Association of obesity in shift workers with the minor allele of a single-nucleotide polymorphism (rs4851377) in the largest circadian clock gene (NPAS2)

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Association of obesity in shift workers with the minor allele of a single-nucleotide polymorphism (rs4851377) in the largest circadian clock gene (NPAS2)

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ABSTRACT
A growing body of evidence has hinted at the involvement of the largest gene of the circadian clock family, NPAS2, in the regulatory mechanisms underlying the link between metabolic diseases and circadian rhythm disruption. We tested whether one of single-nucleotide polymorphisms in NPAS2 (rs4851377) is associated with obesity and alternations of sleep times in 126 male rotational shift workers (bus drivers). We confirmed positive association of Body Mass Index (BMI) with the difference between free and working days in sleep times, but this difference was smaller in the homozygotes for the minor allele. Moreover, BMI above 30 (obesity) was revealed in the majority of these homozygotes and in the minority of homozygotes for the major allele (11 of 21 or 52.4% and 3 of 40 or 7.5%, respectively). Further studies are required to replicate these results and to elucidate the mechanisms linking NPAS2 polymorphism with obesity in shift workers.

Introduction
At the cellular level, the mammalian clocks are regulated by a transcriptional-translational feedback loop (Ko and Takahashi 2006). Namely, BMAL1 and CLOCK or BMAL1 and NPAS2 (neuronal PAS domain protein 2) form heterodimers that 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 BMAL1/CLOCK or NPAS2. The circadian expression of BMAL1 and NPAS2 is additionally influenced by such nuclear receptor 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). In addition to controlling each other’s expression, these regulators also drive rhythmic expression of thousands of target genes by binding
cis-regulatory sites or through downstream transcriptional regulators. Circadian transcription factors also interact with a number of coactivators, corepressors, and chromatin-associated factors that read, write, or erase chromatin histone modification marks to activate or repress transcription (Panda 2016).

Such transcriptional activity of cellular circadian clocks enables a set of transcriptional regulators to temporally couple their activity with the synchronous rhythmic expression of thousands of genes, with peak expression at distinct times of the day (Panda 2016). Therefore, apart from regulating circadian rhythms and rhythmicity of sleep and wakefulness, the circadian clock genes play an important role in many other biological processes including various metabolic processes. In particular, the circadian clock-mediated transcriptional regulation seems to be responsible for coordination of metabolic processes with such key behaviors as sleep and feeding. Normal circadian physiology is maintained by a stable diurnal pattern of sleeping and eating, whereas disruption of the circadian clocks predisposes to metabolic diseases (Ramsey et al. 2007; Laposky et al. 2008; Panda 2016). Experimental animal models and epidemiological data indicate that chronic circadian rhythm disruption increases the risk of metabolic diseases (Karlsson et al. 2001; Arble et al. 2010; Froy 2010; Shi et al. 2013; Jiang, Turek, 2017). For instance, frequent disruptions in daily activity-rest and eating-fasting rhythms occurring in shift workers can lead to the metabolic syndrome (Karlsson et al. 2001). Therefore, shift workers seem to gain weight more often than day workers, and overweight and obesity are more prevalent in shift workers than day workers (Antunes et al., 2010).

A growing body of evidence indicates that the largest of genes of the circadian clock family, NPAS2, might play a crucial role in the mechanism underlying the well-established link of metabolic diseases with circadian rhythm disruption. For instance, Dudley et al. (2003) reported that NPAS2−/− mice show normal overall diurnal activity-rest rhythms under a light-dark cycle, but they lack a siesta-type rest episode in the middle of the active (dark) period and fail to adjust normally to abrupt change in meal timing and availability. It was also demonstrated that NPAS2 plays an important role in sleep homeostasis as indicated by reduction of Non-Rapid-Eye-Movement sleep time in NPAS2−/− mice compared to wildtype (Franken et al. 2006). In a set of experimental studies on both rodent and non-human primate models Aagaard et al. provided several lines of evidence for a crucial role playing by NPAS2 in genetic and epigenetic regulation of responses to dietary and metabolic stresses (Aagaard-Tillery et al. 2008; Suter et al. 2011, 2013; O’Neil et al. 2013, 2017).

