Sleep Science

ORIGINAL ARTICLE

Assessment of biological components associated with sleepiness in young working college students

Avaliação de componentes biológicos associados à sonolência em jovens universitários trabalhadores

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ABSTRACT

Objectives: An association, responsible for affecting circadian rhythms and sleep homeostasis, between *PER3* gene variable number tandem repeat (VNTR) and sleep times has been described in humans. The aim of this study was to evaluate the association between clock genes VNTR and sleep duration, chronotype and melatonin secretion. **Methods:** A hundred forty-six students filled a questionnaire about their sleep habits to determine individual preferences. Salivary samples were also collected for DNA extraction. *PER3* VNTR was genotyped using PCR. **Results:** Seventy subjects were $PER3^{4/4}$ (47.9%), 61 $PER3^{4/5}$ (41.8%) and 15 $PER3^{5/5}$ (10.3%). Mean sleep duration of $PER3^{5/5}$, intermediate chronotype students (8h) was higher than $PER3^{4/4}$, morningness chronotype (5:58h). On days-off, for evening-types, sleep outset was delayed (10:44h) when compared to morning-types (09:38h). Part of the students took part in a study about bright light intervention and its effects upon sleepiness. When exposed to bright light at 19:00h, the students' sleepiness growth went as expected. But, when exposed at 21:00h, sleepiness slightly increased for the intermediates and decreased at 22:00h for the evening-type students. Analyzing PER3 and *HIOMT* genotypes a specific haplotype, associated to melatonin levels at 19:00h and after bright light exposure, at 19:20h, was detected. **Conclusion:** With no social restrictions for sleep onset/outset, chronotypes express different sleep preferences, partly associated to *PER3* VNTR genotype.

Keywords: chronobiology discipline, disorders of excessive somnolence, students.

RESUMO

Objetivos: Em humanos, já foi descrita a associação entre o polimorfismo de repetição (VNTR) do gene *PER3* e os horários de dormir, afetando a ritmicidade circadiana e a homeostase do sono. O objetivo foi avaliar a associação entre polimorfismos nos genes *PER3* e *HIOMT* com a duração do sono, cronotipo e secreção de melatonina individual. **Método:** Cento e quarenta e seis estudantes preencheram um questionário sobre seus hábitos de sono. Também foram coletadas amostras de mucosa oral para extração de DNA. **Resultados:** Setenta jovens eram $PER3^{4/4}$ (47,9%), 61 $PER3^{4/5}$ (41,8%) e 15 $PER3^{5/5}$ (10,3%). A duração média do sono, nos dias letivos, dos estudantes $PER3^{5/5}$ com cronotipo intermediário foi de 8h, maior que os estudantes de cronotipo matutino e $PER3^{4/4}$ (5:58h). Nos dias de folga, para os vespertinos, o fim do sono foi atrasado (10:44h) quando comparado aos matutinos (09:38h). Ao serem expostos à luz intensa às 19:00h, seguiram o padrão esperado para aumento da sonolência quando na ausência de intervenções. Mas, quando a exposição ocorreu às 21:00h, o aumento do nível de sonolência para os intermediários foi menor que o padrão. E, para os vespertinos, redução do nível de sonolência às 22:00h. Ao se analisar os genótipos para os genes *PER3* e *HIOMT*, foi verificado um haplótipo específico para o gene HIOMT, que está associado aos níveis de melatonina às 19h e também após a exposição à luz intensa, às 19:20h. **Conclusão:** Quando não há limitantes sociais para os horários de sono, os cronotipos expressam diferentes perfis de sono, que são associados, em parte, com o genótipo do VNTR do gene *PER3*.

Descritores: disciplina de cronobiologia, estudantes, hipersonia..

INTRODUCTION

The sleep-wake cycle (SWC) is a plastic biological rhythm that changes according to information from internal and external environments, such as socio-cultural factors[1]; seasonal, climatic, and geographical differences[2]; and physiological and psychological data[3]. Daily activities may also reduce the available hours for sleep[4].

The SWC pattern also depends on individual characteristics, such as age, gender, chronotype (morningness, eveningness or intermediary), the ability to tolerate sleepiness, sleep requirements (small and large sleepers), the predisposition for naps (nappers and non-nappers), the number and duration of naps, hormonal changes, and genetic factors[5].

