Retraction and Correction

RETRACTION

GENETICS, SOCIAL SCIENCES
Retraction for “PKNOX2 gene is significantly associated with substance dependence in European-origin women,” by Xiang Chen, Kelly Cho, Burton H. Singer, and Heping Zhang, which published online August 31, 2009, in *Proc Natl Acad Sci USA* (10.1073/pnas.0908521106).

The authors wish to retract this paper because its publication violates the Gene Environment Association Studies Genes and Environment Initiative Study of Addiction: Genetics and Environment (SAGE) dataset’s embargo policy. The SAGE data access agreement states that investigators agree not to submit findings of the SAGE dataset(s) for publication until September 23, 2009. The authors sincerely apologize for this violation of SAGE policy.

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www.pnas.org/cgi/doi/10.1073/pnas.0910252106

CORRECTION

NEUROSCIENCE
Correction for “Stomach ghrelin-secreting cells as food-entrainable circadian clocks,” by Joseph LeSauter, Nawshin Hoque, Michael Weintraub, Donald W. Pfaff, and Rae Silver, which appeared in issue 32, August 11, 2009, of *Proc Natl Acad Sci USA* (106:13582–13587; first published July 24, 2009; 10.1073/pnas.0906426106).

The authors note that on page 13583, in the legend for Fig. 2, an equation appeared incorrectly. The figure and its corrected legend appear below. Additionally, in Fig. 3A on page 13584, the panels labeled “Ghrelin” and “PER1” appeared incorrectly. The corrected figure and its legend appear below. These errors do not affect the conclusions of the article.

Fig. 2. Running wheel behavior of wild-type and GHSR−/− mice during ad libitum feeding, food restriction, and food deprivation conditions. (A) The bar above the actograms shows the light–dark cycle; time of food availability is shown in gray. Actograms depict activity of representative GHSR+/+ and GHSR−/− mice during ad libitum feeding (days 1–4), food restriction ZT6–ZT14 (days 4–15), ad libitum food availability (days 15–18), and food deprivation (day 19). (B) Group activity profiles show the amount of wheel running during the last 7 days of restricted feeding in GHSR+/+ (black) and GHSR−/− (gray) mice. The data are plotted in 10-min bins (mean ± SEM). **, P = 0.002, difference between GHSR+/+ and GHSR−/− in onset time of activity. (C) Line graph of cumulative wheel-running activity (mean ± SEM) from lights on (ZT0) to time of food presentation (ZT6) shows that GHSR−/− mice (solid gray line) ran 42.4% less than GHSR+/+ (solid black line) mice. Superimposed are the curves derived from the Gaussian function \( f(x) = e^{-x^2/2\pi} \) (dashed lines). (D) The anticipation ratios during 7 days of restricted feeding (Top) and the persistence ratio (Middle) on the day of food deprivation are shown for GHSR−/− (gray bars) and control GHSR+/+ (black bars) mice, with significant differences between groups. (Bottom) Daily activity during the period of food restriction. *, P = 0.02; **, P = 0.01.
Fig. 3. Rhythmicity of ghrelin, PER1, and PER2 in oxyntic cells harvested at various ZTs and CTs in animals fed ad libitum or food-deprived. (A) Photomicrographs of a cross-section through the stomach wall show expression of ghrelin, PER1, and PER2, stained with diaminobenzidine (DAB), at 2 times of day—ZT6 and ZT18—in ad libitum-fed animals. (Scale bar: 20 μm.) (B) Quantification of ghrelin, PER1, and PER2 expression shows daily rhythms in these proteins. Note that peak expression of ghrelin occurs at ZT6, in antiphase with peak expression of PER1 and PER2. (C) Expression of ghrelin and PER1 at CT6 and CT18 in animals housed in DD for 48 h and then either fed ad libitum or food-deprived for 48 h. Note: Control sections are shown in Fig. S3. *, P < 0.05.

www.pnas.org/cgi/doi/10.1073/pnas.0909120106
Stomach ghrelin-secreting cells as food-entrainable circadian clocks

