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An ASMT variant associated with bipolar disorder influences sleep and circadian rhythms: a pilot study

Running title: ASMT gene and actigraphy

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ABSTRACT

Patients with bipolar disorder (BD) experience persistent circadian rhythm and sleep abnormalities during periods of remission, and biological studies have shown that these patients have abnormal melatonin secretion profiles or reactivity to light. We previously reported the association with BD of a common polymorphism (rs4446909) of the promoter of the acetylserotonin O-methyltransferase (*ASMT*) gene, encoding one of the two enzymes involved in melatonin biosynthesis. This variant was associated with weaker transcription and lower levels of *ASMT* activity in lymphoblastoid cell lines. Actigraphy, based on the use of a mobile portable device for the analysis of sleep/wake cycles in natural conditions, may be useful for studies of carriers of the at-risk allele. We studied the association between the *ASMT* rs4446909 variant and sleep/activity, as assessed with the Pittsburgh Sleep Quality Index (PSQI) and by actigraphy, in 53 subjects (25 patients with BD in remission and 28 healthy controls). The two groups were similar for age, sex ratio, current mood symptoms, body mass index and risk of sleep apnea syndrome. In the total sample, the GG at-risk genotype was associated with longer sleep duration ($p=0.03$), greater activity in active periods of sleep ($p=0.015$) and greater inter-day stability ($p=0.003$). These associations remained significant when disease status was included in the model. Only the association with inter-day stability remained significant after correction for multiple testing. This pilot study thus shows that a BD-associated functional variant involved in the melatonin synthesis pathway influences sleep and circadian rhythms in bipolar patients in remission and controls.

Introduction

Bipolar disorder (BD) is a severe psychiatric disorder characterized by alternating periods of elevated mood (manic or hypomanic episodes) and depression, interspaced by periods of euthymia. BD affects 1 to 4% of the population worldwide, and has a complex and multifactorial etiology, involving genetic and environmental risk factors (Leboyer & Kupfer, 2010; Geoffroy *et al.*, 2013). Circadian phenotypes and circadian genes have been widely studied in BD, and there is evidence for a deregulation of the circadian rhythm and abnormalities of numerous circadian biomarkers in patients, during both acute episodes and remission (Etain *et al.*, 2011).

BD patients in clinical remission differ from healthy controls in terms of sleep/wake cycles, including phase advance, percentage of nighttime sleep and daytime activity level (McClung, 2007). Actigraphic assessments have confirmed that patients in remission have more sleep abnormalities, spend more time in bed and have a higher frequency of waking shortly after sleep onset than controls, with a lower sleep efficiency and greater variability of rhythms (Millar *et al.*, 2004; Jones *et al.*, 2005; Harvey *et al.*, 2005; Salvatore *et al.*, 2008; Mullin *et al.*, 2011). Biological studies have also revealed numerous abnormalities of circadian rhythms in BD patients: core temperature, blood pressure, plasma cortisol, norepinephrine and TSH (thyroid-stimulating hormone) concentrations and the rhythmicity of hypothalamic-pituitary axis secretion are all affected (Milhiet *et al.*, 2011).

Melatonin is a neurohormone synthesized by the pineal gland from tryptophan, via serotonin. Its secretion is synchronized by day/night cycles, and it has both sleep-promoting and circadian rhythm-regulating activities (Pandi-Perumal *et al.*, 2006). Patients with BD are supersensitive to melatonin suppression by light (Hallam *et al.*, 2006; McIntyre *et al.*, 1989;

Nurnberger *et al.*, 1988). This characteristic has been identified as a possible biomarker “trait” of BD, independent of the subject's mood states, strongly heritable and increasing with the genetic load in a family (Hallam *et al.*, 2006; Pacchierotti *et al.*, 2001).

