Chronic Corticosterone Disrupts The Circadian Rhythm of CRH Expression and m⁶A RNA Methylation In The Chicken Hypothalamus

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Abstract

Background: Corticotropin-releasing hormone (CRH), the major secretagogue of the hypothalamic-pituitary-adrenal (HPA) axis, is intricately intertwined with the clock genes to regulate the circadian rhythm of various body functions. N6-methyladenosine (m⁶A) RNA methylation is involved in the regulation of circadian rhythm, yet it remains unknown whether CRH expression and m⁶A modification oscillate with the clock genes in chicken hypothalamus and how the circadian rhythms change under chronic stress.

Results: Chronic exposure to corticosterone (CORT) eliminated the diurnal patterns of plasma CORT and melatonin levels in the chicken. The circadian rhythms of clock genes in hippocampus, hypothalamus and pituitary are all disturbed to different extent in CORT-treated chickens. The most striking changes occur in hypothalamus in which the diurnal fluctuation of CRH mRNA is flattened, together with mRNA of other feeding-related neuropeptides. Interestingly, hypothalamic m⁶A level oscillates in an opposite pattern to CRH mRNA, with lowest m⁶A level after midnight (ZT18) corresponding to the peak of CRH mRNA before dawn (ZT22). CORT diminished the circadian rhythm of m⁶A methylation with significantly increased level at night. Further site-specific m⁶A analysis on 3'UTR of CRH mRNA indicates that higher m⁶A on 3'UTR of CRH mRNA coincides with lower CRH mRNA at night (ZT18 and ZT22).

Conclusions: Our results indicate that chronic stress disrupts the circadian rhythms of CRH expression in hypothalamus, leading to dysfunction of HPA axis in the chicken. RNA m⁶A modification is involved in the regulation of circadian rhythms in chicken hypothalamus under both basal and chronic stress conditions.

1. Introduction

The hypothalamus plays an important role in the regulation of hypothalamic-pituitary-adrenal (HPA) axis [1], feeding behavior [2], and circadian rhythm [3]. Corticotropin-releasing hormone (CRH) released from hypothalamus stimulates pituitary ACTH secretion to modulate the activity of HPA axis [4]. Moreover, CRH is involved in the regulation of feed intake [5] via interacting with appetite inhibiting proopiomelanocortin (POMC)/cocaine amphetamine-regulated transcript (CART) neurons and the appetite-inducing neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons [6]. Both the HPA axis activity and the feeding behavior exhibit diurnal patterns, which indicates complex interactive networks with the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus [7]. SCN can project to the pineal gland that secretes the hormone melatonin [8]. The core molecular clock consists of a transcriptional-translational autoregulatory “loop” with a positive arm and a negative arm [9]. The clock and *bmal1* genes and their protein products comprise the positive arm, while the period (*per1*, *per2*, and *per3*) and cryptochrome (*cry1*, *cry2*) genes and their protein products comprise the negative arm. An early research reported that the HPA system in the chicken displays a circadian rhythm [10]. Studies in mice indicate that CRH is intricately intertwined with the clock genes to regulate the circadian rhythm of

various body functions [11]. However, the circadian rhythm of CRH expression in chicken hypothalamus has not been characterized.

CRH binds to CRH receptors type 1 (CRHR1) and type 2 (CRHR2) in the pituitary, causing the production and secretion of adrenocorticotrophic hormone (ACTH) [12] to regulate the stress response of the body through corticosterone (CORT) synthesis and secretion from adrenal cortex [13, 14]. CORT exerts a negative feedback regulation on CRH synthesis and secretion through its receptor, glucocorticoid receptor (GR), at different levels including hippocampus and hypothalamus [15]. Chronically elevated circulating CORT has detrimental physiological and cognitive effects [16], including HPA axis dysfunction and neuroinflammation [17], as well as depressive and anxiety-like behaviors in SD rats [18]. In addition, chronic stress causes irregular expression of circadian regulatory clock genes in mouse hippocampus [19], hypothalamus SCN [20] and pituitary [21]. However, it remains unknown how chronic CORT exposure affects the circadian rhythms of clock-related genes in the chicken brain, and how it is related to the circadian rhythm of CRH in hypothalamus.

N⁶-methyladenosine (m⁶A) is the most prevalent modification in RNAs, which plays an important role in RNA splicing, degradation, and translation [22]. M⁶A level is finely balanced through interplay among m⁶A methyltransferases (“writers”, such as METTL3, METTL14 and WTAP), demethylases (“erasers”, such as fat mass and obesity-associated gene FTO and ALKBH5), and binding proteins (“readers”, such as YTHDF1, YTHDF2 and YTHDF3) [23]. Chronic stress is reported to modulate m⁶A modification in the brain [24]. For instance, heat exposure for 6 h increases m⁶A RNA methylation levels in the hypothalamus of 3-day-old chicks [25]. Yet, chronic CORT treatment reduces the m⁶A methylation in chicken liver [26]. Moreover, m⁶A methylation has been reported to have circadian rhythm [27]. Clock gene *cry1/2* knockout mice show significantly lower m⁶A level and lost the circadian rhythm of m⁶A level in RNA [27]. However, studies in the chicken are scarce. Questions remain regarding whether m⁶A modification in chicken hypothalamus show a circadian rhythm, whether the m⁶A rhythmicity, if any, is interrupted by chronic CORT exposure, and whether m⁶A is involved in the regulation of CRH expression in chicken hypothalamus.

Therefore, the objectives of the present study were, firstly, to elaborate the effects of chronic CORT exposure on circadian rhythms of clock-related genes in different brain areas including hippocampus, hypothalamus and pituitary; secondly, to delineate the circadian rhythms of CRH mRNA expression and m⁶A methylation in chicken hypothalamus, and to reveal their responses to chronic CORT exposure; and thirdly, to investigate the possible link between m⁶A modification and CRH expression in chicken hypothalamus.

2. Materials And Methods

2.1 Ethics statement

The experimental protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University. The project number is 31972638. The sampling procedures according to the “Guidelines on Ethical Treatment of Experimental Animals” (2006) No.398 set by the Ministry of Science and Technology, China.

