Feeding and adrenal entrainment stimuli are both necessary for normal circadian oscillation of peripheral clocks in mice housed under different photoperiods

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The mammalian circadian rhythm is entrained by multiple factors, including the light–dark cycle, the organism’s feeding pattern and endocrine hormones such as glucocorticoids. Both a central clock (the suprachiasmatic nucleus, or SCN) and peripheral clocks (i.e. in the liver and lungs) in mice are entrained by photoperiod. However, the factors underlying entrainment signals from the SCN to peripheral clocks are not well known. To elucidate the role of entrainment factors such as corticosterone and feeding, we examined whether peripheral clock rhythms were impaired by adrenalectomy (ADX) and/or feeding of 6 meals per day at equal intervals under short-day, medium-day and long-day photoperiods (SP, MP and LP, respectively). We evaluated the waveform and phase of circadian rhythms in the liver, kidney and salivary gland by in vivo imaging of PER2::LUCIFERASE knock-in mice. In intact mice, the waveforms of the peripheral clocks were similar among all photoperiods. The phases of peripheral clocks were well adjusted by the timing of the “lights-off”-operated evening (E) oscillator but not the “lights-on”-operated morning (M) oscillator. ADX had almost no effect on the rhythmicity and phase of peripheral clocks, regardless of photoperiod. To reduce the feeding-induced signal, we placed mice on a restricted feeding regimen with 6 meals per day (6 meals RF). This caused advances of the peripheral clock phase in LP-housed mice (2–5 h) and MP-housed mice (1–2 h) but not SP-housed mice. Thus, feeding pattern may affect the phase of peripheral clocks, depending on photoperiod. More specifically, ADX + 6 meals RF mice showed impairment of circadian rhythms in the kidney and liver but not in the salivary gland, regardless of photoperiod. However, the impairment of peripheral clocks observed in ADX + 6 meals RF mice was reversed by administration of dexamethasone for 3 days. The phase differences in the salivary gland clock among SP-, MP- and LP-housed mice became very small following treatment with ADX + 6 meals RF, suggesting that the effect of photoperiod was reduced by ADX + 6 meals RF. Because the SCN rhythm (as evaluated by PER2 immunohistochemistry) was not disrupted by ADX + 6 meals RF, impairment of peripheral clocks in these mice was not because of impaired SCN clock function. In addition, locomotor activity rhythm and modifications of the feeding pattern may not be completely responsible for determining the phase of peripheral clocks. Thus, this study demonstrates that the phase of peripheral clocks responds to a photoperiodic lights-off signal, and suggests that signals from normal feeding patterns and the adrenal gland are necessary to maintain the oscillation and phase of peripheral clocks under various photoperiods.

Keywords: Adrenal, biological rhythm, feeding, photoperiod

INTRODUCTION

Circadian clock systems are important for the control of multiple physiological functions and rhythms, including sleep–wakefulness, feeding, autonomic nervous activity and endocrine release (King & Takahashi, 2000). In the mammalian circadian system, a central clock in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus coordinates peripheral oscillators that are present in most peripheral organs (Mohawk et al., 2012). Maintaining synchrony between the SCN and peripheral oscillators is important for normal biological function, and disruption of this synchrony is correlated with several severe pathologies (Filipski et al., 2004; Mahoney, 2010; Marcheva et al., 2010; Schernhammer et al., 2001). At the molecular level, both the SCN and peripheral oscillators consist of interconnected positive and negative transcription–translation feedback loops that regulate the 24-h expression patterns of clock genes (Dunlap, 1999; Reppert & Weaver, 2002; Zhang & Kay, 2010). Clock and Bmal1 are positive elements of the
feedback loop, and Per1, Per2, Cry1 and Cry2 are negative elements.

Peripheral clocks receive information from the SCN through multiple pathways, including the autonomic nervous system, endocrine signals and body temperature rhythms (Brown et al., 2002; Buhr et al., 2010; Guo et al., 2005; Ishida et al., 2005; Le Minh et al., 2001; Terazono et al., 2003). Among the endocrine signals, glucocorticoids are important timing signals for peripheral oscillators. They are rhythmically secreted by the adrenal gland and peak with the onset of its activity (Almon et al., 2008). The daily rhythm of corticosteroid secretion is regulated by the SCN through adrenocorticotropic hormone secretion (Moore & Eichler, 1972; Nader et al., 2010; Pezu¨k et al., 2012). A single injection or daily inhalation of glucocorticoids induced a phase shift of the peripheral clocks in the liver, kidney and lung (Balsalobre et al., 2000a, b; Hayasaka et al., 2007).

Scheduled feeding experiments in mice have suggested that food is also a strong entraining factor for peripheral clocks (Mohawk et al., 2012; Shibata et al., 2010). Feeding after a long fast (e.g. breakfast) caused strong entrainment of peripheral clocks (Hirao et al., 2010; Kuroda et al., 2012), whereas feeding 4 or 6 meals per day at equal intervals failed to produce entrainment (Kuroda et al., 2012). This suggests that the daily feeding pattern is another major time cue for peripheral oscillators. However, several reports have indicated that the dominant entraining factor differs among various peripheral organs and tissues (Sujino et al., 2012; Vujovic et al., 2008). For example, the liver clock could be entrained by a restricted daily feeding regimen even if glucocorticoid was injected daily at anti-phase (Sujino et al., 2012). Furthermore, adrenalectomy (ADX) shifted the phase of Per1 expression in the liver, kidney and cornea but not in the lungs or pineal gland (Pezu¨k et al., 2012). Therefore, in this study, we elucidated whether the adrenal gland and/or feeding are necessary to produce peripheral clock oscillation in mice.

