Differential daily expression of Per1 and Per2 mRNA in the suprachiasmatic nucleus of fetal and early postnatal mice

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Abstract

It is well known that there are circadian rhythms of 2-deoxyglucose uptake and neuronal firing in the rat suprachiasmatic nucleus (SCN) during fetal and early postnatal periods. A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 function as positive regulators and three mPeriod (mPer) genes play a role in negative feedback. Per genes expression occurs not only in the adult SCN but also in the fetal SCN. However, the developmental change in these genes remains unclear. In this experiment, we examined the day–night pattern of expression of Per1 and Per2 mRNA in the mouse SCN and cerebral cortex on embryonic day 17, postnatal day 3, and in young adult mice under a light–dark cycle. Daily rhythms of mRNA content were observed in mPer1 but not mPer2 in the fetal SCN. Interestingly, the expression of mPer2 in the SCN was high throughout the entire day, and a significant daily rhythm of this gene was observed on postnatal day 6. The expression pattern of SCN mPer1 in constant darkness was similar to that seen in the light–dark cycle. The present results suggest that the daily oscillation of mPer1 but not of mPer2 in the SCN in fetal and early postnatal mice may be associated with the daily rhythms of 2-deoxyglucose uptake and neuronal firing.

Introduction

In mammals, the hypothalamic suprachiasmatic nucleus is a pacemaker for both behavioural and hormonal circadian rhythms. A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 function as positive regulators and three mPeriod (mPer) genes, Per1 (Sun et al., 1997; Tei et al., 1997), Per2 (Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998a), and Per3 (Takumi et al., 1998b; Zylka et al., 1998), are involved in negative feedback (for review see Dunlap, 1999). In addition, Kume et al. (1999) determined that two mouse cryptochrome genes, mCry1 and mCry2, act in the negative limb of the clock feedback loop.

The pacemaker components are generated and equipped for a long developmental period. In rats, neurogenesis of the SCN is completed by embryonic day 18 (E18) (Iliff, 1972; Altman & Bayer, 1978). Using 2-deoxyglucose autoradiography, Reppert & Schwartz (1984) reported a circadian rhythm in SCN metabolic activity just after neurogenesis of the SCN. We previously reported that a weak day–night difference in neuronal firing was observed in the rat SCN during the perinatal period, and this difference was obvious 1–3 weeks into the postnatal period (Shibata & Moore, 1987). Ban et al. (1997) reported a phenotypic change in vasoactive intestinal peptide-expressing neurons from postnatal day 10 (P10) to P20 in the SCN. These studies suggest that low-amplitude oscillations during the embryonic stage and early postnatal stage may reflect low-amplitude circadian oscillations in clock genes, low coupling of each oscillatory cell or low amplitude of output signals such as metabolic and firing rhythms.

Although Shearman et al. (1997) have reported a robust expression of mPer in the mouse neonatal SCN, there are no reports on the developmental aspects of the mPer gene in the SCN. Interestingly, these clock genes are expressed not only in the SCN but also in other brain areas such as the cerebral cortex and hippocampus, as well as in peripheral tissues (Albrecht et al., 1997; Shearman et al., 1997; Tei et al., 1997; Zylka et al., 1998), suggesting that the molecular oscillation mechanism exists in most cells. Therefore, in the present experiment we investigated the development of daily expression of the mPer1 and mPer2 genes in the mouse SCN and cerebral cortex under a light–dark cycle.

Recently, it was shown that light-induced Fos-like protein in the mouse SCN initially occurred on postnatal day 4 (Munoz Llamosas et al. 2000). In order to prevent light from directly affecting the rhythm of mPer gene expression, we examined the circadian expression of these genes in P3 mice under conditions of constant darkness.

Materials and methods

Animals and sampling

Pregnant female ICR mice (Takasugi Animal Co, Tokyo, Japan) were obtained at 6–10 days of gestation (timed pregnancy) and were maintained under a light–dark cycle (12-h light : 12-h dark, lights on at 08.00 h). Food and water were given ad libitum. The day after the
matting was designated as E1. Pregnant female or postnatal mice were deeply anaesthetized with ether and intracardially perfused with 0.1 M phosphate buffer (PB) (pH = 7.4) containing 4% paraformaldehyde. The brains of fetuses or postnatal animals were removed, postfixed in 0.1 M PB containing 4% paraformaldehyde for 24 h at 4 °C, and transferred into 20% sucrose in 0.1M PB for 72 h at 4 °C. The time of birth was carefully noted and the following day was designated as P1. We used both male and female fetuses and postnatal mice.

