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Chronobiological characterization of women with primary vasospastic syndrome: body heat loss capacity in relation to sleep initiation and phase of entrainment

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Vollenweider S, Wirz-Justice A, Flammer J, Orgül S, Kräuchi K. Chronobiological characterization of women with primary vasospastic syndrome: body heat loss capacity in relation to sleep initiation and phase of entrainment. *Am J Physiol Regul Integr Comp Physiol* 294: R630–R638, 2008. First published November 28, 2007; doi:10.1152/ajpregu.00609.2007.—Women with primary vasospastic syndrome (VS), but otherwise healthy, exhibit a functional disorder of vascular regulation (main symptom: cold extremities) and often suffer from difficulties initiating sleep (DIS). Diverse studies have shown a close association between distal vasodilatation before lights off and a rapid onset of sleep. Therefore, we hypothesized that DIS in women with VS could be due to a reduced heat loss capacity in the evening, i.e., subjects are physiologically not ready for sleep. The aim of the study was to elucidate whether women having both VS and DIS (WVD) or not (controls) show different circadian characteristics (e.g., phase delay of the circadian thermoregulatory system with respect to the sleep-wake cycle). Healthy young women ($n = 9$ WVD and $n = 9$ control) completed a 40-h constant routine protocol (adjusted to habitual bedtime) before and after an 8-h sleep episode. Skin temperatures [off-line calculated as distal-proximal skin temperature gradient (DPG)] and core body temperature (CBT; rectal) were continuously recorded. Half-hourly saliva samples were collected for melatonin assay and subjective sleepiness was assessed on the Karolinska Sleepiness Scale (KSS). Compared with control, WVD showed no differences in habitual bed times, but a 1-h circadian phase delay of dim light-melatonin onset (hours after lights on: WVD 14.6 ± 0.3 h; control 13.5 ± 0.2 h; $P = 0.01$). Similar phase shifts were observed in CBT, DPG, and KSS ratings. In conclusion, WVD exhibit a phase delay of the endogenous circadian system with respect to their habitual sleep-wake cycle, which could be a cause of DIS.

thermoregulation; sleep-onset insomnia; cold extremities; circadian rhythm

IN HUMANS AND OTHER mammals, circadian rhythms are generated by a self-sustaining circadian pacemaker located in the suprachiasmatic nuclei of the anterior hypothalamus (30). The suprachiasmatic nuclei drives circadian rhythms in physiological processes, which are synchronized to the outside world mainly by the solar light-dark cycle. Core body temperature (CBT) has been investigated as a robust and convenient circadian marker rhythm in humans (4, 64, 67). However, the circadian rhythm of CBT is more than a marker (17, 63); thermoregulatory changes are intimately coupled to sleepiness and sleep induction (see below).

The homeostatic control of CBT is mediated by a hierarchically organized set of neuronal mechanisms, with the preoptic anterior hypothalamus at the top of the hierarchy (59). CBT is regulated within narrow limits around 37°C by a complex feedback system (57). To keep CBT within these limits, preoptic anterior hypothalamus can activate heat loss (e.g., distal vasodilatation, sweating) and heat gain mechanisms (e.g., distal vasoconstriction, metabolic heat production). In addition to the homeostatic regulation, a rostral projection from the suprachiasmatic nuclei to the preoptic areas provides circadian modulation of CBT (pre50). The circadian rhythm of CBT is determined by both changes in heat production and heat loss. CBT declines when heat loss surpasses heat production in the evening and vice versa in the morning (6, 41). There is growing evidence that body heat loss in the evening via increased distal skin temperatures is the crucial thermoregulatory function for induction of sleepiness and sleep (26, 38). The best indirect marker of this readiness for sleep may be the distal-proximal skin temperature gradient (DPG), a measure that has been externally validated to distal skin blood flow (58). The rise of DPG ~90 min before lights off is a good predictor for a rapid onset of sleep (38). Because the circadian regulation of CBT is intimately coupled with the circadian regulation of sleepiness and sleep induction (12), the phase relationships (phase of entrainment) between endogenous circadian rhythmicity of the thermoregulatory system and the sleep-wake cycle are important for good sleep. Disruption of phase of entrainment can profoundly influence human health, being linked, e.g., to mood disorders, jet lag, coronary heart disease, and sleep disorders, such as difficulties initiating sleep (DIS) (22, 43, 47). Therefore, studying DIS in relation to the phase relationship between endogenous circadian rhythmicity of CBT regulation and the sleep-wake cycle may reveal clues to underlying mechanisms.

In a first of a series of studies, we have chosen a strategy to show this relationship in two selected extreme groups of subjects, one with impaired heat loss capacity [i.e., vasospastic syndrome (VS)] in addition to DIS, compared with a group of controls not having these problems. This extreme group comparison has the advantage of allowing a study with relative small sample sizes in both groups, however, with the limitation that no conclusion can be drawn, whether one of the two symptoms (VS or DIS) alone could also produce similar results. Nevertheless, conclusive interpretation can be drawn with respect to subjects showing the combination of VS and DIS. Subjects with VS represent a part of the general population (mostly women before menopause) with a diathesis of responding with spasm, in particular in the distal extremities

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(hands, feet) to stimuli like cold or emotional stress (24). The VS is a similar but weaker form of Raynaud's disease (the classical symptom of Raynaud's phenomenon, the triphasic color changes of the digits of the hands and feet from white to blue to red, is not necessary for its definition). In a recent epidemiological study carried out in a representative urban Swiss population (age: 20–40 yr) it could be shown that ~30% of women exhibit a VS, in contrast to only 7% of men, and that the relative risk of DIS in these subjects is doubled (34 and Kräuchi K, Fontana P, Vollenweider S, von Arb M, Dubler B, Orgül S, Flammer J, Zemp-Stutz E, unpublished observations). This large survey confirmed our previous findings in a small sample of women with VS and normal tension glaucoma who exhibited significantly prolonged sleep-onset latency (SOL) compared with controls (51, 53). Therefore, prolonged SOL in these subjects could be associated with their impaired capacity for distal vasodilatation and heat loss before habitual bedtime (34). Based on the survey findings, we focused on women.

