Article

Circadian Disruption Leads to Insulin Resistance and Obesity

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Summary

Background: Disruption of circadian (daily) timekeeping enhances the risk of metabolic syndrome, obesity, and type 2 diabetes. While clinical observations have suggested that insulin action is not constant throughout the 24 hr cycle, its magnitude and periodicity have not been assessed. Moreover, when circadian rhythmicity is absent or severely disrupted, it is not known whether insulin action will lock to the peak, nadir, or mean of the normal periodicity of insulin action.
Results: We used hyperinsulinemic-euglycemic clamps to show a bona fide circadian rhythm of insulin action; mice are most resistant to insulin during their daily phase of relative inactivity. Moreover, clock-disrupted Bmal1-knockout mice are locked into the trough of insulin action and lack rhythmicity in insulin action and activity patterns. When rhythmicity is rescued in the Bmal1-knockout mice by expression of the paralogous gene Bmal2, insulin action and activity patterns are restored. When challenged with a high-fat diet, arhythmic mice (either Bmal1-knockout mice or wild-type mice made arhythmic by exposure to constant light) were obese prone. Adipose tissue explants obtained from high-fat-fed mice have their own periodicity that was longer than animals on a chow diet.
Conclusions: This study provides rigorous documentation for a circadian rhythm of insulin action and demonstrates that disturbing the natural rhythmicity of insulin action will disrupt the rhythmic internal environment of insulin sensitive tissue, thereby predisposing the animals to insulin resistance and obesity.

Introduction

Many physiological processes display day-night rhythms, including feeding behavior, lipid/carbohydrate metabolism, and sleep. These daily oscillations are controlled by the circadian biological clock [1]. Circadian desynchrony in humans, a characteristic of shift work, jet lag, and/or sleep disruption, can have profound effects on both normal body weight regulation and glucose/lipid homeostasis [2–4]. Improper circadian entrainment is associated with the onset of metabolic syndrome, obesity, and type 2 diabetes. Disruption of circadian rhythmicity may interact with other susceptibility factors to precipitate the disease state [2, 5, 6]. Many hormones that modulate insulin secretion, glucose homeostasis, and feeding are regulated cyclically by the circadian system, including orexin, leptin, glucagon, cortisol, growth hormone, catecholamines, and melatonin [6, 7]. Another linkage between clock-

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regulated sleep and insulin resistance/type 2 diabetes is the association between short sleep duration (due to e.g., insomnia and/or circadian disruption) and the risk of obesity and diabetes [8, 9].

In any given tissue, 3%–10% of messenger RNA transcripts show circadian rhythmicity in vivo [10, 11], including those encoding transcriptional factors such as DBP, Rev-erbα, PPARγ, HLF, and TEF [6, 12, 13]. These transcriptional factors then regulate downstream target genes involved in different biochemical pathways, including those relating to metabolism of glucose and lipids; synthesis of cholesterol, fatty acids, and bile acids; and mitochondrial oxidative phosphorylation [11, 14, 15]. Therefore, polymorphisms of core clock genes or of clock-controlled hormone receptor genes that might influence the regulation of these metabolic pathways could have health consequences in humans. Indeed, polymorphisms of the clock genes Bmal1 (aka Arntl1 in humans) and Clock are associated with obesity, type 2 diabetes, and hypertension [16, 17]. Moreover, genome-wide association studies show an increased risk of type 2 diabetes associated with variants of the gene (mtnr1b) encoding a receptor for the clock-controlled hormone melatonin [18, 19].

Disruptions of the normal light/dark cycle in wild-type mice—analogous to shiftwork disruption in humans—can lead to increased body weight, hyperleptinemia, and elevated insulin secretion [20, 21]. Altering the circadian timing of food intake stimulates weight gain in mice [22]. Conversely, feeding a high-fat diet (HFD) can itself change the period of the circadian activity rhythm and alter the expression of core clock genes in mice in vivo [23]. Genetic mouse models have also elucidated the linkage between metabolism and the circadian system. For example, mice that are homozygous for a loss-of-function mutation in the circadian gene Clock overeat, become obese and develop hyperglycemia and dyslipidemia [24]. These Clock mutant mice develop the adipocyte hypertrophy and excessive accumulation of fat in the liver that are hallmarks of the metabolic syndrome. Regulation of the histone deacetylase Hdac3 by the clock-regulated transcriptional factor Rev-erbα directs a circadian rhythm of histone acetylation and gene expression that is required for normal hepatic lipid metabolism [25, 26]. Knockout of the three circadian Period genes in mice cause arhythmicity in behavior and increased weight gain on high-fat diets [27]. Similarly, Bmal1-knockout mice display arhythmic behavior in constant conditions, increased fat deposition, elevated triglycerides/free fatty acid levels, and disrupted insulin responsiveness [28–32].

Therefore, a preponderance of evidence supports a close relationship between clocks and metabolism. Consequently, manipulation of biological timing could be used to develop noninvasive therapies for metabolic disorders. However, whether insulin action itself is rhythmic is unclear, nor has the impact of clock disruption upon insulin action been well characterized. Using a hyperinsulinemic-euglycemic clamp procedure that was developed at Vanderbilt to eliminate the need to handle, restrain, or stress mice [33, 34], we show herein that mice show a bona fide circadian rhythm of insulin action such that mice are most resistant to insulin during the phase of relative inactivity. Knockout of the Bmal1 gene leads
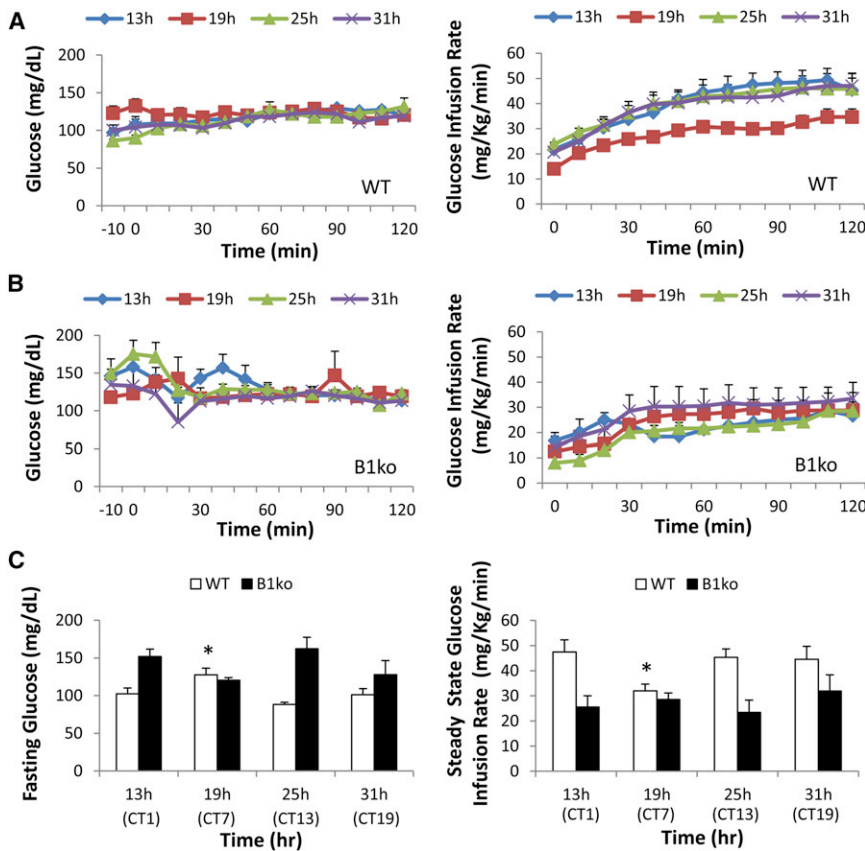
Figure 1. Hyperinsulinemic-Euglycemic Clamps on Conscious, Unrestrained Wild-Type and *Bmal1*-Knockout Mice at Four Circadian Phases under Constant Red Light

(A and B) Arterial glucose levels (left panels) and glucose infusion rates (GIR, right panels) during hyperinsulinemic-euglycemic clamps for wild-type (A) and B1ko (B) mice are shown for four time points under constant red light (CT0 = subjective dawn; CT12 = subjective dusk). The light/dark and fasting protocol prior to the clamps is shown in Figure S1.

