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ORIGINAL ARTICLE

Genotype Influences Day-to-Day Variability in Sleep in *Drosophila melanogaster*

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Abstract

Patterns of sleep often vary among individuals. But sleep and activity may also vary within an individual, fluctuating in pattern across time. One possibility is that these daily fluctuations in sleep are caused by the underlying genotype of the individual. However, differences attributable to genetic causes are difficult to distinguish from environmental factors in outbred populations such as humans. We therefore employed *Drosophila* as a model of intra-individual variability in sleep using previously collected sleep and activity data from the *Drosophila* Genetic Reference Panel, a collection of wild-derived inbred lines. Individual flies had significant daily fluctuations in their sleep patterns, and these fluctuations were heritable. Using the standard deviation of sleep parameters as a metric, we conducted a genome-wide association study. We found 663 polymorphisms in 104 genes associated with daily fluctuations in sleep. We confirmed the effects of 12 candidate genes on the standard deviation of sleep parameters. Our results suggest that daily fluctuations in sleep patterns are due in part to gene activity.

Statement of Significance

Previous studies in humans have observed intra-individual variability in sleep phenotypes, and there may be a connection between this variability and both sleep and mental health disorders. This study, conducted in *Drosophila*, reveals that day-to-day fluctuations in sleep are due, in part, to the underlying genotype of the individual. We identified 104 candidate genes for intra-individual variability, some of which have predicted homology with human genes. We confirmed the effects of 12 of these genes on intra-individual variability. Daily fluctuations in sleep in flies are thus regulated in part by genes, and these results may extend to human sleep as well.

Keywords: intra-individual variability, sleep standard deviation, *Drosophila melanogaster*, sleep

Introduction

Although the need to sleep is persistent within a species, the manner in which that need is fulfilled often varies considerably among individuals. Modern-day human populations living in pre-industrial environments have

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characteristic sleep patterns consistent with ecological needs [1]. Sleep in some groups features a single bout of sleep at night with rare instances of napping [2], whereas others have a combination of night sleep bouts and daily napping [1, 3]. Historical evidence suggests that humans typically had two sleep bouts during the night [4], and this differs from the modern concept of a single night sleep bout. Although different among individuals, some measures of sleep and sleep-related characteristics are remarkably stable within individuals and are characterized by low within-individual variance [5–8].

However, some characteristics of sleep are highly variable within an individual. Often measured as the standard deviation or coefficient of variation [9–14], total sleep time or time in bed [13, 15–17], sleep onset latency [13, 15, 16], sleep quality or efficiency [15, 16], daytime sleepiness [17], and wake after sleep onset [13, 15, 16] can be more variable within a single individual than among individuals. This intra-individual variability has been observed using sleep diaries, actigraphy, and polysomnography in both children [13, 16, 17] and adults [13, 15].

Although the causes and consequences of daily fluctuations in sleep are not understood, one intriguing possibility is that high intra-individual variability in sleep is a hallmark of sleep disorder and mental illness. Greater variability in night-to-night sleep characteristics has been observed in those with chronic insomnia than in controls [18, 19], and patients with post-traumatic stress disorder (PTSD) exhibit exceptionally fragmented sleep patterns [20]. Daily fluctuations in obstructive sleep apnea (OSA) severity class and periodic leg movements have been observed as well [21, 22]. Variability in sleep duration has been observed in those with bipolar disorder [23, 24], as has interdaily stability, the similarity of activity patterns across days [24]. Intra-individual variability in activity patterns, including the amount, predictability, and intricacy, is also associated with bipolar disorder (reviewed in Ref. [25]). Intra-individual variability in sleep onset time and duration in children could be used to classify those with attention deficit hyperactivity disorder (ADHD), a distinction that could not be made using the averages of sleep parameters [14]. Correlates between physiological endophenotypes and high intra-individual variability in sleep characteristics have also been described, including flattening cortisol responses [26], increasing blood concentrations of inflammatory markers [27], and abdominal obesity [28].

Stress, work demands, iron deficiency, and moderate exercise can all mediate intra-individual variability in sleep [10–12, 29, 30]. Thus, intra-individual variability in sleep can be said to be plastic, i.e., it can be altered in response to changes in the environment [31]. The degree to which an individual can adapt to changing environmental conditions is ultimately due to the underlying genotype, however. Few studies have attempted to map genes influencing intra-individual variability in sleep thus far. Linkage mapping in humans identified two candidate genes, JARID1a and CACNA1C, associated with interdaily stability [24]. Spada and colleagues attempted to map sleep onset irregularity, measured as the standard deviation of the timing of sleep onset, but the associations did not reach their genome-wide threshold of significance, though some nominal associations were found [32]. These difficulties occur in part because of the difficulties in estimating trait variability in an outbred population accurately. Sources of heritable genetic variance and environmental variance are confounded in outbred populations, making it challenging to distinguish between the two [33].

Model organism populations have the potential to address this problem. Model organisms can be inbred so that many simultaneous measurements can be made on a single genotype, and environmental conditions can be controlled to a greater extent. Previously, model organism populations have been used to study the genetic basis of inter-individual variability in morphological and fitness traits in *Arabidopsis* and maize [34–36]; gene expression in isogenic strains of yeast [37, 38] and inbred populations of flies [39]; and wing shape [40], bristle number [41], food consumption [42], locomotor handedness [43], chill coma recovery, startle response, starvation resistance [44], and sleep [45] in flies. Measured as the coefficient of environmental variation (CV_E), the differences in sleep parameters among individual flies were found to have a heritable, mappable basis [45]. Thus, model organism populations offer the opportunity to investigate among-animal variability and its genetic underpinnings.

We wanted to determine whether this strategy could also be applied to study intra-individual variability in sleep, and if so, whether this variability had an underlying genetic cause. We used previously collected data from a genome-wide association study of the *Drosophila* Genetic Reference Panel (DGRP) to investigate this possibility [45]. We examined daily fluctuations in the sleep patterns of 10 239 flies. Daily fluctuations in sleep were more variable in some genotypes than in others, revealing that the variation is heritable. Expressing the variability in sleep traits as a standard deviation enabled us to conduct a genome-wide association study of intra-individual variability. We mapped 104 candidate genes to seven sleep parameters varying over days in flies. These genes included plausible candidates from previous studies in flies and mapped to 51 human homologs. Day-to-day fluctuations in sleep characteristics may therefore depend in part upon underlying genotype.

Methods

Assessment of Individual Differences in Day-to-Day Sleep

We reanalyzed previously collected [45] data to determine whether sleep fluctuated across days in individual flies and whether that fluctuation had a heritable

component. In that experiment, 168 lines of flies from the DGRP [46, 47] were randomly divided into four equal blocks with four replicates. Each replicate had eight flies per line per sex, for a total of 64 flies per line. Flies were assayed for rest and activity behavior under standard environmental conditions (cornmeal–molasses–agar medium, 25°C, 60%–75% relative humidity, and 12-hr light:dark cycle). Virgin males and females were assayed in order to avoid any effect of mating status on sleep [48], and 30 same-sex flies were maintained per vial for 4 days to standardize social exposure effects [49]. Sleep and activity recordings were made for each fly using the *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA). Seven days of recordings were made for each fly; the first day was discarded to mitigate any potential effect of CO_2 anesthetization recovery on sleep and activity. The flies were visually inspected after the experiment and data from dead flies were eliminated from the data set. The raw sleep and activity data were used to calculate sleep parameters for each day using an in-house C# program. Sleep duration, numbers of sleep bouts, and average sleep bout length during the day and night were calculated. In addition, waking activity, the number of activity counts per minute spent awake, was determined. We assessed the change in sleep parameters across days using the four-way random ANOVA model, $Y = \mu + D + B + L(B) + R(B) + D \times L(B) + D \times R(B) + L \times R(B) + D \times L \times R(B) + \varepsilon$, where D represents the random effect of day, B represents the random effect of block, L represents the random effect of line, R represents the random replicate effect, and ε represents the error variance. Analyses were performed separately for males and females. A significant D term indicates that the sleep phenotype is different across days, whereas a significant $D \times L(B)$ term indicates that the fluctuation in a sleep parameter across days has a heritable component. In addition, we evaluated males and females of each line separately for day effects using the reduced model, $Y = \mu + D + R + D \times R + \varepsilon$. We used SAS 9.3 (SAS Institute, Cary, NC) to evaluate these models.

