Greased lighting : implications of circadian lipid metabolism for cardiometabolic health

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Chapter 4

Glucocorticoid rhythm regulates the diurnal activity of brown adipose tissue

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ABSTRACT

The biological clock regulates brown adipose tissue (BAT) activity. However, how the timing signal is conferred is poorly understood. We hypothesize that the diurnal rhythm in glucocorticoid levels (corticosterone in rodents) plays a crucial role. To address this hypothesis mice received subcutaneous pellets containing a low-dose of corticosterone, which markedly dampened the rhythm of endogenous circulating corticosterone concentrations. After one week the ability of BAT to take up triglyceride-derived fatty acids from the circulation was determined at AM and PM. In control mice fatty acid uptake by BAT displayed a large AM-PM fluctuation. In mice with dampened corticosterone rhythm this AM-PM fluctuation was abolished due to lowered PM uptake of fatty acids by BAT. This was accompanied by dampened AM-PM differences in the expression of *Lpl* and the phosphorylation of CREB, a downstream β3-receptor signaling target. Unexpectedly, known glucocorticoid-responsive element-controlled genes retained a rhythmic expression, suggesting that glucocorticoids did not directly act on the brown adipocyte. We conclude that short-term dampening of corticosterone rhythm in mice dampens the rhythmicity of BAT activity with respect to the uptake of triglyceride-derived fatty acids. Our data suggest that glucocorticoid rhythms may act as a signal from the biological clock to regulate diurnal rhythm of BAT activity via a yet to be identified mechanism.

INTRODUCTION

Brown adipose tissue (BAT) is currently viewed as a promising therapeutic target to combat metabolic disease due to its ability to dissipate energy into heat [1]. Brown adipocytes contain large amounts of mitochondria that uniquely contain uncoupling protein 1 (UCP-1). UCP-1 uncouples electron transport from ATP synthesis, resulting in heat production [2]. To fuel thermogenesis, intracellular triglyceride (TG) stores are used, which are replenished by LPL-mediated uptake of TG-derived fatty acids (FA) from the circulation [3]. Activation of BAT increases energy expenditure and reduces plasma TG levels; in mice this prevents obesity and reduces dyslipidemia [4]. Low temperature is the physiological stimulus for BAT activity. Cold is perceived by skin; the signal is transmitted to the hypothalamus, which increases sympathetic nervous system output and increases peripheral release of noradrenaline that activates the β3-adrenergic receptor on the brown adipocyte [5]. In addition to adrenergic input, BAT activity is controlled by the endocrine system [6]. It is becoming increasingly clear that the biological clock regulates BAT function.

The mammalian biological clock system generates circadian (i.e. 24 h) rhythms in physiological and behavioral functions. It consists of a central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral clocks within organs, which regulate rhythmicity within the different tissues. Light-dark information is processed by the SCN, which synchronizes peripheral clocks via sympathetic and endocrine output [7]. Disturbances of day-night rhythms in humans, *e.g.* through shift work and artificial light exposure, are associated with an increased risk for obesity, type 2 diabetes and dyslipidemia [8-10]. Studies in rodents demonstrated a causal relationship between a disturbed biological clock and the development of metabolic disease [11-13]. We recently showed that BAT may be the missing link in this causal relationship. Prolonged light exposure duration specifically reduced the uptake of TG-derived FA by BAT resulting in increased adiposity in mice, without affecting food intake [14]. Furthermore, we demonstrated that BAT activity is not only regulated by light exposure duration but also displays a strong diurnal rhythm (Chapter 3). Although we showed that fatty acid uptake by BAT is highly rhythmic, the exact mechanism by which the SCN transfers diurnal information to BAT is not fully understood.

Besides noradrenalin, glucocorticoids are likely candidates for transferring the circadian time-keeping signal to BAT. The release of glucocorticoids, cortisol in humans and corticosterone in rodents, shows a pronounced diurnal rhythm. Glucocorticoids peak before wakening and show a trough before sleeping [15]. The diurnal profile of plasma corticosterone concentrations and FA uptake by BAT are strikingly similar, with a peak just before wakening and a through at the end of the waking period. Moreover, prolonged light exposure duration, which directly affects SCN output [16], shifts the peak time and decreases the amplitude in the diurnal rhythm of both plasma corticosterone concentrations and FA uptake by BAT, as shown by others [17] and us (Chapter 3). Therefore, we hypothesized that the diurnal rhythm of plasma corticosterone confers the diurnal timing signal of the SCN towards BAT. To investigate this hypothesis, we dampened the endogenous diurnal rhythm of corticosterone by implantation of a low dose corticosterone-containing pellet in

mice and evaluated the effects on rhythmicity of whole-body metabolism and BAT activity. The subcutaneously implanted pellets release a continuous dose of corticosterone, which inhibits production of CRH and ACTH through negative feedback on the hypothalamus and the pituitary [18]. In this study, we demonstrate that dampening of the corticosterone rhythm did not flatten whole-body energy metabolism rhythms, but did reduce the evening peak in FA uptake by BAT.

METHODS

Animal husbandry

All animal experiments were performed in accordance with the local ethics committee. Male 8-week-old C57Bl/6J mice (Charles River) were single-housed in clear plastic cages within light-tight cabinets on a 12h:12h light:dark cycle (lights on 9.00h) at constant ambient temperature of 22°C with *ad libitum* access to food (standard chow, Special Diet Services) and water.

