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Original paper

Melatonin modulates monochromatic light-induced melatonin receptor expression in the hypothalamus of chicks

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ABSTRACT

To study the mechanism of the effect of monochromatic light on physiological function in chicken, a total of 192 newly hatched chicks were randomly divided into intact, sham-operated and pinealectomy groups then exposed to white light (WL), red light (RL), green light (GL) and blue light (BL) using a light-emitting diode (LED) system for two weeks. At P14, the hypothalami were immediately collected for immunohistochemical staining of melatonin receptor subtypes (Mel1a and Mel1b) and detection of Mel1a and Mel1b expressions using RT-PCR and western blot. Immunohistochemical staining of the hypothalamus showed that the Mel1a-ir cells were distributed in the preoptic area (POA), nucleus preopticus periventricularis (POP) and suprachiasmatic nuclei (SCN), and the Mel1b-ir cells were presented in the POA and SCN. Analysis of RT-PCR and western blot showed that the mRNA and protein levels of Mel1a and Mel1b in the hypothalamus of chick exposed to GL were increased by 10.7–29.3%, 9.18–35.9% and 8.97–27.3% compared to those in the chicks exposed to WL (P = 0.029–0.002), RL (P = 0.027–0.001) and BL (P = 0.038–0.007) in the intact group, respectively. After pinealectomy, however, these parameters decreased and there were no significant differences among the WL, RL, GL and BL groups. These findings suggested that melatonin plays a critical role in GL illumination-enhanced Mel1a and Mel1b expressions in the hypothalamus of chicks.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), an indole hormone, is mainly synthesized by pineal glands in mammalian and avian as well as in the retina of birds, which plays a major role in circadian and seasonal rhythms (Johnston and Skene, 2015), including affecting animal reproduction (Pang et al., 1998). Melatonin influences many physiological processes via binding to specific transmembrane G-protein-coupled receptors. To date, there are three different melatonin receptor subtypes (Mel1a/MT1, Mel1b/MT2 and Mel1c) in vertebrates (Li et al., 2013). The MT1 and MT2 receptor subtypes are present in mammals (Dubocovich, 1995), whereas the Mel1c subtype was only identified in fish, amphibians and birds (Sugden et al., 2004). In addition, a melatonin binding site called the “MT3” was later characterized as the enzyme quinone reductase 2 (QR2) (Nosjean et al., 2000; Mailliet et al., 2004). Meanwhile, the MT3/QR2 binding site demonstrated that it was widely distributed in mammals (Nosjean et al., 2001). MT3 was also proven to be present in embryonic and post-hatch chick retina (Sampaio et al., 2014). Furthermore, melatonin receptors were found in the chicken brain (Rivkees et al., 1989; Stehle, 1990; Pang et al., 1995) and numerous peripheral tissues including ovary, lung, spleen and kidney of the chicken (Pang et al., 1995; Sundaresan et al., 2009). Fusani and Gahr (2014) studied the three melatonin receptor subtypes in the brain of two songbirds, the blackcap and the zebra finch, and found that melatonin receptors presented the area-specific expression patterns and species-related differences in the brain of birds. However, few papers have been reported on the location and distribution of melatonin receptors in the chick hypothalamus.

Avians have superior vision system compared to mammals, making the exogenous light a key factor in the management of chickens especially their reproduction. For example, different photoperiods have been shown to affect hypothalamic GnRH-I mRNA expression in female turkeys at different reproductive states (Kang et al., 2006). According to previous studies, the melatonin receptor expression can be influenced by many factors such as photoperiod (Moniruzzaman and Maitra, 2012; Mukherjee and Haldar, 2014), daily rhythms (Bayarri et al., 2004), season (Bentley et al., 2013), age and sex (Singh et al., 2015). For example, the photoperiod affected the melatonin receptor expression in avian brain (Aste et al., 2001). In addition, the different light wavelengths affected the behavior of broilers (Sultana et al., 2013). D.Y. Li

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et al. (2015) and J. Li et al. (2015) found monochromatic light affected the expression patterns of melatonin receptors in chicken ovarian follicles. Therefore, we hypothesized that different light wavelengths could influence the melatonin receptor expression in the chick hypothalamus.

If so, an interesting question is: how does light information affect the melatonin receptor expression in the chick hypothalamus? As we all know, melatonin is a pivotal indicator of photoelectric conversion and functions via binding to its corresponding receptor. Furthermore, our previous study showed that monochromatic green light enhanced the level of plasma melatonin in chicks (Jin et al., 2011), and played a role in the influence of monochromatic lights on the expression of three melatonin receptors in the thymus of broilers (Chen et al., 2016a,b). Therefore, we hypothesized that monochromatic light affected the expression of three melatonin receptors via melatonin in the chick hypothalamus.

Hence, this study was designed to: (1) explore the location and distribution of Mel1a and Mel1b in the chick hypothalamus; (2) study the impact of monochromatic light on the expression of Mel1a and Mel1b in the chick hypothalamus; and (3) discuss the role of melatonin in the influence of monochromatic light on the expression of Mel1a and Mel1b in the chick hypothalamus.

2. Materials and methods

All the animal experiments were conducted in accordance with the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Beijing).

