

Constant Light in Early Life Induces Depressive-Like Behavior in Chickens Via Modulation of Hippocampal BDNF/ERK Pathway

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Abstract

Background

Light management plays an important role in broiler growth and behavior. Constant light in early post hatch stage has been a common practice for improving feed intake and body weight gain in broiler chickens, while whether and how constant light in early life affects the behavior in broiler chickens is rarely reported.

Results

In this study, newly hatched chickens were kept in either constant light (24L:0D, LL) or natural photoperiod (12L:12D, LD) for 7 days and maintained in constant light thereafter from 8 d to 21 d of age. Constant light did not affect chicken body weight, while increased average daily feed intake (ADFI) in 7 d and 21 d and every week feed conversion ratio (FCR). Constant light exposure in early life induces depressive-like behaviors, which was associated with higher corticosterone (CORT), lower melatonin and 5-hydroxytryptamine (5-HT) plasma. Concurrently, constant light exposure increased the mRNA expression of clock related genes and suppressed the expression of antioxidative genes in the hippocampus of both 7- and 21-day-old chickens. Moreover, brain derived neurotrophic factor (BDNF)/extracellular signal-regulated kinase (ERK) pathway in hippocampus was suppressed by constant light exposure.

Conclusions

These findings imply that constant light exposure in early life disrupts hippocampal expression of clock genes and BDNF/ERK pathway, which contributes to depressive-like behaviors in the chicken.

1. Introduction

Light management is an important factor affecting broiler growth development, welfare, behavior, immune response, growth rate and behavioral rhythms [1, 2]. Broilers are usually provided with continuous or near-continuous illumination, especially in the United States, because most of the early photoperiodic studies showed that such regimens maximized feed intake and body weight gain, especially during the initial part of the growing period [3]. Photoperiod could affect biological clock *in vivo*, which could drive circadian rhythmicity of mainly all processes of physiology and behavior [4]. Constant light has been reported that leads to circadian disruption, fatigue, irritability, depression and anxiety [5]. Previous study shown that due to chronic constant light disrupt the circadian rhythms and leads to depressive and anxiety-like behaviors in the rat [6], while it still unknown that whether and how constant light in early life affect the welfare and behavior in chickens.

Hippocampus is the key structure governing many important functions including learning and memory, as well as depressive and anxiety-like behaviors [7]. Brain derived neurotrophic factor (BDNF) is critical for the hippocampal neurogenesis and synaptic plasticity [8, 9] through binding to its receptor tropomyosin receptor kinase B (TrkB) and triggering the activation of phosphatidylinositol 3-kinase (PI3K), and/or extracellular signal-regulated kinase (ERK) pathways [10]. Our previous study found that chronic constant light exposure for 3 weeks reduces hippocampal neurogenesis and impairs cognitive behaviors via suppresses BDNF/TrkB/ERK pathway [11]. The core molecular clock consists of a transcriptional-translational autoregulatory “loop” with a positive arm and a negative arm [12]. The clock and *bmal1* genes and their protein products comprise the positive arm, while *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *ROR α* , *Rev-erba* genes and their protein products comprise the negative arm. Previous studies have confirmed that light exposure can influence the clock genes in the pineal gland, hypothalamus and retina of chicks [13, 14]. However, whether constant light in early life affect hippocampal neurogenesis and clock gene expression remain unclear.

Therefore, the objectives of the present study were, firstly, to elaborate the effects of constant light in early life on depressive-like behavior in chicken; secondly, to delineate the expression of BDNF/TrkB/ERK pathway and clock gene in chicken hippocampus, and to reveal their responses to constant light exposure.

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

Eighty one-day-old male Yellow-footed chickens were purchased from Changzhou Lihua Livestock and Poultry Co., Ltd. Chickens were randomly divided into normal photoperiod (LD) and constant light (LL) group in first week. Light regime in LD group was 12 h light: 12 h dark, with light on at 07:00 am and off at 19:00 pm; light regime in LL group was 24 h light: 0 h dark. From 8 d to 21 d, the light regime was maintained 24 h constant light, light intensity about 200 lux, until the experiment end. Feed and water were provided ad libitum. Daily feed consumption was recorded and body weight was recorded every week. By the end of the experiment, the chickens were sacrificed and hippocampus tissue quickly excised, frozen immediately in liquid nitrogen, and stored at -80°C until use.