Many studies have demonstrated that changes in photoperiod modulate the SCN clock as well as peripheral clocks in the liver and adrenal gland (Inagaki et al., 2007; Otsuka et al., 2012; Parkanová et al., 2012; Sosniyenko et al., 2010; Sumová et al., 2007). Under a light–dark cycle, lights-on (dawn) and lights-off (dusk) activities are controlled by morning (M) and evening (E) oscillators, respectively, and several researchers have proposed a model for hamsters and mice in which the E oscillator is located in the rostral SCN and the M oscillator is in the caudal SCN (Hazlerigg et al., 2005; Inagaki et al., 2007; Johnston, 2005).

When mice were subjected to a change from a short-day photoperiod to a long-day photoperiod and fed twice daily during the daytime, clock gene expression profiles in the SCN were not affected, in contrast to the waveforms and phases of those in the liver (Parkanová et al., 2012). Photoperiod affected the diurnal fluctuations in plasma adrenocorticotropic hormone and corticosterone of rats, and these changes resulted in altered waveforms of the clock gene expression profile in the adrenal gland (Otsuka et al., 2012). Although these reports show that photoperiod affects peripheral oscillators, the factors that entrain peripheral clocks in response to photoperiod are still unknown.

In this study, we demonstrate that ADX combined with a feeding regimen consisting of 6 daily meals given at 4-h intervals leads to the loss of two major daily time cues: glucocorticoid secretion and nocturnal feeding, respectively. Using a recently established in vivo imaging system (IVIS) for the peripheral bioluminescence rhythm in PER2::LUC mice (Tahara et al., 2012), we examined the characteristics (rhythmicity, amplitude and phase) of peripheral clocks in mice housed with short-day, medium-day or long-day photoperiods (SP, MP and LP, respectively) before and after ADX and/or restricted feeding of 6 meals per day.

MATERIALS AND METHODS

Ethics statement

All experimental protocols were approved by the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University (permission protocol Nos. 2013-A059, 2013-A060). Experiments were performed in accordance with the law (No. 105) and notification (No. 6) of the Japanese government.

Animals and experimental design

To facilitate the penetration of bioluminescence through skin, we used PER2::LUC knock-in ICR mice, which were backcrossed more than five times with PER2::LUC C57BL/6J mice (courtesy of Dr. Joseph Takahashi, UT Southwestern Medical Center, Dallas, TX) and ICR mice as described previously (Kuroda et al., 2012; Tahara et al., 2012). Mice were maintained on a fixed light-dark cycle at a room temperature of 25 ± 0.5 °C, humidity of 60 ± 5%, and light intensity of 100–150 lux at the cage level. Prior to the start of experiments, mice were provided a standard diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum. Male PER2::LUC ICR mice (aged 9–15 weeks) were used in this study, except that male ICR mice (aged 9–10 weeks) were used for immunohistochemistry to examine the PER2 expression rhythm in the SCN. Detailed information on the mice used for each experiment is provided in the Supplementary Material (Supplementary Figure S1).

Lighting conditions

Mice were separated into three groups. In the MP group, mice were housed under 12-h light–dark conditions (lights on from 5:00 a.m. to 5:00 p.m.). In the SP group, mice were exposed to a 6:18 h light–dark cycle (lights on from 11:00 a.m. to 5:00 p.m.), and in the LP group, mice were exposed to an 18:6 h light–dark cycle (lights...
on from 11:00 p.m. to 5:00 p.m.). These conditions were maintained for more than 2 weeks prior to in vivo imaging. For analysis, data were adjusted to align the start of each dark period, and the dark onset zeitgeber time (real time of 5:00 p.m.) was defined as DZT 0.

Adrenalectomy

Bilateral ADX was performed via the dorsal approach under midazolam/xylazine anesthesia as described previously (Takita et al., 2013). In brief, the skin on the back was shaved and disinfected, and an incision of approximately 1 cm was made above and parallel to the spinal cord. The adrenal glands were removed from the surrounding fat tissue via small openings made in the muscle layer to the left and right of the spinal cord. All ADX mice were given at least 1 week to recover and had free access to a 0.9% saline solution instead of water to maintain an appropriate mineral balance during the study.

Scheduled feeding protocol

Mice assigned to the restricted feeding (RF) schedule were housed individually in separate cages, each equipped with a food dispenser (Pellet Dispenser 45 MG; Med Associates, St. Albans, VT) supplying a 45-mg food pellet (rodent purified diet, Bio-Serv, Flemington, NJ) as determined by a timer. In order to reduce feeding pattern-induced entrainment of the circadian rhythm of peripheral clocks, we gave mice 6 meals per day on an RF schedule (6 meals RF). The mice were given 3.78 g of food per day (84 pellets), which was 93.3% of the total daily free feeding volume, and each meal (0.63 g) was consumed within 30 min. Mice were fed at DZT 3, 7, 11, 15, 19 and 23; this schedule was similar to that reported in our previous paper (Kuroda et al., 2012). Mice were adapted to this feeding schedule for at least 2 weeks before in vivo images were recorded. Both intact and ADX mice had free access to food.

