

doi:10.3969/j.issn.1673-5374.2012.028.008 [http://www.crter.org/nrr-2012-qkquanwen.html]

Sun B, Feng X, Ding X, Bao L, Li YF, He J, Jin MF. Expression of Clock genes in the pineal glands of newborn rats with hypoxic-ischemic encephalopathy. *Neural Regen Res.* 2012;7(28):2221-2226.

Expression of Clock genes in the pineal glands of newborn rats with hypoxic-ischemic encephalopathy[☆]

Bin Sun¹, Xing Feng¹, Xin Ding¹, Li Bao², Yongfu Li³, Jun He⁴, Meifang Jin¹

1 Division of Neonatology, Affiliated Children's Hospital of Soochow University, Suzhou 215003, Jiangsu Province, China

2 Department of General Pediatrics, Yixing People's Hospital, Yixing 214200, Jiangsu Province, China

3 Division of Neonatology, Suzhou Municipal Hospital (Main Campus), Suzhou 215002, Jiangsu Province, China

4 Jiangsu Institute of Hematology and Blood Diseases, First Affiliated Hospital of Soochow University, Suzhou 215006, Jiangsu Province, China

Abstract

Clock genes are involved in circadian rhythm regulation, and surviving newborns with hypoxic-ischemic encephalopathy may present with sleep-wake cycle reversal. This study aimed to determine the expression of the clock genes Clock and Bmal1, in the pineal gland of rats with hypoxic-ischemic brain damage. Results showed that levels of Clock mRNA were not significantly changed within 48 hours after cerebral hypoxia and ischemia. Expression levels of CLOCK and BMAL1 protein were significantly higher after 48 hours. The levels of Bmal1 mRNA reached a peak at 36 hours, but were significantly reduced at 48 hours. Experimental findings indicate that Clock and Bmal1 genes were indeed expressed in the pineal glands of neonatal rats. At the initial stage (within 36 hours) of hypoxic-ischemic brain damage, only slight changes in the expression levels of these two genes were detected, followed by significant changes at 36–48 hours. These changes may be associated with circadian rhythm disorder induced by hypoxic-ischemic brain damage.

Bin Sun[☆], Ph.D., Associate professor, Division of Neonatology, Affiliated Children's Hospital of Soochow University, Suzhou 215003, Jiangsu Province, China

Corresponding author: Xing Feng, Ph.D., Professor, Division of Neonatology, Affiliated Children's Hospital of Soochow University, Suzhou 215003, Jiangsu Province, China
xing_feng66@hotmail.com

Key Words

brain hypoxia; cerebral ischemia; neonatal rats; pineal gland; Clock; Bmal1; mRNA; protein; brain; neural regeneration

Received: 2012-01-09
Accepted: 2012-04-23
(N20111220002/H)

Research Highlights

(1) Clock and Bmal1 genes were expressed in the pineal glands of newborn rats with hypoxic-ischemic brain damage.

(2) At the initial stage (within 36 hours) of hypoxic-ischemic brain damage, only slight changes in the expression levels of these two genes were detected, but expression levels were significantly different after 36 hours. These changes may be associated with circadian rhythm disorder induced by hypoxic-ischemic brain damage.

Abbreviations

HIE, hypoxic-ischemic encephalopathy; HIBD, hypoxic-ischemic brain damage

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) is a type of hypoxic-ischemic brain

damage (HIBD) caused by perinatal hypoxia. Surviving newborns with this condition are at risk of developing psychomotor developmental retardation, sleep-wake cycle reversal, or endocrine

disorders, which seriously affects the quality of life of these children^[1-2]. Extensive studies on the molecular mechanisms underlying the rhythm disturbance after hypoxia-ischemia could provide a theoretical basis for the intervention and treatment of HIE.

