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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

Stephanie Vollenweider[1], Anna Wirz-Justice[1], Josef Flammer[2], Selim Orgül[2] and Kurt Kräuchi[1]

[1]Centre for Chronobiology, Psychiatric University Clinics, CH-4025 Basel, Switzerland

[2]University Eye Clinic, CH-4012 Basel, Switzerland

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Address for correspondence:
Kurt Kräuchi
Psychiatric University Clinics
Thermophysiological Chronobiology
Centre for Chronobiology
Wilhelm Klein - Strasse 27
CH-4025 Basel
Switzerland
kurt.kraeuchi@upkbs.ch
Phone: +41613255508
Fax: +41613255577

Abstract

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, CON) 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 CON) completed a 40-h constant routine protocol (CR, adjusted to habitual bedtime), before and after a 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).

In comparison to CON, WVD showed no differences in habitual bed times, but a 1 h circadian phase delay of dim-light-melatonin-onset (h after lights on: WVD 14.6 ± 0.3h; CON 13.5 ± 0.2h; 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.

Keywords: Thermoregulation, sleep onset insomnia, cold extremities, circadian rhythm

Introduction

In humans and other mammals, circadian rhythms are generated by a self-sustaining circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (30). The SCN 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 (64) (67) (4). 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 (POAH) at the top of the hierarchy (59). CBT is regulated within narrow limits around 37°C by a complex feedback system (57). In order to keep CBT within these limits POAH 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 SCN to the preoptic areas provides circadian modulation of CBT (50). 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 (38) (26). The best indirect marker of this readiness for sleep may be the distal-proximal skin temperature gradient (DPG), a measure which has been externally validated to distal skin blood flow (58). The rise of DPG about 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, jetlag, coronary heart disease and sleep disorders, such as difficulties initiating sleep (DIS) (47) (22) (43). 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, comparing with a group of controls having not these problems. This extreme group comparison has the advantage 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 (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 years) it could be shown that about 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), submitted manuscript 2007). 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) in comparison to controls (53) (51). 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 (CON), using a "constant routine protocol" (CR) that controls for the main masking effects such as locomotor activity, large meals, changes in light condition and posture on the endogenous timing system (48) (15) (20) (41). 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 in comparison to habitual bedtimes.
2) A larger circadian amplitude of distal skin temperature (expressed by DPG).
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 review (62) (37)), providing therefore additional information about mechanisms of phase of entrainment.

Methods

Subjects

Two groups of healthy young women (WVD N = 9, and CON 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 (CON 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% e.g. a relevant between-group difference in DPG of 1°C with expected standard errors of the mean (SEM) of 0.25°C will lead to a power of 80% (e.g. t-test); a relevant between-group difference in sleep onset latency to sleep stage 2 (SOL) of 10 min with expected SEM 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, SEM: 2 min, Δ 5min, power: at least 0.8; DPG, SEM: 0.2°C, Δ 0.5°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 months or transmeridian travel within 1 month prior to the study, excessive caffeine (i.e. > 300mg) 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 sec = WVD, < 12 sec = CON) (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 sleep onset latency to sleep stage 2 (≥ 20 min for WVD, < 15 min for CON, see Table 1).

All selected subjects entered the study between the fourteenth and the first day of their menstrual cycle in order 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®, 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 (EKBB) 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 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 (D1) 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 D1, after having self-inserted the rectal probe, subjects laid down exactly 30 minutes 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 bedcovers to maintain thermal comfort. Isocaloric snacks were given hourly and water was available ad libitum. After a second 8 h sleep episode (Night 1 [N1]), the subjects followed a 40 h CR (Day 2 and 3 [D2, D3]) with constant wakefulness. After a third 8 h sleep episode (recovery night, Night 3 [N3]), the protocol was continued for a further 1.5 h on the morning of Day 4 (D4).

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 9-point Karolinska Sleepiness Scale (KSS) was used to assess subjective sleepiness at half-hourly intervals (1).

Thermometry

Temperature data were continuously recorded by a computerized system (System Hofstetter, SHS Allschwil, Switzerland) in 20-sec 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 P 224, 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 9 body sites: Rectal (46), midforehead (T_{fh}), 1cm 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}, 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 x 0.093, thigh x 0.347, infraclavicular region x 0.266, and stomach x 0.294.

