

Human period-3 gene involvement in diurnal preference among Argentinean bipolar disorders patients

O envolvimento do gene humano período-3 na preferência diurna de uma população argentina de pacientes com transtornos bipolares

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ABSTRACT

Background and objective: Due to circadian disturbances observed among bipolar disorder (BD) patients, several studies have analyzed a possible link with genes involved in the molecular generation of biological rhythms. Some of these genes have been previously associated with diurnal preference (i.e., chronotype) in healthy individuals. In this study, we aimed to establish the influence of two genetic polymorphisms on chronotypes among two local populations of healthy subjects and individuals affected by bipolar disorders. **Methods:** The polymorphisms analyzed were a variable number of tandem repeats (4 or 5-repeat alleles; 4R or 5R) in exon 18 of *hper3*, and a T/C single nucleotide polymorphism in *clock*. Chronotypes of healthy individuals (n=39) and patients (n=37) were determined by the Horne and Ostberg Questionnaire, and the control population was divided into three groups according to results. **Results:** No difference in allele or genotype frequencies was detected between control and bipolar disorder populations. In controls, the 5R allele in *hper3* and the C allele in *clock* were sub-represented among evening-type individuals (p<0.05). Moreover, subjects homozygous for the 4R allele of *hper3* had significantly lower scores (evening preference) than 5R allele carriers among both controls (p<0.05) and patients (p<0.001). **Conclusions:** We have confirmed the strong correlation of *hper3* variable number of tandem repeats (VNTR) polymorphism with diurnal preference on a local population, even when not taking into account chronotype classification. This correlation was replicated with an even stronger robustness, on patients with bipolar disorders. These findings add to the growing body of evidence supporting an important role for *hper3* on human circadian physiology.

Keywords: Chronobiology disorders; Bipolar disorder; Polymorphism, genetic; Sleep/physiology; Phenotype; Minisatellite repeats

RESUMO

Introdução e objetivo: Devido a distúrbios circadianos observados entre pacientes portadores de transtornos bipolares (TBP), vários estudos têm se focado em uma possível associação genética na geração molecular de ritmos biológicos. Alguns genes já foram associados anteriormente à preferência diurna (por exemplo, o cronótipo) em indi-

víduos saudáveis. O enfoque neste estudo foi estabelecer a influência que dois polimorfismos genéticos exercem sob os cronótipos pertencentes a duas populações locais saudáveis e a indivíduos portadores de transtornos bipolares. **Métodos:** Os polimorfismos analisados consistiram em um número variado de repetições em pares (alelos de 4 ou 5 repetições; 4R ou 5R) em exon 18 do *hper3*, e um T/C polimorfismo de nucleotídeo simples em relógio. Os cronótipos de indivíduos saudáveis (n=39) e de pacientes com transtornos bipolares (n=37) foram determinados pelo questionário Home-Ostberg, e a população controle foi dividida em três grupos de acordo com os resultados colhidos. **Resultados:** Nenhuma diferença em alelos de frequência de genótipos foi encontrada entre os pacientes controles e as populações com transtornos bipolares. Nos controles, o alelo 5R no *hper3* e o alelo C no relógio foram representados como indivíduos noturnos (p<0,05). E indivíduos homocigóticos no alelo 4R do *hper3* tiveram pontuações mais baixas (preferência noturna) do que os alelos 5R entre os grupos controle (p<0,05) e indivíduos com transtornos bipolares (p<0,001). **Conclusões:** Confirmamos uma forte relação de polimorfismo do *hper3* VNTR com preferência diurna dentro da população local mesmo desconsiderando a classificação de cronótipos. Tal correlação foi reproduzida com mais robustez entre portadores de transtornos bipolares. Estas conclusões vêm ao encontro do que tem sido levantado em estudos anteriores, que indicam o papel importante que o *hper3* desempenha na fisiologia circadiana humana.

Descritores: Transtornos cronobiológicos; Transtorno bipolar; Polimorfismo genético; Sono/fisiologia; Fenótipo; Repetições minissatélites

INTRODUCTION

Circadian rhythm is a widespread property of living organisms. A number of circadian biological clocks and the genetic machinery involved in the generation and modulation of these rhythms have been extensively described and are still under active study. In mammals, the biological clock that controls circadian rhythms is located at the suprachiasmatic nuclei of the hypothalamus (SCN). The genes that

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govern circadian functioning within SCN neurons are well conserved among mammals, and include clock, *bmal1*, the *per* and *cry* families and a large array of complementary genes^(1,2). It has been demonstrated that mutations affecting these so-called “clock genes” originate circadian alterations in several animal models.

