# It's about time: Circadian rhythm and metabolism

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# Circadian rhythm of glucocorticoids regulates brown adipose tissue activity and is important for maintaining metabolic health

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## Abstract

Circulating levels of glucocorticoids display diurnal fluctuations, and act as synchronizers of circadian rhythm in many peripheral tissues. The primary glucocorticoid in mice, corticosterone, is known to modulate brown adipose tissue (BAT) activity. However, it is not known whether corticosterone also orchestrates the circadian rhythm in BAT activity and thereby contributes to metabolic health. We implanted mice with corticosterone-releasing pellets, resulting in constant circulating corticosterone levels that were exactly in between the morning and evening levels of control mice, thus flattening corticosterone rhythm. Strikingly, flattened corticosterone rhythm was accompanied by a complete loss of circadian rhythm in BAT activity. This effect was independent of glucocorticoid receptor expression in the adipocytes themselves, but our results rather suggest that corticosterone flattening modulates sympathetic innervation of BAT. In APOE*3-Leiden.CETP mice, a model for hyperlipidemia and metabolic syndrome, experimental flattening of the corticosterone and thereby BAT activity rhythm resulted in adiposity. Restoring glucocorticoid rhythm could therefore be a promising strategy to promote BAT activity and reduce metabolic disease associated with a dampened glucocorticoid rhythm, as occurs with glucocorticoid therapy and ageing.

# Introduction

The past decade brown adipose tissue (BAT) has been re-discovered in adult humans as a highly metabolically active tissue [1]. BAT takes up nutrients, mostly fatty acids (FA) and glucose, from the circulation to fuel mitochondrial oxidation. The mitochondria of brown adipocytes express uncoupling protein 1 (UCP1), which dissipates the mitochondrial proton gradient necessary for production of energy in the form of ATP [2]. This generates heat instead of ATP, a process called thermogenesis. Due to its ability to increase energy expenditure through thermogenesis, BAT has emerged as a novel target to reduce adiposity [3]. The natural stimulus of BAT is cold, which results in increased sympathetic outflow to BAT. Sympathetic nerve endings release noradrenalin (NA), to stimulate β3-adrenergic receptors (β3AR) on murine brown adipocytes. The β3AR signals through the second messenger cyclic AMP (cAMP) to, amongst other targets, promote phosphorylation of cAMP response element-binding protein (CREB) [3]. Subsequently, phosphorylated CREB promotes lipolytic activity and stimulates the transcription of key thermogenic genes, such as *Ucp1* and *Pgc1a*, thereby increasing the thermogenic activity of brown adipocytes.

The central biological clock, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, receives light signals from the retina and subsequently synchronizes circadian (i.e. 24 h) rhythms in various tissues throughout the body. The SCN communicates with these tissues through the autonomic nervous system and endocrine signals, thereby regulating appetite and timing of fasting/feeding [4]. Disturbances of the biological clock in humans, e.g. through shift work or artificial light exposure, are associated with an increased risk of obesity, type 2 diabetes and dyslipidemia [5, 6], and studies in rodents suggest a causal relationship [7]. We recently showed that BAT activity is highly dependent on the biological clock and thus may explain the relationship between rhythm disturbances and metabolic disease [8, 9]. BAT exhibits a strong diurnal rhythm in both mice [9, 10] and humans [11], and disruption of this rhythm by prolonged light exposure reduces the metabolic activity of BAT resulting in increased adiposity in mice [8]. However, the exact mechanism by which the SCN transfers diurnal information to BAT is not fully understood. Glucocorticoids, predominantly cortisol in humans and corticosterone in rodents, are likely candidates for transferring the circadian time-keeping signal to BAT. The release of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, which is under strict circadian control [12]. HPA axis activation starts with the release of corticotrophin-releasing hormone (CRH) by neurons of the paraventricular nucleus (PVN) in the hypothalamus. CRH stimulates the pituitary to produce adrenocorticotrophic hormone (ACTH), which in turn promotes secretion of glucocorticoids by the adrenals. SCN nerve endings project to the CRH-expressing neurons of the PVN, thereby directly modulating the activity of the HPA axis [13, 14]. In addition, the SCN connects to the autonomous nervous system to regulate splanchnic innervation of the adrenal gland [15, 16]. The actions of these mechanisms together result in a pronounced diurnal rhythm in plasma levels of glucocorticoids [12]. By acting through the glucocorticoid receptor (GR), glucocorticoids entrain rhythm in peripheral organs [17, 18]. However, whether this is also the case for BAT remains to be elucidated.

In this study, we report that glucocorticoid rhythm dictates circadian BAT activity in mice. In addition, we demonstrate that disruption of glucocorticoid rhythm - and thereby BAT activity - aggravates metabolic disease.

# Materials and methods

## Animal experiments

All mice were housed on a 12 h:12 h light:dark cycle at 21°C and fed *ad libitum.* Experiments were performed in male 8-12 week-old C57BL/6J wildtype (WT) mice (Charles River Laboratories), 10-18 week-old male adipocyte-specific GR-deficient mice (ad.GRKO; Nr3c1$^{tm2Gsc}$Tg(Adipoq-cre)1Evdr: GR$^{\Delta Adip}$; C57BL/6 x FVB/N; breeding University of Ulm) [19] or 12-18 week-old female APOE*3-Leiden.CETP mice (C57BL/6J background; breeding Leiden University Medical Center) [20]. C57BL/6J and ad.GRKO mice were fed standard chow diet. APOE*3-Leiden.CETP mice were fed Western-type diet (WTD) containing 15% fat from cocoa butter and 1% fat from corn oil (diet T, Altromin), enriched with 0.1% cholesterol.



