Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men

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Context: Shift workers are at increased risk of metabolic morbidities. Clock genes are known to regulate metabolic processes in peripheral tissues, e.g. glucose oxidation.

Objective: To investigate how clock genes are affected at the epigenetic and transcriptional level in peripheral human tissues following acute total sleep deprivation (TSD), mimicking shift work with extended wakefulness.

Intervention: In a randomized, 2-period, 2-condition, crossover clinical study, fifteen healthy men underwent two experimental sessions: one-night sleep (2230–0700h) and overnight wakefulness. On the subsequent morning, serum cortisol was measured, followed by skeletal muscle and subcutaneous adipose tissue biopsies for DNA methylation and gene expression analyses of core clock genes (*BMAL1, CLOCK, CRY1, PER1*). Finally, baseline and 2-hr-post oral glucose load plasma glucose concentrations were determined.

Main Outcome Measures: In adipose tissue, acute sleep deprivation vs. sleep increased methylation in the promoter of *CRY1* (+4%, P=0.026) and in two promoter-interacting enhancer regions of *PER1* (+15%, P=0.036; +9%, P=0.026). In skeletal muscle, TSD vs. sleep decreased gene expression of *BMAL1* (-18%, P=0.033) and *CRY1* (-22%, P=0.025). Concentrations of serum cortisol, which can reset peripheral tissue clocks, were decreased (2448 ± 932 vs. 3177 ± 723 nmol/L, P=0.023), whereas postprandial plasma glucose concentrations were elevated after TSD (7.77 ± 0.42 vs. 6.59 ± 1.32 mmol/L, P=0.011).

Conclusions: Our findings demonstrate that a single night of wakefulness can alter the epigenetic and transcriptional profile of core circadian clock genes in key metabolic tissues. Tissue-specific clock alterations could explain why shift work may disrupt metabolic integrity as observed herein.

Trial Registration: clinicaltrials.gov Identifier: NCT01800253.

Animals studies have convincingly demonstrated that the circadian clock allows gene expression to coincide with anticipated metabolic requirements throughout day/night variations via *CLOCK* and *BMAL1* as positive transcriptional regulators and *PERIOD* and *CRYPTOCHROME* as negative transcriptional regulators (1). The lack of clock genes, even when ablated only in skeletal muscle or adipose tissue (2, 3), results in systemic meta-

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bolic perturbations in animal models (4). These metabolic responses include hyperglycemia and insulin resistance, and can also result in obesity and type-2 diabetes in animals (3–5). As reviewed in (6, 7), similar metabolic phenotypes have been observed in humans subjected to experimental paradigms mimicking night shift work, comprising reduced energy expenditure, impaired systemic glucose disposal, and increased food intake. Over time, these conditions may thus result in metabolic dysregulation and weight gain (6, 7). While shortened sleep leads to genome-wide changes in the leukocyte transcriptome comprising clock genes (8), the influence of overnight wakefulness, as it occurs in night shift work, on the circadian machinery in tissues critically involved in whole-body energy homeostasis is however unknown. The importance of this research is highlighted by the fact that today, at least 15% of the workforce – numbering 15 million in the U.S. alone – carry out shift work, with job activities scheduled during the biological night.

With this background, we characterized the effects of one night of sleep deprivation on gene expression and DNA methylation of core circadian clock genes in peripheral tissues. DNA methylation of gene promoters and promoter-interacting enhancers is one epigenetic mechanism involved in the control of gene expression (9) and is a malleable process following acute lifestyle interventions (10). We obtained subcutaneous adipose tissue and skeletal muscle biopsies from fasted healthy young men following both acute sleep deprivation and normal sleep. In addition, fasting serum cortisol and plasma glucose were measured, the latter before and 120 minutes after an oral glucose tolerance test (OGTT).

Materials and Methods

Study Design

This randomized crossover within-subject trial was conducted from March through September 2013 at Uppsala Biomedical Centre, Uppsala University, Sweden. The sessions were relatively evenly distributed across the study period. Study procedures and written consent forms were approved by the Regional Ethical Review Board in Uppsala (EPN 2012/477). The study was conducted in accordance with the Helsinki Declaration. Each enrolled participant voluntarily signed the consent form.

