

Circadian Clock-Related Polymorphisms in Seasonal Affective Disorder and their Relevance to Diurnal Preference

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Disturbed circadian rhythms have been observed in seasonal affective disorder (SAD). The aim of this study was to further investigate this connection, and to test for potential association between polymorphisms in circadian clock-related genes and SAD, seasonality (seasonal variations in mood and behavior), or diurnal preference (morningness–eveningness tendencies). A total of 159 European SAD patients and 159 matched controls were included in the genetic analysis, and subsets were screened for seasonality ($n = 177$) and diurnal preference ($n = 92$). We found that diurnal preference was associated with both SAD and seasonality, supporting the hypothesis of a link between circadian rhythms and seasonal depression. The complete case–control material was genotyped for polymorphisms in the CLOCK, Period2, Period3, and NPAS2 genes. A significant difference between patients and controls was found for NPAS2 471 Leu/Ser ($\chi^2 = 9.90$, Bonferroni corrected $P = 0.035$), indicating a recessive effect of the leucine allele on disease susceptibility ($\chi^2 = 6.61$, Bonferroni corrected $P = 0.050$). Period3 647 Val/Gly was associated with self-reported morningness–eveningness scores ($n = 92$, one-way ANOVA: $F = 4.99$, Bonferroni corrected $P = 0.044$), with higher scores found in individuals with at least one glycine allele ($t = 3.1$, Bonferroni corrected $P = 0.013$). A second, population-based sample of individuals selected for high ($n = 127$) or low ($n = 98$) degrees of seasonality, was also genotyped for NPAS2 471 Leu/Ser. There was no significant difference between these seasonality extreme groups, and none of the polymorphisms studied were associated with seasonality in the SAD case–control material ($n = 177$). In conclusion, our results suggest involvement of circadian clock-related polymorphisms both in susceptibility to SAD and diurnal preference.

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INTRODUCTION

Traits and symptoms of affective disorders appear to interweave with circadian clock work and rhythms in both seasonal and nonseasonal forms of depression (Bunney and Bunney, 2000). Manipulations of the sleep–wake cycle and circadian phase have proven beneficial for some patients; for example, sleep deprivation can give a temporary remission from a depressive episode (Wirz-Justice and

Van den Hoofdakker, 1999) and morning bright light therapy is currently the treatment of choice for recurrent winter depression, or seasonal affective disorder (SAD) (Rosenthal *et al.*, 1984a). SAD is often accompanied with atypical depressive symptoms including increased sleep, overeating, and craving for carbohydrates (Partonen and Lönnqvist, 1998). In addition to hypersomnia, there are also results from physiological and endocrine studies implying circadian irregularities in SAD. Abnormalities in the diurnal rhythm of core body temperature, cortisol, and melatonin secretion have been reported in some, but not all, studies (Lam and Levitan, 2000). Patients with SAD seem to generate a melatonin-dependent signal that is absent in healthy volunteers and that is similar to the signal that mammals use to regulate seasonal changes in their behavior (Wehr *et al.*, 2001). While not proving causality, this recent

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finding agrees with the view that the neural circuits that mediate the effects of seasonal changes in daylight on behavior also mediate effects of light therapy on SAD. It has been suggested that at least a subset of the patients have a phase delay, which can be corrected by morning light exposure (Lewy *et al*, 1987). The antidepressant effect of light appears greatest when adjusting the time of administration to the early morning of the individual circadian time of the patient (Terman *et al*, 2001).

Results from family and twin studies point to genetic components in SAD (Sher, 2001), seasonality (seasonal variations in mood and behavior) (Madden *et al*, 1996; Jang *et al*, 1997), and diurnal preference (morningness–eveningness tendencies) (Vink *et al*, 2001). Genetic variations in genes involved in the endogenous circadian clock could have an influence on the deviations from the normal 24-h daily rhythm found in patients with affective disorder (Bunney and Bunney, 2000; Desan *et al*, 2000). The core of this molecular clock consists of a transcription–translation feedback loop that generates a self-sustained circadian oscillation, and can be adjusted to the environment by responding to external cues, such as new light/dark conditions (for a review see Albrecht, 2002). The accuracy of this synchronization depends on the response of endogenous clocks, and the intrinsic period of the circadian pacemaker is tightly linked to the behavioral trait of morning or evening activity patterns (Duffy *et al*, 2001).