The sleep-activity cycle is the most evident circadian rhythm in humans, and a classification of phenotypes, known as chronotypes, according to diurnal preferences of sleep and activity timing has been proposed. Several polymorphisms in human clock genes have been shown to influence chronotype⁽⁵⁻⁶⁾, and some of them have also been linked to the appearance of sleep disorders like Familial Advanced Sleep Phase Syndrome (FASPS)^(7,8) or Delayed Sleep Phase Syndrome (DSPS)^(5,6,9), and more recently it has been shown that clock genes can also affect sleep architecture⁽¹⁰⁾.

The link between Bipolar Disorders (BD) and the circadian system has been studied for the last decades; indeed, several circadian-related issues have been reported, mainly sleep disorders, both before and after the onset of the illness⁽¹¹⁻¹⁵⁾. Another strong evidence of this link is that lithium, the main drug used in the treatment of BDs, has been shown to affect circadian rhythms in rodents and flies by inhibiting GSK3b, a kinase involved in the secondary loop of the molecular circadian clock^(16,17). Moreover, certain clock gene polymorphisms have been recently linked to diverse BDs features, as age of onset and certain treatment responses⁽¹⁸⁻²³⁾.

Since it has been suggested that photoperiod related to latitude may have an effect on circadian adaptation⁽⁶⁾, we considered it important to try to replicate previous findings related to the effect of clock genes polymorphisms on chronotypes on a local population of healthy individuals. At the same time, we also extended this research onto a BDs patient population in order to assess whether this reported genetic correlation could persist even under this circadian-disturbing psychiatric condition.

We chose two chronotype-related polymorphisms, a variable number of tandem repeats (VNTR) in exon 18 of *hper3* and a single nucleotide polymorphism (SNP) in the 3'UTR region of *clock*, which had been both previously reported to affect diurnal preference^(5,5,6). We analyzed their influence on the diurnal preference of an Argentinean group of healthy volunteers and a population of BD patients, comparing the frequencies of alleles and genotypes present among groups.

METHODS

Volunteers and patients

Subjects with BD ($n=37$; age 46.09 ± 14 years; 30% males) were consecutively selected from the outpatients popula-

tion of the Bipolar Disorder Program of the Favaloro Foundation (Buenos Aires, Argentina) with the following inclusion criteria: age between 18 and 75 years old; diagnosis of BDI or BDII according to Diagnostic and Statistical Manual of Mental Disorders – Fourth edition (DSM-IV) using Structured Clinical Interview for DSM-IV (SCID)⁽²⁴⁾; euthymic (defined by Hamilton Depression Rating Scale ≤ 8 and Young Mania Rating Scale ≤ 6) for at least 8 weeks. Exclusion criteria were: other diagnosis in axis I, antecedent history of substance abuse, history of mental retardation or neurological disease. A total of 53% of patients included in the study suffered from BDI, while the remaining 47% suffered from BDII. All patients signed a special informed consent providing information regarding study objectives, methodologies and secrecy of all personal data. All procedures regarding patients were approved by a local Ethics Committee before starting the study. Patients were on pharmacological treatment at the time of the study: 94.7% of patients were taking mood stabilizers, 18.4% antidepressants, 42.1% antipsychotics and 13.1% benzodiazepines.

A Control Group ($n=39$; age 28.5 ± 9 years; 25% males) was recruited among students at the National University of Quilmes, near Buenos Aires, Argentina (latitude $-58^{\circ}23'$). All of them signed an informed consent and were interviewed to control for psychiatric or sleep disorders before being analyzed. The Horne & Ostberg Morningness-Eveningness Questionnaire (HO)⁽²⁵⁾ was applied to all selected subjects and patients. The HO questionnaire gives a score of 16 to 86 points for each tested subject: low scores indicate evening preference and high scores indicate morning preference.

For the chronotype analysis among the control population, we selected the 9 lower scores in the HO test to conform the Evening-type Group, the 9 higher scores for the Morning-type Group, and the middle 10 scores for the Intermediate Group. Individuals with scores falling between these groups were discarded from chronotype analysis to avoid bias from border behavior, but they were later included in the morningness-eveningness distribution according to genotype analysis.