Participants

Sixteen out of 17 enrolled subjects participated in two sessions of this study. Participants were of self-reported good health, free from chronic medical conditions or chronic medication, nonsmokers and had normal sleeping habits (7–9 hours of sleep/night; Pittsburgh Sleep Quality Index score ≤ 5) (extended screening protocol in **Supplement part 1**).

Study Protocol and Interventions

All 16 participants engaged in two conditions (acute sleep deprivation vs. sleep), in which each condition was separated by at least 4 weeks. Participants came in a semifasted state (fasted since 1500h) to the lab two evenings before each session's final experimental morning, and remained in the laboratory, under constant supervision until the end of the experimental session (ie, approximately a 36-hour laboratory stay).

Participants were provided with breakfast, lunch and dinner during their 24-hour baseline period (each meal providing 1/3 of the participants' individually calculated energy requirements; based on the Harris-Benedict equation factored 1.2 for light physical activity), and had an 8.5-hour sleep opportunity during the first night (2230–0700h). During the first baseline day, participants were provided with two standardized and supervised 15-minute walks. During nonexperimental time periods, participants were confined to their rooms but were free to engage in sedentary-level activities.

Randomization to the first experimental condition (sleep or acute sleep deprivation) was generated by drawing lots, with a fixed block size of 2 and allocation ratio of 1:1. Participants were randomized after having been screened by J.C. as eligible, and were scheduled in pairs for the next available session slot. The allocation sequence was only known by one of the researchers (CB) but was concealed from the participants, with the experimenters only notified two weeks in advance of each new session for experimental preparation. Participants were blinded to the experimental condition (sleep or acute sleep deprivation) until 90 minutes in advance of onset of the nighttime intervention, which took place during the second night (2230–0700h). During this period in the sleep condition, room lights were kept off and sleep was monitored. Contrarily, in the sleep deprivation condition, participants were under constant supervision 2230–0700h to ensure wakefulness, remaining bed-restricted and fasted.

Blood Sampling, Biopsy Collection and Oral Glucose Tolerance Test

After fasting, blood samples were obtained at 0730h. Tissue biopsies were also obtained in the fasted state, 2–3 hours after subject wake-up time, with the collection of the adipose tissue preceding that of the skeletal muscle. Following the biopsy collection, participants completed a 75g OGTT (further details provided in the Supplement).

DNA Extraction and Epigenetic Analyses

DNA extraction and epigenetic analysis with Illumina's HumanMethylation450 BeadChip are further described in **Supplement part 1**.

DNA methylation preprocessing consisted of probe filtering (removal of probes with missing β-values; probes with $< 75\%$ of samples with detection p-value < 0.01; or nonspecific or SNP-coinciding probes), followed by adjustment of type I and type II probes using BMIQ, and removal of batch effects using ComBat. We ran four pairs of technical replicates, including at least one from each experimental condition (sleep and acute sleep deprivation), to estimate the inner variability of each probe. We only considered for further analysis the probes for which at least half of the subjects showed a methylation difference between conditions greater than the mean difference in technical replicates.

CpG sites within 1500 bp of the TSS of *CLOCK*, *ARNTL*, *CRY1* and *PER1* were analyzed (15 CpG sites for adipose tissue

and 9 for skeletal muscle). The promoter is a key part of a gene, but enhancers also prominently contribute to the regulation of gene expression (11). In order to identify putative enhancers of *CLOCK*, *ARNTL*, *CRY1* and *PER1*, we inferred chromatin states in adipose nuclei and skeletal muscle, and mapped long-range interactions in five different cells lines, with three different transcription factors (12). CpG sites located in chromatin states indicative of enhancers in adipose nuclei and skeletal muscle and in regions having long-range interactions with the promoters of *CLOCK*, *ARNTL*, *CRY1* and *PER1*, were also analyzed (6 CpG sites for adipose tissue and 4 for skeletal muscle).

