Biological Rhythm Research

ISSN: 0929-1016 (Print) 1744-4179 (Online) Journal homepage: http://www.tandfonline.com/loi/nbrr20

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To cite this article: Vladimir B. Dorokhov, Alexandra N. Puchkova, Anton O. Taranov, Petr A. Slominsky, Tatiana V. Tupitsina, Igor D. Ivanov, Valentin A. Vavilin, Victor V. Nechunaev, Sergey N. Kolomeichuk, Artem V. Morozov, Elena V. Budkevich, Roman O. Budkevich, Valeriy V. Dementienko, Dmitry S. Sveshnikov, Olga G. Donskaya & Arcady A. Putilov (2018) An hour in the morning is worth two in the evening: association of morning component of morningness–eveningness with single nucleotide polymorphisms in circadian clock genes, Biological Rhythm Research, 49:4, 622-642, DOI: 10.1080/09291016.2017.1390823

To link to this article: https://doi.org/10.1080/09291016.2017.1390823

BIOLOGICAL RHYTHM RESEARCH, 2018
VOL. 49, NO. 4, 622–642
https://doi.org/10.1080/09291016.2017.1390823

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An hour in the morning is worth two in the evening: association of morning component of morningness–eveningness with single nucleotide polymorphisms in circadian clock genes

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ABSTRACT

Sub-constructs of morning–evening preference might be differentially related to polymorphisms in circadian clock genes. We previously reported significant association between a single nucleotide polymorphism in *PER3* (rs2640909) and Morning but not Evening Lateness scale of the Sleep–Wake Pattern Assessment Questionnaire. To further explore such a scale-specific relationship, seven single nucleotide polymorphisms in five circadian clock genes were studied using exploratory and confirmatory samples (in total, $n = 698$). The association of rs2640909 with Morning Lateness scale was not replicated in the confirmatory sample but remained significant in the merged sample. Moreover, we found and confirmed an association of this scale with rs1159814 in *RORα*. The results provided further evidence for differential relationship of polymorphisms in circadian clock genes with morning and evening components of morning–evening preference. We also suggested possibility to take into account the pattern of geographic variation in allele frequency for prioritization of circadian clock polymorphisms in candidate gene studies.

ARTICLE HISTORY
Received 3 October 2017
Accepted 6 October 2017

KEYWORDS
SNPs; RORA; PER3; PER2; CLOCK; NPAS2; candidate gene; chronotype

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Introduction

The universal circadian clock mechanism is organized as a transcriptional/translational feedback loop underlying various endogenous circadian rhythms of an individual (Ko and Takahashi 2006). In mammals, BMAL1/CLOCK (or NPAS2) heterodimers activate the expression of CRY and PER (PER1, PER2, and PER3) genes acting as transcription factors directed to the CRY and PER promoters via E-box elements. PER and CRY proteins complete the feedback loop by forming heterodimers and suppressing the activity of the BMAL1/CLOCK (or NPAS2). The circadian expression of BMAL1 and NPAS2 is additionally influenced by such nuclear receptors as RORα and REV-ERBα. They regulate the expression of BMAL1 and NPAS2 mRNA by targeting a ROR-response element in the promoters of BMAL1 and NPAS2 genes (Crumbley et al. 2010).

The timing of endogenous circadian rhythms is controlled by this circadian clock mechanism and it is well-known that it widely varies in any human population. Some individuals referred to as "owls" or "evening chronotypes" are usually sleepier in the morning than in the beginning of the night and they tend to have late bedtime and late rising time. In contrast, individuals referred to as "larks" or "morning chronotypes" tend to wake up early in the morning and they feel sleepy early in the night. Other individuals fall in between these extremes (Horne and Östberg 1976). Unsurprisingly, most of candidate gene studies are focusing at uncovering genetic variation responsible for the observed differences in behavior of morning and evening chronotypes by examining associations between self-assessments morningness–eveningness scale and polymorphic variants in circadian clock genes. Substantial evidence is accumulating for significant relationships between such self-assessments and polymorphic variants in several circadian clock genes (Zhang et al. 2013; Goel 2017; Kalmbach et al. 2017). However, a common problem of many candidate gene studies is that they are massively plagued by false positive findings (Sullivan 2007). In psychiatry, nominal agreement between initial studies and meta-analyses regarding the presence of a significant effect is not better than chance (Dumas-Mallet et al. 2016). This might be also hold true for the studies of genetic variation underlying chronotypological differences, but not so many studies exploring and confirming relationship between circadian clock genes and chronotype have been published so far to allow evaluation of frequency of false positive findings.

The significant associations were most intensively reported for polymorphisms in *PER3* (PERiod circadian clock 3; e.g. Ebisawa et al. 2001; Archer et al. 2003; Dijk and Archer 2010; Hida et al. 2014; Johansson et al. 2003; Kripke et al. 2014; Mansour et al. 2017; Parsons et al. 2014; Dorokhov et al. 2017). For example, a variable number tandem repeat (VNTR) in the coding region of *PER3* (marker name: rs57875989) was related by Archer et al. (2003) to chronotype and delay sleep phase syndrome in a sample from the UK population. "Owls" and individuals suffering from delay sleep phase syndrome had the longer (5-repeat) allele whereas "larks" had the shorter (4-repeat) allele (Archer et al. 2003). Confirmation was provided in, at least, one study of South African athletes (Kunorozva et al. 2012). When a larger British sample was collected and analyzed, the results suggested that the association was significant only in young subjects and rapidly attenuated with aging (Jones et al. 2007). Although Pereira et al. (2005) confirmed significance and direction of association for sleep phase delay syndrome in a Brazilian sample, the longer allele was found to be more frequent among "larks" than "owls". Such an association in the opposite direction was confirmed by Lázár et al. (2012) and Liberman et al. (2017) in studies of samples from the UK and New York, respectively. Besides, significance of association was not confirmed in analyses of samples

collected from South African (Shawa and Roden 2016), British (Barclay et al. 2011), Norwegian (Osland et al. 2011), Romanian (Voinescu and Coogan 2012), Italian (Turco et al. 2017), Chinese (An et al. 2014), Korean (Kang et al. 2011); Pennsylvanian (Mansour et al. 2017), Californian (Kripke et al. 2014), Columbian (Perea et al. 2014) and some other populations.

We previously suggested (Dorokhov et al. 2017) that low replicability of the results on association between chronotype and polymorphisms in circadian clock genes might be partly explained by multidimensionality of morningness–eveningness scales designed to rank and chronotype people in accord with their preferred times for sleep, wakefulness, and physical and mental activity (Horne and Östberg 1976; Torsvall and Åkerstedt 1980; Smith et al. 1989; Brown 1993; Bohle et al. 2001). To become a reliable estimate of morningness–eveningness, a scale has to include, at least, 6–7 items (Di Milia et al. 2013), but factor analysis of the scales of such size or larger size yields more than one factorial dimensions (Torsvall and Åkerstedt 1980; Smith et al. 1989; Neubauer 1992; Caci et al. 2005; Randler 2009; Konttinen et al. 2014). Several studies suggested that candidate gene might be significantly associated with only one of these factorial dimensions and, consequently, a correlation with the whole scale might be attenuated. Particularly, these studies indicated that a circadian gene polymorphism might be significantly associated with only minority of items of a morningness–eveningness scale (e.g. Ellis et al. 2009) or with only some of subscales but not with the whole morningness–eveningness scale (e.g. Ojeda et al. 2013).

