Effect of Acute Total Sleep Deprivation on Plasma Melatonin, Cortisol and Metabolite Rhythms in Females

Aya Honma¹, Victoria L. Revell², Pippa J. Gunn¹,³, Sarah K. Davies¹,⁴, Benita Middleton¹, Florence I. Raynaud⁵ and Debra J. Skene¹

1. Chronobiology, Faculty of Health and Medical Sciences, University of Surrey, Guildford GU2 7XH, UK
2. Surrey Clinical Research Centre, University of Surrey, Guildford GU2 7XP, UK
3. Radcliffe Department of Medicine, University of Oxford, Oxford OX3 9DU, UK
4. Department of Surgery and Cancer, Imperial College London, London, SW7 2AZ, UK
5. Cancer Research UK, Cancer Therapeutics Unit, Division of Cancer Therapeutics, The Institute of Cancer Research, London SM2 5NG, UK

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ejn.14411

This article is protected by copyright. All rights reserved.
Abstract

Disruption to sleep and circadian rhythms can impact on metabolism. The study aimed to investigate the effect of acute sleep deprivation on plasma melatonin, cortisol and metabolites, to increase understanding of the metabolic pathways involved in sleep/wake regulation processes. Twelve healthy young female subjects remained in controlled laboratory conditions for ~92 h with respect to posture, meals and environment light (18:00-23:00 h and 07:00-09:00 h <8 lux; 23:00-07:00 h 0 lux (sleep opportunity) or <8 lux (continuous wakefulness); 09:00-18:00 h ~ 90 lux). Regular blood samples were collected for 70 h for plasma melatonin and cortisol, and targeted liquid chromatography-mass spectrometry metabolomics. Timepoints between 00:00 and 06:00 h for day 1 (baseline sleep), day 2 (sleep deprivation) and day 3 (recovery sleep) were analysed. Cosinor analysis and MetaCycle analysis were performed for detection of rhythmicity. Night time melatonin
levels were significantly increased during sleep deprivation and returned to baseline levels during recovery sleep. No significant differences were observed in cortisol levels. Of 130 plasma metabolites quantified, 41 metabolites were significantly altered across the study nights, with the majority decreasing during sleep deprivation, most notably phosphatidylcholines. In cosinor analysis, 58 metabolites maintained their rhythmicity across the study days, with the majority showing a phase advance during acute sleep deprivation. This observation differs to that previously reported for males. Our study is the first of metabolic profiling in females during sleep deprivation and recovery sleep, and offers a novel view of human sleep/wake regulation and sex differences.

Introduction

Sleep comprises approximately one-third of our lifetime and is an essential physiological function indispensable for survival. In our 24/7 lifestyle of modern society sleep duration has been significantly reduced and more people experience insufficient sleep (Deng et al., 2017). Sleep deprivation has been shown to impact metabolism and immune systems, leading to increased incidence of obesity, impaired glucose tolerance, metabolic syndrome and cardiovascular disease (Spiegel et al., 2005; Scheer et al., 2009; Huang et al., 2011; Johnston, 2014).

Circadian clocks control the timing of most daily biological processes, behaviour and activity, including changes in metabolism and the sleep/wake cycle (Hastings et al., 2003; Mohawk et al., 2012). The master clock, located in the hypothalamic suprachiasmatic nuclei (SCN), is entrained to the external light-dark cycle by photic signals transmitted via the retinohypothalamic tract, and coordinates timing of peripheral clocks distributed throughout the body (Reppert & Weaver, 2002; Barclay et al., 2012). Melatonin and cortisol show robust circadian rhythms driven by the SCN oscillator (Czeisler et al., 1999; Gunn et al., 2016), and melatonin is considered a reliable marker of the master clock (Klerman et al., 2002). In
entrained conditions, melatonin levels rise in the evening hours, peak in the early hours of the morning, and return to basal levels soon after waking (Arendt, 1988).

Liquid chromatography–mass spectrometry (LC/MS) metabolomics analysis has been employed widely to identify and quantify hundreds of metabolites in complex biological matrices such as blood, urine and saliva. Robust circadian rhythms in rodent (Minami et al., 2009; Eckel-Mahan et al., 2012) and human metabolites have been identified under constant routine conditions (Dallmann et al., 2012; Kasukawa et al., 2012; Skene et al., 2018).

Dallmann et al. (2012) reported that 15% of the metabolites quantified in human plasma and saliva showed circadian variation, particularly amino acids in saliva and fatty acids in plasma. Previously, we reported a significant time of day variation in 64% of the metabolites measured in healthy young men kept under a controlled light/dark conditions (Davies et al., 2014). In spite of numerous studies on sex differences in metabolic profiling (Mittelstrass et al., 2011; Ruoppolo et al., 2014; Rist et al., 2017), surprisingly little is known about the effect of sleep deprivation on metabolite profiles in females. As the composition of metabolites is different between males and females (Pitkanen et al., 2003; Mittelstrass et al., 2011; Ruoppolo et al., 2014), the sex-based differences in metabolite rhythms are of great interest. The aim of this study was to assess the timing, amplitude and phase relationship of the SCN-driven hormone rhythms (melatonin and cortisol) and plasma metabolite rhythms with a sleep/wake cycle, during 24 h of wakefulness and a recovery sleep in healthy young females. Assessing the effect of acute sleep deprivation on circulating metabolites and their rhythms may lead to increased understanding of the metabolic pathways involved in sleep/wake regulation and development of sleep biomarkers.
Materials and Methods

**Clinical study.** Laboratory sessions were conducted at the Surrey Clinical Research Centre (SCRC) at the University of Surrey. Ethical approval for the study was given by the University of Surrey Ethics Committee. All participants provided written informed consent prior to any procedures being performed and participants were allowed to withdraw at any time. All participant information was coded and held in strictest confidence according to the Data Protection Act (UK, 1998). The initial screening phase including eligibility criteria has been reported previously (Ackermann et al., 2012). Briefly, study eligibility was determined by questionnaires including Pittsburgh Sleep Quality Index (PSQI), Epworth Sleepiness scale (ESS), Horne-Östberg (H-O) and Beck Depression Index (BDI), medical and physical assessment, and analysis of blood and urine samples. The participants did not report sleep problems (PSQI score ≤ 5), depression (BDI < 10), daytime sleepiness (ESS < 11) and were not extreme morning or evening types (30 < H-O < 70).

Healthy females (*n* = 12; aged: 25 ± 4 yrs, mean ± SD) with a BMI of 24.9 ± 3.6 kg/m² (mean ± SD) were enrolled into the study. All were non-smokers and unmedicated except that they were taking combined oral contraceptives and were on the active phase during the laboratory session. None had a history of shift work or travel across two or more time zones in the preceding month. Prior to the in-laboratory session, participants maintained a regular sleep/wake schedule (23:00 – 07:00 h) at home for 10 days (confirmed by wrist actigraphy (Actiwatch-L, Cambridge Neurotechnology Ltd., UK) and sleep diaries). They were also required to have exposure to outdoor light each morning for at least 15 min between 07:00 and 08:30 h. For 72 h before the in-laboratory session, participants were requested to abstain from alcohol, caffeine and strenuous exercise. This baseline-at-home period minimised exogenous confounding factors, stabilised circadian phase and ensured participants were not sleep deprived prior to the study.
**In-Laboratory Session.** Participants entered the laboratory at 16:00 h on day 0 and remained until 12:00 h on day 4 in a controlled environment with regards to environmental light, sleep, and meals, with an adaptation night (day 0) followed by an 8 h sleep opportunity (23:00 – 07:00 h) on day 1 (baseline sleep), total sleep deprivation on day 2 (sleep deprivation), and an 8 h recovery sleep opportunity on day 3 (recovery sleep). Participants remained in <8 lux between 18:00 and 23:00 h, and between 07:00 and 09:00 h each day. On days 0, 1 and 3 participants slept between 23:00 and 07:00 h (0 lux), and remained awake during this time in <8 lux on day 2. Between 09:00 and 18:00 h each day participants were free to move about in normal room lighting (~ 90 lux). The same standardised meals were given on each day of the study at 07:00, 13:00, and 19:00 h with a snack at 22:00 h. Blood samples were collected for 70 h at 2 hourly intervals for metabolomics analysis and at 1 – 2 hourly intervals for hormone assays (12:00 h on day 1 – 10:00 h on day 4). Plasma fractions for metabolomics analysis were stored at -80°C until derivatisation, plasma for hormone assays were stored at -20°C until analysis. A detailed scheme of the study protocol is shown in Supplementary Figure S1.