Individuals identified as exhibiting morningness prefer to wake up early in the morning and find it difficult to remain awake beyond their usual sleep time. These individuals exhibit higher levels of alertness upon waking, increased numbers of awakenings during the last 2 hours of sleep, decreased frequencies of paradoxical sleep, and increased frequencies of stage 1 and shorter stage 2 compared to eveningness individuals[6].

Study carried out at Escola Nacional de Saúde Pública, Fiocruz, Rio de Janeiro, RJ.
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Eveningness individuals are characterized by late sleeping and waking, especially on the weekends. The time in bed is reduced during the week, and it is compensated on days off. The SWC is irregular, sleep efficiency is reduced, and naps are more frequent during the day in these individuals[7].

Mecaci & Rocchetti[8] reported that eveningness is associated with greater symptoms of anxiety, depression, neuroticism, psychoticism, stress, and cardiovascular disease. Morningness individuals maintain greater regularity than eveningness individuals, especially in SWC circadian rhythms, in both adolescents[9] and adults[10].

These differences in different chronotypes' behaviors are confirmed by the rhythmic parameters of cortisol, core temperature, heart rate, and melatonin secretion[11].

Differences in mean questionnaire scores between men and women have been reported[12]. However, other investigators observe no correlation between gender and individual preferences[13]. Ontogenetic variations in chronotype differentiation are well known. Young individuals generally exhibit more eveningness, and the elderly exhibit more morningness[14].

Genetic mechanisms regulate circadian rhythms intracellularly, and genetic mutations alter circadian rhythms in mammals[15]. The clock genes (*Clock, BMAL1, Per1, Per2, PER3, Cry1, Cry2, CKε*, and *CKσ*) control circadian rhythms in mammals.

Polymorphisms in some rhythmic expression genes affect the circadian timing system, and these polymorphisms are associated with chronotypes[16].

The *PER3* gene is part of the *PER* gene family, and it is located on chromosome 1. The function of *PER3* is not well understood, but it is highly expressed in the suprachiasmatic nuclei of the hypothalamus where it is likely associated with disturbances in the circadian rhythm[17].

Bae et al.[18] suggested that the *PER3* gene is not essential for circadian rhythmicity. However, its role in SWC regulation is clear. Groeger et al.[19] demonstrated that $PER3^5$ homozygous individuals suffer greater effects of sleep deprivation than $PER3^4$ homozygotes. Taillard et al.[7] observed that eveningness people suffer fewer effects of sleep deprivation.

The genes that encode melatonin synthesis enzymes (e.g., AANAT and HIOMT) are strong candidates for circadian rhythmicity. Variations in these genes may interfere with the expression and control of melatonin synthesis and produce several phenotypes, such as the morningness-eveningness character[20].

The expression of circadian rhythmicity from the SWC and other rhythms that maintain stable phase relationships, such as melatonin, cortisol, core temperature, and alertness and psychomotor performance rhythms, are dependent on biological factors that are influenced by environmental and social surroundings. Exposure to intense light is an important modifying factor of biological circadian rhythmicity. Intense light adjusts biological rhythms in experimental studies, especially in individuals who suffer from seasonal depression, insomnia, and excessive sleepiness shift, as well as night workers[21,22]. Experimental studies using intense light treatment in young individuals have been performed. Duffy et al.[23] found adaptations in body temperature phase with daily activities schedules after light treatment (10,000 lux) for 20 min/hour for 3 consecutive days. Lavoie et al.[24] observed suppression of melatonin secretion and an increase in body temperature in young individuals subjected to light treatment (3000 lux from 12:30 am to 4:30 am). Research on the genetic influence of intense light in daily social surroundings is lacking. Therefore, the present study evaluated the association between *PER3* and *HIOMT* polymorphisms on SWC, subjective chronotype, and individual melatonin secretion during weekdays and on the weekend. We also evaluated differential responses to intense light exposure to reduce the sleepiness that is associated with chronotypes and the *PER3* gene.

MATERIALS AND METHODS

Population

This study included 146 working college students aged 18 to 26 years who studied in a public university in São Paulo from 7:30 pm-11:10 pm. Students who had been working for more than three months with similar work schedules (approximately 40 hours per week) were selected for this research.

Ethical aspects

The students were personally contacted and read the Statement of Informed Consent. All individuals agreed to participate in the study voluntarily by completing and signing the consent document. The Ethics Committee of the School of Public Health, University of São Paulo approved the consent form.

