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Effect of Monochromatic Light on Melatonin Secretion and Arylalkylamine *N*-Acetyltransferase mRNA Expression in the Retina and Pineal Gland of Broilers

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ABSTRACT

The goal of this study is to investigate the effects of various monochromatic lights on plasma melatonin (MT) levels and the expression of arylalkylamine *N*-acetyltransferase (AANAT) mRNA in the pineal gland and retina. A total of 160 newly hatched (posthatching day 1, P1) broilers, including intact, sham-operated, and pinealectomized groups were exposed to blue light (BL), green light (GL), red light (RL), and white light (WL) by light emitting diode (LED) system for short term (24 hr) or long term (2 weeks), separately. For intact and sham-operated birds, the plasma MT level exhibited marked circadian rhythms at P7 and P14 regardless of short-term and long-term exposure to four monochromatic lights. However, WL and BL showed a faint suppression of MT secretion in contrast to GL and RL at either light or dark time points, with the following rank order: GL < RL < WL < BL. Larger circadian amplitude of MT levels was observed in GL group versus BL group (at P14: 87.70 pg/mL vs. 19.85 pg/mL, respectively). Pinealectomy disturbed the MT rhythm under different light colors, especially in RL. Additionally, consistent with the alteration of plasma MT levels, we observed increased AANAT mRNA expression and immunoreactive cell numbers of proliferating cell nuclear antigen (PCNA) and c-Fos in the pineal gland or retina in GL than that of BL, whereas 5-HT immunoreactive cell number was significantly decreased in GL. These data suggested that GL enhanced chick pinealocytes and retinal cells to express AANAT mRNA and to secrete MT, which may be depended on promoting c-Fos expression and cell proliferation. Anat Rec, 294:1233–1241, 2011. © 2011 Wiley-Liss, Inc.

Key words: melatonin; AANAT mRNA; monochromatic light; broiler; pinealectomy

It is well established that melatonin (MT) can regulate many different daily and seasonal cycles or rhythms in various physiological systems of vertebrates, including the cardiopulmonary, reproductive, excretory, thermoregulatory, behavioral, immune, and neuroendocrine systems (Mikami et al., 1983; Pang et al., 1996; Gwinner et al.,

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1997). In birds, MT is rhythmically released by the pineal gland, which wax at night and wane during the day (Underwood et al., 1984; Gwinner et al., 1997). Its synthesis and release can be influenced by many environmental factors such as environmental temperature, magnetic field, light and so on (Csernus et al., 2005). An exposure to the light-at-night inhibits the synthesis and secretion of MT (Lewy et al., 1980; Stevens et al., 1997). Long-term exposure to the constant light markedly suppressed circulating MT levels in the chicken (Zawilska and Wawrocka, 1993). Except photoperiod, light intensities of 0.11 or 0.01 lx failed to depress pineal MT levels in Syrian hamsters, whereas light intensities of 1.08 lx or greater significantly depressed pineal MT levels (Brainard et al., 1982). Additionally, suppression of 170 $\mu W/cm^2$ irradiances red light (RL) to MT secretion is significantly different with that of 1,040 $\mu W/cm^2$ irradiances in the albino rats (Sun et al., 1993). It is thus clear that these studies about light information affecting MT secretion were mainly focused on light duration and intensity. However, the visual systems of avian species are substantially different from those of mammals, particularly with regard to their photopic color vision (Barber et al., 2006). Our previous studies have found that green and blue monochromatic lights promote myofiber growth and immune response in the broilers (Cao et al., 2008; Xie et al., 2008; Liu et al., 2010). A recent study showed that shorter wavelength blue light (BL) was significantly more potent than the longer wavelength green light (GL) for circadian phase shifting (Lockley et al., 2003), but little work has been done to evaluate the effects of different monochromatic lights on MT circadian secretion in the chicken.

Except the pineal gland, the retina is another important organ of MT secretion (Underwood et al., 1984; Zawilska and Nowak, 1992; Arendt, 1995). The constant darkness resulted in significant elevations of MT levels during the subjective light phase in the chick retina (Zawilska and Wawrocka, 1993), whereas exposure of chicken to UV-A light-at-night dramatically decreased the retinal MT levels (Zawilska et al., 2000). The above data suggest that light can affect MT biosynthesis in the pineal gland and retina (Okano et al., 1994; Chaurasia et al., 2005). However, the difference of MT secretion induced by monochromatic light between the chick pineal gland and retina was not clear.

Therefore, this study was conducted to examine the effects of different spectrum of light on MT circadian levels of blood, to compare the arylalkylamine *N*-acetyltransferase (AANAT) activity in the pineal gland and retina, and to evaluate the expression of proliferating cell nuclear antigen (PCNA), immediate early gene (c-Fos), and serotonin (5-HT) in the pineal gland of broilers reared under different monochromatic lights.

