ADDITIVE EFFECTS OF CONSTANT LIGHT EXPOSURE AND DIET ON CIRCADIAN RHYTHMS OF ENERGY METABOLISM AND INSULIN SENSITIVITY

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ABSTRACT

Disturbances in circadian rhythms are associated with increased incidence of obesity and type 2 diabetes. We examined the effects of a disturbed circadian rhythm on energy metabolism and insulin sensitivity by exposing mice to constant light.

Mice were subjected to 12 h/12 h light/dark cycle (LD) or constant light (LL) for 5 weeks, and were fed chow or high-fat diet. Neuronal activity was measured by *in vivo* electrophysiological SCN recordings. During the last 4 days of the experiment, metabolic cage analysis was performed. Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamp in the middle of the resting (ZT 6) and active phases of the LD mice (ZT 18). Diet-independent contribution of the LL regimen to body weight gain was assessed by mixed model analysis.

Constant light exposure reduced the circadian amplitudes of the suprachiasmatic nucleus' (SCN) neuronal activity pattern by 55% and rhythm strength by 54% within three days. The first week in constant light, LL mice had increased total food intake over 24 h and decreased total energy expenditure over 24 h, which resulted in immediate body weight gain on both diets compared to LD mice. Mixed model analysis revealed that weight gain induced by constant light was apparent before high-fat diet resulted in weight gain. After four weeks in constant light, the circadian pattern in feeding and energy expenditure was completely lost. Total food intake and energy expenditure over 24 h was reduced compared to LD mice, which resulted in stabilization of weight gain caused by constant light. LL neutralized the normal circadian variation in insulin sensitivity of liver as well as peripheral tissues on both diets.

In conclusion, constant light exposure reduces SCN rhythm strength instantaneously and results in a complete absence of rhythm in energy metabolism and insulin sensitivity, exacerbating the effect of a high-fat diet. These data indicate that mild rhythm disorders can contribute to the pathophysiology of obesity and T2DM and may be of great relevance for people having reduced SCN rhythmicity such as, for instance, elderly and shift workers.

INTRODUCTION

The increasing prevalence of obesity and type 2 diabetes during the 20th century coincides with environmental light pollution, reduction of sleep duration and/or quality, jet lags and shift work, resulting in disruption of circadian rhythm. 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 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, 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 constant light, which reduces the amplitude of circadian rhythms (23-25). *In vivo* recordings in freely moving mice indeed showed a reduction in SCN rhythm amplitude of ~50% in constant light. Remarkably, we found that the circadian variation in energy metabolism and insulin sensitivity was completely lost in animals housed in constant light, on chow and high-fat diet compared to mice on a light-dark cycle. The results indicate that a reduction in SCN rhythmicity strongly impairs glucose and energy homeostasis, thereby contributing to development of obesity and insulin resistance.

MATERIALS AND METHODS

Animals

Male C57Bl/6J mice (10 weeks old) were housed in a temperature-controlled room on a 12 h/12 h light/dark cycle (LD) or in constant light (LL, ≥ 180 lux) for 5 weeks. The mice had free access

to chow and water throughout the experiment. In the high-fat experiment, mice were fed *ad libitum* high-fat diet (45 energy% of fat derived from lard; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands). Body weight was monitored twice a week for all individual animals throughout the experiment. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures from the Leiden University Medical Center, Leiden, The Netherlands approved the protocol.

In vivo electrophysiological SCN recordings

For electrophysiological SCN recording, an electrode was implanted in the SCN of C57Bl/6J mice. 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 stereotactic 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 µm, Plastics One, Roanoke, Virginia, 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 sacrificed 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 day 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 counter-balanced rotating contact, allowing animals to move around freely. Water and food was available *ad libitum* during the recording. 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 sec and stored on a computer. During the first days of the recording, animals were kept in constant darkness (DD) to test for the presence of a circadian rhythm in the recorded action potential frequency. If a rhythm was detected, light were turned on and animals were exposed to constant light for a minimum duration of 7 days.

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 constant light between animals, the amplitude values were normalized with respect to the amplitude in constant darkness.

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, Illinois, U.S.) that recorded the amount of sensor activation in one min bins. The presence of circadian

rhythms was determined 25 days into the experiment for 10 consecutive days by F-periodogram analysis of activity based on the algorithm of Dörrscheidt and Beck (26).

Plasma corticosterone analysis

Three weeks after initiating the light intervention, blood samples were taken from mice on chow and high-fat diet via tail indentation at two different circadian times: one hour after the start of the subjective day (CT 1) and one hour before the start of the subjective night (CT 11), when corticosterone levels are at their lowest and highest, respectively (27). The activity (subjective night) period was determined for each mouse using passive infrared motion detection sensors (28). The samples were taken into capillaries, placed on ice and centrifuged at 4°C. Total plasma corticosterone concentrations were determined in an assay using a ^{125}I double-antibody kit (ICN Diagnostics). The high and low limits of detectability of the assay are 1,000 and 7.7 ng/ml, respectively.

