Effect of sleep deprivation on rhythms of clock gene expression and melatonin in humans

Running head: Circadian rhythms during sleep deprivation

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Abstract

This study investigated the impact of sleep deprivation on the human circadian system. Plasma melatonin and cortisol, and leukocyte expression levels of 12 genes were examined over 48 h (sleep vs no sleep nights) in 12 young males (mean ± SD, 23 ± 5 yrs). During one night of total sleep deprivation *BMAL1* expression was suppressed, the heat shock gene *HSPA1B* expression was induced, and the amplitude of the melatonin rhythm increased while other high-amplitude clock gene rhythms (e.g. *PER1-3*, *REV-ERBα*) remained unaffected. Our data suggest that the core clock mechanism in peripheral oscillators is compromised during acute sleep deprivation.

Key words:

circadian clock, circadian rhythms, clock genes, sleep deprivation, oxidative stress

Introduction

In our modern 24/7 society, chronic partial sleep deprivation is becoming increasingly common. There is mounting evidence that long-term sleep deprivation has a severe impact on health and enhances vulnerability to both, infectious diseases and pathologies such as diabetes, obesity, or cancer (Lamont et al., 2007; Logan & Sarkar, 2012; Mullington et al., 2010). Restricted sleep has been shown to significantly phase-delay the melatonin rhythm in humans, independent of bedtime (Burgess & Eastman, 2004; Burgess & Eastman, 2006). The acute impact of sleep deprivation on the circadian system, however, has not been fully elucidated in humans. As the circadian system controls most aspects of our daily lives, including cyclic changes in physiology, behaviour, and metabolism (Hastings et al., 2003; Mohawk et al., 2012), profiling of the central and peripheral clocks during sleep deprivation will be key to understanding the molecular basis of how these pathologies develop (Hastings et al., 2003; Kyriacou & Hastings, 2010; Lamont et al., 2007; Logan & Sarkar, 2012).

In mammals, the core clock mechanism is based on a transcriptional-translational feedback loop involving the *Period* (*PER*) and *Cryptochrome* (*CRY*) genes as negative elements, the basic helix-loop-helix Per-Arnt-Sim (PAS) transcription factors CLOCK and BMAL1 as positive components (Hastings et al., 2003; Isojima et al., 2003) and a range of auxiliary loops for stabilisation (Albrecht, 2012; Isojima et al., 2003; Relogio et al., 2011). While there are a number of reports on rhythmic expression of clock genes in human peripheral blood cell oscillators (Archer et al., 2008; Boivin et al., 2003; Fukuya et al., 2007; Kusanagi et al., 2008), studies investigating changes in these rhythms upon sleep deprivation are fewer. A recent study provided some data on clock gene expression during sleep deprivation under light conditions compared to during sleep (Kavcic et al., 2011). The authors found significant

rhythmicity for *BMAL1* expression under both conditions whereas rhythmicity in *PER2* expression was strongly affected by sleep deprivation. The study, however, was limited by a small number of subjects (n = 7), a blood sampling interval of 4 h, and restriction to the analysis of two genes (*PER2* and *BMAL1*). An earlier study in six subjects described comparable rhythms for *PER1* and *PER2* under sleep/wake and constant routine sleep deprivation conditions, and a more variable expression for *BMAL1*, with differences in peak times between the two conditions (James et al., 2007). Studies in mice showed that *cryptochromes* may be involved in the homeostatic regulation of sleep, and that changes in the expression of clock-related genes in the cerebral cortex during sleep deprivation are strain-dependent and parallel the electroencephalographic response (Wisor et al., 2002; Wisor et al., 2008). Using microarrays, a recent study showed that the number of brain transcripts affected by sleep deprivation, including some circadian genes, could be reduced by adrenalectomy (Mongrain et al., 2010).

Similarly, although melatonin and cortisol are two well-characterised central circadian clock-controlled hormones, the effect of acute sleep deprivation on their rhythmicity (in terms of amplitude and timing) has provided conflicting results (Salin-Pascual et al., 1988; von Treuer et al., 1996; Zeitzer et al., 2007). While one study observed increased melatonin levels after sleep deprivation, cortisol levels remained unchanged, and no information was given on the timing of the melatonin rhythm (Salin-Pascual et al., 1988). Another study described opposite results, with elevated cortisol and unchanged melatonin levels on the sleep deprivation night (von Treuer et al., 1996). A more recent study also reported elevated levels of melatonin during extended wakefulness (constant routine conditions) in young subjects, and an age-dependent difference in melatonin concentrations during sleep deprivation (Zeitzer et al., 2007).

The current study aimed to assess the impact of one night of total sleep deprivation under strictly controlled laboratory conditions on markers of both the central circadian pacemaker, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and peripheral blood cell oscillators. An in-depth characterisation of diurnal rhythms in expression levels of 11 clock genes (*PER1-3*, *CRY1-2*, *BMAL1*, *CLOCK*, *DBP*, *DEC1*, *REV-ERBα*, *RORA*), a heat shock gene (*HSPA1B*) known to be involved in the oxidative stress response in peripheral leukocytes, and a comprehensive analysis of the circadian rhythms of plasma melatonin and cortisol was undertaken during a full 24 h sleep/wake cycle and a subsequent 24 h wake/wake cycle (i.e. a night of total sleep deprivation).

Materials and methods

Clinical study

Clinical sessions were conducted at the Surrey Clinical Research Centre (SCRC) at the University of Surrey (UK). Ethical approval was obtained from the University Ethics Committee and all procedures were conducted in accordance with the Declaration of Helsinki. The experimental protocol of the study conformed to international ethical standards (Portaluppi et al., 2010).

A detailed description of the study protocol and the initial screening phase including eligibility criteria have been reported previously (Ackermann et al., 2012). Briefly, study eligibility was determined by questionnaires, medical and physical assessment, and analysis of blood and urine samples. Exclusion criteria included, but were not limited to, smoking, the taking of prescription medication, abnormal blood haematology and testing positive for drugs of abuse. Healthy males (n = 15; mean age ± SD: 24 ± 5 yrs), participated in the study, with 12 participants (23 ± 5 yrs) included in the current study analysis. Written and oral informed

consent was obtained 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).

