Journal of Reproductive Immunology 92 (2011) 54–61

Contents lists available at SciVerse ScienceDirect

Journal of Reproductive Immunology

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

Variation in melatonin receptors (Mel_{1a} and Mel_{1b}) and androgen receptor (AR) expression in the spleen of a seasonally breeding bird, *Perdicula asiatica*

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ARTICLE INFO

Article history:
Received 5 April 2011
Received in revised form 20 July 2011
Accepted 9 August 2011

Keywords:
Melatonin receptors
Androgen receptor
Immunity
Reproduction
Tropical quail

ABSTRACT

Daily variation in the peripheral level of melatonin plays a major role in integrating reproduction and environmental information for seasonally breeding birds. However, the variation in immunity and reproduction has never been assessed in any avian species on a 24 h time scale. Therefore, to understand the relationship between immune function and reproductive phases in a seasonally breeding bird, *Perdicula asiatica*, the Indian jungle bush quail, we studied the daily variation of melatonin and testosterone levels along with expression of their receptors Mel_{1a}, Mel_{1b}, and androgen receptor in the spleen during the reproductively active phase. Immunocytochemistry for the melatonin receptors Mel_{1a} and Mel_{1b} presented a differential distribution pattern. Western blot of splenic protein suggested a daily rhythm of melatonin receptors, while acrophases for the two melatonin receptors Mel_{1a} and Mel_{1b} differed by 4 h, suggesting that the expression of the receptors may peak at different times, causing more of either Mel_{1a} or Mel_{1b} to be available at a particular time to mediate function. The circulatory melatonin level correlated with percentage stimulation ratio of splenocytes and plasma interleukin-2 level, but did not correlate with testosterone or androgen receptor, suggesting that melatonin could be a major hormone imparting a time-of-day effect on the modulation of immune function in a seasonally breeding bird during the reproductively active phase.

1. Introduction

Both immune function and reproductive function are considered of eminent importance in various vertebrate groups. However, only a few studies have investigated the immune capacity and reproduction of birds in relationship to environmental variables under natural conditions (Sorci et al., 1997; Singh and Haldar, 2005). It has been reported that melatonin, an endocrine modulator of the photoperiodic inductions, plays a pivotal role in the seasonal adjustment of immunity in diurnal birds (Skwarlo-Sonta, 1999). Daily variations in the immune response have been positively correlated with pineal function in terms of melatonin secretion and negatively with circulatory testosterone by several researchers in birds (Rodriguez et al., 1999; Brennan et al., 2002). Melatonin binding and receptor mRNA levels vary on a circadian basis, with expression levels affected by light and melatonin concentration in plasma (Bartness et al., 1993). Further, the actions of melatonin are considered to vary with time of the day due to the variations in the availability of melatonin binding sites on target tissues in several vertebrate species (Gauer et al., 1993; Bayarri et al., 2004).

Testosterone has traditionally been considered immunosuppressive (Grossman, 1984; Folstad and Karter, 1992). Testosterone fluctuates on a daily basis being high

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doi:10.1016/j.jri.2011.08.003

during morning hours and low at evening hours (Mock et al., 1978). Further, many studies support a complex trade-off relationship between reproduction and immune function, as the organs responsible for immunity often have specific receptors for gonadal steroids (Grossman, 1990; Avitsur and Yirmiya, 1999; Weil et al., 2006).

Perdicula asiatica, a long day breeding bird of the Indian tropical zone (latitude 25°, 18′N, longitude 83°, 01′E), presents an inversely related seasonal variation in melatonin and testosterone hormone concentration (Singh and Haldar, 2005). On the other hand, these daily changes in both of the hormones are responsible for and lead to seasonal variation in reproduction and immunity. Further, the daily variation in the expression pattern of the above receptors for hormones on target tissue could be of great importance in the fine tuning of the functional strategies showing daily rhythms such as immunity. An interrelationship between gonadal and neurohormone melatonin along with receptor expression has never been reported in any bird. Therefore, in order to understand the interplay of two hormones with opposite functions in the regulation of immunity – melatonin as an immunostimulator and testosterone as an immunosuppressor – we performed a study on the daily variation in melatonin and testosterone levels in plasma and expression of their receptors Mel_{1a}, Mel_{1b}, and androgen receptor (AR) in the spleen of the seasonally breeding species *P. asiatica* during its reproductively active phase.

