Daily rhythmicity in rat white adipose tissue

van der Spek, R.

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Faculteit der Geneeskunde

CONTENTS

General introduction

INTRODUCTION

The two elements in each organism that are absolutely fundamental, i.e., maintaining homeostasis and reproduction, are synchronized to the environmental light dark cycle by the circadian or biological clock. From an evolutionary perspective, natural selection has favoured organisms that could keep track of time. Clock systems can be found in plants, insects and mammals, but originally evolved in single cell organisms. In these single cell organisms, the clock helped to protect replicating DNA from damage by solar radiation and metabolic substances like reactive oxygen species, and to separate incompatible processes such as storing energy and releasing stored energy. These single cell organisms could not separate incompatible processes in space (i.e., over several cells or compartments), and therefore had to separate these processes in time. In multicellular organisms each cell still contains a clock, but many levels of complexity were added to synchronize all these clocks within and between tissues to ensure systemic homeostasis (Gerhart-Hines & Lazar 2015; Chaix et al. 2016). For example in mammals, the biological clock aligns daily patterns of food seeking behaviour and energy metabolism to the light dark cycle and food availability.

However, modern lifestyles provide our clocks with many mixed signals: we spend most of our time indoors where light intensity is much lower than outdoors, we use artificial light when the sun has set, many jobs require people to work in shifts across the 24-hour period, we travel across time zones rapidly, energy dense food is continuously and abundantly present and many people spend their days sedentary. This results in misaligned and reduced daily rhythms of light/darkness, activity/inactivity, sleep/wake and feeding/fasting, which together may significantly contribute to so-called lifestyle diseases; sleep disorders, mood disorders and disorders of metabolism such as obesity, diabetes mellitus type 2 and cardiovascular disease (Stenvers et al. 2019; Vetter 2020).

White adipose tissue is a key player in energy metabolism, as it not only functions as a storage facility for lipids but also produces many factors that regulate energy metabolism systemically. Over the last decades the prevalence of obesity has risen to epidemic proportions globally and contributes to significant morbidity and mortality. Obesity develops when energy intake exceeds energy expenditure. Reducing energy intake or increasing energy expenditure would therefore seem a simple remedy. However, it turns out to be very difficult to maintain energetic balance when food is always available and the necessity for physical activity is limited. Furthermore, both macronutrient content and timing of food intake independently influence metabolism and the circadian clock system. Therefore, maintaining metabolic health may involve more than just restoring the energy balance (Hawley et al. 2020).

A better understanding of the interplay between energy metabolism and the circadian clock system could help to develop strategies to limit the detrimental metabolic effects of circadian misalignment and the corresponding threat to health.

SCOPE OF THESIS

In **chapters 1 and 2** the circadian timing system is introduced as a system of key importance to maintain metabolic health. On a cellular level the clock consists of several feedback loops in gene expression and protein activity that repeat approximately every 24 hours. A number of factors contribute to the regulation of daily rhythms in systemic energy metabolism; the central brain clock in the suprachiasmatic nucleus (SCN), the autonomic nervous system (ANS), and circulating hormones (glucocorticoids, insulin, melatonin) and nutrients. For white adipose tissues (WAT) it was largely unknown whether these factors were involved, and which ones were of prime importance. Furthermore, it was unclear whether obesity is a cause or an effect of disturbed rhythmicity in white adipose tissue.

Therefore, the overall **aim of this thesis** was to improve our understanding of how daily gene expression rhythms in rat white adipose tissue are regulated.

Firstly, we investigated daily gene expression rhythms in undisturbed conditions. In **chapter 3** we analysed daily gene expression rhythms of both clock and metabolic genes, and compared these between several intra-abdominal and subcutaneous compartments.

Secondly, we investigated some of the factors that regulate WAT rhythmicity. In **chapter 4** we assessed the importance of the central brain clock and in **chapter 5** we examined whether adrenal hormones are necessary for maintenance of daily gene expression rhythms.

Lastly, in many obesity models, animals are overeating, which directly influences daily rhythmicity. Neonatal administration of monosodium glutamate (MSG) induces severe obesity while the animals actually eat less than their controls. Therefore, to assess the effect of adiposity without hyperphagia, in **chapter 6** we investigated daily rhythms in WAT gene expression in rats with MSG-induced obesity.

REMARKS ON DATA ANALYSIS

Several methods exist to analyse daily rhythmicity in data. In the studies described in this thesis we used both ANOVA and Circwave v1.4 software (www.hutlab.nl) to evaluate time-of-day differences between time points. Circwave fits a fundamental sinusoidal through the individual sample data points and then tests whether this curve is significantly different from a horizontal line through the data mean (a constant). Circwave provides the following information: number of sines in the fitted curve; data mean, the average of all data points with standard deviation (SD); Centre of Gravity (COG), the phase of the curve with SD; Anova F stat, p-value and R^2; Circwave F stat, p-value and R^2.

We considered a number of factors important when analysing rhythmicity, i.e., statistical differences over time between data groups (measured by ANOVA and Circwave), robustness and amplitude. We define 'robustness' of a rhythm as uniformity between cycles and/or animals measured by three characteristics, i.e., period, phase, and shape of wave. R^2 values indicate the goodness of fit, i.e., how well the curve generated by Circwave describes the data. High R^2 values thus indicate that individual samples deviate very little from the group average curve, and therefore show little variation in period, phase and shape of wave, and can be called robust. Amplitudes of the Circwave curve were calculated as a percentage of the difference between the zenith (highest point) and nadir (lowest point) and divided by the mean (max-min / mean * 100%).

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Circadian rhythms in white adipose tissue

van der Spek R
Kreier F
Fliers E
Kalsbeek A

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ABSTRACT

Adipose tissue is an important endocrine organ. It is involved in the regulation of energy metabolism by secreting factors (adipokines) that regulate appetite, food intake, glucose disposal and energy expenditure. Many of these adipokines display profound day/night rhythms, and accumulating evidence links disruption of these rhythms to metabolic diseases such as obesity and type 2 Diabetes. Here we briefly present the circadian system, describe the development of white adipose tissue (WAT) and its depot specific characteristics and innervation, we discuss energy storage in WAT and, lastly, review recent findings that link circadian rhythmicity to adipose tissue biology and obesity.

INTRODUCTION

The prevalence of obesity is taking on enormous proportions. Obesity is defined by the World Health Organisation as the accumulation of excessive fat tissue, to the point that it may impair health (WorldHealthOrganisation 2011). Fat, or white adipose tissue (WAT), is a metabolically active tissue and a key player in the regulation of glucose and lipid metabolism. Once the amount of fat has accumulated to such an extent that it hinders tissue function, insulin resistance of WAT may occur. Both human and rodent studies point towards an important role for the circadian timing system in energy metabolism. Disturbed day/night rhythms are closely correlated with the development of obesity and type 2 diabetes mellitus (T2DM). Here we briefly present the circadian system and describe the development of WAT and its depot specific characteristics and innervation, we discuss energy storage in WAT and review recent findings that link circadian rhythmicity to adipose tissue biology and obesity.

CIRCADIAN RHYTHMS

Regulation of rhythms in peripheral tissues

Since the discovery of the SCN in 1972, numerous neuro-anatomical tracing studies have been performed to reveal target areas for the circadian information generated in the SCN. Generally, these studies showed that the projection fibers from the SCN are surprisingly limited and mainly restricted to a few hypothalamic nuclei (Watts et al. 1987; Watts & Swanson 1987). In these neuro-anatomical tracing studies the hypothalamic paraventricular nucleus (PVN) showed up as an important target area of the SCN. Although the PVN is considered to be the neuroendocrine “headquarters” of the hypothalamus, it also contains a prominent population of neurons projecting to the autonomic nervous system, i.e., pre-autonomic neurons.

Within pre-autonomic neurons in the PVN there is a clear separation of neurons projecting to the sympathetic and the parasympathetic branch of the autonomic nervous system (ANS) (Buijs et al. 2003; Kreier et al. 2006). Via these ANS projections, the SCN is able to convey rhythmic information to various peripheral tissues. For example, the rhythmic release of melatonin by the pineal gland is controlled by SCN input to the pre-autonomic neurons in the PVN that regulate the sympathetic input to the pineal gland (Moore 1996). Besides this circadian control, the ANS may play a role in resetting peripheral clocks after phase shift-inducing light exposure (Cailotto et al. 2009).

However, peripheral rhythmicity is not solely entrained by the ANS. Although a complete autonomic hepatic denervation (both the sympathetic and parasympathetic branch) abolished the daily rhythm in plasma glucose concentrations, it had no overall effects on the molecular rhythms in the liver (Cailotto et al. 2005). Catecholamines like epinephrine and nor-epinephrine can modulate clock function in vitro (Reilly et al. 2008), however dopamine β-hydroxylase (Dbh, the enzyme that converts dopamine into nor-epinephrine) knockout mice displayed no disrupted molecular rhythms in peripheral tissues (Reilly et al. 2008). Moreover, Dbh-/- mice treated with α- and β-adrenergic receptor antagonists showed undisturbed molecular rhythms in heart, aorta, liver and brown adipose tissue (Reilly et al. 2008), thus excluding the possible compensatory effect of dopamine.

Parabiosis experiments have shown that non-neural signals suffice for the maintenance of circadian rhythms of clock gene expression in liver and kidney, but not in heart, spleen or skeletal muscle (Guo et al. 2005). Moreover, in the submaxillary glands, sympathetic input modulates, but did not determine, the phase of peripheral circadian oscillators (Vujović et al. 2008). Together, these data indicate that peripheral oscillators may receive multiple signals that contribute to their phase of entrainment.

Another example of this multiple signalling principle is found in the hypothalamo-pituitary-adrenal (HPA)-axis, where the SCN use a two-stage mechanism to control the daily rhythm in plasma corticosterone concentrations. Plasma glucocorticoid hormone levels exhibit robust daily oscillations, and glucocorticoids are potent Zeitgebers, both in vitro and in vivo (Balsalobre et al. 2000), by binding to the glucocorticoids response element (GRE) in the promotor region of Period1 (Per1) and possibly Period2 (Per2) (Balsalobre et al. 2000; Yamamoto et al. 2005; So et al. 2009). On the one hand the SCN acts on the corticotrophin-releasing hormone (CRH)-containing neuro-endocrine motorneurons in the PVN to bring about the release of adrenocorticotrophic hormone (ACTH) from the pituitary, while on the other hand it also acts –through the pre-autonomic neurons in the PVN and the ANS– on the adrenals and affects their sensitivity to ACTH (Kalsbeek et al. 2012; Lilley et al. 2012). Adrenal denervation abolishes the circadian corticosterone rhythm, the light-induced corticosterone secretion and the daily change in ACTH sensitivity (Ishida et al. 2005; Engeland & Arnhold 2005; Kalsbeek et al. 2012).

Next to innervation and hormonal control, regulation of the feeding-fasting cycle provides several indirect pathways for the SCN to entrain peripheral tissues. These pathways include daily rhythms in hormones secreted upon feeding and fasting (for example, cholecystokinin, peptide YY, ghrelin, leptin), changes in plasma concentrations of food metabolites (glucose, cholesterol, fatty acids, haem), post-prandial temperature elevations, and intracellular changes in redox state (see below) (Dibner et al. 2009).

At present, it is unclear to what extent each of these Zeitgebers contributes to the regulation of circadian rhythms in peripheral clock systems in general, or to white adipose tissue in particular. Figure 1 summarizes the different pathways via which the SCN could relay its timing information to WAT.

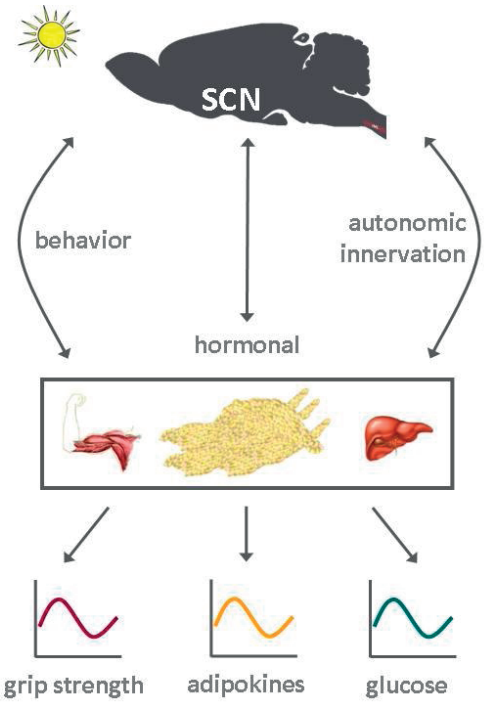

Figure 1. Three pathways via which the SCN could relay timing information to peripheral tissues.

Cellular timekeeping

Transcriptional-translational feedback model

Cellular timekeeping is regulated on several levels. On a genetic level, messenger RNA (mRNA) is synthesised from DNA (transcription). This mRNA may contain coding regions (exons) and non-coding regions (introns). To form a protein, introns are spliced out, so the ribosome can translate the mRNA into an aminoacid sequence, thereby producing a polypeptide (translation). Subsequent posttranslational modification attaches other biochemical functional groups (e.g. phosphates) to the polypeptide, to activate or inactivate the protein, while protein folding gives the polypeptide a 3-dimensional structure, enabling interaction with other proteins. Changes in the transcriptome do thus not necessarily reflect altered protein expression or function, and vice versa, as has been shown for the liver (Reddy et al. 2006).

In the transcriptional-translation feedback model, the molecular clock is based on a core loop formed by Clock:Bmal1 and Period 1-3 (Per1-3) and Cryptochrome 1 and 2 (Cry1-2). The Clock:Bmal1 heterodimer stimulates the transcription of Per1-3 and Cry1-2, and Pers and Crys subsequently heterodimerise, translocate to the nucleus, and inhibit Clock:Bmal1 activity. As a consequence, Clock:Bmal1 transcriptional activity drops, which reduces the transcription of Per and Cry genes, thereby activating Clock:Bmal1 again. Figure 2 shows a simplified model of this molecular clock mechanism.

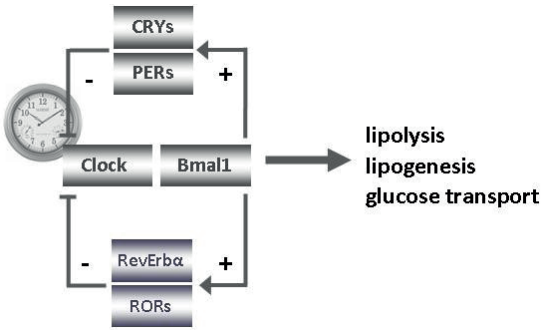

Figure 2. Simplified version of the molecular core clock mechanism. The core loop is formed by Clock:Bmal1 and Period 1-3 (Per1-3) and Cryptochrome 1 and 2 (Cry1-2). The Clock:Bmal1 heterodimer stimulates the transcription of Per1-3 and Cry1-2. Subsequently Pers and Crys heterodimerise, translocate to the nucleus, and inhibit Clock:Bmal1 activity. As a consequence, Clock:Bmal1 transcriptional activity drops, which reduces the transcription of Per and Cry genes, thereby activating Clock:Bmal1 again. Additional loops formed by RevErbs and RORs enhance the robustness of the core loop. Components of the core clock regulate energy metabolism, e.g. lipolysis, lipogenesis and glucose transport.

Additional loops enhance the robustness of the core loop. The retinoic acid-related orphan nuclear receptors, RevErbs and RORs, represent additional regulatory loops by binding to retinoic acid-related orphan receptor response elements (ROREs) on the Bmal1 promotor (Ko & Takahashi 2006).

Non-genetic clock

Cycles of transcription and translation feedback generally operate on a timescale of up to a few hours and maintaining an oscillatory rhythm on a daily basis thus requires a significant delay between activation and repression of transcription. Post-translational modifications such as phosphorylation, histone acetylation, methylation, sumoylation and ubiquitination affect the stability and nuclear translocation of core clock proteins, thereby delaying the cycle to approximately 24 h (Reppert & Weaver 2001; Cardone et al. 2005; Ko & Takahashi 2006; Gallego & Virshup 2007). A more extended review of the genetic clock can be found in (Ko & Takahashi 2006) and (Gallego & Virshup 2007).

Some observations cannot be explained sufficiently by this transcriptional-translational feedback model. In the last few decades several non-genetic pathways were found to play a role in cellular timekeeping.

Nicotinamide adenine dinucleotide (NAD) is involved in reduction-oxidation (redox) reactions; it can accept (becoming NADH), carry and donate electrons (returning to NAD). In vitro experiments have shown that the redox state of NAD can regulate DNA binding activity of the Clock:Bmal1 heterodimer. Interestingly, in vivo NAD levels are subjected to daily variations, thereby giving rhythmic input to the genetic clock (Merrow & Roenneberg 2001; Rutter et al. 2001; Asher & Schibler 2011), but there are also several indirect pathways via which the redox state can be linked to the clock. The enzymes silent information regulator protein (SIRT) and poly (ADP-ribose) polymerase 1 (PARP-1) are both NAD-dependent enzymes. SIRT is expressed rhythmically and interacts with Clock:Bmal1 heterodimers, leading to rhythmic deacetylation of Clock:Bmal1, histone H3 and Per2 (Asher & Schibler 2011). When subjected to daytime feeding, the liver of Parp-1-/- mice when compared with wild type mice showed a significantly delayed phase inversion of clock gene expression, suggesting that PARP-1 activity is indeed implicated in the phase entrainment of peripheral oscillators. However, PARP-1 activity is probably driven by feeding rhythms rather than by local circadian clocks (Asher & Schibler 2011).

The energy status of a cell influences the redox state, implying that via this pathway, food intake might be able to entrain the circadian system (Merrow & Roenneberg 2001; Arble et al. 2010; Maury & Brichard 2010).

Adenosine monophosphate-activated protein kinase (AMPK) activation has an impact on clock function through various mechanisms. AMPK senses the increase in AMP/ATP ratio in conditions that deplete energy, such as hypoxia, ischemia and glucose deprivation. During these conditions AMPK may activate pathways that generate ATP and suppress ATP consuming processes. For instance, in the hypothalamus, AMPK activity stimulates food intake, whereas in the periphery AMPK activity stimulates uptake and oxidation of fatty acids in addition to glucose uptake (Um et al. 2011). AMPK is part of the core clock mechanism by degrading Per2 (via CKIε), phosphorylating Cry1 and thereby decreasing its stability. In line with this concept, studies in mouse embryonic stem cells lacking both isoforms of the catalytic subunit (AMPKα1/α2-/-) and in mice lacking either AMPKα1 or AMPKα2, show altered free running periods, indicating that AMPK plays a fundamental role in the core clock (Um et al. 2011).

Cyclic adenosine monophospate (cAMP) is a further example of an acute signalling pathway that is tightly intertwined with the core clock. cAMP is a second messenger

derived from ATP, and is used for signal transduction in the cell, e.g., transferring the post-synaptic effects of hormones that cannot pass the cell membrane. In a series of elegant experiments, O'Neill et al. showed that cAMP is not solely an output of the SCN, but an integral component of the SCN pacemaker, regulating transcriptional cycles (O'Neill et al. 2008).

Haem has various biological functions; its most common function is as a component of haemoglobin, the red pigment in blood that transports oxygen and carbon dioxide. However, haem also functions as a ligand for the – formerly orphan – RevErbα and –β receptors. Binding of haem to the RevErbs enables the receptors to recruit NCoR, their co-repressor, and subsequently to repress the transcription of target genes (e.g. Bmal1), thereby regulating genes that are essential to the core clock. Furthermore, Per2 contains haem as a prosthetic group (Burris 2008), and Bmal1, Per1 and Per2 regulate the expression of aminolevulinic acid synthase 1 (Alas1), a rate- limiting enzyme in haem production. Moreover, PGC1α (a transcription factor that mediates transition towards FA oxidation and gluconeogenesis when glucose is low) directly regulates the expression of Alas1, identifying haem as another signalling molecule via which nutritional status can influence the core clock mechanism.

Intriguingly, circadian rhythmicity has recently been found in red blood cells, which have no nucleus or indeed any other organelles, and therefore do not undergo transcription or translation. Peroxiredoxins (PRX) are highly conserved anti-oxidant proteins that scavenge cellular reactive oxygen species, and in red blood cells PRX oxidation shows a clear circadian rhythm (O'Neill & Reddy 2011). This illustrates very clearly that in the absence of transcription, circadian rhythms remain observable in basic biochemical reactions. However, when PRX rhythms were measured in an arrhythmic mouse model (Cry1/2-/-), PRX rhythms differed from controls, implying that, although purely biochemical mechanisms are able to sustain 24-hour rhythms, they must normally reciprocally interact with gene expression cycles (Reddy & O'Neill 2011).

Taken together, these observations indicate non-genetic pathways that have a fundamental role in the core clock mechanism and represent molecular routes via which nutritional status can entrain the clock.

WHITE ADIPOSE TISSUE

Adipose tissue is one of the largest organs in the body, making up percentages of bodyweight from 5% in lean men to over 50% in the morbidly obese. In mammals, three

functionally different types of adipose tissue have been described; brown adipose tissue (BAT), white adipose tissue (WAT) and bone marrow adipose tissue (BMAT). The role of BMAT is poorly investigated. It appears to be related to the control of hematopoiesis and osteoblastogenesis by acting as an energy store, but also via the release of paracrine factors (Casteilla et al. 2008). BAT and WAT share the ability to store lipids as triglycerides, but use them for different purposes. BAT produces heat and plays an important role in non-shivering thermogenesis. WAT, besides functioning as mechanical and thermal protection of vital organs and as an important long-term energy store, secretes several proteins that influence processes as diverse as haemostasis, blood pressure, immune function, angiogenesis and energy balance (Christodoulides et al. 2009). Besides adipocytes, WAT comprises a stromavascular fraction including macrophages, mesenchymal stem cells (MSCs), pre-adipocytes, endothelial and epithelial cells.

WAT development

Adipocytes derive from MSCs cells residing in the adipose tissue stroma. The differentiation of MSCs into mature adipocytes (adipogenesis) occurs in two phases. The first phase, known as determination, involves the commitment of a pluripotent stem cell to the adipocyte lineage. Determination – an as yet poorly characterized process - converts the stem cell into a pre-adipocyte. The second phase is terminal differentiation, in which the pre-adipocyte takes on the characteristics of the mature adipocyte: it acquires the machinery that is necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte specific proteins. Terminal differentiation involves a cascade of transcriptional events, mainly regulated by CCAAT/enhancer-binding proteins (CEBPs) and peroxisome proliferator-activated receptor gamma (PPARγ) (Rosen & MacDougald 2006; Christodoulides et al. 2009). Each fat depot (see next paragraph) has a unique pattern of developmental gene expression and these differences are largely independent of the functional state (Gesta et al. 2007).

WAT depots

White adipose tissue is abundantly present throughout the body. The main depots in rodents can be divided in those found underneath the skin (subcutaneous; cervical, dorsal, lumbar, abdominal, mammary and gluteofemoral), and those found in the thoracic and abdominal cavities. These internal fat depots may be further subdivided in visceral and non-visceral. Visceral depots are found intrathoracic, intraperitoneal (omental, mesenteric and umbilical) and extra-peritoneal (retroperitoneal, perirenal, gonadal and urogenital). The non-visceral depots are mainly found in muscle tissue (intramuscular and perimuscular; a.o. orbital).

Among white adipocytes, cells from different depots may have distinct molecular and physiological properties (Rosen & MacDougald 2006; Poulos et al. 2010). A striking example of this is illustrated by the fact that, in humans, accumulation of excess fat in the visceral compartment carries increased metabolic risk, even if subcutaneous fat mass is normal (Yamamoto et al. 2010). Regional differences result from variations in molecular characteristics, such as expression of developmental genes, hormone receptor distribution, adipokine expression pattern and secretory profile, as well as from depot-specific innervation and vascularisation (Frühbeck 2008; Yamamoto et al. 2010; Turer et al. 2011). For instance, adipocytes in visceral depots are sensitive to lipolytic stimuli, whereas adipocytes from non-visceral depots do not release stored lipids easily (Rosen & MacDougald 2006). However, the proteome of visceral and subcutaneous WAT in humans displays more differences in the stromavascular cells than in isolated adipocytes, suggesting that besides intrinsic molecular characteristics in adipocytes, also innervation and vascularisation, and cellular heterogeneity in WAT are important to explain physiological differences between depots (Fain et al. 2004). Furthermore, WAT distribution is influenced by age, sex, and endocrine determinants such as growth hormone, cortisol and sex steroids, leading to an apple-shaped android (visceral) fat distribution in males and post-menopausal females, and a pear-shaped gynoid (subcutaneous) fat distribution in pre-menopausal females (Elbers et al. 1999).

WAT innervation

A role for the autonomic nervous system in the regulation of fat metabolism was proposed as early as the 1920s, as reviewed by Kreier and Swaab (Kreier & Swaab 2010). In 1921, the neurologist Klien presented a case of lipodystrophy (abnormal body fat distribution) and suggested a role for the hypothalamus in the development of this syndrome. Moreover, he hypothesized that dedicated regions of the hypothalamus would be involved in the accumulation of fat tissue while other regions would be involved in the mobilisation of energy stores (Klien-Leipzig 1921). It took nearly a century to provide some neuro-anatomical evidence for this concept. The sympathetic branch of the autonomic nervous system (ANS) regulates lipolysis (Bamshad et al. 1998). However, the proposed role for the parasympathetic innervation of WAT in the storage of lipids is still debated (Giordano et al. 2006; Kreier et al. 2006).

Sympathetic nerve fibers entering WAT mainly innervate blood vessels; only 2-3% of the adipocytes receive parenchymal innervation (Slavin & Ballard 1978). Nevertheless, these sparse nerve-endings might be able to affect many surrounding cells, and thereby potentially account for a functionally significant contribution to WAT regulation (T. J. Bartness, Shrestha, et al. 2010). Neuronal tracing methods have been a valuable tool in unravelling neuronal connections between the brain and WAT. The Bartness lab

reported the connection from brain to WAT via the sympathetic nervous system (SNS) in hamsters using pseudo rabies virus (PRV), which is a neuro-invasive retrograde viral tracer that can be transported between functionally connected neurons (Bamshad et al. 1998). Using two PRV strains with a different reporter, Kreier et al. identified separate neuronal pathways innervating different fat compartments (figure 3) (Kreier et al. 2006). These separate pathways allow a differential ANS drive to internal and subcutaneous

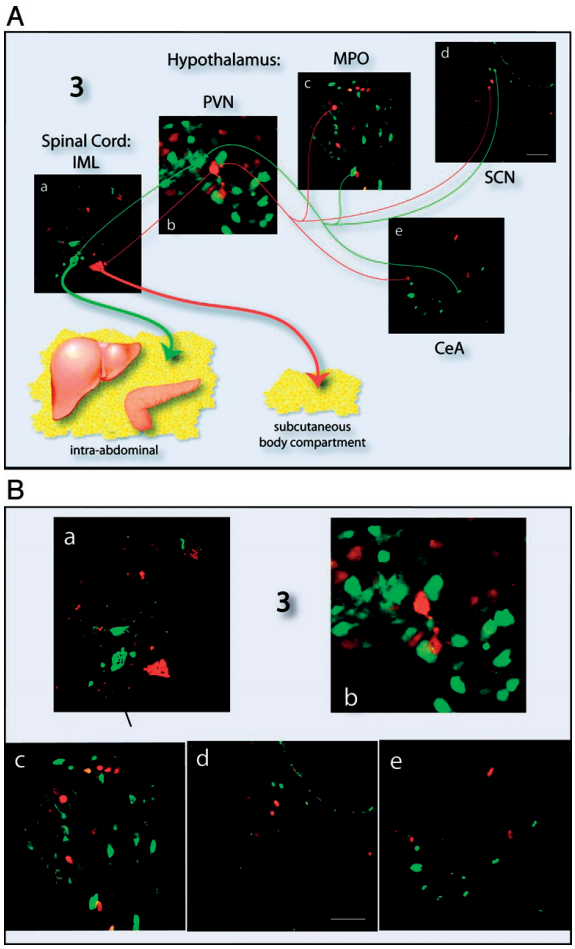

Figure 3. Separate neuron sets in the hypothalamus, amygdala, and spinal cord project to different body compartments. A, Five microliters of PRV B80 and PRV GFP were injected into parasympathetically denervated intraabdominal adipose tissue and in sc adipose tissue. The intermediolateral (IML) cell column of the spinal cord shows a separate control of the compartments; therefore, the survival time of the animals was chosen so that either only second order or third order neurons were labelled. B, In an upstream direction, the PVN of the hypothalamus shows specialised sets of neurons projecting to only one compartment. The same specialisation can be seen in the MPO (C), the central biological clock of the hypothalamus (SCN, D), and the amygdala (E). (Bar in SCN: IML/PVN = 50 µm; MPO/CeA/SCN, 100 µm) (Kreier et al. 2006).

fat compartments. Thus, these tracing data may provide a neuroanatomical basis for depot-specific differences in lipolytic rate among WAT depots, as seen in, e.g., Siberian hamsters exposed to short winter-like days (Bartness 1995), or in patients treated with pharmacological doses of glucocorticoids (Peckett et al. 2011).

Parasympathetic innervation of WAT was reported after injecting a retrograde PRV tracer in retroperitoneal WAT, in combination with surgical sympathetic denervation of the same WAT depot, inducing intense neuronal labelling in the dorsal motor nucleus of the vagus in rats (DMV) (Kreier et al. 2002). The DMV is the main cranial motor nucleus for the vagal nerve, which is the major parasympathetic nerve, and thus neuronal labelling in this nucleus implies parasympathetic innervation of retroperitoneal WAT. However, an experiment using chemical denervation in a different fat pad and in another species did not replicate this finding (Giordano et al. 2006), while immunohistochemical markers of parasympathetic nerves were shown to be absent in various fat pads of several animal models (Giordano et al. 2006).

In order to confirm the existence of parasympathetic innervation of WAT, evidence of the presence of parasympathetic ganglia within the adipose tissue, the presence of biochemical indicators of PNS (peripheral nervous system) innervation or the presence of neurochemical markers of PNS innervation are needed (T. J. Bartness, Shrestha, et al. 2010). Such histological or biochemical evidence is currently not available. However, several physiological studies suggest a role for the PNS in WAT metabolism. In cultured rat eWAT adipocytes acetylcholine decreased insulin-stimulated glucose uptake in a dose-dependent manner. This effect was blocked completely by atropine (non-selective muscarinic receptor agonist), and partially by 4-diphenylacetoxy-N-methylpiperdine methiodide (4-DAMP), a M3 muscarinic receptor antagonist. This suggests a role for the M3 receptor in insulin-mediated glucose uptake (Liu et al. 2004; Yang et al. 2009). Moreover, pharmacological observations in human subcutaneous adipose tissue microdialysis studies showed cholinergic effects on lipolysis (Andersson & Arner 2001). In addition, functional studies provided evidence for the anabolic function of parasympathetic innervation of adipose tissue, as euglycemic hyperinsulinemic clamp studies revealed a >30% reduction in the insulin-mediated uptake of glucose and FFAs in rat adipose tissue after selective removal of its parasympathetic input. Furthermore, the activity of the catabolic enzyme hormone sensitive lipase (HSL) increased by 51% in the denervated adipose tissue (Kreier et al. 2002).

As well as receiving information from the CNS, WAT also provides neural feedback to the brain via sensory nerves. In 1987, Fishman and Dark showed that application of the anterograde tract tracer True Blue in rat inguinal WAT (iWAT) or dorsosubcutaneous WAT

(dWAT) stained neurons in the dorsal root ganglia (Fishman & Dark 1987). Substance P and calcitonin gene-related peptide (CRGP) were identified as neurotransmitters in these neurons (T. J. Bartness, Shrestha, et al. 2010), and by using the H129 strain of herpes simplex virus-1, a viral anterograde transneuronal tract tracer injected into iWAT and eWAT of Siberian hamsters, Song and colleagues identified the spinal cord and brain sites that receive this sensory input (Song et al. 2009). Earlier, labelling in the gracile nucleus – the sensory nucleus in the brain stem involved in the perception of information from the lower body – had been found using cholera toxin B (CTB), which is a monosynaptic anterograde neuronal tracer (Kreier et al. 2006). This observation was later confirmed with the H129 anterograde tracer (Song et al. 2009).

The function of these sensory nerves is not fully understood; however, studies by Song et al. suggest that sensory nerves could be informing the brain about lipid reserves, which might be the neural equivalent of the humoral information that leptin provides to the brain (Song et al. 2009; T. J. Bartness, Vaughan, et al. 2010).

Regulation of energy storage

Lipogenesis

The adipocyte is unique among cells in that one organelle, the lipid droplet, can encompass over 95% of the entire cell body. This lipid droplet serves as a storage vessel for triglycerides produced by lipogenesis and released through lipolysis (Trujillo & Scherer 2006). Lipogenesis is the anabolic process by which simple sugars together with glycerol are converted to fats. Simple sugars such as glucose are converted to pyruvate by glycolysis, and subsequently pyruvate dehydrogenase converts pyruvate into acetyl-CoA. Following the formation of acetyl-CoA, fatty acid synthesis (FAS) combines glycerol with 3 acetyl-CoAs to form triglycerides, which are transported from the liver as very low-density lipoproteins (VLDL), to be stored in the adipose tissues.

Bar the proposed role of the PNS, lipogenesis is regulated by insulin. Insulin plasma levels increase proportionally with rising blood glucose levels. Thus, high insulin levels are associated with the fed state and might therefore be expected to increase the storage of energy. Insulin stimulates lipogenesis in two main ways, namely by 1) the upregulation of pyruvate dehydrogenase and 2) the dephosphorylation (activation) of acetyl-coA carboxylase (ACC).

Much of what we know about lipogenesis has been elucidated through the study of thiazolidinediones (TZDs), a class of anti-diabetic drugs that improve insulin sensitivity. TZDs increase the expression of PPARs and thereby promote energy storage. Upregula-

tion of PPARs in the adipocyte results in upregulation of lipoprotein lipase (LPL), fatty acid transporter protein, adipocyte fatty acid binding protein, malic enzyme, glucokinase and the GLUT4 glucose transporter (Trujillo & Scherer 2006).

In obese patients, lipids may be stored ectopically in non-adipose tissues, including the pancreas, heart, liver, kidney and blood vessel wall. This process and its deleterious consequences, termed lipotoxicity, has been implicated in β-cell loss during the progression of type 2 diabetes, and in the pathogenesis of diabetic complications through loss of cardiomyocytes, hepatocytes, renal parenchymal cells and endothelial cells (Brookheart et al. 2009).

Lipolysis

Lipolysis is the catabolic process leading to the breakdown of triacylglycerols (TAGs) into FFAs and glycerol. After release into the blood, FFAs are transported and taken up by other tissues to be utilized for β-oxidation and subsequent ATP generation. Some FFAs do not leave the fat cell and are re-esterified into intracellular TAG. During lipolysis, intracellular TAG undergoes hydrolysis through the action of three major lipases: adipose triglyceride lipase (ATGL/desnutrin/ phoshopsholipase A2ζ), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL). ATGL hydrolyses TAGs into diacylglycerol (DAG) and one FA, followed by HSL converting DAG into monoacylglycerol (MAG) plus one FA, MSG then hydrolyses MAG to produce glycerol and a third FA (Lafontan & Langin 2009; Ahmadian et al. 2010).

Lipolysis is regulated by the autonomic nervous system (T. J. Bartness, Shrestha, et al. 2010), and by several humoral factors, such as catecholamines (phosphorylation of HSL), glucocorticoids (upregulation of ATGL), natriuretic peptides, and growth hormone (Lafontan & Langin 2009; Ahmadian et al. 2010). While systemic regulation of lipolysis has been relatively well characterized, much remains to be investigated regarding the local regulation of lipolysis in adipocytes by (autocrine/paracrine) factors. Adipocytes secrete several factors able to regulate lipolysis locally, such as TNFα, which stimulates lipolysis, and adenosine, which inhibits lipolysis (Ahmadian et al. 2010).

Circadian rhythms in WAT

Like most other tissues, WAT gene expression shows circadian rhythmicity (Ando et al. 2005; Ptitsyn et al. 2006). Although diurnal variations in adipose tissue metabolism are undisputedly regulated by neurohumoral factors, the circadian clock within the adipocyte probably plays a significant role as well, by altering the sensitivity of the adipocyte to specific stimuli throughout the day (Bray & Young 2007). Indeed, several rodent models with alterations in whole body clock gene expression display disturbed lipid

metabolism (Shimba et al. 2005; Grimaldi et al. 2010; Gimble et al. 2011). For instance, serum leptin levels increased during the light phase in ClockΔ19 mutant mice fed a regular diet, and this increase was enhanced in mice fed a high-fat diet (Turek et al. 2005). Moreover, both in vitro and in vivo, lack of Bmal1 results in reduced differentiation of adipocytes and reduced lipid storage in the adipocytes (Bunger et al. 2005; Shimba et al. 2005; Kondratov et al. 2006; Shimba et al. 2011). ClockΔ19 mutant mice on a C57BL/6J background have hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and hypoinsulinemia. However, the severity of the Clock Δ19 phenotype is dependent on the genetic background of the mice (Oishi et al. 2006; Kennaway et al. 2012). Furthermore, Per2-deficient mice display altered lipid metabolism with a drastic reduction of total triacylglycerol and non-esterified fatty acids. Per2 exerts its inhibitory function by blocking PPARγ recruitment to its target promoters and thereby its transcriptional activation (Grimaldi et al. 2010). Yet RORα mutant mice resist obesity when placed on a high-fat diet (Lau et al. 2008). In sum, these observations demonstrate a tight relationship between genes of the core clock mechanism and lipid metabolism.

WAT plays a central role in the regulation of energy metabolism, mainly via the secretion of factors (adipokines) that regulate appetite, food intake, glucose disposal and energy expenditure (Wang et al. 2008). Adipokines are secreted by adipocytes and/or the stromavascular fraction of WAT. Originally the term adipokine described cytokines secreted specifically from adipocytes; however, as many cell types in adipose tissue have been found to secrete proteins, and other proteins besides cytokines are being produced, the term adipokine now incorporates all proteins secreted from adipose tissue (Wang et al. 2008; Stryjecki & Mutch 2011).

Extensive reviews on the metabolic functions of adipokines can be found in (Trujillo & Scherer 2006; Halberg et al. 2008; Maury & Brichard 2010; Poulos et al. 2010).

Leptin

Leptin is a hormone secreted by adipose tissue in proportion to body fat amount and relays fat storage information to the brain. High levels of leptin signal satiety and reduce food intake, whereas low levels of leptin stimulate food intake (Schwartz et al. 2000). The discovery that leptin could regulate body weight through effects on food intake and energy expenditure represented a major breakthrough in our understanding of the neuro-anatomical and molecular components of the systems involved in energy homeostasis (Farooqi 2011). The prevalence of mutations in this system range from 0,5%-1% in adult obesity up to 6% in subjects with severe obesity starting in childhood (Larsen et al. 2005; Farooqi 2007). For the discovery of this previously unknown endocrine system,

Coleman and Friedman received the Albert Lasker Award for Basic Medical Research in 2010 (Flier & Maratos-Flier 2010).

Plasma levels of leptin are regulated by the biological clock, leading to a clear day/night rhythm. Furthermore, WAT mass and feeding regulate leptin plasma levels; long periods of fasting eliminate the leptin rhythm (Elimam & Marcus 2002). However, under constant and continuous feeding conditions, a circadian rhythm in leptin persists, indicating a role for the circadian clock in regulating leptin levels during fed conditions (Simon et al. 1998; Kalsbeek et al. 2001). In healthy volunteers, misalignment between behaviour and endogenous circadian timing leads to lower overall leptin levels (Scheer et al. 2009), suggesting that leptin responds to the endogenous circadian clock, independent of behavioural factors such as feeding. Although SCN lesions eliminate leptin circadian rhythmicity (Kalsbeek et al. 2001), cultured adipocytes still show rhythmic leptin mRNA expression, implying regulation by an endogenous clock within the adipocytes (Otway et al. 2009). Figure 4 shows plasma leptin levels in rats under varying conditions.

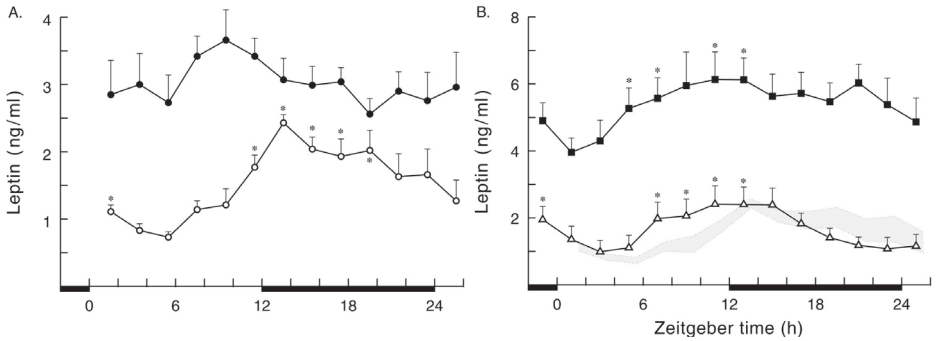

Figure 4. Diurnal plasma leptin profiles in different experimental conditions. The diurnal plasma leptin profile (mean± SEM) of nonoperated control animals (n = 7; ○) is compared with that in SCN-lesioned (n = 9; •; A) or ADX+Cort (n = 8; ▵) and regular-fed (n = 6; ▪) animals (B). Asterisks indicate that plasma leptin values are significantly different ($P < 0.01$) from trough values between ZT0 and ZT6. In B, the shaded area indicates the mean ± SEM for the nonoperated control animals. (Kalsbeek et al. 2001)

In addition to being regulated by the clock, leptin serves as an input factor for the biological clock, as the leptin receptor is expressed in SCN cells, and in vitro leptin can advance the SCN (Prosser & Bergeron 2003).

In sum, leptin is a pivotal factor in the interplay between feeding cues, metabolic state and circadian timing.

Other adipokines

Besides leptin several other adipokines exhibit significant day/night rhythmicity. Adiponectin is an adipokine that is involved in glucose and lipid metabolism by increasing fatty acid oxidation and potentiating insulin-mediated inhibition of hepatic gluconeogenesis, thus promoting insulin sensitivity (Barnea et al. 2010). Interestingly, although adiponectin is produced by adipose tissue, its serum levels and WAT gene expression decrease in obesity and in animals fed a high fat diet (Boucher et al. 2005; Barnea et al. 2010; Turer et al. 2011).

Both in vitro and in vivo, adiponectin has a significant day/night rhythm (Gavrila et al. 2003; Otway et al. 2009; Scheer et al. 2009; Barnea et al. 2010; Scheer et al. 2010; Garaulet et al. 2011), with a trough at night for humans, and a trough during daytime for rats (Oliver et al. 2006; Scheer et al. 2010). In lean men, this rhythm is not driven by the feeding/fasting cycle (Scheer et al. 2010). Clock Δ19 mutant mice that retain melatonin rhythmicity (Clock Δ19 +MEL) show increased eWAT adiponectin gene expression, which may contribute to the improved insulin resistance found in Clock Δ19 ¬+MEL mice compared to Clock Δ19 mice (Kennaway et al. 2012).

