

Detrimental effects of constant light exposure and high-fat diet on circadian energy metabolism and insulin sensitivity

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ABSTRACT Circadian rhythm disturbances are observed in, e.g., aging and neurodegenerative diseases and are associated with an increased incidence of obesity and diabetes. We subjected male C57Bl/6J mice to constant light [12-h light-light (LL) cycle] to examine the effects of a disturbed circadian rhythm on energy metabolism and insulin sensitivity. *In vivo* electrophysiological recordings in the central pacemaker of the suprachiasmatic nuclei (SCN) revealed an immediate reduction in rhythm amplitude, stabilizing at 44% of normal amplitude values after 4 d LL. Food intake was increased (+26%) and energy expenditure decreased (−13%), and we observed immediate body weight gain (d 4: +2.4%, d 14: +5.0%). Mixed model analysis revealed that weight gain developed more rapidly in response to LL as compared to high fat. After 4 wk in LL, the circadian pattern in feeding and energy expenditure was completely lost, despite continuing low-amplitude rhythms in the SCN and in behavior, whereas weight gain had stabilized. Hyperinsulinemic-euglycemic clamp analysis revealed complete abolishment of normal circadian variation in insulin sensitivity in LL. In conclusion, a reduction in amplitude of the SCN, to values previously observed in aged mice, is sufficient to induce a complete loss of circadian rhythms in energy metabolism and insulin sensitivity.—Coomans, C. P., van den Berg, S. A. A., Houben, T., van Klinken, J.-B., van den Berg, R., Pronk, A. C. M., Havekes, L. M., Romijn, J. A., Willems van Dijk, K., Biermasz, N. R., Meijer, J. H. Detrimental effects of constant light exposure and high-fat diet on circadian energy metabolism and insulin sensitivity. *FASEB J.* 27, 1721–1732 (2013). www.fasebj.org

Key Words: constant light • SCN • multiunit activity • electrophysiology

THE INCREASING PREVALENCE of obesity and type 2 diabetes during the 20th century coincides with disruptions of circadian rhythm due to environmental light pollution, reduction of sleep duration and/or quality, jet lags, and shift work. Circadian rhythms are 24-h cycles generated by the suprachiasmatic nuclei (SCN) located in the anterior hypothalamus. The SCN induces daily rhythms in hormone concentrations (1–3), body temperature (4), heart rate and blood pressure (5), feeding behavior, and many other parameters and adjusts these rhythms to local time, mainly by light-dark information received from the eyes (6–8). Disruption of circadian coordination may be manifested by endocrine imbalances, psychological and sleep disorders, cancer incidence, and reduced life span (9). In contrast, the maintenance of robust circadian rhythms is associated with well-being and increased longevity (10, 11).

Many hormones involved in metabolism, such as insulin, glucagon, corticosterone, and leptin, exhibit circadian oscillations (1–3). Furthermore, enzymes and transport systems involved in lipid and glucose metabolism, such as glucose-6-phosphate dehydrogenase and nuclear receptors, are rhythmically expressed (12, 13). Conversely, mice transgenic for clock genes show deficits in several aspects of glucose homeostasis (14, 15). Disturbances in circadian rhythm due to shift work and

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Abbreviations: CT, circadian time; DD, 12-h dark-dark (constant darkness); EGP, endogenous glucose production; GIR, glucose infusion rate; LD, 12-h light-dark; LL, light-light (constant light); R_d , rate of disappearance; RER, respiratory exchange ratio; SCN, suprachiasmatic nucleus; T2DM, type 2 diabetes mellitus; ZT, zeitgeber time

sleep deprivation are associated with an increased incidence of obesity (16). Moreover, chronic disturbances in circadian rhythms have been proposed to be the underlying cause for the adverse metabolic and cardiovascular health effects of shift work (17–20). In part, this may be due to differences in the circadian pattern of energy intake (21).

Understanding of the causal relation between a disturbed circadian rhythm and features of the metabolic syndrome such as insulin resistance could lead to novel therapeutic strategies for patients, the elderly, and shift workers, who are prone to suffer from circadian rhythm disturbances. Importantly, circadian rhythms in these groups are reduced, rather than absent, whereas most animal studies have been performed in mouse models that are characterized by a total absence of circadian rhythmicity (15, 22). Therefore, the aim of the present study was to determine the effect of reduced rather than total loss of circadian rhythm on energy metabolism and insulin sensitivity in mice. Furthermore, we studied the additive effects of high-fat feeding and constant light exposure. To this end, mice were subjected to a 12-h light-light (LL) cycle, which reduces the amplitude of behavioral circadian rhythms (23, 24). *In vivo* recordings in freely moving mice exposed to LL showed a reduction in SCN rhythm amplitude of 56% in constant light, corresponding to values previously observed in aging mice (–50%; ref. 22). Remarkably, we found that the reduction in SCN rhythmicity resulted in complete loss of circadian variation in energy metabolism and insulin sensitivity, in mice on chow as well as high-fat diet. The results indicate that a reduction in SCN amplitude strongly impairs glucose and energy homeostasis, thereby contributing to development of obesity and insulin resistance.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare, and the institutional ethics committee for animal procedures of the Leiden University Medical Center (Leiden, The Netherlands), approved the protocol. Male C57Bl/6J mice (10 wk old) were housed individually in a controlled environment (21°C, 40–50% humidity) on a 12-h light-dark (LD) cycle (7:00 AM to 7:00 PM), in constant darkness [12-h dark-dark (DD)] or in constant light (LL, ≥ 180 lux). Food (chow, 4% energy of fat derived from corn oil, RM3, Special Diet Services, Sussex, UK; or high-fat diet, 45% energy of fat derived from lard; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) and tap water were available *ad libitum* during the whole experiment. Body weight was monitored 2×/wk for all individual animals throughout the experiment.

In vivo electrophysiological SCN recordings

For electrophysiological SCN recording, an electrode was implanted in the SCN. For this, mice were anesthetized using

a mixture of ketamine (100 mg/kg; Aescoket, Boxtel, The Netherlands), xylazine (10 mg/kg; Bayer AG, Leverkusen, Germany), and atropine (0.1 mg/kg; Pharmachemie, Haarlem, The Netherlands) and mounted in a stereotaxic device (Digital Just for Mouse Stereotaxic Instrument; Stoelting Co., Wood Dale, IL, USA). Tripolar stainless steel electrodes were used, with two of the electrodes twisted together and a third, uninsulated electrode for use as reference (125 μ m; Plastics One, Roanoke, VA, USA). The reference electrode was placed in the cortex, and the twisted electrode pair was implanted under a 5° angle in the coronal plane and aimed at the SCN, 0.46 mm posterior to bregma, 0.14 mm lateral to the midline, and 5.2 mm ventral to the surface of the cortex. At the end of the experiment, animals were euthanized, and the electrode placement was determined by histology. Recordings from animals in which the recording location was outside the SCN were excluded from the analysis.

Following a 7-d recovery period, mice were placed in a recording cage, and the electrodes were connected to the recording hardware. The connection consisted of lightweight cables suspended from a counterbalanced rotating contact, allowing animals to move around freely. Neuronal signals from the electrodes were differentially amplified and band-pass filtered (0.5–5 kHz) before being fed into amplitude-based spike triggers that converted action potentials into pulses that were counted every 10 s and stored on a computer. During the first days of the recording, mice were kept in LD to test for the presence of a circadian rhythm in the recorded action potential frequency. If a rhythm was detected, mice were exposed to DD for 3 d to ensure an endogenous rhythm. If the rhythm was maintained in DD, lights were turned on, and animals were exposed to LL for a minimum duration of 7 d.

