The Expression of *Per1* and *Aa-nat* Genes in the Pineal Gland of Postnatal rats

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Background: *The circadian rhythm of melatonin synthesis is controlled by the master clock, suprachiasmatic nucleus (SCN). The level of melatonin changes throughout the aging process. The SCN's rhythm is driven by autoregulatory feedback loop composed of a set of clock genes families and their corresponding proteins. The Period (Per1), one of clock gene develops gradually during postnatal ontogenesis in the rat SCN and is also expressed in the pineal gland.*
Objective: *It is of interest to study the relationship between the postnatal development of Per1 and Aa-nat, genes that produce the rate-limiting enzyme in melatonin synthesis, in the pineal.*
Material and Method: *Daily profiles of mRNA expression of Per1 and Aa-nat were analyzed in the pineal gland of pups at postnatal ages 4 (P4), P8, P16 and P32, at puberty age of 6 weeks; and in 8 week-old adult rats by real-time PCR.*
Results: *As early as P4, Per1 and Aa-nat mRNAs were expressed and existed at relatively high levels during the nighttime. They gradually increased until puberty and decreased at 8 weeks of age. Additionally, the nocturnal changes of Per1 and Aa-nat mRNA levels in the rat pineal gland from P4 to adults were strongly correlated at $r = 0.97$ ($p < 0.01$).*
Conclusion: *The present data indicate that there is a close relationship between the expression pattern of Per1 and that of melatonin synthesis during the development of postnatal rats.*

Keywords: *Pineal gland, Per1, Aa-nat, Postnatal rat*

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The mammal's pineal gland synthesizes and releases melatonin in a circadian rhythm pattern that is controlled by the body's master clock, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus[1]. The SCN conveys day/night information to the pineal gland through a sympathetic noradrenergic innervation. Nocturnal noradrenergic release induces a marked increase in the expression of *arylalkylamine-N-acetyltransferase (Aa-nat).* This is followed by an increase in AA-NAT protein, a rate-limiting enzyme in melatonin synthesis and activity, especially in rats. The rat melatonin level changes according to the rat's stage of maturation which increases during postnatal life and childhood, but rapidly drops at puberty[2]. Together, both *Aa-nat* expression[3] and AA-NAT activity[4] have developmental patterns that parallel the circadian rhythm-guided melatonin rhythm.

The circadian rhythm within the SCN is generated by a transcriptional/translational feedback loops composed of a set of clock genes families and their corresponding proteins[5]. *Per1* has been suggested to be a central component in the loops that plays a critical role in the molecular mechanism underlying circadian rhythmicity in mammals from cells to system levels[6]. Recently, the expression of several clock genes has been demonstrated in the pineal glands. The expression of *Per1* mRNA in the pineal gland significantly increases at night time which corresponded well to that of *Aa-nat* mRNA. Moreover, previous studies demonstrated that the regulation of *Per1* and *Aa-nat* genes is similar and depends on noradrenergic system[7].

The ontogenesis of *Per1* expression in the SCN was detected at the embryonic stage and showed the circadian rhythm at postnatal day 1[8]. However,

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the little is known about the ontogeny of the circadian oscillatory mechanism of *Per1* and the role of clock gene in the rat pineal is also still unknown. The present study, aims to examine the circadian expressions of *Per1* and *Aa-nat* in the rat pineal gland during postnatal, puberty and adults periods and determine the relationship between these two genes during development.

Material and Method

Animals and tissue sampling

Pregnant Wistar rats (National Laboratory Animal Center of Mahidol University, Salaya, Nakornpathom, Thailand) were housed separately for one week under 12 h light/12 h dark with the light on at Zeitgeber time (ZT) 0. After parturition newborn pups were kept with their mothers throughout the experiment. All animals were supplied with access to water and food *ad libitum*. Male rats at postnatal ages of 4, 8, 16, 32 days and the puberty age of 6 weeks, as well as 8 week-old adult were sacrificed at 4-h intervals throughout the daily cycle. Then, pineal glands were dissected, quickly frozen and stored at -80°C until RNA isolation and subsequent real-time PCR. All experiments were performed in accordance with experimental protocols approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (under license No. 2/2550).