Quantitative Genetic Analysis

We found a significant $D \times L(B)$ term for all the sleep traits, indicating that sleep was variable across days and that a portion of this variability was under genetic control (Supplementary Table S1). We used the within-fly standard deviation in sleep across days, σ, to represent the variability in each sleep trait per fly [9–13]. We noted that average day bout length σ and waking activity σ were not normally distributed traits. We normalized these two traits by taking the natural log of σ for each fly. We analyzed σ for each trait using the ANOVA model, $Y = \mu + B + L(B) + S + R(B) + S \times L(B) + L \times R(B) + S \times R(B) + S \times L \times R(B) + \varepsilon$, where $B, L,$ and R are as defined above, and S is sex. We analyzed the data for each sex separately using the reduced model, $Y = \mu + B + L(B) + R(B) + L \times R(B) + \varepsilon$. The variance components for each model were estimated using the restricted maximum likelihood (REML) method. The broad sense heritability for each trait was estimated as $H^2 = (\sigma_L^2 + \sigma_{SL}^2) / (\sigma_L^2 + \sigma_{SL}^2 + \sigma_\varepsilon^2)$ for both sexes combined, where σ_L^2 is the line variance component, σ_{SL}^2 is the line by sex variance component, and σ_ε^2 is the sum of the other variance components [50]. The broad sense heritability was estimated as $H^2 = (\sigma_L^2) / (\sigma_L^2 + \sigma_\varepsilon^2)$ for each sex separately. We calculated the cross-sex genetic correlation as $r_{mf} = (\sigma_L^2) / (\sigma_{LM}^2 + \sigma_{LF}^2)$, where σ_L^2 represents the line variance component for both sexes combined, σ_{LM}^2 represents the line variance component for males, and σ_{LF}^2 represents the line variance component for females [50]. In addition, we computed phenotypic correlations between sleep σ traits and other traits measured in the DGRP having line means available, including mean sleep and sleep CV_E [45]; morphological traits (eye area and size, pigmentation level, wing centroid size, and eye interocular distance) [51–53]; lifespan and metabolic traits [54, 55]; behavior (reproductive or courtship traits, food consumption, chemosensation, startle response, geotaxis, and aggression) [42, 44, 56–61]; environmental responses (oxidative stress, methylmercury tolerance, radiation stress, chill coma, and starvation) [44, 61–64]; and genome size [65]. Phenotypic correlations were calculated as Pearson product moment correlation coefficients. We also estimated the genetic correlations r_G between mean sleep and sleep σ traits as $cov_{12} / \sqrt{\sigma_{L1}^2 \times \sigma_{L2}^2}$ [50]. SAS 9.3 and JMP 13.0.0 (SAS Institute, Cary, NC) were used to evaluate these models.

Genotype–Phenotype Associations

Whole-genome sequence data are available for the DGRP, and a website tool (http://dgrp2.gnets.ncsu.edu/, accessed 21 December 2017) is available to calculate genome-wide associations among variants in this population and any trait of interest [46, 47]. Using the average σ per line for each sleep trait, we computed genome-wide associations with 1 920 276 polymorphisms segregating in the DGRP. Only polymorphic variants with minor allele frequencies of 5% or more were used in the associations. Some lines of the DGRP are known to have *Wolbachia pipientis* infection and large chromosomal inversions. We checked for associations between sleep σ parameters and *Wolbachia* and inversions. No significant associations with *Wolbachia* infection were present, but there were significant associations with chromosomal inversions for average day bout duration σ (associated with the *In(2R)NS* and *In(3R)Mo* inversions, $p = .0003$ and $p < .0001$, respectively), average night bout duration σ (associated with

In(2L)t, $p = .0144$), night bout number σ (associated with *In(2R)NS*, $p = .0051$), and waking activity σ (associated with *In(3R)Mo*, $p = .0008$). We applied the linear mixed model, $y = Xb + Zu + \varepsilon$, where y are the adjusted phenotypes, X is the fixed effect of polymorphisms, and Z represents the covariance matrix accounting for population structure [46]. Associations with a p-value of 1×10^{-5} or less were called significant, a threshold applied in many DGRP studies [42, 52, 57, 66–70]. This threshold was supported by Q–Q plots (Supplementary Figure S1), which generally deviated little from expected values. In addition, false discovery rates (FDRs) were calculated for each polymorphic association using the method of Benjamini and Hochberg [71]. We determined the linkage disequilibrium (LD) (r^2) among significant polymorphisms using PLINK [72]. Finally, human homologs of *Drosophila* genes were identified using the DIOPT website, which compiles homology data from multiple genomic databases [73].

Sleep Assays and Phenotypes

For all sleep assays, flies were maintained in a constant temperature (25°C), constant humidity (60%) incubator and assayed on standard food (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm, accessed 21 December 2017) in standard light:dark cycle (12:12 hr) conditions. Male and female virgins were collected and maintained in single-sex vials of 20 flies for 4 days before sleep measurements to mitigate potential effects of mating status [74] and social exposure [49]. We loaded individual flies into Drosophila Activity Monitors (DAM2, Trikinetics, Waltham, MA) to measure sleep and activity parameters. Numbers of activity counts for each fly were recorded per minute; we defined sleep as 5 min without an activity count [75]. We discarded the first day's data as the flies would be recovering from CO_2 anesthesia and/or adjusting to the monitor tubes during the first day. We examined each fly after the experiment; the data from flies not surviving the experiment were discarded. We used an in-house C# program to calculate sleep parameters for each day: night, day, and 24-hr sleep duration in minutes; night and day sleep bout number; night and day average sleep bout length; sleep latency, the number of minutes before the fly's first sleep bout after lights are turned off; and waking activity, the number of activity counts per minute spent awake. Using these parameters, we calculated the standard deviation of each sleep trait over the 6-day monitoring period for each fly.