We have genotyped European SAD patients and healthy matched controls for four single nucleotide polymorphisms in genes related to intrinsic circadian oscillators; *CLOCK* 3111 C/T (Katzenberg *et al*, 1998), *Period2* 1244 Gly/Glu (NCBI dbSNP database (www.ncbi.nlm.nih.gov): rs934945), *Period3* 647 Val/Gly (Ebisawa *et al*, 2001), and *NPAS2* 471 Leu/Ser (Celera Human SNP Database (www.cds.celera.com): CV2153849). The purpose was to look for association with SAD, seasonality or diurnal preference. All these polymorphisms are located in genes believed to encode for transcription regulatory factors. Mutations in *CLOCK* and the *Period* genes cause circadian rhythm disturbances in mice (King *et al*, 1997; Zheng *et al*, 2001; Shearman *et al*, 2000), although disruption of the *Period3* gene has only a subtle effect (Shearman *et al*, 2000). *NPAS2*, or neuronal PAS domain protein 2, is a functional analogue of *CLOCK*, but unlike the other three genes it is not expressed in the hypothalamic suprachiasmatic nucleus, where the master pacemaker of the mammalian circadian system resides (Shearman *et al*, 1999). However, *NPAS2* is expressed in the frontal association/limbic forebrain pathway, in nuclei essential for sensory processing and emotions such as fear and anxiety, and is believed to be part of a molecular clock operative in the frontal regions of the brain (Reick *et al*, 2001). *NPAS2* is also suggested to mediate hormonal control of a peripheral clock in the vasculature, and to amplify dampened signals in the peripheral tissues (McNamara *et al*, 2001).

To our knowledge, none of these polymorphisms have previously been investigated with regard to SAD. There are studies reporting that *CLOCK* 3111 C/T, which is located in the 3' flanking region of the *CLOCK* gene, is associated with diurnal preference (Katzenberg *et al*, 1998), but not major depressive disorder (Desan *et al*, 2000), and *Period3* 647 Val/Gly is suggested to be the causative polymorphism in a

Period3 haplotype associated with delayed sleep phase syndrome (Ebisawa *et al*, 2001). There are no published association studies for *Period2* 1244 Gly/Glu or *NPAS2* 471 Leu/Ser. However, another *Period2* missense mutation, located in the casein kinase1 ϵ binding region, has been found to cause an autosomal dominant form of familial advanced sleep phase syndrome (Toh *et al*, 2001).

MATERIALS AND METHODS

Subjects

This study was approved by local ethical committees and all participating individuals gave written informed consents. All patients referred to the study (129 women and 30 men) attended outpatient psychiatric services and met the DSM-IV criteria for depressive or bipolar disorder with the seasonal (winter) pattern (American Psychiatric Association, 1994) (for a more detailed description of the inclusion criteria, please see Johansson *et al*, 2001). All individuals were unrelated Caucasians originating from Sweden (88 patients), Finland (41 patients), Austria (19 patients), or Germany (11 patients). The thirteen patients from Finland were diagnosed with bipolar disorder type I, the rest were unipolar. The control individuals ($n = 159$) were matched for ethnicity, nationality, age and sex, and had no history of psychiatric illness. In all, 79 of the patients and 98 of the controls have completed the Seasonal Pattern Assessment Questionnaire (SPAQ) (Rosenthal *et al*, 1984b). It was used for the assessment of seasonal variation in the length of sleep, social activity, mood, weight, appetite, and energy level. The sum of these six scales yields the global seasonality score (GSS), which can range from 0 to 24. The mean GSS was 13.9 ± 2.9 (mean \pm SD) for the patients and 3.7 ± 2.6 for the controls. A total of 46 patients and 46 controls of Swedish or Finnish origin also completed the Horne–Östberg questionnaire (Horne and Östberg, 1976), another self-rating questionnaire that was used for the assessment of preferences for diurnal activity patterns. The sum of this scale gives the global morningness–eveningness score (MES), which can range from 16 to 86. The mean MES was 54.1 ± 5.8 for the patients (when not depressed) and 52.8 ± 4.0 for the controls.

A set of 225 individuals selected for high or low degrees of seasonality (GSS ≥ 10 or ≤ 2 , respectively) from a Swedish population-based sample ($n = 2620$, for a more detailed description, please see Nilsson *et al*, 1997 and Johansson *et al*, in press) was also genotyped for *NPAS2* 471 Leu/Ser.

Genotyping

Genomic DNA was prepared from blood lymphocytes and genotyping for *CLOCK* 3111 C/T was performed as described previously (Desan *et al*, 2000). For the other polymorphisms, standard PCR amplifications were performed using a 50-cycle reaction with annealing temperatures of 63°C for *Period2* 1244 Gly/Glu and 59°C for *Period3* 647 Val/Gly and *NPAS2* 471 Leu/Ser. Primer sequences were: *Period2* 1244 Gly/Glu; CTTCTCTGGGACTCAGCG and CAAGCACACCTGGTGTACCT, *Period3* 647 Val/Gly; CCCAGCCATACCTAAATCAG and AGTGTGGTACCTGT-

CTCTG and for *NPAS2 471 Leu/Ser*; TGGCAGAAG-CAGTGGTAAC and AGACTCACCTGTGCCATGG.

