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A Genome-Wide Association Study of Sleep Habits and Insomnia

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Abstract

Several aspects of sleep behaviour such as timing, duration and quality have been demonstrated to be heritable. To identify common variants that influence sleep traits in the population, we conducted a genome-wide association study of 6 sleep phenotypes assessed by questionnaire in a sample of 2,323 individuals from the Australian Twin Registry. Genotyping was performed on the Illumina 317K, 370K and 610K arrays and the common Single Nucleotide Polymorphisms between platforms were used to impute non-genotyped SNPs. We tested for association with more than 2,000,000 common polymorphisms across the genome. While no SNPs reached the genome-wide significance threshold, we identified a number of associations in plausible candidate genes. Most notably, a group of SNPs in the 3rd intron of the *CACNA1C* gene ranked as most significant in the analysis of sleep latency ($p = 1.3 \times 10^{-6}$). We attempted to replicate this association in an independent sample from the Chronogen Consortium ($n = 2,034$), but found no evidence of association ($p = 0.73$). We have identified several other associations that await replication in an independent sample. Our study had good power to detect common single nucleotide polymorphisms that explain more than 2% of the phenotypic variance in self-report sleep phenotypes at a genome-wide significant level. No such variants were detected.

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Conflict of Interest Declaration

IB Hickie declares the following potential conflicts of interest:

- Director of Headspace, National Youth Mental Health Foundation Ltd.
- Member, Mental Health Expert working group, Department of Health and Ageing, Australian Federal Government.
- Member of Bupa Australia Medical Advisory Panel
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Keywords

insomnia; genetics; mood; sleep; circadian

Introduction

Despite the fact that insomnia is the most common sleep disorder, little is known about the contribution of genetics to its etiology and pathophysiology. Between 6 and 10% of individuals experience insomnia that is chronic in nature, while another 25% report occasional difficulties with sleep¹. Insomnia is associated with a number of negative sequelae including fatigue, irritability and impaired concentration and memory. Longitudinal studies have also repeatedly shown that insomnia is a risk factor for the development of new-onset mood, anxiety, and substance-use disorders². Given the prevalence of insomnia and its associated public health impact, advances in our understanding of the genetic underpinnings of the disorder could lead to prevention and treatment efforts that would benefit a substantial proportion of the population.

One of the difficulties in studying the genetics of insomnia is the lack of standardized phenotypes. Human genetic studies have largely relied on self-report, including one or more questions related to sleep patterns of characteristics such as sleep latency, time spent awake during the night, or total sleep time. A number of studies have demonstrated that part of their variability can be attributed to genetic factors. Several groups have conducted classical twin studies, comparing concordance in MZ and DZ twins³⁻⁶. With only a few exceptions, heritability estimates were consistently in the range of .25 to .45, regardless of the exact insomnia question or phenotype used, indicating that self-reported insomnia has moderate genetic influences. Studies where individuals were asked to report on sleep patterns of family members also provide support for genetic influences⁷⁻¹¹. The search for specific genes that are associated with sleep patterns and insomnia is in its infancy, but initial studies point in a number of directions. Candidate gene studies in animals and humans have found associations between insomnia phenotypes and circadian clock genes such as *BMAL1*/*Mop3*¹², *PER3*¹³, *CLOCK*¹⁴. However, many of these analyses had small sample sizes by comparison to those used in genome-wide association studies. A recent analysis of common variants located in genes known to be involved in the circadian clock revealed an association between *TIMELESS* and symptoms of depression and sleep disturbance¹⁵.

A number of genome wide association studies (GWAS) have been conducted on sleep phenotypes in humans. Gottlieb and colleagues studied a subset (n=749) of the Framingham Heart Study Offspring Cohort using both linkage and association analysis¹⁶. Their survey included assessments of usual bedtime and sleep duration, phenotypes that might have some relevance for insomnia. Linkage analysis failed to find any peaks with $\text{LOD} > 3$, but five peaks with $\text{LOD} > 2$ were found, including a linkage between usual bedtime and *CSNK2A2*, a gene known to be a component of the circadian molecular clock. In population-based association tests, an association between an intronic SNP in the *PDE4D* gene and sleepiness reached the genome-wide threshold of significance. Usual bedtime was associated with the SNP rs324981, located in the gene *NPSR1*, which is a component of the neuropeptide S

receptor. More recently, a genome-wide association study of identified a genome-wide significant SNP in the *ABCC9* gene as influencing sleep duration¹⁷, while a genome-wide scan of insomnia induced by caffeine failed to identify any genome-wide significant signals, but did replicate a previously reported association with a variant in the *ADORA2A* gene¹⁸. These investigations are important in establishing the feasibility of finding genetic associations with self-reported sleep phenotypes.

In order to advance our understanding of the genetics of sleep/wake regulation and insomnia there is a need for gene discovery studies that include a wider range of insomnia phenotypes and that have adequate sample sizes to detect what are likely to be small effects. Here we present the results of a GWAS of sleep and circadian phenotypes in a sample of >2,000 Australian twins. We tested >2,000,000 common genetic polymorphisms for association with a sleep latency, sleep time, sleep quality, sleep depth, sleep duration and an insomnia factor score. We also tested the top hits from the sleep latency analysis for replication in an independent sample of 4 cohorts from the Chronogen Consortium.

Methods

Participants

Between 1980 and 1982 a Health and Lifestyle Questionnaire was administered by mail to 5,867 complete pairs of twins who had been registered with the Australian Twin Registry. Responses were received from a total of 7,616 individuals (2,746 males and 4,780 females) and they had a mean age of 34.5 years (S.D. = 14.3). Phenotypic and genotypic data collection was approved by the Queensland Institute of Medical Research (QIMR) Ethics Committee and informed consent was obtained from all participants. A total of 2,323 individuals provided both phenotypic and genotype information for the study (601 males and 1,721 females). The mean age for the genotyped sample was 31.4 years (S.D. = 11.0). A breakdown of the participants by zygosity is given in Table 1.