**Sample Analysis.** Plasma melatonin and cortisol concentrations were measured by radioimmunoassay (Stockgrand Ltd., University of Surrey) as described previously (Sletten et al., 2009). Targeted liquid chromatography-mass spectrometry (LC/MS) was performed on two-hourly plasma samples to identify and quantify metabolite concentrations, using the AbsoluteIDQ p180 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria), and a Waters Xevo TQ-S mass spectrometer coupled to an Acquity UPLC system (Waters Corporation, Milford, MA, USA) as previously described (Davies et al., 2014; Isherwood et al., 2017; Skene et al., 2017). The kit provides absolute concentrations of 184 metabolites from 6 different compound classes (acylcarnitines, amino acids, biogenic amines, lysosphatidylcholines, glycerophospholipids and sphingolipids). Plasma samples were prepared according to the manufacturer’s instructions. Sample order was randomised.

This article is protected by copyright. All rights reserved.
and three levels of quality controls (QC), run on each 96-well plate. Data were normalised between batches using the quality control level 2 (QC2) repeats across each plate (n = 4) and between plates (n = 5) using Biocrates METIDQ software (QC2 correction). Metabolites where >25% concentrations were below the limit of detection (<LOD) or below lower limit of quantification (<LLOQ) or above limit of quantification (>LOQ) or blank out of range, or the QC2 coefficient of variance was > 30%, were excluded (n = 54).

**Data Analysis.** Dim light melatonin onset (DLMO) for separate study days was calculated for each participant using the 25% threshold method as previously described (Sletten et al., 2009). Night time melatonin levels (00:00 – 06:00 h) were compared between the study nights using two-way repeated ANOVA (R version 6.3.2). Significant p-values were adjusted for multiple comparisons according to the Benjamini-Hochberg false discovery rate (FDR) (Benjamini & Hochberg, 1995). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA-P v.13.0 software (Umetrics, Malmo, Sweden) to visualise the overall variation and components clustering between study days. To examine metabolite levels during the night time sleep periods (00:00 – 06:00 h), a two-way repeated ANOVA was carried out (R6.3.2). To assess 24 h metabolite rhythmicity, cosinor analysis was performed on the mean z-score values of each study day (12:00 – 10:00) for each metabolite profile using MATLAB and Bioinformatics Toolbox Release 2016a (The MathWorks, Inc., Natick, Massachusetts, USA). Peak time (acrophase), amplitude, and significance of a cosine fit (p < 0.05) were determined for each metabolite. Rhythmicity was also assessed using MetaCycle software (Glynn et al., 2006; Hughes et al., 2010) with R6.3.2 which provided acrophase, amplitude, relative amplitude value (FDR adjusted p-value < 0.05). All data are shown as mean ± SEM.
RESULTS

**Hormone rhythms.** Night-time melatonin levels (00:00 – 06:00 h) showed a significant increase during sleep deprivation relative to baseline sleep (19 ± 7%), these levels declining back to baseline during recovery sleep (day 1 sleep, 84.8 ± 8.0 pg/ml; day 2 sleep deprivation, 102.1 ± 5.6 pg/ml; day 3 recovery sleep, 81.5 ± 4.2 pg/ml; FDR adjusted p < 0.001; Figure 1A). On day 1 the DLMO was 21:56 ± 0:14 h and this did not change significantly between study days (day 2, 22:02 ± 0:14 h; day 3, 21:54 ± 0:11 h). No significant differences were observed in the peak night time cortisol levels between the study days (day 1, 268 ± 62 nmol/L; day 2, 309 ± 67 nmol/L; day 3, 287 ± 51 nmol/L; Figure 1B).

**Multivariate analysis of metabolites.** A total of 130 metabolites were detected by targeted LC/MS metabolomics, grouped into amino acids (n = 19), acylcarnitines (n = 10), biogenic amines (n = 7), glycerophospholipids (n = 80) and sphingolipids (n = 14). Principal component analysis (PCA) of all the data showed there was clear time-of-day variation in principal component (PC)1 (amount of variance in the x matrix explained by PC1 (R²) = 0.367, estimate of the predictive ability of the model (Q²) (cumulative) = 0.358) with the mean score on PC1 having a significant fit to a cosine curve across the study days (acrophase: 15:31 h, 14:04 h, 15:20 h; amplitude: 2.14, 2.18, 1.99, day 1, 2, 3, respectively) (Figure 2). OPLS-DA models, validated by permutation analysis and a CV ANOVA p value of the model, showed clear separation between sleep (00:00 – 06:00 h, day 1) and sleep deprivation (00:00–06:00 h, day 2); Q² (cumulative) = 0.641, R²X (cumulative) = 0.707, R²Y (cumulative) = 0.903 (Figure 3A), between sleep (00:00–06:00 h, day 1) and recovery sleep (00:00–06:00 h, day 3); Q² (cumulative) = 0.608, R²X (cumulative) = 0.601, R²Y (cumulative) = 0.790, and between sleep deprivation (00:00–06:00 h, day 2) and recovery sleep (00:00–06:00 h, day 3) (Figure 3B); Q² (cumulative) = 0.439, R²X (cumulative) = 0.592, R²Y (cumulative) = 0.722 (Supplementary Figure S2A). The p(corr) loading plot for the OPLS-DA models are shown in
Figure 3C, D and Supplementary Figure S2B; metabolites with negative values decreased in sleep deprivation and metabolites with positive values increased in sleep deprivation. Plasma levels of histidine, glutamate, glutamine, lysine, carnitine, symmetric dimethylarginine (SDMA) and 6 glycerophospholipids (lysoPC a C18:0, PC aa C36:5, PC aa C40:3, PC aa C42:2, PC ae C36:3, and PC ae C42:2) decreased during sleep deprivation and tetradecenoylcarnitine, taurine, methionine levels increased during sleep deprivation (|p(corr)| >0.15) (Supplementary Table S1).