Methylation levels are presented as β-values (ranging from zero to one, corresponding to zero and 100% methylation, respectively). P-values were adjusted for multiple testing according to the Benjamini-Hochberg (BH) method within each tissue (13).

RNA Extraction and Quantitative PCR Analysis of Gene Expression

Methods used for RNA extraction and quantitative PCR (qPCR) analysis of gene expression are described in further detail in **Supplement part 1.** The gene expression of *CLOCK*, *ARNTL*, *CRY1* and *PER1* was analyzed with qPCR in adipose tissue and skeletal muscle. All analyses were run in duplicates (primer information in **part 2 of the Supplement**). The ΔCt method was used to normalize data (14).

Statistics

Normal-distribution criteria of analyzed data were assessed with Kolmogorov-Smirnov's test of normality. Normally distributed data was analyzed with paired student t-tests, whereas non-normally distributed variables were analyzed with Wilcoxon signed-rank test. Methylation data was analyzed using the software package R (version 3.1); we used the $\log_2$ ratio of the intensities of methylated probe vs unmethylated probe, also called M-value, which is more statistically valid for the differential analysis of methylation levels (15). All other data was analyzed using the software SPSS (version 21, SPSS Inc., Chicago, Ill., USA) and are presented as means ± SD. Two-sided p-values below 0.05 were considered significant. For the adipose tissue, one individual was excluded for all gene analyses (expression values greater than mean + 2 SD for several genes). The significance values were however not changed when the analysis was run with or without this subject (data not shown). For *PER1* in skeletal muscle, an outlier was excluded from both conditions (expression values in the sleep deprivation condition greater than mean + 2 SD), but significance values were not altered when the analysis was run with or without this subject (data not shown).

Results

Out of 17 enrolled subjects, 16 completed participation in both sessions (sleep and acute sleep deprivation). One participant was excluded from later analysis due to insufficient sleep (<7h) in the sleep condition. Fifteen participants were therefore included in the final analysis (age 22.3 ± 1.9 years; BMI 22.6 ± 1.8 kg/m^2). Sleep data are presented in **part 3 of the Supplement.**

Effect of Acute Sleep Deprivation on Methylation and Expression of Circadian Genes in Adipose Tissue and Skeletal Muscle

Methylation levels at cg19308989 (+15%, adjusted p-value = 0.036) and cg20193872 (+9%, adjusted p-value = 0.026; both CpG sites located in enhancers interacting with the promoter of *PER1*), and at cg04674060 (located in the promoter of *CRY1;* +4%, adjusted p-value = 0.026), increased after acute sleep deprivation, as compared with the sleep condition, in adipose tissue (shown in **Figure 1**). In skeletal muscle, the investigated CpG sites were not altered (detailed probe results in **part 4 of the Supplementary**).

In skeletal muscle, mRNA expression of *BMAL1* and *CRY1* was decreased following acute sleep deprivation (-18 and –22% compared to expression levels found after sleep; $P = .033$ and $P = .025$, respectively; see **Figure 2** and **part 4 of the Supplement**). Skeletal muscle *CLOCK* or *PER1* gene expression was unaltered. Moreover, the adipose tissue genes were unaltered following acute sleep deprivation.

Effect of Acute Sleep Deprivation on Fasting Cortisol and Glucose Tolerance

Following acute sleep deprivation, fasting serum cortisol concentrations were decreased at 0730h (2448 ± 932 vs. 3177 ± 723 nmol/L, $P = .023$), compared with after sleep. Plasma glucose concentrations at 120 minutes post-OGTT were higher following acute sleep deprivation, compared with the values obtained after sleep (*Pre-OGTT*: 5.36 ± 0.30 vs. 5.38 ± 0.36 mmol/l, $P = .705$; *Post-OGTT*: 7.77 ± 0.42 vs. 6.59 ± 1.32 mmol/l, $P = .011$).