Phenotypic Measures

As part of the questionnaire, respondents were asked a number of questions about their sleep habits. To assess usual sleep patterns participants were asked the following questions:

“On WEEKDAYS after you go to bed, what time do you usually try to get to sleep?” (Sleeptime)

“On WEEKDAYS, how long in minutes do you think it usually takes you to fall asleep from when you first try to go to sleep?” (Sleep Latency)

“How would you describe the quality of your usual sleep over the last few months?” (Quality)

1=Very good 2=Good 3=Fair 4=Poor 5=Very poor

“In particular, how would you describe the depth of your sleep?” (Depth)

1=Hard to Wake 2=About average 3=Easy to wake

“On WEEKDAYS, how long would you usually sleep for?” (Sleep Duration)

Participants were also asked about how much the quality of their sleep varies and about the frequency with which they wake up in the middle of the night. Further information on the sleep disturbance measures in the questionnaire can be found elsewhere ^{4,5,19}. In total, six variables were analysed – sleeptime, sleep latency, sleep quality, sleep depth, sleep duration and an insomnia factor score (I.F.S.). A factor analysis applied to an independent dataset that used a similar questionnaire also identified a factor that underscored poor sleep to which sleep latency, waking during the night, and sleep quality loaded strongly ²⁰. Principal components analysis applied to this dataset previously showed that among the sleep measures assessed in the questionnaire - sleep quality, variability of quality, sleep latency and frequency of night-time waking - appear to load strongly on a general sleep disturbance component that measures general insomnia ⁴. In the present analysis, principal components analysis was performed on the same variables to derive an overall score for insomnia for each individual. The analysis supported a single factor loading on these variables. Descriptive statistics for each of the traits analysed are given in Table 2. An identical questionnaire (with regard to assessment of sleep habits) was administered to a subsample of 96 individuals who participated in a pilot study several months prior to the main study. This allowed us to test for consistency of responses over time. All of the individual sleep items analysed in this study showed good reliability ($r^2 > 0.71$, Table 2) ⁴. Previous analysis showed that the variables show strong internal consistency – a further indication of the validity of the subjective reports of sleep disturbance ⁴. The bivariate correlations between the variables are shown in Table 3. There were significant age and sex effects, with sex being the most significant by several orders of magnitude. All of the analyses included age and sex as covariates. We also tested for effects of age², age $\times$ sex, and age² $\times$ sex, but there were no significant effects for any of these polynomial terms. Similarly, we tested for the effect of state of residency to check whether sleep patterns were affected by latitude, but no effects were detected. Due to positive skew in the distribution of sleep latency and the insomnia factor score, both traits were natural log transformed (Table 2). In the case of the insomnia factor score, a constant was added to the scores to ensure that all scores were positive prior to transformation. For the sleeptime analysis, individuals who said that they usually try to go to sleep between 3am and 6pm were removed from the analysis ($n = 23$), as there was a high likelihood that they were shift workers who were not choosing to sleep at that time by their own preference, or were individuals with delayed sleep phase syndrome. Individuals who reported regular or recent use of medications or herbal remedies to aid with sleep were also removed ($n = 9$).

Sleep duration has a very low estimated heritability in our data (9%) but was included in our analyses because it was a phenotype included in the only other genome-wide study reported to date ¹⁶. To reduce the effect of outliers on the analysis, individuals whose reported sleep duration was less than 5 hours or greater than 12 hours ($n = 8$) were removed from the analysis. The heritability of the I.F.S. was estimated to be 31%, in line with that found for insomnia phenotypes in other studies. This was also consistent with the estimates of the individual items, indicating there is no increase in heritability when combining information from many sleep phenotypes into a single insomnia phenotype.

Genotyping

Genotype information was collected as part of a number of genotyping projects undertaken by the Genetic Epidemiology group at Queensland Institute of Medical Research. DNA samples were collected in accordance with standard protocols and submitted to different genotype centres using different SNP platforms (Illumina 317K, IlluminaHumanCNV370-Quadv3, and Illumina Human 610-Quad). SNPs were called using the Illumina BeadStudio software. A standard quality control procedure was used for all of the genotyping projects, prior to imputation. A detailed description of the quality control (QC) steps and procedure for detection of ancestry outliers is given elsewhere²¹. A total of 22 individuals were removed from the present analysis due to being ancestry outliers.

A set of 274,604 SNPs that were common to all of the genotyping chips were used for imputation, which was performed using the program MACH²². The imputation process uses information on the haplotype structure in the human genome from the HapMap project (Release22 Build36) to impute non-genotyped SNPs in the sample. The imputed SNPs were screened further for Mendelian errors, minor allele frequency and missingness. Only SNPs with an imputation quality score (R²) greater than 0.3 were retained, which resulted in a total number of 2,380,486 SNPs.

Genome-Wide Association Analysis

Association analysis was performed using a score-test in MERLIN,^{23,24} with each SNP tested in a singlepoint analysis. This association test is appropriate for use with family data and allows inclusion of MZ and DZ pairs. The test combines information from both a within family test and a between family test to give an overall test of association. The analysis utilised the best-guess genotypes from the imputation analysis. A p-value $< 5 \times 10^{-8}$ was considered to be genome-wide significant. Post-GWAS analysis and annotation was carried out using the program WGAViewer²⁵. Owing to the highly correlated nature of the results from imputed data because of linkage disequilibrium (LD) between the SNPs, some regions will have many SNPs with similar p-values. The clumping algorithm in PLINK²⁶ was used to filter results and find the most significant independent signals. SNPs with $r^2 < 0.5$ were considered to be independent signals.

Power

We used a simulation procedure in MERLIN to estimate the power afforded by our sample to detect variants that are associated with traits at the genome-wide significance level. The simulation procedure generates a dataset that has an identical distribution, heritability, marker informativeness, allele frequencies and missingness patterns and then permits testing for association with an allele that accounts for a specified portion of the phenotypic variance. For the present power analyses, we simulated a SNP with minor allele frequency of 0.25 that explains 1% of the variance for each of three traits – insomnia factor score, sleep latency and sleeptime. These traits have estimated heritabilities of 31%, 32% and 42% in our sample. We performed 1,000 replicates for each trait and the power was calculated as the proportion of those replicates for which the simulated variant was associated at a genome-wide significance level. We then used the Genetic Power Calculator to calculate the

equivalent number of unrelated individuals that would be required to have the same power to detect association with the trait.

