A *CLOCK* Polymorphism Associated with Human Diurnal Preference

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Summary: A single nucleotide polymorphism located in the 3' flanking region of the human *CLOCK* gene was investigated as a predictor of diurnal preference in a population-based random sample of 410 normal adults. Morningness-eveningness preferences were determined using the 19-item Horne-Ostberg questionnaire. Subjects carrying one of the two *CLOCK* alleles, 3111C, had a significantly lower mean Horne-Ostberg score. The distribution of scores was clearly shifted toward eveningness for these subjects. The score difference was independent of age, sex and ethnic heritage, thus making population stratification effects unlikely to explain this difference. These subjects had a substantial 10- to 44-minute delay in preferred timing for activity or sleep episodes. We suggest that the identified polymorphism or another tightly linked polymorphism within the *CLOCK* gene or its regulatory elements may be responsible for the finding.
Key words: Circadian; Horne-Ostberg; CLOCK; single nucleotide polymorphism

ENDOGENOUS CIRCADIAN RHYTHMICITY is an almost-universal property of living organisms. It is observed in prokaryotic organisms, microorganisms, plants, invertebrates, and vertebrates.[1,2] The generation of these rhythms is independent of external cues, but can be synchronized by external factors such as light exposure. Recent developments have led to the conclusion that circadian rhythms are regulated intracellularly at the genetic level. A variety of mutations have been reported to alter circadian rhythmicity in Neurospora,[3] Arabidopsis,[4] Drosophila,[5,6] hamsters,[7] and mice.[8-10] In most cases, mutations change the free-running period (τ) as recorded in environmentally constant external conditions.

Cloning studies in mice (*Clock*) and fruit flies (period: *per*, timeless: *tim*) have further shown that two identified circadian genes, *per* and *Clock*, are phylogenetically related.[10] These proteins contain a protein-protein interaction domain, the PAS domain, named for *per* (P), the human aryl hydrocarbon receptor nuclear translocator (A), and the single-minded protein (S).[11] Members of this gene family also typically contain a basic helix loop helix (bHLH) DNA-binding domain, and some are known to be transcription factors.[12-14]

How these genes contribute to the generation of circadian rhythmicity is still uncertain, but translation-transcription autoregulatory feedback loops are believed to be involved. In Drosophila, for example, the PER protein and *per* mRNA levels both display circadian rhythmicity but with a 3-4 hour difference in phase.[15] The expression of *tim* in Drosophila is necessary for these fluctuations to occur.[16-18] It is hypothesized that TIM interacts with PER to enter into the nucleus and directly or indirectly regulate the transcription of the *per* locus with a delay to produce the overall 24-hour rhythmicity.[16-18] In mammals, three puta-

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tive *PER* orthologues have been recently identified.[19-22] Two of the *PER* loci display rhythmic expression within the suprachiasmatic nuclei,[19-22] a brain region known to control most behavioral circadian rhythms in mammals.[23]

Genetic variation within human circadian genes could result in several phenotypes, ranging from arhythmicity to periodic insomnias to difficulties in entraining to external clues (eg, delayed or advanced sleep-phase syndromes). Some of these disorders have been shown to run in families.[24] We hypothesized that human *CLOCK* polymorphisms in the general population could influence circadian phase under entrained conditions, thus resulting in differential morningness-eveningness preferences. In this study, morningness-eveningness preferences were assessed using the Horne-Ostberg questionnaire[25] in 410 individuals and correlated with a polymorphism located in the 3' flanking region of the *CLOCK* gene.

