RESEARCH

BMAL1 but not *CLOCK* is associated with monochromatic green light-induced circadian rhythm of melatonin in chick pinealocytes

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Abstract

The avian pineal gland, an independent circadian oscillator, receives external photic cues and translates them for the rhythmical synthesis of melatonin. Our previous study found that monochromatic green light could increase the secretion of melatonin and expression of *CLOCK* and *BMAL1* in chick pinealocytes. This study further investigated the role of *BMAL1* and *CLOCK* in monochromatic green light-induced melatonin secretion in chick pinealocytes using siRNAs interference and overexpression techniques. The results showed that si-*BMAL1* destroyed the circadian rhythms of *AANAT* and melatonin, along with the disruption of the expression of all the seven clock genes, except *CRY1*. Furthermore, overexpression of *BMAL1* also disturbed the circadian rhythms of *AANAT* and melatonin, in addition to causing arrhythmic expression of *BMAL1* and *CRY1/2*, but had no effect on the circadian rhythms of *CLOCK*, *BMAL2* and *PER2/3*. The knockdown or overexpression of *CLOCK* had no impact on the circadian rhythms of *AANAT*, melatonin, *BMAL1* and *PER2*, but it significantly deregulated the circadian rhythms of *CLOCK*, *BMAL2*, *CRY1/2* and *PER3*. These results suggested that *BMAL1* rather than *CLOCK* plays a critical role in the regulation of monochromatic green light-induced melatonin rhythm synthesis in chicken pinealocytes. Moreover, both knockdown and overexpression of *BMAL1* could change the expression levels of *CRY2*, it indicated *CRY2* may be involved in the *BMAL1* pathway by modulating the circadian rhythms of *AANAT* and melatonin.

Key Words

- pinealocyte
- circadian rhythm
- melatonin
- siRNA interference
- monochromatic light

Endocrine Connections

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Introduction

Avian circadian system is a complex of mutually coupled pacemakers residing in the pineal gland, the retina and the hypothalamic suprachiasmatic nucleus (SCN), which is synchronized to environmental light cycles (1). As one of the biological clock oscillators, the pineal gland secretes melatonin into the blood with high level during the subjective night and low level during the subjective day (2) and is a typical model system for the study of circadian rhythm. Melatonin plays an important role in the circadian and seasonal rhythms, immunomodulation and neurotransmission (3). In birds, pinealectomy abolished the circadian patterns of locomotor activity and body temperature (4, 5), which was attributed to the absence of the rhythmic secretion of melatonin, as cyclic melatonin administration restored the circadian rhythmicity of the pinealectomised birds (6, 7, 8). The change in the duration of melatonin production serves as a biological signal for the organisation of light-dependent functions, such as reproduction and behaviour (9, 10, 11). Light seems to play a definitive role in the rhythmic secretion of melatonin (12, 13). Our previous studies have shown that monochromatic green light (GL) could increase the amount of plasma melatonin in chick with rhythmic

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secretion (14, 15). However, the mechanism underlying the regulation of melatonin secretion by GL remains unclear.

The circadian oscillator in vertebrates is driven by the core circadian-feedback loop that consists of positive and negative components (16). In the nucleus, the positive component brain and muscle ARNT-like-1/2 (*BMAL1/2*) and its paralog circadian locomotor output cycles kaput (*CLOCK*) form a heterodimer via the Per-Arnt-Sim domain. The BMAL-CLOCK heterodimer binds to E-box enhancer elements presented in the promoter of target genes, thereby activating the expression of negative genes, such as *Cryptochrome* (*Crys*) and *Period* (*Pers*) (17, 18, 19). The BMAL1-CLOCK heterodimer also activates the transcription of arylalkylamine N-acetyltransferase (*Aanat*), which is a rate-limiting enzyme for melatonin synthesis (20, 21). Moreover, there was an interesting phenomenon *in vivo*, wherein GL illumination not only promoted the circadian rhythm secretion of melatonin in chick plasma but also enhanced the circadian rhythm expression of *BMAL1* and *CLOCK* in three circadian pacemakers, including the pineal gland, the retina and the SCN (15, 22, 23). This phenomenon suggested that *BMAL1* and *CLOCK* might play critical roles in GL-induced melatonin rhythmic secretion, but further *in vitro* studies were needed to investigate the role of *BMAL1* and *CLOCK* in rhythmic melatonin synthesis in chick pinealocytes exposed to GL.

Because the avian pineal gland can directly receive external photic information and respond to light entrainment, it is often chosen as a prominent model even for cellular analyses (24). Therefore, siRNA interference and overexpression experiments were used in the present study to investigate the role of *BMAL1* and *CLOCK* in monochromatic GL-induced rhythmic synthesis of melatonin in chick pinealocytes.

Materials and methods

Animals

Newly hatched Arbor Acre male broilers (Beijing Huadu Breeding, P. R. China) were housed under 12 h white light: 12 h dark cycles (lights on at 08:30; zeitgeber time (ZT) 0). Food and water were available *ad libitum*. All experimental procedures for this research were approved by the China Agricultural University Animal Care and Use Committee.

Pineal gland cell culture

The 7-day-old chicks were killed by decapitation. The pineal glands were removed and separated from extra tissue in cold D-Hank's solution under sterile conditions, followed by digestion with 0.1% collagenase IV (Worthington, USA) for 30 min at 37°C and incubation with 0.25% trypsin (Gibco) for 10 min at 37°C. After the tissue was filtered through a 200 mesh metal filter, the filtrates were collected and plated onto collagen-coated six-well plastic culture plates (Corning) at a density of 10^6 cells/well in DMEM supplemented with 10% foetal bovine serum (25, 26). The cells were cultured under 12 h GL (560 nm): 12 h dark cycles (lights on at 08:30; zeitgeber time (ZT) 0) supplied by cold light-emitting diode (LED) bulbs. This LED system was placed 15 cm above the cell plates at the top inside a CO_2 incubator. The LED energy output was tuned by changing the lamp's voltage and current by a transformer. The voltage was 13.89 V in GL with an intensity of 0.16 W/m^2.