Experimental design

Mice (n=32) were housed in metabolic cages (Phenomaster, TSE systems) one day prior to the experiment. Mice (n=16/group) were implanted with a subcutaneous pellet containing 2.5% w/w corticosterone (2.5 mg corticosterone, 97.5 mg cholesterol) or 100 mg cholesterol as placebo. After 6 days, stress-free AM and PM blood samples were drawn at Zeitgeber Time (ZT) 1 and ZT11. After 7 days, a TG clearance experiment was performed at either AM (ZT0) or at PM (ZT12). An additional group of 10 animals were also implanted with a corticosterone-containing or placebo pellet (n=5/group) as well as a telemetric system to continuously measure body temperature and activity rhythms.

Surgical procedures

Implantation of the corticosterone pellets was performed under 2.5% isoflurane anesthesia. A small incision was made between the shoulder blades and a subcutaneous pocket was created by blunt end of a scissor. The pellet was inserted subcutaneously in the flank. Mice which also received a telemetric system were implanted with a TA-F10 miniature transmitter (Data Sciences International, MN, USA, kindly provided by Linda van Kerkhof, RIVM) through the same incision in the contralateral flank.

Indirect calorimetry and body composition

Mice were housed in automated metabolic cages to measure oxygen consumption (volume O_2; mL/h), carbon dioxide production (volume CO_2; mL/h), respiratory exchange ratio (RER; VCO_2/VO_2), physical activity via infra-red beam breaks and food intake during 7 days. Means were calculated over 7 days. Mice were weighed and body composition was determined by echoNMR (EchoMRI-100, Houston, TX, USA) the day before acclimatization to metabolic cages and at day 6.

Blood biochemistry

To determine the effects of a corticosterone pellet on circulating corticosterone levels, stress-free (i.e. within 2 minutes of handling the cage) blood samples were taken after the onset of light (AM; ZT 1) and before the onset of dark (PM; ZT11) via tail vein bleeding. To evaluate possible effects of acute stress of the final experiment, blood samples were taken right before the start of TG-clearance experiment from the tail vein into EDTA-coated Eppendorf tubes. Total plasma corticosterone concentrations were determined using ^{125}I-RIA ELISA kit (MP Biomedicals, Orangeburg, NY, USA).

TG clearance experiment

After 7 days of corticosterone or placebo treatment, the kinetics of triglyceride (TG) and cholesterol clearance from plasma and uptake by organs were assessed at AM (ZT0) and PM (ZT12). TG-rich lipoprotein (TRL)-like emulsion particles (80 nm), radiolabeled with glycerol tri[^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO), were prepared as previously described [19]. Mice were fasted for 4h and received an intravenous injection of particles (1 mg TG in total volume of 200 µl) via the tail vein. After 15 minutes, mice were euthanized by cervical dislocation and perfused with ice-cold PBS for 5 min. Organs were harvested, weighed, and ^{3}H and ^{14}C radioactivity was determined by liquid scintillation.

Gene expression analysis

Interscapular brown adipose tissue (iBAT) was snap frozen and stored at -80°C. Total RNA was isolated using TriPure (Roche) and cDNA was reverse-transcribed starting with 1 µg of RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time PCR was performed in triplicate using SYBR-Green (Bio-Rad) on a CFX96 PCR machine (Bio-Rad). Expression levels were normalized to *36b4* housekeeping gene expression.

Protein isolation and Western blot

The iBAT samples stored at -80°C were homogenized and diluted in lysis buffer. The homogenates were centrifuged at 6.5 m/sec for 20 sec (FastPrep-24™ 5G, MP Biomedicals, California, USA) and the protein content was determined using a BCA protein assay kit (Thermo Scientific). Subsequently, after adding Laemmli Sample buffer (1:1 vol/vol; Serva, Heidelberg, Germany), the samples were denatured for 5 min at 95°C. Proteins within homogenates (20 µg) were separated on a 10% SDS-page gel and subsequently transferred onto nitrocellulose blotting membranes. The blotting membranes were blocked with 5% milk powder and incubated overnight at 4°C with the primary antibody rabbit pCREB (Cell Signalling; 1:1000) or mouse β-actin (Sigma; 1:1000). Subsequently, the membranes were incubated for 1 hour with secondary antibodies (anti-rabbit or anti-mouse IgG HRP conjugate; 1:5000; Promega, Madison, WI, USA), after which the protein bands were visualized with SuperSignal Western blot Enhancer (Thermo Scientific, Rockford, IL, USA). The Blots were analyzed with ChemiDoc Touch Imaging System (Bio-Rad) and normalized to β-actin expression levels.

Histology

To assess lipid fraction within BAT the lipid-containing area was determined on Mayer's hematoxylin and eosin (HE) stained formalin-fixed paraffin-embedded iBAT tissue sections (5 µm), using standard protocols. The lipid areas were quantified (as percentage of total area) using ImageJ.

Statistical analysis

Data are presented as means ± SEM. Differences between two groups were determined by T-tests and between more than two groups with a one-way ANOVA. To test for differences between AM-PM groups, post hoc Sidaks for multiple comparisons was used. For repeated measures, repeated measure ANOVA was used. Associations of variables with time were assessed by linear regression analysis. Graphpad Prism v6.0 was used for all analyses. P values < 0.05 were considered statistically significant.

