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# Effect of Suprachiasmatic Nucleus Lesion on Period2 and C-fos Expression in Habenular Nucleus

Haiyan Xu[1], Chengwei Zhang[2], Hua Zhao[1]

[1]Department of Physiology, College of Basic Medical Sciences, Jilin University, Changchun, China

[2]Department of Anesthsiology, The Second Hospital of Jilin University, Changchun, China

Correspondence: Hua Zhao, Department of Physiology, College of Basic Medical Sciences, Jilin University, Changchun, China. Tel: 86-431-85619472. E-mail: zhua@jlu.edu.cn

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**Abstract**

The habenular nucleus (Hb) located in the epithalamus is an important connection between the limbic forebrain and midbrain pathway and it regulates physiological activities including reproduction, sleep-wake, body temperature. Hb is anatomically close to the suprachiasmatic nucleus (SCN), the pacemaker of circadian rhythms in mammals. In vivo data showed circadian oscillation of Period2 (Per2) expression in Hb. However, it remains unknown whether the circadian oscillation of Per2 expression is regulated by SCN. We used semi-quantity RT-PCR methods to examine the relative expression of Per2 and c-fos mRNA in the Hb at 13:00 and 1:00 in the LD (light-dark) cycle either SCN was intact or destroyed. The expression of Per2 and c-fos mRNA in the Hb was higher at 13:00 than at 1:00. However, damaging the SCN abolished the difference of Per2 and c-fos mRNA expression at the two time ponits in the Hb, suggesting circadian oscillation of Per2 and c-fos gene in the Hb may be regulated by SCN under in vivo condition.

**Keywords:** circadian rhythm, habenular nucleus, suprachiasmatic nucleus, Period2 gene, c-fos gene

## 1. Introduction

Habenular nucleus(Hb) is located on the dorsomedial surface of the epithalamus and served as a key position from the limbic forebrain inputs to mid-brain structures(Aghajanian, 1977；Andres，1999). The Hb was reported to play an important role in regulating many biological functions, including reproduction function, cardiovascular activity, pain, stress, themoregulation, sleep-wake and ingestive behavior (Wagner, 1998; Lv, 2012; Aizawa, 2013; Heldt, 2006; Ootsuka, 2015; Christensen, 2013), which all show daily rhythmicity. Some affective disorder diseases deriving from the disrupted circadian rhythms are associated with the Hb (McCathy, 2012; Yang, 2008), which has attracted many scholars' interests. Our previous experiments have manifested that there are activated and suppressed cells by the retinal illumination in the Hb and the Hb neurons also show obvious daily rhythmicity with higher baseline firing rates during the day than the night (Zhao, 2005). Moreover, recent research found that Per 2 circadian gene expression is present in the Hb both in vitro and in vivo (Guilding, 2010; Zhao, 2015). However, it is still unclear that whether Per2 circadian expression in the Hb may be modulated by the suprachiasmatic nucleus (SCN) of the anterior hypothalamus as the major pacemaker of circadian rhythm system (Moore, 1972).

It is well known that the SCN acts a significant role in generating and maintaining daily circadian rhythms in mammals (Moore, 1983; Welsh, 2010). Daily physiological and behavioral rhythmicity, for example, running-wheel and drinking rhythm disappear after the SCN lesioned bilaterally in the rats (Stephan, 1972). The SCN not only possess autonomy circadian rhythm itself but also accept and integrate outside photosignals which lead to synchronize internal rhythm to environmental light-dark cycles (Johnson, 1988; Liu, 2007; Morin, 1994; Pennartz, 2002; Rusak, 1990). However, the maintenance of the circadian rhythm is dependent on the coordination between many brain regions in the central nervous system (Inouye, 1979; Groos, 1982; Schwartz, 1980; Windle, 1992). Significantly, the rhythm characteristics of Per2 expression and firing rates in the Hb were consistent with that of the SCN (zhao, 2005; zhao2015; Groos, 1982; Gillette, 1986). And there is the morphological connection between the Hb and the SCN (Krout, 2002; Morin, 1994). These suggest that the functional connection may occur between the Hb and the SCN.

Per2 gene is considered to play an important role in the feedback loop of auto-excitation oscillation from biological clock genes and their protein production (Bae, 2001; Pendergast, 2010). The c-fos, an immediate early gene, is normally used as a marker of cell excitation (Distel, 1987). In the study, we investigated the expression of Per2 mRNA and c-fos mRNA at two different time points (13:00 and 1:00) with the SCN normal and lesioned rats, in order to clarify whether the expression in the Hb is dependent on the SCN.