We have recently demonstrated an association between BD and genetic variants of the acetylserotonin O-methyltransferase gene (*ASMT*, OMIM 300015/402500) encoding the enzyme responsible for the final step of melatonin biosynthesis (Etain *et al.*, 2012). We first identified several rare deleterious *ASMT* mutations in patients, and showed that these mutations were associated with low levels of *ASMT* activity in B-lymphoblastoid cell lines (BLCLs). We then showed that the level of *ASMT* activity was generally lower in patient than in control BLCL. We showed that rs4446909, a common variant of the *ASMT* promoter region, was significantly associated with BD, and with lower transcript abundance and lower levels of enzymatic activity, providing a partial explanation for the lower than normal levels of *ASMT* activity in patients (Etain *et al.*, 2012).

We report here further characterization of the phenotypic expression of the rs4446909 susceptibility allele. We studied sleep/activity patterns for patients with BD and healthy controls, on the basis of actigraphy and sleep quality self-reports.

Materials and methods

Population

Fifty-three Caucasian adult subjects (25 euthymic patients with BD in remission and 28 healthy subjects) were included in the study at our university-affiliated psychiatric department

(Paris Est University). Written informed consent was obtained from all participants. Institutional review board approval was obtained for this study.

For inclusion in the study, patients had to be (i) diagnosed with bipolar disorder (type I, II or NOS), according to DSM-IV criteria (American Psychiatric Association, 2000), (ii) euthymic, with both a Montgomery–Asberg Depression Rating Scale (MADRS) score (Montgomery & Asberg, 1979) and a Young Manic rating scale (YMRS) (Young *et al.*, 1978) of no more than seven (according to ISBD task force criteria for symptomatic remission (Tohen *et al.*, 2009)) and (iii) in remission, defined as the absence of mood episodes during a period three months immediately preceding participation in the study. Patients were interviewed with the French version of the Diagnostic Interview for Genetic Studies (DIGS) (Nurnberger *et al.*, 1994), which provides lifetime DSM-IV axis I diagnoses.

The unaffected control group was recruited from blood donors attending Henri Mondor Hospital (a general hospital in Paris, France). Unaffected controls were assessed with the DIGS and assessed for family history of psychiatric disorders with the Family Interview for Genetic Studies (FIGS) (Maxwell, 1992). Only subjects with no personal or family history of affective disorders or suicide attempts were included.

Patients and controls were excluded if they presented any of the following during the three months preceding the study: i) severe insomnia, ii) psychostimulant drug use, iii) psychotropic treatment modification or initiation, iv) hospitalization, v) electro-convulsive therapy, or vi) any life event likely to have an effect on sleep patterns (including, for example, shift work, travel with more than three hours of jet lag, pregnancy, child birth, grief, trauma, or somatic disease with associated sleep disturbances).

Assessment procedures

All participants were assessed for a period of 21 consecutive days, with a subjective measure of sleep, the Pittsburgh Sleep Quality Index (PSQI) (Buysse *et al.*, 1989), and an objective measure of sleep, actigraphy.

We used the total score of the PSQI, which is a 19-item self-completed questionnaire requiring the participant to describe patterns of sleep, such as typical bedtime and wake time, sleep latency and actual sleep time (Buysse *et al.*, 1989). The patient is also asked about sleeping habits and quality. The French version of this questionnaire has been validated (Blais *et al.*, 1997).

The American Academy of Sleep Medicine considers actigraphy to be the most relevant non-invasive method for assessing sleep/wake irregularities (Morgenthaler *et al.*, 2007). All participants continuously wore an actigraph (AW-7 CamNtech) on the wrist of the non-dominant hand, making it possible to collect at least 21 consecutive cycles, with each cycle corresponding to 24 hours. The AW-7 actigraph consists of an accelerometer that detects the intensity and amount of movement as a function of time. For this study, a time of 1 minute per epoch and an “average” sensitivity threshold were chosen by default. Instructions were kept to a minimum, to try to guarantee that the study was naturalistic and did not intrude on routine sleep/activity measurements. Participants were instructed to press the actiwatch event marker when they went to bed at night (to go to sleep) and again when they got up (to start the next day); they were also asked to complete a sleep diary. In addition, as this study focused on routine sleep-wake cycles, we avoided the recording of data when participants were on vacation or involved in unusual periods of activity.

