Leptin modulation of daily rhythmicity of blood glucose

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Leptin may affect central and/or peripheral timing, in addition to its well-known regulatory effects on metabolism. Here, we investigated whether leptin can impact rhythmicity of blood glucose and lipids. For that purpose, daily variations of blood glucose and lipids were compared between mice lacking functional leptin receptor (db/db) or deficient for leptin (ob/ob) and controls (db/+ and ob/+, respectively). Next, we investigated whether timed treatment with exogenous leptin in ob/ob mice could modulate blood glucose rhythm. Mice with defective leptin signaling (db/db and ob/ob) have the same phase-opposed timing in glycemia (11 and 9 h shift, respectively) compared to respective controls. By contrast, the phase of plasma lipids rhythms (e.g. triglycerides, non-esterified fatty acid – NEFA, high density lipoprotein – HDL, low density lipoprotein – LDL) remained essentially unchanged, whatever the genotype. Daily injections of leptin (1 mg/kg) in ob/ob mice during nighttime or daytime led to 1–2 h phase-advances of blood glucose rhythm and glucose arrhythmicity, respectively. These injections induced additional phase-dependent shifts of feeding rhythm (ranging from 2.6 h phase-delays to 2.6 h advances). The present study reveals a chronomodulatory role of leptin, and highlights that rhythmic leptin can be a determinant of daily variations of blood glucose and food intake, though not for lipids.

Keywords: Circadian clock, feeding rhythm, glycemia, ob/ob, db/db, plasma lipids,

INTRODUCTION

Leptin, encoded by the ob gene, is secreted by adipocytes. This hormone plays a central role in the regulation of energy metabolism, because it communicates to the brain the level of fat stores available in adipose tissues (Zhang et al., 1994). In particular, leptin acts on the arcuate nuclei of the hypothalamus, where it activates POMC and CART containing neurons and inhibits NPY/AgRP neurons, leading to an overall anorexic effect and stimulation of energy expenditure (Ahima & Lazar, 2008). In peripheral organs, leptin also participates in lipid and glucose metabolism (Chinookoswong et al., 1999; Schwartz et al., 1996; Wang et al., 1999, 2010).

A prominent feature of leptin regulation is its daily rhythmicity. In rodents, levels of circulating leptin are high during the active period, respectively night and daytime in nocturnal and diurnal species (Ahren, 2000; Bodosi et al., 2004; Chacon et al., 2005; Cuesta et al., 2009; Kalsbeek et al., 2001; Zvonic et al., 2006). These daily fluctuations in plasma leptin are thought to be controlled by the master clock in the suprachiasmatic nuclei of the hypothalamus (Kalsbeek et al., 2001; Karakas & Gunduz, 2006) and the secondary circadian clocks in adipose tissues (Otway et al., 2009). Levels of circulating leptin are also modulated by feeding conditions, leptin secretion being enhanced and abolished by food intake and deprivation, respectively (Ahima et al., 1998; Bodosi et al., 2004; Elimam & Marcus, 2002; Martinez-Merlos et al., 2004).

The circadian timing system is based on a network of circadian clocks throughout the brain and the body that are coordinated by the suprachiasmatic nucleus, the site of the master clock and that regulate daily rhythmicity of most cellular, physiological and behavioral processes. A regular daily temporal organization is now considered as important for good health. Conversely, situations of altered internal timing (i.e. circadian desynchronization)
negatively impact on the cardiometabolic health (Delezie & Challet, 2011; Sherman et al., 2012; Zelinski et al., 2014). One key level for internal (de)synchronization is the coupling between the master clock and secondary clocks. Two main routes mediate circadian coupling: (1) nervous pathways innervating peripheral organs via the autonomic system (Buijs & Kalsbeek, 2001; Vujovic et al., 2008) and (2) high-amplitude hormonal rhythms closely controlled by the suprachiasmatic clock. More precisely, experimental data support a role of local tissue time-givers for the circadian rhythms of pineal melatonin and adrenal glucocorticoids (Alonso-Vale et al., 2008; Malek et al., 2007; Pevet & Challet, 2011; Pezuk et al., 2012; Segall et al., 2006). Meal time is another potent synchronizer for peripheral clocks in liver, adipose tissue and other organs. Feeding at unusual times alters internal timing by desynchronizing peripheral clocks while the master clock remains entrained to light cues (Damiola et al., 2000; Zvonic et al., 2006).

In this context, it is noteworthy that rhythmic leptin favors body mass gain of mice challenged with desynchronised feeding, as opposed to the lack of effect of continuous delivery of leptin (Arble et al., 2011). This observation raises the possibility that rhythmic leptin affects central and/or peripheral timing, in addition to its regulatory effects on metabolism reported above. To test the possible implication of leptin as an internal time-giver, we first characterized daily variations of plasma lipids, glucose and metabolic hormones in \(\text{db/db}\) mice lacking leptin receptor. Next, we studied these variations in \(\text{ob/ob}\) mice deficient for leptin and investigated whether timed treatment with exogenous leptin could modulate the observed circadian disturbances.

**MATERIALS AND METHODS**

**Animals, housing and diet**

Eight-week-old male BKS(D)-\textit{Lepr\textsuperscript{db/+}}\textit{OriRj (db/db)} mice and control littersmates (\(\text{db/+}\)), and 8-week-old B6.V-\textit{Lep\textsuperscript{ob/+}}\textit{Rj (ob/ob)} mice and control littersmates (\(\text{ob/+}\)) were purchased from Janvier Labs breeding centre (Le Genest-Saint-Isle, France). They were housed in individual cages, kept at 23 ± 1°C under a 12:12 h light–dark cycle with lights on at 07:00 AM and lights off at 07:00 PM, defining Zeitgeber time (ZT) 0 and ZT12, respectively. Food (standard chow pellets, 105, SAFE, Augy, France) and tap water were available \textit{ad libitum}. All experiments were performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Communities Council Directive 86/609/EEC) and approved by the Regional Ethical Committee of Strasbourg for Animal Experimentation (CREMEAS; AL/01/02/03/10 and AL/01/11/08/11) and in compliance with the ethical standards of the journal (Portaluppi et al., 2010).

**Experimental design**

**Experiment 1**

Twenty-four \(\text{db/db}\) and 24 \(\text{db/+}\) mice were used. Day-night food intake was determined over one day by weighing food at ZT0, ZT12 and ZT24 one week before tissue sampling. Mice were euthanized by overdose of pentobarbital every 6 h, starting at ZT2 (\(n = 6 \text{ db/db}\) or \(\text{db/+}\) mice per ZT). Blood samples were collected with 4% EDTA and centrifuged for 10 min at 5000 rpm at 4°C.

**Experiment 2**

Twenty-four \(\text{ob/ob}\) and 24 \(\text{ob/+}\) mice were used. Daily pattern of food intake was determined over one day by weighing food every 4 h, starting at ZT0 one week before tissue sampling. Mice were euthanized by overdose of pentobarbital every 4 h, starting at ZT0 (\(n = 6 \text{ ob/ob}\) or \(\text{ob/+}\) mice per ZT). Blood samples were collected with 4% EDTA (10 μL for 1 mL of blood) and centrifuged for 10 min at 5000 rpm at 4°C and liver samples were flash-frozen in liquid nitrogen.