Indirect calorimetry

To determine energy metabolism of mice in LD or LL, indirect calorimetry (Phenomaster, TSE Systems, Bad Homburg, Germany) was performed in individually housed animals on high-fat diet. Since exposure to constant light deteriorates circadian rhythm over time, these observations were obtained in two periods. The first period comprised the first 7 days of the continuous light, high-fat diet intervention, and the second period comprised 6 days of measurement, 4 weeks 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 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 (29;30), 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 on chow or high-fat diet was determined in a hyperinsulinemic-euglycemic clamp performed in the middle of the resting (ZT 6) and active phase of the LD mice (ZT 18). Since the LL regimen disrupts the circadian rhythm by elongating the circadian period, the exact circadian time at which a stable hyperinsulinemic-euglycemic infusion rate was established was subsequently calculated for each mouse in the LL group individually. Before the start of the experiment, mice fasted 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). Anaesthesia as well as body temperature was maintained throughout the procedure. At the end of the basal 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 µCi/min) intravenous (i.v.) infusion of 3-^{3}H-glucose (Amersham, Little Chalfont, U.K.) for 60 minutes. Subsequently, insulin (Actrapid, Novo Nordisk, Denmark) was administered i.v. by primed (4.1 mU), constant (6.8 mU/h) infusion, with continuation of infusion of 3-^{3}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-chek, Sensor Comfort; Roche Diagnostics, Mannheim, Germany). In the last 20 min, blood samples were taken with intervals of 10 min. At the day of the hyperinsulinemic-euglycemic clamp, body composition was determined by dual-energy X-ray absorptiometry (DEXA) using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland Strateq, Hampshire, U.K.). Subsequently, the mice were sacrificed.

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 standard according to the instructions of the manufacturer (Instruchemie, Delfzijl, The Netherlands) in 96-wells plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Plasma insulin levels were measured using a mouse-specific insulin ELISA kit (Crystal Chem Inc., Downers Grove, U.S.). Total plasma 3-^{3}H-glucose was determined in supernatant of 7.5 µl plasma, after protein precipitatation using 20% trichloroacetic acid and evaporation to eliminate tritiated water. Turnover rates of glucose (Rd, µmol/min/kg) were calculated in basal and hyperinsulinemic conditions as the rate of tracer infusion (dpm/min) divided by plasma specific activity of 3-^{3}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.

Statistical analysis

To assess the circadian variation of insulin sensitivity, clamp data from chow and high-fat fed mice was used for linear regression analysis. Data from mice in LD and LL was plotted against the calculated circadian time (CT; the time at which stable glucose infusion rates (GIR) were achieved) and linear regression analysis was performed with GIR as dependent and CT as independent variable using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla California U.S.). Slope deviation from 0 was assessed for the LD and LL group 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 on 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 one.

RESULTS

SCN recording and circadian rhythm analysis

The effect of constant light (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 seven mice, showing high activity during the day and low activity during the night. Upon 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 73 ± 5% on day 1, 60 ± 3% on day 2, reaching a steady amplitude of 46 ± 4% from day 3 onwards (Fig. 1C, $P < 0.01$) compared to day 0. F-periodogram analysis over 10 days in LL revealed that the rhythm strength of locomotor activity under LL was 46 ± 6% of the rhythm strength in the LD control mice (n=12 per group, $P < 0.001$).

Body weight gain

Body weights of LD and LL mice on 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, both on chow and high-fat diet. To determine the isolated contribution of constant light to weight development as well as to assess its possible interaction with high-fat diet, a mixed effect

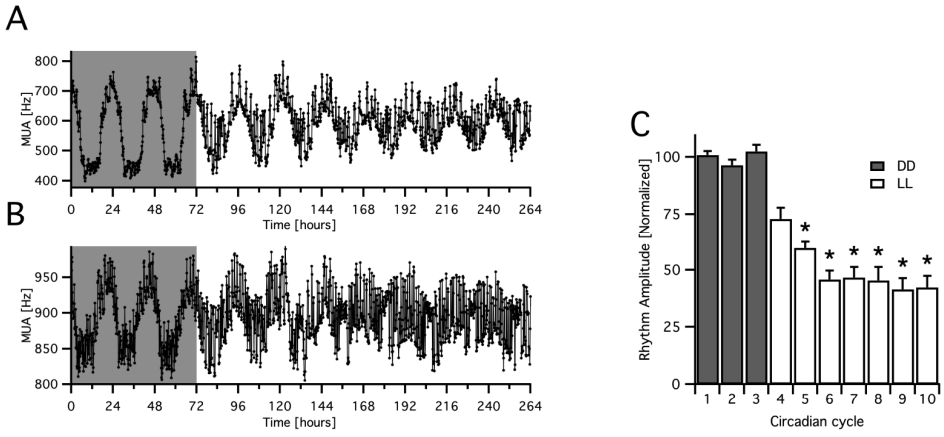

Fig. 1. Longterm *in vivo* multiunit activity recordings were performed in freely moving, awake mice (n=7) using implanted microelectrodes. Representative recordings under constant darkness (DD) followed by constant light (LL) (A, B). Action potential frequency (black trace) is indicated with a 10 min time resolution. Background represents light exposure, with grey representing lights off, and white representing lights on. After 3 cycles in DD, animals were exposed to LL. Analysis of the time course of the SCN rhythm deterioration and stabilization after 3 days of LL (C). Bars show the amplitude of the rhythm, normalized for each animal relative to the average amplitude during the last 3 cycles in DD (grey bars) and 7 cycles in LL (white bars). * $P < 0.01$.