Supplementary Table 1 shows the statistical power of the study for variants explaining different proportions of the phenotypic variance. Our study has >80% power to detect a variant that explains 2% of the phenotypic variance at the genome-wide significance level and approximately 99% power to detect a variant explaining 3%. Further, we estimate that our study had 19.4% power to detect a variant explaining 1% of the phenotypic variation in the insomnia factor score at a genome-wide significant level. We had 16.2% power to detect the same variant in the sleep latency analysis and 19.5% power to detect it in the sleeptime analysis.

Approximately 1,970 unrelated individuals would be needed to have the same power to detect the same variant. Approximately 4,280 unrelated individuals would be needed to have 80% power to detect a variant explaining 1% of the phenotypic variance at a genome-wide significant level ($p < 5 \times 10^{-8}$).

Gene-Based Tests

To determine whether there were any genes that harbour an excess of SNPs with small p-values, a gene-based test of association was performed²⁷. A SNP was considered to be part of a gene if it was located within 50kb of the start or stop site of the gene and so could be allocated to more than one gene. The test uses the p-values from the single SNP association analysis and computes an overall gene-based test statistic by aggregating the individual SNP effects in each gene, accounting for the number of SNPs in each gene, and the correlation between them because of LD. The value of this test depends on the unknown true genetic architecture of causal variants which is likely to differ between genes.

Pathway Analysis

To test whether there was an enrichment of associations in genes that act in the same biological pathway or genes that have strongly related functions, all genes with a p-value < 0.05 from the gene-based test were included in a pathway analysis in the Ingenuity Pathway analysis software (Ingenuity Systems Release 6.0, Ingenuity Systems, Redwood City, CA, USA). The Ingenuity program collates information from published research articles regarding the structure, function, localisation and interactions of genes, proteins and biochemical molecules and assigns them to functional and canonical pathways. This permits testing for enrichment of a particular pathway that may be relevant to the trait of interest. Fisher's Exact Test was used initially to test whether a particular pathway was overrepresented and the Benjamini-Hochberg method was used to correct the p-values for multiple testing. A corrected $p < 0.05$ was considered to be significant.

Candidate Loci and Genes

We attempted to replicate the association findings of Gottlieb et al¹⁶ for sleep duration and usual sleep time. In addition, using the Ingenuity (Ingenuity Systems, Redwood City, CA, USA) software, we identified 86 genes that have been associated with sleep phenotypes in humans or animal models. We then checked whether SNPs within or near these genes

showed evidence of association with the sleep phenotypes or if any of these genes ranked highly in the gene-based test of association.

Replication Sample

For replication of the top hit for sleep latency, the results of a meta-analysis of GWAS performed as a collaborative effort by the Chronogen Consortium were used. This comprised of 4,270 subjects with European ancestry and included samples from the Erasmus Rucphen Family (ERF), Estonian Genome Center (EGCUT), the Co-operative Health Research in the Augsburg Region (KORA), the KORCULA study in Croatia, the Micro-isolates in South Tyrol Study (MICROS), the Netherlands Study of Depression and Anxiety (NESDA) and the Orkney Complex Disease Study (ORCADES). A detailed description of these studies is provided in the supplementary methods. All studies in the replication cohort used the Munich Chronotype Questionnaire²⁸ to assess sleep traits. Sleep information only on free days, when a person's sleep pattern was not influenced by professional duties (use of alarm clock was an exclusion criterion), was analyzed. Persons that used medications that may influence sleep were excluded from the analyses. Informed consents were obtained from all study participants and an appropriate local committee approved study protocols. Descriptive statistics for the replication cohorts are given in Supplementary Table 2.

Replication cohorts were genotyped on a variety of platforms (Affymetrix 250K, Illumina 317K, Illumina 370K; Perlegen 600K; Affymetrix 1000K). Imputations of non-genotyped SNPs in the HapMap CEU v21a or v22 were carried out within each study using MACH²² or IMPUTE²⁹. Quality control was done in each group separately. The overall criteria were to exclude individuals with low call rate, excess heterozygosity, and gender mismatch. Based on sample size and study specific characteristics, different criteria were used.

Individual GWAS was performed using linear regression (under additive model), natural log of sleep latency as the dependent variable, SNP allele dosage as predictor and age and sex as covariates. The association analyses were conducted in ProbABEL³⁰ or SNPTEST³¹. For most cohorts with related individuals (ERF, MICROS), a linear mixed model in ProbABEL was used to account for familial relationships. The software incorporates the FASTA²⁴ method and kinship matrix estimated from the genotyped SNPs to correct for relatedness³².

A fixed effects meta-analysis was conducted using the inverse variance weighted method as implemented in METAL. Genomic control correction was also applied to all cohorts prior to the meta-analysis.

Results

The quantile-quantile (QQ) plots of the observed vs expected $-\log(p)$ from the six association analyses are presented in Supplementary Figure 1. There was no evidence for population stratification as demonstrated by the genomic control λ (the median χ^2 association statistic divided by the median expected under the null) being between 0.99 and 1.02 for all of the analyses. No SNPs passed the genome-wide significance threshold ($p < 5 \times 10^{-8}$) and there is no evidence for an enrichment of associations at the tail of the distribution. Manhattan plots for the analyses are given in Supplementary Figure 2. Table 4

lists the most significant SNPs that represent independent signals for each trait with $p < 10^{-5}$.

