Multi-ethnic GWAS and meta-analysis of sleep quality identify MPP6 as a novel gene that functions in sleep center neurons

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Abstract

Poor sleep quality can have harmful health consequences. Although many aspects of sleep are heritable, the understandings of genetic factors involved in its physiology remain limited. Here, we performed a genome-wide association study (GWAS) using the Pittsburgh Sleep Quality Index (PSQI) in a multi-ethnic discovery cohort (n=2,868) and found two novel genome-wide loci on chromosomes 2 and 7 associated with global sleep quality. A meta-analysis in 12 independent cohorts (100,000 individuals) replicated the association on chromosome 7 between *NPY* and *MPP6*. While *NPY* is an important sleep gene, we tested for an independent functional role of *MPP6*. Expression data showed an association of this locus with both NPY and MPP6 mRNA levels in brain tissues. Moreover, knockdown of an orthologue of *MPP6* in *Drosophila melanogaste*r sleep center neurons resulted in decreased sleep duration. With convergent evidence, we describe a new locus impacting human variability in sleep quality through known *NPY* and novel *MPP6* sleep genes.

Keywords: Sleep quality, genome-wide association study, *MPP6*, sleep centers

Statement of significance: Although many aspects of sleep are heritable, the genetic architecture of sleep quality remains poorly understood. Here, we conduct a genome-wide association study using the Pittsburgh Sleep Quality Index (PSQI) in a multi-ethnic discovery cohort. We discovered and replicated a locus on chromosome 7 between NPY and MPP6 with polymorphisms associated with poor sleep quality. Expression data suggests a higher expression of NPY and MPP6 in the brain. NPY, which codes for neuropeptide Y, has been found to promote sleep in humans and was identified as an important candidate gene in sleep regulation in *Drosophila melanogaster*. We tested the functional role of MPP6 in lateral ventral neurons and confirmed that MPP6 increases sleep duration in *Drosophila melanogaster*.

Introduction

Sleep is essential for brain homeostasis and optimal functioning [1]. Poor sleep has been shown to have a negative impact on multiple biological processes and can have harmful health consequences [2]. There is an intrinsic consequence to poor sleep in the risk of disease; for instance, circadian disruption in prolonged night shift workers increases risk of mortality from heart disease and cancers [3]. An overwhelming majority of chronic pain patients also suffer from poor sleep. The complaints of poor sleep and pain usually co-occur and lead to deteriorating quality of life [4]. Furthermore, poor sleep is associated with major depressive disorders and increased anxiety [5]. The term poor sleep encompasses a wide range of sleep disorders that can include, but are not limited to, insomnia, sleep related breathing disorders, circadian rhythm disorders and sleep quality disturbances. Sleep quality is a complex phenotype that is defined as a construct of sleep duration, sleep latency, number of arousals during sleep and sleep restfulness [6,7]. Laboratory sleep assessment is difficult and costly, but validated questionnaires like the Pittsburgh Sleep Quality Index (PSQI) help capture sleep quality in healthy and clinical populations [6]. Self-perceived poor sleep quality can be difficult to assess. The PSQI captures various components that can potentially affect sleep quality such as sleep latency, sleep duration, and sleep efficiency. The content of PSQI has been validated against measures taken from polysomnography and covers multiple aspects relevant to the sleep quality construct [7]. Although many aspects of sleep are heritable (genetic factors explaining an estimated 17-45% of phenotypic variance), the understanding of genetics involved in its physiology remains limited [8].

Genetic factors have previously been shown to influence multiple sleep traits like circadian rhythms, sleep duration, sleep latency, sleep apnea, and restless leg syndrome [8-14]. For instance, *MEIS1* has been repeatedly associated with restless leg syndrome, whether idiopathic or familial [15,16]. Another gene, *PAX8* has been associated with sleep duration

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and was replicated in independent cohorts [17]. Recently, genome-wide association studies (GWAS) in large datasets on self-reported insomnia and excessive daytime sleeping also identified *MEIS1* and *PAX8* genes, but with further analyses it became clear that the observed signal was likely to be driven by another sleep disorder, namely, restless leg syndrome [9,18]. Overall, we are at the beginning of our understanding of the genotypic architecture of sleep phenotypes, and there is an unmet need to perform standardized GWAS analysis of sleep using validated methodology. Based on the overall heritability of sleep in human populations, we would predict small effects from multiple genetic variants. Despite substantial evidence for the heritability of sleep quality using the PSQI (37%) [19,20], to date, no GWAS has been reported using this tool.

In order to identify genetic factors implicated in sleep quality, here we present results of GWAS using the PSQI among U.S. adults in the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort [21]. Genome-wide significant loci were then carried forward for replication across twelve independent cohorts, combining more than 100,000 individuals. Finally, we performed functional validation of a replicated locus through analysis of expression and by monitoring behavioral sleep patterns in transgenic *Drosophila melanogaster*. Together, our systematic genetic analysis of sleep has identified a novel conserved sleep locus, and these data help provide a better understanding of the underlying biological mechanisms contributing to sleep.

Methods

Cohort description

Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA)

Study participants were selected from the OPPERA study, described in detail elsewhere [22]. In brief, the OPPERA cohort is a large population-based study designed to identify the psychological and physiological risk factors, clinical characteristics, and associated genetic mechanisms that influence the development of temporomandibular disorders (TMD) and related phenotypes. Individuals aged 18 to 44 years were recruited from four demographically diverse US locations (Buffalo, New York; Gainesville, Florida; Baltimore, MD; Chapel Hill, North Carolina). Over 200 pain phenotypes and pain related comorbidities were collected within this study. For the current analysis, the phenotype of interest is the global score of the Pittsburgh Sleep Quality Index (PSQI). The PSQI is a 19-item standardized validated instrument that assesses subject sleep quality over the last month [6]. Global score is derived from the sum of seven sub-components, namely subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleep medication and daytime sleepiness. PSQI global score ranges from 0 to 21, where lower scores denote good sleep quality and higher scores denote poor sleep quality. Other sleep phenotypes, such as insomnia and restless leg syndrome were not assessed in OPPERA.

Ethics statement

All OPPERA participants provided informed, signed consent for all study procedures. The OPPERA study was approved by institutional review boards at each of the four study sites (Buffalo, New York; Gainesville, Florida; Baltimore, MD; Chapel Hill, North Carolina) and at McGill University, Montreal, Canada.

Genotyping, quality control and imputation

DNA samples were extracted from whole blood following purification using a Qiagen™ Extraction Kit. A total of 3,161 samples were genotyped for discovery using the Illumina HumanOmni2.5Exome-8v1A array (Illumina, Inc., San Diego, CA) at the Center for Inherited Disease Research (Johns Hopkins University, Baltimore, MD); from those, there were 2,150 were healthy controls and 1,011 chronic TMD cases. Genetic data cleaning was done by the Genetics Coordinating Center at the University of Washington following their established pipeline [23]. Batch effects were assessed by comparing missing call rate and chi-square test for allelic frequency between genotyping batches. Sample identity and sample quality analyses included checks for missing call rate, chromosomal anomalies, cryptic relatedness, autosomal heterozygosity outliers, gender mismatch, and genetic ancestry. The median call rate was 99.9%. Cryptic relatedness was calculated using kinship coefficient with the R package SNPRelate. Samples were excluded if there was a discrepancy between annotated and genetic sex, the presence of chromosomal abnormalities and higher than second degree relatedness (19 parent-offspring pairs, 8 full siblings, and 11 second-degree relatives). With these criteria, 57 subjects were excluded for a total of 3,104 samples that passed QC. Upon merging with available PSQI scores, a total of 2,868 individuals (1,092 males and 1,776 females) were retained for the analysis.

Consistency of genotyping calls was assessed using 68 duplicates of study samples and 66 stock samples from HapMap reference subjects. SNP quality checks included assessments of missing call rate, duplicate discordance, and Mendelian errors. Because of the mixed population structure of the OPPERA cohort, Hardy-Weinberg equilibrium (HWE) was tested in homogenous European- and African-ancestry groups separately.

Cleaned genotypes were then imputed to the 1000 Genomes Project phase 3 reference panel, [24] using the software packages SHAPEIT2 [25] for pre-phasing and IMPUTE2 [26]

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for imputation. The IMPUTE2 algorithm was selected because it was recommended for use in a genetically diverse study sample using a worldwide reference panel. The IMPUTE2 algorithm uses a "k_hap" value to specify which number of reference haplotypes should be used to impute each study sample. The implementation of this parameter is one of the ways imputation with a worldwide reference panel is made computationally feasible: i.e., by choosing a subset of reference haplotypes to impute each study sample based on perceived genetic similarity [27]. The default k_hap value is 500; however, higher values are recommended when imputing into admixed populations. Thus for this project we set k_hap to 2,000. Following SNP QC and imputation, 35 million high-quality SNPs were retained for the analysis.

Power Calculation

We used the method described in [28] that allows estimating the expected proportion of false positives (expected False Discovery Rate, or "expected FDR") among a specified number of the smallest P-values, U. The expected number of true positives among U top-scoring SNPs is given by $(1-eFDR)*U$. We set U=10, N=2,868, the SNP frequency equal to 0.15, (the minimum frequency used in the present study for association testing was 0.05), and varied the assumed actual number of true positives among about 1.8 million tested SNPs as $M = (10, 50, 100, 250, 500)$. To relate the effect size for the continuous standardized outcome to a commonly used measure for binary outcomes, the odds ratio, we took these effect sizes to correspond to three different values of the odds ratio, 1.2, 1.25, and 1.3. These values give the upper bound for FDR if at least M SNPs have the assumed odds ratio.

