Genetic and functional abnormalities of the melatonin biosynthesis pathway in patients with bipolar disorder

Bruno Etain1,2,4, Anne Dumaine1,4, Frank Bellivier1,2,4,5, Cécile Pagan4,6,7,8, Laetitia Francelle1,4, Hany Goubran-Botros4,6,7, Sarah Moreno4,6,7, Jasmine Deshommes1,2,3,4, Khaled Moustafa1,4, Katia Le Dudal4,9, Flavie Mathieu1,4, Chantal Henry1,2,4,5, Jean-Pierre Kahn4,10, Jean-Marie Launay4,8, Thomas W. Mühliesen11,12, Sven Cichon11,12, Thomas Bourgeron4,6,7, Marion Leboyer1,2,4,5 and Stéphane Jamain1,4,*

1Psychiatrie Génétique, INSERM U 955, Créteil 94000, France, 2Pôle de Psychiatrie and 3Plateforme de Ressources Biologiques, AP-HP, Hôpital H. Mondor – A. Chenevier, Créteil 94000, France, 4Fondation Fondamental, Créteil 94000, France, 5Faculté de Médecine, Université Paris Est, Créteil 94000, France, 6Génétique Humaine et Fonctions Cognitives, Institut Pasteur, Paris 75015, France, 7CNRS URA 2182 “Genes, synapses et cognition”, Institut Pasteur, Paris, France, 8Faculté de Pharmacie, Service de Biochimie, Hôpital Lariboisière, AP-HP, Paris, France, 9Hôpital H. Mondor – A. Chenevier, Pôle Recherche Clinique Santé Publique, INSERM, Centre d’Investigation Clinique 006, Créteil 94000, France, 10Département de Psychiatrie et de Psychologie clinique, CHU de Nancy, Hôpital Jeanne-d’Arc, Toul 54200, France, 11Department of Genomics, Life & Brain Center and 12Institute of Human Genetics, University of Bonn, Bonn D-53127, Germany and 13Institute of Neuroscience and Medicine (INM-1), Research Center Juelich, Juelich D-52425, Germany

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Patients affected by bipolar disorder (BD) frequently report abnormalities in sleep/wake cycles. In addition, they showed abnormal oscillating melatonin secretion, a key regulator of circadian rhythms and sleep patterns. The acetylserotonin O-methyltransferase (ASMT) is a key enzyme of the melatonin biosynthesis and has recently been associated with psychiatric disorders such as autism spectrum disorders and depression. In this paper, we analysed rare and common variants of ASMT in patients with BD and unaffected control subjects and performed functional analysis of these variants by assaying the ASMT activity in their B-lymphoblastoid cell lines. We sequenced the coding and the regulatory regions of the gene in a discovery sample of 345 patients with BD and 220 controls. We performed an association study on this discovery sample using common variants located in the promoter region and showed that rs4446909 was significantly associated with BD (P = 0.01) and associated with a lower mRNA level (P < 10^-5) and a lower enzymatic activity (P < 0.05) of ASMT. A replication study and a meta-analysis using 480 independent patients with BD and 672 controls confirmed the significant association between rs4446909 and BD (P = 0.002). These results correlate with the general lower ASMT enzymatic activity observed in patients with BD (P = 0.001) compared with controls. Finally, several deleterious ASMT mutations identified in patients were associated with low ASMT activity (P = 0.01). In this study, we determined how rare and common variations in ASMT might play a role in BD vulnerability and suggest a general role of melatonin as susceptibility factor for BD.
INTRODUCTION

Affecting 1–4% of the population worldwide, bipolar disorder (BD) is among the most common and devastating psychiatric disorders. It is characterized by alternating periods of elevated mood and severe depression, interspaced with periods of stable mood (1). This disorder is a major public health concern, due to high rates of psychosocial impairments, hospitalizations and suicide. Multiple levels of rhythm abnormalities have been observed in patients with BD, such as circadian preference for eveningness when compared with controls (2–4), seasonality or rapid cycling. These patients also show rhythm abnormalities in secretion of hormones, core temperature and fibroblasts activity (5). Most circadian rhythms and sleep disturbances are supposed to be enduring characteristics of patients with BD and to be underpinned by abnormal function of circadian clocks (6). However, while abnormalities of circadian rhythms and sleep/wake cycles have widely been reported in BD, very little is known whether they represent core elements of the pathophysiological processes underlying the disease (7–9) or symptomatic comorbidities.