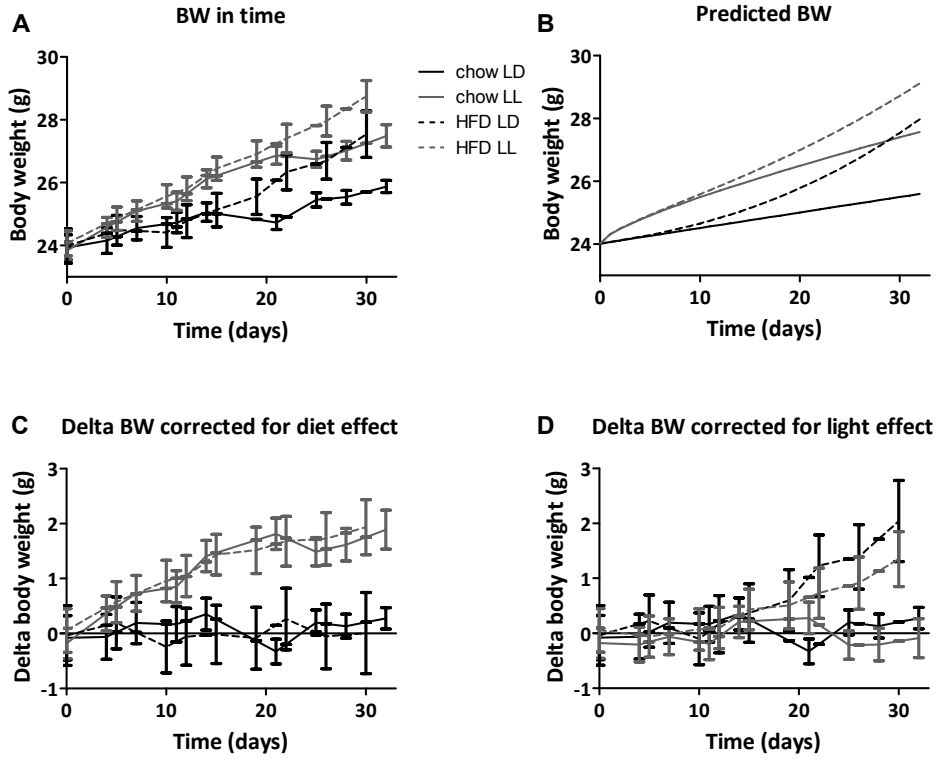

Fig. 2. A mixed effects model to investigate the effect of constant light (LL) and high-fat diet on body weight gain: average body weight (A), average body weight predicted by the mixed effect model (B), time dependent effect of the LL intervention on weight gain (C), the time dependent effect of the high-fat diet intervention on weight gain (D). Values represent means ± SEM for at least 7 mice per group.

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 light-dark regime. The interaction term 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 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 constant light intervention immediately affected weight gain (Fig. 2C) and stabilized later on 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 two different circadian times: one hour after the start of the subjective day (expected lowest corticosterone levels, CT 1) and one hour 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. Indeed, LD mice on chow as well as on high-fat diet had lowest corticosterone levels one hour after the start of the subjective day, which increased dramatically one hour before the start of the subjective night: from 12 ± 2 to 90 ± 32 ng/ml on chow ($P < 0.01$) and from 19 ± 5 to 82 ± 24 ng/ml on high-fat diet ($P < 0.01$) (Fig. 3). In LL mice on a chow diet, corticosterone levels were similar to LD mice one hour before the start of the subjective day but significantly lower one hour before the start of the subjective night (28 ± 16 to 42 ± 34 ng/ml, ns). In addition, corticosterone levels did not differ within the LL group when the two sampling times were compared. On the high-fat diet, the differences between the LL and the LD group were similar, as corticosterone levels were similar one hour before the start of the subjective day but significantly lower in the LL group one hour before the start of the subjective night (high-fat diet from 16 ± 3 to 43 ± 21 ng/ml, $P < 0.01$). Interestingly, in contrast to the chow diet, levels did differ significantly within the LL group when the two sampling times were compared. 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.

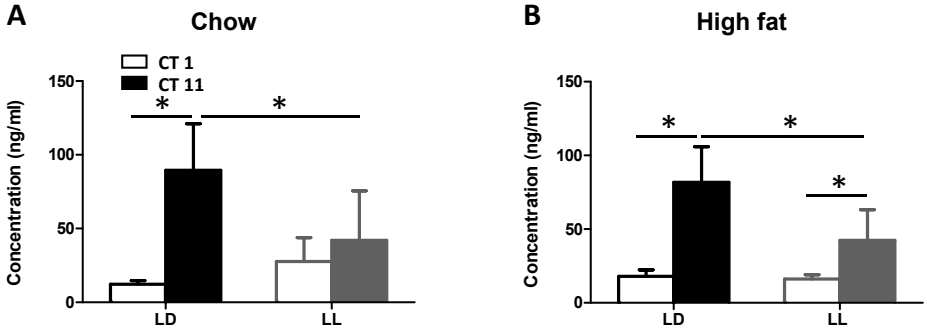

Fig. 3. Corticosterone plasma levels of chow (A) and high-fat (B) fed mice in LD or LL obtained at time of expected lowest (CT 1, open bars) and peak (CT 11, filled bars) corticosterone levels. Values represent means ± SD for 7-12 mice per group. * $P < 0.01$.