Statistical analysis

PLINK v.1.90 software was used for genome-wide association analysis under an additive model of inheritance [29]. Global PSQI scores were used as a dependent variable in a linear regression model. Covariates included in the equation were age, gender, dummy-coded recruitment sites, 3 principal components to account for population stratification, and TMD case status to account for recruitment bias. The three principal components were used as they account for the majority of the variance (Supplementary Figure 1). Furthermore, we also repeat the analysis using five principle components to further increase stringency of a control for population stratification. Genome-wide statistical significance threshold was set at $P < 5 \times 10^{-8}$.

In addition to the analysis in the full cohort, we also performed a stratified analysis by sex and by genetically defined race to account for multi-ancestry. For the race stratified analysis, one principle component generated using each race separately was used in the model, along with age, gender, dummy-coded recruitment sites and TMD case status. Manhattan and quantile-quantile (Q-Q) plots were generated using the R-package qqman [30]. Heritability of PSQI global score was computed using GCTA [31]. A conditional analysis was performed by adjusting for the other SNP that passed genome-wide significance by using its minor allelic count as a co-variable in the linear regression model.

Expression Quantitative trait loci (eQTL) analysis

Gene expression was explored using publicly available eQTL online resources. The BRAINEAC database [32] which contains gene expression data across ten brain regions (cerebellar cortex, frontal cortex, hippocampus, medulla, occipital cortex, putamen, substantia nigra, temporal cortex, thalamus and white matter) was used to identify eQTL with an averaged brain tissues expression. Averaged brain tissues expression was obtained by

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simply averaging expression values across all ten brain regions. In addition to BRAINEAC, the GTEx portal version 6 [33] was used in twelve selected brain tissues (amygdala, anterior cingulate cortex, caudate, cerebellar hemisphere, cerebellum, cortex, frontal cortex, hippocampus, hypothalamus, nucleus accumbens, putamen and substantia nigra).

GO enrichment pathway analysis in biological process

SNPs were assigned to genes based on distance. For *cis*-effects, we considered distances between the SNP and the gene locus up to 10,000 nucleotides, on both positive and negative genomic DNA strands. Analyses were performed using the Gene Ontology (GO) biological processes pathways definitions (file retrieved May 24th, 2016 [34]). To generate a *P*-value for each pathway, we collected all SNP *P*-values in *cis* for all genes pertaining to the pathway. We compared the distribution of PSQI GWAS *P*-values among SNPs belonging to the pathway, with the distribution of *P*-values for SNPs not belonging to the pathway. Comparisons were made with the Kolmogorov-Smirnov test using the ks.test function implemented in R version 3.6.0. The test was performed in a 1-sided fashion, because we searched for pathways enriched with SNPs of lower *P*-values. A total of 1,133 pathways were inspected for enrichment. Adjustment for multiple testing was performed using Bonferroni correction.

Data Availability

Study data have been deposited and made publicly available at the Database of Genotypes and Phenotypes (dbGaP) public repository (accession number phs000796.v1.p1).

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Replication cohorts' description

UK Biobank

The UK biobank is a prospective study that includes more than 500,000 people living in the United Kingdom [35]. In total, over 9.2 million invitations to participate in the study were sent, from which 503,325 individuals were recruited between 2006-2010. Participants were part of the National Health Service registry, aged between 40-69 years and living less than 25 miles from a study center. Recruited study participants gave informed consent and completed questionnaires; underwent a range of physical measures; and blood, urine, and saliva were collected for genetic data. Samples were genotyped on the UK BiLEVE array (~50,000 samples) and the UK Biobank Axiom array (~450,000 samples). Analysis was performed on the interim release of genotype data of 152,000 samples. Arrays contain around 800,000 markers. Following standard QC described elsewhere (http://biobank.ctsu.ox.ac.uk/crystal/refer.cgi?id=155580), SNPs were imputed for a total of 73,355,667 SNPs after phasing the autosomes using a modified version of the SHAPEIT3 program modified to allow for very large sample sizes. Imputation was then carried out using IMPUTE3 according to UK biobank standard described elsewhere (http://www.ukbiobank.ac.uk/wpcontent/uploads/2014/04/imputation_documentation_May2015.pdf). Sleep quality was assessed using a self-reported question: "Do you have trouble falling asleep at night or do you wake up in the middle of the night?" with four possible answers: "never/rarely", "sometimes", "usually", or "prefer not to answer". This phenotype was selected because it is a proxy to the global PSQI score with sensitivity of 0.94, specificity of 0.89 and ROC AUC of 0.947 [12]. The phenotype was dichotomized by using only "usually" as cases and "never/rarely" as controls. Association testing was performed using dosage data with SNPTEST v2.5.2 threshold method. Age, gender, genotyping array and 5

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principal components (PC) to account for population stratification were used as covariates. The current study was conducted under UK biobank application number 20802.

Hispanic Community Health Study /Study of Latinos (HCHS/SOL)

HCHS/SOL is a longitudinal multicenter cohort study of the Hispanic/Latino community in the United States with initial visits between 2008 and 2011. Participants were recruited in a two-stage sampling scheme of individuals from the Bronx, NY; Chicago, IL; Miami, FL; and San Diego, CA for a total of 16,415 individuals enrolled [36]. Of these, 12,803 individuals consented to participate in genetic studies. In the current analysis, n=10,610 individuals participated after applying exclusion criteria. Genotyping was performed with an Illumina custom array (15041502 B3), which consists of the Illumina Omni 2.5 M array (HumanOmni2.5-8v1-1) plus approximately 150k custom SNPs. Genome-wide imputation was carried out using the 1000 Genomes Project phase 1 reference panel, SHAPEIT2, and IMPUTE2 software. The quantitative phenotypic outcome is the WHIIRS (Women's Health Initiative Insomnia Rating Scale), which is derived from form code SLE – HCHS/SOL Sleep questionnaire:

WHIIRS = SLEA4 + SLEA5 + SLEA6 + SLEA7 + SLEA11 − 4

where SLE4 is "Did you have trouble falling asleep?"; SLE5 is "Did you wake up several times at night?"' SLE6 is "Did you wake up earlier than you planned to?"; SLE7 is Did you have trouble getting back to sleep after you woke up too early?" with possible answers being "No, not in the past 4 weeks", "Yes, less than once a week", "Yes, 1 or 2 times a week", "Yes, 3 or 4 times a week" and "Yes, 5 or more times a week". SLEA11 asks "Overall, was your typical night's sleep during the past 4 weeks" with possible answers "Very sound or restful", "sound or restful", "average quality", "restless", "very restless".

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High WHIIRS scores represent poor sleep quality. For this analysis, WHIIRS score was dichotomized with disturbed sleep defined as WHIIRS score of at least 9. Sex, age, recruitment center, 5 PC, and TMD status were used as covariates in the analysis. Subjects were excluded from the analysis if they presented any other sleep disorder, such as restless leg syndrome, narcolepsy, or sleep apnea, or if they were taking sleeping pills for the past 4 weeks.

EA-CRASH

This is a prospective cohort study of adults of European ancestry (EA) who presented to an emergency department (ED) following a motor vehicle collision (MVC) [37]. Patients were enrolled at 9 study sites across Eastern USA. From a total of 10,629 patients screened, 1,416 were eligible, 969 consented to study participation, and 948 were enrolled. Data and blood samples were collected at the initial emergency department visit, and follow-up data was collected at 6 week, 6 month and 1 year assessments. Sleep was assessed using the question: "Prior to the accident, in the past month, please rate your insomnia or difficulty sleeping" with possible answers coded using a 0-10 scale were 0 denotes "no problems" and 10 denotes "major problems" with sleep. DNA was extracted from PAXgene blood DNA tubes and SNPs were genotyped using Sequenom technology. Due to the low frequency of discovery SNPs in EA, minor allele frequency of rs11976703, rs73284230 and rs60869707 was 1%, 0.1% and 6% respectively. Moreover, genotyping rate of rs73284230 was 92.2%, which is lower than the accepted threshold of 95%. The regression analysis included age and gender as covariates in the model.

AA-CRASH

The African American (AA) CRASH study (n=915) is a sister study of EA CRASH. Similar to EA CRASH, AA CRASH is a prospective multicenter observational cohort study

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of AA individuals ≥ 18 and ≤ 65 years of age who presented within 24 hours of MVC to one of eleven EDs in six states/districts (Michigan, Pennsylvania, Florida, Alabama, Massachusetts, and Washington D.C.). The full details of this study have been described previously [38]. DNA was collected in the ED using PAXgene DNA tubes. Sleep quality in the month prior to MVC was assessed with the same question as in the EA CRASH study (above). Following DNA purification (PAXgene blood DNA kit, QIAGEN), genotyping using the Infinium Multi-Ethnic Global (MEG) Array (Illumina) was performed. DNA from an individual with known genotype (NA19819, 1000 genomes) and two repeat samples were included in each genotyping batch (96 samples) to ensure genotypic accuracy and reliability. SNPs rs11976703, rs73284230 and rs60869707 were not included in the MEGA array and were thus imputed using available genotyping data. Following, stringent QC and accounts of relatedness, the regression analysis included age, gender, and study site as covariates in the model.