Two experienced psychiatrists (CB and PAG) analyzed the actigraphic records of the two groups. Markers reported by the patient by the pressing of the actigraph button at bedtime and on getting up were used for signal analysis. The sleep diary completed by the participant for the duration of the recording period was used to correct missing and/or outlier markers. Subsequently, the automatic algorithm program (Actiwatch Activity & Sleep Analysis Ltd CamNtech 7.28) was applied to all records, to calculate the various actimetric measurements of sleep and activity. Finally, visual inspection by a trained observer (PAG or CB) was used to correct incongruences between the definition, by the algorithm, of a rest interval and the schedules provided by the participant's marker recordings and the sleep diary (Boudebesse *et al.*, 2012).

Genotyping

Genomic DNA was isolated from blood lymphocytes or B-lymphoblastoid cell lines from independent cases and controls, with the Nucleon BACC3 kit (GE HealthCare, Chalfont St Giles, UK). The *ASMT* promoter, containing rs4446909, was amplified by polymerase chain reaction (PCR) methods, as previously described (Etain *et al.*, 2012). The sequence of the *ASMT* promoter was analyzed by direct sequencing of the PCR products, with the BigDye® Terminator v3.1 cycle sequencing kit and a 16-capillary ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistics

All analyses were performed with SPSS 19.0.

Non-parametric tests (the Mann-Whitney test for continuous variables and the Chi² test for categorical variables) were used to compare patients and controls for potential confounding

factors, such as sex, age, mood state (MADRS, YMRS), body mass index (BMI) and risk of sleep apnea syndrome (SAS) (assessed with the Berlin Questionnaire (Netzer *et al.*, 1999)).

We explored the association between the rs4446909 genotypes and actigraphic measures, by pooling patients and control subjects. Individuals with the AA and AG genotypes were pooled into a single category and compared with those with the GG genotype (recessive model for the G, at-risk allele). There were two reasons for this approach. First, the AA genotype is infrequent and our sample size was such that it could not be analyzed as a single category in this study. Second, the GG genotype is associated with lower than normal levels of ASMT enzymatic activity, consistent with G being a recessive allele (Etain *et al.*, 2012). Hardy–Weinberg equilibrium was assessed for rs4446909 in our population.

A one-way between-subjects analysis of covariance (ANCOVA) was used to assess the effect of genotype on dependent actigraphic measures, with bipolar/healthy status used as a covariate. The homogeneity of regression and the linear relationship between covariates and the dependent variable were tested and confirmed. We decreased the number of statistical tests required, by focusing on eight candidate actigraphic measurements: four for sleep (sleep duration, sleep latency, sleep efficiency, mean activity in active periods), two for phase (L5 onset, M10 onset), one for stability (inter-day stability) and one for amplitude (amplitude). Other actigraphic measures (time in bed, relative amplitude, intra-day variability, WASO, fragmentation index) were not investigated, as they were strongly correlated with the selected measurements. Assuming that eight independent tests were carried out, we applied Bonferroni correction for multiple testing ($0.05/8 = 0.00625$).

Results

Fifty-three subjects were included in the study: 25 patients with BD in remission, and 28 unaffected individuals.

The two groups were similar in terms of age, sex-ratio, YMRS score, BMI and SAS risk (table 1). MADRS scores differed significantly between patients and controls ($p=0.009$), but the means were very low for both groups, corresponding to a clinical absence of depressive symptoms. We therefore included no covariates in the models.

The GG genotype was found in 11 of the 25 subjects with BD and 12 of the 28 healthy subjects (total $n=23$). The AA genotype was found in 4 of the 25 subjects with BD and 2 of the 28 healthy subjects, whereas the AG genotype was found in 10 of the 25 subjects with BD and 14 of the 28 healthy subjects (giving a total n for these two genotypes of 30: 14 subjects with BD and 16 healthy subjects). The rs4446909 genotype distribution was in Hardy–Weinberg equilibrium ($p=0.94$) and did not differ between the two groups ($p_{\text{exact}}=0.64$).

