

# The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker

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**Temporal restriction of feeding can phase-shift behavioral and physiological circadian rhythms in mammals. These changes in biological rhythms are postulated to be brought about by a food-entrainable oscillator (FEO) that is independent of the suprachiasmatic nucleus. However, the neural substrates of FEO have remained elusive. Here, we carried out an unbiased search for mouse brain region(s) that exhibit a rhythmic expression of the *Period* genes in a feeding-entrainable manner. We found that the compact part of the dorsomedial hypothalamic nucleus (DMH) demonstrates a robust oscillation of *mPer* expression only under restricted feeding. The oscillation persisted for at least 2 days even when mice were given no food during the expected feeding period after the establishment of food-entrained behavioral rhythms. Moreover, refeeding after fasting rapidly induced a transient *mPer* expression in the same area of DMH. Taken in conjunction with recent findings (*i*) that behavioral expression of food-entrainable circadian rhythms is blocked by cell-specific lesions of DMH in rats and (*ii*) that DMH neurons directly project to orexin neurons in the lateral hypothalamus, which are essential for proper expression of food-entrained behavioral rhythms, the present study suggests that DMH plays a key role as a central FEO in the feeding-mediated regulation of circadian behaviors.**

circadian rhythm | feeding | mouse | obesity | period

For animals to survive effectively, the timing of feeding-related behaviors should be appropriately coordinated both with environmental conditions, such as food availability and risk of predation, and internal physiological states, such as gastrointestinal function and energy balance. The circadian oscillator in the suprachiasmatic nucleus (SCN), which is regarded as the master clock in mammals, orchestrates multiple circadian rhythms in the organism and is regulated according to environmental light/dark cues conveyed from the eye (light-entrainable oscillator) (1, 2). However, when food availability is restricted to a single period scheduled at a fixed time of the day [restricted feeding (RF)] animals adapt to this condition within a few days by feeding during the period of food availability and increasing food-seeking activity in the preceding hours [food-anticipatory activity (FAA)] (3, 4). Such anticipatory behaviors are often accompanied by increases in body temperature, adrenal corticosterone secretion, and gastrointestinal motility. These RF-induced biological rhythms persist even when SCN function is physically or genetically ablated, indicating the presence of a food-entrainable oscillator (FEO), that is separate from and independent of SCN.

Expression of certain molecular components of circadian clocks, such as *Per1* and *Per2*, has been found oscillating (usually entrained by the SCN master clock) in many peripheral tissues and brain regions outside of the SCN (1, 2, 5–8). Previous studies demonstrated that RF entrains and shifts the circadian oscillators in the peripheral tissues and certain non-SCN regions of the brain, whereas rhythmicity in the SCN remains phase-locked to the light/dark cycle (9–12). These observations suggest a phase uncoupling of multiple circadian oscillators outside of the SCN from the master clock under RF.

We and others previously reported that neurons producing orexins, neuropeptides critical for promoting wakefulness, convey an efferent signal from the putative FEO in the brain to increase wakefulness and locomotor activity rather than act as the FEO itself (13, 14). Despite the accepted existence of the FEO and efforts to find its anatomical substrate, a long list of lesions to candidate neural and neuroendocrine tissues have failed to identify the locus of the FEO (3, 4). A caveat for lesion-based approach is that these experiments will not tell whether the lesioned area is the site of the FEO, afferent to the FEO, or efferent from the FEO, even if the lesion eliminates food-entrainable rhythms (15, 16). In the present study, we performed an unbiased search for brain region(s) that exhibit a *de novo* rhythmic expression of *mPer1* and *mPer2* under RF to identify a candidate locus for the FEO.