Resistin is a cytokine that is produced in WAT (adipocytes in rodents, macrophages in human) and is a potential mediator of T2DM and cardiovascular disease (Fain et al. 2003; Ando et al. 2005; Oliver et al. 2006; Schwartz & Lazar 2011), with higher expression rates in omental versus subcutaneous WAT of obese female subjects (Fain et al. 2003). Resistin mRNA expression is rhythmic in several WAT compartments in rats, with a peak in the late dark/early light phase (Oliver et al. 2006). Resistin is down-regulated by fasting and up-regulated by (re-)feeding (Oliver et al. 2006). However, WAT gene expression levels of resistin are decreased in obese and high-fat diet-fed mice (Boucher et al. 2005). Rotating shift workers have elevated plasma levels of resistin compared to day work controls (Burgueño et al. 2010).

WAT circadian rhythms in obesity

Obesity and disturbed rhythmicity in WAT are tightly correlated. For example, in humans, chronic desynchrony of internal circadian time with the external environment (e.g. during shiftwork) correlates with increased incidence of obesity and type 2 diabetes (Pan et al. 2011). Furthermore, circadian misalignment in healthy subjects decreases leptin levels (Scheer et al. 2009). In visceral and subcutaneous WAT biopsies of severely obese men, the expression level of clock genes was correlated with waist circumference (Gómez-Abellán et al. 2008). However, when gene expression was measured in human sWAT explants across a 24-h cycle, no differences in expression rhythm of clock genes were observed between overweight study participants and type 2 diabetes participants

or lean controls. The authors suggest this might be due to the early stage of the disease and the high level of glycemic control in the group of participants. However, in cultured WAT of severely obese women, slight differences between visceral and subcutaneous fat compartments in acrophase and amplitude of the expression of several clock and metabolic genes have been found (Martínez-Agustin et al. 2010; Garaulet et al. 2011). Together, these results indicate that circadian WAT rhythms may be disturbed in obesity, and that results from one WAT compartment cannot be downright extrapolated to another WAT compartment.

Due to limited sampling possibilities in human experiments, most of the research on the relationship between obesity and WAT rhythms has been done in animal models. Below, we describe 3 rodent models with obesity due to altered leptin function.

Ob/ob mice

Ob/ob mice exhibit a mutation in the leptin gene, rendering leptin unable to bind to its receptors. As a result, these mice become severely obese, with hyperphagia, hyperglycemia, hyperinsulinemia, high levels of corticosteroids, hypothyroidism, dyslipidemia, decreased body temperature, defective thermogenesis and infertility due to hypogonadotropic hypogonadism. The broad and severe behavioural, metabolic and neuroendocrine changes in these animals represent the classic starvation response designed to protect viability, and can all be remedied by leptin administration (Robinson et al. 2000; Arble et al. 2010).

The mutated leptin gene in ob/ob mice results in disturbed sleep and attenuates diurnal and overall locomotor activity. However, reports are indecisive on the effect of the mutation on the day/night pattern of food intake (Laposky et al. 2006; Ando et al. 2011). Daily mRNA expression rhythms of clock genes in the SCN are unaffected; yet the daily rhythms of these genes were substantially damped down in the liver and eWAT of ob/ob mice (Ando et al. 2011). Daily leptin injections modestly but significantly improved the mRNA expression rhythms of subsets of clock genes in liver and eWAT as they did the activity levels during the dark phase. At present it is unclear whether the increased nocturnal activity is a prerequisite for the improved rhythms in liver and eWAT clock genes.

Db/db mice

Db/db mice have a mutation in the long form of the leptin receptor, leading to ineffective leptin signalling and thereby to a phenotype similar to the ob/ob mutation. These mice are characterised by impaired regulation of core components of the clock mechanism in WAT, possibly due to impaired AMPK and SIRT1 function (Caton et al. 2011). Clock, Bmal1 and Per2 mRNA and/or protein levels are decreased; treatment with the anti-diabetic drug metformin reversed these decreases (Caton et al. 2011).

Obese Zucker rat

A third rodent model manifesting altered leptin signalling is the Zucker rat. These rats are relatively insensitive to leptin, again due to a mutation in the long form of the leptin receptor, and consequently have a similar phenotype to ob/ob and db/db mice. As early as 1977 Becker & Grinker observed that the normal pattern of predominantly nocturnal feeding was absent in the Zucker rat (Becker & Grinker 1977). A more detailed study 10 years later found no significant differences between obese (fa/fa) and lean (Fa/-) Zucker rats in light and dark feeding, expressed as a percentage of 24h intake. The increased food intake of the Zucker was mainly due to an increase in meal size (Alingh Prins et al. 1986; Fukagawa et al. 1988). Nevertheless, some circadian disturbance was noted: only in Zucker rats was a significant difference observed between meal sizes in the light and dark phase, and Zucker rats ate fewer but larger meals during the first half of the dark phase. Body temperature, activity and feeding rhythms were phase-advanced in obese Zucker rats, probably because of altered SCN resetting in response to light (Fukagawa et al. 1992; Mistlberger et al. 1998). All in all, the conclusion after almost 20 years of research was that, although the circadian amplitude of daily temperature and activity rhythms in obese Zucker is depressed, obese rats do exhibit normal entrainment and pacemaker functions in the circadian timing system (Murakami et al. 1995).

Moreover, more recently it was shown that, compared to control animals, clock gene expression rhythms were damped down in the liver of Zucker rats, but not in the SCN, mesenteric WAT, and heart. The authors suggest that leptin may play a role in the regulation of the clock in the liver (Motosugi et al. 2011).

CONCLUSION

The role of the circadian timing system in adipose biology represents an exciting new field of study that will give us a greater insight into the pathogenesis of obesity and its health consequences. The discovery of the ob/ob gene and its product leptin, along with the other adipokines, revealed an intriguing endocrine system that regulates feeding and metabolism via communication between adipose tissue and the biological clock in the brain. The monogenetic mutations in this system leading to obesity have taught us valuable lessons on the regulation of energy metabolism. Moreover, there seems to be a clear link between circadian misalignment and metabolic disorders, such as obesity and type 2 diabetes. In view of the recent development of a 24/7h society, combined with globalisation with its increased inter-time zone travelling, it will be increasingly important to expand our knowledge on the health consequences of circadian misalignment.

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2

Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis

Kalsbeek A
van der Spek R
Lei J
Endert E
Buijs RM
Fliers E

Molecular and Cellular Endocrinology, 2011

ABSTRACT

The pronounced daily variation in the release of adrenal hormones has been at the heart of the deciphering and understanding of the circadian timing system. Indeed, the first demonstration of an endocrine day/night rhythm was provided by Pincus in 1943, by showing a daily pattern of 17-keto-steroid excretion in the urine of 7 healthy males. Twenty years later the adrenal gland was one of the very first organs to show, in vitro, that circadian rhythmicity was maintained. In the seventies, experimental manipulation of the daily corticosterone rhythm served as evidence for the identification of respectively the light- and food-entrainable oscillator. Another 20 years later the hypothalamo-pituitary-adrenal (HPA)-axis was key in furthering our understanding of the way in which rhythmic signals generated by the central pacemaker in the hypothalamic suprachiasmatic nuclei (SCN) are forwarded to the rest of the brain and to the organism as a whole. To date, the adrenal gland is still of prime importance for understanding how the oscillations of clock genes in peripheral tissues result in functional rhythms of these tissues, whereas it has become even more evident that adrenal glucocorticoids are key in the resetting of the circadian system after a phase-shift. The HPA-axis thus still is an excellent model for studying the transmission of circadian information in the body.

HISTORICAL INTRODUCTION

The continuous rotation of the earth around its own axis combined with the earth's continuous revolution around the sun generates alternating 24h-cycles of light and dark. As this has always been a feature of this planet, evolution has equipped almost all organisms with an elaborate intrinsic timing system, the so-called biological clock, to be able to deal with these recurring changes. Biological clock mechanisms evolved both in single-cell algae and fungi that are directly exposed to sunlight, and in animals, such as fruit flies and zebrafish, where light easily penetrates all tissues. However, evolution, with the resulting increase in size and complexity of organisms, led to a greater centralization of the biological clock and its light receptive elements. In mammals, a central pacemaker evolved in which the dominant clock elements are concentrated in the central nervous system (CNS). Initially the phrase "biological clock" was coined to describe the physiological entity responsible for navigation in migratory birds, but it has since come to refer to the central timing system that is responsible for the generation of endogenous rhythms with a period close to 24 hours (i.e. circadian) that persist in constant conditions.

A primary role of the biological or circadian clock is to entrain the organism to environmental cues, so that an animal is able to anticipate fluctuations in the environment and determine key issues such as food availability, predator risk, and the likelihood of reproductive success. Furthermore, the circadian system is critical to the synchronization and relative phasing of various internal physiological processes. Such an internal coordination is essential for the optimization of responses to environmental fluctuations and for the strengthening of homeostatic control mechanisms. The realization that humans and animals are able to maintain a sleep/wake cycle of about 24-hours without any environmental clue led to a search for the location of the "master clock" or "central pacemaker". The description of a diurnal variation in the excretion of urinary ketosteroids by Gregory Pincus in 1943 was the first demonstration of a daily rhythmicity in any endocrine parameter. Observations by Andrews & Folk in 1964 -the endocrine era in the midst of the previous century- that circadian rhythms persisted in isolated endocrine glands, including the adrenal gland, supported the idea that the rhythms of these hormone glands were responsible for driving the rhythm of the organism. However, as removal of individual endocrine glands did not abolish the overall rhythm of the organism, the attention shifted from the endocrine system to the CNS. The common idea evolved that from lower vertebrates to mammals the crucial circadian clock elements had moved into the CNS and had become concentrated close to or within the light-transducing parts of the CNS. The breakthrough in the search for these crucial clock structures was the finding in 1972 that retinal fibers were not only directed to the lateral geniculate nucleus

in the thalamus, but also to a small nucleus in the anterior hypothalamus, the suprachiasmatic nucleus (SCN) (Hendrickson et al. 1972; Moore & Lenn 1972). In the same year, subsequent selective destructions of the SCN resulted in a complete disappearance of, among other things, the circadian rhythm in adrenal corticosterone content (Stephan & Zucker 1972; Moore & Eichler 1972): the CNS master clock had been found! Since then, numerous neuro-anatomical tracing studies have been performed to reveal the target areas for the circadian information generated in the SCN. Generally these studies showed that the projection fibers from the SCN are surprisingly limited and by and large restricted to a few hypothalamic nuclei (Watts et al. 1987; Watts & Swanson 1987). In subsequent studies aimed at unraveling the chemical nature of these timing signals and the pathway by which they were propagated further downstream from the SCN, the circadian activity of the HPA-axis again played an essential role (Kalsbeek et al. 1992). The discovery in the 1990s of molecular clock mechanisms in many peripheral organs, including endocrine glands such as the adrenal (Bittman et al. 2003), caused the attention to revert to the endocrine glands – organs that once were thought to have lost their endogenous rhythmic activity.

The overview of the circadian regulation of the different components of the HPA-axis in the present chapter evidences that the daily rhythm in adrenal activity has been of utmost importance in the history of the circadian timing system. Moreover, nowadays it is becoming increasingly evident that the circadian output of endocrine glands such as the adrenal play an important role in the (re)synchronization of the internal environment with the external environment, for instance during seasonal changes, shift work or rapid travel across several time zones (Cho et al. 2000; Oishi et al. 2005; Kiessling et al. 2010). Moreover, a dysregulated secretion of glucocorticoids is responsible for numerous pathological conditions and alterations in their rhythmicity are frequently found in many human diseases (Chung et al. 2011). Therefore, a better understanding of the mechanism responsible for the entrainment of peripheral clocks as well as of the mechanism that connects the peripheral clock with the functional output of the tissue in which it is situated remains important.

THE DAILY CORTISOL/CORTICOSTERONE AWAKENING RISE

The wide variety of circadian rhythms in hormone release has only become evident since the early 1970s, as the waiting was for the development of highly accurate and specific radioimmunoassays. Before the radioimmunoassay became available, hormones had to be measured either by a bioassay or by chemical analyses. Indeed, for the first demonstration of circadian rhythmicity in adrenal function, Pincus (Pincus 1943) performed

chemical analyses in urine. Once 17-hydroxysteroids could be measured directly, a circadian rhythm of plasma corticosteroids was rapidly recognized (Migeon et al. 1956). Under baseline conditions, plasma concentrations of the glucocorticoid hormones released from the adrenal gland vary in a predictable way across the day/night cycle. In all species, waking-up is preceded by a considerable surge in the release of steroid hormones from the adrenal cortex. Adrenal steroids have highly integrated effects on both energy metabolism and behavior [Dallman et al., 1993]. The adrenal glucocorticoid and mineralocorticoid hormones are considered major stress hormones as they boost energy production and increase blood pressure, respectively. It is thought that the increased levels of glucocorticoids at awakening gear the body up for the impending activity phase, and act to enable foraging behavior by increasing the amount of available energy. This daily change in behavioral status is usually locked to the environmental change from dark to light or light to dark. As, both in nocturnal and diurnal species, the rise in plasma glucocorticoid concentrations is coupled to the time of arousal, there is a 180° phase difference in their peak time, i.e., morning for humans and evening for most rodents. In humans there are prominent circadian rhythms of both plasma cortisol and corticosterone concentration, with an approximate 13:1 ratio of cortisol to corticosterone maintained throughout the 24-hour light/dark-cycle. In rats, corticosterone is the predominant adrenal steroid.

Once an accurate and sensitive radioimmunoassay had become available, many different aspects of the adrenal rhythmicity were uncovered in the following years (Moore-Ede et al. 1982). In short, a daily rhythm in adrenal activity is not yet present at birth. In the rat, a significant circadian variation in plasma corticosterone concentrations is seen only from three weeks of age; in humans, the rhythm is not fully established until two or three years of age. Besides the circadian rhythm, adrenal release is also characterized by a clear ultradian rhythmicity. It is the clustering of six to nine secretory episodes in the latter part of the night and the early part of the light period that accounts for the awakening surge in humans. The daily plasma corticosteroid rhythm is remarkably stable, with little influence from day-to-day behavioral and environmental changes. For example, both ultradian and circadian rhythmicity persist when volunteers are subjected to constant bed rest, sleep deprivation for one or two nights, or to eating identical small liquid diets at hourly intervals. Even when subjects maintained a 3-hour sleep/wake-cycle (i.e., 2 hours of activity and 1 hour of sleep in a repeating cycle) an essential normal rhythm of plasma cortisol was observed. Furthermore, ultradian and circadian cortisol secretory patterns are similar in sighted subjects and in subjects totally blind from birth, except for the free-running nature of their rhythms. These observations confirm that the rhythm is not passively driven by the alternation of light and dark. In the following years, with the

sensitivity of radioimmunoassay further optimized, all of the above observations could be confirmed in experimental animals.

CIRCADIAN RHYTHMS IN THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS

The medial parvocellular part of the hypothalamic paraventricular nucleus (PVN) contains neuroendocrine neurons that synthesize corticotrophin-releasing hormone (CRH). Together they represent the major determinant of the setpoint of the neuro-endocrine pathway known as the HPA-axis (Watts 2005). In about half of the neuroendocrine CRH neurons vasopressin (AVP) is co-expressed, with their axons projecting to the median eminence and releasing CRH and AVP to stimulate the adrenocorticotrophic hormone (ACTH)-producing cells in the anterior pituitary. ACTH, in its turn, controls the release of corticosterone via its stimulatory action on the adrenal cortex.

In the neuro-anatomical tracing studies mentioned above the PVN showed up as an important target area of the SCN. In addition, evidence had been accumulating that the vasopressin-containing neurons in the dorsal SCN were an important component of the SCN output (Kalsbeek et al. 2010). The close proximity of (vasopressin-containing) SCN nerve endings near CRH-containing neurons in the PVN gave rise to the hypothesis that via this projection circadian information would be imprinted onto the HPA-axis.

Microinfusions with vasopressin and its antagonist in different SCN target areas, such as the PVN and the dorsomedial hypothalamus (DMH), demonstrated that vasopressin released from SCN terminals strongly inhibits the release of adrenal corticosterone (Kalsbeek et al. 1992). Further studies on the relation between the circadian release of vasopressin and the control of the daily rhythm in the activity of the HPA-axis revealed that vasopressin release in the rat DMH is important to ensure low circulating levels of corticosterone during the first half of the light period (Kalsbeek, van Heerikhuize, et al. 1996). In addition, the halt of vasopressin release from these SCN terminals in the DMH during the second half of the light period is a prerequisite for the daily surge in plasma corticosterone before the onset of the main activity period of the nocturnal rat, i.e., the dark period (Kalsbeek, van der Vliet, et al. 1996). The important role of vasopressin in the propagation of output signals of the SCN into the PVN was nicely confirmed in a series of experiments using multi-electrode recordings in hypothalamic brain slices (Tousson & Meissl 2004). These experiments showed that the circadian rhythm in spontaneous firing rate of PVN neurons was lost in slices from which the SCN had been surgically removed, but could be reinstated by either co-cultures of SCN tissue or a rhythmic (12-h

on, 12-h off) perfusion of vasopressin. Moreover, simultaneous perfusion with a vasopressin-antagonist abolished PVN rhythms during co-culture and rhythmic vasopressin perfusion experiments, but not in intact slices. Together, these series of experiments clearly showed that vasopressin is an important, but not the sole, SCN signal involved in the control of the daily rhythm in HPA-activity. SCN control over the daily corticosterone rhythm appeared to have its basis in the alternating activity of stimulatory and inhibitory SCN inputs to the appropriate target neurons, i.e. a kind of push-and-pull, or ying-yang mechanism (Fig.1). The existence of an SCN neurotransmitter with a stimulatory effect on the HPA-axis not only follows from the above described infusion experiments, but is also evidenced by the fact that in adrenalectomized animals supplemented with corticosterone pellets the evening rise of ACTH is abolished by an SCN lesion (Cascio et al. 1987). The SCN transmitter responsible for this stimulatory effect on the HPA-axis has not yet been identified, but both vasoactive intestinal peptide (VIP) (Alexander & Sander 1994; Loh et al. 2008) and neuromedin U (Graham et al. 2005) have been proposed as possible candidates.

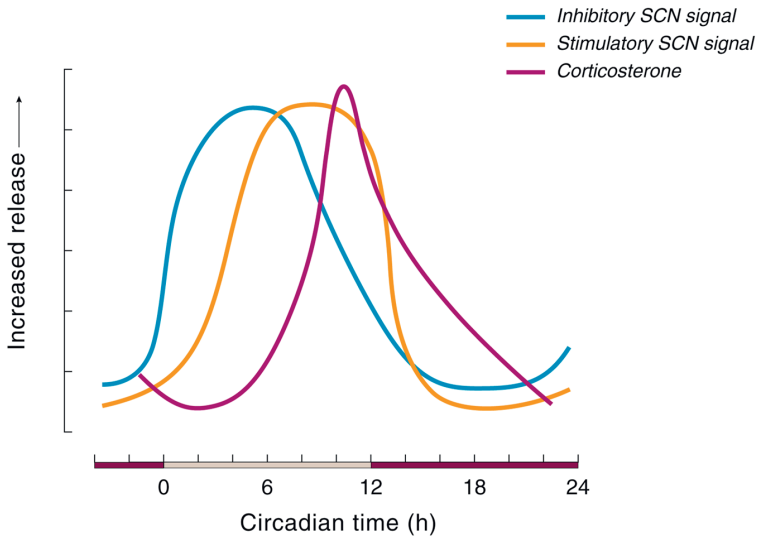

Figure 1. Schematic representation of the diurnal release pattern of SCN transmitters involved in the circadian control of corticosterone release.

Based on the apparent overlap of SCN projection fibers and CRH neurons in the medial PVN, the most likely target neurons for the inhibitory effect of AVP appeared to be the CRH-containing neurons. However, several pieces of evidence did not tally with such a primary role for the CRH neuron. First, a direct effect of vasopressin on the CRH neuron would imply a clear daily rhythm in CRH release and plasma ACTH concentrations. However, often this rhythm is not observed [see below]. Second, the observed inhibitory

effect of vasopressin was not in line with the usual excitatory effect that vasopressin has on its target neurons (Joëls & Urban 1982; Kow & Pfaff 1986). Third, contrary to the expected abundant contacts between SCN-derived (vasopressin) fibers and CRH neurons, only a limited number of such appositions were found (Vrang et al. 1995; Buijs & Van Eden 2000) (Fig.2).

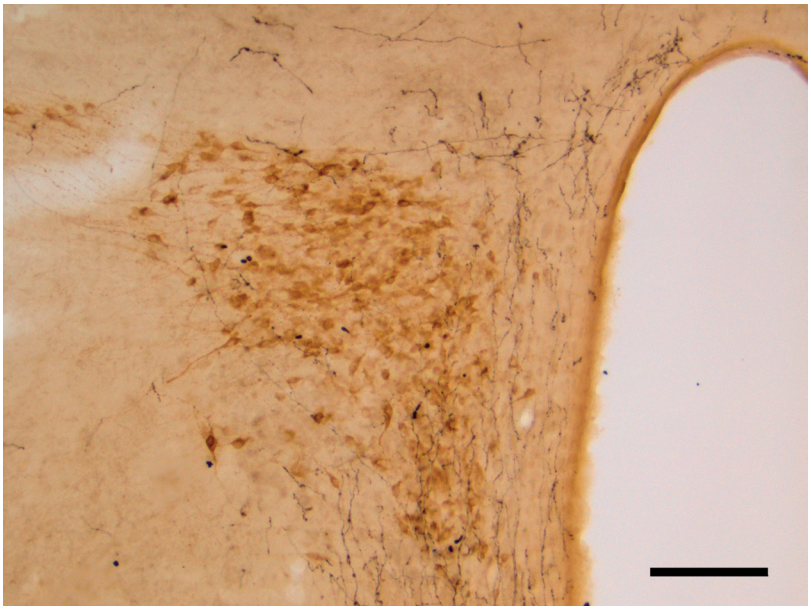

Figure 2. A transversal section of the hypothalamus in the region of the paraventricular nucleus (PVN) shows fibers arising from the suprachiasmatic nucleus (SCN), as labeled by an iontophoretic injection with the anterograde tracer Phaseolus vulgaris leucoagglutinin into the SCN, and penetrating the boundaries of the PVN in which cell bodies immunoreactive for corticotrophin-releasing factor are stained (dark brown). Branching and putative termination of SCN fibres are visible just ventral of the PVN close to the ventricle and in the periventricular and dorsal part of the PVN. Scale bar = 500 µm.

A detailed anatomical scheme explaining the current view on the SCN control of the daily rhythm in HPA-activity is shown in Fig.3. The proposed intermediate role of the gamma-aminobutyric acid (GABA)ergic neurons in the subPVN and DMH in rats is supported by electrophysiological in vitro experiments using hypothalamic slices (Hermes et al. 2000). As shown in the image in the right-hand side panel of Fig.3, the proposed important role for intermediate areas such as the subPVN and DMH also provides a good explanation for the mechanism behind the 12-h reversal of the corticosterone rhythm between nocturnal and diurnal species (Kalsbeek et al. 2008), while apparently the phase of SCN activity (including vasopressin release) is similar for nocturnal and diurnal species (Dardente et al. 2004; Cuesta et al. 2008).

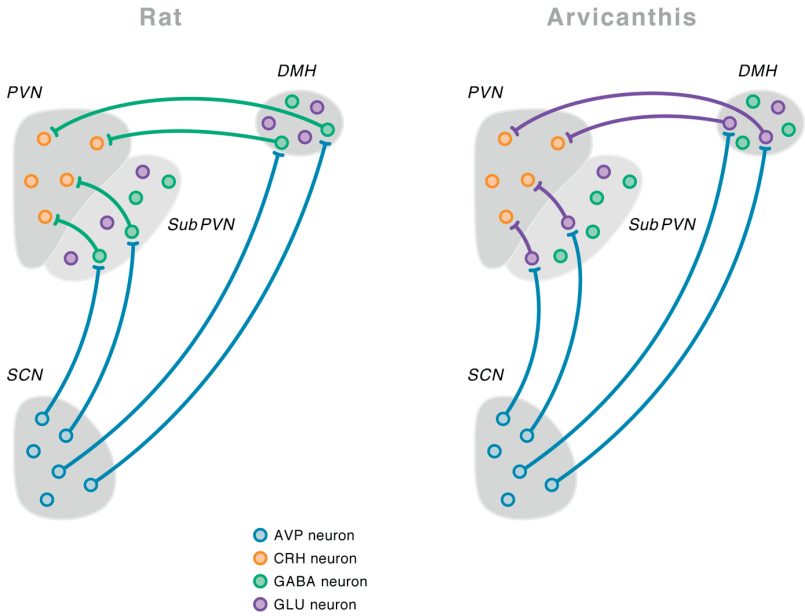

Figure 3. Detailed anatomical scheme of demonstrated and putative connections of the suprachiasmatic nucleus (SCN) in the nocturnal rat and the diurnal Arvicanthis ansorgei brain to explain the opposite effects of VP on the HPA axis in these two species. VP is released during the light period, both in the nocturnal rat and the diurnal A. ansorgei. In rats VP release during the light period will inhibit the corticotropin-releasing hormone (CRH)-containing neurons in the paraventricular nucleus of the hypothalamus (PVN) by contacting gamma-aminobutyric acid (GABA)ergic interneurons in the subPVN and dorsomedial nucleus of the hypothalamus (DMH). On the other hand, in the A. ansorgei, AVP release during the light period will stimulate CRH-containing neurons because it acts on the glutamatergic, instead of GABAergic, interneurons in the subPVN and DMH.

The above-mentioned mismatch between plasma ACTH and plasma corticosterone concentrations made us realize that, in addition to the HPA-axis, other, i.e. ACTH-independent, mechanisms of adrenal regulation might be involved in the cortisol/corticosterone awakening rise. In view of the close connection of the adrenal gland with the sympathetic branch of the autonomic nervous system (ANS) (i.e. the adrenal gland may be considered as a modified sympathetic ganglion), and of the already established essential role of the ANS in the control of the circadian melatonin rhythm (Moore 1996; Perreau-Lenz et al. 2004), we hypothesized that the ANS is important for modulating the sensitivity of the adrenal cortex to ACTH.

Most undergraduate textbooks still state that the adrenal cortex has no nerve supply whereas the medulla does. The innervation of chromaffin cells in the adrenal medulla by preganglionic sympathetic neurons present in the thoracolumbar segment of the spinal cord has already been known since the late 1930s (Charlton 1990). Although

even then there were already speculations about the presence of postganglionic fibers in the adrenal medulla and, conclusive evidence was not presented until the 1980s (Kesse et al. 1988). In addition, more recently a vagal innervation of the adrenal gland was suggested (Coupland et al. 1989; Niijima 1992). The functional significance of the adrenal cortical innervation was demonstrated in an elaborate series of experiments by the groups of W.C. Engeland (Engeland 1998) and A.V. Edwards (Edwards & Jones 1993). For instance, electrical stimulation of the splanchnic nerve increases adrenal cortical sensitivity to ACTH, i.e. the splanchnic innervation acts as an extra-ACTH mechanism controlling adrenal corticosteroid secretion. More recently, transneuronal retrograde viral tracing experiments from the adrenal revealed a direct input from PVN neurons to the preganglionic neurons in the spinal cord (i.e. second-order labeling), and third-order labeling in SCN neurons (Buijs et al. 1999). The functional significance of this multi-synaptic connection between the SCN and the adrenal cortex for the daily rhythm in adrenal corticosterone release was proven by elegant combinations of adrenal microdialysis and denervation experiments (Jasper & Engeland 1994; Ishida et al. 2005; Cailotto et al. 2009). Apparently, the SCN uses a dual mechanism to control the daily rise in plasma glucocorticoids: on the one hand it acts on the neuroendocrine motorneurons to influence the release of hypothalamic releasing factors, while on the other hand it acts - through the ANS - on the adrenal gland to influence the sensitivity of the adrenal cortex to the incoming hormonal ACTH message. It appears, that through the autonomic nervous system, the SCN establishes a window of time during which the adrenal gland is at its most responsive to ACTH.

This SCN – adrenal cortex pathway via the autonomic nervous system presents an anatomical basis for the observations by the Dallman group, showing that changes in the sensitivity of the adrenal cortex to ACTH are dependent on the circadian cycle (Kaneko et al. 1980; Kaneko et al. 1981). Furthermore, a disturbance of daily cortisol rhythms and a dissociation of plasma ACTH and cortisol values in different disease states was already noted in the early days of the description of the daily cortisol rhythm (Fullerton et al. 1968; Sachar et al. 1973; Mortola et al. 1987; Deuschle et al. 1997; Bornstein et al. 2008). More recently it became clear that also during chronic or prolonged phase of critical illness, plasma ACTH and total cortisol levels are dissociated, with low ACTH and elevated cortisol (Vermes & Beishuizen 2001). The SCN - adrenal cortex neural pathway may be one explanation for these discrepancies, and possibly accounts for failing dexamethasone suppression tests during mental depression, schizophrenia and Alzheimer's disease (Linkowski et al. 1985; Lammers et al. 1995; O'Brien et al. 1996).

As indicated above, surprisingly, the pronounced day/night rhythm in plasma corticosterone is not "preceded" by clear rhythms in the release of CRH, AVP or ACTH. Also the

use of CRH mRNA to delineate daily patterns of gene expression yielded equivocal results (Watts & Swanson 1989; Kwak et al. 1992; Cai & Wise 1996), probably because tracking subtle changes in the functionally heterogeneous CRH mRNA expressing neurons is difficult. Using the more sensitive hnRNA technique to monitor transcription rates Watts et al. (Watts et al. 2004) found clear daily rhythms in both CRH and AVP. However, it also became evident that there is a considerable degree of uncoupling between CRH gene transcription on the one hand and CRH and ACTH secretion on the other. Unexpectedly, ACTH release precedes increased CRH expression by several hours. Moreover, in the anterior pituitary no significant day/night rhythm occurs in pro-opiomelanocortin (POMC) hnRNA, despite the presence of clear clock gene rhythms in this tissue (Girotti et al. 2009). Together, these data indicate that the circadian control of the HPA-axis is not directed at the CRH neuron or CRH gene expression exclusively, but also at CRH terminals and CRH release.

THE FOOD-ANTICIPATORY RISE IN CORTICOSTERONE

In all species studied so far, the circadian peak in plasma corticosterone levels is phase locked to the onset of the major activity period of the organism, that is, at dusk in nocturnal species and at dawn in diurnal species. It has been shown that the corticosterone peak can be phase shifted by changes in the light/dark (L/D) cycle (Weinert et al. 1994), whereas in constant dark (D/D) conditions the corticosterone rhythm remains phase locked with the free-running rhythm of locomotor activity (Takahashi et al. 1977; Perlow et al. 1981; Fischman et al. 1988). The daily L/D cycle has long been recognized as the most important Zeitgeber for the circadian rhythms of most organisms. However, numerous studies have since indicated that, in addition to light, a variety of nonphotic stimuli, such as increased locomotor activity, feeding, social cues, and exposure to novelty can phase shift and/or entrain the circadian pacemaker of mammals (Mrosovsky 1996). Perhaps one of the strongest nonphotic stimuli affecting the organization of activity independent of the L/D cycle is forcing nocturnal animals to feed during a limited period in the light phase (Boulos & Terman 1980; Stephan 2002). The first seminal observations on this issue were made by Carl Richter (Richter 1922), i.e., clock driven nocturnal (feeding) activity can be easily overcome by alterations in energy supply, for instance during restricted feeding (RF) schedules, when food is only available to animals during set periods that typically occur outside of their normal feeding period, i.e. during the day for nocturnal rodents. Early studies by Dorothy Krieger showed that RF also has a strong influence on corticosterone release (Krieger 1974). Soon other investigators showed that a bout of increased locomotor activity before the opportunity to feed is accompanied by a prominent anticipatory corticosterone peak in the rat (Honma et al.

1992; Yoshihara, Honma, Katsuno, et al. 1996), but also in mice (Nelson et al. 1975) and primates (Sulzman et al. 1977). This anticipatory peak appears in addition to the already existing circadian rise in corticosterone.

The mechanisms underlying the neural control of the anticipatory peak in corticosterone during RF are clearly different from those regulating the circadian release of corticosterone. For instance, the shift in corticosterone release is not necessarily accompanied by a shift in ACTH peak release, but a shift in the adrenal sensitivity to ACTH has been documented (Wilkinson et al. 1979; Leal & Moreira 1996). Also the effects of RF on CRH activity in the PVN are equivocal: whereas one study reported no changes in CRH peptide content (Leal & Moreira 1996) another study reported a significant shift in CRH hnRNA (Girotti et al. 2009). Finally, it was shown that food-entrained anticipatory corticosterone secretion is associated with hypothalamic release of noradrenaline and neuropeptide-Y (Kalra et al. 1991; Yoshihara, Honma, & Honma 1996a; Yoshihara, Honma, & Honma 1996b; Yoshihara, Honma, Katsuno, et al. 1996). As lesions of the noradrenergic innervation of the PVN abolished the RF-induced rise but not the circadian rise in plasma corticosterone (Honma et al. 1992; Ritter et al. 2003), the noradrenergic hypothalamic innervation clearly differentiates the circadian and the RF control mechanism. However, the most compelling evidence for a separate control mechanism is the fact that the anticipatory corticosterone peak is still present in SCN-lesioned animals (Krieger et al. 1977). Although initially this observation met with great skepticism, the fact that the SCN seems to be indispensable for the development of an anticipatory rise in corticosterone does not mean that in the SCN-intact situation the SCN is “insensitive” or “blind” to the behavioral and endocrine shifts induced by the RF. On the contrary, the daily rhythm in vasopressin release, crucial for the control of the daily rhythm in plasma corticosterone (as explained above), seems to adapt to the changed pattern of activity and corticosterone release. Both a delayed onset of the diurnal rise and a premature decline of the elevated daytime levels were observed, whereas the acrophase of the vasopressin rhythm was not phase shifted. The circadian corticosterone peak did not show a phase shift of its acrophase either (Kalsbeek et al. 1998). The delayed onset and premature decline of the elevated daytime vasopressin release are completely compatible with the previously proposed inhibitory action of SCN-derived VP on the HPA axis, and in fact seem to facilitate the RF-induced anticipatory rise of plasma corticosterone. Moreover, the most recent results show that an inportant function of the increased activity of DMH neurons during RF may be to suppress SCN neuronal activity, allowing increased locomotor activity during the light period (Acosta-Galvan et al. 2011).

CLOCK GENE RHYTHMS WITHIN THE ADRENAL GLAND

From the early days of the awareness of a circadian rhythm in plasma corticosterone concentrations onwards there have been equivocal reports about an intrinsic periodicity in the adrenal gland itself (Andrews & Folk 1964; O'Hare & Hornsby 1975). One publication reported that adrenal corticosteroid periodicity in hypophysectomized rats implanted with ACTH and thyroxine containing pellets was maintained (Meier 1976). Interestingly, these rhythms disappeared when, in addition to the hypophysectomy, an adrenal denervation was performed (Ottenweller & Meier 1982). Moreover, in capuchin monkeys, the suppression of ACTH by dexamethasone reduces the cortisol concentration to 10% of the regular level, but its circadian rhythmicity is not abolished (Torres-Farfan et al. 2008). In addition, some groups reported daily rhythms in the adrenal sensitivity to ACTH, both in vivo (Dallman et al. 1978) and in vitro (Ungar & Halberg 1962). Multiple early studies also reported a circadian rhythm in the responsiveness of the HPA-axis to stress (Zimmermann & Critchlow 1967; Gibbs 1970). Due to the absence of data on ACTH secretion it was not possible to dissociate a circadian variation in stress-induced release of ACTH from a circadian variation in the adrenal responsiveness from stress. In addition, the circadian variation in stress responsiveness was often explained by the circadian variation in basal plasma corticosterone concentrations, although this cannot possibly be the whole explanation (Kalsbeek et al. 2003). However, evidence that daily rhythms of plasma ACTH concentrations remained present in adrenalectomized animals indicated that the daily rhythmicity of the HPA-axis was not (solely) dependent on the adrenal gland. As a consequence the attention shifted from the adrenal gland to the pituitary and hypothalamus. But as described above, after years of research on the master oscillator in the hypothalamus, and the discovery of the clock genes and especially the existence of peripheral clocks, the attention shifted back to the peripheral organs, including the adrenal glands.

On a molecular level, both the SCN and peripheral tissues such as the adrenal gland maintain their intrinsic rhythm via a transcriptional/translational feedback loop that consists of positive and negative regulators. The core loop is formed by the Clock:Bmal1 heterodimer that stimulates the transcription of Period1, Period2, Period3 (Per1-3), Cryptochrome1 and Cryptochrome2 (Cry1-2). Pers and Crys heterodimerise and inhibit Clock:Bmal1 activity. Consequently, Clock:Bmal1 transcriptional activity drops, which reduces the transcription of Per and Cry genes, thereby activating Clock:Bmal1 again. Retinoic acid related orphan nuclear receptors, RevErbs and RORs, form additional regulatory loops that can bind to retinoic acid-related orphan receptor response elements (ROREs) on the Bmal1 promotor (Ko & Takahashi 2006). Cycles of transcription and translation feedback generally operate on a timescale of up to a few hours. Main-

taining a daily oscillation rhythm thus requires a significant delay between activation and repression of transcription. Post-translational modifications such as phosphorylation, histone acetylation, methylation and ubiquitination affect the stability and nuclear translocation of core clock proteins, thereby delaying the cycle to approximately 24h (Reppert & Weaver 2001; Ko & Takahashi 2006; Gallego & Virshup 2007).

The adrenal gland consists of two anatomically and functionally different compartments: the medulla forms the core of the adrenal gland and is surrounded by a three-layered cortex. The medulla produces the catecholamines epinephrine and nor-epinephrine, whereas the different layers in the cortex produce steroids from cholesterol. The outer layer (zona glomerulosa) produces the mineralocorticoids that regulate sodium balance and long term blood pressure. The middle layer (zona fasciculate) produces the glucocorticoids, while the inner layer (zona reticularis) produces the adrenal androgens.

Clock gene expression patterns are clearly tissue-specific (Panda et al. 2002; Ko & Takahashi 2006), but even within the adrenal gland, the expression levels and phasing of the cycling pattern of the clock genes differ between the medulla and the different layers of the cortex (Dickmeis 2009). All clock genes, except for Clock and Cry2, show a robust circadian rhythm in expression in the adrenal cortex (Oster, Damerow, Kiessling, et al. 2006). The adrenal medulla rhythmically expresses Per1, Per3, Cry2 and Bmal1, whereas Per2, Cry1 and Clock are -at best- weakly expressed in this tissue (Bittman et al. 2003; Torres-Farfan et al. 2006; Oster, Damerow, Kiessling, et al. 2006). These data indicate that the corticoid producing outer layers of the cortex are the chief sites of the adrenal circadian pacemaker.

In principle, the central clock in the SCN could entrain the peripheral oscillators in the adrenal gland via the two pathways introduced in the foregoing: 1) the humoral pathway of the HPA-axis, and 2) the neural pathway of the autonomic nervous system. The persistent daily rhythmicity of adrenal clock genes in hypophysectomized animals indicates that the adrenal oscillator acts independent of humoral SCN signaling via the pituitary gland (Fahrenkrug et al. 2008). These data also fit with the early observations of Meier (Meier 1976), i.e., the maintenance of adrenal corticosteroid periodicity in hypophysectomized rats. Indeed, there is more evidence that supports the implication that entrainment of the adrenal oscillator by the SCN is mediated via the adrenal autonomic innervation. First, adrenal denervation causes a disappearance of both the circadian corticosterone rhythm (Jasper & Engeland 1994), as well as the daily change in ACTH sensitivity (Ottenweller & Meier 1982; Ulrich-Lai et al. 2006). Second, light directly affects corticosterone release and adrenal Per1 and Cry2 expression via the autonomic nervous system (Buijs et al. 1999; Ishida et al. 2005; Cailotto et al. 2009), although it is currently

unknown whether this light-induced clock gene expression actually leads to sustained changes in clock gene rhythms. Third, also experiments in which Syrian hamsters were induced to split rhythms of locomotor activity by exposure to constant light support the contribution of neural pathways to the determination of circadian oscillator phase in the adrenal gland (Mahoney et al. 2010).

In addition to the autonomic nervous system, additional pathways and mechanisms may influence the adrenal oscillator. For instance, restricted feeding schedules profoundly affect the adrenal oscillator in rats by reversing the expression profile of clock genes (Girotti et al. 2009). However, at present it is completely unknown how RF is able to affect the adrenal oscillator. Does it affect the adrenal gland via brain mechanisms that control the HPA-axis and/or its autonomic innervation or is there a direct, local influence of metabolic signals or body temperature on the adrenal oscillator? Clock genes in the pituitary do not seem essential, as the pituitary oscillator does not follow meal time during RF (Bur et al. 2010). Finally, there are indications that melatonin may be involved in the control of adrenal cortical function either via direct effects within the adrenal cortex or via its effects on the SCN, as has been shown in the monkey adrenal gland (Torres-Farfan et al. 2006).

The ultimate question is whether the adrenal oscillator is necessary for an intact glucocorticoid rhythm. Although most mouse models with a defect in one or more of the essential clock genes show a clear disturbance of the circadian corticosterone rhythm (Nader et al. 2010), this does not answer the question. However, transplantation studies in which clock mutant adrenal glands are transplanted into wild-type mice, and vice versa (wild-type adrenal glands into clock mutant mice) show that an intact adrenal clock can sustain corticosterone rhythmicity even in the absence of a functional SCN pacemaker (but with intact L/D-induced behavioral rhythms present) (Oster, Damerow, Kiessling, et al. 2006). The most likely explanation for these observations is that under conditions of constant levels of circulating ACTH, the daily rhythm in ACTH sensitivity as induced by the adrenal oscillator results in rhythmic corticosterone release. Surprisingly, the rhythmic corticosterone rhythm could not be maintained in D/D conditions, i.e., arrhythmic hosts without functional SCN carrying wild-type adrenal glands, indicating the necessity of a direct light input of the adrenal gland (probably via a reinstated autonomic innervation) to sustain the intrinsic adrenal rhythmicity. Two years later these results were replicated in another elegant study (Gi et al. 2008). The use of a promoter of the ACTH receptor to express Bmal1 in antisense orientation resulted in an adrenal-specific knock-down of the circadian oscillator. Also, these transgenic animals showed unimpaired corticosterone rhythms in L/D-conditions, but severely dampened rhythms in D/D conditions. Thus, somewhat contrary to the prevailing view, peripheral

clocks may not primarily generate self-sustained rhythms but more likely modulate and stabilize circadian rhythms of physiology.

In conclusion, it appears that the adrenal gland clock directs ACTH input into glucocorticoid synthesis to certain times of day. Indeed, among the many genes showing circadian mRNA expression in the adrenal gland, there is the ACTH receptor itself (Fig.4) as well as components of its downstream signaling pathway, such as Adcy5, several G-proteins, protein kinase A and protein phosphatase 1 subunits (Oster, Damerow, Hut, et al. 2006; Dickmeis 2009). Other genes implicated in the biosynthesis of glucocorticoids, such as regulators of cholesterol transport also show circadian rhythms of expression. Interestingly, the genes previously demonstrated as rate-limiting for synthesis, such as steroidogenic actute regulatory protein (STAR) or cholesterol side chain cleavage monooxygenase (CYP11A1), do not seem to be among these, although a pronounced rhythmic expression of STAR has been reported by (Gi et al. 2008).