Discussion

We determined the effect of one night of wakefulness, as occurs during night shift work, on DNA methylation and mRNA expression of key circadian genes (ie, *BMAL1*, *CLOCK, CRY1*, and *PER1*) in human skeletal muscle and adipose tissue. We provide evidence that acute sleep deprivation increases promoter methylation and reduces transcription of circadian genes in a tissue-specific manner. Our analysis reveals increased methylation of transcription-regulating regions of *PER1* and *CRY1* in adipose tissue and reduced gene expression of *CRY1* and *BMAL1* in skeletal muscle. We also observed an impaired glucose response following an OGTT after acute sleep deprivation. Our results suggest that acute sleep loss alters clock gene regulation, concomitant with deleterious met-

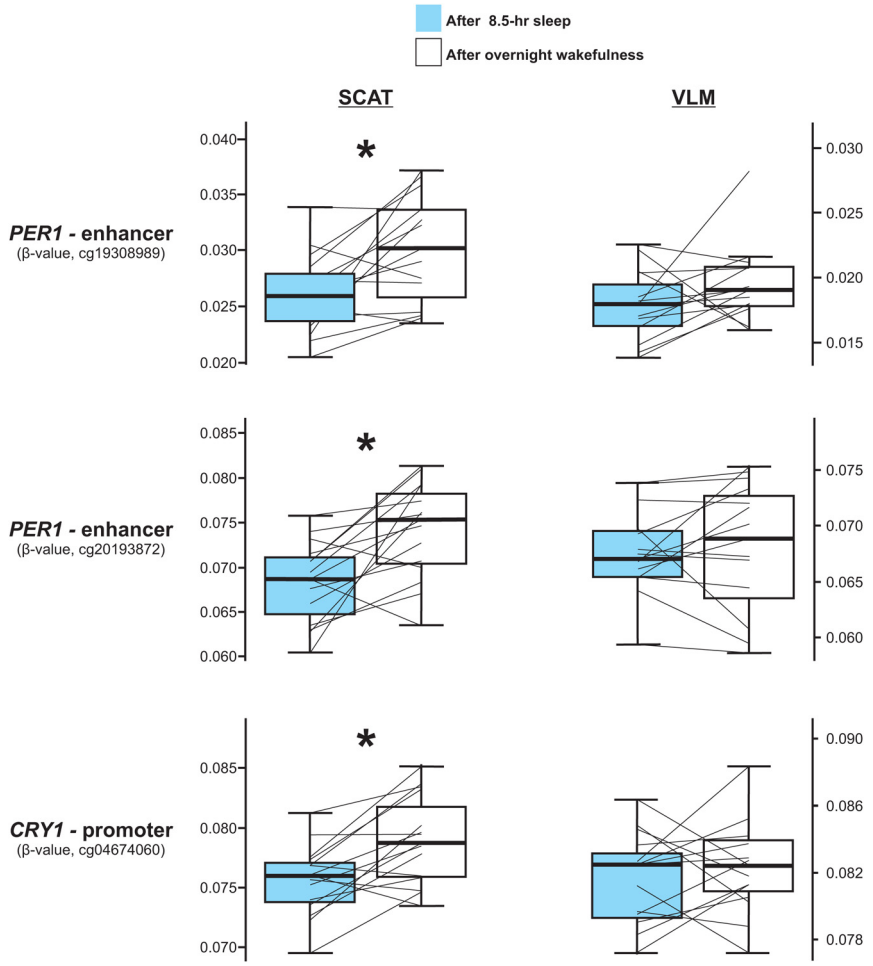

Figure 1. Methylation levels after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. Methylation levels in two putative enhancers interacting with the promoter of *PER1* (probes cg04674060 and cg19308989) were increased following overnight wakefulness (ie, acute sleep deprivation), in adipose tissue (SCAT), as compared with after sleep. Methylation levels in the promoter of *CRY1* (probe cg20193872) were also increased following overnight wakefulness (acute sleep deprivation), as compared with after sleep, in adipose tissue (SCAT). No differences were however seen between the two conditions in skeletal muscle (VLM). Methylation levels are shown as beta values (β-value; ranging from zero to one, corresponding to zero and 100% methylation, respectively). Horizontal line represents median, box interquartile range, 'whiskers' represent spread of remaining values. Two points that are linked by a line show the difference in methylation levels in overnight wakefulness vs. sleep conditions for each individual. *, $P < .05$, n = 15 for all analyses. Abbreviations: SCAT, subcutaneous adipose tissue; VLM, *vastus lateralis* muscle.

abolic effects, which are differential, rather than uniform across key peripheral metabolic tissues in healthy humans.