Feeding and locomotor activity analysis

Some intact and ADX mice carrying PER2::LUC were moved to a feeding monitoring system (FDM System, model FDM-ezl; Neuroscience, Tokyo, Japan) to record their feeding activity under SP (n = 4 for intact; n = 3 for ADX), MP (n = 4 for intact; n = 4 for ADX) and LP (n = 4 for intact; n = 4 for ADX) conditions (Supplementary Figure S1A). In this experiment, intact and ADX mice had free access to food. The food intake rhythm is shown as the change in chow consumption measured every hour over a 24-h period. The peak phase and amplitude of chow consumption data were determined using the single cosinor procedure (Acro.exe, version 3.5; designed by Dr. Refinetti).

General locomotor activity was recorded with an infrared radiation sensor (F5B; Omron, Tokyo, Japan) according to our previous study (Tahara et al., 2012). Double-plotted actograms of locomotor activity are shown with 6-min epochs, and onset of activity was detected automatically using ClockLab software (Actimetrics Ltd., Wilmette, IL). Time of activity onset was examined during the last 10 days of each experiment, and mean time of onset was calculated for each mouse. The same mice used for in vivo imaging were used for locomotor activity analysis. Due to the limited number of chambers, some intact, ADX, 6 meals RF and ADX + 6 meals RF mice carrying PER2::LUC were moved to the activity monitoring chambers to record their locomotor activity under SP, MP or LP conditions (Supplementary Figure S1B).

Drug administration

To examine the effect of daily dexamethasone (DEX) injections on reversal of peripheral clock impairment, we prepared six additional ADX + 6 meals RF mice (Figure 1D). DEX (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water. The dose of DEX was 1 mg/kg (0.1 mL/10 g body weight). As the circadian rhythm of peripheral clocks was strongly disturbed under LP conditions, DEX was injected into the ADX + 6 meals RF mice at DZT 0 under LP conditions for 3 consecutive days before in vivo imaging.

In vivo monitoring of PER2::LUC rhythms

We monitored the PER2::LUC activity rhythm waveform in peripheral tissues (kidney, liver and submandibular salivary gland) of mice using IVIS. After in vivo images had been recorded from mice housed under SP (n = 10), MP (n = 9) or LP (n = 11) conditions, the mice were fed according to the 6 meals RF schedule and then in vivo images were recorded again (Supplementary Figure S1A). ADX was then performed on these mice. Some of the mice died during surgery and/or recovery periods, and in vivo images were recorded from the remaining mice (SP, n = 9; MP, n = 9; LP, n = 7). These ADX mice and additional ADX mice (n = 1 for SP; n = 4 for LP) were maintained on the 6 meals RF schedule for 14 days under SP (n = 10), MP (n = 9) or LP (n = 11) conditions (Supplementary Figure S1A).

In vivo imaging was performed as previously described (Kuroda et al., 2012; Tahara et al., 2012), utilizing an IVIS kinetics system (Caliper Life Sciences, Hopkinton, MA). Mice were placed inside a black box and anesthetized with isoflurane (Mylan Inc., Tokyo, Japan) and concentrated oxygen (SO-005B, Sanyo Electronic Industries Co. Ltd., Okayama, Japan) using a gas anesthesia system (XGI-8, Caliper Life Sciences). While under anesthesia, mice were injected subcutaneously with d-luciferin potassium salt (Promega, WI) on the back, near the neck, at a dose of 15 mg/kg (30 mg in 10 mL PBS; 0.05 mL/10 g body weight). Images were taken in the dorsal-up position for the kidney 6 and 8 min after injection and in the ventral-up position for the liver and submandibular gland 10 and 12 min after injection. Images were captured with a 1-min exposure time. For each time point, the bioluminescence image was merged with the gray scale image.
FIGURE 1. Waveforms of intact PER2::LUC mice under various photoperiods in vivo. Waveforms of bioluminescence rhythms of the kidney, liver and salivary gland in intact mice housed under a short-day photoperiod (SP; L:D = 6:18), medium-day photoperiod (MP; L:D = 12:12) or long-day photoperiod (LP; L:D = 18:6). (A) Waveforms adjusted to align the onset of the dark period. (B) Waveforms adjusted to align the middle of the dark period. (C) Waveforms adjusted to align the onset of the light period. The horizontal white and black bars indicate light and dark periods, respectively. To facilitate understanding of the waveform, data were double-plotted. Each point is the mean for the tested mice. (A) DZT 0 denotes the zeitgeber time at the onset of the dark period. The horizontal scale represents clock time. (B, C) The horizontal scale represents the time course after adjustments (arrows). The numbers in parentheses indicate the number of tested mice for the given condition.
Images were obtained six times/day (DZT 0, 4, 8, 12, 16 and 20) from mice housed under SP, MP and LP conditions. Mice were returned to their home cages after each experiment. The total time under isoflurane anesthesia was approximately 20 min per experiment, from which the mice recovered quickly. Anesthesia and bioluminescence analysis every 4 h did not affect behavior of mice or luciferase activity in the peripheral tissues (Tahara et al., 2012).