C57BL/6J male mice were subcutaneously implanted with a pellet containing either 2.5 mg corticosterone (2.5 mg corticosterone and 97.5 mg cholesterol, 100 mg in total) or vehicle (100 mg cholesterol) ($n$ = 16/group). The continuous corticosterone release from the pellets flattens corticosterone rhythm by providing negative feedback to the HPA axis, as previously described by others [21]. After 7 days, the uptake of triglyceride (TG)-derived FAs by various adipose tissue depots was determined at AM (starting at lights on) and PM (starting at lights off) after a 4 h fasting period ($n$ = 8/timepoint/group). Ad.GRKO mice ($n$ = 13) and WT littermates (adipoq-cre negative; $n$ = 14) were acclimated for 7 days and the update of TG-derived FAs was determined at AM (starting at lights on) and PM (around lights off) after an 8 h fasting period ($n$ = 6-8/timepoint/genotype).

APOE*3-Leiden.CETP female mice were fed a run-in WTD (as described above) for three weeks, after which they were randomized into experimental groups based on plasma TGs and total cholesterol, body weight and age. Mice were subcutaneously implanted with either a 3.75 mg corticosterone pellet, 7.50 mg corticosterone pellet or vehicle pellet ($n$ = 8/group). Flattening of the glucocorticoid rhythm by corticosterone-releasing pellets was confirmed for a period of at least two weeks by measuring plasma corticosterone levels (data not shown), and pellets where replaced every two weeks. Body weight, food intake and body composition (EchoMRI-100-analyzer; EchoMRI) were determined throughout the experiment. After 5 weeks, mice were fasted for 4 h and uptake of TG-derived FAs was determined at PM (around lights off).

All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals after having received approval from the central animal experiments committee.

## Clearance and organ uptake of radiolabeled triglyceride-derived fatty acids

TG-rich lipoprotein (TRL)-like emulsion particles radiolabeled with glycerol tri[$^{3}$H]oleate were prepared as previously described [22]. Mice were fasted for 4-8 h and injected intravenously with particles containing 1.0 mg TG in 200 µl PBS. Blood was collected by a nick in the tail vein at 2, 5, 10 and 15 min after injection, in order to monitor plasma decay of the radiolabels. Afterwards, mice were killed by cervical dislocation and perfused for 5 min with ice-cold PBS. Tissues were harvested to measure the 3H activity by liquid scintillation, and evaluate TG-derived FA uptake by BAT.

## Corticosterone measurement

To determine plasma corticosterone, blood was collected in a stress-minimized manner, i.e. via a nick in the tail vein and within 2 min (before stress-induced corticosterone levels rise)

[23]. Corticosterone concentration was measured by ELISA according to the manufacturer's protocol (Corticosterone EIA, Immunodiagnostics).

## Cell culture

Immortalized preadipocytes were generated and cultured as previously described [24]. Preadipocytes were differentiated into brown adipocytes for 14-15 days and during the last 2 days of differentiation and during the experiments, cells were grown in medium containing hormone-deprived (charcoal-stripped) serum. Cells were pretreated with 1 μM RU486 for 30 min, followed by treatment with 10 nM corticosterone or vehicle. Cells were harvested at T = 0, 8, 16, 24 or 32 h after treatment for gene expression analysis.



## Gene expression analysis

Total mRNA was extracted from brown adipocytes and interscapular BAT (iBAT) by using TRIzol RNA isolation reagent (Thermo Fisher) following manufacturer's protocol. RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher), and 1 μg RNA was reverse transcribed to cDNA by using M-MLV Reverse Transcriptase (Promega). The qRT-PCR was performed using a SYBR Green kit (Promega) on a CFX96 PCR machine (Bio-Rad), and expression levels of genes of interest were normalized to expression of a housekeeping gene.

## Protein isolation and western blot analysis

iBAT samples were homogenized and diluted in lysis buffer. Homogenates were centrifuged and protein content of the supernatant was determined using a BCA protein assay kit (Thermo Scientific). After heating the samples (5 min, 95°C), 20 μg of protein was separated by 10% SDS-PAGE, followed by transfer to a nitrocellulose membrane. Membranes were blocked with 5% milk and incubated overnight at 4°C with the primary antibody rabbit anti-pCREB (Cell Signaling at 1:1000) or mouse anti-β-actin (Sigma at 1:1000), followed by incubation for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse, respectively; Promega at 1:5000). Bands were visualized using SuperSignal Western Blot Enhancer (Thermo Scientific) and analyzed with ChemiDoc Touch Imaging System (Bio-Rad). Expression of pCREB was normalized to expression of the housekeeping protein β-actin.