RESULTS

Subcutaneous corticosterone-containing pellets dampen corticosterone rhythm

Mice were implanted with subcutaneous pellets containing vehicle or 2.5% corticosterone, aimed to flatten the endogenous diurnal corticosterone rhythm. To confirm this, we determined plasma corticosterone levels at AM (ZT1) and PM (ZT11). Mice implanted with placebo pellets showed a 29-fold difference between AM and PM plasma corticosterone levels (4.5 ± 1.2 *vs.* 129.9 ± 9.1 ng/ml, P < 0.0001) (Fig. 1a). In contrast, mice implanted with corticosterone pellets displayed similar plasma corticosterone levels at AM and PM (39.3 ± 3.3 *vs.* 46.9 ± 3.0 ng/ml, P = 0.408) (Fig. 1a). Moreover, corticosterone levels did not reach the physiological trough and peak values at either AM or PM. To further explore whether mice had been exposed to supraphysiological levels of corticosterone during the treatment, the thymus and adrenals were weighted after 7 days of implantation. The thymus and adrenals are sensitive to chronic hypercorticosteronemia and decrease in weight due to apoptosis of thymocytes [20] and lack of trophic effect of ACTH on adrenals. Thymus weight decreased by 34% in the corticosterone-treated group compared to the placebo group (43 ± 2 *vs.* 28 ± 2 mg, P < 0.001) (Fig. 1b). Adrenal weight also decreased by 20% in the corticosterone treated mice compared to placebo (1.4 ± 0.1 *vs.* 1.8 ± 0. 1 mg, P = 0.038) (Fig. 1c). These data indicate that corticosterone pellets may have raised the total corticosterone exposure in treated mice. Therefore, we conclude that corticosterone pellets dampened the diurnal rhythm of endogenous corticosterone treatment, with a possible elevation of total 24h corticosterone exposure.

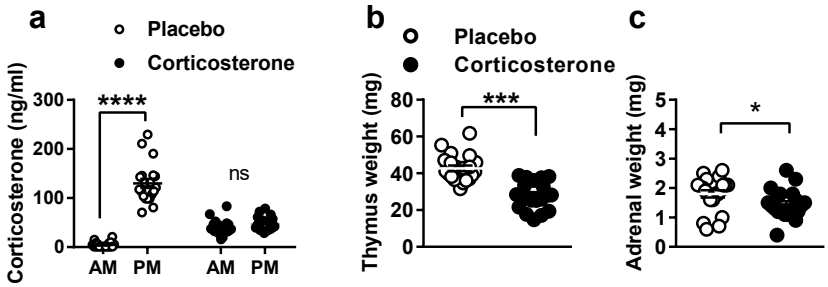


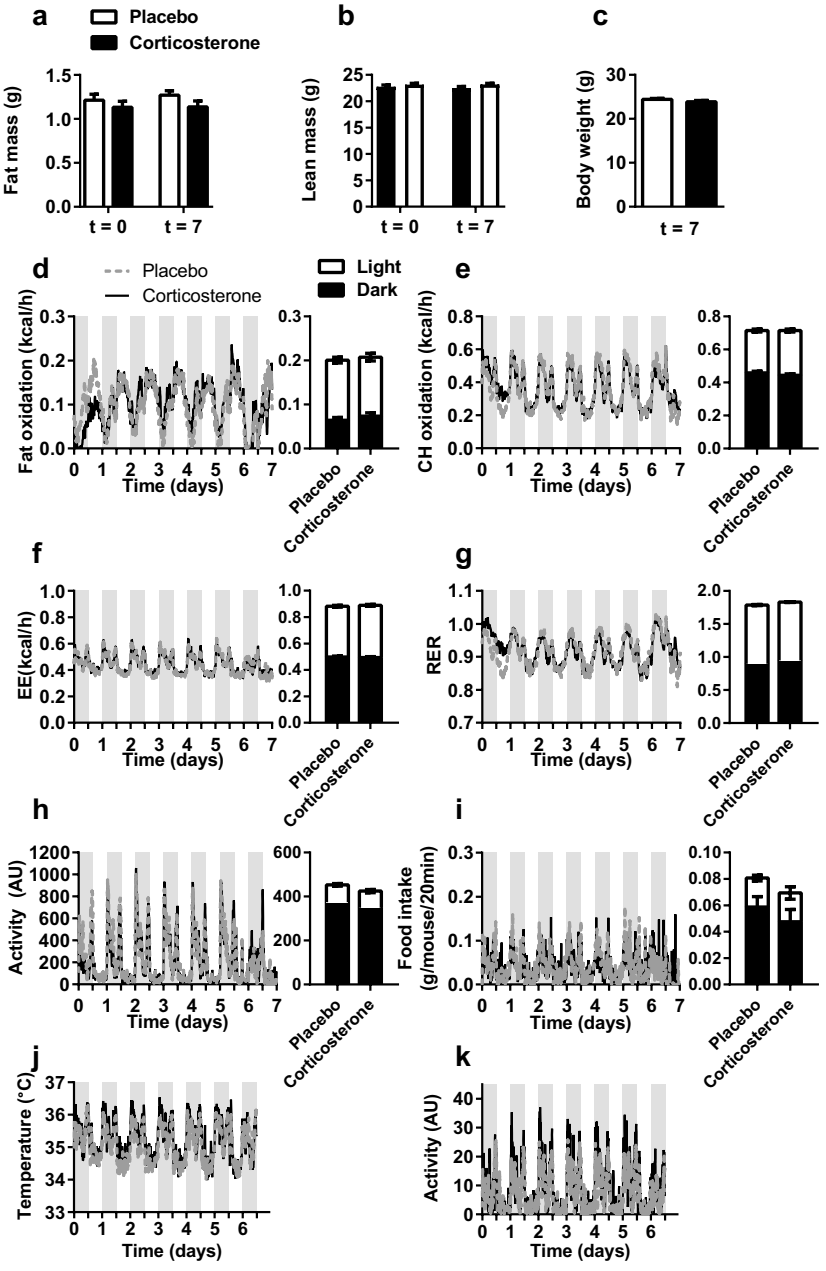
Figure 1. Implantation of corticosterone pellet flattens endogenous glucocorticoid rhythm. *C57BL6/J mice were implanted with placebo pellets (n=16) or low-dose corticosterone pellets (n=16). After 6 days, corticosterone plasma levels were determined at AM (ZT1) and PM (ZT11) (a). After 7 days, mice were sacrificed at AM (ZT0) or PM (ZT12) and thymus (b) and an adrenal (left-sided) (c) were weighed. Data are presented as individual values and the mean ± SEM. *P < 0.05, ***P < 0.001, ****P < 0.0001 (repeated measure ANOVA (a); Student's T-test (b-c)).*