The purpose of the present study was the chronobiological characterization of women with VS and DIS (WVD) compared with control women, using a constant routine (CR) protocol that controls for the main masking effects such as locomotor activity, large meals, changes in light condition, and posture on the endogenous timing system (15, 20, 41, 48). To clarify the relationship between DIS and VS, three a priori hypotheses were tested, all of which could lead to a higher vasoconstriction level (lower DPG values) before their habitual bedtimes and hence leading to a longer SOL: *1*) a circadian phase delay of the thermoregulatory system, leading to a changed phase of entrainment compared with habitual bedtimes; *2*) a larger circadian amplitude of distal skin temperature (expressed by DPG); and *3*) a normal circadian rhythm of the thermoregulatory system with respect to phase and amplitude but on a generally lower 24-h mean level (e.g., of DPG).

All these possibilities could lead to lower DPG levels before habitual bedtimes, i.e., subjects with VS are simply physiologically not ready for sleep. To test these hypotheses, the circadian time course of CBT, distal and proximal skin temperatures, and subjective ratings of sleepiness were compared with an established circadian reference rhythm, that of melatonin production measured during a CR adjusted to their habitual bedtimes. Melatonin production itself is known to be related to circadian thermoregulation and sleepiness (for reviews, see Refs. 37 and 62.), providing, therefore, additional information about mechanisms of phase of entrainment.

METHODS

Subjects

Two groups of healthy young women (WVD, $n = 9$ and control, $n = 9$) were recruited via poster advertisements at the University of Basel and via announcements on the Internet (for their physiological characteristics, see Table 1). Based on the homogenous selection of the two study groups (control vs. WVD) and on our very stringent controlled CR protocol, the main target variables (SOL, DPG, CBT) can be statistically analyzed with $n = 9$ for each group to have a statistical power of at least 80%. For example, a relevant between-group difference in DPG of 1°C with expected means ± SE of 0.25°C will lead to a power of 80% (e.g., *t*-test); a relevant between-group difference in SOL to *sleep stage 2* of 10 min with expected means ± SE of 2 min will lead to a power of 86% (e.g., *t*-test); similar criteria are valid for all the other variables. Intraindividual changes can be detected even with a higher power, or in other words, smaller standardized differences will be significantly detected (SOL, means ± SE : 2 min, Δ5 min, power: at least 0.8; DPG, means ± SE: 0.2°C, Δ0.5°C, power: at least 0.8). All subjects had to successfully complete the following screening questionnaires: The Torsvall-Åkerstedt morning-evening-type questionnaire (61) and two questionnaires covering sleep habits, sleep quality, life habits, physical health, medical history, and thermophysiological behavior. Exclusion criteria were extreme morning or evening types (M/E-types) (ratings: ≤14 and ≥21 points), chronic or current major medical illness or injury, amenorrhea or irregular menstrual cycles, smoking, intake of over-the-counter or prescription medications (including oral contraceptives or other hormonal treatments) or illicit substances, shift work within 3 mo, or transmeridian travel within 1 mo prior to the study, or excessive caffeine (i.e., >300 mg) and alcohol consumption (i.e., >1 beverage per day).

Subjects who fulfilled the described criteria were subjected to a finger nailfold video capillary microscopy to objectively document their self-ratings about cold or warm extremities, respectively (inclusion criteria: blood standstill for ≥12 s = WVD, <12 s = control) (23, 27). Additionally, the nailfold skin temperature was measured. After a physical examination to exclude any medical disorders, a polysomnographically recorded screening night in the laboratory was performed to test their ability to sleep in a new environment, to exclude primary sleep disorders (i.e., insomnia) and to assess the SOL to *sleep stage 2* (≥20 min for WVD, <15 min for control, see Table 1).

All selected subjects entered the study between the 14th and the 1st day of their menstrual cycle to complete the experiment within the luteal phase. During 7 days before their admission to the laboratory (baseline week) subjects were instructed to maintain a regular sleep-wake schedule (bedtimes and wake times within ± 60 min of self-selected target time scheduled 8 h apart). Adherence to this regular schedule was verified with a wrist activity monitor (Cambridge Neurotechnologies, Cambridge, UK) and sleep-wake logs. They were also instructed to abstain from excessive caffeine and alcohol consumption (definition see above) as well as heavy physical exercise. The nature, purpose, and risks of the study were explained before subjects gave their written consent. It was explicitly permitted to stop the experiment at any time. The study protocol, screening questionnaires, and consent form were approved by the local ethical committee (Ethikkommission beider Basel) for research on human subjects and conformed with the Declaration of Helsinki. All 18 subjects completed the study without any complaints.

