Effects of adrenalectomy on daily gene expression rhythms in the rat suprachiasmatic and paraventricular hypothalamic nuclei and in white adipose tissue

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It is assumed that in mammals the circadian rhythms of peripheral clocks are synchronized to the environment via neural, humoral and/or behavioral outputs of the central pacemaker in the suprachiasmatic nucleus of the hypothalamus (SCN). With regard to the humoral outputs, the daily rhythm of the adrenal hormone corticosterone is considered as an important candidate. To examine whether adrenal hormones are necessary for the maintenance of daily rhythms in gene expression in white adipose tissue (WAT), we used RT-PCR to check rhythmic as well as 24 h mean gene expression in WAT from adrenalectomized (ADX) and sham-operated rats. In addition, we investigated the effect of adrenalectomy on gene expression in the hypothalamic SCN and paraventricular nucleus (PVN).

Adrenalectomy hardly affected daily rhythms of clock gene expression in WAT. On the other hand, 48% of the rhythmic metabolic/adipokine genes in WAT lost their daily rhythmicity in ADX rats. Likewise, in the hypothalamus adrenalectomy had no major effects on daily rhythms in gene expression, but it did change the expression level of some of the neuropeptide genes. Together, these data indicate that adrenal hormones are important for the maintenance of daily rhythms in metabolic/adipokine gene expression in WAT, without playing a major role in clock gene expression in either WAT or hypothalamus.

Keywords: Adrenal hormones, circadian, clock genes PVN, SCN, WAT

INTRODUCTION

The daily cycle of light and darkness has a profound influence on the behavior of most living organisms. Therefore, many living organisms have developed a highly conserved circadian clock system to adjust their daily activities to these day/night changes. The mammalian circadian clock system consists of 2 main components: (1) the central master clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph et al., 1990) and connected to the environment via the retino-hypothalamic-tract (RHT), and (2) the peripheral clocks, that are found in most organs and tissues (Yamazaki et al., 2000; Yoo et al., 2004) and influence organ-specific activity. The molecular mechanism of the circadian clock involves two interlocking, regulatory feedback loops: a core loop, consisting of the heterodimer BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein (1) and CLOCK, which can stimulate the transcription of the Per (period) and Cry (cryptochrome) genes by binding to the E-box sequence in their promoters. PER and CRY interact with each other and form a heterodimer that inhibits the transcriptional activity of the BMAL1-CLOCK heterodimer (Okamura et al., 2002). In the second, so-called accessory loop the BMAL1-CLOCK dimer also binds to the E-boxes in the promoter of Rev-erb and Ror (retinoic acid-related orphan receptor). REV-ERB and RORalpha compete for the RORE (retinoic acid-related orphan receptor response element) in the BMAL1 promoter. ROR stimulates the expression of BMAL1, while REV-ERB has an inhibitory effect on BMAL transcription (Guillaumond et al., 2005). By binding to the E-box and RORE sequences in the promoter region of other genes this regulatory mechanism also contributes to the daily rhythmicity of many other, so-called clock-controlled genes.

The circadian rhythm of the peripheral clocks has to be synchronized with the environmental...
light/dark-cycle through the central SCN clock, since these peripheral clocks are not light sensitive themselves. This synchronization is thought to occur through neural, hormonal and/or behavioral connections (Balsalobre et al., 2000; Oishi et al., 1998a; Stokkan et al., 2001), but the details of these connections are far from being elucidated.

Glucocorticoid hormones (mainly cortisol in humans and corticosterone in rodents) are essential for physiological organ function, acting on every organ and tissue and affecting physiological homeostasis in a cell- and gene-specific manner. The pronounced daily rhythm of glucocorticoid release is controlled by SCN, via its neural projections towards the paraventricular nucleus of the hypothalamus (PVN) (Kalsbeek et al., 2006). Glucocorticoid (GR) and mineralocorticoid (MR) receptors can be found in most peripheral organs. Moreover, several researchers have shown that glucocorticoids can phase shift molecular rhythms within a number of peripheral tissues, such as liver, kidney and heart (Balsalobre et al., 2000; Sujino et al., 2012). Therefore, adrenal hormones and in particular glucocorticoids are considered as an important endocrine pathway for the SCN to synchronize peripheral clocks.

In the last decade white adipose tissue (WAT) has come to be known as an important endocrine organ, presenting a highly rhythmic behavior (Ando et al., 2005). It is heavily involved in the regulation of energy metabolism especially by secreting adipokines that regulate appetite, food intake, glucose disposal and energy expenditure. It seems likely that each adipokine should be secreted at the right time and in the right order for the adipose tissue to function in an effective way. Indeed, recently, evidence has been presented that links disruption of daily rhythms in adipokine release to metabolic diseases, such as obesity and diabetes (Ando et al., 2005; Calvani et al., 2004; Saad et al., 1998). In order to further our understanding of the role of glucocorticoid mRNA expression of various clock, metabolic/adipokine and neuropeptide genes in adipose and hypothalamic tissue collected at four different time points along the light/dark-cycle from adrenalectomized and sham-operated male rats.

MATERIALS AND METHODS

Animals
All experiments were performed with adult male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany). Animals (n = 64) were group housed (four rats/cage) in the animal facility. Thirty-two rats with a 12 h light/12 h dark cycle (light on at 7:00) and another 32 rats with a 12 h dark/12 h light cycle (light on at 19:00). All rats were kept under constant temperature (21 ± 2°C) and humidity (60% ± 5) conditions. Food and water were available ad libitum. All experiments were approved by the animal care committee of the Royal Netherlands Academy of Arts and Sciences and in accordance with international ethical standards (Portaluppi et al., 2010).

