The circadian variation of sleep and alertness of postmenopausal women

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Abstract

Study Objectives

Several factors may contribute to the high prevalence of sleep disturbances occurring in postmenopausal women. However, the contribution of the circadian timing system to their sleep disturbances remains unclear. In the present study we aim to understand the impact of circadian factors on changes of sleep and alertness occurring after menopause.

Methods

Eight healthy postmenopausal women and 12 healthy young women in their mid-follicular phase participated in an ultradian sleep-wake cycle procedure (USW). This protocol consisted of alternating 60-minute wake periods and nap opportunities for ≥48 hours in controlled laboratory conditions. Core body temperature (CBT), salivary melatonin, self-reported alertness, and polysomnographically recorded sleep were measured across this procedure.

Results

In both groups, all measures displayed a circadian variation throughout the USW procedure. Compared to young women, postmenopausal women presented lower CBT values, more stage N1 and N2 sleep, and number of arousals. They also showed a reduced amplitude of the circadian variation of melatonin, total sleep time (TST), sleep onset latency (SOL), stage N3 sleep, and alertness levels. Postmenopausal women fell asleep faster and slept more during the biological day and presented higher alertness levels during the biological night than young women.

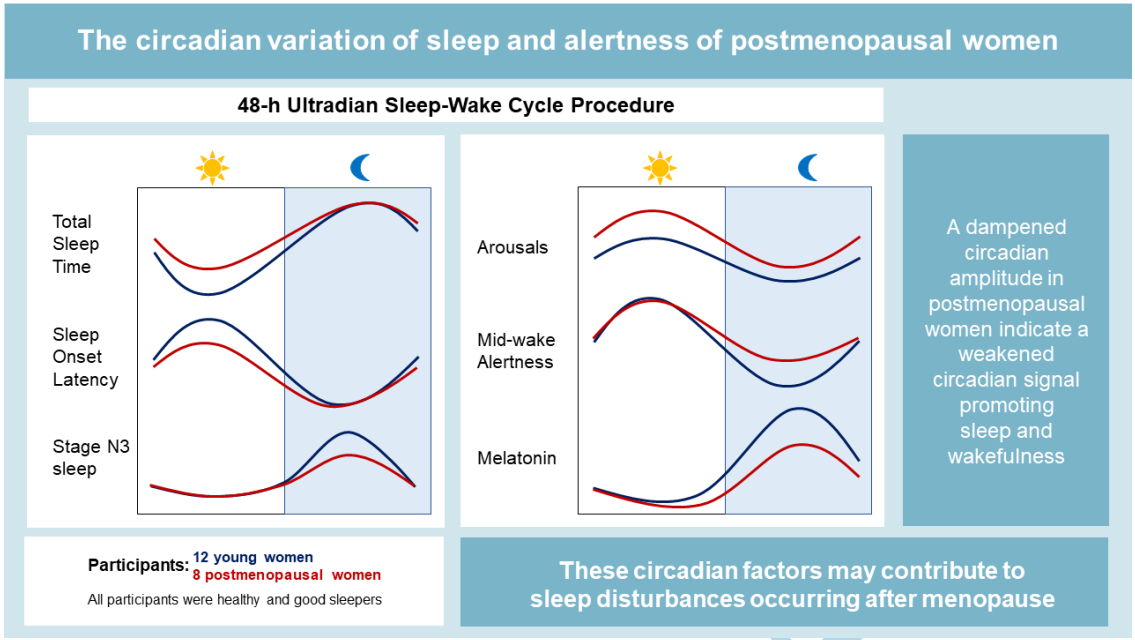
Conclusion

These results support the hypothesis of a weakened circadian signal promoting sleep and wakefulness in older women. Aging processes including hormonal changes may be main contributors to the increased sleep-wake disturbances after menopause.

Keywords

Alertness; circadian; core body temperature; menopause; sleep

Graphical abstract



Statement of Significance

Circadian factors may play an important role in the sleep-wake changes that occur after menopause. We investigated the effect of menopause on the circadian variation of body temperature, melatonin, sleep, and alertness. Our results demonstrate that postmenopausal women have more shallow and fragmented sleep throughout day and night, as well as a reduced circadian variation of sleep, alertness, and melatonin. Postmenopausal women tend to fall asleep faster and sleep more during the biological day and be more alert during the biological night than young women. These results are consistent with a diminished circadian organization of sleep and wakefulness, possibly due to hormonal changes and/or aging. Circadian factors could be main contributors to the high prevalence of sleep-wake disturbances in postmenopausal women.

Introduction

Women present a higher prevalence of sleep complaints than men throughout their life (see [1] for review), and these complaints steeply increase in the peri- and postmenopausal period, affecting 40-60% of women [2-5]. According to epidemiological studies, sleep disturbances at menopause mainly present as sleep fragmentation and difficulties falling and staying asleep [2,3,5]. Several factors have been reported to influence the sleep of postmenopausal women, including vasomotor symptoms, chronic health conditions, and higher incidence of sleep apnea/hypopnea syndrome and periodic limb movements disorder [6-8]. Furthermore, age- and hormone-related changes in the circadian timing system can have an important role in the regulation of sleep and may additionally contribute to the sleep disturbances observed in postmenopausal women.

Menopause is characterized by decreased levels of sex hormones and increased levels of gonadotropins due to loss of ovarian function [9] which may contribute to their sleep disturbances. Indeed, research has identified estradiol and progesterone receptors in brain regions that regulate sleep, wakefulness and circadian rhythms, in female rodents [10], although the precise mechanisms by which sex hormones regulate these systems in women remain to be elucidated.

The circadian timing system, controlled by the suprachiasmatic nucleus, present age-related changes in its physiology that may affect sleep and alertness of aged individuals [11]. Advanced circadian rhythms of core body temperature (CBT), melatonin, and cortisol, have been reported in older compared to younger adults and are consistent with their early morning awakenings and earlier sleep schedules [11]. Few studies have specifically investigated circadian rhythms in postmenopausal women [12,13], but the contribution of the circadian timing system to the regulation of sleep and its circadian variation has not yet been studied in this population. The aim of the present study is to better understand the role circadian factors play in the sleep changes occurring after menopause, as possible contributors to the increased risk of sleep disturbances in this population.

Methods

Participants

Eight healthy postmenopausal women (mean age ± SD: 54.80 ± 3.37 y, range 50 – 61 y) with no sleep complaints were enrolled and their results compared with previously-collected data from 12 healthy naturally ovulating young women in their mid-follicular phase (age: 25.83 ± 3.35 y, range 20 – 30 y; days 5-9 after menses) (see *Supplementary Table S1* for demographic information). At the time of the study, the time elapsed since the last menstrual period of postmenopausal women was between 2 and 11 years and one blood sample was taken on the first morning of the laboratory phase to measure serum levels of FSH, LH, estradiol, and progesterone. Their hormone levels were consistent with their menopausal status (*Supplementary Table S2*). Young women confirmed ovulation via plasma progesterone test on day 21 of their menstrual cycle preceding experimental procedures [14]. Postmenopausal women were free of medications, except for one woman who was using estradiol transdermal patches 25 micrograms every 3 days and micronized progesterone pills 100 mg once a day. Details from 11 of the 12 young women have previously been published [14,15]. All young women were physically healthy, had regular menstrual cycles of 26 to 32 days, showed no evidence of psychiatric or gynecological diseases, and were not using any medications or contraceptives.