ENTRAINMENT OF PERIPHERAL CLOCKS BY GLUCOCORTICOIDS

The search for mechanisms integrating central and peripheral clock regulation of physiology has rekindled the interest in endocrine rhythms. Many hormones show pronounced circadian patterns of release and are therefore well placed as candidate signals that may coordinate central and peripheral rhythms, and glucocorticoids seem to be among the prime candidates. Glucocorticoids regulate a wide variety of functions, including stress responses, development, arousal, cognition, energy metabolism and immune responses. They act via 2 subtypes of receptors; the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which, despite its name, binds glucocorticoids with a higher affinity as compared to the GR. Both receptors are expressed in brain and periphery with some overlap. GR is the most ubiquitously expressed, whereas the MR has a more limited distribution primarily concentrated in the hippocampus and septum (Segall & Amir 2010). GR appears to exert its predominant effect in the pituitary, while MR mediates the brain effects (Dickmeis 2009). The SCN is one of the rare areas that do not seem to express either the GR or the MR, except for a short period around birth (van Eekelen et al. 1987; Cintra et al. 1994). However, likely the glucocorticoid message is able to reach the central clock through an indirect mechanism, as glucocorticoids do modulate neuroglial plasticity in the SCN (Girardet et al. 2010) It is also possible that information from glucocorticoid-dependent circadian gene expression in other brain regions is transmitted to the SCN. Indeed, several brain regions outside the SCN seem to require glucocorticoids for their molecular clock. For example, Per2 expression in the bed nucleus of the stria terminalis (BNST) and in the central nucleus of the amygdala

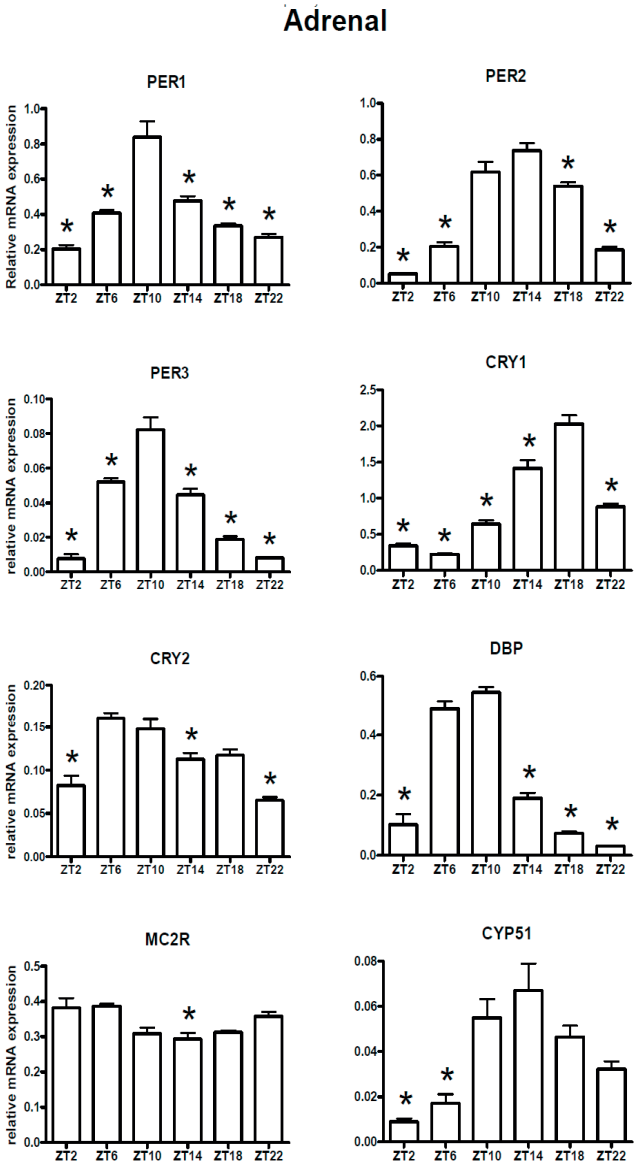

Figure 4. A few examples of (clock) gene rhythms in the adrenal. Daily relative expression profiles for 6 canonical clock genes (PER1,2,3, CRY1,2 and DBP), the ACTH receptor (MC2R) and the steroidogenic enzyme CYP51 in the adrenal gland as determined by RT-qPCR in male Wistar rats (n=5-6) sacrificed at 6 different time points during a regular L/D-cycle. Surprisingly the MC2R expression shows a trough at the circadian peak of corticosterone release (a similar pattern was observed by Oster et al., 2006, Cell Metabolism). On the other hand, the expression profile of the steroidogenic enzyme CYP51 nicely correlates with the daily rhythm of corticosterone release (again a similar pattern was observed by Oster et al., 2006, J Biol Rhythms). One-way ANOVA revealed significant effects of time for all genes ($F(5,28)=28.1$ (PER1), 65.7 (PER2), 62.4 (PER3), 83.0 (CRY1), 19.7 (CRY2), 131.1 (DBP), 6.7 (MC2R) and 10.2 (CYP51). Asterisks indicate a significant difference ($p<0.01$) from the highest value according to Bonferroni post-hoc tests.

is severely blunted in adrenalectomized animals (Amir et al. 2004; Lamont et al. 2005). Interestingly, rhythmic replacement of corticosterone via the drinking water re-instates the daily rhythm in Per2, whereas continuous replacement via a corticosterone pellet does not (Segall & Amir 2010). A possible feedback mechanism for glucocorticoids to influence the SCN might run via projections from the raphe nucleus, as the raphe nucleus is sensitive to glucocorticoids and its serotonergic projections have been suggested to affect light entrainment of the clock (Morin 1999; Sage et al. 2004; Buijs & Escobar 2007; Malek et al. 2007). Because of the high affinity of corticosterone for the MR, most MRs will be occupied even at trough levels of the rhythm in plasma glucocorticoids. This suggests that the actions of corticosterone at basal levels are solely mediated by the MR. The GR becomes active at the onset of the active period, when corticosterone levels are peaking. The distribution of these two receptors, together with the difference in affinity for corticosterone, ensures a high level of temporal and spatial compartmentalization of genomic activation within each cell (Segall & Amir 2010). Unbound GRs reside in the cytoplasm, and once the glucocorticoid binds to the GR, the complex travels towards the nucleus where it can bind the glucocorticoid response element (GRE) in the promotor region of target genes, thereby regulating gene expression. Per1, and possibly Per2, possess a GRE (Balsalobre et al. 2000; Yamamoto et al. 2005; So et al. 2009), and the transcription of RevErbα has been shown to be repressed by glucocorticoids (Torra et al. 2000).

In various tissues, glucocorticoids seem to play a role in the regulation of the rhythmic clock gene expression, as based on both in vitro (Balsalobre et al. 2000) and in vivo (Torra et al. 2000; Yamamoto et al. 2005; Kiessling et al. 2010) experiments. A major part of the liver transcriptome seems to be dependent on the adrenal gland (Oishi et al. 2005), but to a certain extent these results might also be due to low insulin levels because of the adrenalectomy. Also, in muscle tissue roughly 50% of the daily regulated genes have been reported as glucocorticoid responsive genes (Almon et al. 2008). However, when a wild-type adrenal gland was transplanted into the kidney of a Per2/Cry1 double mutant mouse, the kidney clock gene transcription was fully reflected by the genotype of the host, indicating that rhythmic corticosterone per se is not sufficient to re-establish rhythmicity in a mutant kidney clock (Oster, Damerow, Kiessling, et al. 2006). Taken together, glucocorticoids are clearly playing a role in the regulation of the peripheral clocks but the effects are tissue specific and the exact mechanisms involved remain to be elucidated.

Besides entraining peripheral clocks on a molecular level, glucocorticoids are also involved in the entrainment of behavioral rhythmicity. Adrenalectomized rats show an accelerated rate of re-entrainment to a shifted LD cycle (Sage et al. 2004). In an adrenal

specific clock knockdown mouse, no differences were found between mutants and their wild type littermates as long as they were kept under LD conditions. When transferred to DD, not only the amplitude of plasma corticosterone rhythm was severely dampened in the mutants, their behavioral rhythm, too, was significantly diminished (Gi et al. 2008). This indicates that in the absence of light as a timekeeping cue, the rhythm in corticosterone is an important factor driving locomotor activity. In a mouse model of jet lag, behavioral reentrainment was preceded by a shift of the glucocorticoid rhythm (Kiessling et al. 2010). Moreover, injecting mice with an inhibitor of corticosterone production either advanced or delayed behavioral resynchronization, depending on the time of injection and the direction of the phase change (Kiessling et al. 2010). Taken together, these findings suggest that corticosterone acts as a regulator of behavioral adaptation to phase shifts. It has been postulated that glucocorticoids indirectly feed back to the SCN to enhance entrainment, thus preventing inappropriate shifts to 'Zeitgeber noise' (Prasai et al. 2011).

CONCLUSIONS

It appears that the circadian control of adrenal corticosterone secretion predominantly relies on the connections of the biological clock with the autonomic nervous system via its projections to the hypothalamic pre-autonomic neurons, and not so much on the SCN connections with the HPA-axis. The CRH and ACTH dependent control of adrenal corticosterone release may be more important during stress conditions.

Clearly, the hypothalamo-pituitary-adrenal axis has been and continues to be an excellent model for studying the transmission of circadian information throughout the body. In many diseases the daily corticosterone rhythm is altered, but it is still not clear whether this reflects the pathological condition, or whether it contributes to it. Therefore the important question: "Do changes in the daily rhythm of corticosterone release contribute to pathology?", remains unanswered for now.

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Regulation of daily rhythmicity in WAT

3

Daily Gene Expression Rhythms in Rat White Adipose Tissue Do Not Differ between Subcutaneous and Intra-abdominal Depots

Van der Spek R
la Fleur SE
Fliers E
Kalsbeek A

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ABSTRACT

White adipose tissue (WAT) is present in different depots throughout the body. Although all depots are exposed to systemic humoral signals, they are not functionally identical. Studies in clock gene knock-out animals and in shift workers suggest that daily rhythmicity may play an important role in lipid metabolism. Differences in rhythmicity between fat depots might explain differences in depot function; therefore we measured mRNA expression of clock genes and metabolic genes on a 3-hour interval over a 24-hour period in the subcutaneous inguinal depot and in the intra-abdominal perirenal, epididymal and mesenteric depots of male Wistar rats. We analysed rhythmicity using CircWave software. Additionally, we measured plasma concentrations of glucose, insulin, corticosterone and leptin. The clock genes (Bmal1/Per2/Cry1/Cry2/RevErbα/DBP) showed robust daily gene expression rhythms, which did not vary between WAT depots. Metabolic gene expression rhythms (SREBP1c/PPARα/PPARγ/FAS/LPL/Glut4/HSL/CPT1b/leptin/visfatin/resistin) were more variable between depots. However, no distinct differences between intra-abdominal and subcutaneous rhythms were found. Concluding, specific fat depots are not associated with differences in clock gene expression rhythms and therefore do not provide a likely explanation for the differences in metabolic function between different fat depots.

INTRODUCTION

Sustained disturbances in daily rhythmicity (e.g. shift work, jet lag) increase the risk to develop obesity and related metabolic disease (Laermans & Depoortere 2016). Storage in and release of lipids from white adipose tissue (WAT) are regulated processes that anticipate rest-activity and feeding cycles. WAT is abundantly present throughout the body in different fat depots. In male rats, the main depots are located underneath the skin in the inguinal area (subcutaneous white adipose tissue (sWAT)), and in the abdominal cavity (intra-abdominal depots): perirenal- (pWAT, retro-peritoneal, next to the kidney), epididymal- (eWAT, connected to and lining the epididymis) and mesenteric WAT (mWAT, intraperitoneal, lining the gastrointestinal tract).

Interestingly, although all depots are exposed to systemic humoral signals, such as circulating hormones and nutrients, subcutaneous and intra-abdominal WAT depots are not functionally identical (Tchkonia et al. 2013; White & Tchoukalova 2014). For example, retroperitoneal WAT is more responsive to metabolic challenges (fasting / refeeding) compared to subcutaneous WAT (Palou et al. 2010). Additionally, in various lipodystrophy syndromes subcutaneous fat stores are depleted, while simultaneously intra-abdominal WAT accumulates (Jazet et al. 2013), pointing to differential differentiation and proliferation of adipose depots. Moreover, excess storage of intra-abdominal WAT is associated with adverse health effects, whereas subcutaneous WAT accumulation might be beneficial (Snijder et al. 2003; Yamamoto et al. 2010; Berings et al. 2012; Neeland et al. 2012). Moreover, effects of sex hormones (Elbers et al. 1999) and glucocorticoid treatment differ between WAT depots (Fardet et al. 2013). To date it is unexplained where these differences originate, and how they are integrated to ensure that the net effect of the WAT depots results in energy homeostasis.

Like most peripheral tissues, WAT depots encompass an intrinsic molecular clockwork based on a transcriptional-translational feedback loop. Since clock proteins regulate the expression of genes involved in many (metabolic) processes within a cell, clock rhythms play an important role in tissue function. The core loop of the molecular clock is formed by the Clock:Bmal1 heterodimer that up-regulates expression of the Period 1-3 (Per 1-3) and Cryptochrome 1-2 (Cry1-2) proteins. Per's and Cry's subsequently heterodimerize, translocate to the nucleus and inhibit Clock:Bmal1 activity. As a consequence, Clock:Bmal1 transcriptional activity drops, which reduces the transcription of *Per* and *Cry* genes, thereby activating Clock:Bmal1 again. The retinoic-acid related orphan nuclear receptors, RevErb and ROR, represent additional regulatory loops that enhance the robustness of the core loop, by binding to retinoic acid-related orphan receptor response elements on the Bmal1 promotor (Ko & Takahashi 2006).

Studies in clock gene knock-out animals and studies in shift workers suggest daily rhythms play an important role in lipid metabolism. For example, the arrhythmic CLOCKΔ19 C57BL/6J mouse is hyperglycaemic, hyperlipidaemic, hyperleptinaemic and hypoinsulinaemic, with increased body weight and visceral adiposity (Turek et al. 2005; Tsang et al. 2017). Moreover, disruption of the adipocyte clock by adipose tissue specific deletion of Bmal1, results in obesity, temporal changes in plasma concentration of fatty acids, and altered hypothalamic appetite regulation (Paschos et al. 2012). In CLOCKΔ19 C57BL/6J mice, the impaired adipose tissue clock may directly affect diurnal transcriptional regulation of lipid homeostasis, reducing FFA/glycerol mobilization from WAT stores (Shostak et al. 2013).

To determine whether differences in daily rhythmicity between WAT depots could explain differences in depot function, we analysed rhythmicity of clock gene (*Bmal1, Per2, Cry1, Cry2, RevErbα* and *DBP*) and metabolic gene expression (*SREBP1c, PPARα, PPARγ, FAS, LPL, Glut4, HSL, CPT1b, leptin, visfatin* and *resistin*) in different intra-abdominal and subcutaneous WAT depots. We conclude that differences in the molecular clock or clock-controlled genes do not provide a major explanation for the differences in metabolic function between the different fat depots. Furthermore, our results suggest that in ad libitum feeding conditions the timing of subcutaneous WAT clock gene rhythms can be extrapolated to those of intra-abdominal WAT depots.

MATERIALS AND METHODS

Animals

Sixty-four male Wistar rats (Harlan, Horst, Netherlands) were kept on a 12-h/12-h light/dark cycle (lights on at 0700), at a room temperature (20±2 °C), with 4-6 animals per cage. Thirty-two animals were housed in a room with a reversed light/dark cycle. The experiment was carried out in October. After arrival, animals were allowed to adapt to their new environment and the lighting schedule for three weeks before the experiment. Food and water were provided ad libitum. The experiment was conducted under approval of the Local Animal Welfare Committee.

Experiment

To obtain WAT tissues and plasma, animals were anaesthetised with isoflurane and killed by decapitation at a three hour interval starting at ZT2 (ZT14 for reversed light-dark cycle) and ending at ZT11 (ZT23 for reversed light-dark cycle). At every time point, 4 animals were obtained from both rooms, thereby spreading the total sampling period over a 48 hours period.

Intra-abdominal perirenal (pWAT), epididymal (eWAT), and subcutaneous inguinal (sWAT) white adipose tissues were dissected and snap frozen in liquid nitrogen. Intra-abdominal mesenteric (m)WAT was separated from the gastro-intestinal tract and pancreas and snap frozen in liquid nitrogen. Blood was collected in heparinised tubes.

Plasma analyses

Following decapitation trunk blood was collected and kept on ice in heparinised tubes until centrifugation for 15 minutes at 3000rpm at 4°C. Plasma was transferred to a clean tube and stored at -20°C until use. Plasma glucose was measured using a Biosen apparatus (EKF diagnostics, Cardiff, UK). Plasma insulin, leptin and corticosterone were measured using a radio immuno assay (RIA; Merck Millipore, Billerica, MA, USA).

Gene expression analysis

RNA extraction

Total RNA (tRNA) was extracted from approximately 100mg of adipose tissue, using the RNeasy lipid kit (Qiagen Benelux, Venlo, Netherlands), with on-column DNAse treatment using RNAse free DNAse (Qiagen Benelux, Venlo, Netherlands), according to the manufacturers protocol. tRNA was measured on a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to equal concentrations.

cDNA synthesis

cDNA was synthesized with the Transcriptor First Strand cDNA synthesis kit from Roche (Roche, Almere, Netherlands) using anchored oligo (dT)18 primers and 18ng tRNA per µl cDNA. To check for genomic DNA contamination in the extracted RNA, we included several samples for which we replaced reverse transcriptase with PCR grade water (–RT controls). If the fluorescence curve of one of the –RT controls lay within 10 cycles of the cDNA sample with the lowest expression, the PCR assay was rejected because of potential genomic DNA contamination.

RT-qPCR

Gene expression was analysed by real time RT-qPCR on a LightCycler 480 system (Roche, Almere, Netherlands), using SybrGreen I Master, primer pairs, PCR grade water and cDNA. All primer pairs were designed intron-spanning if possible, and amplicon size and specificity was checked on electrophoresis gel. If the amplicon size matched and a single band was found, the PCR product was purified using a QIAquick PCR purification kit (Qiagen Benelux, Venlo, Netherlands). The purified PCR product was diluted and used in subsequent PCRs as a positive control combined with melting peak analysis.

LinRegPCR

For each PCR assay, PCR efficiency was checked for all samples individually using LinRegPCR. LinRegPCR software determines baseline fluorescence sets a Window-of-Linearity to calculate PCR efficiency. The starting RNA concentration expressed in arbitrary fluorescence units, is calculated using the mean PCR efficiency per sample, the Cq value per sample and the fluorescence threshold used to determine the Cq (Ramakers et al. 2003; Ruijter et al. 2009). Samples that differed more than 0,05 from the efficiency median value were excluded from further analysis.

Normalisation

To control for variation in the amount of mRNA input, gene expression levels of the target sequence were normalised to the expression of an endogenous control, hypoxanthine phosphoribosyl transferase (HPRT) gene expression (Sweet et al. 2001).

Several commonly used reference genes show a circadian rhythm in their expression profile (Kamphuis et al. 2005), and these rhythms may vary between tissues, species and strains (Kosir et al. 2010). HPRT was chosen as a reference gene because it expressed no, or only very low amplitude rhythms in our samples (data not shown). Additionally, all PCR data are expressed relative to ZT2, to allow comparison between WAT depots.

Genes of interest

Primer sequences of clock genes *Bmal1, Per2, Cry1* and *Cry2, RevErbα* and *DBP*, and metabolic genes *SREBP1c, PPARα, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, leptin, visfatin*, and *resistin* have been published previously (Su et al. 2016).

Data analysis and statistics

For identification of outliers we used Dixon's Q test with two tailed Q-values (Rorabacher 1991). Samples that were determined outliers were excluded from further analysis.

All data (plasma and PCR) are presented as mean ± SEM unless otherwise stated. P values below 0,05 were considered statistically significant.

Variations between time points within one gene in one depot were evaluated by one-way ANOVA and rhythmicity was assessed using Circwave v1.4 [www.hutlab.nl]. Circwave software fits one or more fundamental sinusoidal curves through the individual data points and compares this with a horizontal line through the data mean (a constant). If the fitted curve differs significantly from the horizontal line, the data set is considered rhythmic. Circwave provides the following information: number of sines in the fitted curve; data mean, the average of all data points with standard deviation (SD); Centre of

Gravity (CoG), representing the general phase of the curve with SD; Anova F stat, p-value and r2; Circwave F stat, p-value and r2. Centre of Gravity (COG) standard deviations were calculated without assuming the data was circular, as rhythmicity of gene expression was one of the outcome measures.

COG data per gene were compared between WAT depots using unpaired two-tailed T-test with F test. Variances did not differ between WAT depots.

Amplitudes of Circwave curves were calculated as percentages of data mean to enable comparison of amplitudes between data sets (difference between the zenith (highest point) and nadir (lowest point) and divided by the data mean (max-min / mean * 100%)).

RESULTS

Overall rhythmicity of gene expression in adipose tissue

To describe rhythmicity, we considered the following factors to be important; peak time (expressed as centre of gravity; COG), robustness, and amplitude. Therefore, we analysed variation between depots for these factors. We defined 'robustness' of a rhythm as: uniformity between cycles and/or animals measured by three characteristics; period, phase, and shape of wave. R2 values indicate goodness of fit on a scale from 0 to 1, i.e., how well the Circwave curve describes the data. Thus, r2 values close to 1 indicate that individual samples deviate very little from the curve, and therefore show little inter-animal variation in period, phase and shape of wave, and can be called robust. Clock gene and metabolic gene expression per WAT depot, r2 (inter-individual variability) and amplitude are plotted for each gene in Figures 1 and 2. For all WAT depots, clock gene expression was highly rhythmic, with large amplitudes (range 125-272) and low variability (r2 range 0,61-0,92) between animals. A clear exception was *Cry 2*, which showed much lower amplitude (range 37-61) and r2 values (range 0,21-0,46) than the other 5 clock genes investigated. Metabolic genes on the other hand exhibited weak rhythmicity with lower amplitude (range 0-97) and high variability (r2 range 0,21-0,71) between animal (Figure 2 and 3), similar to or lower than the values for *Cry 2*. Individual expression curves for each gene and WAT depot can be found in Supplemental Figure 1.

Clock gene expression comparison between WAT depots

Clock gene expression showed pronounced daily rhythms in all WAT depots. R2 values showed little variation between depots, and limited variation between genes (Figure 1). *Cry2* showed the most pronounced variation between WAT depots; r2 values for pWAT (0,26) and eWAT (0,21) were about 50% smaller than for sWAT (0,46) and mWAT (0,44).

Amplitude variations were limited between WAT depots (Figures 1 & 3). Of note, for most clock genes the lowest amplitude was found in pWAT. For *Per2* mRNA the amplitude in sWAT was clearly higher compared to the other depots. Peak time for the different clock gene curves (depicted as COG) was very similar between WAT depots (Figure 1, one way ANOVA: ns). *Bmal1* peaked in the beginning of the light phase (ZT24) and as expected, *Per* and *Cry* rhythms were in antiphase, to *Bmal1*. *Per2* (ZT15-16) and *Cry2* (ZT14-17) peaked in the early dark period, whereas *Cry 1* (ZT19-20) mRNA peaked in the middle of the dark period. *RevERBα* (ZT9-10) and *DBP* (ZT11-12) mRNA were high at the end of the light phase (Figure 1).

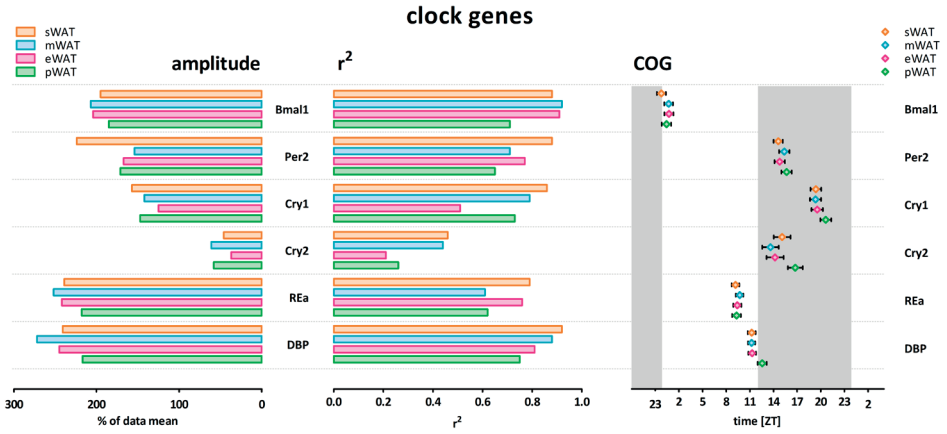

Figure 1. Clock genes show high amplitude, high r2 (low inter-individual variability) and low variability in center of gravity (COG) in subcutaneous and intra-abdominal white adipose tissue (WAT) depots. sWAT, subcutaneous; mWAT, mesenteric; eWAT, epididymal; pWAT, perirenal. Gray bars indicate the dark phase (ZT 12–24). Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

Metabolic gene expression comparison between WAT depots

Daily rhythms in metabolic gene expression were present, however, rhythmicity was not as robust (higher variability and lower amplitudes) as it was for clock genes (Figure 2). Rhythmicity was not apparent for every gene and for some genes not in every WAT depot. Absence of amplitude, R2 and COG values in Figure 2 indicates absence of significant rhythmicity, not absence of gene expression (see Supplemental Figure 1 for individual gene expression curves). R2 values were modest overall; r2 was highest for *visfatin* in eWAT and sWAT (Figures 2 & 3). Similarly, amplitudes in metabolic genes were modest overall, i.e., <100%. Peak time (COG) for most metabolic genes did not differ between WAT depots. However, for *LPL* significant differences were detected (Figure 2).

LPL peaked significantly earlier in pWAT compared to sWAT (two tailed t-test F = 1,133; p = 0, 0342; difference = 3,2h ± 1,5h).

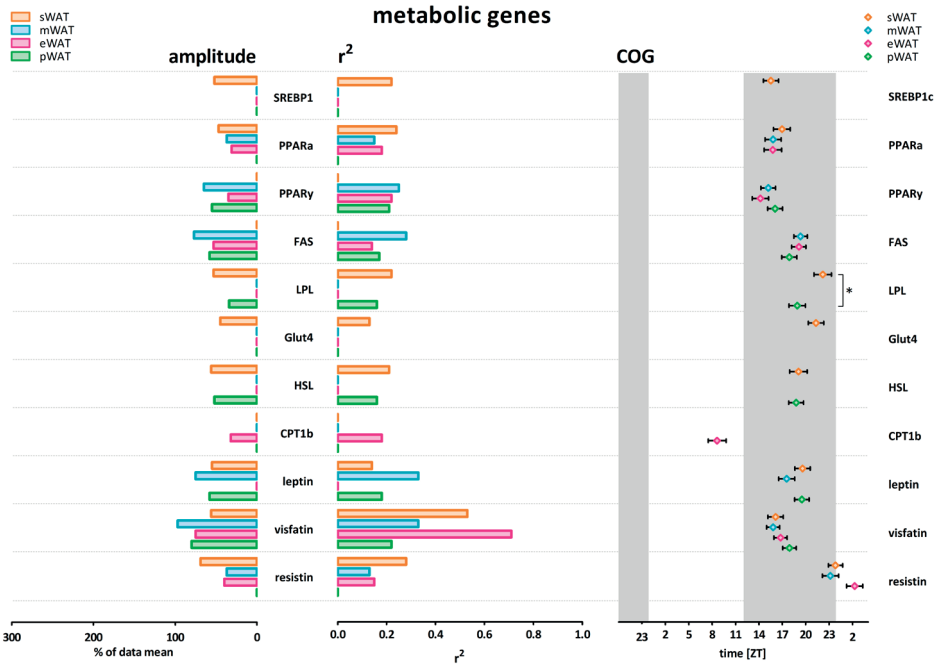

Figure 2. Metabolic genes show modest amplitude, modest r2 (inter-individual variability) and higher variability in center of gravity (COG) in subcutaneous and intra-abdominal white adipose tissue (WAT) depots. LPL peaked significantly earlier in pWAT compared to sWAT (two-tailed t-test F = 1,133; p = 0.0342; difference = 3.2 ± 1.5 h) sWAT; subcutaneous, mWAT; mesenteric, eWAT; epididymal, pWAT; perirenal. Gray bars indicate the dark phase (ZT 12–24). Absence of amplitude, R2, and COG values indicates absence of significant rhythmicity, not absence of gene expression. Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

Daily rhythms in plasma hormone and substrate levels

Plasma levels and COGs of glucose, insulin, corticosterone and leptin are shown in Figure 4. Plasma glucose concentrations were modestly rhythmic and peaked at the transition from light to dark phase (~ZT14, amplitude 21%, ANOVA: F = 4,78; p <0,001, CIRCWAVE: r2 = 0,35; p <0,001). Plasma insulin concentrations were not rhythmic, but showed a greater variation at the end of the light phase (ANOVA: F = 1.867; p = 0,0923). Plasma corticosterone concentrations were highly rhythmic and peaked slightly before the glucose peak (~ZT13, amplitude 237%, ANOVA: F = 8,852; p <0,001, CIRCWAVE: r2 = 0,53; p <0,001). Plasma leptin concentrations were modestly rhythmic and peaked in the middle of the dark phase (~ZT17, amplitude 32%, ANOVA: F = 4,073; p <0,005, CIRCWAVE: r2 = 0,22; p <0,001).

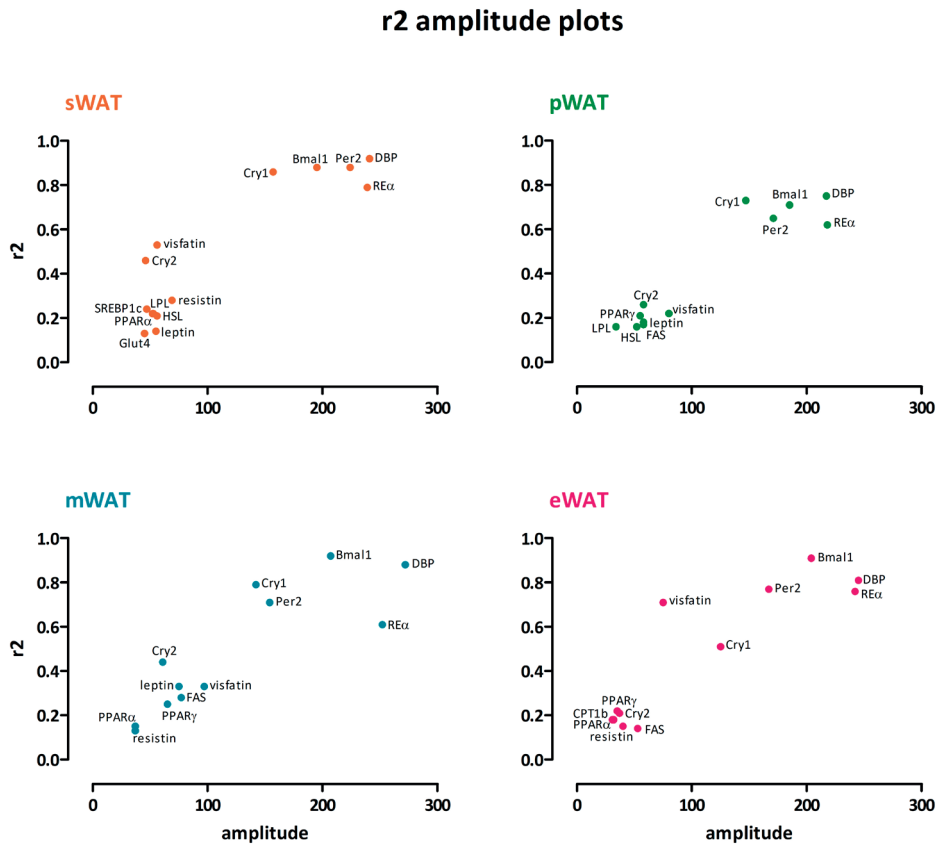

Figure 3. Clock genes show high amplitude together with high r2, whereas for metabolic genes modest amplitudes go along with low r2 values. A clear exception is Cry2, which showed much lower amplitude and r2 values than the other clock genes. We found no distinct differences between subcutaneous and intra-abdominal white adipose tissue (WAT) depots. sWAT, subcutaneous; mWAT, mesenteric; eWAT, epididymal; pWAT, perirenal. Individual expression curves for each gene and WAT depot can be found in Figure S1 in Supplementary Material.

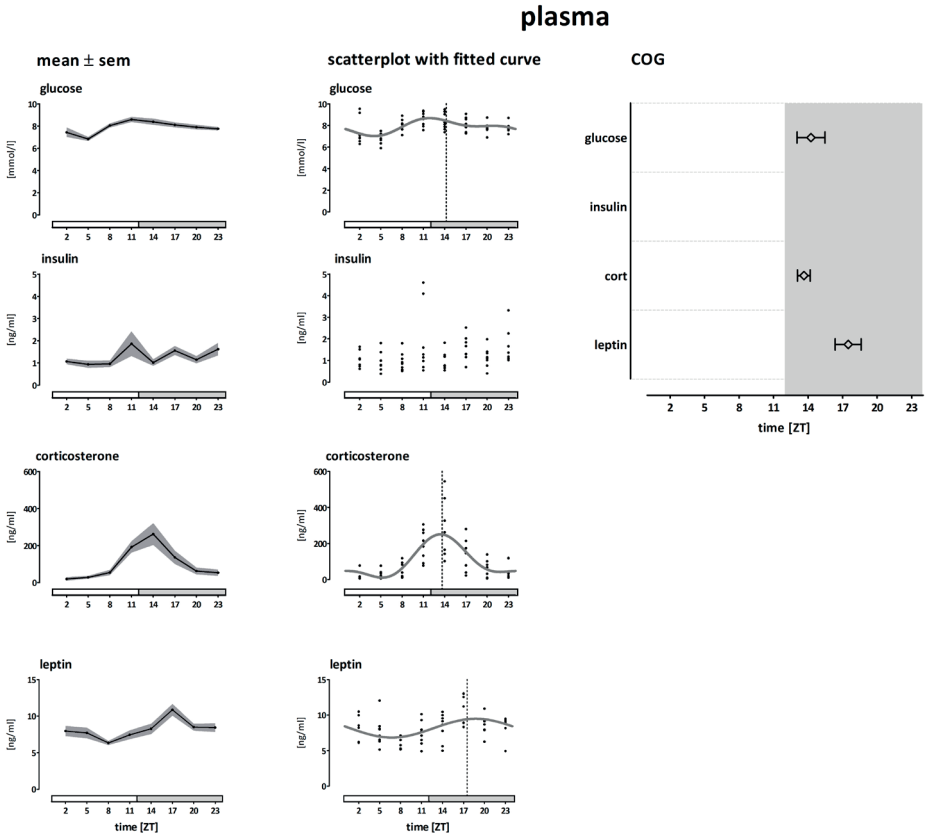

Figure 4. Plasma concentrations of glucose, insulin, corticosterone, and leptin. The left-hand graphs show the mean (±SEM) plasma concentrations. The right-hand graphs show the individual data points and Circwave curves, the dotted line indicates the Center of Gravity (COG). The bottom graph shows the COG (±SD). Absence of the Circwave curve and the COG line indicates absence of significant rhythmicity as analyzed by Circwave. Gray bars indicate the dark phase (ZT 12–24).

DISCUSSION

Different WAT depots have different functions, and increase and reduce their mass differentially, as illustrated by several metabolic disorders that result in loss of mainly subcutaneous or gain of mainly intra-abdominal (visceral) fat mass. Rhythmicity plays an important role in lipid metabolism, and clock gene rhythms have been described for some but not all WAT depots in rodents (Ando et al. 2005; Zvonic et al. 2006; Bray & Young 2007; Ando et al. 2009) and in humans (Gómez-Santos et al. 2009; Johnston 2012). We therefore hypothesized that differences in rhythmicity might explain differences in depot function, and analysed rhythmicity of gene expression in subcutaneous and different intra-abdominal WAT depots. However, in contrast to our hypothesis, we did not observe clear differences in clock gene rhythms between different WAT depots

(Figure 1-3). Moreover, most metabolic genes only showed modest or non-significant rhythmicity. Therefore, differences in the molecular clock or clock-controlled genes do not provide a major explanation for the differences in metabolic function between the different fat depots.

We observed robust rhythms in clock gene expression in all four fat depots studied (*Bmal1, Per2, Cry1, Cry2, RevErbα, DBP*), with a peak time that was similar to what has been described previously for Wistar rats (Ando et al. 2009; De Farias et al. 2015; Su et al. 2015) and other rodent species (Ando et al. 2005; Zvonic et al. 2006; Bray & Young 2007). Only few studies have measured clock gene expression rhythms in both epididymal WAT and subcutaneous (inguinal) WAT; one found lower amplitudes in subcutaneous WAT compared to epididymal (Bray & Young 2007), whereas in the other study, amplitudes were marginally smaller in epididymal WAT compared to subcutaneous WAT (Zvonic et al. 2006). In our data set, amplitude, robustness, or timing (COG) were not significantly different between mesenteric-, perirenal-, epididymal- and subcutaneous WAT depots. This is the first study to extensively compare clock gene rhythms in subcutaneous and different abdominal WAT depots simultaneously. Because we did not observe pronounced differences between depots under these untreated, ad libitum feeding conditions, this suggests that with regard to clock gene expression rhythms the results from subcutaneous inguinal WAT - which in humans is far less invasive to biopsy compared to internal WAT depots - may be extrapolated to other depots.

In contrast to the overt day/night rhythms in clock gene expression, expression of metabolic genes showed no profound rhythmicity. Metabolic genes that did show significant rhythmicity mostly showed peak expression in the active (dark) phase. These findings are in line with data from mice (Kohsaka et al. 2007). Metabolic genes are influenced by multiple circulating factors, such as corticosterone, insulin and nutrients, either directly (e.g., via a glucocorticoid response element; GRE) or via transcription factors (e.g., *SREBP1c, PPARs*) (Peek et al. 2012; Su et al. 2016; Reynés et al. 2017).

The daily rhythms in plasma corticosterone and glucose are independent of the daily rhythm in feeding behaviour, whereas plasma levels of insulin and glucagon are mainly regulated by food intake (La Fleur et al. 1999; Ruiter et al. 2003). Corresponding with previous data, we found that plasma concentrations of corticosterone and glucose peaked at the onset of the active phase. *PPARα* and *–γ* are glucocorticoid sensitive transcription factors (Desvergne et al. 1998), and indeed for *PPARs* we observed an expression peak with a similar timing as that of plasma corticosterone. Plasma insulin concentrations did not show a significant day/night rhythm, but rather followed feeding activity with 3 spikes during the dark phase. Several genes encoding for proteins involved with energy

storage in the fed state (*SREBP1c, PPARγ, LPL, FAS, Glut4, leptin, resistin*) showed a spiky expression pattern similar to the insulin curve (Supplemental Figure 1). These genes are likely influenced by feeding-induced insulin release, or by nutrients directly (e.g. via PPRE) (Reynés et al. 2017).

LPL serves as a gatekeeper that controls local fatty acid uptake into cells by catalysing the hydrolysis of circulating triglycerides. Transcription of *LPL* is upregulated by fatty acids, SREBP1c and PPARγ and downregulated and inactivated in the fasted state by glucocorticoids, catecholamines, and decreased levels of PPARγ and SREBP1c. In our data *LPL* showed a 3h delayed expression in sWAT compared to pWAT. In line with upregulation during the feeding period, we observed peak expression in eWAT when animals are eating. The delayed peak in *LPL* expression in sWAT fits with the hypothesis that intra-abdominal WAT is primarily functional in short term metabolic regulation, and sWAT takes up the lipid overflow for long term energy storage (Tchkonia et al. 2013). LPL protein concentration also peaks in the active period, but it remains to be determined how the rhythms in mRNA and protein content correspond to activity levels, as most physiological variation in LPL activity appears to be driven by posttranslational mechanisms by extracellular proteins (Kersten 2014).

Leptin concentrations peaked in the middle of the active (dark) phase (Figure 4), in line with previous experiments (Kalsbeek et al. 2001). This peak in plasma corresponds with the rhythm in *leptin* mRNA in fat tissue (Supplemental Figure 1 Leptin). We observed the clearest correlation between plasma leptin concentrations and *leptin* mRNA expression in mesenteric WAT (Supplemental Figure 2 Leptin correlation). Although we cannot compare absolute mRNA expression levels between depots (due to the number of samples we had to analyse each depot as a separate batch), others have shown that *leptin* mRNA levels are generally much higher in intra-abdominal depots, compared to subcutaneous depots (Oliver et al. 2001). Furthermore, they found plasma leptin levels correlated only with *leptin* expression in mesenteric WAT, but not any of the other WAT depots (Oliver et al. 2001), which is in line with the correlations we observed between *leptin* mRNA and plasma leptin concentrations. Another study comparing *leptin* mRNA expression rhythms between WAT depots in rats found expression curves quite similar to our data in mesenteric and perirenal (retroperitoneal) WAT. However, they found epididymal WAT to be rhythmic, in contrast to our dataset. These different observations accentuate the modest amplitude of the leptin expression rhythms; hence conclusions should be drawn with caution. In contrast to rodents, in humans subcutaneous fat tissue is the primary source of circulating leptin levels (Montague et al. 1997; Wiest et al. 2010). Therefore, the contribution from subcutaneous leptin mRNA to both plasma leptin levels, and their rhythm would be expected to be more important in humans. Indeed *leptin* mRNA is

rhythmic in human subcutaneous tissue as well (Johnston 2012). One explanation for this discrepancy between rodents and humans could be a different ratio of subcutaneous versus intra-abdominal fat mass.

A number of other factors in our study may have contributed to variation in gene expression, of both clock and metabolic genes. Firstly, our animals had ad libitum access to food, which could have induced small variations in timing of food intake between animals which might have led to less robust rhythms. Secondly, we have used Circwave to analyse rhythmicity in our data. Circwave recognises wave forms using Fourier transformation whereby harmonics are added in a stepwise regression like fashion (using F-testing). This method is based on the assumption that the rhythms consist of one or more sine waves, and that noise variance is Gaussian (normally) distributed and independent of measurement magnitude. Therefore, it limits the recognition of spiky and saw tooth-shaped wave forms (Thaben & Westermark 2014). Although the choice for this method might influence the sensitivity with which we were able to recognise rhythms, it will only affect our main conclusion (no rhythmic differences between depots) if there would be major differences in shape of wave between the WAT depots. Looking at the raw data sets (Supplemental Figure 1) we may underestimate spiky rhythmicity of insulin or nutrient regulated genes. Nevertheless, alternative methods do not allow for estimation of amplitudes and phases (Thaben & Westermark 2014), which were our main outcome measures.

We found no evidence that differences in rhythmicity in clock or metabolic genes underlie the functional differences described for the different WAT depots. Alternative explanations for functional differences are differences in pre-adipocyte lineage (Tchkonia et al. 2013), differences in innervation, or differences in local regulation. Typically, the hypothalamus integrates peripheral signals and ensures energy homeostasis by regulating peripheral energy metabolism via humoral pathways and the autonomic nervous system (ANS). Indeed, intra-abdominal and subcutaneous WAT are innervated by separate sets of neurons (Bartness et al. 2014), all the way up to the pre-autonomic neurons in the hypothalamus (Kreier et al. 2002). Subcutaneous (inguinal) WAT gains more adipose cells after denervation compared to intra-abdominal (retroperitoneal) WAT (Bowers et al. 2004). These data indicate that differential innervation can contribute to functional differences between WAT depots, but apparently do not result in differences in rhythmicity. Whether differences in functionality are indeed depending on differences in autonomic activity at the level of WAT still needs to be proven. Moreover, it could well be that ANS mediated differences in WAT functionality only surface during positive or negative energy balance.