Study Design and Protocol

After the baseline week, subjects reported to the laboratory 2 h before their habitual bedtime for an adaptation night (the timing of their sleep-wake schedule was calculated in such a way that the sleep episode was centered at the midpoint of each subject's habitual sleep episode as assessed by actigraphy during the baseline week). They were prepared for continuous polysomnographic and temperature recording. Subjects were allocated to a sound attenuated, air-conditioned chronobiology room controlled for light [<8 lux (typically 3–5 lux at the angle of gaze) during wakefulness and 0 lux during scheduled sleep], ambient air temperature (22°C), and relative humidity (55%). The following 8 h of wakefulness on *day 1* were used to adjust the subjects to the experimental dim light conditions (<8 lux). They were allowed to walk around the laboratory. To assure no light input stronger than 8 lux when they walked out of the dimmed room, they had to wear sunglasses. In the afternoon of *day 1*, after having self-inserted the rectal probe, subjects lay down exactly 30 min before the start of the protocol (8 h before lights off), and the remaining thermocouples were immediately attached. Subjects were covered with a blanket but could adjust their bed covers to maintain thermal

comfort. Isocaloric snacks were given hourly and water was available ad libitum. After a second 8-h sleep episode (*night 1*) , the subjects followed a 40-h CR (*day 2*, *day 3*) with constant wakefulness. After a third 8-h sleep episode (recovery night, *night 3*), the protocol was continued for a further 1.5 h on the morning of *day 4*.

Physiological Measurements

Salivary melatonin. Saliva collections (1–2 ml) were scheduled every 30 min during wakefulness. The samples were immediately refrigerated at 5°C, centrifuged within 2 days and stored at −20°C. A direct double-antibody radioimmunoassay was used for the melatonin assay [validated by gas chromatography-mass spectroscopy with an analytical least detectable dose of 0.65 pm/ml; Bühlmann Laboratories, Schönenbuch, Switzerland (66)].

Subjective ratings of sleepiness. The nine-point Karolinska Sleepiness Scale (KSS) was used to assess subjective sleepiness at half-hour intervals (1).

Thermometry. Temperature data were continuously recorded by a computerized system (System Hofstetter; Allschwil, Switzerland) in 20-s intervals and collapsed off-line into 15-min intervals. Rectal temperature as a measure of CBT was registered by a thermocouple (polyoxymethylene probe: 2-mm diameter, copper-constantan, accuracy: 0.01°C; Interstar, Cham, Switzerland; Therm, type 5500-3, Ahlborn, Holzkirchen, Germany) inserted 10 cm into the rectum and maintained in place by surgical tape. Skin temperatures were also registered by thermocouples (silver disk: 1-cm diameter, copper-constantan, model P224, Prof. Schwamm, Ahlborn; accuracy: 60.01°C; Therm, type 5500-3, Ahlborn) fixed to the skin with thin air-permeable adhesive surgical tape (Fixomull, Beiersdorf, Hamburg, Germany). The body temperatures were measured on nine body sites: rectal (46), midforehead (T_{fh}), 1 cm above the navel (T_{st}), right infraclavicular area (T_{ic}), center of back of hands (T_{ha}), middle of foot insteps (T_{fo}), and midthigh on musculus rectus femoris (T_{th}). Raw data of temperatures were inspected visually, and data segments that were affected, e.g., by probe slips of malfunctioning of the temperature sensors, were removed. These missing data were replaced by value derived from a linear interpolation procedure. To reduce short-term fluctuations and the number of time segments, data were averaged into 15-min bins. For theoretical reasons (5) and because of similarities to our earlier study (41), we combined T_{ha} and T_{fo} to provide an average for the distal skin temperature, and T_{fh}, T_{st}, T_{ic}, and T_{th} for the average proximal skin temperature (T_{prox}). A weighted average was calculated for T_{prox} (according to Ref. 29) with slight modifications: forehead × 0.093, thigh × 0.347, infraclavicular region × 0.266, and stomach × 0.294.

SOL. Sleep was polysomnographically recorded by a digital recording system using the Vitaport digital ambulatory sleep recorder (Vitaport-3 digital recorder; Temec Instruments, Kerkrade, The Netherlands) and sleep stages were visually scored on a 20-s basis according to standard criteria (for details see Ref. 32). The sleep analysis will be published elsewhere. SOL was defined as the time interval between lights off and the occurrence of the first 20-s sleep epoch of *sleep stage 2*.

Data Analysis

Analysis of the dynamics before, during, and after the CR. Analyses of the time course of *day 1* and *night 1* deliver details of how the thermoregulatory system of WVD compared with control differs with respect to sleep induction and sleep. The time course of the thermoregulatory variables over the time span from 2 h after lying down on *day 1* until end of *night 1* (13 h) was analyzed by a two-way ANOVA (33) for repeated measures (rANOVA) with the factor time (13 × 1-h bins) and factor group (WVD vs. control).

The CR protocol reduces, on the one hand, the most important masking effects (e.g., food intake, activity, postural changes) but, on the other hand, also induces other masking effects (e.g., sleepiness, long-term changes of constant bed rest). To reveal possible influences of the CR protocol on the thermoregulatory system, melatonin, and sleepiness, a two-way rANOVA was performed with factor day (*day 2* vs. *day 3*) and factor group (WVD vs. control), each level comprising an 8-h episode between 3 h and 11 h after habitual lights on. The selected timing of the 8-h episodes allows a comparison between *day 2* and *day 3* without possible influences of circadian phase shifts.

Analyses of the time course of *night 3* and *day 4* delivered details of how the thermoregulatory system of WVD differs with control after a 40-h sleep deprivation with respect to recovery sleep and the 1-h episode afterward. The time course of the thermoregulatory variables comprising the time span between lights off on *day 3* until 1 h after lights on on *day 4* (9 h), was analyzed by a two-way rANOVA (33) with the factor time (9 × 1-h bins) and factor group (WVD vs. control). Melatonin and sleepiness were not measured during sleep.

