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# Effect of the melatonin nuclear receptor RORα on monochromatic light-induced T-lymphocyte proliferation in chicken thymus

Juanjuan Xiong, Zixu Wang, Jing Cao, Yulan Dong, Yaoxing Chen*

*Laboratory of Anatomy of Domestic Animals, College of Animal Medicine, China Agricultural University, Haidian, Beijing 100193, China*

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ABSTRACT

Present study clarified role of melatonin nuclear receptor RORα in monochromatic light-induced T-lymphocyte proliferation in chicks. Green light elevated plasma melatonin level and organ index, T-lymphocyte proliferation and IL-2 production in thymus, but decreased RORα, p-P65 and p-IκB expressions relative to red light. By contrast, pinealectomy decreased the melatonin content and reversed the stimulatory effect of green light, and resulted in that these thymus parameters were not significantly different among the light-treated groups. Exogenous melatonin supplementation enhanced T-lymphocyte proliferation and IL-2 production in cultured thymocytes. This stimulatory effect of melatonin was reversed by RORα agonist but was enhanced by RORα antagonist. In contrast to RORα antagonist, RORα agonist decreased cytoplasmic P65 level and increased nuclear P65 level. Supplementation with P65 antagonist increased T-lymphocyte proliferation. We conclude that RORα could negatively regulate green light-enhanced T-lymphocyte proliferation in chick thymus by upregulating IκB phosphorylation, which promotes P65 nuclear translocation and NF-κB activation.

## 1. Introduction

Light is a critical environment factor for birds because avian have highly specialized visual systems [1]. Thus avian growth, development, physiological function and behavior are influenced by optical information from the environment, which consists of three different aspects: intensity, duration and wavelength [2,3]. For example, splenic T and B lymphocytes from 6-wk-old chickens grown under intermittent lighting have higher activities than those from chickens grown under constant light [4]. Blatchford reported the effect of light intensity on the behavior and immune function of broiler chickens and suggested that a brighter light intensity could improve health and provide opportunities for more normal behavioral rhythms [5]. These studies focused on the effects of the photoperiod or lighting intensity, while less study on wavelength have been reported. Our previous studies indicated that monochromatic green light (GL) illumination effectively elevated the antioxidative capacity to promote B-lymphocyte proliferation in the bursa [6] and T-lymphocyte proliferation in the thymus [7] in young broilers. The combination of monochromatic green and blue lights could enhance splenic T-lymphocyte proliferation in broilers compared to single monochromatic light [8]. Increasing evidence has shown that light color can induce the immune response of chickens; however, little is known about the mechanisms underlying the effect of light color on the immune response.

Melatonin, as the major compound synthesized in the pineal gland, is now considered a component of the neuroendocrine-immunoregulatory system [9]. Melatonin modulates a wide array of physiological processes, including immunity. Many of melatonin's actions are mediated through melatonin membrane receptors such as MT1, MT2 and MT3 [10]. Researchers studied this specific binding site for melatonin in different immune tissues from birds and mammals and found that melatonin can bind to different receptors to trigger the corresponding downstream signals in different species or cell types. In chickens, our previous studies proved that melatonin can signal through the Mel1b and Mel1c receptors to mediate GL-enhanced T-lymphocyte proliferation in the thymus [7] and in the spleen [11]. However, Mel1a and Mel1c are involved in GL-enhanced B-lymphocyte proliferation in the chick bursa [12].

In addition to the membrane receptor, melatonin binding sites also have been characterized in nuclear receptors, a retinoid-related orphan nuclear hormone receptor family (ROR) [13]. Lardone found that melatonin is the natural ligand of RORα in Jurkat cells [14, but see 15]. RORα and two other structurally related members, RORβ and RORγ, are members of the orphan nuclear receptor (ROR) subfamily [13]. Of them, RORα is widely expressed in a variety of tissues and the immune system, where expression has been shown in both murine lymphoid and myeloid cells, including different subsets of T-cells, B-cells and monocytes [9,16]. Melatonin induces a decrease in the RORα levels in the

* Corresponding author.
*E-mail address:* yxchen@cau.edu.cn (Y. Chen).

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nucleus [14], but cAMP mediates melatonin's function and increases the expression of RORα [17]. RORα was shown to suppress cellular apoptosis and oxidative stress via melatonin in myocardial ischemia/reperfusion injury [18]. Moreover, the levels of TNF-α and IL-6 produced by mast cells and macrophages were increased in RORα$^{-/-}$ mice [16]. Interleukin-2 (IL-2) plays an important role in regulating the proliferation and development of lymphocytes [19]. Garcia-Mauriño demonstrated that melatonin, through nuclear receptor signaling, regulates the expression of IL-2 and IL-6 in PBMCs [20]. However, the role of the ROR site remains controversial [21]. It is possible that melatonin can affect RORα but only indirectly [22, but see 23], even some studies reported that RORα is not a receptor for melatonin [15,24,25]. They considered that sterols and their derivatives can interact with the ligand binding domain of RORs and regulate the ROR transcriptional activity. Agez et al. also found that melatonin had no effect on the RORα mRNA expression in the rat hypothalamic suprachiasmatic nucleus [26]. Therefore, whether RORα is involved in regulation of the T-lymphocyte proliferation in chicks under monochromatic light is unclear.

In this study, we analyzed the effect of monochromatic light on the expression of the nuclear receptor RORα and assessed whether RORα is involved in the effect of melatonin on monochromatic light-induced T-lymphocyte proliferation in the chicken thymus. Further, we postulated the mechanism for RORα participation in this regulated process.

## 2. Materials and methods

### 2.1. Animals and treatments

All experimental procedures were approved by the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Approval No. CAU20171114-2). A total of 120 post-hatching day (P) 0 Arbor Acre male broilers (Beijing Huadu Breeding Co., Beijing, China) were used in this study. They were randomly allocated into 4 separate color light rooms and were exposed to blue (BL, 480 nm), green (GL, 560 nm), red (RL, 660 nm) or white (WL, 400–760 nm) lights by a light-emitting diode (LED) system for 2 weeks. The light intensity was 15 ± 0.3 lx at the bird-head level with a light period of 23 h daily (23 L:1 D). Each light treatment was divided into three subgroups: intact, sham-operated and pinealectomy subgroups. Pinealectomy and sham operation were performed on each light treatment group at P3. The ambient temperature was maintained at approximately 32 °C for the first week and later reduced to 30 °C during the second week, and the relative humidity was maintained at 60% for the entire period. The broilers had ad libitum access to feed and water. The diet was formulated to meet or exceed the nutrient recommendations of the National Research Council for poultry (1994).