Recruitment and screening

Eligible postmenopausal women needed to be 45-65 years old and were at least 2 years past their last menstrual period, as described by the Stages of Reproductive Aging Workshop (STRAW) classification [9]. Exclusion criteria included shift work, transmeridian travel of two or more time zones in the past 2 months, and chronic pathologies and medications that might affect sleep and circadian rhythms. Participants were healthy physically and mentally as documented by psychological and medical evaluations. All participants were healthy sleepers as verified by a polysomnographic screening night in the laboratory. Postmenopausal women with Apnea-Hypopnea Index (AHI) ≥ 15/h of sleep, and

young women with AHI ≥ 5/h of sleep were excluded [6,16]. Women with periodic leg movements during sleep (PLMS; ≥ 15/h of sleep) were also excluded [7,17]. Respiratory events and PLMS were identified according to AASM criteria [18,19], and PLMS in young women were scored in accordance with Coleman's criteria [20], as described previously [15]. This project was approved by the Douglas Mental Health University Institute Research Ethics Board (2018-175). Each participant provided informed consent prior to study initiation.

Study design

For at least 2 weeks prior to laboratory entry, participants maintained a regular 8-h nocturnal sleep schedule, as verified by actigraphy for one week prior to laboratory entry. Additionally, participant's compliance to their sleep schedule was visually verified on actigraphy recordings and sleep logs. Bedtime and rise-time for the baseline sleep period at the laboratory was calculated using individual's bedtimes and rise-times of Actiwatch and sleep logs. Upon arrival to the laboratory, participants underwent toxicological screening of urine and training of experimental tasks. Participants were scheduled for one 8-h sleep opportunity at their habitual bedtime to monitor their baseline sleep (*see Figure 1 and Supplementary Figure S1*). This was followed by a 48-h (postmenopausal women) or 72-h (young women) ultradian sleep-wake cycle (USW) procedure in constant conditions (described below). This USW procedure consisted of alternating 60-minute wake episodes in dim light (< 10 lux) and 60-minute nap opportunities in complete darkness. In total, postmenopausal women had 24 wake-nap periods whereas young women had 36 wake-nap periods. All participants were allowed to sleep *ad libitum* during the last nap opportunity of the USW procedure.

Laboratory conditions

While in the laboratory, participants stayed in a windowless time-isolation suite. During wake periods, participants answered pen-and-paper questionnaires and biological samples were collected (saliva, urine, and blood; not reported in this paper). Constant conditions during the USW were as follows: light exposure was maintained at <10 lux during wake periods and at ~0 lux during nap opportunities (use of phone and tablets were not allowed); ambient

temperature was maintained at 22 ± 2.0 °C; food intake comprised isocaloric snacks provided half-way through each wake period. Daily caloric intake was calculated using the Harris-Benedict formula and divided in isocaloric snacks, one per every wake period of the USW procedure. A semi-recumbent position was maintained throughout this procedure in young women. In postmenopausal women, a semi-recumbent position was maintained throughout wake periods, while a supine position was allowed during nap opportunities. To decrease the risk of thrombophlebitis, postmenopausal women were required to use the ensuite bathroom every wake period and to walk around their room every alternate wake period. The period walking around the room started 10 minutes after lights were turned on and was planned every other wake period. Ambulation lasted less than 10 minutes and included the time permitted to use the ensuite bathroom. Young women were required to always remain in bed and use a bedpan. Additionally, three 2-ml doses of tinzaparin sodium pre-filled injection (Innohep), an anticoagulant, were administered to the postmenopausal woman taking hormonal replacement therapy (wake periods 1, 13, and 24) to decrease her risk of thrombophlebitis.

Measures and data processing

Sleep was recorded during the baseline night and during all naps using polysomnography (Harmonie, Stellate Systems, Montreal, QC, Canada), which included electroencephalogram (EEG; C3/A2, C4/A1, O1/A2, O2/A1 leads for all participants, and additionally F1/A2 and F2/A1 for postmenopausal women), electrooculogram, electromyogram, and electrocardiogram. Since the frontal leads of young women were not available, only central and occipital EEG leads were used for scoring. Sleep was visually scored in 30-second epochs according to the American Academy of Sleep Medicine guidelines [18]. Sleep parameters included: sleep onset latency (SOL), REM sleep onset latency (ROL), total sleep time (TST), sleep efficiency (SE), awakenings, number of arousals, arousal index, and stages N1, N2, N3, and REM sleep. SOL was the time from lights-off to the first appearance of any sleep stage; a value of 60 minutes was allocated when participants did not sleep

during a nap opportunity. ROL was defined as the time from SOL to the first epoch scored as REM sleep. TST was defined as the sum of stages N1, N2, N3, and REM sleep. SE was calculated by dividing TST over the time in bed and expressed as a percentage. Awakenings were defined as time spent in ≥ 2 epochs scored as wake (i.e., for ≥ 1 minute). Arousals were defined as abrupt shifts of a sleep stage to theta, alpha, or beta frequencies, lasting 3 to 15 seconds. Arousal index was calculated as number of arousals per hour of sleep. Core body temperature (CBT) was recorded every 15 seconds using a rectal probe, inserted 10-cm into the rectum (DeRoyal General Purpose Temperature Probe, Powell, TN, USA). After visual inspection of CBT recordings, declines in temperature > 0.1 °C within 15 seconds associated with artifacts and probe "slips" were removed by an automatic in-house program and manually confirmed. CBT was not collected in one postmenopausal woman. Melatonin was assayed from saliva samples collected twice every wake period (upon awakening and before lights-off). Saliva was collected using passive drooling technique in young women and Salivettes in postmenopausal women. Melatonin was assayed in duplicate using a specific radioimmunoassay in young women (Stockgrand Ltd, Guilford, UK, coefficient of variation: 8.5%, lower limit of detection: 0.2 pg/ml; Buhlman Alpco Diagnostics, Windham, NH, USA, mean intra and interassay coefficient of variation: 7.9% and 9.8%, respectively, lower limit of detection: 0.2 pg/ml) [15], and ELISA in postmenopausal women (Salimetrics, coefficient of variation: 7.27%, lower limit of detection: 0.78 pg/ml). Self-reported alertness was measured twice per wake period, using a 10-cm bipolar visual analog scale (VAS), anchored by the statements "sleepy" (0 cm) and "alert" (10 cm). Post-nap alertness tests were completed upon awakening, and mid-wake alertness tests were completed 20 (postmenopausal women) or 30 (young women) minutes after awakening. Post-nap alertness was not available for one young woman. Self-reported sleep quality was assessed upon awakening using a Likert scale from 0 to 6 (0 = poor sleep; 6 = good sleep). Mood and stress levels were also evaluated by 10-cm VAS twice every wake period (mood: happy – sad; stress: relaxed – stressed). Chronotype was assessed once with the Morningness-

Eveningness Questionnaire [21], which was administered before starting the ambulatory phase.

Statistical analyses

Statistical analyses were performed in R version 4.0.0 [22]. Normality of all data were verified with the Shapiro-Wilk test. To be able to compare data between postmenopausal women and young women, only the first 48 hours of the USW procedure were used for all analyses. The package “lme4” [23] was used for linear mixed-effect models.

For the baseline sleep period preceding the USW procedure, each sleep parameter was compared between postmenopausal women and young women with a two-sided t-test or Mann-Whitney U tests, when appropriate. Bedtimes and rise-times were compared between groups using circular statistics in the package “circular” [24]. To further observe the variation of sleep parameters across the baseline sleep episode, sleep parameters were additionally calculated for each third of the time spent in bed. Since number and duration of sleep cycles displays a high inter-individual variability, the baseline sleep period was analyzed by thirds of the time-in-bed period. This was selected for a consistent comparison between our groups [25]. Baseline sleep parameters were then compared between groups using linear mixed-effects models. Participant parameters were considered as random effects. “Group” and “time” within the sleep period were considered as fixed effects as sleep architecture changes across the night [14]. Using a forward method to build the model, likelihood-ratio tests were used to test the significance of the fixed effects.