Analysis of phase markers. To ensure that circadian measurements were made under basal conditions, the first 5 h of CR data on *day 2* were excluded from analysis to eliminate any residual effects of sleep on the tested variables (10). To reduce effects of sleep preparations on the tested variables, the last 2 h of data on *day 3* were also omitted. Therefore, data of 33-h CR were analyzed. To determine circadian characteristics, we focused on melatonin production, which provides accurate information about the endogenous circadian rhythm (9).

Dim light-melatonin onset time (DLMO) (44), as determined by linear interpolation of the evening melatonin rise across a 3-pg/ml threshold, was taken to estimate the phase of melatonin production. For analysis, all of the 30-min samples were used. Maximum values were extracted to get information about circadian amplitude of salivary melatonin concentration. As an accurate method to determine phase, amplitude, and mesor of the melatonin rhythm, nonorthogonal spectral analysis was used to fit a three-harmonic model without correlated noise to the data (10, 16, 42). The fitted maximum of the salivary melatonin rhythm was used as a marker of the phase of the endogenous circadian pacemaker. The period of the fundamental component of the model was constrained between 23 and 25 h. For CBT rhythm analysis a two harmonic model with correlated noise was used (10, 16).

The phase relationship between WVD and control regarding CBT, melatonin, DPG, and subjective ratings of sleepiness (KSS), was calculated using cross-correlation analysis. These analyses were performed using the circadian time course during the CR of a 33-h episode starting 5 h after lights on on *day 2* and ending 2 h before lights off on *day 3*. To purify original sleepiness and temperature data from additional long-term trends due to the CR, residuals to a linear regression line were taken for the cross-correlation analysis. Cross-correlations were calculated for time lags of ±480 min. Time lags (Δmin) of maximum or minimum r values were extracted from individual cross-correlation-curves (for details see Ref. 37).

Statistical Analyses

The statistical packages StatView 5.0 and SuperANOVA (Abacus Concepts, Berkeley, CA), and Statistica 6 for Windows (StatSoft) were used.

Analyses of time courses were performed by cross-correlation analyses and by two-way rANOVA with grouping factor group (WVD vs. control) and repeated factor time (or day). All P values derived from rANOVAs were based on Huyhn-Feldt corrected degrees of freedom, but the original degrees of freedom are reported. For post hoc comparisons Fisher's protected least significant differences test with alpha correction for multiple comparisons according to Curran-Everett (14) were calculated. For statistical analyses between WVD and control without an a priori hypothesis, the threshold for alpha errors was set at $P < 0.05$ (two-sided, not especially indicated), otherwise, at $P < 0.1$ (one-sided is indicated in tables). The Mann-Whitney U-test was used to reveal significant differences between WVD and control. Means ± SE values are presented.

RESULTS

Characteristics of Subjects

Table 1 presents the descriptive and inferential statistics for age, body mass index, finger temperature, and data from the sleep/wake diary (including actimetry), sleep questionnaires, and polysomnographic recordings of SOL to *sleep stage 2*. WVD and control do not significantly differ in age and body mass index, whereas the measured finger temperatures of WVD are significantly lower. Neither habitual time of lights off and lights on nor M/E-type differ significantly, indicating no differences between the two groups in their sleep-wake cycle. The subjective rating of DIS in WVD was polysomnographically confirmed by significantly longer sleep-onset latencies not only in the screening night and *night 1* but also in *night 3* even after 40-h sleep deprivation.

Analysis of Salivary Melatonin, CBT, Skin Temperatures, and Sleepiness

Analysis of the time course of the first 5 h CR on *day 1* provides information about the transition phase from daily life to the controlled CR condition. Analysis of the time course of the succeeding night (*night 1*) delivers details as to how the thermoregulatory system of WVD differs compared with control with respect to sleep induction and sleep. For the CR, systematic changes from *day 2* to *day 3* (e.g., caused by long bed rest and sleep deprivation) were tested. Additionally, the influence of recovery sleep on the variables was tested during *night 3* and 1 h afterward.

WVD are compared with control also with respect to circadian amplitude and phase of melatonin and CBT. The time span between 5 h after lights on on *day 2* and 2 h before lights off on *day 3* was analyzed (33 h). DLMO, and sinusoid-based analyses of melatonin and CBT were performed to determine circadian phase, and cross-correlation analyses were used to define phase relationships between the variables (phase of entrainment).

Table 1. *Physiological characterization of the study participants*

Variable	Control	WVD	*P* Values
Age, yr	25.1±1.7	24.2±1.2	0.50
BMI, kg/m^2	20.85±0.6	20.82±0.54	0.96
Morning/evening-type (Ref. 61)	16.7±0.9	16.0±1.6	0.86
Finger temperature, °C	32.83±0.49	28.5±0.99	0.002†
Habitual lights off time, clock time	23:46±0:07	23:25±0:12	0.17
Habitual lights on time, clock time	07:44±0:07	07:24±0:12	0.11
SOL baseline week, subjective ratings, minutes	15.02±3.27	31.59±4.46	0.0025†
SOL screening night, polysomnography, minutes	10.04±1.14	37.41±10.47	0.0001†
SOL *night 1*, polysomnography, minutes	8.82±1.24	19.11±3.54	0.01†
SOL *night 3*, polysomnography, minutes	5.37±1.13	9.78±1.48	0.015†

Values are means ± SE. Polysomnographically obtained sleep-onset latency (SOL) refers to the interval between lights off and the first epoch of *sleep stage 2*. WVD, women with both vasospatic syndrome and difficulties initiating sleep. *P* values are by Mann-Whitney *U*-test and are control vs. WVD. †One-sided.