Dampened rhythm of corticosterone levels does not affect body composition and diurnal energy expenditure

To investigate the effects of a dampened corticosterone rhythm on energy metabolism, we measured body composition before and after 7 days of treatment, and performed indirect calorimetric analysis. Corticosterone treatment did not affect fat mass (corticosterone vs placebo; 1.1 ± 0.1 *vs*. 1.3 ± 0.1 g, P = 0.122) (Fig. 2a), lean mass (22.5 ± 0.2 *vs*. 23.2 ± 0.3 g, P = 0.083) (Fig. 2b), or total body weight (23.8 ± 0.3 *vs*. 24.5 ± 0.2 g, P = 0.135) (Fig. 2c). Also, flattening of the rhythm in corticosterone concentrations did not change the diurnal pattern of fat oxidation, carbohydrate (CH) oxidation, energy expenditure (EE), respiratory exchange ratio (RER), physical activity or food intake obtained over a period of 7 days (Fig. 2d-i). Average values were different between light and dark period, but unaffected by a dampened corticosterone rhythm (Fig. 2d-i). In an additional group of mice equipped with telemetric systems, 24h body temperature (Fig. 2j) and activity (Fig. 2k) were simultaneously recorded. Overall, the diurnal rhythm of metabolic parameters as well as body temperature rhythms were similar between the placebo and corticosterone-treated mice.

Dampened rhythm of corticosterone levels flattens the rhythm of TG-derived FA-uptake specifically by BAT

To investigate the effect of a dampened diurnal corticosterone rhythm on BAT activity, we assessed the ability of BAT to take up TG-derived FA from plasma at two time points. To this end, mice were injected with double-labeled TRL-mimicking particles either at AM (ZT0) or PM (ZT12). The AM and PM uptake of [^{3}H]oleate by interscapular BAT (iBAT) and subscapular BAT (sBAT) was higher than the uptake by subcutaneous white adipose tissue (sWAT), liver, gonadal WAT (gWAT), heart, spleen and muscle, indicating that iBAT and sBAT are highly metabolically active compared to other metabolic organs. The PM uptake of [^{3}H]oleate by


a
Placebo
Corticosterone
Fat mass (g)
t = 0
t = 7
b
Lean mass (g)
t = 0
t = 7
c
Body weight (g)
t = 7
d
Placebo
Corticosterone
Light
Dark
Fat oxidation (kcal/h)
Time (days)
Placebo
Corticosterone
e
CH oxidation (kcal/h)
Time (days)
Placebo
Corticosterone
f
EE(kcal/h)
Time (days)
Placebo
Corticosterone
g
RER
Time (days)
Placebo
Corticosterone
h
Activity (AU)
Time (days)
Placebo
Corticosterone
i
Food intake (g/mouse/20min)
Time (days)
Placebo
Corticosterone
j
Temperature (°C)
Time (days)
k
Activity (AU)
Time (days)

iBAT, sBAT and perivascular adipose tissue (pVAT) was higher compared to AM (iBAT: 35.2 ± 6.4 *vs.* 20.8 ± 2.6 % dose/g, P = 0.029; sBAT: 41.7 ± 6.4 *vs.* 21.0 ± 2.7 % dose/g, P = 0.004; pVAT: 24.1 ± 3.0 *vs.* 14.8 ± 1.8 % dose/g, P = 0.023) (Fig. 3a). Remarkably, corticosterone treatment abolished this difference between AM and PM in iBAT (22.0 ± 2.9 *vs*. 22.8 ± 2.0 % dose/g, P = 0.985), sBAT (23.9 ± 2.7 *vs*. 24.5 ± 3.4 % dose/g, P = 0.993) and pVAT (17.5 ± 2.1 *vs*. 18.1 ± 2.3 % dose/g, P = 0.982). Since the AM uptake decreased and the PM uptake remained the same, the average total uptake of [^{3}H]oleate by brown adipocyte depots was decreased by corticosterone treatment. In placebo treated mice, the liver showed the highest uptake of [^{14}C]CO, reflecting the clearance of cholesterol-rich remnant particles, when compared to all other organs (Fig. 3b). Remnant uptake by the liver was significantly higher PM versus AM in placebo treated mice (61 ± 3 *vs.* 46 ± 2 % dose/g, P=0.002), while in corticosterone treated mice, the PM and AM uptake of remnants was equal (58 ± 3 *vs.* 57 ± 3 % dose/g, P=0.958). In conclusion, corticosterone pellet implantation dampened the diurnal rhythm in TRL clearance with respect to TG-derived FA uptake by BAT depots, as well as the rhythm in remnant uptake by the liver.