The Finnish BrePainGen cohort

The BrePainGen cohort consists of 1000 Finnish women (aged 18-75 years) who had unilateral non-metastasized breast cancer and received surgery at the Breast Surgery Unit, Helsinki University Hospital, between August 2006 and December 2010. The day before surgery, following informed consent acquisition, medical and medication demographic history was taken and background data collected. Patients also underwent experimental cold and heat pain tests and answered psychological questionnaires that included questions about insomnia. This prospective study cohort has been described in detail earlier [39]. Genotyping was done at the Wellcome Trust Sanger Institute (Hinxton, UK) using the Human OmniExpress Illumina BeadChip (Illumina, Inc., San Diego, CA, USA). After stringent sample quality control procedures, a total of 926 samples passed QC. An MDS plots (12 dimensions) was used to remove outliers (N=4), when the rest of the subjects represented a

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homogeneous population. SNPs were filtered based on minor allele frequency (MAF>0.005), Hardy-Weinberg equilibrium (HWE $p>1x10^{-6}$) and success rate (>0.97). Insomnia data was available for 823 participants. The sub-sample used in this study consists of the 757 individuals with both genotype and insomnia questionnaire data. The patients who stated that they never have insomnia were considered as unaffected (n=399) and those who said that they have insomnia at least once a week (n=237) or every night (n=121) were considered affected. during which also MDS.

Post-mastectomy pain syndrome cohort

This cohort (N =1200) was recruited from the Comprehensive Breast Cancer Program's registry of breast cancer patients undergoing total or partial mastectomy at Magee Women's Hospital of University of Pittsburgh Medical Center. University of Pittsburgh Institutional Review Board approval was obtained prior to all data collection, and all patients gave informed consent before participation in the study. The majority were postmenopausal. Ethnic group was primarily white/Caucasian. The percentage of women of ethnic groups other than white/Caucasian in the study was limited, making an assessment of racial/ethnic differences inappropriate. Patients completed study questionnaires a mean of 38.3 ± 35.4 months (range, 2 months–10 years) after surgery. Full cohort description was reported elsewhere [40]. Sleep disturbance was assessed using a short-form instrument from the National Institutes of Health roadmap initiative, Patient Reported Outcome Measurement Information System (PROMIS) [41]. Genotyping was done using the UK Biobank Axiom platform on samples derived from lymph node tissue, blood or saliva. Genotyping was done using the UK Biobank Axiom platform on samples derived from lymph node tissue, blood or saliva at the Genome center at McGill University. Following QC and imputation, a total of 665 samples were used in the replication. Age and 3 principal components were used in the regression model.

Complex Persistent Pain Conditions

The Complex Persistent Pain Conditions (CPPC): Unique and Shared Pathways of Vulnerability study included 745 participants enrolled in a case control study of overlapping pain conditions conducted at UNC Chapel Hill. Subjects were aged 18-64, and included both sexes (86% female) and major ethnic and racial groups (68% non-Hispanic white). All subjects had at least one of four index CPPCs (episodic migraine, irritable bowel syndrome, fibromyalgia, or vulvar vestibulitis), or were otherwise healthy controls with none of these conditions. Sleep quality was assessed using the PSQI. DNA from all subjects was genotyped using the Axiom Precision Medicine Research Array by Genome center at McGill University. PSQI was used as a dependant variable in a linear regression model with age, gender, 3 PCs and CPPC dummy-coded.

OPPERA-S

The OPPERA–Subset (OPPERA–S) cohort was a subset of the original OPPERA cohort that consists of 973 healthy controls that were not genotyped as part of the initial project as they were saved for further replication. Sleep was assessed using the PSQI and the same analytical plan was used as in the discovery cohort. DNA from all subjects was genotyped using the Axiom Precision Medicine Research Array. Genotyping data was cleaned and imputed in the same manner as the discovery cohort. Covariates included in the analysis included age, gender, dummy coded recruitment sites and 3 PCs.

OPPERA-R

The OPPERA-Replication (OPPERA–R) case-control study of chronic TMD (NIDCR protocol 12-052-E) was designed as a replication study from the initial OPPERA discovery GWAS. Recruitment was independent from the discovery cohort as it was done in 2016-2017. Potential subjects were recruited by telephone screening of 166,062 phone numbers listed in counties surrounding the four OPPERA initial recruitment sites, of which, 2,430

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were eligible and 1,342 subjects (66% female, age 18-74) returned complete phenotype and genotype information and were included in the replication analysis. Phenotype was assessed using the question "please rate the quality of your sleep in the past three months on a 0-10 scale" where 0 represented the worst sleep quality and 10 represented the best sleep quality. Saliva samples for DNA genotyping were obtained using Oragene collection tubes (DNA Genotek Inc., Kanata, Ontario). DNA from all subjects was genotyped using the Axiom Precision Medicine Research Array. Sleep scale scores were used as a dependent variable in a linear regression. Covariates included in the equation were age, gender, dummy coded recruitment sites and 3 PCs.

Jackson Heart Study

The Jackson heart study (JHS) is a populational-based longitudinal prospective cohort aiming to investigate cardiovascular disease among African Americans (AA). Recruitment is community-based in the Jackson Mississippi metropolitan area. This study recruited 5,302 AA adults between 2012 and 2016 [42].For this analysis, we used a subset of 2,999 individuals that had both sleep assessment and genetic data. Sleep quality was determined at Exam 1 using the question "How do you rate your overall sleep quality?" with possible answers: Excellent, very good, good, fair, and poor. This cohort was genotyped using Affymetrix and imputed to a 1000 Genomes phase 1 version 3 template [43]. A univariate linear mixed model adjusted for genetic relatedness, age, and sex was used for each analysis using GEMMA version 0.94.1 [44].

Multi-Ethnic Study of Atherosclerosis

The multi-ethnic study of atherosclerosis (MESA) is a multicenter prospective cohort study aiming to study the development of cardiovascular disease. A total of 6,814 individuals between the ages of 45 and 65 were recruited for the first examination between 2000 and 2002. Participants were recruited in six US cities (Baltimore, MD; Chicago, IL; Forsyth

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County, NC; Los Angeles County, CA; Northern Manhattan, NY; and St. Paul, MN) [45]. In the present analysis, 518 participants from AA ancestry with complete data were used for replication (28%). Sleep quality was assessed using the question "In the past 4 weeks, rate the overall typical night of sleep", extracted from the Center for Epidemiologic Studies Depression Scale (CES-D). Possible answers were: Very sound or restful, sound and restful, average quality, restless, very restless. This cohort was genotyped using the Affymetrix 6.0 array, phased using SHAPEIT and imputed using IMPUTE2 with the Haplotype Reference Consortium version 1.1 template [46]. A univariate linear mixed model adjusted for genetic relatedness, age, and sex was used for each analysis using GEMMA version 0.94.1 [44].

Cleveland Family Study

The Cleveland Family Study (CFS) aims to examine the genetic and familial basis of sleep apnea and consists of 2,534 African Americans and European Americans from 356 families. Index probands with sleep apnea were recruited from northern Ohio sleep centers [47]. Sleep was assessed using the question "During the last month, have you had, or have you been told you do the following while asleep or trying to sleep? Toss, turn or thrash frequently over the night". Values ranged from 0 (Never) to 4 (always or almost always, or 5-7 times per week). A total of 719 African American individuals who had genotype data available and non-null values for the outcome variable were used for analysis. This cohort was genotyped using the Affymetrix 6.0 array, phased using SHAPEIT and imputed using IMPUTE2 with the Haplotype Reference Consortium version 1.1 template[46]. A univariate linear mixed model adjusted for genetic relatedness, age, and sex was used for each analysis using GEMMA version 0.94.1 [44].

Meta-analysis

An additive model of inheritance was used to generate summary statistics for each replication cohort. The direction of phenotype scale in each cohort was reverted if necessary to be consistent across all cohorts included in the meta-analysis, i.e. higher score represents poor sleep. Next, genotypic effect statistics were corrected to reflect the effect of the same allele in each cohort. Because sleep was assessed differently in each of the replication cohorts, genotypic effects were standardized according to the method described elsewhere [48]. Briefly, if the phenotype was binary, the effect size was converted to a continuous standardized scale using the following formula:

$$effect\ size = \ln OR\left(\frac{\sqrt{3}}{\pi}\right)$$

If the phenotype was continuous, the regression coefficient or the difference in mean generated was converted to a standardized effect size by dividing the effect size by the residual standard deviation [49].

Meta-analysis of replication studies, excluding OPPERA discovery was computed using the R-package metafor using a fixed-effect method. The p-value shown is a two-sided p-value. Heterogeneity of effects was verified using the heterogeneity coefficient Q for each SNP. A race-specific meta-analysis was also computed to account for ethnicity.

