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ORIGINAL ARTICLE

Relationship between melatonin and bone resorption rhythms in premenopausal women

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Abstract

Although evidence exists for a daily rhythm in bone metabolism, the contribution of factors such as melatonin levels, the light–dark cycle, and the sleep–wake cycle is difficult to differentiate given their highly correlated time courses. To examine these influences on bone resorption, we collected 48-h sequential urine samples under both ambulatory (8-h sleep:16-h wake) and constant routine (CR) (constant wake, posture, nutrition and dim light) conditions from 20 healthy premenopausal women. Urinary 6-sulphatoxymelatonin (aMT6s; ng/h) and the bone resorption marker amino-terminal cross-linked collagen I telopeptide (NTx; bone collagen equivalents nM/h) were assayed and fit by cosinor models to determine significant 24-h rhythms and acrophase. Most participants had significant 24-h aMT6s rhythms during both ambulatory and CR conditions (95 and 85%, respectively), but fewer had significant 24-h NTx rhythms (70 and 70%, respectively). Among individuals with significant rhythms, mean (± SD) aMT6s acrophase times were 3:57 ± 1:50 and 3:43 ± 1:25 h under ambulatory and CR conditions, respectively, and 23:44 ± 5:55 and 3:06 ± 5:15 h, respectively, for NTx. Mean 24-h levels of both aMT6s and NTx were significantly higher during CR compared with ambulatory conditions ($p < 0.0001$ and $p = 0.03$, respectively). Menstrual phase (follicular versus luteal) had no impact on aMT6s or NTx timing or 24-h levels. This study confirms an endogenous circadian rhythm in NTx with a night-time peak when measured under CR conditions, but also confirms that environmental factors such as the sleep–wake or light–dark cycles, posture or meal timing affects overall concentrations and peak timing under ambulatory conditions, the significance of which remains unclear.

Keywords Melatonin · Circadian rhythm · Bone metabolism · Light · Sleep

Introduction

The pineal hormone melatonin has been reported to play a direct role in the regulation of bone metabolism in rodents [1–6]. Melatonin has been shown to act via melatonin receptors on mesenchymal stem cells or preosteoblasts to induce their differentiation into osteoblasts, which are the cells that generate new bone tissue [7–9]. Melatonin has also been shown to induce osteoprotegerin in osteoblast cells [10], induce osteoblast proliferation [1] and inhibit the activity of osteoclasts, which are the cells responsible for bone resorption [10, 11]. Bone formation marker mRNA levels are highest in mice during the dark phase when melatonin levels are also at their highest [6]. In addition, low nocturnal levels of melatonin induced by either ocular light exposure at night [12] or pinealectomy [13] have been shown to be associated with an increase in markers of bone metabolism in rats.

Melatonin may also influence bone metabolism in humans. In perimenopausal women, nocturnal

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supplementation with melatonin for 6 months has been shown to restore the equilibrium in single time point afternoon levels between the bone resorption marker, amino-terminal crosslinked telopeptide of collagen I (NTx), and the bone absorbing marker, osteocalcin [14]. In postmenopausal women with osteopenia, one-year nightly supplementation with melatonin increased bone mineral density (BMD) in the femoral neck in a dose-dependent manner and increased volumetric BMD in the spine at high doses (3 mg melatonin) [15]. In addition, several studies have reported that markers of bone formation [16] and bone resorption [16–25] display daily rhythms with peaks during the night, around the same time that melatonin levels reach their peak. These studies, however, were conducted in sighted individuals studied under normal sleep–wake cycles and, therefore, could not establish whether the rhythm was endogenous and self-sustained (the definition of a circadian rhythm) or was driven by external factors such as the sleep–wake or rest–activity cycles, the light–dark cycle, or feeding cycles.

The constant routine (CR) protocol is a gold-standard method employed in circadian biology to differentiate if a rhythm is intrinsically generated and sustained and, when compared to measures taken under ambulatory baseline conditions, can measure the extent to which it is driven by external factors such as sleep, light, meal timing, or posture [26]. During a CR procedure, participants remain awake in bed for typically 30–50 h (1–2 circadian cycles) in a semi-recumbent posture under dim light conditions and are fed hourly isocaloric meals [26]. This procedure removes the direct impact of sleep, light, activity, and posture on rhythm expression and distributes calorie intake uniformly across the circadian cycle, hence removing or minimizing many invoked effects due to external factors that may mask the underlying endogenous circadian rhythm. The present study was conducted to determine whether (1) the bone resorption marker NTx exhibits an endogenous circadian rhythm under CR conditions and (2) the extent to which it is influenced by environmental conditions experienced during a typical ambulatory 24-h day (i.e., including exposure to sleep–wake and rest–activity cycles, the light–dark cycle, and meal times). We examined data from participants who were studied in the laboratory under both ambulatory conditions in which they kept a habitual sleep/wake cycle, and under CR conditions. We also examined associations between menstrual phase and melatonin and bone resorption markers.

Materials and methods

Ethical considerations

Ethical permission for the study was granted by the Partners Human Research Committee/Institutional Review Board in accordance with HIPAA regulations and the Declaration of Helsinki. Written informed consent was obtained from all participants.