Sleep Deprivation Protocol

The most variable DGRP lines were deprived of sleep using the following procedure. We placed individual flies into the DAM2 Trikinetics monitors using the protocol above. The flies acclimated to the monitors for a single day, and then we recorded 2 baseline days of rest and activity. We used a vortexer (Troemner, Thorofare, NJ) fitted with a custom mounting plate (Trikinetics, Waltham, MA) to apply a mechanical stimulus on the night of the third day. Each minute, the vortexer gently shook the flies for 5 s every minute of the 12-hr night sleep period (shaking speed = 2.0). The flies' recovery was monitored for 2 additional days. An identical set of flies were loaded into Trikinetics monitors on a different shelf of the same incubator to serve as nonsleep-deprived controls. We used this protocol to deprive the following DGRP lines: DGRP_41, DGRP_73, DGRP_153, DGRP_335, DGRP_338, DGRP_409, DGRP_646, and DGRP_892. We sleep-deprived 16 flies of each sex per line. We used an in-house Python algorithm to determine the average minutes of sleep per 30 min. We evaluated differences in 24-hr sleep for each DGRP line separately using the ANOVA model, $Y = \mu + T + D + S + T \times D + T \times S + D \times S + T \times D \times S + \varepsilon$, where T is the control or sleep-deprived treatment, D is day, and S is sex. We analyzed the data for each population or line separately using the reduced ANOVA model, $Y = \mu + D + S + D \times S + \varepsilon$, where D and S are as defined above. We applied a post hoc Tukey analysis to identify significant loss and gain of sleep.

Verification of Candidate Genes

We tested 17 Minos element (Mi{ET1} and Mi{y[+mDint2] = MIC}) insertion lines in 14 candidate genes identified by the GWAS: *5-hydroxytryptamine* (serotonin) *receptor 1A* (*5-HT1A*), *CG42260*, *CG5888*, *CG7985*, fruitless (*fru*), *histone deacetylase 4* (*HDAC4*), *rhomboid* (*rho*), *scab* (*scb*), *sallimus* (*sls*), *solwind* (*sowi*), *super sex combs* (*sxc*), *Transmembrane channel-like* (*Tmc*), *turtle* (*tutl*), and *ZnT35C* (Supplementary Table S2). The inserts for *sls* and *sowi* were not viable as homozygotes and were tested as heterozygotes. Lines with Mi{ET1} insertions were created in an isogenic w^{1118} background (w^{1118}[5905]); hence, we used this background as a control [76]. Lines with Mi{y[+mDint2] = MIC} insertions were crossed to a common background when created: *y*[1] *w*[*]; *nub*[2] *b*[1] *sna*[Sco] *pr*[1] *cn*[1]/*CyO* [3628] [77]. We used this background as a control for lines with the Mi{y[+mDint2]=MIC} insertion. All stocks were obtained from the Bloomington, IN stock center. Sleep phenotypes were measured as described above for 16 flies per sex per genotype in the mutants and their respective controls. Mutant sleep σ phenotypes were compared with the control using the ANOVA model, $Y = \mu + G + S + G \times S + \varepsilon$, where G is genotype, S is sex,

and ε is the error term. Males and females were analyzed separately using the reduced ANOVA model, $Y = \mu + G + \varepsilon$.

Results

Quantitative Genetics of Day-to-Day Variability in Sleep

Daily fluctuations occurred in all sleep traits among individual flies. Figure 1 contrasts night sleep traits among individuals from the least variable DGRP line with those of the most variable line, illustrating how extreme the differences can be (for day sleep traits, see Supplementary Figure S2). Night sleep duration in DGRP_41, the least variable line, was high and changed little across time (Figure 1A). In contrast, sleep in DGRP_153, the most variable line, fluctuated through the full range of possible night sleep values (Figure 1A). Numbers of night bouts were relatively low in DGRP_531, but fluctuated greatly in DGRP_882 (Figure 1B). Stable and fluctuating patterns were also observed for average night bout length (Figure 1C). These daily fluctuations among individuals translated into changes in overall patterns of sleep for some DGRP lines. For example, male flies of DGRP_181 had increasing night nap length over the course of 6 days; simultaneously, the average number of night bouts decreased, whereas overall night sleep duration stayed constant (Figure 2A). Thus, these males had less fragmented sleep patterns over the 6-day observation period. Females of DGRP_761, on the other hand, had increased numbers of nightly naps and decreased average nap times, suggesting an increasingly fragmented

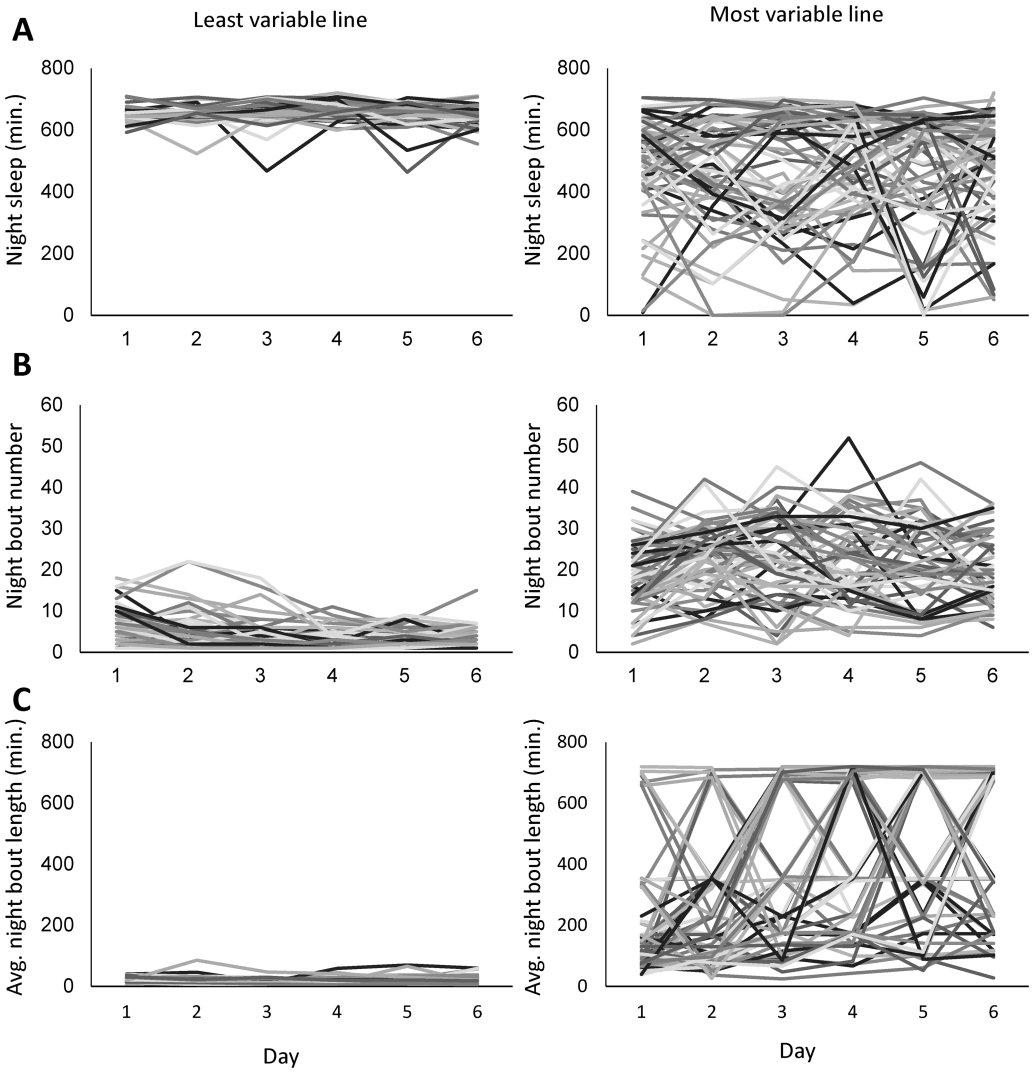

Figure 1. Example of variability in night sleep among individual flies. The plots contrast sleep changes over days for the least variable DGRP line with the most variable DGRP line for that trait. (A) Night sleep duration. The least variable line is DGRP_41 (n = 64); the most variable line is DGRP_153 (n = 61). (B) Night bout number. The least variable line is DGRP_531 (n = 62); the most variable line is DGRP_882 (n = 54). (C) Average night bout length. The least variable line is DGRP_42 (n = 64); the most variable line is DGRP_338 (n = 64).