RNA isolation and reverse transcription

Total RNA from the pineal gland was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (2 µg) was reverse transcribed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions and then stored at -20°C until used.

Real time PCR

The specific primers and probes for the target genes were selected from the TaqMan®Gene Expression Assay (Applied Biosystems). Assay used were as follows: rat *Per1* (NM_001034125.1), assay No. Rn01496753_g1 and rat *Aa-nat* (NM_012818.1), assay No. Rn01461110_m1. In the present study, the rat *beta-actin* gene (*Actb*, NM_031144), assay No. PN 4352931E was used as an endogenous control and for gene normalization. Levels of *Actb* did not vary significantly as a function of time. *Per1, Aa-nat* and *beta-actin* reactions were performed in separate tubes and all samples were run in triplicate for 40 cycles on ABI 7500 real-time PCR system (Applied Biosystems). Quantification was achieved by the SDS software version 1.3.1 by performing the comparative threshold cycle (Ct) method of relative quantification.

Statistical analysis

Daily profiles of *Per1* and *Aa-nat* were presented as means from three animals ± SEM per time point. Data were analyzed by one-way ANOVA for time differences and subsequently by Tukey's post hoc tests. A value of $p < 0.05$ was considered statistically significant.

Results

Developmental expression of Per1 and Aa-nat in the pineal gland

The patterns of daily expression of *Per1* and *Aa-nat* mRNA in the pineal during development were determined (Fig. 1). At P4, the expressions of *Per1* and *Aa-nat* mRNAs were relatively high during the nighttime. At P8, the one-way ANOVA revealed a significant effect of time on expression of *Per1* ($p < 0.001$) and *Aa-nat* ($p < 0.001$). *Per1* expression reached its peak at ZT15, its level increased up to 3-fold compared to the daytime (Fig. 1b). *Aa-nat* mRNA rose significantly from ZT11 to 15 (4-fold) and maintained a plateau until ZT23, when it started to decline ($p < 0.001$, Fig. 1h). A clear circadian rhythm in the expressions of both genes appeared at P16. *Per1* mRNA levels increased up to 9-fold at the peak time at ZT15 (Fig. 1c). *Aa-nat* mRNA peaked at ZT19 (50-fold) and then reached a plateau (Fig. 1i). At P32, *Per1* mRNA level peaked at ZT15, increased up to 10-fold ($p < 0.001$, Fig. 1d) and *Aa-nat* mRNA level peaked at ZT19, a 140-fold nocturnal increase compared to the daytime ($p < 0.01$, Fig. 1j).

At puberty, the peak of *Per1* mRNA at ZT15 was the same as those of P16 and P32, with a 17-fold increase relative to daytime value (Fig. 1e). *Aa-nat* mRNA levels increased after dark onset up to 350-fold at peak (ZT19) and significantly declined at ZT23 ($p < 0.001$, Fig. 1k). The pattern of expression of *Aa-nat* at puberty was different from the pattern at P16 and P32. There was a distinct peak at ZT19 at puberty, while the plateau pattern was found at P16 and P32. In adult rats the Per1 expression was significantly increased at ZT15 ($p < 0.001$, Fig. 1f). The nocturnal increase of *Per1* mRNA levels was 14-fold, which is lower than that of puberty (17-fold). *Aa-nat* mRNA levels increased during the night up to 250-fold, with highest values at ZT19 and