**Experiment 3**

Forty \(\text{ob/ob}\) mice were housed in individual cages, including 32 equipped with infra-red sensors to record general locomotor activity every 5 min with a PC-based acquisition system (Circadian Activity Motor System, INSERM, France). Activity data were analyzed with ClockLab software (Actimetrics, Evanston, IL). During 7 days, mice were injected subcutaneously with vehicle (i.e., PBS 0.1 M + 0.1% BSA) at ZT4, ZT10, ZT16 or ZT22 (\(n = 10 \text{ ob/ob}\) mice per ZT). Following incision of the skin surface of the tail, micro-samples of blood (about 0.5 μL) were collected with heparinized glass capillaries every 4 h, starting at ZT2. During the next 7 days, each group of \(n = 10\) mice was injected subcutaneously with recombinant murine leptin (1 mg/kg; Peprotech, Neuilly-sur-Seine, France) at the same ZTs as before (i.e., ZT4, ZT10, ZT16 and ZT22, respectively). The dose and timing of leptin treatment were chosen based on previous data (Arble et al., 2011). On the 6th day, micro-samples of blood were collected again with heparinized glass capillaries every 4 h, as described above. Due to poor tail cicatization that prevented full blood sampling for the two rhythms, data from 1, 2 and 3 animals were excluded from ZT4, ZT16 and ZT22 groups, respectively.

**Circulating metabolites**

Blood glucose was determined with GOD-PAP Kit (LP80009, Biolabo, Maizy, France) in Experiments 1 and 2, and with a glucometer (Glucotrend premium kit, Roche Diagnostics, Meylan, France) in Experiment 3. Concentrations of non-esterified fatty acids (NEFA) and triglycerides (TG) were measured by NEFA-HR(2) kit (Wako Chemicals GmbH, Neuss, Germany) and serum triglyceride determination kit (TR-0100, Sigma-Aldrich, Saint-Quentin Fallavier, France), respectively. Plasma levels of total cholesterol, low density lipoprotein (LDL)-cholesterol and high density lipoprotein (HDL)-cholesterol were measured by the enzymatic colorimetric method.
(HDL)-cholesterol were evaluated by a colorimetric direct method (LP80106, 90206, 90416, respectively; Biolabo, Maizy, France).

**Plasma hormones**
Leptin concentrations were determined by a mouse leptin ELISA kit (EZML-82K, Millipore). Insulin was determined by an ultra-sensitive mouse insulin ELISA kit (Crystal Chem, Inc., Downers Grove, IL).

**Hepatic glycogen**
Glycogen content in the liver was quantified according to the method of Murat & Serfaty (1974).

**Statistical analysis**
A logarithmic transformation of plasmatic data was performed to ensure normality and homogeneity of variance required for parametric statistics. In Experiments 1 and 2, data of plasmatic parameters are presented as mean ± SEM on a logarithmic scale (left Y axis). Glucose levels in Experiment 3 correspond to repeated measures for each animal, represented on a logarithmic scale. Data of food intake are presented as means (bars) and plots (raw data). For all graphs, the first time-point has been double-plotted to make easier the visualization of daily rhythmicity. However, the duplicate time-point was removed in all statistical analyses.

For assessing daily rhythmicity, we used a cosinor analysis to determine mean level, amplitude and acrophase of the considered parameter with SigmaPlot software (Jandel Scientific, Chicago, IL). Data were fitted to the following regression: \[y = A + B \cos(2\pi(x - C)/24)], where \(A\) is the mean level, \(B\) the amplitude and \(C\) the acrophase of the rhythm. Student’s \(t\) test was used to compare the two groups. One-way analyses of variance (ANOVA) followed by post-hoc comparisons with the Tukey’s test were used to compare changes of body mass and food intake of the vehicle and leptin-treated groups in Experiment 3. Two-way ANOVAs were used to compare the effects of [genotype × time] (Experiments 1 and 2) and the effects of [treatment × ZT] (Experiment 3).

**RESULTS**

Experiment 1: Daily variations of plasma lipids, glucose and metabolic hormones in db/db mice
Body mass was significantly larger in db/db mice, compared to db/+ controls (33.9 ± 0.8 versus 26.2 ± 0.3 g, respectively; \(p < 0.001\)). Blood glucose levels were markedly up-regulated in db/db mice, compared to db/+ animals (effect of genotype: \(p < 0.001\)). There was also a significant [genotype × time] interaction (\(p = 0.003\)), indicating that the inter-genotype differences varied according to time of day (Table 1). This was confirmed by the cosinor analysis that detected significant rhythms for both the genotypes (\(p < 0.05\), with opposite acrophases (ZT18.5 ± 1.4 h and ZT7.5 ± 1.3 h in db/db and db/+ mice, respectively). Plasma insulin was significantly increased and non-rhythmic in db/db mice (effect of genotype: \(p < 0.001\); Table 1), in contrast to the clear rhythmicity in db/+ mice, with an acrophase at ZT17.7 ± 1.2 h. Leptin values were higher across the daily cycle in db/db mice, compared to db/+ animals (effect of genotype: \(p < 0.001\)). Moreover, the [genotype × time] interaction was significant (\(p < 0.001\)), highlighting daily differences between the genotypes (Table 1). Accordingly, a significant daily rhythm was found in both db/db and db/+ mice (\(p < 0.001\)), albeit with phase-opposed peaks (ZT6.9 ± 1.1 h and ZT18.7 ± 0.6 h, respectively; Figure 1).

Triglycerides levels were significantly higher in db/db mice (\(p < 0.001\)), and affected by times of day (\(p < 0.001\); Table 1). A cosinor analysis revealed a significant rhythmicity in both the genotypes (\(p < 0.001\)) with similar acrophases (ZT1.1 ± 0.7 h and ZT0.7 ± 0.6 h in db/db and db/+ mice, respectively). With respect to NEFA, there was a significant rhythm (\(p < 0.001\); Table 1) only in db/+ mice (peak in the morning at ZT4.2 ± 0.9 h; Figure 1). Levels of HDL showed no differences between the genotypes, or times of day. By contrast, LDL levels were increased in db/db mice (\(p = 0.003\)) and modified according to the times of day (\(p = 0.002\)). A significant rhythm was detected in db/db mice (peak in the morning at ZT4.4 ± 0.9 h; \(p < 0.001\), but not in db/+ mice. Finally, total plasma cholesterol did not display daily variations, but was increased by 20% in db/db mice, in comparison with db/+ animals (effect of genotype: \(p < 0.001\); Figure 1, Table 1).