Surgical procedures
After habituation to their respective L/D or D/L schedule, rats were anesthetized using a mixture of Hypnorm (0.8 ml/kg, i.m.) and Dormicum (0.3 ml/kg, s.c.) and received an adrenalectomy (ADX) or sham-operated surgery. In ADX rats, bilateral adrenal glands were removed via a dorsal incision of the skin and a small cut through the muscle layer. Sham-operated rats received a similar surgical procedure but without removal of the adrenal glands. Separate bottles with water and saline solution (0.9% NaCl) were provided ad libitum to all rats after surgery.

Tissue samples collection
Animals were anesthetized with 80% CO2 and sacrificed by decapitation at four time points (ZT2, ZT8, ZT14 and ZT20) 23 days after surgery. After decapitation, trunk blood was collected. The brain was removed, snap frozen on dry ice and stored at −80°C. Epididymal white adipose tissue (eWAT) was collected and frozen in liquid nitrogen, followed by storage at −80°C.

Brain sections were cut with a cryostat into 200 μm slices starting from the most anterior appearance of the SCN. Hypothalamic slices containing SCN and/or PVN were placed on a plate covered with RNAlater (Ambion, Foster City, CA) and punched with a 1-mm diameter needle to isolate PVN and SCN tissue. SCN punches were taken bilaterally and adjacent to the ventral third ventricle above the optic chiasm (approximately from bregma −0.48 to bregma −0.96). For the PVN, punches were taken bilaterally between the dorsal part of the third ventricle and the fornix (approximately from bregma −0.96 to bregma −2.04).

RNA extraction and cDNA synthesis
SCN and PVN tissue was homogenized by MagNA Lyser Green Beads (Roche) with Tissue Lysis Buffer (Roche) before RNA extraction. RNA was extracted from SCN and hypothalamic tissue collected at four different time points along the light/dark-cycle from adrenalectomized and sham-operated male rats.
and PVN using MagNA Pure LC RNA Isolation kit III-Tissue (Roche, Penzberg, Bavaria, Germany) by MagNA Pure LC (Roche, Penzberg, Bavaria, Germany).

eWAT was homogenized by MagNA Lyser Green Beads (Roche, Penzberg, Bavaria, Germany) with QIAzol® lysis reagent (QIAGEN, Hilden, North Rhine-Westphalia, Germany), RNA was extracted by RNeasy mini Kit (QIAGEN, Hilden, North Rhine-Westphalia, Germany) and included a DNase step according to the manufacturer’s instructions.

RNA was reverse transcribed using Transcriptor First cDNA Synthesis Kit (Roche, Penzberg, Bavaria, Germany) with oligo-dT primers (30 min at 55 °C, 5 min at 85 °C). Additional reverse transcriptase minus (−RT) controls were run to check genomic DNA contamination.

**Real-Time PCR (RT-PCR)**

Gene expression was measured by quantitative RT-PCR using the following reaction system: 2 µl cDNA was incubated in a final volume of 20 µl reaction containing 1 × SYBR-Green master mix and 50 ng of each primer (forward and reverse). Quantitative RT-PCR (qRT-PCR) was performed in Lightcycler® 480 (Roche, Penzberg, Bavaria, Germany), the information of primers for each gene is represented in Table 1. The relative amount of each gene in eWAT was normalized against the reference gene hypoxanthine phosphoribosyltransferase (HPRT). The relative amount of each gene in SCN and PVN was normalized against the reference gene heat shock protein 90 alpha (HSP90α). Reference gene expression was not significantly changed by the treatments and time.

**Statistical analysis**

Data are presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using SPSS version 19.0 (IBM, Armonk, NY). One way or two way ANOVAs were performed to detect the effects of Time (4 levels: ZT2, ZT8, ZT14 and ZT20), Treatment (sham-operated versus ADX) or Interaction. P values are considered statistically significant at p < 0.05. In addition, to test the daily rhythmicity of gene expression, data were analyzed using the Circwave 1.4 software (Oster et al., 2006; http://www.euclock.org/results/item/circwave.html). p values reported are the result of the F-test, and the 24 h rhythm was confirmed if p < 0.05.