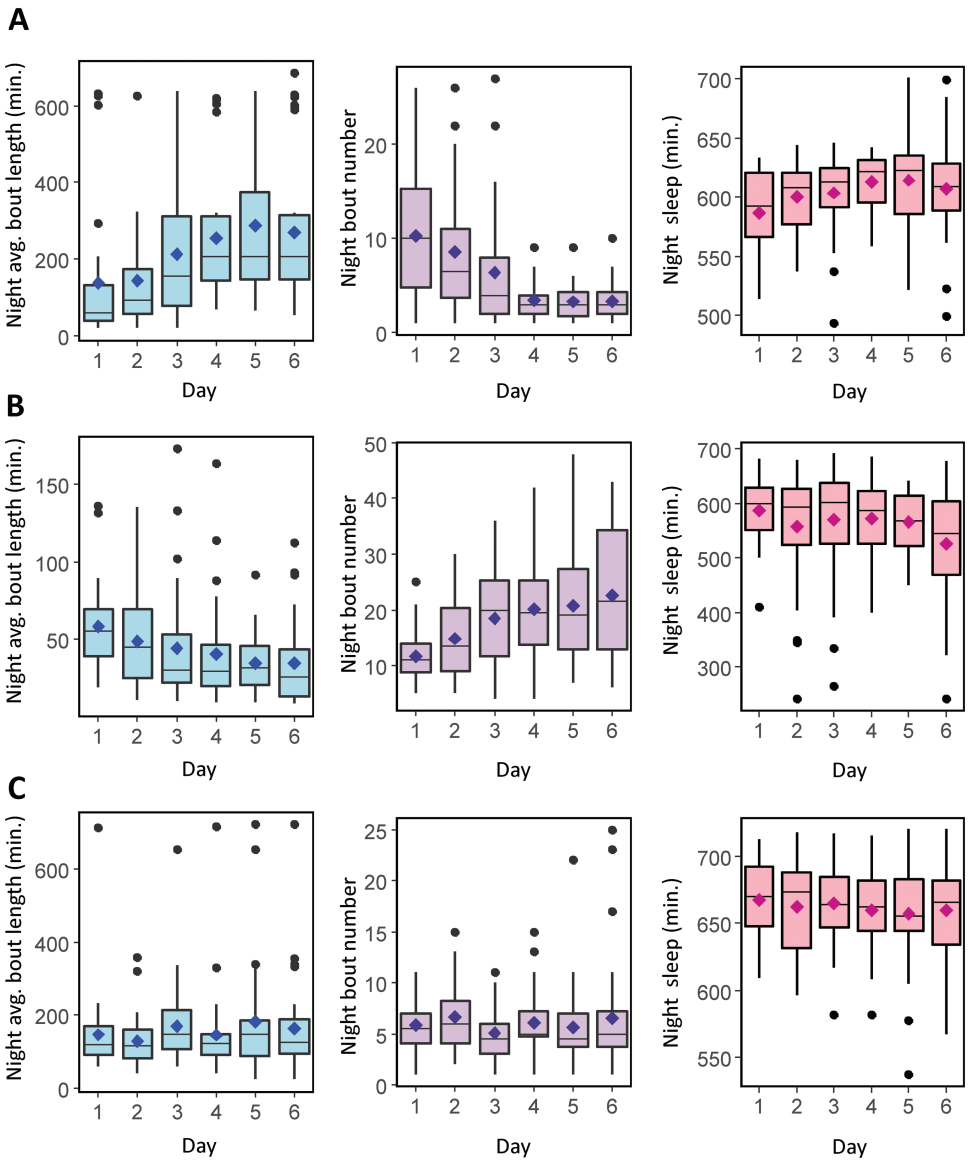

Figure 2. Examples of changes in night sleep parameters over days. Box plots of night sleep duration, night bout number, and average night bout length are plotted for each day. (A) Males of DGRP_181 (n = 32). (B) Females of DGRP_761 (n = 32). (C) Males of DGRP_73 (n = 32).

pattern (Figure 2B). In addition, some flies, such as males of DGRP_73, had stable sleep characteristics over time (Figure 2C). We analyzed the entire population for significant changes across day. With the exception of day sleep duration in females, all sleep traits varied significantly across time (Supplementary Table S1). We observed a highly significant interaction of day and line for all sleep traits (Supplementary Table S1; all $p_{\text{Line×Day(Block)}} < .0001$), suggesting that this variability was heritable. We also used a reduced model ANOVA to determine the numbers of DGRP lines with significant day-to-day fluctuations in sleep (Table 1). Waking activity was variable for nearly every line, with 87.5% and 91% of the lines having significant daily fluctuations in males and females, respectively. Average day bout length was the least variable trait, with only 26.2% of the lines varying across day. These results show that daily fluctuations in sleep parameters are common in flies, with some genotypes exhibiting stability while others are characteristically variable.

Lines with characteristically variable sleep could also potentially have altered responses to disruption of the sleep homeostat. We examined the response of several DGRP lines to a single night of mechanical sleep deprivation: DGRP_338, DGRP_892, DGRP_41, and DGRP_646, which had the most variable average night bout length; DGRP_335, DGRP_409, DGRP_73 (and DGRP_646), which had the most variable waking activity; and DGRP_153, which had the most variable night sleep. Flies from all eight lines were significantly deprived of sleep compared with their baseline sleep times (Figure 3). However, the sleep loss in flies of line DGRP_335 was quite low; only 104.7 min of sleep were lost on average (Figure 3). All of

Table 1. Numbers of Lines With Significant Daily Fluctuations in Sleep Parameters

	Males		Females	
Sleep Trait	No. of variable lines	Percentage	No. of variable lines	Percentage
Day sleep (min)	98	58.3	84	50.0
Night sleep (min)	89	53.0	85	50.6
Day bout number	69	41.1	67	39.9
Night bout number	69	41.1	59	35.1
Avg. day bout length (min)	49	29.2	41	24.4
Avg. night bout length (min)	54	32.1	50	29.8
Waking activity (cts/min)	147	87.5	153	91.1

The table lists the number and percentage of DGRP lines with significant daily variability in the sleep parameter indicated ($p_{Day} < .05$). The total number of DGRP lines with sleep phenotypes is 168.
cts = counts.

the lines had a statistically significant increase in their 24-hr sleep on the day after sleep deprivation compared with their baseline sleep, with the exception of DGRP_335 and DGRP_73. DGRP_335 did not lose much sleep, so one possibility is that the homeostat was not perturbed sufficiently in these flies to elicit a response. However, flies from line DGRP_73 lost 540.5 min of sleep during the night, and only gained 99.8 min during the next 24-hr period. Thus, although the recovery sleep in most of the lines was similar to that of other wild-derived and laboratory strains [78, 79], the rebound sleep of DGRP_335 and DGRP_73 suggests that genotypes with variable sleep may also have an altered homeostatic response.