Analysis of in vivo monitoring data

In vivo monitoring data were analyzed as described previously (Tahara et al., 2012). The bioluminescence emitted from each organ was calculated using Living Image 3.2 software (Caliper Life Sciences). For individual organs, the region of interest was set to the same shape and size throughout all experiments. In the case of the kidney, data from the right and left kidneys were added together before analysis. The average value (photon/s) from the six time points for each day was designated as 100%, and the bioluminescence rhythm for the entire day was expressed as a percentage of each set of six time points for the individual organs. The peak phase and amplitude of this normalized percentage data were determined using the single cosinor procedure (Acro.exe, version 3.5; designed by Dr. Refinetti; Tahara et al., 2012). The strength of the rhythmicity index (goodness of fit) was calculated as the ratio of the sums of squares of the best and worst fits. If a mouse was plotted in the area in which the amplitude in kidney, liver or salivary gland was >50% and the goodness-of-fit score was <0.1, we concluded that it possessed strong rhythmicity. The details of these criteria have been published elsewhere (Tahara et al., 2012).

Immunohistochemistry

Brain tissues of intact and ADX + 6 meals RF mice under LP conditions were collected at DZT 1, 7, 13 and 19 (N=3–5 for each time point) using ICR mice (Supplementary Figure S1E). Brains were fixed with 4% paraformaldehyde in phosphate buffer and stored in 20% sucrose in phosphate buffer overnight. Brains were sliced at 20-μm thickness using a cryostat. After three washes in PBS for 6 min, slices were incubated in blocking solution containing 20% normal goat serum (Vector Laboratories Inc., Burlingame, CA) in carrier solution, 1% bovine serum albumin (Sigma) and 0.3% Triton X in PBS for 1 h at room temperature. Following incubation with rabbit anti-mouse PER2 IgG (diluted 1:1000; Alpha Diagnostic International, San Antonio, TX) overnight at 4°C, slices were washed thrice with PBS and incubated with anti-rabbit IgG Fab2 conjugated with Alexa Fluor 488 (diluted 1:1000; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. After three washes in PBS, brain slices were coverslipped with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories Inc.) for cellular nuclei staining.

Images were taken with an all-in-one fluorescence microscope (BZ-8100; KEYENCE, Osaka, Japan) with a 20× objective lens and a 10× ocular lens. Finally, positive cells in each slice were counted using Image J, and total numbers of PER2 positive cells were averaged from both bilateral SCN using 2 slices per mouse.

Statistical analysis

All data are expressed as means ± SEM (standard error of the mean). Statistical analysis was performed using GraphPad Prism version 6.03 (GraphPad software, San Diego, CA). We determined whether the data showed a normal or non-normal distribution and equal or biased variation using the D’Agostino–Pearson/Kolmogorov–Smirnov and F tests, respectively. We used the Kruskal–Wallis test for non-parametric analysis and the Mann–Whitney or Dunn test for post hoc analysis. Analysis of two proportions was performed with Fisher’s exact test (JavaScript Star ver. 5.5.7), and one-sided p values are shown.

RESULTS

Effect of photoperiod on peripheral clocks

Bioluminescence rhythm waveforms in the kidney, liver and submandibular salivary gland were analyzed after the following adjustments: (1) alignment of the onset of each dark period (Figure 1A), (2) alignment of the middle of each dark period (Figure 1B) or (3) alignment of the onset of each light period (Figure 1C). Data were double-plotted to facilitate understanding of daily rhythms. Although the daily rhythm waveforms of peripheral clocks were the same among mice housed under SP, MP, or LP conditions, the peak time (phase) varied depending on the lighting conditions. We concluded that the phase of peripheral clocks is well adjusted by the onset of the dark period, driven by an E oscillator in every mouse housed under photoperiodic conditions. Therefore, in the following experiments, we analyzed the waveforms and phases of peripheral clocks after adjusting the data to align the onset of dark periods (DZT 0).