(C) Left: Arterial glucose levels in mice subjected to a 5 hr fast (the average of −10 and 0 min prior to the start of clamps, left) during clamps for WT and B1ko mice (left panel; open rectangle, WT; solid rectangle, B1ko) at four time points. Right: Glucose infusion rates for the final 50 min (steady state of glucose levels) during the clamps at four circadian time points under constant red light (right panel; open rectangle, WT; solid rectangle, B1ko). The times or phases of the clamp measurements are plotted as both hours in red light and circadian time (CT; clamp time should not be expressed in CT for B1ko mice since the clock appears to be abolished in these animals). Data are presented as mean ± SEM (WT, six to eight mice/group; B1ko, three to five mice/group). Asterisks denote the phase of the WT data (CT7) that is significantly different ($p < 0.05$) from the other phases of the WT samples for both fasting glucose levels and GIRs as described in the text.

See also Figure S1.

to profound insulin resistance, which can be rescued by constitutive expression of the *Bmal2* gene. In addition to insulin resistance and hyperglycemia, arhythmic mice exhibit metabolic phenotypes related to fat accumulation. By analysis of food intake and activity levels in rhythmic versus arhythmic mice in light/dark and continuous light, these metabolic phenotypes are associated with disruption of rhythmic circadian behavior.

Results

Circadian Rhythm of Insulin Action and Its Elimination in "Clockless" Mice

The hyperinsulinemic-euglycemic clamp, or insulin clamp, is widely considered to be the "gold standard" method for assessing insulin action in vivo. During an insulin clamp, hyperinsulinemia is achieved by constant insulin infusion. Euglycemia is maintained via a concomitant glucose infusion at a variable rate. The glucose infusion rate (GIR) is determined by measurement of blood glucose at brief intervals throughout the experiment and adjustment of the GIR accordingly to maintain constant blood glucose levels. We applied the insulin clamp methodology [33, 34] over the circadian cycle in conscious and unrestrained mice to assess most precisely circadian control over insulin action and glucose homeostasis in wild-type mice (WT C57/BL6) and in mice whose key circadian clock gene *Bmal1* has been knocked out (B1ko) such that the circadian system is abolished or at least severely disrupted [35].

Our protocol measures GIR during a hyperinsulinemic-euglycemic clamp at different phases of the circadian cycle in freely roaming, nonstressed mice whose circadian system is "free-running" in constant dim red light (Figure S1 available online). This protocol revealed a clear circadian rhythm of insulin action in WT mice (Figure 1). In particular, mice are significantly more insulin resistant at hour 19 in constant dim red light as indicated by a lower GIR (Figures 1A and 1C). This phase corresponds with the middle of their subjective day (circadian time 7 [CT7]; see Figure S1), when they are relatively inactive. Constant dim red light is perceived as darkness by the circadian system of mice [30, 36], so mice in constant dim red light express their endogenous "free-running" circadian patterns. Statistical analyses of the data depicted in Figure 1 addressed two questions for both the fasting glucose levels and GIR data sets: (1) are there significant differences among the phases of the WT or B1ko samples, and (2) are the WT and B1ko groups different statistically? One-way ANOVA analysis of the phase data revealed statistically significant phase differences among the WT data for both fasting glucose levels (peak at CT7, $p = 0.004$) and for GIR (trough at CT7, $p = 0.047$). Therefore, there are circadian differences in fasting glucose levels and insulin action in WT mice such that CT7 is the phase that is different for both rhythms; arterial glucose was higher at the same phase (CT7) when the mice were less sensitive to insulin as measured with the hyperinsulinemic-euglycemic clamp (Figures 1A and 1C) [37].

In contrast, in the B1ko mice, one-way ANOVA analyses indicate no significant differences in the phase for either fasting glucose levels ($p = 0.163$) or GIR ($p = 0.675$), consistent with the interpretation of the B1ko strain as being "clockless." Moreover, one-way ANOVA analyses indicate no significant differences between the CT7 values of WT with any of the
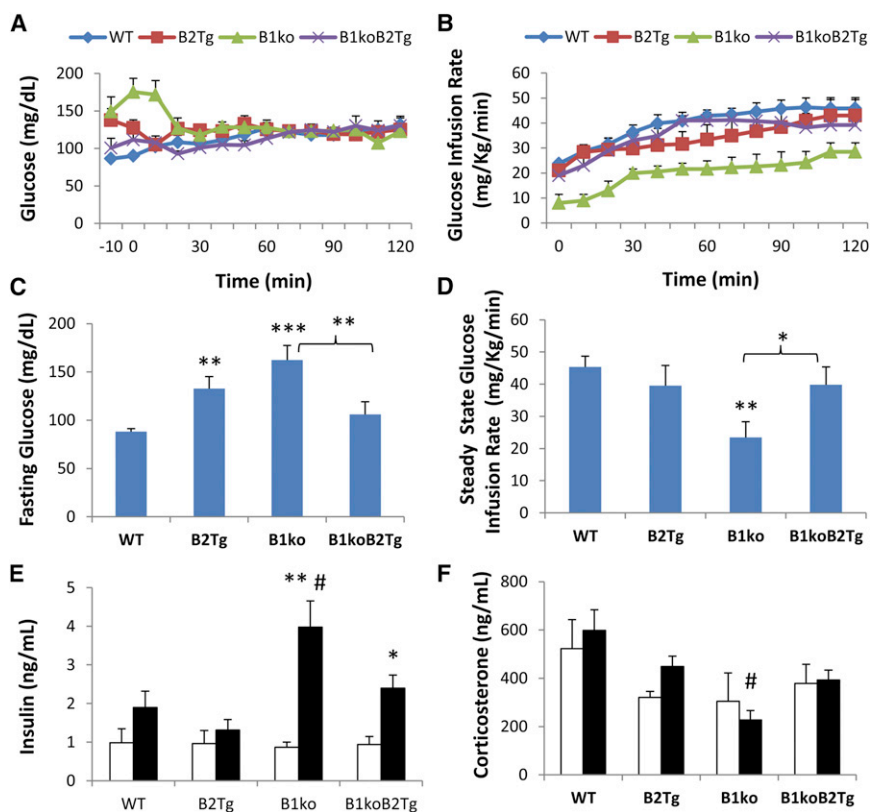
Figure 2. Hyperinsulinemic-Euglycemic Clamps on Mice at 25 hr in Dim Red Light

The 25 hr time point is CT13 for WT, B2Tg, and B1ko/B2Tg mice.

(A and B) Arterial glucose levels (A) and glucose infusion rates (B) during insulin clamps for wild-type (WT), Bmal2 transgenic (B2Tg), Bmal1-knockout (B1ko), and Bmal1ko/Bmal2Tg (B1koB2Tg) mice.

(C) Arterial glucose levels for mice subjected to a 5 hr fast (average of times −10 min and 0 min prior to the initiation of insulin infusion).

(D) Glucose infusion rates during the last 50 min of the clamps.

(E and F) Arterial insulin (E) and corticosterone (F) levels during the clamps (open bars, basal; solid bars, clamp period).