Participants were required to maintain a strict regular sleep-wake schedule for 7 days prior to entering the laboratory, confirmed by activity/light monitors (Actiwatch, CamNtech, Cambridge, UK), sleep logs and time-stamped voicemail, which aimed at stabilising the circadian clock and ensuring that participants were not sleep deprived. In addition, they were required to get at least 15 min of outdoor lighting within 90 min of waking each day. Consumption of caffeine, alcohol and medication was prohibited for the final 3 days of this baseline period.

Participants entered the laboratory at 17:00 h on day 1 and remained there until 18.00 h on day 4. Overnight periods were spent in individual sleep laboratories, with the adaptation (night 1; N1) and baseline (night 2; N2) nights including a normal sleep period (23:00 – 07:00 h), and total sleep deprivation on the third night (night 3; N3). Polysomnography monitoring occurred on nights 2 and 3 to confirm sleep on night 2 and wakefulness on night 3. Blood samples for gene expression and hormone analyses were collected into PAXgene RNA tubes (Qiagen, Crawley, UK) and lithium heparin tubes at hourly intervals for 48 hours (from 12:00 h on day 2 to 12:00 h on day 4) using an intravenous catheter and sampling through a portal in the wall on night 2 so that sleep was not disturbed. Subjects had a recovery sleep from 13:00 – 18:00 h on day 4 before leaving the laboratory. Identical meals (breakfast, lunch, dinner, and snack) were eaten by all participants during the study according to UK dietary guidelines. Meals were identical in composition on each day of the study. A detailed scheme of the study protocol is shown in Figure 1.

Sample analysis

Hormone assays

Radioimmunoassay analysis was performed on hourly plasma samples to determine melatonin and cortisol concentrations (Stockgrand Ltd., University of Surrey, UK) as described previously (Fraser et al., 1983; Sletten et al., 2009).

RNA isolation, reverse transcription and real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from leukocytes using the PAXgene 96 Blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) according to the manufacturer's instructions. RNA concentration was determined by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), and random samples were inspected for RNA integrity using a Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Two-hourly samples from 12:00 h on day 2 to 12:00 h on day 4 (25 samples per subject) were used for cDNA synthesis using the RevertAid^{TM} H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) with random hexamer primers as described in the manufacturer's instructions.

cDNA was subjected to qRT-PCR using the LightCycler 480 SYBR Green I Kit on a LightCycler 480 II platform (Roche Diagnostics, Mannheim, Germany). The protocol was as follows: 10 min at 95°C, 45 cycles of 95°C, 60°C, and 72°C (10 s each), followed by a melting curve step with continuous acquisition from 65 – 97°C. Relative quantification of gene expression was performed using the second derivative maximum method (Roche Diagnostics) followed by the delta-delta-cycle-threshold ($2\Delta^{CT}$) method as described (Ackermann et al., 2007).

Five reference genes (*GAPDH, ACTB, HPRT, PPIB, UBC*) were tested for suitability using NormFinder software (MOMA, Aarhus University Hospital, Aarhus, Denmark) to determine

gene stability values and single cosinor analysis (Minors & Waterhouse, 1989) to ensure constitutive expression levels. Of these reference genes, *ACTB* was determined to be the most suitable, with a stability value of .017 and an average p value of .5 for diurnal rhythmicity, and was therefore used as reference gene in subsequent RT-PCR experiments. Relative expression levels of 11 clock genes and the gene coding for *Homo sapiens* heat shock 70 kDa protein 1B (HSPA1B) were determined: *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *REV-ERB*α, *DEC1*, *RORA*, *DBP* and *HSPA1B*.

All primers were acquired from Metabion (Metabion, Martinsried, Germany) and tested for efficiency and specificity of amplification. Except for the *HSPA1B* gene, which does not contain introns, primers were designed to span at least one exon-exon junction using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), or were taken from published literature (Archer et al., 2008; Kimura et al., 2011; Lavebratt et al., 2010; Visser et al., 2011; Wu et al., 2006), and were checked for transcript specificity using the UCSC In-silico PCR tool (http://genome.ucsc.edu/). Primer sequences are given in Supplementary Table S1.

Data analysis:

Dim light melatonin onset (DLMO) was used as a marker of circadian timing and calculated for each individual for both the sleep (night 2, N2) and sleep deprivation (night 3, N3) nights using the 25%-threshold method as previously described (Sletten et al., 2009). Melatonin and cortisol profiles for each individual were subjected to cosinor analysis (Minors & Waterhouse, 1989) for determination of amplitude and peak time (acrophase) for the sleep and sleep deprivation conditions separately and for the collapsed data (full 48 hours of sampling period). Paired Student's t-tests (two-tailed) were performed to compare melatonin and cortisol rhythm parameters during sleep (N2) and sleep deprivation (N3) (DLMO,

melatonin and cortisol acrophase and amplitude, and melatonin area under the curve (AUC) with a 12 h night-time bin with individual acrophase as midpoint and a 6 h daytime bin as control) (GraphPad Prism 5).

Relative gene expression profiles for each individual were z-scored and subjected to cosinor analysis (Minors & Waterhouse, 1989) with a 24 h period (for *HSPA1B* additional analysis with a 12 h period was performed, as reported previously in mice (Hughes et al., 2009)) for determination of peak time, amplitude, and significance of fit. Cosinor analysis was performed separately for each condition and the collapsed dataset as described for the hormone analysis. Calculated acrophases for melatonin and *PER3* expression on N2 were subjected to Spearman correlation. Non-linear curve fitting using a cosine function with a 24 h period on z-scored normalised gene expression levels was employed to characterise diurnal rhythms for each gene (nls function of the stats package of R software version 2.12.0) as described recently (Ackermann et al., 2012). This analysis was phase-adjusted individually using i) average melatonin peak time and ii) average DLMO on baseline sleep night (N2) (two of the 12 participants exhibited a phase-delay in peak melatonin timing compared to the other subjects and were excluded for calculation of average peak time and DLMO). Gene expression data were phase-adjusted using the calculated time difference between individual melatonin peak time/DLMO and average melatonin peak time/DLMO for all downstream analysis. Equation for non-linear curve fitting: phase-adjusted normalised z-score = α_i + β*cos(2*π*(TP-t)/24); where TP is the sampling time, α_i is the individual term for the individual *i*, β is the amplitude and *t* the peak time of the cosine function. The following null hypotheses were tested for significance: 1) the amplitude and 2) peak times were not different from zero. Analysis was performed separately for the sleep and sleep deprivation condition,

and for the collapsed 48 h dataset. The level of significance was reduced from $p < .05$ to $p < .0042$ (Bonferroni correction for multiple testing).

