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The *dihydropyrimidine dehydrogenase* gene contributes to heritable differences in sleep in mice

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SUMMARY

Many aspects of sleep are heritable, but only a few sleep-regulating genes have been reported. Here, we leverage mouse models to identify and confirm a previously unreported gene affecting sleep duration – *Dihydropyrimidine Dehydrogenase (Dpyd)*. Using activity patterns to quantify sleep in 325 Diversity Outbred (DO) mice – a population with high genetic and phenotypic heterogeneity – a linkage peak for total sleep in the active lights off period was identified on chromosome 3 (LOD score = 7.14). Mice with the PWK/PhJ ancestral haplotype at this location demonstrated markedly reduced sleep. Among the genes within the linkage region, available RNA sequencing data in an independent sample of DO mice supported a highly significant expression quantitative trait locus for *Dpyd*, wherein reduced expression was associated with the PWK/PhJ allele. Validation studies were performed using activity monitoring and EEG/EMG recording in Collaborative Cross mouse strains with and without the PWK/PhJ haplotype at this location, as well as EEG/EMG recording of sleep and wake in *Dpyd* knockout mice and

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AUTHOR CONTRIBUTIONS

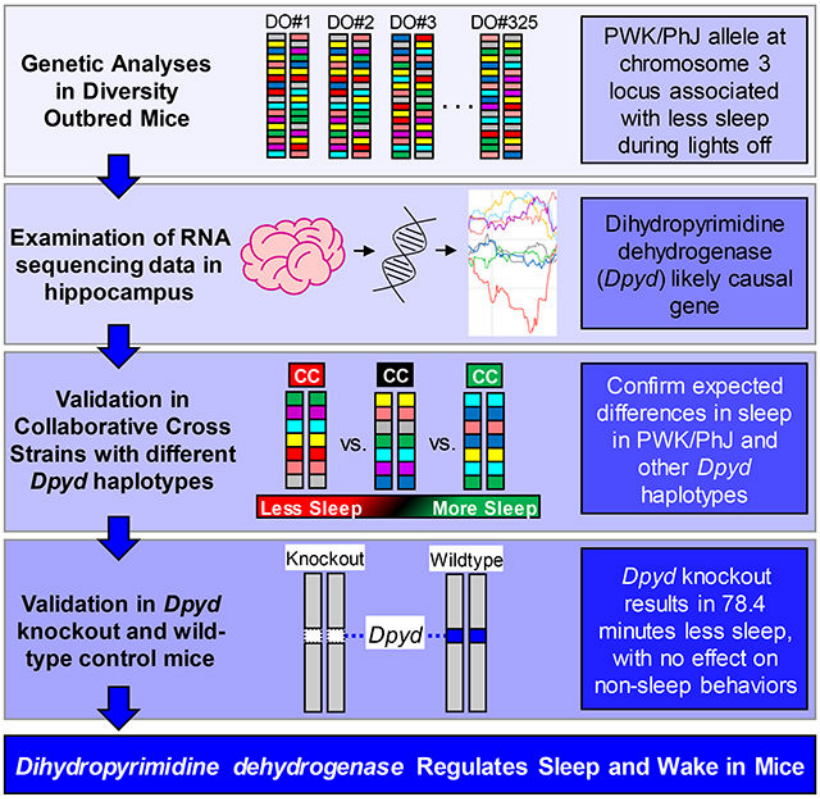
Specific contributions of co-authors include: *Study Design:* KLS, GAC, AIP; *Mouse phenotyping:* RJG, JL, LZ, XG, EJC; *Data analysis:* BTK, GAC, EJC; *Writing:* BTK, XG, OJV, EJC, KLS, GAC, AIP.

DECLARATION OF INTERESTS

Dr. Pack is The John L. Miclot Professor of Medicine at the University of Pennsylvania; funds for this endowment were provided by the Philips Respironics Foundation. Dr. Churchill, Dr. Chesler, and Dr. Svenson disclose that The Jackson Laboratory produces and sells a wide variety of mice for research, including Collaborative Cross and Diversity Outbred mice. All other authors report no financial or non-financial conflicts of interest related to the present manuscript.

wildtype littermate controls. Mice lacking *Dpyd* had 78.4 minutes less sleep during the lights off period than wildtype mice (p=0.007; Cohen's d=−0.94). There was no difference in other measured behaviors in knockout mice, including assays evaluating cognitive, social and affective disorder-related behaviors. *Dpyd* encodes the rate-limiting enzyme in the metabolic pathway that catabolizes uracil and thymidine to β-alanine, an inhibitory neurotransmitter. Thus, data support β-alanine as a neurotransmitter that promotes sleep in mice.