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Increases in arousal and activity in anticipation of a meal, termed “food anticipatory activity” (FAA), depend on circadian food-entrainable oscillators (FEOs), whose locations and output signals have long been sought. It is known that ghrelin is secreted in anticipation of a regularly scheduled mealtime. We show here that ghrelin administration increases locomotor activity in nondeprived animals in the absence of food. In mice lacking ghrelin receptors, FAA is significantly reduced. Impressively, the cumulative rise of activity before food presentation closely approximates a Gaussian function (r = 0.99) for both wild-type and ghrelin receptor knock-out animals, with the latter having a smaller amplitude. For both groups, once an animal begins its daily anticipatory bout, it keeps running until the usual time of food availability, indicating that ghrelin affects response threshold. Oxyntic cells coexpress ghrelin and the circadian clock proteins PER1 and PER2. The expression of PER1, PER2, and ghrelin is rhythmic in light–dark cycles and in constant darkness with ad libitum food and after 48 h of food deprivation. In behaviorally arrhythmic-clock mutant mice, unlike control animals, there is no evidence of a premeal decrease in oxyntic cell ghrelin. Rhythmic ghrelin and PER expression are synchronized to prior feeding, and not to photic schedules. We conclude that oxyntic gland cells of the stomach contain FEOs, which produce a timed ghrelin output signal that acts widely at both brain and peripheral sites. It is likely that other FEOs also produce humoral signals that modulate FAA.

Daily fluctuations of CNS arousal and activity require energy output and reflect the need for energy input. Here, we investigate mechanisms that constitute the intersection among circadian time, eating behavior, CNS arousal, and metabolic state. The body uses an endogenous circadian timing system, termed “food-entrainable oscillators” (FEOs), to predict the availability of food. These activate food-seeking behaviors and enable the synthesis and secretion of enzymes necessary for digestion before mealtime. For regularly scheduled daily meals, the behavioral manifestation of this timing mechanism is the expression of food anticipatory activity (FAA), reflected in an increase in activity several hours before the appearance of food. Food anticipatory behavior provides an experimentally tractable window for exploring phenomena associated with anticipatory behavior and the regulation of eating. Many historical lines of evidence converge to indicate that a circadian rather than homeostatic mechanism controls FAA (1). Among these, even when food is withheld for several days and all other environmental conditions are constant, FAA occurs at the time of day that meals had been available previously. FAA survives ablation of the suprachiasmatic nucleus, indicating that this behavior does not require the master clock in the hypothalamus.

Understanding the nature and localization of FEOs has been both controversial and elusive (2–4). In the search for a nervous system site, it remains unresolved whether FEOs lie at one locus or in a network of multiple loci, and whether they are localized to the CNS or also include peripheral nervous system elements. Multiple food-entrainable circadian oscillators have been discovered in the brain and periphery (5), stimulating the search for the localization and identification of FEOs regulating FAA. Many brain areas have been implicated as the neural locus of FEOs, but each of such claims has been challenged (1, 6). Most recently, the dorsomedial hypothalamus (DMH) has been designated a site of FEOs, based on both an unbiased search for brain region(s) that exhibit a rhythmic expression of the Period genes (7) and on site-specific effects of a viral vector containing the Bmal1 gene (8, 9). However, other work challenges such an interpretation because complete DMH ablations do not eliminate FAA (10). Although ablation studies suggest that there is no single neural locus for FEOs, the data indicate several brain sites where food-derived signals influence FAA. Examination of FOS expression and local cerebral glucose utilization points to involvement of a dynamic circuit (11, 12). Also, the earliest sign of behavioral arousal preceding a change in meal time, measured by FOS expression, occurs in the ventromedial hypothalamus (VMH), suggesting that this hypothalamic brain region contributes to the increased activity seen in anticipation of food (13).

Prior searches for the peripheral loci of FEOs involved adrenalec-tomy (14), subdiaphragmatic vagotomy (15), and capsaicin-induced vagal deafferentation (16), none of which abolish FAA. In fact, the possibility of a peripheral locus was all but dismissed after the demonstration that rhythm expression of Period1-luciferase in esophagus, stomach, liver, and colon remained nocturnal during total food deprivation, indicating that these oscillators are not self-sustained (17). Although such studies lead to the unsatisfactory conclusion that FEOs and FAA survive ablation of each one of the many brain regions and peripheral organs tested, our experiments take into account the foregoing data and lead to a very different conceptualization: we envision a network of CNS sites at which timed secretion of ghrelin and other signals could modulate FAA. We define FEOs and their output signal by requiring that they meet the following criteria: the signal must (i) antecede mealtime, (ii) stimulate activity in the absence of food deprivation, (iii) promote eating behavior, and (iv) elimination of the FEO output signal or its receptor should eliminate or attenuate FAA. The FEOs that produce this putative signal should be (v) under circadian control, (vi) rhythmic in constant photic and nutrient conditions, and (vii) be entrained to the timing of food presentation. Given the evidence that some mutant mice lacking molecular components of circadian clock exhibit FAA (18), the presence of known clock genes/proteins is not a requirement for FEOs. Neverth-

Author contributions: J.L. and R.S. designed research; J.L., N.H., and M.W. performed research; D.W.P. and R.S. contributed new reagents/analytic tools; J.L., N.H., M.W., D.W.P., and R.S. analyzed data; and J.L., D.W.P., and R.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0906426106/DCSupplemental.

