The 5-HTTLPR rs25531 L_AL_A-genotype increases the risk of insomnia symptoms among shift workers

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Abstract

Background: Previous studies indicate that shift work tolerance may be associated with individual factors including genetic variability in the gene encoding the serotonin transporter 5-HTT (*SLC6A4*). The aim of the present study was to explore the interaction between work schedule (shift work versus non-shift work), genetic variability in *SLC6A4* and insomnia symptoms.

Methods: The study was based on a national probability sample survey of 987 Norwegian employees drawn from The Norwegian Central Employee Register by Statistics Norway. Insomnia symptoms were assessed by three items reflecting problems with sleep onset, sleep maintenance and early morning awakenings. Genotyping with regard to *SLC6A4* (the 5-HTTLPR S versus L and the SNP rs25531 A versus G) was carried out using a combination of gel-electrophoresis and TaqMan assay.

Results: Using the L_AL_A genotype as a reference a main effect of the SS genotype (B=.179; 95% CI =.027-.330) was found. Also, a main effect of work schedule (0=non shift, 1=shift work) was found (B=.504; 95% CI =.185-.823). The genotype x work schedule interaction was significant for all genotypes; SL_A (B=-.590; 95% CI =-.954--.216), L_AL_G (B=-.879; 95% CI =-1.342--.415), SL_G (B=-.705; 95% CI =-1.293--.117) and SS (B=-.773; 95% CI =-1.177--.369) indicating higher insomnia symptom scores among L_AL_A-participants compared to participants with other genotypes when working shifts.

Conclusions: The ability to cope with shift work is associated with the combination of the *SLC6A4* variants 5-HTTLPR and SNP rs25531. Our findings demonstrated that the L_AL_A-genotype increases the risk of insomnia symptoms among shift workers.

Key words: Chronobiology; genetics; insomnia symptoms; shift work; shift work tolerance

1. Introduction

Ample evidence suggests that shift work is associated with a wide range of problems and disorders. These may include sick leave [1], low job satisfaction [2], turnover and turnover intention [3, 4], fatigue [5], sleepiness [6], gastrointestinal disorders [7], cardiovascular diseases [8-10], cancers (breast, colorectal, prostate) [11-14], metabolic disturbances [15-17] as well as psychological distress [18, 19]. The most commonly reported problem by shift workers, however, is sleep difficulties [20]. Sleep before morning shift, especially when starting the workday early, is associated with reduced sleep length and subsequent daytime sleepiness [20]. Moreover, daytime sleep following night work is shorter (mean 5 h 51 min) than sleep after evening shifts (mean 8 h 2 min) [21]. Also, sleep duration may be curtailed by short rest time between shift (≤ 11 hours) [22]. Still, large individual differences in terms of the ability to cope with shift work have been reported [23]. Evidence exists that shift work tolerance [24] is associated with individual factors such as age, sex, and personality [23]. In addition, several earlier studies suggest that shift work tolerance may be linked to genetic variability.

For example, genetic polymorphisms in genes involved in circadian rhythm regulation such as CLOCK, NPAS2, PER2, and PER3 have been shown to be associated with outcomes such as alcohol/caffeine consumption and sleepiness, as well as sleep phase, inertia and duration in hospital day-and night-shift nurses [25]. Moreover, screening of genetic variants in clock genes suggests that polymorphisms in CLOCK, CRY1, NPAS3, RORA, and TEF may be relevant with regard to shift work disorder [26]. Previous observations also indicate that genetic variability of CRY1 may influence adaptation to rotating shift work [27].

