Melatonin mediates monochromatic light–induced proliferation of T/B lymphocytes in the spleen via the membrane receptor or nuclear receptor

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ABSTRACT Our studies found that melatonin mediates the monochromatic light–induced lymphocyte proliferation in chickens. However, melatonin receptor subtypes contain membrane receptor (Mel1a/Mel1b/Mel1c) and nuclear receptor (Retinoic acid receptor–related orphan receptor [**ROR**] α/RORβ/RORγ) and are characteristic with cell specificity. This study compared receptor pathway of melatonin, which mediated the monochromatic light–induced T/B lymphocyte proliferations in chickens. Newly hatched chicks were randomly divided into white light, red light, green light (**GL**), and blue light groups. Green light promoted the membrane receptor expression in the spleen but decreased the nuclear receptor level compared with that of red light. These changes were accompanied by increase of T/B lymphocyte proliferation and plasma melatonin level under GL. Pinealectomy reversed aforementioned changes and resulted in no differences among the light-treated groups. Supplementation of exogenous melatonin enhanced GL-induced T/B lymphocyte proliferation in the spleen but was reversed by Mel1c antagonist prazosin and RORα agonist SR1078 and enhanced by RORα antagonist SR3335. However, Mel1b antagonist 4P-PDOT and RORγ antagonist GSK increased the stimulation effect of melatonin on GL-induced T lymphocyte proliferation but no effect on the B-lymphocyte proliferation. These results indicate that melatonin promotes the GL-induced T lymphocyte proliferation through Mel1b, Mel1c, and RORα/RORγ; however, the Mel1a, Mel1c, and RORα may be involved in the B lymphocyte proliferation.

Key words: T-lymphocyte, B-lymphocyte, melatonin receptor, lymphocyte proliferation, monochromatic light

INTRODUCTION

The effect of light on immune function in birds has previously been reported (Kliger et al., 2000; Zhang et al., 2014). The development of immune function includes cytokine secretion, antioxidant and lymphocyte proliferation, and so on. The properties of light include the light intensity, photoperiod, and light wavelength. A greater IgM response was observed from 50 lx than from 5 lx or 200 lx in the broiler (Blatchford et al., 2009). Splenic lymphocyte proliferation was enhanced in vitro when deer mice were exposed to short day compared with long day (Demas and Nelson, 1996).

Prendergast proved that the proliferation of circulating splenocytes also was higher in long-day than in short-day exposed cells from hamsters (Prendergast et al., 2001). These studies investigated the effects of light intensity or photoperiod on lymphocytes in the spleen in different species. However, there are few studies about the light color affecting lymphocyte proliferation. Our previous research found that green light (**GL**) promotes the proliferation of T lymphocytes in the thymus (Chen et al., 2016) or in the spleen (Guo et al., 2017), and it can also promote the proliferation of B lymphocytes in the bursa of Fabricius (Li et al., 2015) of chicks.

As we know, the pineal gland is an endocrine gland that receives light information to secrete melatonin in a 2-step process in which serotonin is N-acetylated by the enzyme, arylalkylamine N-acetyltransferase, and N-acetylserotonin is converted to melatonin by the enzyme hydroxyindole-O-methyltransferase (Moore, 1996). Therefore, the melatonin, which is a neuroendocrine hormone that is secreted by the pineal gland, plays

an important role in regulating immune function by light. Pinealectomy and melatonin administration were used to detect the function of melatonin. The pinealectomy decreased the plasma melatonin levels (Jin et al., 2011) and inhibited monochromatic GL–induced lymphocyte proliferation in the thymus (Chen et al., 2016), spleen (Guo et al., 2017), and bursa of Fabricius (Li et al., 2015) of chicks. In contrast, the addition of melatonin promoted the short-day–induced proliferation of splenocytes when compared with long day in hamsters (Vishwas and Haldar, 2013). It is well-known that melatonin regulates physiological effects via its membrane and nuclear receptors (**NR**), as well as by receptor-independent pathways (Lardone et al., 2009, 2011). Generally, the melatonin receptor expression is tissue specific, therefore, melatonin can combine with different receptors to trigger downstream signals. The melatonin receptor subtype Mel1b, but not Mel1a, is involved in the melatonin-induced enhancement of humoral immune function in mice (Drazen and Nelson, 2001), whereas Mel1b also plays a major role in splenocyte proliferation in *Perdicula asiatica* (Kumar Yadav et al., 2014). Our previous research found that melatonin mediates the effect of GL on the proliferation of T lymphocytes through the Mel1b and Mel1c receptors in the thymus of the chick (Chen et al., 2016); Mel1a and Mel1c participate in the proliferation of B lymphocytes in the bursa (Li et al., 2013). However, it is still unknown which membrane receptor is involved in the melatonin-stimulated, GL-induced proliferation of T or B lymphocytes in the spleen of chicks.