At P14, six chickens were randomly selected from each light treatment group. Their blood samples were collected via a cardiac puncture. The plasma was separated by centrifugation at 3000 × g for 15 min and then stored at −80 °C for ELISA. The thymus was aseptically removed and divided into two parts. One part of the thymus was fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4, 4 °C) for immunohistochemistry staining, and the other part was assessed for expression of mRNA and protein levels by RT-PCR and western blotting. The remaining chicks from each color light group were used for a lymphocyte proliferation assay.

### 2.2. ELISA

A commercial chicken ELISA kit (CEA908Ge, Uscn Life Science, Inc., Wuhan, China) was used to detect the melatonin concentration in plasma. The sensitivity of the ELISA kit was 4.63 pg/ml, and the intra-assay and inter-assay coefficients of variation were less than 10% and 12%, respectively. According to the ELISA kit manufacturer's protocol, the standard curve concentrations were 1000 pg/ml, 333.33 pg/ml, 111.11 pg/ml, 37.04 pg/ml and 12.35 pg/ml. The standard or sample (50 μl) was added to each well and incubated with 50 μl of detection reagent A for 1 h at 37 °C. Next, 100 μl of detection reagent B was added for 30 min at 37 °C, followed by 5 washes. Then, 90 μl of substrate solution was added for 20 min at 37 °C, followed by the addition of 50 μl of stop solution. The OD values were then read at 450 nm. The OD value of the standard was calculated as a standard curve by the log function. The sample's concentration was calculated according to the standard curve.

Separated thymus tissue was stored at −80 °C for ELISA. An ELISA kit (CSB-E06755Ch, CUSABIO, Wuhan, China) was used to detect IL-2 content in thymus tissue homogenates. The 100 mg tissue was rinsed with PBS and stored overnight at −20 °C. Some samples were used for BSA to detect the total protein concentration, while others were used to determine the IL-2 concentration. The standard or sample (100 μl) was incubated for 2 h at 37 °C, followed by the addition of 100 μl of an anti-biotin antibody for 1 h at 37 °C. Next, 100 μl of HRP-avidin was added for 1 h at 37 °C, and 90 μl of TMB substrate was added before the 50 μl stop solution to each well. The OD was read at 450 nm within 5 min. The data were linearized by plotting the log of the IL-2 concentrations versus the log of the OD. The sensitivity of the ELISA kit was 0.04 pg/ml, and the intra-assay and inter-assay coefficients of variation were less than 8% and 10%, respectively.

### 2.3. Immunohistochemical staining

Paraffin-embedded thymus tissue was cut into 5 μm sections. The sections were incubated with a rabbit against RORα primary antibody (OM184933, 1:200, OmnimAbs, China) at 4 °C overnight. After three washes in PBS, the sections were incubated with a biotinylated conjugated goat anti-rabbit IgG secondary antibody (sc-2020, 1:200; Santa Cruz, CA, USA) for 2 h at 37 °C. After washing in PBS, the samples were incubated with streptavidin-horseradish peroxidase (1:300, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The reaction was stopped with the addition of 3, 3-diaminobenzidine (DAB, Sigma)-$H_2O_2$. Finally, the sections were cover slipped after being stained with hematoxylin and dehydrated. The brown portion of cells indicated that RORα expression was positive. Positive cells were counted in 20 random fields from five cross-sections in each sample. The data were analyzed by measurement of the integrated optical density (IOD) using Image-pro Plus software. Immunohistochemistry was simultaneously performed in all thymus samples as well as negative control samples without primary antibody incubation.

### 2.4. RT-PCR

Total RNA was purified using a reverse transcription kit (Thermo Fisher Scientific, Boston, USA). cDNAs were synthesized from RNA by reverse transcription of 2 μg total RNA. Oligo dT and RNase-free $H_2O$ were incubated at 65 °C for 10 min, placed on ice for 5 min, followed by the addition of 4 μl of 5 × buffer, 1.5 μl of a dNTP mixture, 1 μl of RNase inhibitor, and 1 μl of reverse transcriptase to reach a total reaction volume of 20 μL that was incubated at 42 °C for 60 min. cDNA was stored at −20 °C for the general chain reaction (PCR). The general PCR amplification system contained 2 μl of sample cDNA, 10 μl of GoTaq®Green Master Mix (M7122, Promega, USA), 0.2 μl of primers, and 7.2 μl of $ddH_2O$. PCR was performed with an initial incubation step for 5 min at 95 °C, followed by 30–32 cycles of 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s, and extension for 5 min at 72 °C. Melting curve analysis was used to confirm the formation of the expected PCR products, and products from all assays were assessed via 1% agarose gel electrophoresis to confirm the correct lengths. 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*. The experiments were repeated three times. The PCR primers are listed in Table 1.

**Table 1**
Sequences of primers used for PCR.

| Genes | Primer sequences (5'-3') | Accession number | Product size (bp) |
|---|---|---|---|
| *RORα* | F:TGG GCATACCCCTGAAGGTA<br>R:CCG ATGCTGGTGTGTAGTCA | XM_413763.2 | 140 |
| *GAPDH* | F:ATC ACAGCCACACAGAAGACG<br>R:TGA CTTTCCCCACAGCCTTA | NM_204305 | 124 |

### 2.5. Western blotting

Thymus was homogenized and lysed in RIPA buffer containing protease inhibitors. Total protein was determined by a BCA protein assay kit (CW0014, CWBIO, Beijing, China) and was adjusted to the same level for each sample. The protein samples were resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro blotted onto a PVDF membrane (Millipore, Billerica, MA, USA). Nitrocellulose membranes were blocked for 60 min with TBST (a mixture of Tris-buffered saline and 0.05% Tween-20) containing 5% fat-free dry milk and incubated with primary antibodies (RORα, 1:500, OM184933, OmnimAbs, China; P65, 1:1000, ab16502, Abcam, England; p-P65, 1:500, orb6504, Biorbyte, England; IκB, 1:2000, I0505, Sigma, USA; p-IκB, 1:1000, 2859, CST, USA; β-actin, 1:4000, cw0263, Biotech, China; Histone H3, 1:4000, 9715, CST, USA) overnight at 4 ˚C. The membranes were then washed with 3 times of TBST over 10 min. Immunodetection was performed with a horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG-HRP diluted 1: 6000; goat anti-mouse IgG-HRP diluted 1: 4000; CoWin Biotech Co., Inc.) for 2 h at room temperature. Finally, the blot was washed 3 times with TBST. The bands were quantified by measurement of the IOD using Image Analysis software (Gel-Pro Analyzer 4.5; Media Cybernetics, Rockville, MD, USA). The values of the target bands were normalized to the corresponding β-actin values. The results were repeated three times.