No association was observed between rs4446909 genotype and sleep quality, as measured by PSQI total score ($U=378$, $p=0.37$).

For the pooled samples of patients and controls, univariate analyses showed an association between the GG at-risk genotype and each of the following variables: longer sleep duration ($p=0.03$), greater activity during active periods of the night ($p=0.015$) and a higher inter-day stability ($p=0.003$). Sleep latency, sleep efficiency, L5 onset, M10 onset and amplitude did not differ between the rs4446909 genotype groups (table 2). Only the association with inter-day stability remained significant after correction for multiple testing.

Multivariate analyses including disease status (first column) as a covariate confirmed the association between the GG genotype and the three actigraphic measurements (table 2). The disease model (second column of multivariate analyses) showed a main effect of disease status (BD case versus control) on the actigraphic variables. These results confirm that, for two of the three actigraphic measurements (for sleep duration and inter-day stability, but not for mean activity in active periods) the observed effect of the *ASMT* gene variant on the actigraphic variables is not associated with disease status (table 2).

Discussion

This is the first study investigating the relationship between a functional variant involved in the melatonin synthesis pathway and sleep/wake patterns in a sample of bipolar patients in remission and healthy controls. The GG at-risk genotype at rs4446909 in the promoter of the *ASMT* gene is associated with susceptibility to BD and a lower than level of enzymatic activity for the corresponding protein (Etain *et al.*, 2012). We show here that this genotype is also associated with a longer sleep duration and higher levels of activity during active periods of sleep, both consistent with a worse-than-normal objective sleep quality. These results are consistent with the previous finding that these circadian characteristics are associated with BD (McClung, 2007; Harvey *et al.*, 2005; Millar *et al.*, 2004). Longer periods of poorer quality sleep in BD patients have been repeatedly reported in actigraphic studies (Jones *et al.*, 2005; Millar *et al.*, 2004; Mullin *et al.*, 2011; Harvey *et al.*, 2005; Salvatore *et al.*, 2008). *ASMT* may regulate the amplitude of melatonin secretion (Etain *et al.*, 2012) and, consequently, a genetic variant associated with a lower level of enzyme activity may influence actigraphic sleep amplitude-related variables, causing longer sleep duration and shallower sleep.

Melatonin is a synchronizer with several systemic functions, including promoting sleep and its quality by causing drowsiness and lowering body temperature (Srinivasan *et al.*, 2006). The association between the GG at-risk genotype for BD and better inter-day stability was therefore the opposite of what would have been expected. Indeed, inter-day stability in BD patients in remission has been shown in previous studies to be lower than normal, with, for example, the Social Rhythm Metric (Ashman *et al.*, 1999; Jones *et al.*, 2005) and actigraphic studies (Millar *et al.*, 2004; Salvatore *et al.*, 2008; Jones *et al.*, 2005). Our findings suggest that rs4446909 does not itself cause rhythm irregularities. Other variants of this gene or of other genes may be involved in these irregularities (Relógio *et al.*, 2011; Hastings *et al.*, 2007). The phase of melatonin secretion is regulated more strongly by aralkylamine N-acetyltransferase (AANAT) than by ASMT and the stability of circadian rhythms appears to involve the ROR/Bmal/REV-ERB (RBR) loop. Consequently, genes encoding AANAT or the RBR loop may contribute to inter-day stability and instability. Investigations of the entire melatonin pathway and of other circadian genes may explain these preliminary results and disentangle the circadian physiology responsible for the instability of the sleep/wake cycle in BD patients.

In our sample, the common variant of the *ASMT* promoter associated with BD was not associated with subjective sleep quality. This absence of association may reflect the small sample size or the commonly observed discrepancy between subjective assessments and objective actigraphic sleep measurements in patients with BD (Harvey *et al.*, 2005). In the study by Harvey *et al.*, euthymic patients overestimated their sleep latency and underestimated their sleep duration (Harvey *et al.*, 2005). Observations are similar for patients with insomnia, who misperceive their sleep characteristics, by overestimating waking during sleep, for example (Mercer *et al.*, 2002).