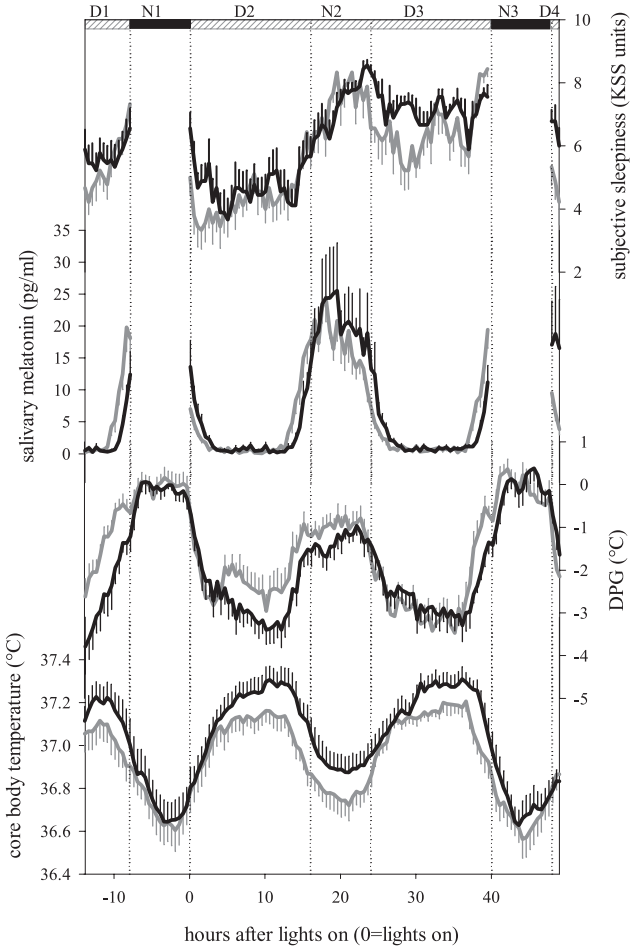

Fig. 1. Subjective sleepiness, melatonin, and core body temperature waveforms averaged with respect to usual wake time for control and women with both vasopatic syndrome and difficulties initiating sleep (WVD). Black wave, WVD (n = 9); gray wave, control (n = 9). Black bar on the top, time of scheduled sleep episode. Data were plotted with respect to scheduled wake time, with scheduled wake time assigned a value of 0 h. Temperature data were first averaged in 15-min bins for each subject; data for all subjects in each group were then averaged, and the mean ± SE is shown. KSS, Karolinska Sleepiness Scale; DPG, distal-proximal skin temperature gradient.

Salivary Melatonin

Half-hour mean values of salivary melatonin concentration with typical high levels during the night are shown in Fig. 1.

Extracted DLMO time of WVD occurs significantly later than in control [pooled DLMO (*days 1*, *2,* and *3*), hours after lights on: 14.63 ± 0.30 h and 13.54 ± 0.23 h, main effect group: $F_{1,16}$ = 8.31; P = 0.01]. WVD and control do not significantly differ with respect to the experimental days (main effect day: $F_{2,16}$ = 0.89; P = 0.42), nor reveal any interaction term significance (day× group: $F_{1,32}$ = 0.04; P= 0.96). This analysis indicates that WVD compared with control exhibit a phase-delayed circadian rhythm in salivary melatonin concentrations.

Two-way rANOVA for *day 1* reveals a significant interaction term time × group (Table 2), which also can be interpreted as a later onset of melatonin production in WVD than in control. This interpretation could be confirmed in further analyses (see below). Additionally, significant main effects time and group are found emphasizing the strong influence of the

Table 2. *Effect of sleep on thermoregulatory variables, subjective ratings of sleepiness (KSS), and salivary melatonin of control and WVD during day 1 and night 1*

Variable	Group		Time		Time × Group	
CBT	$F_{1,16} = 0.35$	$P = 0.56$	$F_{12,192} = 81.15$	$P < 0.0001$	$F_{12,192} = 1.12$	$P = 0.35$
DPG	$F_{1,16} = 3.98$	$P = 0.03$†	$F_{12,192} = 50.63$	$P < 0.0001$	$F_{12,192} = 3.32$	$P = 0.0001$†
KSS	$F_{1,16} = 0.30$	$P = 0.3$†	$F_{6,96} = 4.53$	$P = 0.0002$	$F_{6,96} = 1.39$	$P = 0.115$†
Melatonin	$F_{1,16} = 6.95$	$P < 0.02$	$F_{6,96} = 51.07$	$P < 0.0001$	$F_{6,96} = 6.28$	$P < 0.0001$

Two-way repeated-measures ANOVAs (rANOVAs) with factors group (control vs. WVD), and time (for the thermoregulatory variables: 5 h before lights off until 8 h after lights off [total 13 × 1 h-bins]; for melatonin and KSS: 7 h before lights off until lights off [total 7 × 1 h-bins]). Melatonin and KSS were measured only during the wake phase; therefore, degrees of freedom of factor time is 6 for these variables. CBT, core body temperature; DPG, distal-proximal skin temperature gradient; KSS, Karolinska Sleepiness Scale. †One-sided.

circadian modulation of melatonin production and the phase shift between WVD and control. Neither the CR protocol (including 24-h wakefulness and sustained supine posture; *day 1* vs. *day 2*; Table 3) nor the first hour after recovery sleep (*night 3*) reveals significant differences between WVD and control (Table 4).