We modeled daily fluctuations in sleep traits as the standard deviation (σ) per fly (Supplementary Table S3) in a manner analogous to studies of intra-individual variability in human sleep parameters [9–13]. We observed highly significant differences in σ among lines (all $p_{Line(Block)} < .0001$), and the variability was sexually dimorphic as well (all $p_{Line\times Sex(Block)} < .0001$) (Supplementary Table S4). Histograms of σ for night sleep traits (Figure 4) and day sleep traits (Figure 5) illustrate this variability among lines of the DGRP. The significant differences among lines suggested that sleep σ was heritable; thus, we calculated broad-sense heritabilities to quantify the genetic contribution to each trait. Heritabilities for day and night sleep duration σ were 0.49 and 0.26, respectively; 0.07 and 0.13 for day and night bout number σ, respectively; 0.30 for both day and night average bout length σ; and 0.21 for waking activity σ. With the exception of the low heritability for day bout number σ, heritabilities were moderate, suggesting that daily fluctuations in sleep are partially driven by genotype.

We calculated the phenotypic and genetic correlations among sleep σ phenotypes (Supplementary Table S5). Many of these correlations were significantly different from zero; however, the correlations were not necessarily high (i.e., the correlations were not close to 1). For example, night sleep duration σ had significant genetic correlations with all other sleep σ traits, but genetic correlations with other night-related traits were higher (0.573 and –0.459 with night bout number σ and average night bout length σ, respectively) than day and activity traits (0.256, –0.222, and –0.152 with day bout number σ, average day bout length σ, and waking activity σ, respectively). These correlations suggest a shared

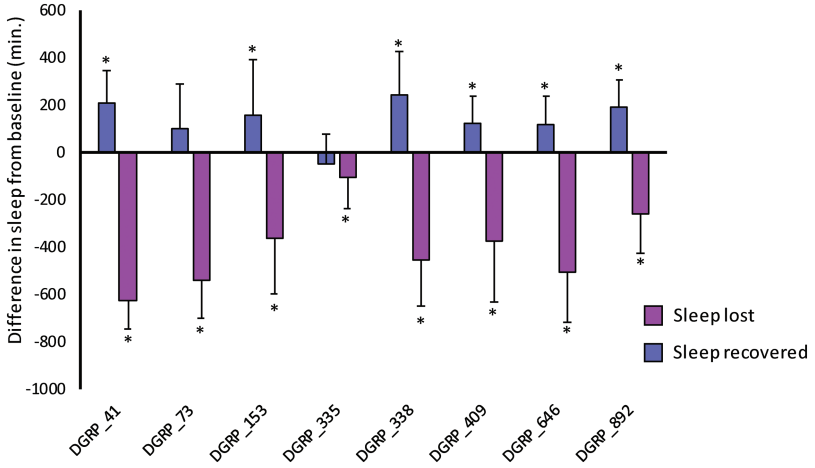

Figure 3. Sleep loss and recovery in DGRP lines with the greatest daily fluctuations in sleep. *$p < .05$. For DGRP_41, $n = 32$; DGRP_73, $n = 29$; DGRP_153, $n = 31$; DGRP_335, $n = 30$; DGRP_338, $n = 30$; DGRP_409, $n = 28$; DGRP_646, $n = 30$; and DGRP_892, $n = 32$.

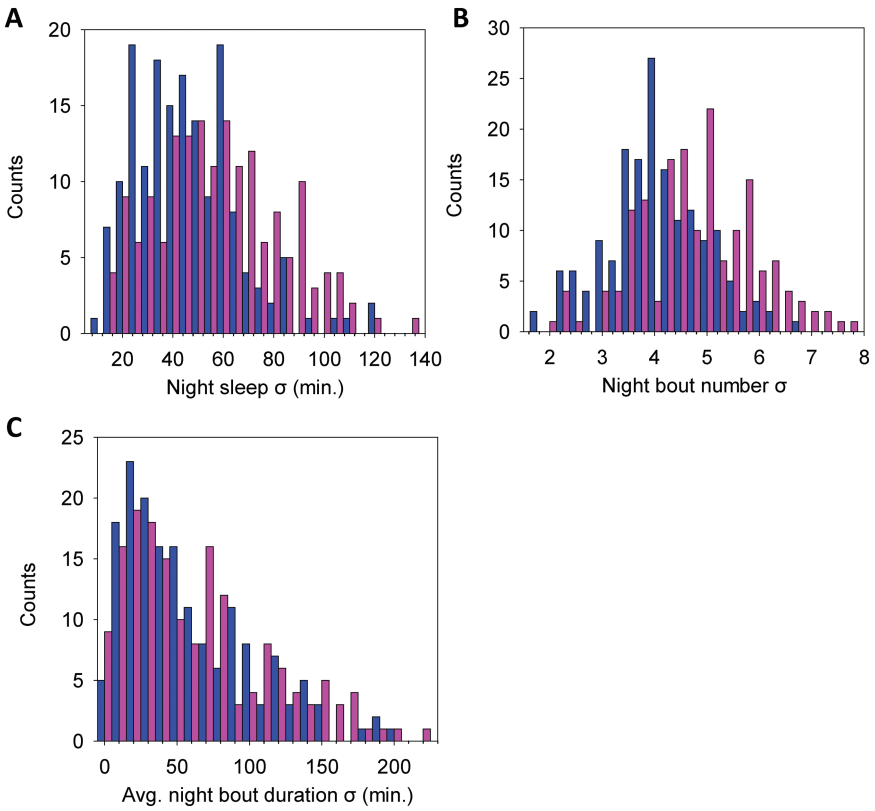

Figure 4. Histograms of night sleep σ traits. The histogram shows male line means in blue, and female line means in pink. (A) Night sleep duration σ. (B) Night bout number σ. (C) Avg. night bout length σ.

genetic architecture underlying daily fluctuations in sleep, but the overlap is far from complete. We also calculated the phenotypic and genetic correlations with the previously published mean and CV_E sleep phenotypes for these lines. Sleep σ traits tended to be correlated with their respective mean and CV_E, and, in general, these correlations were moderate to high (Supplementary Table S6). Night sleep σ and mean night sleep had a negative genetic correlation of $r_G = -0.80$, whereas day sleep σ and mean day sleep had an r_G of −0.44; that is, lower mean sleep duration was more variable. The trend for night sleep σ may also be true of single-gene mutations; we examined data from a previous *p*-element mutagenesis screen [80] and estimated the mutational genetic correlation as $r_G = -1.07$ ($p < .0001$) between night sleep σ and night sleep. Day sleep σ and day sleep had a positive mutational genetic correlation of $r_G = 0.61$ ($p < .0001$), however, suggesting the opposite trend. Day bout number σ and waking activity σ were the least correlated among sleep traits, whereas average day and night bout length σ was highly correlated with their mean. Thus, intra-individual variability in sleep has, in part, some distinctive genetic features.

We wondered if sleep σ traits might be correlated with other types of traits in flies. The DGRP has been used extensively to study many different complex traits; thus, it was possible to assess the phenotypic correlations with these traits and sleep σ. We calculated the phenotypic correlations between sleep σ traits and morphological traits; lifespan and metabolic traits; behavior; responses to environmental stresses; and genome size [42, 44, 51–65]. Many of the correlations with other traits were significantly different from zero, but their magnitudes were quite low (Supplementary Table S7). Only six correlations were higher than 0.25. Night sleep duration σ was negatively correlated with mean lifespan ($r = -0.27$) [55] and inter-individual variability in starvation resistance ($r = -0.26$) [44]. Average night bout length σ was positively correlated with variability in intraocular distance ($r = 0.29$), a morphological measure related to reproductive fitness in flies [53]. Night bout number σ was correlated with eclosion rate ($r = 0.30$) [62]. Waking activity σ was negatively correlated with startle response on control medium ($r = -0.28$) and menadione bisulfite-supplemented medium ($r = -0.30$), an inducer of oxidative stress [61]. Thus, correlations between sleep σ traits and other types of traits were generally quite low, suggesting a distinct genetic basis for sleep σ.