Effects of 6 meals RF, ADX and ADX + 6 meals RF on peripheral clock oscillation

The waveforms and phases of the peripheral clocks of mice in the six meals RF (Figure 2A), ADX (Figure 2B) and ADX + 6 meals RF (Figure 2C) treatment groups were determined by adjusting the data to align the onset of dark periods. The waveforms were not affected by 6 meals RF or ADX. However, waveforms were attenuated in the liver and kidney of mice in the ADX + 6 meals RF treatment group under LP conditions but not MP or SP conditions. These data suggest that impairment of kidney and liver clocks in LP-housed mice may be explained by dampened amplitude, weak rhythmicity or randomized phase of clocks.
To examine the possibility of weak rhythmicity, we next calculated the strength of rhythmicity by evaluating the amplitude and goodness-of-fit values. Figure 3 shows the plot distribution for each sample. Rhythmicity was similarly evaluated in the intact group, as described in our previous paper (Tahara et al., 2012), and strong rhythmicity was observed in the kidney, liver and salivary gland in the intact mice in this study. In comparison with the salivary gland clock, the kidney and liver clocks easily lost rhythmicity (Figure 3). Results of statistical analyses are shown in Table 1. Almost half mice in the 6 meals RF group.
FIGURE 3. Strength of rhythmicity evaluated by goodness of fit and amplitude. Correlation between cosinor amplitude and goodness of fit for PER2::LUC rhythms of (A) kidney, (B) liver and (C) salivary gland (SG). The horizontal dashed lines indicate a cosinor amplitude of 50%. The vertical dashed lines indicate a goodness of fit of 0.1. The numbers in parentheses indicate the number of mice tested for the given condition.
TABLE 1. Number of animals showing greater amplitude values (>50%) and lesser Goodness of fit values (<0.1) in each group.

|                | Kidney |                | Liver |                | Salivary gland |
|----------------|--------|----------------|-------|----------------|----------------|
|                | Amplitude (>50%) and goodness of fit (<0.1) | Amplitude (>50%) and goodness of fit (<0.1) | Amplitude (>50%) and goodness of fit (<0.1) |
| SP             | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) |
| Intact         | 9/10 | 10/10        | 10/10 | 10/10         | 10/10 | 10/10 |
| 6 Meals RF     | 7/10 | 4/10 | 6/10 | 7/9         | 4/10 | 6/10 |
| ADX            | 1/9  | 0.0010*      | 6/9   | 0.0054*      | 7/9   | 0.0054* |
| ADX + 6 meals RF | 3/10 | 0.0099*      | 10/10 | 10/10        | 10/10 | 10/10 |
| MP             | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) |
| Intact         | 9/9   | 9/9          | 9/9   | 9/9          |
| 6 Meals RF     | 6/9   | 5/9          | 6/9   | 6/9          |
| ADX            | 2/9   | 0.0011*      | 6/9   | 6/9          |
| ADX + 6 meals RF | 4/9  | 0.0147b      | 7/9   | 7/9          |
| LP             | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) | Hit/total | p Value (versus intact) |
| Intact         | 11/11 | 11/11        | 11/11 | 11/11        |
| 6 Meals RF     | 8/11  | 7/11 | 11/11 | 11/11        |
| ADX            | 3/7   | 0.0114b      | 7/7   | 7/7          |
| ADX + 6 meals RF | 0/11 | 0.0000a      | 6/11  | 6/11         |

Hit/total indicates the number of mice which exceed the criteria/total number of mice used.
The data are evaluated for one-sided p value by two proportions Fisher’s exact test (versus intact group) in each group *p<0.01; b p<0.05.

retained rhythmicity in the liver (4/10 [6 meals RF] versus 10/10 [intact], p<0.01 for SP; 5/9 [6 meals RF] versus 9/9 [intact], p<0.05 for MP; 7/11 [6 meals RF] versus 11/11 [intact], p<0.05 for LP; Fisher’s exact test) but not lost rhythmicity in the kidney. In the ADX group, few mice retained rhythmicity in the kidney (1/9 [ADX] versus 9/9 [intact], p<0.01 for SP; 2/9 [ADX] versus 9/9 [intact], p<0.01 for MP; 3/7 [ADX] versus 11/11 [intact], p<0.01 for LP) but not lost rhythmicity in the liver or salivary gland. Mice in the ADX + 6 meals RF group were not able to maintain rhythmicity in the kidney (3/10, p<0.01 for SP; 4/9, p<0.05 for MP; 0/11, p<0.01 for LP) or liver (4/10, p<0.01 for SP; 7/9, p<0.05 for MP; 6/11, p<0.05 for LP). Although we did not observe any differences in the strength of rhythmicity among mice housed under SP, MP, or LP conditions in the intact, 6 meals RF, ADX or ADX + 6 meals RF groups (Figure 3 and Table 1), the kidney clock was sensitive to ADX and the liver clock was sensitive to 6 meals RF (Figure 3; Table 1).

As the strength of rhythmicity was similarly impaired in mice in the ADX + 6 meals RF group under SP, MP and LP conditions, the impairment of kidney and liver clocks in LP-housed but not MP- or SP-housed mice (Figure 2) may not be explained by weak rhythmicity of clocks, but rather by randomized phase or dampened amplitude of clocks. In addition, we concluded that clock oscillation was very sensitive to the influence of ADX + 6 meals RF in the kidney and liver but not in the salivary gland.