Among circadian rhythms that seem to be particularly altered in patients with BD, a focus has been made on sleep/wake abnormalities. First, sleep disturbances are observed at early stages in the development of BD (10–12), before the occurrence of the first mood episode, being thus suggested to be an early susceptibility marker. Secondly, sleep/wake disturbances have been observed, not only during mood states, but also in recovered patients (6), including several measures such as a phase advance, a higher percentage of nocturnal sleep, a lower average daily activity phase advances (13) and an abnormal duration of sleep (long/short sleeper) (14). It has been suggested that both sleep disturbances and abnormal circadian functioning are underlain by genetic susceptibility factors, and interact to influence the catecholamine’s circuity leading to mood disturbance (6).

Sleep patterns and circadian rhythms are influenced by melatonin, a neurohormone synthesized primarily in the pineal gland during the dark phase of the night. Melatonin is a multitask molecule with an effective antioxidant property (15–17). Several independent studies have reported that patients with BD have lower melatonin levels on the light night, a later peak time for melatonin on the dark night (18) and a supersensitive melatonin suppression to light (19). These biochemical abnormalities have been suggested to be trait-markers for BD (20–23) and underpinned by genetic factors (24,25).

Melatonin results from the conversion of serotonin to N-acetylserotinin by aroylkylamine N-acetyltransferase (AA-NAT; OMIM 600950) followed by the conversion of N-acetylsertotonin to melatonin by the acetylserotonin O-methyltransferase (ASMT, OMIM 300015/402500), also known as hydroxyindole O-methyltransferase. Variations in the melatonin biosynthesis pathway have recently been reported to be associated with psychiatric disorders, such as autism spectrum disorders (ASD) and depression (26–31). The ASMT gene has never been studied in patients with BD, although its role in the melatonin biosynthesis pathway makes it a compelling candidate gene. Moreover, ASMT is located in the pseudo-autosomal region 1 (PAR1) shared by the X and Y chromosomes, which has been recently linked to BD (32).

In this study, we screened all exons of ASMT for rare and common variations in patients with BD and unaffected subjects and determine the functional consequences of such variations on ASMT enzymatic activity in their B-lymphoblastoid cell lines (BLCL). We used an independent cohort of patients and controls to replicate the association study findings. Both genetic and functional results suggest a role for decreased melatonin biosynthesis in the susceptibility to BD.

RESULTS

Mutation screening

We investigated whether rare variations in ASMT were associated with BD by directly sequencing all coding exons and regulatory sequences of the gene in a discovery sample, which included 345 patients and 220 healthy controls. Six non-synonymous variations (R111K, P243L, Y248H, E288D, L298F, V305M), one splice-site mutation (IVS5+2T>C) and a two base-pair deletion, leading to a frame shift in the open reading frame after N13 (N13fsX22), were detected exclusively in the patient sample (Table 1). The splice-site variation (IVS5+2T>C), E288D and L298F mutations (originally E320D and L326F, respectively) were previously found in patients with ASD and controls (28,30,33). The five other variations were not found in the 220 controls, but four substitutions (E61Q, K219R, C273S, R291Q) were exclusively found in the control sample (Table 1).

Association study

Recent data on ASMT showed that variations in the promoter region were frequently associated with various psychiatric disorders, such as ASD and depression (27–30). We thus used the same four common single nucleotide polymorphisms (SNPs) located in the promoter region, rs4446909, rs5989681, rs56690322 and rs6644635, to perform an association study. In order to increase our statistical power, we added 52 additional French controls to our sample and performed an association study on 345 patients with BD and 272 controls of French origin. Although a difference in allele frequencies was observed between patients and controls for three out of the four SNPs [rs4446909, allele G, P = 0.01, OR = 1.38, 95% CI (1.07;1.76); rs5989681, allele G, P = 0.05, OR = 1.27, 95% CI (1.00;1.60); rs56690322, allele A, P = 0.03, OR = 1.48, 95% CI (1.03;2.13)], only the association with rs4446909 resisted to Bonferroni correction for multiple tests (P = 0.04, Table 2).