2.2 Animals and experimental design

Seventy 45-day-old male bantam chickens were purchased from Changzhou Lihua Livestock and Poultry Co., Ltd. After a three-day adaption, chickens were randomly divided into vehicle (CON) and corticosterone (CORT) group. Light regime was 12 light: 12 dark, with light on at 07:00 as zeitgeber time 0 (ZT0) and off at 19:00 as ZT12. Feed and water were provided ad libitum. CORT (Sigma-Aldrich, St Louis, USA) was sonicated in saline with 0.1% Tween 80 and 0.2% DMSO until dissolved and protected from light. Chickens were injected (twice per day, 9:00-10:00 and 18:00-19:00) intraperitoneally with vehicle or CORT (4 mg/kg BW), according to previous publication [28], for 11 consecutive days. Daily feed consumption was recorded and body weight was recorded every other day. By the end of the treatment, the chickens were sacrificed at the indicated time points (ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22), and hippocampus, hypothalamus and pituitary were quickly excised, frozen immediately in liquid nitrogen, and stored at -80°C until use.

2.3 Measurement of corticosterone and melatonin

Corticosterone concentration was determined by Enzyme Immunoassay (EIA) kit (No. ADI-900-097, Enzo, Farmingdale, NY, USA) following the manufacturer's instructions. Serum melatonin levels were measured using Chicken MT (Melatonin) ELISA Kit (MM-3427801, ImmunoWay Biotechnology, USA) following the manufacturer's instructions.

2.4 RNA isolation and real-time PCR

High quality total RNA was isolated from 30 mg hippocampus, hypothalamus and pituitary using 600 mL TRIzol reagents (Invitrogen, Carlsbad, California, USA). One microgram of RNA was reverse-transcribed according to the manufacturer's protocol (Vazyme Biotech, Nanjing, Jiangsu, China). Four microliter cDNA was diluted (1:25) and then used for real-time PCR in a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, California, U.S.A.). Peptidylprolyl isomerase A (PPIA) was used as an internal control to normalize the technical variations. Data were analyzed using the method of $2^{-\Delta\Delta CT}$ and presented relative to the CON group. All primers (Table 1) were synthesized by Suzhou GENEWIZ Biological Technology Co., Ltd (Suzhou, Jiangsu, China).

Table 1
The primers sequences for RT-PCR and SELECT.

Target genes	Primer sequences (5'to 3')	
<i>Clock</i>	F: GATCACAGGGCACCTCCAATA	R: CTAGTTCTCGCCGCCTTTCT
<i>Baml1</i>	F: GTAGACCAGAGGGCGACAG	R: ATGAAACTGAACCAGCGACTC
<i>Cry1</i>	F: GATGTGGCTATCCTGTAGTTCCT	R: GCTGCTGGTAGATTTGTTTCAT
<i>Cry2</i>	F: GCACGGCTGGATAAACACT	R: AAATAAGCGGCAGGACAAA
<i>Per2</i>	F: ATGAAACGAGCCATCCCG	R: CAGTTGTCGTGATTTTGCCTA
<i>Per3</i>	F: CAGTGCCTTTGTTGGGTAC	R: GATGGATTCACAAAAGTGGAC
<i>Gr</i>	F: CCAAGACGAAACCAGGAA	R: TTGAGAAGTCAGCCAGAGC
<i>Rora</i>	F: GGGGATGTCTCGAGATGCTG	R: TGCTTTGCTACCTTCAGGGG
<i>Rev-erba</i>	F: CAGCGGTTTCCAGTCATCCT	R: TCACTCTTTGGTGCCCCATC
<i>Crh</i>	F: CTCCCTGGGCTGGCTTT	R: CCTCACTTCCCGATGATT
<i>Crhr1</i>	F: CACAGCCTTCATCCTACGCA	R: CGGAGCTTGTCGGTGGAATA
<i>Crhr2</i>	F: TCTTTCCTGGGCTTTCACGG	R: ATTGAAGAACTCCGGGCAGG
<i>Npy</i>	F: ACTCGGCTCTGAGGCACT	R: GGTCTTCAAACCGGGATC
<i>Fto</i>	F: TCACCAAGGCGACCTCTACT	R: GCTGAACCGAGGTGAAAAGC
<i>Mettl3</i>	F: ATCCTGGAGCTGCTCAACAC	R: AGATTTCGTCCGTGTGCTTGT
<i>Mettl14</i>	F: ATTCGACCAGGATGGCTGAC	R: GACTTGGGTGGTGGTGAAGT
<i>Ythdf1</i>	F: AACCAACCAGCTCCGACACAT	R: GATTCTGACGTTCTTCCGC
<i>Ythdf2</i>	F: AAGGCCAAGGCAACAAAGTG	R: ATATGCATTGTTCCGGCCGGG
<i>Ythdf3</i>	F: CGTAATAGGGGTGTGGGCTTC	R: CACTTCCACACCAGAAGGTGA
<i>PPIA</i>	F: TTACGGGGAGAAGTTTGCCG	R: TGGTGATCTGCTTGCTCGTC
SELECT		
<i>CRH N site</i>	F: tagccagtaccgtagtgcgtgGGCGCGCAGCGCGGCCGCTG R: CCCGGTGCTGAAACGCGGCCcagaggctgagtcgctgcat	
<i>CRH X1 site</i>	F: tagccagtaccgtagtgcgtgTTCCCGATGATTTCCATCAG R: TTCCTGTTGCTGTGGGCTTGcagaggctgagtcgctgcat	
<i>CRH X2 site</i>	F: tagccagtaccgtagtgcgtgCTCTGGTGCTGACCGCGGGG R: CCCTTTGGCACGGCGCGGGGcagaggctgagtcgctgcat	

2.5 Analysis of mRNA m⁶A methylation by dot-blotting assay

Dot-blot analysis of mRNA m⁶A methylation was performed following a published procedure with minor modifications [29]. Briefly, total RNAs were isolated using the Trizol method and mRNAs were enriched by using GenElute™ mRNA Miniprep Kit (Sigma). The concentration and purity of mRNAs were measured by NanoDrop 2000. The mRNAs were denatured by heating at 95°C for 5 min, followed by chilling on ice immediately. Next, the mRNA (100 ng) was spotted directly onto the positively charged nylon membrane (GE Healthcare, USA) and air dried for 5 min. The membrane was then UV crosslinked in Ultraviolet Crosslinker, blocked with 5% of nonfat milk in TBST, and then incubated with anti-m⁶A antibody overnight at 4°C. HRP-conjugated anti-rabbit IgG secondary antibody was added to the membrane for 2 h at room temperature with gentle shaking and then developed with enhanced chemiluminescence. Methylene blue staining was used to verify that equal amount mRNA spotted on the membrane.