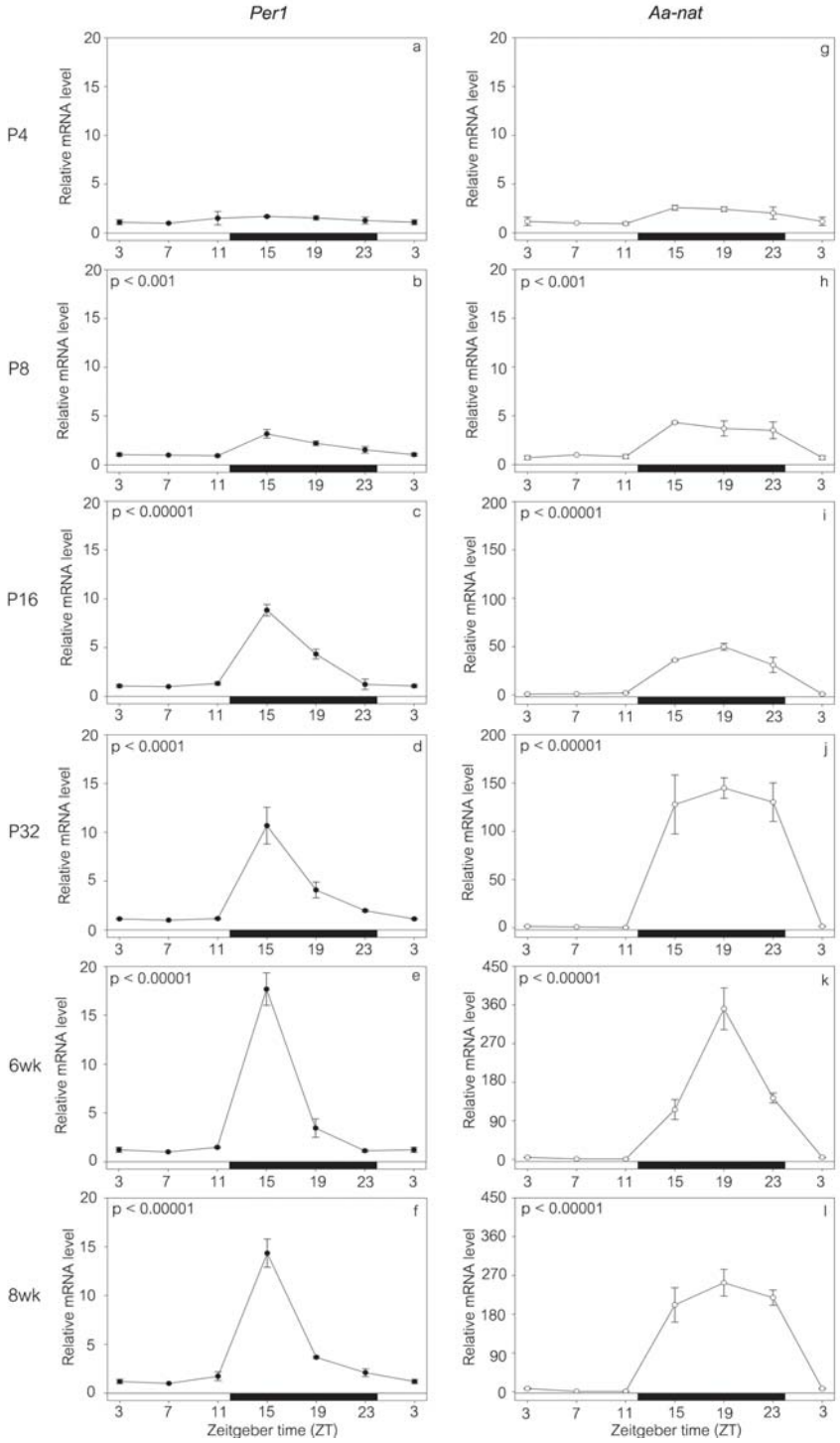

Fig. 1 Development of *Per1* and *Aa-nat* mRNA expression in the pineal gland. The graphs show daily profiles of *Per1* (a, b, c, d, e, f) and *Aa-nat* (g, h, i, j, k, l) mRNAs from rats at P4, P8, P16, P32, puberty (6 weeks old), and adulthood (8 weeks old) analyzed by real-time PCR. The mRNA levels are expressed as relative values with respect to the lowest mRNA amount. Values are means ± SEM (n = 3). White and black bars represent light and dark phases respectively. Global p-values for one-way ANOVA analysis are provided

started to decline at the beginning of the day (Fig. 1l). The nighttime increase of *Aa-nat* mRNA at this age was lower than that at puberty.