Data collection

Data from 192 university students were collected from 08/11/2008 to 10/31/2008. Forty-six of these subjects (23.9%) were discarded due to contamination of the material. Therefore, a total of 146 students were included in the final sample. No data were collected during holiday weeks.

The subjects initially answered the "Survey on the characterization of life, health, sleep, and work conditions." Buccal mucosa cells were collected in the second stage, and the students were instructed to rinse their mouth thoroughly with water. Each participant scraped an individual sterile brush on the inside of their cheeks approximately 20 times on each side. Each brush with buccal mucosa cells was placed in an Eppendorf tube and stored under refrigeration for subsequent DNA extraction.

Records on melatonin and SWC rhythms of the college workers during and after intense light exposure were obtained using subjective (daily activities report and Karolinska Sleepiness Scale) and objective (measurement of salivary melatonin and actigraphy) methods in the third stage. A sub-sample (n = 23) was divided into two groups and exposed to intense light (8,000 lux) for 20 minutes once a week for two weeks starting at 7:00 pm or 9:00 pm, alternately (crossover design). Saliva samples were collected for salivary melatonin measurement at 7:00 pm and 9:00 pm on exposure days and at the end of the exposure period (i.e., 7:20 pm in the week of exposure at 7:00 pm).

Survey of living conditions and health

Information on age, gender, family income, smoking and alcohol habits, health condition, and daily and weekly working hours were obtained. We obtained information on sleep location, sleep and waking hours (weekdays and weekends), strategies for sleep, sleep-related complaints[25], Epworth Sleepiness Scale[26], and the identification survey of morningness and eveningness character[27]. The results of the morningness-eveningness survey were categorized into two types: eveningness (16-41 points) and indifferent (42-58 points).

Genotyping of *PER3* and HIOMT genes

VNTR genotyping of the *PER3* gene was performed using the polymerase chain reaction (PCR). Polymorphism genotyping of the *HIOMT* gene was performed using the TaqMan SNP Genotyping Assays methodology. The genotyped polymorphisms were analyzed for possible linkage disequilibrium and arranged in haplotypes.

Actimetry

Actigraph (MicroMini-Motionlogger Actigraph, Ambulatory Monitoring, Inc®) was used on the non-dominant wrist for 21 consecutive days. The students simultaneously completed the daily activities reports to accurately determine the beginning and end of nocturnal sleep (Sadeh et al., 1989). The Sadeh algorithm (Souza et al., 2003) was used in the present study, and the following SWC variables were analyzed: beginning, end and duration of nocturnal sleep, and the middle of the sleep phase.

Assessment of sleepiness

The Karolinska Sleepiness Scale (Akerstedt & Gillberg, 1990) includes nine points that range from extremely alert (1) to very sleepy, fighting with sleep, and much effort to stay awake (9). For example, an individual responded to the question "How are you feeling now?" with the most appropriate value at that time. The alertness perception self-assessment was performed on Tuesdays, Wednesdays, and Thursdays at 7:00 pm, 8:30 pm, and 9:00 pm.

Data analysis

Data analyses were performed using the following data: gender; age; beginning, end, and middle of the sleep stage; daily and weekly working hours; chronotype[27]; and genetic material.

Two-factors ANOVA analyzed the beginning, end, and middle sleep phase variables. *PER3* gene genotype (4/4, 5/5, 4/5) and subjective chronotypes, which were obtained through the survey of individual preferences (in tertiles), were used as factors. The analyses were performed separately for working days (Monday through Friday) and days off (Saturday and Sunday). The level of significance was 5% in all analyses. Statistics 5.0 software was used.

RESULTS

Description of the population

The participant population was 56% male. The main working environment was an office (47.8%), and the main function was as an intern (65.3%). The daily working hours demonstrated that 30.4% worked more than 8 hours/day and 69.6% worked between 6 and 8 hours.

Analyses of the chronotype data revealed that 32.8% of the population exhibited eveningness, 62.7% were indifferent, and 4.5% exhibited morningness. Seventy subjects were *PER3*^{4} homozygous (47.9%), 61 subjects were heterozygous (41.8%), and 15 subjects were *PER3*^{5} homozygous (10.3%). The morningness-eveningness survey revealed that 31.6% of the population was in the 1st tertile (eveningness), 34.2% were in the 2nd tertile (intermediate), and 34.2% were in the 3rd tertile (morningness). The cutoff points of Horne & Östberg were used[27], for which 28.8% of the population exhibited moderate or extreme eveningness, 62.8% were intermediate, and 8.4% exhibited moderate morningness.