A gene that has been previously associated with bipolar disorder *CACNA1C* (calcium channel, voltage-dependent, L type, alpha 1C subunit) on chromosome 12 showed evidence for association with sleep latency and with sleep quality. A set of SNPs in perfect LD located in the 3rd intron (rs7316184, rs7304986, rs7301906, rs16929275, rs16929276, rs16929278, rs2051990) each with minor allele frequency ~ 0.014 were the most strongly associated SNPs with sleep latency (Table 4, $p = 1.3 \times 10^{-6}$). The SNPs were genome-wide significant when the analysis was performed on the untransformed residuals ($p = 4.9 \times 10^{-10}$), but this did not remain after transforming the distribution to log-normal. These SNPs were not in LD with the validated bipolar variants³³ subsequently found to be associated with schizophrenia and recurrent major depression³⁴ (rs1006737 and rs10848635 also in intron 3, r^2 with rs7316184 etc = 0.018 and 0.006) and so represent an independent signal. We attempted to replicate the association with the *CACNA1C* SNPs in an independent sample comprising 7 cohorts with a total sample size of 4,260 that had collected information on sleep latency. Of these, 4 cohorts had the rs7304986 variant genotyped or imputed (sample size = 2,001). Three of the cohorts had the same direction of effect as found in the initial GWAS, with the minor allele found to increase sleep latency. The association was not nominally significant however ($p = 0.73$) (Table 5). We also performed a meta-analysis of the Australian results and the results from the 4 cohorts in Chronogen in which results for rs7304986 were available. The p-value for the meta-analysis was 0.01 ($\beta = 0.12$, S.E. = 0.05).

For the sleeptime analysis, there were two SNPs located in or near genes with $p < 10^{-5}$. They are intronic SNPs in the *L3MBTL4* and *EBF3* genes respectively (Table 4). *L3MBTL4* is a gene on chromosome 18 whose function is not well annotated. *EBF3* is located on chromosome 10 and is known to be expressed in the brain. It is frequently found to be silenced in brain tumours and other forms of cancer and is thought to be a tumour suppressor gene³⁵. No circadian candidate genes harbor SNPs that show strong evidence of association with timing of sleep from our analysis. One SNP – rs10734107 – located 2kb downstream of the *NPS* gene had a p-value of 1.1×10^{-5} . *NPS* is an interesting candidate gene for association with sleep timing as it encodes a Neuropeptide S, a molecule that is known to stimulate arousal and that has been associated with anxiety and sleep apnea³⁶. In a previous GWAS of usual bedtime¹⁶, a SNP in the Neuropeptide S Receptor gene (rs324981) was among the most associated variants ($p = 4.5 \times 10^{-5}$). That result did not replicate in our study ($p = 0.133$), but the combined findings of the two genome-wide studies implicate a role for the Neuropeptide S system in sleep/wake regulation.

The *CACNA1C* gene also shows evidence of association with sleep quality (Table 4). The most significant SNP from this region – rs2302729 ($p = 4.4 \times 10^{-6}$) – is located in intron 9 of the gene and is not in LD with the variants associated with sleep latency ($r^2 = 0.004$) or with the SNPs associated with bipolar disorder ($r^2 = 0.009$ and 0.034 respectively).

For the sleep depth analysis, there were only two independent regions associated with $p < 10^{-5}$, neither of which were located within or near annotated genes. The most significantly

associated SNP with sleep duration was rs4780805 ($p = 2.66 \times 10^{-6}$). This SNP is located on chromosome 16, 17kb from the nearest gene *TMC5*.

A SNP – rs11174478 ($p = 1.92 \times 10^{-6}$) in the *SLC2A13* gene is the most strongly associated with the insomnia factor score. This gene is located in the same region of the genome as LRRK2, a gene known to be associated with Parkinson's Disease.

Gene-based Tests and Pathway Analysis

At least one SNP mapped to 17,695 autosomal genes. A conservative genome-wide threshold for significance was set at 2.83×10^{-6} , which corresponds to a nominally significant p-value of 0.05 corrected for 17,695 tests. This threshold does not correct for analysing multiple (albeit correlated) traits. No genes reached this significance threshold. A list of the five most significant genes for each trait is given in Table 6. The most significant association across the six traits was *ZNF695* with sleep duration ($p = 1.14 \times 10^{-4}$). None of the most strongly associated genes on the list have a known role in circadian rhythms or have previously been identified as candidate genes for sleep phenotypes.

After correction for multiple testing, no biological functions or pathways were found to be enriched in the gene-based test. Supplementary Table 3 gives the most significant functions and pathways for each of the gene-based analyses.

Candidate Genes

From the Ingenuity Pathway Analysis software, we identified 86 genes that have been associated with circadian rhythms and sleep phenotypes in humans or animal models. In addition, we examined the association statistics for a further 9 genes identified in the study of Gottlieb et al¹⁶. The most strongly associated SNP and results from the gene-based test for each of the candidate genes are given in the Supplementary File. With the exception of *NPS* in the sleep latency analysis, none of the candidate genes ranked among the most associated genes for any of the traits. Strikingly, the *NPS* ranked top of the candidate genes for sleeptime ($p = 0.001$) and fourth in the latency analysis ($p = 0.03$), indicating that variants within the gene may influence several different sleep phenotypes. This result is not unsurprising given that the principal components analysis showed that the variables load on one common factor for insomnia. The number of genes with $p < 0.05$ ranged from 0 for sleep duration to 7 for sleep latency and there was no overall evidence for an enrichment of associations in the candidate genes. A list of SNPs located either in the gene or within 50kb of the start of stop site with $p < 10^{-3}$ for any of the association analyses are listed in Supplementary Table 4.

Gottlieb et al identified 34 SNPs that showed evidence of association - in either a population-based or family-based test – or linkage with sleep duration, sleeptime or sleepiness. We attempted to replicate those SNPs in our sample initially with the phenotypes for which associations had been reported, and then with the other phenotypes in our study. No measure of sleepiness was available in this study and so it was not possible to try to replicate the top SNPs for that phenotype. Only one SNP replicated with the same phenotype – rs2985334 with sleep time ($p = 0.0062$ $\beta = 5.3$ minutes), survived multiple testing. Several

of the other SNPs replicated with other phenotypes in the sample, but none of these results were significant after accounting for multiple testing. A list of SNPs from Gottlieb et al with $p < 0.05$ for association with any of the phenotypes is given in Supplementary Table 4.

Discussion

A GWAS of six insomnia-related traits in a sample of over 2,000 Australian twins and their siblings (with power equivalent to 1,970 unrelated individuals) was performed. One previous GWAS of sleep and circadian phenotypes has been reported, but the analysis was limited to ~71,000 SNPs with minor allele frequency > 0.1 . The present study used $> 2,000,000$ SNPs in the analysis and so surveys a larger fraction of the common variation in the human genome and has a larger sample size. No SNPs reached the genome-wide significance level for any of the traits. The Q-Q plots show that the distribution of the association test statistics closely follows the expected distribution under the null hypothesis of no association. This is not an unexpected finding given the sample size of the study and the effect sizes of variants detected in genome-wide association studies of other complex trait³⁷⁻³⁹. This contrasts with the Q-Q plot for a similar study of hair morphology that used the same sample used in the present analysis and found a genome-wide significant hit²¹.