Participants

Twenty women were chosen at random from a larger sample of >50 participants who completed a 9-day inpatient laboratory study [27]. Urine samples from these 20 women were analyzed for 6-sulphatoxymelatonin (aMT6s) and NTx. Participants were eligible for the inpatient study if they had normal sight and were in good physical and mental health as determined by medical history and examination. Participants were also free of caffeine, alcohol, nicotine, medication, and illicit drug use as determined by urine toxicology during screening and at the time of the inpatient admission. Participants reported a normal menstrual cycle between 23 and 35 days for at least the previous 3 months, and 7 participants were using oral contraceptives at the time of the study. Participants maintained a constant sleep–wake schedule (16 h wake, 8 h sleep) for at least 2 weeks prior to the inpatient admission, confirmed for 7 days prior to admission by continuous actigraphy monitoring (Actiwatch-L; Minimitter, Inc., Bend, OR, USA), and had not traveled across more than one time zone during the previous 3 months. During the inpatient stay, participants lived individually in a suite free of time cues [27]. During the first 3 days of the study, participants were scheduled to sleep and wake at their habitual times. Participants slept in darkness and were exposed to room light (maximum of ~190 lx/48 μW/cm^2 when measured in the horizontal plane at a height of 187 cm and ~88 lx/23 μW/cm^2 when measured in the vertical plane at a height of 137 cm) until midway through study day 3, after which the light was dimmed to <3 lx (~1.5 lx/0.4 μW/cm^2 maximum when measured in the horizontal plane and ~ 0.6 lx/0.1 μW/cm^2 when measured in the vertical plane). On the morning of day 4, participants awoke to a 50-h CR procedure in which participants were kept awake in bed in a semi-recumbent posture under dim light (<3 lx) and fed isocaloric snacks hourly. Ambient light was provided by ceiling-mounted 4100K fluorescent lamps (Philips Lighting, Eindhoven, The Netherlands) and transmitted through a UV-stable filter (Lexan, General Electric Plastics, Pittsfield, MA, USA). Urine samples were collected at every urine void (approximately every 3 h while awake plus 8 h overnight) on the ambulatory days, and at every urine void (approximately every 3 h) during the CR. Urine samples collected under ambulatory conditions on study days 2 and 3 and during the initial CR condition were included in the analysis. Of the 20 participants included in the analysis, 12 were studied during the follicular phase and 8 were studied during the luteal phase of their menstrual cycle. Menstrual phase was determined on the basis of two consecutive cycles of self-report

history and plasma progesterone values at the start of the CR [mean of the first three morning values (at approximately +0, +0.5 and +1 h after waking) at the start of CR ≥6 ng/ml was defined as the luteal phase].

Sample collection and assays

Urine samples were assayed for aMT6s levels by radioimmunoassay [28] (Stockgrand Ltd., University of Surrey, Guildford, UK) and reported in nanograms per milliliter (ng/ml). The inter- and intra-assay coefficients of variation (CVs) were 11.4 and 5.7% at 4.6 ng/ml, 7.0 and 5.9% at 14.0 ng/ml, and 7.7 and 7.3% at 26.1 ng/ml, respectively. To correct for the urine volume and sampling interval between urine samples, the aMT6s level at each time point was multiplied by the total urine volume in the void (in ml) and this product was then divided by the duration of time between samples (in hours) to obtain the level of aMT6s in nanograms per hour (ng/h) [29]. This method therefore assumes that the rate of aMT6s production is constant across each sampling interval (Fig. 1).

NTx was assayed using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Osteomark; Wampole Laboratories, Princeton, NJ, USA) by one of the co-authors (PWE). NTx levels were reported as bone collagen equivalents in nanomoles per liter [bone collagen equivalents (BCE) nM/l]. The inter-assay CV ranged from 0.99−19.8% with a Spearman $\rho = 0.9888$ between log-transformed duplicates. The intra-assay CVs were 3.8% at 393.4 BCE nM/l and 2.6% at 1295.5 BCE nM/l. As with the aMT6s data, the NTx data were corrected for both the urine volume and sampling interval to obtain NTx levels in BCE nM/h (Fig. 1) by multiplying the level at each time point by the total urine volume (in liters) and then dividing this product by the total time interval between samples (in hours).

Cosinor analysis

aMT6s (ng/h) and NTx (BCE nM/h) levels were expressed as a function of the midpoint of each sampling interval. To determine whether aMT6s and NTx exhibited a 24-h rhythm, the 48-h ambulatory and CR profiles of aMT6s and NTx