Graphical Abstract



eTOC Blurb

Many aspects of sleep are heritable, but few genes have been reported. Keenan *et al.* combine discovery in Diversity Outbred mice with validation in both Collaborative Cross and knockout mice to implicate *Dihydropyrimidine dehydrogenase (Dpyd)* as a gene affecting sleep. This, in turn, supports β-alanine as a sleep-promoting neurotransmitter in mice.

Keywords

Dihydropyrimidine Dehydrogenase (Dpyd) ; Sleep; Mice; Diversity Outbred; Collaborative Cross; Knockout; Genetics

INTRODUCTION

Most aspects of sleep are heritable. This includes the timing of sleep,[1-4] the duration of sleep,[5-7] the amounts of different stages of sleep,[8] the electroencephalogram (EEG) characteristics of sleep,[9] and the response to loss of sleep.[8] There are currently multiple approaches to identifying relevant gene variants. Human genome-wide association studies (GWAS) using data from the UK Biobank and 23andMe have identified common variants in genes that are associated with sleep phenotypes.[10-14] However, the phenotype data for these GWAS are based on a limited number of questions, which are subjective,[10-14] or use of accelerometry, which is prone to error.[12,13,15] Moreover, few of the genes implicated as regulating sleep by GWAS have been validated in experimental model systems. In addition to GWAS, which typically identify common genetic variants with smaller effect sizes, targeted studies have found rare variants associated with large effect sizes on human sleep and response to sleep deprivation.[16,17]

Unbiased forward genetics studies in *Drosophila*[18,19], zebrafish[20] and *C. elegans*[21-23] have identified sleep-regulating genes,[24] but the relevance of these genes to mammalian sleep has, in most cases, not yet been demonstrated. A forward genetic screen using EEG/electromyography (EMG)-defined sleep in mice has led to the identification of two genes regulating mammalian sleep.[25] However, the scale of such a screen was limited by the high labor costs of instrumenting each animal for EEG/EMG recording of sleep, and therefore focused only on dominant mutants.

In this report, we describe the application of a cutting-edge approach to identifying genes affecting mammalian sleep by leveraging Diversity Outbred (DO) mice with a high genetic diversity that allows for precise genetic mapping.[26-28] DO mice are derived from the original founder strains of the Collaborative Cross lines,[29] using a randomized outbreeding strategy over multiple generations.[26-28] This outbreeding creates genetic and phenotypic heterogeneity in mice that approaches that in human populations. Since each DO mouse is genetically unique, one can assess the phenotypic contributions of gene variants in numerous contexts. Phenotyping data in DO mice can be combined with genomic data from a mouse genotyping array[26] to identify quantitative trait loci (QTLs). Given the large number of recombination events that result from the multiple generations of outbreeding, QTL regions identified using the DO strategy are small, typically including only a few genes. This greatly improves the ability to identify causal genes when compared to the large QTL regions normally identified using the more traditional two-parent inbred mouse cross approach.[27,28] Along with increased genetic heterogeneity comes the requirement to study a larger number of DO mice to maintain statistical power. Hence, it is optimal to perform high-throughput phenotyping. We recently reported such a phenotyping strategy using activity patterns to assess multiple quantitative sleep and circadian traits in DO mice, which we have shown are heritable.[30]

Here, we report the results of studies in mouse models to identify and confirm a previously unreported gene affecting sleep duration. Initial results are from a large study of sleep behavior in 325 DO mice that identified a linkage peak on mouse chromosome 3 (chr3: 118.4 – 122.6 Mb) associated with the amount of sleep during the active lights off period.