## Results

To reveal behavioral rhythms entrained by feeding, we examined the hourly distribution of locomotor activity under ad libitum feeding (ALF) or 4-hr RF in the middle of the light phase [zeitgeber time (ZT) 4–8, ZT0 is defined as the lights-on time and ZT12 as the lights-off time; see *Methods*]. As reported (3, 4, 13), mice demonstrated under RF a robustly increased locomotor activity for 2 h before food availability (Fig. 1). Temporal pattern of locomotor activity under RF, once established, was maintained with a sharp rise of FAA, even when mice were given no food during the expected feeding period (RF-F1). This finding is consistent with the hypothesis that a circadian oscillator (FEO) regulates food-entrainable rhythms, rather than that FAA is simply triggered each day when energy depletion reaches some threshold before food availability (3, 4).

To seek for location(s) of the putative FEO(s), we then performed an unbiased search for such brain region(s) that exhibit under RF a strong rhythmic expression of *mPer2*, a core component of circadian clocks. We collected brains at four time points (ZT1, ZT7, ZT13, and ZT19) on the ninth day under ALF or RF. *In situ* hybridization using <sup>35</sup>S-labeled antisense riboprobe for *mPer2* was carried out on a series of stereotaxic coronal sections covering the entire rostrocaudal axis from the olfactory bulb to the medulla oblongata.

In the SCN, we observed weak expression of *mPer2* at ZT1, the highest levels at ZT7, moderate levels at ZT13, and essentially no expression at ZT19. As reported (9–12), RF did not appreciably change the *mPer2* rhythm in SCN. In contrast, the diffuse *mPer2* expression in the striatum and cerebral cortex was higher in the dark phase (ZT13 and ZT19) under ALF, whereas

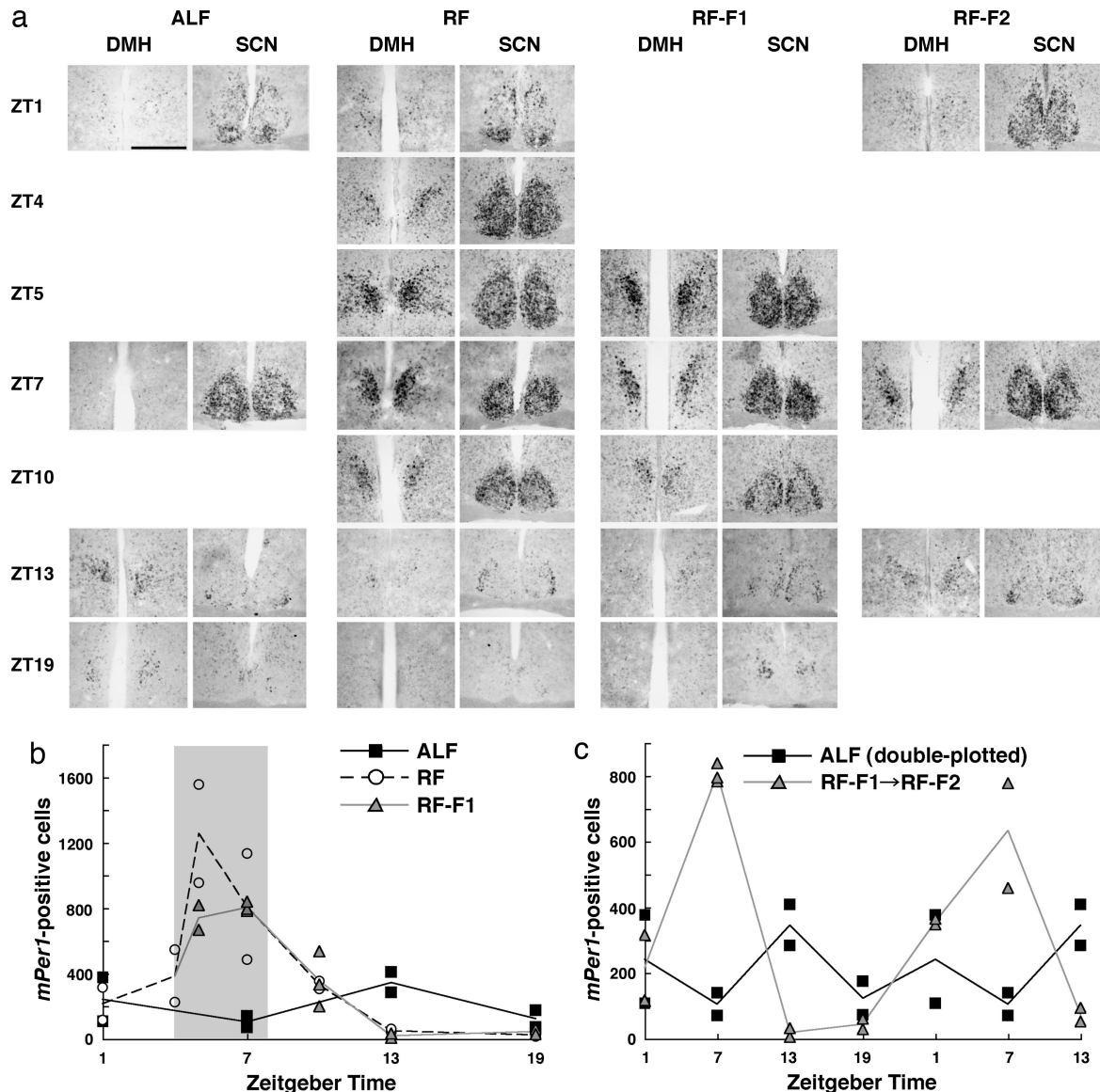
Conflict of interest statement: No conflicts declared.