**Univariate analysis of metabolites.** Of the 130 metabolites, 41 (32%) metabolites showed significant differences in their night time concentrations (0:00 – 6:00 h) in the study days (FDR adjusted p < 0.05); 12 (of 19) amino acids (threonine, glycine, alanine, glutamate, glutamine, histidine, lysine, asparagine, ornithine, methionine, arginine and proline), carnitine, 2 biogenic amines (taurine, SDMA), 25 (of 80) glycerophospholipids (lysoPC a C18:0, PC aa C36:1, PC aa C36:5, PC aa C38:0, PC aa C38:5, PC aa C40:2, PC aa C40:3, PC aa C40:4, PC aa C40:5, PC aa C40:6, PC aa C42:2, PC aa C42:6, PC ae C34:2, PC ae C34:3, PC ae C36:0, PC ae C36:3, PC ae C36:4, PC ae C36:5, PC ae C38:0, PC ae C38:5, PC ae C38:6, PC ae C40:1, PC ae C42:1, PC ae C42:2 and PC ae C42:4) and 1 (of 14) sphingolipid (SM C20:2) (Supplementary Table S2). Only 15 out of 130 metabolites (12%) were significantly different between the sleep and sleep-deprivation periods, 5 amino acids (histidine, glutamate, threonine, lysine, citrulline), carnitine, SDMA, lysoPC a C18:0 and 7 phosphatidylcholines. Except threonine, all of these metabolites exhibited decreased levels during sleep deprivation compared to baseline sleep. Of these, 7 metabolites showed a significant difference between sleep deprivation and recovery sleep; histidine, lysine, citrulline, carnitine and SDMA returned to baseline levels, 2 phosphatidylcholines (PC aa C36:5 and PC ae C42:2) showed a further decrease and threonine showed a further increase during recovery sleep (Supplementary Table S3).
Daily rhythms in metabolites. To assess rhythmic variation of the metabolites, cosinor analysis of the mean z-score metabolite profiles on each study day was performed. Of the 130 metabolites, 33 (25%) metabolites showed no rhythmicity across any of the study days (Figure 4A). Of the 97 remaining metabolites, significant daily rhythms were observed in 78 (60%) metabolites; 3 amino acids (asparagine, glutamate, glycine), valeryl-carnitine, 4 biogenic amines (alpha-Amino acid (alpha-AAA), kynurenine, SDMA, trans-4-Hydroxyproline (t4-OH-Pro)), 60 glycerophospholipids and 10 sphingolipids, with most metabolites (n = 63; 81%) peaking during the day (06:00–18:00 h). Of these, 62 (79%) metabolites maintained their rhythmicity during sleep deprivation, with 58 (74%) maintaining their rhythmicity across all 3 study days. Metabolites (n = 58) showing daily rhythms across all 3 study days included 2 amino acids (asparagine, glutamate), one acylcarnitine (valeryl-carnitine), 2 biogenic amines (SDMA, t4-OH-Pro), 46 glycerophospholipids and 7 sphingolipids (Figure 4B). Of these, the majority (n = 56; 97%), except valerylarnitine and SDMA, showed an advanced acrophase during sleep deprivation (-1.6 ± 0.1 h), with the majority (n = 49; 88%) subsequently returning to baseline timing during recovery sleep (Supplementary Table S4). Metabolite data were also analysed with MetaCycle (JTK-CYCLE). Using this analysis significant daily rhythms were observed in only 18% (n=23) of metabolites on day 1 (normal sleep/wake); namely 2 amino acids (glutamate, glycine), 2 biogenic amines (SDMA, t4-OH-Pro), and 19 glycerophospholipids. Of these, 12 (52%) metabolites maintained rhythmicity on day 2 (sleep deprivation) and only 8 (35%) metabolites maintained rhythmicity across the 3 study days; t4-OH-Pro and 7 glycerophospholipids (Supplementary Figure S3, Supplementary Table S5).
Sex differences

In our previous study diurnal rhythms in plasma metabolites in young males were also observed (Davies et al., 2014). Reanalyzing these data using the same criteria as for the female data as described in the Methods above (Isherwood et al., 2017; Skene et al., 2017), 141 metabolites were quantified. Of these, 77 (55%) exhibited a daily rhythm that had a significant fit to a cosine curve on day 1 (sleep) with most (n = 53; 69%) maintaining their 24 h rhythmicity on day 2 (sleep deprivation). Of these, the majority (n = 45; 85%) showed a delay in acrophase time during sleep deprivation (1.4 ± 0.2 h) (Supplementary Table S6). The mean score on PC1 had a significant fit to a cosine curve on day 1 with an acrophase of 14:47 h, but no significant fit on day 2 (15:50 h, p=0.053) (Supplementary Figure S4). In a subset analysis of both sexes, there were 32 common metabolites that exhibited diurnal rhythms on day 1 and day 2. The mean acrophase time of these metabolites was significantly later in the female group on day 1 (15:48 ± 0:40 h) compared to the male group (14:53 ± 0:42 h, p<0.001, Student’s T-test), and earlier on day 2 (14:29 ± 0:38 h) compared to males (16:52 ± 0:38 h, p<0.001, Student’s T-test) (Figure 5, Supplementary Table S7), although no significant difference in DLMO (female: 21:56 ± 0:14 h, male: 21:58 ± 0:31 h) was observed. Metabolites that were only rhythmic on day 1 and day 2 in females (n = 13) and only rhythmic in males (n = 15) are also presented in Supplementary Table S7.

Sex differences in the metabolites affected by sleep deprivation were also observed (Supplementary Table S8). In females, 15 (12%) of the 130 metabolites were significantly altered during sleep deprivation, all but threonine showing decreased levels. By contrast, in the males 37 out of 141 metabolites (26%) exhibited significant changes during sleep deprivation, all increasing during sleep deprivation.

This article is protected by copyright. All rights reserved.
DISCUSSION

This study aimed to assess the effect of a single night of total sleep deprivation followed by recovery sleep under controlled laboratory conditions on both biomarkers of the central circadian pacemaker and plasma metabolic profiles in young females.

The pineal hormone melatonin showed significantly increased plasma concentrations during sleep deprivation, which is in agreement with our previous study in young males (Ackermann et al., 2013; Davies et al., 2014), and returned to baseline levels during a subsequent night of recovery sleep. Although the mechanism underlying this increase is not known, we previously showed induction of a heat shock protein coding gene HSPA1B expression during sleep deprivation (Ackermann et al., 2012), suggesting a direct consequence of the oxidative stress induced by sleep loss, which may lead to up-regulation of melatonin, a well-known antioxidant (Reiter et al., 2000; Carrillo-Vico et al., 2005).

Increased melatonin levels during sleep deprivation might also reflect increased adrenergic stimulation (Klein & Weller, 1973; Skene et al., 1994). Sleep deprivation may also act directly on SCN neuronal activity reducing SCN output (Deboer et al., 2007) that, in turn, may activate melatonin synthesis via the SCN-pineal multi-synaptic pathway. Whilst we assume that the increased plasma levels arise from the pineal gland, there is also the possibility that the melatonin is derived from the gastrointestinal tract (GIT). The amount of melatonin in the GIT is estimated to be more than 400 times that of the pineal gland (Huether, 1993). There is some evidence that food deprivation and fasting increases melatonin levels in the brain and GIT of mice (Bubenik et al., 1992) and in the serum of humans (Beitins et al., 1985). It can be speculated that the increased energy expenditure and fasting during sleep deprivation may induce melatonin release from the GIT into the circulation.
In our study, only 15 metabolites levels were significantly different between the sleep and sleep-deprivation periods: histidine, glutamate, threonine, lysine, citrulline, carnitine, SDMA, lysoPC a C18:0 and 7 phosphatidylcholines. Apart from threonine, all of these metabolites exhibited decreased levels during sleep deprivation. Both during the sleep period and during sleep deprivation, the participants were in a fasting state. Thus the metabolites that changed during sleep deprivation were not a direct effect of fasting. However, the interaction of increased energy consumption during the sleep deprivation period and the fasting state may contribute to the observed metabolite differences.