Results

Hormone rhythms

Cortisol

Individual cortisol profiles exhibited a significant ($p < .03$) diurnal pattern with peak times in the early morning (Supplementary Figure S1; Supplementary Table S2). There were no significant differences between the sleep and the sleep deprivation condition for either acrophase ($p = .4913$) or amplitude ($p = .1040$) (Figure 2).

Melatonin

Individual melatonin profiles showed a significant ($p < .0001$) diurnally rhythmic pattern with peak times in the second half of the night (Supplementary Figure S2; Supplementary Table S3). Compared to the sleep condition, during sleep deprivation, the melatonin acrophase was significantly delayed (mean ± SEM: 00.42 ± 00.14 h; $p = .0129$; Figure 2 top), amplitude was significantly increased (29.6 ± 7.7%; $p = .0012$; Figure 2 bottom) and the nocturnal melatonin AUC significantly increased (25.4 ± 4.4%; $p = .0003$, Supplementary Figure S3). By contrast, daytime melatonin levels assessed by AUC did not differ significantly between day 2 and day 3 ($p = .7408$, Supplementary Figure S3). DLMO did not differ significantly in timing between N2 (21.08 ± 00.21 h) and N3 (21.38 ± 00.26 h, $p = .1931$, Supplementary Figure S4), but results showed a trend towards significance upon excluding the most phase-delayed subject (Figure S2; $p = 0.0572$).

Gene expression levels

Diurnal rhythmicity in mRNA signatures during the baseline condition was found for most but not all candidate genes tested. An illustration of individual profiles for each gene investigated is provided in Figure 3, and a summary of the cosinor analysis is given in Table 1. The most robust rhythms, displaying clear peaks and high amplitude, were detected for *PER3* and *REV-ERBα*, followed by *BMAL1* and *PER1*. Eight out of the twelve subjects showed significant *PER3* expression rhythms during the sleep condition (N2), and correlation with corresponding melatonin peak times showed a tendency towards significance ($p = 0.069$; $n = 8$). *HSPA1B* expression showed strong 24 h, but not 12 h, rhythmicity. Except for *RORA* and *DEC1*, at least 3 out of the 12 subjects showed significant rhythmicity in expression levels for the 10 remaining genes, and the number of subjects with significant rhythms was found to be equal or lower during sleep deprivation compared to the sleep condition. Non-linear curve fitting revealed significant rhythms in all genes, except *DEC1* and *CRY1/2*, in at least one of the three conditions (sleep, sleep deprivation, collapsed 48 h) for data corrected to individual circadian time using both DLMO and melatonin peak time. Peak times calculated by non-linear curve fitting (Table 2, Supplementary Table S4) were in close agreement to the peak times determined using the cosinor method.

High-amplitude rhythms for all conditions (sleep, sleep deprivation, collapsed 48 h) were detected for *PER1*, *PER3* and *REV-ERBα*, while for *BMAL1* and *HSPA1* such high amplitudes were only found in the sleep condition. Closer examination revealed that the diurnal rhythms in expression levels of *BMAL1* and *HSPA1B* were disrupted by sleep loss, exhibiting strongly reduced amplitudes (~70 % and ~50 % reduction for *BMAL1* and *HSPA1B*, respectively) and loss of rhythmicity (Table 2). While *BMAL1* expression was suppressed (total sum z-scores: 15.33 during sleep compared to -12.29 during sleep

deprivation), *HSPA1B* expression was induced, exhibiting elevated baseline levels during sleep deprivation (total sum z-scores: -3.46 sleep versus 4.49 sleep deprivation). By contrast, other clock genes with high-amplitude diurnal rhythms (e.g. *PER1*, *PER3*, *REV-ERB*α) were not significantly affected by sleep deprivation. Of the genes with lower amplitude, *CRY1*, corrected to melatonin peak time, showed significant rhythmicity only during the sleep condition, with an almost complete loss of amplitude during sleep deprivation (Table 2). In addition, for *CLOCK* and *DBP* a strong reduction in amplitude during sleep deprivation was observed (~40% and ~18%, respectively; Table 2), even though rhythmicity was maintained. In general, the amplitudes of the gene expression rhythms in the sleep deprivation condition were lower and less often reached statistical significance compared to the sleep condition.

Discussion

This study aimed to address the impact of one night of total sleep deprivation under controlled laboratory conditions on both biomarkers of the central circadian pacemaker and peripheral oscillators in blood cells.

Although melatonin is one of the best characterised and reliable markers of human circadian phase and widely used in sleep-related studies, there is surprisingly little conclusive information regarding the acute effects of sleep deprivation on the amplitude and timing of the melatonin rhythm (Salin-Pascual et al., 1988; von Treuer et al., 1996; Zeitzer et al., 2007). Our data are in agreement with the work of Salin-Pascual et al. (1988) which showed an increase in melatonin levels, but not in cortisol levels, during sleep deprivation, but in contrast with the study by von Treuer and colleagues (1996) which reported the opposite effects. Further in agreement with the current data, a phase delay in the melatonin rhythm has previously been reported after one night of sleep deprivation (Cajochen et al., 2003), and an

increase in melatonin levels was found in young subjects during extended wakefulness (up to 50 h) (Zeitzer et al., 2007). The latter study, however, also described an age-dependent difference in melatonin profiles, with young subjects exhibiting reduced and older subjects exhibiting elevated melatonin levels during one night of sleep deprivation compared to habitual sleep.

While melatonin peak times were consistently delayed during sleep deprivation in all but the two phase-delayed subjects, the DLMO did not consistently change to later times on the sleep deprivation night (N3). This finding was predicted as we would not expect the DLMO to show the same change as the melatonin acrophase as in the early evening at DLMO the subjects were not yet sleep deprived and thus DLMO should be the same on both nights (N2 and N3). While the current melatonin data showed both, an increase and a phase-delay during sleep deprivation, similar changes in cortisol levels were not observed. . Even though we cannot fully exclude that the differential responses of melatonin and cortisol to sleep deprivation observed in our study are protocol-related, we hypothesise that the increase in melatonin might be interpreted as a direct consequence of the oxidative stress induced by sleep loss, and thus might be related to melatonin's well-reported antioxidative properties (Reiter et al., 2000).