Some results of questionnaire studies are calling into question the existence of morningness–eveningness as a single trait. Three were, at least, three attempts to implicate conventional psychometric procedures into the process of construction of a chronobiological questionnaire that led to development of separate scales for assessment of morning and evening components of morning–evening preference (Putilov 1990, 2000, 2007; Roberts 1998; Randler et al. 2016). We previously used morning and evening scales of one of such questionnaires, the 72-item Sleep–Wake Pattern Assessment Questionnaire or SWPAQ (Putilov 2000, 2007, 2010) in the pilot association study aimed on replication of one of the findings of Ojeda et al. (2013) who suggested a link of a single nucleotide polymorphism (SNP) in *PER3* gene (rs2640909) with one of the subscales of a morningness–eveningness scale named "morningness". Notably, one of earlier reported results on rs2640909 was negative, but the whole morningness–eveningness scale was applied for testing association with this polymorphism (Dmitrzak-Węglarz et al. 2016). Our analysis of data-set collected in Moscow region from 149 bass drivers supported the expectation of significant association of rs2640909 with only morning scale of the SWPAQ and non-significant association with its evening scale (Dorokhov et al. 2017). This result pointed at possibility to partially overcome the problem of false positive findings by considering morningness–eveningness as a complex trait that might demonstrate differential relationship between a particular polymorphism in circadian clock genes and each of two its major sub-traits.

Consequently, the purpose of the present report was to provide further evidence for difference between the patterns of association of the morning and evening scales of the SWPAQ with polymorphisms in circadian genes. Data collected in four regions of Russia were used to examine whether these two scales differ from each other in strength of their link to seven SNPs in five circadian clock genes, rs228697 and rs2640909 in *PER3*, rs934945 in *PER2*, rs12649507 and rs1801260 in *CLOCK* (Circadian Locomotor Output Cycles Kaput), rs4851377 in *NPAS2* (Neuronal PAS domain protein 2), and rs1159814 in *RORα* (Retinoic acid receptor-related Orphan Receptor α).

Previously, positive finding on the association with morningness–eveningness scales/subscales were reported for rs228697and rs2640909 in *PER3*, rs934945 in *PER2*, and rs1801260 in *CLOCK* (Katzenberg et al. 1998; Mishima et al. 2005; Benedetti et al. 2008; Garaulet et al. 2011; Lee et al. 2011; Ojeda et al. 2013; Hida et al. 2014; Kripke et al. 2014; Song et al. 2016; Dorokhov et al. 2017; Liberman et al. 2017; Turco et al. 2017). For three other polymorphisms evidence for their association with chronotype was not provided, but rs12649507 and rs4851377 were previously tested for their association with such chronobiology-relevant traits as sleep duration(Allebrandt et al. 2010; Lane et al. 2013; Gagulin et al. 2016) and ability to adjust to shift work (Gamble et al. 2011).

Methods

Study participants and DNA sampling

All study participants described themselves as Caucasian. One sample included adult males involved in shift work (drivers public buses) on the territory of Moscow region (55°45′ N 37°37′ E). They volunteered to participate in the study of the role of chronotype and genotype in risk of road accidents. Their mean age ± SD (Standard Deviation) was 46.7 ± 11.3 (range from 23 to 66 years old).

Another sample was collected from student populations in three Russian cities, Novosibirsk (55°03′N 82°57′ E), Stavropol (45°02′ N 41°58′ E), and Petrozavodsk (61°48′ N 34°21′ E). They volunteered to participate in a questionnaire study of individual variation in the domains of chronobiology, personality, and behavioral genetics. The ranges of ages of male and female university students of the Novosibirsk State Pedagogical University were between 17 and 19 years and 17 and 23 years with mean age ± SD of 18.7 ± 0.7 and 19.3 ± 1.6 years, respectively. The ages of students from the North-Caucasus Federal University (Stavropol) ranged from 19 to 22 for males (20.0 ± 0.9) and from 18 to 21 for females (19.4 ± 0.7 years old). The mean age ± SD of the university students from Petrozavodsk State University was 20.1 ± 2.7 for males and 21.6 ± 2.5 for females (ranges 17–27 and 17–28 years, respectively). In total, 698 individuals were genotyped for, at least, one polymorphism (214, 133, 115, and 236 residents of Moscow Region, Novosibirsk, Stavropol, and Petrozavodsk, respectively).

DNA was collected using the well-known standardized protocols. In three of four populations, DNA was extracted from buccal epithelium cells by the method described in detail by Saab et al. (2007). The participants were asked to twirl a sterile cotton swab on each inner cheek for 15 s. Then the swabs were returned to the laboratory to extract DNA from them within 24 h. All samples were processed at room temperature (~24 °C) in accordance with good laboratory practices. In Petrozavodsk, DNA was extracted from either saliva or blood. For 30 min preceding collection of saliva, participants were requested to avoid smoking, drinking, and eating. To collect saliva with cotton swabs (Nuova Aptaca™, Canelli, Italy), participants were asked to rinse mouth with water and wait for 10 minutes before saliva collection. Then, they were asked to spit into the collection tubes until the saliva level reached 1 mL line. Handling solution was added, and the tube was gently shaken by a hand for, at least, 10 seconds. Rest of procedure was done using Syntol K-Sorb DNA Isolation Kit (Syntol; Moscow, Russia) in accord to the manufacturer's instructions. Blood sampling was performed in the course of routine health check-up between 9 am and 10 a.m. after an overnight fasting.

Blood samples (5 ml) were drawn from an antecubital vein with a 19-gauge needle. DNA was extracted from peripheral blood using K-Sorb DNA Isolation Kit (Syntol; Moscow, Russia) in accord with the manufacturer's instructions.

Genotyping on seven SNPs

SNPs genotyping was performed at three research institutes, the Institute of Molecular Genetics in Moscow, the Institute for Molecular Biology and Biophysics in Novosibirsk, and the Institute of Biology in Petrozavodsk. In the Moscow institute, four circadian clock genes were genotyped using validated TaqMan SNP Genotyping Assays (Applied Biosystems) for *CLOCK* (C___1836992_10), *RORA* (C___2095745_10), *NPAS2* (C___9902033_10) and *PER3* (C__16268918_10).TaqMan Genotyping Master Mix was used according to the manufacturer's protocols .The amplification mixture (25 µl) contained 2.5 µl of 10× Polymerase Chain Reaction (PCR) buffer; 2.5 µl of 25 mM $MgCl_2$; 2.5 µl of 2.5 mM dNTP; 0.6 µl of 40x sample; 1.25 unit thermostable DNA polymerase (Taq-polymerase, Fermentas); 0.1–0.2 µg of genomic DNA and deionized water. The amplification for TaqMan SNP Genotyping Assay plates was done in the Applied Biosystems Step One Plus 96-well Real-Time PCR System (Thermo Fischer Scientific, USA) using 100–200 ng of genomic DNA in the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 50 s at 60 °C. Data acquisition and analysis was performed using the allelic discrimination analysis module in SDS v2.4 software (Applied Biosystems).

