Journal of Photochemistry & Photobiology, B: Biology 173 (2017) 476–484

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

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

Effect of monochromatic light on circadian rhythmic expression of clock genes in the hypothalamus of chick

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ARTICLE INFO

Keywords:
Circadian rhythm
Hypothalamus
Clock gene
Monochromatic light
Chick

ABSTRACT

To clarify the effect of monochromatic light on circadian clock gene expression in chick hypothalamus, a total 240 newly hatched chickens were reared under blue light (BL), green light (GL), red light (RL) and white light (WL), respectively. On the post-hatched day 14, 24-h profiles of seven core clock genes (*cClock*, *cBmal1*, *cBmal2*, *cCry1*, *cCry2*, *cPer2* and *cPer3*) were measured at six time points (CT 0, CT 4, CT 8, CT 12, CT 16, CT 20, circadian time). We found all these clock genes expressed with a significant rhythmicity in different light wavelength groups. Meanwhile, *cClock* and *cBmal1* showed a high level under GL, and followed a corresponding high expression of *cCry1*. However, RL decreased the expression levels of these genes. Be consistent with the mRNA level, CLOCK and BMAL1 proteins also showed a high level under GL. The CLOCK-like immunoreactive neurons were observed not only in the SCN, but also in the non-SCN brain region such as the nucleus anterior medialis hypothalami, the periventricularis nucleus, the paraventricular nucleus and the median eminence. All these results are consistent with the auto-regulatory circadian feedback loop, and indicate that GL may play an important role on the circadian time generation and development in the chick hypothalamus. Our results also suggest that the circadian clock in the chick hypothalamus such as non-SCN brain region were involved in the regulation of photo information.

1. Introduction

Most living organisms share a characteristic of endogenous rhythmicity, for which functional mechanisms are organized around biological clocks. Unlike mammals, birds are considered to have a central circadian system comprised of three independent endogenous circadian oscillators such as retina, pineal gland, and the suprachiasmatic nuclei (SCN) of hypothalamus [1,2]. The SCN controls the metabolism of the pineal gland, especially the SCN nocturnally restricts the synthesis of melatonin [3]. Moreover, the molecular oscillators also occur in many other non-SCN brain structures of hypothalamus to regulate physiology activity and behavior both in mammal and avian. For instant, rhythmic expressions of CLOCK and BMAL1 were found in the paraventricular, arcuate and dorsomedial nuclei of the young mouse hypothalamus [4]. Also, the circadian clock genes expressed in the mediobasal hypothalamus (MBH) of Japanese quail hypothalamus [5,6].

Circadian oscillation is generated by a molecular feedback loop with positive and negative limbs formed by a set of circadian clock genes. Briefly, the "positive elements" – *Clock* and *Bmal1* encoded for the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family, CLOCK and BMAL1. CLOCK and BMAL1 heterodimerize and activate transcription of the "negative ones" – *Per* and *Cry* genes containing *E*-box cis-regulatory enhancer sequences; in turn, PERs and CRYs form a complex to inhibit transcriptional activity of CLOCK/BMAL1. In mammal, the negative elements include *Per1*, *Per2, Per3* and *Cry1*, *Cry2* [7]. In bird, a similar transcriptional-translational feedback loop involving *Clock-Bmal1/2* and *Per2/3-Cry1/2* genes has been revealed in circadian time generation (in Japanese quail [8]; the house sparrow [9–10]; chicken [11–15]; the redheaded bunting [16]). The various profiles of circadian clock genes can be found due to different species and pacemakers. For example, the rhythmicity of *qClock* mRNA was detected in eye and pineal gland of Japanese quail [8]. However, the circadian oscillation of *rbClock* mRNA failed to be found in redheaded bunting but robust in hypothalamus [16]. Moreover, the circadian oscillation of clock genes in chick pineal gland was demonstrated under constant dark condition [17,18]. However, there are few reports about the circadian expressions of seven core clock genes in chick hypothalamus.