Dampened rhythm of corticosterone levels flattens the rhythm in clock gene expression in BAT

Corticosterone-containing pellets dampened both the rhythm in endogenous corticosterone levels and the rhythm in BAT activity with respect to TG-derived FA uptake. We aimed to further investigate the underlying mechanism by which corticosterone levels influence FA uptake by BAT. Corticosterone can act through binding of the mineralocorticoid receptor (MR) as well as the glucocorticoid receptor (GR). To investigate whether corticosterone directly acts on brown adipocytes, we determined the gene expression in iBAT of the known MR and GR responsive genes *Fkbp5* (Fig. 4a), *Gilz* (Fig. 4b) and *Hsd11b1* (Fig. 4c). Corticosterone pellets did not influence the AM-PM difference in expression of these responsive genes, showing that the corticosterone-containing pellets do not universally flatten GR and MR dependent gene expression within the brown adipocytes.

We previously demonstrated that diurnal FA-uptake by BAT is accompanied by diurnal clock gene expression (Chapter 3). We hypothesize that corticosterone may act on the cell autonomous clock to regulate diurnal FA-uptake. We studied genes in both the forward and negative feedback loop of the clock machinery. The expression levels of the genes in the positive loop, namely *Clock* (Fig. 4d) and *Bmal1* (Fig. 4e) showed no rhythmicity in

Figure 2. (left page) Dampened rhythm of corticosterone levels does not affect body composition and diurnal energy metabolism pattern. *C57BL6/J mice were implanted with placebo pellets (n=16) or low-dose corticosterone pellets (n=16). Fat mass (a) and lean mass (b) were determined by EchoMRI at baseline and after 7 days and total body mass was determined after 7 days (c). Indirect calorimetry was used to determine fat oxidation (d), carbohydrate oxidation (e), energy expenditure (EE) (f), respiratory exchange ratio (RER) (g), physical activity (h) and food intake (i). Bars show mean value over 7 days. Body temperatures (j) and activity (k) measured every 10 minutes from subcutaneously implanted thermometers in placebo (n=5) and corticosterone pellet treated (n=5) mice. Grey dotted line represents placebo, black line represents corticosterone. Data are presented as spline curve (d-l) or means ± SEM (a-c).*

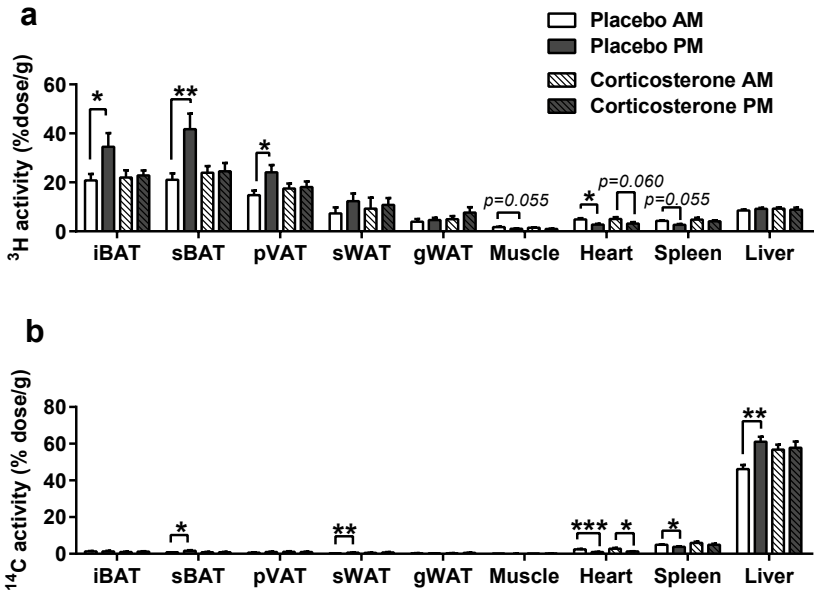
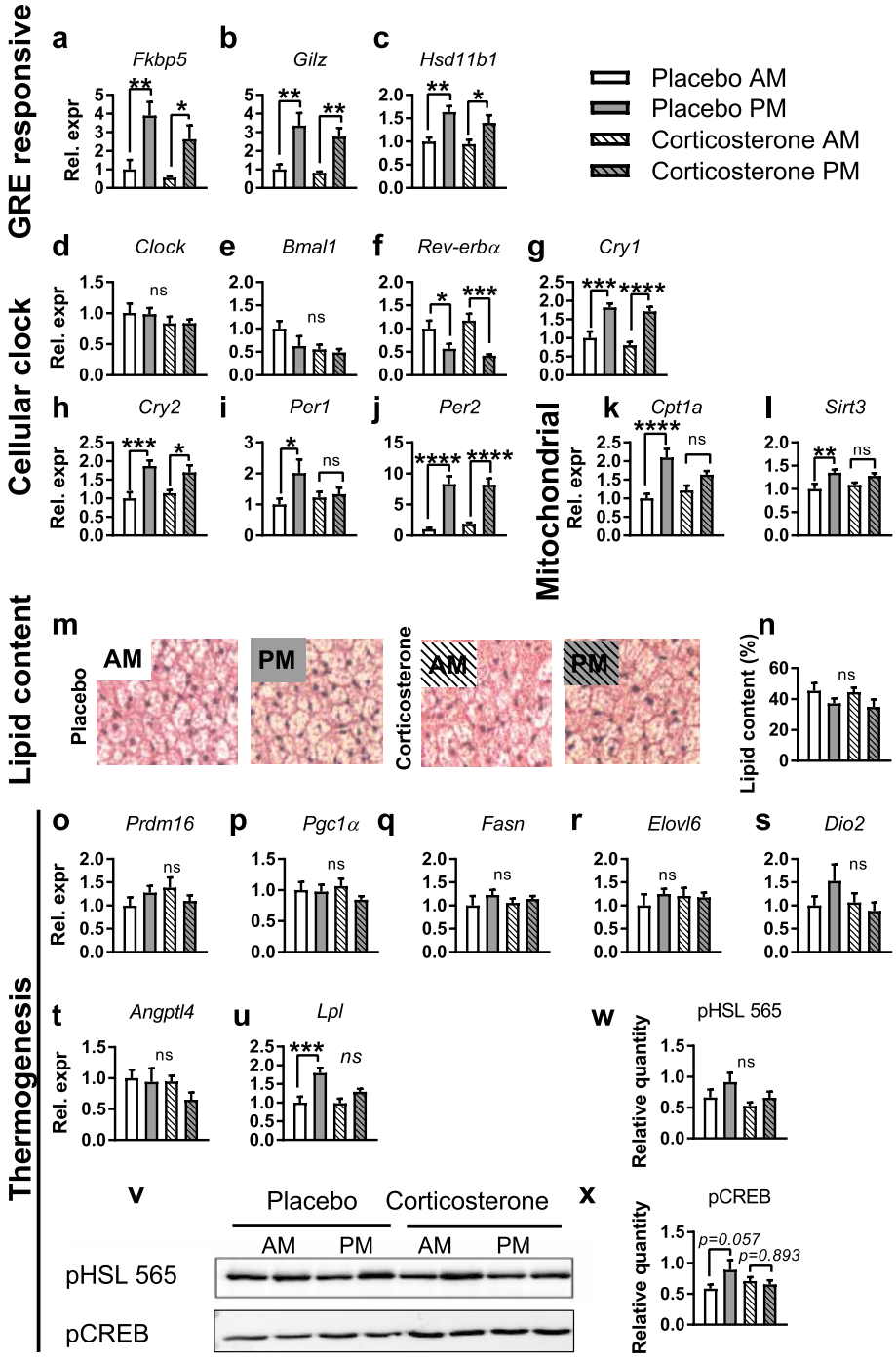