In the Novosibirsk institute genotyping was carried out using methodology of the Allele-Specific Polymerase Chain Reaction (AS-PCR). The primers were designed with the online software OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer). The AS-PCR of *PER2* (rs934945) was carried out using 3 primers: A-specific primer 5-CA CCA AAG AAG ACG AAA ACG AA-3, G-specific primer 5-CA CCA AAG AAG ACG AAA ACG GA-3 _, and a common reverse primer 5-CAC TCT CCA CAG TTT TAA GTC GC C-3. For this *PER2* the program consisted of a 3-min denaturation step at 95 °C (1 cycle), 95 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s (45 cycles), and high resolution melting step. Alleles and genotypes were determined by presence or absence of a melting peak at 85 °C. The method applied for *PER3* (rs2640909) genotyping was very similar to that earlier described by Ojeda et al. (2013). The primers were the following: 501C, 5-TTAAGATCTGATAGGAGAAAAGAAGCCA-3, 501T, 5-TTAAGATCTGATAGGAGAAAAGAAGCTA-3, and 501R, 5'- TGCCAAATGGTGTCCTCCAG-3'. However, thermo-cycling program was slightly changed. It consisted of 3-min denaturation step at 95 °C (1 cycle), three steps at 95 °C for 15 s, 59 °C for 15 s, 72 °C for 15 s (45 cycles per each step) and a high resolution melting step. Alleles and genotypes were determined by either presence or absence a melting peak at 84 °C. The result was confirmed with electrophoresis on a 2% agarose gel by separating the fragments of 130 b.p. The following primers were utilized for the detection of polymorphic variants in *PER3* (rs228697): forward (common), CAC CCT CCG GAA GAG AAT ACG, reverse, GA CAG CAA GAC AGG ACA GGC (for C-variant detection), forward (common), CAC CCT CCG GAA GAG AAT ACG, reverse, GA CAG CAA GAC AGG ACA AGG (for G-variant detection). Genotyping was based on the appearance in fluorescence of the dyes associated with the specific alleles. PCR protocol consisted of a 5-min denaturation step at 95 °C (1 cycle), 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s (45 cycles), and high resolution melting. Amplification was performed with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The averaged times of appearance of the signal

were 25–27 cycles for genotype coincidence with the primer. Five or more cycles and a cycle or less difference in the output signal intensity was regarded as evidence for homozygous and heterozygous genotype, respectively. *RORα* (rs1159814) genotyping was carried out with the following primers: T-specific primer 5-TTAAGATCTGATAGGAGAAAAGAAGCTA-3, C-specific primer 5-TTAAGATCTGATAGGAGAAAAGAAGCCA-3_, and the common reverse primer 5′-TGCCAAATGGTGTCCTCCAG-3′. Amplification solution contained 3 µl of genomic DNA (30–50 ng), 2× reaction buffer HS-qPCR SYBR Blue (Biolabmix, Novosibirsk, Russia), 200 nm of specific pairs of primers and deionized water in a total volume of 20 µl. AS-PCR was performed in LightCycler® 96 tool (2014, Roche Diagnostics GmbH, Swiss). PCR program consisted of a 3-min denaturation step at 95 °C (1 cycle), 95 °C for 15 s, 61 °C for 15 s, 72 °C for 15 s (45 cycles per each step) and high resolution melting step. Alleles and genotypes were determined by either presence or absence a melting peak at 84 °C. The result was confirmed with electrophoresis on a 2% agarose gel by separating the fragments of 224 b.p.

In the Petrozavodsk institute, the 3′-UTR (*CLOCK* rs1801260) was genotyped using *MhlI* (Sibenzyme, Russia) and by applying the restriction fragment length polymorphism (RFLP) technique. This method recognizes C but not T allele. To digest a 221 b.p., product was amplified with forward primer 5′- TCCAGCAGTTTCATGAGATGC-3′ and reverse primer 5′-GAGGTCATTTCATAGCTGAGC-3′. Thermo-cycling program consisted of 3-min denaturation step at 95 °C (1 cycle), then 40 cycles at 95 °C for 15 s, 59 °C for 15 s, 72 °C for 15 s, and 72 °C for 5 min as final elongation step. The RFLP profiles were observed in a 6% acrylamide gel stained with ethidium bromide.

Self-assessment of morningness–eveningness

Two separate 12-item scales named "Morning Lateness" and "Evening Lateness" (M and E, respectively) from the 72-item SWPAQ (Putilov 2007, 2010) was used for self-assessment of two major components of morning–evening preference. Only these two scales were administered to the residents of Moscow region, whereas participants from other regions completed the whole questionnaire consisting of six 12-item scales (Putilov 2007, 2010). Empirical evidence for reliability and validity of these Lateness scales were published previously (e.g. Putilov 2000; Putilov et al. 2015) and reviewed elsewhere (Putilov 2010, 2016; Putilov et al. 2017). Score on each scale ranges from −12 to 12 with negative score signifying earliness and positive score signifying lateness. In addition to scores on M and E scales, we calculated sum and difference between these scores (M + E and E-M, respectively). We also used the previously collected data-sets for testing differences in lateness between the previously and newly collected data-sets (only gender- and age-matched individuals).

Additional data on geographic variation in allele frequencies for studied SNPs

We additionally analyzed latitude-dependent variation in allele frequencies in 16 populations of the 1000 Genomes Project Phase 3 (Sudmant et al. 2015). Five and 11 of them were genotyped in the places of their origin in Africa and Eurasia, respectively. Distance from the Equator (latitude, degree North) was related to the frequencies given for these samples (e.g. http://grch37.ensembl.org/Homo_sapiens/Variation/Sample?db=core;r=15:61431496-61432496;v=rs1159814;vdb=variation;vf=827012#373507_tablePanel).

Statistical analysis

All statistical analyses were performed using SPSS Statistics 22.0 (IBM corp.; Armonk, New York, USA). Chi-square test was applied for checking the Hardy–Weinberg equilibrium for each marker in each sample and subsample. We used the same statistics for examining non-randomness of genotype by phenotype distribution. For each gene, three compared genotypes were homozygotes for the minor allele, heterozygotes, and homozygotes for the major allele. Morning Lateness score was used for division on chronotypes. To predict Lateness scores and their combinations (M, E, M + E, and E – M) from genotypes, linear regression analysis was applied. We also performed MANOVAs or MANCOVAs (with age as the covariate) of E and M scores with the following *post hoc* pairwise comparison for detecting differences between genotypes (with Bonferroni correction for multiple comparisons).

To analyze geographic variation in allele frequencies, t-test for independent samples was used to compare frequency of that allele that was major in 5 African samples with frequency of this allele in 11 Eurasian samples. Principal component analysis was performed to calculate score on the first (largest) component of geographic variation in seven major allele frequencies. Finally, Spearman rank coefficients of correlation were calculated to measure spatial associations between latitude and major allele frequency for each of seven SNPs or for score on this largest principal component.

Level of significance was fixed at $p = 0.05$.

Results

Results on associations with SNPs in brief

An association of each of seven SNPs with the two morningness–eveningness scales was first tested in one of samples/subsamples. If the result was found to be positive, the testing was continued using data of another sample/sample. Positive results were obtained only for Morning Lateness scale (M), whereas all results for Evening Lateness scale (E) were negative.

The examination of association of rs1801260 in *CLOCK* using data collected in Petrozavodsk did not provide positive results. Similarly, the results were negative for rs934945 in *PER2* genotyped in there student's subsamples (from Petrozavodsk, Novosibirsk, and Stavropol). Although ANCOVA of Stavropol subsample yielded significant main effect of factor Genotype for rs228697 in *PER3* ($F_{2/124} = 3.67$, $p < 0.05$), the result was not confirmed in the Novosibirsk subsample. Moreover, frequency of minor allele was too low to provide possibility of pairwise comparison of homozygotes for minor allele with two other genotypes. The analysis of the sample collected in Moscow region did not reveal significant associations for *NPAS2* rs4851377 and *CLOCK* rs12649507. However, the associations with Morning Lateness scale for two other SNPs, rs1159814 in *RORA* and rs2640909 in *PER3* were found to be significant. In order to confirm these positive results, university students from three populations were genotypes for these two SNPs.