Daily food intake in db/db mice was twice higher than in the control individuals (9.5 ± 0.4 versus 5.0 ± 0.1 g/day, respectively; \(p < 0.001\)), with marked increased during daytime (3.5 ± 0.2 versus 0.7 ± 0.1 g/day; \(p < 0.001\)) since db/+ and db/db mice ate 13% and 37% of their daily food intake, respectively.

| Parameter          | ZT      | Genotype  | ZT × genotype |
|--------------------|---------|-----------|---------------|
| Glucose            | 0.25    | 0.86      | 625.42        | <0.001 | 5.44 | 0.003 |
| Insulin            | 1.76    | 0.17      | 24.11         | <0.001 | 2.56 | 0.065 |
| Leptin             | 2.00    | 0.13      | 220.0         | <0.001 | 18.59 | <0.001 |
| TG                 | 23.52   | <0.001    | 80.98         | <0.001 | 0.55 | 0.65  |
| NEFA               | 7.25    | <0.001    | 103.54        | <0.001 | 2.75 | 0.052 |
| HDL                | 1.35    | 0.27      | 1.32          | 0.26   | 0.096 | 0.96  |
| LDL                | 5.73    | 0.002     | 9.85          | 0.003  | 0.89 | 0.45  |
| Cholesterol        | 1.28    | 0.29      | 48.33         | <0.001 | 3.63 | 0.019 |

Results present F and p values of two-way ANOVAs for the effects of Zeitgeber time (ZT2, ZT8, ZT14 versus ZT20), Genotype (db/+ versus db/db) and the interactions between the two factors (ZT × genotype). TG, triglycerides; NEFA, non-esterified fatty acids; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ZT, Zeitgeber time.
intake during the light phase, respectively (data not shown).

**Experiment 2: Daily variations of metabolic parameters in ob/ob mice**

Body mass was much higher in ob/ob mice, as compared to ob/+ controls (43.9 ± 0.4 versus 24.6 ± 0.6g, respectively; p < 0.001). Blood glucose levels were increased in ob/ob mice (effect of genotype: p < 0.001), with larger differences with ob/+ mice at night ([genotype × time] interaction: p = 0.002; Table 2). The cosinor analysis revealed a significant rhythm in both ob/ob and ob/+ mice, with opposite acrophases occurring at ZT20.1 ± 1.1 h and ZT4.6 ± 1.3 h, respectively (p < 0.001). Plasma insulin was dramatically enhanced in ob/ob mice, compared to ob/+ littermates (p < 0.001; Table 2). Even if there was a significant effect of the time of day (p = 0.006), there was no daily rhythm of insulin either for ob/ob or ob/+ genotype, as assessed by cosinor regression. In keeping with the profile observed in db/+ mice, a leptin rhythm was found in ob/+ mice with a peak occurring during late night (ZT23.6 ± 0.9 h; p = 0.003). Liver glycogen showed a clear daily rhythm (p < 0.001 for both the genotypes; Table 2), with similar peaks at lights onset (around ZT0) in both ob/+ and ob/ob mice. The only difference between the genotypes concerns a less marked depletion of hepatic glycogen in ob/ob mice at the end of the resting period ([ZT × genotype] interaction, p = 0.008; Figure 2, Table 2). The same effect has already been identified in db/db, as compared to db/+ mice (Roesler et al., 1985).

Plasma triglycerides were similarly affected by times of day in both ob/ob and ob/+ mice (effect of time:
The daily pattern of food intake in ob/ob mice injected with vehicle was also rhythmic \( (p<0.001) \), the peak of feeding occurring close to midnight (i.e. ZT18; Figure 3; Supplemental Table S2).

Next, mice were injected daily with leptin at ZT4 or ZT10 (i.e. out of phase compared to the endogenous rhythm in the control mice) for one week. For both ZTs, there was a strong reduction in blood glucose levels between saline and leptin treatments \( (p<0.001; \) Table 3). Moreover, the \( [\text{treatment} \times \text{time}] \) interaction was significant \( (p<0.01; \) Table 3), indicating that the effects of leptin at ZT4 and ZT10 on blood glucose depended on the time of day considered. Accordingly, cosinor analysis indicated that these diurnal treatments produced a disappearance of blood glucose rhythm (i.e. arrhythmicity) in both cases (Figure 3; Supplemental Table S1). In mice injected daily with leptin during nighttime at ZT16 or ZT22 (i.e. closer to the timing of endogenous secretion of leptin in the control mice), there was also a strong reduction in blood levels between saline and leptin treatments \( (p<0.001; \) Table 3), while the \( [\text{treatment} \times \text{time}] \) interaction was not significant in both the cases \( (p>0.2; \) Table 3), indicating that the effects of leptin injected at night on blood glucose did not heavily depend on the time of day considered. According to cosinor analysis, the nocturnal treatments with exogenous leptin tended to advance the phase of glucose rhythm only by 1 and 2 h (respectively; Figure 3; Supplemental Table S1).

With respect to food intake, leptin treatments whatever the time of injections led to a major reduction of food ingestion \( (p<0.01; \) Table 4). Moreover, the \( [\text{treatment} \times \text{time}] \) interaction was significant when leptin was injected at ZT4, ZT10 or ZT16 \( (p<0.01; \) Table 3), but not for the treatment at ZT22 \( (p>0.9; \) Table 3). In line with these effects, cosinor analysis detected phase-shifts of feeding rhythm when leptin was injected at ZT4, ZT10 and ZT16, and no phase-shift for treatment at ZT22. Interestingly, the amplitude and direction of the phase-shifts of feeding rhythm were also phase-dependent (-2.2 and -2.6 h phase-delays and +2.6 h phase-advance for leptin treatments at ZT4, ZT10 and ZT16, respectively; Figure 3; Supplemental Table S2). As expected, besides reduced food intake, administration of exogenous leptin also led to reduction in body mass. The same reducing effects were observed whatever the time of treatment (Supplemental Table S3). While day–night distribution of general locomotor activity in ob/ob mice was not significantly modified either by daytime or nighttime leptin treatment (Supplemental Figure S1), daily levels of activity during leptin treatment were significantly increased compared to the period with daily injections of vehicle (effect of treatment: \( p<0.05 \) for levels of daytime, nocturnal and daily activity; Supplemental Table S4), in accordance with the view that leptin stimulates locomotor activity (Coppari et al., 2005; Huo et al., 2009). In addition, activity levels during daytime were found to be higher.

### Experiment 3: Phase-shifts in glucose and food intake rhythms in ob/ob mice injected with leptin

We determined individual daily rhythms of blood glucose in ob/ob mice injected with saline at ZT4, ZT10, ZT16 or ZT22. As expected from Experiment 2, we confirmed that the daily variations in blood glucose were rhythmic \( (p<0.05) \), with a nocturnal peak around ZT16 in all the groups of obese mice treated daily with vehicle (Figure 3; Supplemental Table S1).