A three-harmonic nonorthogonal spectral model without correlated noise was used to estimate the circadian phase, amplitude, and 24-h mean level. This model reveals a significant phase delay for the fitted maximum of the melatonin rhythm of WVD compared with control (19.19 ± 0.37 h and 18.02 ± 0.36 h after lights on, $P < 0.01$). No significant differences regarding fitted amplitude (WVD: 13.0 ± 3.3 pg/ml vs. control: 11.9 ± 1.4 pg/ml; $P = 0.57$) and 24-h mean level (WVD: 8.8 ± 2.0 pg/ml vs. control: 7.7 ± 0.8 pg/ml; $P = 0.76$) were found.

To provide additional information regarding the phase relationship between control and WVD, cross-correlation analyses were performed with averaged melatonin values (control, $n = 9$ subjects) as the reference rhythm (Table 5). A significant phase delay was found between WVD and control (−51.66 ± 12.53 min $P = 0.003$; Table 5).

Core Body Temperature

Typical time courses of CBT (15-min bins) for WVD and control are shown in Fig. 1. The time course of CBT on *day 1* and *night 1* do not statistically differ between WVD and control (interaction term time × group, not significant and main effect group, not significant Table 2). The strong influence of the nightly drop in CBT leads to a significant main effect time (Table 2).

The effect of 24-h wakefulness and sustained supine posture in a CR protocol during a 8-h time segment on *day 2* and *day 3* at a similar circadian phase was tested by a two-way rANOVA. Mean values of 8-h episodes at a circadian phase, which is relatively unaffected by circadian phase shift effects were taken for rANOVA (see METHODS). No significant interaction term day × group and no significant main effects (day and group; both not significant) were found (Table 3). The time course during *night 3* and the first hour afterward does not statistically differ between WVD and control (Table 4).

Possible phase shifts between WVD and control were calculated using a dual-harmonic nonorthogonal spectral model with correlated noise. It yielded a tendency to a difference between WVD and control for the fundamental minimum and maximum of circadian CBT rhythm (21.86 ± 0.40 h vs. 20.78 ± 0.37 h, $P = 0.07$, after lights on, and 9.73 ± 0.41 h vs. 8.57 ± 0.38 h, after lights on, $P = 0.06$). That is, the fundamental minimum of WVD tends to occur later, indicating a phase delay in the circadian CBT rhythm. 24-h mean value tends to remain at a higher level in WVD than control (37.10 ± 0.06°C vs. 36.99 ± 0.05°C, $P = 0.06$). The fitted amplitude of CBT over the CR shows no difference between WVD and control (0.24 ± 0.02°C vs. 0.24 ± 0.02°C, $P = 0.51$).

Cross-correlation analyses exhibit a significant phase delay of CBT rhythm in WVD compared with control (−60.00 ± 21.36 min, $P = 0.02$; Table 5).

DPG

The time course of DPG (15-min bins) for WVD and control are shown in Fig. 1. Table 2 summarizes the results of the two-way rANOVA of *day 1* and *night 1*. A significant interaction term time × group was found. Inspection of the differences between WVD and control reveals significant lower DPG values in the evening before lights off (WVD vs. control mean 5 h segments; −2.65 ± 0.39°C vs. −1.46 ± 0.18°C, $P = 0.015$).

The effect of 24-h wakefulness and sustained supine posture in a CR protocol during a 8-h time segment on *day 2* and *day 3* at a similar circadian phase was tested by a two-way rANOVA. A significant interaction term day × group was found (Table 3). Compared with control, WVD reveal signif-

Table 3. *Effect of 24-h sleep deprivation on thermoregulatory variables, subjective ratings of sleepiness (KSS) and salivary melatonin in control and WVD and comparison of a 8-h episode at the same circadian phase on days 2 and 3 of the constant routine*

Variable	Group		Day		Day × Group	
CBT	$F_{1,16} = 2.13$	$P = 0.16$	$F_{1,16} = 1.02$	$P = 0.33$	$F_{1,16} = 0.16$	$P = 0.70$
DPG	$F_{1,16} = 0.08$	$P = 0.78$	$F_{1,16} = 1.98$	$P = 0.18$	$F_{1,16} = 6.31$	$P = 0.02$
KSS	$F_{1,16} = 1.50$	$P = 0.24$	$F_{1,16} = 78.79$	$P < 0.0001$	$F_{1,16} = 2.04$	$P = 0.17$
Melatonin	$F_{1,16} = 0.36$	$P = 0.56$	$F_{1,16} = 2.90$	$P = 0.11$	$F_{1,16} = 0.04$	$P = 0.84$

Two-way rANOVAs with factors group (control vs. WVD), and day (8 h of *days 2* and *3* for thermoregulatory variables, KSS, and melatonin [3 h after time of lights on until 5 h before lights off].

Table 4. *Effect of sleep on thermoregulatory variables and subjective ratings of sleepiness (KSS) and salivary melatonin of control and WVD during night 3 and the following morning (day 4)*

Variable	Group		Time		Time × Group	
CBT	$F_{1,16} = 0.32$	$P = 0.58$	$F_{8,128} = 8.22$	$P < 0.0001$	$F_{8,128} = 0.81$	$P = 0.471$
DPG	$F_{1,16} = 0.09$	$P = 0.77$	$F_{8,128} = 11.16$	$P < 0.0001$	$F_{8,128} = 2.52$	$P = 0.036$
KSS	$F_{1,16} = 4.73$	$P = 0.045$	$F_{3,48} = 5.72$	$P = 0.0073$	$F_{3,48} = 0.195$	$P = 0.827$
Melatonin	$F_{1,16} = 2.25$	$P = 0.145$	$F_{3,48} = 7.82$	$P < 0.0068$	$F_{3,48} = 1.97$	$P = 0.176$

Two-way rANOVAs with factors group (control vs. WVD), and time (for the thermoregulatory variables: 8 h after lights off and 1 h after lights on [total 9 × 1-h bins]. Melatonin and KSS was measured only during the wake phase: 1.5 h after lights on [total 4 values]).

icant differences only on *day 2* but not on *day 3*. The analysis of the time course during *night 3* and afterward shows 1 h after lights on a significant higher level in WVD than control (significant interaction term: time × group: $P = 0.036$; Table 4).