The pathogenesis of HIBD is complicated. Hypoxia has been recognized as the basic underlying mechanism. In addition, a variety of factors are involved in HIBD, including the newly proven oxygen-free radicals, nitric oxide, calcium overload, excitotoxicity, transient reduction in the number of γ -aminobutyric acid receptors, and changes in expression levels of the glutamate carrier and apoptosis proteins. However, none of these previous studies have investigated the role of circadian rhythm disorders in HIBD or the effect of changes in the expression levels of the clock genes on HIBD. The pineal gland is one of the best models for studying the circadian clock in higher vertebrates^[3-4]. However, the role of the impaired pineal gland in HIBD has never been completely understood. In the present study, we analyzed the expression of several clock genes in the rat pineal gland and also evaluated their association with HIBD, in order to provide further clues to the causes of rhythm disturbance. Clock genes are a group of genes that regulate circadian rhythm, which are critical for maintaining the endogenous circadian clock^[5]. Previous studies on the molecular mechanisms of the circadian clock oscillator mainly focused on clock genes such as Clock, Bmal1/2, Per1/2/3, and Cry, as well as the related transcription/translation regulation feedback loop^[6-7]. The CLOCK and BMAL1 proteins can form dimers, which function as positive regulatory proteins to activate the transcription of the Per1/2/3, Cry, and Clock genes. In addition, Per and Cry proteins can serve as negative regulators of the BMAL1/CLOCK dimers to inhibit their own transcriptional activations. To our knowledge, there has been no research conducted on the expression of these clock genes in HIBD diseases.

RESULTS

Quantitative analysis of experimental animals

Seventy-two newborn Sprague-Dawley rats were initially included in the study, and equally assigned to a control group (without the hypoxia treatment) and a HIBD group (HIBD model). The rats were further studied at 0, 2, 12, 24, 36, and 48 hours, with six rats at each time point. All 72 rats were included in the final result analysis.

Changes in the expression of Clock mRNA in the pineal gland after HIBD

The expression of Clock mRNA (reverse transcription-PCR)

in the pineal gland did not show rhythmic variation in both groups, but a slight decrease of Clock mRNA in the HIBD group was observed ($P > 0.05$; Figure 1).

Changes in the expression of CLOCK protein in the pineal gland after HIBD

As shown in Figure 2, the expression levels of CLOCK protein (western blot detection) in the HIBD group was significantly higher than that of the control group at 48 hours after HIBD ($P < 0.05$).

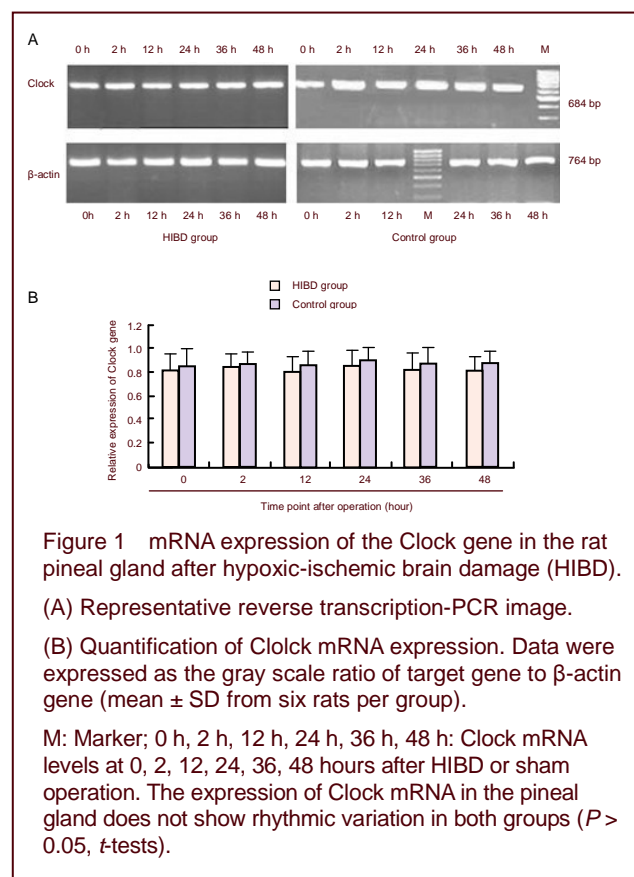


Figure 1 mRNA expression of the Clock gene in the rat pineal gland after hypoxic-ischemic brain damage (HIBD).

(A) Representative reverse transcription-PCR image.

(B) Quantification of Clock mRNA expression. Data were expressed as the gray scale ratio of target gene to β -actin gene (mean \pm SD from six rats per group).

M: Marker; 0 h, 2 h, 12 h, 24 h, 36 h, 48 h: Clock mRNA levels at 0, 2, 12, 24, 36, 48 hours after HIBD or sham operation. The expression of Clock mRNA in the pineal gland does not show rhythmic variation in both groups ($P > 0.05$, *t*-tests).