MATERIALS AND METHODS

Animal Treatment and Sample Collection

A total of 160 newly hatched (posthatching day 1, P1) male broilers (Arbor Acre, Beijing Huadu Breeding, P.R. China) were used in this study. Birds were randomly selected for each treatment group and housed in one of four light-controlled cells, and were exposed to BL (480 nm), GL (560 nm), RL (660 nm), and white light (WL, 400–700 nm) by light emitting diode (LED) system, separately (Cao et al., 2008). Fifteen LED lamps were installed on a plastic board (width = 2 cm and length = 1 m). The distance between lamps was 6 cm. These LED lamps were placed 10 cm above head of broilers by attachment of the plastic board to the cage ceiling. Energy output of LED lamp was tuned through changing lamp's voltage and current by a transformer. Their voltages were 13.36 V in WL, 9.56 V in RL, 13.89 V in GL, and 14.94 V in BL, separately. The illuminance was measured daily using automatic range luminometer (Digital Luxmeter MS6610 from Hong Kong Manufacturer Union Instruments). All light sources were equalized on the illuminance of 15 ± 0.2 lx (10 cm above the head of broilers) and light period of 20 hr daily (20L:4D; light off at 22:00) (Engel et al., 2004). Chicks had *ad libitum* access to feed and water, and diets were formulated to meet or exceed the nutrient recommendations for poultry of the National Research Council (1994). The animal protocol for this research was approved by the China Agricultural University Animal Care and Use Committee.

Experiment 1 was conducted to determine the effects of short-term exposure to monochromatic light on circadian rhythms of MT secretion. Forty broilers (P1) were first exposed to WL with 20:4LD cycle for 6 days, and then transferred to BL, GL, RL, and WL at 12:00 on P7 (N = 10), separately. Their blood was collected at 11:00, 13:00, 21:00, 23:00, 1:00, and 3:00 on the same day, separately.

Experiment 2 was designed to evaluate the effects of long-term exposure to monochromatic light on MT circadian levels of blood after pinealectomy. The remaining 120 broilers (P1) were divided into four groups (N = 30) and exposed to BL, GL, RL, and WL with 20:4LD cycle for 2 weeks, separately. On P3, 15 chicks of each light group were operated to remove pineal glands (N = 10) or were sham-operated (N = 5), and the remaining 15 chicks were used as internal control group. The blood of all groups was collected from brachial vein at 0:30 and 9:30 on P7 and P14, respectively. The pineal glands and retinas of control group were isolated at 10:00 on P7 and P14.

All blood samples were collected and were heparinized with 1,000 UI/mL heparin in avian saline. After centrifuged at 1,000*g* for 30 min, the plasma was decanted and stored at −80°C until assay.

Measurement of Plasma MT Levels

Plasma MT concentrations were measured using a commercial Elisa kit of anti-chicken MT (Randox Laboratories, UK) according to the manufacturer's protocol. The standard curve and regression equation were made. According to fitting degree and conciseness of the regression curves, quadratic equation was selected. On the basis of this equation, the MT concentration in plasma of all samples was calculated. The lower limit of detection was 1.0 pg/mL. Each sample was evaluated in three replicates assay. The intra-assay and inter-assay coefficients of variation were lower than 3.3% and 8.9%, respectively. The concentrations of MT were represented by pg/mL plasma.

Isolation of Pineal Glands and Retinas, RNA Extraction, and RT-PCR

The pineal glands and retinas in the control group were isolated and immediately frozen in liquid N_2, and were kept at −70°C for RNA extraction. Their RNA was extracted by using the Trizol reagents (Invitrogen) and

RNAprep pure animal tissue kit (Tiangen Biotech, Beijing) following the instructions of the manufacturer. The equal aliquots of RNA from five to six pineal glands or retinas collected under a kind of light color were pooled. The concentration of extracted RNA was determined by measuring the optical density at 260 and 280 nm by using Smart Spec plus Nucleic Acid Analyzer (Bio-Rad, Germany).

Extracted RNA was reverse-transcribed by using the following reagents (Takara, China) in a total volume of 50 μL. The mixture containing 2.0 μL RNA sample (500 ng), 1 μL oligo$(dT)_{18}$ primer, and 7 μL RNase-free H_2O was incubated at 70°C for 5 min and was immediately placed on ice. The reaction was added to 10 μL of 50 × buffer, 2.5 μL dNTP mixture, 1 μL RNase inhibitor, 1.5 μL M-MLV reverse transcriptase, and 25 μL RNase-free H_2O; the mixture was incubated at 42°C for 60 min. The cDNA was stocked at −20°C for general polymerase chain reaction (PCR) and real-time-PCR.

General PCR was carried out in a total volume of 20 μL containing 2 μL of 10× buffer, 0.6 μL dNTP (2.5 mM) (Takara, China), 0.2 μL rTaq DNA polymerase, 0.4 μL primer(10 mM) each, 1.0 μL sample cDNA, and 15.4 μL ddH_2O. The PCR amplification was performed by using Eppendorf Mastercycler gradient (Germany) as follows: denaturation for 5 min at 95°C, followed by 27 cycles of 30 sec at 95°C, 30 sec at 62°C, and 20 sec at 72°C, and then the reaction was cooled to 15°C for 5 min. The primers for AANAT cDNA (*Gallus gallus* AANAT cDNA, GenBank accession no. NM_205158) were forward 5′-GGACCAGGACAGGCTCAG-3′ and reverse 5′-CGAAACCACACTTCTCGTAG-3′. Real-time-PCR was carried out in a total volume of 20 μL containing 10 μL SYBR Green Premix Ex Taq (Takara, China), 0.4 μL primer (10 mM) each, 0.4 μL ROX II (Takara, China), 2.0 μL sample cDNA, and 6.8 μL ddH_2O. PCR amplification and quantification was performed by using Applied Biosystems 7500 Real-Time PCR System as follows: denaturation for 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, 34 sec at 62°C, and followed 15 sec at 95°C, 1 min at 62°C, and 15 sec at 95°C. The primers for AANAT cDNA were forward 5′-ACAGGCACCTTTACAGCACGAGA-3′ and reverse 5′-CTGCTTCACGAC AAACCAAGGCAT-3′ (Contin et al., 2006). All amplifications were performed at least three times. The amount of RNA was computed by using 2 method. The data were normalized by the determination of the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (*G. gallus* GAPDH cDNA, GenBank accession no. NM_204305).