Interference experiments

The knockdown of endogenous *BMAL1* and *CLOCK* was performed using the synthesised siRNA (Shanghai GenePharma Co, Ltd). The siRNA sequences used to target chicken *BMAL1* were sense 5′-GGACUACGCAGACCAACAATT-3′ and antisense 5′-UUGUUGGUCUGCGUAGUCCTT-3′ and those used to target chicken *CLOCK* were sense 5′-GCUCGGAAG AUGGAUAAAUTT-3′ and antisense 5′-AUUUAUCCAUC UUCCGAGCTT-3′. The negative control siRNA were sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3′. After cultivation for 2 days, the pinealocytes were transiently transfected with si-*BMAL1*, si-*CLOCK* and negative control siRNA at ZT0 using a Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (11668019, Invitrogen). The cells were continuously cultured for 2 days, and the harvested cells and supernatants were then subjected to dim red light at six separate time points at 4-h intervals (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20).

Overexpression experiments

Full-length *BMAL1* (NM_001001463.1) and *CLOCK* (NM_204174.2) segments in chicken with the homologous arm of the pcDNA3.1 vector were amplified by PCR using chicken pineal gland cDNA. The PCR products were

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cloned into plasmid pcDNA3.1 with *EcoR I* and *Xho I* using the seamless assembly cloning kit (C5891, CloneSmarter, USA). After cultivation for 2 days, the pinealocytes were transiently transfected with pcDNA3.1-*BMAL1*, pcDNA3.1-*CLOCK* or pcDNA3.1 (negative control). The cells were cultured and harvested in accordance with the interference experiment methods. The blank control group was the chick pinealocytes cultured under GL, we only retained the negative control group in order to make the figures clear and concise since the statistical analysis showed that expressions of all genes in blank and negative control groups were no significant difference.

Quantitative real-time PCR

Total RNA was isolated from pinealocytes using TRIzol reagents (CW0580A, CWBIO Company, China). The first-strand cDNA was synthesised according to the RevertAid first strand cDNA synthesis kit (K1622, Thermo, USA). PCR amplification was performed using the AceQ qPCR SYBR green master mix (Q111-02, Vazyme Biotech, China). PCR cycle primers are shown in Table 1. Each sample was assayed in triplicate, and relative mRNA levels were normalised to the expression levels of the housekeeping gene *GAPDH*.

Western immunoblot analysis

Protein concentrations were measured by the bicinchoninic acid assay (CW0014, CWBIO Company) after the total protein extracts were collected from pinealocytes using the radioimmunoprecipitation lysis buffer (CW2333, CWBIO Company). Equal amounts of protein from each sample were separated using SDS-PAGE and transferred onto PVDF membranes using electroblotting. The membranes were incubated in a solution containing 5% skim milk in phosphate buffer saline at room temperature for 1 h. The membranes were incubated with a CLOCK rabbit polyclonal antibody (1:1000, ab461, Abcam) and BMAL1 rabbit polyclonal antibody (1:1000, ab93806, Abcam) overnight at 4°C. After washing the membranes in Tris-buffered saline-Tween, they were incubated with goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin (Ig) G (1:8000, CW0103, CWBIO Company) for 1 h at room temperature. The blot bands were detected using the Ecl Western blot kit (1627003, Millipore) after washing. The intensity of the signals for CLOCK and BMAL1 were quantified using Image-Pro plus software and were normalised relative to the values obtained for β-ACTIN (1:4000, CW0096, CWBIO Company).

Measurement of melatonin levels

The melatonin content in cell supernatants was measured using the competitive inhibition ELISA kit (CEA908Ge, USCN Life Science INC, China) according to the manufacturer's protocol. First, 50 µL of each sample dilution was added into the wells and incubated with 50 µL of detection reagent A for 1 h. Then, 100 µL

Table 1 The primers of clock genes.

Genes	Primer sequences (5′-3′)	Product size (bp)	Accession no.
BMAL1	F:GTAGACCAGAGGGCGACAG R:ATGAAACTGAACCAGCGACTC	215	NM_001001463.1
BMAL2	F:CGGCGTTCCTTCTTCTGTC R:TTCCTCTTCCACTCCACC	156	NM_204133.1
CLOCK	F:GATCACAGGGCACCTCCAATA R:CTAGTTCTCGCCGCCTTTCT	301	NM_204174.2
CRY1	F:GATGTGGCTATCCTGTAGTTCCT R:GCTGCTGGTAGATTTGTTTCAT	281	NM_204245.1
CRY2	F:GCACGGCTGGATAAACACT R:AAATAAGC GGCAGGACAAA	141	NM_204244.1
PER2	F:ATGAAACGAGCCATCCCG R:CAGTTGTCGTGATTTTGCCTA	206	NM_204262.1
PER3	F:CAGTGCCTTTGTTGGGTTAC R:GATGGATTCACAAAACTGGAC	217	XM_417528.2
AANAT	F:GGACCAGGACAGGCTCAG R:CGAAACCACACTTCTCGTAG	224	NM_205158.1
GAPDH	F:ATCACAGCCACACAGAAGACG R:TGACTTTCCCACAGCCTTA	124	NM_204305.1

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of detection reagent B was added and incubated for 30 min at 37°C. Next, 90 µL of the substrate solution was added and incubated for 15–25 min at 37°C. The reaction was stopped with 50 µL of stop solution, and optical density (OD) values were immediately measured using an ELISA analyser at 450 nm (Bio-Rad, Model 680).

Statistical analysis

Significant difference analysis

Data are presented as the mean ± standard error of the mean (S.E.M.).To test the significance of the differences in the expression at the same time point between experimental and negative control groups, and the differences in the expression at each time point between the groups, one-way ANOVA followed by Tukey test using SPSS version 22 (IBM Corp., Armonk) was used.

Unimodal cosinor regression analysis

The 24-h rhythm of clock genes was analysed by MATLAB 7.0 software (MathWorks Inc., USA). The cosinor formula $y=a+b\times\cos\ (x\times\mathrm{pi}/12–c\times\mathrm{pi}/12)$ was used, where a, b and c denote the mesor, amplitude and acrophase levels of the rhythm, respectively and R^2 is the fitting degree. Statistically significant differences in gene rhythms were indicated by a P value of <0.05, which was calculated using the website http://www.danielsoper.com/statcalc3/calc.aspx?id=15 (27).