Despite growing evidence proving that melatonin mediates changes in immunity through membrane receptors, little is known about the mechanisms of the NR in this process. The retinoic acid receptor–related orphan receptors (**ROR**) are members of the NR superfamily. The ROR family comprises 3 members (RORα [NR1F1], RORβ [NR1F2], and RORγ [NR1F3]) (Solt and Burris, 2012). Retinoic acid receptor–related orphan receptor α is widely expressed, especially in the immune organ, and it has been suggested as a mediator of melatonin nuclear signaling (Wiesenberg et al., 1998; Lardone et al., 2011). Lardone et al. suggested that melatonin is able to repress the action of RORα (Lardone et al., 2011). Moraitis and Giguère has suggested that ligand binding is required for the rapid degradation of the RORα (Moraitis and Giguère, 2003). Retinoic acid receptor–related orphan receptor β is mainly expressed in the brain, retina, or bone to regulate circadian rhythm and bone metabolism (Feng et al., 2015). Retinoic acid receptor–related orphan receptor γ is expressed in lymphoid tissues and is essential for the development of thymocytes and lymph nodes (Kurebayashi et al., 2000). Mice that are deficient in RORγ have defective development of thymocytes and lymphoid organs (Santori et al., 2015). However, which melatonin NR plays a role in regulating melatonin-mediated monochromatic light–induced T/B lymphocyte proliferation is still unclear.

In this article, we will determine which melatonin membrane receptors or NR are expressed in the spleen and which ones play a role in the process by which melatonin mediates the monochromatic light–induced proliferation of T/B lymphocytes in the spleen of chicks.

MATERIALS AND METHODS

Animals and Treatments

All of the experimental procedures follow the rules of the Animal Welfare Committee of the Agricultural Research Organization, China Agricultural University (Approval No. CAU20171114-2). A total of 168 post-hatching day (P) 0 Arbor Acre male broilers (Beijing Huadu Breeding Co., Beijing, China) were randomly allocated into 4 separate color light rooms, including white light (**WL**, 400–760 nm, n = 42), red light (**RL**, 660 nm, n = 42), GL (560 nm, n = 42), and blue light (**BL**, 480 nm, n = 42) by a light-emitting diode system (Hongli Tronic Co., Guangzhou, China) for 2 wk. All of the light intensities were 15 ± 0.3 lx at the bird-head level with a light period of 23 h daily (23L: 1D). At P3, each light group was divided into intact (n = 56), the sham-operated (n = 56), and the pinealectomy (n = 56) treatments. Thus, all chicks were divided into 12 subgroups (n = 14). The broilers had ad libitum access to feed and water. The diet was formulated to meet or exceed the nutrient recommendations of the NRC for Poultry (1994). The pinealectomy was performed at P3. The chicks were anesthetized with pentobarbital sodium (30–40 μg/g of BW) by intraperitoneal injection. Their heads were fixed with stereotaxic apparatus after anesthesia, and the skin on the top of the head was cut to expose the skull. The skull was opened in front of the cerebellar and cerebellar junction, and the pineal gland under the skull was immediately removed. Finally, the skin is sewn. The process of sham operation was the same as the pinealectomy but not removed the pineal gland.

At P14, 14 chicks were randomly selected from each subgroup. After weighing, their spleens were dissected out. The spleen index is expressed as spleen weight (g)/BW (g). Then, the spleens (n = 8) were immediately frozen in liquid nitrogen and stored at −80°C until assay and were fixed in 4% paraformaldehyde. In addition, other 6 chicks from each subgroup were used for a lymphocyte proliferation assay.

Immunohistochemical Staining

The spleen (n = 3) was fixed in 4% paraformaldehyde and embedded in paraffin, and the tissue was cut into 5-μmol sections. After dewaxing the slices with xylene and alcohol, the antigen was retrieved by citric acid buffer microwave antigen retrieval. The tissue was treated with 3% hydrogen peroxide methanol for 30 min, washed with PBS 3 times, and rabbit serum was added for 30 min. Then, the sections were incubated with the primary antibody (RORα, OM184933, 1:200; RORβ, OM167645, 1:200, OmnimAbs, China; and RORγ, 13205-1-AP, 1:200; Proteintech) at 4°C overnight. The

slides were rinsed 3 times with PBS for 5 min, after which the sections were incubated with a biotinylated conjugated goat anti-rabbit IgG secondary antibody (1:200; sc-2020, Santa Cruz) for 2.5 h at room temperature. The samples were washed 3 times in PBS and incubated with streptavidin-horseradish peroxidase (1:300; Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Then, 3,3'-diaminobenzidine was added for observation and was stopped with deionized water.

Quantitative Real-time Polymerase Chain Reaction

Total RNA from spleen of each treatment (n = 5) was purified using the reverse transcription kit as per the manufacturer's instruction (Thermo Fisher Scientific, Boston). The cDNA was stored at $-20°C$ until the general chain reaction (polymerase chain reaction [PCR]). The general PCR amplification system contained 2 μL of sample cDNA, 10 μL of AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China), 0.4 μL of primer, and 7.2 μL of ddH$_2$O. The PCR reaction was performed with an initial incubation step for 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. The melting curve analysis was performed with the default program of the LightCycler 480 (Roche, LightCycler 480 System, Switzerland). The relative mRNA levels were normalized to *cGAPDH*. The PCR primers are listed in Table 1.