Data are shown as mean ± SEM (four to seven mice per group), *p < 0.05, **p < 0.01, ***p < 0.001 compared with WT mice (C and D, one-way ANOVA with least significant difference [LSD]), compared with basal levels (E and F, two-tailed unpaired t test). #p < 0.05 compared among four genotypes (one-way ANOVA). Insulin (p < 0.001) but not corticosterone (p = 0.557) levels between the basal and clamp conditions were significantly different among the four genotypes as analyzed by two-way ANOVA. Statistically significant differences among the four genotypes were revealed by one-way ANOVA for both clamp insulin levels (p = 0.011, high in B1ko) and clamp corticosterone levels (p = 0.016, lower in B1ko). Two-way ANOVA analyses of strain X phase interaction indicated a significant difference for insulin levels (p = 0.013), but not for corticosterone levels (p = 0.674). See also Figure S1.

values of the B1ko mice for GIR (p = 0.331) or fasting glucose levels (p = 0.116) (Figure 1C). This means that the disruption of the circadian system in B1ko mice renders them relatively insensitive to insulin and locks them into a circadian phase of insulin action and glucose metabolism that is similar to that of WT mice in the inactive segment of their daily cycle (CT7). WT and B1ko mice are significantly different from each other; two-way ANOVA analyses of the WT and B1ko data together confirm that the data for these two strains are significantly different for both the fasting glucose levels (p < 0.0001) and for GIRs (p < 0.0001). Two-way ANOVA analysis of strain X phase interaction indicated significant difference for the fasting glucose data (p = 0.001), but not for the GIRs (p = 0.182).

Expression of Bmal2 Rescues the Insulin Resistance and Signaling Deficits of B1ko

The circadian clock mechanism in mammals is composed of autoregulatory transcription and translation feedback loops of central clock gene expression. These central clock genes include the positive transcriptional factors BMAL1/BMAL2 and CLOCK/NPAS2 and the feedback repressors PER1/2/3 and CRY1/2 [38–41]. BMAL1 or BMAL2 form heterodimers with CLOCK or NPAS2 and bind to E-boxes in the promoters of a large number of target genes [30, 42, 43], including their own negative regulators Period (Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2). Knockout of the gene encoding BMAL1 (B1ko strain) abolishes the circadian feedback loop as well as the rhythms of gene expression [35]. In a previous study, we reported that expression of Bmal2 from a constitutively expressed promoter (hEF1a promoter) rescues

the clock phenotypes of B1ko mice, including rhythmic locomotor activity and rhythmic oxygen consumption [30]. We concluded from those data and the original Bmal1-knockout report [35] that Bmal1 and Bmal2 form a circadian paralogous pair that is functionally redundant but that in the mouse Bmal2 is regulated by Bmal1 such that knockout of Bmal1 alone results in a functional knockdown of Bmal2.

We find that transgenic expression of Bmal2 (the B2Tg mouse) not only restores rhythmicity, but also rescues the metabolic phenotypes of B1ko that are depicted in Figure 1. Specifically, the insulin resistance and high fasting arterial glucose levels are both restored to wild-type levels in the B1ko mouse when Bmal2 is expressed from a constitutively expressed promoter (the B1ko/B2Tg mouse), as shown in Figure 2. This rescue is also obvious from the arterial insulin levels during the clamp; the high insulin levels and low glucose infusion rate exhibited by the B1ko mice at the end of the clamp are eliminated in the B1ko/B2Tg mice (Figure 2E). It is not clear why basal insulin levels are not high in B1ko mice (Figure 2E), given their resistance to insulin action (Figures 2B and 2D). A likely explanation is that beta cell function is compromised in B1ko mice, resulting in inappropriately low insulin concentrations for the prevailing arterial glucose, as has been shown to be true by Marcheva et al. [44]. Moreover, the GIRs for WT versus B2Tg are not significantly different (Figure 2D), but they are different for fasting glucose (Figure 2C). Because there were differences for fasting glucose but not for GIR, these data suggest that expression of Bmal2 may have some effects on glucose/insulin relationships that are not restricted to insulin action. Nevertheless, the notable result is that transgenic expression of Bmal2 overcame the elevated
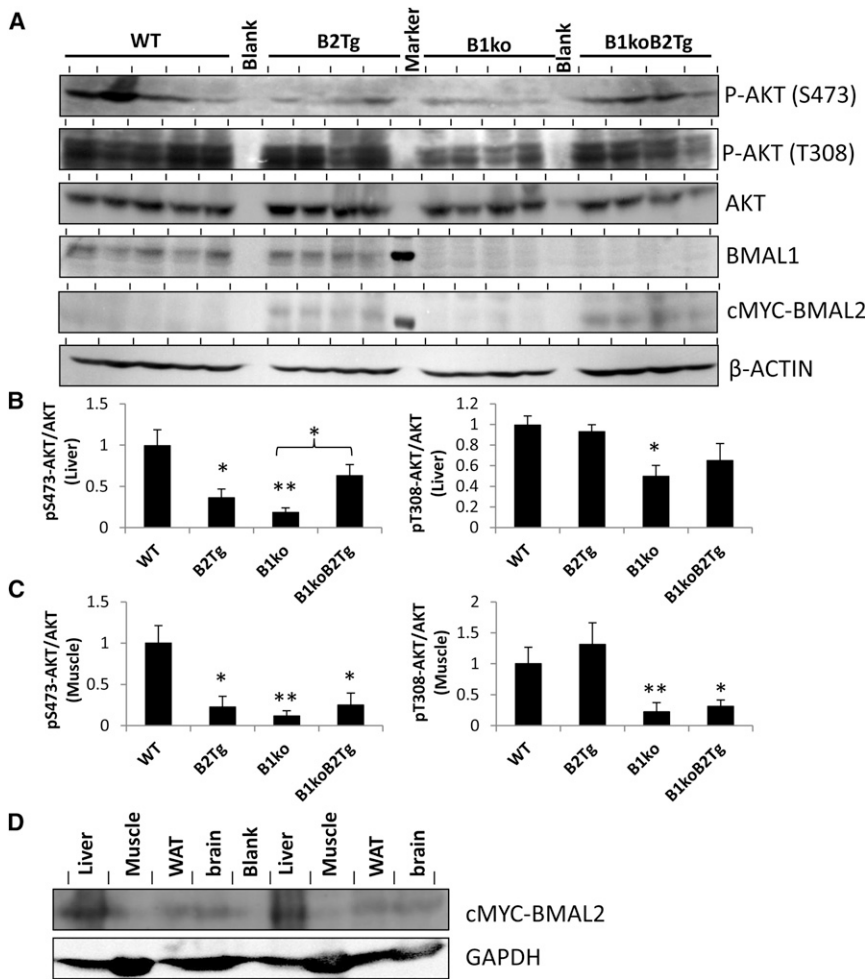
Figure 3. Regulation of AKT Pathway Signaling by Bmal1/2

(A) Immunoblots from liver extracts for phospho-AKT (p-AKT S473 and T308), AKT (total), BMAL1, cMYC-BMAL2 (cMYC-tagged BMAL2 is the version of BMAL2 expressed in the B2Tg and B1ko/B2Tg mice [16]), and β-ACTIN of mice after hyperinsulinemic-euglycemic clamps. Each lane comes from a separate mouse (n = 5 for WT, n = 4 for the other groups). The lane between the B2Tg and the B1ko samples in the BMAL1 and cMYC-BMAL2 blots shows a molecular weight standard indicating 75 kD.

(B) Densitometric analyses of the data shown in (A) for liver extracts. Expression of AKT-pS473 and AKT-pT308 were normalized to total AKT.

(C) Densitometric analyses of the data for muscle extracts analyzed and plotted as in (B) (see Figure S2 for the raw immunoblot data).

(B and C) The value of WT was set as 1.0, and values are expressed as mean ± SEM of integrated intensity.

(D) Expression of cMYC-BMAL2 in various tissues of B1ko/B2Tg mice. Results from two representative mice are shown for liver, muscle, white adipose tissue (WAT), and brain tissue. The blot for cMYC-BMAL2 (upper blot) is compared with a blot for a control protein (GAPDH, lower blot).

In all panels, *p < 0.05, **p < 0.01 compared with WT or as indicated. See also Figure S2.

postclamp insulin deficit and insulin resistance of the B1ko strain. The results shown in Figure 2 are not due to a non-specific stress to the mutant strains. Our clamp procedure differs from other clamp procedures that involve restraint and handling, and it has been demonstrated to be stress free [33]. In this regard, it is notable that serum corticosterone levels (an indicator of stress) are low in the three mutant strains (Figure 2F).

