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Lack of Food Anticipation in *Per2* Mutant Mice

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Summary

Predicting time of food availability is key for survival in most animals. Under restricted feeding conditions, this prediction is manifested in anticipatory bouts of locomotor activity and body temperature. This process seems to be driven by a food-entrainable oscillator independent of the main, light-entrainable clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus [1, 2]. Although the SCN clockwork involves self-sustaining transcriptional and translational feedback loops based on rhythmic expression of mRNA and proteins of clock genes [3, 4], the molecular mechanisms responsible for food anticipation are not well understood. *Period* genes *Per1* and *Per2* are crucial for the SCN's resetting to light [5–7]. Here, we investigated the role of these genes in circadian anticipatory behavior by studying rest-activity and body-temperature rhythms of *Per1* and *Per2* mutant mice under restricted feeding conditions. We also monitored expression of clock genes in the SCN and peripheral tissues. Whereas wild-type and *Per1* mutant mice expressed regular food-anticipatory activity, *Per2* mutant mice did not show food anticipation. In peripheral tissues, however, phase shifts of clock-gene expression in response to timed food restriction were comparable in all genotypes. In conclusion, a mutation in *Per2* abolishes anticipation of mealtime, without interfering with peripheral synchronization by feeding cycles.

Results

To determine whether *Per1* and *Per2* genes are involved in the adaptation to restricted feeding conditions, we exposed wild-type (WT), *Per1*, and *Per2* mutant mice either to a hypocaloric feeding (HF) schedule or to temporally restricted (TR) food access. No difference was found in the amount of wheel-running activity among the three genotypes fed ad libitum (AL) in light-dark (LD) conditions ($p = 0.51$). Daytime temporal food restriction is a potent synchronizer of peripheral clocks in nocturnal rodents held under light-dark conditions and does not alter clock-gene expression in their SCN [8–11]. However, HF can cause significant phase advances of circadian rhythms of locomotor activity and melatonin [12, 13] as well as alterations of both SCN clockwork and circadian responses to light [14]. As previously shown [13–15], WT mice under both TR and HF conditions showed a bout of wheel-running activity before feeding time that occurred at Zeitgeber time (ZT) 4 (3592 ± 1001 and 2208 ± 505 wheel revolutions, respectively; Figure 1A; see also Figure S1A in the Supplemental Data available online); we will refer to this activity as food-anticipatory activity (FAA). FAA is defined as the total number of activity bouts that occur during the 2 hr immediately preceding the daily mealtime. Statistical analysis revealed no difference between TR and HF conditions ($p = 0.67$, not significant [NS]) but a significant difference between AL and both TR and HF conditions ($p < 0.05$) for WT mice. *Per1* mutant mice displayed FAA comparable to that of WT animals (2108 ± 650 and 3208 ± 779 wheel revolutions in TR and HF, respectively; NS; Figure 1B and Figure S1B). No statistical difference could be found when WT and *Per1* mutant mice were compared in the same feeding conditions (AL: $p = 0.98$; TR: $p = 0.52$; and HF: $p = 0.70$). Interestingly, *Per2* mutant mice did not show significant FAA (482 ± 171 and 166 ± 53 wheel revolutions in TR and HF, respectively; $p < 0.05$ compared to WT mice; Figure 1C and Figure S1C). Moreover, no difference was found in wheel-running activity in different feeding conditions for the *Per2* mutants (AL versus TR: $p = 0.50$; AL versus HF: $p = 0.50$; and TR versus HF: $p = 0.9$). Because FAA is supposed to reflect an output of a food-entrainable oscillator (FEO), FAA can be expected to be present during fasting [1, 2]. To avoid masking by light, we tested food anticipation under fasting conditions in constant darkness (DD). Both WT and *Per1* mutant mice displayed FAA under these conditions (arrows in Figures 1A and 1B; see also Figures S1A and S1B). In contrast, fasted *Per2* mutant mice did not show any similar activity bout (Figure 1C and Figure S1C). This supports the finding that lack of activity before feeding time in light-dark conditions corresponds to lack of FAA, indicating that *Per2* plays a critical role in the process of food anticipation. However, a possible reappearance of FAA in *Per2* mutant mice during fasting might have been masked by the intrinsic free-running-activity rhythm (but see below).