Drosophila Melanogaster assay

Fly strain

PDF-Gal4 has been previously described [50]. *Varicose* RNAi hairpins 1&2 (GD #24157, KK104548) was from the Vienna Drosophila RNAi Center VDRC. Flies were reared on a standard cornmeal–yeast–agar medium at 25°C and 70% relative humidity in a 12 hr L:12 hr D cycle. Flies were placed in glass tubes containing standard fly food (2% agar and

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5% sucrose). Flies were acclimated for at least 18 hr at 25°C in Light and Dark (LD) conditions, and then data were collected in LD for 7 days with the Drosophila Activity Monitoring (DAM) System (Trikinetics, Waltham, MA) in 5-min bins. Sleep parameters were measured by averaging 5 days of LD [51]. The sleep parameters tested were: circadian patterns of sleep, percentage of sleep, number of sleep episodes, and the duration of sleep episodes in minutes. These parameters were measured separately for L and D intervals. Group comparison was done between parental controls (PDF-Gal4/+, UASVariIR1-2/+) and varicose knockdown flies (DF-Gal4>UAS-variIR1-2). All statistical analysis was performed in Prism 7.0. Significance levels were determined by one-way ANOVA with Dunnett's multiple comparisons test.

Results

Genome-wide association study in OPPERA

We first performed a primary discovery GWAS of sleep quality measured by the PSQI in the OPPERA cohort. The OPPERA cohort was comprised of self-declared non-Hispanic whites (NHW, 58.4%), African Americans (AA, 25.8%), and other ethnic/racial groups including Asians, Pacific Islanders, Native Americans and individuals with mixed races (15.8%). Racial differences in sleep quality were observed in our data, with AA reporting lower sleep quality compared to NHW (Supplementary Table 1) which is consistent with previous reports [52].

Genome-wide analysis using the PSQI global score identified two loci on chromosomes 2 and 7 at genome-wide significance ($P \leq 5 \times 10^{-8}$), and one locus at suggestive significance ($P \leq 5 \times 10^{-7}$), on chromosome 13 (Table 1, Figure 1, Supplementary Figure 2, Supplementary Table 2). PSQI global score was associated with rs11976703 (effect allele C, β=0.78, standardized effect size=0.23, P=3.78x10^{-8}) and rs73284230 (effect allele G, β=0.95,

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standardized effect size=0.28, P=4.76x10^{-8}) on chromosome 7 and rs60869707 (effect allele G, β=1.09, standardized effect size=0.32, P=5.03x10^{-8}) on chromosome 2. The effect allele (the major allele) in each of these three SNPs was associated with higher global PSQI scores, hence worse sleep quality. Genome-wide significant SNPs were also analyzed for association with four PSQI subscales: subjective sleep quality, sleep efficiency, sleep disturbance, and daytime dysfunction. All SNPs showed the strongest association with subjective sleep quality followed by daytime dysfunction, in the same direction as the global score (Supplementary Table 3).

Table 1 Genome-wide (P ≤ 5x10⁻⁸) and suggestive (P ≤ 5x10⁻⁷) loci associated with PSQI in the OPPERA discovery cohort

SNP	Chr:position	*Nearest genes	EA/OA	EAF	INFO	BETA	SE	P
rs11976703	**7:24549526**	**NPY/MPP6**	**C/A**	**0.886**	**1.059**	**0.780**	**0.141**	**3.78×10^{-8}**
rs73284230	**7:24548947**	**NPY/MPP6**	**G/A**	**0.920**	**1.122**	**0.949**	**0.173**	**4.76×10^{-8}**
rs60869707	**2:86023846**	**ATOH8**	**G/A**	**0.930**	**1.171**	**1.086**	**0.199**	**5.03×10^{-8}**
rs151181914	7:24550927	NPY/MPP6	TATC/T	0.921	1.126	0.917	0.173	1.27×10^{-7}
rs376585198	13:20529278	ZMYM2	ATTATT/-	0.904	0.999	0.776	0.150	2.48×10^{-7}
rs78633772	13:20671080	ZMYM2	T/G	0.909	1.000	0.789	0.154	3.31×10^{-7}
rs9579769	13:20598284	ZMYM2	A/G	0.919	1.024	0.809	0.159	3.79×10^{-7}
rs9579744	13:20529559	ZMYM2	A/C	0.911	1.024	0.781	0.154	4.08×10^{-7}
rs7318279	13:20602148	ZMYM2	C/T	0.911	1.028	0.776	0.154	4.66×10^{-7}
rs115462079	13:20586370	ZMYM2	C/T	0.911	1.028	0.776	0.154	4.66×10^{-7}
rs9578239	13:20539229	ZMYM2	A/T	0.911	1.028	0.776	0.154	4.67×10^{-7}
rs9315234	13:20541404	ZMYM2	C/G	0.911	1.023	0.777	0.154	4.99×10^{-7}
rs9579762	13:20584060	ZMYM2	T/C	0.925	1.017	0.827	0.164	5.10×10^{-7}
rs71803599	13:20549173	ZMYM2	CATTT/C	0.912	1.024	0.777	0.154	5.14×10^{-7}

Chr: Chromosome; EA: Effect allele; OA: Other allele; EAF: Effect allele frequency; INFO: imputation quality from IMPUTE2. Position is based on NCBI Build 37 (hg19). Genome-wide significant results are shown in bold. *Intragenic for ZMYM2.

The top SNPs on chromosome 7 were in linkage disequilibrium (D'=0.99; R^2=0.83) in all populations, highest in Africans (D'=0.99; R^2=0.95) and lowest in Europeans (D'=1; R^2=0.15). The minor allelic frequencies for genome-wide significant SNPs were much higher in AA than in NHW (26% vs. 6% for rs11976703, 25% vs. 1% for rs73284230, and 23% vs. 0.2% for rs60869707 in AA and NHW, respectively). In analyses stratified by self-declared race, these SNPs showed robust effects in AA (rs11976703: β=0.95, P=2.29x10^{-6}; rs73284230: β=0.99, P=1.54x10^{-6}; and rs60869707: β=1.04, P=6.17x10^{-7}) but no effects in NHW (rs11976703: β=0.52; P=0.028; rs73284230: β=0.59; P=0.24; and rs60869707: β=0.99, P=0.99). A meta-analysis of ancestry specific results showed an association for rs11976703 (β=0.75, P=1.03x10^{-6}; Q(2.56,P=0.11), I_{het}=61), rs73284230 (β=0.92, P=2.6x10^{-6}; Q(0.35, P=0.55, I_{het}=0) and rs60869707 (β=1.11, P=1.36x10^{-7}; Q(0.06, P= 0.81, I_{het}=0).

Genome-wide analyses stratified by race did not show any SNP above genome-wide significance in NHW, whereas two loci on chromosomes 8 and 16 near *TSNARE1* and *FAM234A* respectively, were significant in AA only (Supplementary Table 4 and Supplementary Figure 3). A sex-stratified analysis did not identify any significant SNPs in females, whereas in males rs28483449, on chromosome 15, located near *ZNF710,* was associated with PSQI (Supplementary Table 5).

Heritability and pathway analysis of sleep quality

Next, GWAS results were used to measure heritability of sleep quality. It was estimated at 14.37%, using GCTA [31], which was consistent with other heritability estimates of sleep traits [9]. Furthermore, pathway analysis of the full GWAS results using gene ontologies (GO) for "biological process" class function identified many significant biological pathways, of which more than half reflected different aspects of neuronal action potential and activities at the synapse, whether receptor signaling, receptor internalization or

the neuromuscular junction (Figure 2). These analyses are consistent with the current understanding of sleep processes, which largely depend on synaptic plasticity occurring during sleep, reinforcing the validity of results obtained from PSQI GWAS data [53].

Power analysis

In order to assess if the discovery OPPERA cohort was powered to detect genome-wide significant hits, we modeled the proportion of true hits given our sample size (2,868), the number of SNPs tested (1.83M), and three values of the standardized effect size (0.33, 0.40 and 0.48). These values of effect sizes approximately correspond to the odds ratios 1.2, 1.25, and 1.3 in a case-control design [48]. The model showed that among ten top-scoring SNPs, the expected false discovery rate (eFDR) [28] is high for the lowest standardized effect size (0.33) and reached only about 0.42 if the total number of tested SNPs carrying such effect size is 500. Thus, about six true positives were expected, i.e., (1-eFDR)*10 = 5.8. One would need about 200 true positive SNPs in the overall GWAS to bring eFDR below 0.05. However, larger standardized effect sizes (0.40 and 0.48) required smaller numbers of true positives. For example, about eight out of ten top-scoring SNPs are expected to be true positives (eFDR=0.153) for the standardized effect size equivalent to 0.48, assuming 100 true positive SNPs in total (Supplementary Figure 4). Hence, the discovery OPPERA cohort was well powered to detect true positives with the assumed effect sizes and densities in a GWAS using PSQI.

Replication and meta-analysis

To replicate our genome-wide significant associations, we used twelve independent cohorts that assessed sleep quality (Supplementary Table 6). The closest phenotype that captures sleep quality in the absence of PSQI was tested for association in each replication cohort with rs11976703, rs73284230, and rs60869707. Each phenotype was normalized to

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account for different measures of sleep (see Methods). The replication studies' association results were then combined using a fixed-effect weighted meta-analysis for a total sample size of 100,805. Both rs11976703 and rs73284230 had a p-value < 0.05 in more than one individual replication study, whereas rs60869707 was only statistically significant in one replication cohort. In the overall meta-analysis, all three SNPs showed an effect size that is in the same direction as the discovery cohort, but only rs11976703 and rs73284230, on chromosome 7 replicated in a meta-analysis combining all replication studies (rs11976703: standardized effect [95%CI] =0.07 [0.02;0.12]; P=3.50 x10^{-3} and, rs73284230 standardized effect [95%CI] = 0.16 [0.08; 0.25]; P=2.0 x10^{-4}) (Table 2, Figure 3).

