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A Polymorphism at the 3′-Untranslated Region of the *CLOCK* Gene Is Associated With Adult Attention-Deficit Hyperactivity Disorder

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Attention-deficit hyperactivity disorder (ADHD) is frequently found in childhood and persists in about 50% of cases into adulthood. Several studies demonstrate a relationship between ADHD, circadian rhythmicity and sleeping disturbances in unmedicated ADHD patients. Since ADHD is a very complex disease with a high genetic load involving multiple genes of moderate effect, we hypothesized a link between adult ADHD and genes involved in the circadian timekeeping system. A 3′-UTR polymorphism of the circadian locomotor output cycles protein kaput (*CLOCK*) gene, rs1801260, has been linked to disturbed sleep patterns, although both the C-allele and more controversially the T-allele have been proposed as risk factors for different measures of evening preference. This study compared self-rating and interview based measures of ADHD psychopathology of 143 subjects with and without ADHD with their rs1801260 genotype to test the hypothesis that ADHD is linked to one of the alleles of the *CLOCK* polymorphism. The T > C single nucleotide polymorphism rs1801260 was genotyped in DNA isolated from blood samples. The associations between genotype and ADHD-scores were compared using non-parametric ANCOVA with post hoc pairwise comparisons. There was a strong, significant association ($P < 0.001$) between each of the adult ADHD assessments and the rs1801260 polymorphism with at least one T-mutation being the risk allele. This is the first study suggesting that a polymorphism of a gene within the circadian "clock" mechanism is a direct or linked contributing factor in adult ADHD.
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KEY WORDS: adult ADHD; association; *CLOCK* gene; circadian rhythmicity; single nucleotide polymorphism

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INTRODUCTION

Ten to 60% of childhood attention-deficit hyperactivity disorder (ADHD) persists into adulthood [Biederman et al., 1990]. ADHD, in particular "persistent ADHD", shows a high heritability of about 70–80% [Faraone et al., 2005] although few genetic associations with adult ADHD have been confirmed in meta-analyses. In terms of understanding the pathophysiology of ADHD there is much work to be done [Schneider et al., 2006]. However, some strong lines of evidence point toward a role for sleep disturbance in at least contributing to the psychopathology of the condition [Corkum et al., 1998; Ring et al., 1998; Yuen and Pelayo, 1999; Golan and Pillar, 2004; Golan et al., 2004]. Sleep is implicated in brain areas associated with thinking, decision-making, and impulsivity, all processes believed to be disturbed in ADHD. Sleep deprivation can result in mood changes, inattention, delayed reaction time and impaired vigilance, decreased motivation, hyperactivity, aggressive behavior, and impulsivity, symptoms that overlap with those commonly associated with ADHD [APA, 1994]. It is unclear whether these symptoms are secondary effects of ADHD or whether sleep and circadian disorders are causative in the genesis of ADHD. Sleep is also proposed to be important in the consolidation of learning processes in the brain, with slow-wave, non-REM sleep playing a role in the reinforcement of regionally specific neuronal plasticity [Huber et al., 2004]. Thus, inadequate or disturbed sleep may have primary effects that impede physical and cognitive development. In the congenic wiggling (Wig) rat model of human ADHD [Kamimura et al., 2001], there was abnormal impulsive behavior, and a prominent nocturnal hyperactivity of the animals when compared to controls. An increased instability in sleep onset, duration and true sleep was found in a childhood ADHD group compared with healthy controls [Gruber et al., 2000].

The endogenous circadian timekeeping mechanism is believed to be a pivotal regulator of the sleep–wake cycle, interacting with a homeostatic mechanism in determining the onset and duration of sleep [Borbely, 2001]. The molecular

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basis of the circadian timekeeping mechanism appears to involve the interaction of a number of interlocking feedback/feedforward transcriptional loops of so-called "clock" genes and their protein products [Reppert and Weaver, 2002; Gachon et al., 2004]. Polymorphisms in these clock genes have been implicated in a number of human circadian disorders, including delayed sleep phase syndrome (DSPS), and advanced phase sleep disorder [for review see Cermakian and Boivin, 2003]. The circadian clock gene *CLOCK* acts as a transcription factor and is known to play a crucial role in the organization of circadian rhythms in mammals [McClung et al., 2005]. Approximately 10% of gene transcripts have been shown to exhibit significant circadian profiles in their expression, and disruption of the *Clock* locus significantly impairs this regulation [Panda et al., 2002; Oishi et al., 2003].