In spite of the lack of genome-wide significant findings and lack of replication for the top SNPs for sleep latency, we identified a number of regions for each trait that were suggestively associated, and these regions should be targeted for replication in other samples. Studies in model organisms have identified many genes implicated in controlling the circadian clock and regulating sleep patterns. However, the majority of the most strongly identified genes in this study have not previously been implicated. Some of the suggestive association signals represent plausible candidate genes for sleep and circadian phenotypes.

The top ranked region for the sleep latency analysis was the 3rd intron of the *CACNA1C* gene. Variants in this intron have previously been found to be associated with a number of psychiatric disorders including bipolar disorder and schizophrenia.^{33,34} While the SNPs identified here are not in LD with the risk alleles and therefore represent an independent signal, there is widespread evidence to suggest a link between sleep disturbance and mood disorders and several studies have reported associations between circadian genes and mood disorders⁴⁰⁻⁴². It is therefore plausible that variants in genes known to increase risk to mood disorders may also play a role in sleep disturbance. The association did not replicate in an independent sample however, indicating that it may simply be a chance occurrence that these SNPs are associated in our sample.

The lack of replication may be caused by several factors. The replication sample size was 2,001 individuals. Under the assumption that the true causal variant at the *CACNA1C* locus has been detected and the effect size has been estimated without error, the estimated proportion of variance in sleep latency explained is 0.5%. From the Genetic Power Calculator it can be calculated that the replication sample had 89.12% power to detect the same effect with $p < 0.05$. However, because of the “winner’s curse effect”, the true effect size may have been overestimated in the discovery sample and hence the power to replicate the finding may in fact be less than estimated. In addition, there were some differences

between the discovery and replication samples that could have affected the results. Firstly, the questionnaires used to collect latency information were different, with the discovery sample asking about sleep latency on weekdays while the replication sample asked about free days which may have led to slight differences in the phenotypes. Secondly, differences in inclusion criteria between the discovery and replication cohorts and between the individual replication cohorts may have decrease the power to replicate the finding. The NESDA sample removed individuals from the analysis who had major depressive disorder, whereas the Australian questionnaire did not include a diagnostic interview for mood disorders and so could not remove individuals with depression from the analysis. Moreover, the mean age of the discovery cohort (31.28 years) was younger than all of the replication cohorts (Supplementary Table 2), which may have affected the power to replicate. This heterogeneity between cohorts is likely to be an issue in many genetic association studies of sleep and insomnia (not just those that rely on self-report information) and so very large sample sizes may be required to have enough power to have enough power to find variants of small effect.

In spite of the lack of replication, *CACNA1C* represents an interesting candidate gene for sleep phenotypes, not only because of its known association with bipolar disorder. An association study of narcolepsy in a Japanese population implicated another SNP in the 3rd intron of *CACNA1C* (rs10774044, $p = 4.2 \times 10^{-4}$)⁴³. The SNP identified in the narcolepsy study is not in LD in the European population with the SNPs identified in the present study ($r^2 = 0.001$), but it is nominally associated with sleep latency in our sample ($p = 0.035$, $\beta = 0.054$, M.A.F = 0.048). An independent region of *CACNA1C* was also suggestively associated with sleep quality in our sample and it is known that hypocretin 1, a neuropeptide that promotes wakefulness, activates the L-type voltage-dependent calcium channels among other signalling pathways in the brain⁴⁴. There is therefore evidence from a number of sources implicating *CACNA1C* in sleep/wake regulation, and despite the lack of replication for the SNPs identified here, further studies of the role of this gene in regulating sleep are warranted.

Studies in rats have shown that increased concentrations of neuropeptide S can activate the hypocretin-1 system, and this may explain the effect of neuropeptide S on arousal. NPS is a strong candidate gene for circadian phenotypes due to its established effects on wakefulness. Mice exposed to even small amounts of NPS show increased locomotion and NPS has been shown to decrease paradoxical and slow wave sleep in rats³⁶. While the result did not replicate in our study, the finding of a significant SNP located in the gene encoding the receptor for NPS in a previous study also strongly implicates the biological pathway in which NPS acts in controlling timing of sleep in humans.

The single SNP analysis and the gene-based test both implicate a region on chromosome 12 near the *SLC2A13* and *LRRK2* genes as the most strongly associated with the insomnia factor score. This region has previously been identified as being associated in Parkinson's disease⁴⁵. One of the most common features of Parkinson's is sleep disruption however none of the genome-wide significant SNPs from the Parkinson's GWAS were nominally significant in our sample.

While our study represents the largest genome-wide association study of sleep phenotypes published to date, there are several limitations. Foremost among these is the lack of a replication sample for some of the associations. Confirmation of the association in an independent sample is required before an association can be considered “real” rather than simply a chance event. All our associations are at a level expected under the null hypothesis given the extent of multiple testing. However, this study has identified a number of suggestive associations that can be prioritised for replication in other samples.

The somewhat mixed results from candidate gene studies for sleep/wake regulation also highlight the need for replication in association studies. This study also permitted us to attempt replication of candidate genes and polymorphisms identified in candidate gene association studies for sleep, but none of them were replicated in our study.

The second major limitation is that our study is underpowered to detect variants with the small effect sizes that underlie complex genetic traits⁴⁶. We had only ~18% power to detect a variant that explains 1% of the phenotypic variance. Prior to undertaking a GWAS, it is not possible to know the genetic architecture of the trait under analysis. The evidence from genetic association studies however is that for the majority of complex traits, any individual common variant (M.A.F > 1%) is likely to explain only a small fraction of the heritability, and so very large sample sizes (on the order of tens of thousands of individuals) are required to be able to detect such small effects at a genome-wide significance level. Hence, there is a need for many groups that have collected sleep information and genetic data to collaborate and facilitate large meta-analyses in order to establish robust associations with common genetic polymorphisms. Our study will make a significant contribution to future meta-analyses.