2.3 Behavior tests

2.3.1 Open field test

Chicken was placed in the center of a 58 × 58 cm open field apparatus with sides 70 cm high. This OF was made of white wood and the floor was marked off into 25 squares of 12 cm × 12 cm each, illuminated by a 100 W overhead bulb [15]. The following types of behaviors were analyzed for 10 min: the time of first step, the count of grid number, defecation, steps, escapes and tweets. After testing, the floor of the OF apparatus was cleaned with towels wetted with 70% ethanol.

2.3.2 Balance beam test

The test was conducted using an elevated narrow balance beam (6 cm wide and 35 cm long). The balance beam was 22 cm high, so that chicks could jump down onto soft bedding without injuring themselves. The balance beam aimed at representing a novel situation (height and depth) for newly hatched chicks. Thus, we expected using this test to assess fear-related behaviors, particularly fear of heights and the respective avoidance response, similar to tests in rodents [16]. Chicks were tested once only. The test was performed as follows. Each chick was placed on the starting line at an end of the balance beam and then allowed to walk. We recorded the following parameters: (i) the time of the chick stay the beam and (ii) the distance the chick walked on the beam. If a chick stayed on the starting line or was unable to walk on the beam, it received a score of 0. Its time was recorded as 120 s.

2.3.3 Tonic immobility test

Chickens were captured from their home pen and carried to the adjacent room. Hens were placed on their backs in a metal cradle and restrained for 5 s by the experimenter placing one hand on the bird's chest and another over its head with the head hanging down. The single experimenter then removed their hands and stepped aside with eyes averted downwards. The test concluded after 6 min of immobilisation or when the bird righted itself after at least 10 s of immobilisation, whichever occurred first. Duration of tonic immobility was recorded, and the restraining was repeated up to 5 times if the hen righted itself in less than 10 s. All hens were feather-scored again by the same experimenter following conclusion of the testing.

2.4 Measurement of corticosterone, melatonin and 5-Hydroxytryptamine

Corticosterone (CORT) 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 Melatonin (MT) ELISA Kit (MM-3427801, ImmunoWay Biotechnology, USA) following the manufacturer's instructions. Serum 5-Hydroxytryptamine (5-HT) levels were measured using Chicken 5-HT ELISA Kit (E-EL-0033c, Elabscience, USA) following the manufacturer's instructions.

2.5 RNA isolation and real-time PCR

High quality total RNA was isolated from 30 mg hippocampus 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 QuantStudioTM 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.

