Development of Clock Genes Expression in Rat Hippocampus

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Background: *The circadian rhythms in the suprachiasmatic nucleus (SCN), a central clock, are generated by autoregulatory network composed of clock genes that encode transcriptional factors. There is a gradual development of clock gene expression in the SCN during ontogenesis. Moreover, clock genes are expressed in the adult hippocampus with circadian fashion.*
Objective: *It is of interest to examine daily profiles of the clock gene mRNA and protein expressions in rat hippocampus during development.*
Material and Method: *Daily profiles of three clock genes (Per1, Per2, and Bmal1) mRNA, and their protein expressions were analyzed in the rat hippocampus of pups at postnatal (P) day 4 and 8 (P4 and P8), pre-weaning stage (P16), early pubertal stage (P32), and adult (P60) by real-time PCR and immunohistochemistry.*
Results: *The entire studied clock gene mRNAs and proteins did not exhibit circadian rhythm in early postnatal P4-P16. Rhythmic expression of Per1 and Per2 mRNA started at P32, whereas Bmal1 began at adult. However, their proteins showed circadian expression together at adult.*
Conclusion: *The present study suggests that rat hippocampal molecular clock works gradually develop after birth and slower than that in the central clock SCN. It was possible that ontogenetic development of clock gene in hippocampus was waiting for central clock synchronization.*

Keywords: *Hippocampus, Clock gene, Circadian, Postnatal rat*

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The circadian pacemaker that drives most biological rhythms is located in the suprachiasmatic nucleus (SCN) in mammals. The circadian rhythm within the SCN is generated by intracellular molecular machinery that is based on an interlocking positive and negative transcriptional/translational feedback loop of clock oscillations. The positive components of the main feedback loop are set of clock proteins, a heterodimer of the circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein-1 (BMAL1), which activate the rhythmic transcription of three Period genes (*Per1*, *Per2*, and *Per3*) and two Cryptochrome genes (*Cry1* and *Cry2*). Then the mRNAs are translated into PER and CRY proteins in the cytoplasm. These proteins form a complex and act as negative components by translocating back into a nucleus to suppress the transcription of their genes by inhibiting CLOCK: BMAL1 heterodimer activity[1]. In addition, several modification processes of post-transcriptional and post-translational help to maintain the accuracy of the circadian rhythms according to approximately 24 hour of the daily cycle[2].

It is known that the rhythmic expression of clock genes is not limited to cells in the SCN, but has been detected in other brain areas and peripheral tissues with a circadian fashion[3,4]. However, other brain clock oscillations are different from the oscillation in the SCN[5]. Moreover, brain clocks also mediate functional outputs by regulating clock-controlled genes in a tissue-

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specific manner[6]. Interestingly, clock genes are expressed with circadian pattern in hippocampus, which plays a role in learning and memory[7].

Previous studies have demonstrated ongoing circadian clock genes expression in the SCN and peripheral tissues during development[8,9]. There was no report, which showed when the clock molecules started to show oscillation in rat hippocampus. In addition, *Per1* and *Per2* not *Per3* were reported to be essential for core circadian loop[10]. Therefore, the purpose of the present study was to investigate daily expression profiles of three clock gene mRNAs and proteins act in negative and positive feedback loop (*Per1*, *Per2*, and *Bmal1*) in rat hippocampus during development, at early postnatal stage (P4 and P8), pre-weaning stage (P16), early pubertal stage (P32), and adult (P60).

Material and Method

Animals

Pregnant Wistar rat (National Laboratory Animal Center of Mahidol University, Salaya, Thailand) were housed separately at least one week under a 12 hour light/12 hour dark with the light on at six o'clock in the morning or Zeitgeber time (ZT) 0. Animals were given with *ad libitum* access to food and water before parturition. For postnatal studies at postnatal (P) day 4, P8, and P16, newborn pups were kept with their mother through the experiment. The study was performed in accordance with experimental protocols approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (under license No. 10/2551).