Insulin resistance is associated with the activity of the serine/threonine protein kinase Akt pathway through the PI3K/PIP3/Akt signaling cascades, and Akt and TOR signaling are also important for circadian timing [45]. The reduced GIR of the B1ko strain is correlated with attenuated activity of the Akt pathway in response to insulin action, as shown by the significantly decreased Akt phosphorylation at S473 and T308 residues in liver and muscle extracts of B1ko mice (Figure 3). Constitutive expression of Bmal2 can reverse the attenuation of Akt phosphorylation in the liver, particularly on the S473 residue (Figure 3B), which would be predicted to reactivate the Akt pathway. There does not appear to be a significant rescue of Akt phosphorylation in muscle extracts of the B1ko/B2Tg mice (Figures 3C and S2). This observation is consistent with the fact that expression of BMAL2 in the B1ko/B2Tg mouse is significant in liver, brain, and white adipose tissue (WAT), but not in muscle (Figure 3D). Moreover, the low dose of insulin used during the clamp allowed us to detect any modulation of the impact of insulin on adipose tissue and liver, which are more sensitive to insulin than is muscle. It is possible that a higher dose of insulin would have allowed us to detect reversal of insulin signaling defects in muscle of B1ko/B2Tg mice. Therefore, there is a correlation between the rescue of circadian rhythmicity by Bmal2 expression in the B1ko mouse and the restoration of wild-type metabolic function.

Clock-Disrupted Mice Are Obesity Prone

In addition to insulin resistance and elevated fasting arterial glucose (Figures 1 and 2), B1ko mice exhibit abnormal metabolic phenotypes related to fat accumulation. Young mice of the four genotypes placed on a HFD do not show significant differences in total body weight after 2 months, but there are significant differences of body composition in the B1ko strain (Figures 4A–4C). Specifically, the B1ko mice have a significantly elevated percentage of body fat (Figure 4A) with no significant difference in lean mass (Figures 4B and 4C). Constitutive expression of the Bmal2 gene rescued the B1ko phenotype nearly to WT levels (Figures 4A–4C). Food intake was essentially the same among these four genotypes (Figure 4E), but B1ko mice had significantly lower and B2Tg mice had significantly higher levels of activity as compared with WT and B1ko/B2Tg mice (Figure 4D). The restoration of B1ko phenotypes to WT levels in the B1ko/B2Tg strain appears to be correlated with the rescue of rhythmicity and not to some other effect of Bmal2 expression, as can be observed in the B1ko/B2Tg mice. We have previously reported that the rescue of wheel-running rhythmicity in B1ko/B2Tg mice fed with regular chow is complete for some mice, but not for others [30]. We continue to observe this phenomenon of rhythmicity differences with HFD-fed mice whose activity is monitored
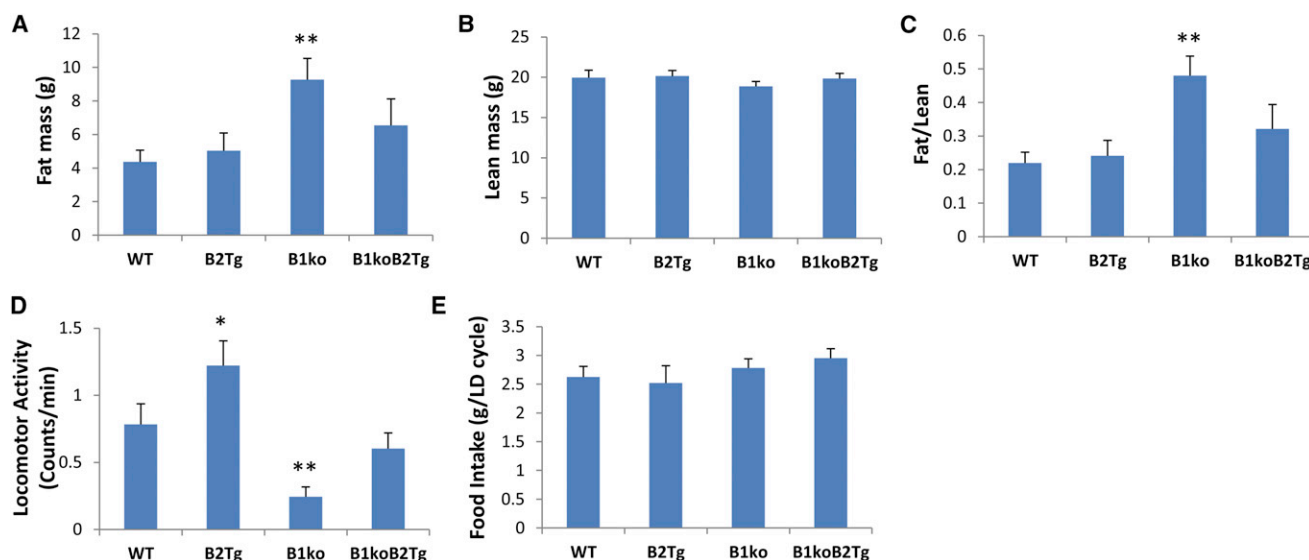
Figure 4. Body Composition, Food Consumption, and Locomotor Activity in Four Different Strains of Mice Fed a High-Fat Diet

Mice were fed HFD starting at an age of 1 month and maintained under LD 12:12 (12 hr light:12 hr dark, lights on 6:00 a.m.–6:00 p.m.).

(A and B) Fat mass of these mice at age 3 months (2 months on HFD) (A) and lean mass of these mice at age 3 months (2 months on HFD) (B). Each bar represents mean ± SEM (n = 12–16/genotype).

(C) Ratio of fat mass to lean mass.

(D) Locomotor activity recorded by infrared sensors in these mice at 3 months of age in LD (mean ± SEM, n = 7–10/genotype).

(E) Daily food intake during one 24 hr LD cycle (mean ± SEM, n = 7–10/genotype).

*$p < 0.05$ and **$p < 0.01$ compared with WT mice (one-way ANOVA with LSD). See also Figure S3 and Table S1.

by the infrared sensor method that measures total activity more accurately than wheel running [30]. Figure S3 depicts this phenomenon as a comparison of rhythmic B1ko/B2Tg-1 mice with arhythmic B1ko/B2Tg-2 mice. When these two groups of mice of the same genotype were separated on the basis of their rhythmicity profiles as assessed by periodogram analyses (Figure S3), we found that the arhythmic B1ko/B2Tg-2 mice exhibited a significantly higher fat mass (p = 0.03) and lower total activity levels (p = 0.04, Table S1).

To confirm the conclusion that clock-disrupted mice are obesity prone [22, 24], we tested another way to disrupt the circadian system in WT animals. In particular, WT mice can be rendered arhythmic (or their rhythms are severely disrupted) by transferring them to constant light (LL), whereas they remain strongly rhythmic in LD or constant darkness (DD) (Figure 5D). Arhythmia induced by light exposure also strongly suppresses the expression of *Bmal1* expression [46]. Therefore, we compared LL as another way to effect rhythmicity and metabolism through *Bmal1* expression. As further support for the association of high body fat with the absence of rhythmicity, WT mice fed HFD were compared in LD 12:12 or LL conditions. The body fat is significantly higher in arhythmic WT mice in LL after feeding with HFD for 3 months as compared with mice in LD (Figure 5A), whereas the lean mass is not significantly altered in mice maintained in LL for 3 months (Figure 5B), resulting in a significant increase of fat mass to lean mass ratio in WT mice exposed to LL (Figure 5C). While the amount of total locomotor activity appears to be lower for mice in LL as compared with LD, this difference is not significant (Figure 5E), and these WT mice in LL accumulated more body fat (Figure 5A) in spite of a trend toward decreased food intake (Figure 5F). Consequently, LL leads to disrupted rhythmicity and possibly to decreased locomotor activity, which results in fat accumulation. This phenomenon

is not restricted to mice; our results confirm observations of a diabetes-prone rat strain that developed metabolic dysregulation after exposure to constant light for 10 weeks [47].