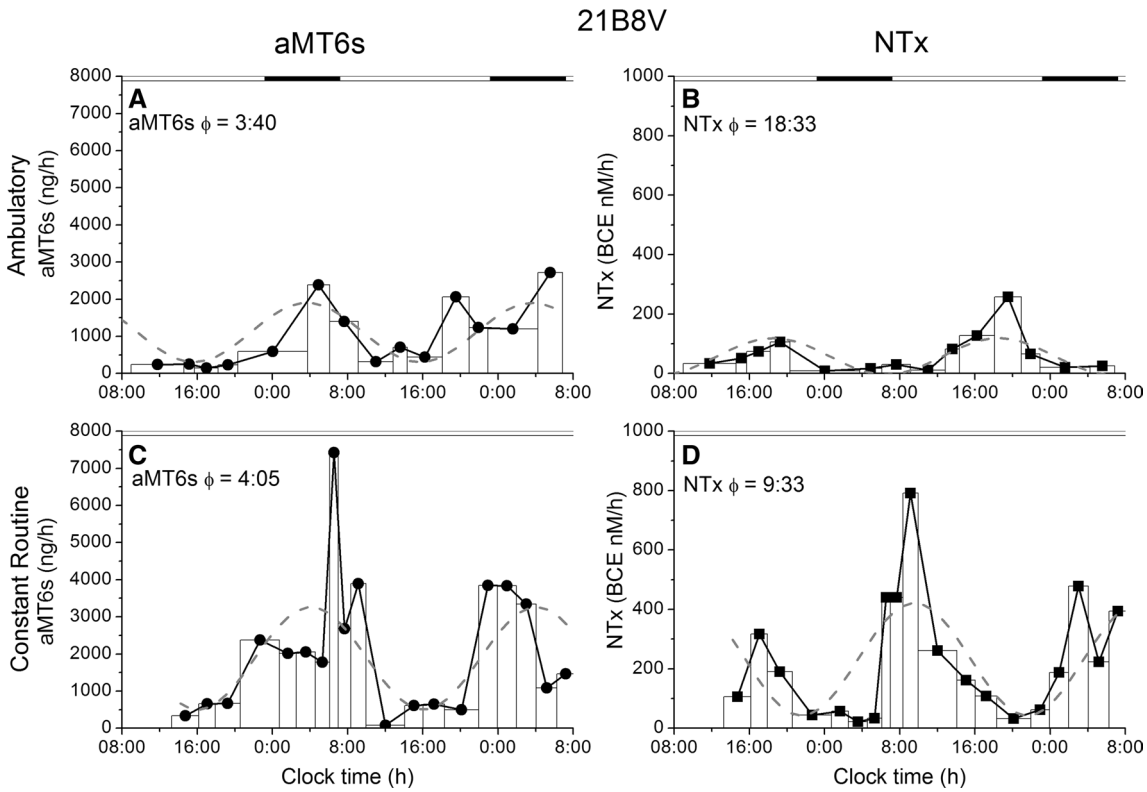

Fig. 1 The 48-h profiles of aMT6s (ng/h; **a**, **c**) and NTx (BCE nM/h **b**, **d**) in a representative subject (21B8V) during ambulatory conditions (**a**, **b**) and constant routine conditions (**c**, **d**). The data (aMT6s, black filled circle; NTx, black filled square) are plotted relative to the midpoint of the sampling interval. The conversion from aMT6s concentration in ng/ml to aMT6s in ng/h and NTx concentration in BCE nM/l to BCE nM/h assumes a constant secretion rate over the sampling interval, which is represented by the rectangular boxes in the figure. The dashed line represents the cosinor fit to the data. The acrophases (ϕ) of the cosinor fit are reported for each profile

levels were each fit separately by the following cosinor function:

$$y = m + A\cos\left(\frac{2\pi(x - t_{ac})}{24}\right),$$

where m represents the mesor (mean) of the 48-h profile, A represents the amplitude, t_{ac} represents the rhythm peak time, or acrophase, y represents the level of aMT6s or NTx and x represents the midpoint of the collection window in which the sample was taken. Cosinor analysis was conducted using PROC NLMIXED (SAS 9.3; SAS, Cary, NC, USA). A p value < 0.05 for the amplitude parameter, A, indicated a significant 24-h aMT6s rhythm, known to be a highly reliable circadian rhythm detectable by this method, whereas a p value < 0.15 indicated a significant 24-h NTx rhythm, accounting for the more exploratory analysis required when assessing novel rhythms that may be less strongly circadian or exhibit a less sinusoidal waveform [30].

Dim light melatonin onset from plasma

Plasma melatonin was assayed using methods described previously [27]. Briefly, blood was sampled every ~30 to 60 min from an indwelling intravenous catheter in a forearm vein during both the ambulatory and CR conditions. To determine the dim light melatonin onset ($DLMO_{25}$), the first melatonin profile during the CR procedure was fit with a three-harmonic regression model to estimate the amplitude. The $DLMO_{25}$ was then defined for each 24-h melatonin profile as the clock time at which the melatonin rhythm crossed a threshold value of 25% of the peak-to-trough fitted amplitude (half the standard amplitude). The $DLMO_{25}$s from the first and second melatonin profiles over the 50-h CR were averaged for comparison to the aMT6s acrophase.

Statistical analysis

Simple linear regressions (Origin 8.5 Pro; OriginLab Corporation, Northampton, MA, USA) were conducted to test for correlations between significant aMT6s and significant NTx acrophases in participants during both ambulatory and CR conditions as well as correlations between aMT6s and $DLMO_{25}$. The Wilcoxon signed-rank test was used to compare the mean 24-h levels and amplitudes of aMT6s and NTx between the ambulatory and CR conditions, which were computed by summing all assayed values within each condition for each participant, dividing by the total number of hours, and multiplying by 24 to obtain the mean 24-h level [29]. We also tested for differences in aMT6s acrophase (continuous variable) and NTx acrophase (continuous variable) with menstrual cycle phase using the Wilcoxon-Mann–Whitney test (PROC NPAR1WAY, SAS 9.3). A p value < 0.05 was considered significant for all statistical comparisons except for the NTx amplitude rhythm, as noted above.

Results

24-h rhythms of aMT6s and comparison to plasma DLMO

Representative aMT6s curves for four participants are shown in Fig. 2a, b, e, f. The aMT6s curves for the remaining 16 participants can be found in Supplementary Figure 1. Data presented in Fig. 2 and supplementary figures are aligned relative to the participant's habitual wake time (e.g., 0 h awake is equal to 08:00 in a participant with a habitual sleep–wake schedule of 00:00–08:00). Significant fits ($p < 0.05$) to the aMT6s data were observed in 19/20 participants during the ambulatory phase of the study and in 18/20 participants during the CR phase of the study, confirming that aMT6s has a strongly endogenous circadian rhythm (Table 1). Among the 19 participants with a significant 24-h aMT6s rhythm during the ambulatory phase, all but one participant had an acrophase that occurred between 01:18 and 07:06 h (mean ± SD 4:05 ± 1:47 h), consistent with a normally phased aMT6s rhythm [29]. One participant (26R1V) had an acrophase that occurred at 09:37 h, which is delayed relative to average onsets in healthy individuals without circadian rhythm disorders [29]. Among the 18 participants with a significant 24-h aMT6s rhythm during the CR, all participants had an acrophase that occurred between 01:18 and 07:06 h (mean ± SD 3:49 ± 1:35 h). The participant who did not have a significant rhythm under ambulatory conditions (2701V) exhibited a normal significant rhythm under CR conditions (04:19). The participant who exhibited a delayed acrophase under ambulatory conditions (26R1V) did not exhibit a significant circadian rhythm under CR conditions.

Plasma $DLMO_{25}$ was assessed in 18 participants; $DLMO_{25}$ was not available in 2 participants due to a significant number of missing blood samples during the CR. The correlation between aMT6s acrophase and $DLMO_{25}$ among the 16 participants who had both a significant aMT6s rhythm and an available $DLMO_{25}$ estimate was $\rho = 0.57$ (adjusted $R^2 = 0.28$). The aMT6s acrophase occurred on average 5.41 h after $DLMO_{25}$ in normally phased individuals (mean ± SD aMT6s acrophase = 3:46 ± 1:19 h; mean ± SD $DLMO_{25}$ = 22:20 ± 1:07 h), which is consistent with previous findings [31].