L-Histidine is an essential amino acid that is a precursor to histamine. Being able to cross the blood brain barrier (Hargreaves & Pardridge, 1988), histidine levels in the blood may reflect brain histamine levels and histaminergic neuronal activity in the tuberomammillary nucleus, where they play a critical role in the maintenance of arousal (Saper et al., 2010). The observed reduction of blood histidine levels during sleep deprivation may reflect its increased degradation into histamine as an acute response to sleep loss, returning to baseline levels during recovery sleep. Glutamate is the most abundant excitatory neurotransmitter in the vertebrate nervous system (Meldrum, 2000) and also serves as the precursor for the synthesis of the inhibitory gamma-aminobutyric acid (GABA) in GABAergic neurons, which plays an important role in the sleep promoting systems in the brain (Sherin et al., 1996; Gallopin et al., 2000). Glutamine is the most abundant free amino acid in blood, making a large contribution to cellular respiration as an energy source following glucose and lactate (Hui et al., 2017). Glutamate and glutamine play important roles in removing excess nitrogen, detoxifying ammonia in the skeletal muscle, brain, kidney and liver (Adeva et al., 2012). In our study, plasma glutamate decreased during sleep deprivation and did not return to baseline levels during recovery sleep. Glutamine also showed a trend to decrease during sleep deprivation, returning to baseline levels during recovery sleep. These results can be attributed to increased energy demands and increased need for removal of metabolic waste products from the brain during sleep deprivation (Xie et al., 2013). Diurnal changes in brain
glutamate and glutamine levels were recently observed in healthy young adults with a significant overnight reduction assessed by proton magnetic resonance spectroscopy (Volk et al., 2018). In addition, a previous study reported reduced glutamine levels associated with lower total sleep time and increased wake-time after sleep onset (Miller et al., 2017), supporting our hypothesis.

Carnitine plays an important role in energy production by conjugating fatty acids for transport into the mitochondria, by forming a long chain acetylcarnitine ester (Rebouche, 2004). Several reports have indicated a role for fatty acid β-oxidation and the carnitine system in sleep/wake regulation. However, the role of carnitine and acylcarnitine in the brain remains unknown. Fasting in systemic carnitine deficient juvenile visceral steatosis (jvs⁻/⁻) mice exhibited a higher frequency of fragmented wakefulness and rapid-eye movement (REM) sleep, and reduced locomotor activity (Yoshida et al., 2006). In these mice, a lower percentage of c-Fos positive orexin neurons, which regulate the sleep/wake cycle and food intake, and reduced orexin-A concentration in the cerebrospinal fluid was observed (Yoshida et al., 2006). It has been reported that supplementation of acetylcarnitine produces releasable glutamate (Toth et al., 1993; Tanaka et al., 2003). Orexin producing cells in the lateral hypothalamus are mainly innervated by excitatory neurons containing glutamate (Li et al., 2002; Horvath & Gao, 2005). These findings indicate that acylcarnitine availability is essential for normal sleep/wake regulation and orexin cell functions. Thus, our results suggest increased acylcarnitine consumption during sleep deprivation to maintain wakefulness via the orexinergic arousal system.

Previous studies have indicated that cholesterol/lipid metabolism is regulated by the sleep/wake cycle and that sleep deprivation may modify it (Jones et al., 2008; Moller-Levet et al., 2013). Detailed characterisation of lipid profiles, however, has remained unclear. In our previous study using LC/MS analysis, increases in blood glycerophospholipids levels were observed during the acute sleep deprivation period (Davies et al., 2014). Elevated phospholipids were also reported after sleep deprivation in the rat and human
Another study with MS-based lipidomic analysis showed increased levels of lipids following sleep restriction (five nights of 4 h/night in bed) compared to controls (8 h in bed) (Aho et al., 2016). These authors reported that sleep loss decreased the expression of genes encoding cholesterol transporters by activating inflammatory responses (Aho et al., 2016). Toll-like receptors suppress the activity of liver X receptor (LXR) regulating cholesterol metabolism (Castrillo et al., 2003; Choi et al., 2015), which leads to decreased reverse cholesterol transport and synthesis of fatty acids and triglycerides (Joseph et al., 2003; Lee & Tontonoz, 2015). These findings suggest that sleep deprivation/restriction modifies cholesterol pathways at the level of gene expression and serum lipoproteins.

SDMA is reported to be an endogenous marker of renal function (Kielstein et al., 2006) and is negatively associated with nitric oxide (NO) production (Bode-Boger et al., 2006). As NO is reported to regulate the sleep/wake state via inactivation of orexin neurons (Cespuglio et al., 2012; Yamakawa et al., 2018), a reduction in plasma SDMA could be related to the disruption of sleep homeostasis during the study.

Previous studies have suggested that amino acid and fatty acid metabolism is under circadian control (Blanco et al., 2007; Bray & Young, 2011; Huang et al., 2011). Reverse-erb alpha (REV-ERβ), a circadian transcription gene encoding the nuclear orphan receptor, regulates hepatic gluconeogenesis, adipocyte differentiation, and lipid metabolism (Duez & Staels, 2008). REV-ERβ also controls oscillations in sterol regulatory element-binding protein (SREBP) activity, through modulation of insulin-induced gene 2, a resident protein of the endoplasmic reticulum, and thereby in the daily expression of SREBP target genes involved in cholesterol and lipid metabolism (Le Martelot et al., 2009). These authors reported that alteration of oxysterol synthesis and LXR activity mediated the effects of REV-ERβ on bile acid metabolism.
Metabolomics and lipidomics studies have analysed plasma samples from participants kept under constant routine laboratory conditions and found that 15% of all identified metabolites, most notably fatty acids (Dallmann et al., 2012) and 13% of lipid metabolites (Chua et al., 2013) were under circadian clock control suggesting a direct effect of circadian timing on fatty acid metabolism. We ourselves have seen 33% rhythmic glycerophospholipids under constant routine conditions following a simulated day shift (Skene et al., 2018) supporting this hypothesis. In the current study, of the 130 metabolites identified, the largest group of compounds exhibiting diurnal variation across the 3 study days, was lipids (n=46 with cosinor analysis and n=7 with MetaCycle analysis), implying a robust circadian timing system underlying the daily rhythms in lipid metabolites. The peak phases of rhythmic phosphatidylcholines and sphingolipids mostly occurred in the day time (15:35 ± 0:14 h, n = 60) which is consistent with previous reports (Ang et al., 2012; Dallmann et al., 2012; Davies et al., 2014). Interestingly, most metabolites (n=56, 97% with cosinor analysis and n=6, 75% with MetaCycle analysis) maintaining rhythmicity across the 3 study days showed a tendency to phase advance during the 24 h of wakefulness, most notably the glycerophospholipids, with the majority returning to baseline timings during the subsequent recovery sleep. Besides the circadian timing system, the behavioural state (e.g. feeding/fasting, sleep/wakefulness, postural changes) may affect the 24 h rhythmicity of metabolites. Currently we are unable to say which of these behaviours contribute most to the observed metabolite rhythmicity. Further systematic experiments are needed to accurately assess the contribution of each.

Recently, sleep restriction was reported to affect circadian rhythmicity of the rodent and human transcriptome (Barclay et al., 2012; Moller-Levet et al., 2013). Employing transcriptome analysis of human blood, Möller-Levet et al. (2013) reported that the number of genes whose transcripts had circadian expression were reduced in sleep restriction. Moreover, during the sleep restriction condition, those genes with peak times during the biological night had later peak times, and genes with peak times in the biological day had
earlier peak times. The peak phases of rhythmic metabolites likely relate to the physiologic pathways in which they are involved. For example, the fat clock promotes lipogenesis and adiponectin production during the wake period (Bass & Takahashi, 2010), which is consistent with our data of most phospholipids peaking during the day.

We performed two distinct analyses for assessing the daily rhythmicity of metabolites: cosinor analysis and MetaCycle. The methods showed large differences in the number of rhythmic metabolites, one possible reason being that MetaCycle performed FDR correction of the p-values. Thus, the data analysed using MetaCycle were more strictly selected, comprising a subset of metabolites which also showed statistical significance with cosinor analysis.