Results

Efficiency of interference and overexpression of *BMAL1* and *CLOCK*

The 85–87% chick pinealocytes were successfully transfected, which clearly observed after 100 pmol/well FAM-siRNA was transfected (Fig. 1A). The mRNA and protein levels of *BMAL1* and *CLOCK* in chick pinealocytes after transfection with siRNAs and overexpression plasmids were examined to evaluate the interference and overexpression efficiencies. Compared with the blank control group, the mRNA and protein levels of *BMAL1* in cells transfected with si-*BMAL1* were decreased by 52.18 and 71.29%, respectively ($P<0.05$, one-way ANOVA) (Fig. 1B and C). pcDNA3.1-*BMAL1* significantly increased the mRNA and protein levels of *BMAL1* by 162.47 and 1.89 fold compared with those of the blank control group, respectively ($P<0.05$, one-way ANOVA) (Fig. 1D and E). Furthermore, compared with the blank control

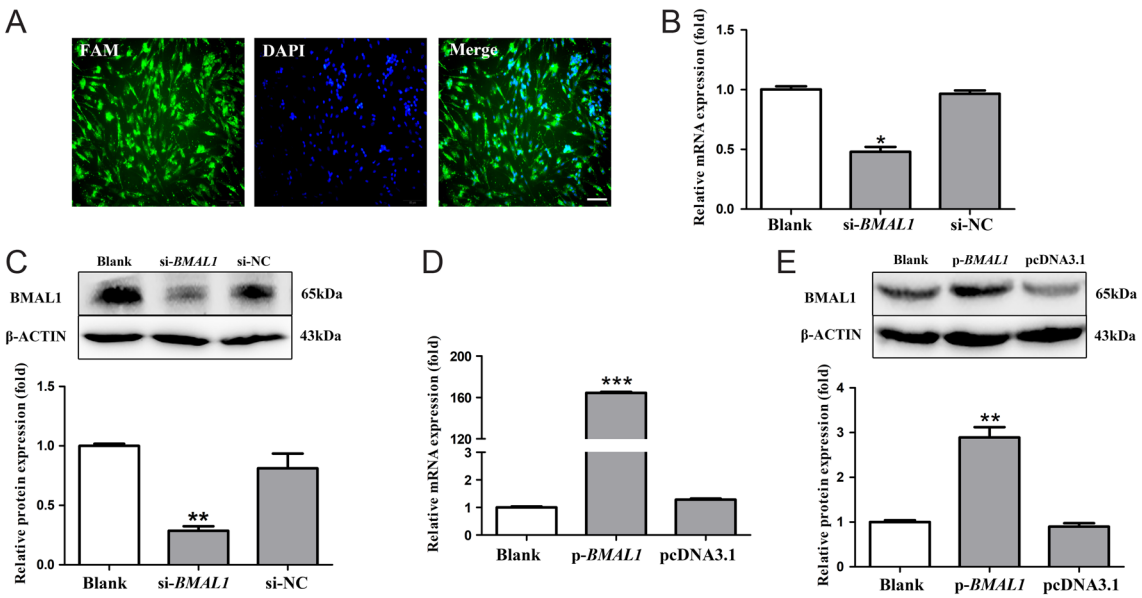

Figure 1
Efficiency of interference and overexpression of *BMAL1* in pinealocytes under green light. (A) Fluorescent dye-labelled siRNA (100 pmol/well; FAM fluorescent) was transfected into chick pinealocytes to evaluate the transfection efficiency. (B and C) The expression of *BMAL1* mRNA and cBMAL1 protein after transfecting si-*BMAL1* and the negative control siRNA. (D and E) The expression of *BMAL1* mRNA and cBMAL1 protein after transfecting pcDNA3.1-*BMAL1* and pcDNA3.1. The results are presented as the mean ± S.E.M. $N = 3$ per time point. Significant differences between different treatment groups are shown with asterisks, where $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (A–C) Scale bar = 50 µm.

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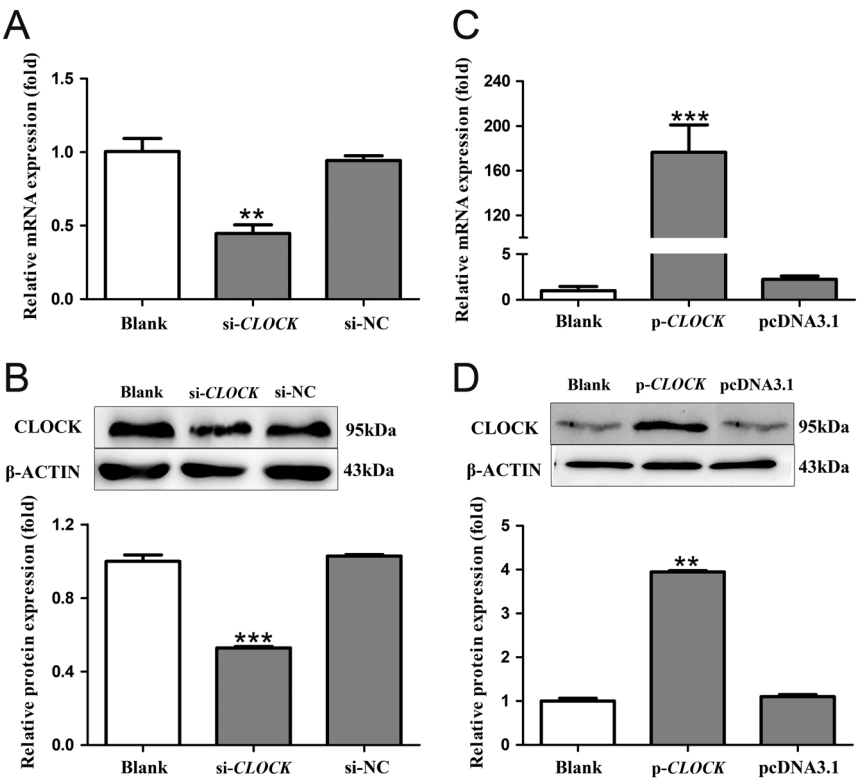