2.6 Single-base elongation and ligation-based qPCR amplification method (SELECT) Assay

The SELECT assay for monitoring site-specific m⁶A levels in the 3'UTR of CRH mRNA was performed as described previously [30]. In brief, total RNA (2 µg) was mixed with 1 µL of 100 µM dNTP (New England Biolabs), 2 µL of CutSmart buffer (New England Biolabs), and 2 µL each of 400 nM up and down DNA probes (Table 1). The total volume was adjusted to 17 µL with water. The DNA probes and RNA were annealed by incubating the mixture with a temperature gradient of 90°C for 1 min, 80°C for 1 min, 70°C for 1 min, 60°C for 1 min, 50°C for 1 min, and 40°C for 6 min. To the mixture was then added a 3 µL solution containing 0.01 U *Bst* 2.0 DNA polymerase, 0.5 U SplintR ligase, and 10 nmol ATP. After incubating at 40°C for 20 min and then at 80°C for 20 min, an aliquot (2 µL) of the reaction mixture was taken out for real-time qPCR analysis to quantify template abundance.

2.7 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The mRNA levels of clock-related genes and melatonin contents were analyzed using one-way analysis of variance (one-way ANOVA) with IBM SPSS Statistics 20 software (United States) to test the statistical significance of the differences among the six daily time points and confirm the daily variation ($p \leq 0.05$), as the premise of cosinor analysis. To determine the circadian rhythmicity of each clock-related gene profile, the mRNA levels of clock-related genes, as well as CORT and melatonin levels were analyzed separately using MATLAB 7.0 (MathWorks Inc., USA) based on unimodal cosinor regression [$y = A + (B \times \cos(2\pi(x - C)/24))$]. A, B and C represent the mesor, amplitude and acrophase, respectively. The results of regression analysis were considered significant at $p \leq 0.05$, which was calculated using the number of samples, R² values and the number of predictors (mesor, amplitude and acrophase) from <http://www.danielsoper.com/statcalc3/calc.aspx?i1/415> [31]. Differences of the mesor, amplitude and acrophase between CON and CORT

group were tested by one-way ANOVA followed by Fisher’s least significant difference (LSD) post hoc test, considering $p \leq 0.05$ to be significant.

3. Results

3.1 Effect of chronic CORT exposure on body weight, food intake, plasma CORT and melatonin concentration.

Chronic CORT exposure leads to growth retardation, with significantly lower body weight, as compared with their control counterparts, from the 5th day of CORT injection (D5) to D11 (Fig. 1A). Interestingly, the feed intake was significantly increased on D3, D4 and D7, leading to significantly increased average daily feed intake (Fig. 1B). Both CORT (Figure 1C) and melatonin (Figure 1D) levels in plasma exhibited diurnal pattern in CON group ($P < 0.05$, one-way ANOVA), which was eliminated in CORT group. The mesors of CORT level were significantly elevated ($P < 0.01$) by CORT injection, while the mesors of melatonin level did not change (Table 2).

Table 2
Circadian rhythm parameters of CORT and melatonin levels in plasma, as determined by cosinor analyses.

Index	Group	CORT	melatonin
Mesor	CON	18.62 ± 0.26	4.37 ± 0.12
	CORT	36.10 ± 1.45**	4.53 ± 0.10
Amplitude	CON	3.13 ± 0.37	0.87 ± 0.17
	CORT	ND	ND
Acrophase (h)	CON	23.18 ± 0.46	18.97 ± 0.68
	CORT	ND	ND
Values are means ± SEM. **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.			

3.2 Effect of chronic CORT exposure on the circadian rhythm of clock genes in hippocampus, hypothalamus, and pituitary.

All the 6 clock genes were expressed in hippocampus (Fig. 2A-F), hypothalamus (Fig. 2G-L), and pituitary (Fig. 2M-R), in gene- and region-specific rhythmic patterns. In CON group, BMAL1, PER2 and PER3 showed more pronounced circadian pattern among 6 clock genes ($P < 0.05$, one-way ANOVA), regardless of the region. Among 3 brain regions, hypothalamus displayed more clearly circadian patterns for all the 6 clock genes ($P < 0.05$, one-way ANOVA) as shown in cosinor analysis. Moreover, chronic CORT exposure abolished the circadian rhythms of all the 6 clock genes in hypothalamus, while hippocampus and pituitary were less affected.

Specifically, chronic CORT exposure significantly delayed ($P < 0.05$) the acrophase of CRY1 mRNA for 2 h (Figure 2C and Table 3), and significantly decreased ($P < 0.05$) the amplitude of PER2 mRNA in hippocampus (Figure 2E and Table 3). However, chronic CORT exposure had no impact on the rhythmicity of CLOCK (Figure 2A), BMAL1 (Figure 2B), CRY2 (Figure 2D) or PER3 (Figure 2F) mRNA expression in hippocampus (Table 3). By contrast, the circadian rhythms of CLOCK (Figure 2G), CRY1 (Figure 2I), CRY2 (Figure 2J) and PER3 (Figure 2L) mRNA in hypothalamus were lost in CORT group (Table 4). Meanwhile, the mesor and amplitude of BMAL1 (Figure 2H) and PER2 (Figure 2K) mRNA were significantly decreased ($P < 0.05$) in CORT group (Table 4). In pituitary, chronic CORT exposure significantly decreased ($P < 0.05$) the mesor of CLOCK (Figure 2M) and CRY1 (Figure 2O) mRNA (Table 5). However, chronic CORT exposure had no impact on the rhythmicity of all the clock genes except CRY2 (Figure 2P, Table 5).

Table 3

Circadian rhythm parameters of all clock genes in hippocampus, as determined by cosinor analyses.