In the present study, CBT and melatonin were used as circadian markers. Circadian parameters of the CBT and melatonin rhythms were obtained using a cosinor analysis based on time of day as well as time elapsed into the USW procedure. Similarly, parameters for the circadian variation of sleep and alertness were calculated based on time of day as well as time elapsed into the USW procedure. Cosinor analysis was performed with a linear mixed-effects model with a modified version of the package “cosinor” [26] to include random

effects. Circadian rhythm parameters included mesor, amplitude, phase, and phase angle. The mesor was defined as the average value of the fitted rhythm and amplitude corresponded to the difference between mesor and trough values. The acrophase, defined as the peak time of a circadian rhythm, was used as a phase marker for sleep parameters and self-reported alertness. The nadir, defined as the time of minimum, was used as the phase marker for the rhythm of CBT. Phase angle was calculated by subtracting the time of the phase marker from the habitual rise-time. Therefore, a positive phase angle represents a phase occurring before habitual rise-time, whereas a negative phase angle represents one occurring after habitual rise-time. Mesor, amplitude, phase, and phase angle were compared between groups using two-sided t-test.

To compare the within- and between-group circadian variations of sleep parameters, self-reported measures (alertness, mood, stress), CBT, and melatonin during the USW procedure, data were first aligned by the time elapsed into the USW (i.e., the habitual rise-time). Data were then collapsed into 2-h bins and compared using linear mixed-effects models with factors “group” and “time” elapsed into the USW, since sleep, alertness, CBT, and melatonin were shown to follow a circadian variation [15]. Participant parameters were considered as random effects. Using a forward method to build the model, likelihood-ratio tests were used to test the significance of the fixed effects. Tukey’s post-hoc tests were performed when group × time interactions were significant.

To exclude a possible bias of ambulation on mid-wake alertness levels in postmenopausal women, further analyses were performed excluding wake periods in which walking around the bedroom was required.

Since we included a participant using hormone replacement therapy in the postmenopausal women group, we evaluated her sleep parameters, alertness, and CBT data for outliers. Using the packages “lme4” and “merTools” [23,27], random effect estimates from these parameters were extracted for each participant. The random effect estimates of the

postmenopausal woman using hormone replacement therapy remained within 2 SD for all parameters, and her data were thus considered adequate to be included in the analyses.

Melatonin correction

Since melatonin was collected and assayed differently between young (n=11: RIA, passive drooling, n=1: ELISA, passive drooling) and postmenopausal women (n=8: ELISA, Salivette), two corrections were calculated to compare melatonin data between groups. A first correction was calculated to convert melatonin assayed with RIA to ELISA equivalent using data provided by Salimetrics on a sample size of n=10. ELISA melatonin value = RIA value * 1.21837 + 3.8767. A second correction was calculated to transform data collected with Salivette to an equivalent of passive drooling. The correction factor was based on data obtained from 8 young and 5 postmenopausal women whose saliva was collected 3 times for one night, using both passive drooling and Salivette. Passive drooling value = Salivette value * 0.8657 + 0.7814. As a result, melatonin data were transformed to ELISA and passive drooling equivalent for statistical analyses.

Results

The 8 postmenopausal women were older, showed higher body mass index, earlier habitual bedtimes, and rise-times compared to the 12 young women (*Supplementary Table S1)*. Chronotype did not differ between groups.

Sleep parameters during baseline sleep.

The results from the PSG recordings obtained during the 8-h baseline sleep period are summarized in *Supplementary Table S3 and S4*. During this nocturnal sleep period, awakenings, number of arousals, arousal index, and time spent in stage N1 sleep were higher in postmenopausal compared to young women. The remaining sleep parameters did not differ between groups. When analyzed by thirds of the period spent in bed, a significant main effect of time was observed in TST, stage N1, N3, and REM sleep, whereas a main

effect of group yielded more arousals and more stage N1 sleep in postmenopausal women when compared to young women (*Figure 2)*.

Sleep parameters during the USW procedure.

The variation of SOL, ROL, TST, arousals, and sleep stages based on time elapsed into the USW procedure is depicted on *Figure 3,* and statistical results are provided in *Supplementary Table S5*. A linear-mixed effects model showed a significant main effect of time on SOL, ROL, TST, number of arousals and stages N1, N2, N3, and REM sleep. A significant main effect of group showed shorter SOL, longer ROL, more TST, a higher number of arousals, more stage N1 sleep and more stage N2 sleep in postmenopausal women than in young women. Group × time interactions were observed on SOL, ROL, TST, and arousals count. Tukey's post-hoc test on significant interactions revealed that postmenopausal women showed shorter SOL, longer ROL, and more TST during the biological day (i.e., the time between the habitual rise-time and bedtime of each individual); as well as a greater number of arousals during the biological day and surrounding the habitual bedtime and rise-time.

Cosinor analysis based on time elapsed into the USW yielded significant circadian variation of SOL, ROL, TST, arousals count, stages N1, N2, N3, and REM sleep, and are evident in *Figure 3*. The circadian parameters of these sleep measures are reported in *Table 1*. In comparison to young women, we observed higher mesor in the rhythms of ROL, TST, arousals count, stage N1 sleep, and stage N2 sleep, and a lower mesor in the rhythm of SOL in postmenopausal women. Postmenopausal women also presented lower amplitude in the rhythms of SOL, TST, and stage N3 sleep, whereas a higher amplitude was observed in the rhythm of arousals count and arousal index. Compared to young women, the phase of SOL, ROL, TST, and stage N1 occurred later in the sleep-wake cycle in postmenopausal women (as reflected by a smaller positive or larger negative phase angles). The phase angle of ROL shows that its acrophase occurred after and before habitual rise-time in postmenopausal and young women, respectively (*Table 1*). When looking at the time-of-day

at which REM sleep acrophase occurred, it was earlier in postmenopausal women compared to young women (*Supplementary Table S6.*). The reverse was observed for ROL. No statistically significant difference was observed for the time-of-day occurrence of other sleep parameters.

The circadian variation of self-reported sleep quality across the USW procedure is depicted on *Supplementary Figure S2* and statistical results are provided in *Supplementary Table S5*. A significant main effect of time was observed in self-reported sleep quality with no significant effect of group and group × time interactions. Cosinor analysis showed a significant circadian variation with no significant between-group differences in mesor, amplitude or phase.

Self-reported measures, CBT and melatonin during the USW procedure.

The circadian variation of post-nap alertness, mid-wake alertness, CBT, and melatonin based on the elapsed time since the start of the USW procedure is depicted in *Figure 4,* and statistical results are provided in *Supplementary Table S5*. Significant main effects of time were observed for post-nap alertness, mid-wake alertness, CBT, and melatonin. A main effect of group revealed higher mid-wake alertness, and lower CBT and melatonin levels in postmenopausal women compared to young women. Importantly, group × time interactions for mid-wake alertness and melatonin were observed. Mid-wake alertness yielded higher levels in postmenopausal women during the first wake episode of the USW procedure, from hours 16 to 26 since start of USW (i.e., at the time corresponding to the first habitual nocturnal sleep), and at 41 hours after starting the USW. Melatonin was shown to be lower during the first 2 hours of USW and during the first 4 hours of the habitual night period in postmenopausal vs young women. No significant interaction was obtained in post-nap alertness and CBT.

Results were similar even when excluding wake periods during which walking around the room occurred. The group effect with higher mid-wake alertness in postmenopausal women remained significant, with a trend for a group × time interaction (*Supplementary Figure S3*).

Circadian parameters based on cosinor analysis of post-nap and mid-wake alertness, CBT, and melatonin are shown in *Table 2*. Post-nap and mid-wake alertness, CBT, and melatonin showed a significant circadian variation based on time of day and time elapsed into the USW. Compared to young women, we observed a higher mesor of the mid-wake alertness rhythm, lower amplitude of the post-nap alertness and melatonin rhythms, and lower mesor of the CBT rhythm in postmenopausal. No other significant group difference was observed for the circadian parameters of alertness, CBT, or melatonin.

