Circadian Clock Gene Per2 Is Not Necessary for the Photoperiodic Response in Mice

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Abstract

In mammals, light information received by the eyes is transmitted to the pineal gland via the circadian pacemaker, i.e., the suprachiasmatic nucleus (SCN). Melatonin secreted by the pineal gland at night decodes night length and regulates seasonal physiology and behavior. Melatonin regulates the expression of the β-subunit of thyroid-stimulating hormone (TSH; Tshb) in the pars tuberalis (PT) of the pituitary gland. Long day-induced PT TSH acts on ependymal cells in the mediobasal hypothalamus to induce the expression of type 2 deiodinase (Dio2) and reduce type 3 deiodinase (Dio3) that are thyroid hormone-activating and hormone-inactivating enzymes, respectively. The long day-activated thyroid hormone T3 regulates seasonal gonadotropin-releasing hormone secretion. It is well established that the circadian clock is involved in the regulation of photoperiodism. However, the involvement of the circadian clock gene in photoperiodism regulation remains unclear. Although mice are generally considered non-seasonal animals, it was recently demonstrated that mice are a good model for the study of photoperiodism. In the present study, therefore, we examined the effect of changing day length in Per2 deletion mutant mice that show shorter wheel-running rhythms under constant darkness followed by arhythmicity. Although the amplitude of clock gene (Per1, Cry1) expression was greatly attenuated in the SCN, the expression profile of arylalkylamine N-acetyltransferase, a rate-limiting melatonin synthesis enzyme, was unaffected in the pineal gland, and robust photoperiodic responses of the Tshb, Dio2, and Dio3 genes were observed. These results suggested that the Per2 clock gene is not necessary for the photoperiodic response in mice.

Introduction

Many organisms living outside of the tropical zone adapt their physiology and behavior in response to seasonal environmental changes. Such adaptations include seasonal reproduction, migration, molting, and hibernation. Among various seasonal time cues (e.g., day length [photoperiod], ambient temperature, and rainfall [1–5]), most organisms use the photoperiod as a calendar because it is the most accurate natural predictor of the annual phase [6–9]. Therefore, this phenomenon is called photoperiodism.

In mammals, the eyes are the only photoreceptive organs, and light information received by them is transmitted to the pineal gland via the suprachiasmatic nucleus (SCN), the master circadian pacemaker [10,11]. Melatonin is synthesized in the pineal gland from serotonin by a rate-limiting enzyme, i.e., arylalkylamine N-acetyltransferase (Aanat), during the night and decodes the night length. Melatonin plays a deterministic role in regulating seasonal reproduction in mammals. For example, pinealectomy prevents seasonal reproduction, whereas melatonin administration mimics the effect of short days on reproductive function [10–12]. Although the mode of action of melatonin was a mystery for a long time, recent studies have uncovered its downstream pathway that regulates seasonal reproduction. Dense melatonin receptors are expressed by thyrotrphs within the pars tuberalis (PT) of the pituitary gland [13,14]. Melatonin suppresses expression of the β-subunit of thyroid-stimulating hormone (TSH; Tshb) in the PT through the MT1 melatonin receptor [15,16].

The duration of melatonin secretion is shorter under long day conditions than under short day conditions, meaning that melatonin cannot suppress Tshb in the PT under long day conditions. Long day-induced PT-derived TSH acts as the “springtime hormone” to alert the mediobasal hypothalamus (MBH) of spring in quail, sheep, and mice [17–19]. PT TSH acts on the TSH receptor located within the ependymal cells (ECs) lining the ventrolateral walls of the third ventricle within the MBH and induces expression of type 2 deiodinase (Dio2) and suppresses expression of type 3 deiodinase (Dio3) genes [17,19]. Dio2 is a thyroid hormone-activating enzyme that converts prohormone thyroxine (T4) to bioactive triiodothyronine (T3), while Dio3 is a thyroid hormone-inactivating enzyme that metabolizes T3 and T2 to inactive rT3 and T2, respectively [20,21]. Long day-induced T3 within the MBH appears to regulate seasonal gonadotropin-releasing hormone secretion from the hypothalamus to the pituitary gland to regulate seasonal reproduction [22]. Recent
studies have demonstrated that Eya3 may regulate Tshb expression with circadian clock-controlled TEF and HLF genes [23,24].