MATERIALS AND METHODS

Subjects

A population-based random sample of 509 middle-aged adults enrolled in the Wisconsin Sleep Cohort Study was used in this analysis.[26] These subjects had undergone nocturnal polysomnography as part of a longitudinal study on the natural history of sleep disorders, and were asked by mail to complete a previously validated instrument, the Horne-Ostberg questionnaire, to determine morningness-eveningness preferences.[25] Questionnaires were received from 410 subjects (mean age ± SD was 50.0±7.9 years, 57.1% male, 95% Caucasians). Caucasian heritages were pooled into five broad geographically based heritage groups (Germany, Great Britain, Scandinavia, Central Europe, South Europe). The heritage group most frequently recorded was German (n=160), followed by British (n=69) and Scandinavian (n=65).

CLOCK Polymorphism Identification and Typing

A single nucleotide polymorphism located in the immediate 3' untranslated region of the human *CLOCK* gene was discovered in the course of sequencing expressed sequenced tag (EST) cDNA clones with partial sequence identity to this region of the *CLOCK* gene. The polymorphism was identified by comparing human *CLOCK* gene cDNA sequences (accession numbers: AF011568, AB002332) to the complete sequences that were obtained from 2 ESTs in this interval (accession numbers H00777 and W45459). The polymorphism is a C to T nucleotide substitution in position 3111 of the *CLOCK* c-DNA sequence; alleles are identified as 3111C in AF011568, AB002332, and W45459, and as 3111T in H00777. DNA extracted from white blood cells was amplified in the region of interest using Clock F TCCAGCAGTTTCATGAGATGC and Clock R GAGGTCATTTCATAGCTGAGC (5 cycles at 95ºC 30 seconds, 58ºC 30 seconds, 72ºC 1 minute followed by 30 cycles at 95ºC 30 seconds, 55ºC 30 seconds, 72ºC 1 minute, followed by 5 minutes at 72ºC). The resulting polymerase chain reaction (PCR) product was dot-blotted onto nylon membranes, hybridized at 42ºC in a standard solution (6x SSPE, 5x Denhardt, 0.1% N-lauroylsarcosine and 0.02%SDS) with either of two labeled sequence-specific oligonucleotides (3111C: TAGGGGCACAGCCAGTTC, 3111T: TAGGGGCATA GCCAGTTC labeled with Digoxin-11-ddUTP), and washed at 55ºC in TMAC solution (50 mM Tris, pH8, 3M tetramethylamonium chloride, 2mM EDTA, 0.1% SDS). Hybridization signals were detected by chemiluminescence after application of antidigoxin antibodies according to the manufacturer's recommendations (Boehringer-Mannheim). Subjects were then categorized into three groups on the basis of their *CLOCK* genotypes (3111 C/C, 3111 C/T and 3111 T/T). The PCR amplicon from a total of 33 randomly selected individuals was also sequenced on an ABI 377, and in all cases (two 3111 C/C, 15 3111 C/T and 16 3111 T/T), sequencing 3111C/T typing results obtained by oligotyping were confirmed. We further did not observe any other additional polymorphism in these PCR products.

Statistical Analysis

Allele frequencies were derived assuming no blanks, as described in Ott, 1985.[27] This calculation assumes that all alleles in the region covered by the probe hybridized with at least one of the two oligonucleotides, a presumption supported by our PCR sequencing data of 33 individuals in the region of interest (see above). Observed genotype frequencies were compared with expected Hardy-Weinberg equilibrium values using χ^2 analysis. Horne-Ostberg scores were calculated in all subjects using pre-established values for each question, as previously described.[25] Factor analysis was performed to compare factor-loading in our sample with previously published data obtained from 477 undergraduate students.[28] Comparison of differences in means or proportions between groups for the individual questions from the Horne-Ostberg survey were assessed using *t* tests or chi-square tests, respectively. Linear regression modeling was used to assess differences between groups for the Horne-Ostberg scores and polysomographic parameters, adjusted for the potential confounding factors of age, sex, and ethnic heritage. The SAS statistical package was used for all analysis, and p values of less than 0.05 were considered to indicate statistical significance.