Sleep onset latency

Sleep was polysomnographically recorded by a digital recording system using the VITAPORT digital ambulatory sleep recorder (Vitaport-3 digital recorder, TEMEC® Instruments BV, Kerkrade, The Netherlands) and sleep stages were visually scored on a 20-sec basis according to standard criteria (for details see (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-sec sleep epoch of sleep stage 2.

Data Analysis

Analysis of the dynamics before, during and after the CR

Analyses of the time course of D1 and N1 deliver details how the thermoregulatory system of WVD in comparison to CON 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 D1 until end of N1 (13 h) was analyzed by a two-way analysis of variance (33) for repeated measures (rANOVA) with the factor *time* (13 x 1-h bins) and factor *group* (WVD vs. CON).

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). In order to reveal possible influences of the CR protocol on the thermoregulatory system, melatonin and sleepiness, a two-way rANOVA was performed with factor *day* (D2 vs. D3) and factor *group* (WVD vs. CON), 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 D2 and D3 without possible influences of circadian phase shifts.

Analyses of the time course of N3 and D4 deliver details how the thermoregulatory system of WVD differs to CON after a 40-h sleep deprivation with respect to recovery

sleep and the 1-h episode afterwards. The time course of the thermoregulatory variables comprising the time span between lights off on D3 until 1h after lights on on D4 (9 h), was analyzed by a two-way rANOVA (33) with the factor *time* (9 x 1-h bins) and factor *group* (WVD vs. CON). 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 constant routine data on D2 were excluded from analysis to eliminate any residual effects of sleep on the tested variables (10). In order to reduce effects of sleep preparations on the tested variables the last 2 h of data on D3 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 in order to get information about circadian amplitude of salivary melatonin concentration. As an accurate method to determine phase, amplitude and mesor of the melatonin rhythm, non-orthogonal spectral analysis was used to fit a three harmonic model without correlated noise to the data (42) (10) (16). 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 CON 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 D2 and ending 2 h before lights off on D3. 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 (37)).

Statistical analyses

The statistical packages StatView™ 5.0 and SuperANOVA™ (Abacus Concepts, Berkeley, California, USA), and STATISTICA 6 ™ for Windows (StatSoft Inc., Tulsa, USA) were used.

Analyses of time courses were performed by cross-correlation analyses and by two - way rANOVA with grouping factor *group* (WVD vs. CON) 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 PLSD with alpha-correction for multiple comparisons according to Curran-Everett (14) were calculated. For statistical analyses between WVD and CON 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, indicated by †). The Mann-Whitney U-test was used to reveal significant differences between WVD and CON. Means ± SEM values are presented.

Results

Characteristics of subjects

Table 1 presents the descriptive and inferential statistics for age, BMI, finger temperature, and data from the sleep/wake diary (including actimetry), sleep questionnaires and polysomnographic recordings of sleep onset latency to sleep stage 2. WVD and CON do not significantly differ in age and BMI, 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 difficulties initiating sleep in WVD was polysomnographically confirmed by significant longer sleep onset latencies not only in the screening night and N1 but also in N3 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 D1 provides information about the transition phase from daily life to the controlled CR condition. Analysis of the time course of the succeeding night (N1) delivers details as to how the thermoregulatory system of WVD differs in comparison to CON with respect to sleep induction and sleep. For the CR, systematic changes from D2 to D3 (e.g. caused by long bed rest and sleep deprivation) were tested. Additionally, the influence of recovery sleep on the variables was tested during N3 and one hour afterwards.

WVD are compared to CON also with respect to circadian amplitude and phase of melatonin and CBT. The time span between 5 h after lights on on D2 and 2 h before lights off on D3 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).

Salivary Melatonin

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

Extracted DLMO time of WVD occurs significantly later than in CON (pooled DLMO [D1, D2 and D3], 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 CON 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 x group*: $F_{1,32} = 0.04$; $P = 0.96$). This analysis indicates that WVD in comparison to CON exhibit a phase-delayed circadian rhythm in salivary melatonin concentrations.

Two-way rANOVA for D1 reveals a significant interaction term *time x group* (Table 2) which also can be interpreted as a later onset of melatonin production in WVD than in CON (see above). 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 circadian modulation of melatonin production and the phase shift between WVD and CON. Neither the CR protocol (including 24-h wakefulness and sustained supine posture; D1 vs. D2; Table 3) nor the first hour after recovery sleep (N3) reveals significant differences between WVD and CON (Table 4).