Abbreviations: FEO, food-entrainable oscillator; RF, restricted feeding; FAA, food-anticipatory activity; ALF, ad libitum feeding; ZT, zeitgeber time; SCN, suprachiasmatic nucleus; DMH, dorsomedial hypothalamic nucleus; DMHC, compact part of DMH; NTS, nucleus of the solitary tract; AP, area postrema; DMV, dorsal motor nucleus of vagus.

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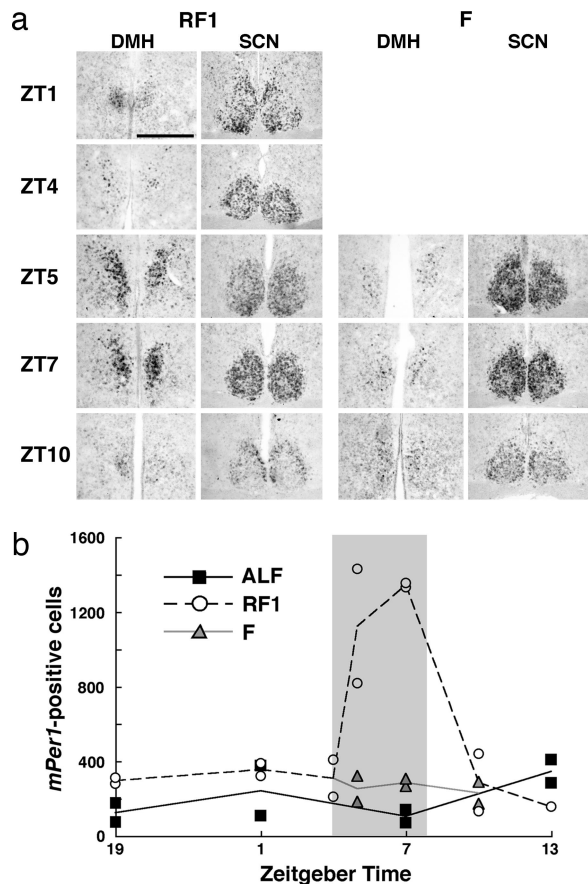