To our knowledge, this is the first report of metabolic profiling during acute total sleep deprivation and recovery sleep conditions in females. Taking data from our previous study of healthy young males (Davies et al., 2014) and reanalysing with the same criteria as the current female study, 37 out of 141 (26%) detected metabolites exhibited significant changes during sleep deprivation compared with during sleep, including tryptophan, serotonin, taurine, 6 acyl carnitines, 24 glycerophospholipids and 4 sphingolipids. All of these metabolites exhibited increased levels during sleep deprivation. By contrast, in the current female study, only 12% (n = 15) of the 130 identified metabolites exhibited significant changes and most of these (n = 14, 93%) exhibited decreased levels during sleep deprivation. Although the same study protocol, research facilities and analytical methodology was used, the differences between the two studies are striking. Although previous studies have suggested that men and women, and male and female rodents respond differently to sleep deprivation (Acheson et al., 2007; Ferrara et al., 2015; Baratta et al., 2018), until now its effect on circulating metabolite profiles have not been investigated.

Sex differences in plasma metabolites have been reported with most studies showing higher levels of amino acids and acyl carnitine in males compared to females.
(Pitkanen et al., 2003; Mittelstrass et al., 2011; Ruoppolo et al., 2014). In addition, the use of oral contraceptives, as used in the current study, induces specific alterations in the serum metabolic status (Ruoppolo et al., 2014). The levels of phosphatidylcholines and sphingolipids show a tendency to be higher in females, supporting an increase in lipid storage rather than metabolism in females (Mittelstrass et al., 2011; Rist et al., 2017).

Sleep disruption can affect peripheral clocks, where each tissue keeps metabolic processes in synchrony (Bass & Takahashi, 2010). As the utilization of energy and metabolic rate are different between genders (Arciero et al., 1993; Volpi et al., 1998), the timing of metabolite rhythms and their alternation during sleep deprivation may vary between the sexes.

DLMO has been reported to be significantly earlier in females than males (Mongrain et al., 2004; Cain et al., 2010; Van Reen et al., 2013), but there was no difference observed in our study. Gunn et al. (2016) demonstrated females exhibited significantly greater levels of plasma melatonin and cortisol than males during a constant routine protocol. Our previous male study also demonstrated increased night time melatonin levels during sleep deprivation (Davies et al., 2014), but the increase was greater in males than females (27 ± 5%, 19 ± 7%, respectively), suggesting a sex difference in reaction to sleep loss/wakefulness. In addition, alteration in blood melatonin levels can affect metabolic profiles (Dubocovich & Markowska, 2005; Bass & Takahashi, 2010), which may relate to different trends (increase/decrease) in metabolite concentrations during sleep deprivation between males and females. Female rodents are reported to have shorter circadian periods than males (Davis et al., 1983; Schull et al., 1989), and in a human study under laboratory conditions to detect intrinsic circadian period (Duffy et al., 2011) females indeed had a shorter tau. Our data showed the mean acrophase of common metabolites occurred significantly later in females, which then advanced to earlier than that of males during sleep deprivation. This tendency was also observed in the acrophase of the mean score on PC1 of all
metabolites; with an advance during sleep deprivation in females but a delay in males (Figure 2 and Supplementary Figure S4).

In the current study the effect of the menstrual cycle was controlled for by only including women on hormonal contraceptives. In follow up studies it will be important to investigate how these metabolites vary across the menstrual cycle (follicular and luteal phases) and whether this is affected by sleep deprivation.

In conclusion, in this first report of metabolic profiling during sleep deprivation and recovery sleep in females, we have identified a panel of plasma metabolites that are significantly altered during acute sleep deprivation and recovery sleep. There appear to be some differences between males and females in the response to sleep deprivation but, as yet, the underlying basis of these cannot be fully explained. These findings will be useful in guiding the design and interpretation of future metabolite-based studies from the point of sex differences.

ACKNOWLEDGEMENTS

This work was funded by a UK Biotechnology and Biological Sciences Research Council (BBSRC) Grant (BB/I019405/1). Melatonin and cortisol measurements were carried out by Stockgrand Ltd. The staff of the Surrey Clinical Research Centre (SCRC) and the Metabolomics Core Facility at the University of Surrey in particular Chris Mitchell, is acknowledged. The authors also thank Dr Anne Skeldon (Department of Mathematics, University of Surrey) and Dr Jeroen Pennings (National Institute for Public Health and the Environment, Bilthoven, The Netherlands) for assistance and writing the analysis scripts for MatLab and R, respectively.

This article is protected by copyright. All rights reserved.
Competing Interests

Debra J. Skene and Benita Middleton are codirectors of Stockgrand Ltd.

Author Contributions

Aya Honma: data analysis, writing MS
Victoria L. Revell: experimental design, data collection, reviewing MS
Pippa J. Gunn: data analysis, reviewing MS
Sarah K. Davies: data collection, data analysis, reviewing MS
Benita Middleton: data analysis, reviewing MS
Florence I. Raynaud: experimental design, data analysis, reviewing MS
Debra J. Skene: experimental design, data analysis, writing MS

Data Accessibility

The datasets analysed during the current study are available from the corresponding author (at d.skene@surrey.ac.uk) on reasonable request.

Abbreviations

alpha-Aminoadipic acid: alpha-AAA
BDI: Beck Depression Index
BMI: body mass index

This article is protected by copyright. All rights reserved.
DLMO: dim light melatonin onset

ESS: Epworth Sleepiness scale

FDR: false discovery rate

GABA: gamma-aminobutyric acid

GIT: gastrointestinal tract

LC/MS: Liquid chromatography–mass spectrometry

LLOQ: lower limit of quantification

LOD: limit of detection

LOQ: limit of quantification

LXR: liver X receptor

lysoPC: lysophosphatidylcholine

NO: nitric oxide

OPLS-DA: orthogonal partial least squares discriminant analysis

PC: principal component

PCA: principal component analysis

PC aa: diacylphosphatidylcholine

PC ae: acyl-alkyl-phosphatidylcholine

PSQI: Pittsburgh Sleep Quality Index

QC: quality controls

REM: rapid-eye movement
REV-ERBα: reverse-erb alpha

SCN: suprachiasmatic nuclei

SDMA: symmetric dimethylarginine

SREBP: sterol regulatory element-binding proteinβ

t4-OH-Pro: trans-4-Hydroxyproline

References

Acheson, A., Richards, J.B. & de Wit, H. (2007) Effects of sleep deprivation on impulsive behaviors in men and women. Physiol Behav, 91, 579-587.

Ackermann, K., Plomp, R., Lao, O., Middleton, B., Revell, V.L., Skene, D.J. & Kayser, M. (2013) Effect of sleep deprivation on rhythms of clock gene expression and melatonin in humans. Chronobiol Int, 30, 901-909.

Ackermann, K., Revell, V.L., Lao, O., Rombouts, E.J., Skene, D.J. & Kayser, M. (2012) Diurnal rhythms in blood cell populations and the effect of acute sleep deprivation in healthy young men. Sleep, 35, 933-940.

Adeva, M.M., Souto, G., Blanco, N. & Donapetry, C. (2012) Ammonium metabolism in humans. Metabolism, 61, 1495-1511.

Aho, V., Olliila, H.M., Kronholm, E., Bondia-Pons, I., Soininen, P., Kangas, A.J., Hilvo, M., Seppala, I., Kettunen, J., Oikonen, M., Raitoharju, E., Hyotylainen, T., Kahonen, M.,

This article is protected by copyright. All rights reserved.
Viikari, J.S., Harma, M., Sallinen, M., Olkkonen, V.M., Alenius, H., Jauhiainen, M., Paunio, T., Lehtimaki, T., Salomaa, V., Oresic, M., Raitakari, O.T., Ala-Korpela, M. & Porkka-Heiskanen, T. (2016) Prolonged sleep restriction induces changes in pathways involved in cholesterol metabolism and inflammatory responses. Sci Rep, 6, 24828.

Ang, J.E., Revell, V., Mann, A., Mantele, S., Otway, D.T., Johnston, J.D., Thumser, A.E., Skene, D.J. & Raynaud, F. (2012) Identification of human plasma metabolites exhibiting time-of-day variation using an untargeted liquid chromatography-mass spectrometry metabolomic approach. Chronobiol Int, 29, 868-881.