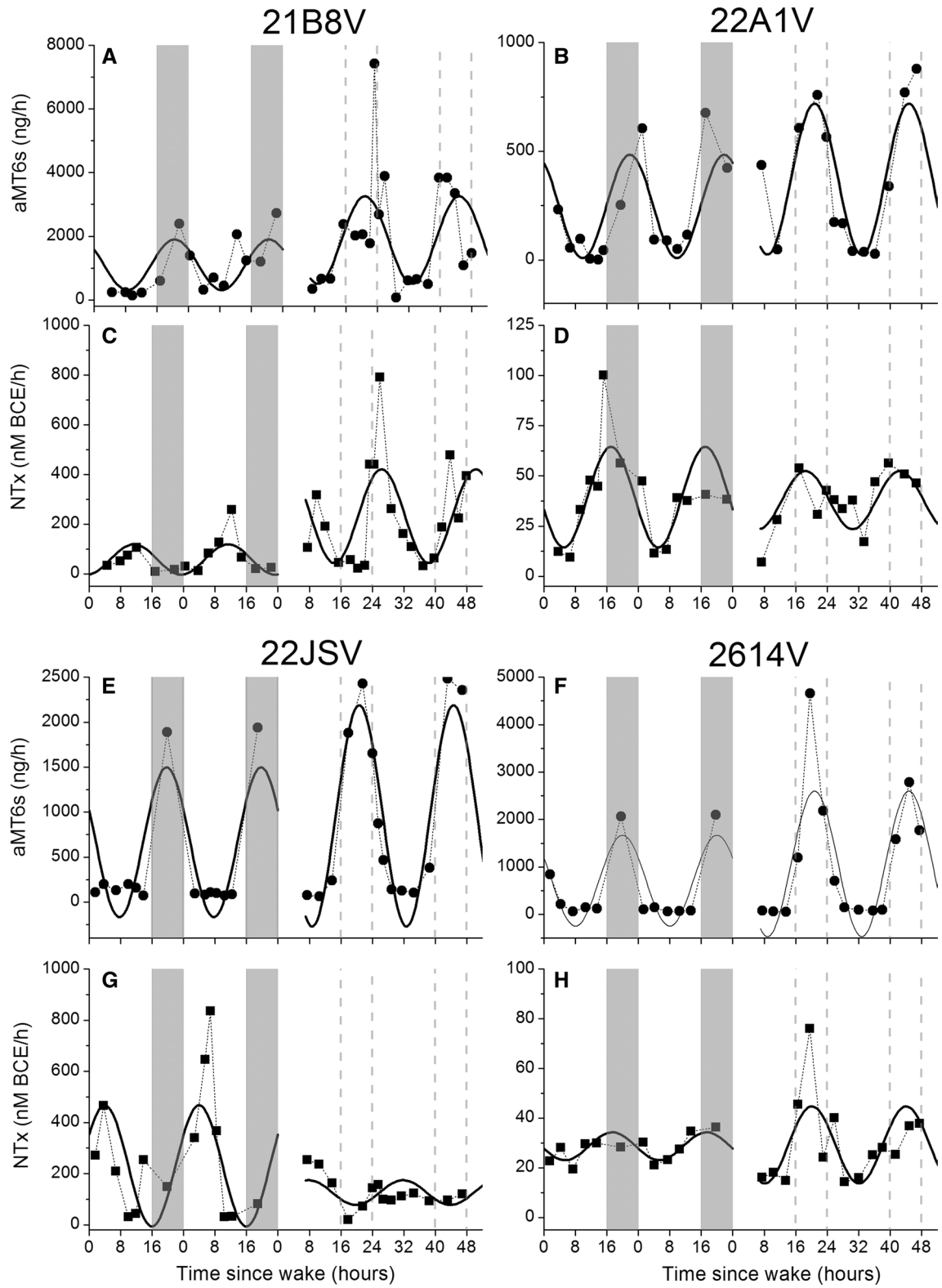

Fig. 2 Data (filled symbols) and cosinor fits (solid lines, separate fits for ambulatory and constant routine conditions) from four representative subjects. aMT6s (**a**, **b**, **e**, **f**, black filled circle) and NTx (**c**, **d**, **g**, **h**, black filled square) are plotted as a function of the midpoint of the time of collection with respect to time since habitual wake. The gray filled bars indicate times of scheduled sleep during the ambulatory condition. The gray dotted bars represent the times at which sleep was scheduled under ambulatory conditions. Gaps represent the break between the ambulatory and constant routine analysis windows, and not missing data

Table 1 aMT6s and NTx acrophases across ambulatory and constant routine conditions

Subject code	BCP use	Menstrual phase	Ambulatory				Constant routine			
			aMT6s acrophase	aMT6s *p* value	NTx acrophase	NTx *p* value	aMT6s acrophase	aMT6s *p* value	NTx acrophase	NTx *p* value
26G6V	Yes	F	**1.45**	**< 0.001**	**0.63**	**0.003**	**1.90**	**0.001**	21.45	0.243
25N6V6T2	No	F	**2.24**	**< 0.001**	**2.13**	**< 0.001**	**2.63**	**0.016**	**19.83**	**0.048**
26G3V	No	F	**2.91**	**< 0.001**	23.62	0.328	**4.87**	**< 0.001**	**6.66**	**< 0.001**
21B4V	Yes	F	**3.31**	**< 0.001**	21.60	0.237	**1.61**	**< 0.001**	21.40	0.376
2692V	No	F	**3.50**	**0.003**	**11.49**	**0.033**	**4.29**	**< 0.001**	**0.24**	**0.062**
2614V	Yes	F	**3.99**	**< 0.001**	**1.37**	**0.002**	**4.83**	**< 0.001**	**4.14**	**0.003**
22JSV	No	F	**4.18**	**< 0.001**	**12.43**	**0.004**	**5.17**	**< 0.001**	**16.35**	**0.020**
22K7V	No	F	**4.37**	**0.003**	**22.07**	**0.032**	**1.47**	**< 0.001**	**4.32**	**0.094**
26T4V	Yes	F	**4.48**	**0.002**	3.37	0.152	**4.22**	**< 0.001**	**7.60**	**0.112**
26H6V	Yes	F	**5.25**	**< 0.001**	**3.08**	**0.06**	**5.01**	**< 0.001**	**6.51**	**0.064**
26F2V	No	F	**5.73**	**0.004**	1.48	0.329	**6.10**	**0.003**	5.22	0.630
2701V	Yes	F	22.09	0.387	18.36	0.203	**4.31**	**< 0.001**	**6.20**	**0.025**
26P1V	No	L	**1.45**	**< 0.001**	**3.19**	**0.033**	**4.43**	**< 0.001**	7.26	0.160
22K3V	No	L	**2.53**	**< 0.001**	**0.91**	**0.001**	**1.39**	**0.001**	**10.14**	**0.103**
2622V	No	L	**3.35**	**< 0.001**	**0.48**	**0.031**	**3.78**	**< 0.001**	23.41	0.399
25Q2V	Yes	L	**3.40**	**< 0.001**	1.82	0.349	**3.87**	**< 0.001**	**1.13**	**0.116**
21B8V	Past	L	**3.67**	**0.003**	**18.55**	**0.004**	**4.09**	**0.006**	**9.55**	**0.003**
22A1V	No	L	**3.83**	**0.001**	**23.05**	**< 0.001**	**2.81**	**< 0.001**	**0.52**	**0.002**
2251V	No	L	**5.71**	**< 0.001**	**3.65**	**0.001**	8.38	0.165	**22.32**	**0.039**
26R1V	No	L	**9.61**	**0.005**	**13.33**	**0.094**	4.68	0.099	22.81	0.553
		Mean ± SD	3:57 ± 1:50		23:44 ± 5:55		3:43 ± 1:25		3:06 ± 5:15	