## 2. Method

### *2.1 General Procedure*

Male Wistar rats (100～120g ) were obtained from the Department of Experimental Animals, Jilin University, Changchun, China) and were maintained in a standard condition (lighting cycle with 12 h light daily, lights on at 07:00; room temperature of 23±2℃; free access to food and water) for 3 weeks and then the samples were considered to obtain.

### *2.2 Surgery*

#### 2.2.1 SCN Lesion

Rats were anesthetized with 10% chloral hydrate (0.4ml/100g) and placed on an S-R stereotaxic instrument (Narishige Corporation, Japan). Bilateral SCN lesions were done by passing a cocenric circle electrode with (outside diameter, 0.4mm; inside diameter, 0.2mm; constant current electric resistance, 40-60kΩ) were placed to the bilateral SCN position (0 mm lateral to the midline, 0.2 mm posterior to bregma, and 8.5-9.0 mm ventral to dura (Paxinos, 1997). The electrodes were passed through a direct current (2 mA, for 3 min) to damage the SCN. The control animals were treated in the same way but without passing current through the electrode. SCN lesions were assessed based on altered water consumption (Buijs, 1993). Samples were extracted 10 days after the SCN lesion.

#### 2.2.2 Sampling

60 rats of normal, SCN lesion and sham group（n = 10）were anesthesized with etherization at 1:00 and 13:00 respectively and then decapitated and Hb were dissected as Eckenrode reported (Eckenrode, 1992). Samples were kept in - 80℃ refrigerator. Dim red light with 10～15 Lux was used in the extraction of samples under the dark phase in order to avoid the effect of light.

### *2.3 Semi-quantity RT-PCR*

Total RNA was isolated according to the manufacturer's protocol (Sigma). cDNA was originated from 5 μg of total RNA for each sample by using the M-MuLV reverse transcriptase (Promega, USA). PCR was performed in a thermal reactor (Takala, Japan) after the primer of Per1, c-fos and GAPDH was added into the reaction system. The volume of reaction was 25μl and the RT-PCR products were gel purified after 30 cycles and sequenced to confirm size and identity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene control. Each primer sequence was as follows: GAPDH Forward 5'-ACC ACA GTC CAT GCC ATC AC-3', Reverse 5'-TCC ACC ACC CTG TTG CTG TA-'(Takumi, 1998); Per2 Forward 5'-AGA CGT GGA CAT GAG CAG CT-3', Reverse 5'-CAG GAT CTT CCC AGA GAC CA-3'(Tosini, 2007); c-fos Forward 5'- ACC CTG AGC CCA AGC CAT-3', Reverse 5'- AGG GTT CAG CCT TCA GCT CC -3'(Robbins, 2008). RT- PCR products of Per2, c-fos and GAPDH were analyzed through 1% agarose gel electrophoresis. Band gray scale was determined through BIORAD gel imaging analytical system. The gray scale ratio of Per2 and c-fos to GAPDH was regarded as the relative expression level of Per2 and c-fos mRNA.

### *2.4 Data Analysis*

Results are expressed as mean±sem. All data were analysed by un-paied t-test. Values of $P<0.05$ were considered significant.

## 3. Results

### *3.1 Expression of Per2 and c-fos mRNA in the Hb of Normal Rats*

20 normal rats were randomly divided into daytime group (13:00, n=10）and nighttime group (1:00, n=10). We evaluated the expression of Per2 and c-fos mRNA in the Hb by semi-quantity RT-PCR. As shown in Fig.1, the transcriptional level of Per2 in the Hb at 13:00 and 1:00 was expressed significantly difference, with higher expression of Per2 mRNA at 13:00 than at 1:00 (t=5.006, P=0.001) under LD conditions（Fig.1）

Moreover, the expression of c-fos mRNA in the Hb showed the same tendency: the level of c-fos mRNA at 13:00 was higher than that at 1:00 (t=3.495, P=0.008) (Fig.1).

*3.2 Expression of Per2 and C-fos mRNA in the Hb of SCN Lesion Rats*

The expression of Per2 and c-fos mRNA in Hb was also evaluated in SCN lesion and sham group rats under LD conditions. In order to determine whether the SCN was destroyed, we measured and compared the water consumption between SCN lesion group and sham group. The results showed that the water consumption of SCN lesion group increased obviously during the day, compared with that of sham group (Fig.2C).