Immunohistochemical Staining for PCNA, c-Fos, and 5-HT

The five pineal glands of the control group were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered (pH 7.4, 4°C) for 48 hr. Serial paraffin cross-sections were made with a thickness of 6 μm.

The sections were immunostained with primary antibodies of PCNA mouse monoclonal antibody (Sigma, 1:2,000), c-Fos rabbit polyclonal antibody (Boster, 1:2,000), and 5-HT rabbit polyclonal antibody (Boster, 1:2,000) overnight at 4°C. Slides were incubated with biotinylated IgG secondary antibodies (Sigma, 1:200) and streptavidin–horseradish peroxidase (Sigma, 1:200) for 2 hr at 25°C, separately. Immunoreactivity was visualized by incubating in 0.01 M PBS (Phosphate Buffered Saline, pH 7.4) containing 0.05% 3′,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.003% hydrogen peroxide for 10 min. Control slides without primary antibody were examined in all cases (figure was not shown). Positive intensity of immunoreactive cells was represented by using integral optical density (IOD) in 25 random fields from five cross-sections of the pineal gland of each bird at each light treatment.

Statistics

The value was expressed as mean ± SD, and was analyzed for statistical significance by one-way ANOVA or Univariate General Linear Model followed by Post Hoc LSD Multiple Comparison test. Significance level was set at 0.05. The data were analyzed using SPSS 11.0.

RESULTS

Circadian Rhythm of MT Secretion in Broiler Under Short-Term Monochromatic Light Condition

Changes in circadian rhythms of plasma MT levels in the AA broilers after exposing to different monochromatic lights for 24 hr are summarized in Fig. 1. In all light-treatment broilers, the MT concentration was still lowest at CT11–CT13 (diurnal, 32.62–37.95 pg/mL), and then gradually increased with time, reaching a maximum at CT1 (nocturnal, 126.60–145.99 pg/mL). The difference between these two points was significant ($P < 0.05$). Thus, the plasma MT level exhibited marked circadian rhythms when broilers were exposed to four monochromatic lights (Fig. 1). However, the secretion rhythms of MT were different in extent under the various light conditions. In GL, the circadian amplitude of plasma MT concentrations was significantly elevated (CT1/CT13 ratio: 3.16; CT23/CT13 ratio: 2.51) when compared with other light treatments (CT1/CT13 ratio: 2.34–2.88; CT23/CT13 ratio: 1.69–2.28).

Change in MT Circadian Levels in Broiler Under Long-Term Monochromatic Light Condition

When broilers were exposed to different monochromatic lights for long term, the changes in circadian rhythms of MT level in the plasma of AA broilers are summarized in Fig. 2A,B. After exposing to monochromatic light for 7 days, the MT secretion still showed circadian rhythm in all light-treatment broilers (Fig. 2A). Compared with the diurnal (CT9:30) level of MT, the nocturnal (CT0:30) levels were significantly increased by 127.48%–138.05% ($P < 0.05$). From light to dark time, however, increasing amplitude of MT secretion was different in various light-treatment groups. Of them, GL group was the highest (138.05%), followed by WL group (134.75%), and the lowest in RL group (127.55%) and BL group (127.47%). Simultaneously, both plasma MT concentrations of daytime and night were the largest in GL group compared with other light groups (diurnal: 54.25 pg/mL vs. 35.78–47.01 pg/mL; nocturnal: 129.14 pg/mL vs. 81.39–106.97 pg/mL, respectively). At daytime, the MT concentration of GL group was respectively larger 34.45%, 15.40%, and 51.62% than that of WL, RL, and BL groups, but no significant difference was observed