Action potential frequency was calculated for each 10-min interval of the recording. Peak and trough levels were quantified by averaging the firing rate over a 2-h interval. To compare changes in rhythm in LL between animals, the amplitude values were normalized with respect to the amplitude in LD.

Circadian rhythm analysis

Behavioral activity of the mice in LD and LL was recorded using passive infrared motion detection sensors (Hygrosens Instruments, Löffingen, Germany) that were mounted underneath the lid of the cage and connected to a ClockLab data collection system (Actimetrics Software, Wilmette, IL, USA) that recorded the amount of sensor activation in 1-min bins. The presence of circadian rhythms was determined 25 d into the experiment for 10 consecutive days by F-periodogram analysis of activity based on the algorithm of Dörrscheidt and Beck (25).

Plasma corticosterone analysis

Three weeks after initiating the light intervention, blood samples were taken from mice fed chow or high-fat diet *via* tail indentation at 2 different circadian times (CTs): 1 h after the start of the subjective day (CT 1) and 1 h before the start of the subjective night (CT 11), when corticosterone levels are at their lowest and highest, respectively (26). The activity (subjective night) period was determined for each mouse using passive infrared motion detection sensors (27). The samples were taken into capillaries, placed on ice, and centrifuged at 4°C. Total plasma corticosterone concentrations were determined in an assay using an ¹²⁵I double-antibody kit (MP Biomedicals, Santa Ana, CA, USA). The high and low limits of detectability of the assay were 1200 and 3 ng/ml, respectively.

Indirect calorimetry

To determine energy metabolism of mice in LD or LL, indirect calorimetric analysis (Phenomaster; TSE Systems, Bad Homburg, Germany) was performed in individually housed animals fed chow or high-fat diet. Since exposure to constant light deteriorates circadian rhythm over time, these observations were obtained in 2 periods. The first period comprised the first 7 d of the constant light, high-fat-diet intervention, and the second period comprised 6 d of measurement, 4 wk after initiation of the light/high-fat-diet intervention.

Individual measurements of oxygen consumption and carbon dioxide production were performed every 9 min. In addition, food and water intake were measured. Furthermore, activity measurements (infrared beam break, inter-beam spacing: 3 cm) were performed, and pooled data were exported every 1 min. Before and after each experiment, animals were weighed to the nearest 100 mg. Since the LL regime lengthens the circadian period (28, 29), activity patterns of individual animals were analyzed and corrected for the subjective day and subjective night periods. Total length of an individual day was determined as the time elapsed between two consecutive periods of high physical activity. Subjective day and night were set at 50% of that elapsed time.

Hyperinsulinemic-euglycemic clamp

Five weeks after initiating the light intervention, insulin sensitivity of mice fed chow or high-fat diet was determined in a hyperinsulinemic-euglycemic clamp performed at 2 time points [zeitgeber time [ZT], with ZT 0 being the time of lights on]: the middle of the resting (ZT 6) and active phase of the LD mice (ZT 18). The hyperinsulinemic-euglycemic clamp was performed simultaneously in LL mice. Since the LL regimen disrupts the circadian rhythm by elongating the circadian period, the exact CT at which a stable hyperinsulinemic-euglycemic infusion rate was estimated for each mouse separately in the LL group (with CT 12 corresponding to activity onset, or ZT 12 for LD mice). Before the start of the experiment, mice unfed for 16 h were anesthetized with acepromazine (6.25 mg/kg; Alfasan, Woerden, The Netherlands), midazolam (6.25 mg/kg; Roche, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg; Janssen-Cilag, Tilburg, The Netherlands). Anesthesia, as well as body temperature, was maintained throughout the procedure. At the end of the basal period, as well as the end of the hyperinsulinemic period, hematocrit values were determined to ensure that the animals were not anemic. First, basal rates of glucose turnover were determined by primed (0.8 μCi), constant (0.02 $\mu\text{Ci}/\text{min}$), intravenous (i.v.) infusion of $3\text{-}^3\text{H}$ -glucose (Amersham, Little Chalfont, UK) for 60 min. Subsequently, insulin (Actrapid; Novo Nordisk, Denmark) was administered by primed (4.1 mU), constant (6.8 mU/h) i.v. infusion, with continuation of infusion of $3\text{-}^3\text{H}$ -glucose for 90 min. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia, as determined at 10-min intervals *via* tail bleeding (<3 μl , Accu-check, Sensor Comfort; Roche Diagnostics, Mannheim, Germany). In the last 20 min, blood samples were taken at intervals of 10 min. Subsequently, the mice were euthanized.

Plasma analysis

During the hyperinsulinemic-euglycemic clamp studies, blood samples were taken from the tail tip into chilled capillaries. The tubes were placed on ice and centrifuged at

4°C. Plasma glucose levels were determined using a commercially available kit and standardized according to the instructions of the manufacturer (Instruchemie, Delfzijl, The Netherlands) in 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Plasma insulin levels were measured using a mouse-specific insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA). Total plasma $3\text{-}^3\text{H}$ -glucose was determined in supernatant of 7.5 μl plasma, after protein precipitation using 20% trichloroacetic acid and evaporation to eliminate tritiated water. Turnover rates of glucose [rate of disappearance (R_d), $\mu\text{mol}/\text{min}/\text{kg}$] were calculated in basal and hyperinsulinemic conditions as the rate of tracer infusion (dpm/min) divided by plasma specific activity of $3\text{-}^3\text{H}$ -glucose (dpm/ μmol) corrected for body mass. Endogenous glucose production (EGP) during the hyperinsulinemic period was calculated as the difference between the tracer-derived rate of glucose turnover and the glucose infusion rate (GIR).

Statistical analysis

To assess the circadian variation of insulin sensitivity, clamp data from chow- and high-fat-fed mice were used for linear regression analysis. Data from mice in LD and LL were plotted against the calculated CT (the time at which stable GIR was achieved), and linear regression analysis was performed with GIR as dependent and CT as independent variable using GraphPad Prism 5.0 for Windows (GraphPad Software, La Jolla, CA, USA). Slope deviation from 0 was assessed for the LD and LL groups individually. Unpaired Student *t* tests were performed to compare GIR, endogenous glucose production, and glucose disposal rates obtained at ZT 6 and ZT 18 of the LD mice.

A mixed-effects model was used to investigate the effect of the constant light and high-fat-diet intervention on body weight gain. The model assumed body weight to increase linearly with time in the control group (LD mice fed chow) and included subject-specific random intercepts and slopes to model individual deviations from the group average. Additional effects of the light and high-fat-diet intervention were modeled by time-dependent covariates. The model allowed possible nonlinear time dependencies in weight gain and a possible interaction between the high-fat diet and the light regimen. The within subject error was assumed to have an autoregressive covariance structure of order 1. SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) was used for the analyses of the mixed-effects model.