Figure 3. Dampened rhythm of corticosterone levels dampens rhythmicity of TG-derived FA-uptake specifically by BAT. *C57BL6/J mice were implanted with placebo pellets (n=16) or low-dose corticosterone pellets (n=16) and the VLDL-TG uptake was assessed after 7 days by injection of glycerol tri[^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO)-labeled emulsion particles. Uptake of [^{3}H]TO-derived (a) and [^{14}C]CO-derived activity (b) by the various organs was determined. Open bars = placebo, hatched bars = corticosterone; grey bars = PM, white bars = AM. Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001*

Figure 4. (right page) Dampened rhythm of corticosterone levels dampens clock gene expression rhythmicity within BAT C57BL6/J mice were implanted with placebo pellets (n=16) or low-dose corticosterone pellets (n=16). *After 7 days, mice were sacrificed at AM (ZT0) or PM (ZT12) and interscapular BAT was isolated. Gene expression was determined by qPCR (a-l, o-u). Histological sections were stained with HE were analyzed for lipid content (m, n). Protein expression was determined by Western blot and quantified (normalized to actin levels, not shown) (v, w, x). Open bars = placebo, hatched bars = corticosterone, grey bars = PM, white bars = AM. Data are presented as means ± SEM. Relative expression was calculated normalized to AM-placebo values. Ns= not significant (P>0.05). *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA, post hoc Sidaks multiple comparisons AM vs. PM).*


GRE responsive
Cellular clock
Mitochondrial
Lipid content
Thermogenesis
Placebo AM
Placebo PM
Corticosterone AM
Corticosterone PM
Rel. expr
Fkbp5
Gilz
Hsd11b1
Clock
Bmal1
Rev-erbα
Cry1
Cry2
Per1
Per2
Cpt1a
Sirt3
AM
PM
Placebo
Corticosterone
Lipid content (%)
Prdm16
Pgc1α
Fasn
Elovl6
Dio2
Angptl4
Lpl
pHSL 565
Relative quantity
Placebo
Corticosterone
pCREB
p=0.057
p=0.893
ns

both the placebo and corticosterone treated groups. *Rev-erbα* displayed a significant AM-PM difference with highest expression at AM (Fig. 4f) in the placebo group ($P<0.05$) and after corticosterone intervention ($P < 0.001$). For the other clock genes, gene expression was highest at PM. The preservation of AM-PM difference in the corticosterone treated groups, was also observed for gene expression of *Per2* (Fig. 4j), *Cry1* (Fig. 4g), *Cry2* (Fig. 4h). Interestingly, the AM-PM difference in gene expression of *Per1* (Fig. 4i) was blunted by corticosterone treatment. Therefore, we conclude that flattening of the plasma corticosterone rhythm specifically dampens the clock gene expression of *Per1*.