Changes in the expression of Bmal1 mRNA in the pineal gland after HIBD

As shown in Figure 3, Bmal1 mRNA expression increased at 36 hours and decreased at 48 hours after HIBD compared to that of the control group ($P < 0.05$). At other time points, such as 0, 2, 12, 24, and 36 hours, no significant difference in the expression level of Bmal1 was noted ($P > 0.05$). At other time points, such as 0, 2, 12, 24, and 36 hours, no significant differences in expression levels were noted ($P > 0.05$).

Changes in the expression of BMAL1 protein in the pineal gland after HIBD

As shown in Figure 4, the expression levels of BMAL1 protein was significantly higher than that of the control group at 48 hours after HIBD ($P < 0.05$). At other time points

such as 0, 2, 12, 24, and 36 hours, no significant difference in the expression levels of BMAL1 was noted ($P > 0.05$). This was consistent with the expression of CLOCK.

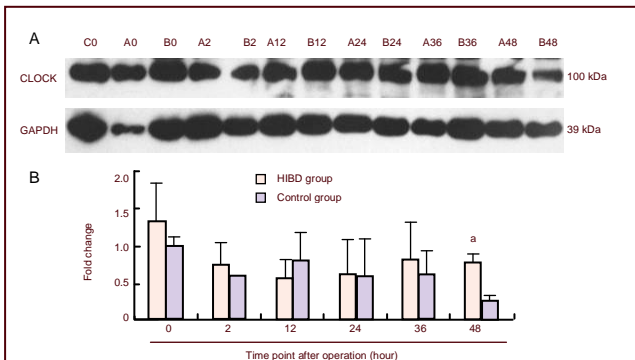


Figure 2 Changes in the expression levels of CLOCK protein in the pineal gland after hypoxic-ischemic brain damage (HIBD).

(A) Representative western blot image. GAPDH was used as an internal control.

(B) Quantification of CLOCK protein expression. The relative expression levels of CLOCK protein are represented as the fold change compared with the control group.

The expression levels of CLOCK protein were significantly higher than that of the control group at 48 hours after HIBD. Data are expressed as mean \pm SD of six independent experiments and were analyzed by *t*-tests.

^a $P < 0.05$, vs. control group. C0: normal group. A0, A2, A12, A24, A36, A48: CLOCK protein levels at 0, 2, 12, 24, 36, 48 hours after HIBD. B0, B2, B12, B24, B36, B48: CLOCK protein levels at 0, 2, 12, 24, 36, 48 hours in the control group.

DISCUSSION

Modern chronobiology studies have shown that, although the rat pineal gland has no circadian pacemaker, all the clock genes are still being expressed in the pineal gland where they play a similar role as does the central circadian oscillator^[8-11]. The pineal gland was known to exert effects on endocrine output. Thus, it was considered the neuroendocrine effector of the circadian system. Therefore, the pineal gland is one of the best models for studying the circadian clock in higher vertebrates.

Clock is involved in the transcriptional regulation of the circadian clock, which is dependent on the chaperone protein BMAL1. Under light-dark conditions, the expression levels of the Clock gene could be sustained for 23.3–23.8 hours^[8, 12]. Mutations in this gene were reported to cause disruption to endogenous and continuous circadian rhythms in humans^[13]. For example, mutant mice with an exon 19 deletion had a shorter circadian cycle than that of normal mice. When the Bmal1 gene was lost, the rhythmic expression of mPer1 and mPer2 in the suprachiasmatic nucleus complex was lost, resulting in a

disturbance in the activities of mice^[14-15].

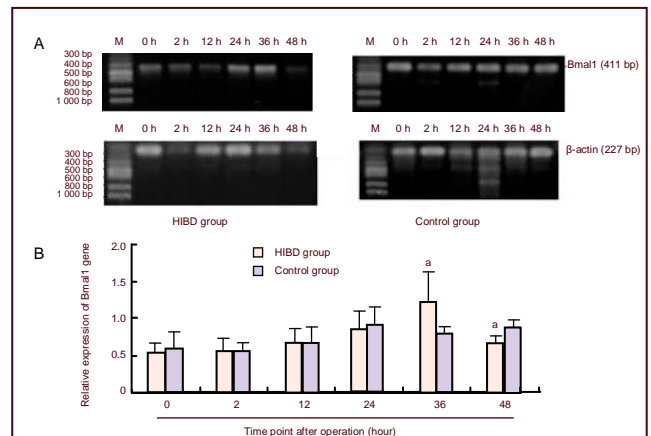


Figure 3 mRNA expression of the Bmal1 gene in the rat pineal gland after hypoxic-ischemic brain damage (HIBD).