The serotonin transporter

In addition to the clock genes mentioned above, earlier reports indicate that shift work tolerance could be associated with genetic variability in the gene SLC6A4 [28] encoding the serotonin (5-HT) transporter (5-HTT). One such genetic variant is the 5-HTT promoter repeated length polymorphic region (LPR) [29]. Two common allelic variants have been described, a short (S) allele of 14 repeats and a long (L) allele of 16 repeats [30]. The S allele leads to decreased 5-HTT expression [31]. In addition, there is a single nucleotide polymorphism (SNP) rs25531 A > G in the promoter region of SLC6A4, which also affects the rate of transcription [29]. This A to G substitution may only be present in the in L allele [32], where the G allele is associated with lower 5-HTT expression [29, 32]. Therefore, there is only one type of the S allele, termed S, but two types of the L alleles, termed L_A and L_G. Environmental stressors may have a more pronounced impact on individuals with the 5-HTT SS and SL_G genotype than other individuals [33]. Workers with higher job-related stress and the SS genotype have increased risk of insomnia, whereas workers with low job-related stress and the SS genotype report reduced sleeping problems [34]. Moreover, the S allele seems to modulate sleep related factors such as anxiety, negative affect [35, 36], and is associated with an increased risk of depression and alcohol dependence [37]. In terms of sleep disorders, the S allele has been found to be significantly more frequent in patients suffering from insomnia than in controls [38], whereas the L allele has been associated with an increased risk of apneas/hypopneas in older subjects compared to the S-allele [39]. In males, the LL-genotype has been shown to be more prevalent among male obstructive sleep apnea patients than controls [40].

Interestingly, previous data indicate that rotating shift workers have an increased frequency of the SS genotype after 60 months of shift work exposure [41]. However, like in most previous studies, the participants were genotyped with regard to the S versus L allele only, not with regard to the SNP rs25531 A > G of SLC6A4. Thus, how shift work-related challenges such

as insomnia may be moderated by each of the five Caucasian 5-HTT variants; SS, SL_G L_AL_G, SL_A and L_AL_A [42] (L_GL_G is usually not found in Caucasians), remain to be investigated. Moreover, previous data shows that SS is associated with reduced 5-HTT expression [29, 41], whereas L_AL_A seems to have the opposite effect and increase the 5-HTT expression [29, 32]. Thus, in particular the influence of the L_AL_A genotype, needs to be examined. The aim of the present study was accordingly to examine how the 5-HTTLPR S/L and SNP rs25531 A > G genotype influence insomnia symptoms in shift workers.

2 Material and methods

2.1 Participants

A random sample of 5000 employees was drawn from the Norwegian Central Employee Register by Statistics Norway, hence representing a probability sampled survey. The Norwegian Central Employee Register is the official register of all Norwegian employees, as reported by employers. Sampling criteria were adults between 18 and 60 years of age employed in a Norwegian enterprise. Questionnaires were distributed through the Norwegian Postal Service during the spring 2015. Subjects who gave consent were also sent saliva collection kits. Altogether, 987 subjects who had satisfactorily completed the questionnaire and provided a saliva sample were included in the present study. The study procedures were carried out in accordance with the Declaration of Helsinki and the Norwegian Health Research Act. The study was approved by the Regional Committee for Medical Research Ethics for Eastern Norway (no. REK 2014/1725). Written informed consent was provided by all participating respondents.

2.2 Instruments

Insomnia symptoms were assessed with three items reflecting problems with sleep onset, maintenance of sleep and early morning awakening, respectively. The time frame was the last

12 months. Response categories ranged from 1 to 4 (‘not bothered’, ‘a little bothered’, ‘considerably bothered’, ‘seriously bothered’). The included symptoms are core nocturnal characteristics of insomnia, in line with modern diagnostic nosology [43, 44]. A composite insomnia symptoms score was calculated by adding the score of the three items and dividing the sum by three. The Cronbach alpha for the insomnia symptoms scale was .81 in the present study. The insomnia symptom items have been used in a previous study [45], still not much is known about their psychometric properties. Therefore, the items were validated in a sample of 190 university students (mean age 22.0, SD= 4.6, 79% ♀) by administering, in addition, the Bergen Insomnia Scale [46] (BIS; higher scores indicate more insomnia symptoms), the Sleep Hygiene Index [47] (SHI: higher scores suggest worse sleep hygiene), and an item asking about lifetime use of prescribed hypnotics. The sum score of the insomnia symptom items had a significant and positive correlation with the composite score of the BIS ($r=0.73$, $p<.01$) and the SHI ($r=0.22$, $p<.01$), where the former correlation was significantly higher than the latter ($Z=6.77$, $p<.01$) attesting to the convergent and discriminative validity of the insomnia symptom items, respectively. Those with lifetime use of prescribed hypnotics scored higher (Mann-Whitney $U = 1862$, $p<.05$) on the insomnia symptom items (mean rank = 90.0) compared to those who never had used such drugs (mean rank = 113.3), thus the insomnia symptom items showed concurrent validity. The mean inter-item correlation of the insomnia items was .32, which is within the recommended range [48]. A total of 45 students participated in a 2-week test-retest of the insomnia symptom items which had an ICC of .79 ($p<.01$) which indicates good reliability [49].