To assess the influence of food restriction on the SCN, we released mice after TR or HF conditions into DD with food provided AL. In accordance with previous findings

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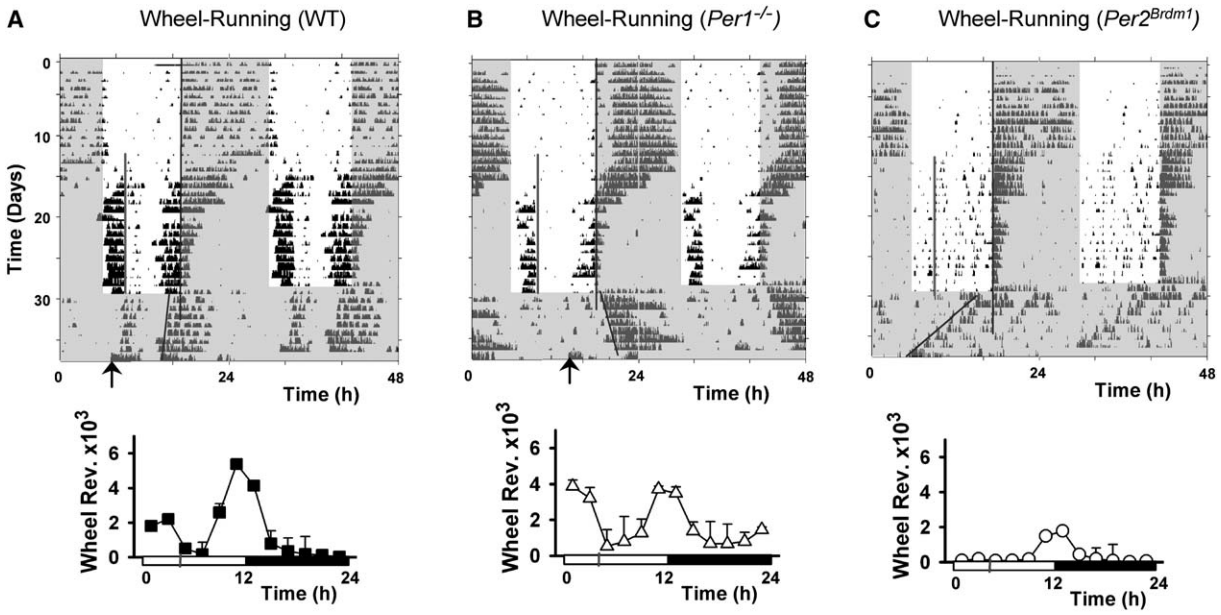

Figure 1. Daily Wheel-Running Activity

Wild-type (A), *Per1* (B), and *Per2* (C) mutant mice with hypocaloric feeding under light-dark conditions. Activity is plotted as an actogram with each horizontal line corresponding to two consecutive days and with the second day being double plotted on the next line. Gray shading indicates lights off. Animals were fed ad libitum, submitted to hypocaloric feeding, and subsequently released into constant darkness with food ad libitum with the exception of the last day (arrow) when no food was accessible. The gray line indicates the time when hypocaloric food was provided. The arrow shows food-anticipatory activity at the expected time in both wild-type and *Per1* mutant mice fed ad libitum in constant darkness. The bottom graph represents the mean daily-activity profile during the last 8 days of hypocaloric feeding period (n = 6 in WT and $Per1^{-/-}$ mice and n = 4 in $Per2^{Brdm1}$ mice; mean ± SEM). The gray line on the X axis indicates time of feeding. Note the lack of food-anticipatory activity in *Per2* mutant mice (C).

[8–10], temporal food restriction did not induce significant phase shifts (−18 ± 24 min) in the locomotor output in WT mice (Figure S2B). In contrast, HF led to phase advances of 82 ± 18 min. Interestingly, *Per1* mutant animals displayed phase delays under temporal food restriction (−71 ± 25 min), whereas under HF, no average phase change was observed (−15 ± 32 min). Furthermore, *Per2* mutant mice exhibited large phase advances under both restricted feeding conditions (TR: 153 ± 26 min and HF: 199 ± 72 min). Note that no phase shifts are observed in control *Per1* and *Per2* mutant mice that were fed AL and transferred from the same light-dark conditions to DD [7, 16, 17].