(A) Representative reverse transcription-PCR image.

(B) Quantification of Bmal1 mRNA expression. Bmal1 mRNA expression increased at 36 hours and decreased at 48 hours after HIBD compared with that of the control group.

Data are expressed as the gray scale ratio of target gene to β -actin gene (mean \pm SD from six rats per group). ^a $P < 0.05$, vs. control group (*t*-tests). M: Marker. 0 h, 2 h, 12 h, 24 h, 36 h, 48 h: Bmal1 mRNA levels at 0, 2, 12, 24, 36, 48 hours after HIBD or sham operation.

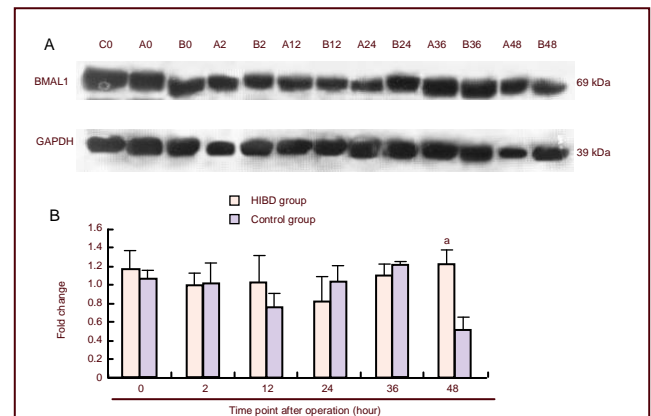


Figure 4 Changes in the expression levels of BMAL1 protein in the pineal gland after hypoxic-ischemic brain damage (HIBD).

(A) Representative image of a western blot analysis. GAPDH was used as the internal control.

(B) Quantification of BMAL1 protein expression. The relative expression level of BMAL1 protein is represented as the fold change compared with the control group. The expression levels of BMAL1 protein were significantly higher than that of the control group at 48 hours after HIBD.

Data were expressed as mean \pm SD of six independent experiments and analyzed by *t*-tests. ^a $P < 0.05$, vs. control group. C0: normal group. A0, A2, A12, A24, A36, A48: BMAL1 protein levels at 0, 2, 12, 24, 36, 48 hours after HIBD. B0, B2, B12, B24, B36, B48: BMAL1 protein levels at 0, 2, 12, 24, 36, 48 hours in the control group.

In the present study, we found that the two major clock genes, Clock and Bmal1, were expressed in the rat pineal gland. The expression levels of Clock mRNA did not change significantly after HIBD. Whereas 48 hours after HIBD, the expression levels of CLOCK protein significantly increased in the HIBD group compared with levels in the control group. The expression levels of Bmal1 mRNA reached a peak at 36 hours after HIBD. The expression levels of BMAL1 protein increased at 48 hours after HIBD. This result was consistent with the findings from a previous study by Valenzuela *et al*^[16]. Our results also confirmed the synchronous expression of these two genes. The delayed increase in the levels of BMAL1 protein expression could be attributed to the time needed for the transition from transcription to translation and for post-translational modification.

In the present study, we found that the expression levels of CLOCK and BMAL1 proteins were lower in the control group. However, the starting time of the rhythmic expressions of the clock genes was not yet clear. It was reported that the circadian rhythm of the AANAT mRNA appeared 10 days after birth in the neonatal rats^[17]. Therefore, we hypothesized that mRNA expression of the Clock, Bmal1, and AANAT genes had similar rhythms, which could be due to their similar E-box enhancer elements. These three proteins could also possibly interact with each other through the bHLH/PAS domain^[18-19]. Thus, these three genes showed similar start times for rhythmic expression. The start of the rhythm was found to be closely associated with light in different regions, feeding conditions in different laboratories, different animal species, and even different circadian rhythms of maternal rats.

At 0, 2, 12, 24, and 36 hours after HIBD, the expression levels of the CLOCK and BMAL1 proteins did not change significantly. This is probably because these genes are more critical for biological rhythm and more conserved compared with other circadian clock genes. Although the expression levels of Clock mRNA was not affected by hypoxia and ischemia, the expression levels of the encoded CLOCK protein showed greater variation^[20]. The cascade effect had already confirmed that slight changes in the expression levels of Clock mRNA could cause significant changes in the expression levels of CLOCK protein. For example, the cascade effect of the norepinephrine β -adrenergic receptor, expressed in the pineal gland, was crucial for the regulation of the rhythmic expressions of many genes, including the pineal clock genes^[21-22].

