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RAPID DAMPING OF FOOD-ENTRAINED CIRCADIAN RHYTHM OF CLOCK GENE EXPRESSION IN CLOCK-DEFECTIVE PERIPHERAL TISSUES UNDER FASTING CONDITIONS

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Abstract—Restricted feeding-induced free-running oscillation of clock genes in the liver was studied in homozygous *Clock*-mutant (*Clock*/*Clock*) mice. Similar to wild-type mice, *Clock*/*Clock* mice showed robust food-anticipatory behavioral activity in accordance with a restricted feeding schedule. Also, the peak of all clock gene mRNAs tested was phase-advanced in the liver of *Clock*/*Clock* mice as well as wild-type mice, although the amplitude of clock gene expression was low in *Clock*/*Clock* mice. The food-anticipatory behavioral rhythm in *Clock*/*Clock* mice maintained a period similar to wild-type mice during 2-day fasting after the cessation of restricted feeding. However, during the fasting days after temporal feeding cues were removed, the oscillation of clock genes in the liver and heart, excluding the suprachiasmatic nuclei, appeared to result in arrhythmicity in *Clock*/*Clock* mice. Thus, although the CLOCK-based molecular mechanism is not required for the expression of food-anticipatory activity, intact CLOCK protein might be involved in sustaining several cycles of peripheral circadian oscillations after restricted feeding-induced resetting. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: circadian rhythm, *Clock*-mutant mouse, restricted feeding, food-anticipatory activity, liver.

Circadian rhythms are intrinsic oscillations which control various physiological and behavioral phenomena. In mammals, the master circadian pacemaker (or circadian clock) is located in the suprachiasmatic nuclei (SCN) of the ventral hypothalamus and slave oscillators can be found in most tissues. Output signals from the master pacemaker drive or synchronize the circadian oscillation of many behavioral and physiological variables. When this pacemaker is defective or the SCN is destroyed, the temporal programs are disturbed (LeSauter and Silver, 1998). Surprisingly, recent findings show that the SCN is not required to sustain all circadian rhythms because the SCN lesions do not abolish circadian rhythmicity in the olfactory bulb and many peripheral tissues, but disrupt phase synchrony among the tissues within individuals, suggesting that the SCN acts as an internal synchronizer within the mammalian circadian system (Granados-Fuentes et al., 2004; Yoo et al., 2004).

Although the environmental light/dark cycle (LD) is the most potent synchronizer of the circadian pacemaker in the SCN, it is well established that daily restricted feeding (RF) can synchronize various circadian oscillations. Under RF conditions, animals exhibit anticipatory behavior, as well as anticipatory changes in physiological rhythmicity (Krieger, 1979; Honma et al., 1983; Mistlberger, 1994). Interestingly, these food-anticipatory activity (FAA) rhythms appear independent of the LD cycle and in SCN-ablated animals and can last for up to several weeks after the cessation of RF (Stephan et al., 1979a,b; Mistlberger and Marchant, 1995). In addition, RF can reset the phase of peripheral molecular oscillation without the participation of SCN clock function (Damiola et al., 2000; Stokkan et al., 2001; Hara et al., 2001). These FAA rhythms are controlled by a food-entrainable oscillator (FEO), but the locus of the FEO is still unknown.

Disruption of *Clock* results in severe phenotypic changes in circadian function (Antoch et al., 1997; King et al., 1997). *Clock*-mutant mice are able to entrain to the environmental LD cycles, but display a lengthened and less stable circadian period in heterozygotes (*Clock*/±) and a gradual loss of circadian rhythmicity under constant dark conditions in homozygotes (*Clock*/*Clock*). In *Clock*/*Clock* mice, RNA rhythms of clock genes such as *Period1* (*Per1*), *Per2*, *Per3*, and *Bmal1* are all significantly blunted in the SCN and periphery under *ad libitum* conditions (Jin et al., 1999; Oishi et al., 2000; Shearman et al., 2000). These results suggest that CLOCK protein might play a critical role in the endogenous molecular oscillations in the SCN and peripheral tissues. Recently, we demonstrated that intact CLOCK protein is not required for the RF-induced resetting of diurnal clock gene expression (Minami et al., 2002), as well as the FAA behavioral rhythm (Pitts et al., 2003). However, it is not yet known whether after RF cessation the food-entrained molecular oscillation is sustained in *Clock*-mutant mice in correspondence with the persistence of FAA that has been seen for several cycles. Therefore, to further clarify the role of *Clock* gene in the food-entrainable circadian system, we assessed the sustainment of food-entrained clock gene expression in the liver of *Clock*-mutant mice during the fasting days after RF establishment.