Arciero, P.J., Goran, M.I. & Poehlman, E.T. (1993) Resting metabolic rate is lower in women than in men. J Appl Physiol (1985), 75, 2514-2520.

Arendt, J. (1988) Melatonin. Clin Endocrinol (Oxf), 29, 205-229.

Baratta, A.M., Buck, S.A., Buchla, A.D., Fabian, C.B., Chen, S., Mong, J.A. & Pocivavsek, A. (2018) Sex Differences in Hippocampal Memory and Kynurenic Acid Formation Following Acute Sleep Deprivation in Rats. Sci Rep, 8, 6963.

Barclay, J.L., Husse, J., Bode, B., Naujokat, N., Meyer-Kovac, J., Schmid, S.M., Lehnert, H. & Oster, H. (2012) Circadian desynchrony promotes metabolic disruption in a mouse model of shiftwork. PLoS One, 7, e37150.

This article is protected by copyright. All rights reserved.
Bass, J. & Takahashi, J.S. (2010) Circadian integration of metabolism and energetics. *Science, 330*, 1349-1354.

Beitins, I.Z., Barkan, A., Klibanski, A., Kyung, N., Reppert, S.M., Badger, T.M., Veldhuis, J. & McArthur, J.W. (1985) Hormonal responses to short term fasting in postmenopausal women. *J Clin Endocrinol Metab, 60*, 1120-1126.

Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol, 57*, 289-300.

Blanco, R.A., Ziegler, T.R., Carlson, B.A., Cheng, P.Y., Park, Y., Cotsonis, G.A., Accardi, C.J. & Jones, D.P. (2007) Diurnal variation in glutathione and cysteine redox states in human plasma. *Am J Clin Nutr, 86*, 1016-1023.

Bode-Boger, S.M., Scalera, F., Kielstein, J.T., Martens-Lobenhoffer, J., Breithardt, G., Fobker, M. & Reinecke, H. (2006) Symmetrical dimethylarginine: a new combined parameter for renal function and extent of coronary artery disease. *J Am Soc Nephrol, 17*, 1128-1134.

Bray, M.S. & Young, M.E. (2011) Regulation of fatty acid metabolism by cell autonomous circadian clocks: time to fatten up on information? *J Biol Chem, 286*, 11883-11889.
Bubenik, G.A., Ball, R.O. & Pang, S.F. (1992) The effect of food deprivation on brain and gastrointestinal tissue levels of tryptophan, serotonin, 5-hydroxyindoleacetic acid, and melatonin. *J Pineal Res*, 12, 7-16.

Cain, S.W., Dennison, C.F., Zeitzer, J.M., Guzik, A.M., Khalsa, S.B., Santhi, N., Schoen, M.W., Czeisler, C.A. & Duffy, J.F. (2010) Sex differences in phase angle of entrainment and melatonin amplitude in humans. *J Biol Rhythms*, 25, 288-296.

Carrillo-Vico, A., Lardone, P.J., Najii, L., Fernandez-Santos, J.M., Martin-Lacave, I., Guerrero, J.M. & Calvo, J.R. (2005) Beneficial pleiotropic actions of melatonin in an experimental model of septic shock in mice: regulation of pro-/anti-inflammatory cytokine network, protection against oxidative damage and anti-apoptotic effects. *J Pineal Res*, 39, 400-408.

Castrillo, A., Joseph, S.B., Vaidya, S.A., Haberland, M., Fogelman, A.M., Cheng, G. & Tontonoz, P. (2003) Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. *Mol Cell*, 12, 805-816.

Cespuglio, R., Amrouni, D., Meiller, A., Buguet, A. & Gautier-Sauvigne, S. (2012) Nitric oxide in the regulation of the sleep-wake states. *Sleep Med Rev*, 16, 265-279.

Choi, J.Y., Seo, J.Y., Yoon, Y.S., Lee, Y.J., Kim, H.S. & Kang, J.L. (2015) Mer signaling increases the abundance of the transcription factor LXR to promote the resolution of acute sterile inflammation. *Sci Signal*, 8, ra21.

This article is protected by copyright. All rights reserved.
Chua, E.C., Shui, G., Lee, I.T., Lau, P., Tan, L.C., Yeo, S.C., Lam, B.D., Bulchand, S.,
Summers, S.A., Puvanendran, K., Rozen, S.G., Wenk, M.R. & Gooley, J.J. (2013) Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. *Proc Natl Acad Sci U S A, 110*, 14468-14473.

Czeisler, C.A., Duffy, J.F., Shanahan, T.L., Brown, E.N., Mitchell, J.F., Rimmer, D.W., Ronda, J.M., Silva, E.J., Allan, J.S., Emens, J.S., Dijk, D.J. & Kronauer, R.E. (1999) Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science, 284*, 2177-2181.

Dallmann, R., Viola, A.U., Tarokh, L., Cajochen, C. & Brown, S.A. (2012) The human circadian metabolome. *Proc Natl Acad Sci U S A, 109*, 2625-2629.

Davies, S.K., Ang, J.E., Revell, V.L., Holmes, B., Mann, A., Robertson, F.P., Cui, N., Middleton, B., Ackermann, K., Kayser, M., Thumser, A.E., Raynaud, F.I. & Skene, D.J. (2014) Effect of sleep deprivation on the human metabolome. *Proc Natl Acad Sci U S A, 111*, 10761-10766.

Davis, F.C., Darrow, J.M. & Menaker, M. (1983) Sex differences in the circadian control of hamster wheel-running activity. *Am J Physiol, 244*, R93-105.

Deboer, T., Detari, L. & Meijer, J.H. (2007) Long term effects of sleep deprivation on the mammalian circadian pacemaker. *Sleep, 30*, 257-262.

This article is protected by copyright. All rights reserved.
Deng, H.B., Tam, T., Zee, B.C., Chung, R.Y., Su, X., Jin, L., Chan, T.C., Chang, L.Y., Yeoh, E.K. & Lao, X.Q. (2017) Short Sleep Duration Increases Metabolic Impact in Healthy Adults: A Population-Based Cohort Study. *Sleep, 40*.

Dubocovich, M.L. & Markowska, M. (2005) Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine, 27*, 101-110.

Duez, H. & Staels, B. (2008) The nuclear receptors Rev-erbs and RORs integrate circadian rhythms and metabolism. *Diab Vasc Dis Res, 5*, 82-88.

Duffy, J.F., Cain, S.W., Chang, A.M., Phillips, A.J., Munch, M.Y., Gronfier, C., Wyatt, J.K., Dijk, D.J., Wright, K.P., Jr. & Czeisler, C.A. (2011) Sex difference in the near-24-hour intrinsic period of the human circadian timing system. *Proc Natl Acad Sci U S A, 108 Suppl 3*, 15602-15608.

Eckel-Mahan, K.L., Patel, V.R., Mohney, R.P., Vignola, K.S., Baldi, P. & Sassone-Corsi, P. (2012) Coordination of the transcriptome and metabolome by the circadian clock. *Proc Natl Acad Sci U S A, 109*, 5541-5546.

Ferrara, M., Bottasso, A., Tempesta, D., Carriero, M., De Gennaro, L. & Ponti, G. (2015) Gender differences in sleep deprivation effects on risk and inequality aversion: evidence from an economic experiment. *PLoS One, 10*, e0120029.

This article is protected by copyright. All rights reserved.
Gallopin, T., Fort, P., Eggermann, E., Cauli, B., Luppi, P.H., Rossier, J., Audinat, E., Muhlethaler, M. & Serafin, M. (2000) Identification of sleep-promoting neurons in vitro. *Nature*, **404**, 992-995.