**TABLE 1. Information of gene primers.**

| Type                     | Gene        | Forward primer                                           | Reverse primer                                           | Tm  |
|--------------------------|-------------|----------------------------------------------------------|----------------------------------------------------------|-----|
| Clock genes and clock-controlled genes | Bmal1       | CCATGAGACAGCCACGGACTGAA                                     | TGGTGATCGGAAAGAGATAGC                                    | 55  |
|                           | Cry1        | AATGGCTATGAGGAACGGACTGAA                                    | TCTATCTGGTCCGAGAACAGA                                    | 55  |
|                           | Cry2        | TGGATAACGGACTGAA                                            | TGTCAAGCTCCCCACGAGCAGA                                    | 60  |
|                           | Per1        | TGTTGCCGTGACACCCATGACTC                                     | TCTGTTAGATCCGAGATGTC                                    | 60  |
|                           | Per2        | CACCCCTGAAAGAAGATCGAGA                                     | CACGCGCAAGAAAGCTCAGA                                     | 55  |
|                           | Rev-erbα    | ACAGCTGACACACCCGATAGC                                      | CATGGCGATATTGAGAAGATTC                                    | 55  |
|                           | Dpb         | CCTTGGAGACACGACACCCGAGC                                    | TCCCTCTTCATGATTGGCAGA                                     | 55  |
| Metabolic genes          | Nampt       | GGGGGATGATAGGTCAGGACAGA                                     | GGCAGCGAGCAGGACTGAGA                                     | 55  |
|                           | Pparα γ     | CAGCAAGAACACAGCAGACATCA                                     | GGGGTAGATAGTGGATATCG                                      | 55  |
|                           | Fas         | CTTGGGTGCTCCGATACCCGAC                                      | GCCCTCCGTCATCCTACCA                                      | 55  |
|                           | Lip         | GAAAATCCACGAGGCTCAGGG                                       | AGCAATCTCCCAGGTTCG                                       | 55  |
|                           | Hsl         | CACACAGCTGAGGACTGAGA                                        | ACCCTCAGAAGCCGAGTCCG                                     | 55  |
|                           | Acc2        | GACGGCTCTGAGGACGAGC                                       | GAGCCCTCTGCTCAGATC                                       | 55  |
|                           | Glut4       | GGCGCTGAGGACGAGCTGAGC                                       | CAGGGCGAAGAAGCTGAGC                                     | 55  |
|                           | Sreb1f      | AACAGATTTGAGGACTGAGC                                       | TGGGCAACAGAGCCAGTTG                                      | 60  |
|                           | Cpt1b       | GTCTGGGAGAGGCTTCTGTC                                       | TGGTGGCAAGGCTTCTGTC                                       | 57  |
|                           | Leptin      | GCTTCTGCTGAGGACTCAGC                                       | GCCCGGTGCTTTGGGACA                                     | 55  |
|                           | Adiponectin  | ATCTCTGGTCGCTGAGGAC                                         | CATCTCTGCTGAGGACTC                                       | 60  |
|                           | Resistin     | ATCAAGCATCTGCTGAGGAC                                       | GTGACGCTTGGTCATGTC                                       | 60  |
|                           | Visfatin     | ACAGATATCTGCTGAGGACTGAGC                                   | TCGACAGATCTGAGGACTGAGC                                   | 60  |
|                           | Mr          | ACCCTGGAGTACCAATGCTGAC                                      | GGGAGGAAGACAGGAGTTG                                      | 55  |
|                           | Lipin1       | TCAATCTCGGTAGGAGGAGG                                       | TCAATCTCGGTAGGAGG                                       | 55  |
| Neuropeptide genes       | Vip         | CCAAGGAGGCGAGGAGGAGGAG                                     | GCCAGCGAGGAGGAGGAG                                      | 65  |
|                           | Oxt         | TGGGCAAGGCGGAAGGAGGAG                                       | AGGCATCGGGGACGAGGAG                                      | 65  |
|                           | Crh         | AAAGGGGAAGGCGAAAGAAA                                       | GTTTAGGGGGCGCATCCTG                                      | 55  |
|                           | Trh         | TCTGCTCAAGTCTCAGTCTC                                       | AGGCGAGGCAAGGACAA                                       | 55  |
|                           | Atp         | TCGCTGCTCAGTCTCAGTCTCAGG                                   | AGGGAGGACAGATTGTCAGTCTCAG                                | 60  |
| Housekeeping genes       | Hprt        | GCAGATACGCGCTGAGGAGGAG                                     | AAGAGGAGGCTGCTGAGGACTCAA                                 | 55  |
|                           | Hsp90αs      | CGGCGCCACCCCCGTCCTCAGTFA                                    | ACCAAATCTGCTGCAAGGACATAA                                 | 55  |

Rev-erbα: Rev-verbalpha (official full name: nuclear receptor subfamily 1, group D, member 1 (Nr1d1)). Dbp: albumin D-box binding protein. Pparα γ: peroxisome proliferator activated receptor alpha (official abbreviation: Ppara). Pparα: peroxisome proliferator-activated receptor gamma (official abbreviation: Pparγ). Fas: fatty acid synthase (official abbreviation: Fasn). Lpl: Lipoprotein lipase. Hsl: hormone sensitive lipase (official full name: lipase, hormone sensitive (Lipe)). Acc2: acetyl-Coenzyme A carboxylase 2 (official full name: acetyl-CoA carboxylase beta (Acacb)). Glut4: glucose transporter 4 (official full name: solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4)). Sreb1f: sterol regulatory element binding transcription factor 1. Cpt1b: carnitine palmitoyltransferase 1b. Visfatin also called Nampt: nicotinamide phosphoribosyltransferase.
RESULTS

Effects of adrenalectomy on body weight and food intake
Plasma corticosterone concentrations in ADX rats above 40 ng/ml (the average concentration of corticosterone at ZT2 in sham-operated animals) were considered to represent an incomplete adrenalectomy (n = 7, out of 32). The results from these animals were therefore discarded from further analysis. Bilateral adrenalectomy resulted in a significant decrease in weight gain. ADX rats only gained about 30 g over 3 weeks compared to about 75 g in Sham-operated animals (Table 2). Bilateral adrenalectomy also affected food intake: ADX rats ate less than sham-operated animals in the dark period, but no difference was observed during the light period (Figure 1D), i.e. both groups presented a clear day/night rhythm in food intake.

TABLE 2. Effects of adrenalectomy on body weight.

|   | Before surgery (g) | 3 weeks post surgery (g) | Body weight gain (g) |
|---|--------------------|--------------------------|----------------------|
| Sham | 256.6 ± 3.1 | 331.6 ± 3.0 | 75.0 ± 2.3 |
| ADX | 254.9 ± 3.6 | 284.6 ± 3.0* | 29.8 ± 3.3* |

Results are presented as mean ± SEM. *indicates p < 0.001 relative to Sham.

Effects of adrenalectomy on corticosterone, insulin and triglyceride levels in plasma
Plasma corticosterone levels were decreased and lost their significant effect of Time, after adrenalectomy (Figure 1A and Table 3). Also plasma insulin levels were lower and lost their significant effect of Time, after adrenalectomy (Figure 1B and Table 3). TG levels in plasma were slightly decreased in ADX rats, especially during the light period (Figure 1C and Table 3).