Tissue preparation

All animals were sacrificed at 4-hour interval throughout the daily cycle (n = 3 animals per time point) beginning at ZT03. For real-time PCR, animals in each group were sacrificed by rapid decapitation under dim red light. The hippocampi were dissected, quickly frozen and stored at -80°C until RNA isolation.

For immunohistochemical study, animals were deeply anesthetized with sodium pentobarbital (40 mg/kg, ip) and transcardially perfused with a fixative (4% paraformaldehyde). The brains were removed and post fixed in the same fixative at 4°C overnight and then transferred to 30% sucrose for 48 hour. The tissue block was cut by a cryostat at 40 μm thick. The free-floating sections were kept at 4°C until use.

RNA isolation and real-time PCR

Total RNA from the hippocampus was extracted with TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Two μg of each RNA sample was reverse-transcribed with High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) according to the users manual, and real-time PCR was performed using with the TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). Each sample was measured in triplicate to ensure the accuracy of data on ABI 7500 real-time PCR system (Applied Bio systems). The relative expression level was calculated using the comparative Ct method to normalize target gene mRNA to β-actin mRNA (*Actb*). The details of TaqMan probe assays are listed in Table 1.

Immunohistochemistry

The immunohistochemical method was performed by the use of goat polyclonal antibodies against of PER1 or rabbit polyclonal antibodies against of PER2 or goat polyclonal antibodies against of BMAL1 (Santa Cruz, CA, USA; 1:100) and either biotinylated rabbit anti-goat IgG antibodies or biotinylated swine anti-rabbit IgG antibodies (DAKO, Glostrup, Denmark; 1:400) with Avidin-biotin complex (ABC Elite, Vector). The protocol of this method has been described previously[11]. The immunoperoxidase activity will be visualized by the light microscopy

Table 1. Assay details of all studied genes for real-time PCR

Interrogated sequence			Translated protein	Exon boundary	Amplicon length
Genes	Assay No.	Ref. sequence			
Per1	Rn01496753_g1	NM001034125.1	NP001029297.1	12-13	142
Per2	Rn01427704_m1	NM031678.1	NP113866.1	22-23	100
Bmal1	Mm01269616_m1	NM007489.3	NP031515.1	9-10	100
Actb	PN4352931E	NM031144.2	NP112406.1	4-5	91

(Olympus BH2, Tokyo, Japan). For semi quantitative densitometric analyzes of the immunoreactions, images were photographed with a digital camera (Olympus DP70, Tokyo, Japan; 8-bit color depth). Densitometric analysis of immunoreactivity was performed using cell Sens Dimension Software (Olympus). Four images per animal were analyzed for each protein and averaged to give a single value for each animal. The relative optical density to background staining was measured within selected areas.

Statistical analysis

All values are expressed as the mean from three animals ± SEM per time point. The experimental data were analyzed using one-way analysis of variance (ANOVA) for time differences and subsequently by Tukey's post hoc tests. A p-value <0.05 was considered statistically significant.

Results

Rhythmic expression of clock genes

Daily expression profiles of clock gene *Per1*, *Per2*, and *Bmal1* mRNA, as well as their corresponding proteins in the hippocampus of P4, P8, P16, P32, and adult rats (P60), are shown in Fig. 1. All the studied clock gene mRNAs and proteins were already expressed at postnatal age; nevertheless, no significant rhythm was found from P4 to P16. At P32, the one-way ANOVA revealed significant time effect on only expression of *Per1* and *Per2* mRNA (p<0.05), but no clear circadian rhythm throughout the day. The mRNA level of *Per1* rose around early midday at ZT07 and reached the maximal level between ZT11 and ZT15 (p<0.05, between ZT03 and ZT15). *Per2* mRNA peaked at ZT11 (p<0.05, between ZT03 and ZT11). However, *Bmal1* mRNA did not show circadian rhythm.