The development of daily rhythms of the mPer1 and mPer2 under a LD cycle was examined using E17, P3, P6, P14, and P50 (adult) animals. Three to five animals from each developmental stage were killed at Zeitgeber times (ZT) 3, 7, 11, 15, 19, and 23 (ZT 0 being 08.00 h). The circadian expression pattern of these genes under constant darkness was also examined using P3 (n = 3–4 for each time point) mice. Animals were adapted to LD conditions until delivery day, then postnatal mice were transferred to constant darkness for 2 days (P1 and P2) with their dam. In this experiment, circadian time zero (CT 0) and CT 12 were at 08.00 h and 20.00 h, respectively. The P3 animals were killed under DD conditions on the third day at CT time points of 3, 7, 11, 15, 19, and 23. The dams were allowed to associate with their litters in the same cage until the time of the experiment. The experimental protocol for the current research was approved by the Committee for Animal Research at Waseda University School of Human Sciences.

In situ hybridization techniques

Slices (30 μm thick), made using a cryostat (Microm, HM505E, Germany), were placed in 2× SSC until processing for hybridization. Slices were treated with 1 μg/mL proteinase K in 10 mM Tris-HCl buffer (pH, 7.5) containing 10 mM EDTA for 10 min at 37 °C, followed by 0.25% acetic anhydride in 0.1 mM triethanolamine and 0.9% NaCl for 10 min. The slices were then incubated in hybridization buffer [60% formamide; 10% dextran sulphate; 10 mM Tris-HCl, pH, 7.4; 1 mM EDTA; 0.6 M NaCl; 1× Denhardt’s solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin); 0.2 mg/mL transferRNA; 0.25% sodium dodecyl sulphate] containing 3P-labelled cRNA probes for 16 h at 60 °C. Radio isotope (RI) [35]SUTP (New England Nuclear, USA) labelled antisense cRNA probes were made from restriction enzyme-linearized cDNA templates [mPer1: nucleotides 538–1752 (Shigeyoshi et al., 1997), mPer2: nucleotides 1–638 (Takumi et al., 1998a)] kindly donated by Dr Okamura (Kobe University, Japan). After a high-stringency post-hybridization wash in 2× SSC (50% formamide) at 68 °C, images for mPer1 and mPer2 mRNA were visualized by autoradiography after 5 days exposure to BioMax MR film (Kodak, USA) and analysed using an image analysing system (MCID, Imaging Research Inc., Canada) after conversion into optical density. Data were normalized with respect to the difference in signal intensities in corresponding areas of the SCN or cerebral cortex and the corpus collosum. In order to confirm the brain areas, brain slices exposed to X-ray film were stained with cresyl violet. The intensities of optical density in sections from the rostralmost to the caudalmost SCN were then summed; this sum was considered a measure of the amount of mPer1 or mPer2 mRNA in this region. We measured the optical density of the motor area of the cerebral cortex using identical sections including the SCN. Preliminary experiments revealed that there were no regional differences of mPer1 expression in the cerebral cortex (data not shown). The values are expressed as means ± SEM. In some cases, we use ‘relative RNA abundance’, which refers to the peak value adjusted to 100.

Statistical analysis

One-way ANOVA was used to detect the daily oscillation of gene expression and changes in developmental time. A post hoc Scheffe’s test was used for comparison purposes.

Results

Figure 1 shows representative fetal and postnatal SCN sections expressing mPer1 and mPer2 mRNAs at various time points. In the SCN of E17 and P3 mice, mPer1 was highly expressed at ZT 7 and ZT 11, with low expression at ZT 15 and ZT 19. In contrast, there was relatively high expression of mPer2 throughout the day. Both mPer1 and mPer2 mRNA signals were observed throughout the entire SCN, including dorsal and ventrolateral areas.