2.3 Genotyping

Collection of saliva and extraction of genomic DNA was done using OrageneRNA sample collection kit (DNA Genotech Inc. Kanata, Ontario, Canada) according to the manufacturer’s

instructions. Genotyping with regard to SLC6A4 tandem repeat length in the promoter (short; S versus long; L), and genotyping with regard to the SNP rs25531 (A versus G) were performed. To determine the length (S versus L) of the polymorphic promoter region of SLC6A4, the DNA sequence was first amplified by polymerase chain reaction (PCR) and then separated by gel electrophoresis. PCR was carried out in a total volume of 25 µl containing ~60 ng of genomic template, 6.25 pmol of each primer and 1x Taq DNA Polymerase Master Mix (VWR international, Dublin, Ireland). The forward primer sequence was 5' –GGCGT TGCCG CTCTG AATGC- 3' and the reverse primer sequence was 5' – GAGGG ACTGA GCTGG ACAAC CAC- 3' (DNA technology A/S, Risskov, Denmark). As previously described [32], samples were amplified on a Perkin Elmer GeneAmp PCR 2400 system following an initial denaturing step for 3 min at 95 °C. The amplification consisted of 40 cycles including denaturing at 95 °C for 40 s, annealing at 60 °C for 20 s and elongation at 72 °C for 80 s. The PCR yielded a long (529 bp) and a shorter (486 bp) fragment. After four hours separation at 100 V on a 2.5% agarose gel (MetaPhor Agarose, Lonza cologne GmbH, Cologne, Germany), GelRed dye was added and the fragments were visualized by UV light (Biotium Inc, California, USA). A PCR 100 bp low ladder (Sigma-Aldrich CO, St. Louis, Mo, USA) was used to determine the length of the fragments.

The SNP genotyping with regard to rs25531 (A versus G) was carried out using custom TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA). Approximately 10 ng genomic DNA was amplified in a 5 µl reaction mixture in a 384-well plate containing 1x TaqMan genotyping master mix (Applied Biosystems) and 1x assay mix, the latter containing the respective primers and probes. The probes were labelled with the reporter dye FAM or VIC to distinguish between the two alleles. Approximately 10% of the samples were re-genotyped and the concordance rate was 100%. Due to poor quality of 47 saliva samples the total number of respondents included in the final analysis was 940.

2.4 Statistical analyses

A hierarchical regression analysis were conducted to test for associations between working arrangement (0=non-shift work, 1=shift work) and the length polymorphism of the serotonin transporter and the SNP rs25531. Educational level was included as a categorical variable with "secondary school or less" as the reference category. For the SLC6A4 genotypes, the L_AL_A comprised the reference category. Deviation from the Hardy-Weinberg equilibrium was tested by the chi-squared test. In order to examine the modifying role of the SLC6A4 genotype on the effect of work schedule on insomnia symptoms, we followed the recommendations for interaction analyses provided by Baron and Kenny [50]. The interaction analysis was conducted in two steps. Control variables, work schedule and the SLC6A4 genotype were entered as predictors in the first step, whereas the interaction term (work schedule * SLC6A4) was entered in the second step. A significant interaction term and a significant increase in explained variance (R^2) in the second step were considered as an interaction effect. The skewness and kurtosis values for the indicator of insomnia were within acceptable range for a normal distribution (between -2 and +2) [51]. Still, all analyses were conducted using bootstrapping (5000 resamples). Bootstrapping is a method for deriving robust estimates of standard error and confidence intervals for estimates such as the mean, median, proportion, odds ratio, correlation coefficient or regression coefficient. The bootstrap method has the advantage that it does not need to meet the assumptions of normality, equal variances, and homoscedasticity that are required in ordinary regression analyses [52]. Multicollinearity was not an issue in the current study as the highest noted variance inflation value was 4.19. All variables had a linear relationship (age) with the insomnia symptoms score or were categorical, hence assumptions about linearity were not violated. Statistical