It is well established that the circadian clock is involved in the regulation of photoperiodism in various vertebrates including fish [25], reptiles [26], birds [27,28], and mammals [29,30]. It has been proposed that the photoperiod is encoded at the neuronal network level of the SCN [31,32]. It has also been suggested that circadian clock genes within the SCN are sensitive to seasonal time, leading to the encoding and decoding of seasonal information [33–37]. Pittendrigh and Minis proposed the “internal coincidence model” for photoperiodic time measurement [38]. This model assumes the existence of 2 internal oscillators that change their phase relationship under changing photoperiods. High-amplitude 24-h cycles of circadian clock gene expression were observed in the ovine PT [39]. Per expression peaked during the day, whereas Cry expression peaked early at night. The phase relationship between the morning Per peaks and the evening Cry peak changed among photoperiods, and the Per–Cry protein–protein interaction (i.e., internal coincidence timer) is proposed to provide a potential mechanism for generating the photoperiodic response [39].

Although mice are generally considered non-seasonal breeders and their testicles do not show seasonal size changes, they were recently shown to be an excellent model for the study of photoperiodism as a marker at the hypothalamic level [19]. Most inbred strains of mice (e.g., C57BL, 129, DBA, BALB) cannot produce melatonin because they genetically lack Aanat enzyme activity [40]. Therefore, these melatonin-deficient mice do not respond to photoperiodic changes. However, they do show clear photoperiodic responses to melatonin administration at the gene expression level (i.e., downregulation of Tshb and Dio2, upregulation of Dio3). In contrast, melatonin-proficient strains (e.g., CBA and C3H) show clear photoperiodic responses of Tshb, Dio2, and Dio3 to changing day lengths, although the gene switches of Tshb, Dio2, and Dio3 are not sufficient to cause photoperiodic gonadal responses.

Although the circadian clock is involved in the regulation of photoperiodism, the involvement of the circadian clock gene and the internal coincidence timer within the PT in the photoperiodic responses of Tshb, Dio2, and Dio3 remains unclear. Among the various clock genes, Per2 appears to be one of the most important genes because Per2 mutant mice show arrhythmic locomotor activity under constant darkness, whereas most of the clock gene null mice do not show circadian rhythms [41–46]. The Per2 gene is also proposed to be a component of the internal coincidence timer [39]. To test whether the circadian clock gene Per2 is involved in photoperiodic response, we generated melatonin-proficient Per2 deletion mutant mice using the speed congenic method and examined the temporal expression profiles of Per1, Cry1, and Cry2 in the SCN, the pineal gland, and the PT. We also examined Tshb, Dio2, and Dio3, key genes in the regulation of the photoperiodic response, under short day and long day conditions.

Materials and Methods

Generation of Melatonin-proficient Per2 Mutant Mice

Melatonin-deficient Per2 deletion mutant mice [B6.129S7-Per2 tm1Brd/J] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Genotyping of the Per2 locus of the progeny was carried out using polymerase chain reaction (PCR) analysis of the genomic DNA by using a separated PCR protocol (version 1;
Figure 2. Expression analysis of clock genes in the suprachiasmatic nucleus (SCN) under short/long day conditions. Temporal changes in Per1 (A, B), Cry1 (C, D), and Cry2 (E, F) expressions in the SCN of the wild-type and Per2 mutant mice under short-day and long-day conditions. The dark and light lines in each graph represent the data of the wild-type and the Per2 mutant mice, respectively. The bars at the bottom of each graph represent the light conditions. Representative autoradiograms of the SCN are also shown. *P<0.05, **P<0.01 Per2+/+ vs. Per2 m/m at the same time point (Student’s t-test), mean ± SEM (n = 4–5). The arrowhead indicates the peak phase determined using cosinor curve fitting. doi:10.1371/journal.pone.0058482.g002
Pineal

**Short day**

- **Per1**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Cry1**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Cry2**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Aanat**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

**Long day**

- **Per2**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Cry1**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Cry2**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences

- **Aanat**
  - mRNA level (nCi)
  - Time of day (hr)
  - * and ** indicate significant differences
by a computer system (The Chronobiology Kit; Stanford Software System, Stanford, CA, USA).

In situ Hybridization

In situ hybridization was conducted as previously described [48]. Sense and antisense 45-mer oligonucleotide probes for each specific gene were labeled with $[^{33}]P$ dATP (NEN Life Science Products, Boston, MA, USA) using terminal deoxyribonucleotidyl transferase (Invitrogen Life Technologies, Inc.). Coronal sections (20-μm thick) of the SCN, the pineal gland, and the MBH were prepared using a cryostat (Leica Microsystems, Inc.). Hybridization was carried out overnight at 42°C. Two high-stringency post-hybridization washes were performed at 55°C. The sections were air dried and apposed to BioMax MR Film (Eastman Kodak) for 2 weeks.