Genotype–Phenotype Associations

Using the average σ of each sleep trait per line, we associated each sleep σ phenotype with all polymorphisms known to be segregating in the DGRP which had a minor

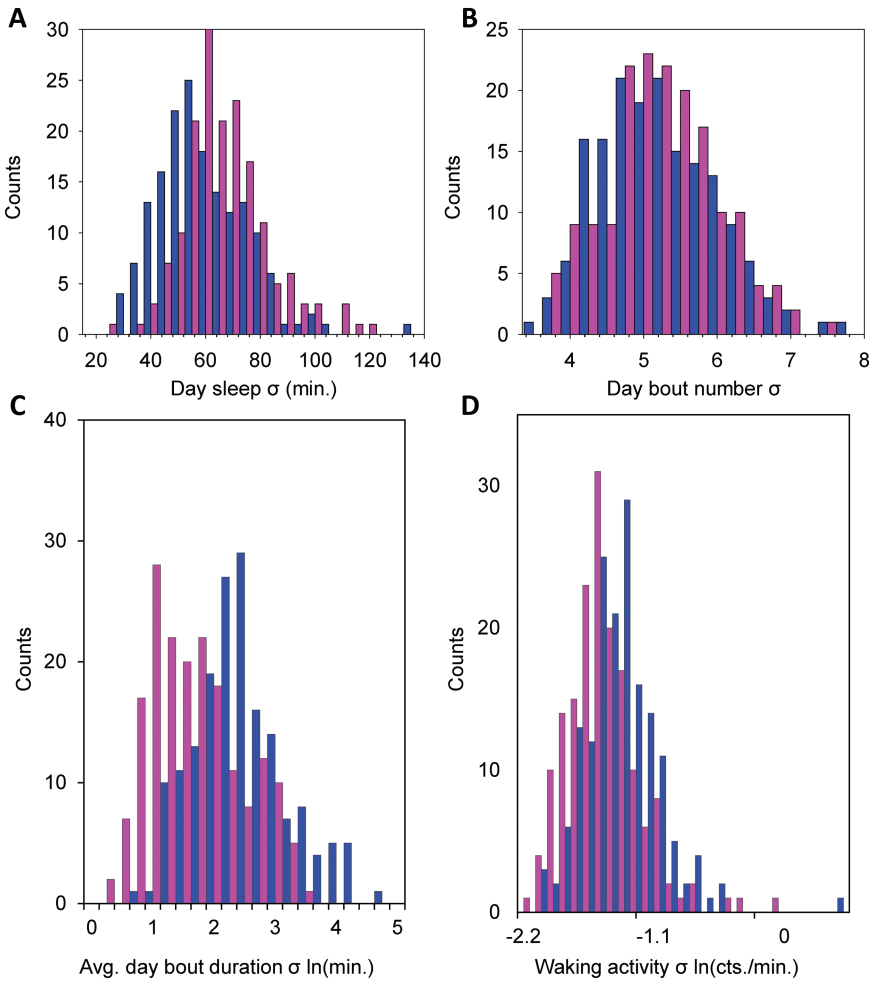

Figure 5. Histograms of day sleep σ traits. The histogram shows male line means in blue, and female line means in pink. (A) Day sleep duration σ. (B) Day bout number σ. (C) Normalized avg. day bout length σ. (D) Normalized waking activity σ.

allele frequency of 0.05 or more: 1 920 276 variants in total [46]. Associations were evaluated for sexes combined, each sex separately, and the difference between sexes (male avg. – female avg.) (see Supplementary Table S8 for details). Table 2 shows the numbers of polymorphisms that were associated with each sleep σ trait at a threshold p-value of 1×10^{-5} or less. Numbers of significant associations were modest, with 14%–26% of the associated polymorphisms located within the coding region of a gene, and the remaining polymorphisms mapping between genes (Supplementary Figure S3). A preponderance of significant associations had low minor allele frequencies as the median allele frequency was 0.12, a typical result in studies using the DGRP [45, 47, 61, 62, 64, 68]. Thus, variants with larger effect sizes tended to be relatively rare. However, significant associations were also observed at more common alleles as roughly 10% of the associations had minor allele frequencies above 0.40. Only 20 of the 663 associations had FDRs of 0.05 or less, and only 34 had FDRs of 0.10 or less. Despite the modest statistical support, effect sizes for some sleep σ traits were very high. We discuss these results in detail below.

Effect sizes for associations with average night bout length σ were very high in magnitude and ranged from –39.2 to 17.7 min. Unlike mammalian genomes, LD decays very rapidly in the DGRP, on the order of 10–30 bp on average [46, 47]. We therefore assumed that a significant variant located within a gene implicated that gene, unless (1) LD

Table 2. Numbers of Polymorphisms Significantly Associated With Sleep σ Traits

Sleep trait	Number of polymorphisms	Percent within genes
Day sleep σ (min)	145	25.5
Night sleep σ (min)	103	16.5
Day bout number σ	43	14.0
Night bout number σ	76	19.7
Day avg. bout length σ [ln(min)]	86	26.7
Night avg. bout length σ (min)	124	13.7
Waking activity σ [ln(cts/min)]	87	18.4

was high ($r^2 \geq 0.8$) among variants, or (2) the trait was associated with a known chromosomal inversion (Methods). LD was generally very low among polymorphisms for average night bout length σ (Figure 6A) except for two regions: one region was on chromosome 2*L* between 5056207 and 5594455 bp; the other was on chromosome 2*R* between 5191057 and 5472092 bp. The first region of LD was also within the *In(2L)t* inversion, which was itself