Effects of adrenalectomy on gene expression in the SCN and PVN
In the SCN, five clock genes, as well as Gr, Mr and two neuropeptide genes (Vip and Avp) involved in efferent projections to other brain areas were analyzed. According to the ANOVA results, Avp, Cry1, Cry2, Per1 and Per2 mRNA levels showed a significant effect of Time (Figure 2 and Table 4). Four out of the 9 genes

Table 3. Effects of adrenalectomy on plasma corticosterone, insulin and TG concentrations.

|   | Treatment | Time   |
|---|-----------|--------|
| Corticosterone | Sham | ADX | Interaction |
|      |         |    |          |
| Insulin |    |    |          |
| TG     |    |    |          |

Results are presented as p value. Significant effects are in bold.

FIGURE 1. Effects of adrenalectomy on plasma corticosterone (A), insulin (B) and TG (C) levels and 24-h food intake (D). Results are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Black bars - Sham-operated animals (n = 32, 8 rats/time point), grey bars ADX animals (n = 25, ZT2 (n = 5), ZT8 (n = 7), ZT14 (n = 7), ZT20 (n = 6)). For ANOVA analysis see Table 3.
studied showed a significant effect of adrenalectomy on their daily mean expression level. Gr and Mr 24-h mean expression was up-regulated in ADX rats compared to sham-operated rats. Cry2 24-h mean expression was up-regulated and lost its significant effect of Time, because of the bilateral adrenalectomy. On the other hand, bilateral adrenalectomy resulted in the appearance of a significant effect of Time in Vip mRNA expression, but did not change its average expression level. Per1, Cry2 and Vip showed a significant Interaction effect. For the Vip gene, this was due to a slight decrease during the light period and a slight increase during the dark period. For the Cry2 gene this was caused by an up-regulation at the end of both the light period and dark period. For the Per1 gene, this was due to a significant decrease at ZT14.

Bilateral adrenalectomy did not affect the expression pattern of any of the other genes tested, i.e. Per2, Bmal1, Avp and Cry1.

According to the results of the Circwave analysis (Table S1), only the Avp, Cry1 and Per2 gene showed a significant daily rhythm in the sham-operated animals. The expression of these three genes remained rhythmic after adrenalectomy. Per1, Bmal1 and Vip gene expression did not show a significant daily rhythm in sham-operated animals, but the expression of these three genes did show a significant daily rhythm in ADX animals. The Cry2, Gr and Mr genes did not show a significant daily rhythm in either sham-operated or ADX animals.

In the PVN we studied six genes involved in corticosterone release and one clock gene. According to the result of ANOVA analysis (Figure 3 and Table 5), only Per1 mRNA expression showed a significant effect of Time. Gr, Mr and Crh 24-h mean expression were...
up-regulated in ADX rats compared to sham-operated rats, whereas Oxt 24-h mean expression was down-regulated. After bilateral adrenalectomy none of the 7 genes investigated showed a significant effect of Time. Bilateral adrenalectomy did not change the expression level of Avp and Trh.

According to the result of the Circwave analysis (Table S2), only the Per1 gene showed a daily rhythm in sham-operated animals. The Per1 gene lost its daily rhythmicity after adrenalectomy.

**Effects of adrenalectomy on clock and clock-controlled gene expression in eWAT**

As expected Time showed a significant effect in all 7 clock genes studied in eWAT (Table 6). Per1 average expression level was strongly down-regulated in ADX rats compared to sham-operated rats. Both Per1 and Rev-erbα showed a significant Interaction effect. For the Per1 expression this was due to the strong down-regulation, but for the Rev-erbα this resulted from the expression levels changing at three time points. Cry2 expression was slightly up-regulated in the ADX rats (Figure 4 and Table 6).

Circwave analysis showed that the expression of all seven clock genes displayed a significant daily rhythm in sham-operated animals (Table S3), the rhythmicity of these seven genes was not changed in ADX animals, but the amplitude of all seven genes expression was decreased after adrenalectomy.

**Effects of adrenalectomy on expression rhythms of metabolic/adipokine genes in eWAT**

We studied 16 metabolic/adipokine genes in the eWAT. According to the result of ANOVA analysis, eleven out of the 16 metabolic genes investigated showed a significant effect of Time in their expression in the sham-operated animals (Figure 5 and Table 7). In eWAT, the average expression of Hsl, Gr and Mr was up-regulated in ADX rats compared to sham-operated rats, whereas Oxt 24-h mean expression was down-regulated. After bilateral adrenalectomy none of the 7 genes investigated showed a significant effect of Time. Bilateral adrenalectomy did not change the expression level of Avp and Trh.

According to the result of the Circwave analysis (Table S2), only the Per1 gene showed a daily rhythm in sham-operated animals. The Per1 gene lost its daily rhythmicity after adrenalectomy.
rats as compared to sham-operated rats; Leptin, Adiponectin and Resistin 24-h mean expression was
down-regulated after bilateral adrenalectomy. Adiponectin and Lpl gene expression showed a weak
effect of Time in sham-operated rats ($p = 0.051$ and $p = 0.064$), but not anymore in ADX rats. For Cpt1b, Glut4, Pparα and Pparγ gene expression, the bilateral
adrenalectomy also resulted in a loss of the significant effect of Time, but it did not affect the overall 24-h
expression level. Finally, bilateral adrenalectomy resulted in a significant down-regulation of Resistin
and Adiponectin average expression. Bilateral adrenalectomy did not affect the expression pattern of the
other six genes investigated, i.e. Fas, Lpl, Acc2a, Srebfl1, Visfatin and Lipin1. Therefore, according to ANOVA
adrenalectomy caused the disappearance of a significant effect of Time in 8 out of 11 genes.

Analysis according the Circwave method produced very similar results, i.e. 13 of the 16 genes studied
showed a significant daily rhythm (Lpl, Hsl, Acc2a, Glut4, Cpt1b, Leptin, Adiponectin, Visfatin, Gr, Mr, Lipin1, Pparα, Pparγ) (Table S4). All of these rhythmic
genes lost their daily expression rhythm after adrenalectomy, except for Acc2a and Visfatin. Resistin gene
expression showed a daily variation in the ADX animals but not in the sham-operated animals. Therefore,
adrenalectomy caused the disappearance of daily rhythmicity in 11 out of the 13 rhythmic genes (85%).