To assess whether the lack of wheel-running anticipation in *Per2* mutant mice is due to a light-masking effect that would directly suppress wheel-running activity in nocturnal mice, we investigated general cage activity and body temperature in WT and *Per2* mutant mice exposed to HF in constant light (LL; Figures 2A–2F) and DD (Figures S3A–S3F). WT mice under both conditions showed FAA during the 2 hr before feeding time, indicated by the shaded area in Figure 2E and Figure S3E. This FAA was not observed in *Per2* mutant mice (LL: 5.6 ± 1.5 versus 1.9 ± 0.7 a.u., $p < 0.05$ and DD: 6.9 ± 1.7 versus 2.3 ± 0.2 a.u., $p < 0.05$; sum in the shaded area of Figure 2E and Figure S3E). The daily amount of total activity was not significantly different in WT compared to *Per2* mutant mice either in LL (18.3 ± 5.6 versus 13.6 ± 4.3 a.u., respectively; NS) or in DD (26.1 ± 9.2 versus 24.0 ± 9.4 a.u., respectively; NS). Note that under both LL and DD conditions, the locomotor activity appeared to be synchronized to mealtime in WT mice, whereas in *Per2* mutant mice, this rhythm seems to free-run, and no FAA can be observed (Figure 2A and 2C; see also Figures S3A and S3C). Under both LL and DD conditions, WT mice showed a daily increase of body temperature during the 2 hr before feeding time, concomitant with FAA, indicated by the shaded area in Figure 2F and Figure S3F. There was also a daily 2 hr postprandial increase of body temperature, corresponding to the so-called diet-induced thermogenesis (DIT). Compared to that in WT mice, body temperature in *Per2* mutant mice was significantly reduced during the 2 hr before mealtime (LL: 36.2 ± 0.4°C versus 34.6 ± 0.3°C, $p < 0.05$; DD: 37.0 ± 0.4°C versus 35.5 ± 0.5°C, $p < 0.05$; average in the shaded area of Figure 2F and Figure S3F). By contrast, DIT was similar between WT and *Per2* mutant mice (LL: +1.7 ± 0.4°C versus +2.6 ± 0.4°C, NS; DD: +1.0 ± 0.5°C versus +1.9 ± 0.5°C, NS; respectively). Similar to the activity rhythms, temperature rhythms appear to be synchronized to mealtime in WT mice, whereas in *Per2* mutants, this rhythm free-runs (Figures 2B and 2D; see also Figures S3B and S3D). Hence, peaks before mealtime every 10–15 days do not correspond to an anticipatory bout of body temperature but rather represent the free-running-activity rhythm. However, it is of note that restricted feeding maintains circadian rhythmicity in the *Per2* mutant mice housed in constant darkness. After the last day of hypocaloric feeding in DD, food was provided AL with a 6 hr delay compared to previous mealtime (horizontal white arrow on Figures S3A–S3D). WT mice displayed both an anticipatory increase in body temperature and a delayed DIT (Figure S3B). By contrast, *Per2* mutants showed only