Analysis of Gene Expression Rhythmicity

When statistically significant differences were observed by one-way analysis of variance (ANOVA), cosinor curve fitting was

**Figure 3. Temporal expression profiles of the circadian clock genes and arylalkylamine N-acetyltransferase (Aanat) gene expressions in the pineal gland.** Temporal gene expressions of Per1 (A, B), Cry1 (C, D), Cry2 (E, F), and Aanat (G, H) in the pineal gland under short-day and long-day conditions. The bars at the bottom of each graph represent the light conditions. Representative autoradiograms of the pineal gland (A, arrowhead) are also shown. *P<0.05, **P<0.01 Per2+/+ vs. Per2 m/m at the same time point (Student’s t-test), mean ± SEM (n = 4–5).

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**Figure 4. Temporal expression profiles of the Aanat gene in wild-type (A) and Per2 mutant mice (B) under short-day and long-day conditions.** Data were replotted from Figure 3. The bars at the bottom of each graph represent the short-day (SD; blue) and long-day (LD; red) conditions. Representative autoradiograms of the pineal gland are also shown. *P<0.05, **P<0.01 LD vs. SD at the same time point (Student’s t-test), mean ± SEM (n = 4–5).

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Jackson Laboratory). The primers used included the following: forward 1, 5′-cttgggtgagatgtcctta-3′; forward 2, 5′-ctatggtagagccagaggt tgtagcactgctgga-3′; reverse 1, 5′-gatcagcta-3′; and reverse 2, 5′-gatcagctgccctca-3′. These mutant mice were backcrossed for 6 generations with the melatonin-proficient CBA/Nslc (Nihon SLC, Shizuoka, Japan) mice. Male wild-type (Per2+/+) and homozygote (Per2 m/m) individuals, which were the most enriched for CBA-type microsatellite markers, were selected using the speed congenic method (Table S1). This study was approved by the Animal Experiment Committee of Nagoya University.

**Animals and Treatment**

Male 4-week-old N7F5 (CBA background Per2+/+ and Per2 m/m) mice were kept under short-day conditions (8 h of light, 16 h of darkness; 8L16D) for 3 weeks in light-tight boxes placed in a room at a temperature of 23±1°C. Food and water were provided ad libitum. At the age of 7 weeks, the mice were divided into 2 groups [19]: the first group was transferred into long-day conditions (16L8D) in which the light onset was advanced by 8 h [24], while the second group was maintained under short-day conditions (8L16D) for 2 weeks. Brains of the 9-week-old mice were collected every 4 h (n = 4–5) under 16L8D or 8L16D, respectively.

**Recording of Circadian Locomotor Activity**

Mice were kept in individual cages equipped with running wheels that were placed in a light-tight box as previously described [47]. Wheel-running activity rhythm was continuously recorded...
Figure 5. No clear internal coincidence timer in the mouse pars tuberalis (PT). Temporal expression profiles of the circadian clock genes (Per1, Cry1, and Cry2) in the PT of wild-type and Per2 mutant mice. The bars at the bottom of each graph represent the light conditions. The dark and light lines in each graph represent the data of the wild-type and the Per2 mutant mice, respectively. *P<0.05, **P<0.01 Per2+/+ vs. Per2 m/m at the same time point (Student’s t-test), mean ± SEM (n=4–5). The arrowhead indicates the peak phase determined using cosinor curve fitting.

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performed as previously described [19]. Genes with values of $R^2 > 0.2$ were classified as rhythmic genes.

**Results**

**Generation of Melatonin-proficient Per2 Deletion Mutant Mice**

We generated melatonin-proficient Per2 deletion mutant mice by using the speed congenic method with microsatellite markers. When we examined the wheel-running activity rhythms, the Per2 mutant mice (Per2 m/m) displayed a shorter free-running period followed by circadian rhythm loss in constant darkness (Figures 1C, 1D), while the wild-type mice (Per2+/+) showed clear circadian rhythms (Figures 1A, 1B).

**Rhythmic Expression of Clock Genes is Attenuated in the SCN of Per2 Mutant Mice**

We examined the expression of the circadian clock genes Per1, Cry1, and Cry2 in the SCN in wild-type and Per2 mutant mice. In the wild-type mice, high Per1 and Cry1 expression levels during the light phase and around the light offset, respectively, were observed under both short-day and long-day conditions (Figures 2A–2D). Although the phases of these rhythmic expressions of Per1 and Cry1 were not altered, amplitude was greatly attenuated in the Per2 mutant mice (two-way ANOVA, $P < 0.01$) (Figures 2A–2D). The expression level of Cry2 was very low in the SCN in both the wild-type and Per2 mutant mice (Figures 2E, 2F). This result was consistent with those of an earlier report [50].