### 2.6. Lymphocyte proliferative activity assay

Cell-mediated immune function was assessed by measuring lymphocyte proliferation in response to a T-cell specific mitogen, concanavalin-A (ConA, Sigma-Aldrich, St. Louis, USA), using a colorimetric assay based on the reduction of tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma). Cells were isolated from the chicken thymus under aseptic conditions at P14. The cells were counted using a hemocytometer, and viability was determined by the trypan blue exclusion method. Viable cells (95%) were resuspended in complete RPMI1640 medium, and $5 \times 10^6$ cells/ml were adjusted per well in a flat-bottom 96 well culture plate. ConA was added to the medium at a concentration of 20 μg/ml. The plates were incubated at 41 ˚C with 5% $CO_2$ for 44 h. Four hours later, 10 μl of MTT was added to the medium (5 mg/ml in complete media RPMI-1640). At the end of 48 h, 100 μl of 10% SDS was added to each well. The OD of each well was determined using a microplate reader (Model 680, Bio-Rad, St. Louis, MO, USA) equipped with a 570 nm wavelength filter. The mean OD values for each triplicate set were used in the subsequent statistical analysis. The proliferative activity of the T-lymphocytes was expressed as the stimulation index (SI) as follows: SI = OD570 (stimulated cells) / OD570 (unstimulated cells). We treated the unstimulated cells as one.

In addition, we added 5 μM SR3335 (a selective RORα inverse agonist; MCE, New Jersey, USA) to the cell suspensions; 10 μM SR1078 (a nonselective agonist of the nuclear receptor RORα and RORγ; MCE, New Jersey, USA) and 1 μM BAY 11-7082 (an antagonist of NF-κB; MCE, New Jersey, USA) were also added for 30 min prior to the addition of ConA and melatonin.

### 2.7. Statistical analyses

All statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). The data were analyzed by performing one-way ANOVA. The values are expressed as the mean ± SEM. A P-value of ≤0.05 was considered statistically significant. Correlation analysis was performed to determine the possible linear relationship between the melatonin concentration and RORα protein expression, which is expressed as the Pearson coefficient ($r^2$).

## 3. Results

### 3.1. Effect of pinealectomy on monochromatic light-induced T-cell proliferation in the thymus

As show in Fig. 1A, the thymus index of GL in the sham operation group was significantly higher than that of RL by 25.83% (P = 0.035) and BL by 29.43% (P = 0.046), but no differences were detected

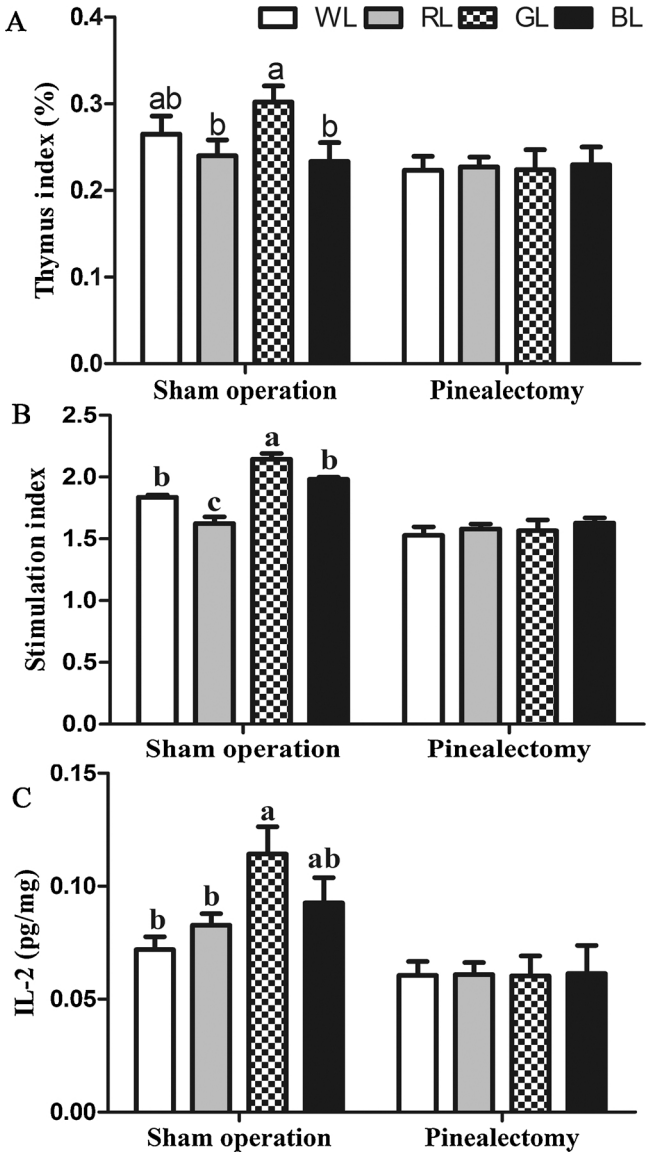


**Fig. 1.** Effect of monochromatic light on thymus index (A), T-cell proliferation (B) and IL-2 expression (C) in the chick thymus after pinealectomy. WL, white light; RL, red light; GL, green light; BL, blue light. Data are presented as the means ± SEM. Bars with different letters are significantly different (P < 0.05) between treatment groups.

between GL and WL (P = 0.254). Similarly, GL increased T-cell proliferation by 8.26–32.11% (P = 0.000-0.007) (Fig. 1B) and the IL-2 concentration by 23.28–58.86% (P = 0.004-0.109) in the thymus (Fig. 1C) compared with the other three light-treated groups. By contrast, RL decreased T-cell proliferation and the IL-2 concentration in the thymus by 13.10–32.11% (P = 0.000-0.001) and 11.93–37.98% (P = 0.047-0.511), respectively, compared to the other light-treated groups.