2.1. Animal treatments

A total of 192 newly hatched Arbor Acre male broilers (0 day old) were procured from Beijing Huadu Breeding Co., Ltd. (Beijing, China). All broilers were randomly selected and divided into four groups (n = 48/group) and exposed to different lights using a light-emitting diode (LED) (Hongli Tronic Co., Guangzhou, China) system for two weeks, group I: white light (WL, 400–760 nm), group II: red light (RL, 660 nm), group III: green light (GL, 560 nm) and group IV: blue light (BL, 480 nm). Each light group contained four separate rooms (12 birds per room) at a density of 11.5 birds/m^2 with a set of independent light sources. The light intensity was 0.25 W/m^2 at the bird-head level, and the light schedule was 23L:1D (lights off at 23:00). Each light group was divided into three subgroups, including intact (n = 16), sham-operated (n = 16) and pinealectomy groups (n = 16). The pinealectomy or sham operation was processed at P3 (D.Y. Li et al., 2015; J. Li et al., 2015). Broilers were kept under constant temperature 32 °C for the first week and then reduced to 25 °C in the second week, and the relative humidity was maintained at 60%. Broilers had access to food and water ad libitum.

2.2. Tissue collection and processing

At age of P14, at 8:30 am, within one hour blood samples were collected via cardiac puncture, treated with heparin (1000 UI/mL), and centrifuged at 100 × *g* for 30 min. At 8:30 am, within one hour the brains were immediately removed, and half of them in each group were quickly fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4, 4 °C) for immunohistochemical staining. The hypothalami were immediately separated from the other half of each group, and rapidly frozen in liquid nitrogen for RT-PCR and western blotting experiments, respectively.

2.3. Immunohistochemical staining

For immunohistochemical studies, brains were immersed in 4% paraformaldehyde in 0.1 M PB for 24 h and cut frozen serial sections (40 μm in thickness) and cut paraffin serial sections (10 μm in thickness). The frozen sections of hypothalami were prepared and immunohistochemically stained for Mel1a; the paraffin sections of hypothalami were prepared and immunohistochemically stained for Mel1b. Endogenous peroxidase activity was eliminated by 3% hydrogen peroxide in absolute methanol for 30 min. Sections were washed with 0.01 M PBST (pH 7.4; thrice for 10 min each time), and incubated with 5% normal donkey serum in 0.01 M PBST (pH 7.4) for 1 h at room temperature. The sections were then incubated with primary antibodies (MEL-1A-R sc-13186, MEL-1B-R sc-13177, Santa Cruz Biotechnology, Inc. USA, dilution 1:100) for 48 h at 4 °C. After rinsing with 0.01 M PBS (pH 7.4; thrice for 10 min each time), the sections were incubated with a biotinylated donkey anti-goat IgG (1:500, ab6884, Abcam, Cambridge MA, USA) for 3 h at room temperature. After rinsing three times, the sections were incubated with streptavidin-conjugated horseradish peroxidase (1:300, Vector Laboratories, Burlingame CA, USA) for 3 h at room temperature. Sections were then washed with PBS, the antigens were visualized using 0.05% 3′,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) and 0.003% hydrogen peroxide in 0.01 M TBS (pH 7.6) for 5 min in the dark. Then, the sections were mounted on the prepared slides. Control sections in which the primary antibodies were replaced with 0.01 M PBS (pH 7.4) were examined in all cases (data not shown). The immunoreactive cells were stained yellow-brown in the cytomembrane of the perikarya. The nuclei of cells were counterstaining by hematoxylin. The localization of the immunoreactive cells in the hypothalami was observed using an Olympus BX51 microscope (Japan) according to a stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988).

2.4. Rt-pcr

Total RNA was extracted according to the manufacturer's protocol by using Trizol Reagent (CW0580A, CoWin Biotech Co., Inc., Beijing, China), and 2 μg of treated RNA was reverse transcribed by the GoScriptTM Reverse Transcription System (A5001, Promega, USA) according to the manufacturer's protocols for cDNA synthesis. RT reactions were performed for 1 h at 42 °C by using 2 μg of total RNA, oligo (dT) primers, and superscript reverse transcriptase in a final volume of 20 μL. *GAPDH*, a housekeeping gene, was used as the internal standard. The sequences of the primers for RT-PCR were listed in Table 1. The primers were designed by Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR amplification was performed in a 10 μL mixture containing 5 μL GoTaq® Green Master Mix (M7123, Promega, Madison, USA), 1 μL cDNA, 0.2 μL primers, and 3.6 μL nuclease-free water. The cycling protocol consisted of initial 5 min at 95 °C followed by the cycles (94 °C for 30 s, annealing at the temperature in Table 1 for 30 s, 72 °C for 1 min) in Table 1, and 72 °C for 5 min. The PCR products were electrophoretically detected on 2% agarose gel electrophoresis after staining with ethidium bromide. The mRNA levels in the hypothalamus were measured by professional

Table 1
Sequences of primers used for RT-PCR.

Genes	Primer sequences (5′-3′)	Accession no.	Product size (bp)	Tm (°C)	Cycles
Mel1a	F:CAA TGG ATG GAA TCT GGG A R:GCT ATG GGA AGT ATG AAG TGG	NM_205362.1	333	58	33
Mel1b	F:TTT GCT GGG CAC CTC TAA AC R:CGC TTG CTC TTC TGT CCA TC	XM_417201.2	259	60	33
GAPDH	F:ATC ACA GCC ACA CAG AAG ACG R:TGA CTT TCC CCA CAG CCT TA	NM_204305.1	124	59	25