## Noradrenalin measurement

NA levels were measured in subscapular BAT (sBAT) tissue using a commercial available ELISA Kit (CatNo. BA E-52000, LND) that was modified for tissue homogenates. Tissue samples were homogenized in a Precellys Tissue homogenizer (Precellys) with ceramic beads in a special buffer to stabilize NA (1 mM EDTA, 4 mM $Na_2S_2O_5$, 0.01 M HCl). BAT samples were homogenized with the tenfold amount of buffer and centrifuged for 10 min at 13000 rpm at 0°C. Thereafter, the infranatant, between cell debris and lipid layer, was centrifuged for 4 min at 13000 rpm at 0°C and the protein content of the lysate was measured using PierceTM BCA protein assay kit (23225, Thermo Scientific). 5 μg of BAT samples in a maximum of 25 μL buffer were applied to the NA Research ELISA (LDN) and measured according to the manufacturer's protocol using the EnSpire®Multimode Plate Reader.

## Histological analysis

Adipose tissues (i.e. gonadal WAT (gWAT), subcutaneous WAT (sWAT) and iBAT) were fixated in 4% formalin, embedded in paraffin, and cut into 5 μm sections. Slides were stained with hematoxylin and eosin (H&E) using standard protocols. UCP1 and tyrosine hydroxylase (TH) stainings were performed as previously described [8]. White adipocyte size, iBAT lipid droplet

content and UCP1/TH expression (relative UCP1/TH staining per area) were quantified using ImageJ software (Version 1.50).

### Statistical analysis

Data are presented as means ± SEM, and group sizes are indicated in the figure legends. Three APOE*3-Leiden.CETP mice from the 3.75 mg corticosterone-pellet group were excluded from the analysis, as corticosterone levels did not flatten following pellet implantation (defined by a rhythm amplitude with less than one SD difference as compared to the rhythm amplitude in the vehicle control group). Statistical analysis was performed using GraphPad Prism (version 7.02). Means were compared using one- or two-way ANOVA, as indicated in figure legends. Differences between groups were considered statistically significant at $P < 0.05$.



## Results

### Corticosterone rhythm regulates circadian BAT activity

To investigate whether glucocorticoid rhythm and circadian BAT activity are linked, we implanted mice with corticosterone-releasing pellets to induce constant corticosterone levels throughout the day, while control mice implanted with vehicle-pellets maintained their natural corticosterone rhythm (Fig. 1A).

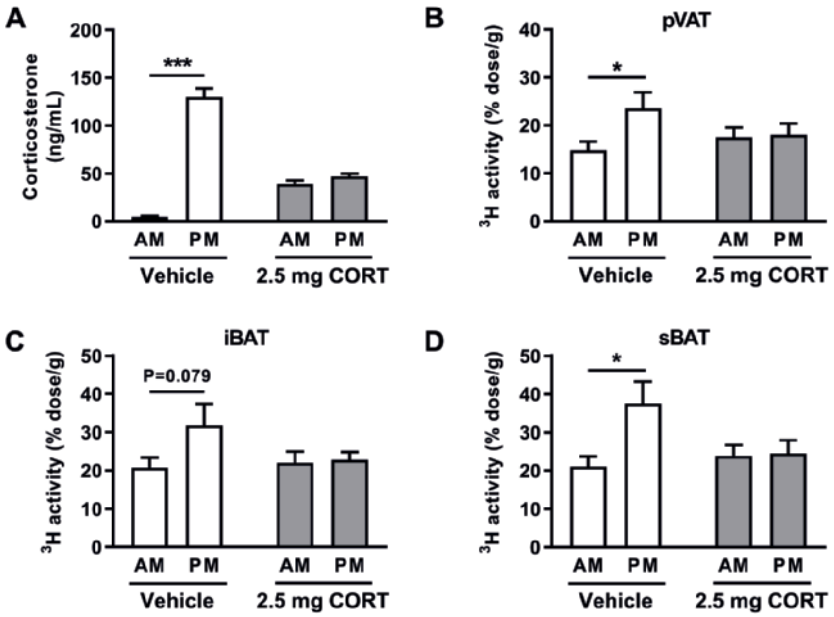



**Figure 1. Flattening of the corticosterone rhythm blunts rhythmic BAT activity.** Male C57BL/6J mice were implanted with either corticosterone- or vehicle-pellets. After 7 days, at lights on (AM) and lights off (PM), plasma corticosterone levels were measured (A) and the uptake of $^3$H-labeled oleate by perivascular adipose tissue (pVAT; B), interscapular brown adipose tissue (iBAT; C) and subscapular brown adipose tissue (sBAT; D) was determined. Data represent means ± SEM ($n$ = 8/group/timepoint). *$P < 0.05$, ***$P < 0.001$ compared to the AM group, according to two-way ANOVA with Sidak's post hoc test.

In line with our previous report [9], control mice exhibited a robust rhythm in the uptake of TG-derived FAs by the thermogenic tissues perivascular adipose tissue (pVAT; Fig. 1B), iBAT (Fig. 1C) and sBAT (Fig. 1D), with a high uptake in the evening (PM) at the time of the endogenous corticosterone peak. Strikingly, flattening of corticosterone levels completely abolished the rhythmic activity of these thermogenic tissues, reflected by a similar FA uptake in the morning versus the evening (Fig. 1B-D).