Figure 2
Efficiency of interference and overexpression of *CLOCK* in pinealocytes under green light. (A and B) The expression of *CLOCK* mRNA and cCLOCK protein after transfecting si-*CLOCK* and a negative control siRNA. (C and D) The expression of *CLOCK* mRNA and CLOCK protein after transfecting pcDNA3.1-*CLOCK* and pcDNA3.1. The results are presented as the mean ± S.E.M. $N = 3$ per time point. Significant differences between different treatment groups are shown with asterisks, where $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

group, si-*CLOCK* suppressed the mRNA and protein levels of *CLOCK* by 55.49 and 47.19%, respectively ($P < 0.05$, one-way ANOVA) (Fig. 2A and B). On the contrary, *CLOCK* overexpression increased the mRNA and protein levels of *CLOCK* by 175.45 and 2.95 fold compared with those of blank control group, respectively ($P < 0.05$, one-way ANOVA) (Fig. 2C and D). These results demonstrated the *BMAL1* and *CLOCK* knockdown and overexpression were successfully made.

The analysis of *AANAT* transcription level and melatonin secretion

Significant daily variations in *AANAT* transcription level and melatonin secretion were observed in the different interference groups ($P < 0.05$, one-way ANOVA). The cosinor analysis showed that si-*BMAL1* not only decreased the levels of *AANAT* mRNA and melatonin (except at ZT12 for melatonin; $P < 0.05$, one-way ANOVA) but also

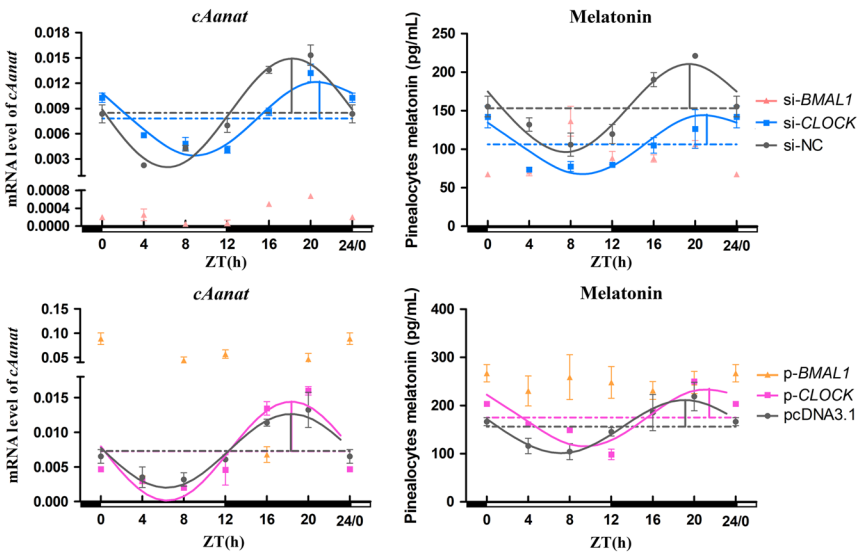

Figure 3
Temporal changes in *AANAT* mRNA and melatonin secretion after *BMAL1* and *CLOCK* interference and overexpression in pinealocytes under green light. Pink points and lines represent the *BMAL1* interference group, blue points and lines represent the *CLOCK* interference group, yellow points and lines represent the *BMAL1* overexpression group and purple points and lines represent the *CLOCK* overexpression group. The horizontal white bar on each figure represents the day, and the horizontal black bar represents the night. The solid points indicate the relative mRNA levels or the plasma melatonin levels. The curved lines indicate the rhythmic levels of genes and melatonin by the cosinor analysis. The horizontal dotted line represents the mesor. The vertical solid line represents acrophase and amplitude (the distance between maximum and mesor). The results are presented as the mean ± S.E.M. $N = 3$ per time point.

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Table 2 Circadian rhythm parameters (mean ± S.E.M.) of all clock genes, *AANAT* and melatonin in interference treatments, as determined by cosinor analyses.

	Group	*BMAL1*	*BMAL2*	*CLOCK*	*BMAL1*	*CLOCK*	*CRY1*	*CRY2*	*PER2*	*PER3*	*AANAT*	Melatonin
Mesor (×10^{-3}) (for gene)	si-*BMAL1*	ND	ND	ND	ND	ND	22.10 ± 1.24	ND	ND	ND	ND	ND
	si-*CLOCK*	4.06 ± 0.21	ND	ND	0.42 ± 0.09	ND	35.93 ± 0.86	ND	3.86 ± 0.01	ND	7.81 ± 0.34	106.41 ± 11.48*
	si-NC	3.70 ± 0.53	4.40 ± 0.90	4.65 ± 0.09	0.43 ± 0.14	0.31 ± 0.06	32.06 ± 8.90	3.49 ± 0.72	2.93 ± 0.52	2.09+0.48	8.47 ± 0.38	153.33 ± 12.53
Amplitude (×10^{-3}) (for gene)	si-*BMAL1*	ND	ND	ND	ND	ND	8.38 ± 0.33	ND	ND	ND	ND	ND
	si-*CLOCK*	1.38 ± 0.12	ND	ND	0.10 ± 0.06	ND	13.94 ± 0.11	ND	1.13 ± 0.04*	ND	4.40 ± 0.12*	38.65 ± 10.69
	si-NC	1.66 ± 0.04	2.22 ± 0.07	3.84 ± 0.18	0.13 ± 0.03	0.22 ± 0.01	12.96 ± 3.90	2.09 ± 0.47	0.96 ± 0.04	1.26+0.46	6.47 ± 3.83	57.12 ± 3.93
Acrophase (h)	si-*BMAL1*	ND	ND	ND	ND	ND	9.39 ± 0.13*	ND	ND	ND	ND	ND
	si-*CLOCK*	12.00 ± 0.76	ND	ND	14.12 ± 1.73	ND	10.77 ± 0.03*	ND	23.64 ± 0.23	ND	20.86 ± 0.02*	21.12 ± 0.38*
	si-NC	12.77 ± 0.34	15.88 ± 0.10	15.07 ± 0.03	14.30 ± 0.39	15.29 ± 0.18	21.77 ± 0.73	16.48 ± 0.25	0.22 ± 0.00	23.64 ± 0.53	18.18 ± 0.06	19.47 ± 0.05
R^2	si-*BMAL1*	0.26	0.34	0.35	0.22	0.25	0.59	0.28	0.00	0.26	0.05	0.22
	si-*CLOCK*	0.52	0.21	0.03	0.80	0.04	0.88	0.21	0.90	0.21	0.93	0.80
	si-NC	0.82	0.93	0.84	0.90	0.87	0.96	0.96	0.76	0.89	0.96	0.91
P	si-*BMAL1*	0.22	0.19	0.10	0.34	0.54	0.02	0.20	-	0.23	0.92	0.38
	si-*CLOCK*	0.02	0.33	0.92	0.00	0.90	0.00	0.34	0.00	0.32	0.00	0.00
	si-NC	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F	si-*BMAL1*	1.66	1.91	2.56	1.21	0.78	5.33	1.79	-	1.64	0.16	1.13
	si-*CLOCK*	4.99	1.24	0.16	11.77	0.19	34.62	1.23	39.00	1.27	60.60	16.26
	si-NC	43.51	127.63	51.67	46.20	25.63	206.59	226.90	15.03	99.97	357.03	52.83