2. Materials and methods

All the experiments were performed in accordance with institutional practice and within the framework of the revised Animals Act (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) of 2007 of the Government of India.

Experiments were conducted on healthy adult male *P. asiatica* (body weight 35 ± 5 g) during the reproductive active phase (May to June) for two consecutive years (2008–2009) and the mean of the data of two years are presented here. The birds were collected from the vicinity of Varanasi (latitude 25°, 18′N, longitude 83°, 01′E) and acclimated to semi-natural conditions for two weeks in an open-air fenced aviary exposed to ambient environmental conditions (May to June, day length – 14L:10D; temperature – maximum 42 °C, minimum 25 °C; humidity – 43%). They were fed with millet seeds (*Pennisetum typhoideum*) along with other seasonal grains and water *ad libitum*.

2.1. Sample collection

Male birds were selected randomly ($n = 7$) and sacrificed by decapitation after complete anesthesia (with anesthetic ether) at 4 h intervals, i.e., 0600 h, 1000 h, 1400 h, 1800 h, 2200 h, 0200 h, and again at 0600 h during a 24 h time scale. Natural sunrise was at 0530 h and natural sunset at 1830 h during the period of study. The blood from the individual birds was taken in heparinized tubes and plasma was separated out after centrifugation at 3000 rpm and stored at −20 °C for hormone analysis and cytokine (IL-2) assay. The spleen of each individual was rapidly dissected on ice and divided into three pieces. One piece was processed for splenocyte culture to observe the blastogenic response. The second piece was utilized for immunohistochemical (IHC) study following fixation in 10% neutral formalin solution and using routine histological procedures. The third piece was kept at −70 °C and later used for protein estimation by Western blot analysis. IHC was performed only for the 1400 h and 0200 h time points, while for other time points the spleen was divided into two halves and processed for splenocyte culture and Western blot analyses.

2.2. Immunohistochemical localization of melatonin receptor types Mel_{1a} and Mel_{1b}

For immunohistochemical localization of melatonin receptor types Mel_{1a} and Mel_{1b}, paraffin sections of spleen (7 μm) were used following the procedure described by Savaskan et al. (2002). The sections were incubated with primary antibody (Mel1aR; R-18, Santa Cruz Biotech, Santa Cruz CA, USA; dilution 1:200, and Mel1bR; T-18, Santa Cruz Biotech; dilution 1:200) overnight at 4 °C, then washed in PBS and incubated with biotinylated secondary antibody (Vectastain ABC Universal Kit; PK-6200, Vector Laboratories, Burlingame CA, USA; dilution 1:50). A counterstain with hematoxylin was then performed for better visualization of the lymphocytes. The preabsorption method was used to test the specificity of the antibodies in splenic tissue. The primary antibodies were replaced by a preabsorbed mixture of Mel_{1a} and Mel_{1b} receptor antisera and their respective antigenic peptides (600 ng/100 μl of Mel_{1a} receptor; sc-13186P and 600 ng/100 μl of Mel_{1b} receptor; sc-13177P peptides; Santa Cruz). For preabsorption, the antigens were added to the same diluted antisera (Mel_{1a} and Mel_{1b}; 1:200) and incubated overnight at 4 °C, centrifuged, and then the supernatant was used (data not shown). Finally, magnified sections were observed and photographed under a Leitz-MPV-3 microscope (Germany) and documented.

2.3. Western blot analysis for melatonin receptor types $Mel_{1a,1b}$ and androgen receptor

Spleens were homogenized and lysed in RIPA buffer and quantified by the Bradford method (1976). Aliquots containing 70 μg of protein for melatonin receptor types (Mel_{1a} and Mel_{1b}) and 100 μg of protein for androgen receptor were resolved with 12% and 10% SDS-polyacrylamide gel electrophoresis, respectively, followed by electrotransfer (Biometra, Goettingen, Germany) to nitrocellulose membranes (Bioscience, Keene NH, USA). Nitrocellulose membranes were blocked with Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM) containing 5% fat-free dry milk and incubated with melatonin receptor antibodies (Mel1aR, R-18, & Mel1bR, T-18, Santa Cruz Biotech; dilution 1:200) and androgen receptor antibodies (AR; N-20, sc-816, Santa Cruz Biotech; dilution 1:250). Immunodetection was performed with horseradish peroxidase conjugated secondary antibody (donkey anti-goat IgG hRP diluted 1:1000) and developed with Super Signal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific, Rockford, IL, USA). Similarly, a blot was