Nocturnal levels of Per1 and Aa-nat mRNAs in the pineal gland (Fig. 2)

To compare the levels of *Per1* and *Aa-nat* mRNA expression at night during development, the mean of the maximum levels at the peak time point was calculated from three animals of each age at ZT15 for *Per1* and at ZT19 for *Aa-nat*. The relative change of mRNA expression was compared with the values of P4 animals. The one-way ANOVA revealed the nocturnal expression of *Per1* ($p < 0.00001$, Fig. 2A) and *Aa-nat* ($p < 0.00001$, Fig. 2B) genes began to increase significantly at P8. The nighttime mRNA levels increased progressively up to their highest value at puberty, for *Per1* with 7-fold changes and at P32 to puberty *for Aa-nat* with 4-fold changes and then they decreased towards in the adult at 8 weeks of age. The *Per1* mRNA level significant decreased at adulthood as compare to the level at 6 weeks ($p < 0.05$). *Aa-nat* mRNA level at 8 weeks old showed a significant lower than the levels at P32 and 6 weeks with $p < 0.05$.

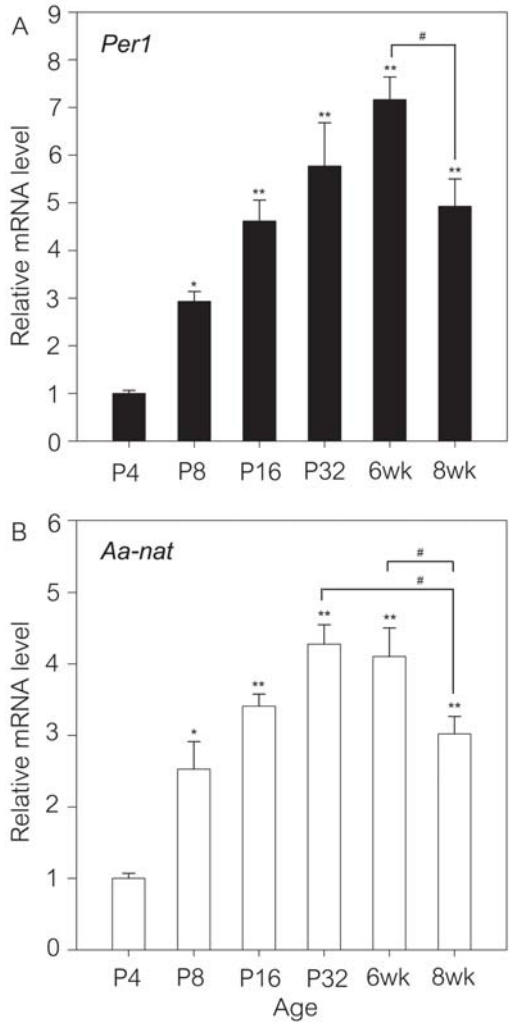

Fig. 2 Relative changes in nocturnal mRNA levels at the peak times of (A) *Per1* (ZT15) and (B) *Aa-nat* (ZT19) in the rat pineal gland of six different ages. Values are mean ± SEM (n = 3) and are expressed as relative mRNA levels with respect to the P4 sample. *$p < 0.05$ and **$p < 0.001$ showed a statistically significant difference with the P4. #$p < 0.05$ shows a statistically significant difference between postnatal age and adulthood

Correlation between Per1 and Aa-nat expression in the pineal gland

The nocturnal changes of *Per1* and *Aa-nat* mRNA levels in the rat pineal gland from P4 to adults were strongly correlated at correlation coefficient (r) = 0.97 ($p < 0.01$, Fig. 3). The changed pattern of *Per1* expression that had an increase before puberty and a decrease after puberty corresponded well to *Aa-nat* expression.