Mean ± SD were computed from participants with significant aMT6s ($p < 0.05$) and NTx ($p < 0.15$) rhythms only

BCP birth control pills, *F* follicular phase, *L* luteal phase

Significant individual acrophases (in decimal hours) are indicated in bold type

24-h rhythms of NTx and comparison to aMT6s

Representative NTx curves for four participants are shown in Fig. 2c, d, g, h. The NTx curves for the remaining 16 participants can be found in Supplementary Figure 2. Significant fits to the NTx data were observed in 14 of 20 participants during the ambulatory phase and a different subset of 14 of 20 participants during the CR phase of the study; a significant 24-h rhythm in NTx was observed during both the ambulatory and CR conditions in 10 participants. Among the 14 participants with significant NTx rhythms under ambulatory conditions, NTx acrophases spanned the entire 24-h period. The average peak across these subjects occurred at 23:44 (± 5:55 h), with the majority of individually determined peaks (10/14 subjects) occurring between 22:00 and 04:00. Similarly, among the 14 participants with significant NTx rhythms under constant routine conditions, the average peak occurred at 3:06 (± 5:15 h), with the majority of peaks (10/14 subjects) occurring between 22:00 and 08:00 h. Among the 10 participants with significant NTx rhythms during both ambulatory and CR conditions, a similarly broad range of NTx acrophases was observed. The correlation between the ambulatory and CR NTx acrophases among these 10 participants was $\rho = -0.44$ (adjusted $R^2 = 0.10$). In contrast, the correlation between ambulatory and CR aMT6s acrophases among 17 participants with significant aMT6s rhythms under both conditions was $\rho = 0.48$ (adjusted $R^2 = 0.18$).

Correlation analyses between aMT6s and NTx acrophases were conducted in participants with significant rhythms for both aMT6s and NTx during ambulatory conditions ($n = 14$) and constant routine conditions ($n = 13$). No significant relationship was observed between the aMT6s and NTx acrophases under ambulatory conditions ($\rho = -0.45$, $R^2 = 0.14$) or constant routine conditions ($\rho = -0.13$, $R^2 = -0.07$) (Fig. 3a, b). The data were then averaged across these individuals and fit by the cosinor function (Fig. 3c, d). The phase angle difference (i.e., time difference in acrophase) between the group-average aMT6s acrophase and NTx acrophase was negligible (the NTx acrophase occurred 0:21 h before the aMT6s acrophase) under ambulatory conditions, indicating that both rhythms peaked at the same time at approximately the midpoint of the sleep episode (4:19 and 3:58 h into the sleep episode for aMT6s and NTx, respectively). Under CR conditions, the aMT6s rhythm peak occurred just after the midpoint of where the scheduled sleep episode would have occurred (i.e., 4:52 h after habitual sleep onset) as under the ambulatory condition, but the NTx rhythm peaked closer to habitual wake onset (i.e., 5:44 h after habitual sleep onset) such that the NTx acrophase occurred 0:52 h after the aMT6s acrophase.

The Wilcoxon signed-rank test showed that mean 24-h levels of both aMT6s and NTx were significantly higher during the CR compared with the ambulatory phase of the study ($p < 0.0001$ and $p = 0.03$, respectively). Mean aMT6s levels were higher during CR compared with the ambulatory phase in all 20 participants; across all participants, aMT6s levels were 1.42 times higher on average during CR (Fig. 4a). Similarly, mean NTx levels were higher during CR in 17 of 20 participants (Fig. 4b); across all participants, NTx levels were 1.16 times higher on average during CR. No correlation between mean 24-h aMT6s and NTx levels was observed under ambulatory conditions ($\rho = -0.06$, $R^2 = -0.05$), but a positive correlation between mean 24-h aMT6s and NTx levels was observed during CR ($\rho = 0.65$, $R^2 = 0.39$) (Fig. 4c, d).

Effect of menstrual phase on aMT6s and NTx

Among the 20 participants in our study, 12 were studied during the follicular phase and 8 were studied during the luteal phase. Significant aMT6s rhythms were observed in all but one subject during the follicular phase and all subjects during the luteal phase under ambulatory conditions, and in all subjects during the follicular phase and all but 2 subjects during the luteal phase under CR conditions. Similarly, 7 of 12 subjects in the follicular phase and 7 of 8 subjects in the luteal phase exhibited significant NTx rhythms under ambulatory conditions, and 9 of 12 subjects in the follicular phase and 5 of 8 subjects in the luteal phase exhibited significant NTx rhythms under CR conditions. There was no significant difference in aMT6s or NTx acrophase (significant acrophases only) during either the ambulatory conditions or the CR conditions between women studied during the follicular phase and women studied during the luteal phase (all $p > 0.05$, Wilcoxon-Mann–Whitney test). Similarly, no significant difference in mean 24-h levels of aMT6s or NTx between the follicular phase and luteal phase was observed for either condition (all $p > 0.05$, Wilcoxon–Mann–Whitney test).