At P60, the entire clock genes mRNAs and proteins were expressed in the circadian manner. There was a significant effect of time on these three clock genes. *Per1* mRNA showed a different pattern of expression when compared to P32. The level of *Per1* mRNA increased slowly between ZT03 and ZT07 then rose faster to ZT11, the peak point (p<0.001). For PER1 protein, the protein expression at ZT19 was significantly higher than other time points (p<0.05 for ZT11 and ZT15; p<0.01 for ZT03 and ZT07) except ZT23. The expression of *Per2* mRNA increased at the beginning of light onset and reached the maximal level at ZT11 (significantly higher than at ZT03 and ZT23; p<0.05). PER2 protein started to rise at ZT07 and reached the maximal level at ZT19 (significantly higher than other time points, p<0.01 for ZT15; p<0.0001 for ZT03, ZT07 and ZT11), which was similar to pattern of PER1 protein expression. According to *Bmal1*, it showed the opposite pattern from *Per1* and *Per2* both mRNA and protein. The level of *Bmal1* mRNA started to rise after ZT15 and then increased rapidly between ZT19 and ZT23.

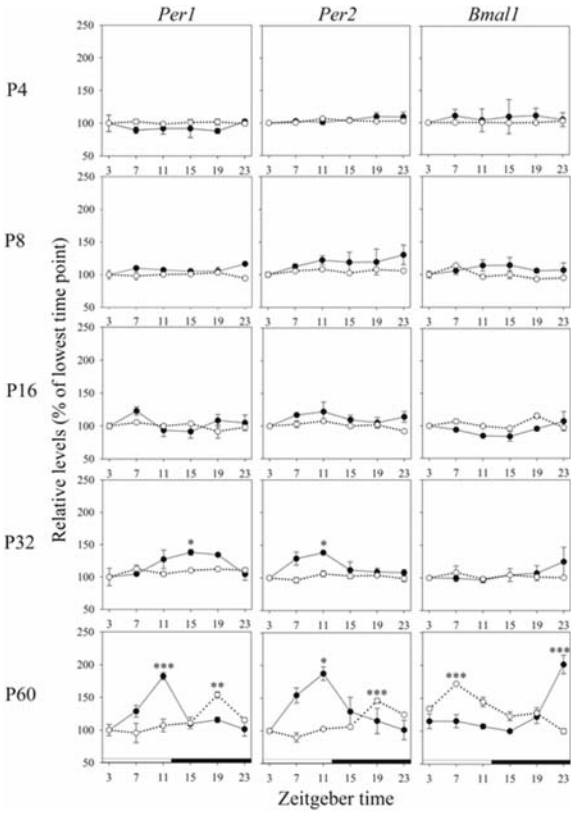

Fig. 1 Developmental expression of clock gene mRNAs and proteins in the rat hippocampus. The graphs show daily profiles of *Per1*, *Per2*, and *Bmal1* mRNAs (closed circles, solid line), as well as PER1, PER2, and BMAL1 proteins (open circles, dashed line) from rats at P4, P8, P16, P32, and P60. The daily profiles for clock gene mRNAs, analyzed by real-time PCR, expressed as % relative values with respect to the mRNA amount at lowest time point. The profiles of clock proteins expression were calculated by semi quantitative densitometric analyzes of immunohistochemistry reactions expressed as % relative optical densities with respect to the protein amount at lowest time point. White and black bars represented the light and the dark phase, respectively. Each point represents the mean ± SEM (n = 3) and considered significant (*) for p<0.05, (**) for p<0.01, and (***) for p<0.001.