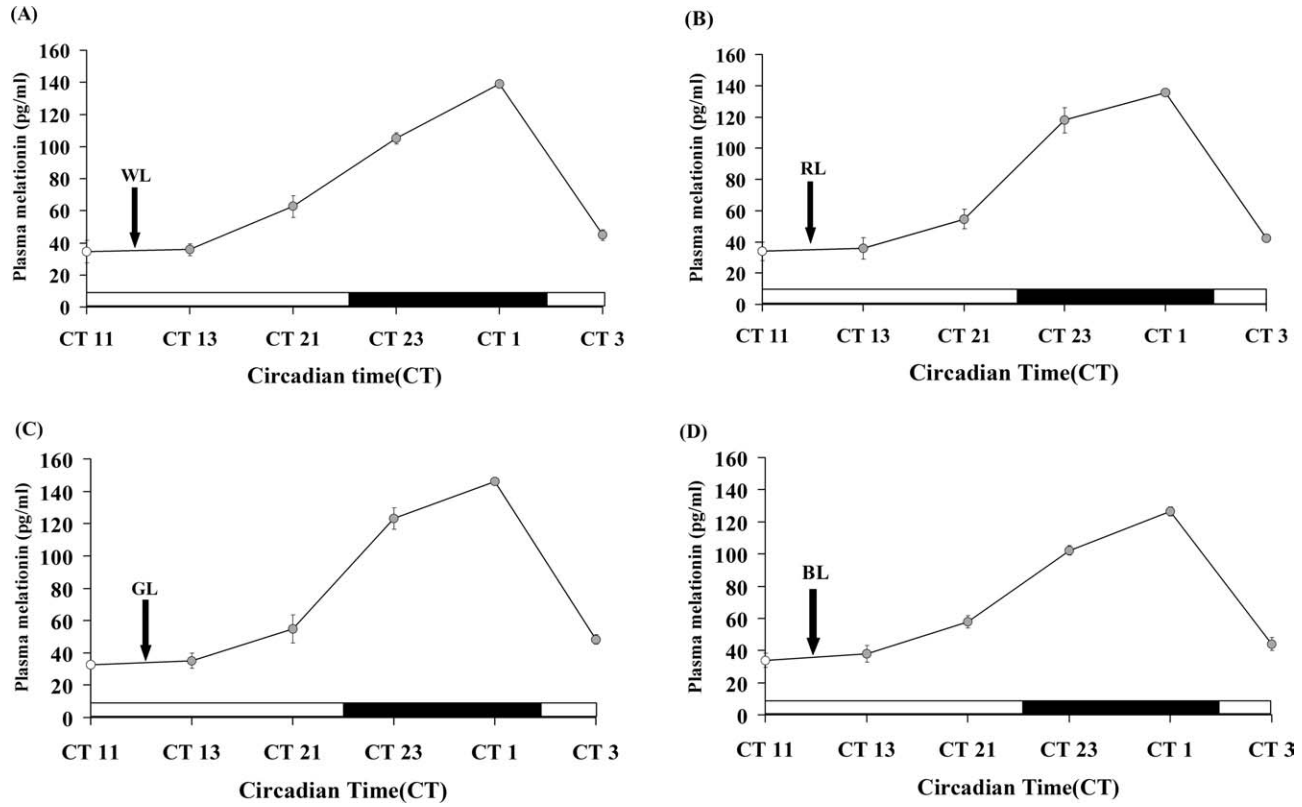

Fig. 1. Circadian rhythm of plasma MT secretion in P7 broiler exposed to monochromatic light for 24 hr. **A**, **B**, **C**, and **D** represented WL, RL, GL, and BL, respectively. The blank (□) and black (■) bars represented the light and dark phase in the 20L:4D cycle, respectively. Values of each point shown are means ± SD (N = 5 animals/time point).

among WL, RL, and GL groups ($P > 0.05$). By contrast, the differences of MT concentrations at night were statistically significant among four light-treatment groups, and GL group was larger 36.34%, 20.73%, and 18.67% than WL, RL, and BL groups, respectively.

After exposing to monochromatic light for 14 days, the MT levels at night were still higher than those of the daytime (Fig. 2B). Compared with the diurnal data, the nocturnal MT concentrations of WL, RL, GL, and BL groups were significantly increased by 52.30%, 76.01%, 85.04%, and 22.46%, respectively. The plasma MT concentrations were significantly affected in P14 among various light-treatment groups. MT levels of GL group were significantly higher than those of WL and BL groups ($P < 0.05$) either at daytime (16.68%–17.82%) or at night (43.15%–76.30%). However, no significant difference was detected between RL and GL groups.

Consequently, compared with WL, a great gradient difference (CT0:30–CT9:30) of day and night MT levels was observed in GL group (74.89 pg/mL at P7 or 87.70 pg/mL at P14), whereas a little gradient difference was found in BL group at P14 (only 19.85 pg/mL).

Effect of Monochromatic Light on MT Secretion in Broiler After Pinealectomy

As shown in Fig. 2A,B, when surgery was performed, pinealectomy produced an obvious reduction of the plasma MT concentrations either at day (P7: 39.78%–62.62%, P14: 42.13%–53.25%, respectively) or at night (P7: 47.93%–58.36%, P14: 40.12%–52.76%, respectively) ($P < 0.05$). At night, however, the absolute reduction was significantly larger than that at day. Consequently, the gradient difference of day and night MT levels was also considerably reduced either at P7 (reduction of 43.97%–59.54%) or at P14 (reduction of 26.56%–55.03%) after pinealectomy. In contrast to pinealectomy, no differences were found between intact and sham-operated broilers ($P > 0.05$) whether at light or at dark time (sham-operated data not shown in table and figure).

On the other hand, compared with various light treatments, the largest reduction was observed in the RL group after pinealectomy. For example, at P14, the gradient difference of MT levels in the RL group was about 2.2-fold lower for the control sham-operated broiler, but other light groups were 1.4–1.9-fold lower.