PNAS | August 11, 2009 | vol. 106 | no. 32 | 13582–13587

www.pnas.org/cgi/doi/10.1073/pnas.0906426106
less, circadian rhythmicity of clock genes within FEOs provides evidence of the presence of oscillators.

In the present studies, we explore the possibility that ghrelin-secreting cells of the stomach oxyntic glands are FEOs. Ghrelin, a 28-amino acid endogenous ligand for growth-hormone secretagogue receptor (GHSR) surges before mealtime (19–21). The numerous E-box elements, known targets of circadian clock proteins, present in the promoter region of the ghrelin gene (22) likely play a role in the timing of ghrelin synthesis. Furthermore, plasma levels of ghrelin fluctuate diurnally, with a peak in the day and a trough at night (23), and exogenous ghrelin administration stimulates eating (24).

Results
Peripheral Ghrelin Administration During the Day Increases Anticipatory Activity and Food Intake. Although ghrelin is known to promote eating, a key question is whether it induces the increased activity that precedes mealtime associated with FAA. To assess the effects of ghrelin on activity in the absence of food deprivation, we injected ad libitum-fed mice with saline or 10 μg of ghrelin i.p. at zeitgeber time 6 (ZT6: lights on at ZT0, off at ZT12) and removed their food from ZT6 to ZT8. General locomotor activity and subsequent food intake were increased after ghrelin treatment. Although it was not quantified, the activity of the animals appeared to be food-oriented, because the animals were seen digging in the bedding on the side of the cage where the food hopper was located. Control undisturbed mice showed little activity at that time of day (Fig. 1). Thus, ghrelin stimulates both activity or arousal and feeding responses.

FAA Is Diminished in Ghrelin Receptor Knockout Mice. To examine the role of ghrelin in FAA, we tested ghrelin receptor knockout (GHSR−/−) and control (GHSR+/+) mice maintained in a 12:12 h light–dark (12:12 LD) schedule, with food access restricted to 8 h—from ZT6 to ZT14—for 12 days. Next, mice were fed ad libitum for 3 days, then food-deprived for 24 h. We found that GHSR−/− mice had normal overall daily activity but reduced FAA.

Individual animal activity records (Fig. 2A) and group data (Fig. 2B) indicate that the GHSR+/+ mice started their activity bout significantly sooner than did the GHSR−/− mice. There was a remarkable regularity in the cumulative anticipatory activity of the mice as a function of time. Results from both GHSR−/− and GHSR+/+ mice closely fit a Gaussian, with correlations between each of the 2 sets of data and the fitted Gaussian = 0.99, and indistinguishable from the Gaussian by using the sensitive Kolmogorov–Smirnov test. The data from GHSR−/− mice had the same shape of curve as controls, but the knockouts had lower amplitude and started their activity bout later (Fig. 2C). The daily initiation of the anticipatory response had a go, no-go property in that once an animal started its daily anticipatory bout of running (monitored in 10-min time bins), it continued to run until food appeared (Fig. S1). Further analysis shows that the anticipation ratio during food restriction and the persistence ratio during food deprivation were lower in GHSR−/− than in GHSR+/+ mice (Fig. 2D), with no differences between groups in amount of activity during food restriction. There was no significant change in body weight during the food-restriction period [GHSR−/− weight, 29.6 ± 1.1 g during ad libitum and 29.9 ± 0.9 g after food restriction; GHSR+/+ weight, 30.0 ± 2.4 g ad libitum vs. 28.9 ± 2.4 g after food restriction, F(3,30) = 0.08].
Rhythms of Ghrelin, PER1, and PER2 in the Stomach. We found that ghrelin-containing oxyntic cells expressed the clock proteins PER1 and PER2 (see Fig. 3 and the high-power view in Fig. S2 A and B), whereas ghrelin adsorption controls and Per1,Per2 mutant mice lacked staining (Fig. S3). We then compared rhythmicity in oxyntic cells in mice housed in a 12:12 LD cycle and fed ad libitum to those in animals kept under constant conditions. Peak and trough expressions of PER1 and PER2 in the oxyntic cells occurred at ZT18 and ZT6, respectively (Fig. 3 A and B). Ghrelin expression is also rhythmic—high during the day and low at night (as in rats; ref. 25)—and is in antiphase to clock protein rhythms. In mice maintained in constant darkness and fed ad libitum or food-deprived for 48 h, ghrelin and PER1 remained rhythmic (Fig. 3 C). The results indicate that stomach ghrelin cells contain the circadian molecular machinery. Furthermore, we found that the behaviorally arrhythmic mPer1,mPer2 double-mutant mouse lacked both rhythmic expression of oxyntic cell ghrelin and premeal decrease in stomach ghrelin (Fig. S4).