The third major limitation of our study is that the phenotypes were assessed by questionnaire. Self-report data may be influenced by perceptual and cognitive biases. Individuals who experience sleep disturbance may be prone to underestimate their usual sleep duration. In addition, in the discovery sample we were unable to adjust the phenotypes for diagnosis of other sleep disorders such as sleep apnea or psychiatric disorders such as major depression which may affect sleep due to them not being systematically examined. However, one of the primary focuses of the analysis was insomnia and owing to the links between insomnia and depression, removing of individuals that have depression may reduce power to detect genetic variants for insomnia. As the primary interest of the replication sample was in chronotype, individuals with a psychiatric diagnosis were removed from the analysis. These differences between the discovery and replication samples may be a factor in the lack of replication of the findings for sleep latency in the discovery sample.

The gold standard for the assessment of sleep is polysomnography, with actigraphy offering another objective measure that also can be used to measure activity rhythms in humans. These methods provide more objective measures of sleep and circadian phenotypes which may be more amenable to genetic analysis. As an example, certain polysomnographic components have been shown to be > 90% heritable⁴⁷. The disadvantage of these methods is that they are expensive and time-consuming and so large genetically informative samples measured for these phenotypes will be difficult to obtain. Studies comparing self-reported

sleep information to objective measures have shown a strong correlation between them and those reporting poorer sleep tend to have increased time to fall asleep, less total sleep duration and increased night waking. The self-report items used in the present study showed good test-retest correlations indicating their stability over time and the items were internally consistent which provides another check of the validity of the self-report items. Moreover, the measures used in this study have been validated against laboratory-based EEG measures of sleep⁴⁸. However, EEG-based measures of sleep remain the most desirable phenotypes for genetic analysis.

In spite of the limitations of the study, we have identified a number of common variants that are suggestively associated with variation in sleep habits in the population, some of which are located in or near candidate genes. These variants should be targeted for replication in other samples. It is likely that large sample sizes (on the order of tens of thousands of individuals) will be required to identify common variants that influence self-report sleep habits in the population. However, any identified variants, genes or biological pathways may have a dramatic impact on our understanding of sleep/wake regulation and will have implications for general medicine, given the link between disturbed sleep and cardiovascular disease⁴⁹, psychiatric illness⁵⁰, life satisfaction and wellbeing⁵¹.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Breakdown of the sample by zygosity

MZ female pairs	492
MZ male pairs	102
DZ female pairs	122
DZ male pairs	59
Opposite Sex DZ pairs	125
Female siblings of twins	33
Male siblings of twins	5
Female non-twin singletons	335
Male non-twin singletons	150
Total Sample Size	2,323

Table 2

Descriptive statistics for each of the phenotypes and test-retest correlations

	No of individuals	Heritability (h ²)	Minimum	Maximum	Mean	Std. Dev	Reliability
Latency raw scores (minutes)	2280	0.32	1	180	20.98	22.94	0.72
Latency (natural log)	2280	0.32	-1.21	5.48	2.67	0.9	-
Quality	2315	0.32	1.00	5.00	1.99	0.87	0.78
Depth	2314	0.21	1.00	3.00	1.78	0.65	0.82
Sleeptime	2322	0.42	8:00PM	2:30AM	10:42PM	52.75 minutes	0.81
Sleep Duration	2278	0.09	5 hours	12 hours	7.73 hours	0.91 hours	0.74
I.F.S. (natural log)	2267	0.31	-1.91	1.72	0.11	0.67	-

* Higher scores for the Quality and Depth variables indicate poorer quality and lighter depth of sleep

Table 3

Annotated list of the most highly associated independent SNPs ($p < 10^{-5}$) each association analysis