Results on associations of rs2640909 in PER3 *and rs1159814 in* RORα *in the driver's sample*

The positive finding for rs1159814 (*RORα*) required confirmation also due to significant difference of genotype frequencies in the Moscow sample from those expected by Hardy–Weinberg equilibrium (Table 1). The frequencies showed significant deviation favoring the

homozygotes, and in the additional subsample taken from the same population, the level of significance of deviation from the expected frequencies was found to be even higher (Table 1). In contrast, genotyping of each of three student's subsamples indicated that frequencies of *RORα* genotypes were not significantly different from those expected by Hardy–Weinberg equilibrium (Table 1).

Results of these analyses suggesting significant association of Morning Lateness scale with rs2640909 and rs1159814 are illustrated in Figures 1–3 and Tables 2–4. For instance, Table 2 shows non-randomness of genotype by M-chronotype distribution for these two SNPs. As illustrated in Figure 1, we found that homozygotes for alleles that were major and minor in this sample prevailed among earliest M-types (*PER3*) and among later M-types (*RORα*), respectively. In other words, C-alleles in *PER3* and *RORα* were found to be more frequent in late and early type, respectively (Figure 1).

Regression analyses of scores as continuous variables corroborated these results. They suggested that *PER3* and *RORα* genotypes significantly predicted M score. These results also held true after controlling for age (Table 3). Collectively, most of the total explained variance in this score was associated with genotypes (19%) and the rest of explained variance was associated with age (Table 3).

Finally, MANOVAs of M score yielded significant main effect of *PER3* genotype ($p < 0.01$) and tendency for significant main effect of *RORα* genotype ($p < 0.1$). The results persisted after including age as the covariate (Table 4). Figure 2 (A and B, right graphs) illustrates that the difference was mainly associated with being homozygote for allele C. Compared to two other genotypes, these homozygotes were more likely to be either a later or an earlier type in their morning habits and behavior (either *PER3* or *RORα*, respectively). Post hoc pairwise comparisons suggested that such difference was statistically significant. Comparison of homozygotes for the minor allele with either heterozygotes or homozygotes for the major allele yielded significant results ($p < 0.05$ or $p < 0.001$, respectively).

Table 1. Observed and expected genotype frequencies.

	Observed			Expected				χ^2-test	
n	C/C	T/C	T/T	C/C	T/C	T/T	MAF	χ^2	*p*
A. Data for *PER3* and *RORα* in all four samples or subsamples									
Gene									
PER3	25	183	236	30.6	171.9	241.6	0.26	1.86	0.172
RORα	93	214	137	90.1	219.8	134.1	0.45	0.31	0.577
B. Data for *PER3* in separate samples or subsamples									
PER3									
Novosibirsk	8	39	49	7.9	39.2	48.9	0.29	0.00	0.951
Stavropol	6	53	72	8.1	48.9	74.1	0.25	0.93	0.334
Petrozavodsk	5	30	33	5.9	28.2	33.9	0.29	0.27	0.606
Moscow	6	61	82	8.9	55.1	84.9	0.24	1.70	0.193
Moscow+	7	80	125	10.4	73.2	128.4	0.22	1.85	0.173
C. Data for *RORα* in separate samples or subsamples									
RORα									
Novosibirsk	17	45	34	16.3	46.5	33.3	0.41	0.10	0.752
Stavropol	29	70	32	31.3	65.5	34.3	0.49	0.63	0.428
Petrozavodsk	12	39	17	14.6	33.8	19.6	0.46	1.60	0.206
Moscow	35	60	54	28.4	73.3	47.4	0.44	4.90	0.027
Moscow+	55	81	76	43.0	105.0	64.0	0.45	11.05	<0.001

Notes: T/T: Homozygotes for the major allele; T/C: heterozygotes; C/C: homozygotes for the minor allele; MAF: Minor Allele Frequency; χ^2-test: Comparison of frequencies expected from the Hardy–Weinberg equilibrium. Moscow+: After including additional 63 genotyped individuals from the same population of shift workers without the SWPAQ self-assessments.

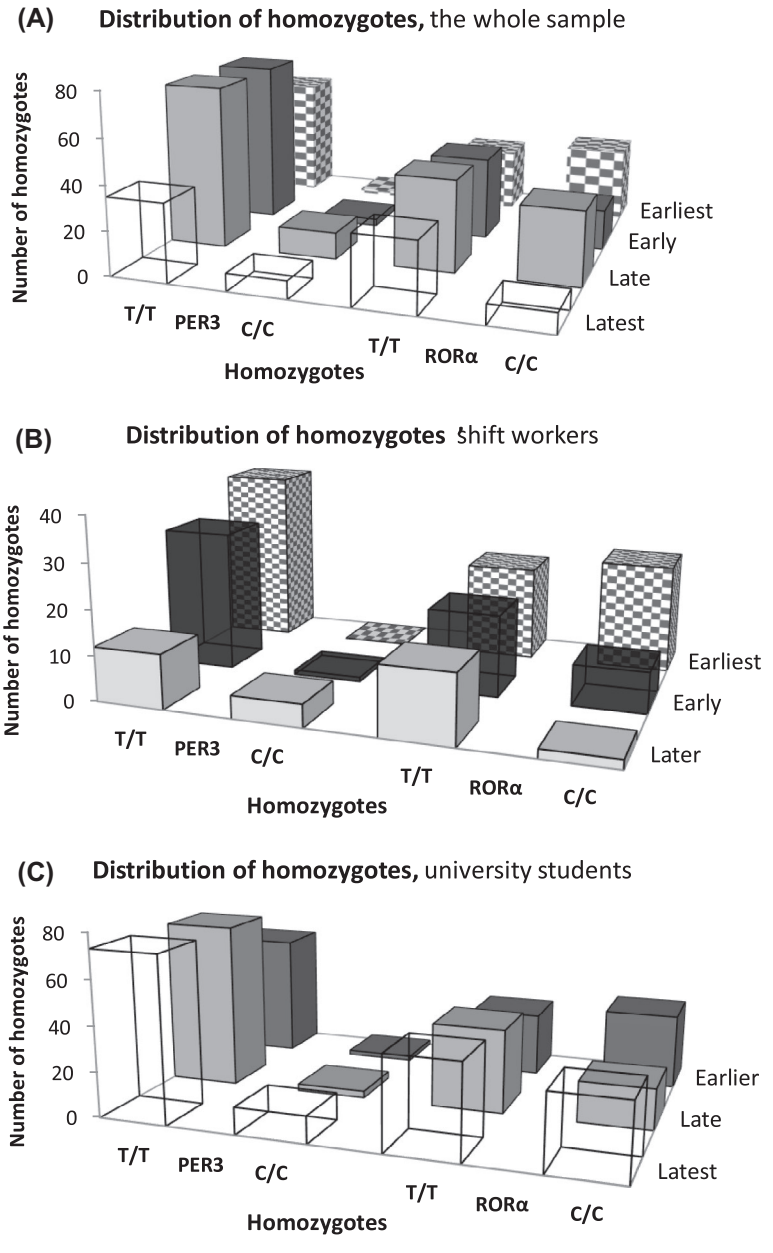

Figure 1. Homozygotes by M-chronotype distribution. Figure illustrates the distributions for homozygotes (see Table 2).