Index	Group	CLOCK	BMAL1	CRY1	CRY2	PER2	PER3
Mesor	CON	1.15 ± 0.03	1.50 ± 0.09	1.36 ± 0.18	0.99 ± 0.08	0.49 ± 0.05	0.54 ± 0.04
	CORT	1.05 ± 0.06	1.60 ± 0.08	1.26 ± 0.06	1.05 ± 0.05	0.44 ± 0.05	0.58 ± 0.06
Amplitude	CON	0.27 ± 0.04	0.58 ± 0.12	0.86 ± 0.27	ND	0.44 ± 0.07	0.45 ± 0.06
	CORT	0.23 ± 0.08	0.65 ± 0.12	0.45 ± 0.27	0.09 ± 0.12	0.27 ± 0.02*	0.41 ± 0.08
Acrophase (h)	CON	10.64 ± 0.55	11.14 ± 0.79	8.53 ± 0.28	5.89 ± 1.87	3.27 ± 0.69	23.80 ± 0.56
	CORT	11.51 ± 1.35	11.43 ± 0.69	10.51 ± 0.70*	ND	3.37 ± 0.32	23.13 ± 0.78
Values are means ± SEM. *p < 0.05, **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.							

Table 4

Circadian rhythm parameters of all clock genes in hypothalamus, as determined by cosinor analyses.

Index	Group	CLOCK	BMAL1	CRY1	CRY2	PER2	PER3
Mesor	CON	1.13 ± 0.05	1.11 ± 0.04	1.13 ± 0.05	0.90 ± 0.02	0.52 ± 0.06	0.63 ± 0.07
	CORT	ND	0.84 ± 0.06*	ND	ND	0.34 ± 0.02*	ND
Amplitude	CON	0.45 ± 0.07	0.44 ± 0.06	0.58 ± 0.07	0.17 ± 0.03	0.36 ± 0.08	0.55 ± 0.09
	CORT	ND	0.15 ± 0.09*	ND	ND	0.19 ± 0.03*	ND
Acrophase (h)	CON	8.94 ± 0.53	8.43 ± 0.48	8.53 ± 0.43	6.21 ± 0.72	2.53 ± 0.92	22.88 ± 0.64
	CORT	ND	7.51 ± 2.01	ND	ND	2.03 ± 0.63	ND
Values are means ± SEM. *p < 0.05, **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.							

Table 5

Circadian rhythm parameters of all clock genes in pituitary, as determined by cosinor analyses.

Index	Group	CLOCK	BMAL1	CRY1	CRY2	PER2	PER3
Mesor	CON	1.15 ± 0.09	1.33 ± 0.06	0.86 ± 0.03	0.86 ± 0.05	0.59 ± 0.02	0.64 ± 0.06
	CORT	0.88 ± 0.05*	1.36 ± 0.08	0.66 ± 0.05*	ND	0.49 ± 0.04	0.65 ± 0.07
Amplitude	CON	0.24 ± 0.13	0.50 ± 0.09	0.21 ± 0.05	0.21 ± 0.06	0.41 ± 0.02	0.52 ± 0.09
	CORT	0.11 ± 0.08	0.66 ± 0.12	0.28 ± 0.07	ND	0.30 ± 0.05	0.51 ± 0.11
Acrophase (h)	CON	18.25 ± 2.00	10.34 ± 0.69	5.04 ± 0.84	4.81 ± 1.15	2.87 ± 0.25	22.53 ± 0.66
	CORT	19.10 ± 2.60	10.57 ± 0.66	7.15 ± 0.84	ND	1.97 ± 0.68	21.92 ± 0.76
Values are means ± SEM. *p < 0.05, compared with CON group. ND represents not determined as there was no circadian rhythm.							

3.3 Effect of chronic CORT exposure on the circadian rhythm parameters of CRH in hypothalamus and CRH receptor genes in pituitary

In line with the abolished rhythmicity of clock genes in hypothalamus, the circadian pattern of CRH mRNA (Figure 3A) in hypothalamus was significantly diminished in CORT group, so was the rhythmic expression

of CRHR1 (Figure 3B) and CRHR2 (Figure 3C) mRNA in pituitary ($P < 0.05$, one-way ANOVA). Chronic CORT exposure significantly decreased the mesor ($P < 0.05$) and amplitude ($P < 0.01$) of CRH mRNA in hypothalamus, as well as CRHR1 and CRHR2 mRNA in pituitary (Table 6). In general, chronic CORT exposure significantly abolished ($P < 0.05$) the rise of CRH (Figure 3A), CRHR1 (Figure 3B) and CRHR2 (Figure 3C) mRNA expression in the dark phase after midnight at ZT18 and ZT22.

Table 6

Circadian rhythm parameters of CRH in hypothalamus, and CRHR1, CRHR2 in pituitary, as determined by cosinor analyses.

Index	Group	CRH	CRH R1	CRH R2
Mesor	CON	1.01 ± 0.05	0.95 ± 0.04	1.01 ± 0.03
	CORT	0.78 ± 0.05*	0.78 ± 0.05*	0.78 ± 0.03*
Amplitude	CON	0.40 ± 0.08	0.43 ± 0.06	0.32 ± 0.05
	CORT	0.13 ± 0.07**	0.17 ± 0.07**	0.15 ± 0.04**
Acrophase (h)	CON	20.81 ± 0.65	20.35 ± 0.44	20.18 ± 0.50
	CORT	ND	ND	ND
Values are means ± SEM. *p < 0.05, **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.				

3.4. Effect of chronic CORT exposure on the circadian rhythm parameters of feeding related genes in hypothalamus

In accordance with the alterations of CRH mRNA, the diurnal patterns of hypothalamic NPY (Figure 4A), AGRP (Figure 4B), POMC (Figure 4C) and CART (Figure 4D) RNA expression were also eliminated in CORT group ($P < 0.05$, one-way ANOVA). The expression pattern of “the hunger genes” NPY and AGRP were opposite to that of the “the satiety genes” POMC and CART, matching the diurnal pattern of feeding behavior in the chicken. Chronic CORT exposure significantly decreased ($P < 0.01$) the mesor and amplitude of all the 4 feeding regulatory genes in hypothalamus (Table 7).

Table 7
Circadian rhythm parameters of NPY, AGRP, POMC and CART in hypothalamus, as determined by cosinor analyses.