The PCR products were analyzed with real-time pyrophosphate DNA sequencing (Alderborn *et al*, 2000), according to standard protocols provided by the manufacturer (Pyrosequencing, Sweden). One of the PCR primers in each pair was 5' biotinylated and after denaturing the single-stranded DNA was separated using streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin from DYNAL, Norway) and sequenced with the following sequencing primers: *Period2 1244 Gly/Glu*; CGATCCTGTGATT-CAAGG, *Period3 647 Val/Gly*; GGTACCTGTCTCTGGGGGT, and for *NPAS2 471 Leu/Ser*; CTGCTGTGTGAGGTCGCAG. Subsets of the samples were also genotyped using ABI 377 sequencing of the *NPAS2* PCR product ($n = 66$) according to standard protocols (Applied Biosystems, USA), or restriction enzyme cleavage with 4 U of Bam HI (Life Technologies, USA) ($n = 138$), or 2 U of Bsm FI (New England Biolabs, USA) ($n = 58$), for selective digestion of the *Gly* allele of *Period2 1244 Gly/Glu* and the *Val* allele of *Period3 647 Val/Gly*, respectively. The digested products were size separated by 10% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. All samples tested showed identical results with both genotyping methods.

Statistical Analysis

χ^2 -analysis was used to test for association between SAD and an allele or genotype, or GSS-extreme group and *NPAS2 471 Leu/Ser* allele or genotype. The same case-control material, as well as the GSS-extreme samples, has previously been genotyped for a polymorphism in the promoter of the serotonin transporter gene (Johansson *et al*, in press), and therefore *P* values were Bonferroni corrected for multiple testing of five or two polymorphisms, respectively. A subset of the material (82 patients and 82 controls) has also been investigated for other serotonin-related polymorphisms (Johansson *et al*, 2001), but because of the limited number of samples this was not corrected for here. The power calculation was performed as described elsewhere (Johansson *et al*, 2001), assuming $\alpha = 0.01$, $\beta = 0.20$, and allele frequencies in the control group identical to a previously published study (Katzenberg *et al*, 1998), or, when there were no previous reports, the observed frequencies. The distribution of GSS or MES within groups under analysis sometimes deviated from the normal distribution (data not shown), and therefore both parametric (one-way ANOVA or unpaired *t*-test) and nonparametric (Kruskal-Wallis or Wilcoxon rank sum) test methods were used when appropriate. Correlation between the MES and the GSS was tested using linear regression. Stata 6 software was used for the statistical analysis and Genepop (<http://wbimed.curtin.edu.au/genepop/>) was used to test the genotype distributions for Hardy-Weinberg equilibrium.

RESULTS

Diurnal Preference, SAD, and Seasonality

Diurnal preference was investigated in a subset ($n = 92$) of the Swedish and Finnish patients and controls. The Horne-Östberg morningness-eveningness questionnaire (Horne

and Östberg, 1976) was used, specified for when the patients are not depressed. In all, 40 matched case-control pairs completed the test and all individuals had intermediate to moderate scores (between 37 and 67). However, the patients had significantly higher MESs, indicating a stronger preference for morning patterns of activity (mean $MES \pm SD = 54.4 \pm 4.9$ for the patients and 52.6 ± 4.0 for the controls, Wilcoxon rank sum test: $z = -2.5$, $P = 0.01$). In addition, there was a significant correlation between the MES and GSS ($n = 78$, linear regression; $R^2 = 0.065$, $P = 0.02$).

Genetic Analysis of SAD and Seasonality

When comparing genotype distributions in SAD patients ($n = 159$) and controls ($n = 159$), a significant difference was found for *NPAS2 471 Leu/Ser* ($\chi^2 = 9.90$, Bonferroni corrected $P = 0.035$, see Table 1), whereas the allele frequencies for this polymorphism were almost identical (80% *Ser* and 20% *Leu* in both groups). Only one individual being homozygous for the leucine allele was found in the control group, compared to nine patients, indicating that this genotype might be a risk factor for SAD ($\chi^2 = 6.61$, Bonferroni corrected $P = 0.050$, when assuming a recessive effect of *Leu*). Gender-specific analysis under a recessive model yielded similar results, although the difference was not significant in men, possibly because of the small number of male participants (women ($n = 129 \pm 129$): $\chi^2 = 6.66$, Bonferroni corrected $P = 0.049$, and men ($n = 30 \pm 30$): $\chi^2 = 4.29$, Bonferroni corrected $P = 0.19$).