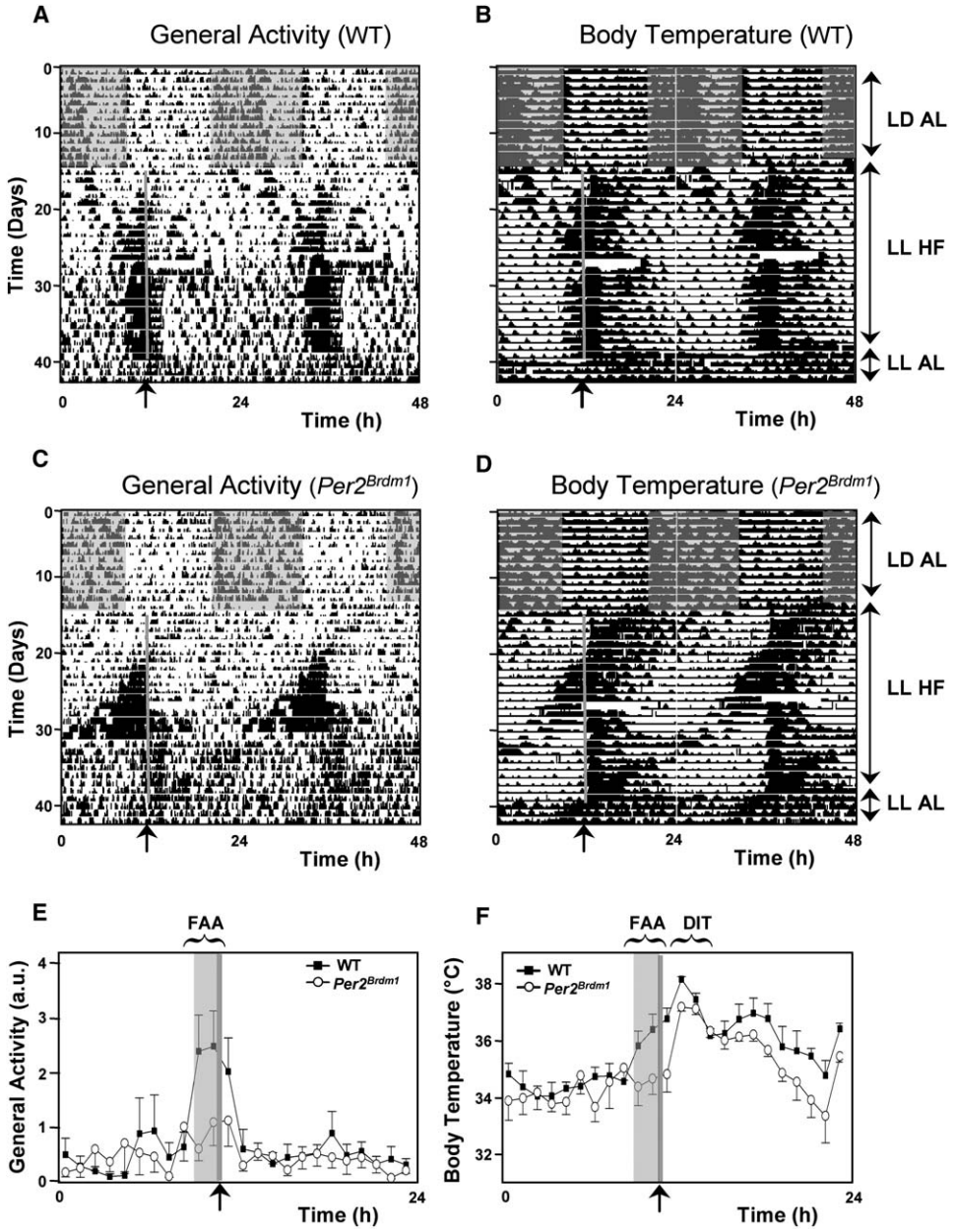

Figure 2. Daily General Cage Activity and Body Temperature

Wild-type (A and B) and *Per2* mutant (C and D) mice under hypocaloric feeding conditions in constant light. General activity (A and C) and temperature (B and D) are double plotted. Gray shading indicates lights off. Animals were fed ad libitum under a light-dark cycle (days 1–14), submitted to hypocaloric feeding, released into constant light (days 15–38), and fed ad libitum for a period at the end (days 39–42). The gray line and vertical black arrow indicate the time when hypocaloric food was provided. Mealtime was followed by a large postprandial increase in body temperature, corresponding to the so-called diet-induced thermogenesis (DIT) in both wild-type (A and B) and *Per2* mutant (C and D) mice. The white area on day 27 is due to a failure in data acquisition. The bottom graphs represent daily profiles of general activity (E) and body temperature (F) in wild-type (black squares) and *Per2* mutant (open circles) mice during the hypocaloric feeding period in constant light (n = 5 for both genotypes; mean ± SEM). The arrow below the X axis indicates time of feeding. Brackets with FAA and DIT show the periods during which FAA and DIT are expected, respectively. The shaded area indicates the 2 hr period prior to mealtime. Note the lack of food-anticipatory general activity (C and E) and temperature (D and F) in *Per2* mutant mice.

a delayed DIT (Figure S3D). This observation rules out the possibility of a masked FAA by free-running rhythms in fasted *Per2* mutant mice in our first experiment.

WT animals exposed to a light-dark cycle display a phase shift under HF conditions, and such finding indicates an effect on the SCN. Therefore, we studied the expression of clock and clock-controlled genes in the SCN by using in situ hybridization (Figure 3). In AL-fed animals, *Bmal1* expression was slightly reduced in *Per1* and *Per2* mutant animals compared to WT mice (Figure 3A) [18, 19]. Under HF conditions, *Bmal1* expression was downregulated in WT mice, whereas in the *Per1* and *Per2* mutant animals, expression was strongly reduced (Figure 3A). *Cry1* expression in *Per1* mutant