Discussion

The results of the present study show evidence for an endogenous circadian rhythm in the levels of urinary NTx in premenopausal women. The group-average rhythm peaks during the biological night at approximately the same time as urinary aMT6s under normal entrained conditions, and about an hour later under CR conditions. These group-average results are consistent with other studies that have observed night-time peaks in group-average bone marker and melatonin rhythms. In contrast to other studies, which have only reported group-average rhythms, in the present study we examined the timing of the NTx acrophase relative to the aMT6s acrophase across individuals and found

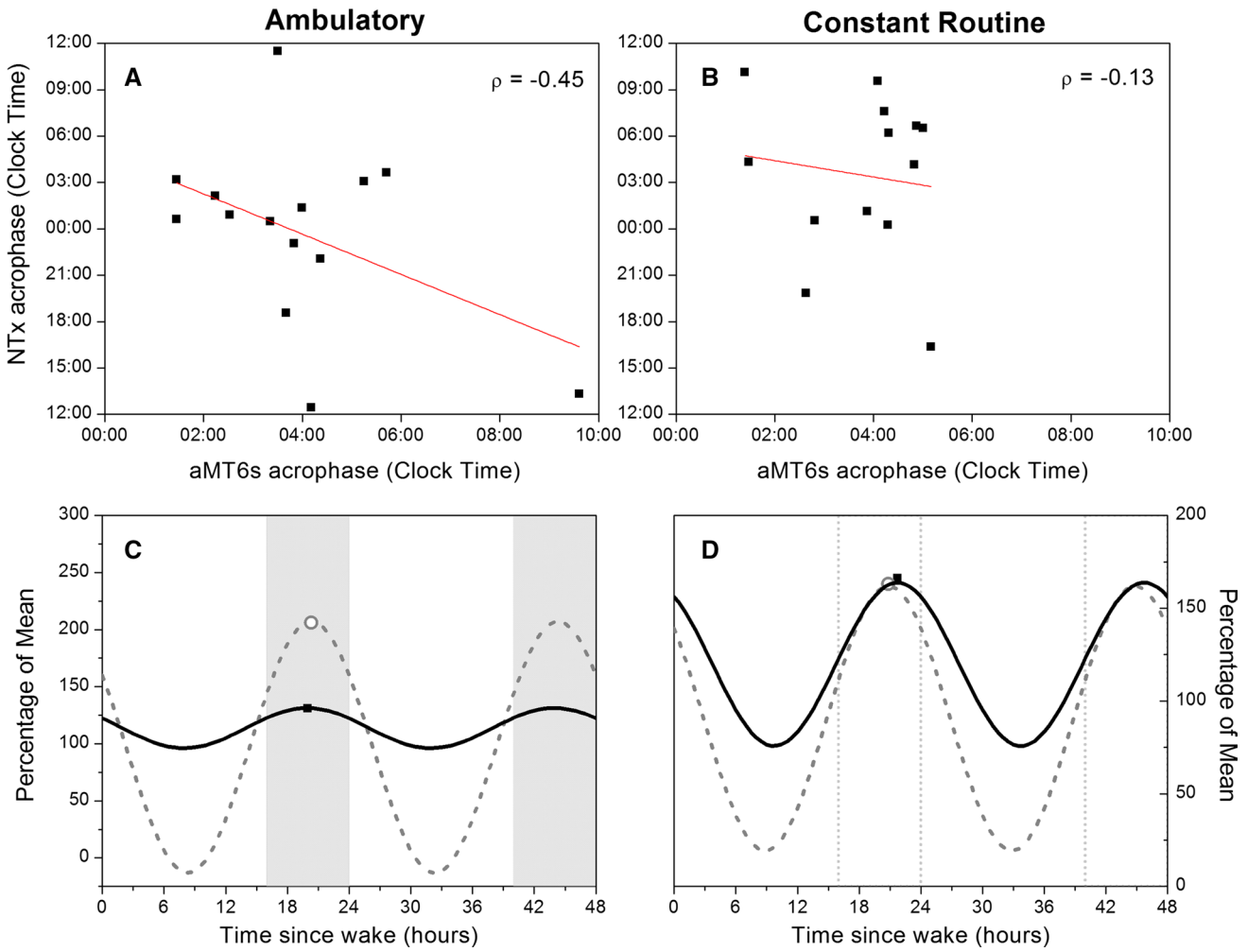

Fig. 3 The correlation between the aMT6s acrophase and the NTx acrophase during ambulatory conditions (**a**) and constant routine conditions (**b**) in participants with significant aMT6s and NTx rhythms ($n = 14$, ambulatory; $n = 13$, constant routine). Non-significant negative correlations were observed between the two measures during ambulatory ($\rho = -0.45$, adjusted $R^2 = -0.14$) and constant routine ($\rho = -0.13$ adjusted $R^2 = -0.07$) conditions. The aMT6s and NTx profiles from the ambulatory condition (**c**) and the constant routine condition (**d**) were averaged across participants with significant rhythms and fit by cosinor functions (dashed gray line = aMT6s fit, gray open circle = aMT6s acrophase, solid black line = NTx fit, black filled square = NTx acrophase). The solid gray bars represent scheduled sleep episodes (**c**) and the dashed gray bars represent the times at which sleep was scheduled under ambulatory conditions (**d**)

no relationship, raising the possibility that bone metabolism may not be tightly coupled to the melatonin rhythm. There was no major effect of menstrual phase on aMT6s or NTx timing and levels although 24-h levels of both metabolites were higher during the constant routine, suggesting suppression by unidentified environmental factors present in the ambulatory conditions.