The association at this region was driven primarily by the PWK/PhJ founder allele, which resulted in reduced sleep. Although the linkage peak contained multiple candidate genes, follow-up analyses and functional experiments demonstrated that *Dpyd* was the causative gene. We identified a strong expression quantitative trait locus (eQTL) for *Dpyd* using available RNA sequencing data from an independent sample of DO mice.[31,32] The PWK/PhJ founder alleles at the implicated region are associated with markedly reduced expression of *Dpyd*. Definitive proof of *Dpyd* as the causative gene is obtained from studies of knockouts of *Dpyd* in mice. Variants of *DPYD*, including loss of function variants, exist in human populations and have pharmacogenomics implications.[33,34] Moreover, common variants of *DPYD* have been shown to be associated with sleep duration in two recent human GWAS.[13,15] Thus, *Dpyd* is a new gene that we have validated to be involved in sleep and wake regulation in mice and may have conserved function in humans.

RESULTS

We evaluated sleep phenotypes in 325 Diversity Outbred mice at the University of Pennsylvania. As previously described,[30] mice were individually housed and maintained in a 12-hour/12-hour light/dark cycle, with food and water available *ad libitum*. Sleep was estimated by tracking breaks in infrared beams projected across the mouse cage (AccuScan IR beam system, AccuScan Instruments, Inc., Columbus, OH). As previously validated,[35] sleep was defined as 40 seconds or more of continuous inactivity (i.e., no beam breaks). Sleep recording was performed over 5 consecutive days to estimate the average amounts of sleep, average number of sleep bouts, and the average duration of sleep and wake bouts in both the lights on (7AM-7PM) and lights off (7PM-7AM) periods, and across the entire day. Each mouse was genotyped using the high-density Mega Mouse Universal Genotyping Array (MegaMUGA)[36] and a hidden Markov model analysis was applied to infer haplotype blocks in each DO genome.[26]

We conducted a genome-wide QTL scan using the *R/qtl2* package[37] to determine if there were any regions with linkage to sleep characteristics. Briefly, genome scans were performed utilizing a linear mixed model that accounts for population structure and evaluates the association between a phenotype of interest and the founder haplotypes present in each DO mouse at each of ~69,000 loci across the mouse genome. Using this approach, we identified a suggestive linkage peak on mouse chromosome 3 (95% Bayesian credible interval = chr3: 118.4 – 122.6 Mb) with a LOD score of 7.14 ($p<0.10$; see Figure 1) that is largely driven by alleles carried on the PWK/PhJ haplotype. Specifically, mice with the PWK/PhJ haplotype at this locus had markedly reduced sleep compared to mice that carried other founder strain haplotypes, while mice with the NOD/LtJ haplotype had the highest amount of sleep (see Figure 1).

The identified linkage peak (chr3: 118.4 – 122.6 Mb) included 18 genes (see Figure S1). Using previously described data from RNA sequencing in the hippocampus of DO mice, we obtained expression data for 16 of these candidate genes[31,32] and evaluated whether there were differences in expression driven by the PWK/PhJ haplotype. As shown in Figure 2, a strong eQTL (LOD = 23.0) was observed for *Dpyd*, with the PWK/PhJ haplotype demonstrating a clear reduction (or absence) in *Dpyd* expression compared to other founder

haplotypes. No other candidate gene in the region demonstrated a strong PWK/PhJ-specific effect on gene expression (see Figure S1). Thus, expression data supported *Dpyd* as the primary candidate causative gene within the identified linkage region.

Having identified *Dpyd* as the likely causative gene, we next studied Collaborative Cross mouse strains with the relevant *Dpyd* alleles to validate the effects on sleep. The majority of Collaborative Cross strains are sequenced and whole-genome haplotypes can be obtained from the University of North Carolina,[38] facilitating this type of validation approach. Specifically, we selected two strains with PWK/PhJ *Dpyd* alleles (CC003/Unc [black] and CC009/Unc [white]), one strain with the NOD/LtJ *Dpyd* allele (CC011/Unc), one strain with the CAST/EiJ *Dpyd* allele (CC040/TauUnc), one strain with the WSB/EiJ *Dpyd* allele (CC051/TauUnc), and one strain with the NZO/HILtJ *Dpyd* allele (CC004/TauUnc). Sleep was initially estimated using infrared beam breaks, as in the DO mice. We observed significant differences among all of these strains in total sleep during lights off ($p=0.0052$; see Figure 3A and Table S1). There was evidence of reduced sleep duration in both PWK/PhJ *Dpyd* strains compared to the NOD/LtJ strain ($p\leq0.008$), as would be hypothesized based on the founder-specific results (see Figure 1), as well as compared to the WSB/EiJ strain ($p\leq0.032$). In addition to total sleep during lights off, differences among Collaborative Cross strains were observed for number of sleep bouts ($p<0.0001$), average sleep bout duration ($p<0.0001$) and average wake bout duration ($p=0.0001$) (see Tables S1 **and** S2). Both PWK/PhJ *Dpyd* strains had longer wake bout durations than the NOD/LtJ strain ($p\leq0.008$). Additional details on sleep/wake traits within Collaborative Cross strains are presented in Tables S1 **and** S2.