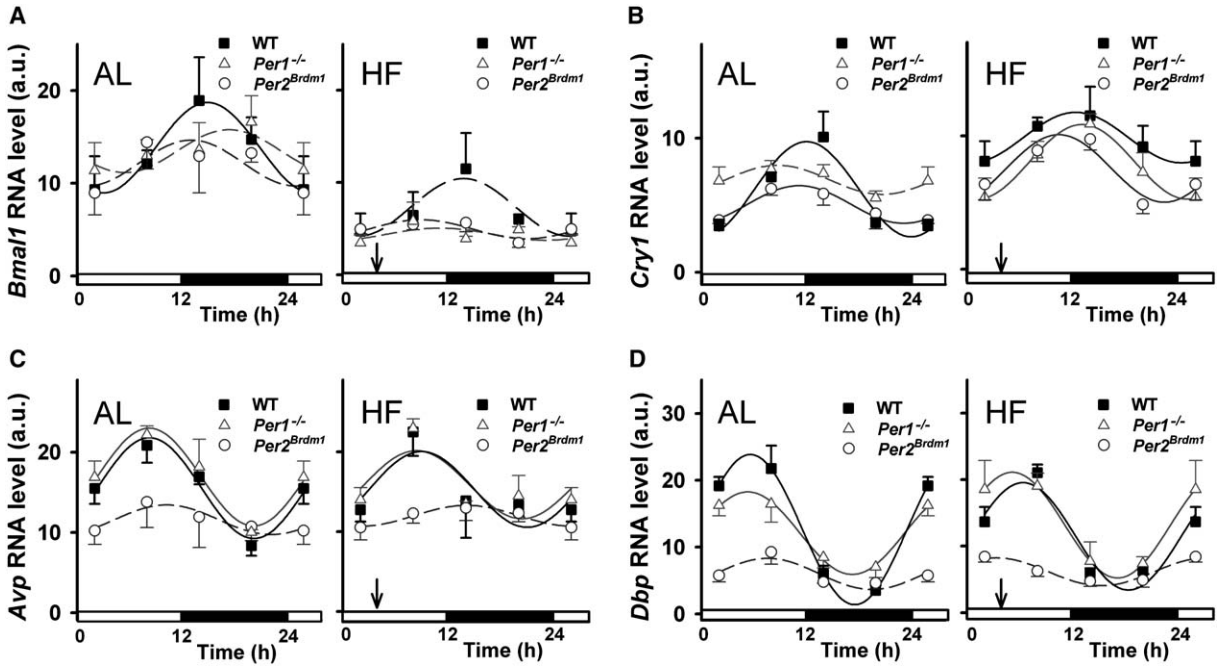

Figure 3. Expression of Clock and Clock-Controlled Genes in the SCN of Mice under Ad Libitum or Hypocaloric-Feeding Conditions as Assessed by In Situ Hybridization

Expression of *Bmal1* (A), *Cry1* (B), *Avp* (C), and *Dbp* (D) under ad libitum (AL, left panel) or hypocaloric (HF, right panel) feeding conditions in wild-type (squares), *Per1* mutant (triangles), and *Per2* mutant (circles) mice (n = 3–4; mean ± SEM). Data for ZT2 were double plotted. Nighttime is indicated by a black bar on the X axis. Significant and nonsignificant regressions are shown with solid and dashed lines, respectively.

mice did not display a diurnal pattern compared with that in WT animals under AL (Figure 3B) conditions, whereas in *Per2* mutant mice, the *Cry1* expression pattern was diurnal with a reduction in amplitude and an advanced phase (Figure 3B) as previously shown [18, 19]. Interestingly, HF not only restored in *Per1* mutant animals the diurnal expression pattern of *Cry1* observed in WT mice, but it also led to upregulated levels of *Cry1* mRNA in WT, *Per1*, and *Per2* mutant mice (Figure 3B). The clock-controlled genes *Dbp* and *Avp* were expressed in a similar diurnal fashion in WT and *Per1* mutant mice under AL conditions. Under HF conditions, this was maintained in the two genotypes (Figures 3C and 3D). In *Per2* mutant mice under either AL or HF conditions, *Avp* and *Dbp* expression did not display a diurnal pattern compared to that in WT animals (Figures 3C and 3D, respectively).