A number of factors have led us to postulate that disruption of molecular components of the circadian mechanism, especially CLOCK, may be important in adult ADHD. In the psychopathology of disorders such as ADHD, dysregulations in dopaminergic neurotransmission have been postulated. Studies on the *Clock* mutant mouse model show these animals to display a pronounced elevation of their locomotor activity [McClung et al., 2005]. Dopamine neurons display elevated impulse activity in the *Clock* mutants compared with wild-type controls [McClung et al., 2005]. These data suggest that dysfunction of the circadian clock mechanisms may contribute to psychiatric disorder by dysregulation of the dopaminergic system. There is evidence [Katzenberg et al., 1998] that a single nucleotide polymorphism in the 3′UTR of the *CLOCK* gene (rs1801260) is a predisposing factor of the diurnal preference in a healthy population of European ancestry, with subjects carrying at least one copy of the C allele having a substantial delay in preferred timing for activity, sleep and higher eveningness scores. Mishima et al. [2004] demonstrated that the rs1801260 polymorphism of *CLOCK* was associated with evening preference and delayed sleep timing in a Japanese population sample and suggest that this single nucleotide polymorphism may influence the human behavioral pattern and lead to altered daytime brain performance. The findings for eveningness preference in Caucasians are disputed however [Robilliard et al., 2002] and the T-allele of the same polymorphism has been suggested as a risk factor for DSPS [Iwase et al., 2002]. Since the 3′-UTR of the gene may contain sequences that regulate translation efficiency, mRNA stability, and polyadenylation signals, polymorphisms of this region may influence the circadian rhythms, which are controlled endogenously.

To test the hypothesis of the association of the SNP rs1801260 of the *CLOCK* gene with ADHD we performed a study in a well-defined group of 143 unrelated male adults with German background. This is the first report of an association of a polymorphism in a circadian "clock" gene with ADHD.

MATERIALS AND METHODS

Subjects

The study enrolled 143 male adults, consecutively referred for psychiatric examination to the Institute of Forensic Psychiatry of the Saarland University. Average age was 31.3 ± 11.7 (SD). ADHD symptoms were assessed according to their relative ADHD-scores following self-report scales, the Wender–Reimherr diagnostic interview and validated background data. Comorbid disorders were diagnosed according to DSM-IV and ICD-10 criteria, using modified, standardized checklists as reported by Rösler et al. [2004].

Since the ethnic heterogeneity of an analyzed sample may notably affect the observed allele frequencies [Dvornyk et al., 2004], all the subjects in this study were Caucasians of western European origin and German background. The study was approved by the local Ethical Committee, as previously outlined [Rösler et al., 2004]. To assure uniformity of data collection, all subjects underwent the same evaluation, after providing written informed consent and explanation of the aims of the study. No subjects with a diagnosis of current substance dependence, acute schizophrenia, major depression/bipolar disorder, or any other severe Axis-I diagnosis according to DSM-IV as well as subjects with the diagnosis of mental retardation (IQ < 70) were admitted to participate to the study.

DNA Analysis

After written informed consent had been obtained from subjects, EDTA-stabilized venous blood samples were taken and stored immediately after collection at -20°C until analysis. Genomic DNA was extracted from 10 ml whole blood of each subject using the commercial Invisorb® Blood Giga Kit (Invitek, Berlin, Germany) and concentration was adjusted using a PicoGreen fluorometric assay (Molecular Probes/Invitrogen, Paisley, UK).