Hypoxia and reperfusion injuries are considered the initiating factors for the regulation of clock gene expression, which possibly act *via* hypoxia-inducible factor (HIF)-1 α -mediated expression of a series of clock genes^[23]. This has been confirmed by the findings of a study by

Theodore and Dmitri^[23]. HIF-1 α is essential for maintaining oxygen stability. The CLOCK, BMAL1, and HIF-1 α proteins belong to the Per/Amt/Sim family. Under hypoxic conditions, HIF-1 α may regulate the transcriptional activation of Bmal1 mRNA by binding to many Per/Amt/Sim-based sites located in the Bmal1 promoter^[24]. Other studies have shown that cell metabolism can directly or indirectly regulate the clock genes through biochemical pathways. For example, the CLOCK/BMAL1 dimer is reported to be regulated by the reduction and oxidation rates of dihydrouracil dehydrogenase, and the coenzyme II electron carriers in the respiratory chain *in vitro*. The rates were known to fluctuate with cell metabolism^[25-26]. HIBD was reported to decrease the oxidative metabolism of brain cells and reduce their energy supply. Subsequently, a large number of brain cells underwent apoptosis and necrosis. These changes were probably the result of altered expression of clock genes. Currently, other mechanisms that regulate the expression of clock genes in hypoxia cases include the generation of reactive oxygen species, the intracellular oxidation-redox buffer system that results in changes to nicotinamide adenine dinucleotide-dependent expression of Bmal1, and the activation of the hypothalamus-pituitary-adrenal axis promoted by increased neuropeptide Y levels^[27].

Considering the circadian clock mode, studying merely the Clock and Bmal1 genes is not enough. The reason is that in this rigorous system, every component is closely related to each other. Comprehensive research on a variety of clock components, including Per and Cry mRNAs, would possibly uncover the real essence of the clock system. Therefore we are currently performing further in-depth research.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed from February 2007 to May 2009 at Pediatric Institute Affiliated to Children's Hospital of Soochow University, China.

Materials

A total of 72 specific pathogen-free Sprague-Dawley rats, aged 7 days and weighing 11–14 g, male or female, were provided by the Experimental Animal Center, Soochow University, China (License No. SCXK (Su) 2007-0008). They were fed with maternal breast milk and kept under alternate light-dark conditions. The room temperature was maintained at 25 \pm 2°C. The light-dark cycle

included 12 hours light and 12 hours dark alternately (Photoperiod, 06:00–18:00; Dark period, 18:00–06:00).

Methods

Establishment of HIBD model

HIBD group: The HIBD model was established according to the improved method from Levine *et al*^[28]. Briefly, newborn Sprague-Dawley rats were anesthetized by ether inhalation. With the rats fixed on surgical boards in the supine position, a vertical incision of approximately 1 cm was made in the central neck. The subcutaneous tissues and muscles were then separated. The left common carotid artery located between the left sternocleidomastoid and the trachea was identified and isolated. A permanent double-layer ligation was made using a No. 3-0 silk thread and then the incision was sutured. After the surgery, the rats were placed in an incubator at 32°C for 1–2 hours to allow them to recover. They were then transferred to a normal-pressure low-oxygen chamber that was already placed in the incubator. A gas mixture of moist 8% nitrogen-oxygen was then supplied for 2 hours (1.5 L/min). The oxygen concentration was monitored using an oxygen analyzer. At the end, the newborns were returned to the maternal rats for feeding. Control group: The left common carotid arteries were isolated without ligation. After being sutured, the newborns were placed under the same incubation conditions without the hypoxia treatment.

Expression levels of Clock and Bmal1 genes detected by reverse transcription-PCR

A pinealectomy was performed using the Kuszak method^[29]. Total RNA was isolated using a TRIzol kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After reverse transcription, semi-quantitative PCR was used to measure mRNA expression levels of Clock and Bmal1 genes in the pineal gland. The expression level of the β -actin gene was used as an internal control. The primers were synthesized by Shanghai Biological Engineering Technology Co., Ltd, China. The primers used are as follows:

Primer	Sequence	Product size (bp)
β -actin	Forward: P1 5'-TTG TCA CCA ACT GGG ACG ATAT GG-3'	764
	Reverse: P2 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'	
Clock	Forward: P3 5'-GCA GTG GAT TTG GCT TCA-3'	684
	Reverse: P4 5'-AGT CCA GGG TTT GAT TGC-3'	
Bmal1	Forward: P3 5'-CTT CCG GGA CAT CGC ATT-3'	411
	Reverse: P4 5'-TGG ACC TTG GAG CCC TTT-3'	

After electrophoresis, the band intensities (gray scale value) were analyzed using the UVIDoc Imaging system. The relative expression levels of the target genes were normalized to the internal reference β -actin.