Changes in gene expression in response to restricted feeding have been observed in peripheral tissues of WT mice [8–10]. To investigate the effects of HF on gene expression in the liver and kidney of *Per1* and *Per2* mutant mice, we performed quantitative PCR analysis. Consistent with previous findings [20], diurnal *Bmal1* expression was greatest close to the dark-to-light transition in the liver of WT mice under AL (Figure 4A) conditions. Also, *Rev-Erbα* expression peaked during daytime (Figure 4B) as previously described [20]. For both *Bmal1* and *Rev-Erbα* genes, expression in the liver of *Per1* mutant mice was close to that in WT mice, whereas it was dampened in the liver of *Per2* mutant mice fed AL (Figures 4A and 4B). The timing of *Per1* expression in *Per2* mutant mice was advanced in comparison to WT profile under AL (Figure 4C) conditions, whereas diurnal expression of *Per2* in *Per1* mutant mice was similar to WT profile under AL (Figure 4D) conditions. In keeping with earlier observations [8], the clock-controlled gene *Dbp* was expressed in a diurnal fashion in WT mice with maximal values in late daytime. A similar pattern of expression was found in *Per1* and *Per2* mutant mice under AL (Figure 4E) conditions.

As described previously [8–10, 21], diurnal expression of *Bmal1*, *Rev-Erbα*, *Per1*, *Per2*, and *Dbp* was shifted in response to diurnal-restricted feeding in WT mice (Figure 4). For *Per1* and *Per2* mutant mice, a hypocaloric feeding during daytime led to comparable phase changes of clock- and clock-controlled-gene expression in the liver (Figure 4).

Discussion

The circadian timing system is thought to be important for an organism to predict daily recurring events such as availability of food and emergence of predators. This appears to be advantageous for survival in a competitive environment. To test this hypothesis, we challenged mice mutant for *Per1* or *Per2*, two genes critical for light-induced clock resetting [5–7] and responses to drugs of abuse [22–24], with limited food availability.

We found that WT and *Per1* mutant animals were able to predict time of food access, whereas mice with a mutation in the clock gene *Per2* did not show food anticipation. Moreover, FAA was not expressed in food-restricted *Per2* mutant mice in either LL or DD, excluding a negative masking effect by light. Reduction in FAA has been observed previously in *Cry* mutant mice [25], and appearance of FAA is delayed in *NPAS2* mutant