Daily light cycle is a dominant environmental component in providing time information to an individual [19]. Among the vertebrates, birds are profoundly affected by light in terms of their physiology and behavior to adapt life cycle processes such as activity, metabolism,

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http://dx.doi.org/10.1016/j.jphotobiol.2017.06.027
Received 17 March 2017; Received in revised form 13 May 2017; Accepted 21 June 2017
Available online 23 June 2017
1011-1344/

Table 1
Primers used for RT-PCR analysis and expected product length.

Gene	Accession No	Primer sequence (5′ to 3′)		Length (bp)
cClock	NM_204174.2	**F:** GATCACAGGGCACCTCCAATA	**R:** CTAGTTCTCGCCGCCTTTCT	301
cBmal1	NM_001001463.1	**F:** GTAGACCAGAGGGCGACAG	**R:**ATGAAACTGAACCAGCGACTC	215
cBmal2	NM_204133.1	**F:**CGGCGTTCCTTCTTCTGTC	**R:**TTCCTCTTCCACTCCCACC	165
cCry1	NM_204245.1	**F:** GATGTGGCTATCCTGTAGTTCCT	**R:** GCTGCTGGTAGATTTGTTTCAT	281
cCry2	NM_204244.1	**F:**GCACGGCTGGATAAACACT	**R:**AAATAAGCGGCAGGACAAA	141
cPer2	NM_204262.1	**F:** ATGAAACGAGCCATCCCG	**R:** CAGTTGTCGTGATTTTGCCTA	206
cPer3	XM_417528.2	**F:**CAGTGCCTTTGTTGGGTTAC	**R:**GATGGATTCACAAAACTGGAC	217
cGapdh	NM_204305.1	**F:** ATCACAGCCACACAGAAGACG	**R:** TGACTTTCCCCACAGCCTTA	124

reproduction and migration by measuring environmental changes in day length [20–23]. Although day length is considered as a major element to provide the light information, daily changes in spectral supplement of light can also influence avian physiology and behavior. For example, green light could increase broilers' growth and productive performance as well as testosterone secretion [24]. Other researches from Jin et al. [25] and Li et al. [26] demonstrated that green light elevated plasma melatonin concentration in the chick under different photoperiod, as 12 h light:12 h dark and 23 h light:1 h dark, respectively. In addition, Zhang et al. [27] reported that green light illumination via melatonin enhanced GHRH expression in the hypothalamus and plasma GH concentration in young broilers. These findings implied that monochromatic light might play a critical role in the optimization of growth and development activity in the chicks.

In our previous study, green light illumination enhanced the expressions of positive clock genes in the chick pineal gland [28]. However, to another circadian pacemaker of avian, it is less known about the integration between center clock and light wavelength in the chick hypothalamus. Therefore, this study was designed to clarify circadian expression profiles of core clock genes (*cClock*, *cBmal1*, *cBmal2*, *cCry1*, *cCry2*, *cPer2* and *cPer3*) in chick hypothalamus with the supplement of various light wavelengths.

2. Materials and Methods

2.1. Animals and Sampling

A total of 240 newly hatched male chicks (*Gallus gallus*, Arbor Acre broilers, Beijing Huadu Breeding Co, Beijing, China) were randomly reared in 4 separate color light rooms, either white light (WL, 400–700 nm), red light (RL, 660 nm), green light (GL, 560 nm) or blue light (BL, 480 nm) illuminated by light emitting diode (LED) system [24,25]. All animals were kept in 15 ± 0.2 lx measured by digital luxmeter (Mastech MS6610, Precision Mastech Enterprises, Hong Kong, China) at the level of the bird's head with 12-h light/12-h dark cycle (LD 12:12). Food and water were provided ad libitum. 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% [28].