Indirect calorimetry

Short term

At the start of the light/high-fat intervention was started, body weight did not differ between the two groups of mice (LD, 25.5 ± 1.8 *vs*. LL, 25.6 ± 1.4 g, ns). LL mice had a higher energy intake 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%, $P = 0.06$, Fig. 4A). Respiratory

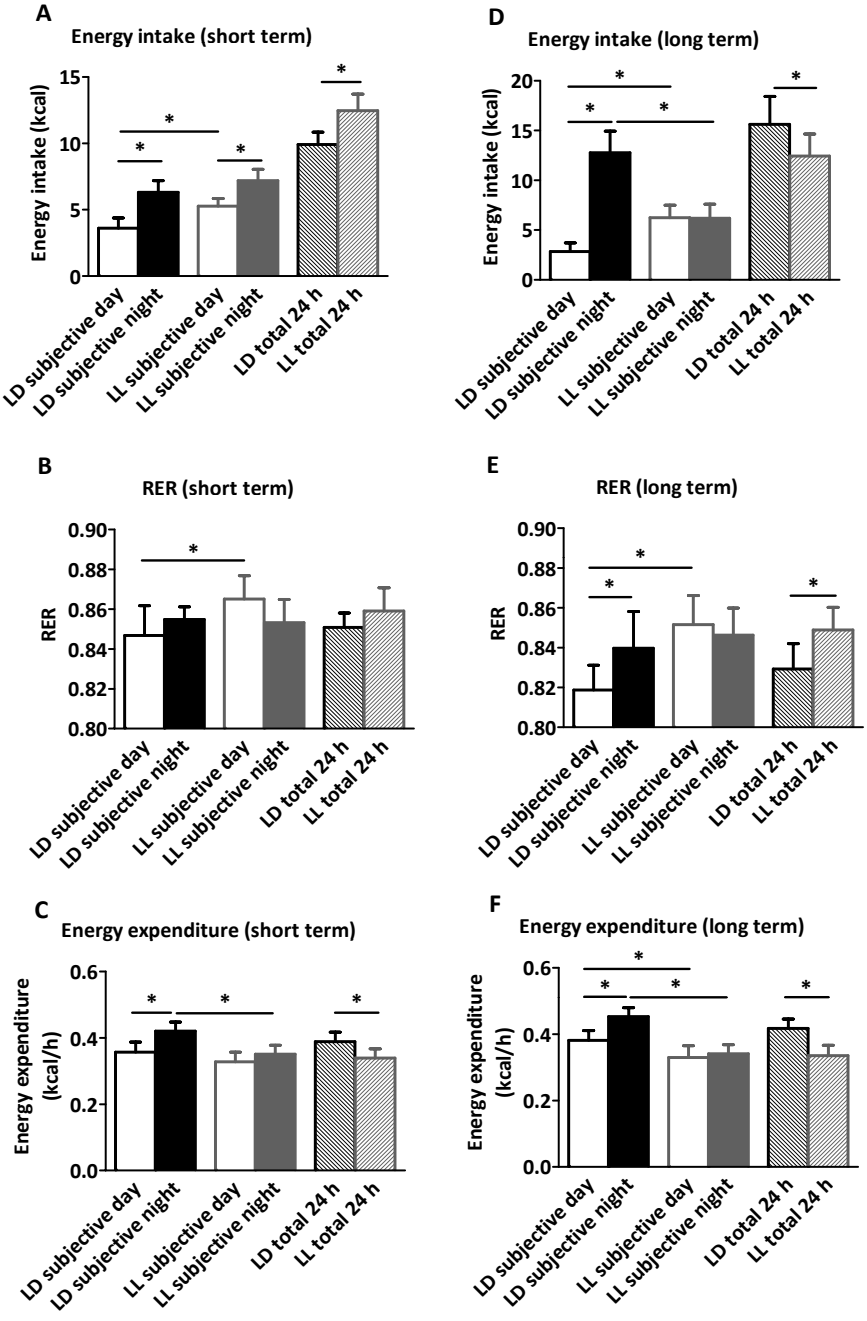

Fig. 4. Indirect calorimetric analysis of mice under light/dark (LD, n=8) and constant light (LL, n=8) conditions on high-fat diet analyzed for subjective day, subjective night and total 24 h levels. Energy intake (A), respiratory exchange rate (RER, B) and energy expenditure (C) during the first 6 days of the intervention. Panels D, E and F represent the same parameters measured in the same mice, for a period of 6 days, after 4 weeks of intervention. Values represent means ± SD. * $P < 0.05$.

exchange rate (RER) was significantly higher in the 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 energy expenditure over a period of a circadian period was significantly lower in the LL mice (Fig. 4C), 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 ($P = 0.06$).