After pinealectomy, the thymus index was reduced by 18.66% in WL (P = 0.185), 5.72% in RL (P = 0.566), 34.82% in GL (P = 0.039) and 1.71% in BL (P = 0.906) compared to the corresponding sham operation group. No significantly differences were observed among the various light-treated groups (P > 0.05). Similarly, pinealectomy decreased T-cell proliferation in the thymus by 20.16% in WL (P = 0.006), 2.72% in RL (P = 0.530), 36.98% in GL (P = 0.000) and 21.77% in BL (P = 0.000) and resulted in no significant differences among the light-treated groups (P > 0.05). The IL-2 concentration of the thymus in the pinealectomy group was reduced by 18.82% in WL (P = 0.202), 43.42% in RL (P = 0.011), 89.57% in GL (P = 0.007) and 51.07% in BL (P = 0.102) compared with the sham operation group. Differences among the light-treated groups disappeared following pinealectomy.

### 3.2. Effect of pinealectomy on monochromatic light-induced RORα expression in the thymus and melatonin level in plasma

RORα expression was detected in the thymus of chicks (Fig. 2). RORα-immunoreactive cells were mainly located in the medulla of the thymus and the joint between the cortex and medulla (Fig. 2A). The RORα-positive cell number was increased in RL than in WL (20.78%, P = 0.001), GL (27.14%, P = 0.000) and BL (31.98%, P = 0.000). However, no statistically significant differences were found among WL, GL and BL in the intact groups (P > 0.05). Similar to the immunohistochemistry results, western blot and RT-PCR analyses showed that RL significantly increased both protein (13.91–30.51%, P = 0.030-0.236) and mRNA (12.42–32.08%, P = 0.000-0.085) expressions of RORα compared with the other light-treated groups (Fig. 2B, 2C).

Consistent with the increase in RORα expression of RL, we observed a 7.11–36.21% (P = 0.000-0.297) reduction in the plasma melatonin level of RL compared with the other light-treated groups (Fig. 2D).

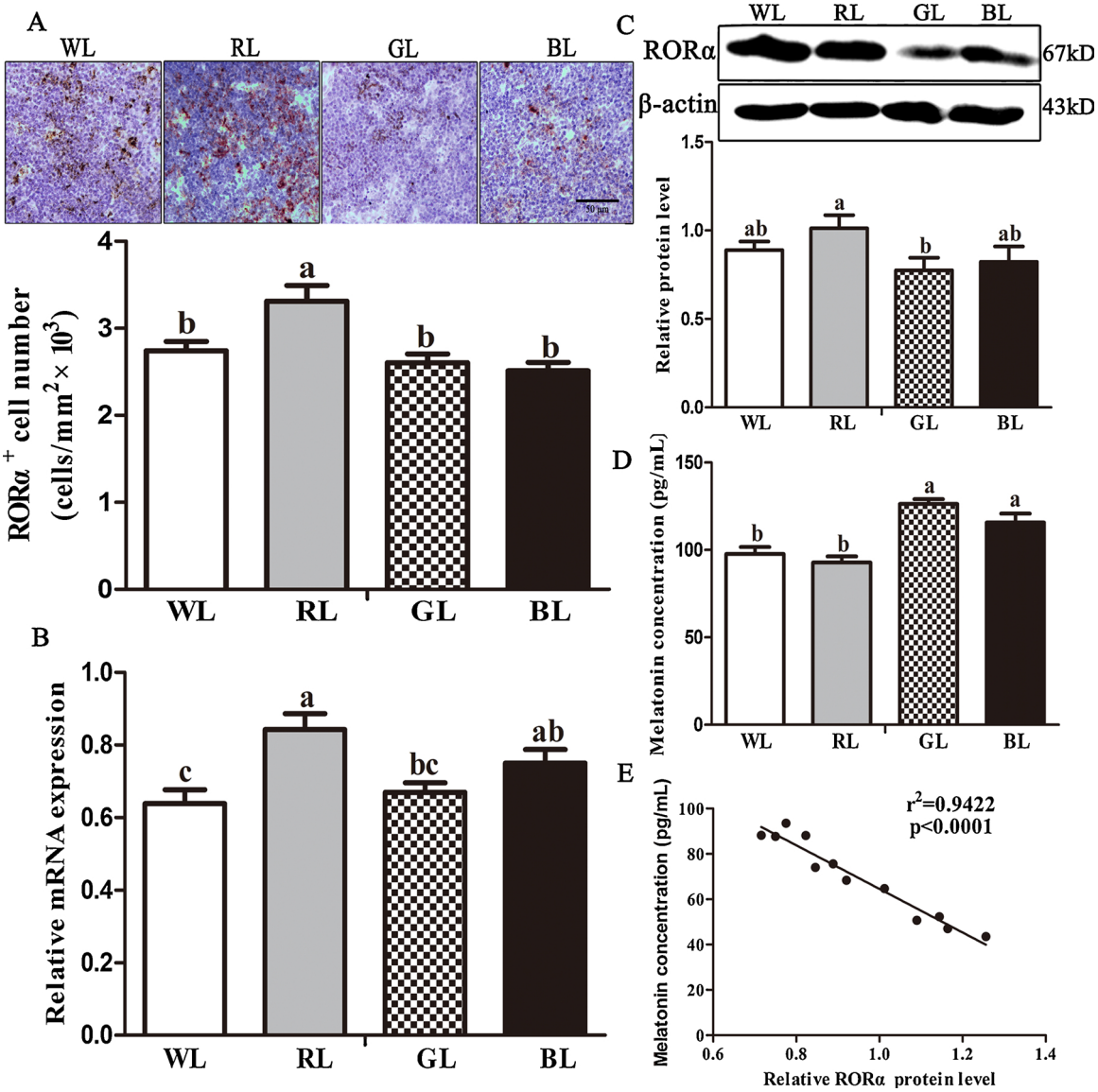


**Fig. 2.** Effect of monochromatic light on RORα in the thymus and the plasma melatonin concentration. A: RORα-positive cells; B: RORα mRNA expression; C: RORα protein expression; D: plasma melatonin concentration; E: correlation between the melatonin level and RORα protein level. WL, white light; RL, red light; GL, green light; BL, blue light. Data are presented as the means ± SEM. Bars with different letters are significantly different (P < 0.05) between treatment groups.