Target genes	Primer sequences (5' to 3')	
<i>Clock</i>	F: GATCACAGGGCACCTCCAATA	R: CTAGTTCTGCCGCCTTCT
<i>Bam1</i>	F: GTAGACCAGAGGGCGACAG	R: ATGAAACTGAACCAGCGACTC
<i>Cry1</i>	F: GATGTGGCTATCCTGTAGTCCT	R: GCTGCTGGTAGATTGTTCAT
<i>Cry2</i>	F: GCACGGCTGGATAAACACT	R: AAATAAGCGGCAGGACAAA
<i>Per2</i>	F: ATGAAACGAGCCATCCCG	R: CAGTTGTCGTGATTTGCCTA
<i>Per3</i>	F: CAGTGCCTTGTTGGTTAC	R: GATGGATTCACAAAATGGAC
<i>Rora</i>	F: GGGGATGTCTCGAGATGCTG	R: TGCTTGCTACCTTCAGGGG
<i>Rev-erba</i>	F: CAGCGGTTCCAGTCATCCT	R: TCACTCTTGGTCCCCATC
<i>5-HTA</i>	F: AGAACACGGAGGCCAAGC	R: ACGGCAACCAGCAGAGGA
<i>5-HTB</i>	F: CACGGACCACGTCCCTACAC	R: TTTCTTGGCGTCTGCTTCA
<i>Maoa</i>	F: ATTCCCTCCTGAAGCACCAT	R: CACTGCCTCACATACCAC
<i>Maob</i>	F: AGGCTGGAGAAAAAGCAGCA	R: CCCGGTACAGATGGCAAGTT
<i>Nrf2</i>	F: GGCCACCCCTAAAGCTCCATT	R: GGCTTCACTGAAC TGCTCCT
<i>Keap1</i>	F: TCAACTGGGTGCAGTACGAC	R: TCTGCGCCAGGTAATCCTG
<i>Sod1</i>	F: GAGCGGGCCAGTAAAGGTTA	R: CCCTTGCAGTCACATTGCC
<i>Sod2</i>	F: TACAGCTCAGGTGTCGCTTC	R: GCGAAGGAACCAAAGTCACG
<i>Nqo1</i>	F: CGCACCCCTGAGAAAACCTCT	R: AAGCACTGGGTTCTTGAG
<i>Cat</i>	F: GCGCCCCGAACTATTATCCA	R: ATACGTGCGCCATAGTCAGG
<i>Gctc</i>	F: GGACGCTATGGGTTGGAA	R: AGGCCATCACAATGGGACAG
<i>Gpx1</i>	F: CTGCAACCAATTGGGCAC	R: CACCTCGCACTTCTCGAAC
<i>Gpx2</i>	F: CGCCAAGTCCTCTACGACC	R: GGTGTAATCCCTCACCGTGG
<i>Gpx3</i>	F: CGAAAGTACGCGGGGAAGAT	R: GGACGACAAGTCCATAGGGC
<i>Bdnf</i>	F: GACATGGCAGCTTGGCTTAC	R: GTTTCCCTCACTGGCTGGA
<i>Trkb</i>	F: TGACTGTGGTGGATTCAAGGC	R: TGGCGAAAAGGCTTCTTGG
<i>Dcx</i>	F: GCAGCTGCCACAGGTAGTAA	R: ACTGCTTGGATTGGCGTA
<i>PPIA</i>	F: TTACGGGAGAAGTTGCCG	R: TGGTGATCTGCTGCTCGTC

2.6 Total Protein Extraction and Western Blotting

Total protein was extracted from about 50 mg frozen hippocampus samples. Protein concentrations were measured using BCA Protein Assay kit (NO.23227, Thermos Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions. Protein (50 µg/lane) was loaded for electrophoresis on a 6-14% SDS-PAGE gel and transferred onto a nitrocellulose membrane. After transfer, the membranes were blocked with 4% milk and then incubated with primary and secondary antibodies. Western blot analysis for NRF2 (16396-1-AP, proteintech, USA, diluted 1:1000), BDNF (ab108319, Abcam, USA, diluted 1:500), TrkB (bs-0288R, Bioss, USA, diluted 1:1000), Doublecortin (DCX; 4604, Cell Signaling Technology, USA, diluted 1:1000), ERK (4695, Cell Signaling Technology, USA, diluted 1:1000), phospho-ERK (4370, Cell Signaling Technology, USA, diluted 1:1000), tubulin a (BS1699, Bioworld, diluted 1:10,000) was used as loading control. Images were captured by VersaDoc 4000MP system (Bio-Rad, USA) and the band density was analyzed with Quantity One software (Bio-Rad, USA).

2.7 Statistical analysis

All data are presented as means \pm SEM and the differences among groups were analyzed using T-Test for independent samples with SPSS 20.0 for windows. The differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Effect of constant light in early life on feed conversion ratio, plasma CORT, melatonin and 5-HT concentration

Constant light did not affect ($P > 0.05$) chicken body weight from 1 d to 21 d (Figure 1A), but significantly increased ($P < 0.05$) average daily feed intake (ADFI) in 7 d and 21 d (Figure 1B). Meanwhile, constant light significantly increased ($P < 0.05$) every week feed conversion ratio (FCR) from first week to third week (Figure 1C). In addition, compared with LD group, 7 d and 21 d chickens plasma CORT levels (Figure 1D) were significantly increased ($P < 0.05$) and plasma melatonin levels (Figure 1E) were significantly decreased ($P < 0.05$) in LL group. Also, in LL group plasma 5-HT levels were significantly decreased ($P < 0.05$) in 7 d, and trend to decreased ($P = 0.06$) in 21 d chickens (Figure 1F).