Long term

As expected from the higher energy intake and lower energy expenditure rates measured during the short term period, body weights in the 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. Interestingly, at the long term, total energy intake measured over a period of a circadian period was significantly lower in the 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 lower energy intake during the day, was absent in LL mice. Reflecting the higher subjective day energy intake, RER was significantly higher in the 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 strongly correlated to energy intake during both the subjective day and night period in the LD mice ($R^2 = 0.70$ and 0.63, $P = 0.01$ and $P < 0.01$, respectively), but not in the 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 subjective night was strongly correlated to energy intake in the LD mice ($R^2 = 0.55$, $P < 0.05$). Interestingly, in the 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 in body-weight matched mice on chow and high-fat diet by hyperinsulinemic-euglycemic clamp analysis at two different circadian times: during the middle of the resting (ZT 6) and during the middle of the active phase (ZT 18) of the LD mice. Since the LL regimen disrupts the circadian rhythm by elongating the circadian period, the exact circadian time at which stable hyperinsulinemic-euglycemic infusion rate was achieved was subsequently determined for each individual mouse in LL.

Chow diet

Basal endogenous glucose production (EGP) did not differ at ZT 6 and ZT 18 between LD or LL mice (Table 1). In LD mice fed a chow diet, glucose infusion rates (GIR) depended on the clock times at which the clamp studies were performed. GIR was significantly higher at ZT 18 compared to ZT 6 (87.5 ± 9.2 *vs.* 51.6 ± 7.4 µmol/min/kg, $P < 0.01$, Fig. 5A). Furthermore, there was a strong correlation between GIR and circadian time (CT) 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 ZT 6 and ZT 18 (65.7 ± 20.8 *vs.* 75.2 ± 9.7 µmol/min/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 approximately 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 ZT 18 compared to ZT 6 (5.4 ± 8.8 *vs.* 20.5 ± 9.1 µmol/min/kg, respectively, $P < 0.05$, Fig. 5B). In addition, hyperinsulinemic-euglycemic glucose disposal rates (Rd) were higher at

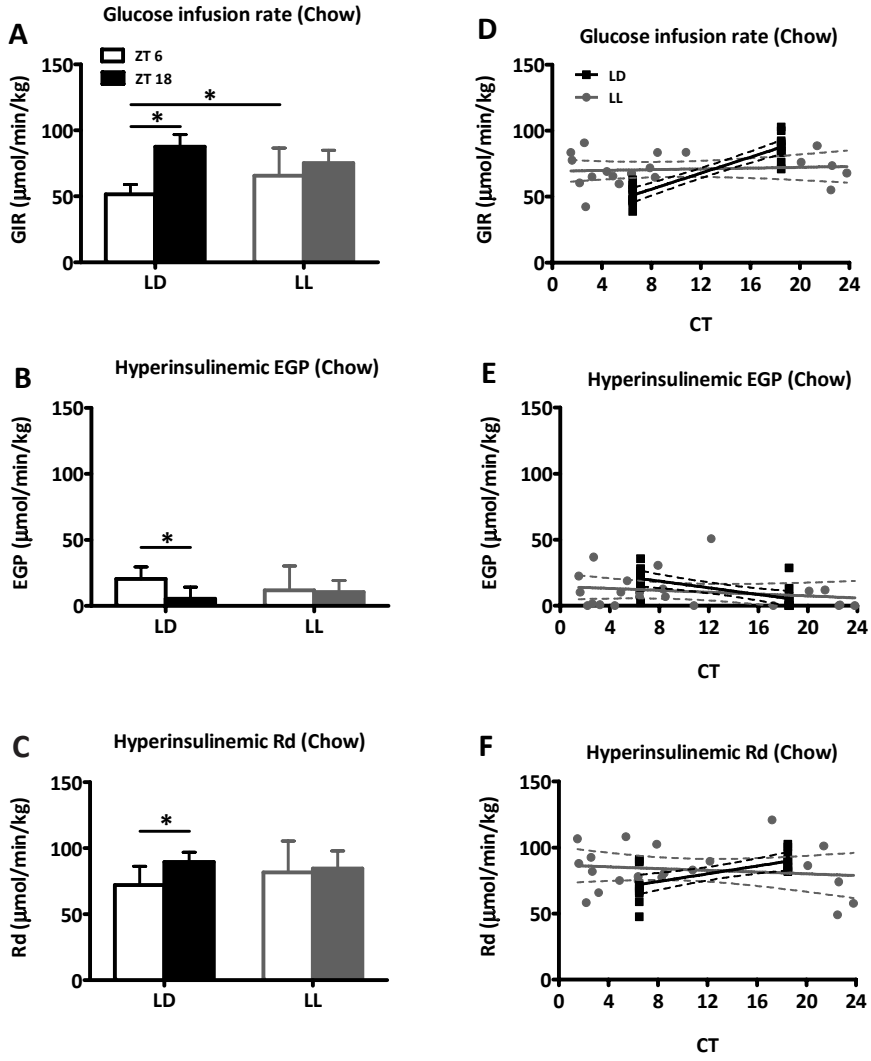

Fig. 5. Glucose infusion rate (GIR, A, D), endogenous glucose production (EGP, B, E) and glucose disposal (Rd, C, F) in chow fed mice under light/dark (LD, black) and constant light (LL, grey) conditions as measured in hyperinsulinemic-euglycemic clamp. GIR (A), EGP (B) en Rd (C) as measured at ZT 6 (open bars) or ZT 18 (filled bars) as determined for LD mice. Linear regression analysis of the GIR (D), EGP (E) and Rd (F) against the circadian time (CT) for LD mice (n=21) and LL mice (n=20). Values represent means ± SD for at least 7 mice per group. * $P < 0.05$ *vs.* control.