The T > C single nucleotide polymorphism rs1801260 occurs in the immediate 3′-UTR of the *CLOCK* gene, 201 bp downstream from the end of the open reading frame (AF011568, position 3111 [Steeves et al., 1999]). A recognition site for Bsp1286I (New England Biolabs, Frankfurt, Germany) is created when the C-allele, but not when the T-allele is present. Amplification of target DNA in 3′-UTR of the gene was carried out using PCR with the following primers: *clockfor*: 5′-TCCAGCAGTTTCATGAGATGC-3′ and *clockrev*: 5′-GAGGTCATTTCATAGCTGAGC-3′ as reported by Katzenberg et al. [1998]. The reaction was performed in a final volume of 25 μl containing 10 ng of genomic DNA in a premixed, ready-to-use master-solution containing *Taq* DNA Polymerase, dNTPs, $MgCl_2$, and reaction buffers (Promega, Mannheim, Germany). DNA containing the polymorphic site was amplified in a PTC-200 Peltier Thermal Cycler (MJ Research/Bio-Rad, San Diego) under the following conditions: 2 min at 95°C followed by 35 cycles at 94°C 30 sec, 58°C 35 sec, 72°C 1 min, followed by 10 min at 72°C. The amplicon (221 bp) was visualized on a 2% agarose gel stained with ethidium bromide. Restriction enzyme digestion was performed on 5 μl of PCR product, digested in a final volume of 12 μl with Bsp1286I at 37°C overnight. The resulting digested samples (unrestricted TT genotype = 221 bp; complete restriction, CC genotype = 125 and 96 bp fragments, respectively) were then visualized on a 2.5% agarose gel stained with ethidium bromide, following separation at 100 V in tris-borate electrophoresis buffer. The gels were viewed under a transilluminator, and recorded for further scoring of genotypes. In a control experiment, a 318 bp fragment containing rs1801260 was amplified in a final volume of 25 μl under the same conditions as mentioned before (but annealing at 59°C) with the primers *CLOCKF*: 5′-GTAGCACACGTGCTTCCTCT-3′ and *CLOCKR*: 5′-TCCCTGGAGGTCATTTCATA-3′. The primers were designed using the Pimer3 program [Rozen and Skaletsky, 2000]. The product was purified with an Invisorb PCR HTS 96 Kit/V (Invitek) and direct sequenced with a multicolor fluorescence-based 16-capillary electrophoresis ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences were analyzed with the Staden processing software package, and the results of the RFLP genotyping were confirmed (Fig. 1).

Statistical Analysis

Allele frequencies of the group were calculated by direct counting from the genotypes observed and tested for Hardy–Weinberg equilibrium (HWE), using a Chi-squared

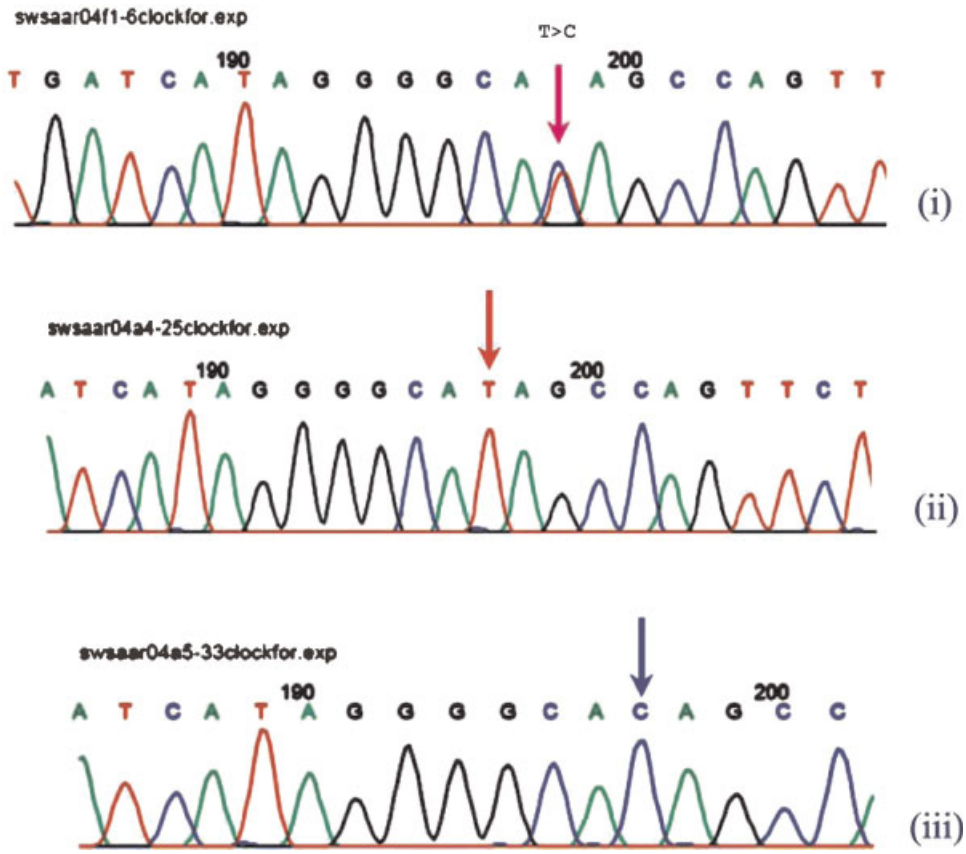

Fig. 1. Sequence analysis of sense strands of the *CLOCK* gene: the edited sequences show (i) an individual heterozygous for the T > C nucleotide variation, (ii) TT, and (iii) CC homozygous, respectively. The arrows indicate the position of SNP rs1801260 on gene. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