Next we investigated which thermogenic pathways within the brown adipocyte may be rhythmic and therefore blunted upon corticosterone rhythm flattening. Since intracellular TG stores are burned upon activation of BAT, the intracellular lipid content of BAT is an indirect measure of its activity. Histologic analysis. of BAT demonstrated that although not significant, BAT tended to contain less lipids at PM than AM, which would be consistent with a higher thermogenic activity at PM (Fig. 4m). However, the same trend was apparent in corticosterone-treated mice (Fig. 4n). Subsequently, we examined the possibility that corticosterone rhythm flattening reduces mitochondrial function which could eventually reduce FA uptake by brown adipocytes. The rhythmic expression of carnitine palmitoyltransferase 1a (*Cpt1a*) (Fig. 4k), which facilitates the transfer of FA into the mitochondrial matrix for oxidation, was blunted in corticosterone-treated mice. Likewise, gene expression of NAD-dependent deacetylase sirtuin-3 (*Sirt3*) (Fig. 4l), which regulates several mitochondrial metabolic enzymes, showed an AM-PM difference in the placebo treated mice, which lost significance in corticosterone treated mice. In contrast, thermogenic genes *Prdm16*, *Pgc1α*, *Fasn* and *Elovl6, Dio2* (Fig 4o-s) did not display any AM-PM difference. LPL is crucial for hydrolysis of TG within TRL and subsequent uptake FA by BAT. We previously found that expression of *Angptl4*, which encodes for the inhibitor of LPL activity, displays a diurnal rhythm within BAT (chapter 3). Although *Angptl4* did not show a AM-PM difference (Fig 4t), *Lpl* expression was rhythmic, and this rhythmicity was dampened after corticosterone treatment (Fig. 4u). Thus, our data suggest that corticosterone rhythms may regulate BAT activity at the level of expression of genes involved in both cellular FA uptake (*Lpl*) and FA oxidation (*Cpt1a*, *Sirt3*).

Since expression of direct GR and MR targets retained their rhythmicity, we hypothesized that corticosterone affects BAT indirectly, *e.g.* via sympathetic output. We studied phosphorylated protein levels of hormone sensitive lipase (HSL), involved in TG hydrolysis, as well as CREB, a downstream target of adrenergic signaling. While HSL phosphorylation was not different between AM and PM (Figs. 4v,w), we found that near-significant rhythmic levels of phosphorylated CREB (pCREB) was dampened upon corticosterone treatment (Fig 4v, x).

DISCUSSION

The uptake of FA uptake by BAT is higher at the onset of the active period (i.e. the dark period for mice, at PM) than at the onset of the resting period (the light period, AM). These data support our recent observations, demonstrating a pronounced diurnal rhythm in TG-derived FA uptake by BAT (Chapter 3). The diurnal rhythmicity of TG-derived FA uptake parallels that of glucose uptake by BAT in mice [21] and supraclavicular temperature in humans [22] (a measure for human BAT activity since it highly correlates to glucose uptake [23]). We showed that implantation of a low dose corticosterone-containing pellet dampens the endogenous diurnal rhythm in plasma corticosterone concentrations in mice; corticosterone plasma levels increased at AM and decreased at PM. Together with the dampened corticosterone rhythm, BAT depots decreased TG-derived FA uptake at PM. Whole body energy expenditure retained a diurnal rhythm and body composition was maintained.

Dampened corticosterone rhythm may act on BAT activity either directly in the brown adipocyte by binding MR and GR, or indirectly, via the central nervous system (CNS). We examined effects on genes with a known high affinity GR-response element (GRE) [24]. Expression of *Fkbp5* and *Gilz* retained rhythmicity, dismissing a direct effect of corticosterone rhythm via GRE-induced expression. Nevertheless, we observed a dampened rhythm in expression of *Per1*, which also has a known high affinity GRE. This phenomenon is in line with recent study in zebrafish with a genetic disturbance of diurnal cortisol levels. In that study, transcriptomic analysis showed a disturbance in rhythmic expression in many metabolic genes. Supplementation with a continuous dose of dexamethasone restored rhythmic expression in part of the arrhythmic genes [25]. In other words, constant glucocorticoid levels restore are sufficient to restore rhythmic gene expression of some genes, but for other genes, the rhythmicity of glucocorticoid levels is necessary to restore the rhythmic expression. The intracellular mechanisms remain obscure, but likely involve non-genomic effects of the GR, such as the interaction with clock proteins.

Implantation of low-dose corticosterone pellet resulted in decreased phosphorylation of CREB at PM. Adrenergic stimulation of BAT increases CREB phosphorylation [26]. Therefore, corticosterone rhythm may centrally regulate BAT activity via modulating the sympathetic outflow. No studies have yet demonstrated a diurnal sympathetic outflow towards BAT. Corticosterone is able to pass the blood-brain barrier, and can bind MR and GR present in the CNS. MR activation in the CNS increases hypertension and sympathetic outflow [27], which represents a possible pathway by which glucocorticoids may affect sympathetic outflow towards BAT.

The difference in AM and PM nutrient uptake by BAT was accompanied by a marked AM-PM difference in *Lpl* expression, which is in line with our previous finding that *Lpl* gene expression and protein levels are highly rhythmic and coincide with FA uptake rhythms by BAT (Chapter 3). Furthermore expression of *Cpt1a* and *Sirt3* was dampened after implantation of low-dose corticosterone pellets. Cold-induced adrenergic stimulation