Concluding, in contrast to our hypothesis, we did not observe clear differences in (clock) gene expression rhythms between different WAT depots. Moreover, we found only modest rhythmicity in metabolic gene expression rhythms, and no results that could explain differences in metabolic function between the different WAT depots. Therefore, functional differences between WAT depots likely stem from other regulatory levels (i.e., translational) or pathways.

ABBREVIATIONS

ANS Autonomic Nervous System; COG Centre Of Gravity (see method section); WAT White Adipose Tissue; ZT Zeitgeber Time

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AUTHOR CONTRIBUTIONS

RS, EF, SF and AK conceived and designed the experiments. RS, SF and AK performed the experiments and analysed the data. RS, EF, SF, AK wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00206/full#supplementary-material.

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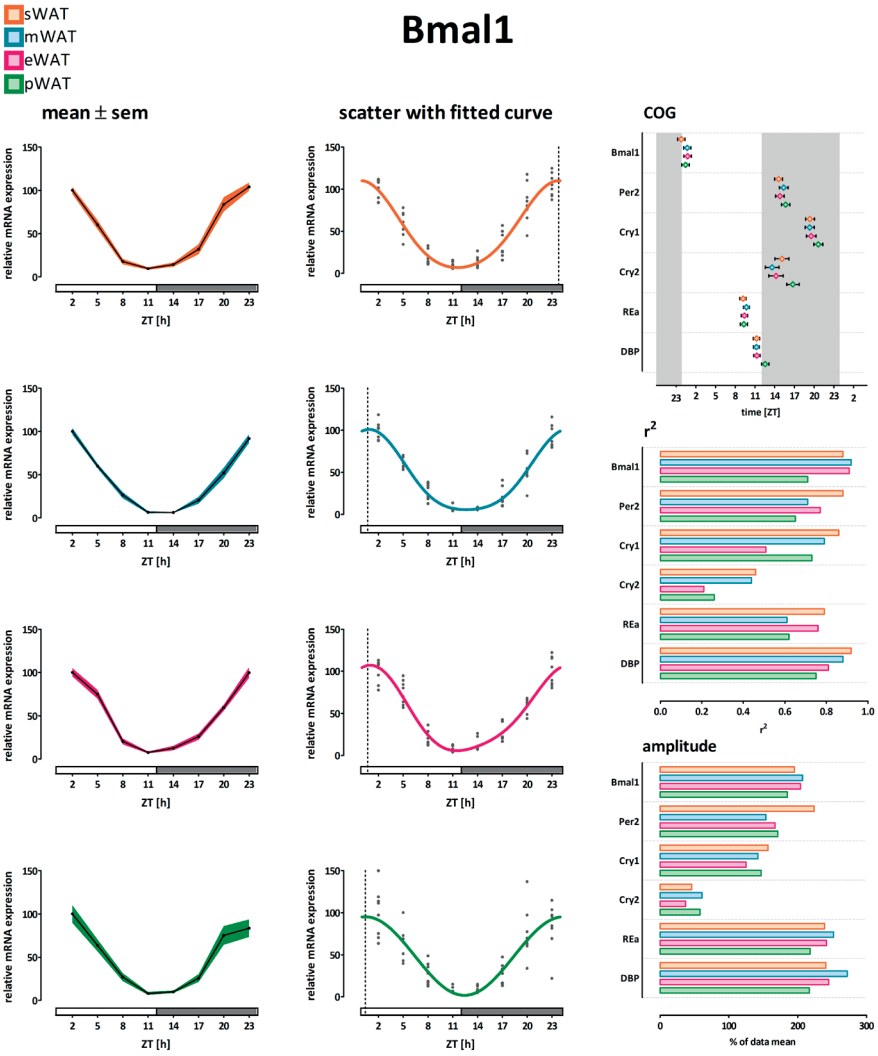
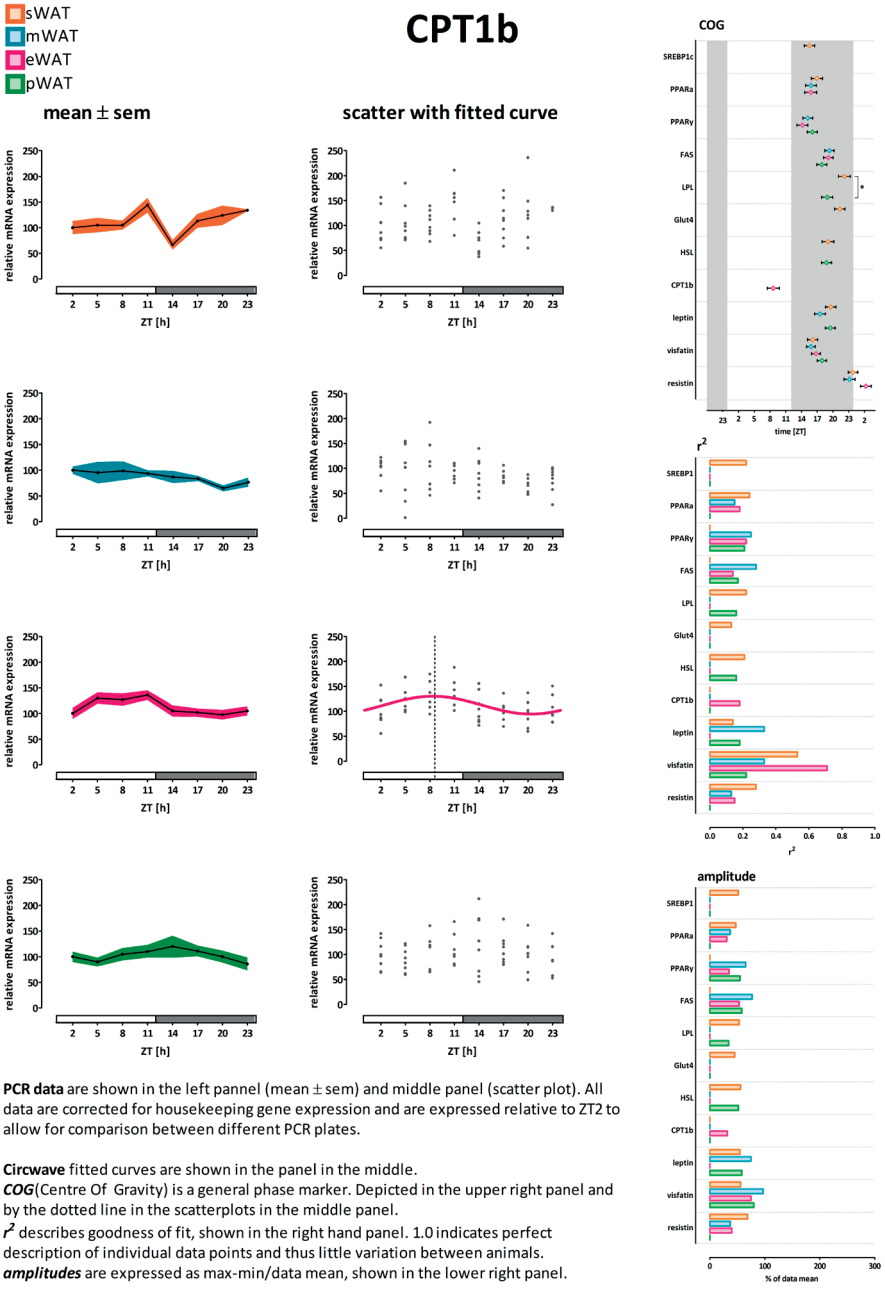
SUPPLEMENTARY MATERIAL



PCR data are shown in the left pannel (mean ± sem) and middle panel (scatter plot). All data are corrected for housekeeping gene expression and are expressed relative to ZT2 to allow for comparison between different PCR plates.

Circwave fitted curves are shown in the panel in the middle.
COG(Centre Of Gravity) is a general phase marker. Depicted in the upper right panel and by the dotted line in the scatterplots in the middle panel.
r^2 describes goodness of fit, shown in the right hand panel. 1.0 indicates perfect description of individual data points and thus little variation between animals.
amplitudes are expressed as max-min/data mean, shown in the lower right panel.



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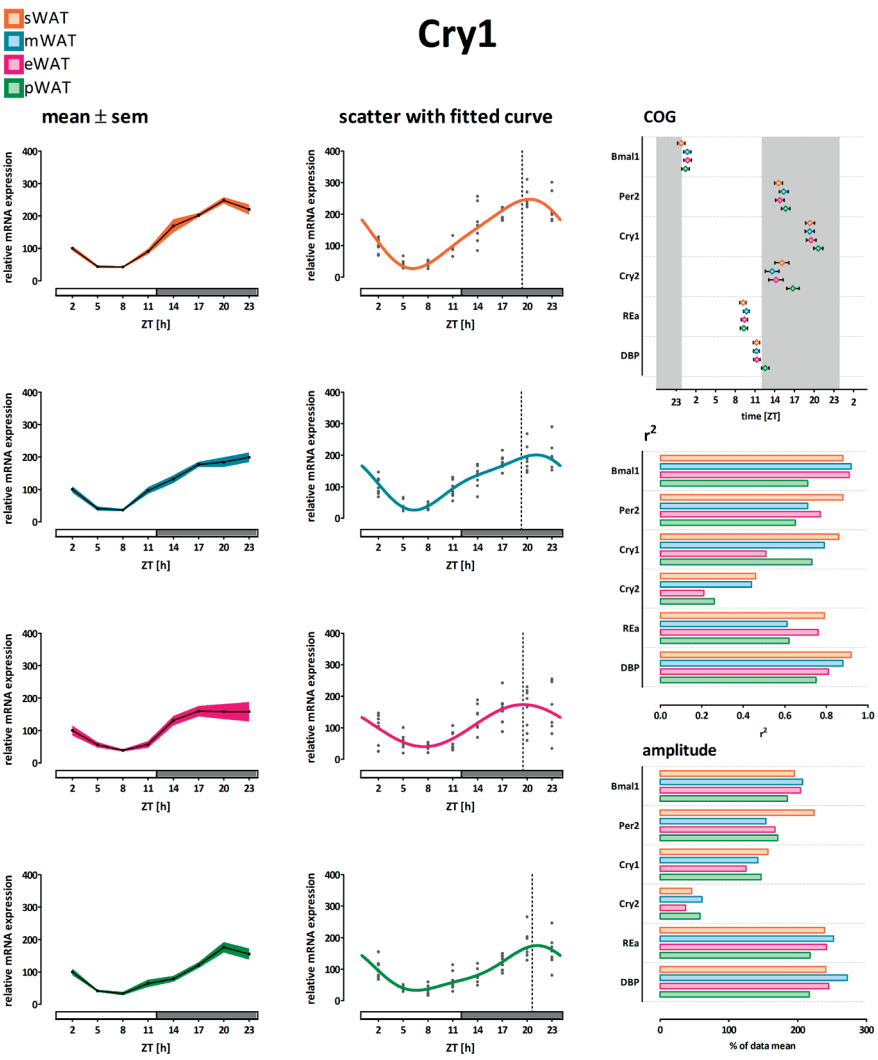

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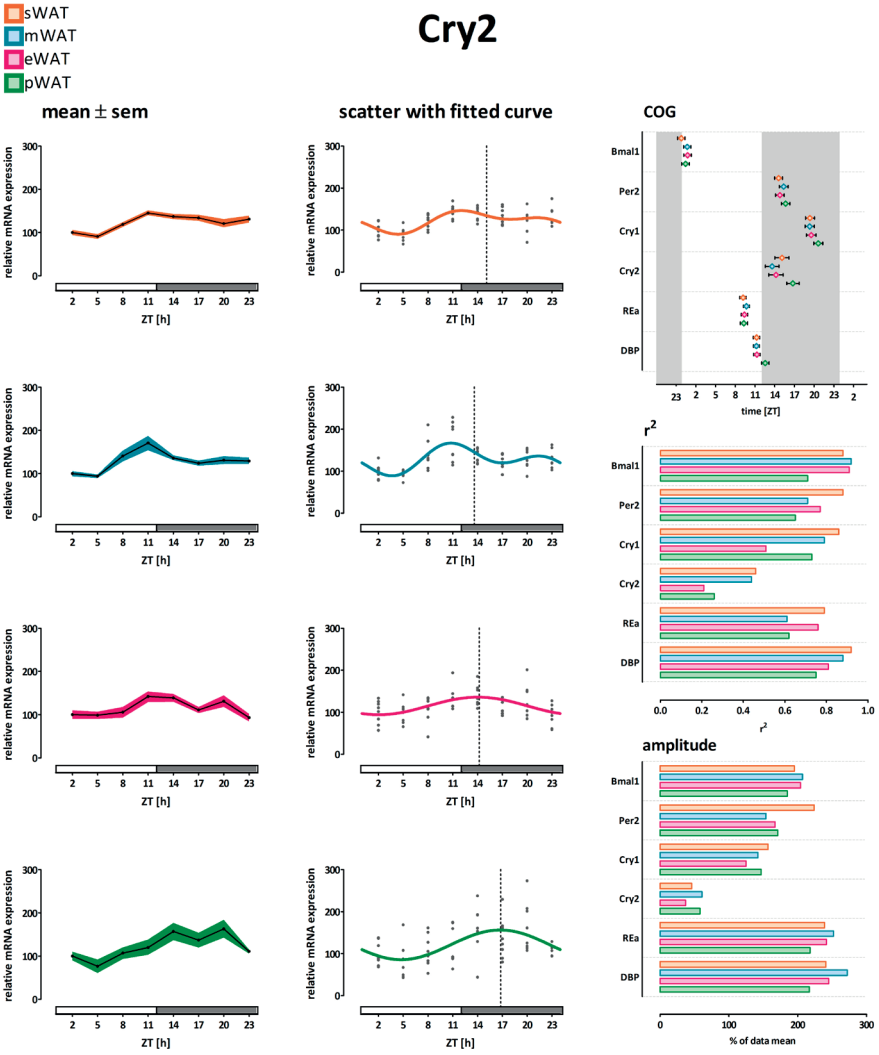

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Circwave fitted curves are shown in the panel in the middle.
COG(Centre Of Gravity) is a general phase marker. Depicted in the upper right panel and by the dotted line in the scatterplots in the middle panel.
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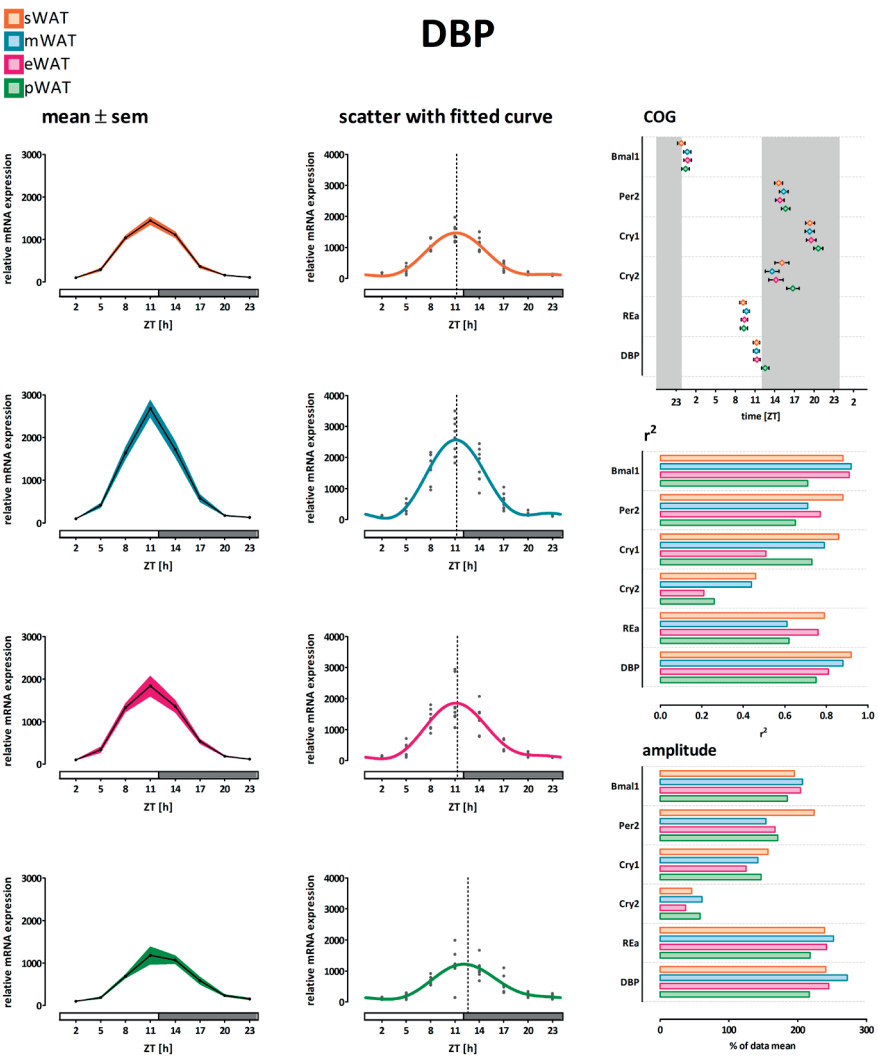

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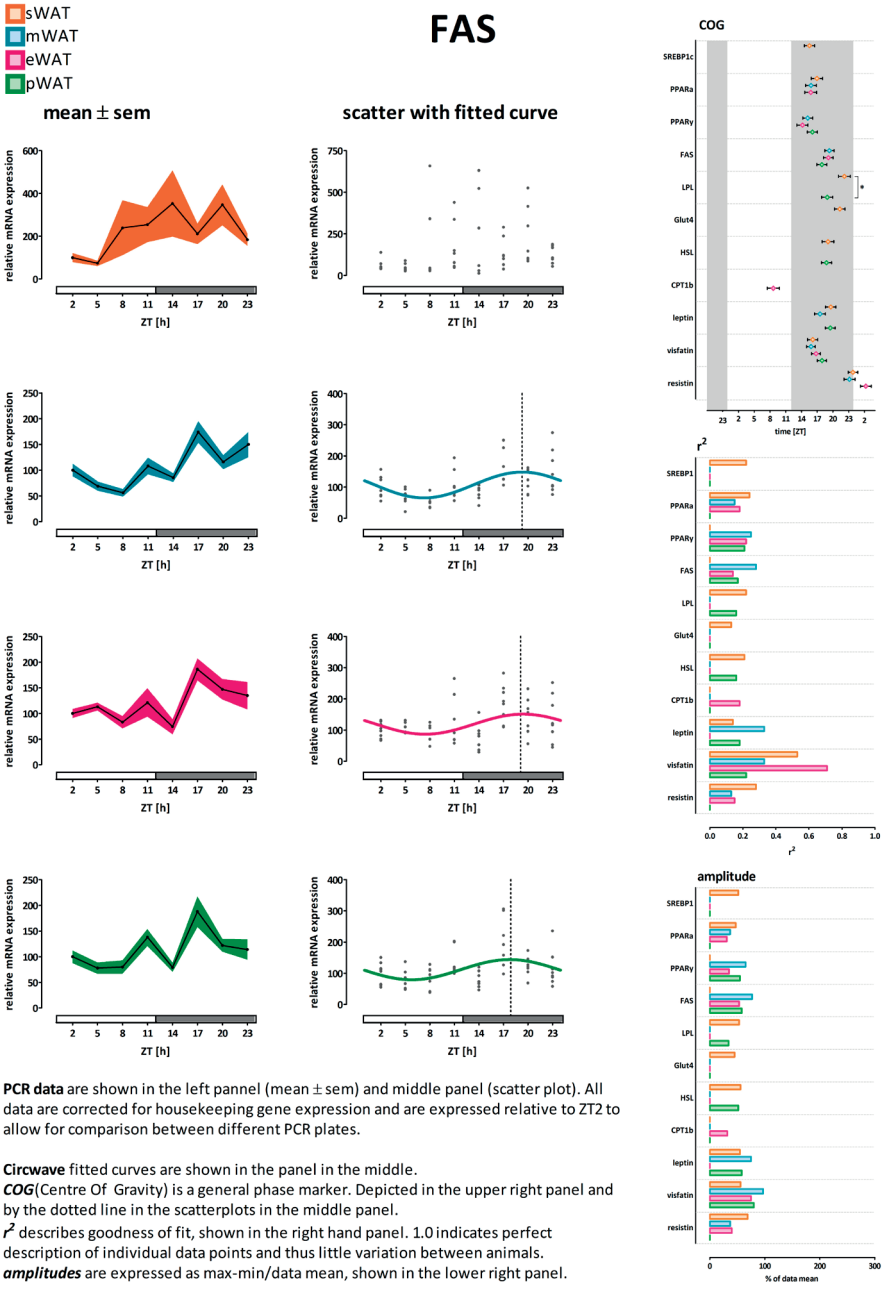

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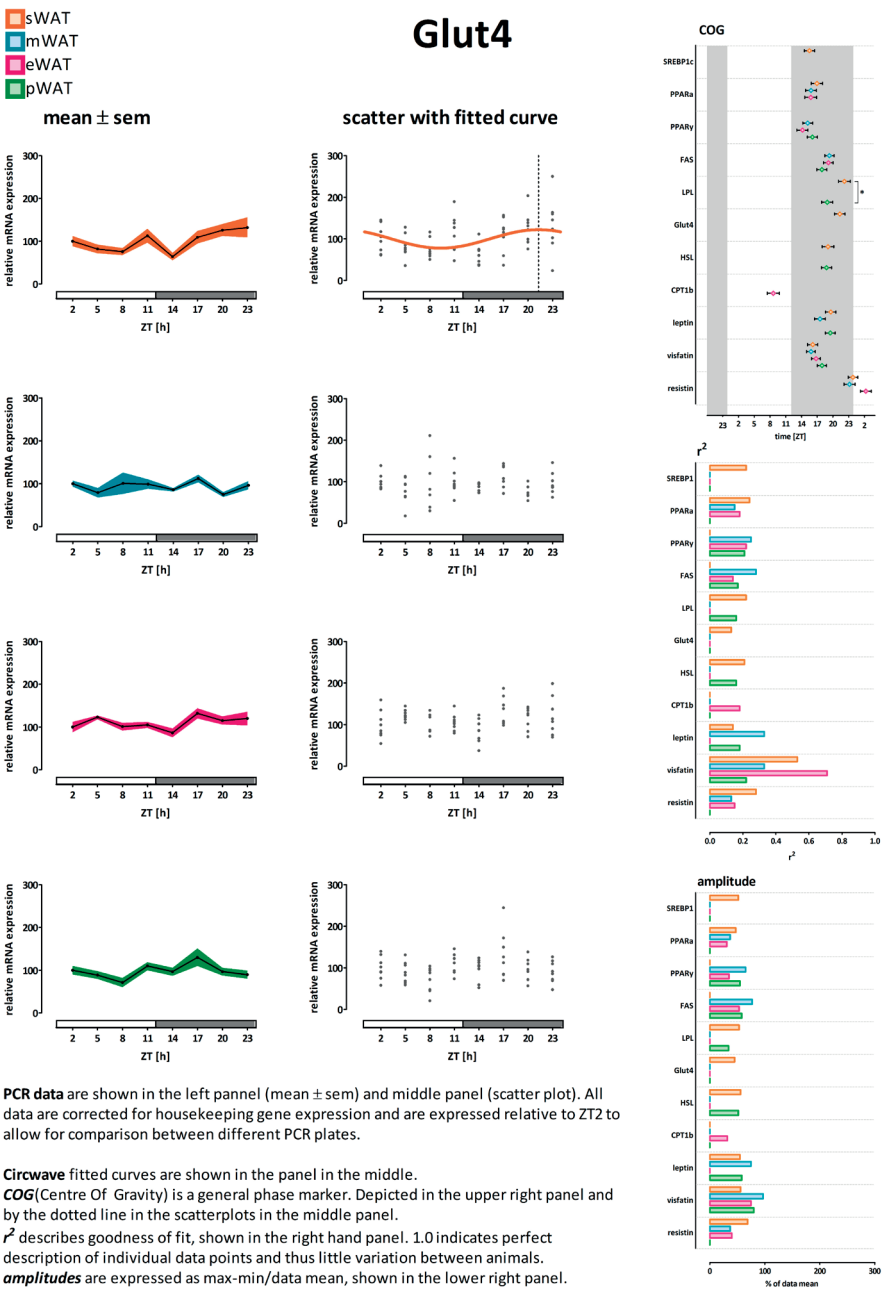

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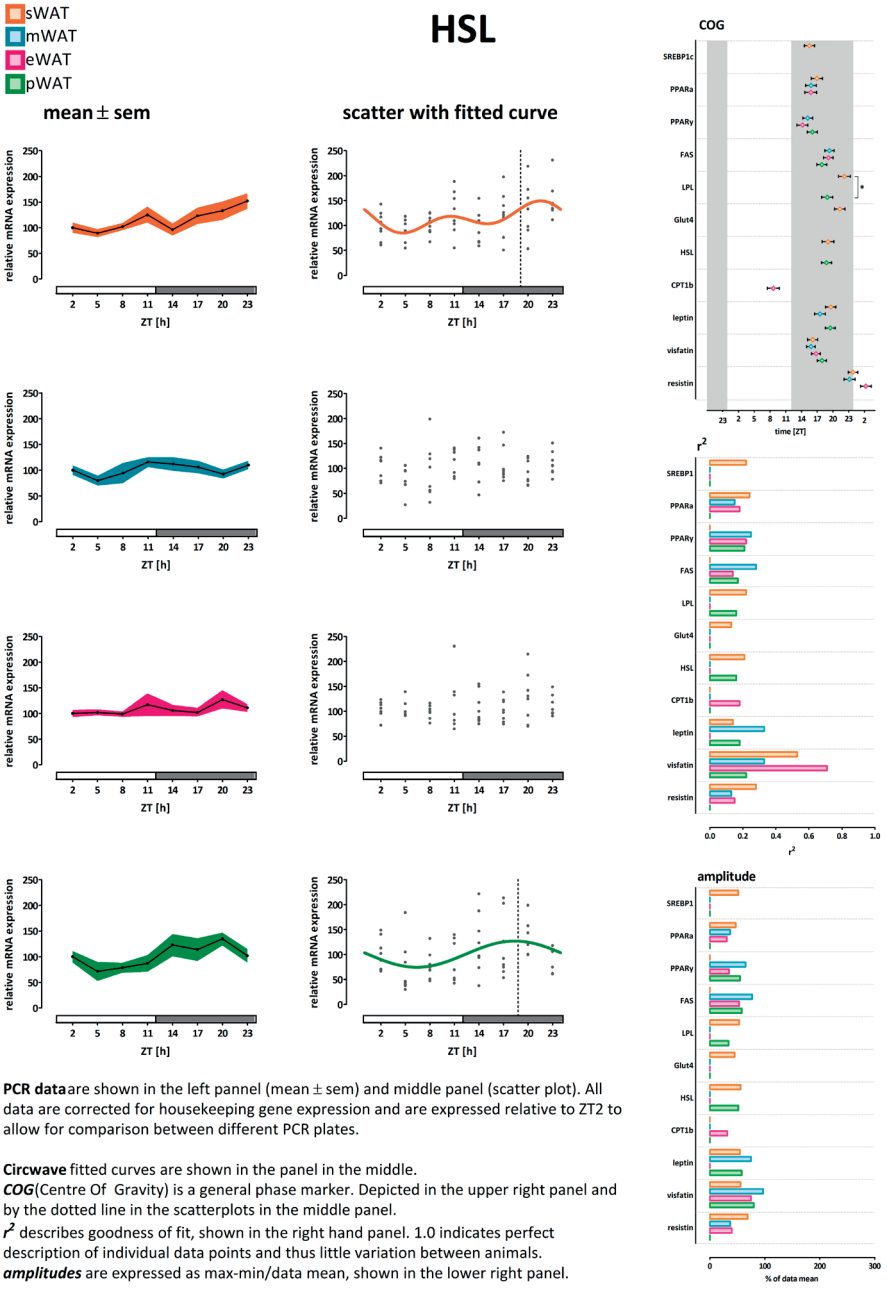

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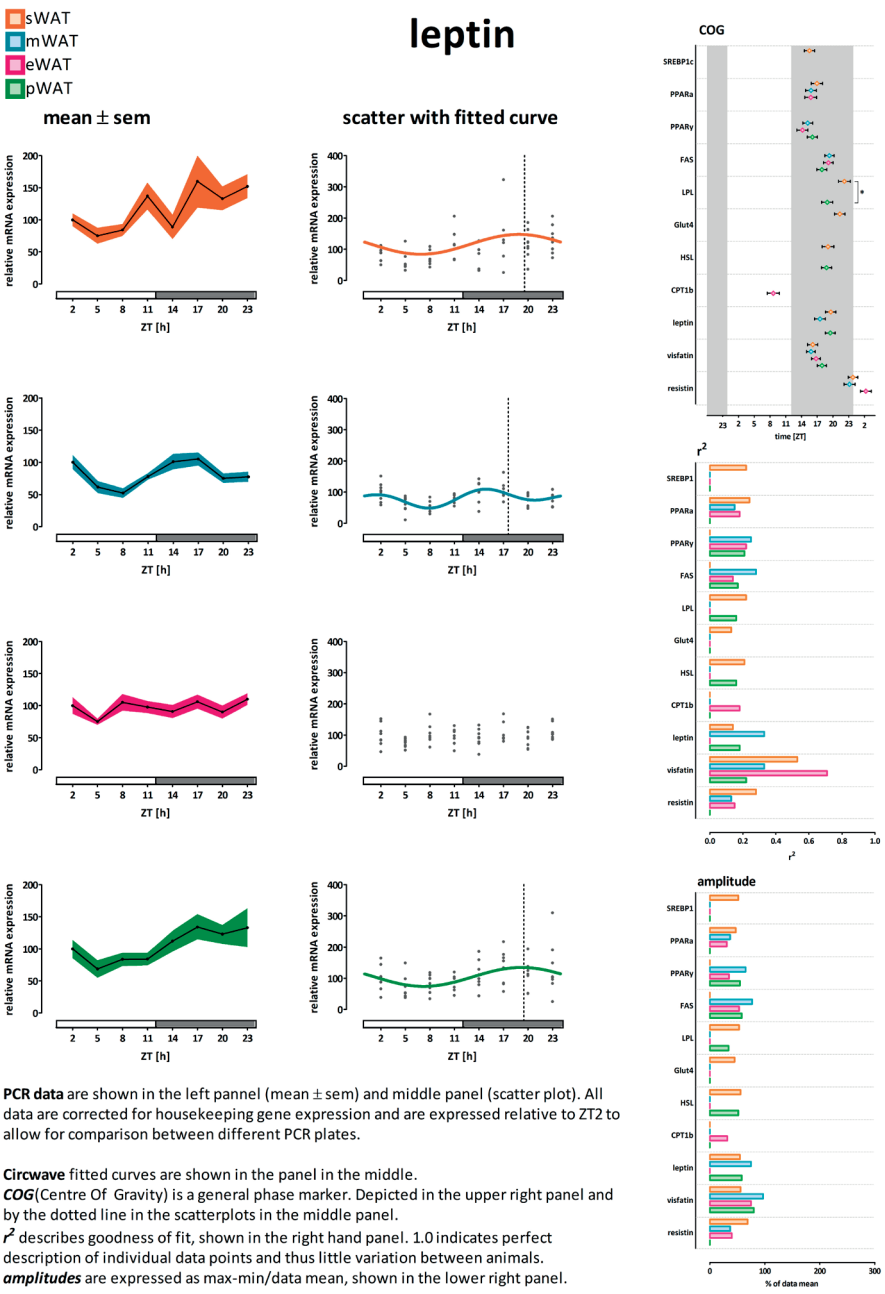

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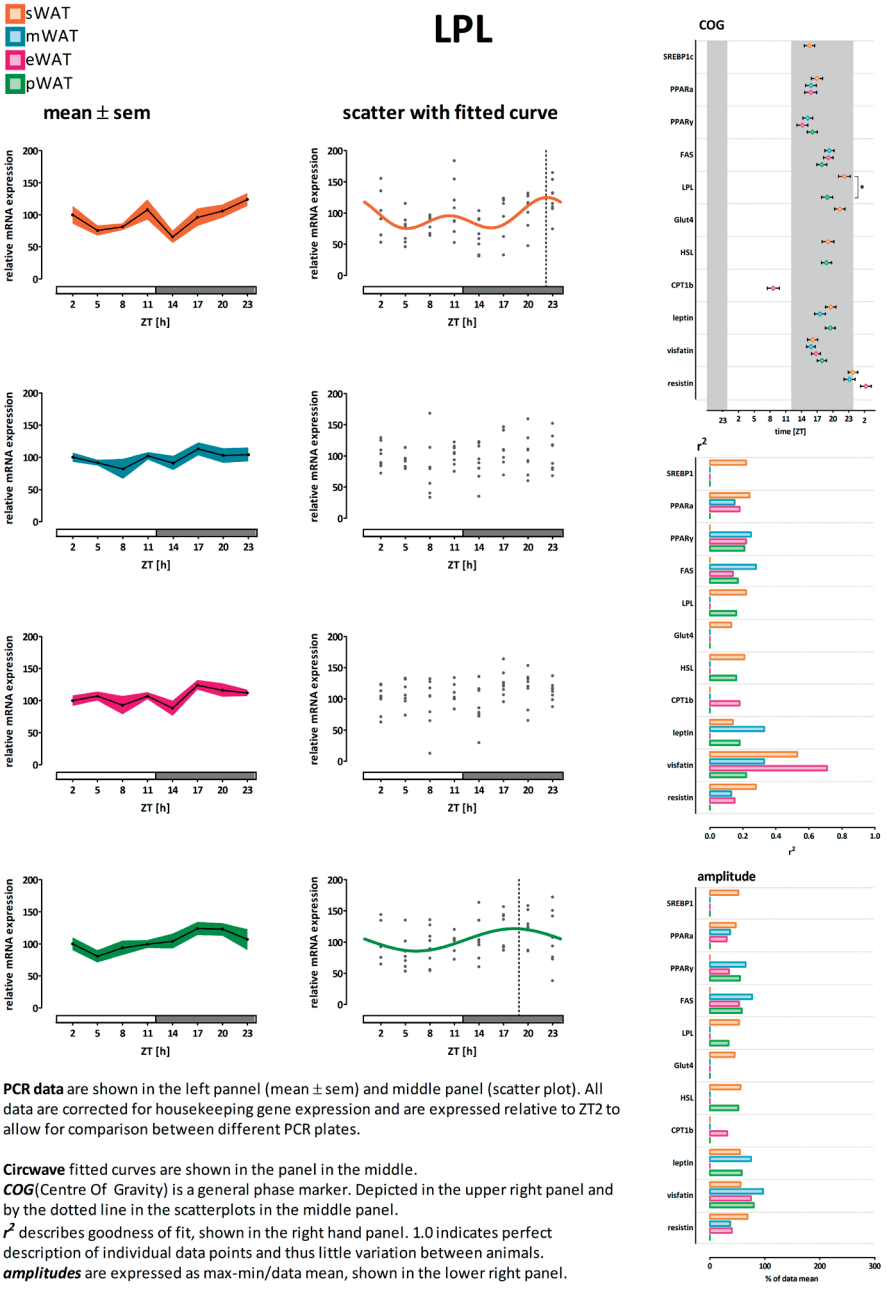

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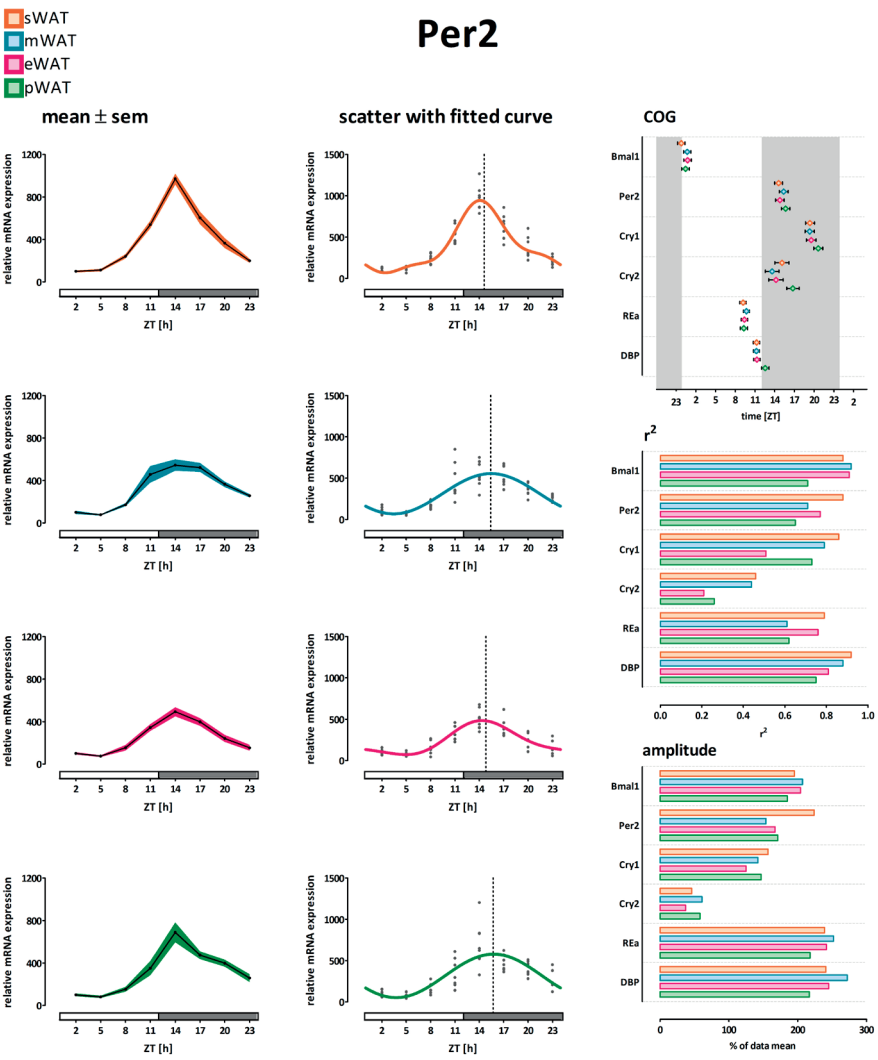

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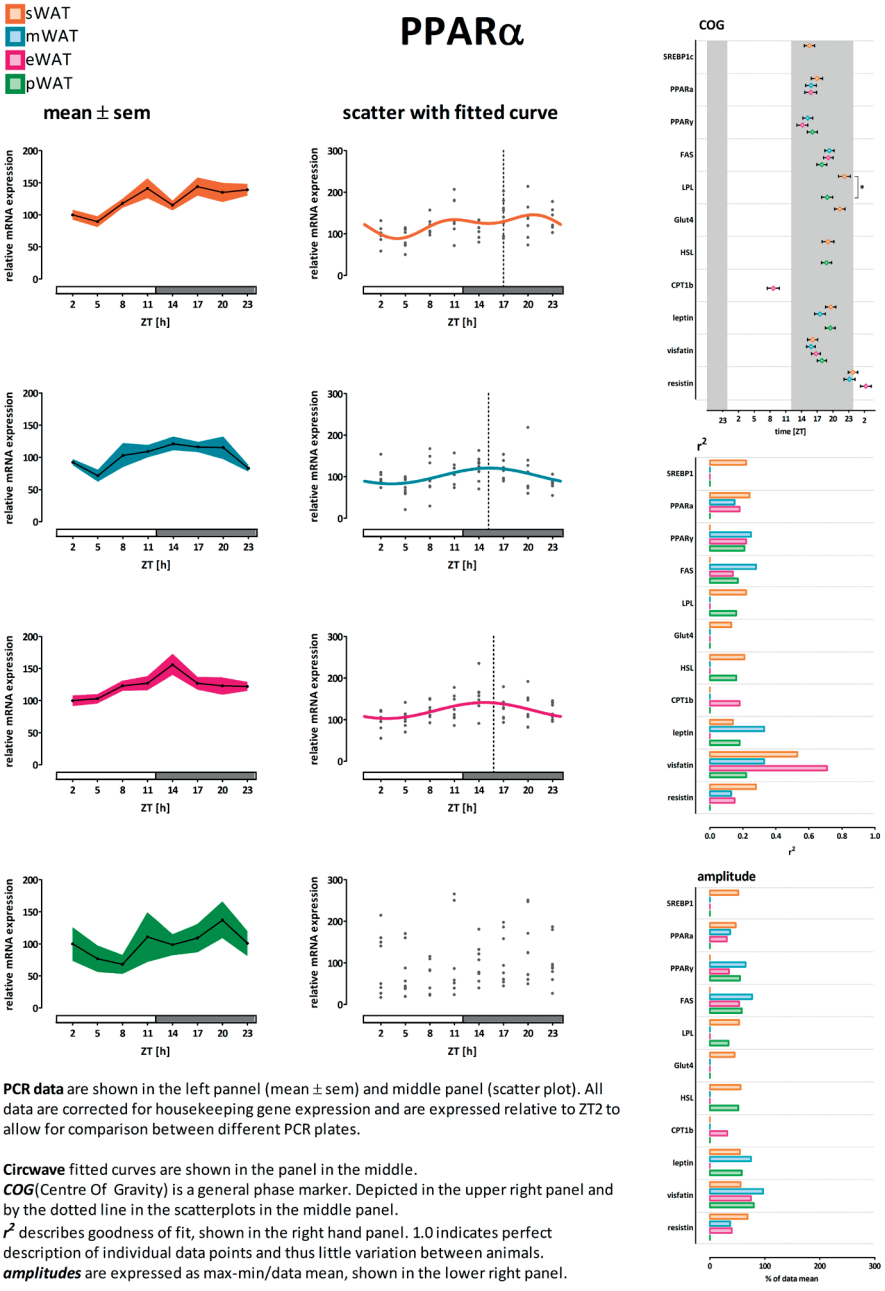