Bmal1 expression at ZT23 was significantly higher than values at any other time points ($p<0.001$), whereas BMAL1 protein level began to rise after ZT03 and reached the maximal level at ZT07 (significantly higher than those at ZT15, ZT19; $p<0.01$, and ZT23; $p<0.001$). As a result of the immunohistochemical method, the immunoreactivities of PER1, PER2, and BMAL1 were found in pyramidal cells in *cornu ammonis* (CA)1-CA3 and granule cells in *dentate gyrus* (DG) of the hippocampus (Fig. 2). As mentioned above, significant circadian rhythm of clock proteins occurred at P60, thus at P32, immunohistochemical images showed no difference in expression between daytime (ZT07) and nighttime (ZT23) of these clock proteins. At P60, the higher expression of PER1 and PER2 at ZT23 was observed than that at ZT07, while the higher expression of BMAL1 at ZT07 was observed than that at ZT23.

Developmental dynamics of clock genes

Clock genes oscillation is able to vary significantly throughout the day, and thus, effects the time required to be taken into consideration when trying to compare developmental dynamics of clock genes. Thus, the authors calculated the overall mean values of mRNA and protein expressions throughout the day in the different postnatal ages from the level of each time point. For comparative purposes, the mean value in adult of each clock gene was set to 1, and the mean at other postnatal ages (P4, P8, P16, and P32) were expressed as a ratio relative to the adult (Table 2). The results showed that the level of expression was not much different between the various ages. For PER1

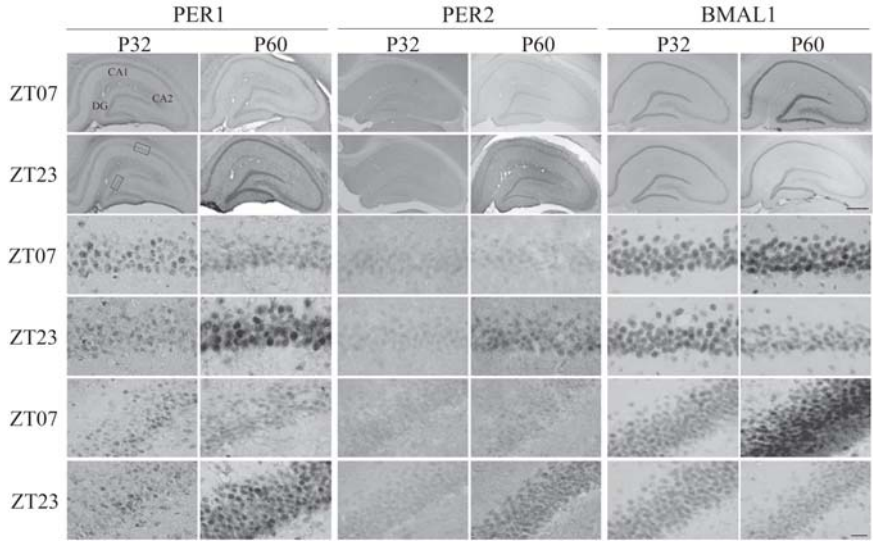

Fig. 2 Photomicrographs of immunohistochemical images of clock proteins expression in the rat hippocampus. Representative images of immunohistochemical staining for PER1, PER2, and BMAL1 in hippocampal coronal sections of early puberty (P32) and adult (P60) rat are shown. Boxed areas are represented as high magnification images of CA1 (third and fourth panels) and DG (fifth and sixth panels). Those immunohistochemical images were selected from investigated time points that showed maximal differences in the clock protein expression. Scale bar: 500 μm (low magnification) and 20 μm (high magnification).

Table 2. Ratio of mean values of clock gene mRNAs and proteins in rat adult hippocampus versus developing hippocampus

Ages	*Per 1*	*Per 2*	*Bmal 1*	PER 1	PER 2	BMAL 1
P4	0.74	0.73	0.76	0.54	0.62	0.71
P8	0.85	0.77	0.78	0.69	0.78	0.73
P16	0.86	0.79	0.79	0.71	0.86	0.84
P32	0.90	0.85	0.83	0.83	0.91	0.87
P60	1	1	1	1	1	1

protein, however, the abundance at P4 was half that of the adult. There was a slight increase in both mRNA and protein levels from the postnatal stage to the adult stage.