No significant effect of time was observed for mood and stress levels ($p>0.33$). Postmenopausal women rated themselves with better mood ($p = 0.04$) and comparable stress levels ($p = 0.08$) than young women.

Discussion

Menopause is associated with fluctuations in sex hormones that may affect circadian physiology, but the role of circadian factors in sleep disturbances after menopause is not well understood. The present study aims to understand the changes occurring after menopause in the circadian variation of sleep and waking. The primary finding in this small group of postmenopausal women with no sleep complaints was a general increase in light sleep and number of arousals during baseline sleep and across circadian phases, as well as a clear dampening of the circadian variation of sleep, alertness, and melatonin rhythms. To our knowledge, our study is the first one to describe the circadian variation of sleep in women after menopause.

Baseline sleep period

Only a few studies have explored the specific changes of polysomnographic sleep after menopause and the changes reported in the architecture of sleep are not homogeneous [4].

In our group of postmenopausal women, the habitual timing of sleep was on average 1.1 h earlier than in young women, possibly as a consequence of aging [11]. Earlier timing of the sleep schedule has been associated with earlier chronotypes [28] which can provide some insight into the circadian timing system [29]. In the present study, chronotype differences were not observed between postmenopausal and young women, possibly due to the less pronounced changes in women towards morningness with aging [30]. In a European cross-sectional population-wide study, age-dependent changes in chronotype using the Munich Chronotype Questionnaire (MCTQ) showed that women and men both become earlier chronotypes with aging [30]. Interestingly, this study revealed that the difference in chronotype with aging is less pronounced in women, since they are earlier chronotypes, on average, than men. Sex differences in chronotype disappears after the age 52, around the time of menopause. The less pronounced changes in women towards a morningness preference with aging might explain the absence of significant differences in chronotype between our groups. In the present study, TST and SE were similar for postmenopausal and young women during the baseline nocturnal sleep period, which is consistent with previous research involving premenopausal, perimenopausal and postmenopausal women aged <60 years [31-33]. Other studies that specifically focused on age-related changes in nocturnal polysomnographic sleep have shown decreased TST and SE with aging, including middle-aged women [34-37]. However, these findings were more pronounced in women over 60 years old, which are older than all but one of the postmenopausal women in our study group. A recent cross-sectional study examined age-related changes in 69,650 adults of both sexes aged 19-67, using wearable activity trackers [38]. Sex differences were observed in night-time awakenings with higher values in women compared to men. The greatest disparities between sex occurred during active-reproductive years of women. Interestingly, when

looking at weekends sleep duration and timing (presumably more influenced by biological factors), clear sex differences were observed in younger and middle-aged women and tended to disappear in older adults. These observations are coherent with a role of ovarian hormones in the circadian regulation of sleep [38].

We observed more stage N1 sleep, more awakenings, a greater number of arousals, and a higher arousal index in postmenopausal compared to young women. These results show that menopause is a period of increased sleep fragmentation with lighter sleep stages, which are in line with previous research showing more time awake, arousals, and stage N1 sleep in menopausal women and aged individuals [31,32,34-36,38-41]. Interestingly, we did not find group differences in stage N3 and REM sleep. Whereas studies including pre-, peri-, and postmenopausal women did not show differences in stage N3 (also called slow wave sleep; SWS) and REM sleep as a function of menopausal status [31-33,40], aging studies have reported an overall reduction of these sleep stages throughout life in women 20-85 years old [34-36]. It is well known that SWS architecture and REM sleep are influenced by sleep disorders and rebound sleep [36,42], which we controlled for with our screening procedures and pre-study instructions. Indeed, since REM sleep and SWS decrease as a function of aging, the lack of group differences in REM sleep and SWS in the present study might be explained by the younger and limited age range of our group of postmenopausal women compared to these aging studies.

Circadian Rhythms

For the present study, we used CBT and melatonin rhythms as reliable circadian markers. We observed that postmenopausal women had a lower CBT mesor and a dampened circadian amplitude of melatonin compared to young women.

Since CBT is influenced by body metabolism, the reduction of CBT in postmenopausal women has been hypothesized to be a consequence of a reduced metabolic resting expenditure due to aging and/or hormonal changes [43,44]. Consistent with our findings,

studies showing age-related changes in circadian rhythms, using either USW or forced desynchrony protocols, also found a lower mesor of the CBT rhythm in a combined group of older men and women compared to younger participants [45-47]. On average, mesor differences in CBT between young and older individuals ranged from 0.14–0.30 °C, of comparable magnitude to the 0.15 °C temperature difference between groups in the present study. Since estrogen and progesterone have hypothermic and hyperthermic effects, respectively, hormonal changes occurring at menopause might be another contributing mechanism to the declining CBT. In naturally cycling women aged 19-29 years, the fluctuation of the progesterone/estradiol ratio across the menstrual cycle has been associated with changes in body temperature. Specifically, women in the luteal phase presented an average progesterone/estradiol ratio of ~120, whereas this ratio was ~30 in the follicular phase, coincidental with lower CBT levels during the night in follicular vs luteal phase [48]. Progesterone and estradiol levels decline during menopause and a progesterone/estradiol ratio of 6.24 was calculated for our group of postmenopausal women. As such, hormonal changes following menopause may additionally contribute to the age-related reduction of CBT.

Group differences were not observed in circadian phase and amplitude of CBT, although an advanced circadian phase and dampened amplitude of CBT with aging have been described in the literature [47,49]. The earlier sleep schedule combined with a similar phase angle between CBT minimum and the habitual rise-time observed in postmenopausal women led us to hypothesize that the lack of group differences in CBT phase might be due to our small sample size. Since CBT declines during sleep [47], the scheduling of naps during our USW might have exerted some masking effect on this rhythm, although it probably had minimal effects on our ability to assess circadian phase since naps were equally spaced across the USW.

The similar phase angle between our groups suggests that postmenopausal and young women slept at similar circadian phases. These results are consistent with prior studies that

found no phase angle differences between young and older adults [50,51], as well as with those comparing pre- and postmenopausal women [12], although shorter phase angles have also been reported with aging [52]. Furthermore, a stable phase angle of entrainment is coherent with the stable circadian period reported with aging [53].

In the present study, the circadian rhythm of melatonin was dampened in postmenopausal compared to young women, with no phase or phase angle differences. The reduced amplitude of melatonin is consistent with prior studies comparing young and older adults, and of comparable magnitude [46,49]. However, prior studies reported an advanced melatonin rhythm with aging, but no between-sex comparisons were performed [46,54]. In a large study of 99 participants using an USW protocol, older adults had earlier melatonin rhythms than younger ones, and women displayed earlier melatonin rhythms than men [54]. Another study employing an USW procedure [46] found that older adults presented an earlier acrophase of aMT6s than younger adults, although no sex differences were observed. In these two studies, aged individuals were older than our group of postmenopausal women (mean ± SD: 66.1 ± 4.7 y; 66.2 ± 4.9 y vs 54.8 ± 3.37 y in our study), and age-related differences by sex were not analyzed. It thus remains difficult to compare our results with these previous studies to address the effects of hormonal changes after menopause.

Interestingly, Walters and colleagues [12] showed, in a constant routine protocol, that postmenopausal women aged between 50 and 60 years presented earlier melatonin rhythms of similar amplitude than those of younger premenopausal women aged between 35 and 50. Compared to our study, the study of Walters and colleagues [12] had an older group of premenopausal women, whereas postmenopausal women were of similar age. It is thus probable that the dampening in melatonin circadian amplitude in our postmenopausal women might be attributed to an age effect other than hormonal changes alone. The dampened amplitude of melatonin observed in postmenopausal women has been described with aging as a consequence of decreased beta-adrenergic receptors and gene expression

of Serotonin N-acetyltransferase in the pineal gland, necessary for melatonin synthesis [55]. However, hormonal changes at menopause may also influence melatonin levels since sex steroid receptors have been found in the pineal gland [56], but the effects of sex steroids on melatonin secretion is complex and requires further experimentation [57].