Our results of altered DNA methylation for promoter and promoter-interacting enhancer regions of core clock genes in adipose tissue suggest that acute sleep deprivation can cause acute epigenetic remodeling of the circadian clock. Similar acute epigenetic changes occur following other types of physiological or metabolic interventions, including acute high-intensity exercise (10). We provide additional evidence that challenges the conventional view that epigenetic regulation is largely a mitotically stable process resistant to the impact of environmental factors. Hypermethylation of core clock genes in humans is linked to insulin resistance in humans (16), and this has also partially been observed in blood of people who chronically work shifts (17). Given that the circadian clock impacts key metabolic processes (1), our results suggests that sleep loss-induced hypermethylation of *PER1* and *CRY1* in adipose tissue may be contribute to glucose intolerance as measured by the 120-minute post-OGTT glucose value.

We found that mRNA expression of the core clock genes *BMAL1* and *CRY1* was decreased in skeletal muscle following acute sleep deprivation. Similar changes occur in circulating leukocytes following longer periods of shortened sleep in humans (8). Skeletal muscle-specific deletion of *Bmal1*, or global deficiency of *Cry1*, impairs insulin sensitivity and glucose metabolism in mouse models (2, 18). Moreover, clock gene expression is altered in peripheral blood cells from type-2 diabetic vs nondiabetic patients (19), with an inverse correlation between clock gene expression (*BMAL1*, *PER1* and *PER3*) and HbA(1c) level noted. Thus, our observed transcriptional changes in circadian clock genes in skeletal muscle in response to acute sleep deprivation may impair glucose tolerance.

Although the design of our study did not allow us to ascertain the molecular cause of the observed epigenetic and transcriptional changes in skeletal muscle and adipose tissue following sleep loss, several putative candidate mechanisms can be implicated. For instance, while glucocorticoid levels may be slightly elevated during nocturnal wakefulness (20), glucocorticoids – as also shown in our study – are reduced during typical awakening hours (eg, between 0700 and 0800h) (21). Glucocorticoids reset circadian rhythms of peripheral circadian clocks (22). Thus, resetting of peripheral circadian clocks may be hampered by a blunted cortisol awakening response after acute sleep deprivation.

At both the epigenetic and transcriptional level, we demonstrate tissue-specific alterations in core clock genes