In human candidate gene studies, single-nucleotide polymorphisms (SNPs) in NPAS2 were most often studied to relate them to depressive disorders (Geoffroy et al., 1015; Nievergelt et al. 2006; Kripke et al. 2009; Mansour et al. 2009; Soria et al. 2010; Shi et al. 2016) and retrospectively reported seasonal variation in well-being and behavior (Johansson et al. 2003; Partinen et al., 2007; Kovanen et al. 2010). One of these SNPs (rs11541353) was also linked to a risk of metabolic diseases as indicated by a significant association of its major allele with hypertension (Englund et al. 2009). Another SNP, rs3768984, was associated with later sleep timing (Evans et al. 2013) whereas homozygotes for the minor allele of rs4851377 had longer sleep duration (Gagulin et al., 2016). However, a possible association of rs4851377 with a risk of metabolic diseases in such obesity-provoking condition as rotational shift work has not yet been tested. The only report on results of genotyping of shift workers (nurses) focused on the associations of
this and other SNPs with chronotype and sleep strategies as indicators of the ability to adapt to night shift work. This study did not yield significant associations of rs4851377 with this ability (Gamble et al. 2011).

In the present report, the polymorphism in rs4851377 was chosen to be genotyped to explore its association with obesity and changes in sleep times in male bus drivers exposed to the stressors of rotating shifts. First, we expected to find that homozygotes for the rare variant (C/C) differ in body mass index (BMI) from homozygotes for the most common variant (T/T). Second, we additionally expected to find that these homozygotes also differ in shift of sleep times in free days relative to working days. Such expectation is based on the reports showing the links of obesity with the so-called “social jet lag” (Wittmann et al. 2006) caused by a mismatch between social rhythms and entrained phase of the sleep-wake cycle (Roenneberg et al. 2012), altered sleep times (e.g. Arble et al. 2010), consumption of food at a “wrong” circadian phase (e.g. Jiang and Turek 2017), etc. Moreover, one of the earlier reports (Gagulin et al. 2016) already revealed a link of this SNP with a sleep parameter (duration of sleep) in adult males.

Methods

Samples of buccal epithelium cells were collected from 126 male bus drivers working in Schatura (Moscow region, 55°45’ N 37°37’ E). Their mean age ± SD (Standard Deviation) was 47.9 ± 11.9 years (range from 23 to 68 years). Mean duration of professional shift work experience was 53.8 months (SD = 49.1). The typical length of the work-rest cycle was 5 or 6 days. Free day was usually followed by one day of “garage work”. Each of the following 4–5 consecutive shifts lasted for approximately 8 h. They started to drive a bus at 3:30 or 6:30, 9:30, 12:30, and 15:30 or 17:30, but such a sequence of gradually delaying shifts was often disturbed by a necessity to replace another driver.

The study was conducted in accordance with the ethical standards laid down in the Declaration of Helsinki. The study protocol was approved by the Ethics Committees of our research institutes. Informed written consent was obtained from each study participant.

Buccal epithelium (aka cheek) cells were sampled using dry buccal mouth swabs. DNA was extracted from buccal epithelium cells by method described by Saab et al. (2007). The participants enrolled in a study were asked to twirl a sterile cotton swab on each inner cheek for 15 sec. Then the swabs were returned to the laboratory to extract DNA from these swabs within 24 h. All samples were processed at room temperature (~24°C) in accordance with good laboratory practices. Questionnaire data were collected and height and weight were measured simultaneously with collection of epithelium cells.

BMI was calculation from height and weight measurements as the body weight divided by the square of the body height, kg/m².

In the present analysis, we additionally used the responses to questions from the Munich ChronoType Questionnaires (Roenneberg et al. 2003; Juda et al. 2013) about most usual times for falling asleep and waking up in working and free days.