The descriptive statistics of sleep patterns (beginning, middle and end phases of sleep and sleep duration) are detailed in Table 1.

Sleep patterns, chronotype and *PER3* on Monday through Friday

The analyses of working day data demonstrated statistically significant interactions between the chronotype and *PER3* on sleep duration ($F = 3.31$, $p = 0.013$) (Table 2). The mean duration and standard deviation of sleep in students in the intermediate tertile and *PER3*^{5} homozygous was 8 hours (± 1.8h), which is significantly higher than the morningness chronotype with the same genotype (mean sleep duration 5.58h ± 1.78h) (Table 3).

Sleep patterns, chronotype and *PER3* on days off

A statistically significant difference between chronotypes and the end of sleep was observed ($F = 3.47$, $p = 0.035$) (Table 2). The end of sleep was delayed in students with the eveningness chronotype (10:44 am ± 1.42h) compared to students with a morningness chronotype (9:38 am ± 94.28h). No associations with *PER3* alone or associated with chronotypes for the days off were observed.

Sleep patterns, chronotype and *PER3* on Sunday through Monday

A statistically significant difference between chronotypes was observed ($F = 3.67$, $p = 0.03$). Students with an intermediate tertile chronotype exhibited delayed values (3:59 am ± 2.16h) compared to students with a 3rd tertile chronotype (3:33 am ± 1.24h) (Figure 1). Significant interactions were observed between the chronotype and genotype in the middle of sleep ($F = 2.71$, $p = 0.03$) and sleep duration ($F = 2.44$, $p = 0.05$). However, these differences could not be detected (Table 3).

Intense light exposure, sleepiness, and individual preferences (morningness-eveningness) on the Horne & stberg questionnaire and the *PER3* gene

Analysis of the Karolinska Sleepiness Scale at 7:00 pm and 9:00 pm on the intervention days revealed a chronotype effect at 9:00 pm ($F = 2.73$, $p = 0.05$). The students followed the expected pattern in the absence of intervention and demonstrated increased sleepiness in the early evening with intense light exposure at 7:00 pm. However, a slight increase in sleepiness levels in the intermediate ($p = 0.02$) and eveningness individuals, and a reduction in the level of sleepiness at 9:00 pm was observed with intense light exposure at 9:00 pm ($p < 0.01$) (Figure 2).

Genes and melatonin secretion

PER3 and HIOMT genotypes were compared to melatonin secretion. An HIOMT-specific haplotype was associated with melatonin concentrations at 7:00 pm and after exposure to intense light at 7:20 pm (Figure 1).

DISCUSSION

Roenneberg et al.[28] identified a trend towards a normal curve distribution for chronotypes in the world population. Alam et al.[29] reported that 32% of college students in the southern region of Brazil exhibited eveningness, 54% were intermediate, and 14% exhibited morningness. A study of 2,135

Table 1. Mean, standard deviation, minimum, and maximum (in hours) of the beginning, middle, and end of sleep stages and duration of sleep on workdays and days off.

	Sleep onset			End of sleep			Middle of sleep			Sleep duration		
	Mean ± SD	Max	Min	Mean ± SD	Max	Min	Mean ± SD	Max	Min	Mean ± SD	Max	Min
Work days	00:37 am ± 1.51h	11:00 am	10:00 pm	06:54 am ± 1.05h	10:00 am	04:30 am	03:46 am ± 0.96h	08:30 am	01:30 am	6,48h ± 1.05h	10,0h	3,25h
Off days	01:52 am ± 1.47h	06:00 am	10:45 pm	10:12 am ± 1.58h	03:00 pm	05:45 am	06:01 am ± 1.35h	09:30 am	03:15 am	8,34h ± 1.33h	12,25h	4,08h
Sunday through Monday	00:32 am ± 2.74h	12:30 pm	10:00 pm	06:56 am ± 1.09h	11:00 am	04:30 am	03:50 am ± 1.78h	02:45 pm	01:30 am	7,03h ± 1.26h	11,00h	3,33h

Table 2. Two-factors analysis of variance (ANOVA) - chronotype (1st tertile, intermediate, and 3rd tertile) and the VNTR polymorphism of the *PER3* gene (4/4, 4/5, 5/5) - of sleep variables (beginning, end and middle of sleep phase, and sleep duration) from **Monday through Friday, on days off**, and from **Sunday through Monday**. São Paulo, 2007-2008.