High-Fat-Diet Feeding Affects Subsequent Clock Properties of Tissues In Vitro

Therefore, lack of rhythmicity is correlated with metabolic dysfunction (Figures 1, 2, 3, 4, and 5) [2, 6], and the expression of BMAL2 can rescue metabolic phenotypes associated with the knockout of *Bmal1* in intact mice. Feeding HFD to mice leads to changes in the period of the (1) locomotor activity rhythm and (2) cycling of central circadian clock genes in vivo [23]. Do isolated tissues that can express circadian rhythms in vitro also show correlations between expression patterns and "after effects" of feeding HFD to the animals from which the tissues are collected? The clockwork in the suprachiasmatic nuclei (SCN) of the hypothalamus is the "master" pacemaker that entrains to environmental cycles and coordinates rhythms throughout the body [38, 48]. Outside of the SCN, circadian molecular machinery exists and oscillates in peripheral tissues—nearly every mammalian tissue expresses circadian rhythms of clock gene expression in vivo as measured by RNA microarrays and also when luminescence reporters of gene activity are recorded from explanted tissues in vitro [10, 14, 49]. Therefore, mammalian organisms are a "clockshop" of circadian oscillators distributed throughout the body [12].

In our previous study, we reported that isolated SCN, lung, and liver slices from chow-fed animals exhibit circadian rhythms of luminescence in vitro from WT and B2Tg animals, but not from B1ko or B1ko/B2Tg mice [30]. However, damped oscillations in liver could be elicited by treatment with glucocorticoids [30] (see Figure S4). That study, however, did not test adipose tissue, nor did it test tissues from HFD-fed
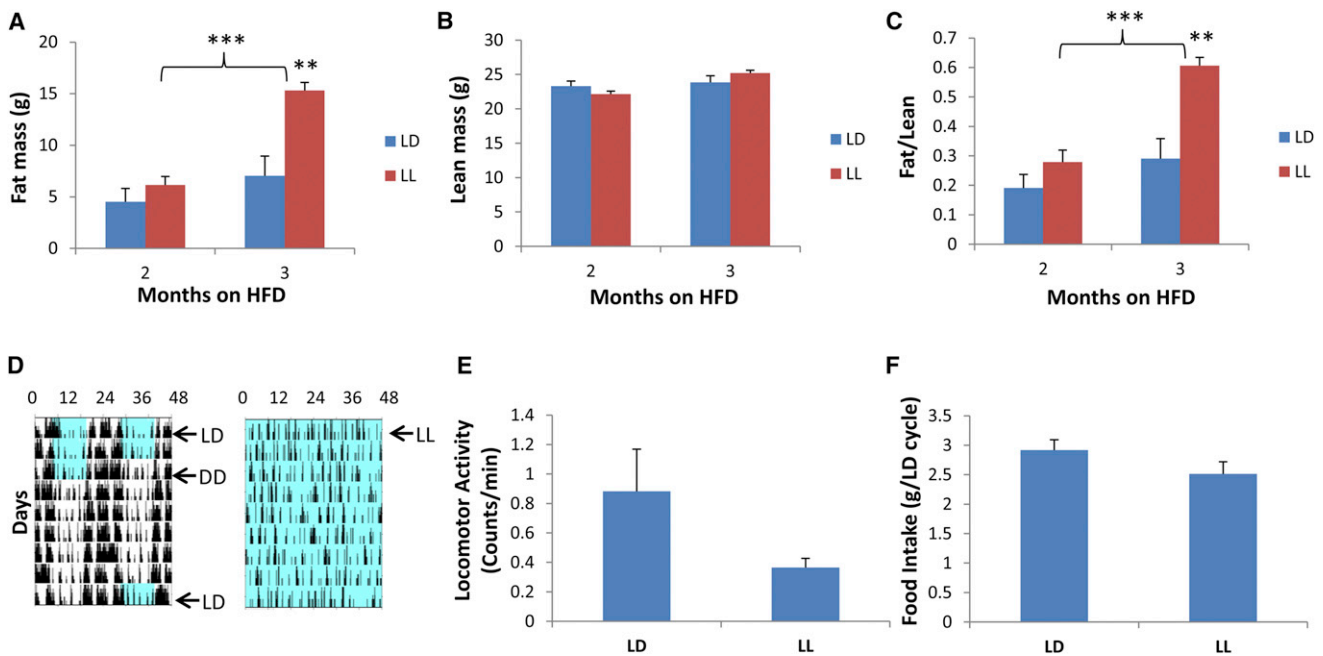
Figure 5. Body Composition, Food Consumption, and Locomotor Activity in High-Fat-Diet-Fed Wild-Type Male Mice under Light/Dark or Constant Light Conditions

Mice were fed regular chow until they were 1 month old, then transferred to HFD at 1 month under either regular LD 12:12 (lights on 6:00 a.m.–6:00 p.m.) or LL.
(A and B) Fat mass (A) and lean mass (B) of 3- and 4-month-old WT male mice. Mice were fed with high-fat diet under either LD 12:12 (blue) or LL (red).
(C) Ratio of fat mass to lean mass. Each bar represents mean ± SEM.
(D) Representative locomotor activity patterns recorded by infrared sensors. Blue shading denotes illumination, while white denotes darkness (left panel is LD 12:12 → DD → LD 12:12, right panel is LL).
(E) Activity levels are expressed as mean counts/min ± SEM.
(F) Daily food intake is expressed as mean grams intake per 24 hr LD cycle ± SEM.
n = 5–7 per treatment, **p < 0.01 and ***p < 0.001 compared with mice in LD (two-tail unpaired t test).

mice. Figure 6 shows that the P_{mPer2}::mPER2-Luc reporter in white adipose tissue (WAT) from the B1ko mouse does not exhibit rhythmicity. Moreover, this was not rescued by Bmal2 expression in the B1ko/B2Tg mouse regardless of whether the mouse was fed chow (Figure 6A) or a HFD (Figure 6B) prior to sacrifice for the real-time luminescence assay. Therefore, even though expression of Bmal2 is able to rescue metabolic phenotypes of intact B1ko mice (Figures 2, 3, and 4), it appears to be less effective in rescuing B1ko phenotypes in isolated tissues of WAT (Figures 6A and 6B) or SCN, lung, or liver [30]. An interesting observation, however, was an effect of HFD on the period of the Per2 rhythm in WAT for wild-type mice; the period in vitro was significantly longer in adipose tissue from WT mice that had been fed HFD as compared with chow (Figure 6C). This effect was not observed in B2Tg mice, suggesting that constitutive expression of Bmal2 might compensate for the HFD effect on period in WAT (Figure 6C). Nonetheless, damped in vitro oscillations can be stimulated by forskolin or glucocorticoid treatment of lung and liver of B1ko/B2Tg mice (Figure S4) [30].

The SCN of the hypothalamus are the central neural pacemaker of mammalian circadian clocks [50]. As we previously reported [30], luminescence activity from SCN slices of B1ko and B1ko/B2Tg mice was not rhythmic in vitro, and treatment with forskolin to synchronize the cells in those tissue slices did not reinitiate rhythmicity from tissues of those genotypes (Figure 6D). In addition, although we found that feeding HFD to mice significantly lengthens the subsequent period of the Per2 rhythm from WAT cultures in vitro (Figure 6C), we did

not observe an effect of prior HFD feeding on the period of Per2 rhythms from SCN slices in vitro (Figures 6D–6F). However, HFD feeding does enhance the amplitude of the Per2 rhythm in WT SCN slices (Figures 6G and 6H). This observation suggests that a diet-induced change in fatty acid metabolism of HFD-fed mice can alter clock properties, and this alteration persists in tissues isolated from those mice and subsequently assayed in vitro; the effect appears to be true for both SCN pacemaker neurons (amplitude but not period) and peripheral tissues such as WAT (period).