### TABLE 2. Effects of genotype and time of day on daily patterns of plasma metabolites and hormones in ob/+ and ob/ob mice.

| Parameter   | ZT   | Genotype   | ZT × genotype |
|-------------|------|------------|---------------|
|             | F    | p  | F    | p  | F    | p  |
| Glucose     | 1.58 | 0.21 | <0.001 | 6.018 | 0.002 |
| Insulin     | 4.83 | 0.006 | 403.12 | <0.001 | 2.15 | 0.11 |
| Leptin      | 5.05 | 0.013 |         |       |      |     |
| Glycogen    | 29.81 | <0.001 | 3.80 | 0.059 | 4.63 | 0.008 |
| TG          | 4.78 | 0.006 | 0.0041 | 0.95 | 0.12 | 0.95 |
| NEFA        | 3.81 | 0.018 | 122.98 | <0.001 | 0.53 | 0.66 |
| HDL         | 13.26 | <0.001 | 11.69 | 0.002 | 1.03 | 0.39 |
| LDL         | 18.39 | <0.001 | 185.44 | <0.001 | 3.42 | 0.027 |
| Cholesterol | 14.99 | <0.001 | 227.11 | <0.001 | 1.94 | 0.14 |

Results present \( F \) and \( p \) values of two-way ANOVAs for the effects of Zeitgeber time (ZT0, ZT6, ZT12 versus ZT18), genotype (ob/+ versus ob/ob) and the interactions between the two factors (ZT × genotype).

\( p = 0.006 \), without significant rhythmic variations (Table 2). By contrast, NEFA levels displayed significant daily variations with a peak at ZT3.8 ± 1.1 h in ob/+ mice, variations that were dampened and up-regulated in ob/ob mice. HDL, LDL and cholesterol levels displayed significant daily variations (effect of time: \( p<0.001 \)), with peaks in the morning in both the genotypes. For the three parameters, the values were significantly enhanced in ob/ob mice (effects of genotype: \( p<0.005; \) Figure 2).

The quantity of food ingested by ob/ob mice per day was increased compared to the control individuals \( (7.5 ± 0.3 \text{ versus } 4.1 ± 0.1 \text{ g/day, respectively; } p<0.001) \). Furthermore, the daily pattern of food intake, albeit oscillating at a higher level in ob/ob mice, showed similar daily timing in both the genotypes (acrophase of food intake rhythm for ob/+ and ob/ob at ZT18.3 ± 0.5 h and ZT19.2 ± 0.4 h, respectively; Figure 2), nocturnal feeding representing respectively 69% and 72% of daily food intake in ob/+ and ob/ob mice.

Of note, the concentrations of plasma metabolites vary between ob/+ and ob/+ mice. This strain effect is consistent with a previous study showing different phenotypes between mice carrying the ob mutation in a C57BL6 or C57BKs genetic background (Coleman & Hummel, 1973). Therefore, the differences between our control groups are likely due to genetic differences in mouse strains.
after injections at ZT10 (effect of ZT: $p < 0.01$), probably due to an arousing effect of treatment close to the usual onset of activity (Supplemental Figure S1). Finally, levels of plasma leptin were clearly higher by 2 h than 4 h after the administration of exogenous leptin (575 ± 83 versus 45 ± 12 ng/mL, $n = 18$; respectively; $p < 0.05$).

DISCUSSION

This study shows that obese and hyperglycemic db/db and ob/ob mice have the same altered timing in blood glucose rhythm (nocturnal peak), opposite to that in respective heterozygous littermates (daytime peak).
FIGURE 3. Daily patterns of glycemia and food intake in ob/ob mice injected subcutaneously with vehicle (white circles and bars) and leptin (dark grey circles and bars), either during early daytime (ZT4), late daytime (ZT10), early night (ZT16) or late night (ZT22). Data at ZT2 are double-plotted for visual comparison only (not for statistical analysis). Significant cosinor regressions are represented with smoothed lines. Shaded area indicates the dark period. ZT, Zeitgeber time.

TABLE 3. Effects of treatment and time of day on daily patterns of plasma glucose in ob/ob mice before and after chronic administration of leptin.

| Time of treatment | ZT | Treatment | ZT × treatment |
|-------------------|----|-----------|----------------|
|                   | F  | p         | F  | p     | F  | p     |
| ZT4               | 7.25 | <0.001 | 1491 | <0.001 | 3.79 | 0.007 |
| ZT10              | 7.96 | <0.001 | 124.9 | <0.001 | 4.37 | 0.002 |
| ZT16              | 8.5 | <0.001 | 262.26 | <0.001 | 1.47 | 0.227 |
| ZT22              | 9.73 | <0.001 | 810.42 | <0.001 | 0.9 | 0.49 |

Results present F and p values of two-way ANOVAs for the effects of Zeitgeber time (ZT), treatment (saline versus leptin) and the interactions between the two factors (ZT × treatment).

TABLE 4. Effects of treatment and time of day on daily patterns of food intake in ob/ob mice before and after chronic administration of leptin.

| Time of treatment | ZT | Treatment | ZT × treatment |
|-------------------|----|-----------|----------------|
|                   | F  | p         | F  | p     | F  | p     |
| ZT4               | 18.51 | <0.001 | 113.22 | <0.001 | 5.33 | <0.001 |
| ZT10              | 16.88 | <0.001 | 81 | <0.001 | 4.49 | 0.002 |
| ZT16              | 25.26 | <0.001 | 46.28 | <0.001 | 11.38 | <0.001 |
| ZT22              | 11.37 | <0.001 | 94.63 | <0.001 | 0.24 | 0.94 |

Results present F and p values of two-way ANOVAs for the effects of Zeitgeber time (ZT), treatment (saline versus leptin) and the interactions between the two factors (ZT × treatment).
Of note, leptin rhythm in \( db/db \) and the control mice also peaks in anti-phase (i.e. midday and midnight, respectively). On the other hand, besides higher levels than in the control mice, impaired leptin signaling does not induce major temporal abnormalities in the daily variations of plasma lipids. Systemic leptin treatment in \( ob/ob \) mice normalizes glycemia and decreases food intake. Furthermore, leptin treatment during the resting period (ZT4 or ZT10) leads to blood glucose arrhythmicity, accompanied by a delayed feeding rhythm. By contrast, the same treatment applied during the active period (ZT16 and ZT22) tends to phase-advance by 1–2 h blood glucose rhythm (i.e. a bit closer to the regular pattern in \( ob/+ \) mice) and phase-advances food intake rhythm in early, but not in late night.