**Fig. 4.** Detailed temporal patterns of circadian *mPer1* expression in the DMH under RF. (a) Coronal brain sections, prepared from mice under ALF, RF, or RF followed by fast (RF-F1) on day 7 or under prolonged fasting on day 8 after RF-F (RF-F2) at time points shown in ZT, were hybridized *in situ* to a digoxigenin-labeled *mPer1* antisense probe. Representative sections from duplicate mice at the level of the DMH and SCN are shown. Note that samples at ZT1 and ZT4 under RF are shared by the RF-F1 condition. (Scale bar: 0.2 mm.) (b and c) Numbers of *mPer1*-expressing cells in the ventromedial part of the DMH. Values from two mice per data point (three mice for ZT7 under RF-F1) are dot-plotted; averages of duplicate counts are line-plotted. (b) Comparison of cell numbers under ALF, RF, and RF-F1. The period of food availability is shaded. (c) Food-entrained circadian oscillation of *mPer1* expression in the DMHc sustains for at least 2 days without food. The preceding four time points (ZT1, ZT7, ZT13, and ZT19) are the same as those of RF-F1 in b, followed by three time points (ZT1, ZT7, and ZT13) on the second day of prolonged fasting (RF-F2). At the end of the experiments, mice under RF-F2 fasted for 53 h since the last food availability. For comparison, values under ALF, the same as those in b, are double-plotted.

specific antigen NeuN demonstrated that this cluster consisted of neurons covering three to four sections 90  $\mu\text{m}$  apart within the ventromedial part of the DMHc (Fig. 3 e-h).

We quantified temporal patterns of *mPer1* expression in the DMH around the period of food availability by counting the number of *mPer1*-positive cells in the ventromedial part of the DMHc (Fig. 4 b and c) (note that the intensity of labeling in each positive cell was not considered in this procedure). Under ALF, *mPer1* expression in the DMHc was relatively low and had relatively little, if any, oscillation, was higher at the onset of dark phase (ZT13), and was lowest in the middle of light phase (ZT7); these results may reflect the nocturnal feeding pattern of mice (Fig. 4 a and b). On the seventh day under RF, robust circadian

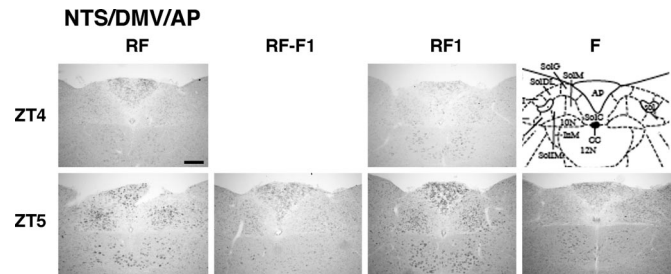
oscillation of *mPer1* expression was observed in the DMHc; expression started to increase immediately before food availability (ZT4), peaked during the period of food availability (ZT5 and ZT7), rapidly decreased after the food availability (ZT10), and reached nadir during the dark phase (ZT13 and ZT19). To exclude the possibility that this pattern of *mPer1* expression was simply a reflection of food availability, we examined *mPer1* expression when mice were given no food during the expected feeding period on the seventh day under RF (RF-F1). The temporal pattern of *mPer1* expression under this condition was similar to that with food (RF). Furthermore, circadian oscillation of *mPer1* in the DMHc persisted under prolonged fasting for an additional day, although peak expression level during the



**Fig. 5.** Rapid induction of *mPer1* in the DMHc by feeding. (a) Coronal brain sections, prepared from mice on the first day under RF (RF1) or fasting (F) at time points shown in ZT, were hybridized *in situ* to a digoxigenin-labeled *mPer1* antisense probe. Representative sections from duplicate mice at the level of the DMH and SCN are shown. Note that samples at ZT1 and ZT4 under RF1 are shared by the fasting condition. (Scale bar: 0.2 mm.) (b) Numbers of *mPer1*-expressing cells in the ventromedial part of the DMHc. Values from two mice per data point (one mouse for ZT13 under RF1) are dot-plotted; averages of duplicate counts are line-plotted. Food was removed at ZT8 preceding time points shown in the graph, and returned, only under the RF1 condition, during the period shaded.

period of expected food availability tended to be slightly reduced (RF-F2, Fig. 4 *a* and *c*). These results indicate that circadian expression of *mPer1* in the DMHc is entrained by feeding and free-runs at least for two cycles without further entrainment.