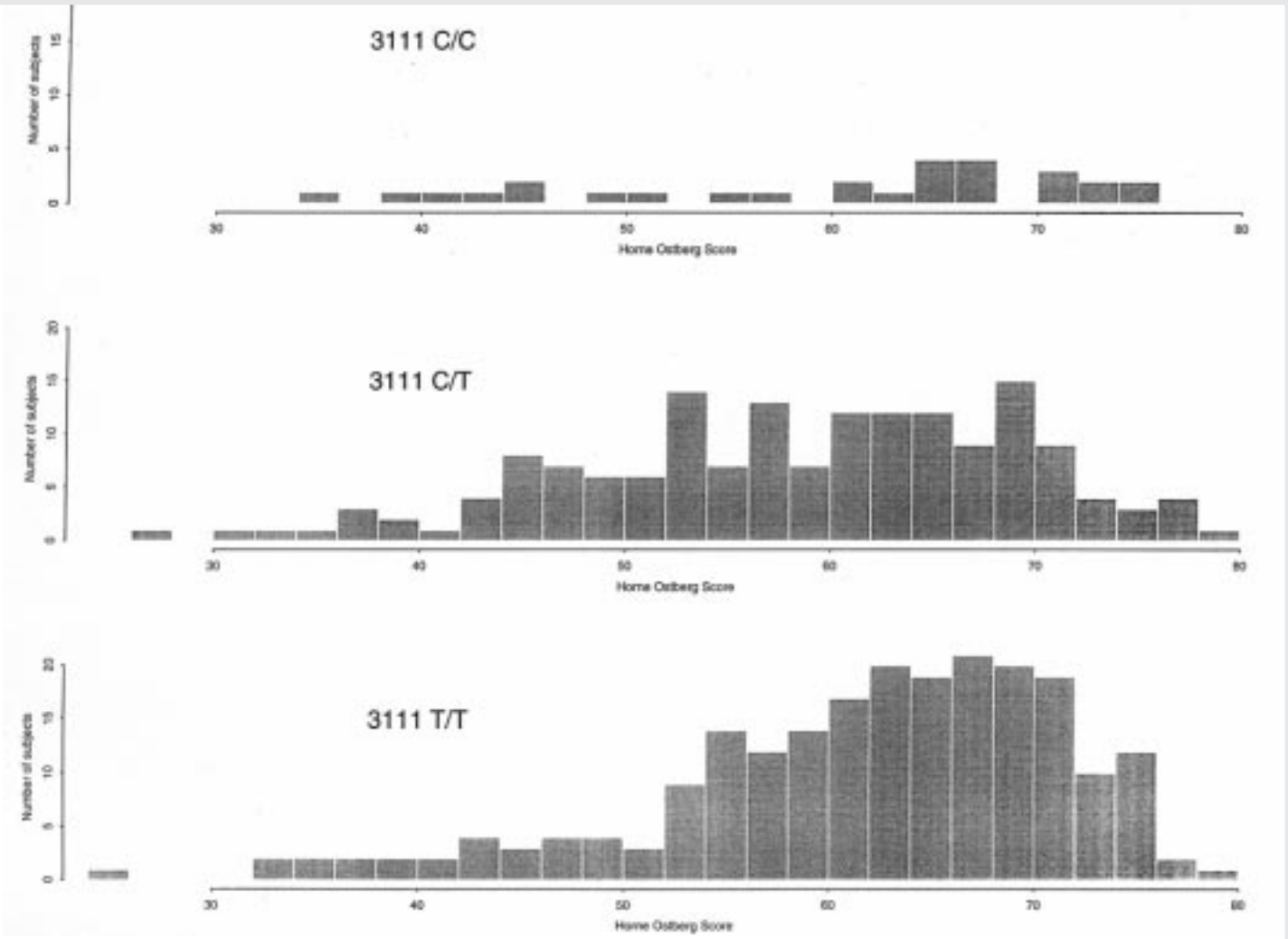

Figure 1.—Distribution of the Horne-Ostberg scores by 3111 *CLOCK* genotype. High scores indicate morningness, low scores eveningness.