Minor disturbances in daily timing of plasma lipids in mice with defective leptin signaling

In accordance with previous studies, \( ob/ob \) mice and, to a lower extent, \( db/db \) mice have increased levels of LDL and HDL, suggesting a role of leptin in lipoprotein metabolism (Camus et al., 1988; Silver et al., 1999). As in the control mice studied here (i.e. \( db/+ \) and \( ob/+ \)), plasma triglycerides and NEFA have already been found rhythmic in nocturnal rodents that show larger values during the resting period (Escobar et al., 1998; Kennaway et al., 2013; Stucchi et al., 2012). Plasma lipid rhythms remained essentially unchanged in \( ob/ob \) and \( db/db \) mice, as compared to the respective controls. While the phase of most lipids rhythms (e.g. triglycerides, NEFA, LDL) was close between genotypes, one exception was daily variations of HDL. In \( ob/ob \) and \( ob/+ \) mice, HDL rhythm peaks during daytime, while in both \( db/db \) and \( db/+ \) mice, plasma HDL tended to be higher at night. This inter-strain mouse difference (BSK versus B6) is difficult to interpret, in particular due to the paucity of previous investigations in mice. Exogenous leptin can trigger lipolytic processes in adipose tissues (Shen et al., 2007). Our data taking into account the daily temporal structure suggest that endogenous leptin (secreted at night in the control mice) does not markedly affect lipolysis that predominates during daytime in mice. Moreover, since the daily changes of most plasma lipids (e.g. NEFA) were hardly modified by defective leptin signaling, leptin rhythm is probably not an internal time-giver for daily variations of lipid metabolism and as such, it does not provide feedback cues onto adipose clocks.

Endogenous leptin rhythm is phase-changed in \( db/db \) mice

In the control heterozygous mice studied here, the nocturnal pattern of leptin secretion matches previous findings in wild-type individuals of this species (Ahren, 2000; Bodosi et al., 2004). This daily rhythm has been shown to depend on the master clock in the suprachiasmatic nuclei of the hypothalamus (Kalsbeek et al., 2001) and the secondary circadian clocks in adipose tissues (Otway et al., 2009), and to be modulated by feeding (Ahima et al., 1998; Martinez-Merlos et al., 2004). In \( db/db \) mice, the inverse rhythm of leptin is unlikely be caused by the master clock because its transcriptional clockwork is hardly affected in these mice (Kudo et al., 2004). Rather, the inverse rhythm of leptin may be due to impaired timing in adipose clocks, as it has been shown for the molecular oscillations in various peripheral clocks of mice bearing the \( db \) mutation (Kudo et al., 2004; Su et al., 2012). Further investigations have to be performed to determine the expression patterns of clock and metabolic genes in white adipose tissues of \( db/db \) mice. Considering the synchronizing effects of feeding cues on peripheral organs including the white adipose tissues (Damiola et al., 2000; Zvonic et al., 2006) and the daytime increase in leptin secretion triggered by food ingestion in rodents fed during the light phase (Ahima et al., 1998; Martinez-Merlos et al., 2004), the inverse rhythm of leptin may in part be due to the very large daytime increase of feeding in \( db/db \) mice (see below for possible effects of rhythmic corticosterone).

Daily rhythmicity of food intake in mice with defective leptin signaling

Leptin is well-known to suppress food intake and augment energy expenditure. It should be pointed out that the anorexic effects of leptin, as often evidenced by pharmacological doses, may be more subtle than generally thought. For instance, the high levels of secreted leptin in the control mice during nighttime, corresponding to their period of activity/feeding, indicate that leptin at physiological levels does not immediately inhibit food intake, at least at night. Nevertheless, it is important to note that the daily variations of food intake coincide in time in mice with \( ob/+ \) and without circulating leptin (\( ob/ob \), thus confirming previous results (Ando et al., 2011). Therefore, the difference between \( ob/+ \) and \( ob/ob \) mice rests with the amount of food ingested during the feeding period. In \( db/db \) mice, increased feeding during daytime accounts for a large part to the hyperphagia of these mice. This confirms previous findings in another model of leptin receptor-deficient rodent: the Zucker (\( Lepr^{+/−} \)) rat (Mistlberger et al., 1998). As noted above, feeding time is a potent synchronizer of extra-SCN clocks, including liver and white adipose tissues (Damiola et al., 2000; Zvonic et al., 2006). Here, we can rule out a major role of feeding synchronization for explaining the temporal differences of plasma glucose rhythm in \( db/db \) mice, because they match those found in \( ob/ob \) mice, which exhibit no change in daily pattern of food intake. In the same line, daily rhythmicity of plasma lipid – likely reflecting the phase of adipose and/or liver clocks – remains essentially unshifted in all genotypes, independent of timing (and amount) of food intake.

When leptin is absent (\( ob/ob \) mice), the daily pattern of food intake is increased in mid-levels, but not altered
in phase. This observation suggests that the endogenous rhythm of leptin plays only a permissive role on the daily rhythm of food intake. By applying exogenous leptin at different times of day, however, our study reveals that leptin treatment during daytime and nighttime differentially modifies the daily rhythm of food intake, leading to respective phase-delays and advances (and no shift after late night treatment). These time-dependent changes can be, in part, explained by direct anorexic effects of leptin acting on the mediobasal hypothalamus and brainstem. An alternative explanation is that leptin in these various cases differentially affects the brain clocks regulating the daily cycle of food intake, for instance the master clock in the suprachiasmatic nuclei and/or the arcuate clock (Bechtold & Loudon, 2013). Further investigations using complementary protocols (e.g. evaluation in constant darkness and/or in the dark-light cycle) are needed to assess the possible involvement of circadian processes.

**Dysregulated rhythmicity of plasma glucose in mice with defective leptin signaling**

The daily pattern of plasma glucose is important for metabolic health. For instance, defective hepatic clock in mice is associated with low plasma glucose during daytime and leads to impaired glucose tolerance (Lamia et al., 2008). The daily rhythm of plasma glucose peaking during daytime in ob/+ and db/+ mice fits with the profiles previously reported in free-fed or fasted male wild-type mice (Ahren, 2000; Rudic et al., 2004). By contrast, the rhythm of plasma glucose is not only up-regulated, but also phase-reversed in both ob/ob and db/db mice, with larger hyperglycemic values at night. This profile of glycemia is reminiscent of what has been already observed in db/db mice and opposite to the rhythm of glycogen stores in the liver (this study, Roessler et al., 1985) for ob/ob and db/db mice, respectively). Glycogen stores in db/db and ob/ob mice are less depleted at the end of the resting phase, which could reflect either decreased glycogenolysis or increased glycogenesis associated with the lack of functional leptin. The daily rhythm of hepatic glycogen in db/db and ob/ob mice being in phase with their heterozygous littermates, the reversion of glucose rhythm cannot be attributed to a shifted glycogen rhythm in these mice. Furthermore, levels of plasma insulin in our control mice are, or tend to be, increased at night, as found in other mouse studies (Ahren, 2000; Kennaway et al., 2013). An inverse day–night relationship between insulin and glucose levels can be noted in the control mice, while these parameters are not inversely correlated either in ob/ob or db/db mice, suggesting dysregulated insulin responses to glucose when leptin signaling is not functional.

The phase-reversion of blood glucose rhythm in both ob/ob and db/db mice (i.e. larger nocturnal and lower diurnal values) compared to ob/+ and db/+ controls may be due to secondary effects of obesity and/or diabetes. If so, phase-reversion of blood glucose should be found in diet-induced obese, as in type 1 or 2 diabetic animals. This is not the case because the phase of blood glucose variations in high-fat fed, obese and diabetic, mice is not markedly altered, in spite of significant overall hyperglycemia (Kohsaka et al., 2007). The same conclusion can be drawn from streptozotocin-induced diabetic rats (Young et al., 2002).