Our results are consistent with other studies that have observed night-time peaks in group-average bone marker and melatonin rhythms [32]. Our results are also in broad agreement with unpublished findings from a study conducted in our laboratory that examined urinary NTx rhythms under ambulatory and CR conditions in 8 young healthy males [33], observing a group-average night-time peak in NTx rhythms under both ambulatory and CR conditions (05:45 vs 05:15, ambulatory vs CR, respectively). It is unknown, however, whether NTx rhythms were significant within an individual. The NTx rhythm was significant only in a subset of individuals in our study regardless of which criteria was used for defining significant circadian rhythmicity. In addition, NTx acrophase occurred across the entire 24-h day, with similar variability among NTx rhythms significant at a criterion of $p < 0.05$ (acrophase range 16:21–09:33) and those significant at a criterion of $0.05 \leq p < 0.15$ (acrophase range 00:14–10:08). Consistent with our results, however, the mean NTx level was higher under CR compared with ambulatory conditions in this previous study [33], which is also consistent with

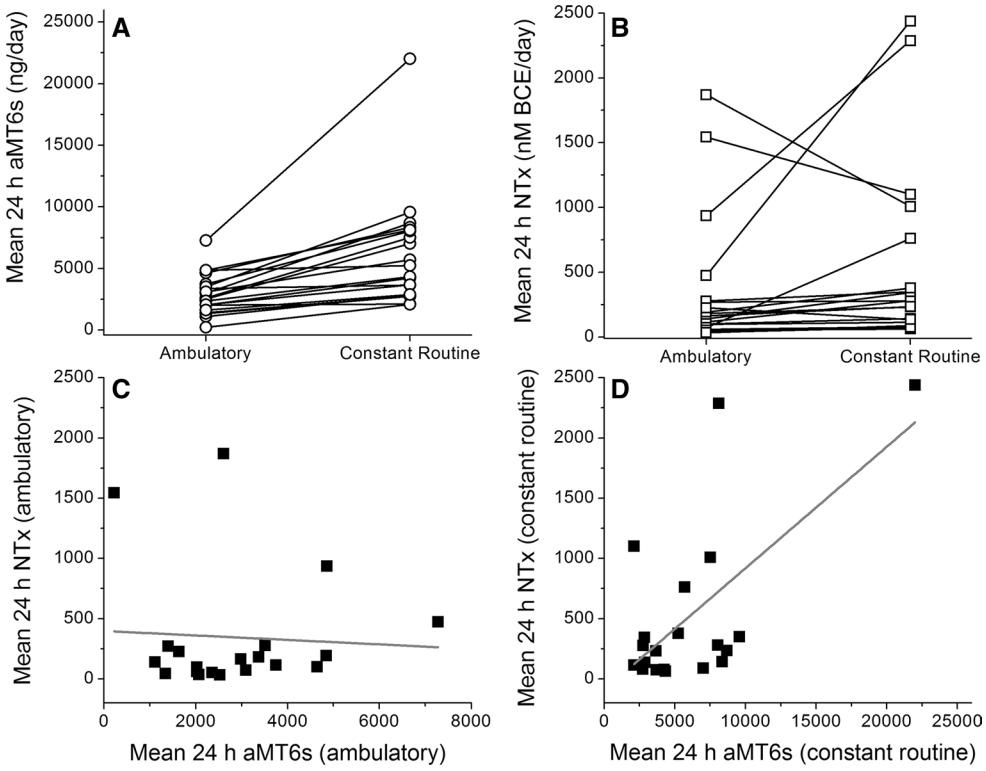

Fig. 4 The mean 24-h concentrations of aMT6s (**a**) and NTx (**b**) under ambulatory and constant routine conditions across participants. Mean 24-h levels of aMT6s increased in all participants from the ambulatory condition to constant routine. Mean 24-h levels of NTx increased in 17 of 20 participants from the ambulatory condition to constant routine. Mean aMT6s and mean NTx levels were not correlated during ambulatory conditions ($\rho = -0.06$, $R^2 = -0.05$) (**c**), but had a positive correlation during the constant routine ($\rho = 0.65$, $R^2 = 0.39$) (**d**)

findings of increased bone resorption during extended bed rest studies [34–36].

The proportions of subjects who exhibited a significant NTx rhythm under ambulatory conditions (14/20) and CR (14/20) are similar to proportions observed for other peripheral rhythms, including plasma lipids [37] and plasma cytokines [38]. Our findings are also similar to a previous study [39] in which significant diurnal variation in urinary NTx levels was observed in 19 of 26 elderly male and female subjects. One limitation of prior studies, however, is that profiles have been examined for 24 h only. Our results (e.g., Fig. 2) reveal substantial day-to-day variation in NTx concentration and timing within an individual. A single 24-h collection window, therefore, may not be representative of the true variation in bone metabolism marker rhythms. Our calculation of NTx acrophase was based on 48-h NTx concentration profiles, accounting for some of this variability. Furthermore, most studies conducted to date have reported results from sighted individuals studied under ambulatory conditions of habitual sleep–wake, rest-activity, light–dark, and meal timing conditions only. Mean 24-h aMT6s and NTx concentrations were not correlated during ambulatory conditions, suggesting that previous investigations into the 24-h rhythms of NTx and their relationship to melatonin rhythms, while relevant to day-to-day conditions under normal entrainment, may not be measuring the underlying endogenous relationships in their circadian rhythms. This distinction is likely to become more important when examining situations where normal timing of sleep, activity, light, or feeding is altered, for example during shiftwork, jet-lag, or fasting. Indeed, a recent study found that mice exposed to constant light exhibited clinical features consistent with the early stages of osteoporosis. It remains unclear from this study, however, whether the observed changes in trabecular bone health were due to the loss of a cyclic environmental cue, exposure to light at adverse circadian phases, or loss of synchrony due to reduction in rhythmic neuronal activity in the suprachiasmatic nucleus, the location of the master circadian clock [40].