Given this confirmation of the observed association using beam-based data in specific Collaborative Cross strains, we then performed EEG/EMG-based sleep assessment, which is the gold-standard to study sleep and sleep architecture, in strains with the PWK/PhJ (CC003/Unc) and NOD/LtJ (CC011/Unc) *Dpyd* haplotypes. Although there was a consistent difference in total sleep over the entire lights off period on average between the strains, this result did not reach statistical significance ($p=0.253$; Cohen's $d = -0.71$; Table S3). When examining the lights off period in more detail using 3-hour windows, there was a clear reduction in total sleep during the first three hours of the lights off period in the PWK/PhJ *Dpyd* strain compared to the NOD/LtJ *Dpyd* strain (41.0±26.4 vs. 80.4±7.7 minutes; $p=0.003$; Cohen's $d = -2.03$) (see Figure 3B and Table S4). Thus, EEG/EMG data supported the overall effect observed using infrared beams, particularly during the early part of the lights off period. Comparisons of other EEG/EMG parameters between these two Collaborative Cross strains are presented in Tables S3 **and** S4. The PWK/PhJ *Dpyd* strain had reduced numbers of sleep and wake bouts and increased wake bout duration during the first 3 hours of the lights off period compared to the NOD/LtJ *Dpyd* strain (Table S4). The PWK/PhJ strain showed reduced number of sleep, NREM, and wake bouts over 24 hours and during lights on, but a small increase in REM bouts during lights on (Table S3). There was also some evidence of increased bout durations in certain time periods among the PWK/PhJ *Dpyd* strain (Table S3).

To further validate the role of *Dpyd* in the regulation of sleep, we obtained a *Dpyd* knockout mouse from the Toronto Center for Phenogenomics through the International

Mouse Phenotyping Consortium (http://www.mousephenotype.org)[39,40] and developed this on the C57BL/6J background. We performed EEG/EMG recording of sleep and wake architecture in male and female knockouts, as well as male and female wildtype littermate controls. On average, mice lacking *Dpyd* had nearly 80 minutes less sleep in the lights off period than wildtype mice (263.5±92.6 vs. 341.9±74.0 minutes; p=0.007; Cohen's d = −0.94; see Table 1 and Figure 4), providing strong validation of our original results from DO mice. The effects of *Dpyd* on sleep/wake phenotypes were similar in male and female mice (interaction p=0.689; see Figure 4). Consistent differences were observed for amounts of NREM and REM sleep, separately, during lights off (see Table 1). There were several differences in other sleep/wake characteristics between *Dpyd* knockout mice and wildtype littermate controls. These include increased sleep bout numbers and decreased average duration of sleep, NREM, and wake bouts during lights on (see Table 1). Decreased total sleep and NREM average bout durations and reduced amounts of sleep and NREM sleep over 24-hours were also observed (see Table S5). Differences were consistent with overall results when examining male and female mice separately (see Table S6); all tests for effect modification by sex resulted in p>0.05 (see Tables 1 **and** S5). As with EEG/EMG data in the Collaborative Cross mice, we also compared sleep and wake characteristics between *Dpyd* knockouts and wildtype littermate controls using 3-hour windows during lights off (see Table S7). While larger reductions in total sleep/wake were again observed in the first three hours of the lights-off period, estimates were generally consistent with reduced sleep in the *Dpyd* knockout mice in each 3-hour window. Thus, evidence strongly supports the involvement of *Dpyd* in regulation of sleep and wake in mice.