To examine the possibility of randomized phase or dampened amplitude, we quantitatively analyzed the phase and amplitude for all IVIS data. Although the phase of the salivary gland clock was unaffected in MP- and LP-housed mice in the 6 meals RF group, the phases of the kidney and liver clocks were significantly advanced in this group (Figure 4). In contrast, in SP-housed mice, the 6 meals RF treatment did not affect the phase of the peripheral clocks. The phase angles of the peripheral clocks were minimally affected by ADX treatment under SP, MP, and LP conditions (Figure 4). However, under all lighting conditions, the phases of the kidney and liver clocks were significantly advanced in mice in the ADX + 6 meals RF group compared with intact mice (Figure 4A). The standard error of phases of the kidney and liver clocks was larger in LP-housed mice than in MP-housed mice. These data suggest that the impairment of kidney and liver clocks in LP-housed but not MP-housed mice (Figure 2) may be explained by randomized phase. Furthermore, SP-housed ADX mice showed a phase delay in the kidney, liver and salivary gland clocks in comparison with intact mice (Figure 4A). A reduction of the waveform amplitude was clearly observed in the kidney and liver of mice treated with ADX + 6 meals RF under SP or LP but not MP conditions (Figure 4B). In contrast, the salivary gland waveform amplitude was only slightly reduced in the ADX + 6 meals RF mice. This suggests that the impairment of kidney and liver clocks in LP-housed but not SP- or MP-housed mice (Figure 2) may also be explained by dampened amplitude.

Based on these results, we conclude that feeding and adrenal entrainment stimuli are both necessary for normal circadian oscillation of liver and kidney clocks in mice housed under different photoperiods.

ADX, 6 meals RF and change in photoperiod may affect feeding volume and/or body weight change, thereby affecting IVIS data. However, we found no differences in body weight change among treatment
groups in the present study (Intact group; 40.9 ± 0.8 g for SP, 41.5 ± 1.4 g for MP, 39.8 ± 0.7 g for LP, ADX + 6 meals RF group; 38.3 ± 1.5 g for SP, 40.7 ± 1.1 g for MP, 37.9 ± 1.3 g for LP) because treatments lasted only 2 weeks and mice in the RF groups received fixed volumes of food.

Feeding rhythm of intact and ADX mice housed under SP, MP or LP conditions

It has been suggested that the daily pattern of meals can determine the phase of peripheral clocks (Kuroda et al., 2012). Under SP, MP and LP conditions, the phase angles of bioluminescence waveforms were larger in
ADX mice than in intact mice (Figures 1, 2 and 4A), suggesting that the feeding pattern may be influenced by ADX. Thus, we measured the feeding activity in intact (Figure 5A) and ADX (Figure 5B) mice under free-feeding conditions after the adjustment to align the onset of dark periods. The cosinor fitting method was applied to find the peak phase of the feeding rhythm. The feeding activities of intact mice showed different patterns among SP, MP and LP conditions (Figure 5A). Intact SP-housed mice tended to take food in the middle of the dark period (DZT 5.5, evaluated by cosinor fitting), whereas intact LP-housed mice did so at the start of the dark period (DZT 0.5, evaluated by cosinor fitting). In contrast, ADX mice exhibited the same feeding pattern under SP (DZT 3.8), MP (DZT 2.0) and LP (DZT 2.0) conditions; under all photoperiods, feeding occurred at the start of the dark period (Figure 5B). These data suggest that the phase angle differences of peripheral clocks in LP-, MP- and SP-housed mice are not due to differences in their feeding activity rhythms.

**Locomotor activity rhythm of intact, ADX, 6 meals RF and ADX+6 meals RF mice housed under SP, MP or LP conditions**

As the peripheral clock phase may correspond to the locomotor activity phase, we examined the onset of locomotor activity. Figure 6 shows representative actograms of intact, 6 meals RF, ADX and ADX+6 meals RF mice housed under SP, MP or LP conditions. The activity onset time was determined using ClockLab software. In all treatment groups, the activity onset time was significantly different among SP-, MP- and LP-housed mice ($p<0.01$; Figure 6B). Intact, 6 meals RF and ADX mice housed under SP conditions exhibited activity...
FIGURE 6. Daily pattern of locomotor activity and activity onset time in intact, ADX, 6 meals RF and ADX + 6 meals RF mice. (A) Representative examples of double-plotted actograms for each condition. The horizontal lines indicate the clock time and the vertical lines indicate the passing day. (B) Average activity onset times. The horizontal white and black bars indicate light and dark periods, respectively. Each point is the mean ± SEM of the tested mice. DZT 0 denotes the zeitgeber time at the onset of the dark period. The numbers in parentheses indicate the number of mice tested for a given condition. Statistical analysis of differences among SP, MP and LP conditions was done using the Dunn’s test (**p<0.01), and statistical analysis of differences among treatments was performed using the Dunn test (#p<0.05).
onset around the time of onset of the dark period, whereas LP-housed mice demonstrated activity 3–4 h before the onset of the dark period. Furthermore, ADX + 6 meals RF mice showed significantly advanced activity onset under both SP and LP conditions compared with intact mice (p<0.05; Figure 6B). However, as in the intact mice, the phase angle of activity onset in ADX + 6 meals RF mice was still significantly different between LP- and SP-housed mice (p<0.01). These data suggest that the phase angle differences of peripheral clocks in LP- and SP-housed mice are not due to differences in their locomotor activity rhythms.

Dexamethasone restored rhythmicity of ADX + 6 meals RF mice housed under LP conditions

We examined whether impaired rhythmicity in ADX + 6 meals RF mice could be restored by exogenous corticosteroid. As the rhythmicity of the kidney clock was severely impaired in ADX + 6 meals RF mice housed under LP conditions, 1 mg/kg dexamethasone was administered to these mice at DZT 0 for 3 days. The impaired amplitude of peripheral clocks, especially in the kidney, was significantly restored by dexamethasone administration (Figure 7). The phases of peripheral clocks were delayed in the dexamethasone-treated group compared with the untreated group (Figure 7).