Variables	Factors	Monday through Friday		Off days		Sunday through Monday	
		F	*p*	F	*p*	F	*p*
Sleep Onset	Chronotype	0.74	0.479	1.60	0.207	0.14	0.864
	PER3	0.37	0.691	0.54	0.579	0.37	0.688
	Chronotype and *PER3*	0.44	0.780	0.61	0.656	0.58	0.677
End of sleep	Chronotype	0.27	0.757	3.47	0.035[b]	0.30	0.741
	PER3	0.62	0.539	0.09	0.909	0.55	0.573
	Chronotype and *PER3*	0.70	0.587	0.22	0.921	0.69	0.596
Middle of sleep	Chronotype	0.25	0.779	3.00	0.054	3.67	0.030[c]
	PER3	2.56	0.081	0.27	0.757	1.77	0.174
	Chronotype and *PER3*	0.91	0.460	0.12	0.972	2.71	0.030[d]
Sleep duration	Chronotype	2.83	0.063	0.33	0.718	0.22	0.799
	PER3	1.57	0.211	0.06	0.940	1.08	0.342
	Chronotype and *PER3*	3.31	0.013[a]	1.68	0.161	2.44	0.050[e]

[a] 2nd tertile 55 > 3rd tertile 55; [b] 1st tertile > 3rd tertile; [c] 2nd tertile > 3rd tertile; [d] no difference was detected; [e] no difference was detected.

Table 3. Mean and standard deviation of sleep duration (in hours) relative to the chronotype and *PER3* interaction.

Chronotype: *PER3*	Mean ± SD
1st tertile: 44	6.14 ± 0.93
1st tertile: 45	6.49 ± 0.75
1st tertile: 55	7.00 ± 1.68
2nd tertile: 44	6.44 ± 0.96
2nd tertile: 45	6.59 ± 0.96
2nd tertile: 55	8.00 ± 1.80
3rd tertile: 44	6.50 ± 1.00
3rd tertile: 45	6.87 ± 0.64
3rd tertile: 55	5.58 ± 1.78

First tertile: eveningness; second tertile: indifferent; third tertile: morningness.

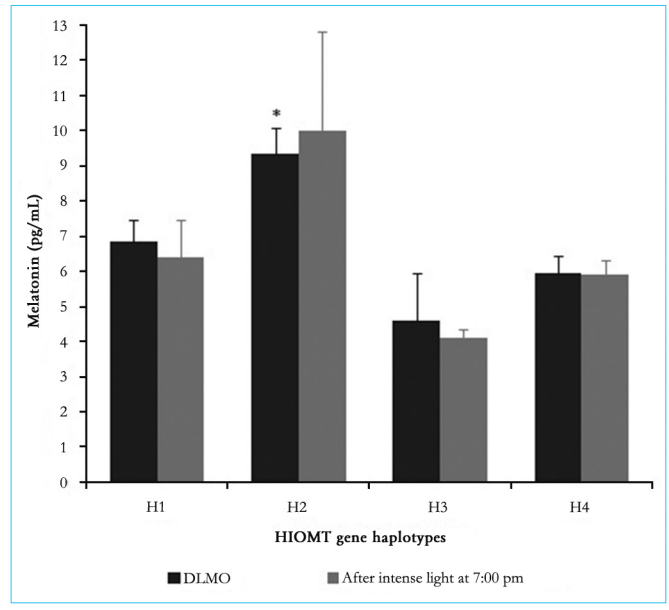

Figure 1. Melatonin levels for the haplotypes (H) H1 (GG), H2 (CG), H3 (AG), and H4 (AC) in gentle light (DLMO) and after intense light exposure for 20 minutes at 7:00 pm. * Statistically significant difference ($p < 0.05$).

Italian and Spanish college students demonstrated that 24.54% of these students exhibited eveningness, 59.62% were intermediate, and 15.84% exhibited morningness[30].

The chronotype is influenced by environmental factors, especially the time of light exposure, age, and genotype[31]. Nadkarni et al.[32] demonstrated that the 4 repetitions allele was the most common in 25 different ethnic groups in Africa, Europe, and Asia. The frequency of this allele was lower than 50% in the populations of Yemen, Ethiopia, and Papua New Guinea.

