Combination of starvation interval and food volume determines the phase of liver circadian rhythm in Per2::Luc knock-in mice under two meals per day feeding

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Submitted 12 July 2010; accepted in final form 14 September 2010

Hirao A, Nagahama H, Tsuboi T, Hirao M, Tahara Y, Shibata S. Combination of starvation interval and food volume determines the phase of liver circadian rhythm in Per2::Luc knock-in mice under two meals per day feeding. Am J Physiol Gastrointest Liver Physiol 299: G1045–G1053, 2010. First published September 16, 2010; doi:10.1152/ajpgi.00330.2010.—Although the circadian liver clock is entrained by the feeding cycle, factors such as food volume and starvation interval are poorly understood. Per2::Luc knock-in mice were given two meals per day with different food volume sizes and/or with different intervals of starvation between two mealtimes with the total food volume per day fixed at 3.6 g (80 food pellets, ~75% of free feeding) per mouse. The bioluminescence rhythm in the liver produced a unimodal peak but not bimodal peak under the regimen of two meals per day over 14–15 days. Peak Per2 expression occurred concurrently with the mealtime of the larger food volume, when the first and second meal were given as different food volume ratios under a 12 h feeding interval. When an equal volume of food was given under different starvation intervals (8 h:16 h), the peak of the Per2 rhythm was close to peak by mealtime after long starvation (16 h). When food volumes for each mealtime were changed under 8 h:16 h, the peak rhythm was influenced by combined factors of food volume and starvation interval. Food intake after the 16-h starvation caused a significant increase in liver Per2, Dec1, and Bmal1 gene expression compared with food intake after the 8-h starvation with 8 h:16 h feeding intervals. In conclusion, the present results clearly demonstrate that food-induced entrainment of the liver clock is dependent on both food volume and the starvation interval between two meals. Therefore, normal feeding habits may help to maintain normal clock function in the liver organ.

FOOD ANTICIPATORY ACTIVITY (FAA) is characterized by locomotor activity that increases 2–3 h before restricted feeding (RF) time (24). FAA rhythm is believed to be controlled by a food-entrainable oscillator (FEO), which might be located in the brain (see Ref. 2 for review). In addition to FAA, daily restricted feeding can also entrain peripheral circadian clocks including the liver clock (1, 9, 37). On the basis of the circadian function, liver clock system has been reported to play critical roles in nutrition metabolism such as carbon hydrate, protein and lipid (see Refs. 3, 8, 13 for review), and also drug metabolism (see Ref. 28 for review). Among peripheral organs, liver is a sensitive organ against RF regimen (5), suggesting that a live clock system may be controlled by the daily feeding pattern in animals.

Most studies of FAA in rats have used a time-limited, once-daily meal regimen that provided an unlimited amount of regular rat chow in pellet or powder form for 2–4 h during the middle of the light period (see Ref. 24 for review). Meal schedules that occur once a day have been a useful tool for investigating the zeitgeber properties of food but may not accurately represent the food availability patterns of nocturnal rodents in the wild. An alternative approach is to schedule small meals more than once a day. Previously, two to six meals were delivered per day in experiments aimed at understanding whether multiple FEO pacemakers are used to track each mealtime. Rats exhibited FAA during two meals per day that were separated by 5 h or more, and both occurrences showed properties of circadian timing rather than interval timing (5, 25, 32, 33). Some of the rats displayed FAA at two of three meals, but no rat anticipated all three meals simultaneously (32). In addition, six meals per day did not lead to the same development of coordinated FAA (22). Interestingly, rats exhibited two episodes of FAA when given two meals per day (daytime and nighttime) and showed entrainment in the liver and gastrointestinal organs that was consistent with only the nighttime meal (5). However, this study did not show why the nighttime meal was the only meal that entrained the peripheral clock. In addition, it was unclear why the peripheral organ clock displayed a unimodal peak but not bimodal peak rhythm under a feeding regimen of two meals per day. On the basis of the feeding styles of wild animals and humans, we wished to examine the effect of multiple feeding times, starvation interval, and food volumes on RF-induced entrainment.

In human studies, increased meal frequency has often been proposed to induce favorable effects on body weight and energy intake (6, 15). In addition, the size of breakfast and whether breakfast is skipped is also related to body mass index (17, 38) and cognitive performance (14). These human feeding pattern habits may affect the circadian rhythm of peripheral organs. As a model of such human studies, we fed mice two meals per day with different food volume, starvation intervals, and zeitgeber times (ZTs) for each meal and examined whether the combination of food volume, starvation interval, and ZTs can affect the restricted feeding-induced entrainment of liver Per2 expression. Per2 gene is one of the clock genes and its expression profile shows a clear circadian pattern in the liver, and good marker gene for evaluation of phase shifts of clock (1, 10, 37, 39).