A three-harmonic non-orthogonal 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 to CON (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. CON: 11.9 ± 1.4 pg/ml; $P = 0.57$) and 24-h mean level (WVD: 8.8 ± 2.0 pg/ml vs. CON: 7.7 ± 0.8 pg/ml; $P = 0.76$) were found.

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

Core body temperature (CBT)

Typical time courses of CBT (15-min bins) for WVD and CON are shown in Figure 1. The time course of CBT on D1 and N1 do not statistically differ between WVD and CON (interaction term *time x group*, n.s., and main effect *group,* n.s., 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 D2 and D3 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 x group* and no significant main effects (*day* and *group*; both n.s.) were found (Table 3). The time course during N3 and the first hour afterwards does not statistically differ between WVD and CON (Table 4).

Possible phase shifts between WVD and CON were calculated using a dual-harmonic non-orthogonal spectral model with correlated noise. It yielded a tendency to a difference between WVD and CON 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 CON (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 CON (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 CON (-60.00 ± 21.36 min, $P = 0.02$; Table 5).

Distal-proximal skin temperature gradient (DPG)

The time course of DPG (15-min bins) for WVD and CON are shown in Figure 1. Table 2 summarizes the results of the two-way rANOVA of D1 and N1. A significant interaction term *time x group* was found. Inspection of the differences between WVD and CON reveals significant lower DPG values in the evening before lights off (WVD vs. CON 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 D2 and D3 at a similar circadian phase was tested by a two-way rANOVA. A significant interaction term *day x group* was found (Table 3). In comparison to CON, WVD reveal significant differences only on D2 but not on D3. The analysis of the time course during N3 and afterwards shows one hour after lights on a significant higher level in WVD than CON (significant interaction term: *Time x group*: $P = 0.036$; Table 4).

Subjective Sleepiness (KSS)

Subjective sleepiness on D1 shows no difference between WVD and CON (no significant main effect *group*, and interaction term *group x 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 D2 and D3 reveals in both groups a similar significant increase in sleepiness during the CR protocol (significant main effect *day*, Table 2). After N3 KSS values are significantly higher in WVD than CON (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 CON regarding the phase relationships between DPG, KSS, CBT and the reference melatonin rhythm.

The phase relationships between CBT (pooled WVD and CON) and melatonin rhythm reveal 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 in comparison to CON 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 CON. The homeostatic aspect of sleepiness regulation is also similar in WVD and CON. DPG, a measure of distal vasoconstriction and

vasospasms, is significantly lower in WVD than CON, at least at the beginning of the CR on D1. During N1 the DPG does not differ between WVD and CON, however, vasoconstriction in WVD reappears during the next day (D2). On D3 and the following night (N3) no differences between WVD and CON are found. The short time segment after the recovery night N3 shows higher DPG and KSS values in WVD than CON.

DISCUSSION

The discussion section 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 vasospastic syndrome and difficulties initiating sleep (WVD) exhibit in comparison to controls (CON) a significant phase delay of the circadian system by ca. 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 sleep onset latency 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 (see below). 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 (see below).

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), jetlag syndrome (65), the non-24 h sleep/wake disorder (60) and extreme M/E-type (49) (21) (7). A condition of marked discrepancy in sleep timing between work and free days is found particularly in E-types (designated “social jetlag”). 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 jetlag (39). All these 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 (37) (35) (55) (26), 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 CON, the time course of diverse phase markers were also analyzed with respect to circadian amplitude and 24-h mean level. As shown in Figure 1 not all measured circadian markers show a

simple phase delay, as salivary melatonin does. E.g. 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 CON, 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 Figure 1). In other words, in comparison to 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 CON 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 CON 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 CON with respect to slow wave sleep (sleep stages 3 and 4) and slow wave activity (SWA) (40). This would indicate no differences in sleep pressure between WVD and CON.

However, further detailed analysis of the build-up and decay rates of SWA 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 vasospastic syndrome 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 h 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 difficulties initiating sleep in WVD. Furthermore, we presently investigate whether VS or DIS alone induces the observed differences between WVD and CON leading to information how the thermoregulatory system and sleep initiation interacts.