Individuals with the intermediate chronotype (and *PER3*$^{5/5}$ homozygous) exhibited longer sleep duration than the morningness chronotype with the same genotype on working days in the present study. Days off analysis revealed differences between chronotypes at the end of sleep. Students with the 1st tertile chronotype (eveningness) exhibited a delayed end of sleep (10:44 am ± 1.42h) compared to the 3rd tertile chronotype (9:38 am ± 1.57h). This result was expected because

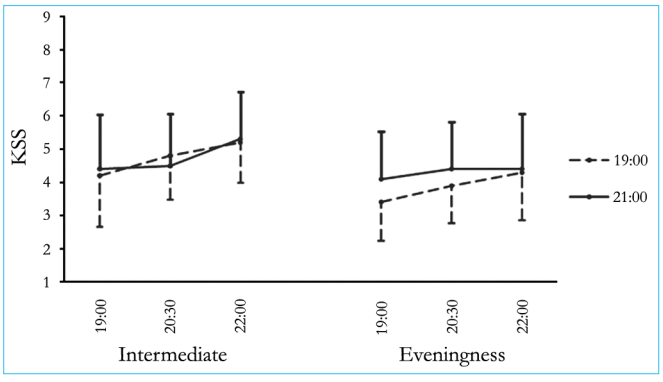

Figure 2. Mean and standard deviation of sleepiness levels (Karolinska Sleepiness Scale - KSS) according to chronotype (indifferent and eveningness). KSS results at 7:00 pm, 8:30 pm, and 10:00 pm on Wednesdays during intense light exposure at 7:00 pm and 9:00 pm. *F = 2.73; $p = 0.05$.

the literature indicates that eveningness individuals exhibit a greater delayed end of sleep tendency compared to other chronotypes[9].

The association between chronotype and the end of sleep on days off may be due to waking and sleeping hours on working days, which is independent of the student's wishes[33]. This schedule is different than days off, which lack the influence of work on the time to sleep or wake-up. Students have greater freedom to express their sleep needs on days off, which are mediated by their chronotypes.

Giannotti et al.[9] discovered differences between chronotypes on weekdays (Monday through Friday) and weekends (Saturday and Sunday) in young adults. Eveningness individuals demonstrated delayed sleep onset and wakening and shorter sleep duration compared to morningness individuals on weekdays and weekends. However, the authors emphasized that the delay of the sleep phase on weekends was more pronounced with the eveningness chronotype.

Korczak et al.[34] conducted a survey on college students in Brazil and analyzed the relationship between chronotypes and school days and days off. The authors observed associations between the chronotype and sleep onset on school days: morningness students exhibited earlier sleep onset (average at 11:00 pm) compared to intermediate and eveningness students (average sleep onsets at 12:17 am and 12:27 am, respectively). Morningness individuals began sleep at approximately the same time on weekends (11:00 pm), but intermediate and eveningness individuals began sleep at later times (approximately 1:20 am and 3:03 am, respectively). The end of sleep occurred at approximately the same time during the week for the three chronotypes. However, the end of sleep occurred later for all chronotypes on days off: 7:51 am for morningness individuals, 9:16 am for the intermediate individuals, and approximately 11:20 am for the eveningness individuals.

The relationship between individual preferences and sleepiness levels prior to and after intense light exposure at 9:00 pm was an interesting result. We observed that this intervention was effective in eveningness students who reported reduced sleepiness levels after the intervention using the Horne & Östberg questionnaire and the *PER3* gene. Griefahn et al.[35] reported a greater phase delay in melatonin secretion in the eveningness individuals who worked in shifts.

We found a significant association between a haplotype in the promoter region of the *HIOMT* gene and the secretion of melatonin prior to and after intense light intervention at 7:00 pm. The HIOMT enzyme is the last enzyme in the synthesis of melatonin from serotonin. Our data suggest that these polymorphisms modulate the effect of light on melatonin secretion.

These data are the first studies in a natural environment that suggest an effect of *HIOMT* gene polymorphism on melatonin secretion.

CONCLUSION

Our results suggest that social activities are important timing agents that should be valued in association studies between circadian genes and phenotypes. Furthermore, the use of intense light in working college students with an eveningness chronotype may reduce sleepiness during class.

Study limitations

Some of the collected samples contained food residues that contaminated the samples. These samples were discarded.

Data on the onset, end, and duration of sleep and the middle period of the sleep phase were obtained through questionnaires, which can produce values that are slightly different from objectively obtained values.

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