AANAT mRNA Expression in Retina and Pineal Gland of Broiler Exposed to Various Monochromatic Lights

The diurnal (CT9:30) expression level of AANAT mRNA was significantly higher in the pineal glands than that of the retinas ($P < 0.05$) (Fig. 3A,C). However, this difference was varied according to various day-old and light-treatments. At P7, the AANAT mRNA levels of

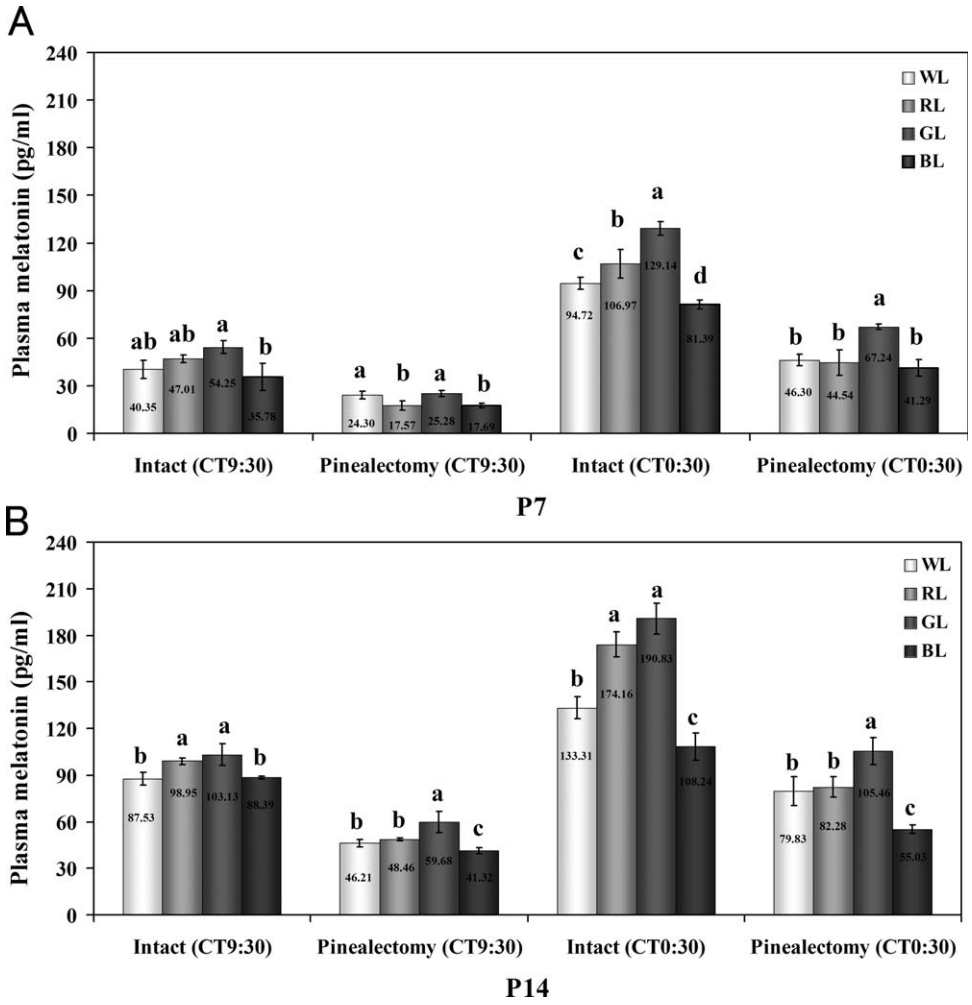

Fig. 2. Change of plasma MT content during the light and dark phase in intact and pinealectomized broilers exposed to monochromatic light at P7 (**A**) and P14 (**B**). WL, RL, GL, and BL represented white light, red light, green light, and blue light, respectively. CT: circadian time. Column marked with different letters are significantly different from each other ($P < 0.05$). Lower case letters (a–c) represented the different light treatment at light or dark. Values shown are means ± SD (N = 5).

pineal glands were higher by 303.28% (WL), 586.42% (RL), 179.78% (GL), and 727.75% (BL) than those of retinas. Until P14, the AANAT mRNA levels of pineal glands were only higher by 184.15% (WL), 136.81% (RL), 108.15% (GL), and 120.77% (BL) than those of retinas (Fig. 3B,D).

In the pineal glands, the expressions of AANAT mRNA were not significantly different at P7 among WL, RL, GL, and BL groups ($P > 0.05$) (Fig. 3B). Until P14, the AANAT mRNA expression of BL group was significantly lower than that of RL (23.19%), GL (19.97%), and WL (13.83%) groups ($P < 0.05$). By contrast, the AANAT mRNA expression of the RL group was significantly higher by 12.18% than that of the WL group ($P < 0.05$); but no significant differences were found between RL and GL groups or BL and WL groups at P14 ($P > 0.05$) (Fig. 3D).

In the retinas of P7, the AANAT mRNA expression of GL group was the highest, followed by the WL group, and that of RL and BL groups were the lowest ($P < 0.05$). The GL group was significantly higher by 39.71%, 125.79%, and 194.86% than that of WL, RL, and BL groups, respectively, whereas the RL and BL groups were significantly lower by 21.37% and 32.54% than that of WL group, but no significant difference was found between RL and BL groups ($P > 0.05$) (Fig. 3B). At P14, however, the AANAT mRNA expression of RL and GL groups was significantly higher than that of BL (21.37% and 32.54%, respectively) and WL (34.60% and 46.99%, respectively) groups ($P < 0.05$), but no significant difference was found between RL and GL groups ($P > 0.05$) (Fig. 3D).