Interestingly, dissociations between behavioral FAA rhythms and those of peripheral clock gene expression have previously been reported. Feillet et al. (7) demonstrated that Per2 mutant mice exhibit weak FAA rhythms but exhibit a strong phase...
shift of peripheral clock gene expression. Davidson et al. (5) also reported that fasting can reestablish FAA in mice that are offered ad libitum feeding for 1 wk after the establishment of an FAA rhythm. However, these mice did not show a phase change of the peripheral clock gene expression rhythm under a reestablished FAA.

Although the dorsomedial hypothalamus (DMH) is not necessary for the induction and persistence of behavioral and peripheral food-anticipatory rhythm (20, 21, 26), behavioral FAA can be attenuated by partial DMH lesions created by a neurotoxin (9) or by very large thermal lesions of the medio-basal hypothalamus (MBH) that encompass the DMH and much of the ventromedial nucleus (VMH) and arcuate nucleus (ARC) (39). In addition to the attenuation of FAA formation, MBH lesions do not affect restricted feeding-induced entrainment of liver Per2 expression rhythm in Per2::Luc knock-in mice (39). MBH-lesioned mice showed the loss of feeding and locomotor activity rhythm provided by suprachiasmatic nucleus (SCN), a main oscillator (see Refs. 31, 39 for review). Therefore, in the major parts of present study, we used MBH-lesioned mice to reduce the influence of FAA on RF-induced entrainment of the liver clock and also to reduce the influence of SCN output function.

MATERIALS AND METHODS

**General conditions of animals and housing.** Per2::Luc knock-in mice (founders courtesy of Dr. Joseph Takahashi, Northwestern University) (41) were bred in house. We prepared Per2::Luc homozygous male mice for mating with female mice from the ICR strain. From this crossing, we obtained F1 hybrid Per2::Luc heterozygous males that weighed 25–35 g each at the start of the experiment. We saw no differences in the peak time of bioluminescence in the livers of the original male or F1 hybrid mice. The animal room had a controlled temperature of 22 ± 2°C, humidity of 60 ± 5%, and a 12-h light:12-h dark (LD) cycle (i.e., lights on from 0800 to 0000). ZTs of ZT0 and ZT12 were used as the lights-on and lights-off times, respectively, and projected ZT (pZT) was used in vitro experiments. The light intensity at the surface of the cages was ∼100 lux. Prior to the restricted feeding experiment, mice were fed normal, commercial rodent chow (catalog no. MF; Oriental Yeast, Tokyo, Japan) and provided with water ad libitum. The pellet for RF was purchased from Bio-Serv (no. F0021-J, Frenchtown, NJ), and the nutritional condition was equivalent to the AIN-93M formula. Experimental animal care was conducted with the permission of the Animal Welfare Committee of Waseda University (permission no. 09A11).

**Feeding activity analysis.** Feeding behavior depends on the rhythm that is related to the amount of food intake. After a recovery period of 10 days from the MBH lesions, mice were moved to the feeding monitoring system (FDM System, model FDM-ez1; Neuroscience, Tokyo, Japan). For this experiment, we also prepared sets of intact and MBH-lesioned mice. Food intake was recorded by measuring the changes in the weight of the food box, and the food intake rhythm is shown as a percent change of chow consumption measured every hour over a 24-h period.

**Food pellet supply and experimental protocol.** With the exception of free-feeding groups, all mice were housed in cages that contained food dispensers (Pellet Dispenser 45 MG, Med-associates, St. Albans, VT) for supplying food pellets. For the first set of experiments, intact, nonlesioned mice were used since the effect of food volume on entrainment of the liver clock may be influenced by a LD cycle through the SCN-dependent clock. For experimental protocol 1, 3.6 g (80 pellets; 80) of food volume was supplied as one meal per day at ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, or ZT21. For experimental protocol 2, 40 pellets of food each were supplied as two meals per day at ZT0 and ZT12 or at ZT6 and ZT18.

In the second set of experiments, MBH-lesioned mice were used to reduce the effect of spontaneous feeding rhythm from output of SCN and also reduce the effect of FAA induced by RF treatments. For experimental protocol 3, the food pellets for the first and second meals were changed (00:00, 20:00, 40:40, 60:20, or 80:00) under a 12-h feeding cycle between two meals. For experimental protocol 4, the food pellets for the first and second meal were fixed (40:40), but feeding intervals between the two mealtimes were changed (8 h:16 h, 12 h:12 h, or 16 h:8 h). For experimental protocol 5, the food pellets for the first and second meal were changed (00:00, 20:00, 40:40, 50:30, 60:20, 10:00, or 80:00) with a long starvation (16 h) or short starvation (8 h) interval between two mealtimes.