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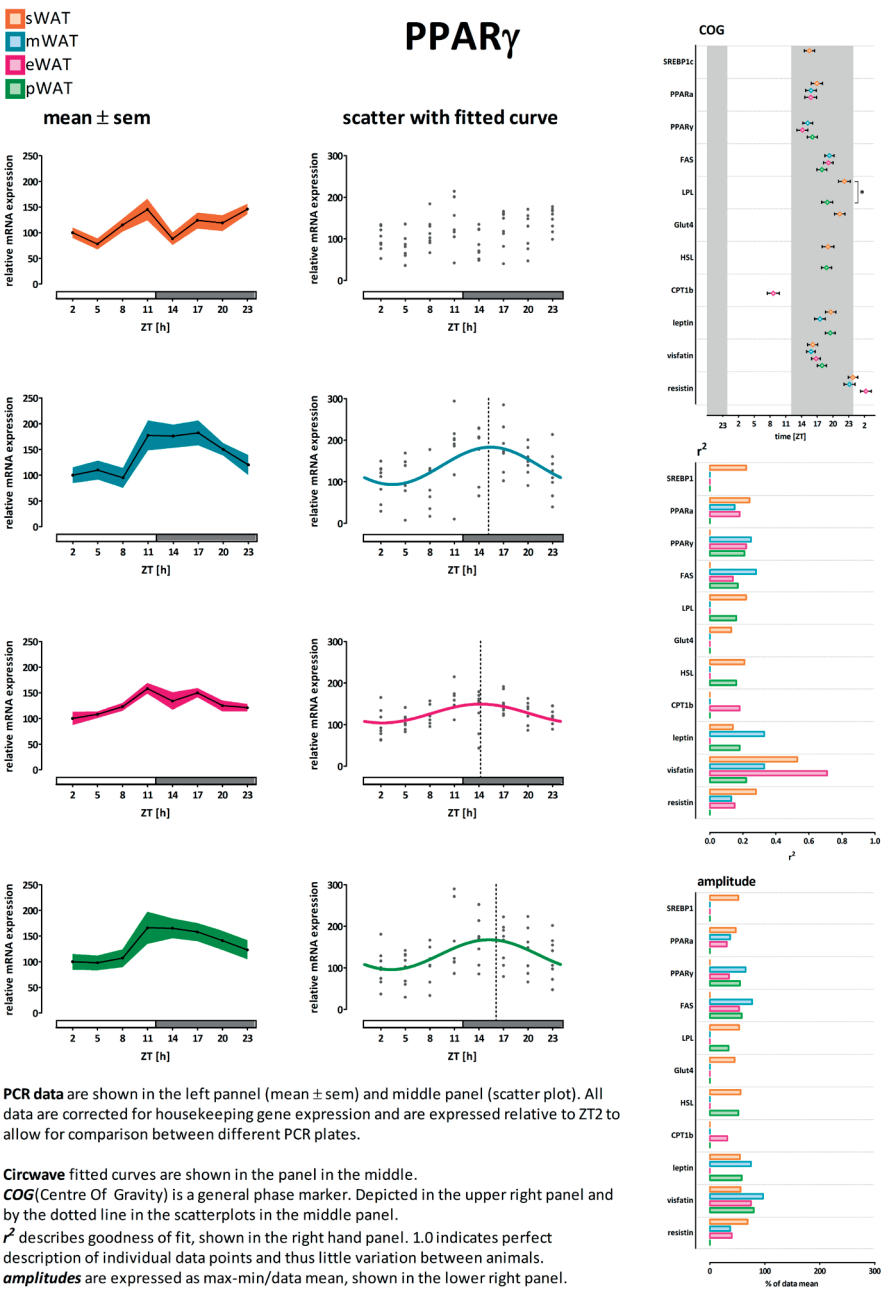

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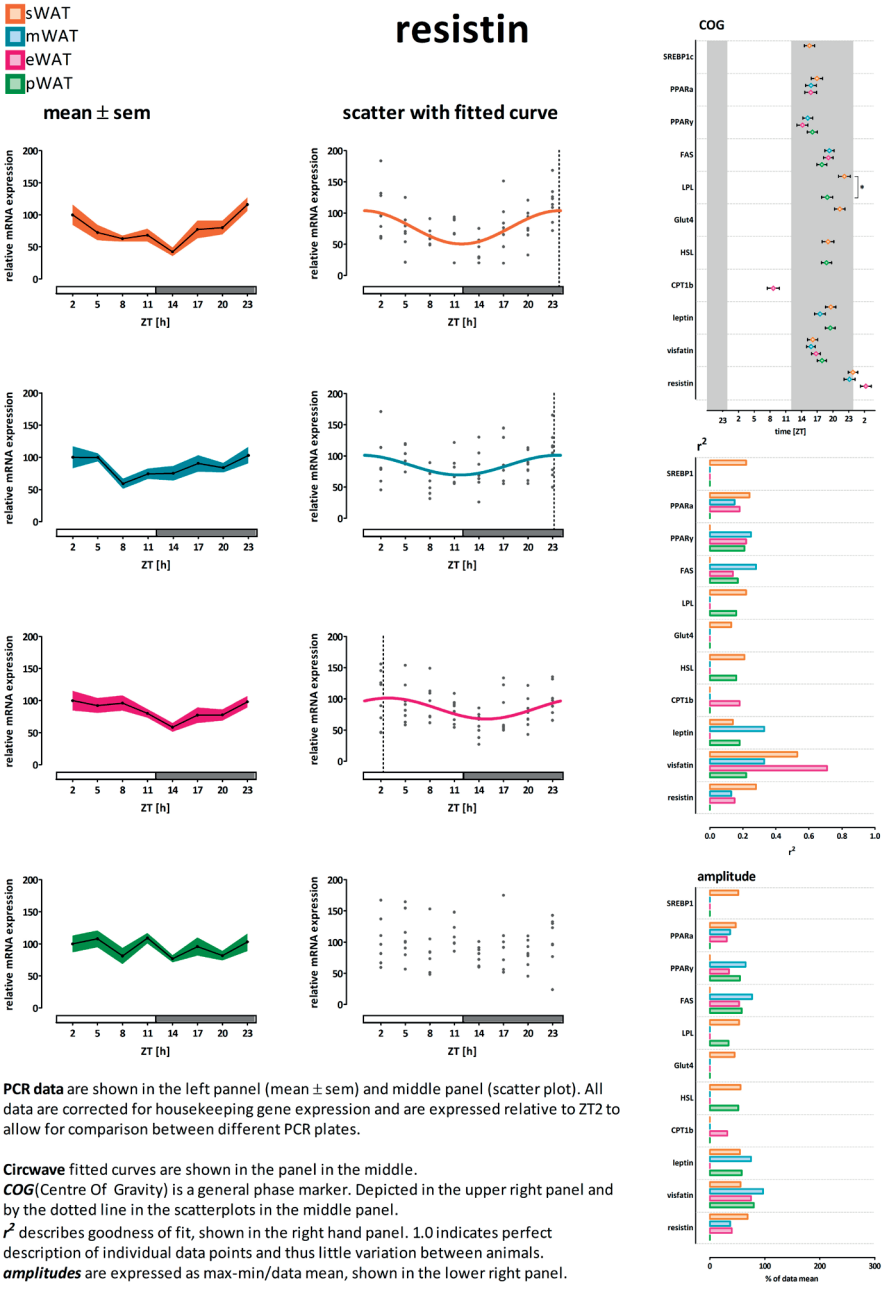

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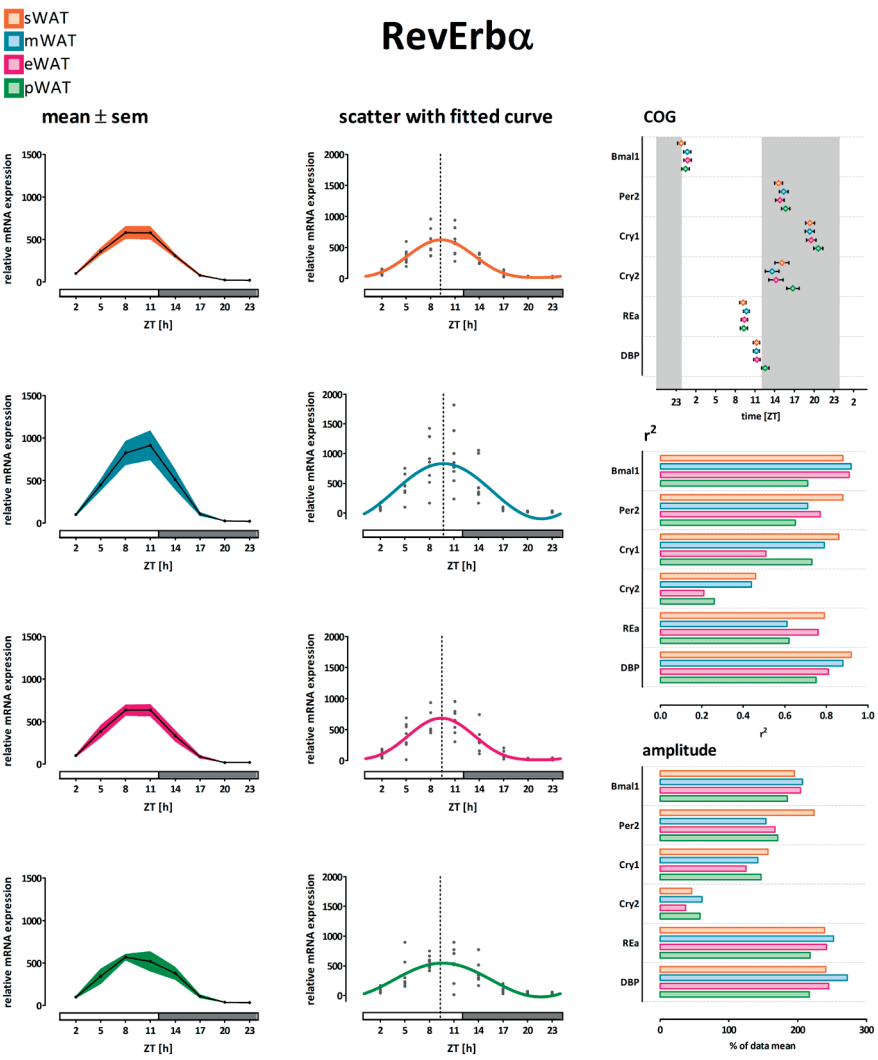

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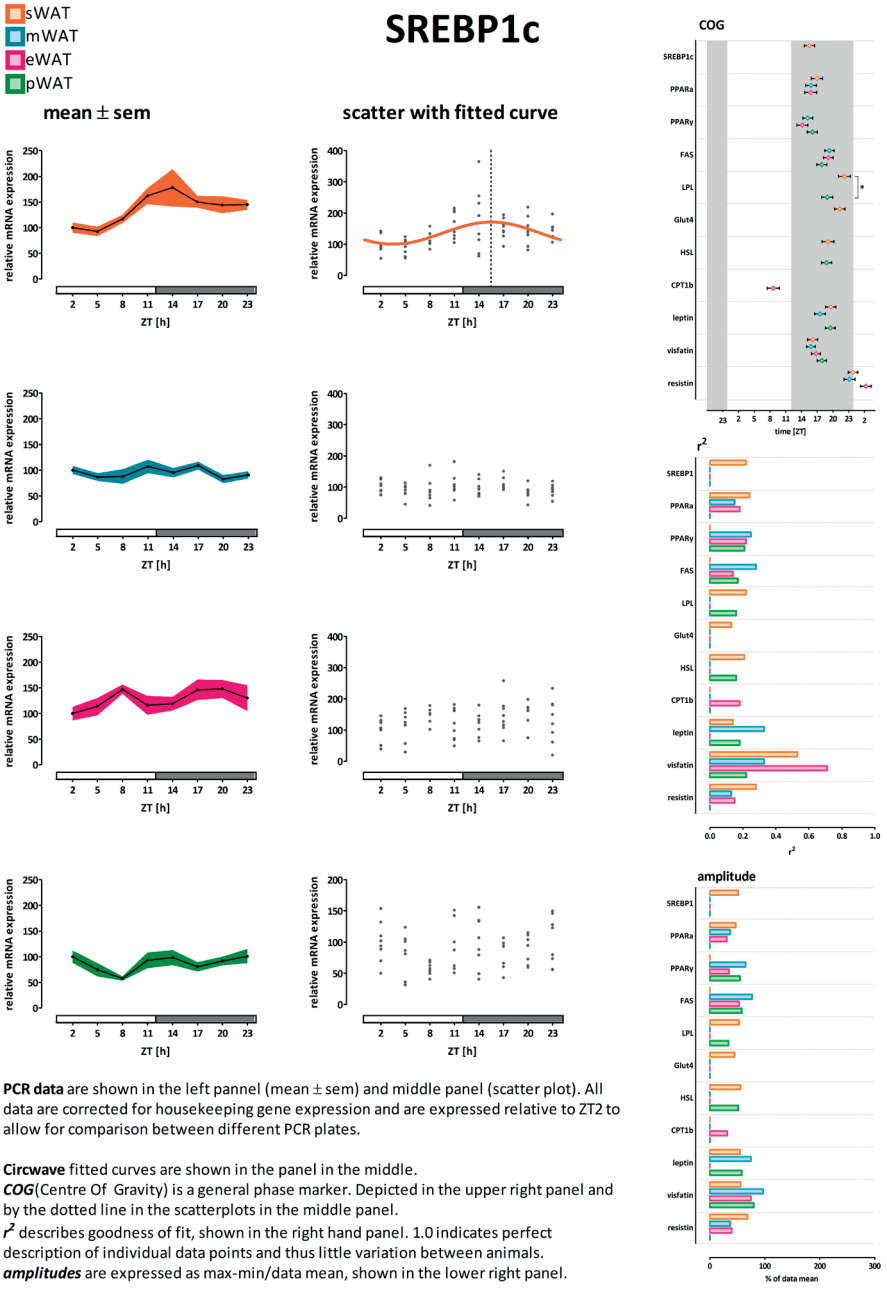

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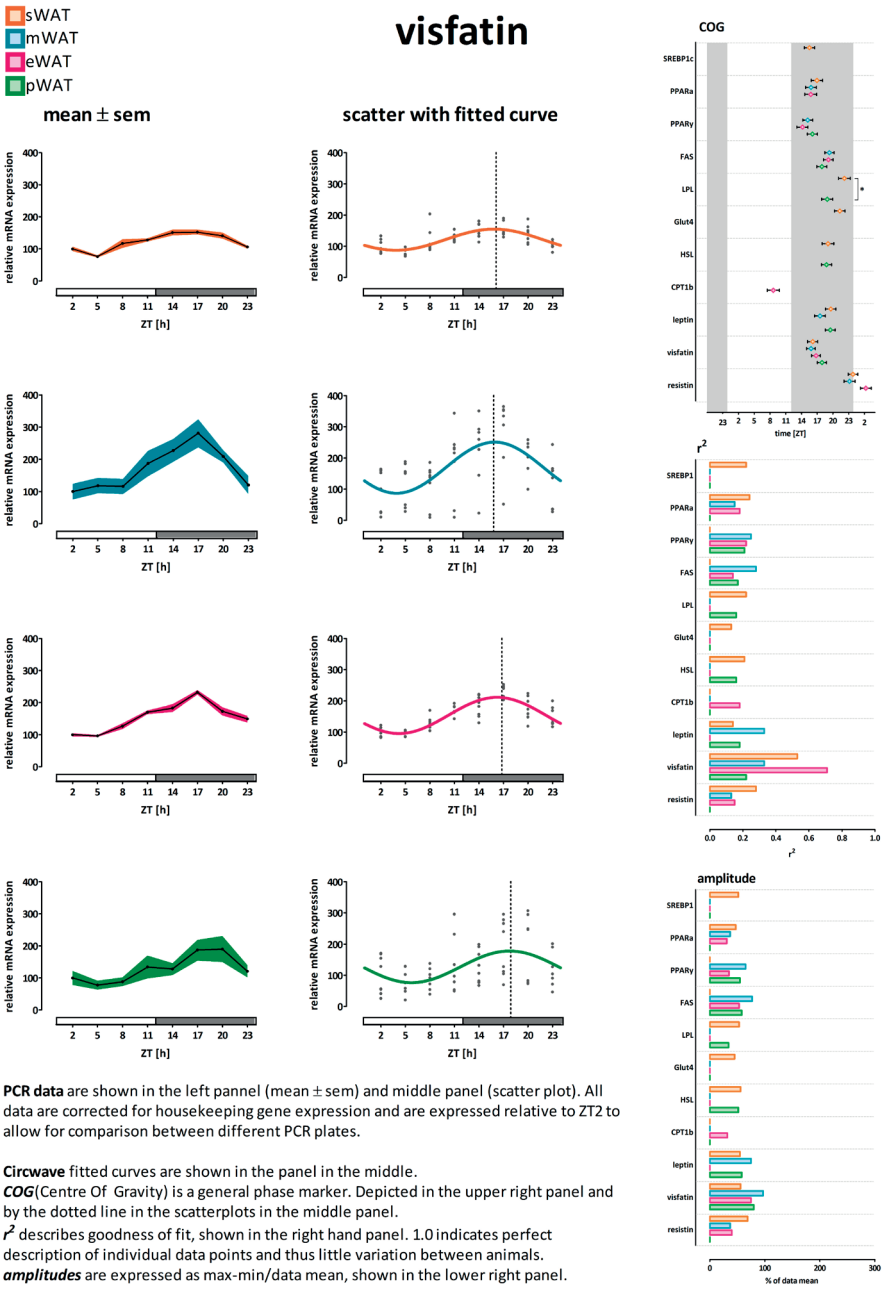

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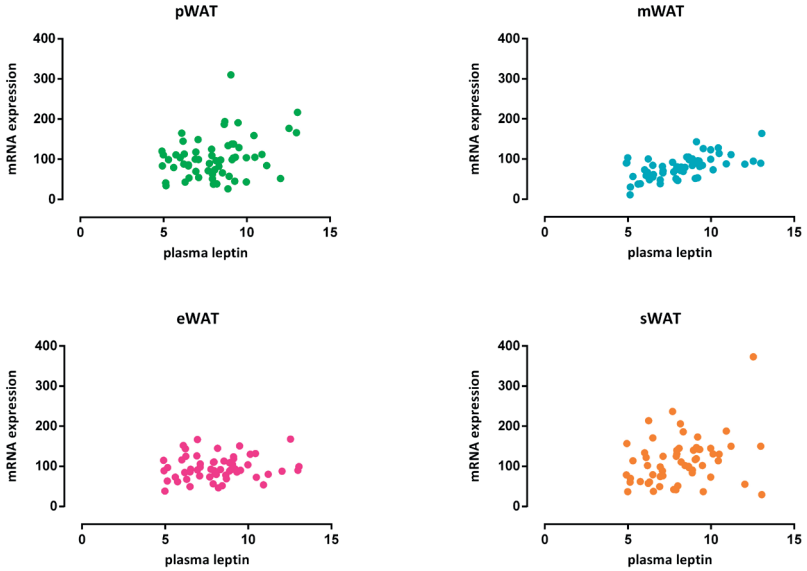
	pWAT	eWAT	mWAT	sWAT
Number of XY Pairs	60	60	60	58
Pearson r	0.3049	0.1352	0.5883	0.2833
95% confidence interval	0.05524 to 0.5188	-0.1230 to 0.3763	0.3930 to 0.7328	0.02693 to 0.5047
P value (two-tailed)	0.0178	0.3029	< 0.0001	0.0312
P value summary	*	ns	***	*
Is the correlation significant? (alpha=0.05)	Yes	No	Yes	Yes
R square	0.09299	0.01829	0.3461	0.08027



4

Thermal lesions of the SCN do not abolish all gene expression rhythms in rat white adipose tissue, NAMPT remains rhythmic

Van der Spek R
la Fleur SE
Fliers E
Kalsbeek A

Chronobiology International, 2021

ABSTRACT

Obesity and type 2 diabetes mellitus are major health concerns worldwide. In obese-type 2 diabetic patients, the function of the central brain clock in the hypothalamus, as well as rhythmicity in white adipose tissue (WAT), are reduced. To better understand how peripheral clocks in white adipose tissue (WAT) are synchronized, we assessed the importance of the central brain clock for daily WAT rhythms. We compared gene expression rhythms of core clock genes (Bmal1, Per2, Cry1, Cry2, RevErbα, and DBP) and metabolic genes (SREBP1c, PPARα, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, leptin, adiponectin, visfatin/NAMPT, and resistin) in epididymal and subcutaneous white adipose tissue (eWAT and sWAT) of SCN-lesioned and sham-lesioned rats housed in regular L/D conditions. Despite complete behavioural and hormonal arrhythmicity, SCN lesioning only abolished Cry2 and DBP rhythmicity in WAT, whereas the other clock gene rhythms were significantly reduced, but not completely abolished. We observed no major differences in the effect of SCN lesions between both WAT depots. In contrast to clock genes, all metabolic genes lost their daily rhythmicity in WAT, with the exception of NAMPT. Interestingly, NAMPT rhythmicity was even less affected by SCN lesioning than the core clock genes, suggesting that it is either strongly coupled to the remaining rhythmicity in clock gene expression, or very sensitive to other external rhythmic factors. The L/D cycle could be such a rhythmic external factor that generates entrainment signals by photic masking via the intrinsic photosensitive retinal ganglion cells in combination with the autonomic nervous system. Our findings indicate that in normal weight rats, gene expression rhythms in WAT can be maintained independent of the central brain clock.

INTRODUCTION

The circadian timing system coordinates physiology and behaviour and enables an organism to anticipate recurring events during the day-night cycle. In mammals, a molecular clock is found in nearly every cell, consisting of a network of transcriptional translational auto-regulatory feedback loops (TTFL) (recently reviewed in (Takahashi 2017). The 'central pacemaker' or 'master clock' in the mammalian brain resides in the suprachiasmatic nucleus (SCN), a bilateral nucleus in the anterior hypothalamus, located just above the optic chiasm. Its endogenously generated circadian rhythm is synchronized to the exact 24h rhythm of the external light-dark (LD) cycle by photic input from the retina via the retinohypothalamic tract (RHT) (Canteras et al. 2011). Although photic input is the main stimulus for synchronizing the SCN to the external environment, information from many other time cues, such as locomotor activity and arousal, variation in body temperature, local energy availability, circulating nutrients and hormones, as well as social signals contribute to this process. The SCN then uses various signalling pathways to relay this integrated temporal information to the 'peripheral clocks' in other brain areas and in the rest of the body (Asher & Schibler 2011; Albrecht 2012; Mohawk et al. 2012).

White adipose tissue (WAT) is an essential metabolic and endocrine tissue, especially given the rise in obesity prevalence worldwide. Circadian disruption disturbs metabolic homeostasis, especially when it is sustained. For example, during shift work the timing of food intake is often not aligned with endogenous clock rhythms in liver, pancreas, and WAT and, therefore, glucose and lipid homeostasis is not ensured. This increases the risk to develop obesity and related metabolic disease (Scheer et al. 2009; Stenvers, Scheer, et al. 2019). Similarly, in rodents, circadian disruption due to genetic mutations or misaligned environmental time cues (repeated shifts of the LD cycle, time restricted feeding) can induce metabolic dysfunction (Johnston et al. 2016; Tsang et al. 2017). On the other hand, rodents with obesity or disturbed glucose regulation show decreased WAT rhythmicity (Ando et al. 2005). Likewise, we recently found that in obese patients with type 2 diabetes, the number of rhythmic transcripts in subcutaneous WAT was reduced by 80% as compared to lean controls (Stenvers, Jongejan, et al. 2019). To better understand via which mechanism circadian disturbance induces metabolic disease, it is first necessary to understand how peripheral clocks in WAT are synchronized to ensure energy homeostasis.

Daily rhythms in feeding behaviour, as well as hormone release, affect daily gene expression rhythms in WAT. Removal of either the daily rhythm in food intake or that of the adrenal hormones abolished rhythmicity in many clock-controlled metabolic genes in

rats. However, clock gene expression rhythms in WAT remained intact. Only when both the feeding and adrenal rhythms were removed, did clock gene expression in WAT (and liver) became arrhythmic (Su et al. 2015; Su et al. 2016). These findings indicate that the daily rhythms of many clock-controlled genes are in fact not solely controlled by local clock genes, but also by outputs from the central clock in the SCN, such as rhythmicity in nutrients, hormones, and behaviour. Therefore, in the current experiments we set out to assess in further detail the importance of the central brain clock for WAT rhythms in rats.

There are different genetic approaches to assess the role of the SCN in WAT rhythmicity; down-regulating the activating TTFL component Bmal1 (Husse et al. 2014; Izumo et al. 2014; Lee et al. 2015), or upregulating the repressing component Per2 (Lee et al. 2015). However, these genetic approaches leave other clock machinery intact (Husse et al. 2014; Izumo et al. 2014; Lee et al. 2015). Although SCN specific Bmal1 ablation severely dampened SCN rhythmicity, the rhythmicity in eWAT clock gene remained, albeit with 30% amplitude reduction and a phase advance of ~4.8h (Kolbe et al. 2016). To exclude that this remaining rhythmicity is an effect of residual SCN function, we employed an alternative approach and used thermal SCN lesions, a well-established method (Kalsbeek et al., 2001; Kalsbeek et al., 1992; La Fleur et al., 1999; La Fleur et al., 2001; Moore & Eichler, 1972; Ruiter et al., 2003; Stephan & Zucker, 1972), which selectively ablates SCN tissue, thus leaving no residual clock gene activity in the SCN. Striving for the smallest lesions possible induces a risk for incomplete lesions. Incomplete lesions often leave the most ventral part of the SCN that is embedded in the optic chiasm intact. If this light recipient part remains intact, this may be sufficient to maintain rhythmicity in L/D conditions, but not constant darkness. Therefore, we housed all animals in L/D conditions. Nevertheless, housing animals in L/D conditions does not enable discrimination between SCN- and non-SCN mediated effects of light for all rhythms. We hypothesized that thermal lesions of the SCN would remove the 24h rhythmicity in all clock- and clock-controlled genes in WAT.

MATERIALS AND METHODS

Animals

Male Wistar rats (Harlan, Horst, Netherlands) were housed with 4-6 animals per cage in a controlled environment, on a 12/12h light/dark cycle (ZT0 is lights on at 07:00h), at room temperature (20 ± 2°C). Food and tap water were provided ad libitum throughout the experiment. The experiment was conducted under approval of the Local Animal Welfare Committee and in accordance with international ethical standards (Portaluppi et al. 2010).

SCN lesions

Bilateral thermal SCN lesions were made in rats weighing 180-210g (n=91). Rats were anaesthetized with Hypnorm (0.8ml/kg i.m.) and Dormicum (0.3ml/kg s.c.), and mounted in a stereotact (David Kopf Instruments, Tujunga, CA), with the toothbar set at 5 mm. After identification of the Bregma, electrodes were introduced bilaterally through a drilled hole in the skull, using the following coordinates: 1.4 mm rostral to Bregma, 1.0 mm lateral to midline, 8.3 mm below the brain surface, at an angle of 6°. The electrode diameter was 0.2 mm and a temperature of 85°C was generated for 1 min (Lesion generator RFG4A by Radionics, Burlington, MA). This temperature was found empirically to result in lesions big enough to eliminate the SCN, yet small enough to leave tissue surrounding the SCN, such as the paraventricular nucleus (PVN), intact, as described and illustrated previously (Kalsbeek et al. 1992; La Fleur et al. 1999; Palm et al. 1999; Kalsbeek et al. 2000; Kalsbeek et al. 2001). Sham animals underwent the same procedure, but no current was passed through the electrodes.

After two weeks, when the animals had recovered from the anaesthesia and surgical procedure, they were housed individually and effectiveness of the lesions was checked by measuring daily rhythmicity of water intake for 3 weeks. If animals drank >30% of their daily water consumption in the middle 8h of the light period (ZT2-ZT10), lesions were considered successful and animals were included in the SCNx group (n=31). This "drinking" measure of arrhythmicity correlates well with immunohistochemical screening of completeness of the SCN lesion by using vasoactive intestinal peptide (VIP) staining as a marker for SCN tissue (La Fleur et al. 1999; Kalsbeek et al. 2000; La Fleur et al. 2001). Sham animals (n=31) typically drank only 15% of their daily water intake in these 8h in the middle of the light period.

Tissue collection

Five weeks after lesioning, animals were anaesthetized with isoflurane and killed by decapitation at a 6h interval starting at ZT2 (N=7-8 per group and time point) to obtain WAT tissues and plasma. Epididymal (eWAT) and subcutaneous inguinal (sWAT) white adipose tissues were dissected and snap frozen in liquid nitrogen, and the contralateral WAT depot was dissected for weighing. Furthermore, perirenal- and mesenteric WAT, bilateral adrenals, and thymus were dissected and weighed. Blood was collected in heparinized tubes.

Plasma analyses

Following decapitation, trunk blood was kept on ice until centrifugation for 15 min at 3000 rpm at 4°C. Plasma was transferred to a clean tube and stored at -20°C until use. Plasma glucose was measured using a Biosen apparatus (EKF diagnostics, Cardiff, UK).

Plasma insulin, leptin, and corticosterone were measured using a Radio Immuno Assay (Merck Millipore, Billerica, MA, USA).

Gene expression analysis

We studied core clock and metabolic gene expression rhythms in epididymal and subcutaneous white adipose tissue (eWAT and sWAT, respectively) in SCN-lesioned and sham-lesioned rats at 4 time points along the L/D-cycle (ZT2, ZT8, ZT14, and ZT20). Gene expression analysis was performed as previously described in detail (van der Spek et al. 2018). In short, total RNA was extracted from approximately 100 mg of adipose tissue, using the RNeasy lipid kit including on-column DNAse treatment (Qiagen Benelux, Venlo, Netherlands). cDNA was synthesized with the Transcriptor First Strand cDNA synthesis kit (Roche, Almere, Netherlands). Gene expression was analysed by real-time RT-qPCR on a Lightcycler 480 system (Roche, Almere, Netherlands). We used additional non-reverse transcriptase samples to control for potential DNA contamination, positive controls, and melting peak analysis for product verification, and analysed individual sample PCR efficiency to exclude all samples that deviated more than 0.05 from mean efficiency. Hypoxanthine Phosphoribosyl Transferase (HPRT) gene expression was used as a housekeeping gene to control for variation in amount of mRNA input. Primer sequences of clock genes Bmal1, Per2, Cry1, Cry2, RevErbα, and DBP, and metabolic genes SREBP1c, PPARα, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, leptin, visfatin/NAMPT, and resistin, have been published previously (Su et al. 2016).

Data analysis and statistics

All PCR data are expressed relative to ZT2, to allow comparison between WAT depots and treatment groups. For identification of outliers, we used Dixon's Q test with two-tailed values using 95% confidence values (Rorabacher 1991). Samples that were determined to be outliers were excluded from further analysis (see supplemental table 4). All data are presented as mean ±SEM unless otherwise stated. P values below 0.05 were considered statistically significant.

Rhythmicity was assessed using Circwave 1.4 (www.hutlab.nl) (van der Spek et al. 2018). Circwave software fits one or more fundamental sinusoidal curves through the individual data points and compares this with a horizontal line through the data mean (a constant). If the fitted curve differs significantly from the horizontal line, the data set is considered rhythmic. Circwave provides the following information: number of sines in the fitted curve; data mean, the average of all data points with SD; Centre of Gravity (COG), representing the general phase of the curve with SD; ANOVA F stat, p-value, and R2; Circwave F stat, p-value and R2. For genes that remained rhythmic after SCN lesioning, a shift in COG was assessed by an unpaired 2-tailed student T-test without assuming

consistent SD using GraphPad Prism 7. Amplitudes of Circwave curves were calculated as percentages of data mean to enable comparison of amplitudes between data sets [difference between the zenith (highest point) and nadir (lowest point) and divided by the data mean ((max – min / mean) * 100%)].

RESULTS

After recovery from surgery, SCN lesioned (SCNx, n=91) and sham operated control rats (SCNsh, n=31) were housed individually and water consumption was measured for the middle 8h (ZT2-ZT10) of the light period for 3 weeks. Sham-lesioned control animals drank on average 16±4% (mean±SD) of their daily water consumption during the light period. Successfully SCN lesioned animals (n=31) drank on average 33±4% during the light period, whereas excluded SCN lesioned animals (n=60) drank on average 22±4% (One-way Anova $P < 0.0001$, $R2 = 0.71$, T-test SCNsh vs SCNx; $p < 0.0001$, Supplemental Figure 1). Furthermore, in line with previous findings, the daily peak in plasma corticosterone was absent in the SCN lesioned animals (Supplemental Figure 2).

Body weight gain in SCN lesioned animals was reduced as compared to sham controls (Supplemental Figure 3, repeated ANOVA; Interaction $p < 0.001$; Time $p < 0.001$; Group $p = 0.06$), as described previously. Sham lesioned animals weighed 6% more at the end of the experiment (BW SCNsh 358±6 g vs. SCNx 337±6 g, T-test $p < 0.05$). Total WAT depot weight corrected for body weight did not differ between groups (tWAT: SCNsh 3.9±0.2% vs. SCNx 5.3±1.0%, T-test $p = 0.11$). Sham lesioned animals had higher thymus weights (thymus: SCNsh 544 ±19 mg vs. SCNx 473±17 mg, T-test $p < 0.01$), but adrenal weight did not differ between groups (adrenal SCNsh 65±1 mg vs. SCNx 65±2 mg, T-test $p = 0.99$) (Supplemenal Figure 4).

SCN lesions reduced amplitude and advanced the phase of clock gene rhythms

In sham-operated animals, clock gene expression showed pronounced daily rhythms in both WAT depots (Figures. 1 & 2), with amplitudes and peak times comparable to our previous findings in unoperated control animals (Su et al. 2015; van der Spek et al. 2018) and also findings by others in their controls (Ando et al. 2005; Sukumaran et al. 2010; De Farias et al. 2015; Kolbe et al. 2016). Cry2, which showed least robust rhythmicity, and DBP lost their rhythmicity in both WAT compartments after SCN lesions, as well as Per2 in eWAT. Clock genes with more robust rhythms, Bmal1, Per2 in sWAT, Cry1, and RevErbα remained rhythmic after SCN lesions, albeit with lower amplitudes and reduced R2 values (Figures. 1 & 2). Amplitudes of clock gene rhythms were reduced by approximately

50% after SCN lesions (Supplemental Table 1). Likewise, R2 values of clock gene rhythms were reduced by approximately 50% after SCN lesions (suppl. table 2). The COG of clock gene rhythms was advanced by ~4 hours after SCN lesions (Supplemental Table 3).

Overall, the effect of SCN lesions on clock gene expression in sWAT and eWAT was similar (on average amplitudes reduced by -53% in sWAT vs. -49% in eWAT; R2 reduced by -47% in sWAT vs. -54% in eWAT, and COG advanced by 3.4h in sWAT vs. 4.4h in eWAT). Two minor differences between sWAT and eWAT were observed: first, the COG of Bmal1 advanced by 3.5h in eWAT ($p < 0.001$), but did not shift in sWAT (-0.1h, $p = 0.91$). Second, Per2 lost rhythmicity in eWAT, but not in sWAT. Per2 expression in eWAT increased at ZT8 and decreased at ZT14 in SCN lesioned animals compared to controls (thus, peak expression advanced) (Figures 1 & 2), effectively flattening the curve and abolishing the rhythm.

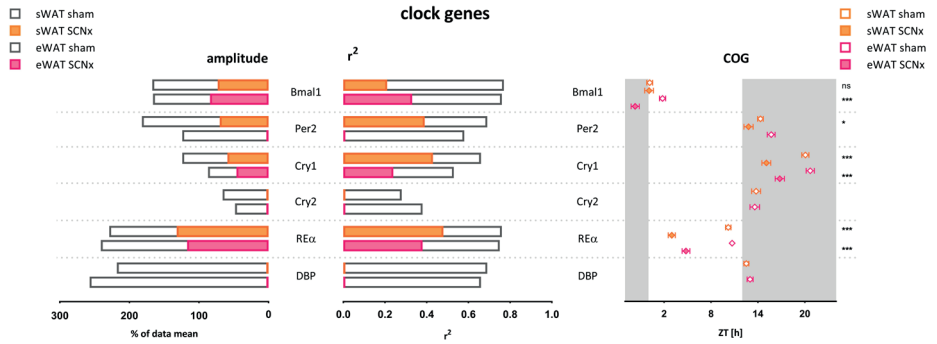


Figure 1. Amplitude, R2, and COG of WAT clock gene rhythms in sham- and SCN-lesioned animals. Amplitudes and R2 of clock gene rhythms were reduced by approximately 50% after SCN lesions. COG was advanced by ~4h after SCN lesions, with the exception of Bmal1 in sWAT. Cry2 and DBP completely lost rhythmicity. Gray bars indicate the dark phase (ZT12-24). SCNx; SCN lesioned, COG; centre of gravity, R2; inter individual variability, eWAT epididymal white adipose tissue, sWAT subcutaneous white adipose tissue.

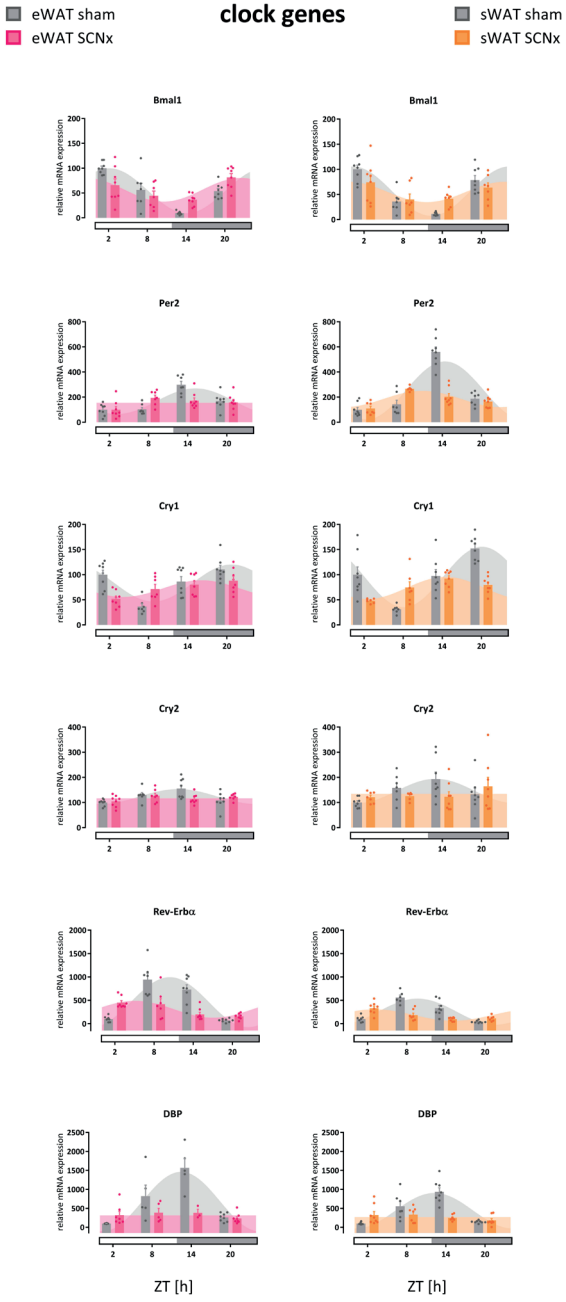


Figure 2. Clock gene rhythms in eWAT and sWAT of sham- and SCN-lesioned animals. Cry2 and DBP lost their rhythmicity after SCN lesions in both WAT compartments, as well as Per2 in eWAT. Bmal1, Per2 in sWAT, Cry1, and RevErbα remained rhythmic after SCN lesions. Gray bars on x-axis indicate the dark phase (ZT12-24). Bar plots represent mean ±SEM, scatter plots represent individual data points, area fills represent the curve as fitted by Circwave (www.hutlab.nl).

SCN lesions abolished diurnal rhythmicity of metabolic genes, NAMPT remains rhythmic

Metabolic genes showed less pronounced 24h patterns in both WAT depots than observed in clock genes, as observed previously (van der Spek et al. 2018). Five out of the 12 metabolic genes measured in sWAT were rhythmic in controls, while 4 of these 5 lost their rhythmicity after SCN lesions (NAMPT remained rhythmic, while PPARα, SREBP1c, LPL, and leptin lost rhythmicity). Similarly, also in eWAT 5 out of the 12 metabolic genes measured were rhythmic in controls, while 4 out of the 5 genes lost their rhythmicity after SCN lesions (NAMPT remained rhythmic, while PPARα, PPARγ, SREBP1c, and HSL lost rhythmicity). Gene expression of FAS, CPT1β, Glut4, adiponectin, and resistin was not rhythmic in controls in either WAT depot. For amplitudes, R2 and COG see Figure3, and Supplement Tables 1-3. For PCR data per gene see Figure4 and Supplemental Figure 5.

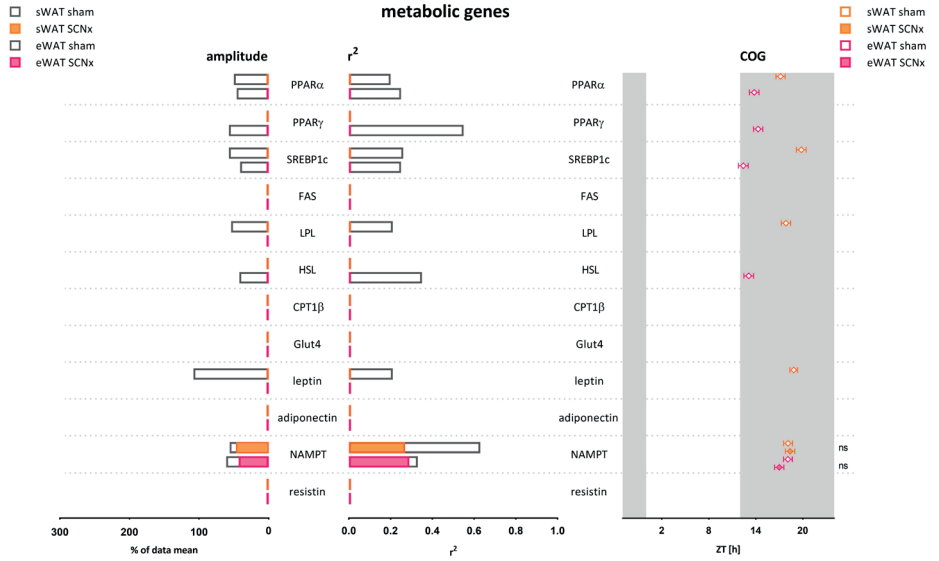


Figure 3. Amplitude, R2, and COG of WAT metabolic gene expression rhythms in sham- and SCN-lesioned animals. NAMPT gene expression remained rhythmic in WAT after SCN lesions, although the amplitude was reduced by 29% in eWAT and 17% in sWAT, and R2 was reduced by 13% in eWAT and 58% in sWAT. The COG remained unaltered. These results indicate that NAMPT rhythmicity was affected less by SCN lesions than any of the other genes measured, including the core clock genes. Absence of data for FAS, CPT1B, GLUT4, adiponectin, and resistin indicates absence of rhythmicity as calculated by Circwave; it does not indicate absence of gene expression.

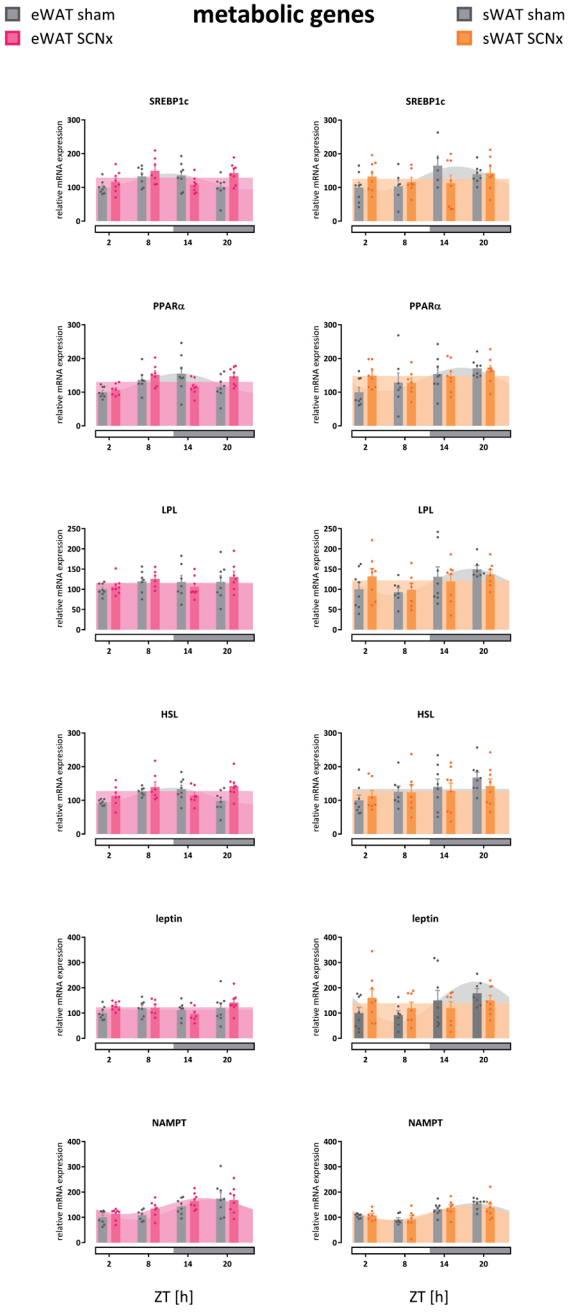


Figure 4. Metabolic gene expression rhythms in eWAT and sWAT of sham- and SCN-lesioned animals. Most metabolic genes lost their daily rhythmicity, with the exception of NAMPT. See supplemental Figure 6 for additional genes. Horizontal gray bars on x-axis indicate the dark phase (ZT12-24). Bar plots represent mean±SEM, scatter plots represent individual data points, area fills represent the curve as fitted by Circwave (www.hutlab.nl).