analyses were performed with Stata 14 (StataCorp). The level of significance was set to $p<.05$.

3. Results

The mean score on the insomnia symptoms across the 940 participants was 1.68 (SD = 0.73). In all, 803 participants had day work, whereas 137 participants reported a work schedule involving some type of shift work. The overall mean age of the sample was 45.18 years (SD = 10.05). The sample comprised 47.2% men and 52.8% females. In all 21.5% of the sample were smokers. In terms of highest completed education, 8.7% had secondary school or less, 29.5% had high school, 33.4% had university/college ≤ 4 years and 28.4% had university/college more than 4 years.

Genotype frequencies of SS, SL_G, L_AL_G, SL_A and L_AL_A were 17.7%, 7.4%, 7.3%, 42.6% and 25.0%, respectively. No deviation from the Hardy-Weinberg equilibrium was observed. The characteristics of the subjects are presented in Table 1. The distribution of the five genotypes did not differ across work schedule (χ^2=4.96, df=4, p=.293). Results from the hierarchical regression analyses of linear associations and interaction effects are presented in Table 2. In the first step, age and female gender were positively associated with insomnia symptoms, whereas educational level were inversely related to insomnia symptoms. The predictor variables explained 6.21% of the variance in insomnia symptoms. The SLC6A4 genotypes and work schedule were both unrelated to insomnia symptoms. The model was significant (Wald χ^2=56.20; p<.001).

In the second step of the analysis, the interaction term (work schedule x SLC6A4) was entered. In this step the SS-genotype (L_AL_A as contrast) turned out significant and comprised a risk factor for insomnia symptoms. A significant work schedule x SLC6A4 interaction was

also observed where the genotypes SS, SL_G, L_AL_G, SL_A (L_AL_A as contrast), were associated with a decreased insomnia symptom score. Moreover, an increased insomnia symptom scores among L_AL_A-participants when working shifts was demonstrated (see Figure 1). The statistical model with the interaction term explained 8.31% of the variance in insomnia symptoms. The model with the interaction term was also significant (Wald χ^2=71.41; p<.001).

4. Discussion

The present data demonstrated a highly significant interaction between work schedule and the SLC6A4 genotype, reflecting higher insomnia symptom scores among L_AL_A-participants compared to the other subjects when working shifts. This indicates that the L_AL_A-genotype may be associated with impaired shift work tolerance. Clearly, shift workers with the L_AL_A-genotype reported higher levels of insomnia symptomatology than other shift workers, including the carriers of two S alleles. Hence, our finding fits well with previously published data showing a higher proportion of individuals with SS among rotating shift workers [41].

According to our data, also individuals with SL_G, L_AL_G, and SL_A had relatively low insomnia symptom scores compared to individuals with L_AL_A when working shifts. Thus, regarding sleeping problems, our results show that SS, but also the SL_G, L_AL_G, SL_A subjects, adapt to shift work better than L_AL_A subjects. This emphasize that examination of the association between shift work, variability in SLC6A4 and health outcomes should be based on combined 5-HTTLPR S/L and SNP rs25531 A > G genotyping. The present observations suggest that the 5-HTTLPR S versus L, but also the SNP rs25531 A versus G, play a crucial role in terms of shift work tolerance and/or adaptation to novel living circumstances. The finding of the present study also support the idea that SLC6A4, through the serotonergic system, may be important for regulation of circadian rhythms. For example, earlier data show that

serotonergic neurons of the midbrain raphe nucleus innervates the master circadian clock, the suprachiasmatic nucleus (SCN) [53], probably modulating the entraining effects of light on the SCN pacemaker [54]. Since the L_A allele is associated with higher 5-HTT expression [55], one hypothesis might be that the L_AL_A-genotype is related to different 5-HT signaling and poorer flexibility in terms of work and sleep times.