The differential temporal organization of blood lipid and glucose rhythms in ob/ob and db/db mice raises the possibility of impaired internal coupling within the circadian system. As mentioned in the Introduction, such circadian coupling is thought to involve both autonomous nervous and endocrine pathways. Ob/ob and db/db mice are known to have a reduced sympathetic nervous system tone. For instance, both display impaired cold-induced thermogenesis (Bates et al., 2004; Reichling et al., 1988). Despite their reduced sympathetic tone, we cannot rule out that the phase-reversion of glucose rhythm relies on altered rhythmic activity of the sympathetic system. This would also explain the phase-shift of leptin rhythm in db/db mice. To test this hypothesis, further studies should investigate clock gene expression in the multi-synaptic pathways of the autonomous nervous system, including the paraventricular hypothalamic nuclei and sympathetic ganglia. In our opinion, an impaired internal coupling within the circadian system is a likely explanation to account for the observed circadian disturbances when leptin signaling is not functional.

Notwithstanding, the phase-reversion of blood glucose rhythm shared by ob/ob and db/db mice is a consequence of impaired leptin signaling. This prompted us to investigate the effects of timed injections of leptin in ob/ob mice. Systemic leptin treatment during their resting or active period normalizes plasma glucose, thus confirming that leptin improves glucose homeostasis. The mechanisms underlying the antidiabetic effects of leptin are not fully characterized yet. They include increased hepatic insulin sensitivity (German et al., 2009), decreased hepatic glucose production, increased peripheral glucose uptake (German et al., 2011) and decreased insulin secretion as shown in ob/ob mice (Seufert et al., 1999). In addition, the present study shows that the timing of leptin treatment leads to differential effects with respect to daily variations of glycemia. Repeated injections of leptin at night (mimicking the timing of endogenous secretion in mice) tends to phase advance the glucose rhythm, while the same injections applied during the light period (out of phase compared to the endogenous rhythm of leptin) cause arrhythmicity of glycemia. Our initial aim was to change the timing of circulating leptin and evaluate its chronobiological impact on rhythm of plasma glucose. For that purpose, ob/ob mice are an ideal model because they lack endogenous secretion of leptin. Therefore, the imposed rhythm by exogenous
leptin accounted for all the circulating leptin. This would not be the case in the control (ob+/+) mice that display a nocturnal peak of plasma leptin that may have interfered with the outcome (i.e. glucose rhythm). Alternatively, the obesity and other alterations due to the lack of leptin in the ob/ob mice we studied may also be other drawbacks. To solve this issue, further studies are warranted in ob/+ or +/+ mice to investigate further the chronobiotic impact of exogenous leptin on glucose metabolism.

**Leptin as a (de)synchronizer of daily rhythm of plasma glucose**

Before considering the effects of leptin on plasma glucose levels, the influence of exogenous leptin on locomotor activity will be briefly discussed. In keeping with previous studies (Coppari et al., 2005; Huo et al., 2009; Ribeiro et al., 2011), leptin treatment increased the global levels of locomotor activity. This increase, however, does not correspond specifically to the so-called food-anticipatory activity that food-restricted animals express prior to the expected time of feeding (Mistlberger, 2011). In the present study, as previously observed in rats fed ad libitum and receiving central injections of leptin on a circadian basis (Martinez-Merlos et al., 2004), leptin treatment appears to stimulate motor activities after the injection without affecting the daily timing of rest–activity rhythm.

By contrast, investigation of daily rhythmicity in lipid and glucose in mice with defective leptin signaling suggests that rhythmic leptin affects timing of plasma glucose via central and/or peripheral targets. Before considering this eventuality according to the observed changes in ob/ob and db/db mice, we will first discuss the possible involvement of hormonal cues known as internal time-givers (namely, the rhythms of pineal melatonin and adrenal glucocorticoids; see “Introduction” section). Actually, melatonin being not synthesized or at very low levels in C57 mice (Vivien-Roels et al., 1998), it is unlikely that this hormone plays a significant role in ob/ob and db/db mice and respective heterozygous litters. Similarly, even if peak levels can be increased by the ob mutation, the phase of corticosterone rhythm, another putative hormonal time-giver able to shift peripheral clocks, has been repeatedly shown to be unaffected in ob/ob mice (Ahima et al., 1998; McGinnis et al., 1992; Saito & Bray, 1983). This regular rhythm of endogenous corticosterone rules out any significant effect of this hormone in the modified temporal organization when circulating leptin is lacking. In db/db mice, the results regarding corticosterone rhythm are more controversial because it has been found to be either unchanged (Saito & Bray, 1983) or phase-reversed due to marked increased values during the normal trough (i.e. daytime) (Ahima et al., 1998). If the rhythm of plasma corticosterone in the db/db mice studied was phase-changed as in the latter study, this could explain, via synchronization by glucocorticoid signaling, the phase-reversion of leptin rhythm in these mice. Such a hypothesis, however, does not give clues on why daily rhythmicity of lipids (e.g. NEFA) would remain unshifted if leptin and NEFA rhythms are controlled by the same adipose clocks.

Because leptin receptors are widely expressed in brain and peripheral tissues, rhythmic leptin may affect timing of plasma glucose at several levels. The first possible target is the master clock in the suprachiasmatic nuclei that expresses leptin receptors and can be shifted by leptin in vitro (Guan et al., 1997; Hakansson et al., 1998; Prosser & Bergeron, 2003). Several arguments do not favor this possibility. Leptin applied in vivo modulates SCN functioning indirectly, most likely via effects in the mediodasal hypothalamus (Grosbellet et al., 2015). Moreover, the daily mRNA expression profiles of the suprachiasmatic clock genes are hardly modified in ob/ob mice (Ando et al., 2011), despite altered timing in blood glucose rhythm (this study).

Considering that leptin promotes glucose uptake in muscles and brown adipose tissue (Toda et al., 2009; Wang et al., 1999), this direct effect is expected to be maximal at the time when daily levels of circulating leptin are highest. When leptin is administered in ob/ob mice, this period would coincide with the hours following the injection. This interpretation partly fits with the apparent phase-advances of glucose rhythm when leptin is injected at night. When leptin is injected during daytime, the glucose rhythm would display high amplitude, as a result of more reduced values of glycemia during afternoon. By contrast, diurnal injections of leptin led to glucose arrhythmicity (due to a disappearance of a significant nocturnal peak), thus not fully supporting an acute effect of leptin.

Alternatively, leptin may affect circadian clocks in peripheral tissues, such as liver and muscles. When leptin is lacking, the molecular clockwork of the liver is altered (reduced amplitude and modified phase), independently of major metabolic disturbances (Ando et al., 2011). Furthermore, repeated treatment with leptin at the light–dark transition partially rescues the hepatic oscillations of clock and clock-controlled genes in ob/ob mice (Ando et al., 2011). In this view, the differential effects of exogenous leptin on timing in blood glucose rhythm according to the time of day (present study) would be due to phase-dependent impact of leptin on the hepatic clock in ob/ob mice. Further investigations are needed to understand the underlying processes.