**DISCUSSION**

Glucorticoids are considered as an important output
signal used by the SCN to entrain and synchronize the
rhythms of peripheral oscillators. However, in the
present study, we found that all the seven clock genes
studied in eWAT did not lose their daily rhythm after
adrenalectomy. These data indicate that the daily
rhythmicity of the adrenal hormones is not very
important for the maintenance of daily clock gene
rhythms in eWAT. On the other hand, more than 80% of
the rhythmic metabolic/adipokine genes in eWAT lost
their daily rhythmicity after ADX. Together these data
dicate that adrenal hormones are important to main-
tain rhythmic metabolic/adipokine gene expression in
eWAT, they also indicate that rhythmicity of peripheral
clock genes is not sufficient to maintain tissue metab-
olism rhythmicity.

All of the seven clock genes we studied in eWAT still
showed a significant daily rhythm after adrenalectomy.
However, the amplitude of all the clock gene rhythms
came smaller after adrenalectomy. This result indi-
cates that adrenal hormones may primarily influence
the amplitude of clock gene rhythms, while their
absence does not obliterate the rhythmicity of clock
genes. Adrenal hormones are thus not necessary to
sustain the daily rhythm of clock genes in eWAT, which
indicates that the SCN synchronizes the daily rhythm of
clock genes in eWAT via other ways than the daily
corticosterone rhythm.

In addition to the seven clock genes we studied 16
other eWAT genes involved in adipocyte development
and energy metabolism. Thirteen out of these 16 genes
showed a significant daily rhythm in their expression
pattern. Eleven out of these 13 genes lost their daily
rhythm after adrenalectomy. These genes are involved
in adipocyte differentiation, fatty acid and glucose
uptake, fatty acid oxidation and triglyceride hydrolysis.

According to studies in mice and rats, PPARα, PPARγ,
LIPIN1, GR and MR are important for adipogenesis.
Previously it has been reported that PPARγ is an
important regulator of adipocyte differentiation, as it stimulates the differentiation from pre-adipocyte to adipocyte (Brun et al., 1997; Chen et al., 2007). Recently, it was found that PPARβ is also a necessary regulator of adipocyte differentiation increasing the rate of adipocyte formation (Goto et al., 2011). Koh et al. (2008) have demonstrated that LIPIN1 is required for the adipocyte differentiation. Corticosterone is also required for the differentiation of adipocytes, and its stimulatory action on adipocyte differentiation is mediated by the GR or MR (Caprio et al., 2007). Our results show that Pparα, Pparγ, Lipin1, Gr and Mr all lose their daily rhythm after adrenalectomy. Some studies found that adrenal hormones have a stimulatory effect on PPARγ expression levels (García-Bueno et al., 2008). Lemberger et al. (1994) reported that Pparγ gene expression is
TABLE 7. Effects of adrenalectomy on metabolic/adipokine gene expression rhythms in eWAT.

| Gene     | Sham | ADX | Treatment | Time | Sham | ADX | Interaction |
|----------|------|-----|-----------|------|------|-----|-------------|
| Fas      | 3.833| 4.769| 0.168     | 0.595| 0.388| 0.687| 0.563       |
| Lpl      | 23.321| 24.318| 0.464     | 0.051| 0.064| 0.514| 0.504       |
| Hsf      | 1.200| 1.364| 0.016     | 0.025| 0.019| 0.546| 0.326       |
| Acaca    | 0.006| 0.006| 0.972     | -0.001| 0.037| 0.007| 0.931       |
| Gt4      | 1.630| 1.772| 0.337     | 0.490| 0.003| 0.940| 0.593       |
| Srebf1   | 0.132| 0.116| 0.090     | 0.422| 0.130| 0.906| 0.323       |
| Cpt1b    | 0.366| 0.395| 0.196     | 0.092| 0.014| 0.716| 0.761       |
| Leptin   | 1.335| 0.540| -0.001    | 0.092| 0.046| 0.634| 0.227       |
| Adiponectin | 68.536| 58.900| -0.001   | 0.123| 0.051| 0.845| 0.297       |
| Resistin | 10.667| 8.038| -0.001    | 0.013| 0.238| 0.055| 0.988       |
| Visfatin | 9.527| 10.483| 0.003    | -0.001| <0.001| 0.009| 0.329       |
| Gr       | 0.134| 0.170| -0.001    | -0.001| <0.001| 0.001| 0.880       |
| Mr       | 0.010| 0.015| -0.001    | 0.003| 0.026| 0.138| 0.984       |
| Lipin1   | 0.226| 0.240| 0.293     | 0.003| 0.030| 0.046| 0.187       |
| Pparγ    | 0.132| 0.138| 0.403     | 0.015| 0.001| 0.767| 0.033       |
| Pparα    | 21.309| 21.201| 0.924    | -0.001| <0.001| 0.129| 0.150       |

Columns 2 and 3 show mRNA values normalized to HPRT. Columns 4–8 show significance levels for the effects of Treatment, Time and Interaction. Results in 4–8 are presented as \( p \) value. Significant effects are in bold.

Stimulated by corticosterone and follows the diurnal rhythm of circulating corticosterone. Zhang et al. (2008) reported that glucocorticoids have a stimulatory effect on Lipin1 gene expression and a GRE is present in the Lipin1 promoter. In addition, the changes in Gr and Mr expression after glucocorticoid withdrawal are well known (Han et al., 2007; Holmes et al., 1995; Noguchi et al., 2010; Svec et al., 1989). All together this makes the observed changes in Ppar γ, Lipin1, Mr and Gr gene expression after ADX not unexpected and suggests that adrenal hormones may be important to maintain the diurnal rhythm of adipocyte differentiation in the eWAT.