Discussion

The present study, the authors demonstrated the ontogenesis of three clock genes *Per1*, *Per2*, and *Bmal1* in rat hippocampus during postnatal stage (P4 and P8), pre-weaning stage (P16), early pubertal stage (P32), and adult (P60). The expression profiles of clock gene mRNAs and proteins have been examined by using real-time PCR and immunohistochemistry, respectively. The results revealed that the entire clock gene mRNAs and proteins have already occurred in hippocampus area as early as postnatal day 4. The present observation suggests that molecular clock genes work efficiently in the rat hippocampus. In addition, the rhythmic expression in each gene and protein in the hippocampus begins at a different age and develops gradually.

The rhythmic expression of *Per1* and *Per2* mRNA started at early pubertal age with different pattern, but developed to similar diurnal patterns in adult rats. However, the significant amplitude oscillation in puberty was lower as compared with that of adult animals. The rhythmic expression of *Bmal1* mRNA was detected at adult, later than that of *Per* mRNA, with maximal level at late dark phase. Concerning the time for peak of clock gene expression, the expression in hippocampus was similar to those in other brain areas such as striatum[12] or peripheral clock such as liver, heart and pineal gland[9,13,14] with peak expression during transition from light to dark for *Per1* and *Per2* and at late dark phase for *Bmal1* mRNA. Interestingly, none of the studied clock proteins exhibited circadian rhythms during developmental stages. The expression of clock proteins displayed a significant rhythm at the adult stage, but the amplitude was lower as compared with that of their mRNAs. In addition, all clock proteins exhibited the opposite expression pattern to their corresponding mRNAs. The present observation confirms the positive and negative transcriptional/ translational machinery in circadian fashion in the rat hippocampus.

Previous studies showed that the rat SCN develops between day 14-17 of embryonic development, but rhythm of clock genes can detect around P3[8,15]. The hippocampus develops both pre- and postnatal; the pyramidal cells in CA1-CA3 hippocampal formation develop before birth, whereas the granular cells in *dentate gyrus* mature postnatal during the first twenty-day after birth[16,17]. It seems that the rhythm of clock genes in the hippocampus occurs after complete hippocampal development. Similar as in peripheral oscillator, circadian rhythm in clock gene expression develop later than the master SCN clock[18,19]. Previous reports suggest that the molecular clockworks in master SCN are mature first and provide the synchronization of other brain and peripheral oscillations[20,21]. However, the mechanism of synchronization requires further elucidation. In addition, the development of clock genes oscillation in different structures able to be described by tissue specificity: development of clock genes expression matures at different ages in different tissues[22]. Another possibility is that food intake, an important zeitgeber for the oscillation of peripheral clock[23], may synchonize the maturation of ontogenetic clock genes development in peripheral tissues and other brain areas. The nurturing behavior of rat mother entrained the circadian rhythms of pups during the first postnatal week[20,24]. The rat mother nurses her offspring during the day, in her rest time, and she feeds during the night[10]. The phase change of *Per1* mRNA expression occurred during P16-P32, which was in between the time of weaning (P19-P22) in the rat. Thus, this changing may be the result of maternal feeding habits and hormone changes, such as lack of maternal melatonin. Hence, it would be of interest to investigate the exact day that rhythmic expression of clock genes occur in rat hippocampus after weaning stage, which help to elucidate the mechanism and factors affecting development of clocks gene expression in the hippocampus.

In conclusion, the results from the present study demonstrate that clock genes in hippocampus gradually develop after birth and the amplitude of oscillation increase during development although no rhythmic expression at postnatal stage. Therefore, they function slower than those in the master clock SCN do. It was possible that ontogenetic development of clock gene in hippocampus was waiting for central clock synchronization. However, further studies are required to elucidate the exact day that rhythmic expression of clock genes occur and the role of clock genes in the hippocampus during development.