Moreover, our results are in favor of chronomodulatory effects of leptin on glucose rhythmicity, depending on the timing of leptin injection. Therefore, one additional prediction would be that continuous delivery of leptin would abolish rhythms of glucose and feeding behavior. Using a mini-osmotic pump could allow to test this hypothesis in a further study.
Based on the present findings, therapeutic treatment with leptin or molecules with leptin-like activity can impact on blood glucose rhythm and the daily pattern of feeding as well. In case of leptin resistance, as occurs during diet-induced obesity due to impaired transport through the blood–brain barrier, the problem can be partly circumvented by intranasal administrations that enhance bioavailability of the compounds for the brain ((Novakovic et al., 2009; Schulz et al., 2012). Adequately timed leptin therapy should therefore be taken into account to maintain/restore rhythmic glucose homeostasis and reduce circadian disturbances related to cardiovascular diseases. Together, these data suggest that rhythmic leptin may act as an internal chronomodulator of rhythms of plasma glucose and food intake, thus opening new avenues for its use in chronotherapeutic strategies of metabolic disorders. In conclusion, the present study demonstrates that a defective leptin signaling is associated with an internal desynchronization between daily variations of plasma lipids and glucose, and highlights that leptin affects not only glucose homeostasis, but also its daily rhythmicity.

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DECLARATION OF INTEREST

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REFERENCES

Ahima RS, Lazar MA. (2008). Adipokines and the peripheral and neural control of energy balance. Mol Endocrinol. 22: 1023–31.

Ahima RS, Prabakaran D, Flier JS. (1998). Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. J Clin Invest. 101:1020–7.

Ahren B. (2000). Diurnal variation in circulating leptin is dependent on gender, food intake and circulating insulin in mice. Acta Physiol Scand. 169:325–31.

Alonso-Vale MI, Andreotti S, Mukai PY, et al. (2008). Melatonin and the circadian entrainment of metabolic and hormonal activities in primary isolated adipocytes. J Pineal Res. 45: 422–9.

Ando H, Kumazaki M, Motosugi Y, et al. (2011). Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. Endocrinology. 152:1347–54.

Arbile DM, Vitaterna MH, Turek FW. (2011). Rhythmic leptin is required for weight gain from circadian desynchronized feeding in the mouse. PLoS One. 6:e25079.

Bates SH, Dundon TA, Seifert M, et al. (2004). LRB-STAT3 signaling is required for the neuroendocrine regulation of energy expenditure by leptin. Diabetes. 53:3067–73.

Bechtold DA, Loudon AS. (2013). Hypothalamic clocks and rhythms in feeding behaviour. Trends Neurosci. 36:74–82.

Bodosi B, Gardi J, Hajdu I, et al. (2004). Rhythms of ghrelin, leptin, and sleep in rats: Effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. Am J Physiol Regul Integr Comp Physiol. 287:R1071–9.

Buijs RM, Kalsbeek A. (2001). Hypothalamic integration of central and peripheral clocks. Nat Rev Neurosci. 2:521–6.

Camus MC, Aubert R, Bourgeois F, et al. (1988). Serum lipoprotein and apolipoprotein profiles of the genetically obese ob/ob mouse. Biochim Biophys Acta. 961:53–64.

Chacon F, Esquifino AI, Perello M, et al. (2005). 24-hour changes in ACHT, corticosterone, growth hormone, and leptin levels in young male rats subjected to calorie restriction. Chronobiol Int. 22:253–65.

Chinookoswong N, Wang JL, Shi ZQ. (1999). Leptin restores euglycemia and normalizes glucose turnover in insulin-deficient diabetes in the rat. Diabetes. 48:1487–92.

Coleman DL, Hummel KP. (1973). The influence of genetic background on the expression of the obese (Ob) gene in the mouse. Diabetologia. 9:287–93.

Coppari R, Ichinose M, Lee CE, et al. (2005). The hypothalamic arcuate nucleus: A key site for mediating leptin’s effects on glucose homeostasis and locomotor activity. Cell Metab. 1: 63–72.

Cuesta M, Clesse D, Pevet P, Challet E. (2009). From daily behavior to hormonal and neurotransmitters rhythms: Comparison between diurnal and nocturnal rat species. Horm Behav. 55: 338–47.

Damiola F, Le Minh N, Preitner N, et al. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14:2950–61.

Delezie J, Challet E. (2011). Interactions between metabolism and circadian clocks: reciprocal disturbances. Ann N Y Acad Sci. 1243:30–46.

El limam A, Marcus C. (2002). Meal timing, fasting and glucocorticoids interplay in serum leptin concentrations and diurnal profile. Eur J Endocrinol. 147:181–8.

Escobar C, Diaz-Munoz M, Encinas F, Aguilar-Roblero R. (1998). Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. Am J Physiol. 274:R1309–16.

German J, Kim F, Schwartz GJ, et al. (2009). Hypothalamic leptin signaling regulates hepatic insulin sensitivity via a neurocircuit involving the vagus nerve. Endocrinology. 150: 4502–11.

German JP, Thaler JP, Wisse BE, et al. (2011). Leptin activates a novel CNS mechanism for insulin-independent normalization of severe diabetic hyperglycemia. Endocrinology. 152: 394–404.

Grosbedet E, Gourmelen S, Pevet P, et al. (2015). Leptin normalizes photic synchronization in male ob/ob mice, via indirect effects on the suprachiasmatic nucleus. Endocrinology. 156:1080–90.

Guan XM, Hess JF, Yu H, et al. (1997). Differential expression of mRNA for leptin receptor isoforms in the rat brain. Mol Cell Endocrinol. 133:1–7.

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Hakansson ML, Brown H, Ghilardi N, et al. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci. 18:559–72.

Huo L, Gamber K, Greesley S, et al. (2009). Leptin-dependent control of glucose balance and locomotor activity by POMC neurons. Cell Metab. 9:537–47.

Kalsbeek A, Fliers E, Romijn JA, et al. (2001). The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. Endocrinology. 142:2677–85.

Karaka S, Gunduz B. (2006). Suprachiasmatic nuclei may regulate the rhythm of leptin hormone release in Syrian hamsters (Mesocricetus auratus). Chronobiol Int. 23:225–36.

Kennaway DJ, Varcoe TJ, Voutsinos A, Boden MJ. (2013). Global loss of bm1 expression alters adipose tissue hormones, gene expression and glucose metabolism. PLoS One. 8: e65255.

Kohsaka A, Laposky AD, Ramsey KM, et al. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. Cell Metab. 6:414–21.

Kudo T, Akiyama M, Kuriyama K, et al. (2004). Night-time restricted feeding normalises clock genes and PAI-1 gene expression in the db/db mouse liver. Diabetologia. 47: 1425–36.