We compared the distributions of the four-SNP haplotypes and showed a significant difference between patients and controls (overall P-value = 0.03) (Table 2). More particularly, we identified a protective haplotype ACGC, significantly more frequent in controls when compared with patients [P = 0.007, OR = 0.71, 95% CI (0.55;0.91)]. A trend in frequency difference between patients with BD and controls was also observed for two susceptibility haplotypes, GGAT [P = 0.07, OR = 1.43, 95% CI (0.98;2.09)] and GGGC [P = 0.11, OR = 1.23, 95% CI (0.95;1.59)], the latter having been previously associated with ASD (30).
Table 1. Rare ASMT variations identified in patients with BD and controls

<table>
<thead>
<tr>
<th>Position in the ASMT gene</th>
<th>Genetic variation</th>
<th>Position in the pseudo-autosomal region 1</th>
<th>Predicted protein change</th>
<th>Allele frequency in cases</th>
<th>Allele frequency in controls</th>
<th>PolyPhen-2 prediction</th>
<th>ASMT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>AT/−</td>
<td>1694130</td>
<td>N13fsX22</td>
<td>1/690</td>
<td>0/440</td>
<td>−</td>
<td>Null</td>
</tr>
<tr>
<td>Exon 2</td>
<td>G/C</td>
<td>1702143</td>
<td>E61Q</td>
<td>0/690</td>
<td>1/440</td>
<td>Possibly damaging</td>
<td>Normal</td>
</tr>
<tr>
<td>Exon 3</td>
<td>G/A</td>
<td>1703249</td>
<td>R111K</td>
<td>1/690</td>
<td>0/440</td>
<td>Benign</td>
<td>NA</td>
</tr>
<tr>
<td>Exon 4</td>
<td>T/C</td>
<td>1708834</td>
<td>IVSS + 2T&gt;C</td>
<td>1/690</td>
<td>0/440</td>
<td>−</td>
<td>Null</td>
</tr>
<tr>
<td>Exon 7</td>
<td>A/G</td>
<td>1712136</td>
<td>K219R</td>
<td>0/690</td>
<td>1/440</td>
<td>Benign</td>
<td>NA</td>
</tr>
<tr>
<td>Exon 8</td>
<td>C/T</td>
<td>1715355</td>
<td>P243L</td>
<td>0/690</td>
<td>0/440</td>
<td>Probably damaging</td>
<td>Null</td>
</tr>
<tr>
<td>Exon 9</td>
<td>G/A</td>
<td>1715445</td>
<td>C273S</td>
<td>0/690</td>
<td>1/440</td>
<td>Probably damaging</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, BLCL of subjects were not available for testing enzymatic activity.

*Based on Hg18.

Based on P46597.

Replication study

We sequenced the ASMT promoter region in 480 additional patients with BD and 672 healthy controls of German origin to perform a replication study. Although the same tendency was observed for the four SNPs of the promoter, a difference was detected only for rs4446909 ($P < 0.05$, OR = 1.22, 95% CI (1.00;1.48)]. A combined meta-analysis using 825 patients with BD and 944 controls identified two susceptibility variants [rs4446909, allele G, $P = 0.002$, OR = 1.28, 95% CI (1.09;1.49)]; rs5989681, allele G, $P = 0.008$, OR = 1.23, 95% CI (1.06;1.42)]. One risk haplotype [GGGC, $P = 0.02$, OR = 1.19, 95% CI (1.03;1.38)] and one protective haplotype [ACGC, $P = 0.0005$, OR = 0.76, 95% CI (0.65;0.89)] (Table 2).

mRNA level of ASMT in BLCL

The ASMT mRNA levels have been estimated in patients and control’s BLCL using quantitative RT–PCR analysis. Two housekeeping genes, ACTB and GAPDH, were used as endogenous controls to perform relative quantification of these levels. A strong correlation was observed between the $2^{−\Delta\Delta CT}$ values of ACTB and GAPDH (Spearman’s correlation $\rho = 0.88$, $P < 10^{-4}$). No difference in ASMT mRNA levels was observed between bipolar patients and controls (Mann–Whitney U-test, $P_{GAPDH} = 0.28$, $P_{ACTB} = 0.26$). When considering genotyping status for the most-associated SNP, rs4446909, a significant lower mRNA level was observed for the G/G genotype (Kruskal–Wallis rank-sum test, $P_{GAPDH} < 10^{-4}$, $P_{ACTB} < 10^{-4}$; Fig. 1), which was more frequently observed in patients with BD when compared with controls. These results are consistent with those previously reported in patients with recurrent depression (27).