Results on associations of rs2640909 in PER3 *and rs1159814 in* RORα *in the student's sample*

The attempt to replicate these positive findings obtained for the sample from Moscow region gave the following results. The same directional effect of *RER3* genotype on Morning Lateness score was noted in data from the three student's subsamples but this effect failed to reach a statistically significant level (Tables 3 and 4). Only a tendency for such a relationship ($p < 0.1$)

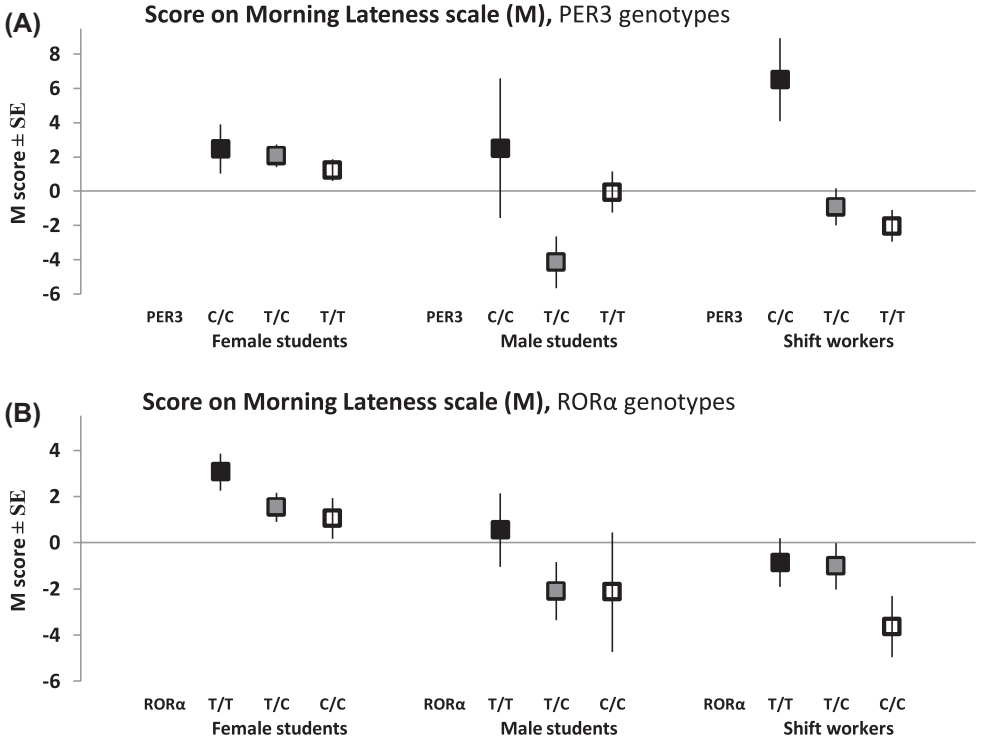

Figure 2. Association of score on Morning Lateness scale with *PER3* and *RORα* genotypes. (A and B) Results for score on Morning Lateness scale (M) from two two-way MANCOVAs of scores on two Lateness scores (M and E). Independent factors were Group (female and male university students and shift workers) and Genotype (either *PER3* or *RORα*, A and B, respectively), and age was the covariate.
Note: Vertical bars show ± Standard Error (SE) of genotype-averaged scores.

was revealed by MANCOVA (Table 4). However, the relationship remained significant in the analysis of the merged sample (Tables 2–4). As for the effect of *RORα* genotype, it was both the same directional and significant in the student's subsamples (Tables 2–4, Figures 1–3). Significant difference between *RORα* homozygotes was found in *post hoc* pairwise comparison ($p < 0.05$). We also found significant interaction of *RER3* genotype with either gender or sample in the analyses of either confirmatory or merged samples, respectively (Table 4). Such results suggested that the effect of this polymorphism might be sex-specific with only M score to be related to two copies of the rare allele in only male study participants (Figures 2(A) and 3(C)).

The analysis of combinations of Lateness scores suggested that, due to the absence of significant association of E score with any of the genotypes, both sum and difference in E and M scores might be, sometimes, significantly related to genotype, e.g. in the sample from Moscow region (Table 3, right side). In the merged sample, the association only tended to reach a statistically significant level for the SNP in *PER3* (Table 3(A), right side).

Results of comparison of lateness scores in sex- and age-matched samples

To further describe chronotypological differences between the study participants, we compared scores of the genotyped drivers from Moscow region ($n = 149$) with scores obtained

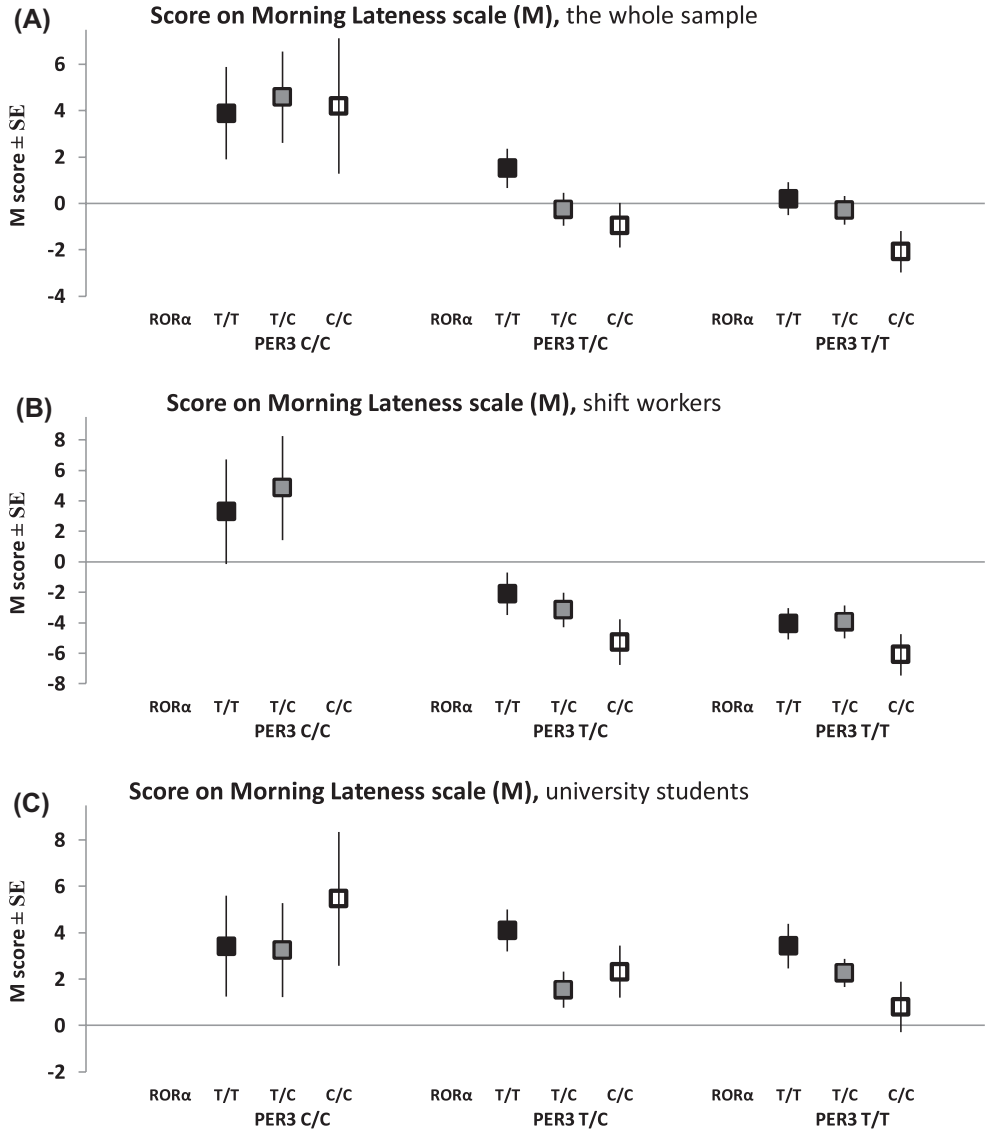

Figure 3. Association of score on Morning Lateness scale with combinations of two genotypes. A. Results for Morning Lateness (M) score from three-way MANCOVA of scores on two Lateness scores (M and E). Independent factors were Sample (exploratory sample of shift workers and confirmatory sample of students) and Genotypes (three *PER3* and three *RORα* genotypes), and age was the covariate. (B and C) Two-way MANCOVAs performed separately on the exploratory and confirmatory data-set (B and C, respectively).