RESULTS

Factor analysis indicates two main factors for morningness and eveningness respectively.—Factor analysis isolated two main factors in the Horne-Ostberg questionnaire, for morningness (factor 1) and eveningness (factor 2) respectively. This result parallels data gathered by previous investigators.[28] Individual questionnaire items factor-loading obtained in the study were then calculated and compared with published data obtained by Smith et al[28] in 477 undergraduate students. Pearson correlation coefficients of r=0.95 (n=19, p<0.0001) and r=0.81 (n=19, p<0.0001) were obtained between our study and that of Smith et al for factor 1 and 2 loading in individual questionnaire items, thus confirming the consistency of the Horne-Ostberg instrument across populations.

***CLOCK* genotype frequency distribution in the sample.**—Overall gene frequencies[27] for 3111C and 3111T alleles in this sample were 0.27 and 0.73 respectively. Observed frequencies for 3111 C/C, C/T, and T/T genotypes were 28/410, 219/410, and 163/410 respectively. These values did not differ significantly from derived Hardy-Weinberg equilibrium values[27] (χ^2=0.032, p=0.98).

3111C allele carriers have lower Horne-Ostberg scores, thus indicating increased eveningness preferences.—Mean Horne-Ostberg scores were compared between genotypes (Table 1). A significant difference was observed whether or not the scores were adjusted for possible confounding factors such as age, sex and ethnic heritage. Score differences were highly significant between 3111 T/T homozygotes vs 3111 C/T heterozygotes or all 3111C carriers. 3111T homozygous subjects had higher scores than did all other groups, thus indicating increased morningness. Subjects homozygous for 3111C had lower values when compared to 3111T homozygotes, but the difference was smaller and not significant, as might be expected with the small sample size (28 subjects). The distribution of the Horne-Ostberg scores in the three genotypes is shown in Fig. 1. It is clearly shifted toward lower scores in 3111 C/T subjects when compared to the 3111 T/T group. The distribution of the scores in the 3111 C/C

Table 1.—Mean Horne-Ostberg score, age, sex and heritage by genotypes

CLOCK Genotypes	Number of subjects	Horne-Ostberg score (unadjusted)	Age (years)	Sex (% male)	Heritage (% German)	Horne-Ostberg score (adjusted+)
3111C positive	191	59.2±0.7	49.9±0.5	59%	42%	58.8±0.8
3111 C/C	28	60.6±1.9	50.6±1.6	64%	46%	60.1±2.0
3111 C/T	163	59.1±0.8	49.8±0.6	58%	41%	58.5±0.9
3111C negative	219	62.2±0.7*++	50.1±0.6	56%	37%	61.7±0.8*++

Data are means ± SEM or %.
+ : Adjusted for age, sex and heritage, *p<0.004 compared to 3111 C/T,++ p<0.005 compared to 3111C positive.

subjects is rather spread across the entire spectrum of scores, but conclusions are not possible due to the small sample size.

Factor and individual item comparisons indicate increased eveningness and decreased morningness tendencies in subjects carrying the 3111C allele.—The observation that 3111 C/C scores were not lower than that of the 3111 C/T group argues against a recessive effect of 3111C on morningness-eveningness. 3111C heterozygotes and homozygotes were merged for further analysis. Factor 1 (morningness), factor 2 (eveningness), and individual questionnaire items were compared between 3111C-positive and -negative subjects. The difference in mean factor 1 score was of borderline statistical significance (p=0.07), while difference in factor 2 was significant (p=0.02). Significant or borderline significant (p≤0.1) differences were observed for 11 of the 19 questionnaire items (Table 2). For all comparisons, differences were in the direction of increased eveningness and decreased morningness in the 3111C carriers. In all questions pertaining to preferred timing for activity or sleep episodes, significant differences ranged from 10 to 44 minutes in delay for 3111C carriers (Table 2).