T/B Lymphocyte Proliferative Activity Assay

Cells were isolated from the chicken spleen (n = 6) on aseptic conditions at P14. The cell counting was performed using a hemocytometer, and viability was determined by the trypan blue exclusion method. Viable cells (95%) were resuspended in complete RPMI 1640 medium, and 5×10^6 cells/mL were added to each well in a flat-bottom 96-well culture plate. The T/B cell–specific mitogen concanavalin A (**ConA**)/lipopolysaccharide (**LPS**) (20 μg/mL) (Sigma-Aldrich, St. Louis) was added to the culture medium. The suspensions were incubated at 41°C with 5% CO$_2$ for 44 h; 4 h later, 10 μL MTT (tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (5 mg/mL) were added to the medium; 2 h later, 100 μL of 10% SDS were added to each well. The optical density (**OD**) was read with a microplate reader (Model 680; Bio-Rad, St. Louis, MO) at 570 nm wavelength. The proliferative activity of the T lymphocytes was expressed as the stimulation index as follows: Stimulation index = OD570 (drug group)/OD570 (control group). The drug group consists of 5 μM SR3335 (a selective RORα inverse agonist; MCE, New Jersey), 10 μmol SR1078 (an agonist of the NR RORα; MCE, New Jersey), 1 μM GSK (a selective RORγ inverse agonist; MCE, New Jersey), 10 μM luzindole (nonselective Mel1a/Mel1b antagonist; Santa Cruz Biotechnology Inc., Dallas, TX), 0.1 μM 4P-PDOT (selective Mel1b antagonist; Tocris Bioscience, Bristol, UK), and 0.1 μM prazosin (selective Mel1c antagonist; Santa Cruz Biotechnology Inc., Dallas, TX) in the cell suspensions. The samples were incubated for 30 min before the addition of ConA/LPS and melatonin.

Statistical Analyses

All of the data are expressed as the mean ± SEM. Using SPSS, version 18.0 (SPSS Inc., Chicago, IL), differences among various monochromatic lights within the different groups (light-treated group, pinealectomy group, and sham operation group) were analyzed by 2-way ANOVA in vivo, followed by a Duncan's multiple comparison post-hoc least significant difference test. One-way ANOVA followed by a multiple comparison post-hoc least significant difference test was performed in vitro to determine whether the melatonin receptor and its signings mediated melatonin-induced lymphocyte proliferation. $P < 0.05$ was considered statistically significant. Using Pearson's correlation coefficient, a correlation analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) to determine the possible linear relationship between the mRNA expressions of ROR and the proliferation of lymphocytes. The correlation was also analyzed and expressed as the Pearson coefficient (r^2).

Table 1. Sequences of primers used for polymerase chain reaction.

Genes	Primer sequences (5′-3′)	Accession no.	Product size (bp)
Mel1a	F: CAA TGG ATG GAA TCT GGG A R: GCT ATG GGA AGT ATG AAG TGG	NM_205362.1	333
Mel1b	F: TTT GCT GGG CAC CTC TAA AC R: CGC TTG CTC TTC TGT CCA TC	XM_417201.2	259
Mel1c	F: AGA TAA GTG GGT TCC TGA TGG R: GCA AAG GTG GAA GAG TAA ATC	NM_205361.1	237
RORα	F: TGG GCATACCCCTGAAGGTA R: CCG ATGCTGGTGTGTAGTCA	XM_413763.2	140
RORβ	F: AAA TCG TTG CCA ACA CTG CC R: AGG TCA ATG ACG TGC CCA TT	NM_205093.1	270
RORγ	F: GTG GGGTAATATCGGGAGCG R: CTT ATCGGGACAACCTGCGT	XM_015280013.1	90
GAPDH	F: ATC ACAGCCACACAGAAGACG R: TGA CTTTCCCCACAGCCTTA	NM_204305	124

RESULTS

Effect of Pinealectomy on Monochromatic Light–Induced Lymphocyte Proliferation in the Spleen

As shown in Figure 1A, the spleen index of GL in the intact operation group was significantly higher than that of RL by 21.57% ($P = 0.008$), BL by 24.61% ($P = 0.003$), and WL by 20.02% ($P = 0.0127$). Similarly, GL increased the T cell proliferation by 32.03–45.73% ($P = 0.000$–0.000) (Figure 1B) and B cell proliferation by 15.43–24.73% ($P = 0.000$–0.000) in the spleen (Figure 1C) compared with the other 3 light-treated groups. As with lymphocyte proliferation, GL increased the melatonin concentrations in plasma compared with those of the other light groups (data not shown). No

Figure 1. Green light increases the proliferation of lymphocytes in the spleen of 3 treatment groups (intact, sham operation, and pinealectomy). (A) Spleen index; (B) T cell proliferation; (C) B cell proliferation. Data are means ± SEM. Bars with different letters are significantly different ($P < 0.05$) in various monochromatic lights within the different groups by 2-way ANOVA. *Significant difference between the corresponding light treatments from the sham-operated and pinealectomy groups ($P < 0.05$). Abbreviations: BL, blue light; GL, green light; RL, red light; WL, white light.

significant differences were found between the intact and sham operation groups that were exposed to the same monochromatic light ($P > 0.05$).