To support our hypothesis on the upregulation of melatonin, the heat shock gene *HSPA1B* was investigated as a representative of genes involved in the oxidative stress response. Notably, expression of *HSPA1B* did not show 12 h rhythmicity, as previously reported for mouse tissues (Hughes et al., 2009), but instead strong 24 h rhythmicity during the sleep condition. The observed induction of *HSPA1B* during sleep deprivation is in agreement with previous work demonstrating its upregulation in obstructive sleep apnoea and disease-associated oxidative stress, and enhanced *HSPA1B* gene expression in several brain regions in mice during sleep deprivation (Lavie et al., 2010; Terao et al., 2003). Interestingly, a recent

study on changes in the brain transcriptome upon sleep deprivation demonstrated that upregulation of *HSPA1B* in mice remains even if the corticosterone arm of the stress response has been eliminated (Mongrain et al., 2010). Thus, our data support a role of *HSPA1B* in the body's immediate response to (oxidative) stress induced by sleep loss. Furthermore, we have recently demonstrated an immediate immune response to sleep deprivation, reflected by elevated circulating granulocyte levels, in blood samples obtained from the same laboratory study (Ackermann et al., 2012). Together, our data support the view that sleep deprivation can be regarded as a strong external stressor and immune challenge (Faraut et al., 2012; Mullington et al., 2010).

The clock gene expression data collected in our study are in agreement with previous reports concerning the timing of clock gene rhythms in a regulate sleep/wake cycle and the proportion of individual significant rhythms for a given gene (see e.g. Archer et al., 2008). Our current data, including the in-depth characterization of the expression levels of 11 clock genes during normal sleep-wake and under sleep deprivation conditions, suggest that the core clock mechanism in peripheral oscillators is compromised under sleep deprivation conditions, as clock gene amplitudes were generally found to be lower compared to the sleep condition. Strongest effects of condition (sleep, sleep deprivation) were found for the expression of *BMAL1*, and to a lesser extent, *CRY1*, both considered core clock components (Hastings et al., 2003), which were acutely suppressed during sleep deprivation. Notably, other high-amplitude clock gene rhythms (e.g. *PER1*, *PER3*, *REV-ERB*α) remained unaffected by sleep deprivation. Our results are in agreement with a study describing diurnal variation of *BMAL1* both under a normal sleep-wake schedule and a night of acute sleep deprivation in light conditions (50 lux) (Kavcic et al., 2011). However, in this study a significant diurnal variation in *PER2* was also reported in the sleep condition, while we found overall

rhythmicity for *PER1* and *PER3*, but not for *PER2*, under both sleep and sleep deprivation conditions. By contrast, James et al. (2007) reported comparable 24 h rhythmicity for both *PER1* and *PER2* under sleep/wake and constant routine sleep deprivation conditions, which is in line with our data on *PER1* and *PER3* (James et al., 2007). However, in contrast to the current results, a change in *BMAL1* peak expression was observed between sleep deprivation and habitual sleep (James et al., 2007), whereas the current study showed no change in *BMAL1* peak time but in amplitude. It should be noted that the sampling intervals chosen for the current study (from 12:00 h on day 2 to 12:00 h on day 4 after the sleep deprivation) must be considered a limitation as it might not have revealed the full effect of the sleep deprivation.

Taken together, our data show that one night of sleep deprivation has a significant impact on both the melatonin profile and the rhythmic expression of certain clock genes in human peripheral leukocytes. Further studies are needed for a better mechanistic understanding of the interactions between sleep deprivation, the circadian system, and the potential impact on immunity.

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Tables

Table 1: Single cosinor analysis of gene expression profiles. Given are the numbers of subjects (out of 12) with statistically significant rhythms ($p < .05$) per condition for each of the genes investigated, and the average amplitude and acrophase (decimal time) of those subjects.

Gene	Sleep	Deprivation	Collapsed	Av. Amplitude	Av. Acrophase
PER1	3	3	8	.87	09.55
PER2	3	3	2	1.09	04.53
PER3	8	7	8	1.09	04.64
CRY1	3	2	0	1.08	03.20
CRY2	5	3	2	1.09	00.07
BMAL1	6	3	6	.99	17.99
CLOCK	5	2	2	1.03	22.20
REV-ERBα	7	6	9	1.01	01.57
RORA	1	2	1	1.21	06.88
DEC1	0	2	1	.98	06.36
DBP	3	1	1	1.14	00.87
HSPA1B	5	1	4	1.00	17.15

Table 2: Summary of non-linear curve fitting results. Given are the calculated amplitudes and acrophases (decimal time) for the three datasets (sleep, sleep deprivation, collapsed data). Calculations for individual phase-adjustment were performed using the average melatonin peak time on the baseline sleep night (N2). Bold numbers indicate significance after Bonferroni correction for multiple testing ($p < .0042$).

Gene	Condition	Amplitude (peak-adjusted)	Acrophase (peak-adjusted)
PER1	Sleep	**.68**	**08.12**
	Deprivation	**.69**	**07.66**
	Collapsed	**.67**	**07.99**
PER2	Sleep	.26	07.52
	Deprivation	.31	**06.39**
	Collapsed	**.26**	**06.57**
PER3	Sleep	**.75**	**03.52**
	Deprivation	**.70**	**04.47**
	Collapsed	**.73**	**03.93**
CRY1	Sleep	**.34**	21.62
	Deprivation	.09	07.61
	Collapsed	.16	22.50
CRY2	Sleep	.17	21.33
	Deprivation	.27	**06.23**
	Collapsed	.11	02.43
BMAL1	Sleep	**.75**	**17.86**
	Deprivation	.22	14.12
	Collapsed	**.45**	**17.17**
CLOCK	Sleep	**.46**	**20.32**
	Deprivation	.10	01.75
	Collapsed	**.28**	21.44
REV-ERBα	Sleep	**.76**	23.60
	Deprivation	**.49**	01.19
	Collapsed	**.66**	00.23
RORA	Sleep	.24	18.98
	Deprivation	.22	22.12
	Collapsed	**.25**	20.78
DEC1	Sleep	.29	13.27
	Deprivation	**.35**	08.85
	Collapsed	.21	10.96
DBP	Sleep	**.34**	23.60
	Deprivation	.27	03.76
	Collapsed	**.28**	01.23
HSPA1B	Sleep	**.66**	**16.77**
	Deprivation	**.32**	**16.36**
	Collapsed	**.50**	**16.63**

Figure legends

Figure 1: Study protocol. Black bars indicate sleep periods, 0 lux; grey bars, wake periods, semi-recumbent position, < 5 lux; white bars, awake and free movement, 100 lux; ▲controlled meals; ▬ blood sampling period. Reprinted with permission from *SLEEP*.