Expression levels of CLOCK and BMAL1 proteins detected by western blot analysis

Pre-cooled protein extracting buffer A (40 μ L) was added to the pineal gland. Tissues were homogenized, and samples were vortexed at 1 600 \times g for 15 seconds and then incubated on ice for 10 minutes.

Phenylmethylsulfonyl fluoride, leupeptin, and aprotinin was added and the mixture was vortexed for 5 seconds at 1 600 \times g, incubated on ice for 1 minute, and then vortexed for 5 seconds at high speed. Next, the samples were centrifuged at 4°C at 1 600 \times g for 5 minutes with 40 μ L of the supernatant transferred immediately to a clean pre-cooled Eppendorf tube. The samples were then stored on ice with 20 μ L of pre-cooled protein extracting buffer B added to the precipitates. The mixture was vortexed at a high speed for 15 seconds. Samples were placed on ice and vortexed for 15 seconds every 10 minutes, four times. Samples were then centrifuged at 4°C at 1 600 \times g for 10 minutes with the supernatant transferred immediately to the previous Eppendorf tube, mixed, and stored at -60°C.

The concentrations of all the extracted protein samples were adjusted to the same level. Next, five times loading buffer was added and samples were lysed in boiling water for 5–7 minutes. Proteins were separated using 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was placed in 5% (w/v) skim milk and rocked on a shaker for 2 hours. With the primary antibody (1:400) added, the membrane was left at 4°C overnight. It was rinsed in Tris-buffered saline containing 0.1% (v/v) Tween-20, four times. The following day, the secondary antibody (1:1 000) was added. The membrane was rocked on a shaker for 1 hour. With the secondary antibody discarded, the membrane was rinsed in Tris-buffered saline containing 0.1% (v/v) Tween-20, four times. Bands were detected using Beyo ECL Plus. The exposed film was photographed using a digital camera and analyzed using the Image Pro-Plus 6.0 image analysis system to determine the gray value bands. The ratio of glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference for the semi-quantitative analysis of the expression levels of CLOCK and BMAL1 proteins.

Statistical analysis

Statistical analysis was performed using SPSS v10.0 software (SPSS, Chicago, IL, USA). All data are represented as mean \pm SD. The differences between two

groups were compared using the *t*-test. A *P*-value less than 0.05 was considered statistically significant.

Acknowledgments: The authors sincerely thank Xindong Wang, Xueming Zhu, and Ying Wang from the Institute of Pediatrics, Soochow University, China for their valuable assistance to the current study.

Funding: This study was supported by grants from the Foundation for Advancing Medical Sciences of the Health Department, Jiangsu Province, No. Z200519, and the Project for Social Development of Suzhou, No. SSZ0230.

Author contributions: All authors participated in experimental design, intervention, and evaluation and approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The study received ethical permission from the Animal Ethics Committee of Soochow University in China.