test between observed and expected values. Descriptive data between genotypes were compared by non-parametric analysis of variance (ANOVA) or χ^2-tests. Possible confounding variables (criterion $P < 0.10$) associated with a specific genotype were controlled for in further analyses. As the distribution of the rating scale values was markedly skewed from a normal distribution, non-parametric analyses were performed to assess the association of the three genotypes with the severity of the ADHD self-assessment and Wender–Reimherr interview. These non-parametric analyses of covariance (ANCOVA) were computed by the SAS-macro *npar* (www.ams.med.uni-goettingen.de/Projekte/makros/index.html) with adjustment for age and history of personality disorder. Bonferroni-adjustment for multiple testing results in critical *P*-value <0.01 indicating significance of the findings.

RESULTS

We studied the effect of a single nucleotide polymorphism (rs1801260) falling into the 3′-UTR of *CLOCK* on adult ADHD symptoms and retrospectively reported childhood ADHD symptoms. A sample of 143 unrelated adult males affected by ADHD was genotyped for rs1801260 only, revealing 73 subjects homozygous for the T-allele (TT), 62 heterozygous (CT) subjects, and 8 CC homozygotes (Table I).

There was no statistical deviation from the HWE in the sample. We collected detailed information on ethnic background to test for an eventual population stratification [Cardon and Palmer, 2003]. Age and history of personality disorder differed between genotypes and therefore were controlled for as possible confounding variates in further analyses (Table I).

TABLE I. Sample Characteristics of the Subjects Separated Into *CLOCK*-Genotype Including Mean Age (±SD) and Relevant Histories

CLOCK-genotype carrier (N = 143)	Age, mean (SD)	History of substance abuse, N (%)	History of personality disorder, N (%)
CC; N = 8	39.3 (13.8)	5 (62.5)	2 (25.0)
CT; N = 62	32.0 (11.3)	21 (33.9)	27 (43.6)
TT; N = 73	35.7 (11.7)	33 (45.2)	17 (23.3)
Statistics: χ^2, df, *P*-value	$\chi^2 = 4.7$ (2), $P = 0.096$	$\chi^2 = 3.4$ (2), $P = 0.187$	$\chi^2 = 5.1$ (2), $P = 0.039$

Sample sizes and statistical significance (incorporating χ^2 values, df, and *P*-value) are included.
N, number of observations; SD, standard deviation; df, degrees of freedom.

TABLE II. Mean ADHD-Scores (±SD) Measured Using the Self-Report Scores, WURS-k and Wender–Reimherr Instruments, Separated Into *CLOCK*-Genotype Carriers Are Shown

	[a]Mean (SD)				
CLOCK-genotype carrier	ADHD-self report total score	ADHD-self report attention problems score	ADHD-self report hyperactivity/impulsivity score	Wender–Reimherr interview score	Childhood ADHD symptoms Wurs-K score
CC	5.5 (3.9)	2.4 (2.1)	3.0 (2.1)	9.6 (4.1)	1 8.2 (5.2)
CT	12.2 (1.4)	5.3 (0.8)	6.9 (0.8)	16.1 (1.5)	25.2 (1.9)
TT	15.8 (1.4)	7.6 (0.7)	8.2 (0.8)	18.8 (1.4)	26.3 (1.9)
Statistics: T (df), *P*-value	22.8 (1.6), $P = 0.00001$	16.9 (1.5), $P = 0.0001$	19.3 (1.6), $P = 0.00004$	21.4 (1.9), $P = 0.00002$	7.4 (1.8), $P = 0.019$

Sample sizes and statistical significance (incorporating T-values, df, and *P*-value) are included.
SD, standard deviation; df, degree of freedom.
[a]Mean adjusted for age differences and history of personality disorder.