Discussion

In the present study, the authors present a complete 24h daily rhythm of *Per1* and *Aa-nat* mRNA

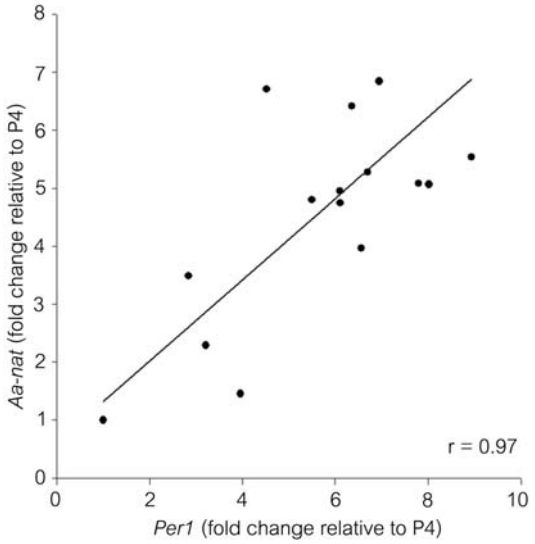

Fig. 3 Correlation between nocturnal *Per1* and *Aa-nat* expression in the rat pineal gland during development at r = 0.97 ($p < 0.01$)

expression in the rat pineal gland during postnatal development, puberty and adulthood. The circadian rhythm of *Per1* and *Aa-nat* expressions started at early postnatal age with similar rhythmic pattern and showed a gradually increase of the amplitudes during the development.

Present study has shown that the rhythmic of *Per1* in the pineal gland was detected later after birth. It corresponded well to the previous report that the overt circadian rhythms of the clock genes of rodent species develop at the postnatal stage but do not oscillate at birth[9]. In the SCN, *Per1* expression starts to express at the embryonic stage, and a marked rhythm is present at P2[10]. In the pineal gland, a significant daily rhythm of *Per1* occurred at P8 which is later than that in SCN. Similarly, the circadian expression of *Per1* in other peripheral oscillators; heart and liver started at P5[11] and P10[12], respectively. These results confirmed that the ontogenesis of the clock gene expression, especially *Per1,* develops first at the master clock and then further develops in the peripheral oscillators. The SCN clock appears to entrain peripheral oscillators by way of neuronal connections or humoral factors[13,14]. The mechanism that drives the peripheral clock to express its clock genes needs to be further elucidated. In addition, the direct influence that mothers have on their pups cannot be overlooked. Maternal entrainment of circadian rhythm in rat pups persists during the first postnatal week[15]. Another possibility is that the time of maturation of peripheral oscillators may involve the transformation of the expression of clock genes into an overt rhythm[9]. The pinealocytes are already mature before birth, although they are not activated to synthesize melatonin until day P8[4]. The present study also showed that the significant circadian rhythm of *Aa-nat* was appeared at P8 as indicated in previous reports that there was no day and night variation of *Aa-nat* mRNA levels present before P5 in rat pineal[3,16]. Nocturnal *Aa-nat* mRNA expression increased progressively at postnatal ages, reaching the highest levels at puberty (6 weeks old) and decreasing from puberty to adulthood. At puberty, the day/night difference observed in this study was a maximum of 350-fold. The decrease of *Aa-nat* expression between the time of puberty and adulthood in rats corresponded well to previous reports of *Aa-nat* mRNA[16] and melatonin levels[17].