RESULTS

SCN recording and circadian rhythm analysis

The effect of LL on the circadian rhythm in SCN neuronal activity was determined by *in vivo* electrophysiological recordings in the SCN of freely moving mice (Fig. 1). A circadian rhythm in neuronal activity was observed in 6 mice, showing high activity during the day and low activity during the night. This rhythm was maintained under DD. On exposure to LL, peak firing rate levels in the SCN decreased, whereas trough firing rate increased, causing a dampening of the amplitude of the rhythm (Fig. 1A, B). In LL, the amplitude was $70 \pm 5\%$ on d 1, $56 \pm 3\%$ on d 2, reaching a steady amplitude of $44 \pm 5\%$ from d 3 onward ($P < 0.01$; Fig. 1C) compared to d 0. F-periodogram analysis was performed over 10 d in LL. The period length, defined as the period between the

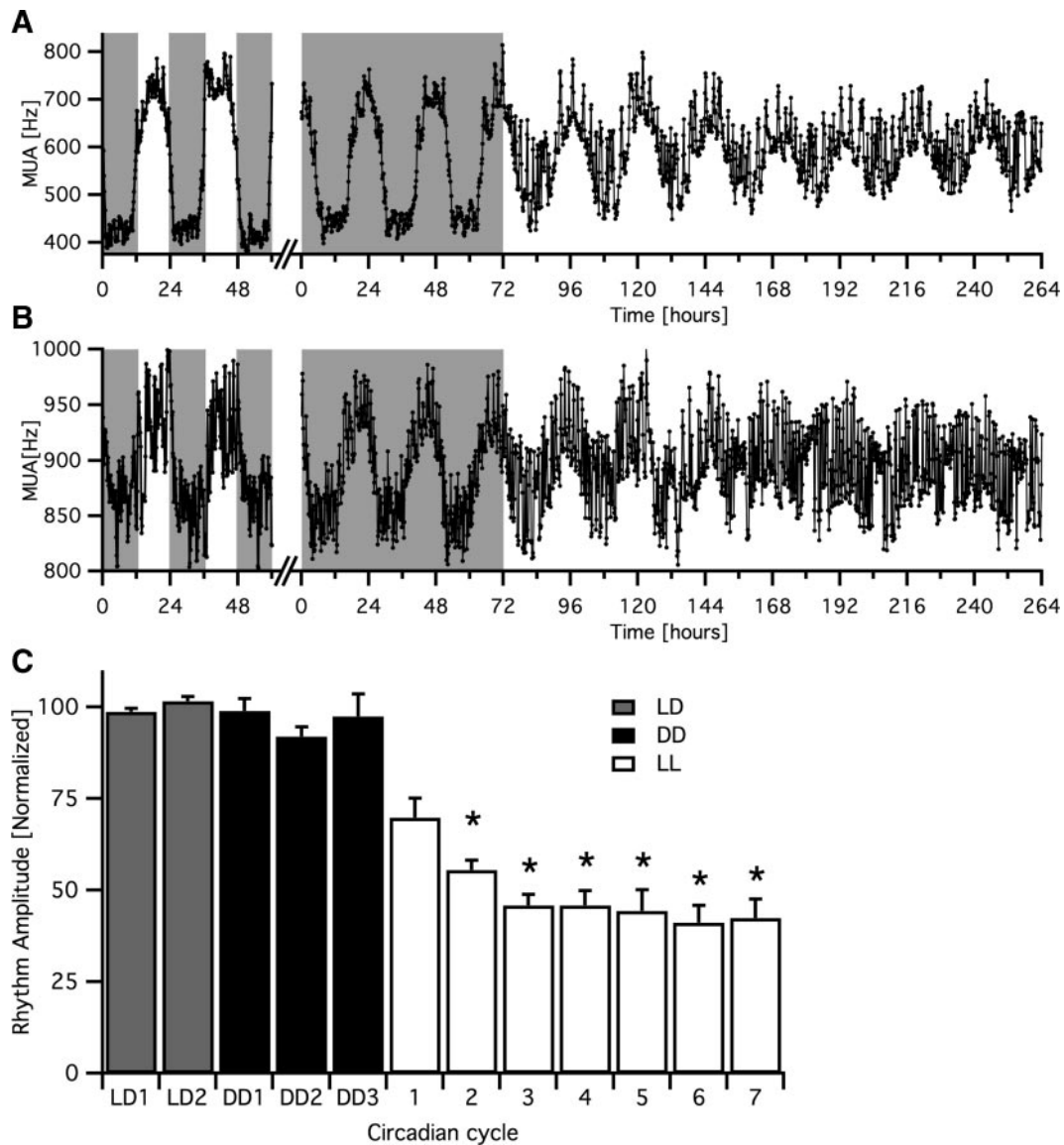


Figure 1. Long-term *in vivo* multiunit activity recordings were performed in freely moving, awake mice ($n=6$) using implanted microelectrodes. *A, B*) Representative recordings under LD followed by DD and LL. Action potential frequency (black trace) is indicated with a 10-min time resolution. Background represents light exposure, with gray shading representing lights off, and no shading representing lights on. *C*) Analysis of the time course of the SCN rhythm deterioration and stabilization after 3 d LL. Bars show the amplitude of the rhythm, normalized for each animal relative to the average amplitude during 2 cycles in LD (shaded bars) for 3 cycles in DD (solid bars) and 7 cycles in LL (open bars). * $P < 0.01$.

onset of the activity phase until the next activity phase, was increased by 8%, and the rhythm strength of locomotor activity, defined as the difference in activity between the active and the inactive phase of the mice, was decreased by 53% in LL compared to LD (Supplemental Fig. S1, $n=12$ mice/group, $P < 0.001$).

Body-weight gain

Body weights of LD and LL mice fed chow or high-fat diets measured during the course of the experiments are shown in **Fig. 2A**. LL mice were heavier compared to LD mice, on both chow and high-fat diet. To determine the isolated contribution of LL to weight development as well as to assess its possible interaction

with high-fat diet, a mixed effect model was developed, including light regimen and diet as covariates. The developed mixed-effects model predicted group averages as shown in **Fig. 2B**.

Both the constant light intervention covariate and high-fat diet intervention covariate were found to be highly significant ($P < 0.00001$ for both covariates), showing that both interventions independently caused additional weight gain compared to the chow diet and LD regime. The interaction term tended to, but did not reach, statistical significance ($P = 0.081$). Interestingly, the time dependency in the LL and high-fat diet covariate was nonlinear, as the exponents of the fitted power functions were significantly different from one ($P < 0.00001$ for both covariates): The LL effect on