In a previous study, the starvation period was slowly shortened by 12, 6, and 4 h to avoid an acute loss of body weight (see Ref. 24 for review). Since we limited food volume but not feeding time in this experiment, the mice were allowed to eat all of the food within 6–8 h after being provided food for first 2–3 days, and finally they could eat all the food within 4–6 h from 6–15 days. Thus our mice did not show acute drop of body weight.

After a 10-day recovery from the surgery, mice were exposed to food deprivation for one night (starting at ZT10) followed by continued RF through the food dispenser for 14–15 days. On the day following RF termination, mice were euthanized at ZT3. In our previous paper, we demonstrated that the euthanasia time is independent of the phase of the mouse liver clock in ex vivo experiments (12).

**Surgery.** The operation protocol of MBH was exactly the same as our previous experiment (39). Mice were anesthetized with ketamine (50 mg/kg administered by intraperitoneal injection) and positioned in a stereotaxic frame (Narishige, Tokyo, Japan) for the placement of stainless steel electrodes (0.35 mm in diameter) into the MBH. The stereotaxic coordinates were 0.8 mm posterior, ±0.3 mm lateral to the bregma, and 6.5 mm under the skull surface. Bilateral lesions were made with a thermal lesion device (RFG-4A; Muromachi, Tokyo, Japan) that heated the electrode tip to 55°C for 4 s via a current path. Sham-operated mice were treated in the same manner but did not receive the current path. All mice were given 10 days to recover from the surgery under a LD cycle with ad libitum food access.

Brain staining and verification of lesion site. The brains of all lesioned animals were collected after being euthanized for the bioluminescence experiments under ether anesthesia. The brains were stored in 4% paraformaldehyde for 3–4 days. Subsequently, 60-µm serial coronal sections from sham and MBH-lesioned mice were stained with cresyl violet and photographed through the MBH area by use of a digital camera attached to a light microscope (BX51, Olympus, Tokyo, Japan). The borders of the DMH, VMH, and ARC were confirmed on the digital images by referencing a mouse brain atlas (30a). Mice with an incomplete lesion of the MBH area were excluded from the data.

**Preparation and measurement of bioluminescence from Per2::Luc mice.** Following the feeding experiment, Per2::Luc mice were euthanized at ZT3 for recordings of the bioluminescence rhythm in the liver. The liver block was rapidly removed from the mice and placed in ice-cold Hanks’ balanced salt solution (pH 7.2; Sigma-Aldrich, St. Louis, MO). Four pieces from randomly five lobes of the liver were taken from one mouse. Each tissue was explanted into a 35-mm petri dish (IWAKI Japan), sealed with paraffilm (Sigma-Aldrich), and cultured with 1.3 ml of DMEM (Invitrogen, Carlsbad, CA) supplemented with NaHCO3 (2.7 mM), HEPES (10 mM), kanamycin (20 g/ml; Sigma-Aldrich), human transferrin (100 µM), and putrescine (100 µM; Sigma-Aldrich), human transferrin (100 µg/ml; Sigma-Aldrich), progesterone (20 nM; Sigma-Aldrich), sodium selenite (30 nM; Sigma-Aldrich), and 0.1 mM o-luciferin Na salt (Invitrogen). The cultures were incubated at 37°C and the bioluminescence was monitored three times over 10-min intervals with a dish-type luminometer (LumiCycle; Actimetrics).
Assessment of circadian periods and liver clock phases. First, original data (1-min bins) were smoothed by an adjusting-averaging method with 2-h running means as previously described (11, 29). The data were then detrended by subtracting the 24 h running average from the raw data using R software (R Development Core Team, http://www.r-project.org/). The program was created by Tsuyoshi Yaita, Naoki Furutani, and Dr. Shigenobu Shibata (Waseda University, Tokyo, Japan). Peaks were defined as points where the bioluminescence was higher than both sides and confirmed by wave form. Typically, the peak phase time (pZT) is evaluated by second peaks, since the first peak is sometimes affected by culture dish movement (4, 12). The averaged peak phase (pZT) was calculated from four liver pieces of one mouse. The period of Per2::Luc activity (recorded from 24 to 72 h in vitro) was assessed for each liver culture and calculated by averaging the period between the first and second peaks and the period between the second and third peaks.

Total RNA isolation and real-time RT-PCR. Tissue mRNA was measured by real-time RT-PCR as previously described (19). Mice (n = 3–5 per time point) were deeply anesthetized with ether and the heart and liver were rapidly isolated. Total RNA was extracted by use of ISOGEN Reagent (Nippon Gene, Tokyo, Japan), and the concentration was determined by SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA). The DNA digestion step was performed by using RQ1 RNase-free DNase (Promega, Madison, WI) after an incubation step at 37°C for 30 min. The enzyme was inactivated by RQ1 DNase Stop Solution (Promega). Fifty nanograms of total RNA was reverse transcribed and amplified using the One-Step SYBR RT-PCR kit (TaKaRa, Otsu, Japan) in the iCycler PCR machine (Bio-Rad Laboratories). Specific primer pairs were designed on the basis of published data for the β-actin, Per2, Dec1, and Bmal1 genes. The β-actin, Per2, Dec1, and Bmal1 primers were designed to cross exon-intron boundaries.