SNP	TRAIT	CHR	POSITION	LOCATION	closest gene	distance to gene (BP)	Allele	FREQ	BETA^	SE	P	Gen/Imp	P males	Beta Males (S.E.)	P Femal Beta Females (S.E)
rs7304986	Latency	12	2438105	INTRONIC	CACNA1C	0	C	0.01	0.49	0.11	1.38E-06	Imp	4.80E-01	0.13 (0.18)	1.13E-06 0.61 (.13)
rs7716813	Latency	5	146651673	INTRONIC	STK32A	0	T	0.44	0.14	0.03	4.11E-06	Imp	2.84E-03	0.15 (0.05)	8.53E-05 0.13 (0.03)
rs949441	Latency	22	44744884	INTERGENIC	N/A	N/A	A	0.2	-0.17	0.04	4.66E-06	Gen	2.47E-04	0.04 (0.09)	1.18E-02 -0.15 (0.06)
rs9922235	Latency	16	79096417	INTRONIC	WWOX	0	G	0.41	-0.13	0.03	5.80E-06	Imp	1.91E-03	0.16 (0.05)	7.33E-04 0.11 (0.03)
rs951896	Latency	10	106428784	INTRONIC	SORCS3	0	C	0.1	-0.21	0.05	6.26E-06	Imp	1.16E-02	-0.21 (0.08)	1.10E-04 -0.21 (0.05)
rs292410	Latency	5	163043385	INTERGENIC	LOC391844	84256	C	0.45	-0.12	0.03	7.37E-06	Gen	3.13E-04	-0.17 (0.05)	2.79E-03 -0.10 (0.03)
rs722258	Sleeptime	10	23369421	INTERGENIC	N/A	N/A	T	0.42	-8.03	1.71	2.85E-06	Gen	6.69E-03	-8.97 (3.31)	3.03E-04 -7.13 (1.97)
rs1478693	Sleeptime	16	7164219	INTERGENIC	N/A	N/A	G	0.27	8.82	1.9	3.61E-06	Imp	1.50E-02	8.525 (3.50)	5.72E-05 8.99 (2.23)
rs1539808	Sleeptime	18	5978931	INTRONIC	L3MBTL4	0	T	0.01	44.44	9.64	4.04E-06	Imp	2.75E-03	51.97 (17.34)	4.95E-04 39.81 (11.43)
rs9804200	Sleeptime	10	131673986	INTRONIC	EBF3	0	C	0.3	8.39	1.86	6.26E-06	Imp	7.03E-03	9.36 (3.47)	3.96E-04 7.65 (2.16)
rs13068101	Sleeptime	3	192772295	INTERGENIC	N/A	N/A	A	0.26	-8.5	1.89	7.13E-06	Gen	2.49E-01	-4.14 (3.59)	4.38E-06 -10.09 (2.19)
rs10734107	Sleeptime	10	129353132	DOWNSTREAM	NPS	2197	T	0.48	7.4	1.69	1.13E-05	Imp	1.37E-02	8.10 (3.28)	1.86E-04 7.23 (1.93)
rs1986116	Quality	14	77513844	WNCG*	AC007686.1	0	T	0.24	0.16	0.03	9.81E-07	Gen	1.60E-01	0.08 (0.06)	2.41E-06 0.18 (0.04)
rs1005956	Quality	12	40341085	INTRONIC	SLC2A13	0	C	0.41	-0.13	0.03	2.61E-06	Gen	7.00E-02	-0.09 (0.05)	1.74E-05 -0.14 (0.03)
rs17071124	Quality	6	142033726	INTERGENIC	N/A	N/A	G	0.01	0.75	0.16	2.78E-06	Imp	7.44E-03	0.93 (0.35)	1.15E-04 0.69 (0.18)
rs2302729	Quality	12	2783972	INTRONIC	CACNA1C	0	T	0.17	0.17	0.04	4.37E-06	Gen	2.30E-01	0.08 (0.06)	4.63E-06 0.2 (0.04)
rs9836672	Quality	3	191912870	INTRONIC	FGF12	0	T	0.05	0.27	0.06	4.56E-06	Gen	3.26E-01	0.11 (0.11)	2.46E-06 0.33 (0.07)
rs2210430	Quality	9	25501297	INTERGENIC	TUSC1	175099	T	0.01	0.65	0.15	8.74E-06	Imp	2.00E-02	0.89 (0.40)	1.77E-04 0.60 (0.16)
rs1949200	Quality	4	67301042	DOWNSTREAM	RPI1-793B9.1	3803	T	0.13	0.18	0.04	9.47E-06	Imp	3.30E-02	0.17 (0.08)	1.24E-04 0.18 (0.05)
rs3110232	Quality	5	38381015	INTRONIC	EGFLAM	0	G	0.09	0.21	0.05	9.56E-06	Imp	1.21E-03	0.28 (0.09)	8.92E-04 0.18 (0.05)
rs9830368	Quality	3	64396264	INTERGENIC	N/A	N/A	A	0.2	0.15	0.04	9.66E-06	Gen	3.00E-02	0.14 (0.06)	1.60E-04 0.16 (0.04)
rs679711	Depth	3	109529550	WNCG*	N/A	0	T	0.43	-0.1	0.02	1.18E-06	Imp	1.18E-02	-0.10 (0.04)	8.24E-05 -0.09 (0.02)
rs12913538	Depth	15	62899160	INTERGENIC	N/A	0	A	0.37	0.1	0.02	6.77E-06	Imp	9.45E-03	0.11 (0.04)	3.75E-04 0.08 (0.02)
rs4780805	Duration	16	19404645	INTERGENIC	TMC5	-17216	A	0.15	-0.19	0.04	7.99E-07	Imp	1.42E-01	0.12 (0.08)	1.03E-04 -0.20 (0.05)
rs17737465	Duration	8	98154441	INTRONIC	PGCP	0	G	0.37	-0.14	0.03	1.50E-06	Imp	7.83E-02	-0.11 (0.06)	4.47E-05 -0.16 (0.04)
rs11640439	Duration	16	57303194	INTRONIC	PLLP	0	A	0.09	0.24	0.05	2.93E-06	Imp	9.51E-02	0.19 (0.11)	1.21E-05 0.27 (0.06)
rs2042126	Duration	3	175055759	INTRONIC	NAALADL2	0	G	0.48	-0.14	0.03	2.76E-06	Imp	6.28E-01	-0.03 (0.06)	1.98E-05 -0.16 (0.03)
rs11987678	Duration	8	81228826	INTRONIC	RPI1-941H19.3	0	C	0.05	-0.32	0.07	4.22E-06	Gen	1.40E-01	-0.23 (0.16)	1.28E-03 -0.28 (0.08)

SNP	TRAIT	CHR	POSITION	LOCATION	closest gene	distance to gene (BP)	Allele	FREQ	BETA^	SE	P	Gen/Imp	P males	Beta Males (S.E.)	P Femal	Beta Females (S.E)
rs10823607	Duration	10	72500763	CODING	ADAMTS14	0	T	0.15	-0.19	0.04	4.57E-06	Imp	5.90E-01	-0.05 (0.09)	2.58E-06	-0.24 (0.05)
rs2278331	Duration	18	71581506	INTERGENIC	FBXO15	159082	A		0.14	0.03	4.72E-06	Imp	7.83E-02	0.11 (0.06)	3.19E-05	0.17 (0.04)
rs11174478	I.F.S	12	40354244	INTRONIC	SLC2A13	0	A	0.4	-0.1	0.02	1.92E-06	Imp	1.10E-01	-0.03 (0.44)	5.42E-06	-0.12 (0.02)
rs2725544	I.F.S	15	48992897	INTERGENIC	CEP152	26298	C	0.23	-0.12	0.03	2.28E-06	Gen	6.00E-01	-0.02 (0.04)	-4.00E-02	-0.15 (0.03)
rs12471454	I.F.S	2	200014483	INTERGENIC	SATB2	-119740	T	0.41	-0.1	0.02	6.21E-06	Imp	7.30E-02	-0.06 (0.03)	5.90E-05	-0.11 (0.03)

WNCG = Within Non-Coding Gene
Effect Sizes for Sleep Duration are given in decimal hours. For sleep time the units are minutes.