We also genotyped for *NPAS2 471 Leu/Ser* in a second material consisting of 225 individuals from the general Swedish population, selected for high or low degrees of seasonality (GSSs were ≥ 10 or ≤ 2 , respectively). No association was found in this sample set ($\chi^2 = 2.5$, Bonferroni corrected $P = 0.58$, see Table 2), nor was there an association between *NPAS2 471 Leu/Ser* and GSS among the SAD patients and controls where this had been measured ($n = 177$, Kruskal-Wallis: $H = 2.05$, Bonferroni corrected $P > 1$). Assuming a recessive effect of the leucine allele, based on the findings from the case-control material,

Table 1 Genotyping of SAD Patients and Matched Controls

	Genotype	SAD ($n = 159$)	Controls ($n = 159$)	χ^2 (<i>P</i> value)
CLOCK 3111	T/T	79 (50%)	75 (47%)	0.21 (0.90)
	C/T	72 (45%)	76 (48%)	
	C/C	8 (5%)	8 (5%)	
Period2 1244	Gly/Gly	120 (75%)	111 (70%)	3.44 (0.18)
	Gly/Glu	34 (21%)	46 (29%)	
	Glu/Glu	5 (3%)	2 (1%)	
Period3 647	Val/Val	95 (60%)	102 (64%)	1.29 (0.52)
	Gly/Val	58 (36%)	49 (31%)	
	Gly/Gly	6 (4%)	8 (5%)	
NPAS2 471	Ser/Ser	105 (66%)	95 (60%)	9.90 (0.007*)
	Leu/Ser	45 (28%)	63 (40%)	
	Leu/Leu	9 (6%)	1 (1%)	

*Bonferroni corrected *P* value = 0.035.

Table 2 Genotype Frequencies of *NPAS2 471 Ser/Leu* in Two Population-Based Groups Selected for Extreme Degrees of Seasonality

	Subjects with GSS ≤ 2 (n = 98)	Subjects with GSS ≥ 10 (n = 127)	χ^2 (P value)
Ser/Ser	70 (71%)	78 (61%)	2.47 (0.29*)
Leu/Ser	27 (28%)	47 (37%)	
Leu/Leu	1 (1%)	2 (2%)	

*Bonferroni corrected *P* value = 0.58.

as well as separate analysis of SAD patients (*n* = 79) and controls (*n* = 98), gave similar results (data not shown). Analysis of the individual SPAQ items in the population-based material revealed slightly higher appetite and weight scores in individuals with the leucine allele, although the differences were not statistically significant (Kruskal–Wallis test, uncorrected *P* values 0.039 and 0.071, respectively).

No significant differences in the genotype or allele frequencies were found between SAD patients and controls for *CLOCK 3111 C/T*, *Period2 1244 Gly/Glu*, or *Period3 647 Val/Gly* (for the genotypes: χ^2 = 0.21–3.44, Bonferroni corrected *P* > 0.9, see Table 1). The minimum detectable allelic effect sizes for the different polymorphisms were estimated to odds ratios between 1.7 and 1.9, after accounting for multiple testing. There was no association with GSS (*n* = 177, Kruskal–Wallis: *H* = 0.66–3.68, Bonferroni corrected *P* > 0.8), nor when analyzing patients (*n* = 79) and controls (*n* = 98) separately (data not shown).

No significant deviations were found when testing genotype distributions in SAD patients, controls and GSS extreme groups for Hardy–Weinberg equilibrium (Bonferroni corrected *P* values > 0.05). However, a *P* value of borderline significance was found for *NPAS2 471 Leu/Ser* in the control group (Bonferroni corrected *P* = 0.054).

In addition to the results described above, we studied two other reported polymorphisms in clock-relevant genes, *Timeless 592 Val/Met* (Sangoram et al, 1998) and *Casein kinase 1 ε415 C/T* (dbSNP rs1803339), that were found not to be polymorphic after having genotyped approximately 100 individuals (data not shown). Therefore, these polymorphisms were not analyzed further.