With respect to the continuous ADHD measures assessed in this study, there was an association between genotypes containing one or both T-alleles and number of present ADHD symptoms either reported by the individual or obtained by the Wender–Reimherr interview (Table II). Interestingly the retrospective analysis of childhood ADHD (WURS-K) in the same subjects revealed an association that was significant but much smaller than that seen for adult ADHD ($0.01 < P < 0.05$).

A more detailed pairwise analysis (Table III) showed the relative difference between one or two T-alleles only reached significance for the ADHD-self report hyperactivity/impulsivity score. The level of significance was greater when comparing both homozygous genotypes (CC vs. TT) compared with the CC versus CT analysis in each case except for the Wender–Reimherr interview score, which was very highly significant for both tests.

DISCUSSION

ADHD is a multifactorial disease in which both a high genetic load and environmental factors play a crucial role. Based on the fact that *CLOCK* is an essential molecular component of the mammalian circadian clock, variations of this gene could be related to sleep disorders and disturbances of circadian rhythmicity. Furthermore, genetic variation of the *CLOCK* could have effects on dopamine-driven adaptive regulatory processes and, in turn, influence the predisposition to ADHD.

Although the SNP rs1801260 in *CLOCK* will not alter the structure of the encoded protein, this mutation outside the coding sequence of the gene could alter the expression or the stability of its message [Bennett et al., 1995; Kennedy et al., 1995]. Such a mechanism has been shown to operate in the post-transcriptional regulation of the clock gene *Per1* in the mouse [Kojima et al., 2003], as well as the *Per* gene in *Drosophila* [Brookes et al., 2006]. A polymorphism (T > C) in the 3′-UTR region of the *CLOCK* gene has been implicated in eveningness preference in humans [Katzenberg et al., 1998], and delayed sleep timing [Mishima et al., 2004] although a separate study failed to show any association of this polymorphism with alterations in diurnal preference in Caucasians [Robilliard et al., 2002]. Further studies have revealed a link between this locus and insomnia in mood disorders [Serretti et al., 2003] and illness recurrence in bipolar depression [Benedetti et al., 2003]. As an alternative, this SNP could be in linkage disequilibrium with more functionally yet unidentified mutations [Savov et al., 1995]. Finally, the possibility exists that the 3′-UTR of *CLOCK* is involved in the production of antisense message that may be involved in producing circadian molecular oscillations [Crosthwaite, 2004]. Whilst such a mechanism has not been demonstrated in mammalian *Clock*, it has been observed for the *frq* gene of the *Neurospora crassa* circadian clock [Kramer et al., 2003].

Interestingly, about 70–80% of individuals with ADHD also suffer from sleep disorders, which can cause additional problems with attention and impulsiveness leading to distraction, thereby increasing some of the main symptoms of ADHD in adults. To the best of our knowledge, no genetic studies of *CLOCK* in adults with ADHD have been previously reported. Our data suggests the T-allele of this polymorphism is a risk factor for adult ADHD. Conversely, given the low prevalence of it in this ADHD population, the C allele may be protective against adult ADHD. This is perhaps surprising since the onset of eveningness has been (controversially) associated with the C-allele of the same SNP [Katzenberg et al., 1998] but reflects a similar finding linking the T-allele to

TABLE III. Pairwise Comparison of the Three *CLOCK*-Genotypes Comparing ADHD-Scores Measured Using the Self-Report Scores, WURS-k and Wender–Reimherr Instruments (*P* Value; One Degree of Freedom)

CLOCK-genotypes compared	ADHD-self report score	ADHD-self report attention problems score	ADHD-self report hyperactivity/impulsivity score	Wender–Reimherr interview score
CT vs. TT	0.060	0.018	0.205	0.408
CC vs. CT	0.001	0.006	0.002	<0.001
CC vs. TT	<0.001	<0.001	<0.001	<0.001

DSPS [Iwase et al., 2002]. This pilot study could be built upon with increased non-patient equivalent studies, further comparisons of ethnicity and the systematic analysis of other polymorphisms within the circadian machinery. A study into the possible gene/environment interactions would also be a valuable endeavor. Clinically the possibility that the C allele of the rs1801260 polymorphism was protective against ADHD in the general population would be a clear avenue of interest of therapeutic or diagnostic value. The current study demonstrates for the first time that the rs1801260 polymorphism is either directly contributing or linked to a causative polymorphism for ADHD in adults.

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