Glynn, E.F., Chen, J. & Mushegian, A.R. (2006) Detecting periodic patterns in unevenly spaced gene expression time series using Lomb-Scargle periodograms. *Bioinformatics*, **22**, 310-316.

Gunn, P.J., Middleton, B., Davies, S.K., Revell, V.L. & Skene, D.J. (2016) Sex differences in the circadian profiles of melatonin and cortisol in plasma and urine matrices under constant routine conditions. *Chronobiol Int*, **33**, 39-50.

Hargreaves, K.M. & Pardridge, W.M. (1988) Neutral amino acid transport at the human blood-brain barrier. *J Biol Chem*, **263**, 19392-19397.

Hastings, M.H., Reddy, A.B. & Maywood, E.S. (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci*, **4**, 649-661.

Horvath, T.L. & Gao, X.B. (2005) Input organization and plasticity of hypocretin neurons: possible clues to obesity’s association with insomnia. *Cell Metab*, **1**, 279-286.

Huang, W., Ramsey, K.M., Marcheva, B. & Bass, J. (2011) Circadian rhythms, sleep, and metabolism. *J Clin Invest*, **121**, 2133-2141.

This article is protected by copyright. All rights reserved.
Huether, G. (1993) The contribution of extrapineal sites of melatonin synthesis to circulating melatonin levels in higher vertebrates. *Experientia*, 49, 665-670.

Hughes, M.E., Hogenesch, J.B. & Kornacker, K. (2010) JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. *J Biol Rhythms*, 25, 372-380.

Hui, S., Ghergurovich, J.M., Morscher, R.J., Jang, C., Teng, X., Lu, W., Esparza, L.A., Reya, T., Le, Z., Yanxiang Guo, J., White, E. & Rabinowitz, J.D. (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature*, 551, 115-118.

Isherwood, C.M., Van der Veen, D.R., Johnston, J.D. & Skene, D.J. (2017) Twenty-four-hour rhythmicity of circulating metabolites: effect of body mass and type 2 diabetes. *FASEB J*, 31, 5557-5567.

Johnston, J.D. (2014) Physiological links between circadian rhythms, metabolism and nutrition. *Exp Physiol*, 99, 1133-1137.

Jones, S., Pfister-Genskow, M., Benca, R.M. & Cirelli, C. (2008) Molecular correlates of sleep and wakefulness in the brain of the white-crowned sparrow. *J Neurochem*, 105, 46-62.

Joseph, S.B., Castrillo, A., Laffitte, B.A., Mangelsdorf, D.J. & Tontonoz, P. (2003) Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med*, 9, 213-219.

This article is protected by copyright. All rights reserved.
Kasukawa, T., Sugimoto, M., Hida, A., Minami, Y., Mori, M., Honma, S., Honma, K., Mishima, K., Soga, T. & Ueda, H.R. (2012) Human blood metabolite timetable indicates internal body time. *Proc Natl Acad Sci U S A*, **109**, 15036-15041.

Kielstein, J.T., Salpeter, S.R., Bode-Boeger, S.M., Cooke, J.P. & Fliser, D. (2006) Symmetric dimethylarginine (SDMA) as endogenous marker of renal function--a meta-analysis. *Nephrol Dial Transplant*, **21**, 2446-2451.

Klein, D. & Weller, J.L. (1973) Adrenergic-adenosine 3′,5′-monophosphate regulation of serotonin N-acetyltransferase activity and the temporal relationship of serotonin N-acetyltransferase activity synthesis of 3H-N-acetylserotonin and 3H-melatonin in the cultured rat pineal gland. *J Pharmacol Exp Ther*, **186**, 516-527.

Klerman, E.B., Gershengorn, H.B., Duffy, J.F. & Kronauer, R.E. (2002) Comparisons of the variability of three markers of the human circadian pacemaker. *J Biol Rhythms*, **17**, 181-193.

Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Lo Sasso, G., Moschetta, A. & Schibler, U. (2009) REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol*, **7**, e1000181.

Lee, S.D. & Tontonoz, P. (2015) Liver X receptors at the intersection of lipid metabolism and atherogenesis. *Atherosclerosis*, **242**, 29-36.
Li, Y., Gao, X.B., Sakurai, T. & van den Pol, A.N. (2002) Hypocretin/Orexin excites hypocretin neurons via a local glutamate neuron-A potential mechanism for orchestrating the hypothalamic arousal system. *Neuron, 36*, 1169-1181.

Meldrum, B.S. (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr, 130*, 1007S-1015S.

Miller, C.B., Rae, C.D., Green, M.A., Yee, B.J., Gordon, C.J., D’Rozario, A.L., Kyle, S.D., Espie, C.A., Grunstein, R.R. & Bartlett, D.J. (2017) An Objective Short Sleep Insomnia Disorder Subtype Is Associated With Reduced Brain Metabolite Concentrations In Vivo: A Preliminary Magnetic Resonance Spectroscopy Assessment. *Sleep, 40*.

Minami, Y., Kasukawa, T., Kakazu, Y., Iigo, M., Sugimoto, M., Ikeda, S., Yasui, A., van der Horst, G.T., Soga, T. & Ueda, H.R. (2009) Measurement of internal body time by blood metabolomics. *Proc Natl Acad Sci U S A, 106*, 9890-9895.

Mittelstrass, K., Ried, J.S., Yu, Z., Krumsiek, J., Gieger, C., Prehn, C., Roemisch-Margl, W., Polonikov, A., Peters, A., Theis, F.J., Meitinger, T., Kronenberg, F., Weidinger, S., Wichmann, H.E., Suhre, K., Wang-Sattler, R., Adamski, J. & Illig, T. (2011) Discovery of sexual dimorphisms in metabolic and genetic biomarkers. *PLoS Genet, 7*, e1002215.

Mohawk, J.A., Green, C.B. & Takahashi, J.S. (2012) Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci, 35*, 445-462.

This article is protected by copyright. All rights reserved.
Moller-Levet, C.S., Archer, S.N., Bucca, G., Laing, E.E., Slak, A., Kabiljo, R., Lo, J.C., Santhi, N., von Schantz, M., Smith, C.P. & Dijk, D.J. (2013) Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci U S A*, **110**, E1132-1141.

Mongrain, V., Lavoie, S., Selmaoui, B., Paquet, J. & Dumont, M. (2004) Phase relationships between sleep-wake cycle and underlying circadian rhythms in Morningness-Eveningness. *J Biol Rhythms*, **19**, 248-257.

Pitkanen, H.T., Oja, S.S., Kemppainen, K., Seppa, J.M. & Mero, A.A. (2003) Serum amino acid concentrations in aging men and women. *Amino Acids*, **24**, 413-421.

Rebouche, C.J. (2004) Kinetics, pharmacokinetics, and regulation of L-carnitine and acetyl-L-carnitine metabolism. *Ann N Y Acad Sci*, **1033**, 30-41.

Reiter, R.J., Tan, D.X., Osuna, C. & Gitto, E. (2000) Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci*, **7**, 444-458.

Reppert, S.M. & Weaver, D.R. (2002) Coordination of circadian timing in mammals. *Nature*, **418**, 935-941.

Rist, M.J., Roth, A., Frommherz, L., Weinert, C.H., Kruger, R., Merz, B., Bunzel, D., Mack, C., Egert, B., Bub, A., Gorling, B., Tzvetkova, P., Luy, B., Hoffmann, I., Kulling, S.E. &
Watzl, B. (2017) Metabolite patterns predicting sex and age in participants of the Karlsruhe Metabolomics and Nutrition (KarMeN) study. PLoS One, 12, e0183228.

Ruoppolo, M., Campesi, I., Scolamiero, E., Pecce, R., Caterino, M., Cherchi, S., Mercuro, G., Tonolo, G. & Franconi, F. (2014) Serum metabolomic profiles suggest influence of sex and oral contraceptive use. Am J Transl Res, 6, 614-624.