To understand how feeding entrains rhythmic expression of *mPer1* in the DMHc, we next examined acute effects of feeding on its expression in this area (Fig. 5). On the first day under RF, scheduled feeding after 20-h fasting rapidly induced *mPer1* expression in DMHc within 1 h (RF1). This expression continued at least for 3 h and then returned to the baseline level within 6 h from the onset of feeding. Fasting by itself did not cause such a robust *mPer1* expression (F in Fig. 5). Interestingly, the basal *mPer1* expression in the DMHc under fasting was higher than that under ALF during the late dark phase and the light phase (ZT19, ZT1, and ZT7; collectively,  $P < 0.05$ , unpaired *t* test); a small number of DMH neurons might sense negative energy balance to up-regulate *mPer1* expression. Although we have not performed systematic quantification of expression, *mPer2* expression detected by *in situ* hybridization using digoxigenin-labeled riboprobe also demonstrated spatial and temporal patterns similar to those of *mPer1* under various feeding conditions (data not shown).



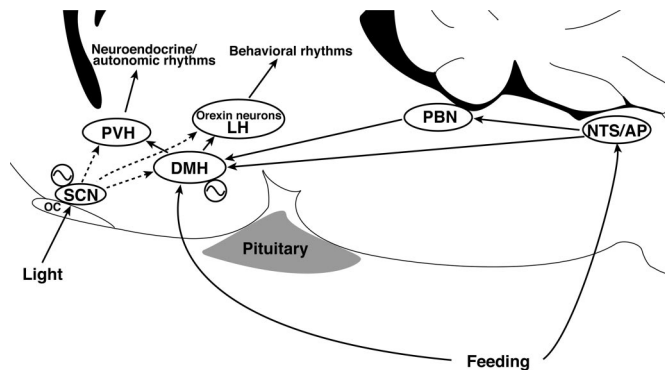
**Fig. 6.** Feeding-dependent up-regulation of *mPer1* in the NTS, DMV, and AP. Coronal brain sections, prepared from mice at ZT4 and ZT5 under RF or RF followed by fast (RF-F1) on day 7, or RF (RF1) or fasting (F) on day 1, were hybridized *in situ* to a digoxigenin-labeled *mPer1* antisense probe. Representative sections from duplicate mice are shown, together with a coronal diagram from the mouse brain atlas (39). Note that samples at ZT4 are shared between RF and RF-F1 and between RF1 and F. (Scale bar: 0.2 mm.)

Consistent with the results of our *in situ* hybridization experiments using  $^{35}\text{S}$ -labeled riboprobe for *mPer2*, we observed rhythmic *mPer1* expression in the NTS and AP under RF by *in situ* hybridization using digoxigenin-labeled riboprobe for *mPer1* (Fig. 6). Expression peaked during the food availability, although the maximum levels were blunted compared with those in the DMHc. Expression in the NTS was highest at the anatomical level of the AP; most *mPer1*-expressing cells were restricted within the medial nucleus of the NTS and the dorsal motor nucleus of vagus (DMV). In contrast to the aforementioned situation in the DMHc, however, rhythmic *mPer1* expression in the NTS/DMV/AP area under RF depends on periodical feeding *per se*; when mice were given no food during the expected feeding period on the seventh day under RF, little induction of *mPer1*, if any, was observed at ZT5 compared with that of fed mice under RF. On the first day under RF (RF1), like in the DMHc, feeding rapidly induced *mPer1* expression in the NTS/DMV/AP area. These results suggest that the NTS/DMV/AP area increases *mPer1* expression in acute response to feeding, but it does not contain an autonomous circadian oscillator entrained by feeding.

## Discussion

Our observations suggest that the DMHc contains a self-sustained, autonomous circadian oscillator entrained by feeding, although additional lines of evidence such as demonstration of sustained rhythmicity in explants of the DMHc *in vitro* will be required to definitively prove this idea. One of the salient characteristics of the DMHc that differentiates it from other peripheral circadian oscillators is that the amplitude of *Per1/Per2* oscillation was much higher under RF than under ALF; whereas RF does shift the phase of other extra-SCN oscillators, their amplitudes do not differ overtly between ALF and RF (9, 10, 12). Notably, the DMHc was found to be the only brain locus other than the SCN that contains densely clustered neurons exhibiting robust *mPer1* and *mPer2* oscillation under RF, whereas *mPer* genes are diffusely expressed in nearly all CNS and peripheral tissues.