Table 2 Association of genome-wide significant SNPs in independent replication cohorts and meta-analysis

		Sample size	Effect allele frequency	effect size (se)	p-value
rs11976703 Effect allele (C)	OPPERA-Discovery	2,868	0.89	0.78 (0.14)	3.78×10^{-8}
	UKBB	79,947	0.95	-0.01 (0.03)	6.70×10^{-1}
	HCHS/SOL	7,247	0.87	0.002 (0.013)	8.87×10^{-1}
	Finnish BrePainGen*	800	0.95	0.12 (0.11)	3.54×10^{-1}
	EA-CRASH	894	0.95	0.018 (0.10)	8.64×10^{-1}
	CPPC	**641**	**0.90**	**0.19 (0.09)**	**3.60×10^{-2}**
	OPPERA-S	**929**	**0.87**	**0.14 (0.06)**	**3.20×10^{-2}**
	OPPERA-R	1,297	0.92	0.01 (0.07)	8.45×10^{-1}
	AA-CRASH	**906**	**0.70**	**0.27 (0.12)**	**2.10×10^{-2}**
	PMPS	399	0.94	0.04(0.15)	7.87×10^{-1}
	JHS	2,999	0.73	0.03(0.03)	2.76×10^{-1}
	MESA	518	0.74	0.04(0.07)	5.99×10^{-1}
	CFS	719	0.74	0.02(0.06)	7.04×10^{-1}
	Meta-analysis	**97,296**		**0.07 (0.03)**	**3.6×10^{-3}**
rs73284230 Effect allele (G)	OPPERA-Discovery	2,868	0.92	0.95 (0.17)	4.76×10^{-8}
	UKBB	**79,947**	**0.99**	**0.18 (0.08)**	**3.50×10^{-2}**
	HCHS/SOL	7,247	0.90	0.007 (0.014)	6.17×10^{-1}
	EA-CRASH	895	0.99	0.29 (0.25)	2.52×10^{-1}
	CPPC	657	0.93	0.16 (0.11)	1.50×10^{-1}
	OPPERA-S	**944**	**0.90**	**0.14 (0.07)**	**4.90×10^{-2}**
	OPPERA-R	1,328	0.96	-0.05 (0.10)	6.04×10^{-1}
	AA-CRASH	915	0.71	0.23 (0.12)	7.80×10^{-2}
	PMPS	**399**	**0.99**	**0.70 (0.32)**	**3.02×10^{-2}**
	JHS	2,999	0.74	0.04(0.03)	2.16×10^{-1}
	MESA	518	0.75	0.04(0.07)	5.65×10^{-1}
	CFS	719	0.75	0.04(0.06)	5.25×10^{-1}
	Meta-analysis	**96,568**		**0.16 (0.04)**	**2.0×10^{-4}**
rs60869707 Effect allele (G)	OPPERA-Discovery	2,868	0.93	1.09 (0.20)	5.03×10^{-8}
	UKBB	79,947	0.99	-0.03 (0.15)	8.28×10^{-1}
	HCHS/SOL	7,247	0.96	0.011 (0.025)	6.67×10^{-1}
	EA-CRASH	896	0.99	0.39 (0.45)	3.88×10^{-1}
	CPPC	622	0.94	0.08 (0.14)	5.98×10^{-1}
	OPPERA-S	**877**	**0.92**	**0.23 (0.10)**	**2.10×10^{-2}**
	OPPERA-R	1,282	0.97	-0.01 (0.15)	9.40×10^{-1}
	AA-CRASH	915	0.76	-0.05 (0.15)	6.32×10^{-1}
	MESA	518	0.79	-0.01(0.08)	8.89×10^{-1}
	CFS	719	0.78	-0.04(0.07)	5.67×10^{-1}
	Meta-analysis	93,023		0.07 (0.06)	2.48×10^{-1}

*All directions are presented with respect to the effect allele. SNPs rs73284230 and rs60869707 were not genotyped in the Finnish and rs60869707 was not genotyped in the PMPS and the JHS cohorts. The OPPERA Discovery cohort was excluded from the meta-analysis calculation. OPPERA: Orofacial Pain: Prospective Evaluation and Risk Assessment; UKBB: UK biobank; HCHS/SOL: Hispanic Community Health Study/Study of Latinos; EA: European American; AA: African American; PMPS: Post mastectomy pain syndrome. CPPC: Complex Persistent Pain Conditions. OPPERA-S: Subset; OPPERA-R: Replication; JHS: Jackson Heart Study; MESA: Multi-Ethnic Study of Atherosclerosis CFS: Cleveland Family Study. Statistically significant results are shown in bold. OPPERA-Discovery cohort was not included in the meta-analysis.

In order to account for the large difference in allelic frequency between Europeans and African ancestries, we undertook a separate race-stratified meta-analysis in racially homogeneous cohorts. In Non-Hispanic whites ancestry only replication cohorts, both rs11976703 and rs73284230 replicated in the same direction as the discovery cohort (effect=0.07 and 0.16; P=0.047 and 2.0×10^{-4}, respectively). The same was also true in African ancestry replication cohorts (effect=0.09 and 0.087; P=0.017 and 0.027, respectively). Overall, we concluded that both SNPs rs11976703 and rs73284230 have a significant effect on sleep quality in both ancestries (Supplementary Table 7).

The following analyses will focus solely on the locus of chromosome 7 given that it is the only locus that replicated in an independent meta-analysis.

Genetic analysis of locus chromosome 7

Using a probabilistic identification of causal SNP (PICS) approach, we determined that the probability for causality was distributed as 51.33% for rs11976703 and 26.53% for rs73284230. A conditional analysis showed that both SNPs are not independently associated with global PSQI score ($\text{rs73284230}_{\text{condit11976703}}$; beta= 0.5147; P=$6.9\times10^{-2}$). The combined annotation-dependent depletion (CADD) scores for rs11976703 and rs73284230 were 0.19 and 1.718, respectively, which does not indicate high deleteriousness [54].

The locus on chromosome 7 is situated between neuropeptide Y (*NPY)* and membrane palmitoylated protein 6 (*MPP6)* genes with a surrounding LD structure that differs between ancestries (Figure 1a,b). Using EUR and AFR as reference panels, it can be observed that SNPs in high LD with rs11976703 are located in *MPP6* (EUR), or upstream of it (AFR). All highly associated SNPs ($P<1\times10^{-4}$) around rs11976703 were upstream of *MPP6* and downstream of *NPY*. Nevertheless, the associated locus is substantially closer to the promoter of *MPP6* and separated by the presence of a recombination hotspot from *NPY*.

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SNP association with other phenotypes

According to the Genome-wide Repository of Associations between SNPs and Phenotypes (GRASP) [55], rs11976703 was previously reported to be associated with coronary artery disease, Parkinson's disease and body mass index (BMI) with p-values of 6.33×10^{-3}, 1.2×10^{-2} and 3.0×10^{-2} respectively [56-58]. By contrast, rs73284230 was not associated with any phenotype in GRASP.

We next evaluated whether the effects of SNPs associated with sleep quality were mediated by phenotypes known to influence sleep quality and available in the OPPERA cohort. Six clinically relevant phenotypes were tested for correlation with the PSQI score: two clinical pain conditions (painful temporomandibular disorders (TMD) and low back pain), two measures of psychological distress (trait anxiety and depression), and two measures of sensitivity to experimental pain (heat pain tolerance and threshold). All six phenotypes correlated with PSQI. SNPs rs11976703, rs73284230, and rs60869707 were also associated with depression, anxiety and experimental pain (Supplementary Tables 8 and 9). After adjustment for each potential mediator individually, the allelic effect was slightly attenuated but remained statistically significant. After inclusion of all potential mediators simultaneously, the effect size for each genome-wide significant SNP was attenuated by around 45% but remained statistically significant ($P < 0.05$) (Supplementary Table 10), even after correcting for seven tests. Consequently, the effect of SNPs on sleep is unlikely to be fully mediated by pain states or psychological distress.