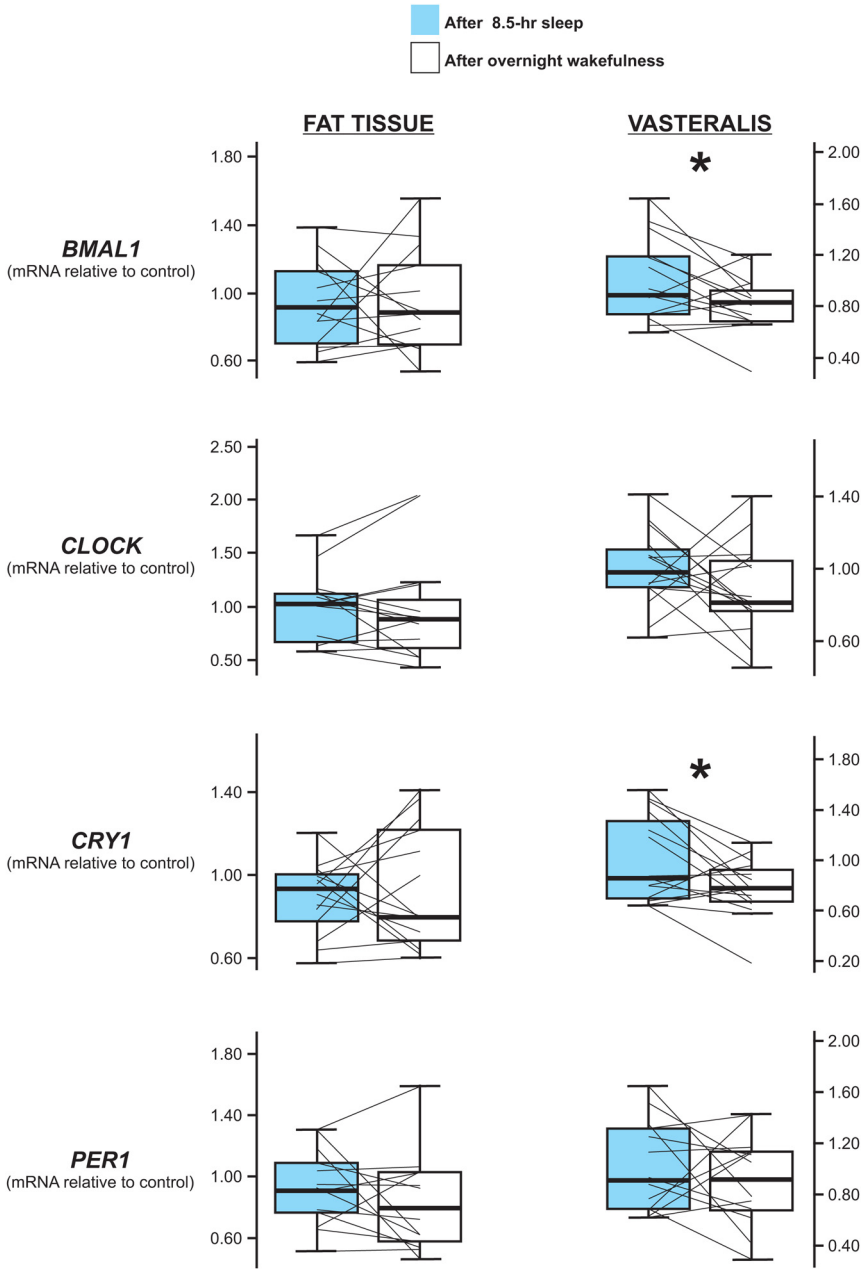

Figure 2. mRNA expression of core clock genes after sleep and acute sleep deprivation in adipose tissue and skeletal muscle. mRNA expression of *BMAL1* and *CRY1* was downregulated in skeletal muscle (VLM) from humans following overnight wakefulness (ie, acute sleep deprivation) compared with after sleep. No differences between the two conditions were found for the other genes or in the adipose tissue (SCAT). Values are shown as expression levels relative to the control condition (sleep). Horizontal line represents median, box interquartile range, 'whiskers' represent spread of remaining values. Two points that are linked by a line show the difference in mRNA expression in overnight wakefulness vs. sleep conditions for each individual. *, $P < .05$; n = 14 for all analyses in adipose tissue; n = 15 for all analyses in skeletal muscle except for *PER1* (see main text for descriptions of excluded values). Abbreviations: SCAT, subcutaneous adipose tissue; VLM, *vastus lateralis* muscle.

under conditions of acute sleep deprivation, consistent with animal studies, in which the circadian machinery exhibits tissue-specific rhythms following shift-work-mimicking sleep loss paradigms (23). Such internal desynchrony has been hypothesized to underlie metabolic effects of shift work (24–26). The physiological relevance of tissue-specific circadian clocks is further supported by aforementioned and other animal studies in which core clock genes have been ablated or rescued in a tissue-specific manner (3, 27), eg, an adipose tissue-targeting *Bmal1* deletion in mice resulting in an obese phenotype (3). Furthermore, insulin-dependent peripheral tissues, ie, adipose tissue and skeletal muscle, shift towards a diabetes-like phenotype following sleep loss (28, 29). Our tissue-differential effects further reinforce the notion that acute circadian misalignment can produce desynchrony of peripheral circadian clocks, with possibly tissue-specific downstream metabolic effects.

