

Polymorphisms in hypocretin receptors and insomnia

Polimorfismos em receptores hipocretinérgicos e insônia

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ABSTRACT

Hypocretin system has been described as one of the most important neurotransmission systems involved in the waking process. The system's lack of function, caused by mutation or neuron death, leads to sharp sleepiness in mammals. It has been proposed that a hyperactive hypocretin system can result in hyperarousal episodes and insomnia. Hypocretins 1 and 2 are bind to two known receptors that are widely distributed in the brain. The current study sought to analyze if either polymorphism in hypocretin receptor 1 or in hypocretin receptor 2 are associated to insomnia. We enrolled 83 insomnia patients, confirmed their clinical insomnia symptoms by means of polysomnographic recordings, comparing single nucleotide polymorphism frequencies in both hypocretin receptors and to those from healthy control patients who had no sleep disorders as confirmed by two nights of sleeping records. Our results show no association to either receptor polymorphism or insomnia.

Keywords: polymorphism genetic, polymorphism single nucleotide, receptors insomnia, sleep.

RESUMO

O sistema de neurotransmissão hipocretinérgico tem sido descrito como um dos mais importantes envolvidos no processo de manutenção do alerta. A ausência da função neste sistema, por mutação ou morte neuronal, leva à sonolência excessiva em mamíferos. Tem sido proposto que um sistema hipocretinérgico hiperativo pode resultar em episódios de alerta e insônia. As hipocretinas 1 e 2 se ligam a dois receptores conhecidos e amplamente distribuídos no cérebro. No presente estudo, buscamos investigar se existe associação entre variações genéticas nos receptores das hipocretinas e a insônia. Foram incluídos 83 pacientes insones, com sintomas clínicos confirmados por registro polissonográfico e investigadas as frequências de polimorfismos em ambos os receptores de hipocretina comparados com uma amostra controle sem distúrbios de sono. Nossos resultados não mostraram associação entre polimorfismos nestes nos dois receptores com insônia.

Descritores: insônia, polimorfismo de nucleotídeo único, polimorfismo genético, sono.

INTRODUCTION

Human sleep depends on two major factors; a circadian regulator and a homeostatic regulator⁽¹⁾. Hyperarousal and homeostatic

sleep dysregulation contribute to the most common sleep disorder, insomnia^(2,3). Insomnia affects nearly 40% of women and 30% of men in the United States⁽⁴⁾. Investigations done in other populations have demonstrated a similarly high prevalence of it worldwide. For example, a study performed by Ohayon⁽⁵⁾ found that 36.3% of people in the United Kingdom suffered from insomnia, based on the DSM-IV criteria. Regarding Brazil, two studies (one in a big city and the other in a mid-sized city) found that approximately 30% of the adult population complain of insomnia^(6,7). Giglio⁽⁸⁾ found evidences that 50% of people have insomnia at a minimum frequency of one episode per week.

In contrast to the “occasional insomnia” experienced by most of the people, insomnia may be a persistent or recurrent problem to some^(9,10), leading to serious medical complications including anxiety, depression and multiple physical ailments^(11,12). Insomnia diagnosis is based on patients' subjective reports or their sleep patterns. Common symptoms include complaints about difficulty in beginning and/or keeping asleep or the presence of non-restorative sleep. Non-restorative sleep is a poor quality sleeping state that is characterized by the inability to keep a high quality of alertness as well as to keep physical and mental well being during daylight, what causes impairment when the individual perform regular daily activities⁽¹³⁾. There are multiple types of insomnia, including transient insomnia which can last a single night or a few weeks; intermittent insomnia happens from time to time and chronic insomnia happens at least three nights a week over a month or longer. Chronic insomnia can be either primary (not related to any medical condition) or secondary (caused by some other factor). Causes of secondary chronic insomnia include cancer, asthma, arthritis, drugs, stress, mental health problems (such as depression) or a poor sleep environment.