Impaired oscillation of peripheral clocks was not due to impaired SCN clock function

In the next experiment, we examined whether impairment of peripheral clocks was due to impairment of the central clock. The circadian pattern of the number of PER2-positive cells in the SCN was compared between intact and ADX + 6 meals RF mice housed under LP conditions (Figure 8). Brains including SCN of intact and ADX + 6 meals RF mice were collected at DZT 1, 7, 13 and 19. The number of PER2-positive cells showed a clear rhythmicity with a peak at DZT 1 in both intact and ADX + 6 meals RF mice (Figure 8A and B). After cosinor fitting of the rhythm of PER2-positive cell numbers, we found similar phases (1.6 for intact, 1.6 for ADX + 6 meals RF) and amplitudes (165.007 for intact, 164.544 for ADX + 6 meals RF) in both groups (Figure 8C). These data suggest that SCN clock function was not involved in impairment of kidney and liver clocks.

DISCUSSION

In this study, we utilized in vivo imaging to examine whether photoperiodic modulation of peripheral clocks is controlled by feeding pattern and/or corticosterone release. We found that waveforms of the liver, kidney and salivary gland clock oscillations were well-fitted by cosinor analysis and were similar among mice housed under SP, MP or LP conditions. Several researchers have proposed a model for hamsters and mice in which the E oscillator is located in the rostral SCN and the M oscillator is in the caudal SCN (Hazlerigg et al., 2005; Inagaki et al., 2007; Johnston, 2005). The role of the E oscillator in clock gene expression rhythm was initially demonstrated by Inagaki et al. (2007), who reported that this oscillator consists of SCN cells located in the anterior region of the SCN. When we determined the peripheral clock waveform after adjustments to align the onset of either the light phase (M oscillator) or the dark phase (E oscillator) or the midpoint of the dark phase under various photoperiods (Figure 1A–C), we found that the three waveforms (SP, MP and LP) were relatively well overlaid after adjustments to align the onset of the dark phase but not after the other adjustments. These results strongly suggest that signal output from the E oscillator may contribute to phase regulation of peripheral clocks. When data were adjusted to align the onset of the dark period, phase angle differences of the PER2::LUC waveform between mice housed under SP and LP conditions (Figures 1A and 2) were similar to those observed for feeding activity rhythms (Figure 5A) and locomotor activity rhythms (Figure 6A). Thus, the phases of these rhythms were advanced approximately 4 h in LP-housed mice compared with SP-housed mice. It is already known that the SCN controls the circadian rhythmicity of feeding and locomotor activity (Shibata et al., 2010). Given these findings and the present results, it is reasonable to conclude that under intact conditions, the phase of peripheral clocks may be driven and determined by information received from the SCN, with special emphasis on the E oscillator.

To clarify the role of regulatory signals from the SCN to peripheral clocks and the role of glucocorticoid secretion, we prepared ADX mice. Many studies have shown that glucocorticoids play a critical role as entraining factors, both in vivo and in vitro (Balsalobre et al., 2000a, b; Hayasaka et al., 2007; Koyanagi et al., 2006; Le Minh et al., 2001). Recently, in an ex vivo experiment, Pezu¨k et al. (2012) determined that ADX caused a phase delay in the peripheral clock in the liver and kidney but not in the lung, pineal gland, or SCN. This suggests that the adrenal gland contributes to phase regulation of some peripheral clocks. As ADX results in the loss of both adrenaline and mineralocorticoid functions, we should consider the roles of such functions in peripheral clock oscillation. In ADX mice in this study, the kidney clock phase was advanced 2–3 h compared with the liver and salivary gland clock phases, and the kidney clock was specifically impaired. Furthermore, the phase angle differences of the activity rhythm and PER2::LUC waveform in mice housed under SP, MP, or LP conditions were not significantly affected by ADX. Thus, these data suggest that the influence of the adrenal gland on the kidney clock is independent of photoperiod.

We reported previously that feeding pattern strongly affects the phase of peripheral clocks in vivo and ex vivo (Hirao et al., 2010; Kuroda et al., 2012). In addition, the onset of locomotor or wheel-running activity has been
shown to determine the phase of peripheral clocks under the condition of light cycle inversion (Castillo et al., 2011). Therefore, SP, MP, or LP conditions may change an organism’s feeding pattern, resulting in a phase change of peripheral clocks. The phase difference of feeding activity in mice housed under SP, MP, or LP conditions was reduced by ADX (Figure 5B), but the phase difference of peripheral clocks was unaffected (Figure 2). As observed in our previous work (Kuroda et al., 2012), mice that were given 6 meals per day with equal food volume and at equal intervals demonstrated phase advance of the peripheral clock and the locomotor activity rhythm. When mice housed under different photoperiods were provided with 6 meals per day, the peripheral clock phase in mice under LP conditions was largely advanced compared with that