Genotyping of rs4851377 (CACAGAACCACCTGCTTGCTTGTCA[C/T]CTGTAAATGGTAG TGCCCTCAGGTA) in position 100905804 (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4851377) was performed at the Institute of Molecular Genetics, Moscow. The method of real-time polymerase chain reaction (PCR) in TaqMan™
technology on a StepOnePlus™ amplifier was applied to analyze DNA from buccal epithelium cells. TaqMan SNP Genotyping Assays (Applied Biosystems) for NPAS2 (C___9902033_10) with TaqMan Genotyping Master Mix was utilized according to the manufacturer’s protocols. Amplification solution (25 µL) contained 2.5 µL 10× PCR buffer solution, 2.5 µL 25-mM magnesium chloride (MgCl2), 2.5 µL 2.5-mM dNTP, 10 pM of TaqMan™ primer, 4pM of DNA probe, 1.25 units of Taq polymerase (Fermentas), 0.1–0.2 µg of genomic DNA, and deionized water, up to 25 µL of a total volume.

For statistical analyses, the SPSS statistical software package was used (IBM, Armonk, NY, USA, version 22.0). Three statistical tests were applied to examine relationship of genotype with BMI and body weight. In order to detect differences between three genotypes in these variables, we performed one-way ANCOVAs with between-subjects factor “Genotype” (homozygotes for the major allele, heterozygotes, and homozygotes for the minor allele) and “Age” as the covariate or with “Age” and “Height” as the covariates (in analysis of BMI or body weight, respectively). Bonferroni’s multiple comparison test was used in the post hoc analysis for examining significance of pairwise differences. Then, Spearman correlation coefficients were computed to test association of genotype with BMI and body weight. Finally, the chi-square statistic was calculated to examine the distribution of three genotypes into three subgroups with normal weight, overweight, and obesity (BMI between 18.5 and 25, 25–30, and above 30).

We additionally examined relationship of genotype and BMI subgroup with sleep times and shifts of these times during free days relative to working days. To compare sleep times, four-way repeated measure ANCOVA (rANCOVA) was applied with between-subjects factors “Genotype” and “BMI”, within-subjects factors “Days” (on and off) and “Phase” (two sleep phases: time of falling asleep and time of awakening), and “Age” as covariate. In order to illustrate this results in terms of shift of phases (calculated as the differences between days off and on), three-way rANCOVA was run with between-subjects factors “Genotype” and “BMI”, within-subjects factor “Phase”, and “Age” as covariate. Degrees of freedom were corrected using Huynh–Feldt correction controlling for type-1 error associated with violation of the sphericity assumption, but the original degrees of freedom are reported in Results.

Finally, four linear regression analyses were performed for predicting genotype or BMI or shift of sleep times or age using three other variables as predictors.

**Results**

Allele frequencies for the genotyped SNP (0.42 vs. 0.58) were consistent with those reported for 1000 genomes (0.46 vs. 0.54; see, for instance, https://www.ncbi.nlm.nih.gov/projects/SNP.snp_ref.cgi?rs=4851377). Genotype frequencies are given in Table 1. They were not significantly different from those expected by Hardy–Weinberg equilibrium.

Figure 1 illustrates BMI and body weight in three genotypes and three subgroups with normal weight, overweight, and obesity. One-way ANCOVA of BMI revealed statistically significant main effect of “Genotype” \(F_{2/122} = 6.749, p < 0.01\). The effect of covariate (“Age”) was non-significant \(p > 0.1\). Similar result was yielded by ANCOVA of body weight \(F_{2/121} = 5.550, p < 0.01\). As expected for this ANCOVA, the effect of one of two covariates (“Height” but not “Age”) was significant.
Table 1. Comparison of frequencies of three genotypes in subgroups with different BMI.