Discussion

Developed countries are facing an epidemic of interrelated metabolic diseases collectively referred to as the metabolic syndrome, the hallmarks of which include obesity, hyperlipidemia, hyperglycemia, insulin resistance, and hepatic steatosis [51]. These symptoms are all independent risk factors of type 2 diabetes. By virtue of its pervasive control over metabolic pathways, the circadian system might be a tractable target for decreasing the prevalence of metabolic diseases, especially since the circadian system serves as the interface between internal rhythms and the cycles of the external world (light/dark, food availability, etc.). Indeed, studies in both mice and humans indicate that relatively noninvasive manipulations of the circadian patterns of activity and feeding can have significant effects on body weight and metabolism [3, 9, 22, 52]. The results reported here conclusively demonstrate the *timing* of insulin action is key; this conclusion is highly
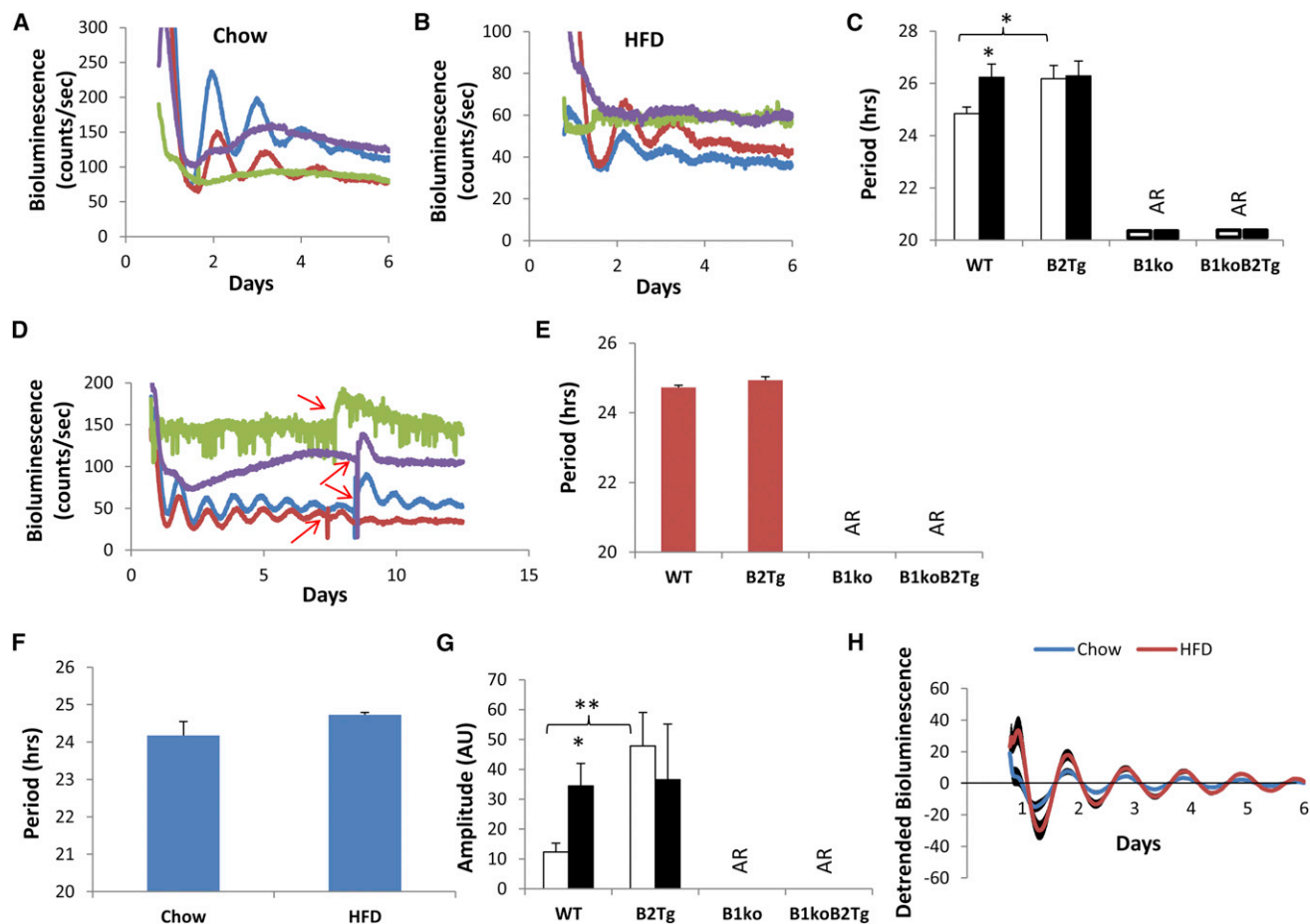
Figure 6. Per2 Expression Measured as Luminescence Emanating from Tissues of the P$_{mPer2}$::mPER2-Luc Reporter Mouse

WAT and SCN explants were dissected and recorded with a LumiCycle apparatus in vitro.

(A and B) Representative raw data of luminescence monitored in vitro from WAT of P$_{mPer2}$::mPER2-Luc knockin mice [49] fed with either chow (A) or HFD (B). Colors denote the following: blue, WT; red, B2Tg; green, B1ko; and purple, B1ko/B2Tg.

(C) Period of Per2::luc luminescence rhythms from WAT explant cultures as mean ± SEM (chow, white, n = 6–8/genotype; HFD, black, n = 5–7/genotype).

(D) Representative data of luminescence monitored in vitro from SCN slices of mice harboring P$_{mPer2}$::mPER2-Luc and fed with HFD. After 7–8 days in culture, the SCN slices were given a 0.5 hr pulse of 10 μM forskolin at the times indicated by red arrows (in vitro cultures were maintained at 36.5°C). Blue, WT; red, B2Tg; green, B1ko; purple, B1koB2Tg.

(E) Period of SCN cultures from HFD-fed mice. Each bar represents mean ± SEM, n = 5–7/genotype.

(F) Period of SCN cultures from WT mice fed with chow or HFD. Each bar represents mean ± SEM (chow, n = 8; HFD, n = 7).

(G) Amplitude of SCN cultures in arbitrary units (AU) from chow (open rectangle, n = 6–8/genotype) or HFD-fed mice (solid rectangle, n = 5–7).

(H) Detrended bioluminescence rhythms from cultured SCN slices of WT mice harboring P$_{mPer2}$::mPER2-Luc and fed with chow or HFD. Each trace represents mean ± SEM (chow, n = 8; HFD, n = 7).

*p < 0.05; **p < 0.01 compared with chow fed mice or as indicated (two-tailed unpaired t test). See also Figure S4.

relevant for human vulnerability to hyperglycemia and hypoglycemia, and could alter strategies for insulin therapy.

While the fundamental concept of homeostasis would predict that insulin activity and glucose levels would be constant over the 24 hr day, our data clearly show that mice are relatively insulin resistant in the middle of their subjective day (CT7), i.e., during the inactive portion of the daily cycle for mice (Figures 1 and S1). Moreover, fasting glucose shows a circadian rhythm with the highest levels occurring during the insulin-resistant phase of inactivity. The "clockless" B1ko mice appear to be locked arhythmically into this insulin-resistant phase, as they demonstrate nonrhythmic insulin-resistance and enhanced fasting glucose levels. When circadian rhythmicity is rescued in the B1ko mouse by transgenic expression of the Bmal1 paralog, Bmal2 [30], the metabolic phenotypes of insulin resistance, fasting glucose

levels, Akt signaling, and fat accumulation are concomitantly rescued (Figures 2, 3, and 4). Another way to induce arhythmicity and suppress Bmal1 expression—exposure to LL—also lowers total activity and stimulates fat accumulation (Figure 5).

The influence of HFD on metabolism not only affects behavioral rhythms in vitro [23], but it also alters molecular clock properties in WAT and SCN by a mechanism that persists for at least several days in these tissues when isolated and assayed in vitro (Figure 6). Moreover, the rescue of metabolic phenotypes by expression of the Bmal2 gene may be mediated by the partial restoration of circadian rhythmicity in peripheral tissues such as liver and lung. In particular, the damped oscillation capability in the isolated tissues of the B1ko/B2Tg mouse (Figures 6 and S4) [30] are likely to return to full-blown rhythmicity in vivo by virtue of systemic signals

in the intact animal that synchronize and amplify the rhythms of peripheral tissues [53]. This is shown in Figure S4A, where cyclic cAMP signals elicited by forskolin restore robust rhythmic gene expression to fibroblasts derived from the B1ko/B2Tg mouse but not to fibroblasts from the B1ko mouse. Because B1ko/B2Tg-1 mice express rhythmic locomotor activity, they are likely to have rhythmic systemic signals so that peripheral tissues are also rhythmic and metabolism is rescued. On the other hand, B1ko mice do not express rhythmic behavior, and therefore B1ko peripheral tissues may not experience rhythmic systemic signals, resulting in metabolic defects.