3.2 Effect of constant light in early life on the depression and anxiety-like behaviors in chickens

In open field test, compared with LD group, the time of first step (Figure 2A) was significantly increased ($P < 0.01$), and the count of grid number (Figure 2B), defecation (Figure 2C), steps (Figure 2D), escape (Figure 2E) and tweets (Figure 2F) were significantly decreased ($P < 0.01$) in LL group. In balance beam test, compared with LD group, the time stayed (Figure 2G) was significantly increased ($P < 0.01$), and distance moved (Figure 2H) was significantly decreased ($P < 0.01$) in LL group. In tonic immobility test, compared with LD group, the immobility time was significantly increased ($P < 0.01$) in LL group (Figure

2I). These behavior results indicated that constant light in early life significantly increased depression and anxiety-like behaviors in chickens.

3.3 Effect of constant light in early life on hippocampal 5-HT receptors and clock genes mRNA expression in chickens

In LL group, hippocampal 5-HT receptor 5-HTA and 5-HTB mRNA expression were significantly decreased ($P < 0.05$) in 7 d chickens (Figure 3A). Also, compared with LD group, in LL group, hippocampal 5-HT receptor 5-HTA mRNA expression was trend to decreased ($P = 0.07$), 5-HTB and MAOB, the key enzymes for 5-HT degradation, mRNA expression was significantly decreased ($P < 0.05$) (Figure 3B). In addition, compared with LD group, in LL group, hippocampal clock-related genes cry1, cry2, per3, rora and rev-erba mRNA expression were significantly increased ($P < 0.05$) in 7 d chickens (Figure 3C). As similarly as 7 d chicken, in LL group, hippocampal clock-related genes cry1, cry2, per3 and rev-erba mRNA expression were significantly increased ($P < 0.05$), per2 mRNA expression was trend to increase ($P = 0.07$) in 21 d chickens (Figure 3D).

3.4. Effect of constant light in early life on oxidative stress related genes mRNA and protein expression chicken hippocampus

In 7 d chickens, constant light significantly decreased ($P < 0.05$) oxidative stress related genes nrf2, keap, sod2 and increased ($P < 0.01$) nqo1, gpx1 mRNA expression (Figure 4A), as well as significantly decreased ($P < 0.05$) NRF2 protein expression in hippocampus (Figure 4B). In 21 d chickens, constant light significantly decreased ($P < 0.05$) oxidative stress related genes nrf2, keap, sod1, nqo1 and increased ($P < 0.05$) gpx1, gpx2 mRNA expression (Figure 4C), as well as trend to decrease ($P = 0.07$) NRF2 protein expression in hippocampus (Figure 4D).

3.5 Effect of constant light in early life on BDNF/TrkB/ERK mRNA and protein expression in chicken hippocampus

In 7 d chickens, constant light significantly decreased ($P < 0.05$) hippocampal BDNF, TrkB and DCX mRNA expression (Figure 5A), and significantly decreased ($P < 0.05$) TrkB, p-ERK protein expression, while BDNF protein expression was trend to decrease ($P = 0.05$) (Figure 5B and C). In 21 d chickens, constant light significantly decreased ($P < 0.05$) hippocampal BDNF and TrkB mRNA expression (Figure 5D), and significantly decreased ($P < 0.05$) BDNF and p-ERK protein expression (Figure 5E and F).

4. Discussion

In this study, we observed that constant light exposure increased ADFI and FCR, but did not affect body weight in 7 d and 21 d chickens. Similar results have been reported by Lewis, who found that compared with photoperiods in 12 h, photoperiods in 24h decreased feeding rate, increased food intake, but there was no significantly differences on body weight in 21 d males Cobb 500 and Ross 308 broiler [3]. However, recent study reported that compared with intermittent lighting, constant lighting in 30 lx decreased ADFI and did not affect FCR in Lingnan Yellow broiler chicks from 1-21 d [17]. The reason why these results were different from the present study may be due to the different broiler breeds and light intensity.

In the present study, behavior tests results found that constant light exposure in early life induced anxiety and depressive-like behavior in 7 d chickens. These results consistent with previous study, which found that constant light exposure increased anxiety and depressive-like behavior in mice [18]. Meanwhile, we found that constant light exposure induces higher plasma CORT levels both in 7 d and 21 d chickens. Higher plasma CORT levels have detrimental physiological and cognitive effects, which were linked with depression-like behavior in weanling mice [19] and adult rats [20] exposed to dim light at night. CORT levels are regarded as an important indicator of depression-like behavior in rodents [21]. Thus, in this study, we speculate that constant light increased anxiety and depressive-like behavior may be directly or indirectly induced by higher plasma CORT levels in chicken.