Subjective Sleepiness

Subjective sleepiness (KSS) on *day 1* shows no difference between WVD and control (no significant main effect group, and interaction term group × time, Table 2). Based on the strong circadian effect on sleepiness in both groups in the evening, a significant main effect time was found (Table 2). A two-way rANOVA for the 8-h time segments on *day 2* and *day 3* reveals in both groups a similar significant increase in sleepiness during the CR protocol (significant main effect day, Table 2). After *night 3* KSS values are significantly higher in WVD than control (significant main effect group, Table 4).

Comparison of Circadian Phase Relationships Between the Variables

To compare circadian phase relationships between the variables (CBT, DPG, and KSS) in relation to melatonin, cross-correlation analyses were performed with residuals after linear detrending (see METHODS; Table 6). Subjective ratings of sleepiness show a significant increase during the CR [two-way rANOVA on KSS: main factor time, $F_{32,512} = 158.98$, $P < 0.0001$] reflecting the effect of sleep deprivation on sleepiness. No difference was found between WVD and control regarding the phase relationships between DPG, KSS, CBT, and the reference melatonin rhythm.

The phase relationships between CBT (pooled WVD and control) and melatonin rhythm revealed a significant phase delay of CBT (-71.3 ± 14.6 min, $P < 0.0001$; Table 6). Similarly, a significant phase delay was found between KSS and melatonin (-110.0 ± 25.8 min, $P = 0.01$; Table 6). In contrast, no phase differences between DPG and melatonin curves were found, indicating phase-locked (inverse) patterns.

Taken together, WVD show, compared with control, a similar circadian phase shift in all variables of about 1 h, whereby the internal phase relationships between them remain constant within the groups. Circadian amplitudes (melatonin and CBT) are not different between WVD and control. The homeostatic aspect of sleepiness regulation is also similar in WVD and control. DPG, a measure of distal vasoconstriction and vasospasms, is significantly lower in WVD than control, at least at the beginning of the CR on *day 1*. During *night 1* the DPG does not differ between WVD and control; however, vasoconstriction in WVD reappears during the next day (*day 2*). On *day 3* and the following night (*night 3*) no differences between WVD and control are found. The short time segment after the recovery night, *night 3*, shows higher DPG and KSS values in WVD than control.

Table 5. *Phase relationship between control and WVD*

Variable	Control	WVD	*P* Values
Melatonin	5.00±10.31	−51.66±12.53	$P = 0.003$†
CBT	0.000±12.99	−60.00±21.36	$P = 0.02$†
DPG	−10.01±8.30	−50.00±15.00	$P = 0.0235$†
KSS	−20.00±16.60	−79.17±36.77	$P = 0.025$†

Values are means ± SE in min ± min. Maximum and minimum lags were extracted from individual cross-correlation curves. The mean time series of control were taken as the reference time series (lag = 0). Individual time series of control and WVD were cross-correlated to the mean time series of control. +lag values, phase advances; −lag values, phase delays. Control values did not statistically differ from 0 (one-sample sign test). *P* values are by Mann-Whitney *U*-test and are control vs. WVD. †One-sided.

DISCUSSION

This DISCUSSION has been structured with respect to the three hypotheses formulated concerning differences in circadian phase, amplitude, and 24-h mean level. The main finding of our study is that women with VS and DIS (WVD) exhibit, compared with controls, a significant phase delay of the circadian system by ~1 h. This finding favors the hypothesis that the circadian physiology in WVD does not sufficiently prepare the body for sleep initiation. This could lead to prolonged SOL found not only in the night directly before the CR, but also after 40-h sleep deprivation in the recovery night, when sleep pressure is high. The phase delay of the circadian system could be measured by diverse variables to a similar extent, i.e., salivary melatonin concentration, CBT, DPG, and subjective ratings of sleepiness (see Table 5). Therefore, a difference in the phase angle between circadian and sleep-wake rhythmicity of WVD [different internal phase of entrainment (56)] could be the cause of DIS in this syndrome.

It is well known that misalignment between the endogenous circadian system and the sleep-wake cycle (difference in phase of entrainment) can lead to sleep disturbances (including DIS), e.g., delayed or advanced sleep phase syndrome (8), shift work sleep disorder (19), jet lag syndrome (65), the non-24-h sleep/wake disorder (60), and extreme M/E-type (7, 21, 49). A condition of marked discrepancy in sleep timing between work and free days is found particularly in E-types (designated "social jet lag"). This leads to a considerable sleep debt on work days, for which they compensate on free days (68). Our large epidemiological survey was able to show that women with VS exhibit not only a prolonged SOL, but also a significant predisposition to E-types and social jet lag (39). All these

Table 6. *Phase relationship between variables*

Variable	Control*	WVD*	*P* Values†	Pooled Control and WVD*	*P* Values‡
CBT	−68.3±16.1	−74.2±25.5	$P = 0.790$	−71.3±14.6	$P < 0.0001$
DPG	−14.2±19.8	−8.34±28.6	$P = 0.534$	−11.3±16.9	$P = 0.45$
KSS	108.3±35.1	−111.7±40.0	$P = 0.965$	−110.0±25.8	$P = 0.01$

Values are means ± SE in min ± min. Maximum and minimum lags were extracted from individual cross-correlation curves. For each variable the mean time series of control and WVD were taken as reference time series, respectively (lag=0). Individual time series of control and WVD were cross-correlated to the mean time series of control and WVD, respectively. *Values are lag to melatonin in minutes. †*P* values are by Mann-Whitney *U*-test and are control vs. WVD. ‡*P* values are by sign test and vs. 0 lag.

disturbances are characterized by differences in internal and external phase of entrainment.