Although insomnia is a prevalent sleep disorder, pathophysiology and brain mechanisms involved in the causative hyperarousal during the night remain unknown. An underlying vulnerability to acute sleep disturbance and subsequent development of chronic insomnia has been proposed by several

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investigators^(14,15), though relatively little data, regarding specific factors that might predispose individuals to insomnia, have been identified.

Genetic approaches aiming to study the sleep-wake cycle allowed the identification of several genes involved in sleep regulation, however concerning insomnia, it has been more difficult.

Insomnia studies examining twins suggest the possibility that vulnerability to acute/transient sleep disturbance in response to stress may have a familial component⁽¹⁶⁾. Serretti et al.⁽¹⁷⁾ hypothesized the possible involvement of a *Clock* gene polymorphism in depressed insomnia patients once significantly higher insomnia recurrences happened to patients who were homozygous for the C variant of the T3111C *Clock* gene polymorphism. Another study suggested that the adenosine A2A receptor gene could be involved in insomnia pathophysiology⁽¹⁸⁾.

Hypocretins are hypothalamic neuropeptides tightly associated to wake/sleep cycle^(18,19) regulation. The hypothalamic hypocretin system is comprised by neurons that synthesize pre-propeptides that form hypocretin-1 (Hcrtr-1) and hypocretin-2 (Hcrtr-2), such neuropeptides act through two G- protein coupled receptors; Hypocretin receptor 1 and 2 (Hcrtr1 and Hcrtr2)^(20,21,22). Receptors genes were mapped on human chromosome 1p33 (Hcrtr1) and 6cen (Hcrtr2) and each receptor gene was found to have seven coding exons with conserved splice junction positions across species⁽²³⁾.

Because hypocretins are involved in wakefulness maintenance, they are natural candidates to be involved to insomnia. Although small in number, neurons containing this neurotransmitter are strongly associated to arousal and receive abundant input from the limbic system^(19,24). This association might be important for increasing arousal during emotional stimuli as it is the case during insomnia. Hypocretin's release into *locus coeruleus* and *raphe nuclei* is required such wake-promoter neuronal areas sustained activation and help stabilizing the sleep to wake switch, thus preventing intermediate states and inappropriate transitions between cycles⁽²⁴⁾.

Recent studies on zebrafish have shown that hypocretin overexpression promotes and consolidates wakefulness and inhibits rest, just as in insomnia^(25,26). Accordingly, Perlis⁽²⁷⁾ suggests that insomnia is a hyperarousal disorder in which the patient has an alertness level that is incompatible to sleep initiation or maintenance. Such hyperarousal is inherent to the insomnia and therefore it is reasonable to infer that alterations in neurotransmission of the postero-lateral hypothalamus, also considered brain's wake center, could be - at least in part - responsible for insomnia⁽²⁸⁾. Reduced sleep time and increased nocturnal awakenings may be related to an increase on circulating hypocretin levels throughout the night⁽²⁹⁾.

There are few studies on genetics of insomnia showing that, possibly, gene polymorphisms involved in sleep regulation could generate or predispose people to such medical condition. Because of the great involvement of hypocretin in sleep regulation, receptors involved in the hypocretin system gene polymorphisms are prime targets when searching genes involved with

insomnia. The current study analyzed two informative, non-synonymous single nucleotide polymorphisms (SNPs) in Hcrtr1 and Hcrtr2 in a group of insomniac patients.

MATERIAL AND METHODS

Subjects

The control group was composed by 74 subjects with mean age of 35 ± 11 years old, and a body mass index (BMI) of 24.1 ± 4.1 . Fifty-four percent of the control subjects were women and 67% were Caucasian. Subjects who had any sleep disturbances (as confirmed in basal polysomnography), subjects that used medicines capable of modifying (inducing or reducing) physiological sleep, or subjects with other health issues, and those having a blood relationship with another volunteer were excluded prior to the beginning of the study.