If a phase advance of the circadian rhythm can normalize DIS in WVD, it will provide a potential non-pharmacological 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 (52) (3) (36), would be its ability to induce distal vasodilatation (37) and its actions on the sympathetic nervous system (54) (11) (2) (52). 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, jetlag, extreme M/E-types, and social jetlag.

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LEGENDS

Figure 1. Subjective sleepiness, melatonin, and core body temperature waveforms averaged with respect to usual wake time for CON and WVD. Black, WVD (N=9); gray, CON (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 mean is shown with + or – SEM. DPG, distal-proximal skin temperature gradient.

Table 1. Values are means ± SEM. Polysomnographically obtained sleep onset latency (SOL) refers to the interval between lights off and the first epoch of sleep stage 2. SOL, sleep onset latency to sleep stage 2. †, one-sided.

Table 2. Two-way rANOVAs with factors *group* (CON vs. WVD), and *time* (for the thermoregulatory variables: 5 h before lights off until 8 h after lights off [total 13 x 1 h-bins]; for melatonin and KSS: 7 h before lights off until lights off [total 7 x 1 h-bins]). Melatonin and KSS were measured only during the wake phase; therefore, *df* of factor *time* is 6 for these variables. CBT, core body temperature. DPG, distal-proximal skin temperature gradient. KSS, Karolinska Sleepiness Scale. †, one-sided.

Table 3. Two-way rANOVAs with factors *group* (CON vs. WVD), and *day* (8 h of D2 and D3 for thermoregulatory variables, KSS, and melatonin [3 h after time of lights on until 5 h before lights off]. CBT, core body temperature. DPG, distal-proximal skin temperature gradient. KSS, Karolinska Sleepiness Scale.

Table 4. Two-way rANOVAs with factors *group* (CON vs. WVD), and time (for the thermoregulatory variables: 8 h after lights off and 1 h after lights on [total 9 x 1-h bins]; melatonin and KSS was measured only during the wake phase: 1.5 h after lights on [total 4 values]). CBT, core body temperature. DPG, distal-proximal skin temperature gradient. KSS, Karolinska Sleepiness Scale.

Table 5. Values are means ± SEM in min ± min. Maximum and minimum lags were extracted from individual cross-correlation curves. The mean time series of CON were taken as the reference time series (lag = 0). Individual time series of CON and WVD were cross-correlated to the mean time series of CON. +lag values, phase advances; -lag values, phase delays. CBT, core body temperature. DPG, distal-proximal skin temperature gradient. KSS, Karolinska Sleepiness Scale. CON values did not statistically differ from 0 (one-sample sign test). †, one-sided.

Table 6. Values are means ± SEM in min ± min. Maximum and minimum lags were extracted from individual cross-correlation curves. For each variable the mean time series of CON and WVD were taken as reference time series, respectively (lag=0). Individual time series of CON and WVD were cross-correlated to the mean time series of CON and WVD, respectively. + lag values, phase advances; - lag values, phase delays. CBT, core body temperature. DPG, distal-proximal skin temperature gradient. KSS, Karolinska Sleepiness Scale. MEL, melatonin.