Note: ND represents not determined as there was no circadian rhythm. Significant differences ($P < 0.05$) between the treatment and si-NC group are shown by an asterisk (*).

destroyed their circadian rhythms (Fig. 3). However, si-*CLOCK* significantly reduced melatonin secretion for the whole day, except ZT0, as well as the mRNA expression of *AANAT* during the night ($P<0.05$, one-way ANOVA); however, it did not abolish the rhythms of *AANAT* mRNA and melatonin (Fig. 3). si-*CLOCK* decreased the amplitude of *AANAT* mRNA and mesor of melatonin and delayed the acrophases of *AANAT* mRNA and melatonin by 2.68 and 1.65 h, respectively (Table 2).

BMAL1 overexpression caused the loss of day–night variations in melatonin secretion ($P=0.871$, one-way ANOVA), although significant daily variations in the level of *AANAT* mRNA and melatonin were observed in other overexpression groups ($P<0.05$, one-way ANOVA). The cosinor analysis showed that the overexpression of *BMAL1* increased the *AANAT* mRNA level and secretion of melatonin, except at ZT16 for *AANAT* and melatonin and ZT 20 for melatonin ($P<0.05$, one-way ANOVA) but abolished the circadian rhythms of *AANAT* mRNA and melatonin (Fig. 3). However, *AANAT* mRNA and melatonin still maintained a remarkable circadian rhythm during the overexpression of *CLOCK* (Fig. 3). Simultaneously, the amplitude in *AANAT* expression was increased by the overexpression of *CLOCK*, and the acrophases of melatonin were delayed by 2.32 h (Table 3).

Transcription level analysis of positive clock genes

Transcripts of all the positive clock genes (*BMAL1*, *BMAL2* and *CLOCK*) in the interference groups displayed day–night variations ($P<0.05$, one-way ANOVA). Compared with si-NC, si-*BMAL1* reduced the mRNA levels of positive genes throughout the day, except at ZT4 ($P<0.05$, one-way ANOVA), whereas si-*CLOCK* only significantly declined its own expression throughout the day ($P<0.05$, one-way ANOVA) (Fig. 4). Thecosinor analysis showed that the knockdown of *BMAL1* and *CLOCK* resulted in a circadian arrhythmy of all positive genes, except *BMAL1* mRNA in si-*CLOCK*-transfected cells that still presented a significant circadian rhythm (Table 2).

The mRNA expression of all positive clock genes in pinealocytes showed a significant diurnal variation after *BMAL1* and *CLOCK* overexpression ($P<0.05$, one-way ANOVA). Moreover, the overexpression of *BMAL1* significantly promoted the mRNA expression of *BMAL1/2* throughout the day ($P<0.05$, one-way ANOVA), whereas the overexpression of *CLOCK* only significantly enhanced its own expression throughout the day except ZT16 ($P<0.05$, one-way ANOVA) (Fig. 4). The cosinor analysis showed that the overexpression of *BMAL1* and *CLOCK*

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Table 3 Circadian rhythm parameters (mean ± S.E.M.) of all clock genes, *AANAT*, and melatonin in overexpression treatments, as determined by cosinor analyses.