Given the observed differences in sleep and wake between *Dpyd* knockout mice and the wildtype strain in which the knockout was generated, along with reported associations between *DPYD* variants and other neuropsychiatric traits in humans,[41-44] we performed additional phenotyping assays for multiple behaviors comparing *Dpyd* knockout mice and wildtype littermate controls. First, we assessed circadian period during constant darkness within a subset of male and female *Dpyd* knockout (n=9; 23.64±0.16 hour circadian period) and wildtype (n=8; 23.62±0.27 hour circadian period) mice. There was no difference between the two genotypes (p=0.885), indicating loss of *Dpyd* does not modify circadian period. Moreover, to assess the effect of *Dpyd* on other behavioral domains we performed additional assays including elevated zero maze, open field activity, object location memory, social preference, accelerating Rotarod, and acoustic startle response (ASR) / pre-pulse inhibition (PPI). While there were overall differences observed between male and female mice, we did not find any significant differences in these behaviors between the *Dpyd* knockout genotype and wildtype littermate control mice (see Table 2 and Figures S2-S5).

DISCUSSION

In this study, we describe a novel role for the *Dpyd* gene in sleep and wake control in mice. Identification of the effects of variation in this gene was based initially on studies in Diversity Outbred mice,[26-28] followed by validation with beam-based sleep measurements and EEG/EMG recording in relevant Collaborative Cross strains,[29] and subsequent EEG/EMG recording in knockout mice. Together, these results demonstrate a role for *Dpyd* in regulation of sleep and wake in mammals. While we found consistent

effects of *Dpyd* on sleep and wake measurements, studies in *Dpyd* knockout mice did not reveal differences in circadian period or in multiple other behavioral traits. Thus, while many genes that affect sleep have pleiotropic effects on other behaviors,[45] the effect of *Dpyd* appears relatively specific to sleep based on our data.

We utilized Diversity Outbred mice to initially identify the associated candidate gene. Diversity Outbred mice have a level of genetic diversity comprised by allelic perturbations in virtually every gene in the genome – a key advantage of the approach. By leveraging the increased genetic heterogeneity, studies of phenotypic variations among DO mice have proven to be effective in identifying novel roles for various genes.[46-48] Our phenotyping strategy in DO mice was based on high-throughput sleep phenotyping[30] using a validated algorithm to assess sleep/wake based on activity patterns.[35] Studies in mice using chemical mutagenesis have employed EEG/EMG recording in 1,100 mice, and identified two novel genes.[25] Given the burden of large-scale EEG/EMG recording, but also recognizing that assessing the phenotypic differences by EEG/EMG recording is ultimately essential, we used a hybrid approach for discovery and validation. First, cost-effective and high-throughput phenotyping was performed for discovery of a candidate gene. Then, for robust confirmation we employed EEG/EMG recording in both selected Collaborative Cross lines and in male and female knockout mice. This hybrid approach provides an ideal balance between efficient discovery and strong validation.

Using this approach in DO mice, we were able to identify a quantitative trait locus with a small number of candidate genes under the linkage peak. Evaluation of expression of these genes using available RNA sequencing data from the hippocampus in an independent sample of DO mice indicated that one gene—*Dpyd*—was the likely causative gene. *Dpyd* encodes dihydropyrimidine dehydrogenase, the first and rate-limiting enzyme in the pyrimidine metabolic pathway that catabolizes the reduction of uracil and thymine to β-alanine and β-aminoisobutyric acid (β-AIB), respectively. Both β-alanine and β-AIB are structural analogs of γ-aminobutyric acid (GABA) and glycine, two important inhibitory neurotransmitters in the central nervous system.[49] It has been proposed that β-alanine meets the criterion for being a neurotransmitter.[50] Thus, there is a plausible biological mechanism for the role of *Dpyd* in sleep/wake control.

The main source of β-alanine synthesis, including in the brain, is believed to be uracil degradation.[50,51] Neurological disorders are common in patients with enzymatic deficiencies in the pyrimidine catabolic pathway, including patients with deficiencies in dihydropyrimidine dehydrogenase.[52-55] Clinical studies of patients with a dihydropyrimidine dehydrogenase deficiency revealed decreased levels of β-alanine in plasma and urine and indicated an altered homeostasis in β-alanine as the underlying mechanism of the clinical abnormalities.[51,56] β-alanine is a naturally occurring β-amino acid. Unlike its chemical enantiomer α-alanine, β-alanine is not used in the synthesis of proteins.[50] It is found widely throughout the brain, with regional differences in concentration,[57] and is present in both neurons and glia.[58] β-alanine acts as a small molecule neurotransmitter.[59] It has five recognized receptor sites, including GABA-A receptors.[60,61] The response at the GABA-A receptor to β-alanine is similar to that for GABA.[62,63] Given that GABA is the predominant neurotransmitter of sleep-promoting neurons in ventrolateral preoptic area

(VLPO)[64] and in the parafacial region,[65,66] our data suggest that β-alanine may be a "parallel neurotransmitter" involved in sleep/wake regulation. This postulate needs to be assessed in future studies.