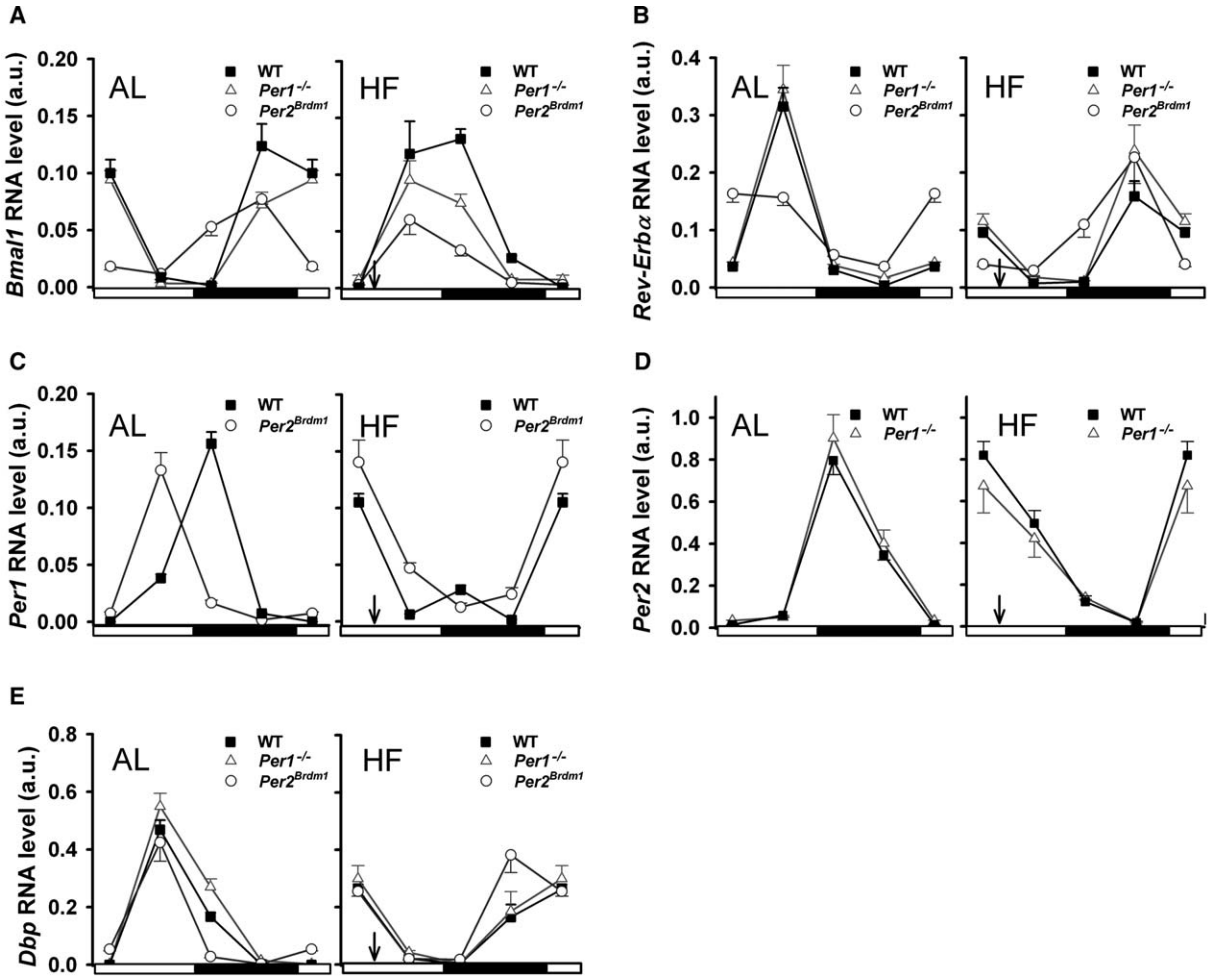

Figure 4. Expression of Clock and Clock-Controlled Genes in the Liver of Mice under Ad Libitum or Hypocaloric-Feeding Conditions as Assessed by Real-Time Polymerase Chain Reaction

Expression of *Bmal1* (A), *Rev-Erbα* (B), *Per1* (C), *Per2* (D), and *Dbp* (E) under ad libitum (AL, left panel) or hypocaloric (HF, right panel) feeding conditions in wild-type (squares), *Per1* mutant (triangles), and *Per2* mutant (circles) mice (n = 3; mean ± SEM). Data for ZT2 were double plotted. Nighttime is indicated by a black bar on the X axis.

animals [26]. However, FAA in *Clock* mutant mice is normal [21, 27]. These investigations demonstrate that not all clock components are essential for regulation of FAA. It is believed that food anticipation is regulated by an FEO independent of the SCN [1, 2]. Although dorsomedial hypothalamic nuclei (DMH) have been implicated in that timing mechanism [28] (but see also [29]), the precise neuronal substrate of the putative FEO remains to be clarified [30]. In the DMH, a robust oscillation of *Per2* gene expression is only observed under restricted feeding conditions, suggesting a role of this gene in DMH function [31]. Our results provide the first evidence that food-entrainable oscillations require functional *Per2*, which will be a tool of choice to target the locations and clockwork of the FEO.

Considering that the *Per2* mutation could lead to pleiotropic effects, the lack of FAA in these mutants might be due to other causes than a circadian defect. Impaired regulation of hunger and altered mobilization of energy stores are relevant possibilities. Several arguments do not support the hypothesis of a reduced feeling of hunger in *Per2* mutant mice. Animals with lowered feelings of hunger tend to be aphagic even with food available ad libitum. This is not the case for *Per2* mutant mice whose daily food intake with regular chow pellets or a high-fat diet perfectly matches that of WT littermates (Figure S4). Moreover, food-restricted *Per2* mutant mice that look usually drowsy before the mealtime wake up and start to eat as soon as food is available (C.A.F. and E.C., unpublished data). Only an EEG recording would allow the exact vigilance state of *Per2* mutant mice prior to meal time to be defined. Our behavioral observation is clearly confirmed by the timing and amplitude of DIT in these mutant mice even when mealtime was delayed by 6 hr. A reduction of FAA has been observed in rats fed with a high-fat diet [32], pointing to possible interactions of body-fat composition or feeding efficiency with FAA, or both. However, it is very unlikely that this causes absence of FAA in *Per2* mutant mice because their body-fat composition and feeding efficiency are similar compared to those of WT animals under normal and high-fat diet (Figure S4).