Table 1. Results obtained from the hyperinsulinemic-euglycemic clamp performed in LD and LL mice on chow diet. Data is represented as mean ± SD, * $P < 0.05$ LL *vs.* LD, $ $P < 0.05$ ZT 6 *vs.* ZT 18.

	LD		LL	
	ZT 6	ZT 18	ZT 6	ZT 18
Body weight (g)	25.4 ± 1.2	22.7 ± 0.9 $	25.0 ± 2.5	23.9 ± 1.3
Basal hematocrit (%)	39.8 ± 1.7	42.4 ± 1.2	39.8 ± 0.9	41.1 ± 1.9
Clamp hematocrit (%)	36.9 ± 1.1	38.8 ± 1.0	37.0 ± 1.9	38.7 ± 1.1
Basal insulin (ng/ml)	0.4 ± 0.1	0.4 ± 0.2	0.6 ± 0.3 *	0.4 ± 0.1
Clamp insulin (ng/ml)	7.6 ± 1.4	6.7 ± 0.9	7.0 ± 1.4	5.2 ± 1.6 *
Basal glucose (mmol/l)	4.0 ± 0.3	5.0 ± 0.5	4.6 ± 0.6	5.0 ± 0.9
Clamp glucose (mmol/l)	4.3 ± 0.5	4.6 ± 0.5	4.7 ± 0.7	5.0 ± 0.6
Basal EGP (µmol/min/kg)	47.3 ± 20.5	43.3 ± 5.6	56.5 ± 20.3	43.9 ± 7.6

ZT 18 compared to ZT 6 (89.7 ± 7.2 *vs.* 72.1 ± 14.2 µmol/min/kg, respectively, $P < 0.05$, Fig. 5C). Both EGP and Rd correlated with CT in the LD group ($R^2 = 0.44$ and 0.41, respectively, $P < 0.01$ for both parameters, Fig. 5E, F). In LL mice, hyperinsulinemic-euglycemic EGP and Rd rates did not differ significantly between ZT 6 and ZT 18 (EGP, 11.8 ± 18.4 *vs.* 10.4 ± 8.8 and Rd, 81.7 ± 23.8 *vs.* 84.7 ± 13.2 µmol/min/kg, respectively, ns, Fig. 5B, C). Furthermore, EGP and Rd did not correlate with CT in chow fed 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 constant light.

High-fat diet

LD mice fed a high-fat diet were more insulin resistant as GIR was lower at both time points compared to LD mice on chow diet (GIR -45% at ZT 6, $P < 0.01$ and -76% at ZT 18, $P < 0.01$). The lower insulin sensitivity was associated with diet-induced liver and peripheral insulin resistance, as hyperinsulinemic EGP was higher (EGP +48% at ZT 6, $P = 0.07$ and +117% at ZT18, $P = 0.09$) and hyperinsulinemic Rd was lower (Rd -7% at ZT 6, not significant and -35% at ZT 18, $P < 0.01$), when compared to chow fed LD mice. Basal EGP did not differ significantly at ZT 6 and ZT 18 between LD or LL mice (Table 2). GIR for LD mice on high-fat diet was significantly higher at ZT 18 compared to ZT 6 (49.7 ± 13.0 *vs.* 35.7 ± 7.4 µmol/min/kg, $P < 0.01$, Fig. 6A), but the magnitude of difference between ZT 6 and ZT 18 in LD mice on high-fat diet was less compared to LD mice on chow diet. Linear regression of GIR against CT 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 ZT 6 and ZT 18 (31.1 ± 13.1 *vs.* 39.1 ± 8.4 µmol/min/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 ZT 18 compared to ZT 6 (11.7 ± 6.5 *vs.* 30.4 ± 12.9 µmol/min/kg, $P < 0.05$, Fig. 6B). Hyperinsulinemic Rd rates were not different between ZT 6 and ZT 18 (67.4 ± 18.8 *vs.* 58.2 ± 11.9 µmol/min/kg, ns, Fig. 6C). Linear regression analysis confirmed these data, as EGP was correlated with CT ($R^2 = 0.50$, $P < 0.01$, Fig. 6E), but Rd was not ($R^2 = 0.09$, ns, Fig. 6F). In LL mice, there was a small difference in EGP during the clamp studies at ZT 6 and ZT 18 (24.6 ± 4.9 *vs.* 17.0 ± 7.2 µmol/min/kg, respectively,

$P < 0.05$, Fig. 6B). However, there was no relation between EGP and CT ($R^2 = 0.05$, ns, Fig. 6E). Rd rates did not differ at ZT 6 and ZT 18 for LL mice (54.3 ± 11.1 *vs.* 50.2 ± 12.7 µmol/min/kg, respectively, ns, Fig. 6C) and Rd did not correlate with CT ($R^2 = 0.00$, ns, Fig. 6F). These data show that, even though high-fat feeding results in an obesity/insulin resistance phenotype, insulin sensitivity was still under circadian control. Furthermore, even in in obese, insulin resistant mice, LL resulted in a further deterioration of the circadian rhythm of insulin resistance.