Nocturnal polysomnography in 3111C 1 positive and negative subjects.—Sleep variables were compared in 3111C positive and negative subjects (Table 3). None of the variables explored differed significantly between groups.

Consistent differences in scores are observed across heritage groups.—Horne-Ostberg scores adjusted for age and sex were compared between genotypes for seven heritage categories (Table 4). Consistently lower scores (increased eveningness) were observed across all heritage groups for 3111C carriers, and differed significantly for two ethnic groups (see Table 4 for p values). 3111C allele frequencies were also calculated for each group and found not to vary substantially across heritage categories (Table 4).

DISCUSSION

In this study, morningness-eveningness preferences were shown for the first time to correlate with a circadian gene polymorphism. The observation that some individuals prefer evening or morning hours is rooted in popular culture. These differences are usually considered constitutional, and our result suggests a genetic component for this behavior. Our finding agrees well with previous data from a study in 238 twin pairs indicating substantial heritability (H=0.48-0.56) for morningness-eveningness tendencies as measured using the Horne-Ostberg questionnaire.[29] Furthermore, no significant association between *CLOCK* genotypes and nocturnal sleep parameters were found (Table 3), making our observation selective for morningness-eveningness.

A limitation in this study might be the use of a subjective instrument, the Horne-Ostberg questionnaire, to measure morningness-eveningness tendencies. It could also be argued that the effect observed in this study is small. A three-point difference in the Horne-Ostberg questionnaire, however, corresponds to a 10- to 44-minute difference in preferred timing, a rather substantial effect. Although admittedly less robust than the differences observed in circadian gene mutant animals, one must remember that the effect observed here is reported for a single circadian gene polymorphism in an unselected human population adapting to societal constraints. Results obtained with the Horne-Ostberg questionnaire have been previously shown to correlate with timing of the temperature nadir and dim-light melatonin onset.[25,30] Scores were found to be stable over several-month intervals, and to be only partially masked by evening-work schedules[31-34] and age.[35] To further validate the questionnaire in our cohort, factor analysis was conducted on individual questionnaire items. Main factors for morningness (factor 1) and eveningness (factor 2) were isolated.[28] These two factors were identical to those previously obtained in the study by Smith et al[28] in 477 undergraduate students. Furthermore, our factor-loading for individual items was highly correlated with that reported by Smith et al,[28] confirming the consistency of the Horne-Ostberg instrument across populations.

In this study, we hypothesized that variation at the level of human *CLOCK* would correlate with morningness-

Table 2.—Individual questionnaire items in 3111C positive and negative subjects

Horne-Ostberg Question	3111C positive (n=191)	3111C negative (n=219)	p value
Q1. Preferred rise time	07:25±5.6	07:05±4.8	0.006
Q2. Preferred bed time	22:55±5.8	22:41±4.7	0.059
Q3. Need alarm clock			0.146
Not at all	12%	13%	
Slightly	33%	39%	
Fairly	20%	23%	
Very dependent	35%	25%	
Q4. Easy to get up			0.560
Not at all	6%	6%	
Not very	22%	17%	
Fairly easy	50%	50%	
Very easy	23%	28%	
Q5 Alert on rising			0.087
Not at all	8%	6%	
Slightly	29%	19%	
Fairly	46%	55%	
Very	17%	19%	
Q6. Appetite on rising			0.540
Very poor	28%	23%	
Fairly poor	28%	26%	
Fairly good	33%	39%	
Very good	11%	11%	
Q7. Tired half an hour after waking up			0.416
Very	8%	5%	
Fairly	32%	29%	
Fairly refreshed	46%	53%	
Very refreshed	14%	12%	
Q8. Bed time before free day versus usual			0.088
Seldom later	12%	19%	
< 1 hr later	38%	35%	
1-2 hrs later	41%	42%	
> 2 hrs later	9%	5%	
Q9. Performance for exercise at 7 am			0.145
Good form	32%	42%	
Reasonable form	35%	35%	
Difficult	23%	17%	
Very difficult	10%	7%	
Q10. Time at night when tired	21:38±6.9	21:34±7.0	0.661
Q11. Best time for mental work			0.006
8-10 am	61%	74%	
11 am-1 pm	26%	22%	
3-5 pm	8%	2%	
7-9 pm	5%	2%	
Q12. How tired at 11 pm			0.488
Not at all	8%	5%	
A little	27%	23%	
Fairly	40%	43%	
Very	25%	29%	