ASMT activity in BLCL of patients

In order to determine how the rare and common variants identified in patients might affect melatonin biosynthesis, we measured ASMT activity in families of bipolar patients carrying mutations and showed a significant decrease (Mann–Whitney U-test, $P = 0.01$) between carriers and non-carriers (Fig. 2, Supplementary Material, Fig. S1). In addition, we measured ASMT activity in BLCL of 41 patients with BD (not carrying rare variations) and 18 controls and showed a significant decreased activity (Mann–Whitney U-test, $P = 0.001$) in patients (Fig. 2). We assumed that more frequent variants in regulatory sequence of ASMT or in ASMT regulating genes might influence the expression level of ASMT and thus decrease the ASMT enzymatic activity. Individuals homozygous G/G for rs4446909, for which a significant lower expression level was observed for ASMT, showed a lower ASMT activity compared with those carrying A/A or A/G genotypes (Mann–Whitney U-test, $P < 0.05$; Fig. 1B). However, this vulnerability genotype explained only 7% of the general decrease in ASMT activity observed in patients with BD, suggesting that additional variants in ASMT or in other genes of the melatonin biosynthesis should be explored.

DISCUSSION

In this study, we report for the first time convergent genetic and functional data suggesting that deleterious variants of ASMT may act as susceptibility factor for BD. Rare ASMT variants, leading to amino acid changes or to truncated protein, were identified in >2% of patients with BD and 1% of unaffected controls. No enrichment could be detected, but carrier individuals showed a lower ASMT enzymatic activity compared with non-carriers. Such decrease in ASMT activity was also observed among patients with BD who did not carry rare variations when compared with controls, suggesting that unidentified regulatory variations might also influence this activity. We found one frequent polymorphism located in the ASMT promoter, rs4446909, which was significantly associated with BD, a lower mRNA level of ASMT and a lower enzymatic activity, partly explaining the general decrease in ASMT activity that we observed in patients. A replication study on an independent sample of patients and controls of German origin as well as a meta-analysis on the combined sample confirmed the association observed for rs4446909 with BD. It also revealed two risk haplotypes and one
protective haplotype. These findings suggest that ASMT alterations might explain the modification in melatonin secretion observed in patients with BD, in terms of amount and/or of sensibility to light/dark cycle (18–23,34).

Patients with BD exhibit multiple abnormalities in circadian rhythms including sleep–wake irregularities (6,13,14), abnormal actimetric parameters (35,36) and circadian preference for evening (2–4). These might be partly induced by an abnormal melatonin secretion (in terms of phase, amplitude and delayed onset). This abnormal clock setting may lead to inability to adapt circadian rhythms to social environment, such as seasons, stress levels, sleep schedules or jet-lag, which are strong triggers of mood episodes in patients with BD (37).

In addition, low melatonin might also alter brain functions by modulating synaptic plasticity, hippocampal long-term potentiation, antioxidative action and immunomodulatory functions (16). One limit of our study is the lack of data on the melatonin level and the circadian/sleep pattern of the patients. A low ASMT activity observed in patients might be associated with high serotonin and N-acetylserotonin concentrations, as well as with a low melatonin level. This is a difficult phenotyping project, but in the future, patients with BD should receive extensive clinical characterization of their sleep patterns, actimetric characteristics, circadian preference and serotonin and melatonin salivary or plasmatic levels in order to further explore phenotype–genotype correlation.

The potential therapeutical implications of these results deserve some comments. Several non-pharmacological treatments seem to be effective in BD, such as sleep deprivation, interpersonal and social rhythm therapy (38) or light therapy. These clinical interventions, termed chronotherapeutics, are based on controlled exposures to environmental stimuli that act on biological rhythms and are mainly designed for bipolar patients during depressive states. Moreover, the efficacy of specific melatonin-focused therapies, such as melatonin or agomelatine (a potent agonist at melatonin receptors), although not fully explored yet in BD, are encouraging (39). It remains to determine whether these treatments might be useful during stabilization phases to provide better outcomes in BD.

In this line, our results provide interesting information to identify patients with BD who might be good responders to chronotherapeutics.