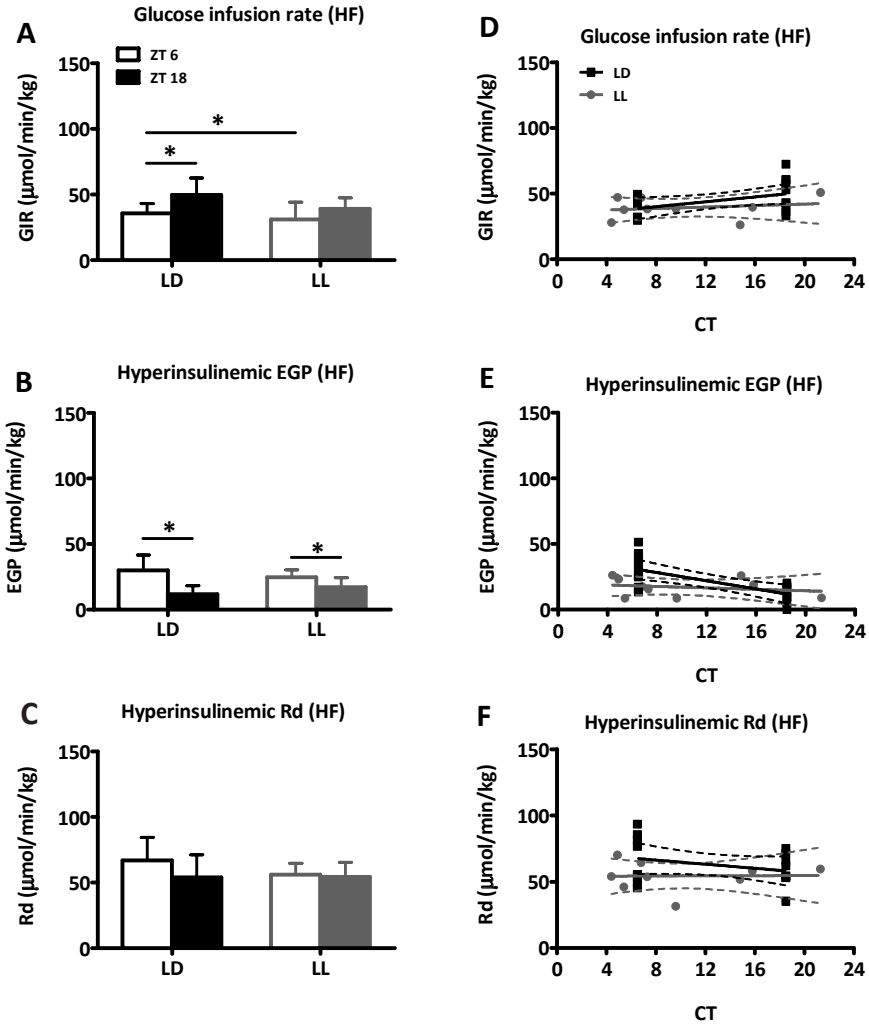

Fig. 6. Glucose infusion rate (GIR, A, D), endogenous glucose production (EGP, B, E) and glucose disposal (Rd, C, F) in high-fate fed mice under light/dark (LD, black) and constant light (LL, grey) conditions as measured in hyperinsulinemic-euglycemic clamp. GIR (A), EGP (B) en Rd (C) as measured at ZT 6 (open bars) or ZT 18 (filled bars) as determined for LD mice. Linear regression analysis of the GIR (D), EGP (E) and Rd (F) against the circadian time (CT) for LD mice (n=19) and LL mice (n=10). Values represent means ± SD for at least 7 mice per group. * $P < 0.05$ *vs.* control.

Table 2. Results obtained from the hyperinsulinemic-euglycemic clamp performed in LD and LL mice on high-fat diet. Data is represented as mean ± SD, * $P < 0.05$ LL *vs.* LD, $ $P < 0.05$ ZT 6 *vs.* ZT 18.