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Table 2, continued

Q13. Experience waking up after bedtime later than usual			0.018
Usual	12%	13%	
Usual but then doze	26%	40%	
Usual then fall asleep	33%	23%	
Wake later	29%	25%	
Q14. Strategy to prepare to work from 4 to 6 am			0.826
No sleep before	3%	4%	
Nap before, sleep after	5%	6%	
Sleep before, nap after	42%	38%	
Sleep before	50%	52%	
Q15. Best time for physical work			0.086
8-10 am	51%	63%	
11am - 1 pm	37%	27%	
3-5 pm	10%	7%	
7-9 pm	2%	2%	
Q16. Performance if exercise at 10-11 pm			0.514
Good form	19%	18%	
Reasonable form	34%	29%	
Difficult	24%	31%	
Very difficult	24%	23%	
Q17. Preferred time to start work	08:38±10.6	08:12±9.7	0.078
Q18. Peak time	10:49±14.8	10:05±11.4	0.021
Q19. Self rated type			0.383
Definitively morning	30%	35%	
More morning	34%	37%	
More evening	25%	21%	
Definitively evening	11%	7%	

Times in Q1, Q2, Q10, Q17 and Q18 are reported as hours ± minutes. Data is reported as % or mean ± SEM. Statistical comparisons were performed using *t* tests or χ^2 whenever appropriate.

Table 3.—Polysomnographic measures of nocturnal sleep in 3111C-positive and -negative subjects

Sleep parameters	3111C positive (n=251)	3111C negative (n=256)
Total sleep time (min)	379.9±5.0	377.3±4.6
Sleep efficiency (%)	85.8±0.7	84.5±0.6
Percent stage 1 sleep	9.2±0.4	8.9±0.4
Percent stage 2 sleep	61.5±0.8	59.9±0.7
Percent stage 3/4 sleep	11.2±0.7	12.2±0.6
Percent REM Sleep	17.8±0.5	18.9±0.4
Sleep onset latency (min)	9.7±1.0	11.0±0.9
REM latency (min)	113.2±4.8	114.8±44

Data are means ± SEM adjusted for age, sex, and heritage.
n = number of subjects. None of the comparisons were statistically significant.

Table 4.—Allele 3111C frequencies and morningness-eveningness scores by ethnic heritage

	Allele 3111C frequency	Horne-Ostberg Scores (adjusted for sex and age)	
Ethnic heritage		**3111C positive (n)**	**3111C negative (n)**
Germany	0.29	60.7±1.2 (80)	61.6±1.1 (80)
Great Britain	0.27	56.8±1.8 (33)	61.8±1.7* (36)
Scandinavia	0.25	61.4±1.8 (32)	62.2±1.8 (33)
Central Europe	0.25	53.3±2.8 (13)	59.1±2.3 (19)
Southern Europe	0.22	53.6±3.8 (7)	64.2±3.0* (11)
Other	0.21	59.9±2.7 (15)	64.4±1.9 (28)
Not specified	0.30	57.9±3.1 (11)	63.6±2.9 (12)