Index	Group	NPY	AGRP	POMC	CART
Mesor	CON	1.06 ± 0.03	1.12 ± 0.06	1.11 ± 0.06	1.01 ± 0.03
	CORT	0.66 ± 0.02**	0.69 ± 0.03**	0.69 ± 0.07**	0.67 ± 0.01**
Amplitude	CON	0.36 ± 0.05	0.42 ± 0.10	0.46 ± 0.08	0.40 ± 0.04
	CORT	0.10 ± 0.03**	0.10 ± 0.05**	0.16 ± 0.10**	0.10 ± 0.01**
Acrophase (h)	CON	8.25 ± 0.44	8.86 ± 0.77	18.72 ± 0.63	20.03 ± 0.35
	CORT	ND	ND	ND	ND
Values are means ± SEM. *p < 0.05, **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.					

3.8 Effect of chronic CORT exposure on the circadian rhythm parameters of m⁶A level and m⁶A related genes in hypothalamus

Interestingly, the global RNA m⁶A levels (Figure 5A) exhibited diurnal pattern in CON group ($P < 0.05$, one-way ANOVA), higher m⁶A levels were detected in light phase. Chronic CORT exposure significantly disrupted this pattern with significantly decreased ($P < 0.05$) m⁶A levels in light phase at ZT6 and ZT10, but significantly increased ($P < 0.05$) m⁶A levels in dark phase at ZT14, ZT18 and ZT22. Meanwhile, chronic CORT exposure significantly ($P < 0.01$) decreased the amplitude of m⁶A levels and delayed the acrophase of m⁶A levels for 13.48 h (Table 8). Concurrently, chronic CORT exposure significantly increased ($P < 0.05$) the mesor of FTO (Figure 5B) mRNA and decreased ($P < 0.05$) the mesor of YTHDF2 (Figure 5F) and YTHDF3 (Figure 5G) mRNA in hypothalamus (Table 8).

Table 8

Circadian rhythm parameters of m⁶A level and m⁶A related genes in hypothalamus, as determined by cosinor analyses.

Index	Group	m ⁶ A	FTO	METTL3	METTL14	YTHDF1	YTHDF2	YTHDF3
Mesor	CON	99.31 ± 3.52	0.97 ± 0.04	1.13 ± 0.04	1.07 ± 0.05	0.92 ± 0.02	0.83 ± 0.02	0.94 ± 0.02
	CORT	104.6 ± 1.92	1.12 ± 0.03*	0.91 ± 0.09	ND	ND	0.54 ± 0.04*	0.73 ± 0.05*
Amplitude	CON	49.76 ± 5.04	0.24 ± 0.06	0.31 ± 0.05	0.25 ± 0.07	0.13 ± 0.03	0.18 ± 0.03	0.12 ± 0.03
	CORT	20.76 ± 2.84**	0.28 ± 0.05	0.17 ± 0.07	ND	ND	0.11 ± 0.55	0.09 ± 0.06
Acrophase (h)	CON	4.31 ± 0.45	19.82 ± 0.83	9.93 ± 0.67	9.58 ± 0.95	23.73 ± 0.98	0.87 ± 0.75	3.76 ± 1.10
	CORT	ND	7.56 ± 0.58**	15.10 ± 3.43*	ND	ND	2.12 ± 1.90	1.31 ± 2.87
Values are means ± SEM. *p < 0.05, **p < 0.01, compared with CON group. ND represents not determined as there was no circadian rhythm.								

3.9 Effect of chronic CORT exposure on the site-specific m⁶A levels in the 3'UTR of CRH mRNA in hypothalamus

To explore the possible link between the site-specific m⁶A modification on CRH mRNA and CRH mRNA expression in hypothalamus, RNA samples from hypothalamus on ZT18 and ZT22 with significant changes in CRH mRNA were subjected to single-base elongation and ligation-based qPCR amplification method (SELECT) assay. Two specific m⁶A sites (Figure 6A) were identified in the coding sequence (CDS) close to 3'UTR (X1) and 3'UTR (X2) of CRH mRNA, respectively, from published MeRIP-seq database [26]. N site located in the 5'UTR without consensus m⁶A motif was selected as a negative control. Chronic CORT exposure did not change the CT value on N site at either ZT 18 (Figure 6B) or ZT 22 (Figure 6E), compared with CON group. However, chronic CORT exposure significantly increased ($P < 0.05$) the CT value on both X1 (Figure 6C, F) and X2 (Figure 6D, G) at both time points (ZT18 and ZT22), which was in accordance with the significant decrease of CRH mRNA in hypothalamus at the same time points.

4. Discussion

In this study, we observed that chronic CORT exposure completely abolished the circadian rhythm of plasma melatonin levels in the chicken, indicating a disruption of the endogenous rhythmicity. The effects of CORT on plasma melatonin are biphasic, being stimulatory in the light phase when the melatonin levels are low, while inhibitory in the dark phase when the melatonin levels are high. The avian pineal gland receives circadian input through the release of norepinephrine during the day [32], and the

dual effects of CORT on pineal melatonin synthesis are determined by the activation of different adrenoceptors (β or $\beta + \alpha 1$) during GR activation [33].

The circadian rhythms in birds are controlled by multiple circadian pacemakers in the central nervous system. Here we show, for the first time, the circadian expression of clock genes in chicken hippocampus, hypothalamus, and pituitary. All the 6 core clock genes show circadian rhythms in all the 3 brain areas, although the amplitude and the pattern of oscillation vary among genes and brain areas. It is noted that *bmal1* oscillates in an opposite pattern from *per2* and *per3*, may be because they belong, respectively, to “negative arm” and “positive arm” of the circadian clock gene network [9]. Among 3 brain areas, hypothalamus shows more clear and significant rhythmicity and higher susceptibility to CORT treatment. This agrees with a previous publication that long-term administration of dexamethasone resulted in loss of the expression rhythms in *Bmal1* and *Clock* genes in rat SCN [34].

CRH is essential for stress adaptation by mediating HPA axis [1] and involved in the regulation of circadian rhythms [2]. Circadian variations of CRH neuron activity are driven by the SCN and likely mediate the characteristic circadian pattern of HPA axis activity [35]. Previous study reported that chronic CORT exposure increased CRH and CRHR1 expression in rat's amygdala [36] and medial prefrontal cortex [37]. In the present study, we found, for the first time, that chronic CORT exposure destroyed the circadian rhythms of CRH expression in hypothalamus and its receptors CRHR1 and CRHR2 expression in pituitary, with significantly decreased expression in dark phase. Many factors may contribute to the disparity of the findings, including animal species (nocturnal rats vs. diurnal chickens), time points of the sampling, and the brain regions.