Diurnal variation of sleep

We observed a significant diurnal variation of SOL, ROL, TST, arousals, sleep stages N1, N2, N3, REM sleep, and self-reported sleep quality throughout the USW procedure in both postmenopausal and young women.

The rhythm of TST and SOL provided an insight into the diurnal variation of sleep propensity. Contrary to our expectations, postmenopausal women, on average, fell asleep more rapidly and slept more across all circadian phases than young women. These group differences can be attributed to shorter SOL and more TST during the biological day for postmenopausal women, as no group differences were observed during the biological night. These differences resulted in lower circadian amplitudes for these sleep parameters in postmenopausal compared to young women.

Our findings might initially seem contradictory of the current literature describing longer SOL and less TST with aging in USW protocols [45,46,51], and during the night in forced desynchrony protocols [58]. There are some factors that may account for these discrepancies. First, previous aging studies regarding the circadian variation of sleep did not address sex differences [45,46,51,58,59] and therefore, did not specifically explore the age-related changes in women. Geisler and colleagues [60] used a multiple sleep latency test with naps scheduled between 9:00 h and 17:00 h to show that sleep latency was shorter for middle-aged women than for younger and older women. Our results showing shorter SOL for postmenopausal women are in line with these results considering that our participants were all middle-aged, except for one who was 61 years old. Second, the group differences in TST and SOL were only observed for nap opportunities occurring during the day and resulted in a

lower amplitude of the diurnal rhythm of these sleep parameters. These results in postmenopausal women are consistent with those of other investigations of aging and additionally supported by the observed reduced amplitude of the circadian variation of stage N3 sleep in postmenopausal women. Other studies employing USW or forced desynchrony procedures have shown older adults with smaller circadian amplitudes of sleep latency, time spent asleep, and slow wave sleep than younger adults [45,51,58], while one study found the opposite [46]. Altogether, these findings support the hypothesized impaired output of the circadian pacemaker in older adults [61]. However, sex differences were not analyzed and disentangling age-related sleep differences by sex remains difficult.

The diurnal variation of number of arousals indicated that sleep was more disrupted for postmenopausal women than young women. Postmenopausal women consistently presented more arousals per nap, whether scheduled in the day or night, which is consistent with aging studies performed in USW procedures and forced desynchrony protocols [45,62,63]. In young women, arousals count was very low throughout circadian phases, thus leading to a smaller rhythm than that of postmenopausal women. In terms of sleep stages, subtle differences were observed between our study groups in the diurnal variation of stages N1, N2, N3, and REM sleep. Postmenopausal women presented, on average, more stage N1 and N2 sleep (i.e., a higher mesor) than young women but no group differences in stage N3 and REM sleep, similar to our observations during the baseline sleep period. The increased duration of lighter stage N1 sleep throughout the USW may reflect the increased frequency of arousals and subsequent transitions from wake to light sleep in postmenopausal women. Despite these observations, self-reported sleep quality remained similar between groups across the USW procedure. This confirmed that both groups reported themselves as good sleepers, but that unperceived changes in the macrostructure of sleep occur after menopause. As for phase angle results, we observed that the phase of SOL, ROL, TST, and N1 sleep occurred later in the sleep-wake cycle of postmenopausal than young women. These show a delay shift in the timing of sleep propensity, with the

highest values of TST slightly closer to the habitual time of awakening in postmenopausal compared to young women. Although subtle changes were observed in the timing of sleep propensity at menopause, the clinical implication of these observations remains unclear. When results were analyzed based on time of day, the circadian variation of REM sleep propensity occurred earlier in postmenopausal women. This observation is consistent with their earlier habitual sleep period and the very sharp pattern in the circadian variation of REM sleep.

Alertness across the USW procedure

Alertness measured upon awakening and at 20-30 minutes after waking exhibited significant diurnal variations for both postmenopausal and young women, and both rhythms followed a similar pattern throughout the USW procedure, with a peak and nadir observed in the early evening and late night, respectively. As SOL, the lowest alertness values occurred late at night and close to the habitual time of awakening in postmenopausal and young women. In comparison to young women, postmenopausal women were significantly more alert at mid-wake (i.e., a higher mesor across the USW procedure), with no differences in post-nap alertness. Based on the mid-wake alertness questionnaire, postmenopausal women were more alert than young women during the biological night and early morning, whereas alertness levels were comparable between groups during daytime hours. These results are in line with previous research performed in constant routine and forced desynchrony protocols indicating that older adults are more resilient to the effects of exposure to adverse circadian phases on alertness and performance than younger adults [64-68]. Controversial results have been observed in USW protocols [45,51], in which sleep inertia may possibly have impaired the ability to detect age-related differences in the circadian variation of alertness since alertness was measured only upon awakening. In the present study, group differences were only observed in mid-wake alertness levels and these differences persisted even when removing the wake periods during which postmenopausal women were allowed to walk around the room. Although higher alertness levels at night in postmenopausal

women are consistent with reports in the literature, we cannot completely exclude an effect of postural changes on the mid-wake alertness of postmenopausal women. However, it is unlikely that such postural effect accounts for between-group differences at specific times of day. These differences are also not related to between group differences in perceived stress levels and hardly explained by better perceived mood in postmenopausal women. Importantly, most of the prior studies on alertness did not address sex differences or age-specific differences in women [45,51,64,65,67]. In the present study, we did not find differences in the timing of alertness acrophase or phase angle. This is consistent with a prior study that compared premenopausal and postmenopausal women under constant routine conditions and found no differences in the timing of alertness offset [12].

In the present study, the amplitude of the circadian rhythm of alertness was lower in postmenopausal women than in young women, but only reached significance when measured upon awakening. It remains unclear why the between-age difference in amplitude we observed are limited to the post-nap assessment. These lower amplitudes of melatonin, post-nap alertness, and sleep parameters in postmenopausal women support the hypothesis of a weakening of the circadian signal promoting sleep and wakefulness with aging [45,61]. Interestingly, between-age differences were more consistent at night for alertness and during the day for sleep. These times of day correspond to the habitual sleep and wake periods, respectively. Higher alertness at night and higher sleep propensity during the daytime in postmenopausal women is also suggestive of a disruption in the temporal organization of the sleep-wake cycle with aging. Overall, the higher mesor of alertness, stage N1 sleep, and number of awakenings during sleep in older women suggest an increase in the strength of the arousal signal and a reduction in the strength of the sleep signal that represent physiological differences potentially contributing to the higher prevalence of insomnia complaints in postmenopausal women.

It is well known that the circadian variation of alertness and neurobehavioral performance parallels the rhythm of CBT, with observed crests and nadirs close to those of the CBT

rhythm [48]. Interestingly, constant routine studies in young adults have shown that women in their follicular phase have poorer performance and lower CBT at night than both men [69] and women in luteal phase [48,69,70]. It was thus proposed that lower CBT could impair neurobehavioral performance, but differences in self-reported alertness were not observed [14,48,69]. In the present study, postmenopausal women reported higher alertness at night despite having lower CBT compared to young women. As previously discussed, CBT declines with aging, and research has shown that older individuals present higher performances than younger ones in psychomotor vigilance test, and less sleepiness measured by Karolinska Sleepiness Scale [64,66]. Although performance was not measured in the present study, the relationship between CBT and alertness levels remains unclear, even if CBT and alertness follow a similar diurnal variation.

Physiological mechanisms

The mechanisms by which the circadian variation of sleep and alertness differ between postmenopausal and young women may include the effect of hormonal changes associated with aging and other aging processes. In both sexes, a drop in gonadal hormones is part of the normal aging process. These hormonal changes in women are concentrated across the menopausal transition, whereas in men a gradual drop of testosterone levels occurs progressively starting in their middle age [71]. It remains difficult to disentangle the effects of hormonal changes from other aging processes, although there is increasing evidence that the drop of ovarian hormones after menopause can have an important effect on sleep-wake organization.