The inclusion of both euthymic BD patients and control subjects in the study group is one of the strengths of our study, also revealing an influence of the variant studied on actigraphic measurements in the general population. Of particular interest, this variant is associated with both the disorder (Etain *et al.*, 2012) and with actigraphic markers that appear to be trait markers of BD (Milhiet *et al.*, 2011).

This study has several limitations. First, the sample was small (53 subjects), although of a reasonable size for an actigraphic study (Millar *et al.*, 2004; Jones *et al.*, 2005; Harvey *et al.*, 2005; Salvatore *et al.*, 2008; Mullin *et al.*, 2011). A larger sample would increase the power for detecting small effects and should probably be recommended for future studies combining actigraphic and genetic data. However, this study was not based on genetic and phenotypic screening (which would have increased the risk of type I errors). Our strategy was hypothesis-driven and based on a specific aim. The ASMT promoter gene variant is a good candidate for association with circadian phenotypes, hence the design of this study. This so-called “reverse phenotyping” strategy is a recognized and increasingly common approach to evaluating the biological significance of signals obtained in genetic association studies (Arif *et al.*, 2013). We also carried out a *post-hoc* calculation of power. The multivariate analyses demonstrated adequate power in all instances (e.g. 78% power to detect between-group differences for activity (for genes and disease status, controlling for age and sex), with an effect size of 0.36 for the model). Both the power and effect sizes are estimated *post-hoc*, but we consider them to be reasonable and they suggest that our results are reliable and potentially valid. As the primary goal of our work was to demonstrate a feasible new method for assessing the associations between sleep parameters and circadian genes, we feel that this study constitutes an important first step in this direction.

We did not control for psychotropic medication or for professional status, both of which are possible confounding factors, in this study. However, the type and dose of psychotropic medication were previously found to be unrelated to actigraphic measurements in similar patients with BD in remission (Salvatore *et al.*, 2008). In addition, the associations between rs4446909 and actigraphic measurements remained significant in the multivariate analyses including disease status as a covariable. We therefore think that it is unlikely that psychotropic drugs or professional status would have a major influence.

Finally, as supersensitivity to melatonin suppression by light has been demonstrated only for patients with BD and patients with seasonal affective disorder (SAD), but not in patients with major depressive disorder (MDD), it might be advisable for future studies to include only patients with BD subtype I or at least with seasonal patterns of mood episodes (McIntyre *et al.*, 1989; Nathan *et al.*, 1999).

If confirmed, these results have various implications. Theoretically, sleep disturbances in patients with BD in remission could be treated by manipulating the circadian system with chronobiotic drugs (*e.g.* melatonin), chronotherapeutics (*e.g.* bright light therapy or sleep deprivation) or psychological interventions focusing on rhythms and sleep (Livianos *et al.*, 2012; Pacchierotti *et al.*, 2001; Wu *et al.*, 2009; Benedetti *et al.*, 2005a; St-Amand *et al.*, 2012). Patients carrying this at-risk genotype might show different patterns of therapeutic response to treatments of this type acting directly on the circadian system or, more generally, to treatments involving mood stabilizers. Indeed, it has been suggested that circadian genes may modulate the therapeutic response to mood stabilizers (Benedetti *et al.*, 2004, 2005b). These findings contribute to our understanding of the circadian pathophysiology of BD and its

complex multifactorial heritability. They also indicate a new area for research to identify treatment targets.

Conclusion

We report here, for a sample of patients with BD in remission and healthy subjects, an association between a BD susceptibility allele (*ASMT* rs4446909) and sleep/activity patterns, as assessed by actigraphy. This reverse phenotyping approach helps shed light on the mechanisms associated with sleep disturbances and circadian rhythm instability, both frequent in patients with BD in remission. These findings also suggest new avenues of research for elucidating the complex interactions between the biology of circadian rhythms and susceptibility to BD. Our preliminary results require confirmation in independent samples of patients with BD and of patients with other disorders, such as recurrent depression or insomnia.