Accordingly, genes involved in feeding regulation, including satiety genes POMC and CART and hunger genes NPY and AgRP [38], show concerted circadian expression pattern, which is in agreement with a previous report that AgRP, NPY, POMC and CART genes are expressed in a circadian rhythm in the hypothalamus [39]. The same as CRH and its receptors, the circadian rhythm of these appetite-related genes is also destroyed in chickens subjected to chronic CORT exposure. These CORT-induced alterations in hypothalamic gene expression may contribute, at least partly, to the disrupted feeding behavior in the chicken.

The m^6A methylation plays important roles in the regulation of neurogenesis, circadian rhythm, cognitive function, and stress responses [40]. Here, we provide the first evidence that the global m^6A level in chicken hypothalamus oscillates in a day, being higher in light phase and lower in dark phase. Interestingly, this pattern is opposite to that reported in nocturnal animals [27]. This makes sense as m^6A is reported to participate in many stress responses [41], and higher m^6A level corresponds to higher body activity. However, chronic CORT exposure disrupted the circadian rhythms of m^6A methylation levels in hypothalamus. Based on the observation that significant decrease of CRH mRNA in the dark phase corresponds to the significant increase in m^6A level changes at the same time points, we speculated that m^6A may be involved in the post-transcriptional regulation of CRH mRNA in chicken hypothalamus. Indeed, the two predicted m^6A sites X1 and X2 were both hypermethylated at detected time points (ZT18

and ZT22). Therefore, it is likely that the decrease of CRH expression was due to m⁶A-mediated mRNA degradation [42]. Nevertheless, a functional verification study is required to elucidate the role of m⁶A on these sites in CRH gene regulation in chicken hypothalamus.

5. Conclusion

In conclusion, our study shows that chronic CORT exposure eliminated the diurnal patterns of plasma CORT and melatonin levels in the chicken. Hypothalamus is the most susceptible brain region to CORT treatment, as almost all the genes, including clock genes, CRH, and feeding-related genes, lost their circadian rhythmicity together with the global m⁶A level. Higher m⁶A on 3'UTR of CRH mRNA coincides with lower CRH mRNA at night, indicating a possible role of m⁶A in the post-transcriptional regulation of CRH expression in chicken hypothalamus. These findings provide evidence of CORT-induced disruption of central circadian rhythmicity in CRH expression that leads to dysfunction of HPA axis in the chicken, and also imply a role of RNA m⁶A modification in the regulation of circadian rhythms in the chicken.

Abbreviations

AgRP: agouti-related protein;

CART: cocaine amphetamine-regulated transcript;

CORT: corticosterone;

CRH: Corticotropin-releasing hormone;

CRHR1: CRH receptors type 1;

CRHR2: CRH receptors type 2;

Cry: Cryptochrome;

GR: glucocorticoid receptor;

HPA: hypothalamic-pituitary-adrenal;

m⁶A: N6-methyladenosine;

NPY: neuropeptide Y;

Per: Period;

POMC: proopiomelanocortin;

SCN: suprachiasmatic nucleus;

SELECT: single-base elongation and ligation-based qPCR amplification method

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University. The project number is 31972638. The sampling procedures according to the “Guidelines on Ethical Treatment of Experimental Animals” (2006) No.398 set by the Ministry of Science and Technology, China.

Consent for publication

The corresponding author and all of the authors have read and approved the final submitted manuscript.

Availability of data and materials

Not applicable.

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Competing interests

The authors declare no competing financial interest.

Authors' contributions

YY contributed to behavior tests, data analysis, and drafting of the manuscript. YY, AZ and WH were responsible for animal care, breeding and sampling. MZ and WC provided technical support. RZ and YJ contributed to conception, experimental design and data interpretation. RZ and DW contributed to critical revision of the manuscript.

References

1. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*. 1981;213:1394–7.

2. Zou H, Shi M, He F, Guan C, Lu W. Expression of corticotropin releasing hormone in olive flounder (*Paralichthys olivaceus*) and its transcriptional regulation by c-Fos and the methylation of promoter, Comparative biochemistry and physiology. Part B. Biochemistry molecular biology. 2021;251:110523.
3. Challet E. The circadian regulation of food intake. *Nat Rev Endocrinol*. 2019;15:393–405.
4. Kuenzel WJ, Kang SW, Jurkevich A. The vasotocinergic system and its role in the regulation of stress in birds. *Vitamins hormones*. 2020;113:183–216.
5. Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake, Nutrition, metabolism, and cardiovascular diseases. 18: NMCD; 2008. pp. 158–68.
6. Van Drunen R, Eckel-Mahan K. Circadian Rhythms of the Hypothalamus: From Function to Physiology. *Clocks sleep*. 2021;3:189–226.
7. Sanchez REA, Kalume F, de la Iglesia HO. Sleep timing and the circadian clock in mammals: Past, present and the road ahead. *Seminars in cell & developmental biology*; 2021.
8. Giudice A, Crispo A, Grimaldi M, Polo A, Bimonte S, Capunzo M, Amore A, D'Arena G, Cerino P, Budillon A, Botti G, Costantini S, Montella M. The Effect of Light Exposure at Night (LAN) on Carcinogenesis via Decreased Nocturnal Melatonin Synthesis. *Molecules*; 2018. p. 23.
9. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Human molecular genetics* 15 Spec No. 2006;2:R271–7.
10. Majsa Z, Mihaly K, Peczely P. Circadian rhythm of hypothalamo-hypophyseal-adrenal activity in the chicken. *Acta physiologica Academiae Scientiarum Hungaricae*. 1976;47:101–9.
11. Muglia LJ, Jacobson L, Weninger SC, Karalis KP, Jeong K, Majzoub JA. The physiology of corticotropin-releasing hormone deficiency in mice. *Peptides*. 2001;22:725–31.
12. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, Myers B. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. *Comprehensive Physiology*. 2016;6:603–21.
13. Darnaudery M, Maccari S. Epigenetic programming of the stress response in male and female rats by prenatal restraint stress. *Brain Res Rev*. 2008;57:571–85.
14. Torres DB, Lopes A, Rodrigues AJ, Cerqueira JJ, Sousa N, Gontijo JAR, Boer PA. Anxiety-like behavior and structural changes of the bed nucleus of the stria terminalis (BNST) in gestational protein-restricted male offspring. *Journal of developmental origins of health disease*. 2018;9:536–43.
15. Laryea G, Muglia L, Arnett M, Muglia LJ. Dissection of glucocorticoid receptor-mediated inhibition of the hypothalamic-pituitary-adrenal axis by gene targeting in mice. *Front Neuroendocr*. 2015;36:150–64.
16. Wolkowitz OM, Burke H, Epel ES, Reus VI. Glucocorticoids. Mood, memory, and mechanisms. 1179: *Annals of the New York Academy of Sciences*; 2009. pp. 19–40.
17. Zhao J, Gao X, Wang A, Wang Y, Du Y, Li L, Li M, Li C, Jin X, Zhao M. Depression comorbid with hyperalgesia: Different roles of neuroinflammation induced by chronic stress and hypercortisolism. *J*