Furthermore, using Genome-wide Complex Trait Analysis (GCTA), we calculated the genetic correlation (Rg) between PSQI and the above-mentioned phenotypes. We did not find evidence for genetic correlation of PSQI with TMD, back pain, anxiety and depression, whereas heat pain threshold and tolerance approached statistical significance of such

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correlation (heat pain threshold, Rg=0.68, *P*=0.09 and heat pain tolerance Rg=0.53, *P*=0.07) (Supplementary Table 11a). Furthermore, a LDHub [59] screen with 173 disease/traits from publicly available summary GWAS did not show any genetic correlations, with inflammatory bowel disease and Crohns disease showing nominal significance at *P*=0.05 (Supplementary Table 11b). This might be due to the fact that we were underpowered to detect any association. Because the findings are not sufficiently robust, we hesitate to draw firm conclusions.

eQTL analysis

The two SNPs on chromosome 7 that replicated in the meta-analysis were tested for evidence of functional effects through the expression quantitative trait loci (eQTL) analysis using BRAINEAC and Genotype-Tissue Expression (GTEx) databases in twelve brain tissues. At *P*=0.05, rs73284230 was not an eQTL in any brain tissues in any dataset. However, because of its low allelic frequency this analysis was underpowered. In BRAINEAC the effect allele (C) of rs11976703 was associated with lower mRNA levels of MPP6 in brain tissues averaged, with a p-value of 3.6×10^{-4} [32]. Furthermore, rs11976703 was an MPP6 eQTL in the GTEx dataset in the same direction in the frontal cortex with p-values of 9.56×10^{-3} (Supplementary Figure 5). Although this association P-value did not cross the strict Bonferroni correction for 12 tissues ($P=4.2 \times 10^{-3}$), substantial correlation of expression in GTEx brain tissues suggests employment of this threshold to be very conservative [33]. Additionally, some brain tissues are duplicates of each other in the GTEx dataset (for example, cortex and frontal cortex) [33]. Finally, in the GTEx dataset this same allele (C) of rs11976703 is also associated with lower mRNA levels of NPY in the anterior cingulate cortex, the cerebral hemisphere, the cerebellum and the frontal cortex with p-values ranging from 0.05 to 1.4×10^{-4} (Supplemental Figure 6).

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Functional Validation in vivo

To investigate the functional role of genes on chromosome 7 in regulating sleep quality *in vivo*, we used the fruit fly *Drosophila melanogaster* [60]. In previous studies, *NPF,* the fly ortholog for *NPY*, has been implicated in suppression of sleep during starvation [61]; however, nothing is known about the role of *Drosophila* ortholog of *MPP6* (*varicose*) in sleep. In the fruit fly, sleep quality is controlled by lateral ventral neurons (LNv) in the brain which can be specifically manipulated using the driver *PDF-Gal4* [50]. Using transgenic RNAi (*UAS*-Inverted repeat; *UAS-IR*), we generated *varicose* knockdown flies specifically within LNv neurons (*PDF-Gal4> UAS-vari$^{IR1/2}$*). Sleep patterns for these flies were then compared to parental controls (*PDF-Gal4/+* or *UAS-vari$^{IR1/2}$/+*). Firstly, we did not observe any difference in circadian behavior between parental controls and transgenic flies (Figure 4a). Next, although no difference was observed in daytime sleep behavior, *PDF-Gal4>UAS-vari$^{IR1/2}$* flies showed a marked reduction in overall nighttime sleep duration (Figure 4b) without a difference in sleep fragmentation assessed by the total number of sleep episodes (Figure 4c). Accordingly, we observed longer sleep episode duration in day time in *PDF-Gal4>UAS-vari$^{IR1/2}$* animals (Figure 4d). Together, our results show reduced sleep time during the night and increased durations of sleep episodes during day in *MPP6* RNAi flies which represents poor sleep homeostasis [62] and this may be a proxy for poor sleep quality. The poor sleep during the night appears to be compensated by longer sleep duration bouts during the day that can serve as consolidation. This effect supports the GWAS finding since the effect allele associated with worse sleep was an eQTL with lower mRNA levels of both *MPP6* and *NPY*. Overall, we concluded that *varicose* (*MPP6*) expression plays a major role in night time sleep maintenance *in vivo*.

Conclusion/Discussion

In this study, a GWAS analysis revealed novel loci and genes associated with sleep quality, measured by the validated PSQI questionnaire. Overall, 15% of the variation in the global score of PSQI was explained by the combined additive effects of assessed SNPs. Previous twin studies showed that the PSQI global score is highly heritable (34-37%) [19,63]. In this study, the heritability estimate for PSQI global score was lower, commonly observed when comparing SNP-based heritability estimates with twin studies. However, the reported SNP-based heritability estimate was within the range of what was previously reported from GWAS for other sleep traits; i.e. 11.5% for insomnia disorder [64] and 10.3% for self-reported sleep duration [9].

The two loci reported here, on chromosomes 2 and 7, were not previously reported to be linked to any sleep phenotype in human association studies. *ATOH8* has been implicated in the development of the nervous system and muscles but has not been reported to be related to sleep [65]. *ATOH8* is a transcription factor that recognized an E-box element and regulates transcription of genes [66]. More than 9 circadian clock genes are E-box-regulated genes and occur in a rhythmic fashion [67]. Whether *ATOH8* specifically binds a circadian clock gene is unknown, but our genetic association could open the door to new research avenues, though we recognize that this finding was not replicated in independent cohorts.

The locus situated downstream of *NPY* and upstream of *MPP6* was replicated in independent cohorts. The *NPY* gene codes for Neuropeptide Y, the most abundant peptide of the central nervous system and a master regulator of stress response, circadian and feeding rhythms through afferent projections from the hypothalamus [68,69]. Neuropeptide Y has been found to promote sleep in humans and zebrafish [70,71]. *NPY* was also identified as an important candidate gene in sleep regulation in *D. melanogaster* and *C. elegans* [72,73].

Through a genetic screen in zebrafish, it was reported that the overexpression of NPY increases sleep by inhibiting noradrenergic signaling [71], while in the fly NPF integrates feeding and sleep behavior [61]. Importantly, no genetic association between *NPY* and sleep quality in humans has been reported previously.

MPP6 codes for membrane palmitoylated protein 6, a member of the peripheral membrane-associated guanylate kinase (MAGUK) family. This family includes synaptic scaffolding proteins DLG1 and PSD95 [74], which have never been previously associated with any sleep related phenotype. In *D. Melanogaster*, this gene was previously shown to regulate the formation of the fly respiratory system [75] but has not yet been implicated in regulating sleep.

Interestingly, the collective evidence suggests that the locus on chromosome 7 identified in our study affect functions of two genes simultaneously, *NPY* and *MPP6,* where *NPY* is a known sleep gene and *MPP6* is a novel sleep gene. This possibility would be in line with the relatively strong effect of the identified locus on sleep phenotypes. In tissue-expression analysis, the effect allele of the top SNP was found to be associated with lower *MPP6* and *NPY* expressions in many brain tissues, including the cerebral hemispheres, the anterior cingulate cortex, the cerebellum and the frontal cortex. The direction of association was consistent between brain tissues and validated in two independent datasets. Taking into account our genetic findings, where the effect allele was associated with higher PSQI global scores, hence worse sleep quality, our genetic results suggest that lower levels of MPP6 and NPY expression are associated with worse sleep, which is in agreement with what is known for NPY [68,71].

We then decided to take an additional step and test the functionality of MPP6 *in vivo*, demonstrating that genetic knockdown of the fly *MPP6* ortholog *varicose* resulted in altered

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sleep homeostasis (worse sleep) compared to parental controls. Although the behavioral translation between species is difficult to interpret, the direction of the effect is consistent. The association of *MPP6* RNA with chromosome 7 locus can be a consequence of genetic co-regulation of these two genes resulting from the physical proximity in the human genome, but in *D. Melanogaster* these two genes are not situated at the same gene locus. Thus, our fly experiments reemphasize the independent functional role of the *MPP6* gene in sleep.

MPP6 acts centrally as the conditional knockdown was done in the sleep center neurons (LNv) of the fly brain. In humans, *MPP6* is known to be expressed in the central nervous system; however, its function is still unknown. *MPP6,* previously named VAM-1, is expressed across the central nervous system, with highest expression in the cerebellum, the caudate and in the pituitary gland. Its sequence is predicted to contain a conserved PDZ domain that binds to veli-1. Veli-1 is a protein that helps couple synaptic vesicle exocytosis with neuronal cell adhesion, suggesting that it promotes the assembly of veli-1 containing protein complex in neurons [76]. Veli-1 also binds PDZ-motif containing proteins that are known to contribute to receptor clustering complexes at the post-synaptic levels [77].

Our pathway analysis of the full GWAS revealed pathways that are in line with MPP6's function. Pathways such as regulation of synaptic structural plasticity and receptor internalization point towards an important genetic contribution of processes occurring at the synaptic level and towards a role for receptor anchoring at the synapse. Many previously identified molecules such as neuroligin, neuropeptides, ion channels, vesicle proteins and scaffolding molecules have been shown to be regulated by sleep homeostasis and circadian rhythms [78]. Indirect evidence from our pathway analysis, the role of MPP6 in synaptic receptor clustering and ATOH8 as a transcription factor for clock genes, support this hypothesis. These findings open the door to future research that should focus on the role of *MPP6* in neuronal communication in brain structures that are important in sleep processes as

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well as sleep homeostasis using complementary methods such as electroencephalography in mouse models.

In this study, in line with many other reports [4], we show that there is a significant relationship between the report of pain conditions, like TMD and back pain, with poor sleep quality. Moreover, we show that there is a positive correlation between psychological factors, like anxiety and depression, with poor sleep quality, as well as a positive correlation between experimental heat pain sensitivity and poor sleep. These epidemiological associations did not translate into genetic correlation findings in our study, probably due to modest size of a discovery cohort. On the other hand, based on publicly available data, our lead SNP was previously shown to be associated with cardiovascular disease and BMI. This finding is in line with poor sleep being a predisposing factor to poor cardiovascular health. The pathway by which NPY and MPP6 participate in the manifestation of cardiovascular disease remains unknown. Further work is needed to determine if variations in NPY and MPP6 are independent or show causal links between poor sleep quality and cardiovascular disease.