On the other hand, the expression of AANAT mRNA was similar in pineal glands between P7 and P14 (Fig. 3B,D), but the expression in retinas of P14 was significantly increased by 47.50%, 220.87%, 55.19%, and 245.24% under WL, RL, GL, and BL compared with retinas of P7, respectively ($P < 0.05$) (Fig. 3D). However, total AANAT mRNA expression of both pineal glands and retinas was largest in the GL group than in other light groups, but no significant differences were found among GL, RL, and WL groups at P7 or between GL

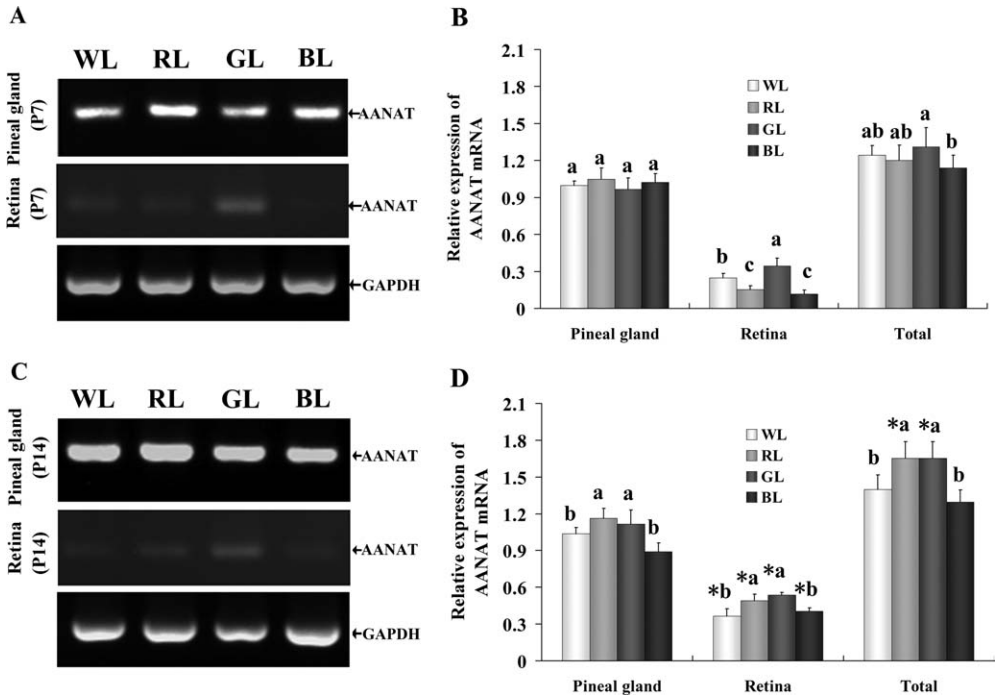

Fig. 3. Reverse transcription (**A** and **C**) and real-time quantitative PCR (**B** and **D**) analysis of mRNA expression of AANAT and GAPDH in the pineal gland and retina of broilers exposed to monochromatic light at P7 (A and B) and P14 (C and D). Total represented sum of AANAT mRNA in the pineal gland and retina. WL, RL, GL, and BL represented white light, red light, green light, and blue light, respectively. Column marked with different letters are significantly different from each other ($P < 0.05$). Lower case letters (a–c) represented the different light treatment. *Significant difference between at P7 and P14. Values shown are means ± SD (N = 5).

and RL groups at P14 (Fig. 3B,D), which was similar to diurnal variation of plasma MT levels.

Expression of 5-HT, c-Fos, and PCNA in Pineal Glands of Broiler Exposed to Various Monochromatic Lights

Most of 5-HT-immunostaining cells, which presented yellow-brown staining in the cytoplasm, were observed around the pineal follicular tissue or on the verge of the follicle (Fig. 4A–D). IOD value of positive cells in the BL group was significantly higher by 39.22%, 13.17%, and 11.89% than that of GL, RL, and WL groups, respectively ($P < 0.05$). However, positive cells in the GL group was significantly decreased by 23.02% and 24.43% compared with RL and WL groups, respectively ($P < 0.05$), but no significant difference was detected between RL and GL groups (Fig. 4M).

PCNA, as an index of cell proliferation was detected in this study. A positive reaction for PCNA in the nucleus presented dark-brown staining. Most of PCNA-immunostaining cells were observed in the pineal follicle (Fig. 4E–H). The IOD value of PCNA-immunoreactive cells in the BL group was significantly lower by 18.12%, 26.58%, and 25.34% than that of WL, RL, and GL groups, respectively ($P < 0.05$). However, no significant difference was detected among WL, RL, and GL groups (Fig. 4N).

Positive cells of c-Fos expression presented yellow-brown staining in the nucleus or cytoplasm (Fig. 4I–L). IOD value of c-Fos-immunostaining cells of GL group was significantly higher by 18.36%, 20.18%, and 116.58% than that of WL, RL, and BL groups, and WL and RL groups were significantly higher by 82.99% and 80.22% than that of the BL group, respectively ($P < 0.05$). However, no significant difference was detected between WL and RL groups (Fig. 4O).

DISCUSSION

The avian species have their own unique visual system, which differs substantially from that of humans, particularly with regard to their photopic color vision. Some experiments have provided behavioral evidence that chick, domestic ducks, and turkeys are able to perceive a broad range of radiation, extending from UV-A (λ = 360 nm) to red (λ = 694 nm) light (Prescott and Wathes, 1999; Barber et al., 2006). Zawilska et al. (1995) found that short monochromatic light pulse could suppress the nocturnal AANAT activity of the chick retina and pineal gland. The potency of the tested lights to suppress AANAT activity was white > blue (434 nm) > green (548 nm) > red (614 nm). This result was similar to that of Brainard et al. (1984), who discovered that monochromatic light could deeply suppress AANAT activity in the rat pineal gland.