Interestingly, the correlation analysis revealed a relationship between the expressions of *Per1* and *Aa-nat* genes during development. The regulation of both genes in the pineal glands was dependent on the adrenergic control originated from the SCN[18]. Superior cervical ganglionectomy or exposure to light during the nighttime inhibited the expression of *Per1*. The administration of adrenergic agonists increases while nighttime administration of an adrenergic antagonist inhibits *Per1* gene expression[7]. Similar to the *Aa-nat* gene, induction of *Per1* mRNA and PER1 protein involves cAMP/PKA-dependent mechanisms[19,20]. Furthermore, the promoter for the *Per1* gene contains a CRE site[21,22]. Thus, the present study shows a close correlation between the expression of *Per1* and *Aa-nat* not only in adult rats, but also during postnatal development. The high peak of *Per1* mRNA at puberty and the declining pattern during adulthood suggest that this gene may have some relationship to the regulation/setting time of the onset of puberty. *Per1* was the only clock gene for which an increase in mRNA level was temporary related to an up-regulated noradrenaline-release from sympathetic nerves[23]. During puberty, although no changes in noradrenaline content were observed, beta-adrenergic receptor density decreased by 30% between the first and the third month of age in rats[24]. Moreover, the peak time point of *Per1*'s expression (ZT15) was prior to that of *Aa-nat*'s expression (ZT19) in all ages. A recent study in *Per1* deficient mice reported a role for PER1 in the modulation of the rhythmic melatonin synthesis. The differences of *Aa-nat* mRNA levels, AA-NAT activity and concentration of plasma melatonin during night time between the *Per1* knockout and wild type mice have been observed[25].

In conclusion, the present study demonstrated the expression pattern of *Per1* in the pineal of postnatal rats. Together, the close correlation of *Per1* and *Aa-nat* expression during postnatal development and puberty reveals the importance of *Per1* in melatonin synthesis especially during puberty. The role of *Per1* in the pineal gland during development still has to be further elucidated.

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Potential conflicts of interest

None.

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การแสดงออกของยีนควบคุมเวลาชนิด Per1 และยีน Aa-nat ในต่อมไพเนียลของหนูหลังคลอด

ประพิมพรรณ วงศ์จิตรัตน์, ปิยะรัตน์ โกวิทตรพงศ์, ปานสิริ พันธุ์สุวรรณ

ภูมิหลัง: *ต่อมไพเนียลทำหน้าที่สังเคราะห์ฮอร์โมนเมลาโทนินที่มีลักษณะขึ้นลงตามจังหวะรอบวันโดยมีสมองส่วน suprachiasmatic nucleus (SCN) ที่เป็นศูนย์กำกับเวลากลางวันและกลางคืนของร่างกายเป็นตัวควบคุม โดยการแปลรหัสและถอดรหัสของยีนควบคุมเวลา (clock gene) ยีนควบคุมเวลาชนิด Period1 (Per1) นอกจากจะมีการแสดงออกที่ SCN แล้ว ยังพบที่ต่อมไพเนียลด้วย สำหรับปริมาณเมลาโทนินนั้นพบว่ามีการเปลี่ยนแปลงไปตามอายุด้วย*

วัตถุประสงค์: *มีความน่าสนใจที่จะศึกษาความสัมพันธ์ระหว่างการพัฒนาการของการแสดงออกของยีน Per1 และยีน Aa-nat ซึ่งเป็นยีนของเอนไซม์สำคัญในการสังเคราะห์เมลาโทนินของต่อมไพเนียล*

วัสดุและวิธีการ: *ตรวจวัดปริมาณ mRNA ของทั้ง Per1 และ Aa-nat ในรอบวันจากต่อมไพเนียลของหนูแรทอายุ 4, 8, 16, 32 วัน หนูวัยเจริญพันธ์อายุ 6 สัปดาห์ และหนูโตเต็มวัยอายุ 8 สัปดาห์ โดยเทคนิค real-time PCR*

ผลการศึกษา: *มีการแสดงออกของยีนทั้งสองในต่อมไพเนียลของหนูแรทตั้งแต่อายุ 4 วัน โดย Per1 ค่อยๆ เพิ่มการแสดงออกขึ้นจนถึงระยะเริ่มเจริญพันธุ์ และจะลดระดับเมื่อถึงอายุ 8 สัปดาห์ ที่เป็นระยะโตเต็มวัย ซึ่งสอดคล้องกับการแสดงออกของ Aa-nat โดยมีค่าสัมประสิทธิ์สหสัมพันธ์ (r) = 0.97*

สรุป: *การแสดงออกของยีนควบคุมเวลา Per1 และการสังเคราะฮอร์โมนเมลาโทนินในระหว่างการเจริญพัฒนาของหนูหลังคลอดนั้นมีความสัมพันธ์กัน*