Accumulating lines of evidence suggest that the DMH is a critical locus for integration of circadian information from the SCN and feeding information from the peripheral organs to modulate a wide variety of behaviors and physiology (Fig. 7) (17). The DMH receives direct and indirect projections from the SCN (18, 19) and senses metabolic and feeding cues such as leptin (20). Furthermore, the DMH sends projections to regions critical for vigilance state regulation, such as the ventrolateral preoptic area (GABAergic projections) (19, 21), the locus coeruleus (22), and the orexin neurons (glutamatergic projections) (19, 23). The DMH also projects to the paraventricular hypothalamic nucleus (20), which



**Fig. 7.** The DMH as a food-entrainable circadian oscillator. When food is freely available (ALF), the light-entrainable oscillator in the SCN predominates in orchestrating circadian behavioral and physiological rhythms according to the environmental photic cycle. The DMH mediates circadian information from the SCN and also receives feeding information directly and indirectly via humoral and neural pathways. When food availability is restricted to a period scheduled at a fixed time of the day (RF), a FEO in the DMH entrains to feeding information, uncouples from the SCN, and starts regulating food-entrainable circadian rhythms. Dotted lines from the SCN designate that the predominance of SCN-derived signals is contingent on food availability. LH, lateral hypothalamus; OC, optic chiasm; PBN, parabrachial nucleus; PVH, paraventricular hypothalamic nucleus.

regulates hypothalamic outflow to the autonomic nervous systems and endocrine systems. Indeed, studies have implicated the DMH in the SCN-entrainable circadian regulation of feeding, drinking, corticosterone secretion, locomotor activity, and vigilance states (19, 24). In the present study, we also found that the food-entrained oscillation of *mPer2* in the DMHc and NTS/AP under RF was unaffected in the orexin/ataxin-3 transgenic mice (13) in which orexin neurons are genetically ablated (data not shown). This result further supports the idea that orexin neurons are located downstream of the DMH within the neural pathway governing food-entrained circadian behaviors.

Collectively, the DMH seems well suited to be a central locus to regulate behaviors and physiology under such situations in which the SCN and peripheral clocks are phase-uncoupled, as under RF (Fig. 7). Recent reports showed that c-Fos immunoreactivity (an indicator of neuronal activity) is increased in the DMH before and during food availability under RF (15, 25). Most importantly, during the course of our independent study, Gooley *et al.* (15) demonstrated that lesions in DMH neurons in rats blocked food entrainment of wakefulness, locomotor activity, and core body temperature. However, although they demonstrated a critical role of the DMH in the expression of food-entrainable circadian rhythms, their findings did not discriminate among the possibilities: (i) that the DMH itself acts as the primary FEO (in other words, a pacemaker under RF), (ii) that the DMH is a key relay site from a still-unknown primary FEO (output pathway), and (iii) that it is a key relay site on the entrainment pathway to an FEO (input pathway). The present study provides support for the first possibility (Fig. 7). Interestingly, Gooley *et al.* showed food-anticipatory increases in c-Fos expression throughout the DMH under RF, whereas we demonstrated a robust oscillation of *mPer1* mostly in a subregion of the DMH with its peak during the food availability. This finding may not necessarily be a contradiction, because c-Fos is an indicator of cumulative neuronal activity, whereas *Per1* is a core component of neuronal circadian oscillators, which may influence the activity of these and surrounding cells quite indirectly. Thus, it would be important to delineate local networks surrounding the *mPer1*-oscillating cells within the DMH.