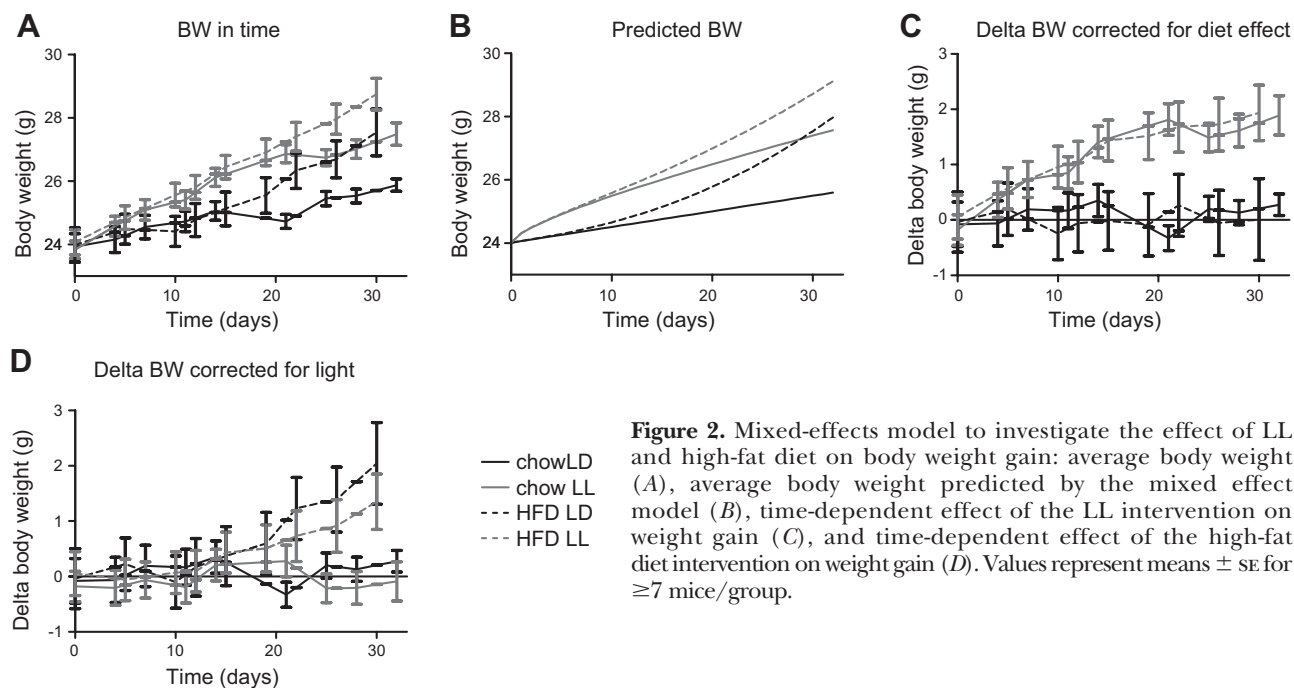


Figure 2. Mixed-effects model to investigate the effect of LL and high-fat diet on body weight gain: average body weight (A), average body weight predicted by the mixed effect model (B), time-dependent effect of the LL intervention on weight gain (C), and time-dependent effect of the high-fat diet intervention on weight gain (D). Values represent means \pm SE for ≥ 7 mice/group.

weight gain was described by a power function with an exponent of 0.6, while the high-fat diet effect had an exponent of 2.4. This indicates that the onset and speed of weight gain is different for both interventions. Indeed, the LL intervention immediately affected weight gain (Fig. 2C) and stabilized later, whereas the effect of high-fat diet became manifest at a later stage (Fig. 2D), indicating that exposure to constant light increases body weight faster than high-fat diet. These data clearly show that LL and high-fat diet have independent and additive effects during the development of weight gain.

Glucocorticoids

Corticosterone levels in plasma were determined at 2 different CTs: 1 h after the start of the subjective day (expected lowest corticosterone levels, CT 1) and 1 h before the start of the subjective night (expected peak in corticosterone levels, CT 11) as determined for each mouse individually using passive infrared motion detection sensors. LD mice fed chow as well as high-fat diet had low corticosterone levels at CT 1, which increased dramatically at CT 11: from 12 ± 2 to 90 ± 32 ng/ml for chow ($P < 0.01$) and from 19 ± 5 to 82 ± 24 ng/ml for high-fat diet ($P < 0.01$; Fig. 3). LD mice fed high-fat diet had higher corticosterone levels at CT 1 compared to LD mice fed chow (19 ± 5 vs. 12 ± 2 ng/ml, $P < 0.01$), whereas LL mice fed high-fat diet had lower corticosterone levels at CT 1 compared to LL mice fed chow (16 ± 3 vs. 28 ± 16 ng/ml, $P < 0.05$). In LL mice fed a chow diet, corticosterone levels were similar to LD mice at CT 1, but significantly lower at CT 11. In addition, corticosterone levels in LL mice fed chow were not significantly different between CT1 and CT11 (28 ± 16 to 42 ± 34 ng/ml, ns). In LL mice fed a high-fat diet, corticosterone levels were

similar to LD mice at CT 1, but significantly lower at CT 11. Interestingly, in contrast to the chow diet, corticosterone levels in LL mice fed high-fat diet were significantly increased at CT 11 compared to CT 1 (16 ± 3 to 43 ± 21 ng/ml, $P < 0.01$). These data show that the LL regimen did not result in a chronic stress response, and that corticosterone levels were lower compared to LD mice during the subjective night.

Indirect calorimetry

Chow diet

The energy intake of LL mice during the subjective day was higher, whereas the energy intake during the subjective night was lower compared to LD mice, resulting in equal 24-h levels of energy intake (Fig. 4A). Respiratory exchange ratio (RER) was significantly higher in LL mice during the subjective day period, indicating a higher relative carbohydrate to fat oxidation ratio (Fig. 4B). RER did not differ between groups during the night period. Total 24-h RER levels were significantly higher in LL mice compared to LD mice. Energy expenditure during the subjective night period was significantly lower in LL mice and, together with similar energy expenditure levels during the subjective day, resulted in significantly decreased total energy expenditure levels (Fig. 4C).

High-fat diet

During the first few days of LL intervention, LL mice had a higher intake of high-fat food measured over a circadian period compared to LD mice and consumed a larger fraction of the total circadian intake during the subjective day (44 vs. 36%, $P = 0.06$; Supplemental Fig. S1A). RER was significantly higher in LL mice during

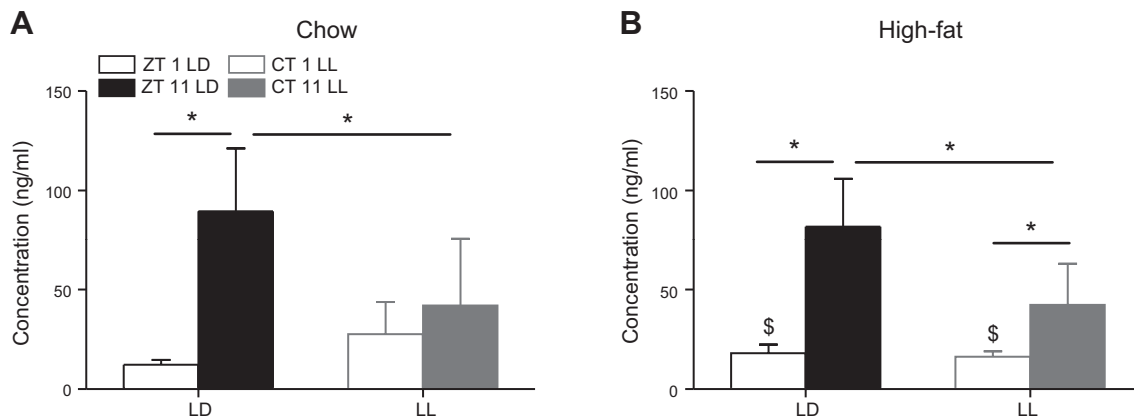


Figure 3. Corticosterone plasma levels of chow-fed (A) and high-fat-fed (B) mice in LD or LL, obtained at time of expected lowest (CT 1, open bars) and peak (CT 11, solid bars) corticosterone levels. Values represent means \pm SD for 7–12 mice/group. * $P < 0.01$. \$ $P < 0.05$ chow vs. high-fat diet.

the subjective day period, whereas the RER did not differ between groups during the subjective night period (Supplemental Fig. S1B). Total energy expenditure over a circadian period was significantly lower in LL mice (Supplemental Fig. S1C), due to a significant reduction in energy expenditure during the subjective night period. Energy expenditure levels also tended to be lower during the subjective day period ($P=0.06$). As expected from the higher energy intake and lower energy expenditure rates measured in the first few days,

body weights of LL mice (33.8 ± 2.5 vs. 29.4 ± 1.8 g, $P < 0.01$) as well as body-weight gain (8.1 ± 1.4 vs. 4.0 ± 1.6 g, $P < 0.01$) were significantly higher compared to LD mice after 4 wk. Interestingly, in the long term, total energy intake measured over a period of a circadian period was significantly lower in LL mice, whereas energy intake during the subjective day was higher compared to LD mice (55 vs. 19%, $P < 0.01$; Fig. 4D). The circadian rhythm in energy intake present in LD mice, with higher energy intake during the night and