In nocturnal rodents, estradiol administration to ovariectomized animals have been shown to promote wakefulness and sleep during the active and rest phases, respectively, thus consolidating the sleep-wake cycle [1,10]. It was proposed that estradiol could promote wakefulness by inhibiting somnogens such as adenosine and lipocalin-type prostaglandin D in the ventrolateral preoptic area, a sleep-promoting nucleus [10]. Since menopause is characterized by declining levels of estradiol and progesterone, it is plausible that the

reduction of these hormones in postmenopausal women contributes to their decreased wake propensity during the day and fragmented sleep. This could account for the therapeutic effects of hormonal replacement therapy on sleep disturbances of postmenopausal women, although it remains unclear whether these drugs exert a direct effect on sleep, or improve it by reducing vasomotor symptoms, or both [72,73]. The mechanisms by which reduced levels of sex hormones in postmenopausal women might produce higher alertness at night remains unclear. Besides hormonal changes, other aging processes could be involved. With aging, the number of neurons in the preoptic area and the lateral hypothalamus declines [74,75]. In postmenopausal women, it is thus possible that age-related changes occurring in the preoptic area promoting sleep and the lateral hypothalamus promoting wakefulness may also contribute to the reduced amplitude of sleep and alertness propensity, respectively. This hypothesis is consistent with the reduced circadian amplitudes of TST, SOL, and self-reported alertness of postmenopausal women in the current study. Further experimentation will be necessary to clarify the effects of ovarian hormone changes on sleep and circadian rhythms after menopause.

Strengths and limitations

The small sample size in the present study is an important limitation that may have not allowed us to observe group differences in circadian parameters of CBT. Additionally, we must acknowledge that we used an USW procedure, and not a constant routine protocol, in which the sleep opportunities may mask the CBT. However, naps were scheduled regularly at 2-hour intervals, minimizing their masking effect on CBT. On the other hand, the USW procedure allowed us to minimize the effects of sleep deprivation by maintaining constant wake periods lasting one hour each. Our results need to be taken with caution, since postmenopausal women included in this study were thoroughly screened and thus only represent a minor healthy proportion of this population. However, this also represents a strength of the study as it allowed us to study the specific effect of menopause rather than comorbidities associated with it.

We cannot completely exclude the possibility that slight differences in the scheduling of the baseline sleep period could have affected between-group comparisons. However, the absence of group differences in N3 sleep as well as similar TST and SE suggest its effect is minimal.

In the current study, we accounted for menopausal status, screened for sleep disorders, asked participants to maintain regular nocturnal sleep/darkness at night for 2 weeks prior to entering the laboratory, and confirmed that participants did not nap via actigraphy, thus reducing the possible confound of an irregular sleep schedule.

The group of postmenopausal women also included one participant taking hormone replacement therapy. Nevertheless, her results were within 2 SD of the group data and were deemed adequate to be included in the analyses.

Due to the exploratory design of our study, adjustment for false discovery rate was not performed. Therefore, subsequent studies will be necessary to further explore the role of menopause on sleep and circadian rhythms.

Conclusion

In the present study, we observed significant changes in the sleep of postmenopausal women at baseline and during the USW procedure. During the baseline sleep, postmenopausal women presented shallower and more fragmented sleep than young women. In the USW procedure, postmenopausal women presented more sleep propensity during the day and higher alertness levels at night, which resulted in a dampening of their circadian variation along with a dampened rhythm of melatonin. It remains difficult to disentangle whether the observed effects in sleep and alertness are due to hormonal changes occurring after menopause, other aging processes, or a combination of both. The described disruption of the temporal organization of the sleep-wake cycle evident at menopause might be one of the many factors contributing to the sleep disturbances occurring at menopause.

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Data and Material availability:

The data underlying this article cannot be shared publicly because participants did not agree that their data be placed in a publicly accessible database. Therefore, for ethical and confidentiality reasons, the authors cannot provide public access to these data. Nevertheless, materials, data, and protocols will be made available for investigation of scientific integrity if necessary. Readers are free to contact the principal investigator if they wish to initiate discussions regarding research collaborations to build on these published data. The data will be shared on reasonable request to the corresponding author.

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Figure captions list

Figure 1. Ultradian sleep-wake cycle (USW) procedure used in postmenopausal women. Polysomnographic (PSG) screening for sleep disorders was performed prior to laboratory entry. For the laboratory phase, recording equipment were installed upon arrival to the laboratory on the evening of Day 1 (light levels: ~150 lux). Participants slept for an 8-h sleep period (light levels: ~0 lux), based on their habitual bedtimes and rise-times for the past 2 weeks. Upon awakening, participants either began a 48-h (postmenopausal women) or 72-h (young women) USW procedure, consisting of alternating 60-minute wake periods and 60-minute nap opportunities in constant conditions. All participants were allowed to sleep *ad libitum* during the last nap opportunity of the USW procedure. The figure illustrates a hypothetical participant with a sleep schedule from 0:00 h to 8:00 h. More details of the USW procedure of young women are provided in *Supplementary Figure S1.*

Figure 2. Variation of total sleep time (TST), arousals count, stage N1, stage N2, stage N3, and REM sleep duration during the 8-h baseline sleep period of postmenopausal women and young women. All baseline sleep parameters were divided into thirds of time spent in bed. Data are presented as mean ± SEM. T = significant main effect of time. G = significant main effect of group. *p < 0.05, *p < 0.01, ***p < 0.001. Statistics provided in *Supplementary Table S4.*

Figure 3. Variation of sleep onset latency (SOL), and REM sleep latency (ROL), total sleep time (TST), arousals, stage N1, stage N2, stage N3, and rapid-eye-movement (REM) sleep, in postmenopausal women and young women. Data were aligned based on the time elapsed into the ultradian sleep-wake cycle procedure (USW, bottom x axis). Black (~0 lux) and white (~10 lux) small squares above the bottom X axis represent the nap and wake periods across the first 48 h of the USW procedure. The top X axis depicts the corresponding time of day for a participant with a bedtime of 0:00 h to 8:00 h. As reported in *Table A.1*, habitual sleep times are not the same for both study groups. Large grey rectangles depict the projected

time of the habitual nocturnal sleep period, which corresponds to the time between habitual bedtime and rise-time. The asterisks (*) along the top x axis of SOL, ROL, TST, and arousals indicate significant group differences by time ($p < 0.05$). T = significant main effect of time. G = significant main effect of group. G×T = significant group-by-time interaction. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Values are presented as mean ± SEM. Statistics provided in *Supplementary Table S5*.