Genetic Analysis of Diurnal Preference

We also found an association between *Period3 647 Val/Gly* genotype and diurnal preference (*n* = 92, one-way ANOVA: *F* = 4.99, Bonferroni corrected *P* = 0.044). The MES difference between genotype groups was most pronounced in patients (*n* = 46, *F* = 4.81, Bonferroni corrected *P* = 0.065), and in general, individuals with at least one *Gly* allele tended to have higher MES (mean MES ± SD = 55.5 ± 4.9, compared to 52.2 ± 4.8 for *Val/Val* homozygotes, two-tailed unpaired *t*-test: *t* = 3.1, Bonferroni corrected *P* = 0.013), again especially in the patient group (*n* = 46, mean MES ± SD = 57.2 ± 4.2 and 52.1 ± 5.9, respectively, *t* = 3.13, Bonferroni corrected *P* = 0.016). There was no association with MES for any of the other polymorphisms

studied (one-way ANOVA: 0.2–1.6, Bonferroni corrected *P* > 1).

DISCUSSION

The finding that SAD patients during remission had higher MES than controls support the hypothesis of SAD being associated with circadian rhythms, and is in line with a previous study reporting more eveningness in depressed SAD patients and a shift towards morningness after light therapy (Elmore et al, 1993).

We found a significant difference between SAD patients and controls for the *NPAS2 471 Leu/Ser* polymorphism, suggesting a recessive effect of the leucine allele. The polymorphism is located outside of the conserved motif domains in the *NPAS2* gene and its potential biological effect remains to be elucidated. Since no association was found when studying seasonality in a subset of the sample set, as well as in a second, population-based material, this indicates that *NPAS2 471 Leu/Ser* might influence the predisposition to SAD rather than the degree of seasonality in general. Even when selecting the most extreme groups in the general population (in this case ca. 5% from each end of the spectrum), the high seasonality group still had lower scores (mean GSS = 11) than the SAD patients (mean GSS = 14), so maybe this group was not extreme enough to detect an effect of *NPAS2 471 Leu/Ser*. Also, the polymorphism might only influence some aspects of seasonality and SAD, as indicated by the tendency of higher seasonality in appetite and weight, in individuals with the leucine allele.

Although strictly speaking, the control group did not deviate significantly from Hardy–Weinberg equilibrium with regard to *NPAS2 471 Leu/Ser*, the result could indicate an effect of the population structure. However, no deviations were found for the four other polymorphisms tested in the same material. In addition, the *NPAS2 471 Leu/Ser* genotype distributions did not differ significantly from the Hardy–Weinberg equilibrium when testing each nationality separately, and pairwise comparison (χ^2 -tests) between all nationalities revealed no significant differences in control group *NPAS2 471 Leu/Ser* genotype distribution (all *P* > 0.59, not corrected for multiple testing). The samples were genotyped at least twice, cases and controls together, using a thoroughly validated technology (Nordfors et al, 2002). In addition, a subset of the samples (*n* = 66) was resequenced for *NPAS2 471 Leu/Ser* using another method (for details see Materials and Methods), minimizing the risk for genotyping errors.

Period3 647 Gly was associated with self-reported MESs in the subset of the samples screened for diurnal preference (*n* = 92). Separate analysis of patients and controls gave similar results, but not always with significant *P* values, probably because of lack of power. Interestingly, the *Val* allele is conserved in most *Period* homologues, and so is the surrounding amino-acid sequence that contains putative casein kinase 1ε target sites (Ebisawa et al, 2001). Casein kinase 1ε-dependent phosphorylation, of at least *Period1* and *Period2*, is believed to be important regulatory steps in the circadian system (Albrecht, 2002). The function of *Period3* in the circadian clock is less well defined, but the *Period3 647 Gly* allele has been suggested to play a role in delayed sleep phase syndrome (Ebisawa et al, 2001).

The lack of association between MES and *CLOCK 3111 C/T* in this study is in contrast to an earlier report of association between the *C* allele and eveningness tendencies in a population-based sample ($n = 410$) (Katzenberg *et al*, 1998), and might be explained by our limited sample size ($n = 92$). Indeed, our results do not exclude minor effects on SAD, GSS, or MES, of the polymorphisms where no associations were found. Also, the findings for *NPAS2 471 Leu/Ser* and *Period3 647 Val/Gly* should be considered preliminary until replicated in a larger material.

In conclusion, diurnal preference was associated with both SAD and seasonality in our material, supporting the hypothesis of a link between circadian rhythms and seasonal depression. Our results suggest that two circadian clock-related polymorphisms, *NPAS2 471 Leu/Ser* and *Period3 647 Val/Gly*, may be implicated in SAD and diurnal preference, respectively. Whether these polymorphisms are functional in themselves, or whether they reflect linkage disequilibrium with other causative polymorphisms remains to be elucidated. Further work is needed to confirm these findings, including replication in independent sample sets, preferably using methods to control for the risk of population stratification, as well as studies of the possible biological effects of *NPAS2 471 Leu/Ser* and *Period3 647 Val/Gly*.

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