In contrast, increased frequency of the S allele has been observed in insomnia patients [38]. Our observation that SS non-shift workers had a higher mean insomnia symptom score than the L_AL_A non-shift workers support these earlier observations. In line with this it has been suggested that the S allele modulates anxiety and negative affect [35, 36]. Therefore, the S allele in the general population, without any stratification, may be associated with sleep problems. This also explains the present observation that individuals with SS overall had higher mean insomnia symptom score than individuals with L_AL_A (second step in the regression analysis; main effect without taking into account work schedule). As 803 participants were non-shift workers, whereas only 137 participants were shift workers, the effect of SS among non-shift workers seems to explain the main effect of SS on insomnia symptoms. Our data also support earlier findings indicating that the SS-variant, possibly associated with an uncoupling of the amygdala-cingulate feedback circuit implicated in the extinction of negative affect [56], is associated with job-related stress induced sleep problems [34]. Learned associations between bedroom and negative affects have been proposed at another important pathway for the development of insomnia [57]. Moreover, evidence exists that in the face of stressors, subjects with the SS-variant show stronger hypothalamus-pituitary-adrenal cortex (HPA) axis activation that L-allele carriers [58], which resonates well with studies linking HPA-axis hyperactivity to sleep difficulties [59].

In addition, our data showed that age was positively related to insomnia symptoms which most likely reflects more arousals during sleep with increasing age [60], as well an increase in somatic conditions that may disturb sleep [61]. Women had a higher mean insomnia symptom score than men, which is in line with studies showing higher insomnia prevalence among women compared to men [62]. Educational level was inversely related to the insomnia symptom score and is in line with studies showing that low socioeconomic status is associated with insomnia [63]. The overall response rate for the questionnaire survey was 32%. This rate is lower than the average response rate established for survey studies [64]. Moreover, not more than 20% returned the saliva samples. This may have affected the results and it is for example known that ill-health is associated with non-response [65]. Thus, it is questionable if the final sample is representative for the overall population or survey pool. Still, the observed genotype frequencies were in accordance with previous findings [66]. Also, earlier data indicate that the response rate and representativity seems to have limited impact on the internal validity [67]. Still, given a mean age of 44-45 years, a selection bias or "healthy worker effect" [68] is possible. It is likely that those who do not cope well with shift work would either never start with such a work schedule or would leave such working arrangement after a short time. This may explain the finding that the shift workers in the present study report less insomnia symptoms than others. However, "the healthy worker effect" should reduce, not increase, the difference between the subjects with L_AL_A and other shift workers. The association between the L_AL_A-genotype and insomnia symptoms among shift workers reported here is arguably therefore not overestimated. It should be noted that despite several significant findings in the present study, the overall explained variance was of a relatively small magnitude.

4.1 Conclusion

In conclusion, the SLC6A4 L_AL_A-genotype significantly increases the risk of insomnia symptoms among shift workers. The positive relationship between the L_AL_A-variant and insomnia symptoms among shift workers suggests that the L_AL_A-genotype is associated with impaired shift work tolerance. Thus, future theoretical models of shift work and sleep (tolerance to shift work) should include genetic factors. In particular, analyses of the interaction between work schedule and the SLC6A4 genotype SS, SL_G, L_AL_G, SL_A versus L_AL_A with regard to insomnia is necessary. Importantly, the present study showed that the negative impact of shift work with regard to health in vulnerable subjects (individuals with L_AL_A), may be more potent than previously reported. A practical implication is that employers, organizations, and health support should acknowledge that workers differ in their tolerance to shift work and that interventions directed toward shift workers should take these differences into consideration.

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Table 1.
Characteristics of the subjects grouped by genotype: SS, SL_G, L_AL_G, SL_G and L_AL_A.