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Conflicts of interest

PA Geoffroy received a prize from Bayer whilst at the Faculty of Medicine of Lille.

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A. Henrion and S. Jamain have no conflict of interest to declare.

Authors' contributions

Dr PA Geoffroy, Dr C Boudebese, Ms A Henrion, Dr S Jamain, Prof. C Henry, Prof. M Leboyer, Prof. F Bellivier and Dr B Etain contributed to and have approved the submitted draft of the paper. FB & BE are the principal investigators of the study. PAG, CB, FB & BE designed the study. PAG & CB assessed the bipolar and control subjects. PAG & CB performed the actigraphy analyses. AH & SJ performed the genotyping. PAG & BE performed the statistical analyses. PAG, CB, AH, SJ, CH, ML, FB & BE contributed to interpreting the results and writing the manuscript, and have approved its final version.

Table 1: Description of the samples of patients with BD and unaffected controls

	Patients with BD (n=25) n (%) or mean (±SD)	Controls (n=28) n (%) or mean (±SD)	Chi² or Mann Whitney	p
Sex: women / men	17 (68%) / 8 (32%)	12 (43%) / 16 (57%)	3.37	0.07
Age (years)	53.20 (±11.62)	53.93 (±9.23)	331	0.74
MADRS	1.92 (±2.83)	0.43 (±1.35)	475.5	0.009
YMRS	0.68 (±1.41)	0.14 (±0.45)	402.5	0.15
BMI	26.89 (±5.79)	26.55 (±4.22)	340.5	0.87
SAS Risk: High / Low	6 (24%) / 19 (76%)	2 (7%) / 26 (93%)	2.93	0.09

BMI= body mass index, MADRS= Montgomery–Asberg Depression Rating Scale, NS= not significant, SAS= sleep apnea syndrome, YMRS= Young Manic Rating Scale, BD= bipolar disorder.

Table 2:

Association between rs4446909 and actigraphic variables, as identified by univariate and multivariate (covariation with disease status) analyses

Actimetric measurements Mean (\pm SD)	UNIVARIATE ANALYSES				MULTIVARIATE ANALYSES (ANCOVA)	
	AA /AG genotype (n=30)	GG genotype (n=23)	U	<i>p</i>	Genotype <i>P</i> value (F)	Disease status* <i>P</i> value (F)
Mean activity in active periods	93.57 (\pm 29.3)	115.09 (\pm 36.3)	480	0.015	0.012 (6.725)	0.004 (9.371)
Sleep duration (min)	452.23 (\pm 53.3)	484.17 (\pm 65.3)	463	0.03	0.04 (4.262)	0.14 (2.334)
Inter-day stability	0.47 (\pm 0.12)	0.57 (\pm 0.12)	508	0.003*	0.007 (8.046)	0.77 (0.083)
Sleep latency (min)	22.47 (\pm 31.7)	12.30 (\pm 8.9)	279	0.24		
Sleep efficiency (%)	82.37 (\pm 9.9)	84.39 (\pm 5.9)	352 .5	0.89		
L5 onset (H:min)	00:40 (\pm 01:36)	00:49 (\pm 00:59)	321	0.65		
M10 onset (H:min)	08:50 (\pm 02:02)	08:18 (\pm 01:00)	298	0.39		
Amplitude	15253.13 (\pm 4393.1)	16424.83 (\pm 3949.2)	402	0.31		

*Main effect

** Significant after correction for multiple testing

Table S1: Genotype distribution in subjects with bipolar disorder (BD) and healthy controls

Gene (SNP)	Genotype	Controls (n=28)	Patients with BD (n=25)	Pooled genotypes	Total sample (n=53)
<i>ASMT</i> (rs4446909)	AA	2	4	AA and AG	30
	AG	14	10		
	GG	12	11	GG	23