REFERENCES

- [1] de Vries LS, Jongmans MJ. Long-term outcome after neonatal hypoxic-ischaemic encephalopathy. *Arch Dis Child Fetal Neonatal Ed.* 2010;95(3):F220-224.
- [2] Perlman M, Shah PS. Hypoxic-ischemic encephalopathy: challenges in outcome and prediction. *J Pediatr.* 2011;158(2 Suppl):e51-54.
- [3] Kommedal S, Bódis G, Matkovits A, et al. Expression pattern of clock under acute phase-delay of the light/dark cycle in the chicken pineal model. *Gen Comp Endocrinol.* 2011;172(1):170-172.
- [4] Okano T, Yamamoto K, Okano K, et al. Chicken pineal clock genes: implication of BMAL2 as a bidirectional regulator in circadian clock oscillation. *Genes Cells.* 2001;6(9):825-836.
- [5] Shearman LP, Sriram S, Weaver DR, et al. Interacting molecular loops in the mammalian circadian clock. *Science.* 2000;288(5468):1013-1019.
- [6] Asher G, Schibler U. Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab.* 2011;13(2):125-137.
- [7] Bellet MM, Sassone-Corsi P. Mammalian circadian clock and metabolism - the epigenetic link. *J Cell Sci.* 2010;123(Pt 22):3837-3848.
- [8] Simonneaux V, Poirel VJ, Garidou ML, et al. Daily rhythm and regulation of clock gene expression in the rat pineal gland. *Brain Res Mol Brain Res.* 2004;120(2):164-172.
- [9] Bustos DM, Bailey MJ, Sugden D, et al. Global daily dynamics of the pineal transcriptome. *Cell Tissue Res.* 2011;344(1):1-11.
- [10] Klein DC, Bailey MJ, Carter DA, et al. Pineal function: impact of microarray analysis. *Mol Cell Endocrinol.* 2010;314(2):170-183.
- [11] Wu T, Dong Y, Yang Z, et al. Differential resetting process of circadian gene expression in rat pineal glands after the reversal of the light/dark cycle via a 24 h light or dark period transition. *Chronobiol Int.* 2009;26(5):793-807.
- [12] Karolczak M, Burbach GJ, Sties G, et al. Clock gene mRNA and protein rhythms in the pineal gland of mice. *Eur J Neurosci.* 2004;19(12):3382-3388.
- [13] Piggins HD. Human clock genes. *Ann Med.* 2002;34(5):394-400.
- [14] Zheng B, Larkin DW, Albrecht U, et al. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature.* 1999;400(6740):169-173.
- [15] Toh KL, Jones CR, He Y, et al. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science.* 2001;291(5506):1040-1043.
- [16] Valenzuela FJ, Torres-Farfan C, Richter HG, et al. Clock gene expression in adult primate suprachiasmatic nuclei and adrenal: is the adrenal a peripheral clock responsive to melatonin? *Endocrinology.* 2008;149(4):1454-1461.
- [17] Garidou ML, Ribelayga C, Pevet P, et al. Syrian hamster and rat display developmental differences in the regulation of pineal arylalkylamine N-acetyltransferase. *J Neuroendocrinol.* 2002;14(11):861-868.
- [18] Bae K, Jin X, Maywood ES, et al. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron.* 2001;30(2):525-536.
- [19] Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Hum Mol Genet.* 2006;15(Spec No 2):R271-277.
- [20] Chilov D, Hofer T, Bauer C, et al. Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain. *FASEB J.* 2001;15(14):2613-2622.
- [21] Bailey MJ, Coon SL, Carter DA, et al. Night/day changes in pineal expression of >600 genes: central role of adrenergic/cAMP signaling. *J Biol Chem.* 2009;284(12):7606-7622.
- [22] Wang GQ, Du YZ, Tong J. Daily oscillation and photoresponses of clock gene, Clock, and clock-associated gene, arylalkylamine N-acetyltransferase gene transcriptions in the rat pineal gland. *Chronobiol Int.* 2007;24(1):9-20.
- [23] Kung TA, Egbejimi O, Cui J, et al. Rapid attenuation of circadian clock gene oscillations in the rat heart following ischemia-reperfusion. *J Mol Cell Cardiol.* 2007;43:744-753.
- [24] Chu K, Jung KH, Kim SJ, et al. Transplantation of human neural stem cells protect against ischemia in a preventive mode via hypoxia-inducible factor-1alpha stabilization in the host brain. *Brain Res.* 2008;1207:182-192.
- [25] Koch-Nolte F, Haag F, Guse AH, et al. Emerging roles of NAD+ and its metabolites in cell signaling. *Sci Signal.* 2009;2(57):mr1.
- [26] Hou J, Lages NF, Oldiges M, et al. Metabolic impact of redox cofactor perturbations in *Saccharomyces cerevisiae*. *Metab Eng.* 2009;11(4-5):253-261.
- [27] Kim HJ, Harrington ME. Neuropeptide Y-deficient mice show altered circadian response to simulated natural photoperiod. *Brain Res.* 2008;1246:96-100.
- [28] Levine S. Anoxic-ischemic encephalopathy in rats. *Am J Pathol.* 1960;36:1-17.
- [29] Kuzsak J, Rodin M. A new technique of pinealectomy for adult rats. *Experientia.* 1977;33(2):283-284.

(Edited by He Y/Yang Y/Song LP)