This study presents some limitations that should be addressed. First, even though we demonstrated that we can identify true positives in our discovery cohort, we have to acknowledge that the sample size of our discovery cohort is small. A larger cohort would have more power to identify additional loci and stronger associations. Second, the phenotype that we used to assess sleep quality, the PSQI, is based on self-report although through a validated questionnaire and not on objective assessment of sleep with polysomnography. Our study's phenotype is a compromise between the use of a self-reported unspecific question derived from a large dataset of hundreds of thousands of individuals and a smaller study with comprehensively assessed polysomnography but lacking power for a genome-wide analysis. In an ideal scenario, our results should be validated with more objective measures including actigraphy and polysomnography. Third, in a majority of replication cohorts, the phenotypes

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chosen for replication were closer to insomnia than sleep quality. While the PSQI has a number of questions related to insomnia, it also has non-insomnia questions and has only modest correlations with insomnia questionnaires. The heterogeneity in the phenotypes might explain why replication was weaker in certain cohorts.

In summary, we performed a discovery GWAS of sleep quality and identified two novel sleep loci. In a meta-analysis of twelve independent cohorts, we replicated the association with one locus on chromosome 7, situated between two genes, *NPY* and *MPP6*. Our eQTL analysis established the association of this locus in an allelic-dependent expression of both *NPY* and *MPP6*. While *NPY* is thought to be important for sleep [68], *MPP6* has not previously been implicated in regulation of sleep. Using sleep center-specific gene knockdown in the fly, we showed that decreasing levels of fly ortholog of *MPP6* leads to altered sleep homeostasis (i.e. worse sleep) *in vivo*, establishing its functionality. Overall, these data are consistent with the observed allelic association between *MPP6* and human sleep quality. Our results have broad biological significance by recognizing the role of *MPP6* as a novel sleep gene, potentially involved in synaptic processing in sleep centers. This work provides new insights into sleep biology and our findings should spur future investigation of mechanisms involving *MPP6* in sleep.

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Author contributions

SK, LD: scientific conception, drafted manuscript, analyzed data. QPW, MP, PG, AVB, SL, AT, TS, TL, JL, MAK, HMM, SBS, GGN: analyzed data. SAM, EAK, SR, IB, AN, GDS, RBF, RO, JDG, WM: data acquisition and interpretation. All authors revised the manuscript.

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Figure captions list

Figure 1: Regional association plots of discovery GWAS for PSQI global score in OPPERA (a-b) Regional association plots for genome-wide significant loci at chromosome 7 using EUR as a reference panel (a) and AFR panel (b). (c-d) Regional association plot at chromosome 2 using EUR as reference panel (c) and AFR panel (d). (e-f) Regional association plots for suggestive loci at chromosome 13 using EUR as reference panel (e) and AFR panel (f) Chromosomal position (Mb) is indicated on the x axis, and the –log10 p-value is indicated on the y axis. Each SNP is plotted as filled circle and the lead SNP is shown in purple. The genes within each region are shown in the lower panel. Recombination sites and rates are shown in blue. Additional SNPs in the locus are colored according to linkage disequilibrium (r2) with the lead SNP. rs78633772 instead of rs376585198 as the latter is not in the reference panel.

Figure 2: Pathway analysis of sleep GWAS using Gene ontology's biological process. Horizontal bar plots represent –log10 p-value enrichment of pathways. The red line represents Bonferroni threshold for statistical significance.

Figure 3: Forest plots in meta-analysis. Forest plots of standardized effect size with 95% confidence interval for each replication study as well as for the fixed-effect meta-analysis for genome-wide significant SNPs. Higher effect sizes represent worse sleep quality. The discovery cohort was excluded from the meta-analysis calculation. The test of heterozygosity for each SNP was Q(df 11)=16.29 P=0.13 for rs11976703; Q(df 10)=20.52 P=0.03 for rs73284230 and Q(df 8)=6.74 P=0.57 for rs60869707. The sample sizes for the meta-analysis is 100659, 99931, and 96386 for rs11575542, rs73284230, and rs60869707 respectively.