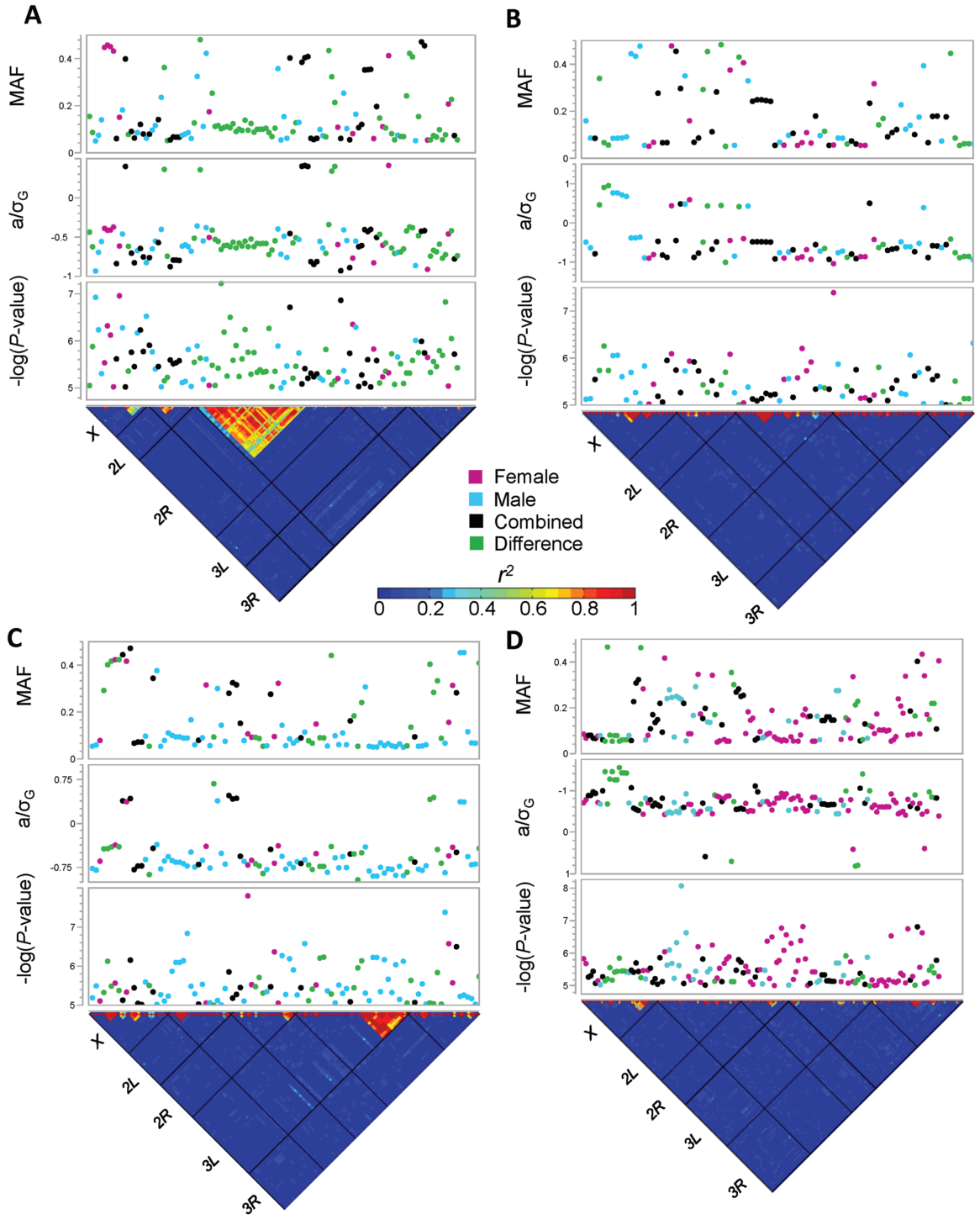

Figure 6. Genome-wide association results for sleep σ traits. Significant polymorphisms ($p < 1 \times 10^{-5}$) are plotted for each trait. The points are color-coded to indicate the most significant association (sexes combined, male, female, or sex difference) as indicated in the legend. The top panel shows the minor allele frequency (MAF) for each polymorphism plotted across chromosomal position. The middle panel plots the effect size *a* normalized by the genetic standard deviation σ_G. The lower panel shows the *p*-values for each polymorphism plotted as the -log of the *p*-value. The bottom triangle is the LD among polymorphisms calculated as r^2. Black lines denote chromosome boundaries. (A) Avg. night bout length σ. (B) Waking activity σ. (C) Night sleep duration σ. (D) Day sleep duration σ.

significantly associated with average night bout length σ (Methods). Variants within candidate genes in these two regions, *CG31918* and *sxc*, were therefore not distinguishable from the other polymorphisms in LD around them (8 and 21 variants, respectively). Most of the remaining 93 polymorphisms associated with average night bout length σ were intergenic, but 15 additional polymorphisms were in 12 candidate genes. These candidate genes included *5-HT1A*, *flower*, *fru*, and *HDAC4* (Supplementary Table S8).

We also observed large effect sizes for associations with waking activity σ; these ranged from 0.76 to 1.25 counts per minute. For waking activity σ, 87 significant polymorphisms were found, and virtually no LD among these polymorphisms was observed. As with average night bout length σ, most of these variants were intergenic, but 16 polymorphisms were in 13 genes (Figure 6B; Supplementary Table S8), including *rho*, *roughoid* (*ru*), and *Heat shock protein 26*. Seventeen polymorphisms had very low FDRs (<0.0001), including one in *roughoid* and two in *Heat shock protein 26*.

Variants associated with night and day sleep duration σ had smaller effect sizes. Night sleep σ effects ranged from −17.6 to 12.1 min and day sleep σ effects ranged from −14.3 to 7.5 min. 103 polymorphisms were significantly associated with night sleep σ. A large region on Chromosome *3L* was in LD (23531038 to 24006114 bp) among 10 intergenic polymorphisms significantly associated with night sleep σ (Figure 6C). Eighteen variants implicated 14 candidate genes, including *sls*, *Secretory 8*, and *unpaired 1* (Supplementary Table S8). One hundred forty-five polymorphisms were associated with day sleep σ. There were 29 genes identified for day sleep σ, including *skittles*, *minibrain*, and *kirre* (Supplementary Table S8). Little LD was evident for day sleep σ (Figure 6D).

One possibility is that the genetic architecture for sleep σ overlaps with mean/CV_E sleep as the genetic correlations among these traits were generally significant. We therefore compared our results with the previous genome-wide association study of sleep in *Drosophila* [45]. We found 22 unique polymorphisms that overlapped with sleep σ traits, which was 3.3% of the total identified in this study (Supplementary Table S9). Most of the overlap was of intergenic variants, but polymorphisms in the genes *CG13699*, *CR45711*, *flower*, and *rho* overlapped among traits. Many polymorphisms identified in this study are therefore unique to sleep σ traits.

Finally, we searched for human homologs of candidate sleep σ genes using the DIOPT tool. DIOPT searches for homologous genes across 12 databases; DIOPT scores indicate the number of databases that predicted a given homologous gene [73]. A total of 438 human genes had predicted homology with 78 *Drosophila* genes, though in many cases the homology was not high. We found 51 human genes that were predicted to have the highest homology with 42 *Drosophila* genes (Supplementary Table S10). Twenty-seven *Drosophila* genes had no known human homolog. Thus, a portion of the genes we identified for sleep σ traits have plausible human homologs.

Verification of Candidate Genes

We tested 17 *Minos* element constructs in 14 candidate genes for their effects on sleep σ traits (Supplementary Table S2). We chose sleep σ traits with the largest effect sizes to confirm: night average bout length σ, night sleep σ, and waking activity σ (Supplementary Table S8). We tested all genes with available *Minos* constructs that were identified for these three traits. We found that 12 of the candidate genes confirmed the GWAS prediction (Table 3). *5-HT1A*, *CG42260*, and *fru* had two *Minos* insertions available. Both *5-HT1A* insertions had significant

Table 3. Verification of Candidate Genes

Gene	Allele tested	Sleep trait	Diff. from control	No.
5HT1A	MB09812	Night avg. bout length σ (min)	14.32***	32
5HT1A	MB09978	Night avg. bout length σ (min)	10.61**	31
CG7985	MI00275	Night avg. bout length σ (min)	−113.93***	31
fru	MB02472	Night avg. bout length σ (min)	28.29*	32
fru	MB01996	Night avg. bout length σ (min)	7.09*	30
HDAC4	MI05513	Night avg. bout length σ (min)	−103.05***	32
sxc	MI02286	Night avg. bout length σ (min)	−125.68****	32
CG42260	MB00428	Night sleep σ (min)	−27.87****	31
sls	MI011177 Het.	Night sleep σ (min)	30.78****	32
rho	MI08786	Waking activity σ (cts/min)	−0.85****	32
scb	MI010788	Waking activity σ (cts/min)	−0.82****	32
sowi	MI03342 Het.	Waking activity σ (cts/min)	−0.97****	32
Tmc	MI02041	Waking activity σ (cts/min)	−0.78****	32
tutl	MI08144	Waking activity σ (cts/min)	−0.90****	31