Genotyping

Fingertip, blood or mouth epithelium tissue samples were collected from healthy volunteers and BD patients and placed on Whatman FTA Cards (Whatman, UK), which were later processed for DNA extraction. Polymorphisms studied were a VNTR (4-repeat or 5-repeat possible alleles; 4R or 5R, respectively) in exon 18 of *hper3*, previously reported by Ebisawa et al.⁽⁹⁾, and a T/C SNP in the 3'UTR region of *clock* reported by Katzenberg et al.⁽³⁾, and PCR

primers and conditions were replicated. Determination of hper3 VNTR alleles was performed by settling the molecular weight of the PCR product on 2% agarose gels. The clock SNP genotypes were obtained by restriction fragment length polymorphism technique (RFLP) using Bsp1286i restriction enzyme (Promega, WI), which cleaves exclusively the C allele, leaving the T allele intact.

Statistical analysis

Differences in allele frequencies and genotype between groups were analyzed by two-tailed Fisher's Exact test (Graph Pad Software). Differences in HO scores between genotype groups in control volunteers and BD patients were analyzed by two-tailed Student's t test or Two-Way ANOVA using Graph Pad Prism software. Results are expressed as Mean \pm SEM.

RESULTS

Allele and genotype frequencies in healthy controls and bipolar disorders patients

No significant difference in age or gender distribution was detected between the control group and the population of patients. Table 1 shows the allele and genotype frequencies found in controls and in BD patients for both hper3 and clock polymorphisms. No significant differences were detected between groups for alleles or genotypes distributions, even when a slightly reversed trend in genotype frequencies, as determined by presence or absence of the less frequent allele for each gene (i.e. the 5R allele for hper3 and the C allele for clock), were appreciated.

Table 1: Allele and genotype frequencies in both Control and Patient Group

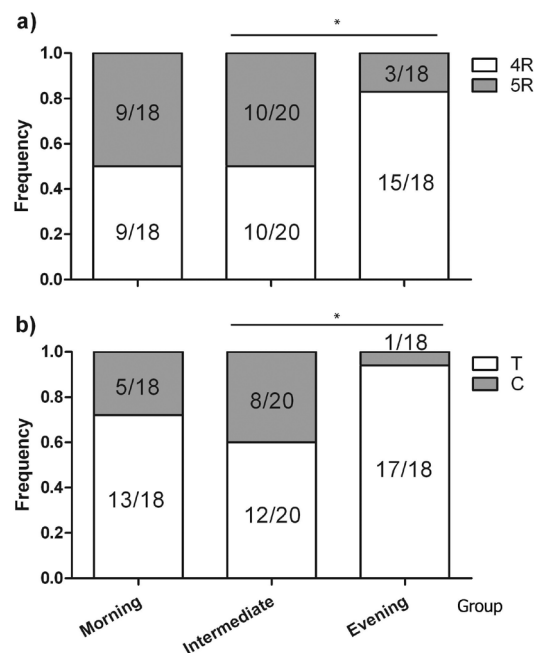
Group	n	Allele		p value	Genotype			p value
		4R	5R		4/4	4/5	5/5	
Control	39	0.63	0.37	0.29	0.41	0.44	0.15	0.16
		(49/78)	(29/78)		(16/39)	(17/39)	(6/39)	
BD	34*	0.72	0.28		0.59	0.26	0.25	
		(49/68)	(19/68)	(20/34)	(9/34)	(5/34)		
Clock								
Control	39	0.77	0.23	0.35	0.59	0.36	0.05	0.25
		(60/78)	(18/78)		(23/39)	(14/39)	(2/39)	
BD	36*	0.69	0.31		0.44	0.50	0.06	
		(50/72)	(22/72)	(16/36)	(18/36)	(2/36)		

Values in brackets represent absolute values (allele/genotype appearances over total present). Two-tailed Fisher's exact test p values are presented for both allele and genotype (as 5R or C allele carriers and non-carriers) frequencies.

*Not all BDs patients could be genotyped for both polymorphisms since we were unable to get a new biological sample after a failure in the genotyping process.

Allele frequencies among chronotypes

We found that both hper3 5R allele and clock C allele were significantly sub-represented in evening-types as compared to intermediates (two-tailed Fisher's exact test $p < 0.05$; Odds Ratio: 5.00; 95% confidence interval 1.095-22.828). No differences were detected between Morning and Intermediate groups (Figure 1).



Two-tailed Fisher's exact test, * $p < 0.05$; Odds Ratio: 5.00; 95% confidence interval 1.095-22.828; see text for details;

Numbers inside bars indicate specific allele appearances over total alleles present.

Morning-types, $n=9$, Intermediates, $n=10$ and Evening-types, $n=9$.

Figure 1: Comparison of allele frequencies of (A) hper3 4R/5R VNTR polymorphism and (B) clock T/C SNP among chronotype groups, as described in the Methods section. A clear significant sub-representation of the hper3 5-repeats and the clock C alleles in evening-types, as compared to intermediates, can be appreciated.