Figure 2: Individual cortisol and melatonin acrophase (top) and amplitude (bottom) values calculated by cosinor analysis for the 12 study participants during sleep (N2) and sleep deprivation (N3).

Figure 3: Individual relative expression levels (n = 12) of *HSPA1B* and 11 clock genes (*PER1-3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *REV-ERBα*, *DEC1*, *RORA* and *DBP*) during the 48 h sampling protocol including a night of sleep and sleep deprivation. The black bars indicate sleep periods, 0 lux; grey bars, wake periods, semi-recumbent position, < 5 lux; white bars, awake and free movement, 100 lux. For each gene the normalized z-scored values are plotted for each participant. Note the delayed peak times for *PER1* in 2 of the subjects, who also showed delayed timing of their melatonin rhythms.

Figures

Figure 1

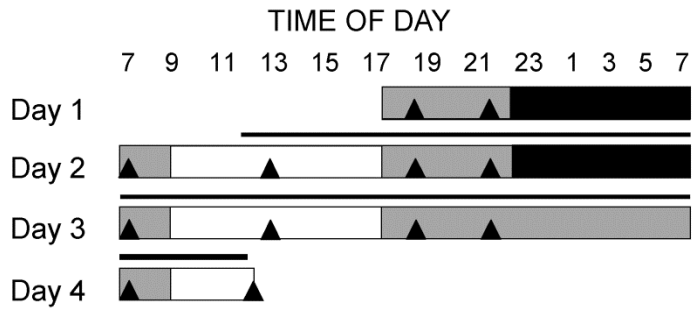


Figure 2

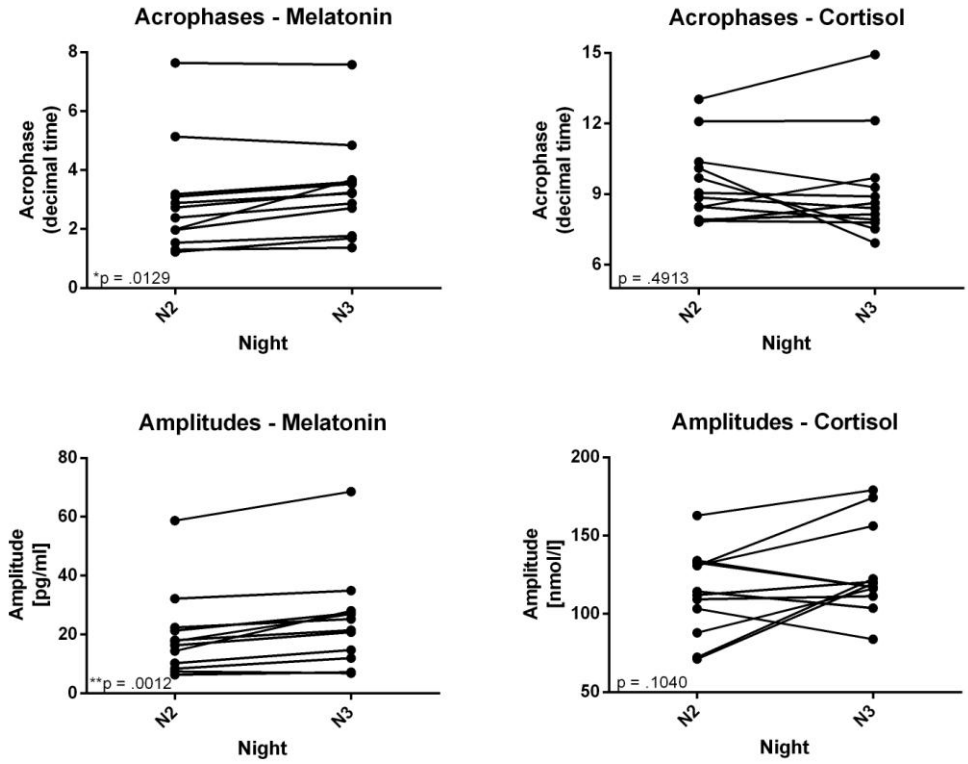
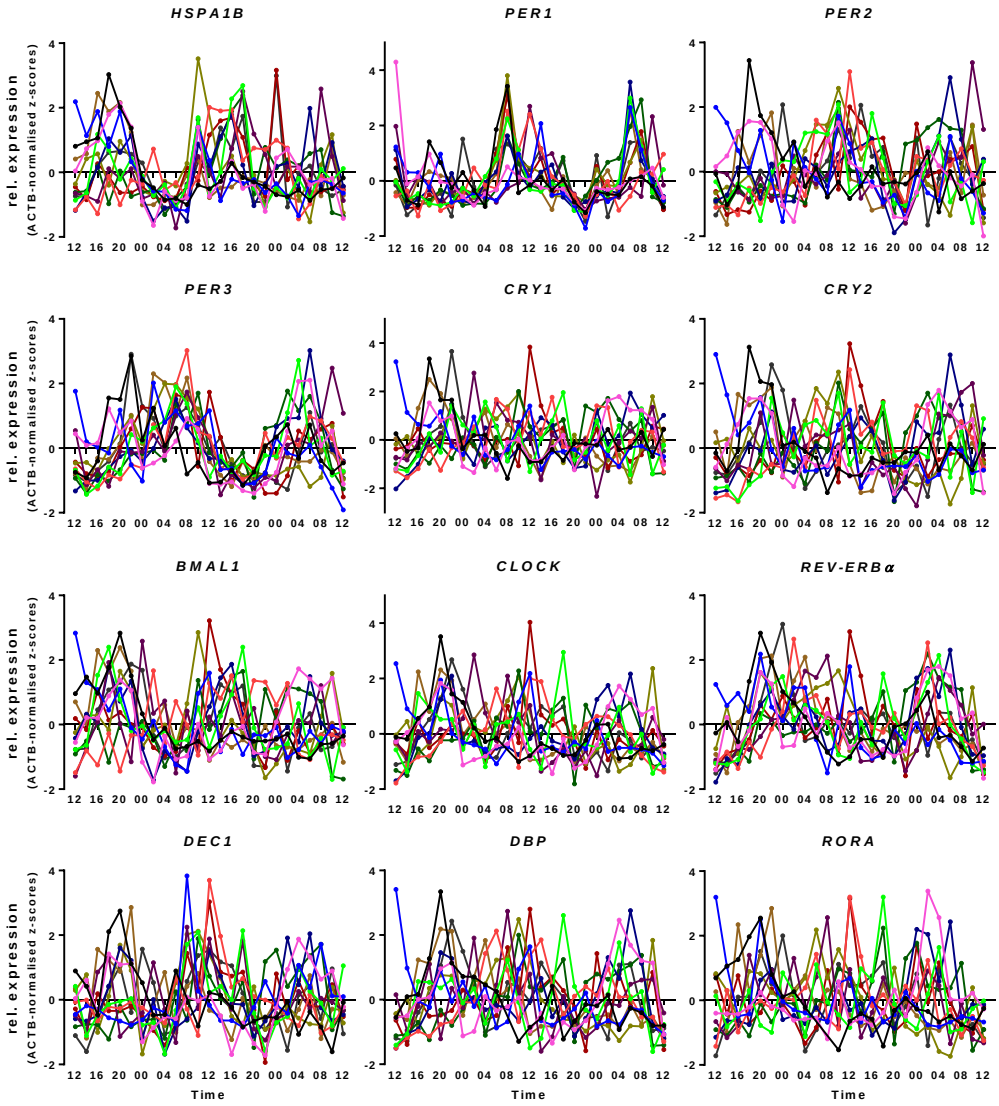