LPL hydrolyzes plasma TGs from chylomicrons and very low density lipoproteins (VLDL) and provides the released fatty acids to the adipocytes for storage and reesterification. Mice studies (Kim et al., 2013; Koh et al., 2008) have shown that LIPIN1 induces Ppar γ transcriptional activity and affects the function of PPARγ. PPARγ directly stimulates the expression of genes which are important for fatty acid uptake, such as Lpl, adipocyte fatty acid binding protein (aP2) and fatty acid transporter protein. Our results show that Ppar γ, Lipin1 and Lpl gene expression lose their daily rhythm after adrenalectomy. Glucose is an important substrate for the formation of glycerol and fatty acid in adipose tissue, so the uptake of glucose into adipose tissue is important for fat storage. Glut4 is one of the most important transporters for glucose uptake in adipose tissue and is also one of the genes that lost its daily expression rhythm without adrenal hormones. Piroli et al. (2007) reported that corticosterone inhibits the translocation of Glut4. Taken together the data suggest that adrenal hormones may be an important link in the mechanism necessary to maintain the daily rhythmicity of fatty acid and glucose uptake in the eWAT.

Fatty-acid oxidation in mitochondria is an important mechanism to provide energy for the body. CPT1b is one of the enzymes necessary to transfer long-chain fatty acid into mitochondria as a substrate for β-oxidation. ACC2 is located at the mitochondrial surface and catalyzes the acetyl-CoA to form malonyl-CoA, which inhibits the activity of CPT1b. Increased levels of ACC2 inhibit CPT1b activity and fatty acid oxidation (Wakil & Abu-Elheiga, 2009). Both LEPTIN and ADIPONECTIN can inhibit activity of ACC2 and increase fatty acid oxidation (Minokoshi et al., 2002; Yamauchi et al., 2002). Another important gene involved in fatty-acid oxidation is Ppar α. Ppar α stimulates the expression of Cpt1b and other genes involved in fatty-acid oxidation and increases fatty-acid oxidation (Goto et al., 2011; Zhou et al., 1999). HSL is one of the key enzymes for intra-adipocyte lipolysis as it governs the breakdown of TGs and the reduction of fat stores. As all of these genes lost their daily rhythm except for ACC2, the implication is that adrenal hormones are also required to maintain the daily expression rhythm of genes that are involved in fatty-acid oxidation and release.

Corticosterone is the best known and most studied rhythmically produced adrenal hormone. Several studies (Balsalobre et al., 2000; De Vos et al., 1998; Torra et al., 2000) have shown that corticosterone can affect the expression and daily rhythmicity of different genes in peripheral tissues, such as Per1, Per2, Rev-erb-α, Leptin. Therefore, corticosterone absence may contribute to the changes in gene expression pattern in WAT observed in the current study. Other studies (Campbell et al., 2011; Sakoda et al., 2000; Slavin et al, 1994; Xu et al., 2009) showed the effect of corticosterone on adipocyte differentiation, lipolysis and glucose uptake in a concentration dependent fashion. Our study showed that the genes involved in these functions lost their daily rhythm after adrenalectomy, therefore we speculate that the daily rhythm of glucocorticoid release is very important for the maintenance of a daily expression pattern in some of the metabolic genes in WAT. Besides corticosterone, the adrenal glands also produce many other hormones, such as aldosterone, catecholamines and androgens that may be released in a daily rhythm as well. Thus, based on the current set of experiments we cannot yet assign the effects observed to corticosterone alone, but also have to consider a possible contribution of other adrenal hormones. Also it is not clear yet whether it is the absence of corticosterone per se or the absence of its rhythmic release that is responsible for the effects observed. Therefore, for a definitive answer additional experiments using continuous and rhythmic corticosterone replacement regimens have to be performed.

Many studies (Dziribiková et al., 2011; Yambe et al., 2002) found that Agrp mRNA levels in the SCN show a 24-h rhythmic profile. In correspondence with these
results, we also found *Avp* mRNA expression to show a daily rhythm. *Avp* gene expression did not lose its rhythmicity after adrenalectomy, indicating that the daily AVP rhythm in the SCN is generated independently from the daily rhythm in adrenal hormones. AVP release from the SCN has an inhibitory effect on the HPA-axis, probably via gamma amino butyric acid (GABA)-ergic interneurons in the subparaventricular nucleus (subPVN) and dorsomedial nucleus of the hypothalamus (DMH) (Hermes et al., 2000; Kalsbeek et al., 2008). On the other hand, only a few studies indicated that circulating corticosterone levels may also affect AVP expression in the SCN (Isobe & Isobe, 1998; Larsen et al., 1994). Our results only show a trend towards increased AVP mRNA expression in the SCN after adrenalectomy.

Gozes et al. (1994) reported that *Vip* expression in the SCN was down-regulated by adrenalectomy. In the present study, we did not find a change in the 24-h average amount of *Vip* mRNA after adrenalectomy. However, we did find *Vip* expression to be reduced during the light period in ADX as compared to sham-operated rats and higher in the dark period in ADX compared to sham-operated rats. Together these changes resulted in an increased daily rhythmicity of *Vip* expression in the ADX rats. Gozes et al. (1994) sacrificed all animals during the light period, 10 days after the operation, whereas we sacrificed our rats at four different time points 23 days after the operation. These experimental differences might explain why our results are a little different, although both studies agree in showing a reduced expression of VIP during the light period after adrenalectomy.