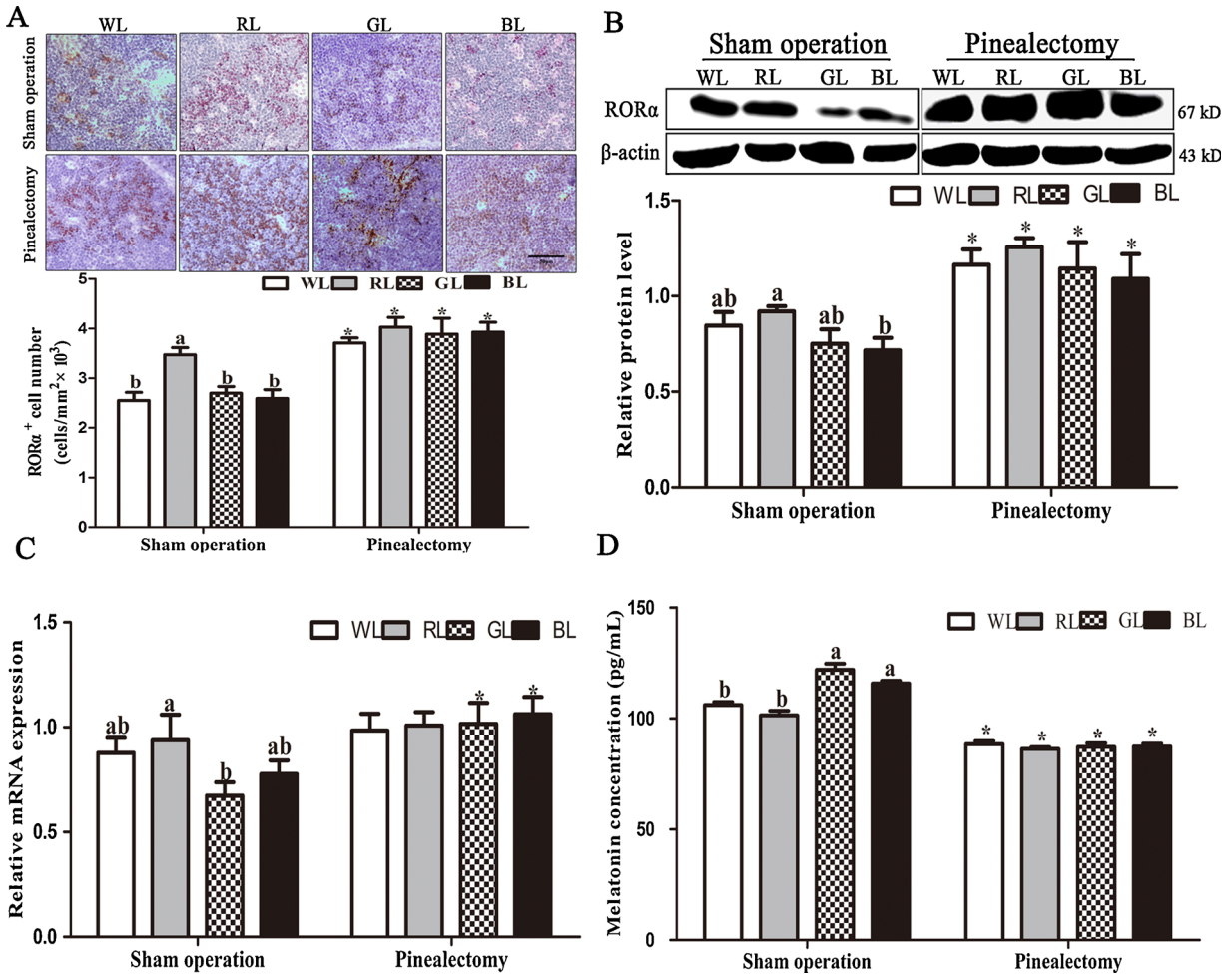


**Fig. 3.** Effect of pinealectomy on RORα expression in the thymus and melatonin level in the plasma. A: RORα-positive cells; B: RORα protein expression; C: RORα mRNA expression; D: plasma melatonin concentration. WL, white light; RL, red light; GL, green light; BL, blue light. Data are presented as the means ± SEM. Bars with different letters are significantly different ($P < 0.05$) between treatment groups.

Pearson correlation analysis showed a negative correlation between the changes in the protein expression of thymus RORα and the plasma melatonin concentration ($r^2 = 0.9422$, $P < 0.0001$) (Fig. 2E) and thymus T-cell proliferation ($r^2 = 0.7873$, $P = 0.0001$).

As shown in Fig. 3, the RORα expression and the melatonin level were altered by pinealectomy, but no significant differences were detected among the various light-treatments of the pinealectomy group ($P > 0.05$). However, immunohistochemical examination showed that pinealectomy increased the RORα-positive cell number in the thymus of chicks under WL (45.47%, $P = 0.000$), RL (15.96%, $P = 0.028$), GL (44.01%, $P = 0.002$) and BL (51.60%, $P = 0.000$) compared with the sham operation group (Fig. 3A). Similarly, protein and mRNA expressions of RORα were also increased by 36.47–52.55% ($P = 0.000$-$0.032$) and 7.62–51.09% ($P = 0.022$-$0.614$) after pinealectomy, respectively (Fig. 3B and 3C). By contrast, pinealectomy reduced the plasma melatonin concentration in WL (20.09%, $P = 0.000$), RL (17.61%, $P = 0.000$), GL (39.89%, $P = 0.000$) and BL (32.55%, $P = 0.000$) compared with the sham operation groups (Fig. 3D), and resulted in that no differences were observed in RORα expression in the thymus and the melatonin level in plasma between the sham operation group and intact group ($P > 0.05$).

### 3.3. Effect of RORα on GL-induced T-lymphocyte proliferation

To investigate the role of RORα in GL-induced T-lymphocyte proliferation, cell suspensions of cultured T-lymphocytes from the chick thymus under GL were prepared for preincubation with RORα agonist SR1078 (10 μM) or RORα antagonist SR3335 (5 μM) for 30 min prior to the addition of ConA (20 μg/ml) and melatonin ($10^{-9}$ M). The controls were incubated with cells only in RPMI 1640 medium, SR1078, SR3335 and 0.01% ethanol or 0.01% dimethyl sulfoxide (DMSO).

The MTT assay results showed that melatonin effectively promoted T-lymphocyte proliferation in response to ConA (39.85%, $P = 0.000$) (Fig. 4A); however, pretreatment with SR3335 increased T-lymphocyte proliferation by 13.75% ($P = 0.049$), whereas SR1078 pretreatment reduced T-lymphocyte proliferation by 30.99% ($P = 0.038$). Similar to the MTT assay results, the combination of melatonin and ConA significantly increased the concentration of IL-2 in the cultured T-lymphocyte supernatant by 17.58% ($P = 0.001$) (Fig. 4B). Compared with treatment with only ConA + melatonin, the IL-2 level was increased by 9.09% ($P = 0.033$) with pretreatment with SR3335 and markedly decreased with SR1078 pretreatment (8.58%, $P = 0.016$). No significant differences were detected between the DMSO (Ethanol) only and cell only experiments ($P > 0.05$).