<table>
<thead>
<tr>
<th>Table 2. Allele and haplotype association studies between patients with BD and controls for the four SNPs located in the ASMT promoter region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNPs</strong></td>
</tr>
<tr>
<td>rs4446909</td>
</tr>
<tr>
<td>P-value (P&lt;sub&gt;c&lt;/sub&gt;)</td>
</tr>
<tr>
<td>OR [95% CI]</td>
</tr>
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</table>

rs5989681                                    | **f(G)** | 0.68 | 0.62 | 0.73 | 0.70 | **0.008** |
| P-value (P<sub>c</sub>) | **0.05 (0.13)** | 1.48 [1.03–2.13] | 1.08 [0.79–1.46] | 1.23 [0.98–1.55], Q = 0.18 |
| OR [95% CI]                                  | 0.03 (0.20) | 0.09 | 0.10 | 0.09 | 0.17 |

rs56690322                                   | **f(A)** | 0.13 | 0.10 | 0.10 | 0.10 | 0.06 |
| P-value (P<sub>c</sub>) | **0.13 (0.20)** | 0.63 [1.00] | 0.63 [1.00] | 0.63 [1.00] |
| OR [95% CI]                                  | 0.03 (0.20) | 0.09 | 0.09 | 0.09 | 0.09 |

rs6446635                                    | **f(T)** | 0.37 | 0.36 | 0.39 | 0.39 | 0.96 |
| P-value (P<sub>c</sub>) | **0.06 (0.13)** | 1.06 [0.84–1.33] | 0.96 [0.80–1.15] | 1.00 [0.87–1.15], Q = 0.53 |
| OR [95% CI]                                  | 0.04 (0.13) | 0.09 | 0.09 | 0.09 | 0.09 |

Haplotypes                                        |          |      |      |      |      |      |
| **GGGC**                                      | 0.29     | 0.25 | 0.33 | 0.29 | 0.02 |
| P-value (P<sub>perm</sub>)                   | **0.11 (0.46)** | 1.23 [0.95–1.59] | 1.17 [0.97–1.41] | 1.19 [1.03–1.38] |
| OR [95% CI]                                  | 0.10 (0.39) | 0.27 | 0.30 | 0.30 | 0.56 |
| 0.33 (0.85) | 1.00 [1.00] | 0.00 [1.00] | 0.00 [1.00] | 0.00 [1.00] |
| OR [95% CI]                                  | 0.88 [0.68–1.14] | 0.32 | 0.22 | 0.22 | 0.96 [0.82–1.11] |
| **GGGT**                                      | 0.25     | 0.25 | 0.22 | 0.22 | 0.96 |
| P-value (P<sub>perm</sub>)                   | **0.007 (0.03)** | 0.71 [0.55–0.91] | 0.71 [0.64–0.97] | 0.76 [0.65–0.89] |
| OR [95% CI]                                  | 0.71 [0.55–0.91] | 0.09 | 0.08 | 0.08 | 0.54 |
| 0.33 (0.85) | 0.09 (0.10) | 0.09 (0.10) | 0.09 (0.10) | 0.09 (0.10) |
| OR [95% CI]                                  | 1.43 [0.98–2.09] | 0.05 | 0.05 | 0.05 | 1.08 [0.85–1.37] |
| **ACGC**                                      | 0.25     | 0.25 | 0.22 | 0.22 | 0.96 |
| P-value (P<sub>perm</sub>)                   | **0.007 (0.03)** | 1.24 [0.77–1.99] | 1.50 [0.98–2.30] | 1.37 [1.00–1.89] |
| OR [95% CI]                                  | 1.24 [0.77–1.99] | 0.06 | 0.06 | 0.06 | 1.37 [1.00–1.89] |

**BD**, bipolar disorder; OR, odds ratio; CI, confidence interval; f(N), frequency of the N allele.
P-values < 0.05 are indicated in bold; P<sub>c</sub> indicates corrected P-values after Bonferroni correction; P<sub>perm</sub> indicates corrected empirical P-values after 10<sup>6</sup> permutations.