	Group	*BMAL1*	*BMAL2*	*CLOCK*	*BMAL1*	*CLOCK*	*CRY1*	*CRY2*	*PER2*	*PER3*	*AANAT*	Melatonin
Mesor ($\times10^{-3}$)	p-*BMAL1*	ND	70.64 ± 6.62*	2.36 ± 0.14*	ND	0.31 ± 0.07	ND	ND	1.22 ± 0.05($\times10^{3}$)*	23.27 ± 4.18*	ND	ND
	p-*CLOCK*	3.44 ± 0.18	3.52 ± 0.56	ND	0.46 ± 0.07	ND	ND	ND	4.21 ± 0.00*	2.55 ± 0.11*	7.27 ± 0.66	175.17 ± 3.51
	pcDNA3.1	3.49 ± 0.15	3.43 ± 0.30	4.04 ± 0.02	0.43 ± 0.15	0.29 ± 0.09	30.39 ± 1.65	2.93 ± 0.29	2.62 ± 0.20	1.98 ± 0.13	7.32 ± 1.10	156.13 ± 19.14
Amplitude ($\times10^{-3}$)	p-*BMAL1*	ND	38.21 ± 0.87*	1.83 ± 0.09*	ND	0.24 ± 0.03	ND	ND	0.16 ± 0.02($\times10^{3}$)*	22.56 ± 3.92*	ND	ND
	p-*BMAL1*	1.03 ± 0.05*	1.74 ± 0.61	ND	0.18 ± 0.03	ND	ND	ND	1.12 ± 0.00	0.98 ± 0.10	7.17 ± 0.32*	60.08 ± 1.97
	pcDNA3.1	1.53 ± 0.06	1.75 ± 0.02	3.88 ± 0.17	0.14 ± 0.05	0.23 ± 0.04	10.41 ± 1.8	1.19 ± 0.10	1.13 ± 0.05	1.01 ± 0.08	5.33 ± 0.24	55.31 ± 9.65
Acrophase (h)	p-*BMAL1*	ND	9.12 ± 0.08*	12.70 ± 0.32*	ND	14.04 ± 0.12*	ND	ND	2.74 ± 0.09*	5.67 ± 0.23*	ND	ND
	p-*CLOCK*	15.31 ± 0.72*	15.95 ± 0.77	ND	19.14 ± 0.20*	ND	ND	ND	19.86 ± 1.01*	22.16 ± 0.09	18.36 ± 0.42	21.45 ± 0.09*
	pcDNA3.1	13.51 ± 0.17	15.35 ± 0.25	15.05 ± 0.18	14.45 ± 1.05	15.39 ± 0.23	21.71 ± 0.27	16.54 ± 0.46	22.18 ± 0.59	22.34 ± 0.36	18.31 ± 0.50	19.13 ± 0.31
R^2	p-*BMAL1*	0.37	0.80	0.63	0.37	0.85	0.28	0.27	0.65	0.77	0.06	0.08
	p-*CLOCK*	0.62	0.57	0.34	0.56	0.17	0.38	0.15	0.53	0.52	0.86	0.81
	pcDNA3.1	0.80	0.97	0.86	0.83	0.72	0.86	0.68	0.85	0.83	0.95	0.99
P	p-*BMAL1*	0.13	0.00	0.00	0.48	0.01	0.21	0.24	0.00	0.00	0.86	0.80
	p-*CLOCK*	0.00	0.01	0.13	0.05	0.52	0.07	0.51	0.01	0.01	0.00	0.00
	pcDNA3.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F	p-*BMAL1*	2.31	17.32	7.88	0.96	11.71	1.72	1.61	7.58	15.30	0.25	0.34
	p-*CLOCK*	7.72	6.18	2.26	3.80	0.80	2.86	0.80	5.21	5.15	29.75	15.67
	pcDNA3.1	41.67	371.68	42.12	35.60	21.49	39.79	12.18	43.48	36.08	194.50	254.87

Note: p-*BMAL1* represents the overexpression *BMAL1* group, p-*CLOCK* represents the overexpression *CLOCK* group. ND represents not determined as there was no circadian rhythm. Significant differences ($P < 0.05$) between the treatment and pcDNA3.1 groups are shown by an asterisk (*).

only abolished their own rhythm (Fig. 4). Moreover, *BMAL1* overexpression significantly increased the mesors and amplitudes of *BMAL2* and *CLOCK*. Compared with the pcDNA3.1 group, the acrophases of *BMAL2* and *CLOCK* in the *BMAL1* overexpression group were increased by 6.23 and 2.35 h, respectively. However, the acrophase of *BMAL1* in the *CLOCK* overexpression group was delayed by 1.80 h (Table 3).

Protein level analysis of positive clock genes

The protein expression of BMAL1 displayed evident diurnal oscillations in the *BMAL1* interference group (si-*BMAL1*) and *CLOCK* interference group (si-*CLOCK*) ($P < 0.05$, one-way ANOVA), but the daily variation of CLOCK was destroyed by si-*CLOCK* ($P = 0.124$, one-way ANOVA). The protein expression of both CLOCK and BMAL1 throughout the day were decreased by si-*BMAL1* except ZT4 for CLOCK ($P < 0.05$, one-way ANOVA), while si-*CLOCK* only decreased its own protein levels ($P < 0.05$, *t*-test) (Fig. 5). The cosinor analysis showed that the circadian rhythms of BMAL1 and CLOCK protein were disturbed by si-*BMAL1*, whereas si-*CLOCK* had no impact on the rhythmicity of the BMAL1 protein (Fig. 5 and Table 2).

The BMAL1 and CLOCK protein levels also displayed a remarkable diurnal oscillation after *BMAL1* and *CLOCK* overexpression ($P < 0.05$, one-way ANOVA). The overexpression of *BMAL1* and *CLOCK* remarkably increased their own protein levels ($P < 0.05$, one-way ANOVA). The cosinor analysis showed that the overexpression of *BMAL1* and *CLOCK* disrupted their own circadian rhythms of protein levels (Fig. 5). The acrophase of BMAL1 protein was delayed by 4.69 h after the overexpression of *CLOCK*, while the acrophase of CLOCK protein was advanced by 1.35 h after the overexpression of *BMAL1* (Table 3).

Transcription level analysis of negative clock genes

The results showed that there were significant diurnal variations at 24 h for the mRNA level of negative genes in the interference groups ($P < 0.05$, one-way ANOVA). si-*BMAL1* significantly decreased the levels of *CRY2* mRNA and *PER3* mRNA in one entire day, except at ZT20 for *PER3* ($P < 0.05$, one-way ANOVA) (Fig. 6). The circadian rhythms of *CRY2* mRNA and *PER3* mRNA were destroyed by si-*BMAL1* and si-*CLOCK*, and the cosinor analysis showed that the mRNA level of *CRY1* still retained a robust circadian rhythm in si-*BMAL1* and si-*CLOCK* accompanying an opposite phase change with

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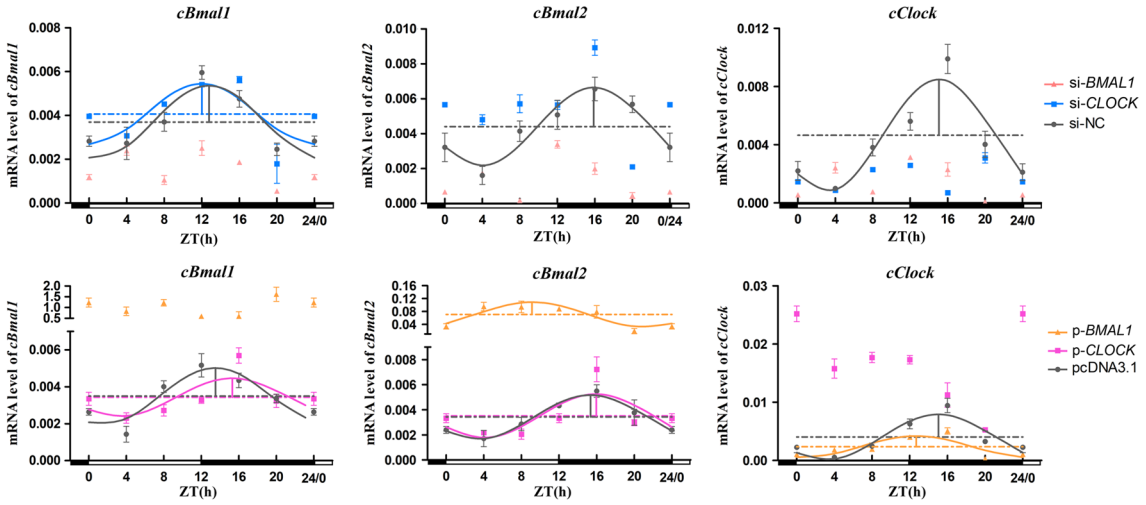