Figure 4. Variation of post-nap alertness, mid-wake alertness, melatonin, and core body temperature (CBT) on postmenopausal women and young women. The top and bottom X axes are as in Figure 3. The asterisks (*) along the top x axis of mid-wake alertness, indicates significant group by time differences ($p < 0.05$). Values are presented as mean ± SEM. Statistics provided in *Supplementary Table S5.*

	Circadian parameters	PMW **Mean** ± SEM [95% CI]	YW **Mean** ± SEM [95% CI]	*p-values*
SOL	Mesor (minutes)	19.15±1.59 [16.03, 22.28]	29.88±2.07 [25.83, 33.93]	**<0.001**
	Amplitude (minutes)	11.71±1.80 [8.17, 15.25]	18.79±1.46 [15.92, 21.66]	**0.002**
	Phase angle (h)	11.80±0.52 [12.82, 10.79]	13.72±0.27 [14.25, 13.18]	**0.001**
ROL	Mesor (minutes)	35.21±1.85 [31.58, 38.83]	22.29±2.40 [17.58, 26.99]	**<0.001**
	Amplitude (minutes)	6.43±1.58 [3.33, 9.52]	9.65±1.32 [7.06, 12.23]	0.12
	Phase angle (h)	-1.67±0.95 [0.20, -3.54]	2.57±0.52 [3.60, 1.54]	**<0.001**
TST	Mesor (minutes)	34.81±1.37 [32.12, 37.49]	27.65±1.78 [24.17, 31.13]	**<0.001**
	Amplitude (minutes)	12.83±1.68 [9.55, 16.12]	18.79±1.43 [15.98, 21.60]	**0.007**
	Phase angle (h)	0.61±0.56 [1.71, -0.49]	2.04±0.31 [2.64, 1.43]	**0.026**
Arousals count	Mesor (count)	6.87±0.53 [5.82, 7.92]	3.62±0.71 [2.23, 5.01]	**<0.001**
	Amplitude (count)	2.28±0.34 [1.61, 2.95]	0.51±0.35 [-0.16, 1.19]	**0.001**
	Phase angle (h)	-3.00±0.55 [-1.91, -4.08]	-4.24±2.54 [0.74, -9.21]	0.52
Arousal index	Mesor (count/h)	4.32±0.37 [3.59, 5.05]	2.11±0.49 [1.15, 3.08]	**<0.001**
	Amplitude (count/h)	2.10±0.21 [1.68, 2.52]	0.70±0.23 [0.26, 1.14]	**<0.001**
	Phase angle (h)	-1.42±0.38 [-0.68, -2.16]	0.58±0.36 [2.85, -1.69]	0.35
Stage N1	Mesor (minutes)	5.35±0.50 [4.36, 6.34]	3.66±0.65 [2.39, 4.94]	**0.010**
	Amplitude (minutes)	2.33±0.36 [1.62, 3.04]	1.66±0.28 [1.12, 2.21]	0.15
	Phase angle (h)	-2.87±0.61 [-1.68, -4.06]	-0.30±0.75 [1.16, -1.76]	**0.007**
Stage N2	Mesor (minutes)	23.55±1.24 [21.12, 25.97]	16.01±1.60 [12.86, 19.15]	**<0.001**
	Amplitude (minutes)	5.23±1.27 [2.74, 7.72]	8.20±1.15 [5.95, 10.45]	0.08
	Phase angle (h)	0.47±1.17 [2.77, -1.83]	2.34±0.58 [3.48, 1.21]	0.15
Stage N3	Mesor (minutes)	1.87±0.59 [0.71, 3.02]	2.96±0.76 [1.47, 4.46]	0.15
	Amplitude (minutes)	0.95±0.53 [-0.09, 1.99]	2.67±0.46 [1.78, 3.56]	**0.014**
	Phase angle (h)	3.25±2.07 [7.30, -0.80]	4.59±0.58 [5.73, 3.44]	0.53
REM sleep	Mesor (minutes)	4.08±0.61 [2.89, 5.28]	4.95±0.79 [3.40, 6.50]	0.27
	Amplitude (minutes)	5.69±0.86 [4.00, 7.39]	7.19±0.72 [5.78, 8.60]	0.18

	Phase angle (h)	1.62±0.58 [2.74, 0.49]	1.31±0.38 [2.05, 0.57]	0.66
Self-reported sleep quality	Mesor (Likert)	3.70±0.32 [3.07, 4.33]	3.09±0.42 [-1.43, 0.23]	0.15
	Amplitude (Likert)	0.77±0.26 [0.26, 1.28]	0.77±0.22 [0.34, 1.21]	0.98
	Phase angle (h)	0.11±0.62 [1.32, -1.09]	1.09±0.60 [2.26, -0.08]	0.25

Table 1. Circadian sleep parameters of postmenopausal women (PMW) and young women at mid-follicular phase (YW) based on time elapsed into the ultradian sleep-wake cycle procedure. SOL = sleep onset latency. ROL = REM sleep latency, TST = total sleep time. Phase angle was calculated by subtracting the acrophase from the habitual rise-time. *P*-values for were based on two-tailed t-test. Data are expressed as mean ± SEM.

	Circadian parameters	PMW **Mean** ± SEM [95% CI]	YW **Mean** ± SEM [95% CI]	*p-values*
Post-nap alertness[a]	Mesor (cm)	6.01±0.75 [4.54, 7.48]	4.57±0.99 [2.64, 6.50]	0.14
	Amplitude (cm)	1.22±0.28 [0.68, 1.76]	2.08±0.24 [1.60, 2.55]	**0.019**
	Phase angle (h)	-10.25±0.87 [-8.54, -11.95]	-10.30±0.44 [-9.44, -11.17]	0.95
Mid-wake alertness	Mesor (cm)	7.76±0.59 [6.62, 8.91]	6.22±0.76 [4.74, 7.70]	**0.042**
	Amplitude (cm)	0.97±0.30 [0.39, 1.56]	1.68±0.24 [1.21, 2.15]	0.07
	Phase angle (h)	-10.46±1.10 [-8.29, -12.62]	-9.27±0.54 [-8.21, -10.33]	0.33
CBT[b]	Mesor (°C)	36.98±0.05 [36.87, 37.08]	37.12±0.07 [36.99, 37.26]	**0.033**
	Amplitude (°C)	0.28±0.03 [0.22, 0.34]	0.29±0.02 [0.25, 0.34]	0.80
	Phase angle (h)	2.91±0.41 [3.71, 2.10]	2.94±0.30 [3.53, 2.35]	0.95
Melatonin[a]	Mesor (pg/ml)	10.38±1.72 [7.01, 13.75]	14.75±2.27 [-0.09, 8.83]	0.055
	Amplitude (pg/ml)	3.15±1.30 [0.60, 5.70]	7.00±1.19 [4.66, 9.33]	**0.029**
	Phase angle (h)	4.26±1.41 [16.97, 22.51]	4.85±0.56 [5.95, 3.75]	0.69

Table 2. Circadian parameters of post-nap alertness, mid-wake alertness and core body temperature (CBT) of postmenopausal women (PMW) and young women at mid-follicular phase (YW) based on time elapsed into the ultradian sleep-wake cycle procedure. [a]YW: n=11. [b]PMW n=7. Phase angle was calculated by subtracting the acrophase from the habitual rise-time. *P*-values for were based on two-tailed t-test. Data are expressed as mean ± SEM.