	SS	**SL_G**	**L_AL_G**	**SL_A**	**L_AL_A**	***Sum***
Subjects n (%)	166 (17.7)	70 (7.4)	69 (7.3)	400 (42.6)	235 (25.0)	940 (100)
Insomnia, mean±SEM	1.75±0.05	1.64±0.08	1.71±0.10	1.66±0.04	1.68±0.05	
Working schedule[a]	140/26	63/7	59/10	339/61	202/33	
Age, mean±SEM	45.93±0.81	43.87	44.52±1.26	45.44±0.51	44.79±0.64	
Male/female	84/82	33/37	31/38	187/213	109/126	
Tobacco (n smokers)	36	14	9	81	62	
Education[b]	13/50/59/44	9/24/23/14	11/16/18/24	29/119/138/114	20/68/76/71	

a. Daytime/other
b. Secondary school or less/High school/University 4 years or less/University 4 years or more

Table 2.
Hierarchical regression with genotype L_AL_A as reference. The analyses were adjusted for the covariates age, sex, tobacco use and education.

	Insomnia symptoms	B	std. err	p-value	95 % conf. interval
Step 1	**R^2=0.0621**				
	Age	0.012	0.002	0.000	0.007 – 0.016
	Sex (0=♂, 1♀)	0.118	0.048	0.014	0.024 – 0.212
	Tobacco[1]	0.040	0.059	0.492	-0.075 – 0.156
	Education[2]				
	High school	-0.132	0.100	0.185	-0.328 – 0.064
	University ≤4y	-0.316	0.097	0.001	-0.505 – -0.127
	University >4y	-0.329	0.098	0.001	-0.521 – -0.137
	5-HTT				
	SL_A	-0.017	0.059	0.771	-0.134 – 0.099
	L_AL_G	0.028	0.102	0.783	-0.172 – 0.228
	SL_G	-0.045	0.094	0.635	-0.229 – 0.140
	SS	0.065	0.073	0.370	-0.078 – 0.209
	Work schedule[3]	-0.007	0.070	0.921	-0.144 – 0.130
Step 2	**R^2=0.0831**				
	Age	0.011	0.002	0.000	0.007 – 0.016
	Sex (0=♂, 1♀)	0.124	0.048	0.009	0.031 – 0.218
	Tobacco[1]	0.034	0.058	0.558	-0.080 – 0.149
	Education[2]				
	High school	-0.140	0.099	0.161	-0.335 – 0.055
	University ≤4y	-0.302	0.096	0.002	-0.491 – -0.113
	University >4y	-0.321	0.097	0.001	-0.512 – -0.130
	5-HTT				
	SL_A	0.066	0.061	0.281	-0.054 – 0.186
	L_AL_G	0.152	0.110	0.168	-0.064 – 0.369
	SL_G	0.047	0.098	0.632	-0.145 – 0.239
	SS	0.179	0.077	0.021	0.027 – 0.330
	Work schedule[3]	0.504	0.163	0.002	0.185 – 0.823
	5-HTT x Work schedule[3]				
	SL_A	-0.590	0.097	0.002	-0.964 – -0.216
	L_AL_G	-0.879	0.236	0.000	-1.342 – -0.415
	SL_G	-0.705	0.019	0.021	-1.293 – -0.117
	SS	-0.773	0.206	0.000	-1.177 – -0.369

[1]non-use=0, use=1; [2]secondary school or less is the reference; [3]non-shift work=0, shift work=1
Step 1 cons: 1.206±0.198; Step 2 cons: 1.126±0.183

Figure legend

Figure 1. The relationship between shift work status and mean insomnia symptom score. Participants were divided into five groups based on *SLC6A4* genotype: SS, SL_G, L_AL_G, SL_A and L_AL_A (used as reference for the regression analysis). Note the difference between L_AL_G and L_AL_A among shift workers. Data are shown as means ± SEM.


Mean insomnia score
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
Non-shift workers
Shift workers
SS
SL_G
L_AL_G
SL_A
L_AL_A
SS
SL_G
L_AL_G
SL_A
L_AL_A