What is already known on this topic ?

The development of clock genes expression has been reported in the master clock, suprachiasmatic nucleus, and other peripheral tissues, e.g. heart, liver, pineal etc.

What this study adds ?

The ontogenetic expression of three clock genes (*Per1*, *Per2*, and *Bmal1*) and their proteins were reported in rat hippocampus. These clock genes gradually develop after birth and the amplitude of oscillation increased during development although no rhythmic expression at postnatal stage. The rhythm of these clock proteins occurred significantly at adult. The expression of the clock gene mRNAs and their proteins developed slower than that in the central clock SCN. It was possible that ontogenetic development of clock gene in hippocampus waiting for central clock synchronization.

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Potential conflicts of interest

None.

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การพัฒนาการแสดงออกของจีนควบคุมเวลาในสมองส่วนฮิปโปแคมปัสของหนู

รัชฎาภรณ์ ประมงค์, ประพิมพรรณ วงศ์จิตรัตน์, ปิยะรัตน์ โกวิทตรพงศ์, ปานสิริ พันธุ์สุวรรณ

ภูมิหลัง: *จังหวะรอบวันหลักของสัตว์เลี้ยงลูกด้วยนมถูกควบคุมด้วยสมองส่วน suprachiasmatic nucleus (SCN) โดยการทำงานเป็นวงจรของจีนและโปรตีนควบคุมเวลาการพัฒนาของจีนควบคุมเวลาใน SCN เกิดขึ้นอย่างค่อยเป็นค่อยไปสัมพันธ์กับช่วงเวลาของการเจริญพัฒนา นอกจากนี้ยังพบการแสดงออกของจีนควบคุมเวลาในสมองส่วนฮิปโปแคมปัสเป็นจังหวะรอบวันอีกด้วย*

วัตถุประสงค์: *เพื่อศึกษาการแสดงออกของจีนและโปรตีนควบคุมเวลาระหว่างการเจริญพัฒนาของสมองส่วนฮิปโปแคมปัส*

วัสดุและวิธีการ: *ตรวจวัดปริมาณ mRNA และโปรตีนของจีนควบคุมเวลา Per1, Per2 และ Bmal1 ในรอบวันจากสมองส่วนฮิปโปแคมปัสของหนูแรทหลังคลอดอายุ 4 และ 8 วัน หนูที่เริ่มหย่านมอายุ 16 วัน หนูวัยเจริญพันธุ์อายุ 32 วัน และหนูโตเต็มวัยอายุ 60 วัน โดยเทคนิค real-time PCR และ immunohistochemistry*

ผลการศึกษา: *มีการแสดงออกของจีนและโปรตีนในสมองส่วนฮิปโปแคมปัสของหนูแรทตั้งแต่อายุ 4 วัน แต่ยังไม่เป็นจังหวะรอบวัน เมื่อหนูมีอายุ 32 วัน พบมีการแสดงออกเป็นจังหวะรอบวันของจีน Per1 และ Per2 ในขณะที่พบการแสดงออกของจีน Bmal1 เป็นจังหวะรอบวันในหนูโตเต็มวัย ส่วนการแสดงออกของโปรตีนนั้น พบมีการแสดงออกเป็นจังหวะรอบวันของทั้ง 3 จีนในหนูโตเต็มวัย*

สรุป: *การแสดงออกของจีนควบคุมเวลาในสมองส่วนฮิปโปแคมปัสมีการพัฒนาไปอย่างช้า ๆ และเกิดขึ้นหลังจากการพัฒนาของจีนควบคุมเวลาใน SCN ซึ่งอาจเป็นไปได้ว่าการพัฒนาของจีนควบคุมเวลาในสมองส่วนฮิปโปแคมปัสต้องอาศัยจังหวะการควบคุมจาก SCN*