After pinealectomy, the plasma melatonin level was reduced (data not shown), whereas the spleen index was reduced by 7.01% in the WL ($P = 0.345$), 4.28% in the RL ($P = 0.694$), 24.55% in the GL ($P = 0.076$), and 11.52% in the BL ($P = 0.402$) groups compared with those of the corresponding intact and sham operation groups. No significant differences were observed among the various light-treated groups ($P > 0.05$). Similarly, pinealectomy decreased the T cell proliferation in the spleen by 15.07–63.61% ($P = 0.000$–0.072) compared with those of the intact and sham groups. In addition, no significant differences were observed among the light-treated pinealectomy groups ($P > 0.05$) (Figure 1B). The B cell proliferation activity in the spleen of the pinealectomy group was reduced by 12.79% in the WL ($P = 0.001$), 7.35% in the RL ($P = 0.173$), 25.14% in the GL ($P = 0.000$), and 5.16% in BL ($P = 0.100$) groups compared with those of the corresponding intact and sham operation groups (Figure 1C). The differences among the light-treated groups disappeared after pinealectomy.

Immunohistochemical Localization of Receptors

The immunohistochemical results showed the expression levels of 3 NR and their locations in the spleens of the broilers. In addition, the expression levels of the 3 melatonin membrane receptors were determined in the spleen (Guo et al., 2015) (data not shown). In the structure of spleen, the white pulp is composed of many lymphocytes, macrophages, dendritic cells and so on, and it is the center of immune activity, whereas the red pulp contains splenic cords and venous sinuses, including red blood cells, reticular fibers, reticular cells, macrophages, and lymphocytes. Furthermore, the marginal zone was in a unique region in the spleen and was located at the interface of the red pulp and the white pulp (Cesta, 2006). The RORα receptor was widely distributed in the spleen, which contains the white pulp, marginal zone, and red pulp (Figure 2A). Similarly, the RORγ receptor was widely detected in the spleen (Figure 2C). The numbers of cells that were positive cell for the RORα and RORγ receptors were high. In contrast to RORα and RORγ, the RORβ NR was mainly found in the red pulp of the spleen (Figure 2B).

Effect of Pinealectomy on Monochromatic Light–Induced Receptor mRNA Expression in the Spleen

The melatonin NR and membrane receptor mRNA expression were detected in the spleen of broilers (Figure 3). The expression levels of NR (Figure 3A–3C) and membrane receptors (Figure 3D–3F) were different under different light colors. The results showed that RL
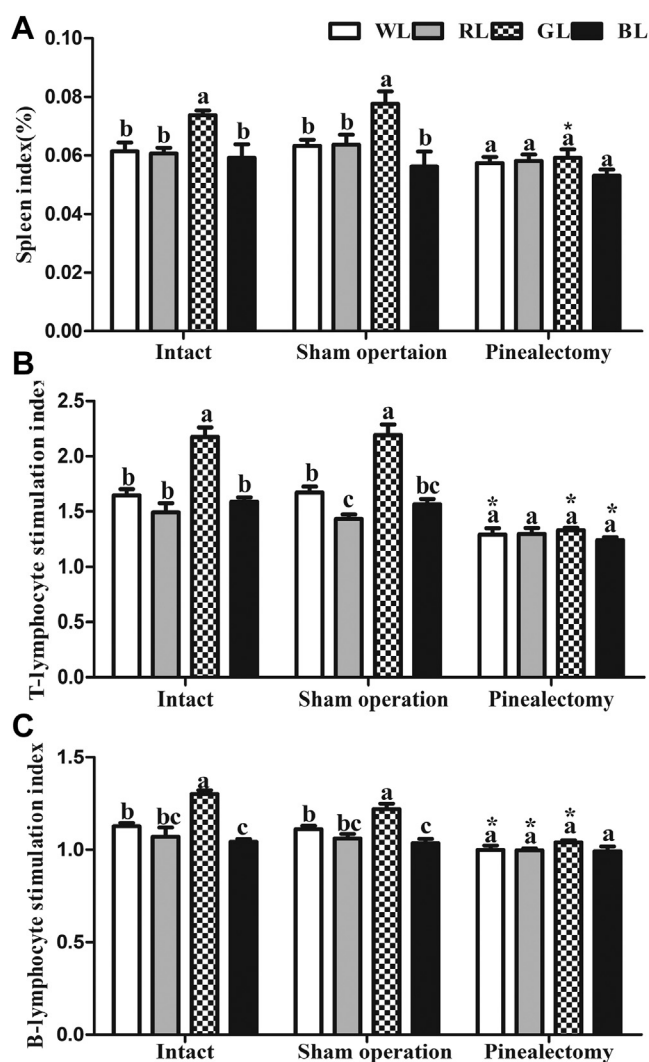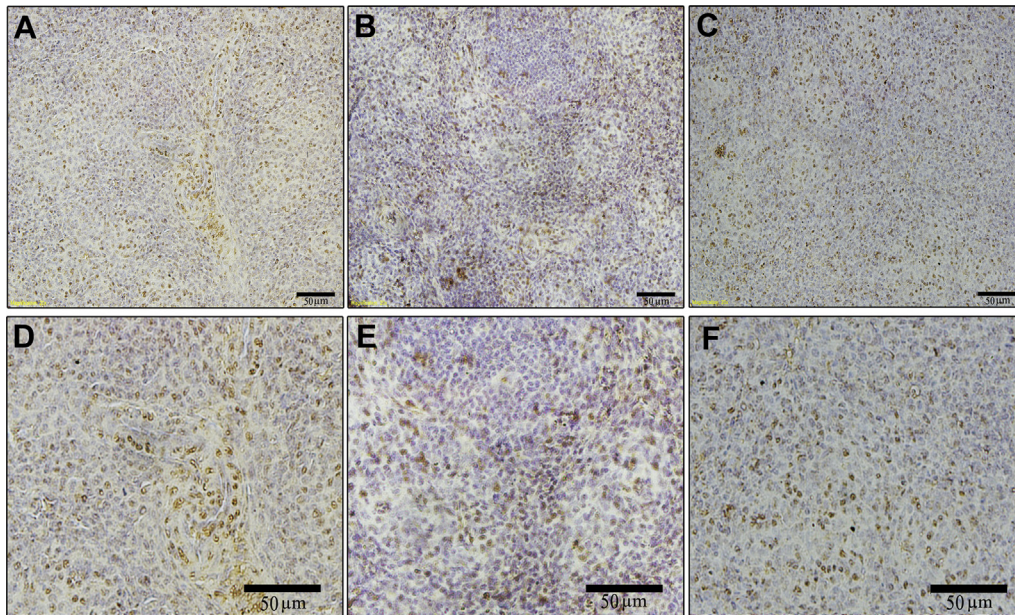
Figure 2. The immunostaining of the 3 melatonin nuclear receptors RORα (A, D), RORβ (B, E) and RORγ (C, F) in the spleen of broilers at 14 D. Scale bar = 50 μm. Abbreviation: ROR, retinoic acid receptor–related orphan receptor.