Here, we unveiled that, for intact and sham-operated birds, the circadian rhythm of MT was not impacted by four monochromatic illuminations for either short term (Fig. 1) or long term (Fig. 2A,B), but the GL had a significant upregulation on MT secretion in contrast to BL and WL. A great gradient difference of MT levels between light and dark time points was observed in the

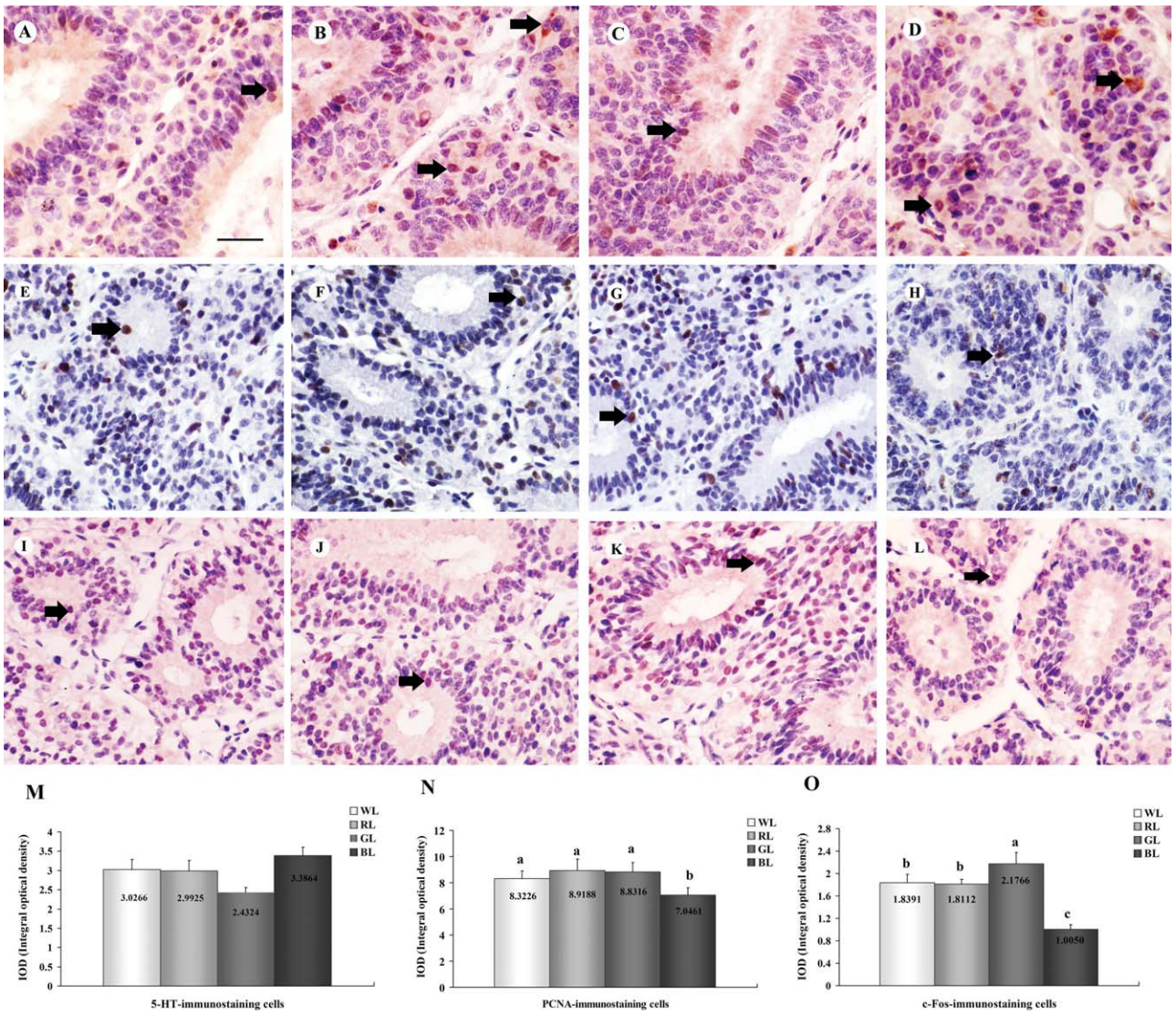

Fig. 4. Immunohistochemistry staining for 5-HT (**A–D**, **M**), PCNA (**E–H**, **N**), and c-Fos (**I–L**, **O**) in the pineal glands of broilers at P7. WL: A, E, and I; RL: B, F, and J; GL: C, G, and K; and BL: D, H, and L. Arrows indicated immunoreactive cells, bar = 30 μm. M–O: Quantitative analysis (IOD value) of 5-HT, PCNA, and c-Fos immunopositive cells, respectively. Column marked with different letters are significantly different from each other ($P < 0.05$). Lower case letters (a–c) represented the different light treatment at light or dark. Values shown are means ± SD (N = 5).