Melatonin plays a key role in controlling circadian behavioral responses [22]. It also acts as a potent antioxidant by scavenging reactive oxygen species [23]. Previous studies reported that constant light induced depressive-like behavior in rodents is usually associated with low melatonin and high oxidative stress levels [24, 25]. Indeed, we detected lower plasma melatonin and 5-HT levels, accompanied by lower hippocampal 5-HT receptor mRNA expression both in 7 d and 21 d of constant exposed chickens. Similar results have been reported by Lauber, who found that compared with photoperiods in 12 h, photoperiods in 23 h significantly reduced plasma melatonin in 7-week-old broiler chickens [26]. NF-E2-related factor 2 (Nrf2) and its endogenous inhibitor, Kelch-like ECH-associated protein 1 (Keap1) plays a critical role in counteract oxidative stress [27]. In this study, constant light exposure reduced Nrf2/Keap1 and superoxide dismutase (SOD) mRNA expression, as well as Nrf2 protein expression both in 7 d and 21 d chickens hippocampus. Agree with our present study, it was reported that constant light induced higher oxidative stress in rat hippocampus, cortex and cerebellum [28], and lower Nrf2 expression in rat thymus which could rescued by melatonin [29].

A number of studies indicate that constant light exposure influences the mRNA expression of clock-related genes, including Clock, Bmal1, Cry, and Per [30, 31], which is associated with impaired hippocampal neurogenesis and depressive-like behavior [32, 33] in mice. Similar with previous reported, we also found that constant light exposure significantly increased the mRNA expression of clock-related genes Cry1, Cry2 and Rev-erba both in 7 d and 21 d chickens hippocampus. In mammals, Crys are important clock genes involved in the regulation of circadian rhythm [34]. Besides, Crys serve as

photoactive pigments and important circadian photoreceptors for light entrainment of the circadian rhythm [35]. Mice lacking Cry1 and Cry2 genes lose completely the free-running circadian rhythmicity in wheel-running behavior [36] and the circadian oscillations in the electric activity of the suprachiasmatic nucleus under constant darkness [37]. Thus, we speculate that higher photoreceptor Cry expression may be involved in

BDNF/TrkB/ERK pathway is critical for the hippocampal neurogenesis [8], which plays an important role in regulated depressive-like behavior in animal [7]. In the present study, we found that constant light exposure significantly suppressed BDNF/TrkB/ERK pathway both in 7 d and 21 d chicken's hippocampus. These results consistent with our previous study, which reported that constant light suppressed BDNF/TrkB/ERK pathway in mice hippocampus [11]. In addition, melatonin could rescue chronic restraint stress induced depressive-like behavior via activated the BDNF/TrkB signaling pathway [38]. And CORT exposure reduced hippocampal neurogenesis through suppressed BDNF/ERK pathway [39]. Thus, combined with the results in present study, we speculate that constant light exposure induces depressive-like behavior in chickens via modulation of hippocampal BDNF/TrkB/ERK pathway.

5. Conclusion

In conclusion, our study shows that constant light exposure in early life induces depressive-like behaviors, accompanied by higher plasma CORT, lower plasma melatonin and 5-HT levels in the chicken. Meanwhile, constant light exposure increased the mRNA expression of clock related genes and suppressed the expression of Nrf2/keap1 both in 7 d and 21 d chickens hippocampus. Moreover, BDNF/TrkB/ERK pathway in chickens hippocampus were suppressed by constant light exposure. These findings provide evidence of constant light exposure in early life is harmful to animal welfare, and also imply a role of clock gene and BDNF/TrkB/ERK pathway modification in the regulation of depressive-like behaviors in the chicken.