## Corticosterone rhythm does not dictate the molecular clock in BAT

We next explored whether glucocorticoid regulation of BAT rhythm is mediated by a direct action on the molecular clock in brown adipocytes, as others have shown that GR activity drives rhythm of circadian clock genes *in vitro* [25]. We exposed cultured brown adipocytes to a low concentration of corticosterone (10 nM), and harvested samples every 8 h to evaluate gene expression. This revealed a robust rhythm in the expression of circadian clock genes *Rev-erbα* (Fig. 2A) and *Per2* (Fig. 2B) in corticosterone-treated cells. This process was at least partly GR-dependent, as the GR antagonist RU486 attenuated synchronization. Expression of *Rev-erbα* (Fig. 2C) and *Per2* (Fig. 2D) was also highly rhythmic in BAT of mice, but not affected by flattening of the corticosterone levels. These results indicate that glucocorticoids can induce a rhythm in brown adipocytes *in vitro*, but do not modulate the molecular clock in BAT *in vivo*.

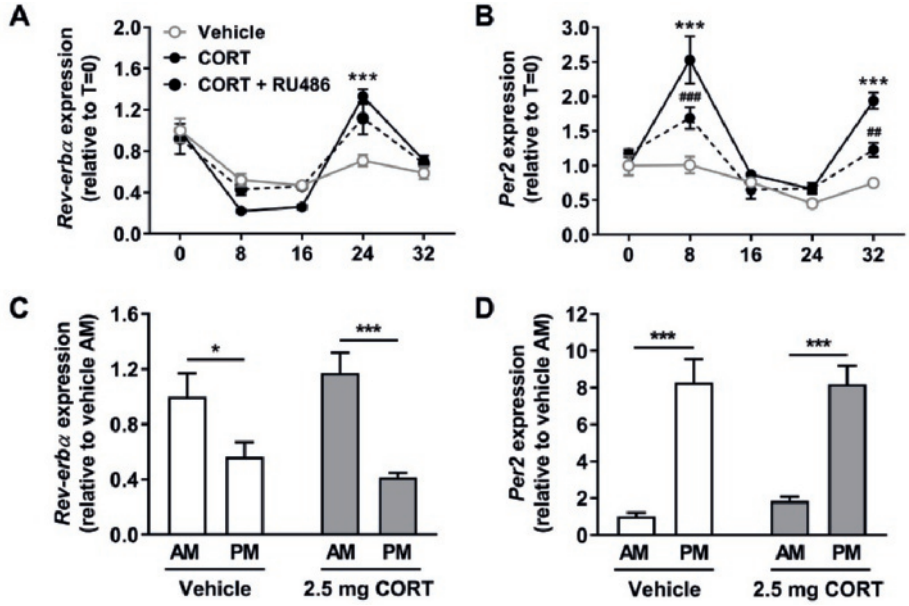



**Figure 2. Corticosterone induces rhythm in cultured brown adipocytes, but does not dictate rhythmic clock gene expression in BAT.** Rhythmic expression of the clock genes *Rev-erbα* (A) and *Per2* (B) was determined in immortalized brown adipocytes treated with vehicle, corticosterone (CORT; 10 nM) or CORT with pretreatment with the GR antagonist RU486 (1 μM). Expression of *Rev-erbα* (C) and *Per2* (D) was measured in interscapular BAT (iBAT) of mice implanted with either corticosterone- or vehicle-pellets, after 7 days at lights on (AM) and lights off (PM). Data represent means ± SEM ($n$ = 4/group/timepoint for A&B; $n$ = 7-8/group/timepoint for C&D). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the vehicle or indicated control group and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the group treated only CORT, according to two-way ANOVA with Sidak's post hoc test.

## Adipocyte GR expression is dispensable for circadian BAT activity

We continued by investigating whether the GR on brown adipocytes mediates circadian BAT activity. To this end, we evaluated the uptake of TG-derived FAs by brown fat in ad.GRKO mice that lack GR in adipocytes, and compared this with WT littermates. Plasma corticosterone levels are not affected by GR-deficiency in adipose tissue, and ad.GRKO mice demonstrate a similar body weight and composition as compared to WT mice at the age in which mice were used for our experiment [19]. TG-derived FA uptake by iBAT (Fig. 3A) and sBAT (Fig. 3B) was rhythmic in both WT and ad.GRKO mice, and both genotypes displayed a similar plasma decay of TGs with faster clearance at PM as compared to AM (Fig. 3C). Comparable lipid droplet sizes were observed in iBAT of WT and ad.GRKO mice, with smaller lipid droplets in ad.GRKO mice at PM when BAT is more active (Fig. 3D). These data demonstrate that GR activity in adipose tissue is dispensable for BAT activity rhythm.