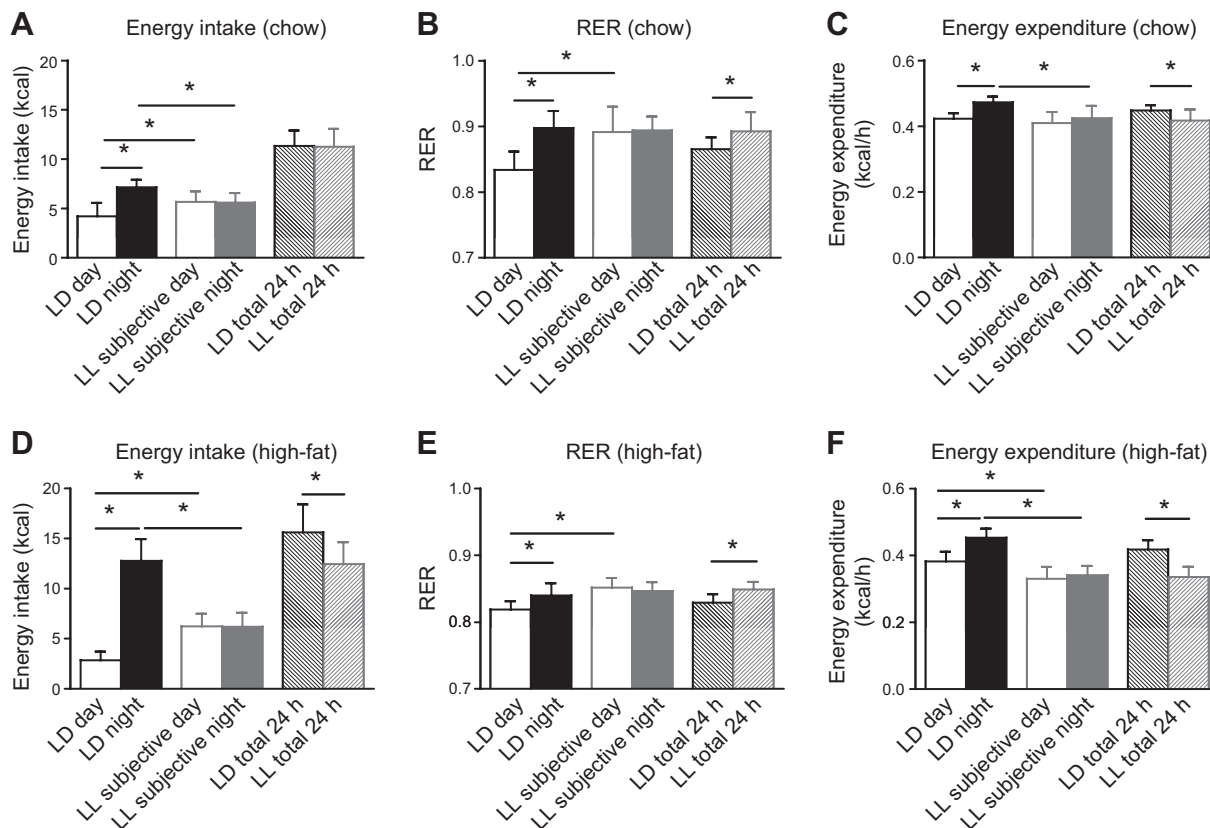


Figure 4. Indirect calorimetric analysis of mice under LD and LL conditions fed chow (A–C) or high-fat diet (D–F) analyzed for (subjective) day, (subjective) night, and total 24 h levels. Energy intake (A, D), respiratory exchange ratio (RER; B, E), and energy expenditure (C, F) after 4 wk of intervention. Values represent means \pm SD for 8 mice/group. * $P < 0.05$.

lower energy intake during the day, was absent in LL mice. Reflecting the higher subjective day energy intake, RER was significantly higher in LL mice (Fig. 4E). Interestingly, RER did not differ between groups during the night period, even though energy intake was higher in the LD group. Furthermore, RER was strongly correlated with energy intake during both the day and night period in LD mice ($R^2=0.70$ and 0.63 , $P=0.01$ and $P<0.01$, respectively), but not in LL mice ($R^2=0.03$ and 0.03 , ns for both periods). Total energy expenditure over a period of a circadian period was still significantly lower in the LL mice (Fig. 4F), due to a reduction during the subjective night period as well as subjective day period. Energy expenditure during the night was strongly correlated to energy intake in LD mice ($R^2=0.55$, $P<0.05$). Interestingly, in LL mice, the higher energy intake during the subjective day was not associated with an increase in energy expenditure.

Hyperinsulinemic-euglycemic clamp analysis

Insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp analysis of mice fed chow or high-fat diet at 2 different times: during the middle of the resting phase (ZT 6, day) and during the middle of the active phase (ZT 18, night) of the LD mice. Since the LL regimen disrupts the circadian rhythm by elongating the circadian period, the exact CT at which stable hyperinsulinemic-euglycemic infusion rate was achieved was subsequently determined for each individual mouse in LL. The clamp results of the LL mice were analyzed separately for CT 0–12 (subjective day) and for CT 12–24 (subjective night).

Chow diet

Plasma levels of LD and LL mice during the hyperinsulinemic-euglycemic clamp are shown in **Table 1**. Basal EGP did not differ during (subjective) day and (subjective) night between LD or LL mice. In LD mice fed a chow diet, GIRs were significantly higher at night compared to day (87.5 ± 9.2 vs. 51.6 ± 7.4 $\mu\text{mol}/\text{min}/\text{kg}$, $P<0.01$; **Fig. 5A**). Furthermore, there was a strong correlation between GIR and ZT in chow-fed LD mice

($R^2=0.84$, $P<0.01$; **Fig. 5D**). In chow-fed LL mice, there was no difference in the GIR between subjective day and subjective night (70.2 ± 212.2 vs. 72.3 ± 12.3 $\mu\text{mol}/\text{min}/\text{kg}$; **Fig. 5A**). There was no relation found between GIR and CT in LL mice ($R^2=0.01$, ns; **Fig. 5D**). Interestingly, not only was the circadian rhythm of insulin sensitivity absent in LL mice, the GIR in LL mice was set at $\sim 50\%$ of the minimal to maximal GIR seen in LD mice. This is in agreement with the similar reduction in SCN output (**Fig. 1**). In LD mice, EGP was significantly lower during the hyperinsulinemic-euglycemic clamp conditions at night compared to day (5.4 ± 8.8 vs. 20.5 ± 9.1 $\mu\text{mol}/\text{min}/\text{kg}$, respectively, $P<0.05$; **Fig. 5B**). In addition, hyperinsulinemic-euglycemic glucose disposal rates were higher at night compared to day (R_d : 89.7 ± 7.2 vs. 72.1 ± 14.2 $\mu\text{mol}/\text{min}/\text{kg}$, respectively, $P<0.05$; **Fig. 5C**). Both EGP and R_d correlated with ZT in LD mice ($R^2=0.44$ and 0.41 , respectively, $P<0.01$ for both parameters, **Fig. 5E, F**). In LL mice, hyperinsulinemic-euglycemic EGP and R_d did not differ significantly between subjective day and subjective night (EGP: 11.2 ± 11.5 vs. 10.6 ± 18.5 , and R_d : 83.8 ± 14.6 vs. 82.7 ± 24.9 $\mu\text{mol}/\text{min}/\text{kg}$, respectively, ns, **Fig. 5B, C**). Furthermore, EGP and R_d did not correlate with CT in LL mice ($R^2=0.04$ and 0.02 , respectively, ns; **Fig. 5E, F**). These data show that the circadian variation in tissue-specific insulin sensitivity that is normally present in chow-fed LD conditions was absent in mice subjected to LL.