Notes: Independent factors were Genotypes (three genotypes per each polymorphism, *PER3* and *RORα*), and age was the covariate.

for two additional samples. One of them represented the same population ($n = 63$) and another represented age-matched day/evening workers who were earlier chronotyped in Novosibirsk studies ($n = 206$). The results of one-way MANCOVA with the independent factor Sample yielded significant difference in M score but not E score between any shift workers from Moscow region and day/evening workers from Novosibirsk. In particular, results of *post*

Table 2. Genotype by phenotype distributions.

	M score	C/C	T/C	T/T	C/C	T/C	T/T
A. Merged sample							
	Gene		*PER3*			*RORα*	
Type	M score	C/C	T/C	T/T	C/C	T/C	T/T
Earliest	From −12 to −6	2	49	55	33	45	28
Early	From −4 to 0	3	43	73	18	64	37
Late	From 2 to 6	12	55	73	33	66	41
Latest	From 8 to 12	8	36	35	9	39	31
	Statistics	χ^2	df	*p*	χ^2	df	*p*
		14.28	6	0.027	15.22	6	0.019
B. Exploratory sample							
	Gene		*PER3*			*RORα*	
Type	M score	C/C	T/C	T/T	C/C	T/C	T/T
Earliest	From −12 to −6	0	30	39	24	24	21
Early	From −4 to 0	1	17	31	9	22	18
Later	From 2 to 12	5	14	12	2	14	15
	Statistics	χ^2	df	*p*	χ^2	df	p
		17.38	4	0.002	10.99	4	0.027
	Gene		*NPAS2*			*CLOCK*	
Type	M score	C/C	T/C	T/T	A/A	A/G	G/G
Earliest	From −12 to −6	7	29	14	9	30	30
Early	From −4 to 0	4	20	15	8	20	20
Later	From 2 to 12	4	9	9	4	11	16
	Statistics	χ^2	df	*p*	χ^2	df	*p*
		2.59	4	0.628	1.10	4	0.895
C. Confirmatory sample							
	Gene		*PER3*			*RORα*	
Type	M score	C/C	T/C	T/T	C/C	T/C	T/T
Earlier	From −12 to 0	2	49	55	33	45	28
Late	From 0 to 6	3	43	73	18	64	37
Latest	From 8 to 12	12	55	73	33	66	41
	Statistics	χ^2	df	*p*	χ^2	df	*p*
		3.76	4	0.500	11.24	4	0.024

Notes: χ^2-test: χ^2-statistics, df: degree of freedom, p: level of significance; Type: Typing of study participants using scores on Morning Lateness scale (M) ranged from −12 to +12. Distribution for homozygotes is illustrated in Figure 1.

hoc multiple comparison (with Bonferroni correction) were significant with $p = 0.005$ and 0.029 for the difference of two samples of shift workers from the sample of day/evening workers. Such results indicated a shift toward morning earliness in the population of shift workers relative to population of day/evening workers.

As for the subsamples of male and female students from three different cities, they did not differ in M score from the previously collected sample of young residents of Novosibirsk ($n = 272$ with 120 males). We found that $p > 0.120$ for each of *post hoc* comparison following two-way MANCOVA of M score with the independent factors Sample and Gender.

As we expected, this and the previous analyses (Tables 3 and 4) also pointed at significant gender difference in M score. It indicated a shift toward morning earliness in male adults as compared to female adults.

Results on geographic variation in allele frequencies

Comparison of allele frequencies in 5 African and 11 Eurasian populations of the 1000 Genomes Project yielded significant out-of-African reduction of the allele that was major in Africa in 5 of 7 SNPs. The same tendency was evident for two remaining SNPs, rs2640909 in

Table 3. Prediction of scores on Lateness scales.

A. Merged sample

Score	M		E		M + E		E – M	
Summary	*R*	R^2	*R*	R^2	*R*	R^2	*R*	R^2
	0.481	0.232	0.110	0.012	0.308	0.095	0.444	0.198
ANOVA	$F_{4/439}$	*p*	$F_{4/439}$	*p*	$F_{4/439}$	*p*	$F_{4/439}$	*p*
	33.07	<0.001	1.36	0.249	11.48	<0.001	27.14	<0.001
Predictor	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*
PER3	−0.093	0.027	−0.009	0.856	−0.070	0.124	0.071	0.097
RORα	0.090	0.033	0.020	0.677	0.074	0.103	−0.060	0.161
Age	−0.491	<0.001	−0.033	0.658	−0.362	<0.001	0.385	<0.001
Group	0.043	0.517	−0.132	0.008	0.110	0.128	0.059	0.381

B. Exploratory sample

Score	M		E		M + E		E – M	
Summary	*R*	R^2	*R*	R^2	*R*	R^2	*R*	R^2
	0.446	0.190	0.348	0.121	0.462	0.214	0.282	0.079
ANOVA	$F_{5/105}$	*p*	$F_{5/105}$	*p*	$F_{5/105}$	*p*	$F_{5/105}$	*p*
	4.94	<0.001	2.89	0.018	5.70	<0.001	1.81	0.117
Predictor	*β*	*p*	*β*	*p*	*β*	*p*	*β*	p
PER3	−0.239	0.008	0.037	0.685	−0.148	0.091	0.248	0.001
RORα	0.213	0.019	0.117	0.214	0.213	0.017	−0.114	0.236
CLOCK	−0.053	0.551	−0.030	0.747	−0.053	0.542	0.027	0.770
NPAS2	0.048	0.587	0.096	0.298	0.086	0.325	0.024	0.797
Age	−0.257	0.005	−0.290	0.002	−0.338	<0.001	0.031	0.743

C. Confirmatory sample

Score	M		E		M+E		E – M	
Summary	*R*	R^2	*R*	R^2	*R*	R^2	*R*	R^2
	0.215	0.046	0.036	0.001	0.129	0.017	0.180	0.033
ANOVA	$F_{4/290}$	*p*	$F_{4/290}$	*p*	$F_{4/290}$	*p*	$F_{4/290}$	*p*
	3.50	0.008	0.09	0.985	1.22	0.304	2.44	0.047
Predictor	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*
PER3	−0.027	0.636	−0.019	0.748	−0.029	0.619	0.006	0.920
RORα	0.118	0.045	−0.015	0.806	0.063	0.289	−0.107	0.073
Age	0.030	0.607	0.024	0.694	0.034	0.569	−0.004	0.942
Gender	0.186	0.001	−0.005	0.932	0.112	0.059	−0.153	0.009

Notes: Results of linear regression analyses aimed on predicting scores on Morning and Evening Lateness scales (M and E, left side), their sum and difference (M + E and E-M, right side). R: Coefficient of linear correlation; R^2: Explained variance; F and p: Results of ANOVA; *β*: Standardized beta for each of predictors; Homozygotes for the minor allele, heterozygotes, and homozygotes for the major allele were numbered as 0, 1, and 2, respectively; Group: Groups of male shift workers, male and female students were numbered as 1, 2, and 3, respectively; Gender: numbers 1 and 2 were assigned to male and female students, respectively.