FIGURE 7. Effect of daily injections of dexamethasone on in vivo PER2::LUC waveforms of ADX+6 meals RF mice housed under LP conditions. Peak phase and amplitude were calculated for individual mice by cosinor fitting analysis and the average peak phase and amplitude are shown for (A) kidney, (B) liver and (C) salivary gland. Each point is the mean±SEM of the tested mice (N=6 for dexamethasone; N=11 for no-drug-treatment). The horizontal white and black bar indicates day and night time, respectively. DZT 0 denotes the zeitgeber time at the onset of the dark period. Data for the non-drug-treated group are identical to the data for the ADX+6 meals RF group (N=11) in Figure 4. Statistical analysis of differences between drug treatment and non-drug-treatment was done using the Mann–Whitney test (*p<0.05, ##p<0.01).
in mice under SP conditions (Figure 4). However, under the same conditions, the locomotor activity rhythm phase was only slightly advanced and the phase differences in mice under SP or LP conditions were similar to those in intact mice (Figure 6). Currently, it is unclear why a feeding schedule of 6 meals per day preferentially affects the phase of peripheral clocks in mice housed under LP conditions but not SP conditions, but the phase of locomotor activity rhythm does not explain it. In two previous studies, the waveforms of the liver clock were evaluated ex vivo by determining the mRNA expression levels of clock genes that were differentially expressed between mice housed under LP or SP conditions (Parkanová et al., 2012; Sosniyenko et al., 2010). In mice subjected to a change from SP to LP conditions and fed twice daily during the daytime, the clock gene expression profiles in the SCN were not affected, whereas the waveforms and phases of the profiles in the liver were affected (Parkanová et al., 2012). In the present experiment, the liver clock was strongly impaired by the 6 meals RF treatment. Thus, the data presented here, together with the previous findings described above, suggest that activity rhythm and modifications of the feeding pattern may not be completely responsible for determining the phase of peripheral clocks. However, the present data suggest that feeding pattern affects the liver clock independently of photoperiod.

Finally, the ADX mice in this study were provided with 6 meals per day. Impairment of rhythmicity was observed in the liver and kidney clocks but not the salivary gland clock (Figure 3A–C). In addition, the phase angles of the peripheral clocks were almost the same in mice housed under SP, MP or LP conditions (Figure 4A–C), suggesting that the effect of photoperiod was reduced by ADX + 6 meals RF. Although the phase of

FIGURE 8. PER2 immunohistochemistry in the SCN of ADX + 6 meals RF mice housed under LP conditions. SCN tissues from intact and ADX + 6 meals RF mice housed under LP conditions were sampled at DZT 1, 7, 13 and 19 (N = 3–5 for each DZT). (A) Representative images of PER2-positive cells (green) and DAPI-positive cells (blue) in the SCN. (B) Daily rhythms of PER2-positive cell numbers in each SCN slice. To facilitate see daily rhythm, data were double-plotted. (C) Amplitude of rhythm after cosinor fitting.
the liver clock was more variable among SP- and LP-housed mice than among MP-housed mice (Figure 4A), we do not know the reason for these differences at present. Furthermore, the phase difference of locomotor activity rhythm among SP, MP, and LP conditions was unaffected in ADX mice fed 6 meals per day, suggesting that the phase of peripheral clocks is not simply reflective of the phase of locomotor activity. These results strongly suggest that a normal feeding pattern and intact adrenal gland are necessary to maintain peripheral clock oscillation in the liver and kidney but not the salivary gland. Le Minh et al. (2001) reported that glucocorticoid hormone inhibited restricted feeding-induced phase shifting of peripheral circadian oscillators, suggesting that feeding pattern-induced phase resetting may become dominant under ADX conditions. However, the present experimental data demonstrate that feeding and corticosterone signals work cooperatively to help keep normal clock function in peripheral organs, because daily injections of dexamethasone rescued the impairment of amplitude of peripheral clocks in ADX + 6 meals RF mice.

In the current study, we also examined whether the impairment of rhythmicity in peripheral clocks, especially the kidney clock, of ADX + 6 meals RF mice was due to impairment of the SCN clock. The PER2 expression rhythm indicated that the SCN clock was unaffected by the ADX + 6 meals RF treatment. Although photoperiod affects SCN clock function and thereby affects peripheral clocks, feeding and corticosteroids may strongly modify peripheral clocks but not the SCN clock. The signals that regulate and maintain the phase relationship between the SCN and peripheral clocks come from the autonomic nervous system, the pineal gland, and adrenal glands, behavioral cycles of feeding and activity, and the rhythm of body temperature (Menaker et al., 2013). Therefore, it is likely that there are many yet unidentified signals specific to individual organs as well. These unidentified signals may also participate in relaying circadian information from the SCN to peripheral organs.

In summary, the phase of the peripheral clocks in PER2::LUC mice was observed using an in vivo imaging system. We demonstrated that SP, MP and LP conditions entrained the phase and that the E oscillator may play an important role in phase regulation. We also showed that both normal adrenal gland function and feeding patterns are necessary for maintaining peripheral clock oscillation in the liver and kidney as well as the photoperiod responses of peripheral clocks.

DECLARATION OF INTEREST

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