Acrophases - Melatonin
Acrophase (decimal time)
8
6
4
2
0
*p = .0129
N2
N3
Night
Acrophases - Cortisol
Acrophase (decimal time)
15
12
9
6
p = .4913
N2
N3
Night
Amplitudes - Melatonin
Amplitude [pg/ml]
80
60
40
20
0
**p = .0012
N2
N3
Night
Amplitudes - Cortisol
Amplitude [nmol/l]
200
150
100
50
p = .1040
N2
N3
Night

Figure 3


HSPA1B
PER1
PER2
PER3
CRY1
CRY2
BMAL1
CLOCK
REV-ERBα
DEC1
DBP
RORA
rel. expression
(ACTB-normalised z-scores)
4
2
0
-2
12 16 20 00 04 08 12 16 20 00 04 08 12
Time

Supplementary Material

Effect of sleep deprivation on rhythms of clock gene expression and melatonin in humans

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Supplementary Figure S1: Individual cortisol rhythms

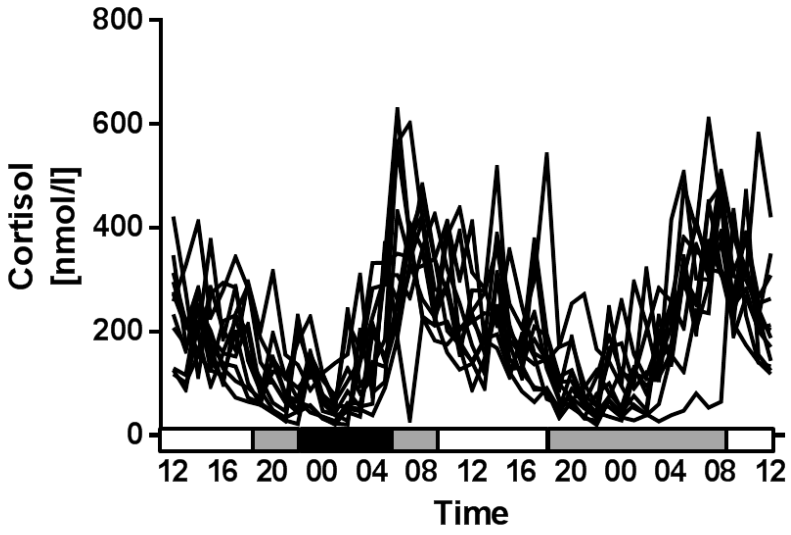

Figure S1: Individual cortisol rhythms (n = 12) over the 48 h sampling protocol. The black bars indicate sleep periods, 0 lux; grey bars, wake periods, semi-recumbent position, < 5 lux; white bars, awake and free movement, 100 lux.

Supplementary Figure S2: Individual melatonin rhythms

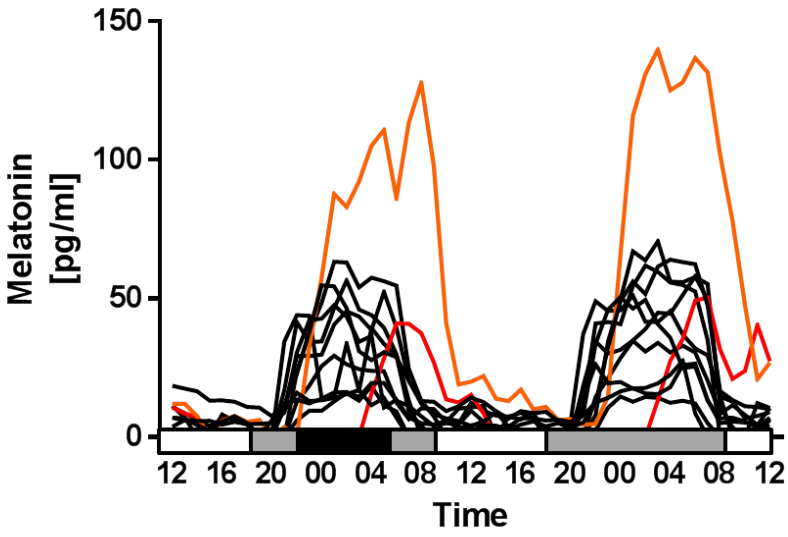

Figure S2: Individual melatonin rhythms (n = 12) over the 48 h sampling protocol. The two subjects which are phase-delayed compared to the other participants are highlighted in orange/red. The black bars indicate sleep periods, 0 lux; grey bars, wake periods, semi-recumbent position, < 5 lux; white bars, awake and free movement, 100 lux.