Figure 4
Temporal changes in positive clock genes after *BMAL1* and *CLOCK* interference and overexpression in pinealocytes under green light. The figure is marked the same as Fig. 3.

a negative control group (Fig. 6). si-*BMAL1* caused rhythm disturbances of *PER2* mRNA, whereas si-*CLOCK* had no effect on the circadian rhythm of PER2 mRNA (Fig. 6). si-*BMAL1* and si-*CLOCK* advanced the acrophase of *CRY1* mRNA by 12.38 and 11.00 h, respectively (Table 2).

The mRNA level of all negative clock genes in pcDNA3.1-*BMAL1*-and pcDNA3.1-*CLOCK*-transfected cells showed circadian oscillations ($P<0.05$, one-way ANOVA), but only the overexpression of *BMAL1* significantly promoted the transcription levels of *CRY2*, *PER2* and *PER3* ($P<0.05$, one-way ANOVA) (Fig. 6E). According to the cosinor analysis, the overexpression of *BMAL1* and *CLOCK* abolished the circadian rhythms of *CRY1/2* mRNA, did not disturb the circadian rhythms of *PER2/3* mRNA and increased the mesors and amplitudes of *PER2/3* mRNA (Fig. 6 and Table 3).

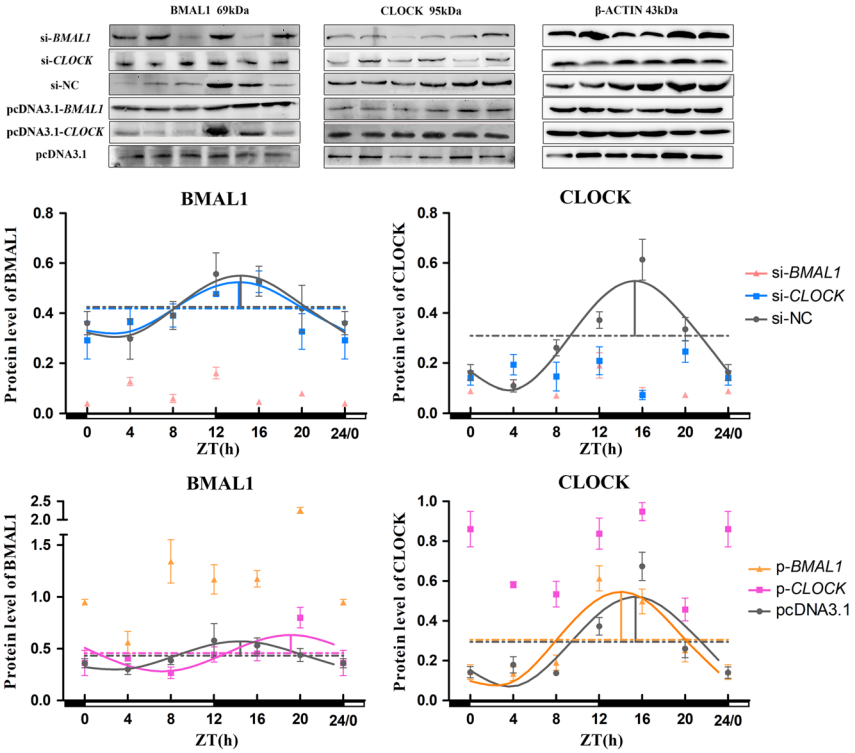

Figure 5
Temporal changes in positive clock proteins after *BMAL1* and *CLOCK* interference and overexpression in pinealocytes under green light. The figure is marked the same as Fig. 3.

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Discussion

The circadian release of avian melatonin can be controlled by the optical environment (13). Previous studies showed that monochromatic GL could enhance melatonin synthesis and expression of positive clock genes, including *CLOCK* and *BMAL1/2*, in the pineal gland of chicks (14, 15, 28). These studies suggested that the positive clock genes were involved in the modulation of circadian release of melatonin by the stimulation of monochromatic light. The present study further clarified the roles of positive clock genes in the monochromatic GL-induced melatonin secretion in chick pinealocytes. Both the knockdown and overexpression of *BMAL1* resulted in arrhythmicity in the circadian levels of *AANAT* mRNA and melatonin in the pinealocytes exposed to GL. Our results corroborated previous study results in which the overexpression of *BMAL1* in chick pineal cells caused deregulation of the melatonin rhythm in a continuous dark condition (29). Rhythm disturbances caused by the deletion of *BMAL1* have also been reported in mammals. The loss of *mBmal1* in mice resulted in an immediate and complete loss of rhythmic behaviour under free-running conditions (30). On the contrary, the knockdown and overexpression of *CLOCK* had no effect on the circadian rhythms and release levels of melatonin. A similar finding was reported in mice that *mClock*-deficient mice continued to exhibit robust behavioural and molecular rhythms (31). However, *AANAT* also maintained an obvious circadian rhythm, but its amplitude was changed by the knockdown and overexpression of *CLOCK*. This was consistent with previous studies in which the knockdown of *CLOCK* significantly reduced the circadian expression of *AANAT* in chicken cone photoreceptors (32). Consequently, our data demonstrated that *BMAL1* rather than *CLOCK* plays a critical role in the regulation of monochromatic GL-induced melatonin synthesis in chicken pinealocytes. However, an additional study as to whether the predominant role of *BMAL1* in regulation of light-induced melatonin synthesis is green light specific or not is still required.