The primer sequences were as follows: mouse β-actin 5’-tgacag-gatgcagaagaga-3’, 5’-gtggaagtggtggagct-3’; mouse Per2 5’-gtggtcttaacgggtgtccta-3’, 5’-aactgggtggtgecatgaa-3’; mouse Dec1 5’-cagcggcgggaataac-3’, 5’-tcagctggcaatgcactc-3’; mouse Bmal1 5’-ccaccaaatgagcact-3’, 5’-gacagtctgtttcactgtgt-3’. Real-time RT-PCR was performed under the following conditions: cDNA synthesis at 42°C for 15 min followed by 95°C for 2 min, PCR amplification for 40 cycles with denaturation at 95°C for 5 s, and annealing and extension at 60°C for 20 s. The relative levels of the target gene PCR product were normalized to that of β-actin. In this experiment, normalized PCR product from free-feeding mice was set as 100%. Data were analyzed by the delta-delta Ct method. A melt curve analysis was performed to check nonspecific products and indicated that there was no amplification of nonspecific products.

Statistical analysis. All values are expressed as means ± SE. For statistical analysis, a one-way or two-way ANOVA using StatView software was applied and a post hoc analysis was conducted with a Student’s t-test or a Fisher’s protected least significant difference (PLSD) test.

RESULTS

Body weight and feeding rhythm under the RF conditions in intact and MBH-lesioned mice. Food pellets (45 mg for each pellet) were supplied through a food dispenser with a total of 3.6 g of food (45 mg × 80 pellets) given per day for each
mouse. Under free-feeding conditions, intact mice ate 5.2 ± 0.2 g/day (N = 24) and MBH-lesioned mice ate 4.9 ± 0.2 g/day (N = 21), where the food volume was set at ~69–73% (3.6 g per day) of free feeding as previously described (3.6 g/day at 66–75% of free feeding) (7, 23).

When we measured the change in body weight in intact mice under an RF schedule of 3.6 g/day of food, the mean body weight after 2 wk of one-meal RF was 92.3 ± 1.2% (N = 61), whereas after 2 wk of two meals (ZT0/ZT12 or ZT6/ZT18) RF the mean body weight was 94.3 ± 1.5% (N = 24) compared with the previous RF. In MBH-lesioned mice, the mean body weight after 2 wk of RF was 91.7 ± 1.6% (N = 48) with no observed difference between intact and lesioned groups. Similar to previous findings (7), intact or MBH-lesioned mice receiving one or two meals per day were able to consume all of the pellets within 4 h (usually within 3 h). Intact mice exhibited a clear daily rhythm in their feeding pattern under the LD cycle, whereas MBH-lesioned mice displayed a strongly attenuated pattern of daily feeding under the LD cycle (Fig. 1B).

To avoid an incomplete lesion in the DMH area, we applied an identical lesion protocol of MBH area lesions from our previous study (39). We created a relatively large lesion area in the ventral and dorsal portions of the medial hypothalamus. In many cases, lesion sites included portions of the VMH and ARC (Fig. 1A). In Fig. 1A, four examples of lesion sites are shown as representatives and indicate that the present surgical procedure did not cause damage to the SCN. Therefore, we concluded that all of the SCN was intact even if the lesion site in the DMH area was large.

One meal per day on RF-induced entrainment of liver bioluminescence rhythms in intact mice. RF was applied to Per2::Luc knock-in mice at ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, or ZT21. We first confirmed that this experimental protocol (3.6 g/day; 80 pellets administered by food dispenser; F1 hybrid mice of C57 black × ICR) could entrain the liver clock. Mice were euthanized to determine the phase of the liver clock (Fig. 2). When a line was drawn between ZT0 and ZT21 to see the direct interaction between ZT and phase shift (pZT), the phase of the liver clock was strongly and directly dependent on the ZT of RF. There were no significant differences of phase among the free-feeding group (pZT9.6 ± 0.38) and RF of ZT15 (pZT9.1 ± 0.69) or ZT18 groups (pZT9.2 ± 0.52). When difference of phase was examined among five lobes of the liver were taken from intact vs. lesioned and/or one-meal vs. two-meal RF mice, we never found the lobe difference in Per2 gene expression rhythm (data not shown).