- Affect Disord. 2019;256:117–24.
18. Lee B, Sur B, Shim I, Lee H, Hahm DH. Angelica gigas ameliorate depression-like symptoms in rats following chronic corticosterone injection. *BMC Complement Altern Med*. 2015;15:210.
 19. Clement A, Pedersen MM, Stensballe A, Wiborg O, Asuni AA. Chronic stress induces NPD-like behavior in APPPS1 and WT mice with subtle differences in gene expression, Genes, brain, and behavior, (2021) e12766.
 20. Jiang WG, Li SX, Zhou SJ, Sun Y, Shi J, Lu L. Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. *Brain research*. 2011;1399:25–32.
 21. Razzoli M, Karsten C, Yoder JM, Bartolomucci A, Engeland WC. Chronic subordination stress phase advances adrenal and anterior pituitary clock gene rhythms. *Am J Physiol Regul Integr Comp Physiol*. 2014;307:R198–205.
 22. Maity A, Das B. N6-methyladenosine modification in mRNA: machinery, function and implications for health and diseases. *FEBS J*. 2016;283:1607–30.
 23. Cao G, Li HB, Yin Z, Flavell RA. Recent advances in dynamic m6A RNA modification. *Open biology*. 2016;6:160003.
 24. Chokkalla AK, Mehta SL, Vemuganti R, Epitranscriptomic Modifications Modulate Normal and Pathological Functions in CNS, *Translational stroke research*, (2021).
 25. Kisliouk T, Rosenberg T, Ben-Nun O, Ruzal M, Meiri N, Early-Life m(6)A RNA Demethylation by Fat Mass and Obesity-Associated Protein (FTO) Influences Resilience or Vulnerability to Heat Stress Later in Life, *eNeuro*, 7 (2020).
 26. Hu Y, Feng Y, Zhang L, Jia Y, Cai D, Qian SB, Du M, Zhao R. GR-mediated FTO transactivation induces lipid accumulation in hepatocytes via demethylation of m(6)A on lipogenic mRNAs. *RNA Biol*. 2020;17:930–42.
 27. Wang CY, Yeh JK, Shie SS, Hsieh IC, Wen MS. Circadian rhythm of RNA N6-methyladenosine and the role of cryptochrome, *Biochemical and biophysical research communications*, 465 (2015) 88–94.
 28. Luo JW, Zhou ZL, Zhang H, Ma RS, Hou JF. Bone response of broiler chickens (*Gallus gallus domesticus*) induced by corticosterone, *Comparative biochemistry and physiology. Part A. Molecular integrative physiology*. 2013;164:410–6.
 29. Nagarajan A, Janostiak R, Wajapeyee N. Dot Blot Analysis for Measuring Global N(6)-Methyladenosine Modification of RNA, *Methods in molecular biology*, 1870 (2019) 263–271.
 30. Xiao Y, Wang Y, Tang Q, Wei L, Zhang X, Jia G. An Elongation- and Ligation-Based qPCR Amplification Method for the Radiolabeling-Free Detection of Locus-Specific N(6) -Methyladenosine Modification. *Angew Chem*. 2018;57:15995–6000.
 31. Singh D, Rani S, Kumar V. Daily expression of six clock genes in central and peripheral tissues of a night-migratory songbird: evidence for tissue-specific circadian timing. *Chronobiology international*. 2013;30:1208–17.

32. Cassone VM, Takahashi JS, Blaha CD, Lane RF, Menaker M. Dynamics of noradrenergic circadian input to the chicken pineal gland. *Brain research*. 1986;384:334–41.
33. Fernandes PA, Tamura EK, D'Argenio-Garcia L, Muxel SM, Cruz-Machado SdaS, Marcola M, Carvalho-Sousa CE, Cecon E, Ferreira ZS, Markus RP, Dual Effect of Catecholamines and Corticosterone Crosstalk on Pineal Gland Melatonin Synthesis, *Neuroendocrinology*, 104 (2017) 126-134.
34. Wu T, Yang L, Jiang J, Ni Y, Zhu J, Zheng X, Wang Q, Lu X, Fu Z. Chronic glucocorticoid treatment induced circadian clock disorder leads to lipid metabolism and gut microbiota alterations in rats. *Life sciences*. 2018;192:173–82.
35. Nicholson S, Lin JH, Mahmoud S, Campbell E, Gillham B, Jones M. Diurnal variations in responsiveness of the hypothalamo-pituitary-adrenocortical axis of the rat. *Neuroendocrinology*. 1985;40:217–24.
36. Gray JM, Wilson CD, Lee TT, Pittman QJ, Deussing JM, Hillard CJ, McEwen BS, Schulkin J, Karatsoreos IN, Patel S, Hill MN. Sustained glucocorticoid exposure recruits cortico-limbic CRH signaling to modulate endocannabinoid function. *Psychoneuroendocrinology*. 2016;66:151–8.
37. Merali Z, Anisman H, James JS, Kent P, Schulkin J. Effects of corticosterone on corticotrophin-releasing hormone and gastrin-releasing peptide release in response to an aversive stimulus in two regions of the forebrain (central nucleus of the amygdala and prefrontal cortex). *Eur J Neurosci*. 2008;28:165–72.
38. Mitchell CS, Begg DP. The regulation of food intake by insulin in the central nervous system. *J Neuroendocrinol*. 2021;33:e12952.
39. Stutz AM, Staszkiwicz J, Ptitsyn A, Argyropoulos G. Circadian expression of genes regulating food intake. *Obesity*. 2007;15:607–15.
40. Chokkalla AK, Mehta SL, Vemuganti R. Epitranscriptomic regulation by m(6)A RNA methylation in brain development and diseases. *Journal of cerebral blood flow metabolism: official journal of the International Society of Cerebral Blood Flow Metabolism*. 2020;40:2331–49.
41. Engel M, Eggert C, Kaplick PM, Eder M, Roh S, Tietze L, Namendorf C, Arloth J, Weber P, Rex-Haffner M, Geula S, Jakovcevski M, Hanna JH, Leshkowitz D, Uhr M, Wotjak CT, Schmidt MV, Deussing JM, Binder EB, Chen A, The Role of m(6)A/m-RNA Methylation in Stress Response Regulation, *Neuron*, 99 (2018) 389-403 e389.
42. Ke S, Pandya-Jones A, Saito Y, Fak JJ, Vagbo CB, Geula S, Hanna JH, Black DL, Darnell JE Jr, Darnell RB. m(6)A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes Dev*. 2017;31:990–1006.