The insomnia group included 83 patients (mean age 52 ± 13 years, BMI 24.1 ± 5.0 , 67% women and 68% Caucasian) that were submitted to an interview and were judged in order to meet the criteria of primary insomnia⁽³⁰⁾ at the Sleep Institute, São Paulo, Brazil. Judging criteria required that patients had at least three insomnia nights a week, and that their sleep disturbances had a significantly negative impact on subjects' social and professional lives⁽³¹⁾. Exclusion criteria included patients having any other sleep disturbance besides insomnia, or any patient who was blood relative to another volunteer.

At a glance, 157 people took part in the experiment, 83 of whom had insomnia and 74 were controls that did not have any sleep disorder.

Polysomnographic recordings

Polysomnograms were taken throughout a night of sleep from the experimental group members aiming to confirm their sleep complaints and to obtain measurements of their sleep patterns. Two nights of sleep from the control group were analyzed, including one habituation night, so patients could get used to sleeping in the laboratory, and a subsequent test night. All subjects were requested to be at the sleep laboratory by 20:00h, but went to bed at their usual bedtime and were told to avoid daytime naps (except when diurnal sleep was usual for the subject). Alcohol consumption was not allowed and caffeine was restricted to one cup a day, in the morning. Control subjects were submitted to the same recording conditions, in the same laboratory settings as the experimental group. During habituation night, the absence of other sleep disorders (sleep apnea syndrome, etc.) was confirmed both for control and insomnia patients.

Sleep parameters

Sleep stages were tabulated for 30-second epochs using the Rechtschaffen and Kales International scoring manual⁽³²⁾. The following sleep parameters were analyzed: total sleep time (TST), sleep efficiency (as described by dividing TST by the total recording time), sleep latency (as defined by the first three consecutive epochs of any sleep stage), REM latency (time from sleep onset to the first epoch of REM sleep), number of arousals (16 s or longer) and its index per hour of sleep, wake time after sleep

onset (WASO), the REM, NREM sleep and the sleep time percentage taken by its four stages (stages 3 and 4 were condensed together and called delta sleep).

Genotyping the polymorphisms in hypocretin receptors 1 and 2

Blood samples were collected from all participants in the experiment and DNA was extracted from the white cells. Genotyping was performed using PCR-RFLP and PCR products for polymorphisms, in both receptors, were digested by *BclI* (New England Biolabs, USA) at 37°C for three hours. The fragment (280-bp) containing hypocretin receptor 1 SNP G1222A (rs2271933) was amplified using designed forward primer 5'-ATTCCGGGAGCAGTTTAAGG-3' and the reverse primer 5'-GATGAAGCCACAGCCTTTC-3'. Regarding hypocretin receptor 2 SNP G922A (rs2653349), the amplification of a 330-bp product was achieved by forward primer 5'-AGAGAAAATGGAAGCCCTG-3' and the reverse primer 5'-AGTCATCTGGCCTGACAAGG-3'. Digestion fragments were analyzed via electrophoresis on a 2% agarose gel.

Statistical analysis

Groups' genotypic frequencies were compared using a χ^2 analysis. Hardy-Weinberg equilibrium was verified in the control and in the insomnia groups as well. Polysomnographic parameters were compared using a Student *t* or Mann-Whitney test with significance levels set at $p = 0.05$.

Ethics issues

All the participants signed an informal consent and the study was approved by UNIFESP Ethics Committee, approval n° 0484/04.

RESULTS

Polysomnographic recordings

Sleep parameters' analysis showed that the patients who complained of insomnia have high deleterious polysomnographic parameters indicating high alertness and poor restorative sleep throughout the night (Table 1).

Sleep parameters observed in the control group are within the expected values for a sample of healthy subjects without sleep disorders. However, sleep parameters observed in the insomnia experimental group corroborate data described in the literature⁽³⁰⁾. It was confirmed that the experimental group had reduced total sleep time, increased latency of sleep onset or REM sleep in relation to the control group. There was also a wake time increase after sleep onset and the index of awakenings, demonstrating that the experimental group had difficulty in initiating sleep or reinitiating sleep after waking during the night, thus jeopardizing sleep efficiency ($72.9 \pm 0.9\%$; $p < 0.01$). Statistically significant differences found between the control group and the insomnia group as well as the absence of any other sleep disorders, show that individuals who took part in the current experiment were correctly diagnosed as insomniac.