Ghrelin, PER1, and PER2 Rhythms Are Controlled by Feeding Time. We asked whether ghrelin, PER1, and PER2 rhythms in oxyntic cells are entrained by time of feeding or by the temporal cues of the LD cycle. In mice with food access restricted to the last 6 h of the light period from ZT6 to ZT12 (food-restricted ZT6–ZT12), the phase of the rhythms of all 3 proteins was 6 h earlier than the one of animals fed at ZT12–ZT18 (Fig. 4). The precision of the mechanisms generating the 6-h difference is such that use of 4 time points during 18 h was sufficient to produce a clear result. This indicates that oxyntic cell rhythms are entrained by food-related signals rather than by the LD cycle.

Discussion

Summary of Results. The results converge to show that stomach oxyntic cells fulfill several essential criteria of an FEO, and the findings provide an avenue for understanding the previous literature on FAA. Ghrelin stimulates both the appetitive (anticipatory locomotor behavior) and the consummatory component (food intake). Administration of ghrelin in the absence of food in a nondeprived animal increases activity/arousal and increases subsequent food intake. In the absence of ghrelin receptors, food anticipatory behavior is diminished. This suggests that ghrelin increases the drive to consume food. Because FAA is not completely abolished, either other ghrelin receptors are activated in this GHSR−/− mouse or, more likely, oxyntic cells are not the only FEOs. Both ghrelin and clock genes are expressed rhythmically within oxyntic cells. The phase of this rhythm is controlled by the time of food availability. Although mice cannot be deprived for many days, in humans, ghrelin release timed to previous mealtimes persists after 1 (26) or 3 (27) days of fasting. In mutant mice lacking a functional circadian clock, ghrelin and clock protein rhythms cease, and the premeal decrease in glandular ghrelin content is abolished. The present series of studies suggest that ghrelin is a signal for FAA and that the stomach oxyntic gland cells are FEOs. Discovery of the brain mechanisms modulating ghrelin effects on activity and eating will further the understanding of this system in the generation of FAA.

Functions and Sites of Ghrelin Action. As expected for complex physiological systems that coordinate circadian time, arousal, and metabolism, the relevant regulatory endocrine and neuronal functions are distributed. Ghrelin of gastric origin can signal the brain through neural afferents from the periphery by a direct action in the CNS. Consistent with the former possibility, ghrelin receptors are present in the vagus (28). There are, however, conflicting results on whether or not the vagus plays a necessary role in ghrelin-induced feeding (28, 29). Circulating ghrelin can also reach the brain via circumventricular organs or by crossing the blood-brain barrier (30).
the blood–brain barrier (BBB) (30). Neurons of the subfornical organ (SFO), a circumventricular organ lacking a BBB, have ghrelin receptors, and ghrelin affects the electrical activity of SFO neurons (31). The SFO projects to hypothalamic nuclei, such as the arcuate (32), paraventricular, supraoptic nuclei, and lateral hypothalamus (33–35), and these are possible sites for the effects of peripheral ghrelin on the brain.

