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Role of monochromatic light on daily variation of clock gene expression in the pineal gland of chick

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ABSTRACT

The avian pineal gland is a master clock that can receive external photic cues and translate them into output rhythms. To clarify whether a shift in light wavelength can influence the circadian expression in chick pineal gland, a total of 240 Arbor Acre male broilers were exposed to white light (WL), red light (RL), green light (GL) or blue light (BL). After 2 weeks light illumination, circadian expressions of seven core clock genes in pineal gland and the level of melatonin in plasma were examined. The results showed after illumination with monochromatic light, 24 h profiles of all clock gene mRNAs retained circadian oscillation, except that RL tended to disrupt the rhythm of *cCry2*. Compared to WL, BL advanced the acrophases of the negative elements (*cCry1*, *cCry2*, *cPer2* and *cPer3*) by 0.1–1.5 h and delayed those of positive elements (*cClock*, *cBmal1* and *cBmal2*) by 0.2–0.8 h. And, RL advanced all clock genes except *cClock* and *cPer2* by 0.3–2.1 h, while GL delayed all clock genes by 0.5–1.5 h except *cBmal2*. Meanwhile, GL increased the amplitude and mesor of positive and reduced both parameters of negative clock genes, but RL showed the opposite pattern. Although the acrophase of plasma melatonin was advanced by both GL and RL, the melatonin level was significantly increased in GL and decreased in RL. This tendency was consistent with the variations in the positive clock gene mRNA levels under monochromatic light and contrasted with those of negative clock genes. Therefore, we speculate that GL may enhance positive clock genes expression, leading to melatonin synthesis, whereas RL may enhance negative genes expression, suppressing melatonin synthesis.

1. Introduction

Circadian rhythms are fundamental to the physiology and behavior of organisms from *Drosophila* to human, with 24 h oscillations that are driven by a circadian pacemaker [1,2]. In mammals, the circadian pacemaker is located in the hypothalamic suprachiasmatic nucleus (SCN) [3]. However, the circadian system is more complex in non-mammalian vertebrates than in mammals because it is composed not only of SCN but also the retina and the pineal gland [4–6]. In the case of birds, the chick pineal gland as a pacemaker is suggested to function similarly to the mammalian SCN [7]. In all these species, the pineal gland has the primary role of synthesizing and releasing the hormone melatonin [8]. In addition, relying on opsin-based photopigments and two cryptochromes, the chick pineal gland directly receives external photic information and responds to light entrainment [9,10]. All these features make the chick pineal gland an excellent model for studies on biological clock systems.

The molecular mechanism of the circadian clock is based on a feedback transcriptional and translational loop composed of a panel of clock genes broadly characterized as the "positive limb" – *Clock* and the *Bmal* genes (*Bma11*, *Bmal2*) and the "negative limb" – the *Per* genes (*Per1*, *Per2*, *Per3*) and the *Cry* genes (*Cry1*, *Cry2*) [11]. Unlike in mammals, there is no avian *Per1*, and birds express only *Per2* and *Per3*, along with a novel avian cryptochrome gene (*Cry4*) that has been reported in the house sparrow brain [7,12–15]. This indicated that avian are unique among phyla in circadian organization. Further, the core clock genes possess their own characteristics of rhythmicity expression and sensitivity to light. In the quail, *Clock* mRNA showed remarkably rhythmic oscillation in the eye and the pineal gland [15]. In the chick, however, *Clock* is expressed throughout the day, with only weak or absent rhythmicity in the pineal gland [13,16]. Regarding the negative elements, *Per2* has been well demonstrated to be up-regulated by light in the quail pineal gland and the mouse SCN. However, *Per3* mRNA is not light inducible in the quail [15,17]. In contrast to mouse, in which only *Cry1* is rhythmic without response to acute light pulse, chicken *Cry1* and *Cry2* mRNA are rhythmically expressed in the pineal gland, and both are light inducible [10,18]. Therefore, despite the detection of homolog of known mammalian core clock genes in the avian pineal

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gland, the expression of these clock genes has been considered to be variable across species.

As a major environmental cue, light has a substantial impact on circadian physiological rhythms such as seasonal reproductive activity, migration and diurnal activities [19,20]. In many avian species, for example, the photoperiod is the dominant environment component providing time information [21,22]. Moreover, light intensity can affect the seasonal timing of birds by changing melatonin profiles [23,24]. Apart from these two photo-supplementations, light wavelength also plays an important role in the avian circadian system. Previously, Malik et al. [25] suggested that wavelength-dependent responses of the photoperiodic clock could be part of an adaptive strategy in the evolution of seasonality in reproduction and migration. Recently, the role of light wavelengths in synchronization of circadian physiology in songbirds was demonstrated [26]. Furthermore, recent studies have shown that different spectral compositions of light can also affect the melatonin rhythm in human, fish and chick [27–30]. These results implied that the shift in the wavelength of light is an essential signal that entrains the endogenous circadian clock, including the pineal gland. Although many studies have detected some responses of clock genes to changing light conditions, there are relatively few reports regarding whether shifts in the wavelength of light can influence the rhythmical changes in clock gene expression in the avian pacemaker – pineal gland.

Therefore, in the present experiments, by using the chick pineal gland as a convenient model, we describe the temporal pattern of the avian core clock genes (c*Clock*, c*Bmal1*, c*Bmal2*, c*Cry1*, c*Cry2*, c*Per2* and c*Per3*) and further characterized the effect of the wavelength of light on the expression of these molecular clock components as well as the level of melatonin in plasma. Our findings suggest a primary role of monochromatic light information to the chick pineal gland circadian system.