Interestingly, NAMPT gene expression remained rhythmic in both sWAT and eWAT after SCN lesions, albeit with reduced amplitude. In sWAT, the amplitude was reduced from 57% to 47% of the data mean (Δ-17%), and in eWAT from 61% to 43% of the data mean (Δ-29%). R2 was reduced from 0.63 to 0.27 (-58%) in sWAT, and it was reduced from 0.33 to 0.29 (-13%) in eWAT. COG did not change in either WAT depot (sWAT +0.2h, p = 0.76; eWAT -1.1h, p = 0.18). These results indicate that NAMPT rhythmicity was affected less by SCN lesions than any of the other genes measured, including the core clock genes.

DISCUSSION

Aim and findings

In line with previous work we observed pronounced daily rhythmicity in core clock genes in WAT of sham-lesioned animals, and less pronounced rhythms in metabolic genes (Ando et al. 2005; Zvonic et al. 2006; Sukumaran et al. 2010; De Farias et al. 2015; Su et al. 2015; Kolbe et al. 2016; Su et al. 2016). Despite hormonal and behavioural arrhythmicity, SCN lesions induced loss of rhythmicity in only two out of the six core clock genes investigated (Cry2, DBP) (Figures 1 & 2). Unlike Cry1, Cry2 gene expression is only modestly rhythmic in WAT, and it may, therefore, be more susceptible to lose statistically significant rhythmicity (Khan et al. 2012; Anafi et al. 2014). In contrast, DBP gene expression is highly rhythmic in WAT, as well as in many other tissues, but it lost rhythmicity completely in eWAT and sWAT after SCN lesions. Together, these findings suggest Cry2 and DBP do not form part of the most robust feedback loops in the core clock in WAT, corresponding with their absence in a proposed top 20 ‘core clock genes’ (Anafi et al. 2014).

The other four clock genes that we investigated retained statistically significant rhythmicity; however, amplitudes and R2 were both reduced by approximately 50%, and their phase was advanced by ~4h. These findings are in line with the study by Kolbe et al. (Kolbe et al. 2016), reporting a 30% reduction in the amplitude of the eWAT clock genes and a mean phase advance of ~5h. We found a more pronounced effect, including complete loss of rhythmicity of Cry2 and DBP, which might be explained by the fact that the mice in the study by Kolbe et al. were released into DD 2 days before measuring gene expression (Husse et al. 2014; Kolbe et al. 2016).

We previously showed that gene expression rhythms in intra-abdominal and subcutaneous WAT depots only show minor differences (van der Spek et al. 2018). In the current study, we also found no differential effects of SCN lesions on WAT depots. Only minor differences were detected in the response to SCN lesions between sWAT and eWAT. For

example, Bmal1 advanced by 3.5h in eWAT, but showed no significant phase change in sWAT, and Per2 lost rhythmicity in eWAT, but not sWAT. The latter is in line with our previous study and our current observation that Per2 expression shows stronger daily rhythmicity in sWAT than in intra-abdominal WAT depots (van der Spek et al. 2018).

In line with our findings in WAT, other peripheral tissues have been shown to retain core clock gene rhythmicity after SCN lesions as well (Akhtar et al. 2002; Yoo et al. 2004; Tahara et al. 2012; Husse et al. 2014; Izumo et al. 2014). For example, in mice, thermal SCN lesions merely reduced the amplitude of clock gene expression rhythms in liver for Bmal1 and Per2 (Akhtar et al. 2002). Genetic SCN lesions reduced the amplitude and phase advanced Bmal1, Per1, Per2, RevErbα, and DBP expression rhythms in adrenal, liver, kidney, and heart in mice (Husse et al. 2014). Furthermore, BMAL1, PER2, and REVERBα bioluminescence remained rhythmic in several peripheral tissues after thermal SCN lesions, both ex vivo (Yoo et al. 2004) and in vivo (Tahara et al. 2012). Equally, after genetic SCN lesions, daily rhythms in PER2 bioluminescence showed a decreased amplitude and increased phase distribution in several peripheral tissues (Izumo et al. 2014). Some earlier studies reported that SCN lesioning completely abolished the rhythm of Per2 in the liver of rats (Sakamoto et al. 1998) and mice (Hara et al. 2001), but these studies only compared two time points, which is insufficient to assess rhythmicity accurately. Together, these studies provide accumulating evidence that after SCN lesions peripheral tissues do retain some rhythmicity, although these rhythms are more variable due to decreased amplitudes and increased phase distribution. Thus, despite behavioural arrhythmicity and contrary to the general assumption, SCN lesions do not abolish all rhythmicity in gene expression.

What mechanism may cause peripheral core clock rhythms to remain after SCN lesions?

Light is the main Zeitgeber for the circadian timing system, and in our current study all animals were housed under regular L/D conditions. Several studies have shown that an L/D-cycle can synchronize peripheral clocks in the absence of a functioning genetic clock in the SCN (Husse et al. 2014; Izumo et al. 2014; Lee et al. 2015; Koronowski et al. 2019; Welz et al. 2019). These data indicate that either the intact neuronal structures within the SCN, or extra-SCN pathways, enable photic masking to synchronize peripheral rhythms.

By using thermal ablation of the SCN, we excluded the possibility of photic masking via neuronal structures within the SCN. However, extra-SCN pathways are unaffected by these lesions. The discovery of the melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) provided evidence for the existence of such a light-sensi-

tive non-SCN neural pathway, with direct projections to many brain areas, including both SCN target and non-SCN target areas (Hattar et al. 2006; Canteras et al. 2011; Noseda et al. 2017). The autonomic nervous system (ANS) could then serve to communicate these light signals from the brain to peripheral tissues, including white adipose tissue (Bartness & Song 2007). Indeed, several examples exist of light-induced changes in peripheral tissues that are mediated via the ANS (Cailotto et al. 2005; Ishida et al. 2005; Fan et al. 2018; Aras et al. 2019; Zhang et al. 2020). Exposure to light may directly increase lipolysis and decrease leptin production in subcutaneous white adipose tissue, indicating that the L/D cycle may also provide a direct rhythmic input to sWAT (Ondrusova et al. 2017; Nayak et al. 2020). Moreover, light might also entrain peripheral clocks via non-ANS pathways as recently we showed that nocturnal light exposure affects the liver transcriptome via both ANS and non-ANS pathways (Opperhuizen et al. 2019). Notable, however all the above experiments were performed in SCN-intact animals.

Interestingly, whereas behavioural and humoral rhythms desynchronise quickly in SCN lesioned animals in D/D, this occurs at a much slower rate for the peripheral tissue clocks, as illustrated by animals that were kept in DD for 2 days (Akhtar et al. 2002; Kolbe et al. 2016), 1 week (Husse et al. 2014), 3 weeks (Yoo et al. 2004), or up to 1 month (Tahara et al. 2012; Izumo et al. 2014); they and still showed core clock rhythmicity in peripheral tissues. This has been explained by the fact that in absence of the master clock, autonomous feedback loops in organs and individual cells within tissues persist. Over time their amplitude reduces, and they increasingly run out of phase with each other, but, thereby, their rhythms only slowly desynchronize.

Systemic factors, such as locomotor activity, food intake and humoral factors can entrain and synchronize rhythmicity, and might explain the rhythmicity observed in WAT of our SCN lesioned animals. Locomotor activity, however, is completely arrhythmic in SCN lesioned animals (Stephan & Zucker 1972; Akhtar et al. 2002; Yoo et al. 2004; Tahara et al. 2012; Izumo et al. 2014), as is body temperature (Refinetti et al. 1994), food intake (Stoynev et al. 1982; Stoynev et al. 1986; Janssen et al. 1994), and as we show here, drinking behaviour (Supplemental Figure 1). Additionally, we did not observe rhythmicity in plasma concentrations of insulin, corticosterone, or glucose, which can directly influence clock gene expression (Balsalobre et al. 2000; So et al. 2009) (Supplemental Figure 2). In conclusion, behavioural or humoral time cues are unlikely to preserve daily rhythmicity in peripheral clocks in the absence of the master clock, but in L/D conditions, photic masking via retinal projections to the ANS might keep peripheral clocks cycling.

Most metabolic gene expression rhythms are abolished after SCN lesions

We found that SCN lesions abolished daily rhythmicity in metabolic output genes (Figures 3 & 4). In intact animals, rhythmicity of metabolic genes is generally modest and less pronounced than that of clock genes (Schwanhüusser et al. 2011; van der Spek et al. 2018). Daily rhythms of metabolic genes are regulated by clock genes (Asher & Schibler 2011), as well as feeding behaviour, circulating nutrients, and hormone fluctuations. Since all of these cues are arrhythmic in SCN lesioned animals, this likely explains most of the effect of SCN lesions on the loss of metabolic gene expression rhythms. Moreover, core clock proteins (directly or indirectly) function as transcription factors for many metabolic genes; thus, since the amplitudes of clock gene expression rhythms were reduced after SCN lesions, this will further contribute to the diminished rhythmicity of metabolic genes.

NAMPT retains more rhythmicity than the core clock after SCN lesions

Surprisingly, NAMPT remained rhythmic in both sWAT and eWAT, with only a modest amplitude reduction (-17% in sWAT and -29% in eWAT) and without phase shift (Figures 3 & 4). Since SCN lesioning did alter clock gene rhythmicity more severely, this raises the question of which factor(s) maintained the rhythmicity of NAMPT.

NAMPT is the rate-limiting enzyme in the NAD+ salvage pathway; reduction of NAD+ to NADH is essential for transfer of energy from nutrients to ATP. NAMPT expression is regulated directly by BMAL1, and forms an additional TTFL through Sirtuin1 (SIRT1) (Nakahata et al. 2009; Ramsey et al. 2009), thus linking cellular metabolism to the circadian clock. In our dataset, Bmal1 rhythms were reduced and phase advanced in eWAT, but not sWAT. NAMPT amplitude was slightly more affected in eWAT than in sWAT; thus, the reduced Bmal1 rhythm may still be sufficient to drive the rhythm in NAMPT gene expression. Indeed, in arrhythmic Bmal1-null mice with reconstituted Bmal1 expression in the liver specifically, oscillation of redox-related metabolites and hepatic NAMPT expression was partly restored (Koronowski et al. 2019). This suggests that NAMPT is either very sensitive to the rhythmic regulation by BMAL1, and this loop keeps cycling through SIRT1, or NAMPT is regulated by other rhythmic factors in addition to BMAL1.

NAMPT is, indeed, regulated by several more factors involved in cellular energy metabolism. Firstly, AMPK is a key sensor of ATP resources in the cell, and is essential for rhythmicity of NAMPT in WAT. Furthermore, the ratio NAD+/NADH determines the production of reactive oxygen species (ROS), and oxidative stress increases NAMPT release (Lin et al. 2015; Lu et al. 2019). Consequently, NAMPT inhibition induces susceptibility to oxidative stress (Hong et al. 2016; Xu et al. 2017). Anti-oxidant proteins that eliminate ROS, such as peroxiredoxins, may generate rhythmicity in absence of the TTFL (Neill et

al. 2011; Bass & Takahashi 2011; Edgar et al. 2012), and may even couple the light signal to the molecular clock (Bodvard et al. 2017). Surprisingly, sWAT contains light sensitive melanopsin channels (Ondrusova et al. 2017; Nayak et al. 2020), raising the suggestion that light could, indeed, reach sWAT. Therefore, the direct effects of light on cellular metabolism pathways could provide an explanation for the remaining rhythmicity in NAMPT, suggesting it is rhythmicity in NAMPT that keeps the molecular clock ticking. On the other hand, this mechanism would not explain the retained rhythmicity in eWAT.

Remarkably, in SCN-lesioned animals on a feeding schedule, only hepatic NAMPT levels lost their rhythmicity; whereas, all of the other clock genes investigated remained rhythmic in these conditions (Sabath et al. 2014). These results show NAMPT levels are not directly regulated by food intake; in fact, the enforced feeding schedule seems to disturb its rhythmicity, since in fasted conditions NAMPT levels in SCN-lesioned animals retained their rhythmicity. Moreover, in intact animals NAMPT rhythms were also lost when feeding times were not in line with the L/D cycle (Salgado-Delgado et al. 2013). Since SCN-lesioned animals in the Sabath et al. (Sabath et al. 2014) study were also housed in L/D conditions, these results agree with the idea that the L/D cycle determines NAMPT rhythmicity, instead of feeding rhythms or the molecular clock.

Study limitations

Although the regular L/D cycle may seem a limitation, as it provides rhythmic input, the housing in L/D conditions does allow differentiation between the pathways responsible for the endogenous SCN-controlled rhythms and those responsible for the non-SCN masking effects of the L/D cycle. Furthermore, we mainly focussed on gene expression, whereas within every regulatory stage from gene expression to protein activity additional layers of circadian control have been revealed (Lee et al. 2015). Nevertheless, Janich et al. concluded that whenever both mRNA abundance and ribosome occupancy cycled, they globally did so in synchrony (Janich et al. 2015). In the current study, we have sampled every 6h over a 24h period to assess rhythmicity. This limited sampling frequency could cause underestimation of rhythmicity. However, the rhythmicity we found in sham lesioned animals corresponded well to the rhythmicity in our earlier study that sampled every 3h (van der Spek et al. 2018). Finally, Circwave recognises wave forms using Fourier transformation, thus assuming rhythms consist of (multiple) sine waves, and could thereby underestimate spiky or saw-tooth shaped wave forms (Thaben & Westermark 2014), as previously discussed (van der Spek et al. 2018), causing underestimation of rhythmicity.

Conclusions

We found that SCN lesions, despite behavioural and hormonal arrhythmicity, reduced but did not abolish clock gene rhythms in rat eWAT and sWAT, with decreased amplitudes and advanced phase. We observed no major differences in the effect of the SCN lesions between WAT depots. Cry2 and DBP completely lost their rhythm, suggesting they are part of less robust, or more adaptable, feedback loops in the core clock in WAT. Metabolic genes lost their modest rhythmicity in WAT, with the exception of NAMPT, which was even less affected than the core clock genes, suggesting that it is either strongly controlled by the remaining rhythmicity in clock gene expression, or it is regulated by an unknown other rhythmic factor. The ANS could provide such an extra-SCN pathway that generates entrainment signals by photic masking, and, thereby, may have contributed to the persisting WAT rhythmicity as observed in our study.

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DISCLOSURE OF INTEREST

The authors report no conflict of interest.

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SUPPLEMENTAL MATERIAL

Supplemental material can be found at https://www.tandfonline.com/doi/full/10.1080/07420528.2021.1930027

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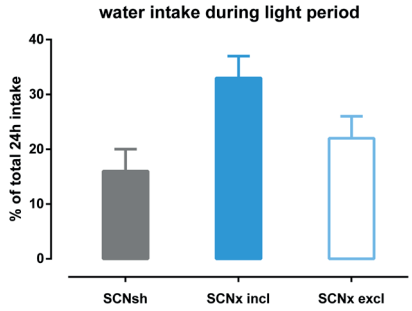
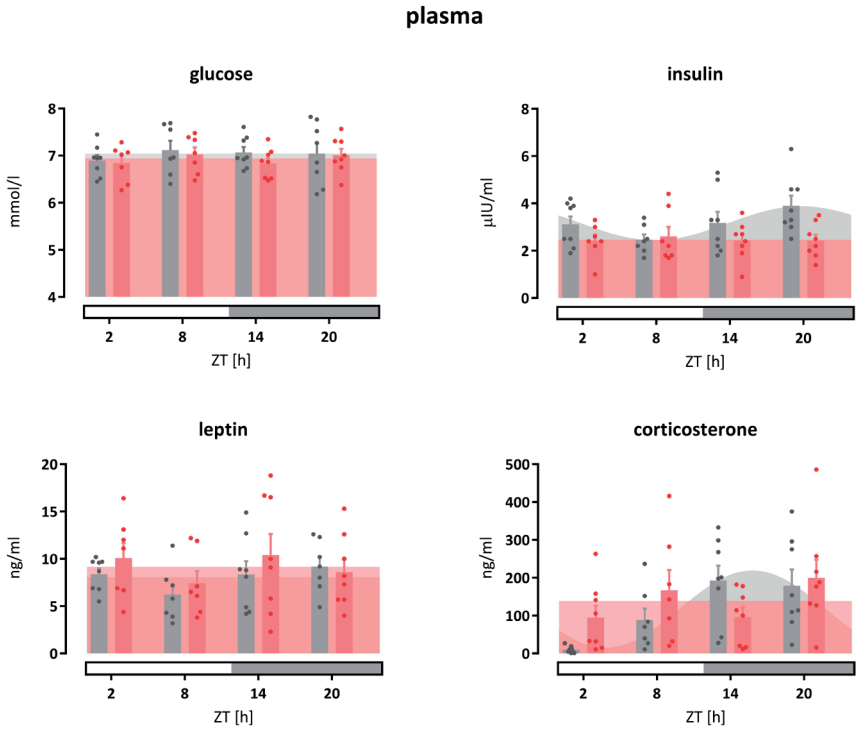
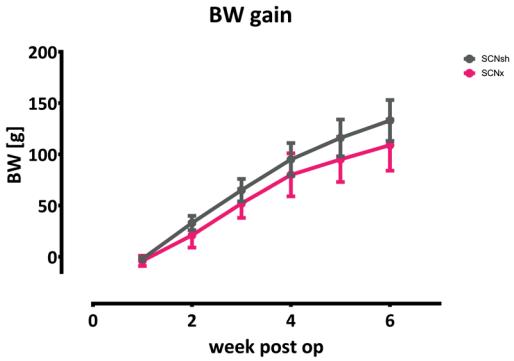


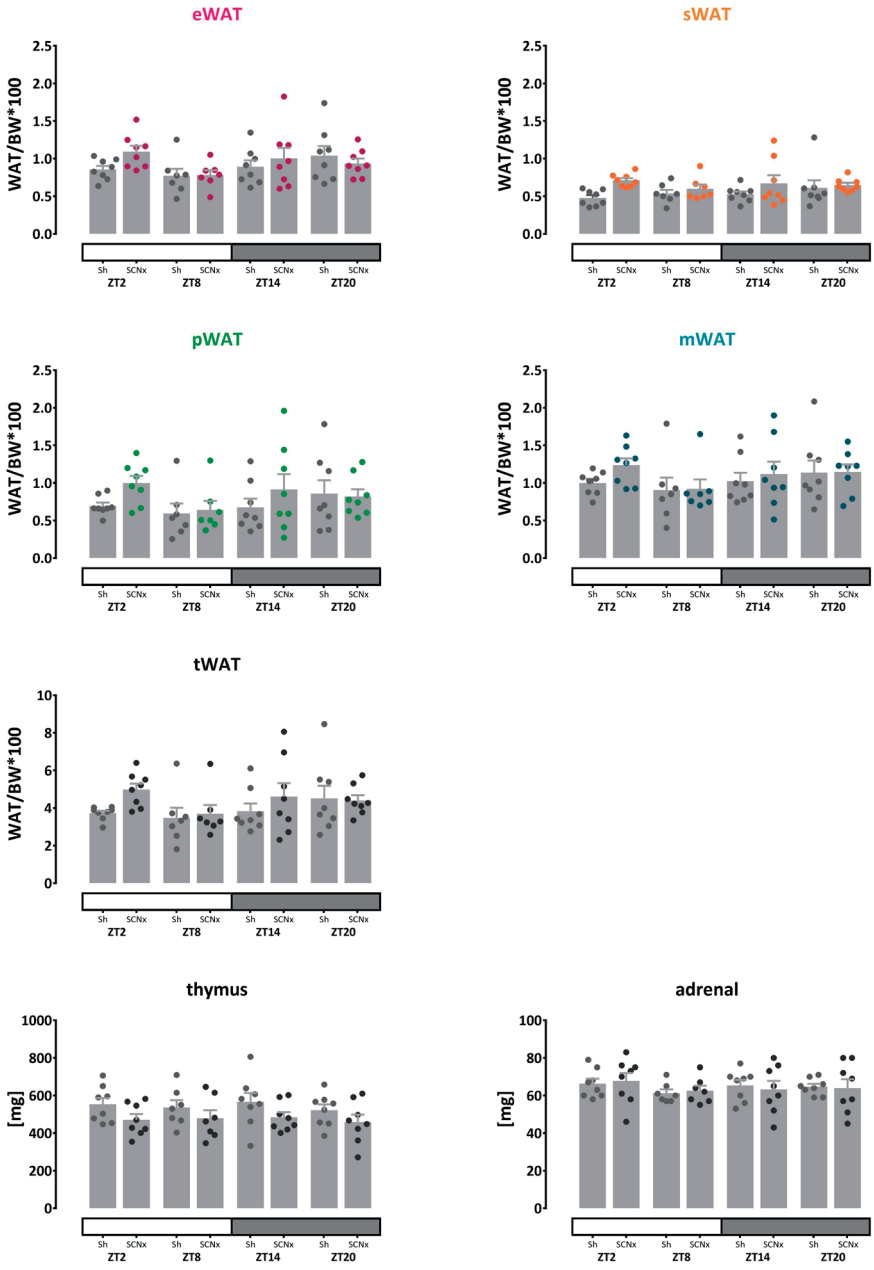
Supplemental Figure 1. After recovery from surgery, SCN lesioned (SCNx, n=91) and sham operated control rats (SCNsh, n=31) were housed individually and water consumption was measured for the middle 8h (ZT2-ZT10) of the light period for 3 weeks. Sham-lesioned control animals drank on average 16±4% (mean±SD) of their daily water consumption in the light period. Successfully SCN lesioned animals (n=31) drank on average 33±4% during the light period, whereas excluded SCN lesioned animals (n=60) drank on average 22±4% (One-way Anova $P < 0.0001$, $R2 = 0.71$, T-test SCNsh vs. SCNx; $p < 0.0001$). SCNsh = sham lesioned; SCNx = SCN lesioned; SCNx incl = data of animals included in analysis, i.e., effectively lesioned animals; SCNx excl = data of animals excluded from analysis, i.e., animals with non-complete lesions.



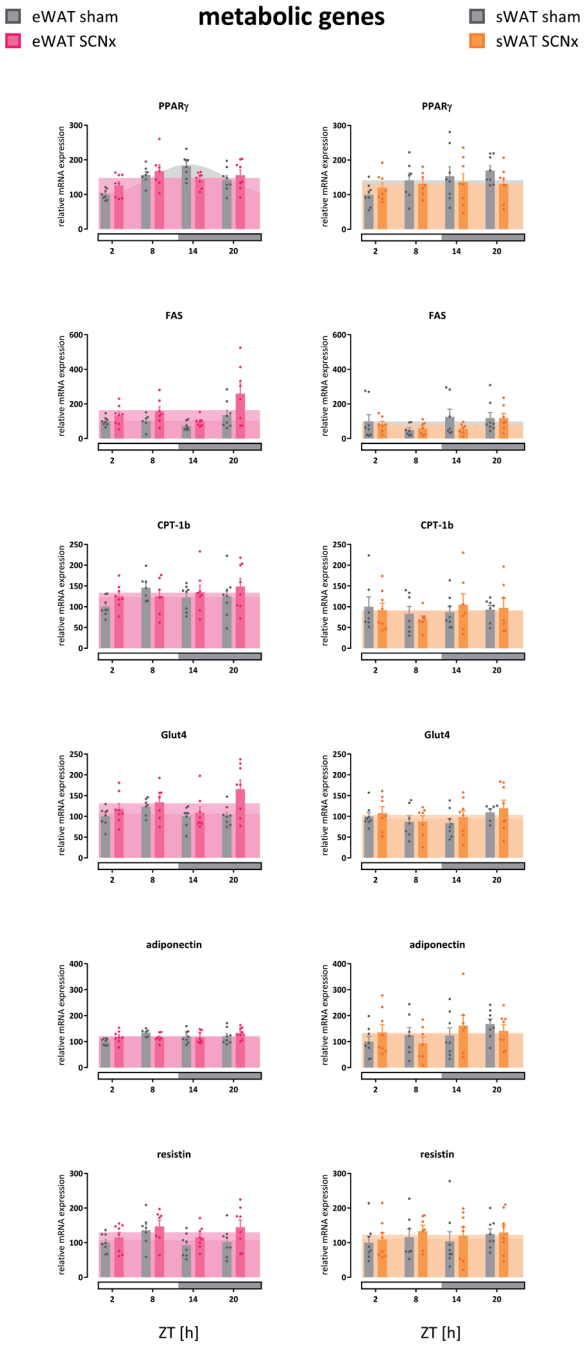
Supplemental Figure 2. Plasma concentration of glucose, insulin, corticosterone, and leptin. The daily peak in plasma corticosterone and insulin as observed in sham-lesioned animals was absent in the SCN lesioned animals. Horizontal gray bars on x-axis indicate the dark phase (ZT12-24). Bar plots represent mean±SEM for the 4 sampling points, scatter plots represent individual data points, area fills represent the curve as fitted by Circwave (www.hutlab.nl).



Supplemental Figure 3. Body weight gain in SCN lesioned animals was reduced as compared to sham controls (repeated ANOVA; Interaction $p < 0.001$; Time $p < 0.001$; Group $p = 0.06$). Sham lesioned animals weighed 6% more at the end of the experiment (BW SCNsh 358±6 g vs. SCNx 337±6 g, T-test $p < 0.05$).



Supplemental Figure 4. Total WAT depot weight corrected for body weight was not different between groups (tWAT: SCNsh 3.9±0.2% vs. SCNx 5.3±1.0%, T-test p = 0.11). Sham lesioned animals had higher thymus weights (thymus: SCNsh 544±1 mg 9 vs. SCNx 473±17 mg, T-test p < 0.01), but adrenal weights did not differ between groups (adrenal SCNsh 65±1 mg vs. SCNx 65±2 mg, T-test p = 0.99). Horizontal gray bars on x-axis indicate the dark phase (ZT12-24). Bar plots represent mean ±SEM, scatter plots represent individual data points.



Supplemental Figure 5. Metabolic gene expression rhythms in eWAT and sWAT of sham- and SCN-lesioned animals. Most metabolic genes lost their daily rhythmicity, with the exception of NAMPT. Horizontal gray bars on x-axis indicate the dark phase (ZT12-24). Bar plots represent mean ±SEM, scatter plots represent individual data points, area fills represent the curve as fitted by Circwave (www.hutlab.nl).

5

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

Su Y
van der Spek R
Foppen E
Kwakkel J
Fliers E
Kalsbeek A

Chronobiology International, 2014

ABSTRACT

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 24h 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, >80% 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.

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 BMAL, 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 promotor 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 (Oishi et al. 1998; Balsalobre et al. 2000; 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 (Saad et al. 1998; Calvani et al. 2004; Ando et al. 2005). In order to further our understanding of the role of glucocorticoids in the regulation of daily rhythms in WAT activity, we investigated the effect of adrenalectomy on the daily rhythm of gene expressions in WAT.

The negative feedback of glucocorticoids on the corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) containing neurons in the PVN is well known (Young et al. 1986; Swanson & Simmons 1989; Ferrini et al. 1997). However, some studies reported that glucocorticoids may also change neuropeptide expressions in the SCN (Larsen et al. 1994; Isobe et al. 2004), although it is unclear whether glucocorticoids can feedback to the SCN (Rosenfeld et al. 1988). In order to check whether glucocorticoids could affect peripheral rhythms via their effects on hypothalamic neuropeptide expression, we also investigated the effects of adrenalectomy on gene expression in the PVN and SCN.

To examine the influence of the adrenal hormones on gene expression in WAT, PVN and SCN, we analyzed the mRNA expression of various clock, metabolic/adipokine and neuropeptide genes in adipose and hypothalamic tissue collected at 4 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 (Harlan, Horst, The Netherlands). Animals (n=64) were group housed (4 rats/cage) in the animal facility. Thirty two rats with a 12h light/12h dark cycle (light on at 7:00) and another 32 rats with a 12h dark/12h 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 200µm 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) 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 PVN using MagNA Pure LC RNA Isolation kit III- Tissue (Roche) by MagNA Pure LC (Roche).

eWAT was homogenized by MagNA Lyser Green Beads (Roche) with QIAzol® lysis reagent (QIAGEN), RNA was extracted by RNeasy mini Kit (QIAGEN) and included a DNase step according to the manufacturer's instructions.

RNA was reverse transcribed using Transcriptor First cDNA Synthesis Kit (Roche) with oligo-dT primers (30min at 55 ºC, 5min at 85 ºC). 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 20µl reaction containing 1×SYBR-Green master mix and 50ng of each primer (forward and reverse). Quantitative RT-PCR (qRT-PCR) was performed in Lightcycler®480 (Roche), 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.

Table 1 Information of gene primers

Type	Gene	Forward Primer	Reverse Primer	Tm
Clock Genes and Clock-Controlled Genes	Bmal1	CCGATGACGAACTGAAACACCT	TGCAGTGTCCGAGGAAGATAGC	55
	Cry1	AAGTCATCGTGCGCATTTCA	TCATCATGGTCGTCGGACAGA	55
	Cry2	TGGATAAGCACT TGGAACGGA A	TGTACAAGTCCCACAGGCGGT A	60
	Per1	GTGGGCTTGACACCTCTTCT	TGCTTTAGATCGGCAGTGGT	60
	Per2	CACCCTGAAAAGAAAGTGCGA	CAACGCCAAGGAGCTCAAGT	55
	Rev-erbα	ACAGCTGACACCACCCAGATC	CATGGGCATAGGTGAAGATTTCT	55
	Dbp	CCTTTGAACCTGATCCGGCT	TGCCTTCTTCATGATTGGCTG	55
	Pparα	TCACACAATGCAATCCGTTT	GGCCTTGACCTTGTTCATGT	55
	Pparγ	CAGGAAAGACAACAGACAAATCA	GGGGGTGATATGTTTGAACTTG	55
Metabolic Genes	Fas	CTTGGGTGCCGATTACAACC	GCCCTCCCGTACACTCACTC	55
	Llp	CAAAACAACCAGGCCTTCGA	AGCAATTCCCCGATGTCCA	55
	Hsl	CACACAGCATGGATTTACGCA	ACCTGCAAAGACGTTGGACAG	55
	Acc2	GACGAAGGCGAG TCAGGTAT	GAAGCCTCTCCTGCAATCAT	55
	Glut4	GGGCTGTGAGTGAGTGCTTTC	CAGCGAGGCAAGGCTAGA	55
	Srebf1	ACAAGATTGTGGAGCTCAAGG	TGCGCAAGACAGCAGATTTA	60
	Cpt1b	GTGCTGGAGGTGGCTTTGGT	TGCTTGACGGATGTGGTTCC	57
	Leptin	GCTCTCTGCAGGACATTCTTCA	GCCCGGTGGTCTTGGAA	55
	Adiponect	AATCCTGCCCAGTCATGAAG	CATCTCCTGGGTCACCCTTA	60
	Resistin	ATCAAGACTTCAGCTCCCTACTG	GTGACGGTTGTGCCTTCTG	60
	Visfatin	ACAGATACTGTGGCGGGAATTGCT	TCGACACTATCAGGTGTCTCAG	60
	Gr	ACCTGGATGACCAAATGACCC	GGAGCAAAGCAGAGCAGGTTT	55
	Mr	AGAGCCGTGGAAGGGCA	AGTTCTTTCGCCGAATCTTATCA	55
	Lipin1	TCACTACCCAGTACCAGGGC	TGAGTCCAATCCTTTCCCAG	55

Table 1 Information of gene primers (*continued*)

Type	Gene	Forward Primer	Reverse Primer	Tm
Neuropeptide Genes	Vip	CCCAAGGAGGCACCGAGATGGA	GCCAGGCCAGCGACTGTGAG	65
	Oxt	TGCGCAAGTGTCTTCCCTGCG	AGCCATCCGGGCTACAGCAGA	65
	Crh	AAAGGGGAAAGGCAAAGAAA	GTTTAGGGGCGCTCTCTTCT	55
	Trh	TCTGCAGAGTCTCCACTTCG	AGAGCCAGCAGCAACCAA	55
	Avp	TGCCTGCTACTTCCAGAACTGC	AGGGGAGACACTGTCTCAGCTC	60
Housekeeping Genes	Hprt	GCAGTACAGCCCCAAAATGG	AACAAAGTCTGGCCTGTATCCAA	55
	Hsp90α	CGGGCCCACCCTGCTCTGTA	ACCGAATCTTGTCCAGGGCATCA	60

Abbreviations used: Rev-erbα: Rev-verbalpha (official full name: nuclear receptor subfamily 1, group D, member 1 (Nr1d1)). Dbp: albumin D-box binding protein. Pparα: peroxisome proliferator activated receptor alpha (official abbreviation: Ppara). Pparγ: peroxisome proliferator-activated receptor gamma (official abbreviation: Pparg). 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)). Srebf1: sterol regulatory element binding transcription factor 1. Cpt1b: carnitine palmitoyltransferase 1b. Visfatin also called Nampt: nicotinamide phosphoribosyltransferase

Statistical analysis

Data are presented as mean±SEM (standard error of the mean). Statistical analysis was performed using SPSS version 19.0. One way or two way ANOVAs were performed to detect the effects of Time (4 levels: ZT2, ZT8, ZT14 and ZT20), Treatment (sham-operated vs 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. P values reported are the result of the F-test, and the 24h rhythm was confirmed if $p<0.05$.

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 grams over 3 weeks compared to about 75 grams 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 (Fig.1D), i.e., both groups presented a clear day/night rhythm in food intake.

Table 2 Effects of adrenalectomy on body weight

group	Body weight (g)		Body weight gain(g)
	Before surgery	3 weeks post surgery	
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 (Fig.1A and Table 3). Also plasma insulin levels were lower and lost their significant effect of Time, after adrenalectomy (Fig.1B and Table 3). TG levels in plasma were slightly decreased in ADX rats, especially during the light period (Fig.1C and Table 3).

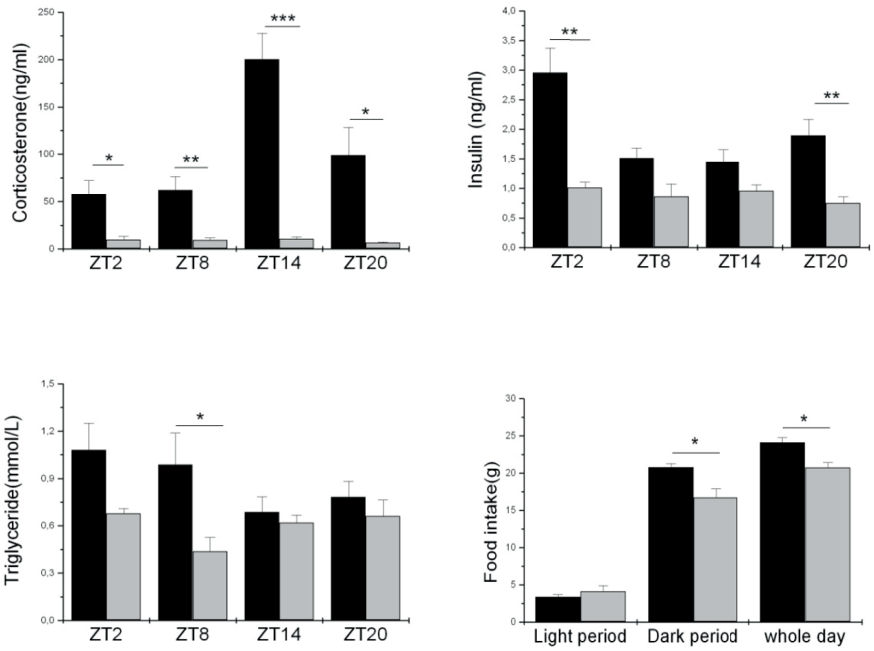


Figure 1. Effects of adrenalectomy on plasma corticosterone (A), insulin (B) and TG (C) levels and 24-h food intake (D). Results are presented as mean ±SEM. * p<0.05; ** p<0.01; *** p<0.001. Black bars – Sham-operated animals (n=32, 8 rats/ time point), grey bars – ADX animals (n=25,ZT2 (n=5), ZT8 (n=7), ZT14 (n=7), ZT20 (n=6)). For ANOVA analysis see Table 3.

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

	Treatment	Time		Interaction
		Sham	ADX	
corticosterone	**<0.001**	**0.002**	0.436	**0.005**
insulin	**<0.001**	**0.008**	0.684	**0.004**
TG	**0.006**	0.274	0.162	0.125

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

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 (Fig.2 and Table 4). Four out of the 9 genes 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

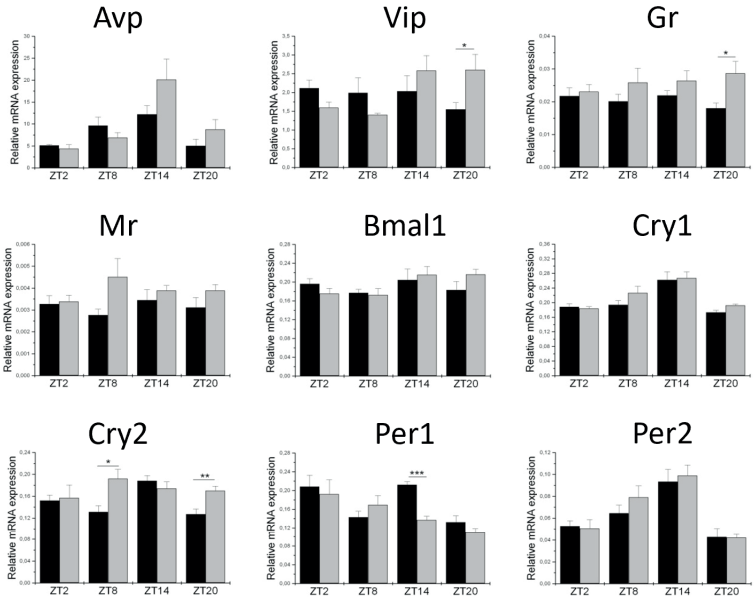


Figure 2. The mRNA expression pattern of 9 genes in the SCN. Data are expressed as the mean ±SEM. mRNA values were normalized to HSP90α. * p<0.05; ** p<0.01; *** p<0.001. Black bars – Sham-operated animals, grey bars – ADX animals. For ANOVA analysis see Table 4. For Circwave V1.4 analysis see Table S1.

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.

Table 4 Effects of adrenalectomy on gene expression rhythms in the SCN

Gene	Mean (24h)		Treatment	Time	Time		Interaction
	Sham	ADX			Sham	ADX	
Avp	0.780	1.040	0.215	**<0.001**	**0.011**	**0.006**	0.096
Vip	0.191	0.208	0.597	0.249	0.577	**0.023**	**0.033**
Gr	0.021	0.026	**0.009**	0.937	0.494	0.734	0.470
Mr	0.003	0.004	**0.025**	0.894	0.710	0.525	0.340
Bmal1	0.190	0.195	0.693	0.132	0.658	0.067	0.404
Cry1	0.203	0.221	0.199	**<0.001**	**<0.001**	**0.002**	0.597
Cry2	0.149	0.174	**0.013**	0.075	**0.001**	0.498	**0.019**
Per1	0.173	0.150	0.078	**<0.001**	**0.008**	**0.001**	**0.037**
Per2	0.062	0.07	0.442	**<0.001**	**0.001**	**0.001**	0.690

Columns 2 and 3 show mRNA values normalized to Hsp. 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.

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 6 genes involved in corticosterone release and 1 clock gene. According to the result of ANOVA analysis (Fig.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.

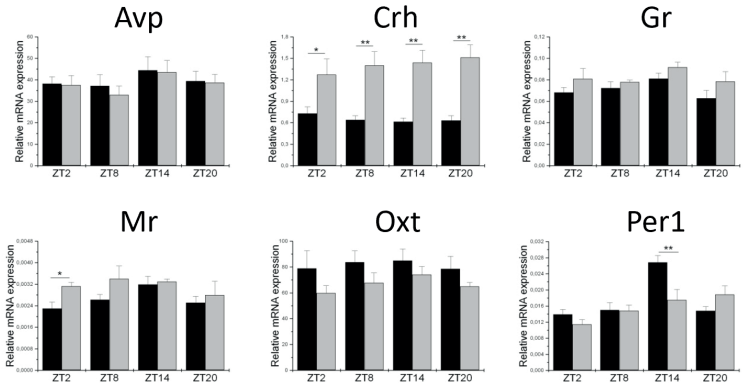


Figure 3. The mRNA expression pattern of 6 genes in the PVN (TRH data are not shown). Data are expressed as the mean ± SEM. mRNA values were normalized to HSP90α. * p<0.05; ** p<0.01. Black bars – Sham-operated animals, grey bars – ADX animals. For ANOVA analysis see Table 5. For Circwave V1.4 analysis see Table S2.

Table 5 Effects of adrenalectomy on gene expression rhythms in the PVN

Gene	Mean (24h)		Treatment	Time	Time		Interaction
	Sham	ADX			Sham	ADX	
Avp	39.9	38.4	0.651	0.348	0.755	0.459	0.984
Crh	0.648	1.41	**<0.001**	0.965	0.674	0.868	0.661
Gr	0.071	0.083	**0.018**	0.078	0.188	0.407	0.888
Mr	0.00267	0.00317	**0.030**	0.208	0.104	0.683	0.573
Oxt	81.48	66.28	**0.029**	0.712	0.962	0.491	0.973
Per1	0.018	0.016	0.115	**<0.001**	**<0.001**	0.111	**0.003**
Trh	0.844	0.835	0.852	0.934	0.986	0.901	0.929

Columns 2 and 3 show mRNA values normalized to Hsp. 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.

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 3 time points. Cry2 expression was slightly up-regulated in the ADX rats (Fig.4 and Table 6).

Circwave analysis showed that the expression of all 7 clock genes displayed a significant daily rhythm in sham-operated animals (Table S3), the rhythmicity of these 7 genes was

not changed in ADX animals, but the amplitude of all 7 genes expression was decreased after adrenalectomy.

Table 6 Effects of adrenalectomy on clock gene expression rhythms in eWAT

Gene	Mean (24h)		Treatment	Time	Time		Interaction
	Sham	ADX			Sham	ADX	
Bmal1	0.117	0.099	0.145	**<0.001**	**<0.001**	**<0.001**	0.164
Cry1	0.105	0.101	0.620	**<0.001**	**<0.001**	**<0.001**	0.390
Cry2	0.211	0.235	**0.045**	**<0.001**	**0.003**	0.053	0.569
Per1	0.612	0.380	**<0.001**	**<0.001**	**<0.001**	**0.001**	**0.003**
Per2	0.069	0.055	0.088	**<0.001**	**<0.001**	**0.001**	0.088
Reverb	1.353	1.288	0.680	**<0.001**	**<0.001**	**<0.001**	**0.014**
Dbp	1.830	1.852	0.939	<0.001	**<0.001**	**<0.001**	0.365

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

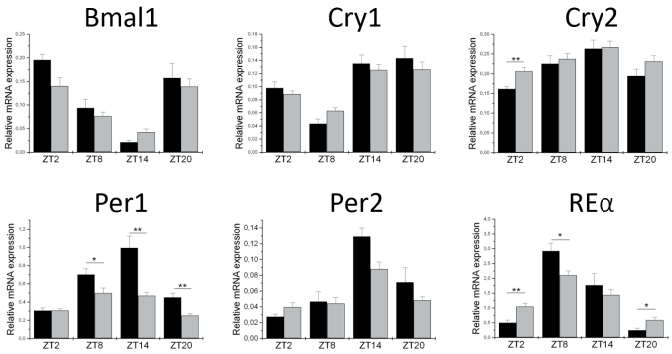


Figure 4. The expression pattern of 6 clock genes in eWAT (DBP data are not shown). Data are expressed as the mean ± SEM. mRNA values were normalized to HPRT. * p<0.05; **p<0.01. Black bars – Sham-operated animals, grey bars – ADX animals. For ANOVA analysis see Table 6. For Circwave V1.4 analysis see Table S3.