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Abbreviations: ANOVA, analysis of variance; *Clock*/+; heterozygous *Clock*-mutant; *Clock*/*Clock*; homozygous *Clock*-mutant; FAA, food-anticipatory activity; FEO, food-entrainable oscillator; LD, light/dark; PCR, polymerase chain reaction; *Per*, *Period*; RF, restricted feeding; SCN, suprachiasmatic nuclei; SSC, saline sodium citrate; ZT, zeitgeber time.

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EXPERIMENTAL PROCEDURES

Animals and housing

Clock-mutant mice were purchased from Jackson Laboratory (Stock No. 002923) (Bar Harbor, ME, USA) and interbred in our laboratory. Genotypes were determined by polymerase chain reaction (PCR) (Jin et al., 1999). Animals were maintained on a 12-h LD cycle (12 h light at an intensity of 100–150 lux at cage level, 12 h dark) with lights on at 8:30 a.m. (room temperature of 23±2 °C) and allowed access to water and commercial chow (Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum*. The amount of food consumed in 1 whole day did not differ regardless of genotype (data not shown). To assess locomotor activity, mice were housed individually in transparent plastic cages (31×20×13 cm) and their locomotor activity rhythm was measured by area sensors (model FA-05 F5B; Omron, Tokyo, Japan) with a thermal radiation detector. Data were stored on a personal computer. All animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government. Experiments were conducted under the permission of the Experimental Animal Welfare Committee of Waseda University. Every effort was made to minimize animal suffering and the number of mice required for each experiment.

Sample preparation

Mice were deeply anesthetized with ether and intracardially perfused with ice-cold saline. After perfusion, to analyze clock gene expression by RT-PCR, the liver and heart of each animal were rapidly isolated, frozen in liquid nitrogen, and stored at −80 °C until RNA purification. Total RNA was isolated using ISOGEN Reagent (Nippon Gene, Tokyo, Japan), and the remaining DNA was completely removed by RNase-free DNase treatment. Also, for *in situ* hybridization, the brain was quickly removed after perfusion, post-fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for at least 24 h at 4 °C, and cryoprotected in 20% sucrose in phosphate-buffered saline overnight at 4 °C.

Semi-quantitative RT-PCR analysis

For RT-PCR, 100 ng of total RNA was reverse transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen, CA, USA) and GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Specific primer pairs were designed based on the following GenBank published data on *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, and *beta-Actin* genes: *mPer1* [289 bp]: 5′-CAAGTGGCAATGAGTCCAACG-3′ (forward) and 5′-CGAAGTTTGAGCTCCCGAAGT-3′ (reverse); *mPer2* [381 bp]: 5′-CAGACTCATGATGACAGAGG-3′ (forward) and 5′-GAGATGTACAGGATCTTCCC-3′ (reverse); *mBmal1* [344 bp]: 5′-CACTGACTACCAAGAAAGTATG-3′ (forward) and 5′-ATCCATCTGCTGCCCTGAGA-3′ (reverse); *mNpas2* [243 bp]: 5′-CTCAGTGGTCAGTTACGCAG-3′ (forward) and 5′-TGGAGGTGGGTTCTGACATG-3′ (reverse); *beta-Actin* [452 bp]: 5′-GAGGGAAATCGTGCGTGACAT-3′ (forward) and 5′-ACATCTGCTGGAAGGTGGACA-3′ (reverse). To validate the semi-quantitative aspect of our RT-PCR method, we used different PCR cycles (data not shown). Consequently, the following PCR protocol was developed: cDNA synthesis at 50 °C for 30 min followed by 94 °C for 2 min, PCR amplification for 28 cycles with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min. The target clock gene cDNA was always co-amplified with *beta-Actin* cDNA in a single PCR tube. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and analyzed by an EDAS-290 system (Kodak, NY, USA). The intensity of the PCR product of the target gene was normalized to the intensity of *beta-Actin*.