PER3 and rs1801260 in *CLOCK* ($p = 0.059$ and 0.055, respectively). Coefficient of correlation between latitude and major allele frequency in samples from 16 populations reached statistically significant level for 6 of 7 SNPs, and the tendency for such correlation was shown by rs12649507 in *CLOCK* ($p = 0.097$).

Principal component analysis yielded three principal components with eigenvalues higher than 1. They collectively explained more than 90% of total variance in major allele frequencies, and the first (largest) component explained 45% of this variance. The highest loadings on this component (0.739–0.912) were shown by rs228697 and rs2640909 in *PER3*, rs1159814 in *RORα*, and rs1801260 in *CLOCK*. The coefficient of correlation of latitude with score on the first principal component was found to be stronger (−0.920, $p < 0.001$) than with any of major allele frequencies (e.g. −0.911 for rs1159814 in *RORα* and −0.814 for rs228697 in *PER3*). Adding allele frequencies obtained for four Russian samples led to further increase of

Table 4. Results of MANCOVAs of scores on two Lateness scales.

A. Merged sample

Polymorphism	*PER3*				*RORα*			
Score	M		E		M		E	
Explained variance	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor
	0.241	0.230	0.026	0.013	0.236	0.225	0.027	0.014
F-ratio	$F_{1-2/437}$	*p*	$F_{1-2/437}$	*p*	$F_{1-2/437}$	*p*	$F_{1-2/437}$	*p*
PER3 or *RORα*	6.39	0.002	0.68	0.934	5.05	0.018	0.23	0.794
Sample	0.04	0.848	7.64	0.006	6.15	0.014	7.17	0.008
Interaction	3.17	0.044	0.15	0.857	1.35	0.260	0.23	0.795
Age (covariate)	12.00	0.001	3.77	0.053	8.41	0.004	3.17	0.076

B. Exploratory sample

Polymorphism	*PER3*				*RORα*			
Score	M		E		M		E	
Explained variance	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor
	0.137	0.119	0.035	0.015	0.097	0.079	0.040	0.020
F-ratio	$F_{1-2/145}$	*p*	$F_{1-2/145}$	*p*	$F_{1-2/145}$	*p*	$F_{1-2/145}$	*p*
PER3 or *RORα*	6.02	0.003	0.10	0.901	2.56	0.081	0.45	0.639
Age (covariate)	11.00	0.001	4.90	0.028	7.75	0.006	4.21	0.042

C. Confirmatory sample

Polymorphism	*PER3*				*RORα*			
Score	M		E		M		E	
Explained variance	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor	R^2	R^2 cor
	0.062	0.043	0.003	−0.018	0.060	0.041	0.002	0.019
F-ratio	$F_{1-2/288}$	*p*	$F_{1-2/288}$	*p*	$F_{1-2/288}$	*p*	$F_{1-2/288}$	*p*
PER3 or *RORα*	2.81	0.062	0.06	0.944	2.47	0.086	0.24	0.789
Gender	2.36	0.125	0.16	0.692	8.09	0.005	0.03	0.869
Interaction	4.31	0.014	0.14	0.869	0.74	0.840	0.24	0.785
Age (covariate)	0.15	0.696	0.21	0.646	0.00	0.996	0.02	0.876

Notes: Results of MANOVAs of scores on Morning and Evening Lateness scales (M and E) with one of two polymorphisms as an independent factor (either *PER3* or *RORα*, left or right, respectively). *R*: Coefficient of linear correlation; R^2 and R^2 cor: Explained variance measured as *R*-square and corrected *R*-square; F-ratio: Results of MANOVA (either main effects of factors and covariates or Interaction between two factors); *PER3* or *RORα*: Three genotypes; Sample: Either exploratory or confirmatory sample (male shift workers or students, respectively). Gender: Either male or female students.

strength of such correlation. The linear relationships with latitude shown be the first principal component score and C allele in *RORα* are illustrated in Figure 4.

Discussion

Although the genetic basis of the circadian rhythmicity is well-established, it remains unknown which of hundreds or thousands of particular polymorphic loci in each of more than a dozen circadian clock genes might underlie individual variation in the preferred phase of the sleep–wake cycle. We suggested that a SNP might be significantly associated with only one of two scales designed to self-assess morning–evening preference. The present results revealed for the first time and confirmed using the confirmatory sample significance of association of SNP in *RORα* with Morning but not with Evening Lateness scale. One more SNP, in *PER3*, was previously revealed to "morning" subscale of another questionnaire tool in a sample of 209 Columbian students (Ojeda et al. 2013), and we confirmed such result showing an association with Morning Lateness scale in the sample from Moscow region (Dorokhov et al. 2017). When the present analysis was limited to the confirmatory sample from the student populations of three Russian cities, the results failed to further confirm this