The data reported here are consistent with a model derived from studies of epigenomic regulation of metabolic switching in mice [26]. During the night (active/feeding phase for mice), metabolic intermediates are largely channeled to lipid synthesis and storage, and we find this phase to be characterized by enhanced insulin activity and lower fasting glucose (Figure 1). Conversely, during the day (inactive/fasting phase for mice), metabolism switches into a calorie-restricted, glucose-producing mode that is characterized by a higher fasting glucose and insulin resistance. Clockless B1ko mice appear to be locked in a metabolic state that resembles the day phase of WT mice. This metabolic day/night switching may be accomplished in WT mice by circadian regulation of the histone deacetylase HDAC3 [26].

Our results have important implications for studies of insulin action in mice. In particular, we show that insulin action as measured by hyperinsulinemic-euglycemic clamps, performed in the absence of stress (i.e., mice are not handled or restrained and do not have their tails cut) varies over the time span of the 24 hr day, confirming and extending some previous studies of the interaction between circadian systems and metabolism in which stress of handling and blood sampling might have been a factor [28, 29, 31, 54]. Our current studies emphasize the need to apply precise control to the timing of metabolic experiments and to consider timing in the interpretation of results. Some of the variance of results among published studies that have been ascribed to diet, mutant background, etc. could be at least partially due to differing times of day when the hyperinsulinemic-euglycemic clamps were performed [54].

Because most previous hyperinsulinemic-euglycemic clamp studies of mice have been done in the day phase, it is possible that gene-dependent effects on insulin action and glucose metabolism that occur during the night phase have been missed. Therefore, the conclusions of this investigation indicate that results obtained from hyperinsulinemic-euglycemic clamps and other measurements of insulin action and glucose tolerance need to be considered in the context of the time of day/circadian phase to facilitate comparison to other studies. It would be most comprehensive to test insulin action and glucose tolerance at different times of day, e.g., mid-day and mid-night to improve the chance of capturing clock-dependent effects on metabolism.

From the work of Claude Bernard in the 19th century, the concept of homeostasis (Bernard's milieu intérieur) as the maintenance of a constant internal environment is deeply ingrained in our thinking about how organisms work. The question then arises why is such a fundamental component of internal environment as glucoregulation rhythmic rather than constant? It is likely to be an inevitable and unavoidable consequence of evolving in an external environment that is profoundly rhythmic over the course of the daily cycle. Consequently, an organism whose responses are merely constant will be outcompeted by organisms that anticipate the predictable changes in their external environment by rhythmically preparing and altering their internal environment. To optimally adapt to a rhythmic environment, organisms must rhythmically regulate their behavior, physiology, and gene expression [55]. These studies show that insulin action and glucose metabolism are intertwined with an internal timekeeping system that evolved to accommodate the environmental rhythmicity.

Experimental Procedures

Complete experimental procedures are described in the Supplemental Experimental Procedures.

Animal Care
Mice were housed on a 12:12 hr light/dark cycle unless otherwise stated and fed chow (5001; Purina Mills, St. Louis, MO) containing 13.5% calories from fat. For HFD (F3282; BioServ, Frenchtown, NJ) experiments, mice were fed HFD containing 60% calories as fat, beginning at 1 month of age. All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted according to that committee's guidelines.

Hyperinsulinemic-Euglycemic Clamp
Detailed clamp procedures have been previously reported [33, 34] except for the entrainment and circadian procedures shown in Figure S1 that are unique to this study.

Determination of Akt Phosphorylation by Immunoblotting
Samples of liver and of vastus muscle harvested from mice of the CT13 group at the end of the hyperinsulinemic-euglycemic clamp procedure were processed for immunoblotting and probed for total Akt, phosphorylated Akt (S473 and T308), MYC-BMAL2, BMAL1, β-ACTIN, and GAPDH.

Locomotor Behavior, Body Weight and/or Fat Composition, and Food Intake Assays
Mice were fed HFD beginning at 1 month of age. After 2 months on HFD, some mice were singly housed in cages equipped with infrared sensors. Locomotor activity and food intake of the mice was measured at 2 months on HFD. Body fat composition was determined with an mq10 nuclear magnetic resonance analyzer (Bruker Optics).

Tissue Culture and In Vitro Luminescence Recording
The mice for tissue culture were fed HFD for 3 to 4.5 months or chow. The cultures of SCN and peripheral tissues were prepared as previously described [30, 49].

Statistics
Data are expressed as means ± SEM. Statistical analyses were performed by two-tail unpaired t test and by one-way and two-way ANOVA as indicated.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.048.