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 (Fig.5 and Table 7). In eWAT, the average expression of Hsl, Gr and Mr was up-regulated in ADX rats as compared to sham-operated rats; Leptin, Adiponection 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-hour 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 6 genes investigated, i.e., Fas, Lpl, Acc2a, Srebf1, Visfatin and Lipin1. Therefore, according to ANOVA adrenalectomy caused the disappearance of a significant effect of Time in 8 out of 11 genes.

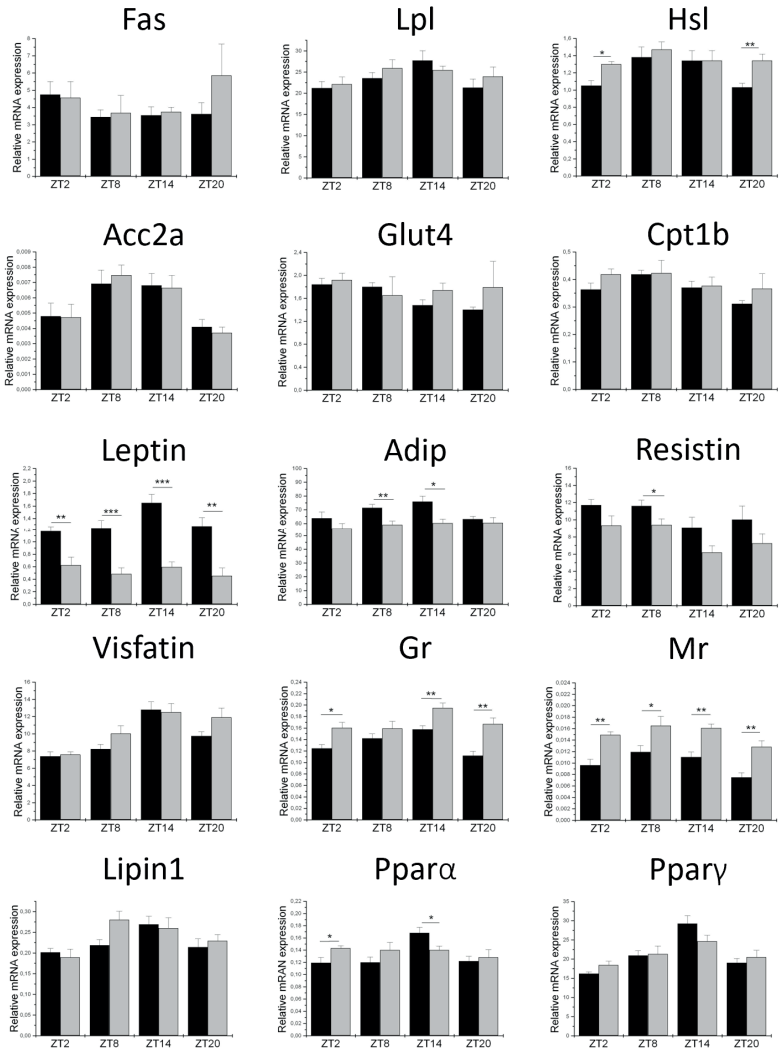


Figure 5. The expression pattern of 15 metabolic/adipokine genes (Srebf1 data not shown). Data are expressed as the mean ± SEM. mRNA values were normalized to HPRT. * p<0.05; ** p<0.01; *** p<0.001. Black bars – Sham-operated animals, grey bars – ADX animals. For ANOVA analysis see Table 7. For Circwave V1.4 analysis see Table S4.

Table 7 Effects of adrenalectomy on metabolic/adipokine gene expression rhythms in eWAT

Gene	Mean (24h)		Treatment	Time	Time		Interaction
	Sham	ADX			Sham	ADX	
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
Hsl	1.200	1.364	**0.016**	**0.025**	**0.019**	0.546	0.326
Acc2a	0.006	0.006	0.972	**<0.001**	**0.037**	**0.007**	0.931
Glut4	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**	0.654	0.227
Adiponectin	68.536	58.900	**<0.001**	0.123	0.051	0.845	0.297
Resistin	10.607	8.038	**0.001**	**0.013**	0.238	0.055	0.988
Visfatin	9.527	10.483	0.093	**<0.001**	**<0.001**	**0.009**	0.329
Gr	0.134	0.170	**<0.001**	**<0.001**	**0.001**	0.080	0.233
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
Pparα	0.132	0.138	0.403	**0.015**	**0.001**	0.767	**0.033**
Pparγ	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.

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 7 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 indicate that adrenal hormones are important to maintain rhythmic metabolic/adipokine gene expression in eWAT, but

they also indicate that rhythmicity of peripheral clock genes is not sufficient to maintain tissue metabolism rhythmicity.

All of the 7 clock genes we studied in eWAT still showed a significant daily rhythm after adrenalectomy. However, the amplitude of all the clock gene rhythms became smaller after adrenalectomy. This result indicates 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 7 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, PPARr, 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 PPARα is also a necessary regulator of adipocyte differentiation increasing the rate of adipocyte formation (Goto et al. 2011). Koh et al. (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. (Lemberger et al. 1994) reported that Pparα gene expression is stimulated by corticosterone and follows the diurnal rhythm of circulating corticosterone. Zhang et al. (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 (Svec et al. 1989; Holmes et al. 1995; Han et al. 2007; Noguchi et al. 2010). All together this makes the observed changes in Pparα, Pparγ, 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 re-esterification. Mice studies (Koh et al. 2008; Kim et al. 2013) have shown that LIPIN1 induces Pparγ 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 Pparγ, 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. (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 Pparα. Pparα stimulates the expression of Cpt1b and other genes involved in fatty acid oxidation and increases fatty acid oxidation (Zhou et al. 1999; Goto et al. 2011). 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 (De Vos et al. 1998; Balsalobre et al. 2000; 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, Leptin. Therefore, corticosterone absence may contribute to the changes in gene expression pattern in WAT observed in the current study. Other studies (Slavin et al. 1994; Sakoda et al. 2000; Xu et al. 2009; Campbell et al. 2011) 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 (Yambe et al. 2002; Dzirbíková et al. 2011) found that Avp 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 (Larsen et al. 1994; Isobe & Isobe 1998). Our results only show a trend towards increased AVP mRNA expression in the SCN after adrenalectomy.

Gozes et al. (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. (Gozes et al. 1994) sacrificed all animals during the light period, 10 days after the operation, whereas we sacrificed our rats at 4 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 (Sutanto et al. 1988; Aronsson et al. 1988; Sousa et al. 1989). 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, Zoli, et al. 1994; Cintra, Bhatnagar, et al. 1994; Morimoto et al. 1996). Morimoto et al. (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 4 time points to check the daily rhythmicity of clock genes, but we just used 4 time points. Indeed when using the same conditions, i.e., L/D and brain punches, but sampling 8 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 (Wuarin & Schibler 1990; Torra et al. 2000; Surjit et al. 2011), 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 Avp, 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 (Young et al. 1986; Ma et al. 2001), thus the upregulation of Crh expression in the ADX groups is completely in line with our expectations. Using in situ hybridization Cai & Wise. (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 (Swanson & Kuypers 1980; Swanson & Simmons 1989; Cai & Wise 1996). 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. (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 Avp in the pPVN is upregulated by adrenalectomy, but unchanged in the mPVN (Swanson & Simmons 1989; Ferrini et al. 1997). However, AVP expression in the pPVN is much lower than in the mPVN (Kiss et al. 1984), thus although Avp expression in the pPVN may be up-regulated after adrenalectomy 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 AVP in the PVN, similar to earlier studies (Kalsbeek et al. 1995; Dzirbíková et al. 2011).

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; Dzirbíková 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 behaviour (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 was 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. 1993).

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. (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 (Girotti et al. 2009; Dzirbíková et al. 2011), 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 (Young et al. 1986; Itoi et al. 1987; Ma et al. 2001). In addition, more recent studies (Segall et al. 2006; Gilhooley et al. 2011) 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. (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. (Oishi et al. 2005) found in the liver and Fujihara et al. (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; Cailotto et al. 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 behavioural pathways.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by a grant from the China Scholarship Council.

SUPPLEMENTALS

Table S1 Circwave V1.4 analysis of gene expression in the SCN

Gene	Acrophase in ZT		Difference of acrophases (vs Sham)	Amplitude%	
	Sham	ADX	ADX	Sham	ADX
Avp	12.19	13.45	1.26	111.42	184.02
Vip	ns	16.35	—	—	78.83
Gr	ns	ns	—	—	—
Mr	ns	ns	—	—	—
Bmal1	ns	17.87	—	—	34.77
Cry1	15.36	12.35	-3.01	39.34	42.83
Cry2	ns	ns	—	—	—
Per1	ns	7.93	—	—	52.34
Per2	12.76	11.72	-1.04	74.27	92.80

ns = not rhythmic according to Circwave

Table S2 Circwave V1.4 analysis of gene expression in the PVN

Gene	Acrophase in ZT		Difference of acrophases (vs Sham)	Amplitude%	
	Sham	ADX	ADX	Sham	ADX
Avp	ns	ns	—	—	—
Crh	ns	ns	—	—	—
Gr	ns	ns	—	—	—
Mr	ns	ns	—	—	—
Oxt	ns	ns	—	—	—
Per1	14.44	ns	—	75.55	—
Trh	ns	ns	—	—	—

ns = not rhythmic according to Circwave

Table S3 Circwave V1.4 analysis of clock genes expression in eWAT

Gene	Acrophase in ZT		Difference of acrophases (vs Sham)	Amplitude%	
	Sham	ADX	ADX	Sham	ADX
Bmal1	0.81	22.73	-2.08	160.41	122.95
Cry1	18.45	17.01	-0.92	101.29	72.54
Cry2	13.08	12.84	-0.24	53.29	26.71
Per1	12.84	11.2	-1.64	120.61	83
Per2	14.58	14.59	-0.01	154.1	98.67
Reverb	9.89	9.99	-0.1	221.90	125.20
Dbp	12.46	11.61	-0.85	228.24	183.45

ns = not rhythmic according to Circwave

Table S4 Circwave V1.4 analysis of metabolic/adipokine genes expression in eWAT

Gene	Acrophase in ZT		Difference of acrophases (vs Sham)	Amplitude%	
	Sham	ADX	ADX	Sham	ADX
Fas	ns	ns	—	—	—
Lpl	12.95	ns	—	30.19	—
Hsl	10.95	ns	ns	38.02	—
Acc2a	10.53	10.39	-0.14	61.73	73.97
Glut4	5.05	ns	—	34.41	—
Srebf1	ns	ns	—	—	—
Cpt1b	6.93	ns	—	29.09	—
Leptin	14.23	ns	—	35.83	—
Adiponectin	10.45	ns	—	21.80	—
Resistin	ns	7.91	—	—	48.51
Visfatin	14.23	13.83	-0.4	59.95	47.24
Gr	11.44	ns	—	33.25	—
Mr	9.32	ns	—	46.21	—
Lipin1	13.77	ns	—	30.03	—
Pparα	13.00	ns	—	36.85	—
Pparγ	12.88	ns	—	63.52	—

ns = not rhythmic according to Circwave

Table S5 Statistical analysis of the effect of *Time* on gene expression in the SCN and PVN in the 8 time point study

GENE	Area	TIME
Avp	SCN	**0.045**
Vip	SCN	0.143
Gr	SCN	0.107
Mr	SCN	0.462
Bmal1	SCN	0.134
Per1	SCN	**0.003**
Per1(without ZT2)	SCN	**0.028**
Per2	SCN	**0.009**
Cry1	SCN	0.073
Cry2	SCN	0.054
Crh	PVN	0.273
Avp	PVN	0.089
Oxt	PVN	0.529
Trh	PVN	0.944
Gr	PVN	0.844
Mr	PVN	0.174
Per1	PVN	**<0.001**

Column 3 shows significance levels for the effect of *Time*. Significant effects are in bold.

Table S6 Circwave V1.4 analysis of gene expression in the SCN in the 8 time point study

Gene	Acrophase in ZT	Amplitude%
Avp	15.93	75.37
Vip	ns	—
Gr	20.25	33.29
Mr	ns	—
Bmal1	22.46	37.07
Cry1	19.05	29.15
Cry2	ns	—
Per1	ns	—
Per2	15.83	67.43
Per1(without ZT2)	15.32	54.74

ns = not rhythmic according to Circwave

Table S7 Circwave V1.4 analysis of gene expression in the PVN in the 8 time point study

Gene	Acrophase in ZT	Amplitude%
Avp	ns	—
Crh	ns	—
Gr	ns	—
Mr	ns	—
Oxt	ns	—
Per1	15.18	56.42
Trh	ns	—

ns = not rhythmic according to Circwave

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6

Obesity induced by neonatal monosodium glutamate does not disturb gene expression rhythmicity in rat white adipose tissue

van der Spek R
Pei L
la Fleur SE
Kalsbeek A

Submitted

ABSTRACT

Purpose Obesity prevalence has risen to epidemic proportions globally, causing significant morbidity and mortality. The circadian timing system is of key importance to maintain metabolic health. Currently, it is unclear why clock gene expression rhythms in white adipose tissue (WAT) are reduced in obesity. Several rodent models with obesity display hyperphagia, which could directly influence clock gene expression, but in neonatal monosodium glutamate (MSG) induced obesity animals are hypophagic. Therefore, we investigated WAT gene expression rhythmicity in MSG-induced obesity.

Methods We measured daily variation in clock gene expression (Bmal1, Per2, Cry1, Cry2, RevErbα and DBP) and metabolic gene expression (SREBP1c, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, and leptin) in subcutaneous inguinal (sWAT), peri-renal (pWAT) and perigonadal (eWAT) WAT at time points ZT2, ZT14 and ZT20 in MSG-induced obese female Wistar rats.

Results MSG-induced obesity caused a slight overall reduction in WAT Per2 and Cry1 expression and reduced peak expression of DBP in sWAT and pWAT, but daily rhythmicity of clock gene expression was not affected. We found an overall upregulation (leptin, CPT1b, LPL) or downregulation (SREBP1c) of several genes involved in energy metabolism, but no effects on daily rhythmicity were observed.

Conclusions These results indicate that reduced rhythmicity of gene expression in WAT as observed during obesity is not an intrinsic feature of increased adiposity, but most likely primarily caused by reduced rhythmicity of behaviour and/or circulating metabolic hormones such as insulin and leptin.

INTRODUCTION

Over the last decades the prevalence of obesity has risen to epidemic proportions globally. This obesity epidemic contributes to significant morbidity and mortality. For example, visceral adiposity is an independent risk factor for developing critical illness in patients with COVID-19 (Yang et al. 2020). Furthermore, obesity is a major risk factor for diabetes mellitus, cardiovascular disease and cancers (Abdelaal et al. 2017).

The circadian timing system is of key importance to maintain metabolic health (Laermans & Depoortere 2016). This system ensures energy homeostasis across a range of daily recurring and often opposing events, such as locomotor activity and sleep, food intake and fasting. In mammals, a 'central brain clock' in the suprachiasmatic nuclei (SCN) of the hypothalamus integrates external and internal signals (zeitgebers, e.g. daylight or nutrients), to align the internal circadian timing system with external time. Most mammalian cells, including the neurons in the SCN, contain a 'molecular clock' that regulates many processes in the cell. The molecular clockwork consists of several transcriptional and/or translational feedback loops generating a rhythm in gene expression and protein synthesis and activity of approximately 24 hours. In peripheral tissues, such as liver, muscle, pancreas and adipose tissue, these peripheral clocks are synchronized by the central brain clock via its humoral and neuronal outputs. Simultaneously, nutrients (e.g. glucose), metabolic receptors (e.g. PPARs) and hormones (e.g. leptin) feed back into the circadian system (Reinke & Asher 2019). Disruption of this alignment is a risk factor for developing obesity and other metabolic disturbances such as type 2 diabetes mellitus (Laermans & Depoortere 2016; Reinke & Asher 2019).

Several studies reported decreased rhythmicity of gene expression in white adipose tissue (WAT) in obese rodents (Ando et al. 2005; Ando et al. 2011) and sometimes also people with obesity (Stenvers et al. 2019). Currently, it is not clear why gene expression rhythms in WAT are attenuated in obesity. Both rodent models, i.e. ob/ob (Ando et al. 2011) and KK-Ay mice (Ando et al. 2005; Adachi et al. 2017) display marked hyperphagia. Nutrients and their downstream metabolic effects can affect especially peripheral clock function, therefore in these mice the reduced WAT rhythmicity could be due to hyperphagia and not obesity per se. Neonatal treatment with monosodium glutamate (MSG) causes a neurotoxic lesion of neurons in the arcuate nucleus of the hypothalamus, inducing severe obesity without hyperphagia (Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999). Therefore, here we investigated whether MSG-induced obesity would affect rhythmic gene expression in rat white adipose tissue.

METHODS

Animals

Female offspring of pregnant Wistar dams (Harlan Nederland), were treated with MSG (4mg/g s.c.; Sigma, St. Louis, MO) or saline on postnatal days 1, 3, 5, 7 and 9. MSG lesions were checked by neuropeptide Y (NPY) immunohistochemistry as described by Bruinstroop et al (Bruinstroop et al. 2012). Animals were housed in group cages with their littermates, in a controlled environment on a 12h/12h=light/dark cycle (ZT0 is lights on at 0700 hours), at room temperature (20±2°C). Standard rodent chow and tap water were available ad libitum throughout the experiment. The experiment was conducted under approval of the Local Animal Welfare Committee (Animal Experimentation Committee KNAW).

Tissue collection and analysis

To obtain WAT tissues and plasma, 60 animals were anaesthetized with pentobarbital and killed by decapitation at ZT2, ZT14 and ZT20 (N = 9-11 per group and time point). Subcutaneous inguinal (sWAT), peri-renal (pWAT) and peri-gonadal (eWAT) white adipose tissues were quickly dissected and snap frozen in liquid nitrogen for RNA extraction. Depots from the contralateral side were dissected and weighed, pWAT and eWAT were weighed together as they were fused in some cases. Furthermore, the mesenteric (mWAT) depot was dissected and weighed.

Plasma analysis

Following decapitation, trunk blood was collected in heparinized tubes and kept on ice until centrifugation for 15min at 3000rpm at 4°C. Plasma was transferred to a clean tube and stored at -20°C until use. Plasma glucose was measured using a Biosen apparatus (EKF diagnostics, Cardiff, UK). Plasma insulin, leptin and corticosterone were measured using a Radio Immuno Assay (Merck Millipore, Billerica, MA, USA), according to manufacturer's protocol.

Gene expression analysis

Gene expression analysis was performed as previously described in detail (van der Spek et al. 2018). In short, total RNA was extracted from approximately 100mg of adipose tissue, using the RNeasy lipid kit including on-column DNAse treatment (Qiagen Benelux, Venlo, Netherlands). cDNA was synthesized with the Transcriptor First Strand cDNA synthesis kit (Roche, Almere, Netherlands). Gene expression was analysed by real-time RT-qPCR on a Lightcycler 480 system (Roche, Almere, Netherlands). We used additional no reverse transcriptase samples to control for potential DNA contamination, positive controls and melting peak analysis for product verification, and analysed individual

sample PCR efficiency to exclude all samples that deviated more than 0.05 from efficiency mean. Hypoxanthine Phosphoribosyl Transferase (HPRT) gene expression was used as a housekeeping gene to control for variation in amount of mRNA input. Primer sequences of clock genes Bmal1, Per2, Cry1, Cry2, RevErbα and DBP, and metabolic genes SREBP1c, PPARγ, FAS, LPL, HSL, CPT1b, Glut4, and leptin have been published previously (Su et al. 2015).

Data analysis and statistics

All PCR data are expressed relative to ZT2, to allow comparison between WAT depots and treatment groups. For identification of outliers, we used Dixon's Q test with two-tailed values using 95% confidence values. Samples that were determined outliers, were excluded from further analysis. All data are presented as mean ± S.E.M. unless otherwise stated. Group differences were analyzed by 2-way-Anova using Graphpad Prism 8. P values below 0.05 were considered statistically significant.

RESULTS

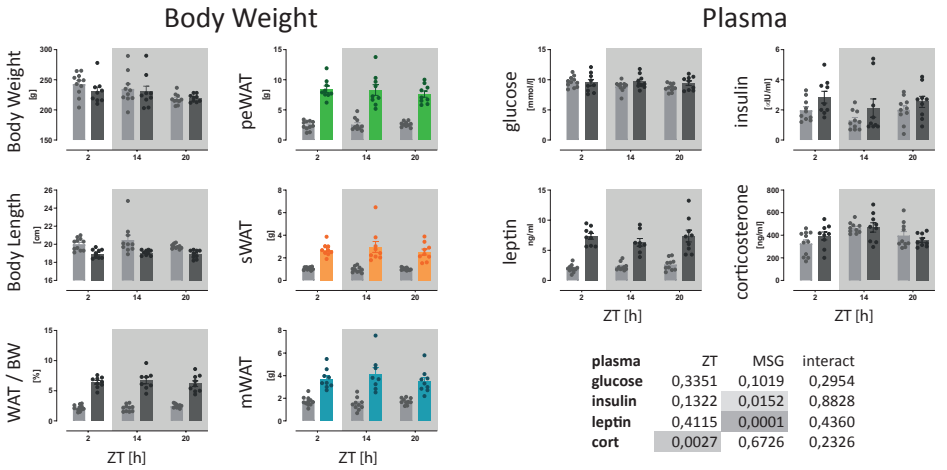
Body weight of all saline and MSG treated animals was on average 230 ± 4g, ranging from 196g to 290g. There was no difference in body weight between saline and MSG treated animals. There was a difference between the time groups (ZT2 vs ZT14 vs ZT20, 2way-Anova: $p = 0.018$), with animals sacrificed at ZT20 being slightly lighter than those at the other 2 time points (figure 1).

Body length differed significantly between groups. MSG treated animals were shorter than saline treated animals (MSG 19.0 ± 0.1cm versus saline 20.1 ± 0.2cm, 2way-Anova: $p < 0.0001$) (figure 1).

Body composition differed significantly between groups. MSG treated animals had approximately 3 times more adipose mass in all three WAT depots analyzed, i.e. peWAT, sWAT and mWAT (figure 1). In total MSG treated animals had on average 6.2 ± 0.3% WAT, versus 2.2 ± 0.1% for saline treated animals (all dissected WAT depots/BW*100%; 2way-Anova: $p < 0.0001$) (figure 1).

Plasma

Plasma glucose levels were on average 9.3 ± 0.1mmol/l and did not differ between groups or time points. Plasma insulin levels were higher in MSG treated animals (MSG 2.5 ± 0.3µU/ml vs saline 1.0 ± 0.2µU/ml; 2way-Anova: p = 0.0152). Plasma corticosterone levels peaked at ZT14, but did not differ between MSG and saline treated animals (ZT2 394 ± 19ng/ml, ZT14 466 ± 20ng/ml, ZT20 378 ± 14ng/ml; 2way-Anova: p = 0.0027). Plasma leptin levels were significantly higher in MSG treated animals (MSG 7.4 ± 0.6ng/ml vs saline 2.3 ± 0.1ng/ml, 2way-Anova: p < 0.0001) (figure 1).

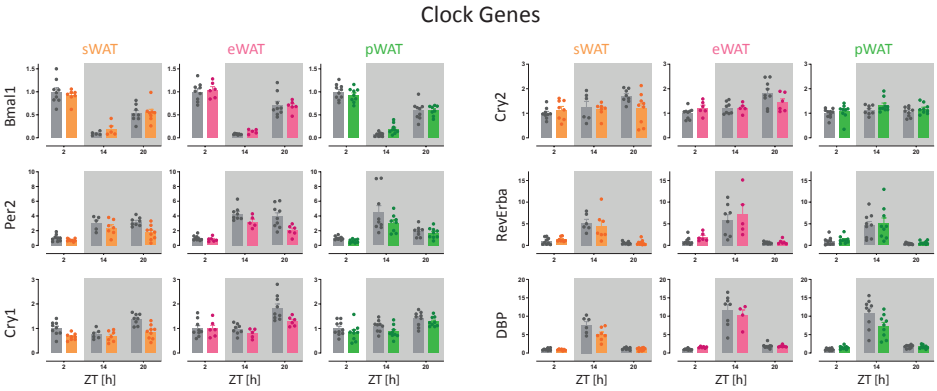


plasma	ZT	MSG	interact
glucose	0,3351	0,1019	0,2954
insulin	0,1322	0,0152	0,8828
leptin	0,4115	0,0001	0,4360
cort	0,0027	0,6726	0,2326

Figure 1. Weight and plasma measurements in saline (grey bars) and monosodium glutamate (MSG, coloured bars) treated animals. Bar plots represent mean ± SEM, scatter plots represent individual data points, grey background fill indicates lights off (ZT12-ZT24). The table shows 2way-Anova p values of time, MSG treatment and their interaction, coloured values are p < 0.05 (darker shades correspond with lower p-values). MSG treatment significantly increased adiposity, insulin and leptin plasma concentrations

Gene expression

All clock genes showed pronounced differences in expression level between time points in both saline and MSG treated animals, except for Cry2 in pWAT. Per2 expression was reduced in MSG treated animals in sWAT (2way-Anova: p =0.0012) and eWAT (2way-Anova: Treatment effect, p = 0.0009) and Cry1 expression was reduced in all WAT depots in MSG treated animals (2way-Anova: Treatment effect, sWAT p < 0.0001; eWAT p = 0.0302; pWAT p = 0.0251). DBP was the only gene that showed an Interaction effect, with reduced peak expression in sWAT (2way-Anova: Interaction effect, p = 0.0063) and pWAT (2way-Anova: Interaction effect, p = 0.0100) of MSG treated animals (figure 2).



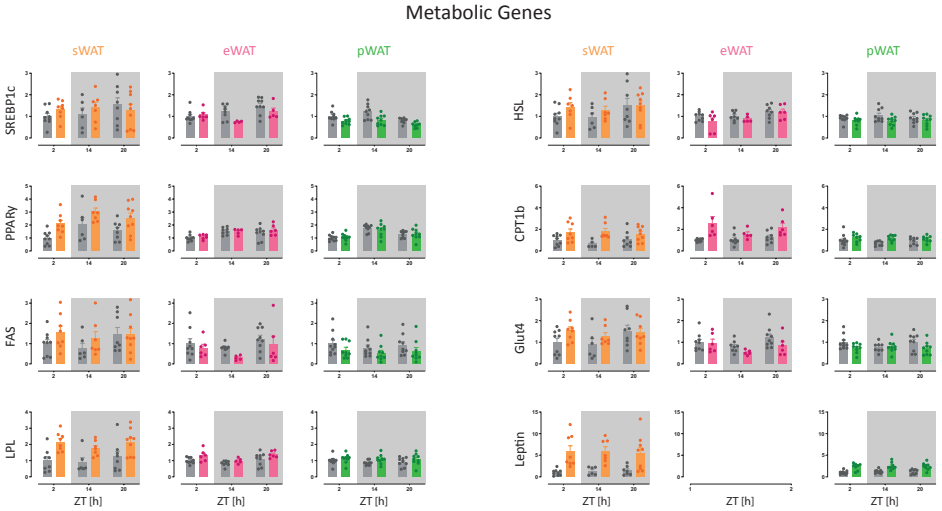
sWAT	ZT	MSG	interact
Bmal1	0,0001	0,8548	0,4803
Per2	0,0001	0,0012	0,0655
Cry1	0,0001	0,0001	0,5490
Cry2	0,0379	0,2043	0,0874
REa	0,0001	0,7981	0,5830
DBP	0,0001	0,0068	0,0063

eWAT	ZT	MSG	interact
Bmal1	0,0001	0,6160	0,7992
Per2	0,0001	0,0009	0,0600
Cry1	0,0001	0,0302	0,1165
Cry2	0,0003	0,5947	0,0566
REa	0,0001	0,2831	0,8241
DBP	0,0001	0,6580	0,4917

pWAT	ZT	MSG	interact
Bmal1	0,0001	0,6322	0,1057
Per2	0,0001	0,0774	0,3807
Cry1	0,0001	0,0251	0,8196
Cry2	0,0556	0,4480	0,7424
REa	0,0001	0,5299	0,9858
DBP	0,0001	0,0667	0,0100

Figure 2. Clock gene expression in subcutaneous (sWAT), gonadal (eWAT) and perirenal (pWAT) white adipose tissue of saline (grey bars) and monosodium glutamate (MSG, coloured bars) treated animals. Bar plots represent mean ± SEM, scatter plots represent individual data points, grey background fill indicates lights off (ZT12-ZT24). The table shows 2way-Anova p values of time, MSG treatment and their interaction, coloured values are $p < 0.05$ (darker shades correspond with lower p values). MSG-induced obesity caused a slight overall reduction in WAT Per2 and Cry1 expression, and reduced peak expression of DBP in sWAT and pWAT, but daily clock gene expression rhythms were not affected

Daily variation was less pronounced in metabolic gene expression, only in eWAT most metabolic genes tested showed significant daily variation. On the other hand, metabolic gene expression was clearly affected by Treatment and generally increased in MSG treated animals, with the most pronounced effects found in sWAT and pWAT. No Interaction effects were observed. Leptin data are missing for eWAT due to a technical error (figure 3).



sWAT	ZT	MSG	interact
SREBP	0,5510	0,5549	0,3898
PPARγ	0,0171	0,0004	0,9815
FAS	0,3410	0,1678	0,5195
LPL	0,4229	0,0001	0,8084
HSL	0,2044	0,1863	0,5754
CPT1b	0,8798	0,0005	0,4494
Glut4	0,2241	0,1223	0,3127
Leptin	0,9699	0,0001	0,8802

eWAT	ZT	MSG	interact
SREBP	0,0119	0,0434	0,1074
PPARγ	0,0008	0,3571	0,5614
FAS	0,0873	0,0902	0,8316
LPL	0,0117	0,0048	0,7399
HSL	0,0108	0,1573	0,5544
CPT1b	0,2254	0,0001	0,1772
Glut4	0,0561	0,0759	0,5144
Leptin			

pWAT	ZT	MSG	interact
SREBP	0,0010	0,0001	0,4125
PPARγ	0,0001	0,7855	0,6094
FAS	0,5028	0,0334	0,9810
LPL	0,6117	0,0256	0,8879
HSL	0,7094	0,0207	0,4896
CPT1b	0,3999	0,0056	0,4476
Glut4	0,6612	0,0466	0,2476
Leptin	0,6600	0,0001	0,9492

Figure 3. Metabolic gene expression in subcutaneous (sWAT), gonadal (eWAT) and perirenal (pWAT) white adipose tissue of saline (grey bars) and monosodium glutamate (MSG, coloured bars) treated animals. Bar plots represent mean ± SEM, scatter plots represent individual data points, grey background fill indicates lights off (ZT12-ZT24). The table shows 2way-Anova p values of time, MSG treatment and their interaction, coloured values are $p < 0.05$ (darker shades correspond with lower p values). Leptin data in eWAT missing due to technical errors. There was an overall upregulation (leptin, CPT1b, LPL) or downregulation (SREBP1c) but no effects on daily rhythmicity were observed

DISCUSSION

In line with previous work we observed pronounced daily variation in core clock gene expression in WAT of saline treated control animals (van der Spek et al. 2018). However, despite severe obesity, hyperinsulinemia and hyperleptinemia and reduced expression of Per2 and Cry1, we found no indication for disturbed (clock) gene rhythmicity. Apart from a reduced peak in DBP expression in sWAT and pWAT, no Interaction effects of MSG treatment on white adipose tissue clock gene expression were observed. As expected, we found an overall upregulation (leptin, CPT1b, LPL) or downregulation (SREBP1c) of several genes involved in energy metabolism, but also in these genes no changes in daily rhythmicity, i.e. no Interaction effects, were observed.

Neonatal MSG administration has several effects that could potentially have disturbed WAT gene expression rhythmicity in our experiment. Firstly, it induces substantial and ir-

reversible loss of neurons in the retina, nevertheless sufficient retinal circuitry is retained to mediate entrainment of circadian rhythms to standard light/dark cycles (Miyabo et al. 1985; Mistlberger & Antle 1999). Secondly, SCN volume is reported to be either normal (Mistlberger & Antle 1999), or reduced (Miyabo et al. 1985) following neonatal MSG. Finally, neonatal MSG administration has been reported to disturb daily rhythms of well-known zeitgebers such as food intake and locomotor activity in adulthood (Dawson et al. 1989; Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999). Nevertheless, we found no major effects on daily rhythmicity of gene expression of core clock and metabolic genes in white adipose tissue.

Two previous studies did report reduced WAT gene expression rhythmicity in obese rodents (Ando et al. 2005; Ando et al. 2011). Interestingly, both these models are hyperphagic (Ando et al. 2011; Adachi et al. 2017), whereas MSG treated rats are hypophagic (Stricker-Krongrad et al. 1998). Food intake has been reported to rapidly decrease Per2 and Bmal1 gene expression in rat liver (Wu et al. 2010), and has been hypothesized to cause a similarly decreased clock gene expression in WAT. Nevertheless, in the experiment with ob/ob obese mice, hypocaloric feeding did not restore WAT gene expression rhythmicity, whereas leptin administration partially did (Ando et al. 2011). In our MSG-treated hypophagic animals, we also see a slight overall reduction in Per2 expression, suggesting other factors than food intake might regulate Per2 levels in obese WAT.

All together, we showed that although neonatal monosodium glutamate injections induced severe obesity in female rats, this metabolic disturbance did not dampen daily variation in clock or metabolic gene expression in white adipose tissue. Clearly, obesity on itself is thus not the reason for the reduced rhythmicity in WAT gene expression. Therefore, it seems more likely that the disturbed rhythms in behaviour and/or hormones that often accompany obesity are to be held responsible for the disturbed rhythms in WAT gene expression.

DECLARATIONS

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Summary

In this thesis 'Regulation of daily gene expression rhythms in rat white adipose tissue', **chapters 1 and 2** introduce the circadian timing system as a system of key importance to maintain metabolic health. Circadian disruption by shift work or (social) jetlag is correlated with an increased prevalence of obesity and related metabolic disturbances like diabetes mellitus type 2. White adipose tissue (WAT) plays a central role in regulation of energy homeostasis and hence it is important to understand the circadian regulation of WAT. Previous work from our group has shown that in the liver the central brain clock in the suprachiasmatic nucleus (SCN) and the autonomic nervous system (ANS) contribute to regulation of daily gene expression rhythms. For white adipose tissue it was largely unknown which factors were involved. Therefore, the overall aim of this thesis was to improve our understanding of how daily gene expression rhythms in rat white adipose tissue are regulated.

In the first experiment, we investigated daily gene expression rhythms in undisturbed conditions. In **chapter 3** we analysed rhythmicity of expression of clock genes (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, and *DBP*) and metabolic genes (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *Glut4*, *HSL*, *CPT1b*, *leptin*, *visfatin/NAMPT*, and *resistin*) in three intra-abdominal (mesenteric; mWAT, perirenal; pWAT, epididymal; eWAT) and the subcutaneous WAT (sWAT) depots. We found robust rhythms in clock gene expression in all four depots. Rhythms in metabolic gene expression were less robust (lower amplitudes and lower R^2). We did not observe clear differences in gene expression rhythms between the different WAT depots. This was the first study to extensively compare clock gene rhythms in subcutaneous and several abdominal WAT depots simultaneously. Because we did not observe pronounced differences between WAT depots, we suggest that in studies with human subjects clock gene expression rhythms in intra-abdominal depots can be extrapolated from subcutaneous WAT, which is much less invasive to take biopsies from.

Next, we investigated potential drivers for the control of daily rhythmicity in WAT. In **chapter 4** we assessed the importance of the central brain clock in the suprachiasmatic nucleus (SCN), for gene expression rhythms in WAT. We compared rats with bilateral thermal lesions of the SCN to sham controls. We measured clock gene expression (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, and *DBP*) and metabolic gene expression (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, *leptin*, *adiponectin*, *visfatin/NAMPT*, and *resistin*) in eWAT and sWAT. We found that SCN lesions did not abolish clock gene expression rhythms completely, but did reduce amplitude and R^2 values by approximately 50%, and advanced the phase with approximately 4 hours. Only *Cry2* and *DBP* were no longer rhythmic in both WAT depots after SCN lesions. All metabolic gene expression rhythms were abolished after SCN lesions, with the sole exception of *visfatin/NAMPT*, which remained rhythmic in both sWAT and eWAT, without phase shift and with less reduc-

tion in amplitude than the clock genes. Overall, the effect of SCN lesions on clock gene expression was similar between both WAT depots. These results show that in absence of the central brain clock, white adipose tissues retain some clock gene rhythmicity.

This raises the (unexpected) question what mechanisms are involved in WAT clock gene expression rhythms in absence of the rhythmic output of the SCN?

Adrenal hormones (glucocorticoids) are a well-known regulator of rhythmicity in a variety of peripheral tissues, and are also known to regulate many metabolic processes in white adipose tissue. Therefore, in **chapter 5** we examined whether adrenal hormones are necessary for the maintenance of daily gene expression rhythms in WAT. We compared rats with bilateral adrenalectomy (ADx) to sham controls. We measured clock gene expression (*Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, and *DBP*) and metabolic gene expression (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, *ACC2a*, *leptin*, *adiponectin*, *visfatin/NAMPT*, *resistin*, *Lipin1, glucocorticoid receptor and mineralocorticoid receptor*) in eWAT. We found that adrenalectomy reduced the amplitude of all clock gene rhythms with 20-50%, without major changes in phase. *DBP* changed the least (-20%), *Cry2* (-50%) and *RevErbα* (-44%) the most. Most metabolic genes lost their rhythmicity after adrenalectomy, with the exception of *ACC2a*, *resistin* and *visfatin/NAMPT*. These results show that adrenal hormones, or their downstream effects, are important for regulating clock gene rhythmicity in white adipose tissue, but other factors contribute too.

Lastly, a number of human and animal studies have shown that obesity is correlated with reduced gene expression rhythms in white adipose tissue. The causality of this correlation is unknown. Many of these models display hyperphagia (overeating) and nutrients directly influence many factors involved in the circadian clock. Therefore, in these models it is difficult to differentiate between the direct effects of hyperphagia and effects of increased adipose mass. Neonatal administration of monosodium glutamate (MSG) induces severe obesity while the animals actually eat less than their controls (Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999).

Therefore, to assess the effect of obesity without hyperphagia, in **chapter 6** we investigated daily rhythms in WAT gene expression in MSG-induced obesity compared to normal weight controls. We measured clock gene expression (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα* and *DBP*) and metabolic gene expression (*SREBP1c*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, and *leptin*) in sWAT, pWAT and eWAT. We found that MSG-induced obesity caused a minor overall reduction in *Per2* and *Cry1* expression, and reduced peak expression of *DBP* in sWAT and pWAT, but in general daily rhythms in clock gene expression were not affected. We found an overall upregulation (*leptin*, *CPT1b*, *LPL*) or downregula-

tion (*SREBP1c*) of several genes involved in energy metabolism, but no effects on daily rhythmicity were observed. Besides causing obesity, neonatal MSG administration has several other effects that in principle could have disturbed gene expression rhythmicity in WAT; it has been reported to disturb daily rhythms of well-known zeitgebers such as food intake and locomotor activity in adulthood (Dawson et al. 1989; Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999). Nevertheless, we found no major effects on daily gene expression rhythms in WAT. Together these data suggest that the reduced gene expression rhythmicity in WAT observed in other obesity models is explained by other factors than increased adipose mass.

Concluding, we found pronounced clock gene expression rhythms in several WAT depots, without differences in rhythmicity between WAT depots (**chapter 3**). These rhythms are regulated by multiple factors, as absence of the SCN and its output reduces rhythmicity but does not completely abolish gene expression rhythms in WAT (**chapter 4**), and neither does the absence of adrenal hormones (**chapter 5**). Lastly, obesity itself does not reduce gene expression rhythms in rat WAT (**chapter 6**).

General discussion

Two main questions arise from the experiments described in this thesis. First, the central brain clock has many rhythmic outputs, in **chapter 5** we show glucocorticoids are one important factor, but other factors must be involved too. Thus, what other SCN output factors contribute to the regulation of gene expression rhythms in WAT? Second, in the absence of the SCN we still find remaining clock gene expression rhythms in WAT (**chapter 4**). This raises the question what mechanisms are involved in WAT clock gene expression rhythms in absence of the rhythmic output of the SCN?

SCN OUTPUT FACTORS THAT REGULATE GENE EXPRESSION RHYTHMS IN WHITE ADIPOSE TISSUE

As reviewed in **chapter 1**, multiple pathways are known to be involved in the regulation of rhythmicity in peripheral tissues; the autonomic nervous system, circulating hormones (e.g., glucocorticoids, insulin, melatonin) and the sleep-wake cycle with subsequent daily rhythms in energy expenditure (e.g., locomotor activity, basal metabolic rate), energy intake (e.g., circulating nutrients like glucose and lipids), body temperature and processes involved in cellular metabolism.

Glucocorticoids are involved in regulating energy homeostasis and regulate many transcriptional and post-transcriptional processes (**Chapter 2**). The effects of glucocorticoids on regulation of peripheral tissue rhythmicity are tissue specific (Sujino et al. 2012; Ikeda et al. 2015), and were largely unknown for WAT. In **chapter 5** we found that after adrenalectomy, clock gene rhythms in eWAT remain, without major changes in phase, albeit with 20-50% reduction in amplitude. This indicates that (the downstream effects of) adrenal hormones contribute to regulation of clock gene rhythmicity in WAT, but other factors must be contributing too.

The reductions in clock gene amplitude after SCN lesions (**chapter 4**) and after adrenalectomy (**chapter 5**) are quite similar. Therefore, one could speculate that the reduction in rhythmicity seen after an SCN lesion might be in large part due to the loss of rhythmicity of circulating glucocorticoids or its downstream effects, i.e. changes in daily rhythms in (locomotor) activity, feeding behaviour or body temperature. To test this hypothesis, SCN lesions could be combined with timed administration of glucocorticoids to reinstate the daily peak.

Meanwhile, several other studies have looked into the effects of adrenalectomy on WAT clock gene rhythms. One study combined adrenalectomy with 7 days of constant darkness and found slightly more pronounced reductions in clock gene rhythmicity in eWAT,

but no phase alterations either (Soták et al. 2016), indicating that the light dark cycle may have a small contribution to WAT clock gene rhythmicity in absence of adrenal hormones.