<table>
<thead>
<tr>
<th>N</th>
<th>Observed</th>
<th>Expected</th>
<th>χ² test*</th>
<th>χ² test**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T/T</td>
<td>T/C</td>
<td>C/C</td>
<td>T/T</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>40</td>
<td>65</td>
<td>21</td>
</tr>
</tbody>
</table>

A. Comparison of genotype frequencies in subgroups with different BMI

<table>
<thead>
<tr>
<th>BMI</th>
<th>T/T</th>
<th>T/C</th>
<th>C/C</th>
<th>T/T</th>
<th>T/C</th>
<th>C/C</th>
<th>MAF</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25</td>
<td>20</td>
<td>33</td>
<td>5</td>
<td>18.4</td>
<td>29.9</td>
<td>9.7</td>
<td>0.37</td>
<td>17.42</td>
<td>0.002</td>
</tr>
<tr>
<td>25–30</td>
<td>17</td>
<td>18</td>
<td>5</td>
<td>20.7</td>
<td>20.6</td>
<td>6.7</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;30</td>
<td>3</td>
<td>14</td>
<td>11</td>
<td>8.9</td>
<td>14.4</td>
<td>4.7</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T/T, homozygotes for the major allele; T/C, heterozygotes; C/C, homozygotes for the minor allele; BMI, further subdivision of three genotypes into subgroups with normal weight (<25), overweight (25–30), and obesity (>30); MAF, minor allele frequency; χ² test*, comparison of frequencies expected from the Hardy–Weinberg equilibrium; χ² test**, comparison of frequencies expected for nine cells (three genotypes x three subgroups with different BMI).

Figure 1. BMI and body weight in subgroups with different genotypes and BMI. a and b: BMI and body weight. T/T, T/C, and C/C: Homozygotes for the major allele, heterozygotes, and homozygotes for the minor allele; BMI: further subdivision of three genotypes into subgroups with normal weight (<25), overweight (25–30), and obesity (>30). Vertical bars: ±95% confidence interval (CI).

(p < 0.001). Post hoc pairwise comparisons are reported in Table 2. They suggested that the most profound pairwise difference in BMI and body weight was the difference between homozygotes (p < 0.01).
Correlation analysis corroborated the results of ANCOVAs. Spearman rank correlation coefficient between genotype and BMI or body weight attained the values of 0.233 or 0.230, respectively (df = 124, \( p < 0.01 \) for both).

The results reported in Table 1 suggested that minor alleles were overrepresented in the subgroup with BMI higher than 30 (obesity) and underrepresented in the two other subgroups. For instance, obesity was found in the majority (11 of 21 or 52.4%) of homozygotes for the minor allele and in only 3 of 40 (7.5%) homozygotes for the major allele. BMI was within a normal range (19–25) either in less than a quarter (5 or 23.8%) or in a half (20 or 50%) of homozygotes for the minor or major allele, respectively. According to chi-square test, the difference between nine cells (three genotypes x three BMI subgroups) was significant at \( p < 0.01 \) (Table 1).

Figure 2 illustrates sleep times on days on and off in three genotypes and three subgroups with normal weight, overweight, and obesity. Three-way rANCOVA not yielded significant main effects of between-subjects factors. Such a result suggested the absence of differences between sample’s subdivisions in sleep times. The main effect of the covariate “Age” was significant (\( p < 0.001 \)) indicating the expected earlier sleep times in older male drivers compared to younger male drivers. Three significant interactions were revealed for within-subjects factor “Days”: with the covariate and with between-subjects factors. The significant interaction with “Age” suggested the expected smaller shift of sleep times in older adults compared to younger adults (\( F_{1/113} = 5.875, p < 0.05 \)). The significant interaction with “Genotype” pointed at the association of smaller shifts of sleep times with the minor allele (\( F_{2/113} = 3.637, p < 0.05 \)) whereas the significant interaction with “BMI” indicated the expected from the earlier published reports association of larger shifts with obesity (\( F_{2/113} = 4.016, p < 0.05 \)).