Whether *Gr* and *Mr* are expressed in the SCN is still a controversial topic. Many studies investigated the expression of GR and MR in the brain, but did not report GR or MR expression in the SCN (Aronsson et al., 1988; Sousa et al., 1989; Sutanto et al., 1988). Two studies found GR expression only to be present in the SCN during the early neonatal period (Rosenfeld et al., 1988; Yi et al., 1994). In contrast, studies using immunohistochemistry to check GR or MR protein expression did find some signals in the SCN (Cintra et al., 1994a, 1994b; Morimoto et al., 1996). Morimoto et al. (1996) used *in situ* hybridization to check *Gr* and *Mr* mRNA expression and with this method found a signal in the SCN as well. Also in the current study, using qRT-PCR, we found *Gr* and *Mr* mRNA expression in the SCN. Because qRT-PCR is more sensitive than *in situ* hybridization (Bates et al., 1997), we assume that GR and MR mRNA are expressed at low levels also in the SCN. This idea is supported by the fact that the average expression level of GR and MR mRNA was up-regulated in the SCN after adrenalectomy.

In the SCN, *Cry1* and *Per2* gene expression showed a significant daily variation in sham-operated animals, similar to what was found in other studies (Asai et al., 2001; Girotti et al., 2009). Adrenalectomy did not affect the rhythmicity of these 2 genes in any way. However, surprisingly, we failed to find significant daily rhythms in *Per1* and *Bmal1* expression in sham-operated animals. The primary reasons for the results of *Per1* and *Bmal1* being different from what we expected are: (1) Most studies used D/D conditions at the time of sacrifice, we sacrificed our animals under L/D condition; (2) Most studies checked the daily rhythm of clock gene expression in the SCN by using *in situ* hybridization, whereas we used brain punches and q-PCR; (3) Most studies used more than four time points to check the daily rhythmicity of clock genes, but we just used four time points. Indeed when using the same conditions, i.e. L/D and brain punches, but sampling eight time points we do find significant rhythms in *Per1* and *Bmal1* expression (Supplemental Table S5–S7 and Supplemental Figure S1–S2). Surprisingly, in the ADX animals we did find a significant daily rhythm in *Bmal1* and *Per1* gene expression in the SCN, indicating that adrenalectomy increases the daily rhythmicity of these two clock genes. In fact, ADX strengthened the amplitude of all genes that showed a daily rhythm in the SCN of ADX animals. Interestingly, several clock genes are under the negative control of glucocorticoids (Surjit et al., 2011; Torra et al., 2000; Wuarin & Schibler, 1990), indicating that a removal of glucocorticoids may enhance their intrinsic rhythmicity and consequently that of related genes. However, this does not explain why similar ADX-induced changes were not observed in the PVN and eWAT. On the other hand, it is well known that even within the brain glucocorticoids can have differential effects on *Per2* rhythms in separate nuclei (Segall et al., 2006).

In the SCN, the expression of *Cry2* did not show a significant daily rhythm in sham-operated and ADX animals. The expression level of *Cry2* was slightly increased after adrenalectomy. AMP activated protein kinase (AMPK) is a mediator of metabolic signals and it sends this metabolic information also to clock genes, therefore it is considered as one of the factors that may affect the expression level of the *Cry2* gene after adrenalectomy. Corticosterone stimulates AMPK activity in the hypothalamus (Christ-Crain et al., 2008) and AMPK activation increases CRY protein degradation (Lamia et al., 2009), hence the AMPK activity may be affected after adrenalectomy, leading to changes in the *Cry2* expression.

In conclusion, we found that the expression pattern of *Atp*, *Per2* and *Cry1* in the SCN was not changed after adrenalectomy. The level of *Cry2* mRNA was increased in the SCN after adrenalectomy. The level of *Gr* and *Mr* mRNA were increased in both the SCN and PVN because of the adrenalectomy. In the PVN the levels of *Crh* and *Oxt* mRNA showed the expected changes with a significant increase and decrease, respectively, in the ADX animals. These data indicate that the SCN might be rather “protected” from changes in the adrenal hormones, but is certainly not completely blind to these changes.
The inhibitory effect of corticosterone on CRH synthesis in PVN neurons is well known (Ma et al., 2001; Young et al., 1986), thus the up-regulation of Crh expression in the ADX groups is completely in line with our expectations. Using in situ hybridization Cai & Wise (1996) found a daily rhythm in Crh mRNA expression in the dorsomedial PVN (dmPVN), but not in the whole PVN. In our study, we used qPCR to check the punched PVN, and did not find a significant daily variation in Crh mRNA expression. It is well known that the CRH neurons in PVN can be subdivided into several groups based on their anatomic location, projections and function (Cai & Wise, 1996; Swanson & Kuypers, 1980; Swanson & Simmons, 1989). The CRH neurons in the dmPVN project to the median eminence and release the CRH into the portal circulation to stimulate ACTH secretion. Other CRH neurons in the PVN project to the spinal cord and brain stem and possibly regulate the activity of the ANS. Apparently the rhythmic nature of the CRH neurons in the dmPVN is overwhelmed by the non-rhythmic nature of other CRH neurons in the PVN.

AVP in the PVN is produced in two types of neurons: the magnocellular (mPVN) and the parvocellular (pPVN) neurons. The magnocellular AVP neurons respond to osmotic stimuli, such as dehydration and salt loading, by releasing AVP via the posterior pituitary to function as an antidiuretic hormone. In part of the parvocellular neurons AVP is amongst others co-expressed with CRH. Itoi et al. (1987) showed that corticosterone suppresses the immunoreactivity of AVP in the pPVN, but not in the mPVN. Some other studies showed that the expression of Agrp in the pPVN is upregulated by adenectomy, but unchanged in the mPVN (Ferrini et al., 1997; Swanson & Simmons, 1989). However, AVP expression in the pPVN is much lower than in the mPVN (Kiss et al., 1984), thus although Agrp expression in the pPVN may be up-regulated after adenectomy in our study, using PVN punches, this pPVN increase will be masked by the much higher expression in mPVN. In addition, we did not find a significant daily fluctuation in the expression of Agrp in the PVN, similar to earlier studies (Dziribiková et al., 2011; Kalsbeek et al., 1995).