Based on the results mentioned above, the RNA of the Hb was isolated for RT-PCR. The results indicated that the the difference of Per2 expression in the Hb was kept in sham group, with higher at 13:00 and lower at 1:00 ($t=12.59$, $P=0.0001$). However, the difference of Per2 mRNA expression in SCN-lesioned rats disappeared, which showed that there was no significant difference between these two time points ($t=2.104$, $P=0.07$) (Fig.2A&B).

Moreover, c-fos mRNA expression in the Hb in SCN-lesioned rats was also detected and got the same trend as Per2 mRNA expressed. The rhythmicity of c-fos mRNA in the Hb did not change in sham group ($t=4.963$, $P=0.001$), while the difference was found to disappear in SCN-lesioned group ($t=0.62$, $P=0.55$) (Fig.2A&B).

## 4. Discussion

Our previous study found that Per2 gene and protein expression in the LHb at six zeitgeber time points in a 12h light and 12h dark(LD) condition had rhythmic oscillation with higher expression at the daytime and lower expression at the nighttime (Zhao, 2015). The cells in both the lateral habenular nucleus (LHb) and medial hebenular nucleus (MHb) also showed higher baseline firing rates during the day than during the night(zhao,2005). Similarly, the rhythmic oscillation of Per2 expression and firing rates with higher at the day and lower at the night have also been found in the SCN neurons (Amir, 2004; Oishi, 1998; Groos, 1982; Inouye, 1979), suggesting that there may be functional connections between the SCN and the Hb. Therefore, in this experiment, the higher expression time point (13:00) during the light phase and lower expression time point of Per2 (1:00) during the dark phase based on zhao's study were selected to test the effects of SCN lesion on Per2 and c-fos mRNA expression The result in normal rats showed that there was high Per2 and c-fos mRNA level on 13:00 and low level on 1:00, which was coincident with the Zhao's (Zhao, 2015) and had similar trend with firing rates of neurons in Hb (Zhao, 2005). The characteristics of this rhythm are also consistent with that of the SCN neurons under LD condition (Harbour, 2013; Inouye, 1982; Rocha, 2014). Further, in the SCN lesioned experiment we found the difference of Per2 and c-fos mRNA expression in the Hb at the 13:00 and 1:00 disappeared after the SCN lesion, suggesting that the rhythmic expression in the Hb may denpend on the presence of the SCN. The SCN lesion was assessed by measuring and comparing the water consumption between SCN lesion group and sham group in the experiment. The results showed that the water consumption of SCN lesion group increased obviously during the day, compared with that of sham group, suggesting that the SCN was successfully destroyed in the experiment.

It has been widely accepted that SCN is the dominant circadian pacemaker in mammals. The SCN lesion causes the rhythmic disorder of running-wheel and drinking (Stephan, 1972), and SCN transplantation makes circadian rhythms restore in the behavior in SCN-lesioned animals (Ralph, 1990). The circadian rhythm is basically regulated via the release of Melatonin (MT) in retina-SCN-pineal gland axis (Moore, 1996). However, the maintenance of the circadian steady rhythm is not limited to the role of these two brain areas. It has been detected that there are some circadian oscillators outside the SCN, such as arcuate nucleus, paraventricular nucleus of the hypothalamus and so on (Abe, 2002). Recently, it was demonstrated that the Hb is also a member of these oscillators (Zhao, 2005; Zhao, 2015). Anatomical studies have provided the evidence that the vasopressinergic fibers from the SCN appear in the medial part of lateral habenula (LHbM) in different animals (Sofroniew, 1978), which are echoed with the results of our experiment. The SCN mainly receives a direct retinal projection and a serotonergic projection from the raphe nuclei. It was reported that the Hb is a key position to control the raphe neclei(Wang, 1980;Wang,1977). Thus, the Hb not only receives the projection and control of the SCN, and may also modulate the SCN function by influencing the activity of raphe nucleus.

This study showed that the different expression of Per2 and c-fos mRNA in the Hb at 13:00 and 1:00 under LD condition is dependent on SCN. The Hb may participate in the center circadian regulation as a secondary structure of SCN.