GL group, whereas the BL group only presented a little gradient difference (at P14: 87.70 pg/mL vs. 19.85 pg/mL, respectively). The cause leading to these different results might attribute to the light schedule/regime. Nevertheless, Prescott and Wathes (1999) confirmed a peak sensitivity between 380 nm $< \lambda <$ 507 nm by their electro-physiological findings in the chick, although they did not study the relationship between different colors in the spectrum and MT secretion. Besides, a BL sensitive (470 nm), pineal-specific photopigment in chick has been proposed to acutely suppress MT synthesis (Okano et al., 1994). Chaurasia et al. (2005) proved that another photoreceptive pigment, melanopsin existed in the chick pineal gland. This 450–470 nm sensitive photopigment was thought as a major component of the photoreceptive system for entrainment in mammals (Ruby et al., 2002). Localization and regulation of melanopsin mRNA in the retina and pineal gland proved that this novel photopigment might play a role in photic regulation of circadian function in the chick. Furthermore, Brainard et al. (2001) and Thapan et al. (2001) have revealed a short-wavelength peak in spectral sensitivity (λ_{max} 446–483 nm) for light-induced MT suppression in humans. Short-wavelength light, such as UV-A light (320–400 nm) could also suppress AANAT activity in the chick pineal gland. So, considering BL at 480 nm being stronger than other lights in suppressing MT was reasonable. The reason of WL (400–700 nm) showing higher restraining effect should attribute to its comprising of more short-wavelength component.

Herljevic et al. (2005) found that MT suppression was significantly reduced in the elderly human following exposure to short-wavelength (456 nm) light compared with the young subjects, but no statistical difference was found after exposure to medium-wavelength (548 nm) light. However, present results showed that the plasma MT level of 14-day-old chick was about 1.5-fold (diurnal) or 2-fold (nocturnal) larger than that of 7-day-old chick under all light treatments. This age-related change in MT levels showed no statistical difference in the broilers among WL, RL, GL, and BL groups. The different results between Herljevic's and our study were probably caused by the intensity of illumination and species, as Herljevic used methods of 62 μW/cm illumination intensity.

Our results further showed that the circadian rhythm of plasma MT levels in the broilers under different lighting colors was considerably disturbed by pinealectomy, especially in the RL. Under RL condition, the circadian amplitude of plasma MT was 2.2-fold lower in pinealectomized broilers versus the control sham-operated broilers. This might indicate that RL was more important for sustaining circadian clock of pinealectomized chick. Moreover, Osol et al. (1985) proved that MT content in chick after pinealectomy could reach up to 38%–70% of intact values 6 weeks later. So, the other interpretation is that RL seemed to be more conducive in restoring MT rhythm of the pineal gland than three other light colors.

After the removal of the broiler pineal gland, although circadian amplitude of the plasma MT was markedly attenuated, but the rhythmicity persisted with high levels during the subjective night and low levels during the subjective day phase. This observation is in contrast to the data reported for the rat (Agez et al., 2009). The reason for this discrepancy is probably because of the fact that in the broiler, there is another important source of circulating MT that contributes significantly to the circulating levels of the hormone. Previous studies confirmed that apart from the pineal gland, the retina is another important organ of MT secretion (Zawilska et al., 2003) and AANAT is a key rate-limiting enzyme in the MT biosynthetic pathway (Iveta et al., 2001). Thus, we further studied the effect of light wavelength on expression of AANAT mRNA in the pineal gland and retina of broilers. Our results showed that total AANAT mRNA expressions of both pineal gland and retina were larger in the broilers rearing under GL versus other light treatments. This alteration of AANAT mRNA expression was in accordance with that of the plasma MT level. However, the expression of AANAT mRNA in the pineal gland was markedly larger than that of the retina. A similar finding has also been reported in the turkey (Zawilska et al., 2006). Moreover, our results also showed that GL significantly promoted the AANAT mRNA expression in the broiler retina at P7 and P14. By contrast, in the pineal gland, the AANAT mRNA expression was not significantly different among WL, RL, GL, and BL groups at P7; but at P14, the expression was significantly larger in RL and GL than that of BL and WL. This indicates that, for AANAT mRNA expression, the retina seemed to be more sensitive on exposure to GL than other three light colors while in broiler, the pineal gland seemed to be more sensitive with respect to light color than the retina during early posthatch period (P14 vs. P7, respectively).

As described above, our study confirmed that the GL significantly promoted MT secretion from the pineal gland. Therefore, an additional study as to how these external light signals affect intracellular events underlying AANAT mRNA expression and MT secretion is interesting. Recently, we reported external light signals were sent into tectum, thalamus, and hypothalamus of chick by various retinal ganglion cell types (Chen and Naito, 2009) and then into the pineal gland (Jia et al., 2009). In this study, consistent with the alterations of AANAT mRNA expression and MT secretion, we observed an increase in the immunoreactive cell number of PCNA and c-Fos in the pineal gland of GL group compared with the BL group (Fig. 4N,O). By contrast, the 5-HT immunoreactive cell number (IOD value) of the pineal gland was significantly decreased in the GL group than the BL group (Fig. 4M). The higher IOD value of 5-HT reflected less amount of MT synthesis in the pineal gland (Tatsuo et al., 1996). This result of 5-HT further demonstrated that GL enhanced MT synthesis and secretion in the chick pinealocytes than BL. Consequently, our findings suggested that GL induced pinealocytes to express AANAT mRNA and to secrete MT, which may be depended on promoting c-Fos expression and cell proliferation in the chick pineal gland.

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