Figure 3 illustrates how pronounced were the shifts of sleep times on days off compared to days on in three genotypes and three subgroups with normal weight, overweight, and obesity. As expected, three-way ANCOVA of these shifts with within-subjects factor “Phase”
provided the same results as four-way rANCOVA with within-subjects factors “Days” and “Phase”. In particular, this analysis yielded the same F-ratios but for main effects of the covariate and between-subjects factors instead of their interactions with within-subjects factor “Days” ($F_{1/113} = 5.875$ for “Age”, $F_{2/113} = 3.637$ and $F_{2/113} = 4.016$ for “Genotype” and “BMI”, respectively, $p < 0.05$ for all). The results of post hoc pairwise comparisons of the shifts of sleep times are reported in Table 3. They indicated that the homozygotes for the major and minor allele as well as the subgroups with normal weight and obesity significantly differed in the extent of sleep delay during free days ($p < 0.05$). Larger delays were associated with the major allele and obesity (Table 3; Figure 3).

The associations of genotype and/or BMI with neither sleep duration nor its change during free days were significant ($>0.1$).

Finally, the results of all previous analyses were confirmed by four linear regression analyses reported in Table 4 and partly illustrated in Figure 4. The results suggested a possibility of highly significant prediction of genotype ($p < 0.001$) from data on the BMI and shift of sleep times. It turns out that a prediction of BMI was also highly significant ($p < 0.001$) with all three predictors being significant (genotype, delay, and age). They collectively explained almost 17% of the total variation in BMI (Figure 4(a)). All three predictors (genotype, BMI, and age) also significantly predicted a shift of sleep times ($p < 0.01$). They explained 11% of the total variation (Figure 4(b)). The BMI and shift of sleep times but not genotype were found to be significant predictors of age of study participants ($p < 0.05$).
Figure 3. Shift of sleep times in subgroups with different genotypes and BMI. a. Filled and open squares: shifts of times of falling asleep and awakening calculated as the differences between working and free days. b. Grey squares: mean shift for both times.

Table 3. Comparison of delays of sleep times in subgroups with different genotypes and BMI.

<table>
<thead>
<tr>
<th>B. Pairwise differences between mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>T/C</td>
</tr>
<tr>
<td>C/C</td>
</tr>
<tr>
<td>C/C</td>
</tr>
</tbody>
</table>

Results of post hoc pairwise comparisons from three-way rANCOVA with between-subjects factors “Genotype” and “BMI”, within-subjects factor “Phase” (mean shift of falling asleep and awakening), and “Age” as the covariate. Mean: Shift of sleep times in free days relative to working days, hours, for either three Genotypes or three BMI subgroups; CI: Confidence Interval; Difference: Pairwise difference between the averaged shifts for three subgroups; p for t: significance of t-statistic in post hoc pairwise comparison of difference between either genotypes or BMI subgroups (with Bonferroni’s correction for multiple comparison).
Discussion

A growing body of evidence points at a crucial role playing by NPAS2 in the genetic and epigenetic mechanisms that underlie the link between metabolic diseases and circadian rhythm disruption. Therefore, polymorphism in this gene might be found to be an important predictor of the risk of metabolic diseases in people exposed to the stressors of night and shift work. However, such possibility has not been yet tested in populations of shift workers, and, to our knowledge, this is the first attempt to associate one of NPAS2’s SNPs (rs4851377) with obesity in male shift workers. The present results supported our expectation that the homozygotes for the rare and most common allele differ in BMI. It might be suggested that the homozygotes for the minor allele become obese more often than other homozygotes due to a genetic or epigenetic predisposition for a more rapid weight gain in response to “wrong” time eating forced by shift work.

Given that earlier published reports have pointed at the links of obesity with social jet lag (Renneberg et al. 2012), alternations of sleep times (e.g. Arble et al. 2010), eating at inappropriate circadian times (Jiang and Turek 2017), etc. and that one of the earlier published reports has suggested the link of this SNP with sleep duration in adult males (Gagulin et al. 2016), we additionally expected to find differences between the homozygotes for major and minor allele in responses of their sleep times for falling asleep and awakening to rotational shifts. Such expectation was supported, for the first time, by the results suggesting a relatively small delay of sleep times during free days in the homozygotes for the minor allele and a relatively large delay of sleep times in the homozygotes for the major allele.