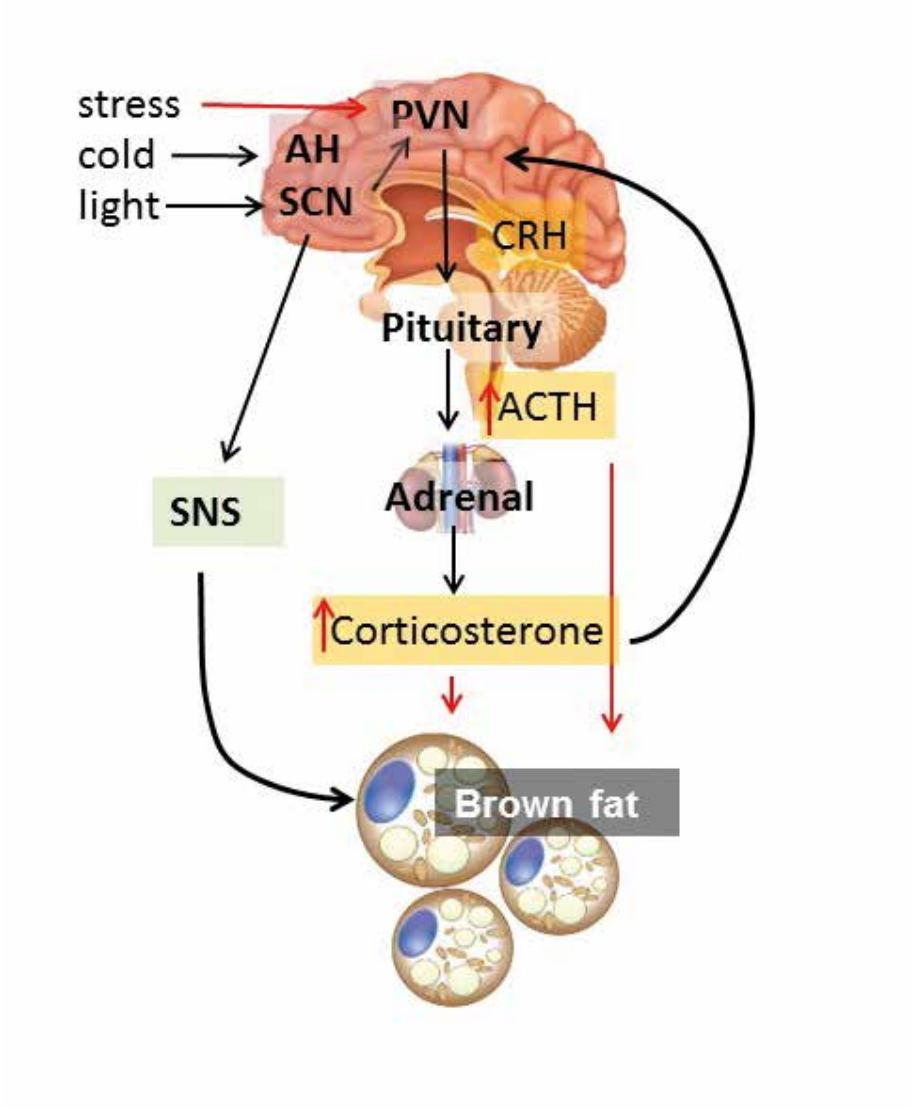


Figure 5. Hypothetical model. *Light information is processed by the SCN, which regulates rhythmic output of the HPA axis via the PVN. Under physiological circumstances, peak corticosterone levels do not directly regulate brown fat rhythm but rather feed back to the brain (possibly via the AH) and via the SNS, BAT rhythmicity is regulated. If a stressor is perceived, the PVN is activated, high levels of ACTH and corticosterone are reached in the circulation, which directly affect BAT activity. AH = anterior hypothalamus, SNS = sympathetic nervous system, PVN = paraventricular nucleus, SCN = suprachiasmatic nucleus.*

increases expression of these genes [28]. Therefore, the dampened expression support the hypothesis that glucocorticoid act indirectly via the CNS to modulate sympathetic outflow.

A few publications reported on the role of glucocorticoids on BAT activity. Increased glucocorticoid levels have been shown to inhibit thermogenic BAT activity *in vitro* in murine brown adipocytes [29]. On the other hand, elimination of endogenous glucocorticoids by adrenalectomy increases UCP-1 protein content and mitochondrial content in BAT in rats, which again decreases with increasing substitution dosages of corticosterone [30]. However, due to the ablation of negative feedback in these models, they are also characterized by increased ACTH levels, which has recently been demonstrated to activate BAT [31, 32]. Therefore, the question remains whether adrenalectomy increases BAT activity due to reduced glucocorticoid levels or because of increased ACTH levels. Furthermore, flattened glucocorticoid levels could affect BAT activity by lack of peak levels or by an increase of the through levels. *In vitro* experiments have demonstrated that GR agonism inhibits *Ucp1* expression in murine brown adipocytes [33, 34]. In these studies, supraphysiological doses of glucocorticoids or dexamethasone were administered. Gene expression responses as well as behavioral responses to glucocorticoids are context and dose dependent [35, 36]. Notably, two human studies recently demonstrated that infusion of glucocorticoids increased BAT thermogenesis, as measured by supraclavicular skin temperature [29, 37]. Taken these data together, we propose that glucocorticoids may serve both as a signal for diurnal BAT activity and as a signal for stress, depending on the plasma levels.

A limitation of the current study is the use of only two time points to determine rhythmicity. Although our study confirmed previous finding of a large amplitude between AM and PM corticosterone rhythm as well as FA uptake by BAT, the detection of rhythms in *e.g.* expression of genes that have other peak and trough times may be missed. It has been previously shown that diurnal oscillations in gene expression in many of the peripheral circadian clocks can be shifted by the administration of glucocorticoids or by adrenalectomy [38]. Additionally, we cannot exclude that total 24h corticosterone plasma levels may be different between the groups only based on AM and PM corticosterone levels. Indeed, adrenal and thymus weight were slightly lowered upon corticosterone treatment. However, we did not observe metabolic effects associated with hypercorticosteronemia such as decreased lean mass [39].

In conclusion, we show that dampening of glucocorticoid levels specifically dampens FA uptake rhythm by BAT. Our study may help to understand how disturbed circadian rhythms can cause metabolic disorders. Chronic disruption of circadian rhythm in BAT may be such a mediator, as lower BAT activity is associated with lower energy expenditure, dyslipidemia and obesity in rodents [40]. Disturbed day-night rhythms, such as through shift work, may disturb circadian BAT function. Glucocorticoid administration may help to restore disturbed circadian BAT function and thereby treating metabolic disease.

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