Table 4

Descriptive statistics for cohorts in Chronogen Consortium

Population	sample	Average sleep latency (sd)	Average [LN(sleep latency + c)](sd)	Average age (sd)
EGP	Total	15.47(17.76)	2.31(1.02)	39.85(16.08)
	Male	14.38(15.74)	2.25(1.01)	38.38(16.24)
	Female	16.54(19.70)	2.37(1.04)	41.03(15.88)
ERF	Total	17.69(18.63)	2.56(0.85)	45.67(13.00)
	Male	15.53(14.99)	2.48(0.80)	47.42(12.98)
	Female	19.59(21.16)	2.64(0.88)	44.12(12.83)
KORA	Total	10.33 (9.72)	2.08(0.79)	54.29 (5.49)
	Male	8.71 (7.75)	1.95 (0.77)	54.64(5.66)
	Female	11.97 (11.16)	2.21 (0.81)	53.96 (5.32)
KORCULA	Total	19.55(12.15)	2.56(1.02)	56.41(12.2)
	Male	17.58(13.86)	2.52(0.94)	57.61(12.97)
	Female	20.69(18.71)	2.59(1.07)	55.72(11.7)
MICROS	Total	13.31(14.29)	2.22(0.94)	40.26(14.52)
	Male	11.94(12.06)	2.16(0.89)	41.24(14.50)
	Female	14.50(15.88)	2.28(0.98)	39.44(14.53)
NESDA	Total	18.52 (18.52)	2.60 (0.90)	41.25 (12.26)
	Male	16.63 (16.95)	2.52 (0.85)	44.15 (12.40)
	Female	19.49 (19.22)	2.64 (0.93)	39.77 (11.94)
ORCADES	Total	15.95(15.61)	2.36(1.00)	51.08(11.08)
	Male	12.67(11.26)	2.22(0.89)	51.08(13.18)
	Female	18.67(18.2)	2.47(1.08)	51.26(13.85)
QIMR	Total	20.98 (22.94)	2.67(0.90)	31.28 (10.88)
	Male	17.67 (16.26)	2.56 (0.86)	28.17 (8.12)
	Female	22.14 (24.78)	2.71 (0.92)	32.37 (11.51)

Table 5

Results from replication analysis of rs7304986 with sleep latency

Cohort	SNP	A1	Freq AL1	Imputation Quality	Sample Size	beta_SNP_add	sebeta_SNP_add	P
ERF	rs7304986	C	0.023	0.99	746	-0.01	0.07	0.86
KORA	rs7304986	C	0.013	0.99	510	0.07	0.21	0.11
Orkney	rs7304986	C	0.019	0.99	205	0.42	0.34	0.22
NESDA	rs7304986	C	0.029	0.97	540	0.09	0.16	0.57

SNP	Allele 1	N	Effect Size	Standard Error	P-value	Direction of effect by cohort	
Meta-analysis replication sample	rs7304986	C	2001	0.02	0.06	0.73	+++

Table 6

The 5 most significant genes from the gene-based test for each of the traits analysed

Gene	Trait	Chr	P-value	nSNPs	Start	Stop	Best-SNP	SNP-pvalue
RSPRY1	Duration	16	1.30E-05	69	55777741	55830448	rs1640439	1.50E-06
NIP30	Duration	16	2.00E-05	84	55743878	55777477	rs767505	5.77E-05
CPNE2	Duration	16	4.40E-05	127	55684010	55739377	rs767505	5.77E-05
ARL2BP	Duration	16	9.20E-05	36	55836538	55845046	rs1640439	1.50E-06
GPR68	Duration	14	1.74E-04	76	90768628	90789977	rs2540871	1.93E-06
SIP1	Latency	14	3.12E-04	82	38653238	38675928	rs8011494	6.23E-05
LOC284009	Latency	17	3.48E-04	61	2257024	2265480	rs898751	8.86E-04
TRAPPC6B	Latency	14	4.97E-04	73	38686765	38709385	rs8011494	6.23E-05
SEC23A	Latency	14	5.23E-04	121	38570873	38642188	rs8011494	6.23E-05
C13orf39	Latency	13	6.06E-04	151	102136097	102144855	rs679331	3.75E-04
NGRN	Sleeptime	15	4.53E-04	87	88609898	88616447	rs1044813	5.29E-05
TTLL13	Sleeptime	15	6.41 E-04	84	88593767	88603316	rs1044813	5.29E-05
FLT3	Sleeptime	13	6.83E-04	136	27475410	27572729	rs9554235	2.01E-04
CIB1	Sleeptime	15	6.84E-04	80	88574480	88578283	rs1044813	5.29E-05
C15orf58	Sleeptime	15	7.66E-04	82	88578490	88586316	rs1044813	5.29E-05
ZNF695	Quality	1	1.14E-04	82	245215248	245237978	rs10802457	8.56E-05
SLC2A13	Quality	12	1.22 E-04	568	38435089	38785928	rs1005956	2.61E-06
TNMF20	Quality	2	3.52E-04	107	227935117	227952266	rs1693555	1.28E-03
SLC39A2	Quality	14	4.30 E-04	111	20537258	20539870	rs1889774	6.71E-05
METTL1D1	Quality	14	4.39 E-04	105	20527804	20535034	rs1889774	6.71E-05
CLNS1A	Depth	11	1.29 E-04	50	77004846	77026495	rs17135809	9.74E-05
RSF1	Depth	11	1.32 E-04	115	77054921	77209528	rs1544274	9.74E-05
PNMA2	Depth	8	1.71 E-04	174	26418112	26427400	rs1372882	3.14E-05
LRRN2	Depth	1	4.26 E-04	142	202852925	202921220	rs2772232	1.50E-04
LYPLA3	Depth	16	5.12E-04	38	66836747	66852462	rs6499163	7.15E-04
LRRK2	I.F.S	12	3.64E-04	583	38905079	39049353	rs1564146	1.33E-04
IERSL	I.F.S	9	5.07E-04	122	130977651	130980361	rs107329	5.71E-04
CSDA	I.F.S	12	5.89 E-04	114	10742954	10767171	rs797168	1.43E-04

Gene	Trait	Chr	P-value	nSNPs	Start	Stop	Best-SNP	SNP-pvalue
CRAT	I.F.S	9	6.18E-04	110	130896893	130912904	rs12346996	4.66E-04
DOLPP1	I.F.S	9	6.53E-04	82	130883226	130892538	rs12346996	4.66E-04