Table 1. Control and insomniac groups' sleep measurement.

Sleep Parameters	Control Group (n = 74)	Insomniac Group (n = 83)
Sleep latency (min)	10.2 ± 0.9	49.5 ± 2.3*
REM latency (min)	82.4 ± 3.8	150.0 ± 7.4*
TST (h)	6.3 ± 0.1	4.9 ± 0.1*
Sleep efficiency (%)	92.1 ± 0.0	72.9 ± 0.9*
Stage 1 (%)	2.4 ± 0.1	7.3 ± 0.5*
Stage 2 (%)	57.8 ± 0.5	61.3 ± 1.1*
Stage Delta (%)	19.2 ± 0.6	16.4 ± 0.8*
REM stage (%)	20.3 ± 0.6	14.9 ± 0.8*
WASO (%)	26.8 ± 1.6	109.1 ± 4.6*
Arousal #	59.1 ± 2.7	82.0 ± 5.0*
Arousal index	9.3 ± 0.4	16.4 ± 0.9*

Sleep latency: Falling asleep (in minutes); REM Latency: Falling into REM sleep (in minutes); TST: Total sleep time (in hours); Sleep efficiency: Percentage of time asleep throughout the night; Stages 1, 2, and delta: Percentage of time in each stage of slow-wave sleep; REM stage: Percentage of the time elapsed in REM sleep; WASO: Wake time after sleep onset; Number of arousals that have occurred throughout the night; Arousal index (Arousals/Total Sleep Time). Values expressed as mean ± standard error. Student *t* test was performed; * indicates that $p < 0.05$.

Aiming to explore whether insomnia sleep parameters were different in males and females we analyzed both genders separately, as seen in Table 2. When comparing the main sleep parameter differences between insomniac males or insomniac females to their respective controls, we observed that female individuals have a much more increased latency to the first REM episode while they exhibit no sharp differences in the second and in delta stages.

Genotyping the polymorphisms in hypocretin receptors 1 and 2

Receptor 1 polymorphism G1222A

The genotypic frequencies observed in the control group and in the insomniac group are shown in Table 3 for the hypocretin receptor 1 polymorphism. We do not observe statistically significant differences between the two groups. Once insomnia was more prevalent in female subjects we also have analyzed individuals separated by gender and no differences were found (data not shown).

Receptor 2 polymorphism G922A

The genotypic frequencies observed in the control and in the insomniac groups are shown in Table 4. We do not observe any statistically significant differences between groups in regards to polymorphism, even when analyzing samples separated by gender.

DISCUSSION

A strong genetic basis for normal sleep has been shown in both humans and animals. Studies due to normal human adults indicate that slow wave sleep rates are strongly influenced by genetic factors. It can be estimate a 50% heritability average, which is higher than most other human traits. In addition, several sleep disorders have familial or genetic and environmental components⁽³³⁾.

Table 2. Control and insomniac groups by gender sleep measurements.