Two meals per day on RF-induced entrainment of the liver bioluminescence rhythms in intact mice. Since mice usually take food during nighttime (~80% at night and 20% at daytime) (Fig. 1B), we set two meals per day at ZT0/ZT12. This procedure is similar to the skeleton photoperiod method in which a short-period light pulse is given at ZT0 and ZT12 (29). Therefore, same amounts of pellets (40 pellets for each time) were given at ZT0 and ZT12 (Fig. 3, A and B). At first, the shape of expression rhythm of the liver Per2::Luc bioluminescence in mice under two-meal RF schedule was similar to those from mice under the one-meal RF schedule (Fig. 3, A and B). In the present experiments, we never found a bimodal pattern of liver bioluminescence rhythm in mice under the two-meal schedule. Since the amplitude of each rhythm is dependent on the size of liver piece and the individual mouse, it is not easy to compare the amplitude of bioluminescence rhythm. There were no difference of amplitude of expression rhythms between the one-meal and two-meal groups (Table 1). Amplitude of expression rhythm in peripheral tissue is shown to be dampened along with culture periods (Fig. 3, A and B); however, the damping rate was almost same between the two-meal group and the one-meal group (Table 1). The free-running period of expression rhythm was also similar between the two groups (Table 1), and the values of the free-running period were indistinguishable from previous studies that had used the same culture medium and protocols (27, 40). The phase of liver Per2 clock from two meals of RF at ZT0 and ZT12 varying with food volume (pellet ratio of ZT0:ZT12 was 80:00, 60:20, 40:40, 20:60 or 00:80) was examined. The phase of liver clock under the ratio of 40:40 or 20:60 was located closest the phase from mice receiving one meal at ZT12 (00:80) (Fig. 3, A–D).

In the previous experiments, rats given two meals per day (ZT6/ZT18) were reported to have two FAA episodes at approximately ZT6 and ZT18 and a peak of the peripheral clock at approximately ZT18 (5). To confirm these previous results with our present mouse protocol, 80 pellets were given at ZT6 or ZT18 or 40 pellets were given at ZT6 and ZT18 (Fig. 3, E and F). The phase (pZT7.7 ± 0.37) of the liver Per2 clock from two meals of RF at ZT6ZT18 (40:40) was located approximately midway between the phases from mice receiving RF at ZT6 only (80:00) (pZT4.5 ± 0.58) and RF at ZT18 only (00:80) (pZT10.2 ± 0.5) (Fig. 3D).

A previous paper by Davidson et al. (5) employed the limitation of access time of food at ZT6 and ZT18, but in this experiment we gave same amount of food at ZT6 and ZT18. When we employed the protocol of limitation of food access time (2 h for each ZT 6 and ZT18), mice ate approximately twice the food volume at ZT18 compared with ZT6 (1.65 ± 0.22 g for ZT6, 3.47 ± 0.38 g for ZT18), and the phase of the liver clock (pZT9.1 ± 0.53, N = 6) was closed to that from mice receiving RF at 18 only (pZT10.2 ± 0.5, N = 5).
Two meals per day on RF-induced entrainment of the liver bioluminescence rhythms in MBH-lesioned mice. In intact mice, the two-meal protocol caused a different result when mice were employed to the meals at ZT0/ZT12 or at ZT6/ZT18. To avoid the effect of daily feeding rhythm and the effect of FAA on entrainment of liver clock by two meals, we prepared the MBH-lesioned mice following the method described in our previous article (39). Similar to our previous paper (39), one-meal RF produced a unimodal rhythm both in intact and MBH-lesioned mice. The amplitude of unimodal rhythm was similar between intact (4,602 ± 575 counts/min for second peak) and MBH-lesioned (4,512 ± 222 counts/min for second peak) mice.

We next applied the two meals per day schedule at ZT0 and ZT12 to lesioned mice with changing food volume for each meal. The ratios of food volumes for the first and second mealtime at ZT0/ZT12 (00:80, 20:60, 40:40, 60:20, or 80:00) were changed under a 12-h feeding interval between two meals (Fig. 4). We found unimodal pattern of liver bioluminescence rhythm in MBH-lesioned mice under a one-meal or two-meal RF schedule (Fig. 4, A and B). The phase of liver clock (pZT6.2 ± 0.65) from mice receiving two meals per day with equal food volume at ZT0/ZT12 (40:40) located exactly midway of the phase from mice receiving one meal at ZT0 (pZT1.9 ± 0.67) or at ZT12 (pZT9.9 ± 0.9). This observation was very different from that in intact mice (Fig. 3, C and D). Finally, a direct positive association was observed between the phase of the liver clock and food volume in MBH-lesioned mice (Fig. 4D).