The SCN regulate the rhythm in food intake. To investigate the influence of the rhythm in food intake, a regular feeding schedule can be used. This provides six identical meals every twenty-four hours, i.e. one meal every four hours, so food intake is evenly spread over the light dark cycle. The six meals feeding schedule has minor influence on clock gene rhythmicity in WAT (Su et al. 2016). However, when adrenalectomy was combined with this regular feeding schedule, all clock gene rhythms were abolished in eWAT (Su et al. 2016). This effect does not seem to be mediated via major effects on rhythmicity of the SCN, as the rhythm in PER2 immunohistochemistry in the SCN was not altered after adrenalectomy with a regular feeding schedule (Ikeda et al. 2015). In conclusion, clock gene rhythms in eWAT do not solely depend on adrenal hormones or the daily feeding rhythm, but at least one of these signals should be present to maintain WAT gene expression rhythmicity. The redundancy in factors that entrain WAT clock gene expression rhythms ensures a robust system that integrates multiple entraining factors and thus can adapt to varying conditions and is not dependent on a single synchronizing factor.

Metabolic gene expression rhythms in WAT are much less robust than clock gene rhythms, even in undisturbed animals (**chapter 3**). They are largely abolished after SCN lesions (**chapter 4**), adrenalectomy (**chapter 5**), and adrenalectomy plus a regular feeding schedule (Su et al. 2016). After a regular feeding schedule also most metabolic genes lose rhythmicity, however, a few others gain rhythmicity (Su et al. 2016).

FACTORS THAT MAINTAIN CLOCK GENE RHYTHMICITY IN WHITE ADIPOSE TISSUE IN ABSENCE OF THE SCN

Light via extra-SCN pathways

As described in **chapter 1**, since the discovery of the relevance of the SCN in time keeping in 1972, the SCN have been considered as the master clock that generates endogenous rhythms and distributes these to the rest of the brain and body. Light is the major zeitgeber for the SCN. However, in absence of the SCN light/dark signals can probably still be communicated to other brain areas and subsequently peripheral tissues via the autonomic nervous system. For example, the melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) also project directly to pre-autonomic nuclei in the hypothalamic paraventricular nuclei (Hattar et al. 2006; Canteras et al. 2011; Noseda et al. 2017; Rupp et al. 2019).

Furthermore, exposure to light may directly affect white adipose tissue through the skin. Adipocytes contain light sensitive photopigments, amongst others melanopsin, that can be activated through the skin by the light intensity on a bright sunny day (Ondrusova et al. 2017; Nayak et al. 2020). What this means for a nocturnal rodent in laboratory conditions is unclear. Alternatively, light could affect the skin to release local signals that reach the underlying adipose tissues. However, if indeed light affects sWAT directly, this would be expected to result in differences between subcutaneous and intra-abdominal tissues, as the latter are located much deeper underneath the skin and thus receive less light or local signals from skin.

As we kept our animals in a regular light dark cycle, light may have contributed to persisting rhythmicity in absence of the SCN (**chapter 4**). Either via a central non-SCN pathway, or directly through the skin. When looking at clock gene expression rhythms after SCN lesion, we observed two minor differences between sWAT and eWAT. *Bmal1* does not phase shift in sWAT, whereas it does in eWAT. *Per2* remains rhythmic in sWAT whereas it does not in eWAT. This might indicate a small effect of light directly through the skin. To investigate whether indeed the light dark cycle contributes to maintain clock gene rhythmicity in WAT, repeating the SCN lesion experiment in constant darkness would allow to assess whether indeed the daily light/dark cycle is responsible for the remaining rhythmicity. It would however not allow for differentiation between a central or direct peripheral pathway. To differentiate between central and direct effects ipRGCs could be targeted directly, for example using immuno-induced ablation (Ingham et al. 2009).

Nevertheless, as discussed in **chapter 4**, other studies looking at peripheral gene expression rhythms in WAT and other peripheral tissues after genetic (Husse et al. 2014; Izumo et al. 2014; Lee et al. 2015; Kolbe et al. 2016) or thermal SCN ablation (Akhtar et al. 2002; Tahara et al. 2012), found reductions in peripheral gene expression rhythms comparable to our data. These rhythms remained most strongly in presence of the light dark cycle. However, two studies reported even after 1 month of constant dark conditions that peripheral tissues still show clock gene expression rhythms (Tahara et al. 2012; Izumo et al. 2014), indicating other factors than rhythmic SCN output and the light dark cycle must keep these rhythms oscillating.

Cellular metabolism

Interestingly, metabolic gene expression rhythms were all abolished after SCN lesions, with the sole exception of *visfatin/NAMPT*, which remained rhythmic in both sWAT and eWAT, without phase shift and with less reduction in amplitude than the clock genes. As discussed in **chapter 4**, the reduced *Bmal1* rhythm, and the TTFL through SIRT1, may have been sufficient to keep driving the rhythm in *visfatin/NAMPT* expression. However,

accumulating evidence suggests cellular metabolism pathways could function both as output as well as an independent input of the molecular clock (Gerhart-Hines & Lazar 2015; Morris et al. 2020). For example, oxidative stress regulates NAMPT (Lin et al. 2015; Lu et al. 2019) and the rhythmic genomic binding of CLOCK:BMAL1 to regulate the transcription of other genes is profoundly influenced by NAD+/NADH status (reviewed by (Gerhart-Hines & Lazar 2015)). Furthermore, proteins that eliminate reactive oxygen species such as peroxiredoxins may generate rhythmicity even in absence of any transcriptional activity in a cell (O'Neill & Reddy 2011; Edgar et al. 2012). This then raises the question what is driving the daily rhythms in cellular metabolism; are these rhythms depending on rhythmic input, or are these oscillating autonomously?

Autonomous feedback loops not involving the SCN

A third mechanism that may keep the white adipose clock oscillating in absence of the SCN, is that the autonomous feedback loops persist in individual cells within tissues. Whereas coupling within the SCN is stronger than in other tissues, cellular oscillators may be coupled within a tissue and peripheral tissues may be coupled to each other independently of the SCN pacemaker as proposed in (Koronowski & Sassone-Corsi 2021). Recent studies suggest that autonomous tissue clocks can indeed exist in a systemically arrhythmic animal. These autonomous clocks can keep a 24h period depending on the light dark cycle in skin (Welz et al. 2019) and liver (Koronowski et al. 2019), and even in constant dark conditions in liver (Sinturel et al. 2021). While in the presence of synchronizing signals from the SCN, these cellular and tissue clocks may have only a small contribution, their contribution could become more significant when synchronizing signals from the SCN are absent, although different tissue clocks do not seem capable of entraining each other. To figure out if these extra-SCN clocks would keep oscillating or if they would eventually dampen *in vivo*, the SCN lesion experiment should be repeated in constant dark conditions with a longer duration.

A network of clocks

In conclusion, the findings in **chapter 4** suggest the hierarchical model of the master clock that confers top-down synchronisation to peripheral tissues does not suffice. Instead, a model comprising a network of clocks with crosstalk over several levels of organisation (cell, tissue, systemic) (Pilorz et al. 2020; López-Otín & Kroemer 2021; de Assis & Oster 2021), would better explain why absence of the SCN reduces rhythmicity, but does not completely abolish it.

Evolutionary, clock systems may have evolved in single cell organisms (e.g., cyanobacteria) to protect replicating DNA from damaging agents such as solar radiation or ROS produced by metabolic activity, and to separate incompatible processes such as

anabolic and catabolic pathways (reviewed in (Chaix et al. 2016)). These single cell organisms cannot separate incompatible processes in space, and therefore had to separate these processes in time. Many levels of complexity are added in a multicellular organism, whereby (Koike et al. 2012) even suggested that circadian transcription factor binding is tightly correlated with gene expression *per se*, rather than rhythmicity of gene expression. In light of development from single cell organisms, it would seem sensible for processes involved in cellular homeostasis to be an integral part of a clock network.

A number of examples illustrate this concept (reviewed in (Chaix et al. 2016); first, the nuclear envelope regulates the spatial organization of the genome and thus plays a role in the circadian control of transcription. Second, unfolded proteins can cause cellular toxicity and activate the unfolded protein response, and the unfolded protein response targets components of the cellular clock system. Furthermore, autophagy, an important cytoplasmic recycling mechanism (López-Otín & Kroemer 2021), can both be entrained by feeding cues and provide micronutrients that regulate the cellular clock. Lastly, cells can release factors that entrain other cells locally or systemically. For example via the cellular energy state, whereby intracellular NAMPT directly influences the clock system within the cell via BMAL1 (Rutter et al. 2001; Nakahata et al. 2009; Ramsey et al. 2009), and extracellular NAMPT regulates locomotor activity rhythms systemically (Yoon et al. 2015; Imai 2016; Stromsdorfer et al. 2016). Hence evolution from single cell to multicellular organism may have added layers of integration on tissue and systemic level to the cellular clock system, which may explain why the central brain clock has widespread systemic influence on peripheral clocks, but is not the sole factor driving peripheral rhythmicity.

Thus, summary of **chapter 3-5** suggests that:

1. Clock gene rhythms in WAT do not fully depend on the SCN, but the SCN does contribute to phase synchronization and amplitude of rhythmicity.
2. Clock gene rhythms in WAT do not solely depend on adrenal hormones or the daily feeding rhythm, but at least one of these signals should be present to maintain WAT gene expression rhythmicity.
3. Light could influence clock gene expression rhythms in WAT via the SCN, via central pathways not involving the SCN, via the skin or directly at the level of the adipocyte. However, in absence of a rhythm in adrenal hormones and food intake, the light dark cycle is not sufficient to maintain rhythmicity in WAT.
4. A network of autonomous clocks may sustain a degree of tissue rhythmicity in absence of the SCN. However, in absence of the synchronizing signals from adrenal hormones and the rhythm in food intake, autonomous clocks in WAT cannot maintain rhythmicity.

OBESITY DOES NOT DAMPEN WAT CLOCK IN FEMALES

In the last chapter (**chapter 6**) we investigated gene expression rhythms in WAT in obesity. Both human and animal studies show obesity is correlated with reduced gene expression rhythms in white adipose tissue. It is unknown whether this is due to the consequences of the increase in fat mass - that can for example induce low grade inflammation - or to other factors related to obesity. Obesity develops when energy intake exceeds energy expenditure over longer periods of time. Both energy intake and energy expenditure are regulated by the circadian clock and show marked daily rhythmicity. Furthermore, on cellular, tissue and systemic level, factors involved in energy metabolism feed back into the circadian clock system (**chapter 1**). In many obesity models, the distribution of food intake over the light dark cycle is altered and food acts a potent synchroniser for peripheral clocks (reviewed in (Greco & Sassone-Corsi 2019)). For example, restricting food intake to the light (sleep) phase can uncouple peripheral tissues like the liver from the SCN; the liver clock entrains to food availability, whereas the SCN remains entrained to the light dark cycle (Stokkan et al. 2001). However, not every tissue shows such a complete synchronization to feeding time as the liver (Opperhuizen et al. 2016). Mice receiving a high fat diet increase their food intake during the light (sleep) phase and show blunted gene expression rhythms in peripheral tissues (Kohsaka et al. 2007). Restricting food intake to the dark (active) phase can revert the adverse metabolic effects of this high fat diet.

Therefore, we hypothesized the blunted WAT rhythmicity might be due to the effects of hyperphagia and not obesity per se. In **chapter 6** we used neonatal administration of monosodium glutamate (MSG) to induce severe obesity. With MSG-induced obesity male rats eat less than their controls. We found that MSG-induced obesity in female rats caused a slight reduction in the expression of some clock genes in WAT, but no major disturbance of clock gene rhythmicity.

However, several factors complicate interpretation of these data. Besides causing obesity, neonatal MSG administration has several other effects that could have disturbed gene expression rhythmicity in WAT; such as disturbing daily rhythms of well-known zeitgebers such as food intake and locomotor activity in adulthood (Dawson et al. 1989; Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999). Nevertheless, we found no major effects on daily gene expression rhythms in WAT.

An additional complicating factor in the interpretation of these data is that we used female animals in this study, whereas most data in literature is based on males. Males and females might respond differently to metabolic or circadian disturbances as observed in

rodents (Revollo et al. 2007; Yoon et al. 2015; Palmisano et al. 2017; Omotola et al. 2019) and humans (Qian et al. 2019; Mulè et al. 2021) and females may be less susceptible to diet induced disturbances (Palmisano et al. 2017; Omotola et al. 2019). On the other hand, the first study to show reduced rhythmicity in obese diabetic mice was done in females (Ando et al. 2005), suggesting females are also susceptible. Furthermore, female mice and hamsters actually gain more fat mass than males after neonatal MSG treatment (Olney & Sharpe 1969; Leitner & Bartness 2008). Of larger concern for the interpretation of our results however, might be sex and species dependent differences in food intake after MSG treatment. In male rats, MSG treated animals ate less than their controls (Stricker-Krongrad et al. 1998; Mistlberger & Antle 1999), although others report an increase in food intake in MSG treated male rats (Sun et al. 1991). In hamsters, females become hyperphagic after neonatal MSG treatment, whereas males do not (Leitner & Bartness 2008). Although in another study no difference was observed in female rats for total food intake or diurnal percentage of food intake after neonatal MSG treatment (Sun et al. 1991). In our study we have not measured food intake, which makes interpretation of our results rather difficult.

Altogether, the data in **chapter 6** show that clock gene rhythmicity in WAT is not disturbed by obesity per se, but given the complicating factors in interpretation of the data, we cannot conclude much more for now.

CLINICAL RELEVANCE

Modern lifestyles give our clock system many mixed or misaligned signals, which contribute to an increased risk for a wide range of pathologies including sleep and mood problems, as well as metabolic disorders, such as obesity, diabetes mellitus type 2 and cardiovascular disease. A better understanding of the interplay between energy metabolism and the circadian clock system could help to develop strategies that limit the detrimental effects of circadian misalignment on metabolism and the corresponding threat to health.

Our results show that gene expression rhythms in WAT can be impaired by absence of the central clock, (downstream effects of) absence of adrenal hormones, and are abolished when both the rhythm in adrenal hormones and food intake are absent. Although we cannot directly translate our results to humans due to the invasive nature of our experiments – there is no human equivalent for absence of the SCN or absence of adrenal hormones – extrapolation of our results suggests that simultaneous disturbance of the rhythm in adrenal hormones and food intake induces severely disturbed clock gene

expression rhythms in WAT. Because clock genes function as transcription factors for many processes in WAT, this could indeed result in dysfunction of WAT and consequently in metabolic disorders such as insulin resistance. In situations where circadian misalignment cannot be avoided, strategies to limit the detrimental metabolic effects could include chrono-nutrition intervention (reviewed in Hawley et al. 2020 and Stenvers et al. 2019) and pharmacological agents that target clock genes (reviewed in Stenvers et al. 2019).

Interestingly, in one specific group of hospitalized patients the rhythm in glucocorticoids and food intake are severely disturbed. Critically ill patients are often treated with glucocorticoids at times other than early morning and receive continuous enteral or parenteral nutrition. This will likely severely disturb the clock in white adipose tissue and other tissues (Ikeda et al. 2015; Su et al. 2016). Indeed, metabolomic profiles in Intensive Care Unit (ICU) patients show significant desynchronization and highly disordered metabolism (Lusczek et al. 2020). However, a host of additional desynchronizing influences are present in critical illness, including unregulated light dark cycles and sound levels in ICU units, poor sleep or continuous sedation, as well as the influence of pharmacological treatments and the illness itself (McKenna et al. 2018; Gao & Knauert 2019; Lusczek et al. 2020). In the acute phase it is challenging to distinguish between a beneficial response to illness that may overrule normal circadian physiology and a detrimental response, given the complex interactions between the circadian clock, metabolism and the immune system. However, only half of the patients rapidly recover from critical illness, the other half develops chronic critical illness or 'persistent inflammation, immunosuppression and catabolism syndrome' (PICS) (Hawkins et al. 2018), characterized by prolonged ICU stay, low-grade organ dysfunction and persistent dysregulation of immune function and metabolism. We hypothesize these patients could benefit from optimising daily rhythmicity to restore metabolic and immunologic homeostasis. Based on the results in this thesis and other experiments performed by our group we hypothesize that restoring a daily rhythm in food intake and providing glucocorticoids in a single bolus in the early morning could help to re-synchronize clocks in chronically critically ill patients. Furthermore, exposure to daylight and early mobilization during daylight hours will help to reinstate a daily rhythm in physical activity, whereas limiting the use of sedatives and reducing light and noise pollution at night will help to reinstate the sleep-wake cycle. So far, some small studies using chronobiological interventions in critical care medicine (reviewed in (McKenna et al. 2018)) have not found any effect. However, these studies were done in extremely heterogenous groups of patients, including those with acute stress responses to critical illness. To our knowledge, so far no studies have been done using chronobiological interventions in chronically critically ill patients specifically.

Of note, both the circadian system and energy metabolism are complex integrated systems with many interactions; therefore, generalisation of findings should be done cautiously. For example, there may be differences between species and sexes. In the studies described in this thesis we have mainly studied the clock at the gene expression level using PCR techniques measuring messenger RNA. Approximately 90% of genes bound by circadian transcription factors are expressed (Koike et al. 2012), indicating that the clock system is highly involved in the regulation of transcriptional activity. However, virtually every regulatory stage from gene expression to protein activity (e.g., transcription, splicing, termination, polyadenylation, nuclear export, microRNA regulation, translation, RNA degradation and posttranslational modification) has revealed layers of circadian control (Takahashi 2017; Mauvoisin 2019). Thus, to fully understand circadian regulation multiple stages need to be studied.

CONCLUDING REMARKS

In conclusion, the experiments described in this thesis improve our understanding of how daily gene expression rhythms in rat white adipose tissue are regulated. We found that clock gene rhythms in WAT do not fully depend on the central brain clock, although the central brain clock does contribute to phase synchronization and increases the amplitude of its daily rhythmicity. Furthermore, clock gene rhythms in WAT do not solely depend on adrenal hormones or the daily feeding rhythm, but at least one of these signals should be present to maintain WAT gene expression rhythmicity. We discussed that light could influence clock gene rhythms in WAT via multiple pathways; the SCN, central pathways not involving the SCN, via the skin or even directly at the level of the adipocyte. However, in absence of a rhythm in adrenal hormones and food intake, the light dark cycle cannot maintain rhythmicity in WAT. Furthermore, we discussed autonomous clocks may sustain a degree of tissue rhythmicity in absence of the SCN. However, in absence of both adrenal hormones and the daily rhythm in food intake, autonomous clocks in WAT cannot maintain rhythmicity for extended periods.

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Appendices

NEDERLANDSE SAMENVATTING

In dit proefschrift 'Regulatie van dagelijkse gen expressie ritmes in wit vetweefsel in de rat', bespreken we in **hoofdstukken 1 en 2** dat de biologische klok essentieel is voor het behoud van (metabole) gezondheid, doordat de biologische klok zorgt voor afstemming van metabole processen in het lichaam. De biologische klok kan verstoord raken door bijvoorbeeld werk in ploegendienst of (sociale) jetlag. Deze verstoring is gecorreleerd met een verhoogde prevalentie van obesitas en verwante metabole aandoeningen, zoals type 2-diabetes mellitus (DMII). Wit vetweefsel speelt een centrale rol in het energiemetabolisme; het zorgt voor opslag van energie die niet direct gebruikt wordt, en het produceert hormonen die bijdragen aan behoud van de energiebalans in het lichaam, bijvoorbeeld leptine. Om beter te begrijpen hoe verstoring van de biologische klok zou kunnen resulteren in obesitas en DMII, is het belangrijk om te begrijpen hoe de biologische klok in wit vetweefsel gereguleerd wordt. Onze groep heeft eerder laten zien dat de dagelijkse ritmes in genexpressie in de lever gereguleerd worden door de centrale breinklok, gelokaliseerd in de suprachiasmatische nucleus (SCN) in de hypothalamus, via het autonoom zenuwstelsel. Voor wit vetweefsel was het onbekend welke factoren verantwoordelijk zijn voor de regulatie van dagelijkse ritmes in genexpressie. Het doel van de studies in dit proefschrift was dan ook om onze kennis te vergroten over de regulatie van genexpressie ritmes in wit vetweefsel.

In het eerste experiment hebben we de dagelijkse genexpressie ritmes in vetweefsel van ratten bestudeerd onder normale omstandigheden. In **hoofdstuk 3** hebben we de ritmes van klokgenen (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, en *DBP*) en metabole genen (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *Glut4*, *HSL*, *CPT1b*, *leptine*, *visfatin/NAMPT*, en *resistin*) in verschillende vet depots vergeleken, zowel intra-abdominaal (mesenterisch white adipose tisssue; mWAT, perirenaal; pWAT, epididymaal; eWAT) als subcutaan (sWAT). We vonden robuuste ritmes voor alle klokgenen in alle vier depots. De dag/nacht ritmes in metabole genen waren minder robuust (lagere amplitudes en lagere R^2). We vonden hierbij geen verschillen tussen de depots. Deze uitgebreide vergelijking van genexpressie ritmes tussen verschillende vet depots was nog niet eerder gedaan. Omdat we geen belangrijke verschillen gevonden hebben tussen de vet depots, stellen we voor dat de genexpressie ritmes die gevonden worden in (humaan) subcutaan vetweefsel, geëxtrapoleerd kunnen worden naar intra-abdominale vetweefsels. Dit is relevant omdat het bij mensen veel minder invasief is om biopten te nemen van het subcutane vetweefsel, dan van de intra-abdominale vetweefsels.

Daarna hebben we een aantal factoren bestudeerd die zouden kunnen bijdragen aan de regulatie van genexpressie ritmes in vetweefsel. In **hoofdstuk 4** hebben we onderzocht

welke rol de SCN, de meester klok in de hersenen, speelt in de regulatie van genexpressie ritmes in vetweefsel in de rat. We hebben ratten met een bilaterale laesie van de SCN vergeleken met ratten die verder dezelfde operatie ondergaan hebben, maar waarbij de SCN intact is gelaten (sham controledieren). We hebben klokgenexpressie ritmes (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, en *DBP*) en metabole genexpressie ritmes (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, *leptine*, *adiponectine*, *visfatin/NAMPT*, en *resistin*) vergeleken in eWAT en sWAT. We vonden dat na een laesie van de SCN weliswaar de amplitude en R^2 waarden van deze ritmes waren gereduceerd met ongeveer 50%, en de piek van de genexpressie met ongeveer 4 uur vervroegd was in vergelijking met de sham controledieren, maar desondanks bleef er toch nog een significant ritme in expressie bestaan in de meeste klokgenen. Enkel *Cry2* en *DBP* verloren hun ritmische expressie in beide vetweefsels. De ritmes in metabole genen waren allemaal verdwenen na SCN-laesies, met uitzondering van *visfatin/NAMPT*. *Visfatin/NAMPT* bleef ritmisch in zowel sWAT als eWAT, met zelfs een beter behoud van amplitude dan de klokgenen en zonder verschuiving van de piek in expressie. Over het algemeen was er geen verschil in de klokgenexpressie ritmes tussen de verschillende vet depots na SCN-laesies. Deze resultaten laten dus zien dat er ondanks de afwezigheid van de centrale klok in de hersenen toch dagelijkse ritmes in expressie van klokgenen in vetweefsel blijven bestaan. Dit was een onverwacht resultaat, en roept de vraag op via welke mechanismes de ritmes in vetweefsel gereguleerd worden in afwezigheid van de centrale klok in de hersenen?

Eerder onderzoek van anderen heeft laten zien dat in andere weefsels dan vetweefsel dagelijkse ritmes in genexpressie vaak worden beïnvloed door hormonen die geproduceerd worden in de bijnieren (glucocorticoïden). Ook is bekend dat veel processen in vetweefsel beïnvloedt door deze bijnierschorshormonen. Het was echter nog niet bekend of deze bijnierschorshormonen ook een rol spelen in de regulatie van genexpressie ritmes in vetweefsel. In **hoofdstuk 5** hebben we genexpressie ritmes in vetweefsel van ratten waarbij de bijnieren operatief waren verwijderd (bilaterale adrenalectomie; ADx) vergeleken met die van ratten die een controle operatie ondergingen (sham controledieren). We hebben klokgenexpressie ritmes (*Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *RevErbα*, en *DBP*) en metabole genexpressie ritmes (*SREBP1c*, *PPARα*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, *ACC2a*, *leptine*, *adiponectine*, *visfatin/NAMPT*, *resistin*, *Lipin1, glucocorticoïd receptor en mineralocorticoïde receptor*) bestudeerd in eWAT. We vonden dat na ADx de amplitude van ritmes in klokgenexpressie in vetweefsel met 20-50% afgenomen was, maar dat het tijdstip van piekexpressie niet verschoven was. *DBP* was het minst beïnvloed van de klokgenen (-20%), *Cry2* (-50%) en *RevErbα* (-44%) het meest. Het grootste deel van de metabole genen verloor het dagelijkse ritme in genexpressie, met uitzondering van *ACC2a*, *resistin* en *visfatin/NAMPT*. Deze resultaten laten zien dat de bijnieren, en/of de effecten van de hormonen die zij produceren, belangrijk zijn voor de regulatie van

klokgenexpressie ritmes in wit vetweefsel, maar dat er ook andere factoren betrokken moeten zijn.

Verschillende studies in mensen en proefdieren hebben laten zien dat obesitas gecorreleerd is met verminderde genexpressie ritmes in vetweefsel. Het is echter onbekend of dit een causaal verband is. Veel van de obese diermodellen eten te veel (hyperfagie), en vaak is daardoor ook het dag/nacht ritme in hun eetgedrag verstoord. Dit maakt het lastig om te onderscheiden of de verstoorde ritmes in genexpressie in obees vetweefsel een gevolg zijn van de obesitas of het verstoorde eetgedrag. Neonatale toediening van mono natrium glutamaat (MSG) veroorzaakt ernstige obesitas in ratten, terwijl deze dieren minder eten dan controledieren. Daarom hebben we in **hoofdstuk 6** naar genexpressie ritmes in vetweefsel gekeken in dieren met MSG geïnduceerde obesitas. We hebben de dagelijkse ritmes in klokgenexpressie (*Bmal1*, *Per2*, *Cry1*, *Cry2*, *RevErbα* en *DBP*) en metabole genexpressie (*SREBP1c*, *PPARγ*, *FAS*, *LPL*, *HSL*, *CPT1b*, *Glut4*, en *leptine*) vergeleken in sWAT, pWAT en eWAT. We vonden een geringe reductie in *Per2* en *Cry2* expressie bij de dieren met MSG geïnduceerde obesitas, en een verminderde piekexpressie van *DBP* in sWAT en pWAT, maar over het algemeen waren de dagelijkse ritmes in klokgenexpressie onveranderd. We vonden een verhoogde expressie van leptine, *CPT1b* en *LPL*, en een verminderde expressie van *SREBP1c*, maar ook voor de metabole genen geen verstoorde ritmiek. Naast het veroorzaken van obesitas, heeft neonatale toediening van MSG ook andere effecten die potentieel de genexpressie ritmes in vetweefsel hadden kunnen beïnvloeden. Er is bijvoorbeeld gerapporteerd dat het de dagelijkse ritmes in voedsel inname en lichamelijke activiteit verstoord. Desondanks vonden wij geen verstoring van genexpressie ritmes in vetweefsel. Deze resultaten suggereren dan ook dat de verstoring van genexpressie ritmes die gezien wordt in andere obesitas modellen door andere factoren dan toename van de vetmassa verklaard moet worden.

Samenvattend hebben we in vetweefsel in de rat uitgesproken klokgenexpressie ritmes gevonden, waarbij we geen grote verschillen tussen intra-abdominale en subcutane vet depots hebben geobserveerd (**hoofdstuk 3**). Deze ritmes worden gereguleerd door meerdere factoren, want de ritmes zijn gereduceerd maar nog wel bestaand wanneer de centrale klok in de hersenen afwezig is (**hoofdstuk 4**), en ook wanneer de bijnieren afwezig zijn (**hoofdstuk 5**). Daarnaast vonden we dat obesitas in zichzelf geen vermindering van genexpressie ritmes in vetweefsel in ratten veroorzaakt (**hoofdstuk 6**).

In de **algemene discussie** bespreken we twee vragen naar aanleiding van deze resultaten. Als eerste, de centrale klok in de hersenen stuurt naast de ritmes in bijnierschorshormonen ook andere processen ritmisch aan, waaronder inname van voedsel. In hoeverre zijn deze andere processen van belang voor de ritmes in genexpressie in

vetweefsel? Door dieren 6 identieke maaltijden gelijkmatig verdeeld over 24 uur te geven kan bestudeerd worden wat de invloed is van het dagelijkse ritme in voedsel inname. In vervolgstudies heeft onze groep laten zien dat wanneer het ritme in voedsel inname afwezig is, dit slechts een gering effect heeft op de klokgenexpressie ritmes in vetweefsel. Echter, wanneer zowel het ritme in voedselinname als de bijnieren afwezig zijn, dan verdwijnen alle klokgenexpressie ritmes in vetweefsel in de rat. Dit betekent dus dat de genexpressie ritmes in vetweefsel afhankelijk zijn van de aanwezigheid van tenminste één van deze factoren.

Ten tweede bespreken we een aantal mechanismen die zouden kunnen bijdragen aan het behoud van genexpressie ritmes in vetweefsel in afwezigheid van de centrale klok in de hersenen. Nadat in 1972 bekend werd dat de centrale meester klok zich in de hersenen in de SCN bevindt, werd dit deel van de hypothalamus gezien als de drijvende factor voor het genereren en synchroniseren van alle ritmes in de rest van het lichaam. Een aantal recente studies heeft echter laten zien dat hoewel in afwezigheid van de SCN dagelijkse ritmes in perifere weefsels sterk verminderd zijn, er toch wel degelijk ook ritmes kunnen blijven bestaan.

Daglicht is de belangrijkste factor die ervoor zorgt dat het circadiane ritme dat gegenereerd wordt in de centrale klok in de hersenen gesynchroniseerd wordt met het 24-uurs licht/donker ritme in de buitenwereld. De SCN geeft dit gesynchroniseerde ritme vervolgens weer door aan de rest van het lichaam via hormonen en het autonome zenuwstelsel. Enige jaren geleden is duidelijk geworden dat de speciale lichtgevoelige, melanopsine bevattende cellen in de retina die deze lichtinformatie doorgeven aan de SCN, die informatie mogelijk ook kunnen doorgeven aan een beperkt aantal andere structuren in de hersenen. Mogelijk kunnen deze hersengebieden op die manier via het autonome zenuwstelsel ook dag/nacht informatie doorgeven aan perifere weefsels. Daarnaast worden er in vetweefsel in ratten ook lichtgevoelige fotopigmenten gevonden (melanopsine), wat zou kunnen betekenen dat licht direct invloed zou kunnen hebben op vetweefsel. Het is echter onduidelijk hoe groot deze effecten zijn in kunstmatig licht, dat een sterk gereduceerde lichtintensiteit heeft in vergelijking met daglicht. Er zijn echter ook een paar publicaties waarin de ritmes in genexpressie in vetweefsel ook blijven bestaan wanneer de dieren zonder SCN in constant donker worden gehouden, wat suggereert dat ook andere factoren dan de licht/donker cyclus van belang zijn.

Opvallend in onze SCN-laesie experimenten was dat het ritme in *visfatin/NAMPT* expressie maar zeer gering beïnvloed werd door de afwezigheid van de centrale breinklok. *Visfatin/NAMPT* is een essentieel onderdeel van het basale energiemetabolisme in een cel, en wordt onder andere gereguleerd door klokgenen, maar bijvoorbeeld ook door

oxidatieve stress in de cel. Mogelijk was de sterk afgenomen ritmiek van de klokgenen in vetweefsel nog net voldoende om het ritme in *visfatin/NAMPT* in stand te houden. Er komen echter steeds meer aanwijzingen dat processen die betrokken zijn bij cellulair metabolisme ook een regulerende invloed hebben op de biologische klok in de cel. Er zijn zelfs cellen waarin het genetische klok systeem geheel ontbreekt, zoals rode bloedcellen, die desondanks ritmiek laten zien in eiwitten die betrokken zijn in het basale energiemetabolisme in de cel. Het is nog onbekend waardoor deze basale processen ritmisch blijven, en of zij ook ritmes in vetweefsel zouden kunnen genereren.

Een derde mechanisme waardoor de genexpressie ritmes in vetweefsel zou kunnen blijven bestaan in afwezigheid van de centrale klok in de hersenen, is dat er autonome feedback loops zijn tussen cellen in het vetweefsel, of tussen perifere weefsels. Cellen in de SCN zijn sterk aan elkaar gekoppeld, veel sterker dan cellen in perifere weefsels, waardoor de SCN-cellen buiten het lichaam (bijvoorbeeld in celkweek) hun ritme vrijwel ongewijzigd kunnen behouden. In een recent review wordt echter voorgesteld dat in afwezigheid van de SCN, perifere cellen en weefsels voldoende aan elkaar gekoppeld kunnen zijn om enige ritmiek te behouden. Mogelijk dat deze perifere koppeling in aanwezigheid van de SCN slechts een kleine bijdrage levert, maar dat deze bijdrage groter wordt wanneer de SCN afwezig is.

Samenvattend suggereert de discussie van onze bevindingen dat:

1. Klokgenexpressie ritmes in vetweefsel niet volledig afhankelijk zijn van de centrale klok in de hersenen, maar dat de SCN wel belangrijk is voor de synchronisatie en amplitude van deze ritmes.
2. Klokgenexpressie ritmes in vetweefsel niet volledig afhankelijk zijn van de aanwezigheid van de bijnieren of een ritme in voedselinname, maar tenminste één van deze twee signalen wel aanwezig moet zijn om dagelijkse ritmes in vetweefsel te behouden.
3. Daglicht klokgenexpressie ritmes kan beïnvloeden via de centrale klok in de hersenen, via andere structuren in de hersenen, of direct via fotopigmenten in vetcellen. Echter, in afwezigheid van een ritme in voedselinname én de bijnieren kan is de aanwezigheid van een licht/donker cyclus onvoldoende om de ritmes in vetweefsel in stand te houden.
4. Autonome klokken een bepaalde mate van ritmiek in stand kunnen houden in afwezigheid van de centrale klok in de hersenen. Echter, in afwezigheid van een ritme in voedselinname én de bijnieren zijn deze autonome klokken niet in staat om de ritmes in vetweefsel in stand te houden.

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AUTHOR AFFILIATIONS

Buijs, Ruud
Hypothalamic Integration Mechanisms, Department of Physiology, Instituto de Investigaciones Biomedicas, UNAM, Mexico

Endert, Erik †
Laboratory of Endocrinology, Department of Clinical Chemistry, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

Fliers, Eric
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

Foppen, Ewout
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Kalsbeek, Andries
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

Laboratory of Endocrinology, Department of Clinical Chemistry, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Kreier, Felix
Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Kwakkel, Joan
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

La Fleur, Susanne
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

La Fleur Group, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Lei, Jun
Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Pei, Lei
Department of Neurobiology, Tongji Medical College, The Institute for Brain Research, Huazhong University of Science and Technology, Wuhan, Hubei province, China

Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

Su, Yan
Kalsbeek Group, Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands

van der Spek, Rianne
Department of Endocrinology and Metabolism, Amsterdam UMC, location AMC, University of Amsterdam, The Netherlands

PHD PORTFOLIO

Name of PhD student: Rianne van der Spek

PhD period: 2009-2022

Names of PhD supervisors and co-supervisors: prof A Kalsbeek, prof SE la Fleur, prof E Fliers

PhD training	Year	ECTS
General Courses		
BROK herregistratie	2015	0.1
Scientific Writing in English for Publication	2011	1.5
Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK)	2011	0.9
Practical Biostatistics	2010	1.1
AMC World of Science	2009	0.7
Proefdierkunde	2009	3.9
Stralingsbescherming, deskundigheidsniveau 5B	2008	1.7
Seminars, workshops, master classes		
Trainee Professional Development Day, SRBR conference	2012	0.25
Rat surgical training, dr Shi, Miami University, Oxford, Ohio, USA	2012	0.25
Trainee Professional Development Day, SRBR conference	2010	0.25
Masterclass with prof Hogenesh	2010	0.25
Weekly research meeting Endocrinology and Metabolism	2009-2012	4.0
Oral Presentations		
Hypothalamic and circadian control of adipose tissue, Annual Dutch Diabetes Research Meeting, Oosterbeek, Netherlands	2012	0.5
Hypothalamic and circadian control of adipose tissue, XII Congress of the European Biological Rhythms Society, Oxford, UK	2012	0.5
Increased fat mass per se does not alter gene expression rhythms in rat WAT, NASO symposium, Zwolle, Netherlands	2011	0.5
Food intake regulates clock gene expression in white adipose tissue, Nutrition, Metabolism and the Brain, Groningen, Netherlands	2011	0.5
Regulation of circadian gene expression in rat WAT, Endo-neuro-psycho Meeting, Lunteren, Netherlands	2011	0.5
Regulation of circadian gene expression in rat WAT, Clock Club Meeting, Guildford, United Kingdom	2011	0.5
Regulation of circadian gene expression in WAT, Dutch Center for Timing Research, Drachten, Netherlands	2010	0.5
Regulation of circadian gene expression in WAT, Annual Dutch Diabetes Research Meeting, Oosterbeek, Netherlands	2010	0.5
Poster Presentations		
Increased Fat Mass Per Se Does Not Alter Gene Expression Rhythms in Rat WAT, 13th meeting Society for Research on Biological Rhythms, Destin, Florida, USA	2012	0.5
Increased fat mass does not disturb gene expression rhythms in rat WAT, XII Congress of the European Biological Rhythms Society, Oxford, UK	2011	0.5
Food intake regulates clock gene expression rhythms in rat perirenal WAT, European Society of Endocrinology, Rotterdam, NL	2011	0.5
Food intake regulates clock gene expression rhythms in rat perirenal WAT, Nutrition, Metabolism and the Brain, Groningen, NL	2011	0.5
Rhythmic expression of clock genes and metabolic genes in rat WAT, 12th meeting Society for Research on Biological Rhythms, Destin, Florida, USA	2009	0.5

(Inter)national Conferences		
13th meeting Society for Research on Biological Rhythms, Destin, Florida, USA	2012	1.0
Annual Dutch Diabetes Research Meeting, Oosterbeek, Netherlands	2012	0.5
NASO symposium, Amsterdam, Netherlands	2012	0.25
Dutch Center for Timing Research, Rotterdam, Netherlands	2012	0.25
XII Congress of the European Biological Rhythms Society, Oxford, UK	2011	1.75
Annual Dutch Diabetes Research Meeting, Oosterbeek, Netherlands	2011	0.5
Endo-neuro-psycho Meeting, Lunteren, Netherlands	2011	0.75
European Society of Endocrinology, Rotterdam, Netherlands	2011	1.25
Nutrition, Metabolism and the Brain, Groningen, Netherlands	2011	0.75
NASO symposium, Zwolle, Netherlands	2011	0.25
Clock Club Meeting, Guildford, United Kingdom	2011	0.25
Dutch Center for Timing Research, Groningen, Netherlands	2011	0.25
Dutch Endocrine Meeting, Noordwijkerhout, Netherlands	2010	0.5
12th meeting Society for Research on Biological Rhythms, Destin, Florida, USA	2010	1.0
Dutch Center for Timing Research, Drachten, Netherlands	2010	0.25
NASO symposium, Utrecht, Netherlands	2010	0.25
Clock Club, Stevenage, United Kingdom	2010	0.25
Novel insights in adipose cell functions, IPSEN, Paris, France	2009	0.25
Annual Dutch Diabetes Research Meeting, Oosterbeek, Netherlands	2009	0.5
Other		
Session moderator Endo-neuro-psycho Meeting	2011	0.25
Teaching and Supervising		
MSc thesis, Jeffrey Schaap (4 months)	2012	1.3
MSc thesis, Elisa Gritsch (8 months)	2011	2.7
Student supervisor Mastercourse Endocrinology, UvA	2010	0.25
Research internship, Laura Kervezee (2 months)	2009	0.7
Parameters of Esteem		
Travel grant Spinoza Foundation	2011	

LIST OF PUBLICATIONS

IN THIS THESIS

van der Spek R, Pei L, La Fleur S, Kalsbeek A. Obesity induced by neonatal monosodium glutamate administration does not disturb gene expression rhythmicity in rat white adipose tissue. *Submitted*.

van der Spek R, Foppen E, Fliers E, La Fleur S, Kalsbeek A. Thermal lesions of the SCN do not abolish all gene expression rhythms in rat white adipose tissue, NAMPT remains rhythmic. *Chronobiol Int*. 2021 jun;1:1-13

van der Spek R, Fliers E, la Fleur SE, Kalsbeek A. Daily Gene Expression Rhythms in Rat White Adipose Tissue Do Not Differ Between Subcutaneous and Intra-Abdominal Depots. *Front Endocrinol.* 2018;9:206.

Su Y, **van der Spek R**, Foppen E, Kwakkel J, Fliers E, Kalsbeek A. Effects of adrenalectomy on daily gene expression rhythms in the rat suprachiasmatic and paraventricular hypothalamic nuclei and in white adipose tissue. *Chronobiol Int.* 2015;32:211-224.

Kalsbeek A, **van der Spek R**, Lei J, Endert E, Buijs RM, Fliers E. Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. *Mol Cell Endocrinol.* 2012;349:20-29.

van der Spek R, Kreier F, Fliers E, Kalsbeek A. Circadian rhythms in white adipose tissue. *Prog Brain Res.* 2012;199:183-201.

OTHER

van der Spek R, van den Goorbergh T, Mijné G, Buijs E, Spronk P. Flipping the GABA switch: a proposed mechanism for adverse paradoxical agitated reactions to midazolam. *Submitted.*

Hillebrandt D, Gurtoo A, Küpper T, Richards P, Schöffl V, Shah P, **van der Spek R**, Wallis N, Milledge J. UIAA Medical Commission Recommendations for Mountaineers, Hillwalkers, Trekkers, and Rock and Ice Climbers with Diabetes. *High Alt Med Biol.* 2018;00:1-17.

Tannheimer M, **van der Spek R**, Lechner R, Steinacker J, Treff G. Reply to Comment 'Nocturnal decrease of arterial oxygen content-hidden stimulus for erythropoietin secretion at altitude by Böning et al. on Oxygen saturation increases over the course of the night in mountaineers at high altitude (3050m-6354 m) by Tannheimer et al.' *J Travel Med.* 2018;25:(1)93.

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Donegani E, Paal P, Küpper T, Hefti U, Basnyat B, Carceller A, Bouzat P, **van der Spek R**, Hillebrandt D. Drug Use and Misuse in the Mountains: A UIAA MedCom Consensus Guide for Medical Professionals. *High Alt Med Biol.* 2016;17:157-184.

Tannheimer M, Angelini C, Faulhaber M, Hillebrandt D, Szygula Z, Ulmer H, Usaj A**, van der Spek R**, Küpper T. Results and Recommendations of the 7th European Hypoxia Symposium 2014. *Medicina Sportiva* 2014;18:192-194. Ergebnisse und Empfehlungen des 7th European Hypoxia Symposiums 2014. *Dtsch Z Sportmed.* 2015;66:78-80.

Oosterman JE, Foppen E, **van der Spek R**, Fliers E, Kalsbeek A, la Fleur SE. Timing of fat and liquid sugar intake alters substrate oxidation and food efficiency in male Wistar rats. *Chronobiol Int.* 2015;32:289-298.

Coomans CP, van den Berg SAA, Lucassen EA, Houben T, Pronk ACM, **van der Spek RD**, Kalsbeek A, Biermasz NR, Willems van Dijk K, Romijn JA, Meijer JH. The suprachiasmatic nucleus controls circadian energy metabolism and hepatic insulin sensitivity. *Diabetes.* 2013;62:1102-1108.

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