The finding that NTx is under the control of the endogenous circadian clock has several important implications. Disruption of the circadian clock will also disrupt the NTx rhythm, and therefore individuals more frequently exposed to circadian disruption (e.g., shift workers and regular transmeridian travelers) or those with chronic circadian misalignment (e.g., 'early riser' workers, adolescents, circadian sleep/-wake phase disorders) may have altered bone metabolism. Consistent with this hypothesis, two studies have reported lower bone mineral density, which is a significant risk factor for osteoporosis, in shift workers who are exposed to chronic circadian, sleep and melatonin disruption [41, 42]. In addition, compared with women who have never worked night shifts, 20+ years of night shift work was associated with a significantly increased risk of hip and wrist fractures [43]. Circadian rhythm and sleep disruption are also common during space flight [44, 45],

an environment where bone health is of major concern [46, 47]. Countermeasures to improve circadian organization and sleep may also therefore benefit bone metabolism during long-duration space missions. Aging, another factor associated with risk of bone fractures, is also associated with changes in sleep and circadian rhythm organization, particularly circadian amplitude [48]. For example, reported associations between poor sleep quality (i.e., delayed bedtime, excessive daytime sleep, and daytime napping) and decreased BMD are observed in postmenopausal but not premenopausal women [49]. Understanding the role of the circadian system in mediating bone metabolism in aging might therefore facilitate the identification of new therapeutic pathways.

The broad inter-individual variation in NTx timing and levels across the 24-h period has significant implications for clinical tests of bone metabolism. Due to this variation, a clinical value measured at one time of day may be significantly higher or lower than a clinical value measured at another time, which is why clinical guidelines suggest that serum markers (e.g., CTx) be measured from a morning fasting sample and urinary markers (e.g., NTx) be measured from a second morning void sample for diagnostic purposes [50]. It is important to note, however, that despite these guidelines, clinical tests based on time of day might occur at very different circadian times between individuals, which vary by up to 5 h in healthy individuals [51–53], making interpretation of the data difficult and potentially misleading. A fuller understanding of the role of time of day and external influences is required to interpret clinical NTx results appropriately.

A limitation of the present study is that NTx concentrations measured from each urine sample were not normalized by creatinine levels as has been performed in prior studies. Creatinine clearance, however, may exhibit its own 24-h rhythm [54–56] and may also be sensitive to sleep deprivation [57], thus complicating this apparent normalization. In this study, NTx levels were corrected for measured urine volume, a method that has been used successfully to determine significant 24-h rhythms of melatonin from aMT6s in thousands of individuals and, to a lesser extent, from cortisol [29, 30, 58]. The number of participants included in the analysis, particularly when stratified with respect to menstrual cycle phase is another potential limitation of the present study. In addition, only premenopausal female participants were included in this analysis. In previous studies that have reported a diurnal rhythm of bone metabolism, the timing and magnitude of these rhythms was dependent on age, sex, disease, and hormonal status [17, 39, 59–61]. For example, the amplitude of the rhythms of bone resorption markers has been shown to be higher in postmenopausal women compared with premenopausal women [25, 61]. However, we did not observe any differences in NTx acrophases or mean 24-h levels between the follicular and luteal phases of the menstrual cycle in this limited group.

In conclusion, our study found that the NTx rhythm is generated by the endogenous circadian clock, the expression of which is influenced by environmental factors present during a standard 24-h day. The apparent relationship between NTx and melatonin rhythms changes between ambulatory and CR conditions, although the factor(s) influencing these changes are as yet unidentified. Therefore, while there is growing evidence that the circadian system plays an important role in bone metabolism in humans, the direct roles of sleep, activity, light, and/or melatonin on markers of bone resorption and bone formation remain unclear. Future studies are needed to differentiate the relative influence of environmental factors on NTx rhythm expression. To this end, we intend to examine bone metabolism rhythms in blind individuals with and without light perception and with different circadian entrainment states to determine the influence of the melatonin rhythm and circadian phase independent from the influence of the light–dark cycle, and sleep–wake, rest-activity and feeding patterns [62].

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Compliance with ethical standards

Conflict of interest In the last 24 months, MAStH has provided consulting services to The MathWorks Inc., MentalWorkout, and the Cooperative Research Centre for Alertness, Safety and Productivity, Australia. None of these commercial interests are related to the research or topic reported in this article. SAR, JJG, and PWE report no conflicts of interest. SWL has had a number of commercial interests in the last 24 months. None of them are directly related to the research or topic reported in this article; however, in the interests of full disclosure, are outlined below. In the past 2 years (2014–2016), SWL has received consulting fees from the Atlanta Falcons, Atlanta Hawks, Carbon Limiting Technologies Ltd on behalf of PhotonStar LED, Perceptive Advisors, and Serrado Capital; has current consulting contracts with Akili Interactive, Delos Living LLC, Environmental Light Sciences LLC, Focal Point LLC, Headwaters Inc., Hintsa Performance AG, Light Cognitive, OpTerra Energy Services Inc., Pegasus Capital Advisors LP, PlanLED, and Wyle Integrated Science and Engineering; owns equity in iSleep pty, Australia; has received unrestricted equipment gifts from Bioilluminations LLC, Bionetics Corporation, and F. Lux Software LLC; has received royalties from Oxford University Press; has received honoraria plus travel, accommodation or meals for invited seminars, conference presentations or teaching from Estee Lauder, Informa Exhibitions (USGBC), and Lightfair; travel, accommodation and/or meals only (no honoraria) for invited seminars, conference presentations or teaching from FASEB, Lightfair and USGBC. Through Brigham and Women's Hospital, SWL has ongoing investigator-initiated research Grants from Biological Illuminations LLC and F. Lux Software LLC; has completed service agreements with Rio Tinto Iron Ore and Vanda Pharmaceuticals Inc; and had completed three sponsor-

initiated clinical research contracts with Vanda Pharmaceuticals Inc. SWL holds process patents for the use of short-wavelength light for resetting the human circadian pacemaker and improving alertness and performance, and for a novel method to measure sleep, which are assigned to the Brigham and Women's Hospital per Hospital policy. SWL has also served as a paid expert in arbitrations related to sleep, circadian rhythms and work hours and legal proceedings related to light, sleep and health. SWL is also a Program Leader for the Cooperative Research Centre for Alertness, Safety and Productivity, Australia.

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