General and Comparative Endocrinology 204 (2014) 158–165

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effects of different colors of light on melatonin suppression and expression analysis of *Aanat1* and melanopsin in the eye of a tropical damselfish

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ARTICLE INFO

Article history:
Received 24 July 2013
Revised 7 April 2014
Accepted 1 May 2014
Available online 20 May 2014

Keywords:
AANAT1
Damselfish
Eye
Melanopsin
Melatonin
Photoreceptor

ABSTRACT

Ocular melatonin production exhibits a daily rhythm with a decrease during photophase and an increase during scotophase (nocturnal pattern) in teleost fish due to day–night changes in the activity of the rate-limiting melatonin synthesizing enzyme arylalkylamine *N*-acetyltransferase (AANAT). Acute light exposure during scotophase suppresses AANAT activity and melatonin production in the eyes, suggesting that external light signals are a principal regulator of ocular melatonin synthesis. To better understand the photic regulation of ocular melatonin synthesis in teleost fish, this study sought to characterize the effect of light on ocular melatonin synthesis in the sapphire devil *Chrysiptera cyanea*, which shows a nocturnal pattern and light-induced inhibition of ocular melatonin production during scotophase. Exposure to three different wavelengths of light (half-peak bandwidth = 435–475 nm with a peak of 455 nm, 495–565 nm with a peak of 530 nm, and 607–647 nm with a peak of 627 nm for the blue, green, and red LEDs) for 2 h during scotophase resulted in the blue wavelength significantly decreasing ocular melatonin content within 30 min after light exposure. This result clearly indicates that the effective range of visible light on ocular melatonin suppression is distributed within the wavelengths of blue light and that a blue light-sensitive opsin is involved in ocular melatonin suppression in the fish. A PCR-based cloning method revealed the expression of melanopsin, a putative blue light-sensitive nonvisual opsin, in the eyes. Furthermore, *in situ* hybridization using the sapphire devil *Aanat1* and melanopsin RNA probes showed mRNA expressions of both genes in the inner nuclear and ganglion cell layer of the fish retina. These results suggest that melanopsin is a possible candidate photoreceptor involved in ocular melatonin suppression by an external light signal in the sapphire devil.

1. Introduction

Melatonin is an indole-derived neurosecretory product synthesized mainly in the pineal gland and retina of vertebrates (Reiter, 1991). Melatonin is considered as a transducer of photic information in the environment because its production responds to external light conditions and exhibits a daily rhythm with an increase during scotophase and a decrease during photophase both in the pineal gland and retina of most vertebrates (Falcón and Collin, 1991; Ekström and Meissl, 1997; Iigo et al., 2007; Falcón et al., 2010). Although the regulation of melatonin production in the pineal gland and retina by the external light cue appears to operate in the same manner, the functional significance of pineal and retinal melatonin differs; melatonin synthesized in the pineal gland is released into the blood stream and cerebrospinal fluid, and plays a role in the daily periodicity of physiological, biochemical, and behavioral processes in the central and peripheral tissues, except in the retina (Grace et al., 1991; Klein et al., 1997; Falcón et al., 2010), while retinal melatonin contributes to the regulation of ocular physiology, such as retinomotor movements, modulation of neurotransmitter release, or neuronal electrical activity (Grace et al., 1991; Besseau et al., 2006; Siu et al., 2006; Ping et al., 2008; Sauzet et al., 2008; Falcón et al., 2010).

The daily variation in melatonin results from changes in the activity of the melatonin synthesizing enzyme arylalkylamine *N*-acetyltransferase (AANAT), which is driven by external photic signals (Iigo et al., 2007; Falcón et al., 2010; Migaud et al., 2010). A single *Aanat* gene is expressed in the pineal gland and retina of higher vertebrates, including mammals, birds, and anurans (Zilberman-Peled et al., 2011), while two *Aanat* genes (*Aanat1*

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http://dx.doi.org/10.1016/j.ygcen.2014.05.015
0016-6480/

and *Aanat2*) have been found in teleosts (Coon et al., 1999; Benyassi et al., 2000; Shi et al., 2004; Zilberman-Peled et al., 2004; Vuilleumier et al., 2007). In nonmammalian vertebrates, both the pineal gland and retina are well known to have photoreceptor cells and directly perceive photic cues, and involvement of the photoreceptor cells in melatonin production has been suggested in both organs (Cahill et al., 1991; Falcón and Collin, 1991). The expression of AANAT and nonvisual opsins, such as vertebrate ancient (VA) opsin and melanopsin, however, were recognized in the inner nuclear and ganglion cell layers of teleost fish (Cahill and Besharse, 1995; Drivenes et al., 2003; Jenkins et al., 2003; Foster and Bellingham, 2004; Bellingham et al., 2006; Falcón et al., 2010; Davies et al., 2011). These imply that these cells cannot be excluded from the site of ocular melatonin production and the photic regulation of ocular melatonin levels in teleost fish.

Acute light exposure during scotophase was reported to suppress ocular melatonin synthesis in many vertebrates, including teleost fish (Binkley et al., 1979; Iigo et al., 2007). In the European seabass *Dicentrarchus labrax*, which exhibits a diurnal ocular melatonin rhythm with an increase during photophase and a decrease during scotophase (García-Allegue et al., 2001), ocular melatonin content is upregulated by light during nighttime (Iigo et al., 1997a; Bayarri et al., 2002). Furthermore, only blue light wavelengths were effective in increasing ocular melatonin levels in the European seabass (Bayarri et al., 2002). This suggests that the wavelength of light influences the regulation of melatonin production in fish eyes. However, in contrast to the amount of information on the regulatory mechanisms of melatonin synthesis in the pineal gland (Iigo et al., 1994, 2007; Ekström and Meissl, 1997; Falcón, 1999), photic regulation of ocular melatonin production is unclear.