2. Materials and Methods

2.1. Animals and Sampling

A total of 240 newly hatched male chicks (Arbor Acre broilers) were purchased from Beijing Huadu Breeding Co. Ltd. (Beijing, P.R. China) and randomly divided into four groups. Each group allocated in a separated room was exposed to either white light (WL, 400–700 nm, control group), red light (RL, 660 nm), green light (GL, 560 nm) or blue light (BL, 480 nm) using a light emitting diode (LED) system from the post-hatching day (P) 0 [27,31]. All chicks were reared for 2 weeks under a 12/12 h cycle of light/dark environment (light on at ZT 0, zeitgeber time 0) and illuminated with 15 ± 0.2 lx measured by digital luxmeter (Mastech MS6610, Precision Mastech Enterprises, Hong Kong, China) at the level of the bird's head. The room temperature was maintained at 32 ± 2 °C in the first week and then reduced by 1 °C every 2 days until it reached to 30 °C in the second week. The mean relative humidity was 55–60%. Food and water were available to the chicks ad libitum. The diet was formulated to meet or exceed the nutrient recommendations of the National Research Council for poultry (1994).

At the beginning of the fifteenth day light-dark illumination cycle, light was off from ZT0. Chicks were kept in constant dark condition (DD). To quantify circadian expression of clock genes, chicks were quickly sacrificed by decapitation under dim red light at 6 separate time points between CT 0 and CT 20 in 4 h intervals (CT 0, CT 4, CT 8, CT 12, CT 16, and CT 20, circadian time). For each time point, ten animals (n = 10) per group were used. Blood samples were collected from cardiac puncture and heparinized with 1000 UI/mL heparin in avian saline, followed by centrifugation (1000 × *g*, 20 min, 4 °C), and then the plasma fractions were stored at −80 °C until melatonin measurement. Their pineal glands were rapidly dissected out and then frozen in liquid nitrogen and stored at −80 °C prior to total RNA isolation. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Beijing, China).

2.2. Semi-quantitative RT-PCR

Total RNA was extracted with TRIzol reagent (CW0580A, CWBIO Company, China). cDNA synthesis was generated using the Revertaid™ first strand cDNA synthesis kit (#K1622, Fermentas, USA). Each 12 μL transcription system included 2 μg total RNA, oligo $(dT)_{18}$ primer, and nuclease-free water, was incubated for 5 min at 65 °C, and then mixed with 4 μL 5× reaction buffer, 1 μL RNase Inhibitor (2 U/μL), 2 μL 10 mM dNTP mix, and 1 μL reverse transcriptase (200 U/μL). The mixtures were then incubated for 1 h at 42 °C and then 15 min at 70 °C. Polymerase chain reaction (PCR) amplification was performed with GoTaq®green master mix (M7123, Promega, USA). Briefly, 1 μL of cDNA was mixed with 5 μL 2× green master mix, 1 μL forward primer (10 μm), and 1 μL rearward primer (10 μm) in a final volume of 10 μL per reaction. The proceeding of PCR amplification was 95 °C for 5 min, 28–32 cycles of 94 °C for 30 s, 55–57 °C for 30 s, and 72 °C for 30 s, 72 °C for 10 min. The PCR primers are listed in Table 1. The PCR products were evaluated using electrophoresis on 2.0% agarose gels (EEO 0.15, Biowest, USA). The maximum OD value of the bands was analyzed using the Gel-Pro Analyzer 4.5 (Media Cybernetics, Rockville, MD, USA). The relative mRNA levels were normalized to the maximum OD value of *GAPDH* in Fig. 4I. The experiments were repeated three times.

2.3. Melatonin Measurement

Plasma melatonin rhythms were measured using the competitive inhibition Enzyme-linked Immunosorbent Assay Kit (CEA908Ge, USCN Life Science INC., Wuhan, China) for melatonin. The detection range of the assay was 12.35 pg/mL–1000 pg/mL, and the intra- and inter-assay coefficients of variations were <10% and <12%, respectively. Briefly, according to the manufacturer's protocol, 50 μL samples were incubated with 50 μL Detection Reagent A for 1 h and then 100 μL Detection Reagent B for 30 min at 37 °C. Then, 90 μL Substrate Solution (TMB, 3,3′,5,5′-Tetramethylbenzidine) was micropipetted for 20 min at 37 °C, and the reaction was terminated by the addition of 50 μL stop solution. OD values were immediately measured using an ELISA analyzer (Bio-Rad, Model 680, USA) at 450 nm.

2.4. Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). To test the significance of the differences in mRNA levels among the six daily time points, the mRNA levels of clock genes were analyzed using one-way analysis of variance (one-way ANOVA) followed by Fisher's least significant difference (LSD) post hoc test using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences between light-condition groups at each time point were considered significant at $p < 0.05$. To describe the circadian rhythmicity of each clock gene profile, the mRNA level of clock genes was analyzed separately using MATLAB 7.0 (MathWorks Inc., USA) based on unimodal cosinor regression [$y = A + (B \times \cos(2\pi(x - C) / 24))$]. A, B and C represent the mesor, amplitude and acrophase, respectively, of the circadian rhythm. The results of regression analysis were considered significant at $p < 0.05$, which was calculated using the number of samples, R^2 values and the number of predictors (mesor, amplitude and acrophase) from http://www.danielsoper.-com/statcalc3/calc.aspx?i1/415 [32].

Additionally, the effects of different monochromatic lights on the level of mRNA expression at each time point were analyzed using one-way ANOVA and Fisher's least significant difference (LSD) post hoc test in SPSS 16.0 software. Differences between light-condition groups at each time point were considered significant at $p < 0.05$.