It is not just *Dpyd* that affects β-alanine levels. There are also transporters in brain for β-alanine. These include GABA transporters (GATs) – both GAT1 and GAT4 are concentrated in the central nervous system.[67] Since β-alanine is transported by these GATs, and subsequently inhibits the transport of GABA, it follows that the transport of GABA is controlled by the levels of β-alanine. It has been argued that the existence of transporters for β-alanine in brain supports the concept that β-alanine is a neurotransmitter.[68]

β-alanine is also transported by the taurine/β-alanine transporter, Slc6a6.[69] *Slc6a6* is widely expressed throughout the body and in the brain,[70,71] including in both neurons and glial cells.[72] In our previous RNA sequencing study of the medial prefrontal cortex that compared C57BL/6J mice under spontaneous sleep and sleep deprivation, expression of *Slc6a6* was highly down-regulated in sleep-deprived animals and up-regulated in animals allowed to sleep.[73] This was true in both young (3 months) and old (18 months) mice.[73] Thus, there is evidence that mechanisms other than *Dpyd* that affect β-alanine levels are driven by sleep/wake differences.

Our observation of an effect of *Dpyd* on total sleep primarily during lights off, and exclusively during the first three hours when comparing the PWK/PhJ and NOD/LtJ Collaborative Cross strains, is consistent with prior literature in transgenic mice, including in the histidine decarboxylase knockout mice[74] and in mice lacking *Homer1a*.[75] Although the largest difference between *Dpyd* knockout and wildtype mice was observed in the first three hours of lights off, there was evidence of differences across the entire lights off period. There was also evidence of increased number of sleep bouts and shorter sleep bout duration during lights on when comparing knockout mice to wildtype controls, although no difference in total sleep. This suggests *Dpyd* could play a role in sleep consolidation.

Results of the present study in mice are likely to be relevant in human populations. Two recent GWAS conducted in approximately 90,000 individuals from the UK Biobank found associations between single nucleotide polymorphisms (SNPs) in *DPYD* and accelerometer-measured sleep duration.[13,15] The minor alleles of both lead SNPs (rs75641275 and rs2660302) were associated with shorter sleep duration. Interestingly, common and rare frequency human variants near *DPYD* have also been associated with a number of neuropsychiatric conditions, including autism spectrum disorders,[41] intellectual disability,[42] and schizophrenia.[43] This is consistent with existing evidence of pleiotropic genetic effects on sleep and neurological disorders.[12,45] A potential mechanism underlying the relationship between genomic variation near *DPYD* and these disorders relates to down-regulation of *DPYD* gene expression that may result in deficiency of the encoded enzyme.[34]

Given these implications beyond sleep,[41-44] we performed a battery of procedures to assess cognitive, social and affective disorder-related behaviors. While we observed differences between male and female mice in our behavioral assays, there were no significant differences between *Dpyd* knockout and wildtype control mice for these traits. *Dpyd*

knockout mice demonstrated intact balance on the Rotarod, spontaneous exploration of the open field arenas and elevated zero maze, with no anxiety-related behavior detected. Auditory startle response, pre-pulse inhibition and novel object memory were not affected by *Dpyd* deletion. Thus, the effect of *Dpyd* appears relatively specific to sleep in our dataset. However, we cannot rule out the potential of finding pleiotropic effects of *Dpyd* on non-sleep traits, as has been commonly seen with other sleep genes[45], with expanded behavioral testing in larger samples. Ultimately, the specificity of *Dpyd* should be followed up in future studies.

To ultimately decipher whether or not *DPYD* variants have pleiotropic effects on expression of distinct clinical traits in humans, further research is necessary. Notably, pharmacogenetic variants of *DPYD* have been described in human populations.[76] In particular, deleterious variants in *DPYD* are associated with dihydropyrimidine dehydrogenase deficiency and increased risk for toxicity following treatment with 5-fluorouracil, which is used as a chemotherapeutic drug.[77,78] As such, assessment of *DPYD* activity is now routine before this therapy is started. Furthermore, a query of human reference populations data resources[79-81] support a number of potentially damaging variants in *DPYD*. At the time of this study, there were 91 predicted loss of function variants identified in the reference populations included in the Genome Aggregation Database (https://gnomad.broadinstitute.org) and 81 copy number variants encompassing *DPYD* that were reported in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home) and Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (https://www.deciphergenomics.org/). Notably, other more commonly occurring variants in *DPYD* (e.g., expression quantitative trait loci) could affect gene expression and, in turn, influence sleep and wake. Specifically, our data suggest that individuals with loss of function variants in *DPYD* will have altered sleep.