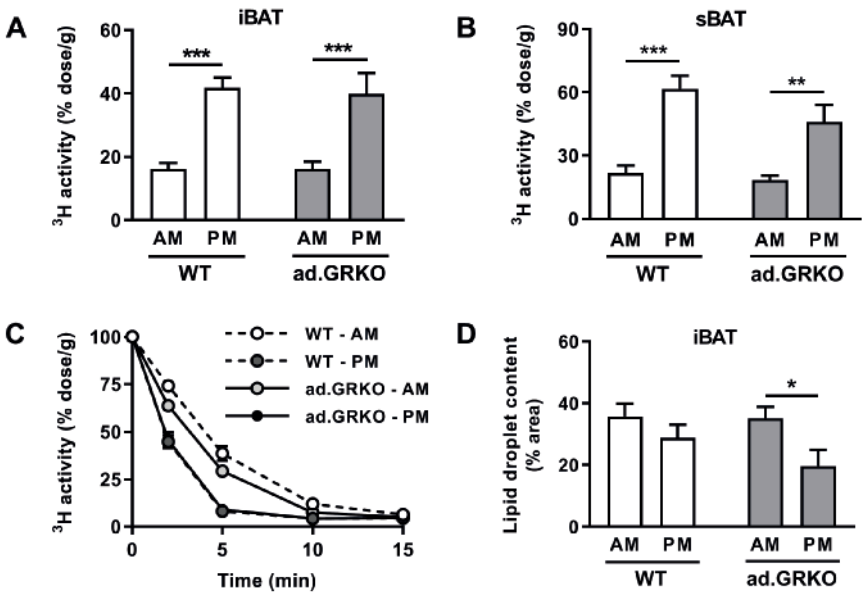



**Figure 3. GR activity in adipose tissue is dispensable for BAT activity rhythm.** At lights on (AM) and lights off (PM), the uptake of $^3$H-labeled oleate was measured by the interscapular brown adipose tissue (iBAT; A) and subscapular brown adipose tissue (sBAT; B) of wildtype (WT) and adipose-specific GR-deficient (ad.GRKO) mice. Plasma decay of $^3$H-labeled oleate was compared between male WT and ad.GRKO mice (C), as well as the lipid droplet content of iBAT (D). Data represent means ± SEM ($n$ = 6-8/group/timepoint). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the indicated control group, according to two-way ANOVA with Sidak's post hoc test.

## Corticosterone rhythm modulates sympathetic input of BAT

As our results so far suggest that corticosterone does not modulate BAT activity rhythm via local GR-dependent effects, we hypothesized an indirect mechanism of action. We previously found that sympathetic denervation blunts circadian BAT activity [9], and therefore aimed to investigate whether centrally mediated sympathetic input of BAT links corticosterone rhythm and BAT activity. To this end, we analyzed tissue expression of tyrosine hydroxylase (TH), the rate-limiting enzyme for local NA synthesis, tissue NA levels and CREB phosphorylation, an

important downstream event of NA-induced β3AR activity. BAT was collected from mice at AM and PM, which revealed rhythmicity in TH (Fig. 4A) and NA (Fig. 4B) in BAT of vehicle-treated mice. While BAT from corticosterone-treated mice retained a rhythm in NA levels, rhythm in TH was completely abolished. Levels of pCREB tended to be rhythmic in vehicle-treated, but not corticosterone-treated mice (Fig. 4C), suggesting that corticosterone may influence sympathetic signaling by desensitizing NA-induced β3AR activation. Indeed, *Abdr3*, the gene that encodes for β3AR, was downregulated upon flattening of corticosterone rhythmicity (Fig. 4D), providing a possible explanation for the decreased BAT activity.



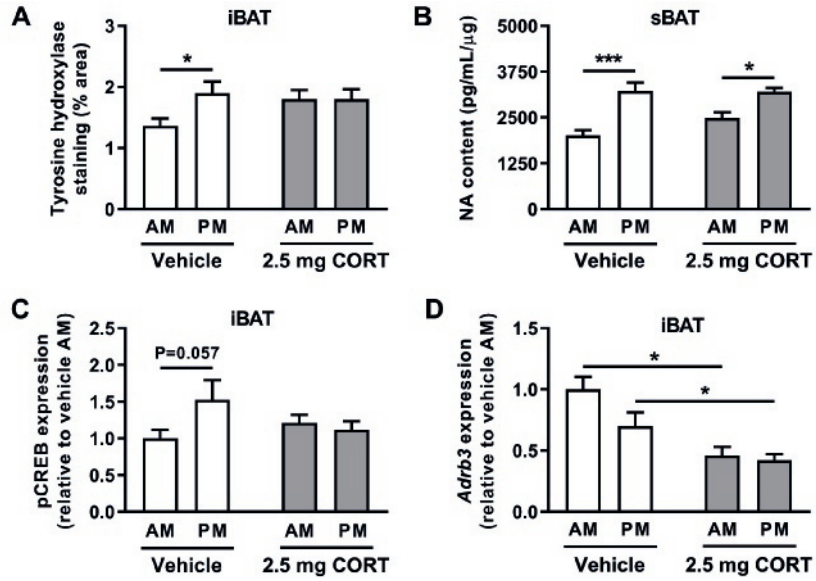



**Figure 4. Corticosterone rhythm modulates adrenergic signaling in BAT.** Male C57BL6/J mice were implanted with either corticosterone-releasing or vehicle pellets. After 7 days, at lights on (AM) and lights off (PM), mice were killed and interscapular brown adipose tissue (iBAT) and subscapular brown adipose tissue (sBAT) were collected to analyze tyrosine hydroxylase (TH) staining (A), tissue noradrenalin (NA) levels (B), phosphorylated CREB levels (C) and *Adrb3* expression (D). Data represent means ± SEM ($n$ = 3-8/group/timepoint). *P $<$ 0.05, ***P $<$ 0.001 compared to the indicated control group, according to two-way ANOVA with Sidak's post hoc test.