**Per2 is not Essential for Pineal Clock Function**

We next examined the expression of Per1, Cry1, Cry2, and Anaat in wild-type and Per2 mutant mice. Although statistically significant differences were observed between wild-type and Per2 mutant mice at some time points (Student’s t-test, $P < 0.05$), clear rhythmicity also was observed in the Per2 mutant mice (one-way ANOVA, $P < 0.05$) (Figure 3). This result was consistent with that of an earlier report using C3H mice kept under 12L:12D conditions [51]. In contrast, the Per1 and Cry1 expression rhythmicities were not robust under long-day conditions in the wild-type mice (Figures 3B, 3D) (one-way ANOVA, Per1 $P < 0.05$, Cry1 $P > 0.05$). Although high Cry1 expression levels were also observed in the Per2 mutant mice at midnight under the short-day conditions (Figure 5C) (one-way ANOVA, $P < 0.05$), its rhythmicity was not clear under long-day conditions (Figure 5D) (one-way ANOVA, $P > 0.05$). Although low-amplitude rhythmicity in Per1 expression was observed under both short-day and long-day conditions in the Per2 mutant mice (one-way ANOVA, $P < 0.05$), its peak phase was not consistent with that in the wild-type mice (Figures 5A, 5B). The Cry2 expression level was very low and robust rhythmicity was not observed in the PT of wild-type or Per2 mutant mice (Figures 5E, 5F) (Per2+/+ long day and short day, Per2 m/m short day; one-way ANOVA, $P > 0.05$; Per2 m/m long day; one-way ANOVA, $P < 0.05$), a finding consistent with those of previous in rodents [54,55].

**Per2 Mutant Mice Show Robust Photoperiodic Responses at the Gene Expression Level**

The ability of mice to show a photoperiodic response was tested after 2 weeks of either short or long days. When we examined the expression of Eya3 and Tshb in the PT and Dio2 and Dio3 in the ECs, long-day induction of Eya3, Tshb, and Dio2 and suppression of Dio3 expression were observed in both wild-type and Per2 mutant mice (Figure 6) (two-way ANOVA, $P < 0.01$, short day vs. long day). Interestingly, Tshb and Dio2 expression induction under long-day conditions and Dio3 expression under short-day conditions were more robust in Per2 mutant mice than in wild-type mice (Figures 6D, 6F, 6G) (two-way ANOVA, $P < 0.01$, wild-type vs. Per2 mutant). Statistical analysis demonstrated rhythmic expression of the Dio2 gene in both wild-type and Per2 mutant mice under short- and long-day conditions (Figure 6F). However, its peak phase was different between the 2 genotypes under long-day conditions (Figure 6F). The temporal expression profiles of Eya3 and Tshb were also different between the 2 genotypes under long-day conditions (Figure 6B, 6D).

**Discussion**

It is well established that the circadian clock is involved in photoperiodic time measurement in various organisms [25–30]; however, the involvement of the circadian clock genes in the photoperiodic response has remained unclear; to elucidate the involvement, we decided to use the mouse model because gene-targeting techniques are unavailable in other photoperiodic mammals such as hamsters and sheep. Although the original Per2 deletion mutant mice have shown arrhythmicity under constant darkness [42], they were unable to produce melatonin. Therefore, we first generated melatonin-proficient Per2 mutant mice using the speed congenic method. Although a different genetic background sometimes alters the circadian phenotype [56], the wheel-running activity rhythms of melatonin-proficient Per2 mutant mice were similar to those of original melatonin-deficient Per2 mutant mice (Figure 1). When we examined the temporal expression profiles of Per1 and Cry1 expression within the SCN and found that the gene expression amplitude was greatly attenuated (Figure 2). This result is consistent with the results seen in the original melatonin-deficient Per2 mutant mice [42]. Recent studies have suggested that core circadian clock elements within the SCN encode and decode seasonal information [33–37]. Although clock gene expression amplitude was greatly attenuated in the Per2 mutant mice, phase relationships of Per1/Cry1 appeared to be conserved (Figure 2). Unexpectedly, the temporal expression profiles of Per1, Cry1, and Cry2 did not differ greatly in the pinacl...
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Aanat in the pineal gland. Although we found clear rhythmicity of the clock genes and Aanat in the pineal gland of Per2 mutant mice, it is possible that the photoperiodic response in Per2 mutant mice in fact reflects the acute suppression of Aanat by light. To test this possibility, it is important to measure Aanat expression under constant dark conditions. It is also possible to speculate that the conserved phase relationship between Per1/Cry1 in the SCN of Per2 mutant mice enabled the pineal gland to retain seasonal information about melatonin even though the Per1/Cry1 amplitude was greatly attenuated.