Q, P-value for Cochrane’s Q statistic for heterogeneity.
Finally, our results converge with those associating ASMT with recurrent depressive disorders (27,29) and ASD (28,30,40), for which the same risk ASMT alleles and haplotype were identified. Recurrent depressive disorders and BD are well-known overlapping disorders with probable shared genetic susceptibility factors. Although the overlap between ASD and BD remains anecdotic (41), these disorders are characterized by a high level of circadian and sleep/wake perturbations (42,43), those being possibly related to shared anomalies in the melatonin pathway. Sleep disturbances and abnormalities of circadian rhythms are increasingly recognized as an important mechanism in the complex and multifactorial causation of the symptoms associated with psychiatric disorders and have been proposed to be transdiagnostic (44). This hypothesis requires further investigation.

Our results and those reported in depression and ASD strongly suggest that the melatonin biosynthesis might play a significant role in susceptibility to psychiatric disorders. Nevertheless, further studies are required to understand the impact of a melatonin/clock defect on human behaviour and to determine how this intermediate clock related phenotypes might orientate therapeutic strategies.

MATERIALS AND METHODS

Subjects

Protocols and procedures were approved by local ethics committees and written informed consent was obtained from all subjects prior to study participation.

Discovery sample

Three hundred and forty-five euthymic patients fulfilling DSM-IV criteria (45) for BD type I or II (162 males and 197 females) were included into three French university-affiliated psychiatry departments (Paris-Crétel, Bordeaux and Nancy) as described elsewhere (46). Two hundred and twenty controls (126 males and 94 females) without personal or first-degree relatives’ history of affective disorders or suicidal behaviours were recruited among blood donors at the Pitié-Salpêtrière and Henri Mondor Hospitals (France). All patients and controls were of French descent, with at least three grandparents from mainland France. Fifty-two (25 males and 27 females) additional healthy French controls, for whom few DNA was available, were sequenced only for the promoter region and used for association studies in order to increase statistical power.

Replication sample

All patients received a DSM-IV diagnosis of BD. Four hundred and eighty patients (230 males and 250 females) were selected from a larger sample from Germany as previously described (47). Six hundred and seventy-two German controls (329 males and 343 females) were drawn from a pool of controls as described elsewhere (48). Controls were screened to exclude a diagnosis of BD. Ancestry was assigned to patients and controls on the basis of self-reported ancestry.

Mutation screening and genotyping

Genomic DNA was isolated from blood lymphocytes or BLCL from independent cases and controls. In the discovery sample, we sequenced the open reading frame, the 5'- and 3'-untranslated regions and 300 bp upstream of the first coding exon of the ASMT gene. The nomenclature of the ASMT variations was based on the functionally active isoform ASMT P46597 and thus led to a change in amino acid numbering when compared with previous articles (30,33). Primers and PCR conditions have been described in details elsewhere (30) and are available on request. The sequence of the ASMT gene was analysed by direct sequencing of the PCR products, using the BigDye® terminator v3.1 cycle sequencing kit and a 16-Capillary ABI PRISM® 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). All mutations
PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Common threshold fluorescence for all samples was set into the exponential phase of the amplification and determined the Ct, corresponding to the number of amplification cycles needed to reach this threshold. All reactions were performed in triplicate and the mean value of Ct was used for subsequent analysis. Relative gene expression quantification was performed using the 2−ΔΔCt method (49). cDNAs from all controls were pooled and used as calibrator. ASMT activity in BLCL was estimated from ~1 million cells according to the previously described method (50). Briefly after incubation in RPMI-1640 culture medium containing 10 μM [3H]-5-HT (5-[1,2-3H(n)]hydroxytryptamine, NET 498), the medium and BLCL pellets were adjusted to pH 10 with sodium borate buffer and the radioactivity was extracted with chloroform. The alkaline-chloroform extracts were pooled, evaporated to dryness and subjected to HPLC as described (50). The radioactivity corresponding to the melatonin peak was then measured. Further proof of peak identity was obtained by mass spectrometry as described (50).

Statistical analyses

Statistical testing for allelic and haplotypic associations was carried out using the PLINK v1.07 software (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml) (51). Statistical analyses of the expression level have been performed using the statistical software package R v2.9.2 (http://cran.r-project.org). Group-wise comparisons included parametric tests (Student’s t-test/ANOVA) and non-parametric tests (Mann–Whitney U-test/Kruskal–Wallis test), according to normality of distribution, tested using the Shapiro–Wilk method.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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