## Flattening of corticosterone rhythm increases adiposity

To investigate whether flattened corticosterone and perturbed BAT activity rhythm results in metabolic disturbances, we performed a long-term experiment in APOE*3.Leiden-CETP mice, which were exposed to low (3.75 mg) or high (7.50 mg) dose corticosterone pellets for 5 weeks. The doses of corticosterone pellets used in female APOE*3.Leiden-CETP mice were higher than the one used in male C57BL6/J mice, due to sex differences in the responsiveness to glucocorticoids. To estimate the total corticosterone exposure, we collected blood at various times throughout the day and night. This revealed constant plasma levels of corticosterone in mice treated with both low and high dose corticosterone pellets (Fig. 5A). Both doses also increased the area under the curve (AUC) reflecting higher corticosterone exposure, but did not exceed the peak value in corticosterone of the vehicle-treated mice (Fig. 5A). Along with

a flattened corticosterone rhythm, we found a dose-dependent reduction in the uptake of TG-derived FAs in both iBAT and sBAT of corticosterone-treated mice (Fig. 5B). In line with a decreased BAT activity, corticosterone exposure resulted in increased body weight (Fig. 5C), elevated fat mass (Fig. 5D) and increased weight of liver, gWAT, sWAT and iBAT (Fig. 5D). Increased tissue weights are likely attributed to enhanced lipid deposition, as corticosterone pellets increased adipocyte size of gWAT (Fig. 6A) and sWAT (Fig. 6B), and increased iBAT lipid droplet content (Fig. 6C). Furthermore, corticosterone pellets tended to reduce UCP1 expression (Fig. 6D) and significantly diminished TH expression (Fig. 6E), in line with a decreased BAT activity due to an impaired sympathetic innervation.



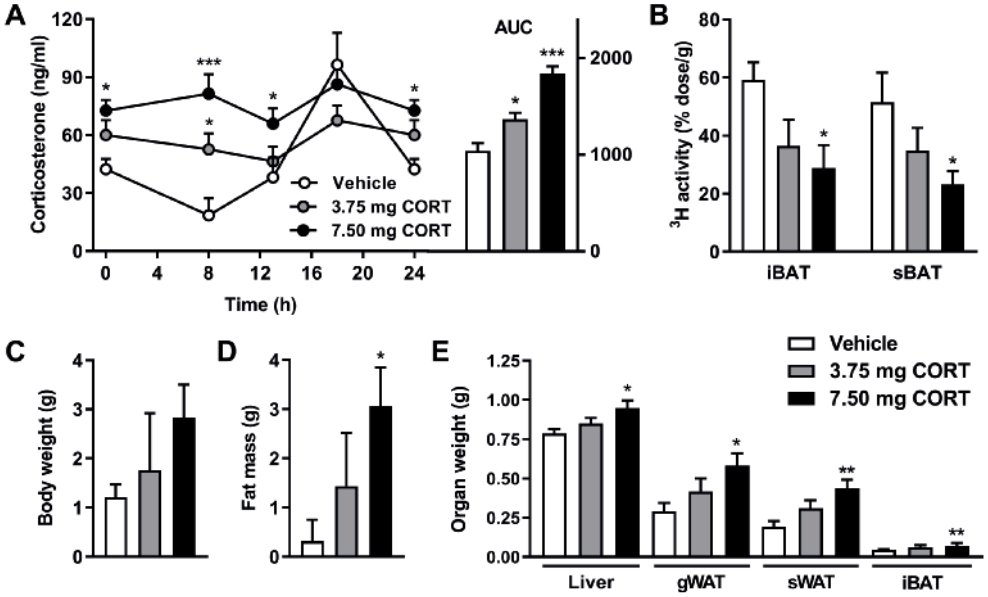
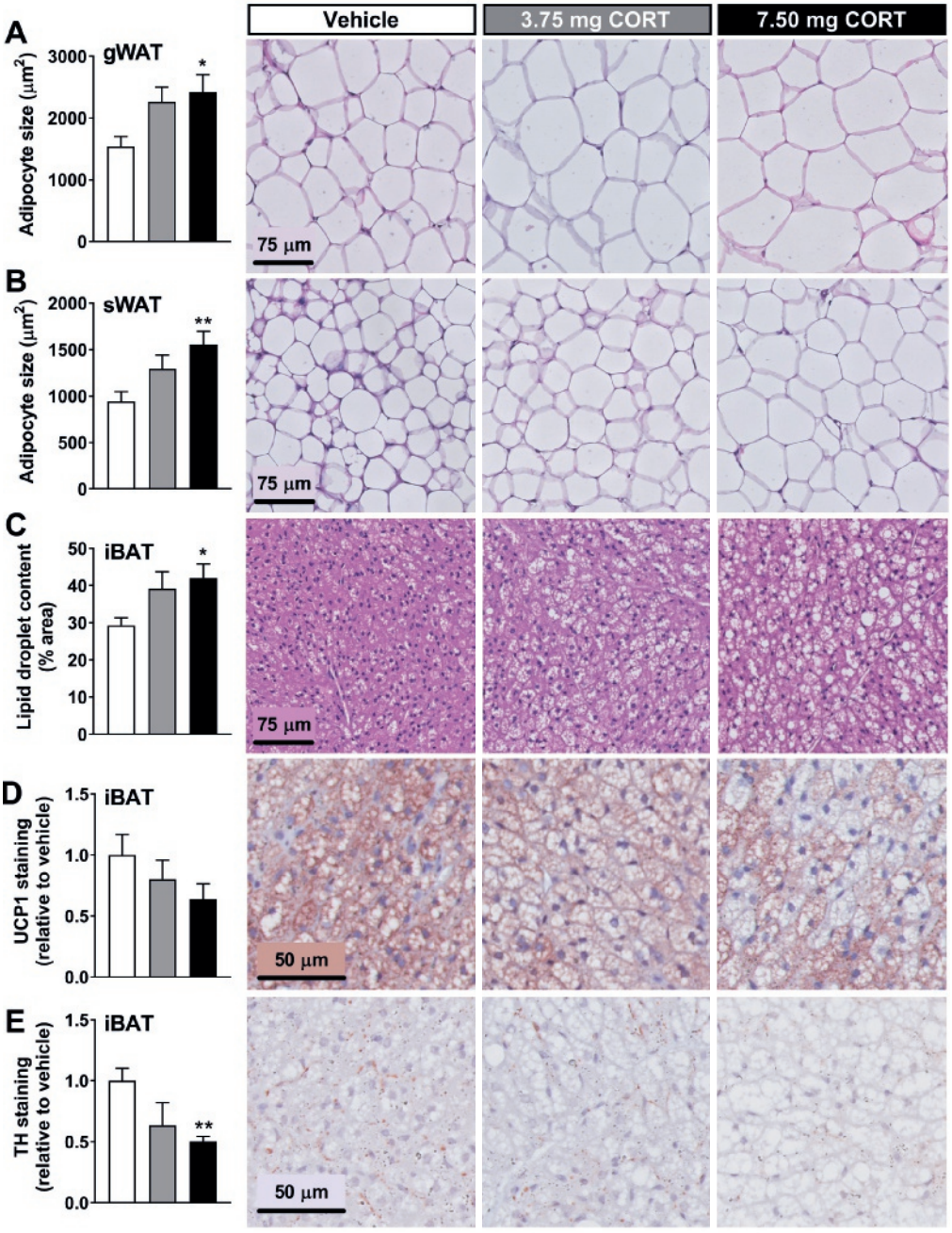