In the SCN core, phase-shifting light pulses rapidly induce expression of *Per1* and *Per2* in the light-responsive cells, and this induction has been suggested as a critical step for photic entrainment (26). Our observations that refeeding after fasting rapidly induces *mPer1* (and *mPer2* as well; data not shown) expression in the DMHc suggest that an analogous mechanism exists in the DMH for feeding-triggered entrainment of *Per* oscillation. Thus, this step may play a key role in resetting circadian rhythms by feeding. The SCN is a heterogeneous network of functionally and phenotypically differentiated neurons; cells directly responsive to the light are shown to represent a population distinct from those with robust circadian oscillation (26). It is currently unclear whether cells in the DMHc that acutely responded to feeding by increasing *mPer1* expression are the same ones as those with robust *mPer1* rhythmicity under RF.

Rhythmic expression of *mPer1* and *mPer2* in the NTS/DMV/AP area of the brainstem under RF is consistent with a recent report showing a feeding-dependent up-regulation of c-Fos expression under RF in the NTS, DMV, and AP (27). The NTS is a relay nucleus for visceral and gustatory sensory afferents, and it has also been suggested as a site of integration for visceral autonomic and sensory responses (28). In the rat, vagal afferents responding to gastric and duodenal distension tend to map to the medial and commissural divisions of the NTS (29), the former found to be the major area of *mPer1* expression in the present study. The motor nucleus of the vagus, located just ventral to the NTS, is the primary site of motor efferents to the gut and is densely innervated by fibers from the NTS. The AP lacks the blood-brain barrier, resulting in its ability to sense circulating factors directly, and it is known to be involved in the regulation of autonomic functions and feeding (30). The NTS and AP also have reciprocal projections (28). Notably, the DMH receives direct projections from the NTS and the parabrachial nucleus (18), which in turn receives dense projections from the NTS/AP.

Not only the locus of FEO but also its molecular components have been elusive. It has been demonstrated that FAA is sustained in arrhythmic *Clock* mutant mice (31). However, each component of the molecular clock has multiple paralogs whose expression patterns are distinct but overlapping throughout the brain and peripheral tissues: *Clock-Npas2*, *BMAL1-BMAL2*, *Cry1-Cry2*, *Per1-Per2-Per3*, etc. (2). Thus, tissue-specific combinations of clock genes may be forming molecular clocks outside of the SCN (1, 32). In accordance with this idea, mice deficient for *Npas2*, which is expressed mainly in the forebrain but not in the SCN, has been demonstrated to have defects in food-anticipatory behaviors (33). Furthermore, *mCry1<sup>-/-</sup>;mCry2<sup>-/-</sup>* double knockout mice demonstrate markedly attenuated FAA (34). The molecular substrate of the DMH oscillator may consist of partly overlapping paralogous members of the same gene families that comprise the core clock in the SCN; in particular, the *Cry* and *Per* genes may be shared by both oscillators.

Disturbed eating patterns are often associated with human obesity (35). Dissociation of the circadian control of feeding relative to sleep/wake has been suggested in night eating syndrome, which is frequently seen in obese individuals (36). Moreover, *Clock* mutant mice were recently reported to demonstrate not only a defect in diurnal feeding rhythm but also hyperphagia, obesity, and metabolic syndrome, for which altered *Clock* expression in CNS feeding centers and/or peripheral tissues involved in metabolism may be a causal factor (37). Understanding the neural mechanism of reciprocal interactions between biological clocks and feeding centers is of potential practical importance, and the DMH might play a key role in these interactions as a putative FEO.

## Methods

The methods we used are described in detail in *Supporting Methods*, which is published as supporting information on the PNAS web site.

RF protocols were essentially the same as described (13). Tissues were collected in duplicate (two mice per data point, except for a data point in triplicate shown in figure legends). Coronal brain sections (30  $\mu\text{m}$ ) were collected in four series. *In situ* hybridization was performed as described (13, 38) with modifications. The numbers of stained cells were counted bilaterally in four coronal sections that were spaced 90  $\mu\text{m}$  apart through the ventromedial part of the DMHc (39). We estimated the number of *mPer1*-expressing cells in the ventromedial DMHc per mouse.

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