High-fat diet

Plasma levels of LD and LL mice during the hyperinsulinemic-euglycemic clamp are shown in **Table 2**. LD and LL mice fed a high-fat diet were more insulin resistant, as GIR was lower during (subjective) day and (subjective) night compared to mice fed chow diet (LD: GIR -45% during day, $P<0.01$, and -76% during night, $P<0.01$; LL: GIR -44% during subjective day, $P<0.01$, and -46% during subjective night, $P<0.01$). The lower insulin sensitivity in LD mice was associated with diet-induced liver and peripheral insulin resistance, as hyperinsulinemic EGP was higher (EGP: $+48\%$ during day, $P=0.07$, and $+117\%$ during night, $P=0.09$) and

TABLE 1. Results from hyperinsulinemic-euglycemic clamp performed in LD and LL mice fed chow diet

Parameter	LD		LL	
	ZT 6	ZT 18	CT 0–12	CT 12–24
Basal hematocrit (%)	39.8 ± 1.7	$42.4 \pm 1.2^*$	40.4 ± 1.7	$41.0 \pm 1.6^\#$
Clamp hematocrit (%)	36.9 ± 1.1	$38.8 \pm 1.0^*$	37.9 ± 1.5	38.0 ± 2.2
Basal insulin (ng/ml)	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.3
Clamp insulin (ng/ml)	7.6 ± 1.4	6.7 ± 0.9	$5.6 \pm 1.7^\S$	6.8 ± 1.8
Basal glucose (mM)	4.0 ± 0.3	$5.0 \pm 0.5^*$	$4.8 \pm 0.9^\S$	4.8 ± 0.7
Clamp glucose (mM)	4.3 ± 0.5	4.6 ± 0.5	$4.9 \pm 0.6^\S$	4.6 ± 0.7
Basal EGP ($\mu\text{mol}/\text{min}/\text{kg}$)	47.3 ± 20.5	43.3 ± 5.6	47.0 ± 10.7	55.2 ± 23.8

Clamps were performed at 2 different times: during the middle of the resting phase (ZT 6, day) and during the middle of the active phase (ZT 18, night) of the LD mice. The exact CT at which stable hyperinsulinemic-euglycemic infusion rate was achieved was subsequently determined for each individual mouse in LL and analyzed separately for CT 0–12 (subjective day) and for CT 12–24 (subjective night). Data are presented as means \pm SD. * $P<0.05$ vs. LD ZT 6; $^\S P<0.05$ vs. LD ZT 6; $^\# P<0.05$ vs. LD ZT 18.

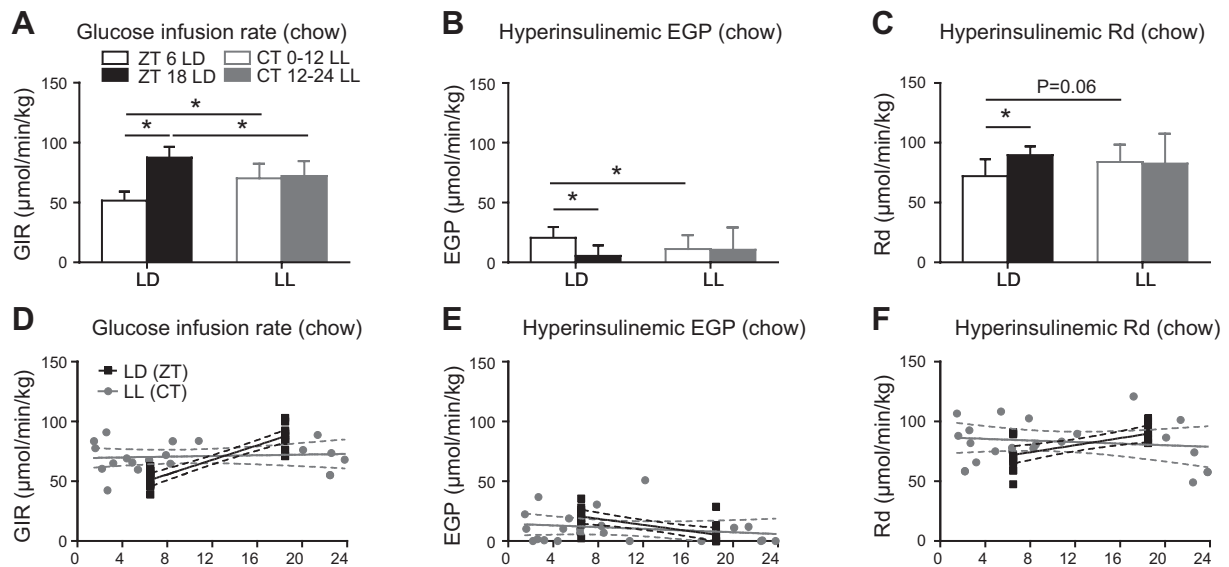


Figure 5. GIR (A, D), EGP (B, E), and glucose disposal R_d (C, F) in chow-fed mice under LD (solid) and LL (shaded) conditions, as measured in hyperinsulinemic-euglycemic clamp. A–C) GIR (A), EGP (B), and R_d (C) in LD mice measured at ZT 6 (day) or ZT 18 (night) and in LL mice measured at CT 0–12 (subjective day) or CT 12–24 (subjective night). D–F) Linear regression analysis of the GIR (D), EGP (E), and R_d (F) against ZT for LD mice ($n=21$) and against CT for LL mice ($n=20$). Values represent means \pm SD for ≥ 7 mice/group. * $P < 0.05$.

hyperinsulinemic R_d was lower (R_{di} : -7% during day, not significant, and -35% during night, $P < 0.01$), when compared to chow-fed LD mice. The lower insulin sensitivity in LL mice was mainly the result of peripheral insulin resistance, as hyperinsulinemic R_d was significantly lower (R_{di} : -36% during subjective day, $P < 0.01$, and -32% during subjective night, $P = 0.1$), when compared to chow-fed LL mice.