When we measured food anticipation by using wheel-running activity, alterations in mean activity levels may

have influenced our read-out. Therefore, we quantified locomotor activity in all genotypes under the different feeding conditions (Figure S2A). No significant differences could be detected between ad libitum and restricted feeding conditions. We noticed a tendency for reduced wheel-running activity in *Per2* mutant mice exposed to hypocaloric feeding conditions under a light-dark cycle (Figure S2A), raising the possibility that impaired FAA might be due to altered capability to mobilize stored energy such as glycogen to sustain locomotor activity. In the liver, we noticed that under ad libitum conditions, the amount of glycogen changed in a diurnal fashion with minimal levels around dusk. This pattern was comparable in all three genotypes measured (WT, *Per1* mutant, and *Per2* mutant, see Figure S5A). In response to hypocaloric feeding during daytime (ZT4), this pattern was inverted with the minimal levels being at dawn in all genotypes (Figure S5A). Moreover, free glucose levels in the liver were not different in *Per2* mutant mice compared to WT and *Per1* mutant animals (Figure S5B). These findings indicate that lack of FAA in *Per2* mutant mice is probably not caused by altered glucose mobilization. Additionally, body-weight changes in response to temporally restricted and hypocaloric feeding were similar between *Per* mutant and WT mice (Figure S6), indicating comparable overall energy mobilization.

Per2 mutant mice display no detectable FAA as assessed in particular by wheel-running activity. However, this lack might be masked by place preference. *Per2* mice might stay in front of the food bin rather than run in the wheel in expectation of food. This is unlikely because previous studies with cocaine as a reward showed that place preferences are similar between WT and *Per2* mutant mice [22]. Moreover, *Per1* mutant mice exhibit reduced place preference [22], although FAA is not strongly affected (Figure 1B and Figure S1B). Taking these findings together with the absence of FAA assessed by general cage activity and the lack of anticipatory thermogenesis, we conclude that *Per2* mutant mice have no capacity to anticipate 24 hr cycles of food availability. Considering the short endogenous period in *Per2* mutant mice, further experiments under both long and short T cycles would reveal whether 24 hr cycles of feeding fall outside the range of entrainment of the FEO in these mice.

When challenged by a hypocaloric feeding or temporal food restriction under a light-dark cycle, *Per2* mutant mice show large phase advances of the locomotor-activity rhythm, and this indicates an effect on the SCN. In WT mice, such advances are usually absent under temporal food restriction [8, 9], although they can be observed under hypocaloric conditions [13, 14] (but see also [15]). Surprisingly, *Per1* mutant animals displaying a phase shift only show delays comparable to that of *Clock* mutant mice [33]. Our observations that *Per1* and *Per2* mutant mice show no or increased phase advances, respectively, are reminiscent of the differential light response of these mutants [7]. The altered shifting effects also reveal the involvement of *Per1* and *Per2* genes in the nutritional synchronization of the SCN. Hypocaloric feeding imposed alterations of gene expression in the SCN [14]. Here, we show that hypocaloric feeding led not only to upregulated *Cry1* expression in the SCN but also to downregulated *Bmal1* expression, with lowest levels in *Per1* and *Per2* mutant mice. Because *Bmal1* and *Cry1* are positive and negative regulators of the molecular clockwork, respectively, these transcriptional modifications under HF conditions may partly explain the apparent reduction in amplitude of two molecular outputs, *Avp* and *Dbp*, at least in the WT mice. Therefore, under certain food-restriction conditions, phase shifts as well as changes in gene expression can occur, suggesting a feedback of nutritional cues to the SCN in which both *Per1* and *Per2* genes may play an important role. Such cues could involve glucose metabolism or 5′-AMP plasma levels [34].

Daytime feeding changes the phase of circadian gene expression in the liver [8–10, 21, 35]. Our data show that hypocaloric feeding during daytime led to similar phase changes in the liver of WT mice. Interestingly, comparable phase shifts were noted in the liver and kidney of *Per1* mutant and *Per2* mutant mice (Figure 4 and Figure S7), suggesting that the synchronizing effects of daytime food restriction on circadian oscillators in peripheral organs do not directly involve the *Per1* and *Per2* genes.

In conclusion, resetting of peripheral oscillators by feeding time does not critically rely on *Per1* or *Per2* genes. Moreover, food-restricted *Per1* and *Per2* mutant mice show altered phase shifts of the locomotor output controlled by the SCN clock, suggesting an involvement of *Per1* and *Per2* in the synchronizing effects of nutritional cues to the SCN. Finally, anticipatory bouts of wheel-running activity, general cage activity, and body temperature, used as outputs of food-entrainable oscillations, are not expressed in food-restricted *Per2* mutant mice, demonstrating the essential involvement of *Per2* in the anticipation of mealtime.

Supplemental Data

Supplemental Data include Experimental Procedures and seven figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/20/2016/DC1/.

Acknowledgments

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