Figures

Figure 1

Effect of chronic CORT exposure on body weight, food intake, plasma CORT and melatonin concentration (A) Body weight (n = 6); (B) Feed intake (n = 6) and average daily feed intake (n = 10); (C) Plasma corticosterone content; (D) Plasma melatonin content. The curves represent the 24-hour period determined by cosinor analysis. N = 6 chicks per time point. Data from CT2 are double-plotted. R2 values represent the degree of fitting. Values are mean $\pm$ SEM, *P < 0.05, **p < 0.01, compared with control.

Figure 2

Effect of chronic CORT exposure on the circadian rhythm parameters of clock genes in chicken hippocampus, hypothalamus and pituitary (A-F) The circadian rhythms of clock gene mRNA expression in chicken hippocampus. (A) CLOCK gene; (B) BMAL1 gene; (C) CRY1 gene; (D) CRY2 gene; (E) PER2 gene; (F) PER3 gene. (G-L) The circadian rhythms of clock gene mRNA expression in chicken hypothalamus. (G) CLOCK gene; (H) BMAL1 gene; (I) CRY1 gene; (J) CRY2 gene; (K) PER2 gene; (L) PER3

gene. (M-R) The circadian rhythms of clock gene mRNA expression in chicken pituitary. (M) CLOCK gene; (N) BMAL1 gene; (O) CRY1 gene; (P) CRY2 gene; (Q) PER2 gene; (R) PER3 gene. The relative mRNA levels of clock gene are normalized to PPIA. The data markers in the graphs indicate the clock gene mRNA expression levels, and the results are expressed as the mean $\pm$ SEM. The curves represent the 24-hour period determined by cosinor analysis. N = 6 chicks per time point. Data from CT2 are double-plotted. R2 values represent the degree of fitting.

Figure 3

Effect of chronic CORT exposure on the circadian rhythm parameters of CRH in chicken hypothalamus and CRH receptors gene in chicken pituitary (A) CRH gene; (B) CRHR1 gene; (C) CRHR2 gene. The relative mRNA levels of CRH and CRH receptors gene are normalized to PPIA. The data markers in the graphs indicate the CRH and CRH receptors gene mRNA expression levels, and the results are expressed as the mean $\pm$ SEM. The curves represent the 24-hour period determined by cosinor analysis. N = 6 chicks per time point. Data from CT2 are double-plotted. R2 values represent the degree of fitting. *P < 0.05, compared with control.

Figure 4

Effect of chronic CORT exposure on the circadian rhythm parameters of feeding related genes in chicken hypothalamus. The circadian rhythms of feeding related gene mRNA expression in chicken pituitary. (A) NPY gene; (B) AGRP gene; (C) POMC gene; (D) CART gene. The relative mRNA levels of feeding related gene are normalized to PPIA. The data markers in the graphs indicate the feeding related gene mRNA expression levels, and the results are expressed as the mean $\pm$ SEM. The curves represent the 24-hour period determined by cosinor analysis. N = 6 chicks per time point. Data from CT2 are double-plotted. R² values represent the degree of fitting.

Figure 5

Effect of chronic CORT exposure on the circadian rhythm parameters of m6A level and m6A related genes in chicken hypothalamus. The circadian rhythms of m6A level and m6A related genes mRNA expression in chicken pituitary. (A) M6A level (N=4); (B) FTO gene; (C) METTL3 gene; (D) METTL14 gene; (E) YTHDF1 gene; (F) YTHDF2 gene; (G) YTHDF3 gene. The relative mRNA levels of m6A related genes are normalized to PPIA, N = 6 chicks per time point. The data markers in the graphs indicate the m6A related genes mRNA expression levels, and the results are expressed as the mean $\pm$ SEM. The curves represent the 24-hour period determined by cosinor analysis. Data from CT2 are double-plotted. R² values represent the degree of fitting. *P < 0.05, compared with control.

Figure 6

Effect of chronic CORT exposure on the site-specific m⁶A levels in the 3'UTR of *CRH* mRNA in chicken hypothalamus Validation of m⁶A modification in *CRH* 3'UTR using single-base elongation and ligation-based qPCR amplification method (SELECT) when treatment with CORT in chicken hypothalamus. (A) Schematic graph of N, X1 and X2 site in *CRH* gene; (B) Amplification curve and qPCR CT value in *CRH* N site at ZT18; (C) Amplification curve and qPCR CT value in *CRH* X1 site at ZT18; (D) Amplification curve

and qPCR CT value in CRH X2 site at ZT18; (E) Amplification curve and qPCR CT value in CRH N site at ZT22; (F) Amplification curve and qPCR CT value in CRH X1 site at ZT22; (G) Amplification curve and qPCR CT value in CRH X2 site at ZT22. Values are mean $\pm$ SEM, *P < 0.05, **p < 0.01, compared with control.

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