Basal EGP did not differ significantly during (subjective) day and (subjective) night between LD or LL mice (Table 2). GIR for LD mice fed high-fat diet was significantly higher at night compared to day (49.7 ± 13.0 vs. 35.7 ± 7.4 $\mu\text{mol}/\text{min}/\text{kg}$, $P < 0.01$; Fig. 6A), but the magnitude of difference between day and night in LD mice fed high-fat diet was less compared to LD mice fed chow diet. Linear regression of GIR against ZT still revealed a strong association with time in LD mice ($R^2 = 0.31$, $P < 0.01$; Fig. 6D). This dependency of GIR to time was absent in LL mice, as there was no difference

in the GIR between subjective day and subjective night (39.3 ± 7.1 vs. 38.7 ± 12.4 $\mu\text{mol}/\text{min}/\text{kg}$, ns; Fig. 6A) and no correlation of GIR with CT ($R^2 = 0.01$, ns; Fig. 6D). In the LD mice, EGP during clamp conditions were significantly lower at night compared to day (11.7 ± 6.5 vs. 30.4 ± 12.9 $\mu\text{mol}/\text{min}/\text{kg}$, $P < 0.05$; Fig. 6B). Hyperinsulinemic R_d rates were not different during day and night (67.4 ± 18.8 vs. 58.2 ± 11.9 $\mu\text{mol}/\text{min}/\text{kg}$, ns; Fig. 6C). Linear regression analysis confirmed these data, as EGP was correlated with ZT ($R^2 = 0.50$, $P < 0.01$; Fig. 6E), but R_d was not ($R^2 = 0.09$, ns; Fig. 6F). In LL mice, EGP and R_d rates did not differ between subjective day and subjective night for LL mice (EGP: 16.5 ± 7.3 vs. 17.8 ± 8.5 $\mu\text{mol}/\text{min}/\text{kg}$, and R_d : 54.3 ± 11.1 vs. 50.2 ± 12.7 $\mu\text{mol}/\text{min}/\text{kg}$, respectively, ns; Fig. 6B, C) and neither EGP nor R_d correlated with CT ($R^2 = 0.05$ and 0.00 , respectively, ns; Fig. 6E, F). These data show that even though high-fat feeding results in an obesity/insulin resistance phenotype, insulin sensi-

TABLE 2. Results from hyperinsulinemic-euglycemic clamp performed in LD and LL mice fed high-fat diet

Parameter	LD		LL	
	ZT 6	ZT 18	CT 0–12	CT 12–24
Basal hematocrit (%)	n.a.	44.1 ± 1.8	43.6 ± 1.4	43.3 ± 0.4
Clamp hematocrit (%)	n.a.	40.6 ± 1.6	40.5 ± 1.7	39.2 ± 1.2
Basal insulin (ng/ml)	0.7 ± 0.3	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.1
Clamp insulin (ng/ml)	5.9 ± 0.8	6.7 ± 0.9	$7.2 \pm 1.1^{\S}$	7.1 ± 1.5
Basal glucose (mM)	5.0 ± 0.7	$5.7 \pm 0.7^*$	5.1 ± 0.5	4.9 ± 0.7
Clamp glucose (mM)	5.6 ± 0.7	5.7 ± 1.1	5.6 ± 0.5	5.2 ± 0.8
Basal EGP ($\mu\text{mol}/\text{min}/\text{kg}$)	38.1 ± 12.9	41.5 ± 8.7	31.8 ± 1.6	$38.0 \pm 2.0^*$

Clamps were performed at 2 different times: during the middle of the resting phase (ZT 6, day) and during the middle of the active phase (ZT 18, night) of the LD mice. The exact CT at which stable hyperinsulinemic-euglycemic infusion rate was achieved was subsequently determined for each individual mouse in LL and analyzed separately for CT 0–12 (subjective day) and for CT 12–24 (subjective night). Data are presented as means \pm SD. * $P < 0.05$ vs. LD ZT 6; $^{\S}P < 0.05$ vs. LD ZT 6.

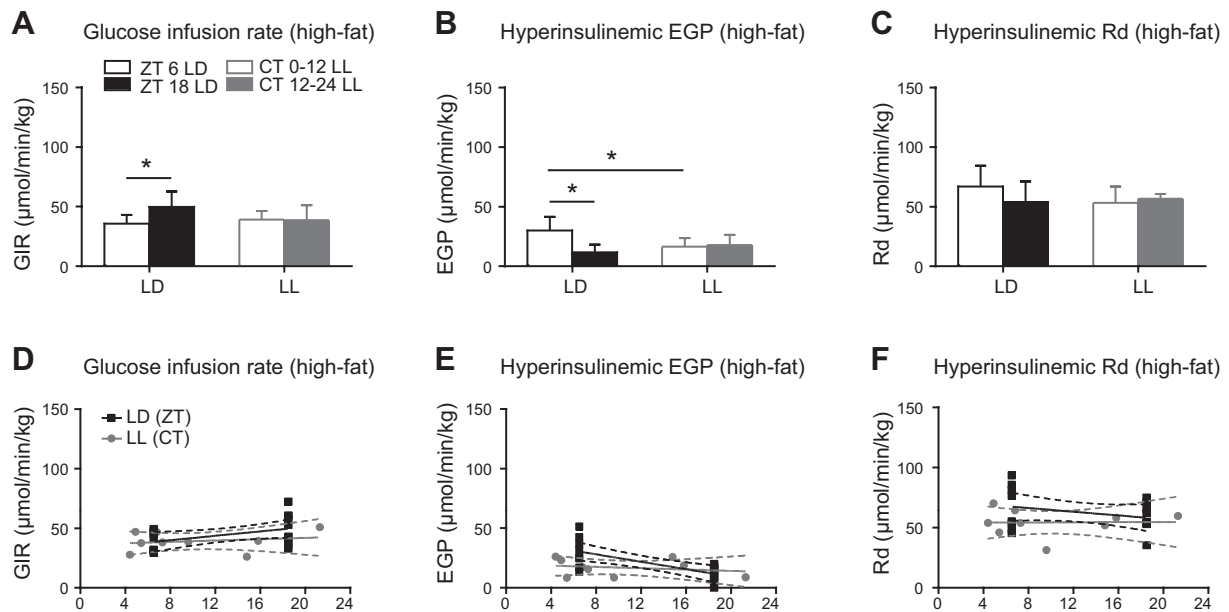


Figure 6. GIR (A, D), EGP (B, E), and glucose disposal R_d (C, F) in high-fat -fed mice under LD (solid) and LL (shaded) conditions, as measured in hyperinsulinemic-euglycemic clamp. A–C GIR (A), EGP (B), and R_d (C) in LD mice measured at Zeitgeber time (ZT) 6 (day) or ZT 18 (night) and in LL mice measured at CT 0–12 (subjective day) or CT 12–24 (subjective night). D–F Linear regression analysis of the GIR (D), EGP (E), and R_d (F) against ZT for LD mice ($n=21$) and against CT for LL mice ($n=20$). Values represent means \pm SD for ≥ 7 mice/group. * $P < 0.05$.

tivity was still under circadian control. Furthermore, even in obese, insulin-resistant mice, LL resulted in a further deterioration of the circadian rhythm of insulin resistance.

DISCUSSION

This study addressed the effect of a decline in rhythm amplitude on energy metabolism and insulin sensitivity. C57Bl/6J mice were exposed to LL or an LD cycle as control. *In vivo* recordings showed that LL resulted in an immediate decrease in SCN amplitude, which stabilized within 3 d at a level of 44%. Furthermore, LL stimulated weight gain, even before high-fat diet resulted in weight gain. Finally, a reduced SCN amplitude resulted in a complete loss of circadian rhythm in energy metabolism and hepatic and peripheral insulin sensitivity. Collectively, these data indicate that reduction of the circadian function of the central clock, as observed in aging and neurodegenerative diseases, can bring about weight gain and insulin resistance.