**Table 1. Amplitude and free-running period of Per2::luciferase rhythm in the liver of intact mouse with 1-meal or 2-meals RF paradigms**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amplitude, counts/min</th>
<th>Free-Running Period, h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2nd peak</td>
<td>3rd peak</td>
</tr>
<tr>
<td>1-Meal RF (14)</td>
<td>4,602 ± 575</td>
<td>3,366 ± 237</td>
</tr>
<tr>
<td>2-Meals RF (14)</td>
<td>4,820 ± 662</td>
<td>3,588 ± 555</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to number of mice. There are no statistical significant differences between the 1-meal restricted feeding (RF) and 2-meals RF groups.
protocol, the interval between the first and second meal was changed to either ZT0/ZT8, ZT0/ZT12, or ZT0/ZT16 (Fig. 5A). The phase of \( \text{Per2} \) rhythm was \( \text{pZT}2.4 \pm 0.69 \) for the ZT0/ZT8 group and was close to the phase of the one-meal RF at ZT0 alone (\( \text{pZT}1.9 \pm 0.67 \)) (Fig. 5B). In contrast to the ZT0/ZT8 group, however, the phase of \( \text{Per2} \) rhythm for the ZT0/ZT16 group was \( \text{pZT}3.0 \pm 0.5 \) and was close to the phase of the one-meal RF at ZT16 alone (\( \text{pZT}11.2 \pm 0.44 \)). As shown in Fig. 4D, the phase of \( \text{Per2} \) rhythm for ZT0/ZT12 was midway of the phase from mice receiving one meal at ZT0 or at ZT12 (Fig. 5B).

**Combination of food volume and starvation interval effects on RF-induced entrainment of liver bioluminescence rhythms in MBH-lesioned mice.** To assess the combined effect of food volume and starvation intervals on two-meal protocol-induced entrainment of liver bioluminescence rhythms in MBH-lesioned mice, we altered both the food volume and the starvation interval. Mealtime for the first and second meal was set at ZT0 and ZT16, respectively, and food volume ratio for each mealtime was altered as follows: 00:80, 20:60, 40:40, 50:30, 60:20, 70:10, or 80:0 (Fig. 6). In Fig. 6, A and B, the example of liver \( \text{Per2} \) bioluminescence rhythm from RF mice with food ratio of 00:80, 40:40, or 80:0 was shown. The shape of the rhythm was similar among groups. The phases of the liver \( \text{Per2} \) rhythm in the 20:60, 40:40, and 50:30 groups were similar to the results produced by 00:80, and the phase of the liver \( \text{Per2} \) rhythm in the 70:10 group was similar to the phase produced by 00:80 (Fig. 6, C and D). In the 60:20 group, the mean phase of the \( \text{Per2} \) rhythm (\( \text{pZT}5.9 \pm 1.7 \)) occurred midway between the phases produced by one-meal RF at ZT0 or ZT16. Individual data of the 60:20 group showed large standard error compared with other experimental conditions as shown in individual data (small triangle symbols in Fig. 6D).

Fig. 5. Effect of starvation interval on 2-meal RF (ZT0/ZT8, ZT0/ZT12, ZT0/ZT16)-induced entrainment of liver bioluminescence rhythms in MBH-lesioned mice. A: protocol of 2-meal RF treatment. \( \text{Per2::Luc} \) knock-in mice were given an RF schedule for 14–15 days and then euthanized for a recording of their liver bioluminescence rhythm. Numbers in parentheses represent number of food pellets. Horizontal white and black bars indicate the light and dark periods for MBH-lesioned mice. Arrows indicate feeding times. B: detrended data of the liver bioluminescence rhythm were obtained for 1 full meal at ZT0 (solid line) or ZT16 (broken line), or 2 half meals at ZT0 and ZT16 (dashed line). C: protocol of starvation intervals for RF treatment. \( \text{Per2::Luc} \) knock-in mice were given an RF schedule for 14–15 days and then euthanized for a recording of the liver bioluminescence rhythm. Numbers in parentheses represent number of food pellets. Horizontal white and black bars indicate the light and dark periods for MBH-lesioned mice. Arrows indicate feeding times. D: experimental, detrended data of liver bioluminescence rhythm in RF-treated mice were obtained and the peak time (pZT) of this rhythm was calculated. Numbers in parentheses represent number of food pellets. Horizontal white and black bars indicate the light and dark periods for MBH-lesioned mice. Arrows indicate feeding times.

**DISCUSSION**

We found a unimodal but not bimodal peak of the bioluminescence rhythm in the liver of \( \text{Per2::Luc} \) knock-in mice under...
the RF schedule of two meals per day in intact and also MBH-lesioned mice. Previously, two or three meals per day regimen were applied to understand whether multiple FEO pacemakers are used to track each mealtime. Rats exhibit FAA with two meals per day, separated by 5 hr more, and both activity episodes exhibit properties of circadian timing rather than interval timing, suggesting that there are at least two FEOs for FAA expression (5, 25, 32, 33). However, Davidson et al. (5) also reported one peak of bioluminescence rhythm in the liver; Per1::Luc transgenic rats under the RF regimen of two meals per day. Therefore, expression of Per1 and Per2 genes may be masked by a bimodal feeding pattern. The mechanism for these observations is currently unknown; however, the liver clock may only possess one oscillator, and the phase of this oscillator may be determined by the combination of the entraining strength of each RF regimen under the RF conditions of two meals per day. Although the oscillation of Per2 is still driven by RF in ex vivo experiments, we do not know whether the effect of RF on the liver clock requires input from other tissues in vitro. In a previous study using Per1::Luc transgenic rats, RF was shown to cause a phase shift of the liver, stomach, and colon circadian rhythm in an organ-dependent fashion (5). In addition, our results from the present study show that the liver and kidney circadian rhythm was phase advanced by RF at ZT0 in an organ-dependent fashion (pZT = 0.2 ± 0.11 for liver; pZT = 1.5 ± 0.9 for kidney). Taken together, these findings support our hypothesis that the liver clock may not require input from other tissues in vitro after RF.