In situ hybridization analysis

To determine the quantity of *mPer1* and *mPer2* mRNA levels in various brain regions, *in situ* hybridization was performed as described in Horikawa et al. (2000). In brief, frontal sections (40 μm thick) were collected and placed in 2× saline sodium citrate (SSC) (33.3 mM NaCl, 33.3 mM $C_6H_5O_7Na_3$, pH 7.0). The sections were then treated with proteinase K [1.0 μg/ml, 10 mM Tris buffer (pH 7.5), 10 mM EDTA] for 10 min at 37 °C, 4% paraformaldehyde in 0.1 M phosphate buffer for 5 min, and 2× SSC for 5 min, followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and 2× SSC for 2×5 min. A radio isotope [α[^{33}P]UTP (New England Nuclear, USA)] -labeled antisense cRNA probe was made using a standard protocol for cRNA synthesis. cDNA templates [nucleotide positions: *mPer1* (538–1752), *mPer2* (1–638)] were kindly provided by Dr. Okamura (Kobe University, Kobe, Japan). Sections were incubated in hybridization buffer [50% formamide, 6× SSC, 0.1 mg/ml denatured salmon sperm DNA, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin) and 10% dextran sulfate] containing labeled cRNA probes overnight at 60 °C. After hybridization, the sections were rinsed in 2× SSC/50% formamide for 45 min followed by a 15-min rinse at 60 °C. Next, sections were treated with RNase A (10 μg/ml) for 30 min at 37 °C, 2× SSC/50% formamide for 2×15 min at 60 °C, and 0.4× SSC for 30 min at 60 °C. *In situ* hybridization images were visualized by autoradiogram and BioMax film (Kodak), then analyzed using an image analyzing system (MCID, Imaging Research Inc., Canada) after conversion into optical density by ^{14}C-autoradiographic microscales (Amersham, UK).

Feeding schedule

To explore the adaptive response of *Clock*-mutant mice to altered food availability, we conducted an RF experiment (see Wakamatsu et al., 2001). Briefly, after 1 day of fasting (termed day 0), mealtime was shifted on day 1. Mice were allowed access to food for only 4 h from ZT5 to ZT9 for 6 consecutive days (day 1 to day 6) (Zeitgeber time; ZT0 is defined as the lights-on time and ZT12 as the lights-off time). From day 7, food was again withdrawn. Animals were killed at ZT5, 11, 17, and 23 on days 6, 7, and 8. During the RF experiment, food intake per body weight was measured in *Clock*-mutant mice.

Statistical analysis

Data from the experiments are expressed as means±S.E.M. These values were analyzed with a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. A $P<0.05$ implied statistical significance.

RESULTS

RF-induced entrainment of locomotor activity in *Clock*-mutant mice

As shown in Fig. 1A, the behavioral rhythm of *Clock*-mutant mice entrained to RF, and RF-induced FAA also appeared. The onset of FAA occurred earlier in *Clock* mutants than in wild-type mice (wild-type: ZT4-5, $P<0.05$ versus *ad libitum* feeding; *Clock*/+, *Clock*/*Clock*: ZT3-4, $P<0.05$ versus *ad libitum* feeding). FAA in the mutants persisted with a period similar to that observed in wild-type mice during fasting days 7 and 8 after RF treatment (Fig. 1B). Food intake and change in body weight during RF did not differ among the three genotypes (data not shown). These results were highly consistent with Pitts's behavioral data (Pitts et al., 2003).