### 3.4. Changes in P65 and IκB expression in chick thymus under different monochromatic lights

The phosphorylation of P65 in the chick thymus under the different monochromatic lights was detected by western blot analysis (Fig. 5). The expression of p-P65 was higher in RL than in WL (35.36%, $P = 0.047$), GL (41.20%, $P = 0.028$) and BL (7.43%, $P = 0.582$)

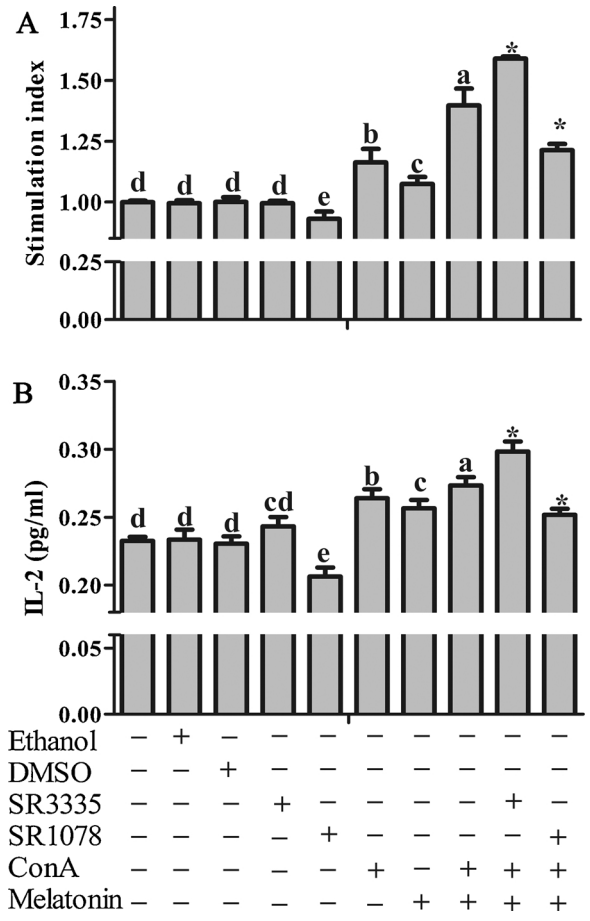


**Fig. 4.** Effects of RORα antagonist and agonist on T-lymphocyte proliferation (A) and level of IL-2 in the T-lymphocyte supernatant (B). SR3335 is a selective RORα inverse agonist. SR1078 is an agonist of RORα; a–e represent significant differences ($P < 0.05$) between treatment groups; * represents a significant difference from the ConA + Mel group.

(Fig. 5A), but no statistically significant differences were found among WL, GL and BL in the intact group ($P > 0.05$). Similar to the p-P65 protein expression results, RL significantly upregulated the p-IκB protein levels (3.53–72.69%, P = 0.000-0.696) compared with the other light-treated groups (Fig. 5C).

Pinealectomy increased the protein expression of p-P65 and p-IκB in the thymus of chicks under the four light treatments (9.98–39.17%, P = 0.052-0.318) (Fig. 5B, 5D). However, no significant differences were observed among the various light-treatments of the pinealectomy group ($P > 0.05$) or between the sham operation group and intact group ($P > 0.05$).

### 3.5. Effect of RORα on the expression of P65 in T-lymphocytes

To detect the regulation of RORα on the expression of P65 in T-lymphocytes, we added melatonin to the T-lymphocyte culture, which increased the P65 protein level in the cytoplasm (14.77%, P = 0.003) (Fig. 6A) and reduced its expression in the nucleus (23.03%, P = 0.011) (Fig. 6B). Pretreatment with SR1078 decreased the P65 protein level in the cytoplasm compared with the ConA + melatonin group (37.52%, P = 0.000) (Fig. 6A) and increased the level in the nucleus (30.39%, P = 0.000) (Fig. 6B). SR3335 increased P65 expression in the cytoplasm (22.37%, P = 0.000) and reduced its expression in the nucleus (16.72%, P = 0.000). These results demonstrate that melatonin reduced while RORα promoted P65 nuclear translocation.

### 3.6. Role of P65 in T-lymphocyte proliferation

To directly prove the role of P65 in T-lymphocyte proliferation, we used the P65 antagonist BAY 11–7082 in an vitro experiment. The addition of different concentrations of BAY 11–7082 to the culture did not result in significant differences compared with the control group (except for the high concentration of BAY 11–7082, which may have toxic effects on cells). As show in Fig. 7, compared with the cell only experiments, the ConA + melatonin treatment caused a significant increase in T-lymphocyte proliferation; however, preincubation with BAY 11–7082 reduced the effect of ConA + melatonin. The cells showed the greatest proliferative activity when 1 μM BAY 11–7082 was added (10.91%, P = 0.042). These results demonstrate that P65 attenuates the effect of melatonin on T-lymphocyte proliferation.

## 4. Discussion

Lymphocyte development is affected by many factors, such as stress [27], medicine [28], radiation and light [29]. Here, we studied the effects of various optical wavelengths on T-lymphocyte proliferation in the chick. The results demonstrated that compared with other lights, monochromatic green light significantly promoted the organ index, T-lymphocyte proliferation and IL-2 concentration in the chick thymus. The increases of the thymus index and IL-2 level reflect enhanced immune function [19,30]. These results corroborate previous studies showed that GL enhanced B-lymphocyte proliferation in the chicken bursa [6].

Along with increased T-lymphocyte proliferation, GL increased the plasma melatonin concentration in chicks. Correlation analysis suggested that melatonin may regulate GL-induced lymphocyte development. The pinealectomy experiment further proved that the thymus index, T-lymphocyte proliferation and IL-2 level were reduced following the downregulation of the melatonin level in chicks under four light-treatments; however, the results were not significantly different among the various light-treated groups. In vitro experiments showed that exogenous melatonin supplementation to cultured thymocytes from GL-treated chickens enhanced T-lymphocyte proliferation and IL-2 production. In hamsters, which are exposed to short photoperiods, have increased levels of serum melatonin [31]. Previous in vitro studies reported that melatonin promotes lymphocyte activity in chickens [4]. The results presented here indicate that melatonin modulates GL-induced T-lymphocyte proliferation in chicks.