	LD		LL	
	ZT 6	**ZT 18**	**ZT 6**	**ZT 18**
Body weight (g)	32.4 ± 3.6	27.4 ± 2.5 $	32.5 ± 3.2	27.5 ± 1.8 $
Basal hematocrit (%)	n.a.	44.1 ± 1.8	n.a.	43.6 ± 1.1
Clamp hematocrit (%)	n.a.	40.6 ± 1.6	n.a.	40.5 ± 2.0
Basal insulin (ng/ml)	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.7	0.9 ± 0.2
Clamp insulin (ng/ml)	5.9 ± 0.8	6.7 ± 0.9	5.4 ± 1.0	7.2 ± 1.2
Basal glucose (mmol/l)	5.0 ± 0.7	5.7 ± 0.7 $	5.8 ± 1.0	5.0 ± 0.5 $*
Clamp glucose (mmol/l)	5.6 ± 0.7	5.7 ± 1.1	6.2 ± 1.1	5.5 ± 0.6
Basal EGP (µmol/min/kg)	38.1 ± 12.9	41.5 ± 8.7	32.6 ± 15.3	38.1 ± 11.8

DISCUSSION

This study addressed the effect of a decline in rhythm amplitude on energy metabolism and insulin sensitivity. C57Bl/6J mice were exposed to constant light (LL) or a light-dark cycle (LD) as control. *In vivo* recordings showed that constant light resulted in an immediate decrease in SCN amplitude, which stabilized within 3 days at a level of 46%. Furthermore, constant light stimulated weight gain, even before high-fat diet resulted in weight gain. Finally, constant light exposure resulted in a complete loss of circadian rhythm in energy metabolism and hepatic and peripheral insulin sensitivity. Collectively, these data indicate that constant light exposure strongly reduces the circadian function of the central clock, as observed in aging and neurodegenerative diseases, associated with 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 (31). Under regular 12 h/12 h light/dark 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 constant light causes a desynchronization among neurons of the SCN, which results at the tissue level in a dampened circadian rhythm (32;33). 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 (34). To quantify the amplitude decrease and time course of the rhythm deterioration of the SCN output rhythm 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;33), which did not allow for a quantitative estimation of the effect of constant light on the SCN rhythm *in vivo*. Our SCN recordings show that the desynchronizing effects of constant light occur

immediately, resulting in an amplitude reduction of 73% and 60% on days 1 and 2, and stabilizing at an amplitude of 54% of the original value from day 3 onwards. Importantly, 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 (32;33). 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.

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 line with previous studies showing that constant light exposure 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 twice a week. Disturbing circadian rhythm by constant light exposure resulted in higher body weights compared to mice on a normal light regime, independent of the diet. A mixed effects model was developed to determine the isolated contributions of constant light exposure and high-fat diet to weight gain as well as to assess the possible interaction between constant light exposure and high-fat feeding. Constant light exposure immediately affected weight gain, which stabilized later on, whereas the high-fat diet effect on body weight gain became manifest at a later stage. Our data are in line with 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 two weeks. 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 constant light exposure to weight gain in chow and high-fat fed mice and shows that the weight gain as a result of constant light exposure is evident before high-fat diet affects body weight gain. This immediate effect of constant light exposure on body weight coincides with the direct reduction in SCN output, suggesting that reduced SCN rhythmicity instantaneously affects energy homeostasis.

Disturbing circadian rhythm by constant light exposure aggravated diet-induced obesity and resulted in a shift of energy intake towards the period when energy intake is normally low. Furthermore, we show that constant light 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 constant light 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 constant light over time. Furthermore, LL mice showed an impaired oxidative response towards food intake, as was indicated by the absence of correlation between the respiratory exchange rate and energy intake. A blunted response of metabolism towards food intake, also known 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 on chow as well as on high-fat diet. We determined insulin sensitivity by hyperinsulinemic-euglycemic clamp analysis at two 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 the LL mice, the individual CT at the time of the clamp analysis was obtained afterwards on the basis of the activity period of each individual mouse. In line with studies form La Fleur *et al.* (41), LD mice on chow show a circadian variation in insulin sensitivity, with higher hepatic insulin sensitivity and higher insulin-stimulated glucose uptake by peripheral organs in the subjective night. Remarkably, the circadian variation of insulin sensitivity was lost in constant light. Moreover, the insulin sensitivity of LL mice was approximately 50% of the variation in insulin sensitivity of LD mice, which is in agreement with the ~50% reduction in SCN output.

In a previous study it was shown that disturbing circadian rhythm by light intervention results in deregultation of glucoregulatory genes in liver, such as phosphoenolpyruvate carboxykinase (PEPCK), glucose transporter 2 (GLUT2) and glucose-6-phosphatase (G6PC) (42). In muscle, glucoregulatory gene expression is dependent on circadian time (43) and glucose uptake in isolated muscle from rats show a circadian pattern (44). Deregulation in glucoregulatory genes in liver and muscle may underlie the disturbed insulin sensitivity we found in constant light exposed mice. Furthermore, disturbing circadian rhythm using constant light also accelerates loss of beta-cell mass and function (45). Together with the results obtained in our study, more insight is gained in how disturbances in circadian rhythm, for instance due to shift work, may lead to the development of obesity and T2DM.

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 about 50% 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 constant light coincides with an instantaneous increase in body weight. These findings indicate that a relatively mild decrease in rhythm amplitude, which is observed in sleeping disorders, degenerative diseases and aging, are a serious concern for health (46), as they may lead to secondary metabolic pathophysiology. SCN recordings in old and middle aged mice have in fact shown a reduction in SCN rhythm amplitude of about 50% (22). The data indicate that new avenues for prevention and treatment programs for patients with mild rhythm disturbances should include life style or light treatment programs to improve rhythm amplitude.

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