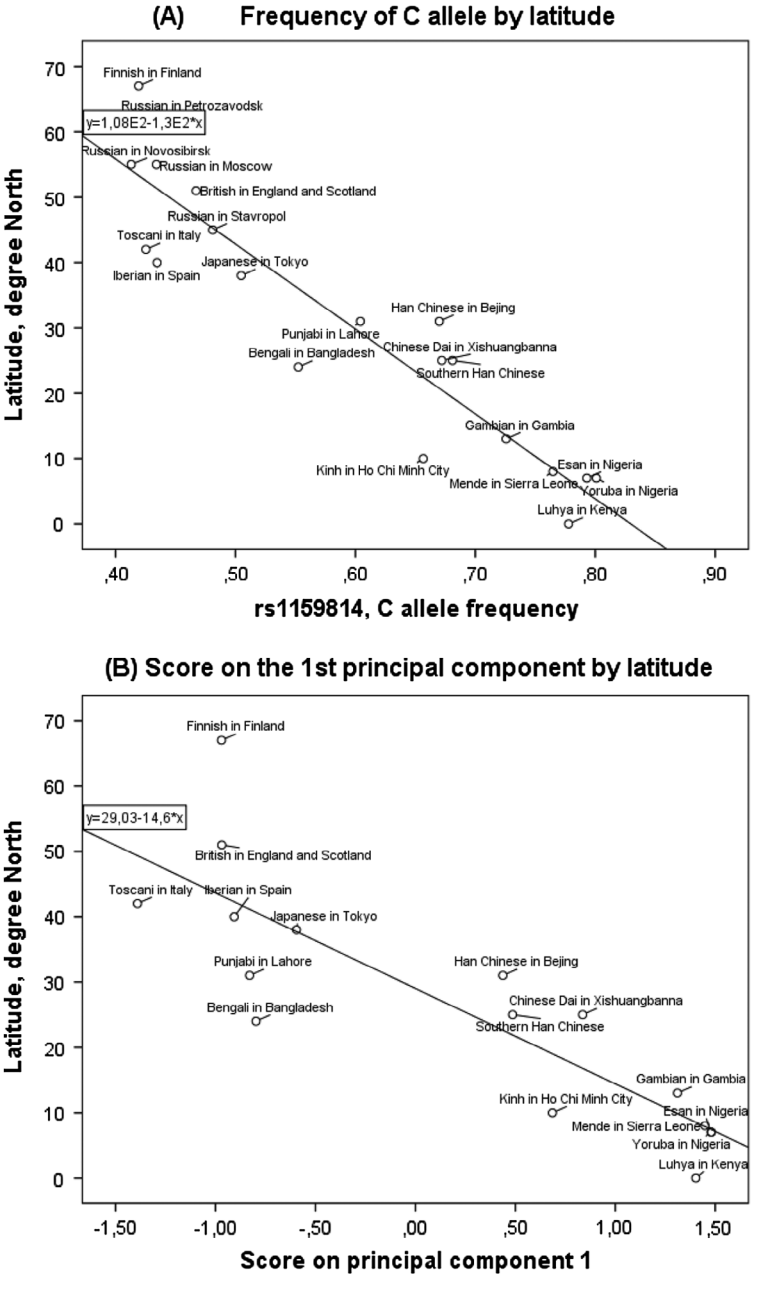

Figure 4. Latitudinal clines of major allele frequency. (A) Major allele frequency for polymorphism in *RORα* was calculated for each of 20 samples (including 4 Russian samples). Spearman coefficient of correlation attained the values of −0.911 and −0.928 for samples from 16 and 20 populations, respectively ($p < 0.001$ for both). (B) Score on the 1st principal component of 7 major allele frequencies was obtained by principal component analysis of samples from 16 African and Eurasian populations of the 1000 Genomes Project Phase 3.

Note: Spearman coefficient of correlation attained the value of −0.920 ($p < 0.001$).

association at the fixed level of significance ($p = 0.05$). However, the relationship remained significant in the results of analysis of the whole sample.

Moreover, we found significant interaction between the effects of gender and this polymorphism. Such interaction might hint at one of possible explanations suggesting that the effect of this polymorphism might be sex-specific. At least, a possibility of sex-depended expression of *PER3* was earlier proposed in several publications (Bailey and Silver 2014; Hida et al. 2014; Shi et al. 2016). However, a bigger sample of male students is required for testing such sex-specific effect.

One of the reports on the most intensively studied VNTR polymorphism in *PER3* suggested age-related differences in its contribution to chronotypological variation (Jones et al. 2007). Our present results on SNP in the same gene, *PER3*, indicated that the association was not attenuated in older adults. Instead, it was not found to be significant in the sample of university students but it was significant in the sample of older shift workers from Moscow region.

The analysis of SNP in *RORα* in this sample revealed that genotype frequencies deviated from those expected by Hardy–Weinberg equilibrium. Negative selection of individuals with morning lateness might be a reason for overrepresentation of homozygotes for C allele in this sample of shift workers. At least, possibility of such negative selection was supported by a higher prevalence of morning earliness among shift workers suggested by comparison with the sample of previously studied Novosibirsk day/evening workers. Given the revealed association of C allele of *RORα* with morning lateness, the reason for possible overrepresentation of homozygotes for T allele in the population of male shift workers remains unknown.

However, there is a general reason for higher prevalence of such homozygotes in the studies populations. It seems that higher latitudes are associated with higher eveningness in both Northern (Randler and Rahafar 2017) and Southern Hemispheres (Leocadio-Miguel et al. 2017). Moreover, racial differences in chronotype were found in multi-ethnic communities of the USA and Brazil. In the USA, non-Hispanic European Americans reported a more pronounced evening preference than African Americans (Eastman et al. 2016; Malone et al. 2017) and showed a longer circadian period (Eastman et al. 2012, 2016, 2017). In Brazil, Amerindian rather than African or European ancestry was related a higher morningness (Egan et al. 2017). The present analysis provided correlative evidence for latitudinal clines in frequencies of alleles of those SNPs that were associated with chronotype in the present and previous studies.

Such results might be explained by polygenic adaptation of Eurasian populations to seasonal variations in day length and other environmental factors. The out-of-African expansion of these populations might be accompanied by the latitude-driven polygenic selection in favor of eveningness and longer circadian period. In other words, at the level of phenotype, such selection is favoring evening preference and longer than 24 h free-running period, whereas, at the level of genotypes, it is producing small latitude-dependent shifts in allele frequency spread across many loci in circadian genes.

Like other studies of candidate genes (Sullivan 2007; Dumas-Mallet et al. 2016), a search for association of these loci with morning–evening preference suffers from increased likelihood of false positive results. Most probably, many genetic variants in circadian clock genes have only tiny effect on such complex trait as chronotype, and such polygenic contribution

remains undetected by genome-widely significant loci. It seems that genome-wide association studies (GWAS) cannot help much in identification of chronotype-associated loci within genes of the circadian clock family. Most GWAS peaks map to non-protein-coding sequences, where their molecular consequences can be difficult to evaluate. Although significant association with chronotype was reliably identified at DNA regions located in closed proximity to several circadian genes, such as PER2 and PER3 (e.g. Hu et al. 2016; Jones et al. 2016; Lane et al. 2016), their mapping near but not within these structural genes pointed at their regulatory rather than protein-coding function. Since most of causal variants within circadian clock genes might explain just a small amount of variation, their effects do not reach a stringent significance threshold in GWAS. One possible way to overcome this problem might be to prioritize the variants in the circadian clock genes by taking into account the pattern and magnitude of their geographic variation. Therefore, implication of such approach to prioritization of genetic markers for morningness–eveningness into further candidate gene studies might be recommended.

In the present study, we replicated and extended the previous results suggesting significant associations between genetic variation and only one of two components of morningness–eveningness. Moreover, we previously also showed that only Morning Lateness scale was reliably associated with seasonality measured with the Seasonal Pattern Assessment Questionnaire or SPAQ, and depression assessed with the Center for Epidemiological Studies –Depression scale or CES-D (Booker et al. 1991; Putilov et al. 1994; Putilov 2017a, 2017b). A growing body of reports on candidate genes pointed at significant association of polymorphic loci in circadian clock genes with depression (e.g. Kripke et al. 2014; Shi et al. 2016) and seasonality (e.g. Johansson et al. 2003). Moreover, the latitude-dependent changes in the allele frequencies of several polymorphisms in circadian clock genes were linked to psychiatric disorders (Forni et al. 2014). Therefore, such latitude-dependent shifts at many loci of the circadian clock genes might underlie not only adaptive changes in regulation of sleep–wake behavior, but they can also play more or less important role in a very wide spectrum of psychic and biological adaptations to seasonal variations in environmental factors at moderate latitudes.

Conclusions

For 2 of 7 examined SNPs (rs2640909 in *PER3* and rs1159814 in *RORα*) findings on association with morning component of morningness–eveningness were positive, whereas all findings on association with evening component of morningness–eveningness were negative. Several difficulties faced by the attempts to replicate positive findings were highlighted. In particular, it is likely that latitude-related genetic variation in circadian clock genes was shaped by polygenic selection. Since the key feature of adaptation driven by such selection is that most of genetic variants have only tiny effect on a complex trait, implication of prioritization of genetic markers for morningness–eveningness into further candidate gene studies might be recommended.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The studies of VBD, ANP, AOT, and PAS were supported by grants from the Russian Foundation for Humanities [grant numbers 15-06-10909a, 15-06-10874a, and 14-06-00963a]; SNK received support from the Federal Research Program [grant number 0221-2014-0034]; The studies of AAP and OGD were supported by grants from the Russian Foundation for Basic Research [grant numbers 07-06-00263-a, 10-06-00114-a, 13-06-00042-a, and 16-06-00235-a] and the Russian Foundation for Humanities [grant numbers 06-06-00375-a, 12-06-18001-e, and 15-06-10403-a].

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