In the previous study, Davidson et al. (5) reported that the peak of bioluminescence under the two meals per day regimen at ZT6/ZT18 was identical to the peak at a ZT18 feeding alone, but not at ZT6 feeding alone. In contrast, our present results demonstrate that the phase of liver clock from mice receiving two meals per day with equal food volume at ZT6 and ZT18 located exactly midway of the phase from mice receiving one meal at ZT6 or at ZT18. The discrepancy between our data and the Davidson data may be a result of differences in experimental protocols. We gave exactly the same amount of food at each mealtime, whereas Davidson et al. controlled the length of feeding time at each mealtime. Therefore, rats may eat more food at ZT18 than at ZT6, because our present experiment revealed that mice with 2-h feeding periods at ZT18 ate more food than those with 2-h feeding periods at ZT6 under the two-meal paradigm, resulting in a similar phase of liver clock to that obtained by one-meal RF at ZT18. On the other hand, the present results demonstrate that the peak of bioluminescence under a two meals per day regimen at ZT0/ZT12 in intact mice was similar to the peak of mice feeding alone at ZT12 or free feeding. Thus data of two meals at ZT0/ZT12 were very different from those at ZT6/ZT18. Intact mice usually eat more food during the night period (80% of total feeding) than daytime period (20%). Thus the feeding pattern provided by two meals per day at ZT0 and ZT12 was relatively similar to that by free feeding and resulted in a similar phase of liver clock to that provided by free feeding. Interestingly, the skeleton photoperiod method in which a light pulse is applied for 1 h at around ZT0 and ZT12 caused a unimodal peak of Per1 and Per2 gene expression in the skin, and the phase of clock caused by the skeleton photoperiod was almost identical to that by normal LD cycle (30). This article strongly supports our present results showing that the unimodal peak of the liver rhythm by RF of ZT0/ZT12 was similar to the rhythm from the free-feeding paradigm. Furthermore, one-meal RF at ZT12 produced the same effect of Per2-luciferase oscillation as the two-meal RF at ZT0/ZT12 in the intact mice. Since ZT12 mice eat a large amount of food under free-feeding conditions (Fig. 1B), mice receiving one-meal RF at ZT12 may show a similar peak of bioluminescence rhythm to mice receiving two-meal RF at ZT0/ZT12.
In our present study, we have found a positive relationship between the phase shifts of the liver clock (pZT) and the mealtime ZT under a one meal per day schedule. Thus the phase of liver clock produced by RF of one meal per day is determined by clock time of each RF treatment. Alternately, under our present experimental conditions, the euthanasia time of mice for the preparation of liver tissue may not affect the phase of liver clock.

On the other hand, feeding at ZT6 may provide entraining strength under two meals per day at ZT6/ZT18 and peak phase located midway from phase by RF at ZT6 or at ZT18 alone. When MBH-lesioned mice were employed to a two-meal schedule, the peak phase of liver clock was determined by summation of entraining strength by food volume at each ZT, because MBH-lesioned mice lost the daily feeding pattern.

The next important finding of this study is that the phase of the liver clock entrained by two meals per day is dependent on the combination of food volume and the feeding interval between the first and second mealtime. When we used MBH-lesioned mice that had arrhythmic locomotor (37) and feeding activity (present data), the phase of the liver clock was positively associated with the food volume of each meal under the conditions of two meals per day. We previously reported that feeding caused food-volume-dependent phase advancement of the mouse liver clock (12). In our present study, we again observed that the phase shift of the liver clock was related to food volume, although the present experimental protocol was different from the previous one. In addition to the liver clock, a previous study has shown that food can produce phase shifts of the rat FAA rhythm in a food volume-dependent manner, whereas substitution of the diet with nonnutritive bulk or lipid failed to produce phase shifts (34, 35, 36). Taken together, our present results strongly demonstrate that nutritive food volume is a key factor for resetting the phase of the liver clock under conditions of several meals per day.