Ghrelin receptors are found in a number of brain regions and neural circuits implicated in FAA, locomotor activity, and/or feeding. The arcuate nucleus contains ghrelin receptors, and genetic or chemical ablation of agouti-related protein and neuropeptide Y arcuate neurons inhibits ghrelin-induced feeding (36, 37). The nucleus accumbens controls motivated behaviors and reward, including food-associated reward (38). It receives input from the ventral tegmental area (39), which contains ghrelin receptors (40), and has been implicated in ghrelin-induced feeding (41). Lesions of the nucleus accumbens core attenuate FAA (42). The parabrachial–DMH–lateral hypothalamus (PBN–DMH–LH) circuit may serve as another pathway upon which ghrelin acts to regulate FAA. The PBN, DMH, and LH are all critical for the regulation of feeding, body weight, and metabolism. These regions either have ghrelin receptors (40) or express FOS after ghrelin administration (43), and ablation of each of these regions attenuates or abolishes at least some index of FAA (7, 9, 44, 45). The ventromedial nucleus of the hypothalamus is also a good candidate for the effect of ghrelin on FAA. It is the only nucleus (of 16 food- and arousal-related brain sites) to show activation at the start of FAA (13). The VMH contains ghrelin receptors (40, 46–49), and ghrelin application increases the firing rate in a large proportion (~65%) of VMH neurons in young rats brain slices (Yanagida et al; 50). Moreover, microinjection of ghrelin into the lateral hypothalamus, the medial preoptic area, or the paraventricular nucleus induces wakefulness and eating (51). Finally, ghrelin application to a brain slice phase shifts the suprachiasmatic nucleus (52).

**Multiple Potential Signals for FAA.** There are several possible mechanisms that can account for the attenuated FAA in the ghrelin receptor knockout mice, because numerous signals can influence FAA, and many central and peripheral sites may serve as FEOs. Pfluger et al. (53) suggest that novel ghrelin receptors remain to be discovered. Among the hormones that influence food intake, increase before meals, and could signal FAA are apolipoprotein A-IV and corticosterone. Intestinal apolipoprotein A-IV is rhythmically released and is controlled by the timing of meals, with levels increasing before meal onset. Adrenal corticosterone levels are rhythmic and peak in anticipation of feeding (54), but adrenalectomy does not abolish FAA (14, 55). Multiple sites of action for ghrelin compounded by multiple potential signals for FAA argue strongly for the existence of distributed physiological systems—endocrine and neuronal—that regulate the intersection among arousal and activity, metabolic state, and time of day to coordinate them adaptively.

**Timing of Anticipatory Activity.** Interestingly, we show that the daily initiation of the anticipatory response has a go, no-go property and that once the mice start their daily anticipatory bout of running, they continue to run until food appears; this finding lends itself to quantitative analysis of its organization and points to possible loci for exploration of its neural basis. The cumulative activity of GHSR<sup>−/−</sup> and GHSR<sup>+/+</sup> mice increases systematically as a function of time since lights on, as well as in advance of the daily appearance of food. This pattern of responses of both GHSR<sup>−/−</sup> and GHSR<sup>+/+</sup> mice closely fits a Gaussian curve (Fig. 2C). The group data indicated that once the animals started their daily running bout, they continued to run until the food appeared. Upon examination of individual animal's data (Fig. S1), the standard error around each data point was revealed to reflect variability in the threshold for arousal and initiation of activity. Because the binomial distribution of yes/no choices of a discrete random variable approximates—with large Ns of choices—the Gaussian, we note that the results are consistent with the speculation that the mechanisms underlying these data include a large number of individual neuronal go, no-go decisions with an increasing proportion of go decisions as feeding time draws near. The excellent fit of the data to a binomial distribution suggests that the decision to activate this appetitive behavior can be understood as a series of repetitive binary choices in which the probability of a positive decision is only about half as large in the ghrelin knockout animal.

**Conclusion.** The present results integrate information across several levels of analysis to show that stomach gland oxyntic cells are loci of FEOs. These cells contain the machinery that constitutes an FEO: They bear circadian clock genes and ghrelin as their timed output signal. Ghrelin affects both activity (in the absence of food) and eating behavior. In the absence of the circadian clock genes Per1 and Per2, ghrelin is no longer rhythmically expressed, and the premeal glandular decrease is abolished. In the absence of the ghrelin receptor, FAA is diminished but not lost, arguing for the existence of other FEOs and output signals. At the mechanistic level, it remains to be determined how gastric clock genes regulate ghrelin synthesis/secretion and how/where ghrelin...
acts in the CNS to increase arousal related to food anticipation in the CNS.