The SCN generates a circadian rhythm in neuronal activity and neurotransmitter release that serves as a timing signal by downstream target nuclei. The SCN receives input on environmental light levels by a direct projection from a subset of ganglions in the retina (30). Under regular 12-h LD cycles, this input pathway keeps individual SCN neurons synchronized to each other and the environment with a majority of neurons that are active during the day and silent during the night. Exposing animals to LL causes a desynchronization among neurons of the SCN, which results at the tissue

level in a dampened circadian rhythm (31, 32). Metabolic processes that manifest circadian fluctuations are regulated by a combination of excitatory and inhibitory inputs, with rhythmic SCN output regulating the balance between excitation and inhibition as a function of the time of day (33). To quantify the amplitude decrease and time course of the rhythm deterioration of the SCN in our mice, we performed longitudinal behavioral activity recordings, as well as a series of *in vivo* SCN electrophysiological recordings. These *in vivo* recordings are of great relevance, as previous investigations on LL influence on SCN rhythm were exclusively performed in the isolated SCN *in vitro* (1, 32), which did not allow for a quantitative estimation of the effect of LL on the SCN rhythm *in vivo*. Our SCN recordings show that the desynchronizing effects of LL occur immediately, resulting in an amplitude reduction of 70 and 56% on d 1 and 2, and stabilizing at an amplitude of 44% of the original value from d 3 onward. Notably, this dampening of SCN function was caused by the combined effects of increased trough levels and decreased peak levels. This is consistent with the finding that dampening is caused by a desynchronization among SCN neurons (31, 32). These effects lead to stronger output signals during the night and weaker signals during the day. In agreement with the effects within the SCN, home cage locomotor activity recordings show that the rhythm strength is reduced by 54% under constant light. Assessment of the melatonin rhythm under constant conditions has shown that in humans, the melatonin amplitude is reduced by 52–54% after constant light exposure (34), suggesting a comparable effect of LL on SCN function in humans. It

has long been questioned whether the amplitude of the SCN rhythm, apart from its phase and period, is of functional significance for the animal. The present data support the view that the amplitude of the SCN is of functional significance and should be incorporated as an important parameter in future studies.

Alterations in light regime typically are considered stressful. In our study, constant light disturbed corticosterone rhythm, resulting in lower corticosterone peak levels, which is in agreement with previous studies showing that LL does not lead to increased corticosterone levels (35–37). As corticosterone release is mediated by hypothalamic nuclei that receive strong input from the SCN (38), the dampening of the corticosterone rhythm may be a direct result of the dampening of SCN neuronal activity.

Body-weight development of all mice was measured $2\times/wk$. Disturbing circadian rhythm by LL resulted in higher body weights compared to control mice, independent of the diet. A mixed-effects model was developed to determine the isolated contributions of LL and high-fat diet to weight gain as well as to assess the possible interaction between LL and high-fat feeding. Constant light exposure immediately affected weight gain, which stabilized later, whereas the high-fat diet effect on body weight gain became manifest at a later stage. Our data correspond to a previous study showing that exposing chow-fed Swiss-Webster mice to LL increases body weight compared to mice in LD only for the first 2 wk. After this period, body weight gain in LL mice is equal to LD mice (35). The present study shows for the first time the independent effect of LL to weight gain in chow- and high-fat-fed mice and shows that the weight gain as a result of LL is evident before high-fat diet affects body weight gain. This immediate effect of LL on body weight coincides with the direct reduction in SCN output, suggesting that reduced SCN rhythmicity instantaneously affects energy homeostasis.

Disturbing circadian rhythm by LL aggravated diet-induced obesity and resulted in a shift of energy intake toward the period when energy intake is normally low. Furthermore, we show that LL is associated with a reduction in energy expenditure. These disturbed circadian rhythms in energy metabolism deteriorated over time. Within the first days of the experiment, exposure to LL led to lengthening of circadian rhythm in food intake, RER and energy expenditure. During the experiment, the circadian rhythm weakened further. This is in agreement with our findings that the amplitude of rhythmic SCN output dampened in LL over time. Furthermore, LL mice showed an impaired oxidative response toward food intake, as was indicated by the absence of correlation between RER and energy intake. A blunted response of metabolism toward food intake, also known as a metabolic inflexibility (39), has been shown to be associated with impaired insulin sensitivity and obesity (40). It is therefore likely that the reduced metabolic flexibility of the LL mice is a reflection of reduced insulin sensitivity.

We assessed the effect of disturbed circadian rhythm

on insulin sensitivity in mice fed chow as well as high-fat diet. We determined insulin sensitivity by hyperinsulinemic-euglycemic clamp analysis at 2 different time points that corresponded to the middle of the resting phase (ZT 6) and middle of the active phase (ZT 18) for the LD mice. For LL mice, the individual CT at the time of the clamp analysis was obtained afterward on the basis of the activity period of each individual mouse, and results were analyzed separately for the subjective day (CT 0–12) and subjective night (CT 12–24). In line with previous studies (41), LD mice fed chow show a circadian variation in insulin sensitivity, with higher hepatic insulin sensitivity and higher insulin-stimulated glucose uptake by peripheral organs at night. Remarkably, the circadian variation of insulin sensitivity was lost in LL mice. Moreover, the insulin sensitivity of LL mice was $\sim 50\%$ of the variation in insulin sensitivity of LD mice, which is in agreement with the 56% reduction in SCN output. As we have recently shown that bilateral microlesions of the SCN lead to complete loss of circadian energy metabolism and severe hepatic insulin resistance (42), the effect of LL on energy metabolism and insulin sensitivity is most likely the result of a reduction in SCN output.

In a previous study it was shown that disturbing circadian rhythm by light intervention results in deregulation of glucoregulatory genes in liver, such as phosphoenolpyruvate carboxykinase (PEPCK), glucose transporter 2 (GluT2), and glucose-6-phosphatase (G6PC) (43). In muscle, glucoregulatory gene expression is dependent on CT (44), and glucose uptake in isolated muscle from rats shows a circadian pattern (45). Deregulation in glucoregulatory genes in liver and muscle may underlie the disturbed insulin sensitivity we found in LL mice subjected to LL. Furthermore, disturbed circadian rhythms also accelerate loss of beta-cell mass and function (46). In combination with the current findings, the picture emerges that disturbances in circadian rhythms alone can lead to the development of obesity and type 2 diabetes mellitus (T2DM).

Aging in humans and other mammals has been associated with deterioration of circadian rhythms, as evidenced by fragmented sleep episodes and disrupted patterns in activity (47). Recently, SCN recordings in middle-aged mice showed a reduction in SCN rhythm amplitude of $\sim 50\%$ compared to young mice, which coincided with reduced circadian activity rhythm (22). The LL-induced reduction of circadian amplitude of the SCN activity observed in the current study remarkably resembles the dampening of SCN rhythm amplitude in aged mice. Therefore, the detrimental metabolic effects as a result of reduced SCN rhythmicity we describe here could also in part explain the increased prevalence of T2DM in older adults.

Our studies provide more insight on the relation between disturbances in endogenous rhythms and the increase in the risk of obesity and T2DM. Specifically, we have shown that a decrease in SCN rhythm amplitude of 56% is sufficient to completely abolish circadian

rhythm in energy metabolism and insulin sensitivity. Furthermore, we have shown that the immediate reduction in SCN output by LL coincides with an instantaneous increase in body weight. These findings indicate that a naturally occurring decrease in rhythm amplitude, as observed in sleeping disorders, degenerative diseases and aging, are a serious concern for health (48), as they may lead to secondary metabolic pathophysiology. The data indicate that new avenues for prevention and treatment programs for patients with metabolic disorders should include lifestyle programs, aimed to improve SCN rhythm amplitude, such as mild exercise (49, 50), proper light exposure (51, 52), and stable sleep-wake cycles (53). **FJ**

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