Mutations were evaluated for their effect on the sleep σ traits predicted by the GWAS. Mean differences from the respective control line are given. The number of w^{1118}[5905] control flies was 32; the number of y[1] *w*[*]; *nub*[2] *b*[1] *sna*[*Sco*] *pr*[1] *cn*[1]/*CyO* [3628] control flies was 27. Waking activity σ differences reflect un-transformed values; statistical tests were ln-transformed. Asterisks reflect FDRs to correct for multiple tests.
*0.01 < FDR ≤ 0.05; **0.001 < FDR ≤ 0.01; ***0.0001 < FDR ≤ 0.001; ****FDR < 0.0001.
FDRs = false discovery rates; Het. = heterozygote.

differences in night average bout length σ from the control; likewise, both *fru* insertions were significantly different for night average bout length σ. Only one of the *CG42260* insertions had significant differences in night sleep σ from the control line, however. All the remaining genes had one *Minos* construct available for test, and the following genes were confirmed: *CG7985*, *HDAC4*, and *sxc* for night average bout length σ; heterozygous *sls* for night sleep σ; and *tutl*, *Tmc*, *rho*, heterozygous *sowi*, and *scb* for waking activity σ. In addition, some of these constructs had pleiotropic effects on other sleep traits (Supplementary Table S11), consistent with previous observations of gene-level pleiotropy in *Drosophila* genome-wide studies [45, 81]. These mutant tests validate these genes as candidates affecting daily fluctuations in sleep.

Discussion

Here, we demonstrated that individual flies experience daily fluctuations in sleep characteristics. Some flies altered their sleep patterns dramatically from day to day, whereas others were remarkably stable. This intra-individual variability, which we quantified as the standard deviation (σ) of each sleep trait over time, differed among genotypes and was heritable. Importantly, sleep σ traits were partially but not completely genetically correlated with summary sleep traits (mean sleep and sleep CV_g). Likewise, sleep σ traits were relatively uncorrelated with other complex traits studied in the DGRP. Daily fluctuations in sleep therefore have a distinctive genetic basis, with a partial overlap with summary sleep traits. Fourteen polymorphisms overlapped between sleep σ traits and their respective summary traits, demonstrating this partial overlap. Although further experimentation is required to understand the mechanistic basis of the predicted associations here, some of the candidate genes were previously reported to affect summary sleep phenotypes. *5-HT1A* was identified as a gene regulating sleep duration and bout length in flies via signaling in the mushroom bodies [82]; here, it was implicated in average night bout length σ, and tests of flies with two different *Minos* insertions in *5-HT1A* verified these effects. *VAChT* was associated with day bout number σ; mutations in this gene were previously reported to affect mean day bout number [68]. Two other genes known to affect summary sleep phenotypes, *rho* [83] and *kirre* [68], were also associated with waking activity σ and day sleep σ, respectively. We confirmed the effects of *rho* on waking activity σ. The associations with sleep σ traits differ somewhat from the sleep summary traits previously reported, indicating that these genes may be pleiotropic. The activity of these genes may therefore vary from day to day to produce the summary effects observed previously.

Other genes we identified are plausible candidates for daily fluctuations in sleep. Interestingly, several of these genes have known effects on circadian rhythms. *super sex combs* was associated with average night bout length σ; this gene interacts with the GSK3β gene *sgg* to affect circadian rhythms [84]. We confirmed the effect of *sxc* on average night bout length σ. *HDAC4* was also identified in the GWAS and verified for average night bout length σ. It is an intriguing candidate as it is involved in epigenetic modification and transcription [85], which might be anticipated characteristics of a gene affecting daily fluctuations in sleep. *HDAC4* also functions in long-term memory [86], and mutations of this gene affect circadian rhythmicity in flies [87]. RNAi-mediated knockdown of *unpaired1*, which was associated with night sleep σ in this study, results in arrhythmicity [88]. *minibrain* was identified for day sleep σ; double-mutant flies *minibrain/small optic lobes* increased instability in rest/activity rhythms [89]. In addition, *roughoid*, like *rhomboid*, is a serine protease functioning in the EGFR pathway [90]; this gene was associated with waking activity σ.

About 80% of the polymorphic variants that we identified were in intergenic regions. This observation is consistent with other GWA studies [91], but reveals the difficulty in fully understanding the genetic basis of complex traits such as sleep. Intergenic variants might alter gene function via changes in enhancer or DNA binding sites, which in turn may alter the transcription or translation of a gene or genes. Enhancer or promoter effects can occur far upstream or downstream of the gene they affect, however. As a first step, these variants could be studied as potential cis-regulatory genes by using genome editing to perturb each position and by examining the impact of these perturbations on transcript abundance in neighboring genes. Polymorphisms within the coding region of candidate genes are more tractable, as they can be tested directly with allelic replacement via genome editing. Overall, we identified 663 polymorphisms and 104 candidate genes for intra-individual variability in sleep.

Our study has several limitations. First, the statistical support for polymorphisms associated with intra-individual variability in sleep was relatively low. This is partly due to the relatively small size of the DGRP and relatively low heritabilities, both of which make the detection of significant associations more difficult. Thus, the associations reported here probably represent a subset of the true associations, and for some of the traits such as day and night bout number σ, FDRs were high, decreasing the confidence in those predictions. However, quantile–quantile plots supported the 1×10^{-5} threshold *p*-value for night sleep σ, day sleep σ, average night bout length σ, and waking activity σ, indicating that there was adequate statistical power for these measures (Supplementary Figure S1). This makes sense

as the effect sizes for these four traits were the largest we observed. Accordingly, our follow-up mutational tests focused on the genes with the largest predicted effect sizes. This was an effective strategy for confirming candidate genes as 12 of 14 genes had significant effects on the predicted sleep σ trait. Second, the variability in sleep patterns that we observed is confounded with age. The original study evaluated sleep in flies that were 4 to 14 days old, so it is not known how sleep patterns in these lines might change in either very young or very old flies. We observed both increasing and decreasing patterns of fragmentation in these relatively young flies. A previous longitudinal study of sleep in flies documented a monotonic trend for increased fragmentation across the entire lifespan, with some fluctuation in sleep patterns over short periods [92]. Varying trends have been observed in human sleep as well over time, from inverted U-shaped distributions [93] to declines in intra-individual variability with age [13]. It would therefore be interesting to examine daily fluctuations in sleep across the entire lifespan in this population. Finally, our study focuses on behavioral correlates for sleep and does not address any potential fluctuation in sleep intensity, which vary with genotype in flies [94] and are critical to understanding sleep need [95].

Conclusion

We have demonstrated that *Drosophila* sleep patterns fluctuate from day to day. We defined the standard deviation as a measure of the daily fluctuations in sleep for each fly and found it to be heritable. Using all available variants in the DGRP, we mapped 104 candidate genes, some of which have known roles in sleep and circadian rhythms. We also confirmed the role of 12 genes on sleep intra-individual variability. Many of these genes have predicted homology with human genes, suggesting that the underlying genetic architecture of daily fluctuations in sleep may be conserved.

Supplementary Material

Supplementary material is available at *SLEEP* online.

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Disclosure Statement

None declared.

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