Saper, C.B., Fuller, P.M., Pedersen, N.P., Lu, J. & Scammell, T.E. (2010) Sleep state switching. Neuron, 68, 1023-1042.

Scheer, F.A., Hilton, M.F., Mantzoros, C.S. & Shea, S.A. (2009) Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci U S A, 106, 4453-4458.

Schull, J., Walker, J., Fitzgerald, K., Hiilivirta, L., Ruckdeschel, J., Schumacher, D., Stanger, D. & McEachron, D.L. (1989) Effects of sex, thyro-parathyroidectomy, and light regime on levels and circadian rhythms of wheel-running in rats. Physiol Behav, 46, 341-346.

Sherin, J.E., Shiromani, P.J., McCarley, R.W. & Saper, C.B. (1996) Activation of ventrolateral preoptic neurons during sleep. Science, 271, 216-219.

Skene, D.J., Bojkowski, C.J. & Arendt, J. (1994) Comparison of the effects of acute fluvoxamine and desipramine administration on melatonin and cortisol production in humans. Br J Clin Pharmacol, 37, 181-186.

This article is protected by copyright. All rights reserved.
Skene, D.J., Middleton, B., Fraser, C.K., Pennings, J.L., Kuchel, T.R., Rudiger, S.R., Bawden, C.S. & Morton, A.J. (2017) Metabolic profiling of presymptomatic Huntington's disease sheep reveals novel biomarkers. Sci Rep, 7, 43030.

Skene, D.J., Skornyakov, E., Chowdhury, N.R., Gajula, R.P., Middleton, B., Satterfield, B.C., Porter, K.I., Van Dongen, H.P.A. & Gaddameedhi, S. (2018) Separation of circadian- and behavior-driven metabolite rhythms in humans provides a window on peripheral oscillators and metabolism. Proc Natl Acad Sci U S A, 115, 7825-7830.

Sletten, T.L., Revell, V.L., Middleton, B., Lederle, K.A. & Skene, D.J. (2009) Age-related changes in acute and phase-advancing responses to monochromatic light. J Biol Rhythms, 24, 73-84.

Spiegel, K., Knutson, K., Leproult, R., Tasali, E. & Van Cauter, E. (2005) Sleep loss: a novel risk factor for insulin resistance and Type 2 diabetes. J Appl Physiol (1985), 99, 2008-2019.

Tanaka, M., Nakamura, F., Mizokawa, S., Matsumura, A., Matsumura, K. & Watanabe, Y. (2003) Role of acetyl-L-carnitine in the brain: revealed by Bioradiography. Biochem Biophys Res Commun, 306, 1064-1069.

Toth, E., Harsing, L.G., Jr., Sershen, H., Ramacci, M.T. & Lajtha, A. (1993) Effect of acetyl-L-carnitine on extracellular amino acid levels in vivo in rat brain regions. Neurochem Res, 18, 573-578.

This article is protected by copyright. All rights reserved.
Van Reen, E., Sharkey, K.M., Roane, B.M., Barker, D., Seifer, R., Raffray, T., Bond, T.L. & Carskadon, M.A. (2013) Sex of college students moderates associations among bedtime, time in bed, and circadian phase angle. *J Biol Rhythms, 28*, 425-431.

Volk, C., Jaramillo, V., Merki, R., O’Gorman Tuura, R. & Huber, R. (2018) Diurnal changes in glutamate + glutamine levels of healthy young adults assessed by proton magnetic resonance spectroscopy. *Hum Brain Mapp, 39*, 3984-3992.

Volpi, E., Lucidi, P., Bolli, G.B., Santeusanio, F. & De Feo, P. (1998) Gender differences in basal protein kinetics in young adults. *J Clin Endocrinol Metab, 83*, 4363-4367.

Weljie, A.M., Meerlo, P., Goel, N., Sengupta, A., Kayser, M.S., Abel, T., Birnbaum, M.J., Dinges, D.F. & Sehgal, A. (2015) Oxalic acid and diacylglycerol 36:3 are cross-species markers of sleep debt. *Proc Natl Acad Sci U S A, 112*, 2569-2574.

Xie, L., Kang, H., Xu, Q., Chen, M.J., Liao, Y., Thiyagarajan, M., O’Donnell, J., Christensen, D.J., Nicholson, C., Iliff, J.J., Takano, T., Deane, R. & Nedergaard, M. (2013) Sleep drives metabolite clearance from the adult brain. *Science, 342*, 373-377.

Yamakawa, T., Kurauchi, Y., Hisatsune, A., Seki, T. & Katsuki, H. (2018) Endogenous Nitric Oxide Inhibits, Whereas Awakening Stimuli Increase, the Activity of a Subset of Orexin Neurons. *Biol Pharm Bull, 41*, 1859-1865.
Yoshida, G., Li, M.X., Horiuchi, M., Nakagawa, S., Sakata, M., Kuchiiwa, S., Kuchiiwa, T., Jalil, M.A., Begum, L., Lu, Y.B., Iijima, M., Hanada, T., Nakazato, M., Huang, Z.L., Eguchi, N., Kobayashi, K. & Saheki, T. (2006) Fasting-induced reduction in locomotor activity and reduced response of orexin neurons in carnitine-deficient mice. *Neurosci Res, 55*, 78-86.

**Figure Captions**

**Figure 1.** Mean (± SEM) plasma hormone concentrations (n = 12) over the 70-h sampling protocol (A, melatonin; B, cortisol). The black bar indicates the sleep period, 0 lux, supine; grey bars, wake periods, semi-recumbent position, <8 lux; white bars, awake and free movement, 90 lux.

**Figure 2.** PCA of all metabolites data were carried out, and the significant time-of-day variation in mean score (± SEM) across all subjects on PC1 is shown. The black bar indicates the sleep period, 0 lux, supine; grey bars, wake periods, semi-recumbent position, <8 lux; white bars, awake and free movement, 90 lux.

**Figure 3.** OPLS-DA models of selected time points (00:00–06:00 h) separated according to sleep status (A, day 1, sleep (black circle) vs. day 2, sleep deprivation (red circle); B, day 1, sleep (black circle) vs. day 3, recovery sleep (blue circle)). Loading plots for the OPLS-DA models (C, day 1, sleep vs. day 2, sleep deprivation; D, day 1, sleep vs. day 3, recovery sleep) coloured according to class: amino acids and biogenic amines (blue), acylcarnitines (green), glycerophospholipids (PC aa (yellow), PC ae (light orange), lysoPC (dark orange)) and sphingolipids (brown). Positive P (loading) values represent metabolites with higher concentrations, and negative P (loading) values represent metabolites with lower concentrations.

This article is protected by copyright. All rights reserved.
concentrations during sleep deprivation (C) and recovery sleep (D) compared with sleep. For lists of metabolites and their corresponding P (loading) values, see Supplementary Table S1.

Figure 4. Metabolites with a significant cosine rhythm during the 3 study days. (A) Venn diagram showing the number of metabolites exhibiting a significant fit to a cosine curve on day 1 (sleep, grey circle), day 2 (sleep deprivation, red circle), day 3 (recovery sleep, blue circle). (B) Pie charts showing the proportion of metabolites from each metabolite class exhibiting a significant fit to a cosine curve all days.

Figure 5. Peak times (acrophase) of 31 common metabolites with a significant fit to a cosine curve in both males and females on day 1 (black circle) and day 2 (red circle). Dim light melatonin onset (DLMO) times on both days (day 1: black triangle, day 2: red triangle) are shown. Labelled metabolites had a difference in acrophase time of 4 hours or more between males and females. For the details of metabolite acrophase times, see Supplementary Table S7.