With respect to medicine and public health, the results point to a role for the stomach in regulating the timing of meals, in promoting postprandial arousal, and in inducing eating behavior. Obesity has been associated with a failure in the regulation of the timing of food intake in night eating syndrome (56) and compulsive overeating (57). Ghrerin administration triggers appetite, and ghrelin levels in the blood rise before meals and drop afterward (58). People given ghrelin injections feel voraciously hungry, and they eat more buffet-style meals than otherwise (59). In people who diet and lose weight, ghrelin levels are elevated over their preceding baselines. In contrast, people with morbid obesity treated with bariatric surgery produce less ghrelin and more orexigenic gut peptides; they report feeling less hungry, eat less, and lose weight (60). These studies converge with our results to suggest that ghrelin has a role in the anticipation of eating, including timing of meals, and long-term regulation of body weight.

Materials and Methods

Animals and Housing

Animals were male C57BL/6 mice (Charles River Laboratories) and ghrerin receptor knockout (GHSR<sup>+/−</sup>) and control (GHSR<sup>+/+</sup>) mice (gift from Tamas Horvath, Regeneron Pharmaceuticals, Tarrytown, NY) (Fig. 55). They were housed at 21 ± 1 °C in translucent propylene cages (29 × 19 × 12.5 cm) and adapted to a 12:12 LD (300 lux) schedule for 4 weeks before being used in the experiments. To maintain constant conditions, we used constant darkness (DD) with a dim red light (1 lux) and a white-noise generator (91 dB sound pressure level). All animals were cared for in accordance with the Columbia University Institutional Animal Care and Use Committee and Animal Welfare regulations.

Experimental Design. Monitoring eating, general activity, and wheel running.

To explore the effect of ghrelin administration on activity and food intake (Fig. 1), male C57BL/6 mice weighing 30–36 g (n = 6) were implanted with a transmitter (MiniMitter), and their general activity was monitored by using Datacol 3 software (MiniMitter) (see SI Text). Two weeks later, they were adapted to the procedure with 0.1 mL of saline i.p. (day 1, ZT4; day 2, ZT11; and day 3, ZT0) and placed in a new cage. During the experiment, the mice received datacol 3 software (MiniMitter) (see


**Supporting Information**

LeSauter et al. 10.1073/pnas.0906426106

**S1 Text**

**Results. Colocalization of ghrelin and clock proteins in stomach.** Because oxyntic cells of the stomach are the primary source of plasma ghrelin, we assessed whether they bear a molecular circadian clock machinery. The oxyntic gland tissue was harvested from the stomach’s corpus region (Fig. S2A). The results indicate that ghrelin-containing oxyntic cells express the clock proteins PER1 and PER2 (Fig. S2B). At CT18, when PER proteins peak (see below), most ghrelin-containing cells coexpress PER1 (84.0% ± 3.4%) and PER2 (88.5% ± 2.1%).

**Rhythms of ghrelin in cells in the stomach of clock mutant mice.** We surmised that if the molecular clockwork machinery was mutated, rhythmic ghrelin should be disrupted. Thus, we characterized the pattern of ghrelin expression in mPer1mPer2 double-mutant mice that are arrhythmic in constant darkness (1). In wild-type animals, ghrelin expression was higher at CT6 than at CT18 in both ad libitum and food-deprived conditions (Fig. S3A). In contrast, there was no difference between these time points in ghrelin expression in the mPer1mPer2 double-mutant. We conclude that a mutation in the circadian clock mechanism abolishes rhythmic expression of ghrelin.

**A functional circadian clock is necessary for food anticipatory ghrelin release.** Given that the stomach is the source of most of the plasma ghrelin, we used another group of animals to ask whether oxyntic cell ghrelin content decreases as normal before food presentation in animals with a dysfunctional circadian clock. In wild-type mice, but not in mPer1mPer2 double-mutant animals, when food availability is restricted to ZT12–ZT18, expression of ghrelin is decreased just before food presentation, compared with ZT6 (Fig. S3B). This suggests that the circadian system times the release of ghrelin. The question of whether clock mutant mice show FAA is not resolved at present. The Per2dcrd mouse maintained on an 8-h food availability schedule does not show FAA (2). The mPer1mPer2dcrd mutant (among others) maintained on a 3-h food availability schedule shows FAA (3). The difference among studies may be the result of homeostatic signals resulting from short duration of food availability. It is noteworthy that the mice used in the present study differ from those of Storch and Weitz (3). Given strain differences, as well as the possibility that compensatory mechanisms may emerge in mice with a null mutant allele (as in the mPer2dcrd) versus those with in-frame deletions (as in the mPer2dcrd), the results of the Storch and Weitz experiment cannot be extrapolated to the animals used here.