Figure 2 shows development of the daily rhythm of mPer1 and mPer2 mRNAs in the SCN as well as the cerebral cortex. The results of one-way ANOVA revealed that mPer1 expression showed a significant daily rhythm in E17 (F5,14 = 4.6, P < 0.05), P3 (F5,16 = 7.9, P < 0.01), P6 (F5,15 = 5.1, P < 0.01), P14 (F5,18 = 22.1, P < 0.01), and P50 (F5,18 = 5.8, P < 0.01) mice. On the other hand, there was no significant daily expression of mPer2 in E17 (F5,18 = 0.4, P > 0.05) and P3 (F5,16 = 1.8, P > 0.05) mice, although, a robust daily rhythm was observed in P6 (F5,13 = 45.0, P < 0.01), P14 (F5,18 = 23.5, P < 0.01) and P50 (F5,18 = 30.6, P < 0.01) mice. The right panel of Fig. 2 illustrates the development of a daily fluctuation of mPer1 and mPer2 in the cerebral cortex under LD conditions. There are no significant daily fluctuations of either mPer1 or mPer2 in E17 (F5,17 = 2.5, P > 0.05 for mPer1; F5,18 = 0.9, P > 0.05 for mPer2), P3 (F5,17 = 0.7, P > 0.05 for mPer1; F5,18 = 1.5, P > 0.05 for mPer2), P6 (F5,17 = 1.6, P > 0.05 for mPer1; F5,18 = 1.9, P > 0.05 for mPer2) and P14 (F5,17 = 2.6, P > 0.05 for mPer1; F5,18 = 2.2, P > 0.05 for mPer2) mice. However, a significant daily rhythm was observed in mPer1 (F5,18 = 9.6, P < 0.05) and mPer2 (F5,17 = 4.0, P < 0.05) in P50 mice. The peak level in the cerebral cortex occurred between ZT11 and ZT15 and fell to the lowest level between ZT23 and ZT7.

Figure 3 shows the developmental changes in maximum and minimum values of daily mPer1 and mPer2 expression in the SCN under LD conditions. The maximum mPer1 level is 1.6-fold higher than the minimum corresponding to age development from E17 to P50. One-way ANOVA revealed a significant increase in maximum levels of mPer1 (F4,14 = 27, P < 0.01), but not in minimum levels (F4,12 = 2.0, P > 0.05). Interestingly the maximum mPer2 level was approximately similar in all developmental stages, but minimum levels significantly lowered along with developmental stage (F4,12 = 16.3, P < 0.01 by one-way ANOVA).

Fig. 1. Representative in situ hybridization autoradiograms of mPer1 and mPer2 showing the SCN of E17 and P3 mice at various time-points under LD conditions (A), and cerebral cortex of P50 (B) mice. Numbers on the right panel of autoradiograms indicate the sampling zeitgeber time (ZT). (A) Hybridization signals of SCN. (B) Hybridization signals of cerebral cortex. Note the daily changes in signals of mPer1 but not of mPer2 in the E17 and P3 SCN.
In order to prevent light from having an effect on the expression of mPer1 and mPer2 in the SCN, we transferred the P0 mice into DD conditions for 3 days along with their dam. Even under DD conditions, a significant circadian rhythm of mPer1 expression was observed in the SCN (F_{5,15} = 8.4, P < 0.01 by one-way ANOVA). mPer2 (F_{5,15} = 1.7, P > 0.05) expression, on the other hand, was

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FIG. 2. Development of daily expression of mPer1 and mPer2 mRNA levels in the SCN and cerebral cortex during LD conditions as examined by in situ hybridization method. RNA abundance was determined by quantitative in situ hybridization using isotope-labelled probes with the peak value under LD conditions being adjusted to 100. Each value represents the mean ± SEM of three to four animals. Closed circles and open circles indicate mPer1 and mPer2 expression, respectively. Significance of daily rhythm was determined by one-way ANOVA (*P < 0.05, **P < 0.01).
stable throughout the entire day, an occurrence similar to the expression pattern seen in LD conditions (Fig. 4). There was an approximately 1.6-fold difference in the amplitude of day-night changes in mPer1 both under DD and LD conditions.