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**Appendix A**

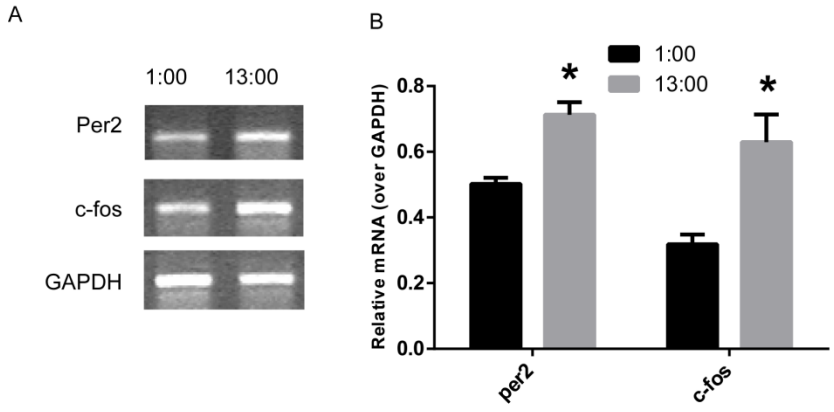


Figure 1 The expression of Per2 and c-fos gene in the whole Hb. (A) Representative gel electrophoresis of semi-quantitive polymerase chain reaction products stained with ethidium bromide (EB), demonstrating that Per2 and c-fos gene expressed more strongly in the daytime than that in the nighttime; and (B) Relative quantitative analysis of Per2 and c-fos mRNA in the LHb at two different time points. All values are normalized against the housekeeping gene GAPDH. Data are presented as mean ± SEM (n = 10) (**P*<0.05, compared with 13: 00).

**Appendix B**

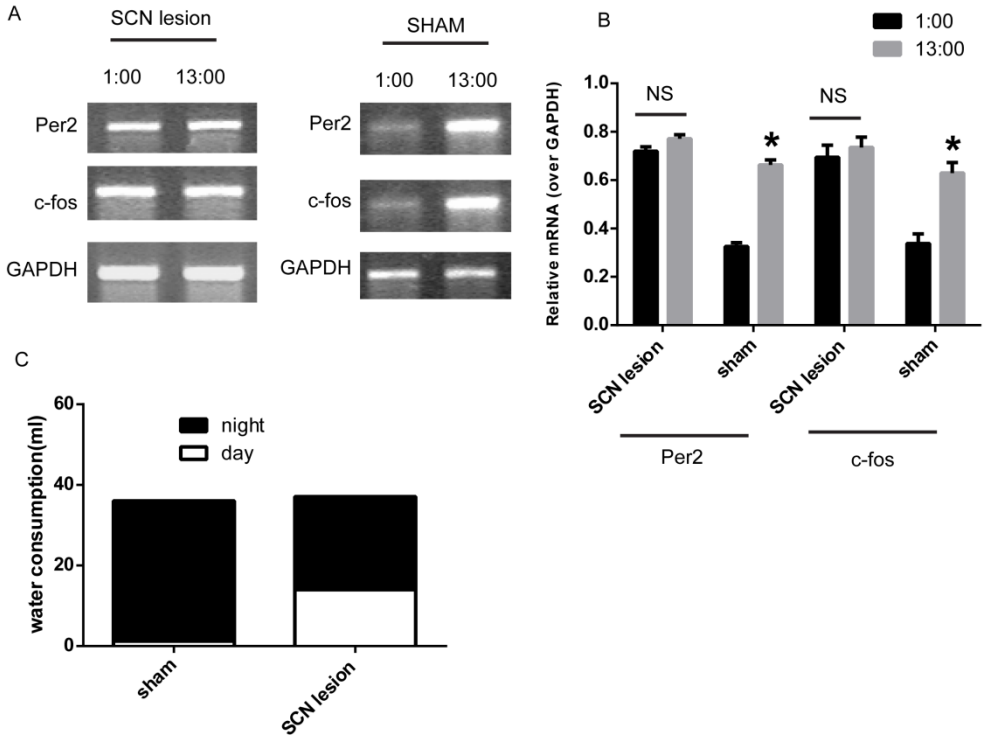


Figure 2 The expression of Per2 and c-fos gene in the whole Hb in the SCN lesion group and sham group rats. (A) Results of semi-quantitive PCR products showed that the rhythmicity of Per2 and c-fos mRNA in the Hb rats disappeared after SCN was destroyed (SCN lesion). Whileas the rhythmic vibration of these genes maintained in the sham group rats. (B) The density of Per2 and c-fos mRNA was normalized against GAPDH at two different time points. The water consumption of rats was measured in the SCN lesion group and sham group. (C) The water consumption grew up markedly in the daytime compared with sham group. Data are presented as mean ± SEM (n = 10) (**P*<0.05, compared with 13: 00).