**Figure 5. Corticosterone-releasing pellets decrease BAT activity and increase adiposity in APOE*3-Leiden.CETP mice.** Female APOE*3-Leiden.CETP mice were implanted with pellets containing vehicle, 3.75 mg or 7.50 mg corticosterone (CORT). After 1 week, plasma corticosterone levels were measured throughout the day and night, and an area under the curve (AUC) of all data points was calculated (A). After 5 weeks, the uptake of $^3$H-labeled oleate was determined in the evening, arounds lights off, in iBAT and sBAT. Total body weight (C), fat mass (D) and organ weight (E) was determined at endpoint. Data represent means ± SEM ($n$ = 5-8/group). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the vehicle control group, according to one-way ANOVA (AUC of A; B-E) or two-way ANOVA (A) with Dunnett's post hoc test.

≫ **Figure 6. Corticosterone-releasing pellets induce lipid accumulation in adipose tissue of APOE*3-Leiden.CETP mice.** Female APOE*3-Leiden.CETP mice were implanted with pellets containing vehicle, 3.75 mg or 7.50 mg corticosterone (CORT). After 5 weeks, mice were killed, and adipose tissues were collected. Hematoxylin and eosin (H&E) stainings were performed to analyze adipocytes size of gWAT (A) and sWAT (B), and lipid droplet content of iBAT (C). Immunohistochemistry was used to stain uncoupling protein 1 (UCP1) and tyrosine hydroxylase (TH) and determine relative UCP1 (D) and TH (E) expression in iBAT. Data represent means ± SEM ($n$ = 5-8/group). *P < 0.05, **P < 0.01 compared to the vehicle control group, according to one-way ANOVA with Dunnett's post hoc test.


A
gWAT
Adipocyte size (μm²)
3000
2000
1000
0
*
B
sWAT
Adipocyte size (μm²)
2000
1500
1000
500
0
**
C
iBAT
Lipid droplet content (% area)
50
40
30
20
10
0
*
D
iBAT
UCP1 staining (relative to vehicle)
1.5
1.0
0.5
0.0
E
iBAT
TH staining (relative to vehicle)
1.5
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Vehicle
3.75 mg CORT
7.50 mg CORT
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## Discussion

In the current study, we aimed to investigate the importance of glucocorticoid rhythm for (circadian) brown fat activity and metabolic health. We demonstrated that flattening of the corticosterone rhythm in mice completely blunts rhythmic BAT activity, and may contribute to metabolic disturbances.

While we are the first to study effects of a merely flattened physiological glucocorticoid rhythm on metabolic health, effects of supraphysiological glucocorticoid levels that inherently flatten glucocorticoid rhythm have been well-described. Patients with hypercortisolism, due to endogenous overproduction of glucocorticoids or therapeutic administration of exogenous glucocorticoids, demonstrate profound metabolic disturbances [26]. In mice, chronic treatment with glucocorticoids also results in metabolic aberrations [27, 28], along with reduced UCP1 levels and increased lipid accumulation in BAT [29, 30]. On the other hand, depletion of endogenous glucocorticoids by adrenalectomy reduces weight gain and prevents lipid accumulation in BAT of mice that are genetically predisposed to obesity and therefore experience hypercortisolism [31-33]. These results demonstrate that an excess in glucocorticoids negatively impacts metabolic health. It is tempting to assume that a reduction in BAT activity contributed to this effect, but this is discredited by a recent study showing that glucocorticoid-induced obesity develops independent of UCP1 expression [34]. However, all above-mentioned studies have focused on the role of excess glucocorticoids, while little was known about the effects of a physiological glucocorticoid rhythm on (circadian) BAT activity.