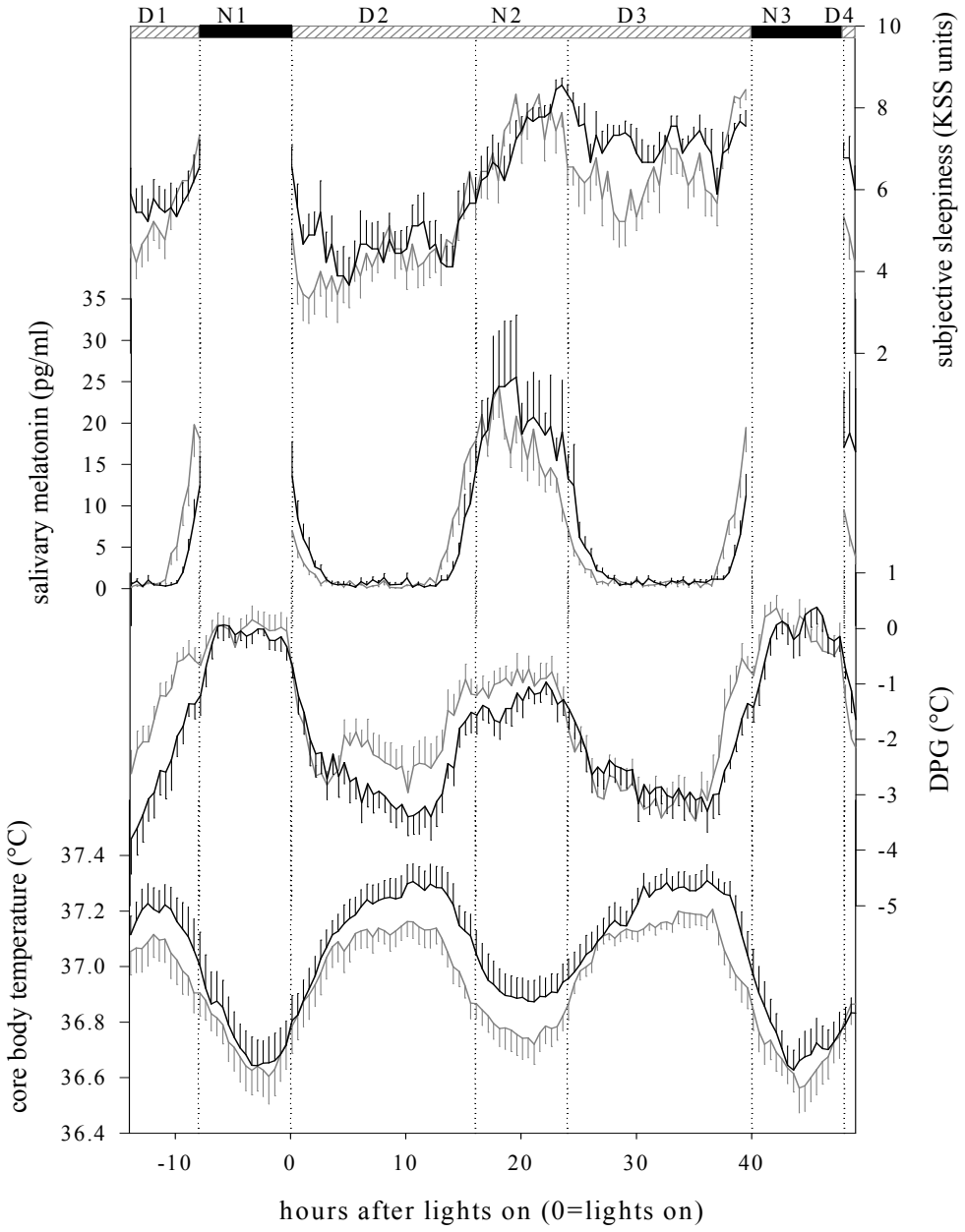

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Table 1. Physiological characterization of the study participants

Variable	CON	WVD	*P* value (U- test) CON vs. WVD
Age, years	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 (61)	16.7 ± 0.9	16.0 ± 1.6	0.86
Fingertemp, °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, min	15.02 ± 3.27	31.59 ± 4.46	**0.0025** †
SOL screening night, polysomnography, min	10.04 ± 1.14	37.41 ± 10.47	**0.0001** †
SOL N1, polysomnography, min	8.82 ± 1.24	19.11 ± 3.54	**0.01** †
SOL N3, polysomnography, min	5.37 ± 1.13	9.78 ± 1.48	**0.015** †

Table 2. Effect of sleep on thermoregulatory variables, subjective ratings of sleepiness (KSS) and salivary melatonin of CON and WVD during D1 and N1

Variable	*Group*		*Time*		*Time x 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**

Table 3. Effect of 24-h sleep deprivation on thermoregulatory variables, subjective ratings of sleepiness (KSS) and salivary melatonin in CON and WVD: Comparison of a 8 h episode at the same circadian phase on D2 and D3 of the CR

Variable	*Group*		*Day*		*Day x 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

Table 4. Effect of sleep on thermoregulatory variables, subjective ratings of sleepiness (KSS) and salivary melatonin of CON and WVD during N3 and the following morning (D4)

Variable	*Group*		*Time*		*Time x 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 (3.98) = 1.97	P = 0.176

Table 5. Phase relationship between CON and WVD

Variable	CON	WVD	*P* (U-test)
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** †

Table 6. Phase relationship between the variables

Variable	CON Lag to MEL (min)	WVD Lag to MEL (min)	*P* (U-test) CON vs. WVD	Pooled CON and WVD Lag to MEL (min)	*P* (sign-test) vs. 0 lag
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**