In contrast, we could show that WVD exhibit a selective difference in internal phase of entrainment with no differences in sleep timing (e.g., lights off time) compared with controls. This could indicate that a difference in internal phase of entrainment is crucial for DIS. Furthermore, a difference in internal phase of entrainment includes also changes in the thermoregulatory system relative to the sleep-wake cycle.

Earlier studies have shown that an increase in distal vasodilatation, and hence body heat loss (e.g., induced by exogenous melatonin, mild skin warming etc.), induces sleepiness and sleep initiation (26, 35, 37, 55), thereby changing internal phase of entrainment between the thermoregulatory system and the sleep-wake cycle. Thus, a different internal phase of entrainment, as found in WVD, could be caused by a difference in thermoregulatory heat loss capacity before habitual sleep onset. We have confirmed these controlled laboratory findings in a week-long ambulatory study. Under real-life conditions, WVD showed a lower DPG throughout the day and most relevantly in the evening before sleep onset, together with a prolonged SOL (28).

In addition to the circadian phase difference between WVD and control, the time course of diverse phase markers were also analyzed with respect to circadian amplitude and 24-h mean level. As shown in Fig. 1 not all measured circadian markers show a simple phase delay, as salivary melatonin does. For example, CBT exhibits, in addition to a phase delay (as measured by cross-correlation analysis, Table 5), a tendency to an increase in 24-h mean level. DPG, a measure of distal vasodilatation and heat loss, shows an even more complex pattern. On the first day, 8 h before lights off (which corresponds most closely to real-life conditions), DPG was markedly reduced in WVD compared with control; however, this difference decreases during the following sleep episode. This finding demonstrates the functional vascular disorder in WVD, i.e., vasospasms disappear during the night sleep episode but reappear the next morning. This new vasoconstriction disappears completely during the course of the subsequent CR (see Fig. 1). In other words, compared with the relaxed state of a CR, WVD exhibit an increase in the diurnal amplitude of DPG during normal life. This could be caused by, e.g., an increased activity of the sympathetic branch of the autonomous nervous system. It is well known that the sympathetic innervation of the vascular muscles located in distal arterioles and arteriovenous anastomoses is the main determinant of distal skin blood flow, and hence body heat loss. Therefore, the difference between distal skin blood flow in WVD and control could be caused by changed sympathetic nervous activity.

In previous studies under controlled CR conditions, it has been shown that the homeostatic aspect of sleepiness and sleep regulation does not affect the thermoregulatory system (35). In this study no significant differences between WVD and control could be found in the homeostatic aspect of subjective ratings of sleepiness (KSS) suggesting, conversely, no influences of the thermoregulatory system on the long-term build-up process of sleepiness. First analyses of the sleep EEG before and after the 40-h sleep deprivation reveals no differences between WVD and control with respect to slow wave sleep (*sleep stages 3* and *4*) and slow-wave activity (40). This would indicate no differences in sleep pressure between WVD and control. However, further detailed analysis of the build-up and decay rates of slow-wave activity are necessary to draw final conclusions (18).

Because the study subjects were measured during their luteal phase, it is possible that the described effects are different during the follicular phase. It is well known that estrogens and gestagens exhibit specific effects on the thermoregulatory system; e.g., progesterone increases sympathetically mediated vasoconstriction (13). In a recent survey, women with VS reported that cold hands and feet were not limited to their luteal phase, indicating rather an independence of VS of hormonal status (39) (data not shown). To draw a final conclusion regarding a hormonal influence on the thermoregulatory system in WVD, a replication study during the follicular phase would be needed.

Perspectives and Significance

One implication of our results is that if WVD would delay their bedtimes sufficiently, by at least 1 h, they would have less or little trouble falling asleep. However, most people usually have constraints on their schedules that necessarily require wakening between 0600 to 0800 most mornings. A delayed bedtime would result in even less sleep than usual and be less desirable than suffering from DIS. More reasonably, manipulation of the circadian temperature rhythm by resetting the phase position to earlier could alleviate the vasospasms prior to sleep-onset and concomitant DIS in WVD. Furthermore, we presently investigate whether VS or DIS alone induces the observed differences between WVD and control leading to information of how the thermoregulatory system and sleep initiation interact.

If a phase advance of the circadian rhythm can normalize DIS in WVD, it will provide a potential nonpharmacological therapy to shift the endogenous rhythm using the appropriate stimulus at the right time (e.g., temperature, light, melatonin). Additional and beneficial effects of administering melatonin to

WVD, besides its phase-shifting properties (3, 36, 52), would be its ability to induce distal vasodilatation (37) and its actions on the sympathetic nervous system (2, 11, 52, 54). Another way to relieve WVD of their clinical symptoms is to focus on the putative increased influence of sympathetic activity in this population. That could be done through relaxation techniques, such as suggestion of warmth (31), autogenic (25), and biofeedback training (25, 45).

More knowledge of human circadian thermoregulatory function can be of future relevance for the simple treatment of disorders related to a circadian disturbance, such as delayed sleep-phase syndrome, non-24-h sleep/wake disorder, shift work sleep disorder, jet lag, extreme M/E-types, and social jet lag.

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