Discussion

Our present investigations demonstrate a similar daily expression of mPer1 in the SCN in fetal, postnatal, and young adult mice. In contrast, a daily expression of mPer2 in the SCN was not observed on fetal and postnatal day3. Instead, a significant daily rhythm of this gene appeared on postnatal day 6. A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 function as positive regulators and Per and Cry genes participate in negative feedback (Dunlap, 1999; Kume et al., 1999). Therefore, the present results strongly suggest that the negative limb of the clock feedback loop using mPer1 in the SCN is functional during fetal and early postnatal ages. Thus, the present results are consistent with previous papers, which demonstrate that rhythms such as 2DG uptake (Reppert & Schwartz, 1984; Shibata & Moore, 1988) and neuronal firing (Shibata et al., 1983; Shibata & Moore, 1987) within the SCN itself show apparent daily rhythms in fetal and postnatal rats. In the present experiment, the daily rhythm of mPer1 expression appeared in the cerebral cortex during postnatal days P14–50. At present, we do not know why the establishment of a daily rhythm of mPer1 in the cerebral cortex was delayed. One possible explanation is that efferent signals from the SCN to the cerebral cortex are immature. A recent paper demonstrated that the re-entrainment of Per1 gene expression to a phase-shift in the LD cycle was faster in the SCN than in the cerebral cortex and liver (Yamazaki et al. 2000). Thus, it may take an additional several cycles to convey the information from the SCN to the cerebral cortex. Alternatively, positive regulators for the clock feedback system such as CLOCK and BMAL1 may not be mature, especially in the brain rather than the SCN. Further experiments are required to clarify the amount of CLOCK and BMAL1 in developing animals. The flattened oscillation of Per1 and Per2 in the cerebral cortex may be associated with a decrease in the activity-to-rest ratio observed in the early stages of development in animals. It is of course possible that the expression of mPer1 and mPer2 in areas other than the SCN is unrelated to circadian clock function.

Interestingly, we found a delayed onset in the daily oscillation of mPer2 gene expression in the SCN in fetal and postnatal mice. In addition, the lowest level of mPer2 expression was high in the SCN on fetal day E17 and P3, compared to mPer2 on P6 or P14. The present results strongly suggest that the negative limb of the clock feedback loop using Per2 is immature, and that a weak inhibition of feedback may result in high levels of Per2 mRNA in the SCN throughout the entire day. Therefore, the amount of PER2 itself and/

![Fig. 3. Developmental changes in peak and bottom levels of mPer1 and mPer2 mRNA as examined by in situ hybridization method. Vertical bars indicate optical density. Each value represents the mean ± SEM of three to four animals. Closed circles and open circles indicate peak and bottom levels of expression, respectively. Significance of developmental changes in peak and bottom values was determined by one-way ANOVA (*P < 0.05, **P < 0.01) and Scheffe’s test (***P < 0.01 vs P50).](image-url)
or the partner proteins for dimerization (Kume et al., 1999; Yagita et al. 2000) may be inadequate to inhibit Per2 expression. Alternatively, the daily circadian expression of mPer1 in the fetal and postnatal SCN may reflect responses to their dam. A robust daily rhythm of mPer2 expression in the SCN of P6 mice may reflect their clock oscillation, as mice with a deletion mutation of mPer2 displayed a loss of circadian rhythmicity under DD conditions (Zheng et al., 1999). Taken together, these results indicate that the daily expression of Per1, but not Per2, in the SCN is responsible for daily rhythms of 2DG uptake and firing activity in the fetal and early postnatal SCN.

In the present experiment, we found a circadian rhythm of mPer1 gene expression in the SCN under DD conditions. The oscillation pattern of mPer1 mRNA was similar in LD and DD conditions. Therefore, at least until P3, environmental day–night signals are not necessary for circadian rhythmicity of mPer1 mRNA expression in the SCN. There are no previous studies establishing when retinal efferents arrive in the mouse SCN. Nevertheless, the gestation period in mice (21 days) is similar to that of the rat (22 days). Thus, one might predict that retinohypothalamic innervation of the SCN in the mouse could begin by P1 (Leard et al., 1994). The present results are well supported by a recent paper which demonstrated that light-induced Fos-like protein in the SCN initially occurred on P4 in mice (Munoz Llamosas et al. 2000). As periodic treatment with D1-dopamine receptor agonist SKF38393 is capable of entraining the fetuses of dams and early postnatal rats (Viswanathan et al., 1994; Weaver & Reppert, 1995; Grosse & Davis, 1999), it would be of interest to know the role of D1 receptor systems in the daily oscillation of Per1 in the SCN. Further experimentation is required to examine the effect of acute injection of these chemicals or light treatment on Per expression in the fetal and postnatal SCN.

In summary, we have demonstrated a robust daily oscillation of mPer1 in the SCN of fetal and postnatal mice, but that of mPer2 in the SCN appeared after a delayed onset. Thus, the daily oscillation of Per1 mRNA may be associated with daily rhythms of 2DG uptake and firing in the SCN.

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Abbreviations

CT, circadian time; 2DG, 2-deoxyglucose; DD, constant darkness; E, embryonic day; LD, light–dark cycle; P, postnatal day; PB, phosphate buffer; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

References