**Methods. Transmitter implants: recording of general activity.** Mice were deeply anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine. A 2-cm midline lower abdominal laparotomy was performed. The transmitter (VM-FH; MiniMitter) was coated with a silicone elastomer outer layer and was inserted into the abdominal cavity, and abdominal muscle and fascia were closed. To monitor general activity, the animals’ cages were placed on an RTA-500 telemetry receiver plate (MiniMitter) connected to a computer. An activity event was scored whenever a change in signal strength from the transmitter exceeded a threshold. General activity was recorded in 2-min bins by using Datacol 3 software (MiniMitter)

**Expression of ghrelin and clock proteins.** To determine whether oxyntic cells contain the circadian clock molecular machinery, the corpus aspect of the stomach was stained for ghrelin and the clock proteins PER1 and PER2 in C57BL/6 mice killed at ZT18 (n = 5), a time of high PER protein expression.

To test whether a functional circadian system is necessary for rhythmic ghrelin expression, mPer1mPer2 double-mutant mice (mPer1mPer2dcrd strain) (ref. 1; gift from Zhong Sun; Cornell Weil Medical Center, New York) and control C57BL/6 mice, fed ad libitum, were maintained in a 12:12 LD cycle, and then housed in DD for 42 or 54 h and killed at CTs 6 or 18 (projected from ZT6 or ZT18), respectively (n = 4 per group). To assess whether ghrelin, PER1, and PER2 protein rhythms could be entrained by periodic food intake, animals were housed in DD and food-deprived for exactly 24 h before being killed at the projected CT6 and CT18 (n = 4 per time point). To test for premeal gastric ghrelin contents, mPer1mPer2 double-mutant mice were maintained in an LD 12:12 and fed ZT12–ZT18 for 1 week, and then killed at ZT6 and ZT12.

**Perfusion and immunohistochemistry.** Animals were deeply anesthetized (200 mg/kg pentobarbital, i.p.). Mice killed in the dark were anesthetized under the dim red light and their heads covered with a light-proof hood until they were perfused. Mice were perfused intracardially with 50 mL of saline followed by 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Stomachs were postfixed for 18–24 h at 4 °C and cryoprotected in 20% sucrose in 0.1 M PB overnight.

The corpus region of the stomach was dissected and blocked for sectioning. Sections cut on a cryostat (15 μm) were processed on slides. Rhythms in ghrelin, PER1, and PER2 expression were detected in single-labeled sections stained with an avidin–biotin–immunoperoxidase technique using DAB (Sigma) as the chromogen. Colocalization was analyzed in double-labeled sections that were incubated in donkey serum for 1 h, then in ghrelin antibody made in goat (polyclonal; 1:5,000; Santa Cruz Biotechnology) and either PER1 (rabbit polyclonal; 1:8,000; gift from S. M. Reppert and D. R. Weaver, University of Massachusetts, Worcester, MA) or PER2 (mouse monoclonal; 1:5,000; Alpha Diagnostic) for 48 h. After washes, sections were incubated in donkey anti-goat and donkey anti-rabbit (for PER1) or anti-mouse (for PER2) secondary antibodies conjugated to the Cy2 and Cy3 fluorophores (1:200; Jackson ImmunoResearch) for 2 h. Sections were coverslipped with Permount (Fisher Scientific) for DAB or Krystalon (EMD Chemicals) for Cy2 and Cy3.

To test for specificity, sections were stained with fluorescent Cy2 by (i) omitting the primary antibodies (ghrelin, PER1, or PER2), or (ii) by preincubation with ghrelin (1:500; Santa Cruz Biotechnology) or PER2 peptide (1:500; Alpha Diagnostic), and (iii) by staining sections for ghrelin in a ghrelin-knockout mouse (tissue provided by Tamas Horvath) and staining for PER1 and PER2 in mPer1mPer2 double-mutant mice. Staining was not detected after omission of primaries. Ghrelin and PER2 staining were eliminated by preincubation. Ghrelin staining was not detected in the ghrelin-knockout mouse, nor was PER1 or PER2 staining detected in mPer1mPer2 double-mutant mice. (Fig. S5).