significantly increased the RORα mRNA (6.82–43.36%, $P = 0.000$–0.3523) and RORγ mRNA (37.72–118.05%, $P = 0.000$–0.000) expression levels compared with those of the other light-treated groups (Figure 3A, 3B). The data from sham operation were not significantly different from those of the intact group ($P > 0.05$). However, the expression of RORα and RORγ was altered by pinealectomy, as the RORα mRNA was increased by 10.03–43.43% ($P = 0.000$–0.102) and RORγ increased by 1.66–118.05% ($P = 0.000$–0.766) after pinealectomy, but no significant differences were detected among the various light treatments of the pinealectomy group for either of these 2 NR ($P > 0.05$). The Pearson correlation

analysis showed a negative correlation between the changes in the mRNA expression levels of spleen RORα and T lymphocyte proliferation ($r^2 = 0.7636$, $P = 0.0002$); in addition, a negative relationship between RORα and B lymphocyte proliferation was found ($r^2 = 0.7584$, $P = 0.0002$). A negative correlation was observed between the changes in the expression of spleen RORγ and T lymphocyte proliferation ($r^2 = 0.8153$, $P < 0.0001$); however, there was no correlation between RORγ and B lymphocyte proliferation ($r^2 = 0.6283$, $P = 0.0021$). Although monochromatic treatment affects the mRNA expression of RORβ, it was not as highly expressed as either RORα or RORγ.
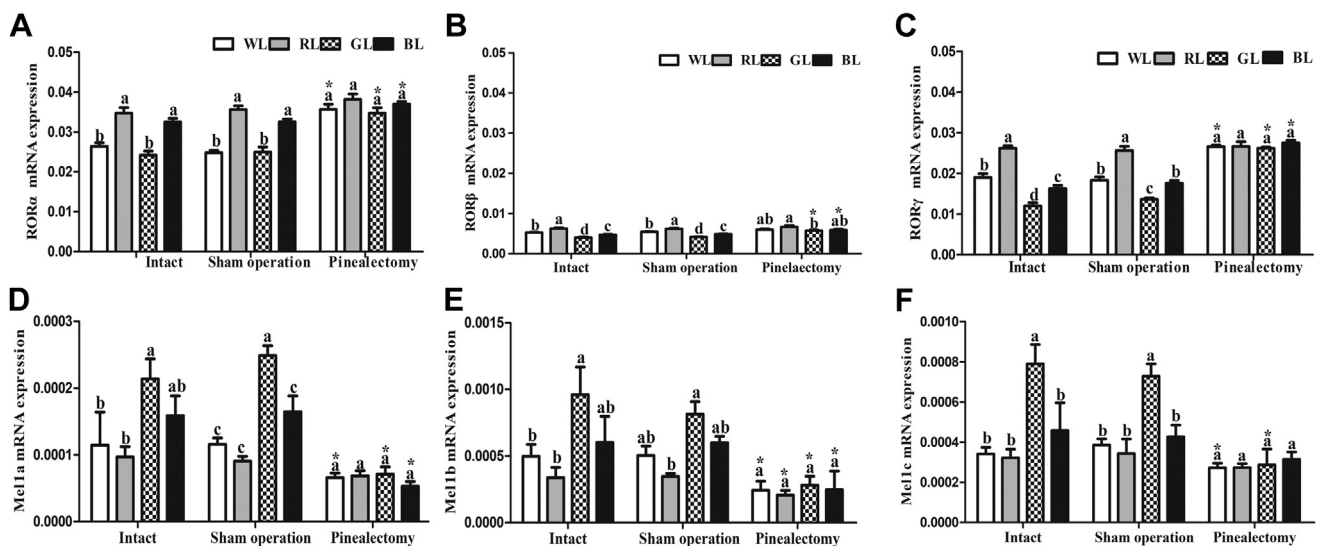

Figure 3. Effect of monochromatic light on mRNA expression of RORα (A), RORβ (B), RORγ (C), Mel1a (D), Mel1b (E) and Mel1c (F) in the spleen of 3 treatment groups (intact, sham operation, and pinealectomy). Data are means ± SEM. Bars with different letters are significantly different ($P < 0.05$) in various monochromatic lights within the different groups by 2-way ANOVA. "*" represents a significant difference between the corresponding light treatments from the sham-operated and pinealectomy groups ($P < 0.05$). Abbreviations: BL, blue light; GL, green light; RL, red light; ROR, retinoic acid receptor–related orphan receptor; WL, white light.