Applying information on these known *DPYD* variants to data from large biorepositories linked to electronic health records could lead to identification of individuals that are very likely to exhibit deficiency of *DPYD*. These individuals, in turn, could be re-contacted for deep, objective phenotyping of sleep. This approach may help better define the effects of *DPYD* deficiency on sleep and other behaviors in humans. We expect that this knowledge will be useful for informing new precision medicine approaches to treatment, particularly for individuals with sleep disturbances and neuropsychiatric disorders, and may lead to insights that are useful in the general population.

Overall, this study provides a roadmap for utilizing a cutting-edge paradigm in mice for discovery and validation of a novel gene affecting sleep. Analyses incorporated high-throughput and gold-standard phenotyping across different mouse models, including Diversity Outbred mice with high genetic diversity, Collaborative Cross recombinant inbred mice selected based on their *Dpyd* founder haplotypes, and *Dpyd* knockout mice to functionally validate the predicted association. However, there are also limitations in our approach that should be considered in future studies. First, gene expression analyses were based on available RNA sequencing data in the hippocampus. While this is not a primary brain region affecting sleep, similar data in DO mice was not available for more relevant regions, such as the pre-optic area of the hypothalamus. Thus, these analyses presumed

that strong founder-specific effects on *Dpyd* expression would generalize to other areas of the brain. However, future RNA sequencing analyses in brain regions more directly relevant to sleep are warranted to test this assumption and understand specificity of these effects. Second, for validation in Collaborative Cross lines we selected strains with predicted decreased (two lines with PWK/PhJ *Dpyd* alleles), intermediate or neutral (NOD/LtJ and CAST/EiJ *Dpyd* alleles) and increased (NZO/HILtJ and WSB/EiJ *Dpyd* alleles) expression of *Dpyd*. While this covers the spectrum of *Dpyd* expression, future studies examining multiple Collaborative Cross strains with the same *Dpyd* founder haplotypes would be useful to understand the potential for genomic variation at other locations to influence the sleep phenotypes. Finally, while our experiments in *Dpyd* knockout mice clearly demonstrate an effect of loss-of-function on sleep, it would be helpful to also study the effect of *Dpyd* gain-of-function on sleep. Possible approaches could include studying many additional Collaborative Cross strains with *Dpyd* founder haplotypes predicted to increase expression or employing a *Dpyd* gain-of-function mutant mouse, which does not currently exist. Ultimately, gain-of-function experiments are an important next step to determine whether *Dpyd* alone is sufficient to drive sleep.

In conclusion, using a rigorous discovery and validation approach including Diversity Outbred, Collaborative Cross, and knockout mice, we have identified and confirmed a previously unreported gene regulating sleep and wake in mice – *Dpyd*. This finding suggests that β-alanine plays a role as a neurotransmitter in sleep/wake control. These results, when combined with the recent implications through GWAS, evidence of effects of variation in *DPYD* on neuropsychiatric conditions with commonly coexisting sleep abnormalities, and existence of deleterious pharmacogenetic variants of *DPYD*, strongly suggest the effects of variation in this gene are relevant to sleep alterations in humans.

STAR METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests should be directed to and will be fulfilled by the lead contact, Dr. Allan I Pack (pack@pennmedicine.upenn.edu).