Data are mean ± SEM. n=number of subjects.
*p<0.05 versus 3111C positive. Allele frequencies were calculated as described in Ott,[21] assuming no blanks

eveningness preferences in the general population. The rationale for this study stems from the observation that circadian gene mutations that alter the free-running period also change the phase angle of entrainment under light:dark conditions in both fruit flies and mammals.[7,36-38] Specifically, circadian gene mutations that have been shown to reduce the free-running period (eg, *tau* hamster mutants) result in animals that anticipate their activity period earlier than controls, while the converse is observed in animals with abnormally long free-running periods (eg, *Clock* mouse mutants) (references 7, 36-38 and unpublished results obtained using *Clock* mouse mutants). Even in normal animals, several investigators have shown an inverse relationship between the length of the free-running period and the phase relationship of entrainment to external cues.[39, 40]

Equivalent effects in humans would result in differential circadian phases under entrained conditions, and thus possibly altered diurnal preferences. Work conducted in the 1970s by Wever has suggested significant interindividual variations in human free-running periods with free-running circadian periods ranging from 23.8 to 27.1 hours in 147 individuals (mean $\tau \pm SD = 25.0 \pm 0.5$ hours).[41-43] Although these early studies are now known to be partially confounded by uncontrolled artificial-light exposure [see Klerman et al[44] for discussion], these data suggest interindividual differences in the endogenous circadian period. More recently, Hall et al[30] used data collected in 68 young men studied under a constant routine protocol and with the Horne-Ostberg questionnaire. This study showed highly significant relationships between morningness-eveningness scores and body temperature or melatonin phase under constant routine. Interestingly, this study also showed that the interval between the phase of the temperature cycle and habitual waketime was significantly longer in morning types vs evening types; morning types slept better than evening types during the part of their circadian cycle most sensitive to light in term of producing phase advances and thus morningness. This last result suggests that the difference in entrained phase between types was not the result of a simple difference in the timing of exposure to light, but that it rather reflects intrinsic interindividual differences in the circadian oscillator, such as changes in intrinsic period.[30] Together with recent data from the same laboratory demonstrating a statistically significant correlation between entrained circadian and free-running period under strictly controlled environmental conditions (C. Czeisler, personal communication), these results support the hypothesis that genetically determined changes in the circadian oscillator could result in significant variation in eveningness-morningness preferences.

The finding that 3111C allele carriers have increased eveningness tendencies could be the result of a direct effect of the polymorphism studied on the expression of CLOCK. Polymorphisms in the 3' flanking region have been shown to affect mRNA stability and half-life,[45,46] and polymorphism in this region could thus have significant effects on the level of CLOCK protein finally being translated. In mouse, the mRNA 3' untranslated region of *Clock* is unusually long (6 kb), and contains several functional polyadenylation signals[10]; it may thus have important regulatory function. It is also interesting to note that the 3' polymorphism tested in this study is in a region well conserved between mice and humans (100% identity over an 8 bp sequence and >85% across 60 bp around the polymorphism). More likely, however, is that this polymorphism is only a marker for one or several other polymorphic changes within the *CLOCK* gene or its regulatory elements. In mouse, the *Clock* gene spans over 100 kbs, has 24 exons and potential alternative splicing variants,[10] thus offering numerous other possibilities for functionally significant polymorphisms. Sequencing studies within the coding region and the testing of other polymorphisms in the region should in time provide an answer to this question.

The association observed in our study could be the result of population stratification effects[47,48] in the sample. To investigate this possibility, scores were compared by genotypes within seven heritage groups (Table 4). Consistent differences between *CLOCK* genotypes were still observed, thus reducing the possibility of population stratification as a confounding factor. Furthermore, differences in overall mean scores remained statistically significant, and did not diminish in magnitude after adjustment for heritage (Table 1). Additional studies in other populations with larger sample sizes and using sib pair analysis or other intrafamilial designs will be needed to better define the genetic model involved. If confirmed and extended, our finding of an association of *CLOCK* polymorphism and diurnal preference may have far-reaching scientific and societal implications in areas as diverse as insomnia research and therapy and work schedule organization.

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