Figure 1

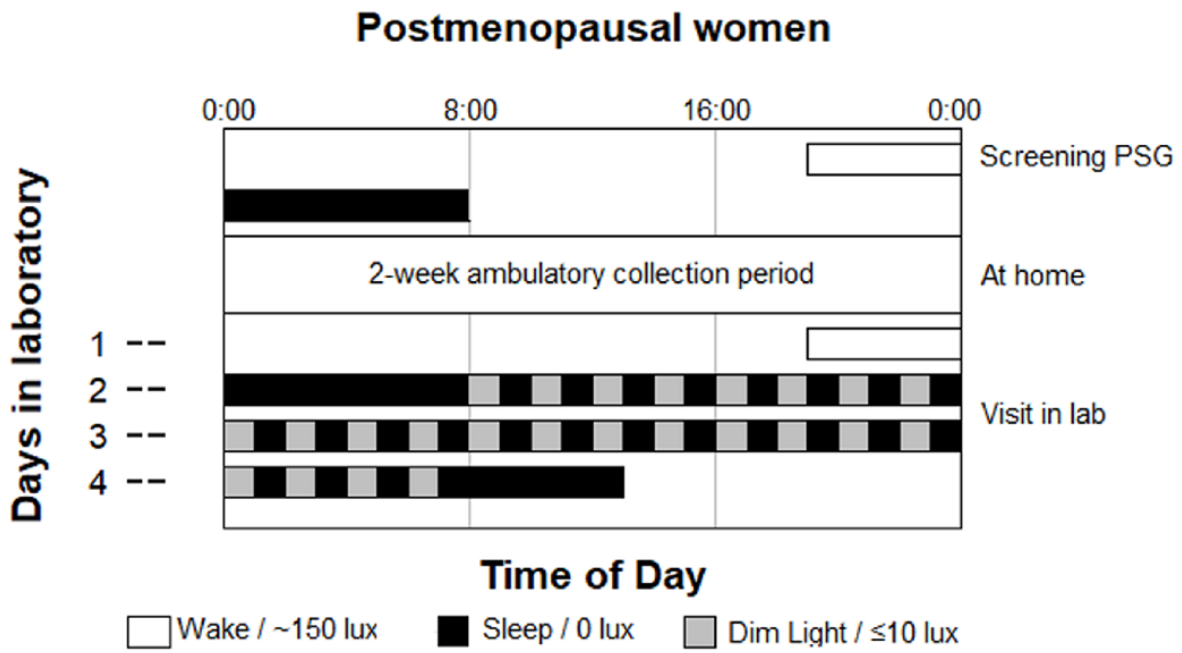

Figure 2

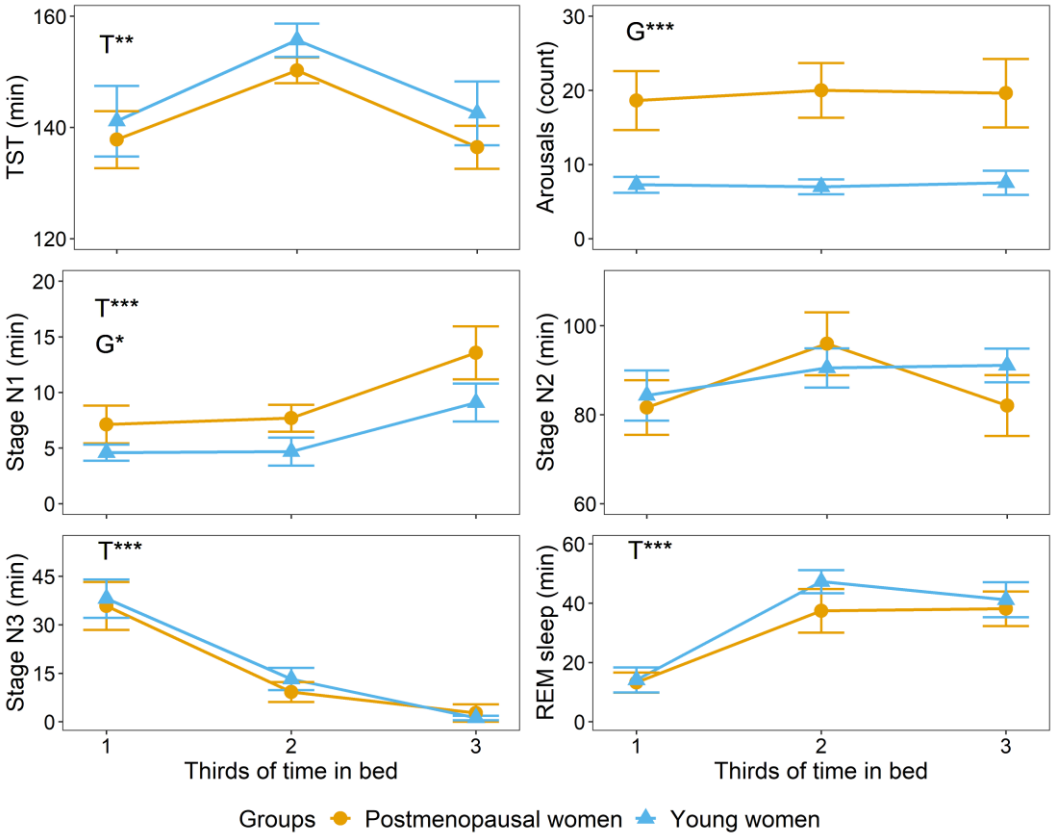

Figure 3

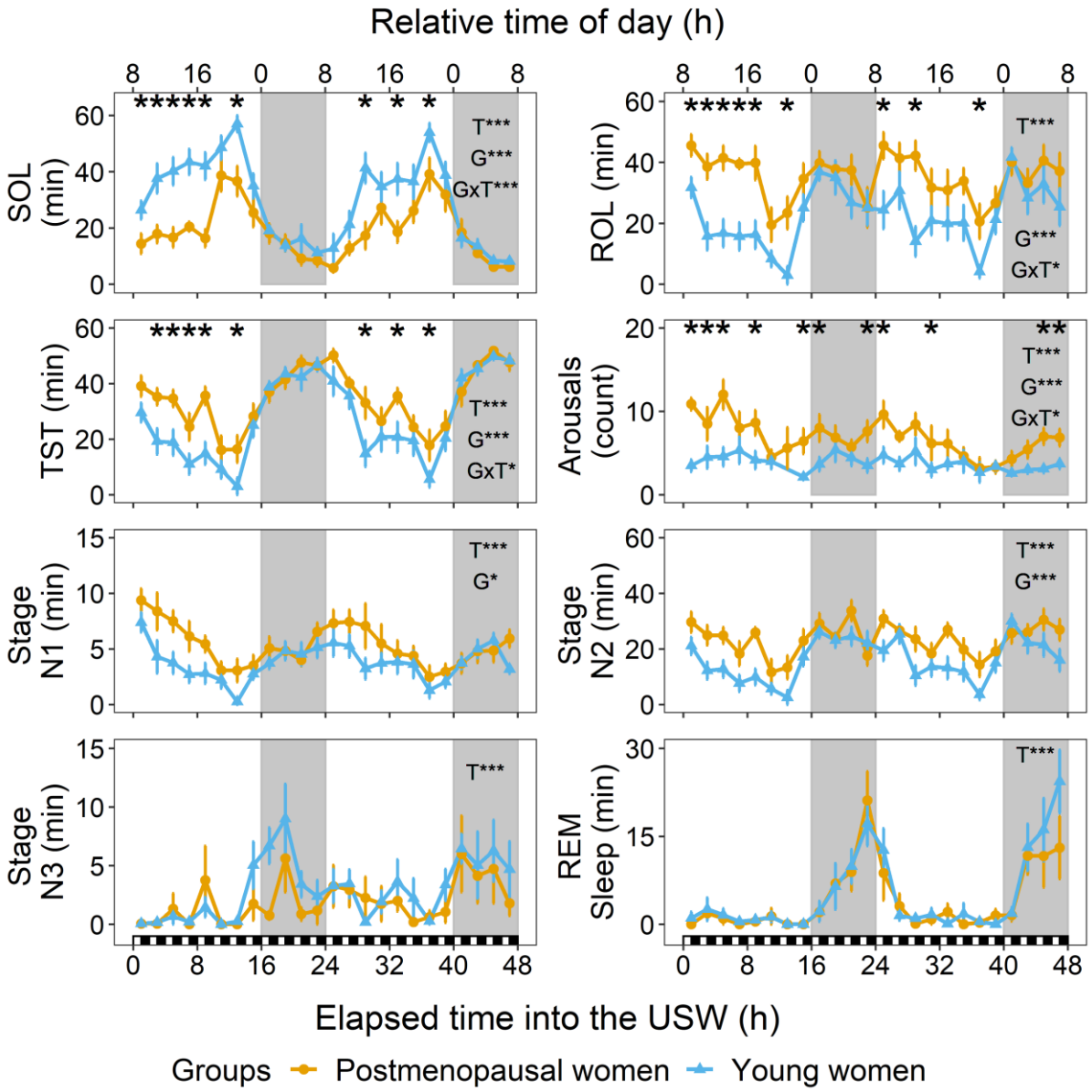

Figure 4