Parameters	Male		Female	
	Control (n = 36)	Insomniac (n = 32)	Control (n = 38)	Insomniac (n = 51)
Sleep Lat (min)	9.1 ± 1.3	49.9 ± 3.6*	11.1 ± 1.4	49.2 ± 3.0*
REM Lat (min)	79.5 ± 6.1	120.3 ± 7.0*	85.1 ± 4.7	168.1 ± 10.3*
TST (h)	6.2 ± 0.1	5.1 ± 0.1*	6.3 ± 0.1	4.7 ± 0.1*
Sleep effic (%)	92.7 ± 0.5	73.1 ± 1.4*	91.4 ± 0.5	72.7 ± 1.3*
Stage 1 (%)	2.7 ± 0.2	6.8 ± 0.8*	2.2 ± 0.1	7.6 ± 0.6*
Stage 2 (%)	57.4 ± 0.9	63.1 ± 1.7*	58.1 ± 0.7	60.1 ± 1.4
Stage Delta (%)	18.4 ± 0.9	14.8 ± 1.1*	20.0 ± 0.8	17.3 ± 1.1
REM stage (%)	21.3 ± 0.8	15.0 ± 1.3*	19.4 ± 0.9	14.8 ± 1.1*
WASO	25.4 ± 2.2	114.3 ± 7.6*	28.2 ± 2.3	105.9 ± 5.8*
Arousal #	62.7 ± 4.1	91.0 ± 9.2*	55.7 ± 3.5	76.4 ± 5.8*
Arousal index	9.9 ± 0.6	17.4 ± 1.4*	8.6 ± 0.5	15.9 ± 1.1*

Sleep latency: Falling asleep (in minutes); REM Latency: Falling into REM sleep (in minutes); TST: Total sleep time (in hours); Sleep Efficiency: Percentage of the time in sleep throughout the night; Stages 1, 2, and delta: Percentage of time in each stage of the slow-wave sleep; REM stage: Percentage of the time elapsed in RIM sleep; WASO: Wake time after sleep onset; Number of arousals throughout the night; Arousal index (Arousals/Total Sleep Time). Values expressed as mean ± standard error. Student *t* test was performed for analysis. * *p* < 0.05.

Table 3. Genotypic frequencies of the receptor 1 polymorphism.

Genotypes	Control Group (n)	Insomniac Group (n)
AA	0.14 (10)	0.10 (8)
GA	0.58 (43)	0.66 (55)
GG	0.28 (21)	0.24 (20)

n = number of individuals.

Table 4. Genotypic frequencies of the hypocretin receptor 2 polymorphism.

Genotypes	Control Group (n)	Insomniac Group (n)
AA	0.03 (2)	0.01 (1)
GA	0.26 (19)	0.22 (18)
GG	0.72 (53)	0.77 (64)

n = number of individuals.

The hypocretin system is one of the major arousal systems in the brain and it is a potential site for genetic variations identifications associated to insomnia. Recent data in a zebrafish model of sleep show that a null mutation in the hypocretin receptor causes a sleep/wake pattern that resembles insomnia⁽²⁶⁾. However, in our study, no association was observed between either hypocretin receptor 1 or hypocretin receptor 2 and insomnia.

The observation of our results suggest that the non-synonymous polymorphisms found In hypocretin receptors do not affect hypocretin neurotransmission or perhaps our sample size is not large enough to reveal an association. Significant associations among other hypocretin receptor polymorphisms (besides the ones studied here) and cluster headaches have been reported^(34,35). It is thus a worthwhile endeavor to further explore these polymorphisms and their relationship to sleep, since there is a reciprocal relationship between sleep and headaches⁽³⁶⁾.

Another hypocretin neurotransmission area that should be examined by further genetic studies is hypocretin's synthesis and release. It may be the key element producing the waking state, instead of receptors.

One of the deepest difficulties faced when studying insomnia is the problem of identifying endophenotype. Different endogenous brain processes can lead to insomnia and a too heterogeneous group comprised of insomniacs without similar insomnia causes can hamper proper genetic analysis. Based on our sample, we could realize that despite our efforts to control it, insomnia profiles are not the same in all patients. Some of the patients have lengthy sleep latency, while others wake too often during the night. More rigid group stratification may be needed in order to achieve positive results.

The search for candidate genes to understand primary insomnia has not yet yielded satisfactory results, most likely due to the disease's complex nature. The causes of sleep loss are many. In spite of the negative results from our study, hypocretin system still holds great promise for a possible association to insomnia. Additional studies with larger samples, more rigid criteria for patient stratification and a search for new polymorphisms involved in hypocretin synthesis and release machinery are important future steps.

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