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

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Figures

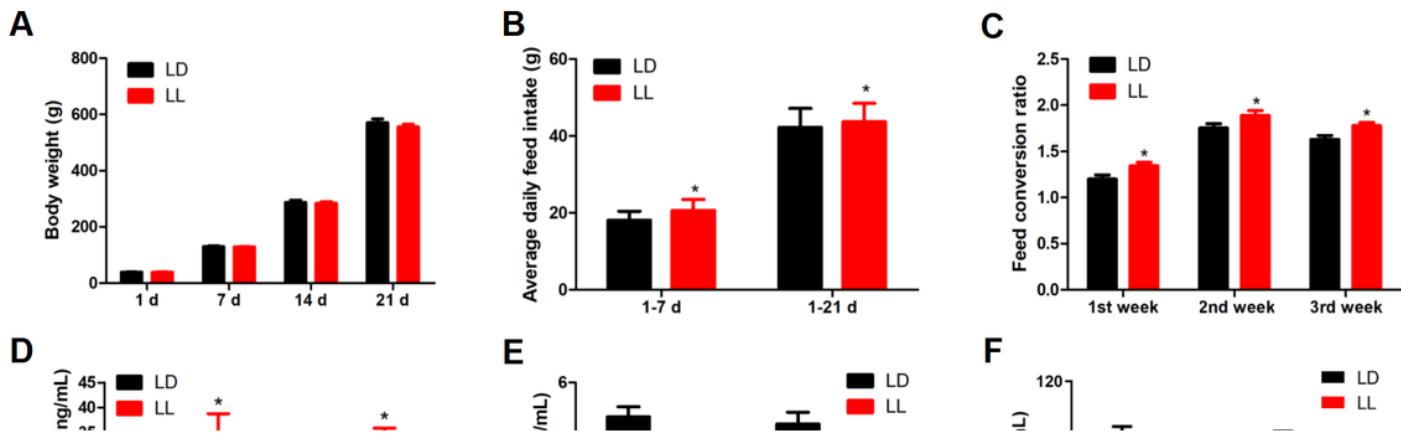


Figure 1

Effect of constant light exposure on growth performance and plasma hormone concentration (A) Body weight (1d, 7d, n = 40, 14d, 21d, n=20), (B) Average daily feed intake (7d, n = 7, 21d, n=21), (C) Feed conversion ratio, (D) Plasma corticosterone content (n = 10), (E) Plasma melatonin content (n = 10), (F) Plasma 5-HT content (n = 10). Values are mean ± SEM, *P < 0.05, **p < 0.01, compared with LD.

Figure 2

Effect of constant light exposure on anxiety and depressive-like behavior In order to elaborate the effect of constant light exposure on locomotor activity, anxiety and depressive-like activity, the open-field test, balance beam test, and tonic immobility test were performed. (A) The times of first step, (B-F) The counts of grid number, defecation, steps, escape and tweets in the open field test, (G) Times stayed in balance beam test, (H) The distance moved in balance beam test, (I) The immobility time in tonic immobility test. Values are mean ± SEM (n = 40), **p < 0.01, compared with LD.

Figure 3

Effect of constant light exposure on 5-HT receptor and clock related gene mRNA expression in chicken's hippocampus (A) 5-HT receptor in 7 d chickens, (B) 5-HT receptor in 21 d chickens, (C) Clock related genes in 7 d chickens, (D) Clock related genes in 21 d chickens. Values are mean ± SEM (n = 12), *p < 0.05, compared with LD.

Figure 4

Effect of constant light exposure on oxidative stress related gene mRNA and protein expression in chicken's hippocampus (A) Oxidative stress related gene mRNA expression in 7 d chickens (n = 12), (B) Nrf2 protein expression in 7 d chickens (n = 6), (C) Oxidative stress related gene mRNA expression in 7 d chickens (n = 12), (D) Nrf2 protein expression in 7 d chickens (n = 6). Values are mean ± SEM, *p < 0.05, compared with LD.

Figure 5

Effect of constant light exposure on BDNF/TrkB/ERK pathway mRNA and protein expression in chicken's hippocampus (A) BDNF, TrkB and DCX mRNA expression in 7 d chickens (n = 12), (B) BDNF, TrkB and DCX protein expression in 7 d chickens (n = 6), (C) ERK and p-ERK protein expression in 7 d chickens (n = 6), (D) BDNF, TrkB and DCX mRNA expression in 21 d chickens (n = 12), (E) BDNF, TrkB and DCX protein expression in 21 d chickens (n = 6), (F) ERK and p-ERK protein expression in 21 d chickens (n = 6). Values are mean ± SEM, *p < 0.05, **p < 0.01, compared with LD.