References

1. Dunlap, J.C., Loros, J.J., and DeCoursey, P.J. (2004). Chronobiology: Biological Timekeeping (Sunderland, MA: Sinauer Associates).
2. Green, C.B., Takahashi, J.S., and Bass, J. (2008). The meter of metabolism. Cell 134, 728–742.
3. Scheer, F.A., Hilton, M.F., Mantzoros, C.S., and Shea, S.A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc. Natl. Acad. Sci. USA 106, 4453–4458.
4. Gimble, J.M., Sutton, G.M., Bunnell, B.A., Ptitsyn, A.A., and Floyd, Z.E. (2011). Prospective influences of circadian clocks in adipose tissue and metabolism. Nat Rev Endocrinol 7, 98–107.
5. Antunes, L.C., Levandovski, R., Dantas, G., Caumo, W., and Hidalgo, M.P. (2010). Obesity and shift work: chronobiological aspects. Nutr. Res. Rev. 23, 155–168.
6. Bass, J., and Takahashi, J.S. (2010). Circadian integration of metabolism and energetics. Science 330, 1349–1354.
7. Van Cauter, E., Polonsky, K.S., and Scheen, A.J. (1997). Roles of circadian rhythmicity and sleep in human glucose regulation. Endocr. Rev. 18, 716–738.
8. Knutson, K.L., Ryden, A.M., Mander, B.A., and Van Cauter, E. (2006). Role of sleep duration and quality in the risk and severity of type 2 diabetes mellitus. Arch. Intern. Med. 166, 1768–1774.
9. Buxton, O.M., Cain, S.W., O'Connor, S.P., Porter, J.H., Duffy, J.F., Wang, W., Czeisler, C.A., and Shea, S.A. (2012). Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. Sci. Transl. Med. 4, 29ra43.
10. Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H., and Kyriacou, C.P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr. Biol. 12, 540–550.
11. McCarthy, J.J., Andrews, J.L., McDearmon, E.L., Campbell, K.S., Barber, B.K., Miller, B.H., Walker, J.R., Hogenesch, J.B., Takahashi, J.S., and Esser, K.A. (2007). Identification of the circadian transcriptome in adult mouse skeletal muscle. Physiol. Genomics 31, 86–95.
12. Stratmann, M., and Schibler, U. (2006). Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J. Biol. Rhythms 21, 494–506.
13. Grimaldi, B., Bellet, M.M., Katada, S., Astarita, G., Hirayama, J., Amin, R.H., Granneman, J.G., Piomelli, D., Leff, T., and Sassone-Corsi, P. (2010). PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab. 12, 509–520.
14. Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., and Hogenesch, J.B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320.
15. Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., and Evans, R.M. (2006). Nuclear receptor expression links the circadian clock to metabolism. Cell 126, 801–810.
16. Woon, P.Y., Kaisaki, P.J., Bragança, J., Bihoreau, M.T., Levy, J.C., Farrall, M., and Gauguier, D. (2007). Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. Proc. Natl. Acad. Sci. USA 104, 14412–14417.
17. Scott, E.M., Carter, A.M., and Grant, P.J. (2008). Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. Int J Obes (Lond) 32, 658–662.
18. Lyssenko, V., Nagorny, C.L., Erdos, M.R., Wierup, N., Jonsson, A., Spégel, P., Bugliani, M., Saxena, R., Fex, M., Pulizzi, N., et al. (2009). Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. Nat. Genet. 41, 82–88.
19. Prokopenko, I., Langenberg, C., Florez, J.C., Saxena, R., Soranzo, N., Thorleifsson, G., Loos, R.J., Manning, A.K., Jackson, A.U., Aulchenko, Y., et al. (2009). Variants in MTNR1B influence fasting glucose levels. Nat. Genet. 41, 77–81.
20. Fonken, L.K., Workman, J.L., Walton, J.C., Weil, Z.M., Morris, J.S., Haim, A., and Nelson, R.J. (2010). Light at night increases body mass by shifting the time of food intake. Proc. Natl. Acad. Sci. USA 107, 18664–18669.
21. Karatsoreos, I.N., Bhagat, S., Bloss, E.B., Morrison, J.H., and McEwen, B.S. (2011). Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. Proc. Natl. Acad. Sci. USA 108, 1657–1662.
22. Arble, D.M., Bass, J., Laposky, A.D., Vitaterna, M.H., and Turek, F.W. (2009). Circadian timing of food intake contributes to weight gain. Obesity (Silver Spring) 17, 2100–2102.
23. Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W., and Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metab. 6, 414–421.
24. Turek, F.W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D.R., et al. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. Science 308, 1043–1045.
25. Alenghat, T., Meyers, K., Mullican, S.E., Leitner, K., Adeniji-Adele, A., Avila, J., Bućan, M., Ahima, R.S., Kaestner, K.H., and Lazar, M.A. (2008). Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology. Nature 456, 997–1000.
26. Feng, D., Liu, T., Sun, Z., Bugge, A., Mullican, S.E., Alenghat, T., Liu, X.S., and Lazar, M.A. (2011). A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. Science 331, 1315–1319.
27. Dallmann, R., and Weaver, D.R. (2010). Altered body mass regulation in male mPeriod mutant mice on high-fat diet. Chronobiol. Int. 27, 1317–1328.
28. Rudic, R.D., McNamara, P., Curtis, A.M., Boston, R.C., Panda, S., Hogenesch, J.B., and Fitzgerald, G.A. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol. 2, e377.
29. Lamia, K.A., Storch, K.F., and Weitz, C.J. (2008). Physiological significance of a peripheral tissue circadian clock. Proc. Natl. Acad. Sci. USA 105, 15172–15177.
30. Shi, S., Hida, A., McGuinness, O.P., Wasserman, D.H., Yamazaki, S., and Johnson, C.H. (2010). Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. Curr. Biol. 20, 316–321.
31. Sadacca, L.A., Lamia, K.A., deLemos, A.S., Blum, B., and Weitz, C.J. (2011). An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. Diabetologia 54, 120–124.
32. Shimba, S., Ogawa, T., Hitosugi, S., Ichihashi, Y., Nakadaira, Y., Kobayashi, M., Tezuka, M., Kosuge, Y., Ishige, K., Ito, Y., et al. (2011). Deficient of a clock gene, brain and muscle Arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation. PLoS ONE 6, e25231.
33. Ayala, J.E., Bracy, D.P., McGuinness, O.P., and Wasserman, D.H. (2006). Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. Diabetes 55, 390–397.
34. Ayala, J.E., Samuel, V.T., Morton, G.J., Obici, S., Croniger, C.M., Shulman, G.I., Wasserman, D.H., and McGuinness, O.P.; NIH Mouse Metabolic Phenotyping Center Consortium. (2010). Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis Model Mech 3, 525–534.
35. Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., and Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009–1017.
36. Lall, G.S., Revell, V.L., Momiji, H., Al Enezi, J., Altimus, C.M., Güler, A.D., Aguilar, C., Cameron, M.A., Allender, S., Hankins, M.W., and Lucas, R.J. (2010). Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. Neuron 66, 417–428.
37. Molusky, M.M., Li, S., Ma, D., Yu, L., and Lin, J.D. (2012). Ubiquitin-specific protease 2 regulates hepatic gluconeogenesis and diurnal glucose metabolism through 11β-hydroxysteroid dehydrogenase 1. Diabetes 61, 1025–1035.
38. Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature 418, 935–941.
39. Sancar, A. (2004). Regulation of the mammalian circadian clock by cryptochrome. J. Biol. Chem. 279, 34079–34082.

40. Lowrey, P.L., and Takahashi, J.S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu. Rev. Genomics Hum. Genet. *5*, 407–441.

41. Ripperger, J.A., Jud, C., and Albrecht, U. (2011). The daily rhythm of mice. FEBS Lett. *585*, 1384–1392.

42. Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

43. Ueda, H.R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat. Genet. *37*, 187–192.

44. Marcheva, B., Ramsey, K.M., Buhr, E.D., Kobayashi, Y., Su, H., Ko, C.H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M.H., et al. (2010). Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. Nature *466*, 627–631.

45. Zheng, X., and Sehgal, A. (2010). AKT and TOR signaling set the pace of the circadian pacemaker. Curr. Biol. *20*, 1203–1208.

46. Grone, B.P., Chang, D., Bourgin, P., Cao, V., Fernald, R.D., Heller, H.C., and Ruby, N.F. (2011). Acute light exposure suppresses circadian rhythms in clock gene expression. J. Biol. Rhythms *26*, 78–81.

47. Gale, J.E., Cox, H.I., Qian, J., Block, G.D., Colwell, C.S., and Matveyenko, A.V. (2011). Disruption of circadian rhythms accelerates development of diabetes through pancreatic beta-cell loss and dysfunction. J. Biol. Rhythms *26*, 423–433.

48. Silver, R., and Schwartz, W.J. (2005). The suprachiasmatic nucleus is a functionally heterogeneous timekeeping organ. Methods Enzymol. *393*, 451–465.

49. Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., et al. (2004). PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. USA *101*, 5339–5346.

50. Welsh, D.K., Takahashi, J.S., and Kay, S.A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. Annu. Rev. Physiol. *72*, 551–577.

51. Wang, Y., Beydoun, M.A., Liang, L., Caballero, B., and Kumanyika, S.K. (2008). Will all Americans become overweight or obese? estimating the progression and cost of the US obesity epidemic. Obesity (Silver Spring) *16*, 2323–2330.

52. Hatori, M., Vollmers, C., Zarrinpar, A., DiTacchio, L., Bushong, E.A., Gill, S., Leblanc, M., Chaix, A., Joens, M., Fitzpatrick, J.A.J., et al. (2012). Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab. *15*, 848–860.

53. Kornmann, B., Schaad, O., Bujard, H., Takahashi, J.S., and Schibler, U. (2007). System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol. *5*, e34.

54. McGuinness, O.P., Ayala, J.E., Laughlin, M.R., and Wasserman, D.H. (2009). NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse. Am. J. Physiol. Endocrinol. Metab. *297*, E849–E855.

55. Johnson, C.H., and Kyriacou, C.P. (2005). Clock evolution and adaptation: whence and whither? In Endogenous Plant Rhythms, A.J.W. Hall and H.G. McWatters, eds. (Oxford, UK: Blackwell Publishing), pp. 237–260.

Note Added in Proof

While this manuscript was in production, we became aware of a recently published study reporting that clock disruption by lesioning the suprachiasmatic nuclei can also induce weight gain and insulin resistance, thereby confirming our conclusions using a different methodology. The complete citation for that paper is as follows: Coomans, C.P., van den Berg, S.A., Lucassen, E.A., Houben, T., Pronk, A.C., van der Spek, R.D., Kalsbeek, A., Biermasz, N.R., Willems van Dijk, K., et al. (2012). The suprachiasmatic nucleus controls circadian energy metabolism and hepatic insulin sensitivity. Diabetes. Published online December 28, 2012. http://dx.doi.org/10.2337/db12-0507.