On the post-hatched day 14, chicks were kept under constant darkness condition (DD) and sacrificed every 4 h (for 6 time points, CT 0, CT 4, CT 8, CT 12, CT 16 and CT 20, circadian time) [28]. Five animals (n = 5) per group/each time point were used for total RNA and protein isolation. The hypothalami were rapidly dissected out, instantly frozen in liquid nitrogen, and stored at − 80 °C. Another 5 animals (n = 5) for each light group per time point were killed and theirs heads were fixed in 4% paraformaldehyde for immunohistochemical staining.

All of the experimental procedures were approved by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Beijing).

2.2. Semi-Quantitative RT-PCR

The total RNA was extracted using TRIzon reagent (CW0580A, CoWin Biotech Co., Inc., Beijing, China). cDNA synthesis in the volume of 20 μL was generated by RevertAid™ first strand cDNA synthesis kit-product (#K1622, Fermentas, Waltham, MA, USA). NanoPhotometer (P330, Implen, Germany) was used to measure the quality of total RNA for each sample. 2 μg total RNA from each sample was taken with the component of 12 μL transcription system including oligo $(dT)_{18}$ primer, nuclease-free water to be incubated for 5 min at 65 °C. And then the component was mixed with 4 μL 5 × reaction buffer, 1 μL RNase inhibitor (2 U/μL), 2 μL 10 mM dNTP mix and 1 μL RT (200 U/μL) for 1 h at 42 °C and then 15 min at 70 °C. The cDNA was stored at − 20 °C for polymerase chain reaction (PCR) amplification.

PCR amplification was performed as follow. 1 μL of cDNA was mixed with 5 μL 2 X GoTaq®green master mix (M7123, Promega, Madison, WI, USA), 0.2 μL forward primer (10 μm), 0.2 μL rearward primer (10 μm) and 3.6 μL distilled water in a final volume of 10 μL reaction system. The proceeding of PCR amplification was 95 °C for 5 min, 25–32 cycles of 94 °C for 30 s, 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 performed by electrophoresis on a 2% agarose gel (EEO 0.15; Bio-Rad, Hercules, CA, USA). The MaxOD values of the bands were analyzed by the Gel-Pro Analyzer 4.5 (Media Cybernetics, Rockville, MD, USA). Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was chosen as a control for semi-quantitative PCR [28,29]. The data were expressed as the MaxOD of the bands, normalized to the MaxOD of the corresponding *cGapdh*, and the results were obtained from 3 separate experiments.

2.3. Western Blot

Tissue protein was extracted by RIPA lysis buffer (CW2333S, CoWin Biotech Co., Inc., Beijing, China). A sample of 70 μg of protein was separated in 10% SDS-PAGE, followed by electrotransfer onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk/0.5% Tween - 20 in phosphate-buffered saline (PBS) for 2 h at room temperature. The primary antibody was probed with β-actin antibody (1:4000; CW0096M, CoWin Biotech Co., Inc., Beijing, China) or anti-KAT13D/CLOCK antibody (Synthetic peptides: FEGTIQRTHRPSYEDRVC, dilution 1:1000; ab461, Abcam, Cambridge, MA, USA)/anti-BMAL1 antibody (Synthetic peptides: DMIDNDQGSSSPS, dilution 1:1000; ab3350, Abcam, Cambridge, MA, USA) overnight at 4 °C. Then, the membranes were washed in PBS, followed by a horseradish peroxidase-conjugated goat anti-mouse IgG (1:6000; CW0102M; CoWin Biotech Co., Inc., Beijing, China) for β-actin or goat anti-rabbit IgG (1:6000; CW0103M; CoWin Biotech Co., Inc., Beijing, China) for 2 h at 37 °C. Immunoblot was visualized with an eECL Western Blot Kit (CW0049M, CoWin Biotech Co., Inc., Beijing, China). The bands obtained in the blots were scanned and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA). The data were expressed as the IOD of the bands, normalized to the IOD of the corresponding β-actin bands.