When we looked at Per1 and Cry1 expressions in the PT, strong Per1 expression late at night and strong Cry1 expression at midnight were observed in wild-type mice under short-day conditions (Figure 5). Although the timings of the Per1 and Cry1 peaks were consistent with those in earlier reports in C3H mice [51,58], those timings were slightly different from the results of hamsters [59,60]. Per is believed to be expressed at dawn in response to declines in melatonin signal. Therefore, differences in gene expression profiles among species are probably caused by differences in temporal melatonin secretion profiles among species [61,62]. In contrast to the short-day condition, no robust rhythmicity was observed in either Per1 or Cry1 expression under long-day conditions in either genotype (Figure 5). Since clock gene expression in the PT is melatonin dependent [52,63], and the duration of Aanat expression was slightly shorter under long-day than under short-day conditions (Figure 4), the long-day secretion profile of melatonin appeared to be insufficient for generating rhythmic clock gene expression in the PT under long-day conditions (Figure 5). In the Per2 mutant mice, we observed robust rhythmic Cry1 expression under short-day conditions only and Per1 expression rhythmicity was weak (Figure 5). The Cry2 expression level was very low in the PT and the SCN under both short-day and long-day conditions (Figures 2, 5).

In this study, despite the lack of clear evidence of the existence of a Per–Cry internal coincidence timer in the PT, we found robust photoperiodic responses of the Eya3 and Tshb genes in the PT and of the Dio2 and Dio3 genes in the ECs (Figure 6). These results were in marked contrast with those in the sheep PT, which showed clear phase angle differences in the Per and Cry genes under both short-day and long-day conditions [39], suggesting that the Per–Cry internal coincidence timer in the PT is not necessary for the photoperiodic responses of the Eya3, Tshb, Dio2, and Dio3 genes in mice. If the internal coincidence timer within the PT mediates the photoperiodic control of summer or winter physiology and is central in the regulation of photoperiodism, the internal coincidence timer could be highly conserved among various species. The differences observed in PT clock gene expression profiles among species suggest that the internal coincidence timer in the PT observed in or study is not a universal mechanism. Although we failed to find clear internal coincidence timer in the mouse PT, we found different expression profiles of the Per1, Eya3, Tshb, and Dio2 genes between the 2 genotypes under long-day conditions. Different temporal expression profiles of the clock genes (Per1) and Eya3 between the 2 genotypes may reflect the different temporal expression profiles of Tshb and Dio2 gene expression under long-day conditions. Generation of a floxed allele of circadian clock gene driven by a PT-specific gene Cre driver line will clarify the functional significance of circadian clock in the PT in the regulation of photoperiodism.

The circadian clock gene Per2 plays a critical role in the regulation of circadian locomotor activity rhythms. However, our present study showed that Per2 is not necessary for photoperiodic responses in mice, at least within 2 weeks of photoperiodic exposure. Although we have previously reported clear photoperiodic responses within 2 weeks in birds and mammals [17,19,20], 2 weeks is a rather short period of time compared to durations that are usually used for sheep. Therefore, we cannot exclude the possibility that a study duration longer than the current one may reveal some differences between the 2 genotypes. The tau mutation of the circadian clock of the Syrian hamster responded to programmed systemic infusions of melatonin in a manner comparable to that observed in wild-type hamsters [64]. However, this mutation altered the photoperiodic responsiveness of the gonadal axis to melatonin signal frequency, suggesting a role for the circadian clock in the interpretation of a series of signals and the subsequent generation of a photoperiodic response [65]. Thus, the circadian clock is known to be involved in photoperiodic time measurement in various organisms [25–30]. However, we cannot totally exclude the possibility of the involvement of a completely different set of “clock genes” for the photoperiodic time measurement. The existence of a circadian clock mechanism that lacks a transcription-translation feedback loop was recently suggested in some studies [66,67], but how the circadian clock measures day length (i.e., how it defines the photoinducible phase or critical photoperiod—the heart of the photoperiodic time measurement—remains unanswered.

Supporting Information

Table S1 Microsatellite markers used to generate speed congenic mice. Microsatellite loci and polymerase chain reaction annealing temperature (°C) are shown. (TIF)

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Author Contributions

Conceived and designed the experiments: KI TY. Performed the experiments: KI. Analyzed the data: KI. Contributed reagents/materials/analysis tools: KI MI TY. Wrote the paper: KI TY.

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