Figure 4: LNv-specific knockdowns of *Varicose* in D. Melanogaster. PDF-Gal4 is a neuron-specific driver. UAS-IR represents transgenic RNAi inverted repeats. PDF-Gal4/+, UAS-VariIR1-2/+ are parental control flies. PDF-Gal4>UAS-variIR1-2 are *varicose* knockdown flies within LNv neurons. (a) Circadian pattern of sleep (n=28-32). (b) Percentage of sleep in day time and night time (n=28- 32). (c) Number of sleep episode in day time and night time (n=28-32). (d) Sleep episode duration in minutes in day time and night time (n=28-32). Data presented as mean ± S.E.M. Statistics were determined one-way ANOVA with Tukey's multiple comparisons test. n.s., not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.001$.
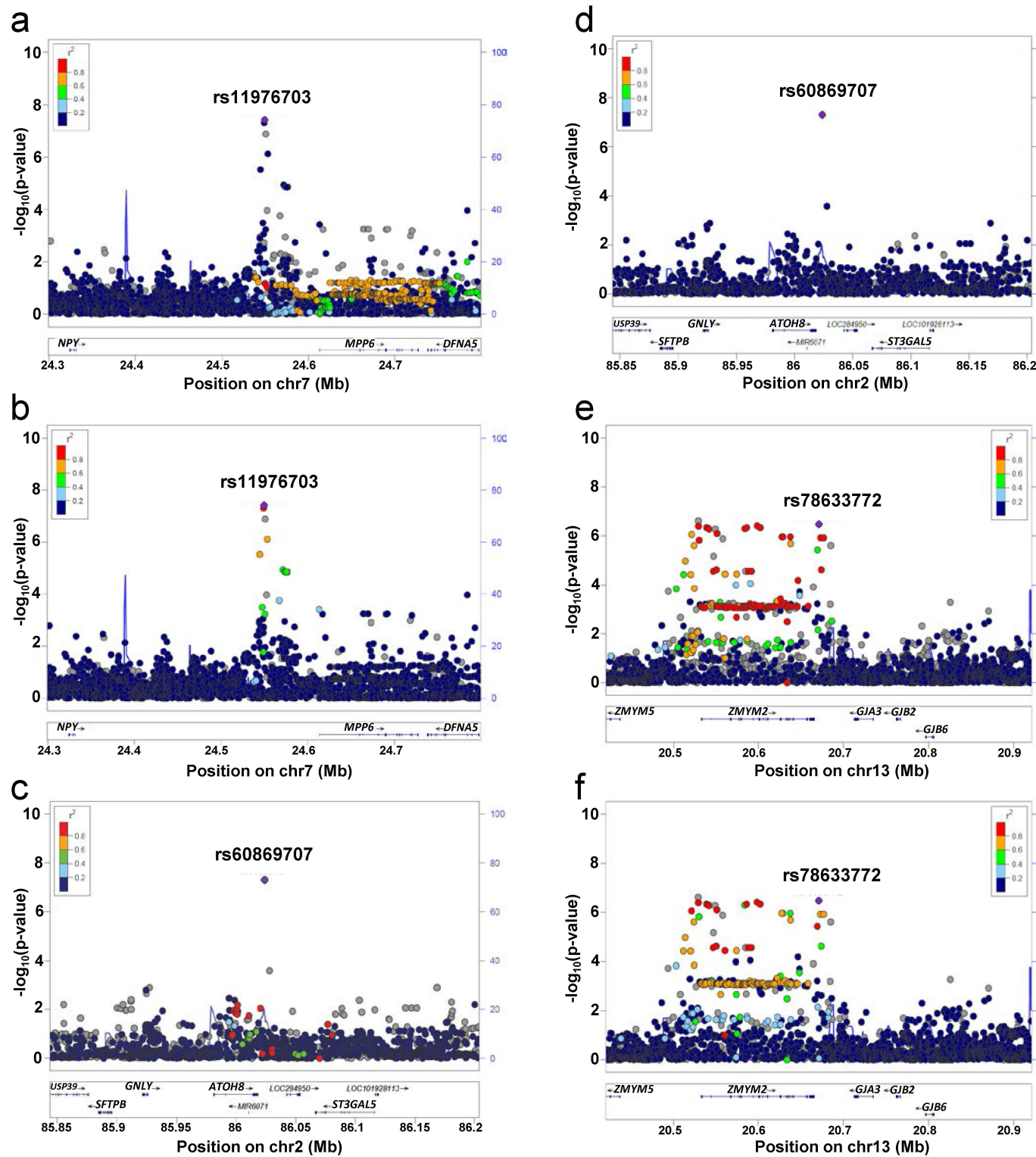
Figure 1: Regional association plots of discovery GWAS for PSQI global score in OPPERA
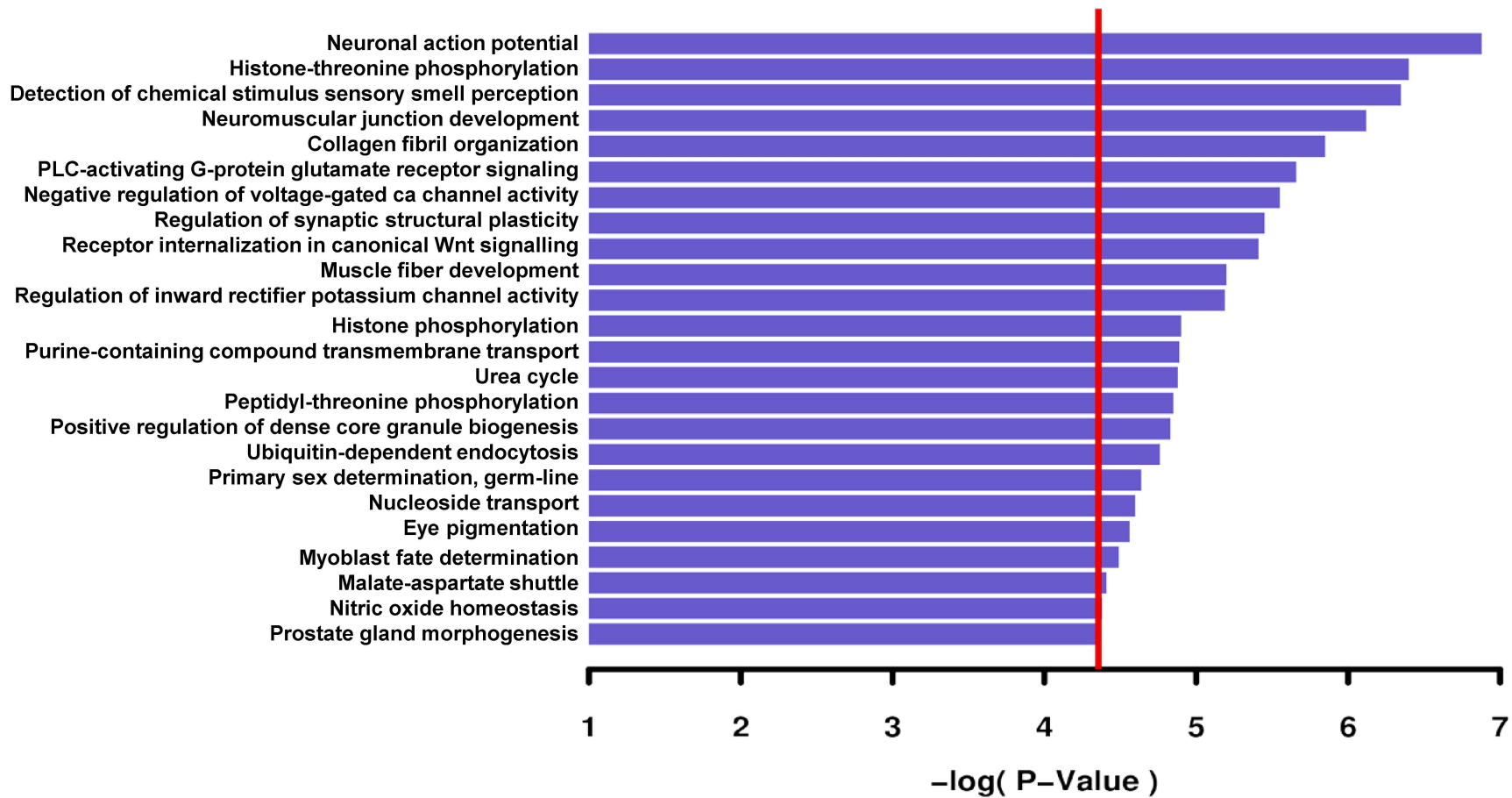
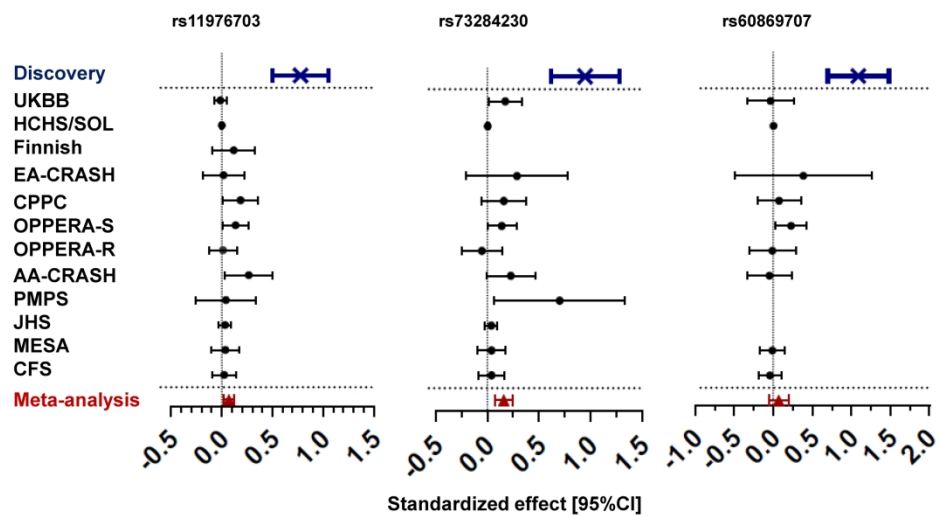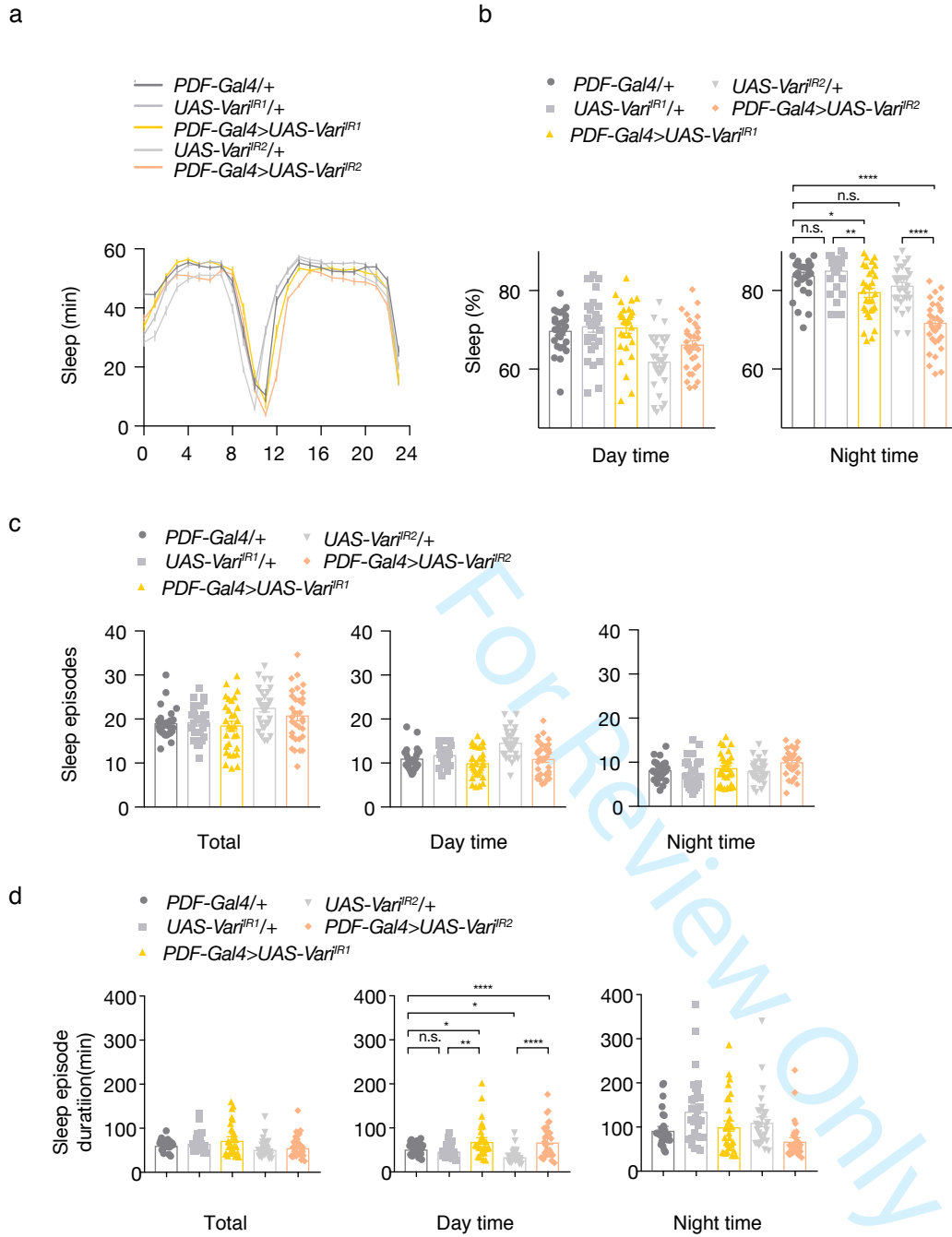**Figure 2: Functional annotation of discovery GWAS.**

Figure 3: Forest plots of replication meta-analysis.

Figure 4

Figure 4: LNv-specific knockdowns of *Varicose* in *D. Melanogaster.*