We previously observed a potent rhythm in BAT activity in mice [9], which coincides with circadian rhythm in plasma corticosterone [35]. To specifically evaluate effects of a physiological rhythm in corticosterone, we used a corticosterone concentration *in vitro* that did not downregulate *Ucp1* expression, which is usually observed with high concentrations of glucocorticoids [36]. In addition, in our animal models we used a low corticosterone dose that resulted in constant plasma levels that did not exceed the normal peak in the diurnal rhythm. In fact, the corticosterone peak at the start of the wakeful period was decreased in the corticosterone-treated mice, along with a decrease in BAT activity at this timepoint. This might indicate a positive effect of a physiological peak in corticosterone levels on BAT activity, as has been observed in humans [37]. By flattening corticosterone levels in mice, we observed similar metabolic effects as observed in people with hypercortisolism (e.g. increased adiposity), suggesting that the negative effects of glucocorticoids on metabolic health could be partly due to an absence in glucocorticoid rhythm, rather than supraphysiological levels alone. Therefore, modulation of glucocorticoid rhythm could be a promising strategy to promote BAT activity and reduce metabolic disease, in particular for individuals with a dampened glucocorticoid rhythm due to exogenous glucocorticoid therapy or ageing [38, 39].

GR activity in adipose tissue was dispensable for BAT activity rhythm. This is in line with a recent study showing that the GR in brown adipocytes is not required for BAT-dependent energy homeostasis [40]. Alternative mechanisms thus exist through which corticosterone can modulate BAT activity *in vivo*. Aside from the GR, brown adipocytes express another receptor for glucocorticoids, namely the mineralocorticoid receptor (MR) [41]. Although the MR has been suggested to modulate BAT activity [42-44], the MR on brown adipocytes is most likely continuously occupied by endogenous corticosterone levels due to its high affinity, and therefore not expected to regulate a rhythm in BAT activity.

Endogenous glucocorticoids not only signal directly to peripheral tissues like BAT, but also pass the blood-brain barrier to act on corticosteroid receptors within the central nervous system [45-

47], thereby regulating sympathetic innervation of tissues [48, 49]. Differences in sympathetic outflow to BAT have been observed before in obese rodents that suffer from hypercortisolism [50, 51], and adrenalectomy restores sympathetic outflow to BAT in obese animals [52]. Therefore, sympathetic input of BAT may also be affected by flattening of the corticosterone rhythm. In support of this notion, we found a reduced expression of phosphorylated CREB in BAT, an important downstream mediator of NA activation, when the corticosterone peak was flattened in the evening. While we did not find differences in the actual NA levels in BAT, reduced expression of the NA receptor β3AR suggests a diminished responsiveness of the tissue towards sympathetic innervation. Whether this desensitization is regulated through action of corticosterone on the central nervous system remains to be confirmed. Of note, we have previously shown that BAT activity rhythm is completely blunted upon sympathetic denervation, but maintained upon adrenalectomy [9]. Together with the data from our current study, this suggests that sympathetic innervation is essential for generating BAT rhythms, and that glucocorticoids play an important role in finetuning this rhythm.



A limitation of our study is the modest increase in total corticosterone exposure observed in the APOE*3-Leiden.CETP mice implanted with corticosterone-pellets. We cannot fully exclude that this increased corticosterone exposure contributed to the metabolic disturbances observed in these mice. Nevertheless, a recent study observed similar metabolic effects in mice with a disrupted glucocorticoid rhythm, while glucocorticoid excess timed at the right time of day (i.e. at the natural peak of glucocorticoids) did not further exacerbate metabolic symptoms [53]. These data corroborate our hypothesis that a disrupted glucocorticoid rhythm contributes to the adverse metabolic effects observed in subjects with hypercortisolism.

Another drawback of our study setup is the continuous negative feedback by the corticosterone pellets on the HPA axis. Although necessary to blunt endogenous corticosterone production, this also diminishes endogenous ACTH levels. ACTH has been shown to activate BAT by signaling through the same cAMP pathway that is involved in adrenergic stimulation of brown adipocytes [30]. In our study, reduced ACTH levels could have diminished CREB phosphorylation, thereby mimicking effects of a reduced sympathetic innervation of the tissue. However, the effects of ACTH on BAT seem to occur at supraphysiological doses only [30, 54], making this possibility unlikely.

In conclusion, we demonstrate that a physiological glucocorticoid rhythm is essential for rhythmic BAT activity and metabolic health. Disruption of glucocorticoid rhythm, and thereby BAT activity rhythm, could (in part) underlie the relationship between rhythm disturbances and metabolic disease in humans. We thus anticipate that activation of BAT by enhancement of glucocorticoid rhythmicity could be a promising strategy to prevent and/or treat metabolic disease related to a dampened glucocorticoid rhythm. As species differences in glucocorticoid action on BAT have been reported [37], further research is required on the interplay between glucocorticoids, BAT rhythm and metabolic health in humans.

## Acknowledgements

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