The expression of melatonin membrane receptors was different from that of the NR, as GL obviously increased 3 membrane receptor mRNA levels (Figure 3D–3F). The Mel1a level in the GL group was 34.66–120.68% ($P = 0.002$–0.267) higher than those of the other lights (Figure 3D). No significant differences were found between the WL, RL, and BL intact groups ($P > 0.05$). Similar results were found for Mel1b and Mel1c. The expression of Mel1b in the GL group was 59.98–183.87% ($P = 0.000$–0.295) higher than those of the others (Figure 3E), and the expression of Mel1c in this group was 72.44–145.18% ($P = 0.000$–0.002) higher than those of the others (Figure 3F).

The result of sham group was similar with that of the control group for the 3 membrane receptors, and there were no significant differences in the sham operation groups compared with those of the corresponding intact groups ($P > 0.05$). However, in the pinealectomy group, the mRNA expression levels of the 3 membrane receptors were decreased under the 4 lights compared with those of the corresponding intact and sham groups; also, no significant differences were observed among those lights (Figure 3) ($P > 0.05$).

Effect of Receptor on GL-Induced Lymphocyte Proliferation in the Spleen

To investigate the role of the receptor in GL-induced lymphocyte proliferation, cell suspensions of cultured T and B lymphocytes from the chick spleen were prepared under GL for preincubation with the receptor antagonist. In the suspension, exogenous ConA stimulated T lymphocyte proliferation and exogenous LPS stimulated B lymphocyte proliferation (Yang et al., 2015).

As shown in Figure 4A and 4B, the RORα agonist SR1078 (10 μM) or RORα antagonist SR3335 (5 μM) were added 30 min before the addition of ConA (20 μg/mL) or LPS (35 μg/mL) and melatonin (10^{-9} M). The control was incubated with cells only in RPMI 1640 medium, SR1078, SR3335, and 0.01% ethanol or in 0.01% dimethyl sulfoxide (**DMSO**).The MTT assay results showed that melatonin effectively promoted T lymphocyte proliferation in response to ConA (55.48%, $P = 0.000$) (Figure 4A); pretreatment with SR3335 increased T lymphocyte proliferation by 12.48% ($P = 0.000$) compared with the ConA + melatonin group, whereas SR1078 treatment reduced the T lymphocyte proliferation by 30.37% ($P = 0.000$). Similar to the T lymphocyte results, the combination of melatonin and LPS significantly increased B-lymphocyte proliferation by 25.02% ($P = 0.000$) (Figure 4B). Compared with the melatonin and LPS groups, B lymphocyte proliferation was increased by 7.88% ($P = 0.000$) after pretreatment with SR3335 and was markedly decreased with SR1078 pretreatment (48.90%, $P = 0.000$). No significant differences were detected between the DMSO (ethanol)-only and cell-only experiments ($P > 0.05$).

The RORγ antagonist GSK (5 μM) was added 30 min before the addition of ConA (20 μg/mL) or LPS (35 μg/mL) and melatonin (10^{-9} M) in GL-induced lymphocytes. The controls were incubated with cells only in RPMI 1640 medium, GSK, and 0.01% ethanol or 0.01% DMSO. The results showed that pretreatment with GSK had no effect on the T lymphocyte proliferation ($P = 0.818$) in response to ConA, whereas GSK increased this effect by 14.98% ($P = 0.001$) in response to melatonin and ConA (Figure 4C). In contrast to the T lymphocyte results, the combination of melatonin and LPS significantly increased the B lymphocyte proliferation by 21.24% ($P = 0.000$) (Figure 4D). Compared with the melatonin and LPS groups, B lymphocyte proliferation was increased by 3.19% ($P = 0.438$) after pretreatment with GSK, which had no significant difference on B lymphocyte proliferation. No significant differences were detected between the DMSO (ethanol)-only and cell-only experiments ($P > 0.05$).

In addition to the interaction of melatonin with NR, the membrane receptors are also involved in the proliferation of T and B lymphocyte in the spleen; here, antagonists of the 3 membrane receptors were used. The results showed that the function of melatonin on T lymphocyte proliferation was decreased with the addition of 4P-PDOT and prazosin (33.22%, $P = 0.000$; 36.67%, $P = 0.000$) (Figure 4E) compared with that of the melatonin + ConA group. The combined action of 4P-PDOT and prazosin further reduced the proliferation activity of T lymphocytes (46.28%, $P = 0.000$), but luzindole had no effect on the cell activity ($P > 0.05$). In B lymphocytes, melatonin also increased the cell proliferation in response to LPS (22.65.00%, $P = 0.000$) (Figure 4F), whereas luzindole and prazosin reduced the effect of melatonin (14.30%, $P = 0.010$; 15.92%, $P = 0.002$), which is different from T-lymphocytes, where 4P-PDOT had no effect on B lymphocytes. Therefore, melatonin regulates the GL-induced T lymphocyte proliferation via Mel1b and Mel1c, whereas Mel1a and Mel1c were involved in B lymphocyte proliferation in the spleen.