Melatonin modulates a wide array of physiological events that have pleiotropic effects on the immune system through its receptor subtypes, which consist of a melatonin membrane receptor (Mel1a, Mel1b and Mel1c) [32,33] and a melatonin nuclear receptor (RORα, RORβ and RORγ) [18,34; but see 25]. Of the melatonin nuclear receptors, RORα plays an important role in many physiological processes, especially in the immune system [35,36]. In the present study, immunohistochemical examination showed that RORα-immunoreactive cells were mainly located in the medulla and the joint between the cortex and medulla of the thymus. Although no changes in the distribution pattern of RORα were observed following the various light-treatments, western blot and RT-PCR analyses detected an increase of RORα protein and mRNA expressions in the thymus exposure to RL compared with the other light-treated groups. A similar finding was also reported in the Indian palm squirrel [35]. Another study reported that LED light increased the RORα level compared with night time in mouse hippocampal neuron cells [37]; however, RORα mRNA expression was higher in night than the day in the rat pancreas [38]. The conflicting results may be caused by the use of different methods or different cell types and species.

As mentioned in the introduction, many studies have reported that RORα is not a receptor for melatonin [15,24,25], but our study found a negative correlation between RORα expression and melatonin level or T-lymphocyte proliferation in the thymus. Our results were also

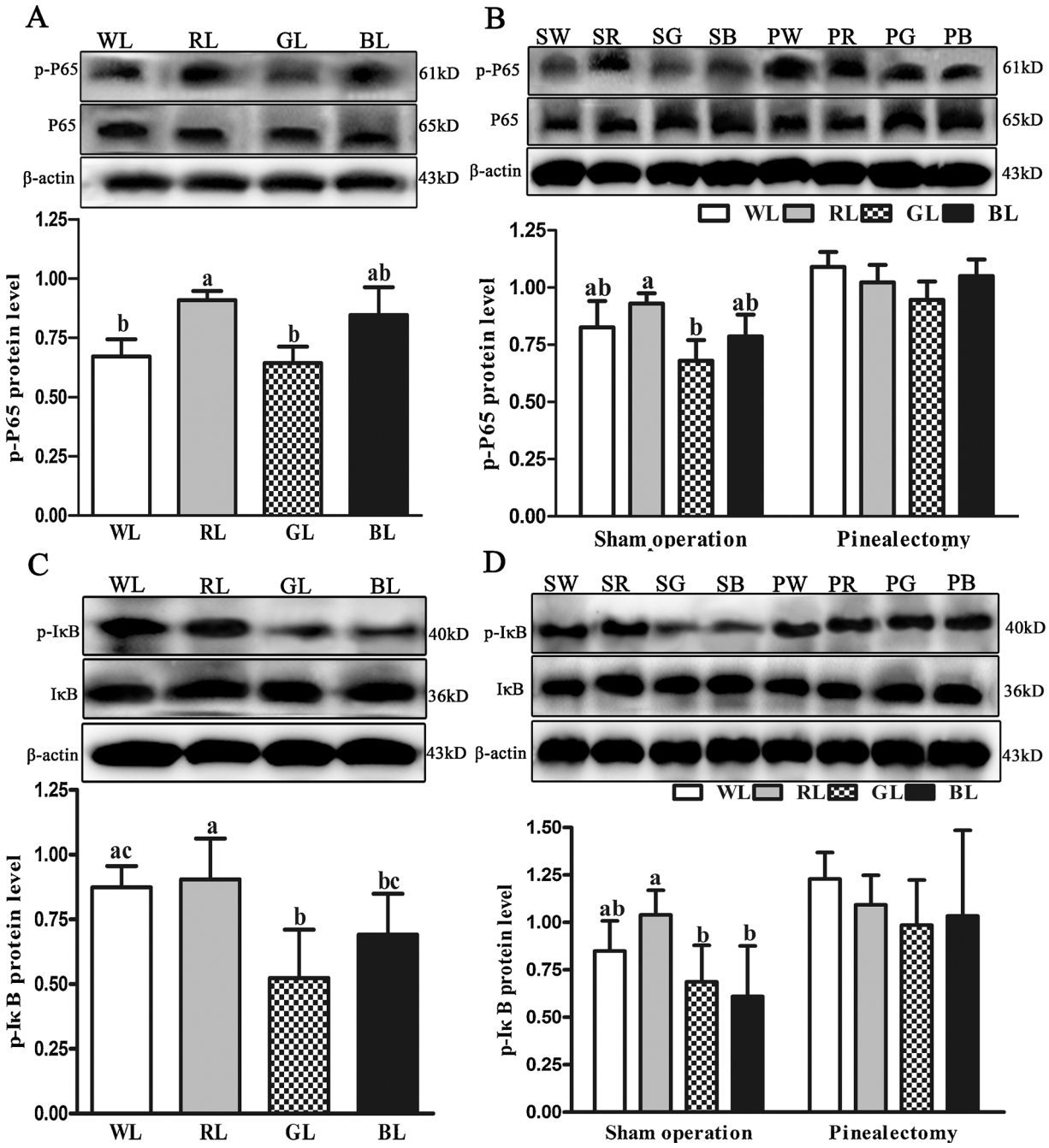


**Fig. 5.** Effect of monochromatic light on p-P65 and p-IκB expressions in the chick thymus. WL, white light; RL, red light; GL, green light; BL, blue light. SW, SR, SG and SB represent sham operation under white, red, green and blue lights, respectively. PW, PR, PG and PB represent pinealectomy under white, red, green and blue lights, respectively. Data are presented as the means ± SEM. Bars with different letters are significantly different ($P < 0.05$) between treatment groups.

supported by studies that RORα expression in the lymphoid organs (spleen and thymus) of seasonally breeding, tropical squirrels, *Funambulus pennant*, presented an inverse correlation with the plasma melatonin level [35], and RORα transactivation and DNA-binding activity were repressed by melatonin in human breast cancer cells [39]. After pinealectomy, consistent with the decrease in the melatonin concentration and T-lymphocyte proliferation, we observed a 24.13–52.55% increase in RORα expression in the chick thymus under different monochromatic lights but no significant differences among the various light-treatments of the pinealectomy group. A previous report showed that RORα reversed the effect of melatonin on the antioxidant status in birds [40]. These data suggest that RORα plays a key role in the melatonin modulation of monochromatic light-induced T-lymphocyte proliferation in the chick thymus. Despite a study reported that melatonin can affect RORα but only indirectly [22], our in vitro experiments further showed that the stimulatory effect of melatonin on T-lymphocyte proliferation was reversed by SR1078 but enhanced by SR3335. This result corroborates the previous report which melatonin can directly down-regulate the expression of ROR in human gastric cancer cells [23]. These data demonstrate that melatonin mediates monochromatic light-induced T-lymphocyte proliferation via repressing the action of RORα.

