8 mM glucose, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.7 mM NaH_2PO_4, and 0.88 g/l Eagle's MEM (Nissui Seiyaku, Tokyo, Japan)] containing antibiotics (0.1 g/L of streptomycin and 0.01 g/L of polymyxin B sulfate, Sigma, St. Louis, MO, USA). The light intensity at the surface of the microplate was set at approximately 1000 lx during the light phase of incubation. The microplate containing the pineal gland was incubated at 24±0.5 °C in an incubator (MIR-153, Sanyo, Tokyo, Japan) under LD 12:12 (lights on at 07:00 h) for 2 days. The medium was renewed every 12 h. For assessing daily variations in Mel_{1c} mRNA expression, the pineal gland ($n=5$–6) was collected at 3-h intervals from ZT3 to ZT24, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

The effect of light on Mel_{1c} mRNA expression in the cultured pineal gland was also examined in the present study. After dissection, the pineal gland was cultured in a 24-well microplate (Iwaki glass) with 1 mL of the ice-cold medium under LD 12:12 conditions. The microplates with the pineal gland ($n=7$–8) were exposed to light (1000 lx) at ZT17 or covered with a black sheet at ZT5 and maintained at 24±0.5 °C under the LD 12:12 (light on at 07:00 h) conditions. There was no change in the light conditions in the control. After 2 h of incubation, the pineal gland was collected from the microplate, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. The duration of incubation (2 h) was decided by preliminary experiments in which the maximum effects of light on Mel_{1c} mRNA expression were obtained.

2.3. Extraction of RNA and synthesis of cDNA

Total RNA was extracted from the brain by using the TRI reagent kit (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The samples for real-time quantitative PCR were further treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to avoid genomic DNA contamination. The first strand cDNA was synthesized from 1 μg and 0.5 μg total RNA, using the