Oxt mRNA levels in the PVN did not show a daily rhythm. This finding is consistent with other studies that reported no daily rhythmicity in Oxt mRNA expression in the PVN and supraoptic nucleus (SON) (Burbach et al., 1988; Dziribiková et al., 2011). The lack of daily rhythmicity in the OXT system in the PVN is also supported by a microdialysis study (Kalsbeek et al., 1995). OXT from the magnocellular neurons of the PVN and SON is released from the posterior pituitary in the general circulation in response to a variety of stimuli (Kasting, 1988). More and more studies indicate that OXT release from the PVN also plays an important role in the stress response (Nishioka et al., 1998; Wotjak et al., 2001) and in feeding behavior (Arletti et al., 1990). Because OXT secretion is increased by stressful stimuli, and the upstream region of the rat Oxt gene contains a consensus sequence of the corticosterone enhancer region (Mohr & Schmitz, 1991), corticosterone may have a stimulatory effect on Oxt gene expression. This would explain why Oxt mRNA levels are slightly decreased in the ADX rats in our study.

Corticosterone has a modulatory effect on many brain functions. Besides the negative feedback action on the HPA axis, it also regulates the levels of neurotransmitter and neuronal excitability in many other brain areas (Meyer, 1985). All these functions of corticosterone in the brain are mediated by MRs and GRs. A number of studies found that GR and MR protein and mRNA expression in the brain is increased after adrenalectomy (Holmes et al., 1995). Other studies found that GR and MR protein and mRNA expression in the brain are reduced by stressful stimuli (Noguchi et al., 2010). In our study, Gr and Mr mRNA was up-regulated in both the SCN and PVN after adrenal removal, verifying the negative feedback of corticosterone on GR and MR expression. We did not find a significant daily rhythm in Gr or Mr mRNA expression in the SCN or PVN, even though some other studies found a circadian rhythm in GR and MR expression in the hippocampus (Herman et al., 1987).

As to Per1 it has been shown in several instances now that its rhythmic expression in the brain is affected by changes in circulating levels of adrenal hormone (Gilhooley et al., 2011). Yamamoto et al. (2005) reported that a glucocorticoid-responsive element (GRE) exists in the Per1 promoter region. Indeed Per1 gene expression is up-regulated by dexamethasone injections (Balsalobre et al., 2000). Thus, corticosterone seems to have a stimulatory effect on Per1 mRNA expression. Per1 gene expression in PVN showed a significant daily rhythm, with the peak expression at the early dark period (Dziribiková et al., 2011; Girotti et al., 2009), nicely correlating with plasma corticosterone levels. In the adrenalectomized animals, Per1 expression remained low at ZT14, causing the Per1 gene to lose its circadian rhythm. Also in the eWAT the daily expression pattern of Per1 was changed by the ADX. Corticosterone thus seems to be an important signal for the maintenance of a strong circadian rhythmicity of Per1 gene expression.

The pronounced effects of corticosterone on neuropeptide expression in the PVN, such as CRH and AVP, are well known (Itoi et al., 1987; Ma et al., 2001; Young et al., 1986). In addition, more recent studies (Gilhooley et al., 2011; Segall et al., 2006) have shown that the corticosterone rhythm is important for the maintenance of daily rhythms of Per1 and Per2 gene expression in several brain areas outside the SCN. Therefore, the absence of corticosterone in the ADX animals clearly has contributed to the currently observed changes in PVN gene expression.

Many studies have shown that a disturbed feeding rhythm changes the expression rhythm of peripheral (clock) genes (Balakrishnan et al., 2010; Salgado-Delgado et al., 2013). In our study, the daily rhythm of
food intake was not changed after adrenalectomy. This indicates that the loss of gene expression rhythms in eWAT is not caused by a change in the daily food intake rhythm. However, Kobayashi et al. (2004) showed that the mRNA levels of clock genes in the fasting group recovered to those in ad libitum group after refeeding, indicating that the amount of food intake may have some effect on clock gene expression. Food intake of ADX animals was decreased during the dark period, resulting in a lower amplitude of the daily feeding rhythm. Therefore, we cannot exclude that the decrease in the amplitude of the daily feeding rhythm contributed to the changes in the amplitude of the daily clock gene expression rhythms.

In summary, in this study we found that >80% of the genes that are important for adipose metabolism in eWAT lost their daily rhythm after adrenalectomy, but that the clock genes still kept their daily rhythmicity. This result is very similar to what Oishi et al. (2005) found in the liver and Fujihara et al. (2014) in bone after ADX. Together these results indicate that after adrenalectomy the daily rhythms of many metabolic genes are dissociated from that of clock genes in eWAT and liver. Apparently, the daily expression rhythm of many metabolic genes in eWAT and liver is synchronized by adrenal hormones without a necessary and obligatory role for the clock genes. Previously, we reported a similar finding for the autonomic nervous control of hepatic glucose metabolism, i.e. hepatic denervation caused a loss of the daily rhythm in plasma glucose concentrations, whereas hepatic clock gene rhythms were not affected (Cailotto et al., 2005, 2008). Thus many clock-controlled genes might be only clock-controlled in an indirect way. On the other hand, clock gene rhythms in eWAT and liver do not seem to depend solely on adrenal hormones or the autonomic nervous system to be synchronized with the environment, and thus may be synchronized via other (combinations of) hormonal, autonomic and behavioral pathways.

DECLARATION OF INTEREST

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Supplementary material available online

Supplementary Tables S1–S7

Figures S1–S2.