DISCUSSION

The visual system of poultry is more developed than that of mammals, as it possess 5 types of cones that can sense light information (Yang et al., 2016). Therefore, lighting can affect many physiological functions of poultry, including the immune system (Olanrewaju et al., 2006; Archer, 2019). Majewski et al. showed that light promotes the mitogen-stimulated proliferation of splenocytes of chickens (Majewski et al., 2005). In addition, the combination of green and blue monochromatic light enhances immune function (Zhang et al., 2014). Our results showed that GL increased the plasma melatonin levels and the proliferation of T/B lymphocytes in the spleen. Based on this result, GL also increased the organ index of the spleen. Similar to our results, GL stimulation during hatching significantly resulted in more weight gain than in birds that were incubated under dark conditions (Rozenboim et al., 2013). In addition,
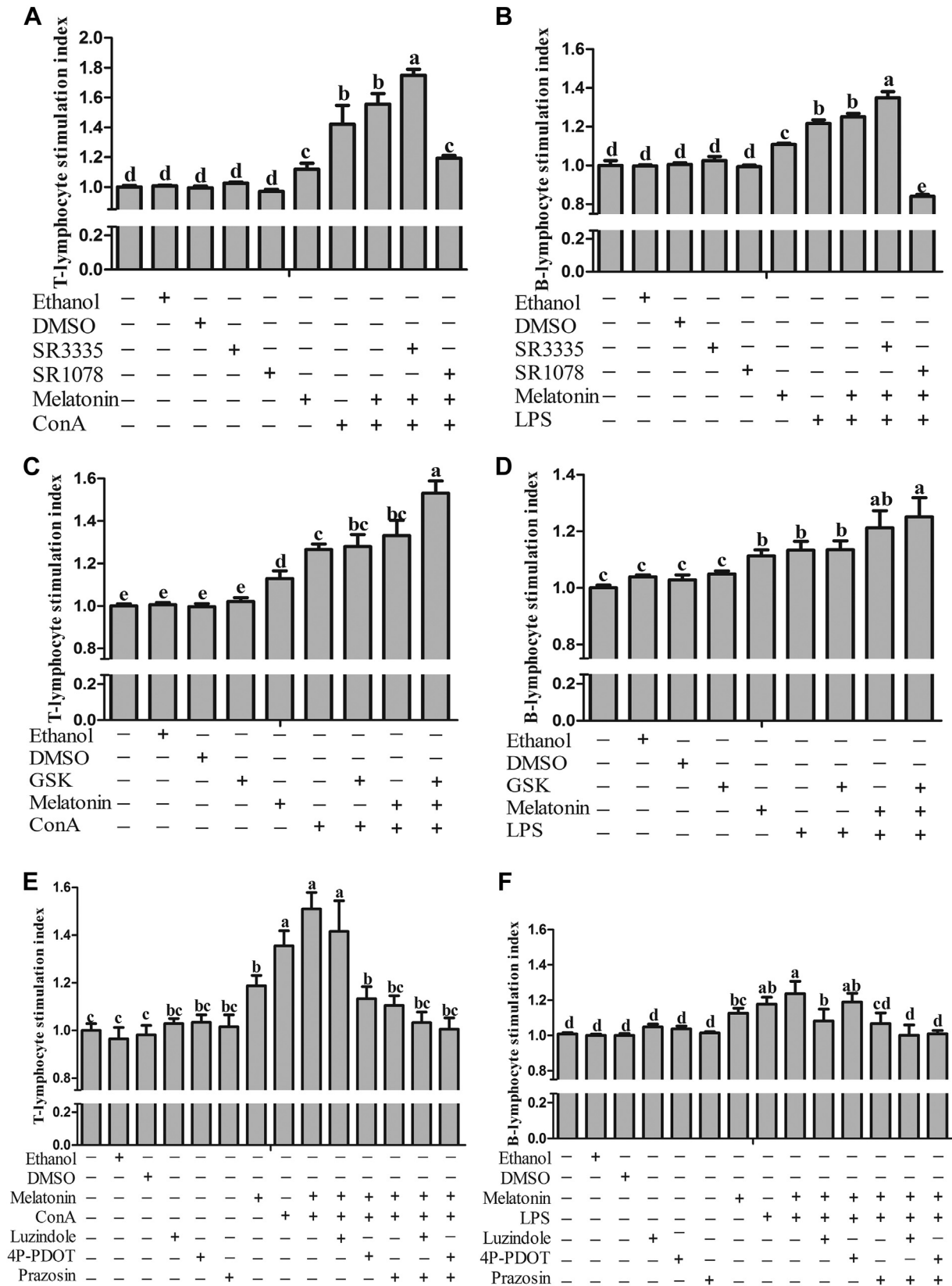
Figure 4. Role of RORα (A, B), RORγ (C, D), and melatonin membrane receptor (E, F) on lymphocyte proliferation in the GL-treated spleen. (A, C, E) T lymphocyte proliferation; (B, D, F) B lymphocyte proliferation. "SR3335" is a selective RORα inverse agonist. "SR1078" is an agonist of RORα; "GSK" (GSK2981278) is a RORγ antagonist. "Luzindole" is a Mel1a/Mel1b antagonist; "4P-PDOT" is a Mel1b antagonist; "prazosin" is a Mel1c antagonist. ConA acts as a T lymphocyte proliferation stimulator; LPS acts as a B lymphocyte proliferation stimulator. Bars with different letters are significantly different ($P < 0.05$) between different treatments by 1-way ANOVA. Abbreviations: ConA, concanavalin A; DMSO, dimethyl sulfoxide; GL, green light; LPS, lipopolysaccharide; ROR, retinoic acid receptor–related orphan receptor.