When mice were given two meals with 8 h:16 h, 12 h:12 h, and 16 h:8 h intervals, the phase of the liver clock was similar to that of the phase of the liver clock produced by meal with a longer starvation interval. Therefore, we call this effect the “breakfast effect,” since a meal for breaking fast is an effective signal for resetting the liver clock. The mechanism of this breakfast effect may be complex and involve many factors. For example, food digestion and nutritional absorption and metabolism may be more active under the conditions of strong fasting than weak fasting. Recently, it has been reported that fasting can downregulate Per2, Dec1, and Bmal1 expression in the mouse liver and that refeeding prevents fasting-induced changes in gene expression (16, 18). Therefore, we compared Per2, Dec1, and Bmal1 gene expression after 8-h or 16-h starvation intervals. Expression of these genes was strongly increased by the 16-h starvation but not the 8-h starvation. Expression of Per2 and Dec1 was slightly higher in the 16-h starvation no-food group than in the 8-h starvation no-food group. Moreover, Bmal1 expression was slightly lower in the 16-h starvation no-food group, suggesting that a weak difference of the phase angle of gene expression existed between the two groups at this time of euthanasia (Fig. 7B). Therefore, the difference in gene expression level induced by food intake after 8 or 16 h of starvation may be due to a change in the absolute level of gene expression but not the phase shift. These results suggest that the higher expression of these clock genes induced by food consumption may be a critical signal(s) for the entrainment of the liver clock after a long starvation.

When we changed the food volume and starvation interval simultaneously, the phase of the liver clock was dependent on the combination of these two factors. These results suggest that the breakfast effect is not observed when food volume for breakfast is too small compared with the food volume for “dinner.” When there is strong competition of two factors (food volume and starvation length), such as the case of 60:20 pellets for mealtime at ZT0:ZT16, the phase of the liver clock for each animal showed strong individual differences, suggesting that the phase of the liver clock in an individual mouse is controlled under subtle interactions of these volume and starvation factors. The present experiment demonstrated that the phase of liver clock is controlled not only by light-dark cycle through the SCN function but by multiple feeding cycles. Taken with the present results and above papers, it is suggested that circadian clock function of liver may be related with feeding pattern habits in animals and in humans as well. In summary, the phase of the liver clock under multiple restricted feeding conditions is dependent on the combination of factors of food volume, starvation intervals, and ZTs. In consideration of human lifestyles, appropriate volumes of breakfast may be important for maintaining the position of the liver clock phase under two or perhaps three meals per day conditions. On the other hand, abnormal feeding pattern habits may cause undesirable phase of the liver clock.

ACKNOWLEDGMENTS
We thank M. Itokawa and M. Shimoda for care of Per2::Luc knock-in mice.

GRANTS
This study was partially supported by grants awarded to S. Shibata from grants-in-aid for Scientific Research (20390065, 19659058) as well as from Nakashima Foundation (2008) and Urakami Shokuhin Foundation (2009), Asahi Beer Foundation, and Fuji Foundation for Protein Research (2010).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
9. Gooley JJ, Schomer A, Saper CB. The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. 


13. Hoogerwerf WA. Role of biological rhythms in gastrointestinal health and disease. 


17. Kent LM, Worsley A. Breakfast size is related to body mass index for men, but not women. 


19. Kudo T, Kawashima M, Tamagawa T, Shibata S. Clock mutation facilitates accumulation of cholesterol in the liver of mice fed a cholesterol and/or cholic acid diet. 


21. Landry GJ, Yamakawa GR, Webb IC, Mear RJ, Mistlberger RE. The dorsomedial hypothalamic nucleus is not necessary for the expression of circadian food-anticipatory activity in rats. 

22. Mendoza J, Drevet K, Pévet P, Challet E. Daily meal timing is not necessary for resetting the main circadian clock by calorie restriction. 


24. Mistlberger RE. Food-anticipatory circadian rhythms: concepts and methods. 


27. Nishide SY, Honma S, Nakajima Y, Ikeda M, Baba K, Ohmiya Y, Honma K. New reporter system for Per1 and Bmal1 expressions revealed self-sustained circadian rhythms in peripheral tissues. 


32. Stephan FK. Entrainment of activity to multiple feeding times in rats with suprachiasmatic lesions. 

33. Stephan FK. Forced dissociation of activity entrained to T cycles of food access in rats with suprachiasmatic lesions. 

34. Stephan FK. Calories affect zeitgeber properties of the feeding entrained circadian clock. 

35. Stephan FK, Becker G. Entrainment of anticipatory activity to various durations of food access. 

36. Stephan FK, Davidson AJ. Glucose, but not fat, phase shifts the feeding-entrained circadian clock. 

37. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M. Entrainment of the circadian clock in the liver by feeding. 

38. Szajewska H, Ruszczynski M. Systematic review demonstrating that scheduled exposures to a novel environment with a running-wheel differentially accelerate re-entrainment of mice peripheral clocks to new light-dark cycles. 


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