Limitations

Several limitations should be kept in mind when interpreting our results. Lights were on in the TSD condition but not in the sleep condition (~300 lux vs darkness). Since light can entrain the human circadian clock (30), our experimental design does not allow us to disentangle if the observed effects of overnight wakefulness on core circadian genes were either driven by loss of sleep, light exposure, or both. However, it is important to note that our experiment aimed at mimicking night shift work, which is typically performed under ambient light exposure. Another limitation of our study is that expression and methylation of clock genes was measured only at a single time point, ie, under fasting conditions in the morning following each sleep intervention. Thus, our study does not allow firm conclusions on how the circadian pattern of expression and methylation pattern of clock genes if influenced by overnight wakefulness. This would however have required repeated tissue sampling, which is far more feasible utilizing animal models; models that can also be maintained longer in a fasted state to avoid the entraining effect of meals on clock genes. Finally, hypermethylation of transcription-regulatory re-

gions of core circadian genes in the adipose tissue were not paralleled by concomitant reduced expression of these genes. Given that we only sampled biopsies at one time point, a possible explanation might be that acute promoter hypermethylation altered circadian gene mRNA expression at subsequent points in the sleep deprivation condition, ie, following biopsy collection. Supporting this assumption, a separate study examined the effects of an acute bout of exercise on skeletal muscle promoter methylation and corresponding gene expression (10). There, remodeling of promoter methylation of *PGC-1α*, a gene involved in the circadian machinery of the skeletal muscle (31), was accompanied by a delayed, but not concomitant (ie, 3 hrs later) change in gene expression (10).

Conclusions

One night of sleep loss results in hypermethylation of regulatory regions of key clock genes. These effects are tissue-specific, and occur in adipose tissue, but not in skeletal muscle. Gene expression differences were observed for the investigated clock genes in skeletal muscle, but not in adipose tissue. Shift work is associated with many of the same phenotypes observed in transgenic animal models in which the circadian clock is disrupted, eg, glucose intolerance (32–34). This suggests that our findings of peripheral clocks at the epigenetic and transcriptional level following acute sleep loss may contribute to metabolic disruptions typically observed in humans with activities regularly scheduled during times that produce chronic desynchrony between tissue-specific clocks.

Perspectives

Since recurrent partial sleep deprivation decreases insulin sensitivity at the systemic and adipose tissue level in humans (28, 35), future studies to examine whether similar changes occur under conditions of recurrent partial sleep deprivation are of interest. Using repeated biopsy collection, eg, also under insulin-stimulated conditions, may decipher the time-dependent dynamics of peripheral circadian misalignment and how this might relate to metabolic perturbations, including impaired glucose tolerance. Whether our findings can be extrapolated to females or older participants is currently unknown and warrants investigation. Large interindividual differences were observed in our data for how sleep deprivation altered peripheral tissues' clock gene methylation and gene expression. Contributing factors may be subjects' chronotype – linked to differential responses' to sleep deprivation – or seasonality; recent studies demonstrating seasonal circadian clock gene variability in animals as well as humans (36), supported by summer-winter variation in human adipose tissue. Ambient light can influence circadian rhythms (37). Thus, light can also resynchronize peripheral circadian rhythms in the absence of a functioning central pacemaker (38); and enhance the cortisol awakening response (39). The mechanism by which different ambient light exposures influences the peripheral clock under conditions of extended wakefulness remains to be investigated. Finally, although the absence of nighttime meals in our sleep deprivation condition precludes the synchronizing influence from such a zeitgeber on peripheral clocks (40), nighttime meal intake is common in shift workers and may thereby modulate effects on tissue-specific circadian clocks.

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Author Contributions: Dr. Cedernaes had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. JC and CB designed the study; JC and CB wrote the protocol; JC and JEB collected the data; JC, SV, JEB, HV, and CB conducted the analyses; JC, MO, SV, JEB, HV, SD, JZ, HS, and CB interpreted the data; and all authors contributed to writing. All authors have approved the final manuscript.

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