GL induced the proliferation of T lymphocytes in the thymus of chickens (Chen et al., 2016).

Melatonin plays an important role in the effects of light on immune function (Haldar and Ahmad, 2010). Lymphocyte proliferation was suppressive by melatonin in female Siberian hamsters under long-day conditions but not short-day conditions (Prendergast et al., 2001). Our previous research proved that GL increased B lymphocyte proliferation through melatonin in the bursa of Fabricius (Li et al., 2015) and also the proliferation of T-lymphocytes in the thymus (Chen et al., 2016). The experiments in our article demonstrate that the significant differences disappeared under various light conditions after pinealectomy, and the addition of exogenous melatonin also promoted the proliferation of T/B lymphocytes. Many of the functions of melatonin are mediated by interaction with the melatonin receptors, including membrane receptors and NR (Slominski et al., 2012). The melatonin type 1 and 2 receptors belong to the category of G protein–coupled receptors in mammals, where these 2 receptors are called Mel1a and Mel1b in chicks; Mel1c has also been found in chicks (Liu et al., 1995; Reppert et al., 1995). Our results found that melatonin interacts with Mel1a and Mel1c to promote T lymphocyte proliferation, but Mel1b and Mel1c participate in B lymphocyte proliferation in the spleen. Similar to our results, in mice, melatonin enhanced splenocyte proliferation through melatonin type 2 receptor- but not melatonin type 1 receptor(Drazen and Nelson, 2001). In addition, Mel1b and Mel1c participate in the GL-induced T lymphocyte activity in the thymus of chicks (Chen et al., 2016), but Mel1a and Mel1c are involved in the proliferation of B lymphocytes in the bursa of Fabricius (Li et al., 2013). These different results may be explained by different experimental conditions, species, or cell types, which all could have contributed to the differences in the findings.

The NR RORα/RORγ are reported to be involved in the role in which melatonin mediates the immune system (Dzhagalov et al., 2004). Immunohistochemical staining showed the expression of the 3 melatonin NR in the spleen. Retinoic acid receptor–related orphan receptor α and RORγ are widely expressed in the red pulp and white pulp of spleen, whereas RORβ is mainly expression in the red pulp. The RORβ mRNA expression level was less than those of RORα and RORγ. The RORα/RORγ expression changed under different light conditions in the spleen of chicks. Red light treatment obviously promoted the mRNA levels of RORα and RORγ compared with GL. These findings are similar to our results showing that white light-emitting diode light increased the protein expression of NR RORα in HT-22 cells (Yang et al., 2019). The correlation analysis showed that RORα was almost negatively correlated compared with T and B lymphocyte proliferation. In vitro, treatment with SR3335 increased both T and B lymphocyte activity, but SR1078 decreased lymphocyte activity in response to melatonin. These results showed us that melatonin mediated the GL-induced T and B lymphocyte proliferation in the spleen by decreasing the RORα pathway. Similar to our results, Wang et al. studied the antitumor activity of melatonin by reducing the expression of the melatonin NR RZR/ROR in human gastric cancer cells (Wang et al., 2016). The difference is that some researchers have said that RORα is not a receptor for melatonin (Slominiski et al., 2014; Slominski et al., 2016). They said the cholesterol was the natural ligand for RORα. The finding that intrigued us was that GSK obviously increased the proliferation of T lymphocytes in response to melatonin and ConA in the spleen but had no effect on B lymphocyte proliferation. This finding means that melatonin mediates the GL-induced T lymphocyte proliferation by negatively regulating the NR RORγ expression but that it has no effect on B-lymphocytes. This is likely because of the difference in RORγ expression between T lymphocytes and B lymphocytes in the spleen. Eberl and Littman found that the RORγ is expressed at the double positive stage of T cell development but is absent in the mature thymocytes and in mature splenocytes (Eberl and Littman, 2004). Several studies provide evidence that RORγt negatively regulates cytokine secretion by thymocytes (He et al., 1998). In addition, melatonin treatment reduced the expression of RORγ in human cancer cells (Wang et al., 2016).

In conclusion, melatonin mediates the monochromatic light–induced T/B lymphocyte proliferation by positively binding to different receptors. The membrane receptors Mel1a and Mel1c were involved in T lymphocyte proliferation, but Mel1b and Mel1c were involved in B lymphocyte proliferation; by contrast, melatonin negatively regulates the expression of RORα to modulate the light-induced T/B lymphocyte proliferation in the spleen. However, RORγ negatively participated in melatonin modulation of T lymphocyte activity but not in that of B lymphocytes in the spleen.

ACKNOWLEDGMENTS

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