Supplementary Figure S3: Melatonin AUC

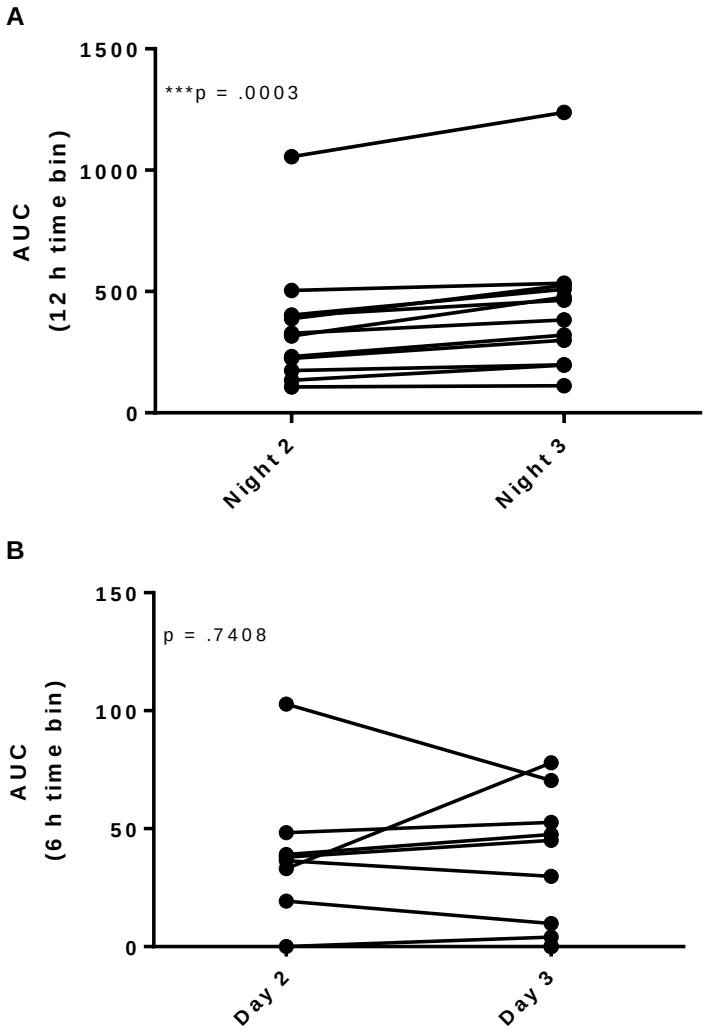

Figure S3: Area under the curve (AUC) as integrated from individual melatonin profiles for a 12 h night-time bin (A) and a 6 h daytime bin (B) on day 2/night 2 versus day 3/night 3.

Supplementary Figure S4: Melatonin DLMO

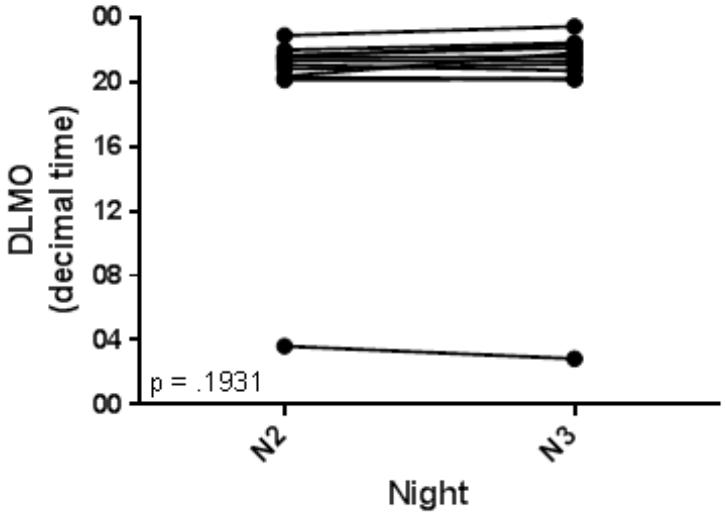

Figure S4: Calculated dim light melatonin onsets (DLMO) on N2 and N3. One of the subjects shows a very late DLMO in the morning hours on both nights.

Supplementary Table S1
Primer sequences and amplicon lengths of all genes investigated, and the reference gene *ACTB*.

Gene	Primer forward (5' → 3')	Primer reverse (5' → 3')	amplicon [bp]
HSPA1B	CTGTACCAGGGTGCCGGTGGT	AGTCCCAACAGTCCACCTCAAAGAC	148
PER1	GGACACTCCTGCGACCAGGTACTG	GGCAGAGAGGCCACCACGGAT	126
PER2	CGCAGGGTGCGCTCGTTTGA	GGGCTCTGGAACGAAGCTTTCG	105
PER3	GGTCGGGCATAAGCCAATG	GTGTTTAAATTCTTCCGAGGTCAAA	143
CRY1	TCCCAGGTTGTAGCAGCAGTGGA	TGCCCATGGAGCTTCTTCCTTGC	114
Cry2	CCTACCTGCGCTTTGGTTGT	TGCTGTTCCGCTTCACCTTT	84
BMAL1	GCCTACTATCAGGCCAGGCTCA	AGCCATTGCTGCCTCATCATTAC	149
CLOCK	TTGGCAAAATGTCATGAGCAC	TTGCCCCTTAGTCAGGAACCT	78
REV-ERBα	GCGGCGATCGCAACCTCTAGT	GTAGGTGATGACGCCACCTGTGTT	115
DEC1	GCACGGAGACCTACCAGGGATGT	CGATGAGCCGGTGCGGCAAT	119
DBP	GCCGGAGGAGCAGAAGGATGAGAAA	AAGGCCGCCCGCACCGATAT	120
RORA	GAGGCAAGAGTGCCGTGGTCAA	GGTTGAAGAGCTTCTGGCTCCTTC	108
ACTB	TGACCCAGATCATGTTTGAG	CGTACAGGGATAGCACAG	74

Supplementary Table S2
Single cosinor analysis of cortisol profiles. Given are amplitude, acrophase (in decimal time), p-value and % rhythm for the sleep condition (first 24 h of sampling), the sleep deprivation condition (second 24 h of sampling), and the combined dataset (collapsed, 48 h of sampling) for the 12 study participants. Note the delayed phase in subjects R29 and R30.