Lamia KA, Storch KF, Weitz CJ. (2008). Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci USA. 105: 15172–7.

Malek ZS, Sage D, Pevet P, Raison S. (2007). Daily rhythm of tryptophan hydroxylase-2 messenger ribonucleic acid within raphe neurons is induced by corticoid daily surge and modulated by enhanced locomotor activity. Endocrinology. 148:5165–72.

Martinez-Merlos MT, Angeles-Castellanos M, Diaz-Munoz M, et al. (2004). Dissociation between adipose tissue signals, behavior and the food-entrained oscillator. J Endocrinol. 181:53–63.

Maginnis R, Walker J, Margules D, et al. (1992). Dissociation of the hypothalamic–pituitary–adrenal axis in male and female, genetically obese (ob/ob) mice. J Neuroendocrinol. 4:765–71.

Mistlberger RE. (2011). Neurobiology of food anticipatory circadian rhythms. Physiol Behav. 104:355–45.

Mistlberger RE, Lukman H, Nadeau BG. (1998). Circadian rhythms in the Zucker obese rat: Assessment and intervention. Appetite. 30:253–67.

Murat JC, Serfaty A. (1974). Simple enzymatic determination of glucocorticoids (glycogen) content of animal tissues. Clin Chem. 20:1576–7.

Novakovic ZM, Leinung MC, Lee DW, Grasso P. (2009). Intranasal administration of mouse [D-Leu-4]OB3, a synthetic peptide amide with leptin-like activity, enhances total uptake and bioavailability in Swiss Webster mice when compared to intraperitoneal, subcutaneous, and intramuscular delivery systems. Regul Pept. 154:107–11.

Otway DT, Frost G, Johnston JD. (2009). Circadian rhythmicity in murine pre-adipocyte and adipocyte cells. Chronobiol Int. 26: 1340–54.

Pevet P, Challet E. (2011). Melatonin: Both master clock output and internal time-giver in the circadian clocks network. J Physiol Paris. 105:170–82.

Pezuk P, Mohawk JA, Wang LA, Menaker M. (2012). Glucocorticoids as entraining signals for peripheral circadian oscillators. Endocrinology. 153:4775–83.

Portuluppi F, Smolensky MH, Toutou Y. (2010). Ethics and methods for biological rhythm research on animals and human beings. Chronobiol Int. 27:1911–29.

Prosser RA, Bergeron HE. (2003). Leptin phase-advances the rat suprachiasmatic circadian clock in vitro. Neurosci Lett. 336: 139–42.

Reichling S, Patel HV, Freeman KB, et al. (1988). Attenuated cold-induced increase in mRNA for uncoupling protein in brown adipose tissue of obese (ob/ob) mice. Biochem Cell Biol. 66: 193–8.

Ribeiro AC, Cecchinari G, Dupre C, et al. (2011). Contrasting effects of leptin on food anticipatory and total locomotor activity. PLoS One. 6:e23364.

Roesler WJ, Helgason C, Gulkas M, Khandelwal RL. (1985). Aberrations in the diurnal rhythms of plasma glucose, plasma insulin, liver glycogen, and hepatic glycogen synthase and phosphorylase activities in genetically diabetic (db/db) mice. Horm Metab Res. 17:572–5.

Rudic RD, McNamara P, Curtis AM, et al. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol. 2:e377.

Saito M, Bray GA. (1983). Diurnal rhythm for corticosterone in obese (ob/ob) diabetes (db/db) and gold-thioglucose-induced obesity in mice. Endocrinology. 113:2181–5.

Schulz C, Paulus K, Johnen O, Lehnert H. (2012). Intranasal leptin reduces appetite and induces weight loss in rats with diet-induced obesity (DIO). Endocrinology. 153:143–53.

Schwartz MW, Baskin DG, Bukowski TR, et al. (1996). Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes. 45:531–5.

Segall LA, Perrin JS, Walker CD, et al. (2006). Glucocorticoid rhythms control the rhythm of expression of the clock protein, Period2, in ovum nuclei of the bed nucleus of the stria terminalis and central nucleus of the amygdala in rats. Neuroscience. 140:753–7.

Seufert J, Kieffer TJ, Habener JF. (1999). Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. Proc Natl Acad Sci USA. 96:674–9.

Shen J, Tanida M, Nijjima A, Nagai K. (2007). In vivo effects of leptin on autonomic nerve activity and lipolysis in rats. Neurosci Lett. 416:193–7.

Sherman H, Genzer Y, Cohen R, et al. (2012). Timed high-fat diet resets circadian metabolism and prevents obesity. FASEB J. 26: 3493–502.

Silver DL, Jiang XC, Tall AR. (1999). Increased high density lipoprotein (HDL), defective hepatic catabolism of ApoA-I and ApoA-II, and decreased ApoA-I mRNA in ob/ob mice. Possible role of leptin in stimulation of HDL turnover. J Biol Chem. 274: 4140–6.

Stucchi P, Gil-Ortega M, Merino B, et al. (2012). Circadian feeding drive of metabolic activity in adipose tissue and not hyperphagia triggers overweight in mice: Is there a role of the pentose-phosphate pathway? Endocrinology. 153: 698–99.

Su W, Xie Z, Guo Z, et al. (2012). Altered clock gene expression and vascular smooth muscle diurnal contractile variations in type 2 diabetic db/db mice. Am J Physiol Heart Circ Physiol. 302: H621–33.

Toda C, Shiuchi T, Lee S, et al. (2009). Distinct effects of leptin and a melanocortin receptor agonist injected into medial hypothalamic nuclei on glucose uptake in peripheral tissues. Diabetes. 58:2757–65.

Vivien-Roels B, Malan A, Rettori MC, et al. (1998). Daily variations in pineal melatonin concentrations in inbred and outbred mice. J Biol Rhythms. 13:403–9.

Vujovic N, Davidson AJ, Menaker M. (2008). Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. Am J Physiol. 295:R355–60.

Wang JL, Chinooskosong N, Scully S, et al. (1999). Differential effects of leptin in regulation of tissue glucose utilization in vivo. Endocrinology. 140:2117–24.

Wang MY, Chen L, Clark GO, et al. (2010). Leptin therapy in insulin-deficient type 1 diabetes. Proc Natl Acad Sci USA. 107: 4813–19.
Young ME, Wilson CR, Razeghi P, et al. (2002). Alterations of the circadian clock in the heart by streptozotocin-induced diabetes. J Mol Cell Cardiol. 34:223–31.
Zelinski EL, Deibel SH, McDonald RJ. (2014). The trouble with circadian clock dysfunction: Multiple deleterious effects on the brain and body. Neurosci Biobehav Rev. 40C:80–101.
Zhang Y, Proenca R, Maffei M, et al. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature. 372:425–32.
Zvonic S, Ptitsyn AA, Conrad SA, et al. (2006). Characterization of peripheral circadian clocks in adipose tissues. Diabetes. 55:962–70.

Supplementary material available online
Supplementary Table S1-S4 and Figure S1 and S2