**Analysis of immunostaining.** Images of gastric sections were captured by using a CCD video camera (Sony XC77) attached to a light microscope (Olympus BH-2) using National Institutes of Health (NIH) Image 1.61. Three sections were analyzed per stomach. Two observers blind to the experimental conditions processed the photographs with NIH Image 1.61 software to provide a quantitative estimate of the number of immunoreactive ghrelin cells. The images were analyzed in grayscale without any adjustment of brightness or contrast. The density slice threshold was adjusted for 1 section and kept constant thereafter. To measure the number of ghrelin cells, the particle size was.
adjusted to 100–500 pixels (the size of a ghrelin-expressing cell is ~150–350 pixels). The “analyze particles” command gave the number of cells in each photograph. Because dense packing of PER1- and PER2-stained oxyntic cells prevented counting of individual cells, the intensity of staining was assessed as relative optical density (ROD) measured as optical density of staining within the region of oxyntic cells minus optical density of background. The staining for each animal was expressed as the average ROD in 3 stomach sections. One-way ANOVA, or t test, was used to test for time of day effects.

Coexpression of ghrelin and PER1 or ghrelin and PER2 was analyzed on confocal optical images. Slides were observed under a Zeiss Axiovert 200 MOT fluorescence microscope (Carl Zeiss) with a Zeiss LSM 410 laser scanning confocal attachment. The sections were excited with an argon-krypton laser using the excitation wavelengths 488 nm for Cy2 and 543 nm for Cy3. The images were collected as 1-μm optical images with sequential excitation by each laser to avoid cross-talk between wavelengths. Each cell was examined in ~10 sequential optical images to verify that it was double-labeled, by using the LSM 3.95 software (Zeiss) to separate/superimpose green and red. Three stomach sections were analyzed per animal at each time point.

Fig. S1. Cumulative wheel running data for individual GHSR\textsuperscript{+/-} (A) and GHSR\textsuperscript{-/-} (B) animals. In Fig. 2C, the standard errors for group data around each point reflect the differences (shown here) among individual animals in thresholds for initiating activity. The data, averaged over the 7 last days of food restriction, show that once that "go" decision is made, the animal continues to run as feeding time approaches.
Fig. S2. Low- and high-magnification views of the stomach, oxyntic cells, and localization of ghrelin and PER proteins. (A) *(Upper)* Image of the mouse stomach, indicating the corpus region where tissue was harvested. (Scale bar: 500 μm.) *(Lower)* Photomicrograph of a cross-section of the stomach wall. (Scale bar: 100 μm.) (B) Photomicrographs of the stomach oxyntic gland stained for ghrelin, PER1 *(Upper)* or PER2 *(Lower)* and the overlay in optical sections *(z axis = 2 μm).* (Scale bar: 10 μm.)
Fig. S3. Photomicrographs of the stomach oxyntic cells stained for ghrelin (CY2) (Top Left) or after preincubation with ghrelin peptide (1:500; Santa Cruz Biotechnology) (Top Right); PER1 in wild-type (Middle Left) and mPer1,mPer2 double-mutant (Middle Right) mice; PER2 in wild-type (Bottom Left) and in mPer1,mPer2 double-mutant (Bottom Right) mice. (Scale bar: 20 μm.)
Fig. S4. (A) Comparison of ghrelin immunoreactive cells in wild-type and mPer1,mPer2 clock mutant animals. Number of oxyntic cells expressing ghrelin in animals housed in LD and then placed in DD for 2 days and then either fed ad libitum (Left), food-deprived for 24 h (Middle), or food-restricted (Right; see Materials and Methods for details). * P < 0.05.
Fig. S5. Generation of ghrelin receptor knockout (GHSR<sup>−/−</sup>) and control GHSR<sup>+/+</sup> mice (provided by M. W. Sleeman, Regeneron Pharmaceuticals). Bacterial artificial chromosome for Ghsr was isolated, and targeting vectors in which exon 1 of the Ghsr locus was precisely deleted and replaced with an in-frame lacZ reporter gene and neomycin-selectable marker were electroporated into embryonic stem cells. Correctly targeted embryonic stem cells, as well as eventual heterozygotes and homozygous mice derived from these embryonic stem cells, were identified by a real-time PCR-based loss-of-native-allele assay, as described previously [Valenzuela DM, et al. (2003) Nat Biotechnol 21:652–659]. After germ-line transmission was established, F<sub>1</sub> heterozygous mice were backcrossed to C57BL6/J to generate N3 breeding heterozygote pairs that were used to generate homozygous GHSR<sup>−/−</sup> and GHSR<sup>+/+</sup> mice used in these experiments. Deletion of Ghsr was also confirmed by the absence of exogenous ghrelin-induced feeding response (M. W. Sleeman, Regeneron Pharmaceuticals).