To better understand the photic regulation of ocular melatonin synthesis in teleost fish, we profiled ocular melatonin content under a light–dark cycle and investigated the effect of light on ocular melatonin production in the sapphire devil *Chrysiptera cyanea*, a reef-associated damselfish widely distributed in the shallow waters of the western Pacific Ocean. Previously, as a result of PCR-based cloning of comprehensive opsin family genes from the eyes of the sapphire devil, four types of visual cone opsin, rhodopsin, extra-retinal rhodopsin (exo-rhod), and long form of VA opsin (VAL-opsin) were successfully isolated, while cloning of blue light-sensitive cone opsin (SWS2) cDNA, which contributes to blue vision (Yokoyama, 2000), was failed (Takeuchi, 2012). To identify a candidate opsin involved in ocular melatonin regulation, we additionally cloned melanopsin and two forms of *Aanat1* cDNAs and localized their expression in the retina by *in situ* hybridization in the present study.

2. Materials and methods

2.1. Animals

Sexually mature sapphire devils with body masses between 1.9 and 3.1 g were collected from coral reefs around Irijima, Urasoe City, Okinawa, Japan, using hand nets during daytime low tide out of the breeding season (August–September). The fish were transferred to the Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Japan, and reared in plastic aquaria (stock tank; approximately 20 L capacity) equipped with filtered and recycled seawater and an aeration system under the following conditions: LD = 12:12 using a fluorescent bulb (5000 K with peaked at approximately 430, 545, 611 nm, 8.9 μmol m^{-2} s^{-1} around the water surface) and water temperature averaging 26 °C. The fish were fed with commercial pellets (Fry Feed Kyowa C1000; Scientific Feed Laboratory, Tokyo, Japan) daily around 12:00 h. The daily pattern of ocular melatonin content was examined in the end of October, 2012. After 2 weeks of acclimatization, the fish (n = 6) were taken from the aquaria every 4 h starting at Zeitgeber time (ZT) 6. After anesthetization and decapitation, the eyes were removed from the fish and frozen at −80 °C, and their melatonin content was measured.

The effect of acute light exposure during scotophase on ocular melatonin content was evaluated in the middle of May, 2013. After 2 weeks of acclimatization under the above-mentioned light–dark conditions (LD = 12:12), fish (n = 6) were exposed to light with a fluorescent bulb (5000 K, 30.4 μmol m^{-2} s^{-1} around water surface) at ZT16 for 2 h. To examine the influence of the light spectrum, fish (n = 6) were also exposed to three colored lights (half-peak bandwidth = 435–475 nm with a peak of 455 nm, 495–565 nm with a peak of 530 nm, and 607–647 nm with a peak of 627 nm for the blue, green, and red LEDs; all colored lights were adjusted to approximately 30.4 μmol m^{-2} s^{-1}) created with 2.5 W LEDs (OPTILED; Optiled Lighting International Ltd., Kwun Tong, Hong Kong) at ZT16 for 2 h. Collection of the eyes was done at 30 and 120 min after the onset of acute light exposure following anesthetization and decapitation. The eyes of fish kept without acute light exposure were also collected at the same sampling time as controls.

All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the University of the Ryukyus and the regulations for the care and use of laboratory animals in Japan. The fish were deeply anesthetized with 2-phenoxyethanol (Kanto Chemical Co., Tokyo, Japan) and euthanized by decapitation.

2.2. Melatonin measurement

The removed eyes were homogenized in 1 ml of 20 mM phosphate-buffered saline (PBS; pH 7.3) containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 13,000×g for 20 min at 4 °C. The supernatant was then used for extraction of the melatonin-containing fraction. A Sep-Pak Vac C_{18} cartridge (Waters Corporation, Milford, MA, USA) was equilibrated with 1 ml of 100% methanol and the same volume of distilled water (DW). After application of the supernatant (500 μl) and DW (500 μl), the cartridge was washed twice with 1 ml of 10% methanol and hexane. The melatonin-containing fraction was then eluted with 1 ml of 100% methanol and dried using a vacuum evaporator centrifuge system (Eyela, Tokyo, Japan). The fraction was dissolved in 500 μl of assay buffer, which consisted of 0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN_3, 0.01% Tween 40, and 20 μM diethylenetriaminepentaacetic acid (DTPA; pH 7.75; Kanto Chemical Co.).

The melatonin content in the supernatant was measured by time-resolved immunofluoroassay (TR-FIA) according to the method of Takemura et al. (2006). Rabbit anti-melatonin antiserum (anti-melatonin) (HAC-AA92-03RBP86) was obtained from the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University. Goat anti-rabbit immunoglobulin G antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and labeled with europium (Eu) using an Eu-labeling kit (PerkinElmer, Waltham, MA, USA). Melatonin–BSA conjugate (100 μl) diluted to 5 ng/ml with 0.1 M carbonate buffer (pH 9.6) was added to each well of a 96-well plate (AGC Techno Glass, Funabashi, Japan) and immobilized onto its surface. After 2 h of incubation at 24 °C, the plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) using Immuno Wash 1575 (Bio-Rad, Hercules, CA, USA). Residual binding sites on the surface of each well were blocked with PBS-T containing 0.1% BSA and 0.01% thymerosal for 1 h. After washing three times with PBS-T, 50 μl of the standard (7.8 to 4000 pg/ml) or extracted samples and 50 μl of anti-melatonin (1:200,000) diluted with assay buffer were dispensed into the wells of the plate. The plate was left overnight at 4 °C. After washing, the 2nd-IgG diluted to 1:2000 with assay buffer was added to the wells. After incubating at 25 °C for