Morningness-eveningness scores according to genotype

We classified both control and BD populations into genotypes for each gene according to the presence or absence of the less frequent allele of each polymorphism and analyzed the mean HO score for each group. We found a significant effect of genotype of hper3 VNTR on mean scores. 5R allele carriers ($n=23$) had significantly higher scores, i.e. morning type scores, than non-carriers ($n=16$; 50.23 ± 1.69 versus 42.93 ± 2.78 ; $p < 0.05$) (Figure 2). No differences were found between clock C allele carriers and non-carriers (not shown). It should be noted that in this analysis no subjects are discarded as in the chronotype groups study.

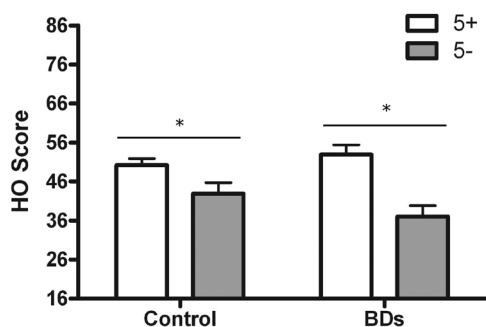


Figure 2: Effect of *hper3* genotype on Horne-Ostberg questionnaire score in control and BD groups. Mean HO score for both populations classified in genotypes according to the presence (5+ genotype) or absence (5- genotype) of the 5-repeat allele of *hper3*. Significantly higher scores (i.e. morning preference) are found in 5+ individuals, as compared to 5-subjects. A very significant (* $p < 0.0001$ for effect of genotype) effect of 5R allele presence on scores is detected, but no effect of health status and no interaction, as reported by a two-way ANOVA.

Among BD patients, we found an even stronger correlation of *hper3* genotype on mean HO scores. The 5R allele carriers ($n = 14$) had a mean score of 52.93 ± 2.46 , while non-carriers ($n = 20$) had a 37.00 ± 2.86 mean score ($p < 0.001$) (Figure 2). Again, no differences were detected regarding the presence of the clock C allele (not shown).

A two-way ANOVA revealed a significant ($p < 0.0001$) effect of *hper3* genotype on HO score, but no effect of bipolar disorders condition.

DISCUSSION

Our finding about the influence of both *hper3* VNTR and clock SNP polymorphisms on diurnal preference among healthy individuals confirms previously published results^(3,5,6). The importance of the first study of this kind on Argentinean populations lies on the hypothesis first proposed by Pereira et al.⁽⁶⁾, based on their work on the length polymorphism *hper3* and DSPS, which states that differences in latitude (i.e., in seasonal photoperiods) may have different effects on circadian functioning in relation to this gene variants. There have been some confronting results with regard to the influence of clock 3111 T/C polymorphism on diurnal preference^(26,27), but, as previously noted, differences in photoperiod may account for apparently opposing results.

Regarding our *hper3* results, it is important to highlight the fact that, even without taking into account chronotype classification, we have shown that the length polymorphism studied has an effect *per se* on diurnal preference determined by HO questionnaire, suggesting that *hper3* variants effect may exist not only on extreme phenotypes. These findings add to several recent studies that attribute an important role for *hper3* in human physiology in relation to sleep and circa-

dian functioning^(10,28-30), which may also include a relationship with cancer processes^(31,32).

The effect on diurnal preference of *hper3* VNTR polymorphism was replicated on a population of bipolar disorder patients, with more robust score differences between 5R allele carriers and non-carriers. It is very interesting that BD patients, who are known to suffer from several types of circadian disturbances (mainly sleep problems), and who are under pharmacological treatments that affect circadian rhythms, show a similar diurnal preference to that found in healthy individuals. Previous works by other groups have looked for an effect of *hper3* variability on BD features and predisposition^(19,22), and recently some groups have studied chronotypes among BD patients and reported that they display an evening preference as compared to controls^(33,34). Our present results should be taken into account in such analysis, as the eveningness reported may hold relation with *hper3* variants.

A probable cause of the strong correlation among BD patients could be the above mentioned circadian disturbances. It can be hypothesized that, although all patients are treated in order to minimize these disturbances, these internal disturbances are driving individual subjects more strongly to evening or morning-like behavior. We are currently evaluating if these polymorphisms affect the need of bipolar disorder patients for pharmacological treatment for sleep disorders.

Altogether, the present paper confirms previous findings related to the effect of clock and *hper3* polymorphisms on chronotypes, and shows for the first time that the correlation between *hper3* VNTR variants and diurnal preference is maintained on BD patients.

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