We also confirmed the association of obesity with a relatively large delay of sleep times on free days (Renneberg et al. 2012; Parsons et al. 2015). Direction of causal relationship between obesity and such an enlarged delay remains to be established. It seems that this association became evident already in adolescents (Olds et al. 2011; Golley et al. 2013) and it has been earlier clarified that social jetlag seems to be enlarged only in metabolically unhealthy individuals whereas in metabolically healthy obese individuals it appears to be similar to that in the rest of population (Parsons et al. 2015).

Table 4. Prediction of genotype, BMI, shift of sleep times, and age in liner regression analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th>BMI</th>
<th>Shift</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>0.378</td>
<td>0.409</td>
<td>0.336</td>
<td>0.257</td>
</tr>
<tr>
<td>R²</td>
<td>0.143</td>
<td>0.167</td>
<td>0.113</td>
<td>0.066</td>
</tr>
<tr>
<td>F₃/122</td>
<td>6.553</td>
<td>7.899</td>
<td>4.995</td>
<td>2.784</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Results of four linear regression analyses aimed on predicting genotype or BMI or shift or age. R, coefficient of linear correlation; R², explained variance; F₃/122 and p, results of ANOVA; significance of predictor (t-test) at *** p < 0.001, ** p < 0.01, and * p < 0.05. Genotype was numbered as 0, 1, and 2 (T/T, T/C, and C/C, respectively).
Figure 4. Results of linear regression analysis aimed on predicting BMI and shift of sleep times. a and b: BMI and shift of sleep times during free days relative to working days as dependent variables (significant predictors were genotype, shift of sleep times, and age for BMI and genotype, BMI, and age for shift of sleep times).
In the light of such results of cross-sectional questionnaire studies, one can speculate that prevention of delay of sleep times on free days (Figure 3B) might protect the homozygotes for the minor allele from overweight and obesity. It has to be stressed that a stronger circadian control of the sleep-wake cycle might underlie a smaller delay of sleep times during free days in these homozygotes, and, on the other hand, such a stronger circadian control in these homozygotes might also be responsible for a more rapid weight gain after eating at “wrong” circadian phase during working days. However, further studies are necessary to elucidate the regulatory mechanisms underpinning the association of polymorphisms in NPAS2 with BMI and delay of sleep times during free days.

We did not support the earlier published result indicating significant difference of the homozygotes for the major and minor allele of rs4851377 in sleep duration (Gagulin et al. 2016). However, it is likely that many of adult males from that study (Gagulin et al. 2016) were not engaged in night and shift work, and a possibility cannot be fully excluded that the homozygotes for the minor allele genotyped in our study would exhibit significant difference from other genotypes in sleep duration when they are not involved in rotating shift work.

This is an exploration study and it has several limitations. Only bus drivers were studied, and, therefore, it remains unknown whether the study results are applicable to other than drivers shift workers or to day-working drivers. The sample size is rather small and therefore the present positive results require replication using a bigger independent sample. Other limitations of this exploration study include the application of cross-sectional and non-repeated measures design, the absence of a control group (e.g. professional day-working drivers), lack of any information on medical examinations of health status of the study participants, the using subjective rather than objective measures for determining sleep times, and the absence of measurements of amplitudes and phases of the rhythms-markers of the circadian pacemaker as well as the absence of any other data that can provide a deeper insight into specific genetic and epigenetic mechanisms underlying the associations of the minor allele of rs4851377 with BMI and shifts of sleep times.

**Conclusion**

The results of genotyping of professional bus drivers exposed to the stresses of rotating shift work indicated that having two copies of the minor allele of rs4851377 is associated with obesity, possibly, due to a genetic or epigenetic predisposition to a more rapid weight gain in response to “wrong” time eating forced by shift work.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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