Subject	Condition	Amplitude	Acrophase	P-value	% Rhythm	
R04	Sleep	71.22	8.46	.013418	32.4	*
	Deprivation	119.39	7.91	.000052	59.2	***
	Collapsed	96.32	8.20	.000001	46.0	***
R06	Sleep	134.10	10.10	.000019	62.8	***
	Deprivation	117.17	6.92	.000304	52.1	***
	Collapsed	116.13	8.67	.000000	47.3	***
R07	Sleep	112.10	8.85	.000063	58.5	***
	Deprivation	120.54	8.40	.000022	62.4	***
	Collapsed	118.52	8.71	.000000	61.8	***
R08	Sleep	109.34	9.69	.000013	65.8	***
	Deprivation	111.27	7.52	.000074	59.6	***
	Collapsed	105.91	8.59	.000000	55.3	***
R10	Sleep	132.67	10.37	.000020	62.6	***
	Deprivation	117.06	9.29	.000693	48.4	***
	Collapsed	127.76	9.94	.000000	56.2	***
R12	Sleep	130.77	9.05	.000503	49.9	***
	Deprivation	174.31	8.90	.000015	63.6	***
	Collapsed	154.10	9.01	.000000	56.9	***
R29	Sleep	114.35	13.03	.000357	51.4	***
	Deprivation	103.73	14.93	.001164	45.9	**
	Collapsed	104.04	13.97	.000002	44.1	***
R30	Sleep	162.81	12.09	.000000	84.7	***
	Deprivation	179.10	12.12	.000021	62.4	***
	Collapsed	170.16	12.11	.000000	69.4	***
R32	Sleep	130.98	7.91	.000002	70.0	***
	Deprivation	156.22	8.15	.000000	79.6	***
	Collapsed	144.51	8.09	.000000	74.2	***
R33	Sleep	103.30	7.87	.030655	27.2	*
	Deprivation	83.83	7.79	.004386	39.0	**
	Collapsed	96.46	8.05	.000107	32.8	***
R39	Sleep	87.96	8.45	.032099	26.8	*
	Deprivation	116.20	9.69	.000012	64.2	***
	Collapsed	102.45	9.21	.000009	39.6	***
R41	Sleep	72.37	7.81	.002721	41.5	**
	Deprivation	122.44	8.62	.000205	53.8	***
	Collapsed	98.55	8.41	.000000	47.0	***

Supplementary Table S3
Single cosinor analysis of melatonin profiles. Given are amplitude, acrophase (in decimal time), p-value and % rhythm for the sleep condition (first 24 h of sampling), the sleep deprivation condition (second 24 h of sampling), and the combined dataset (collapsed, 48 h of sampling) for the 12 study participants. Note the delayed phase in subjects R29 and R30.

Subject	Condition	Amplitude	Acrophase	P-value	% Rhythm	
R04	Sleep	21.30	1.97	.000002	70.3	***
	Deprivation	27.25	2.71	.000001	73.0	***
	Collapsed	24.48	2.35	.000000	69.8	***
R06	Sleep	22.36	3.10	.000000	84.2	***
	Deprivation	25.25	3.53	.000000	84.3	***
	Collapsed	23.77	3.33	.000000	83.1	***
R07	Sleep	17.69	1.54	.000027	61.6	***
	Deprivation	26.97	1.77	.000000	88.0	***
	Collapsed	22.45	1.67	.000000	72.2	***
R08	Sleep	7.36	2.38	.000001	72.3	***
	Deprivation	6.85	2.87	.000010	66.7	***
	Collapsed	7.09	2.62	.000000	69.0	***
R10	Sleep	32.19	2.88	.000000	84.3	***
	Deprivation	34.92	3.21	.000000	81.0	***
	Collapsed	33.57	3.05	.000000	81.9	***
R12	Sleep	10.25	3.18	.000021	62.5	***
	Deprivation	14.72	3.60	.000000	73.6	***
	Collapsed	12.59	3.38	.000000	66.3	***
R29	Sleep	16.27	7.64	.000003	68.5	***
	Deprivation	20.76	7.58	.000000	73.5	***
	Collapsed	18.58	7.64	.000000	69.7	***
R30	Sleep	58.72	5.14	.000000	85.7	***
	Deprivation	68.54	4.84	.000000	84.8	***
	Collapsed	63.43	5.01	.000000	83.6	***
R32	Sleep	8.37	1.30	.000000	81.8	***
	Deprivation	11.99	1.37	.000000	75.0	***
	Collapsed	10.27	1.33	.000000	73.0	***
R33	Sleep	18.05	1.22	.000002	69.6	***
	Deprivation	21.45	1.69	.000000	78.1	***
	Collapsed	19.91	1.46	.000000	73.2	***
R39	Sleep	14.38	1.99	.000085	57.3	***
	Deprivation	28.03	3.67	.000000	78.5	***
	Collapsed	20.86	3.09	.000000	62.5	***
R41	Sleep	6.27	2.73	.000001	71.3	***
	Deprivation	7.20	3.24	.000011	64.6	***
	Collapsed	6.83	2.94	.000000	66.8	***

Supplementary Table S4
Summary of non-linear curve fitting results. Given are the calculated amplitudes and acrophases (decimal time) for the three datasets used (sleep, sleep deprivation, collapsed data). Calculations for individual phase-adjustment were performed using the average DLMO on N2. Bold numbers indicate significance after Bonferroni correction for multiple testing ($p < .0042$).

Gene	Condition	Amplitude (DLMO-shifted)	Acrophase (DLMO-shifted)
PER1	Sleep	**.73**	**08.17**
	Deprivation	**.68**	**07.58**
	Collapsed	**.69**	**08.00**
PER2	Sleep	.32	**07.30**
	Deprivation	.29	**06.27**
	Collapsed	**.28**	**06.47**
PER3	Sleep	**.78**	**03.83**
	Deprivation	**.70**	**04.42**
	Collapsed	**.75**	**04.04**
CRY1	Sleep	.30	22.01
	Deprivation	.07	06.40
	Collapsed	.16	22.97
CRY2	Sleep	.12	22.21
	Deprivation	.23	**05.87**
	Collapsed	.12	02.71
BMAL1	Sleep	**.69**	**17.85**
	Deprivation	.17	14.85
	Collapsed	**.41**	**17.41**
CLOCK	Sleep	**.40**	**20.65**
	Deprivation	.14	01.34
	Collapsed	**.27**	21.94
REV-ERBα	Sleep	**.74**	23.85
	Deprivation	**.52**	01.15
	Collapsed	**.66**	00.35
RORA	Sleep	.22	19.28
	Deprivation	.23	21.96
	Collapsed	**.25**	20.98
DEC1	Sleep	.24	13.11
	Deprivation	.30	08.46
	Collapsed	.17	10.42
DBP	Sleep	**.34**	00.41
	Deprivation	.28	03.38
	Collapsed	**.31**	01.53
HSPA1B	Sleep	**.63**	**16.79**
	Deprivation	**.33**	**16.73**
	Collapsed	**.49**	**16.76**