One question arises: how does melatonin enhance monochromatic light-induced T-lymphocyte proliferation through a mechanism mediated by RORα? Prior research reported that melatonin promoted a time-dependent decrease in the nuclear RORα level in Jurkat T cells [14]. We

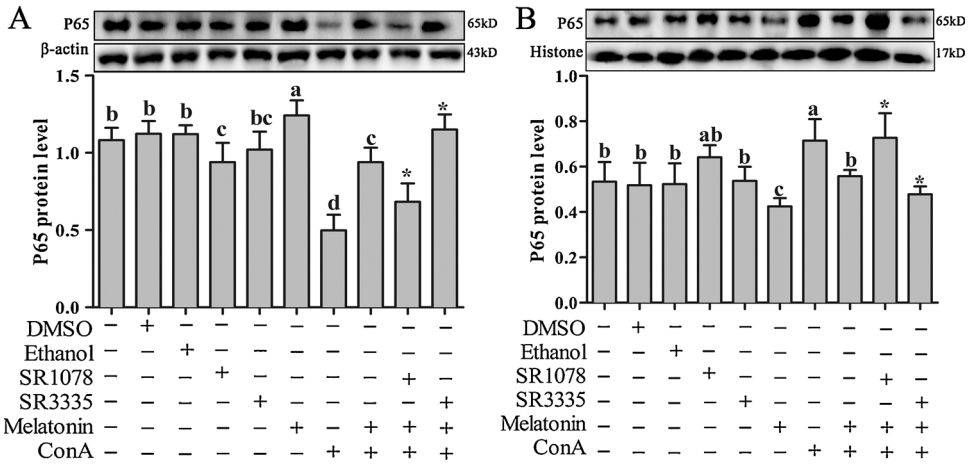


**Fig. 6.** Role of RORα in the expression of P65 protein in the cytoplasm (A) and in the nucleus (B). SR3335 is a selective RORα inverse agonist; SR1078 is an agonist of RORα. a–d represent significant differences ($P < 0.05$) between treatment groups; * represents a significant difference from the ConA + melatonin group.

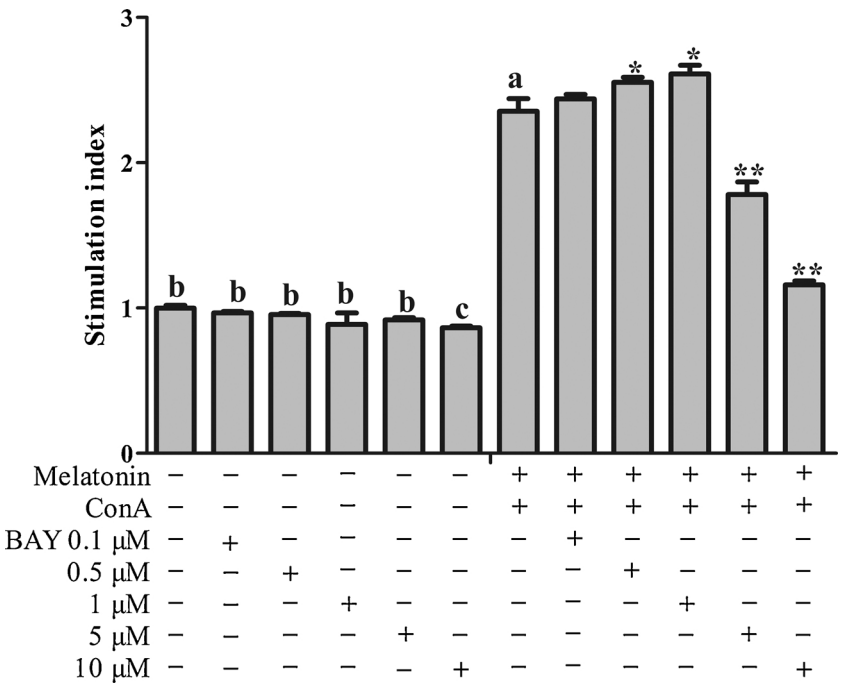


**Fig. 7.** Effect of P65 on T-lymphocyte proliferation in the chick thymus. BAY (BAY 11–7082) is an antagonist of NF-κB; a–c represent significant differences ($P < 0.05$) between treatment groups; * represents a significant difference from the ConA + melatonin group ($P < 0.05$); ** represents a significant difference from ConA + melatonin group ($P < 0.01$).

found that RL promoted the protein expression of p-IκB/p-P65 accompanied with an increase in the RORα level and a decrease in T-lymphocyte proliferation in the chick thymus relative to GL. Our in vitro experiments showed that the RORα antagonist SR3335 induced an increase in the cytoplasmic P65 protein level and a decrease in the nuclear P65 protein level in cultured thymocytes. By contrast, the RORα agonist SR1078 decreased the cytoplasmic P65 level and increased the nuclear P65 level. The P65 antagonist BAY 11–7082 increased T-lymphocyte proliferation in response to the effects of melatonin. A similar report found that melatonin suppressed the production of nitric oxide and interleukin-6 in LPS-activated murine macrophage cells by inhibiting NF-κB transcriptional activity [41]. IκB was reportedly able to block P65 nuclear entrance, but this effect was reduced when following phosphorylation [42]. These results indicate that RORα negatively regulates monochromatic light-induced T-lymphocyte proliferation by increasing the expression of p-IκB, which releases P65 for nuclear translocation. In the human aorta, however, RORα1 negatively interferes with the NF-κB signaling pathway by upregulating the IκBα level [43]. The difference in results may be caused by the use of different cells types or treatment methods.

## 5. Conclusion

In summary, monochromatic green light promotes melatonin secretion and T-lymphocyte proliferation and IL-2 production in thymus of chickens, but decreased the protein and mRNA levels of RORα relative to red light. RORα negatively mediates the GL-enhanced T-lymphocyte proliferation in the thymus of chickens by the up-regulation of the IκB phosphorylation and promoting p65 into the nucleus, which actives NF-κB activation.

## Declaration of Competing Interest

No potential conflict of interest was reported by the author.

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