Our qPCR and Western blot results showed that si-*BMAL1* broke the circadian rhythm of *CLOCK* in chick pinealocytes exposed to monochromatic GL, while si-*CLOCK* had no effect on the circadian rhythm of *BMAL1* mRNA and BMAL1 protein. These results were consistent with the changes in the circadian expression of *AANAT* and the release of melatonin. The stability of *BMAL1* rhythm in the si-*CLOCK* group may be due to the effect of orphan nuclear receptors RORs and REV-ERBα, which directly activate and repress the transcription of *BMAL1*, respectively, by binding with an Rev-erb/Ror-binding element in the *BMAL1* promoter (33, 34, 35, 36). Therefore, our results demonstrated that compared with *CLOCK*, *BMAL1* had a more predominant role in maintaining the circadian rhythms of melatonin synthesis and secretion in chick pinealocytes when stimulated by monochromatic GL, although some reports have suggested that *AANAT* transcription could be promoted by Bmal1/Clock heterodimers (20, 21).

The circadian rhythms of chicks were regulated by the transcription–translation feedback loop, which consists of a highly conserved set of genes including positive (*BMAL1/2*, *CLOCK*) and negative (*CRY1/2*, *PER2/3*) genes (18, 37). The deletion of *mBmal1* caused rhythm disturbances and a decrease in the expression

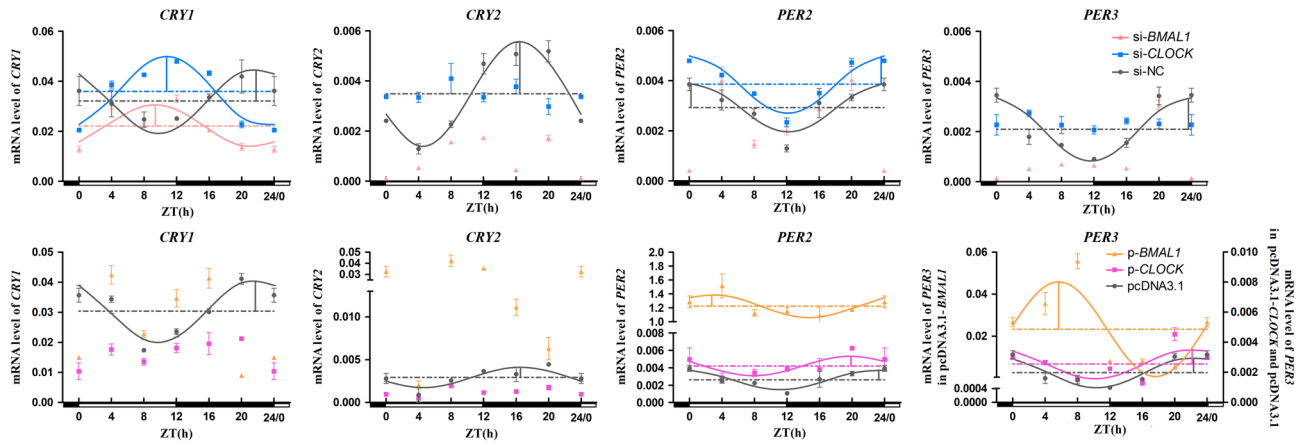

Figure 6
Temporal changes in negative clock genes after *BMAL1* and *CLOCK* interference and overexpression in pinealocytes under green light. The figure is marked the same as Fig. 3.

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of *mBmal2* in mice (30), whereas the overexpression of *BMAL2* deregulated the melatonin rhythm in chick pineal cells (29). However, our study showed that both si-*BMAL1* and si-*CLOCK* disrupted the circadian rhythm of *BMAL2*, but the overexpression of *BMAL1* and *CLOCK* had no such effect. Compared with the changes in the circadian rhythms of *BMAL2*, *AANAT* and melatonin, *BMAL2* was not synchronised with the rhythms disturbance of *AANAT* and melatonin in both si-*CLOCK* (*BMAL2* with arrhythmicity vs *AANAT* and melatonin with rhythmicity) and pcDNA3.1-*BMAL1* (*BMAL2* with rhythmicity vs *AANAT* and melatonin with arrhythmicity) treatments. It indicated that *BMAL2* was not an essential requirement for the modulation of circadian rhythm of melatonin synthesis and secretion in chick pinealocytes stimulated by monochromatic GL.

In mammals, *mBmal1*-deficient mice showed extremely low expression levels and loss of rhythmicity of *mPer1/2* in SCN; however, the expression of *mCry1* and *mPer1/2* continued to oscillate in *mClock*-deficient mice (30, 31). In the present study, the knockdown of *BMAL1* destroyed the circadian rhythms of negative clock genes, except *CRY1*, while the overexpression of *BMAL1* abolished the circadian rhythms of *CRY1/2*. For negative clock genes, however, we found that the circadian rhythm of *CRY2* mRNA was always consistent with the changes of *AANAT* and melatonin when *BMAL1* was downregulated or upregulated. Previous studies have reported that *mCry2* plays an important role in maintaining the biological rhythms of mammals because mice lacking *mCry2* showed a delay in the free-running periodicity of locomotor activity (38, 39, 40). These data suggested that *CRY2* is involved in *BMAL1*-mediated modulation of the circadian levels of *AANAT* and melatonin in chick pinealocytes stimulated by monochromatic GL. Consistent with the changes in *AANAT* and melatonin rhythmicity, both knockdown and overexpression of *CLOCK* had no effect on the circadian rhythm of *PER2* mRNA, which was contrary to that in *CRY1/2* and *PER3*. Different regulation mechanisms for circadian expression among Period family genes under light stimulation have also been reported in mammals (41, 42, 43). Therefore, combined with previous findings, our results indicated that *PER2* may have a role in the regulation of *AANAT* expression when *CLOCK* expression is altered.

Taken together, our study showed that *BMAL1* and *CLOCK* have different output pathways to regulate the cellular mechanism of chick pinealocytes cultured under GL. Moreover, *BMAL1* rather than *CLOCK* plays a predominant role in the regulation of diurnal rhythms of melatonin secretion, and *CRY2* may be involved in this regulatory pathway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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