Materials availability—Mouse lines leveraged in this study are available through existing resources, including the Jackson Laboratory for Diversity Outbred mice (https://www.jax.org/strain/009376)[26-28], the Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py) for Collaborative Cross mice, and the Toronto Center for Phenogenomics through the International Mouse Phenotyping Consortium (C57BL/6N-Dpyd$^{tm1b(KOMP)Wtsi>}$/Tcp; http://www.mousephenotype.org).[39,40]

Data and code availability

- Phenotypic data on mice analyzed in this manuscript have been uploaded to *figshare* (https://figshare.com/projects/The_dihydropyrimidine_dehydrogenase_gene_contributes_to_heritable_differences_in_sleep_in_mice/122094) and are publically available as of the date of

publication. Genetic data on the 325 DO mice are available through the Jackson Laboratory Diversity Outbred database (https://www.jax.org/research-and-faculty/genetic-diversity-initiative/tools-data/diversity-outbred-database; *project:* 209_DO_Pack_Sleep). Data from RNA sequencing in the hippocampus are available as described by Skelly *et al.*[32]

- This paper does not report original code. Analyses described were performed using available statistical software, including R (www.r-project.org), primarily the *qtl2* package,[37] and Stata/SE 14.2 (StataCorp LLC, College Station, TX). RNA sequencing data were analyzed using the *DO Hippocampus QTL Viewer* created by the Churchill Lab and available at https://churchilllab.jax.org/qtlviewer/DO/hippocampus. Stata programs used to analyze specific datasets are provided through *figshare* (https://figshare.com/projects/The_dihydropyrimidine_dehydrogenase_gene_contributes_to_heritable_differences_in_sleep_in_mice/122094) as examples.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All studies were done in accordance with guidelines published by the National Institutes of Health and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The following provides details on specific mouse models used in these experiments.

Diversity Outbred Mice—Studies were done in male Diversity Outbred mice (n=325).[26-28] These mice were ordered 3 times/year in groups of 50 non-siblings (mice are generated in waves), following the Diversity Outbred production schedule (https://www.jax.org/strain/009376). All mice were approximately 6-8 weeks of age when delivered to the University of Pennsylvania. Mice were approximately 2-3 months of age at the time of sleep phenotyping.

Collaborative Cross Mice—We obtained male Collaborative Cross (CC) mice with specific founder alleles at the identified *Dpyd* locus. These mice were obtained from the Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py).[38] The specific mice we obtained included two strains with PWK/PhJ *Dpyd* alleles (CC003/Unc [black] and CC009/Unc [white]), one strain with the NOD/LtJ *Dpyd* allele (CC011/Unc), one strain with the CAST/EiJ *Dpyd* allele (CC040/TauUnc), one strain with the WSB/EiJ *Dpyd* allele (CC051/TauUnc), and one strain with the NZO/HILtJ *Dpyd* allele (CC004/TauUnc). Mice were approximately 2-3 months of age at the time of sleep phenotyping.

Dpyd *Knockout Mice—**Dpyd* knockout (KO) mice were obtained from the Toronto Center for Phenogenomics through the International Mouse Phenotyping Consortium (http://www.mousephenotype.org).[39,40] Briefly, gametes of strain C57BL/6N-Dpyd$^{tm1b(KOMP)Wtsi>}$/Tcp were received at the Jackson Laboratory (Bar Harbor, ME) and

used to generate breeders. Breeders were transferred for expansion and phenotyping to the research team at University of Pennsylvania. Mice were bred with C57BL/6J to generate heterozygous mice. Heterozygous mice were then bred to obtain both homozygous knockout mice and wildtype littermate controls. Both male and female knockout and wildtype mice were studied. Mice were approximately 2-3 months of age at the time of sleep phenotyping.

Mice were genotyped using an assay provided by the Toronto Center for Phenogenomics to select homozygous and wildtype (non-carrier) animals for phenotyping. Genotyping was performed using PCR followed by gel electrophoresis for DNA amplicon detection. Briefly, DNA extractions from tails or ear punches and PCR amplifications were performed using Extract-N-Amp Tissue PCR kit (Sigma) following manufacturer's instructions. The following PCR primers were used in the reaction: DPYD1: 5'-TGT ACA GTG CAT CTG GGC TAC −3'; DPYD2: 5'- CTC TCA CCT TGA GCT GTT CCT −3'; DPYD3: 5'- CAC ATC TCA CTG CCA ACT TCA −3'; DPYD4: 5'- CGG TCG CTA CCA TTA CCA GT −3'. Wildtype mice yielded a single 366bp band, mutant mice yielded a single 472bp band, and heterozygous mice yielded both bands.

METHOD DETAILS

Sleep Phenotyping Studies in Mice—Sleep and wake phenotyping was performed in mice using both non-invasive high-throughput methods and gold-standard electroencephalogram/electromyography (EEG/EMG). Estimates of circadian period during constant darkness were also obtained. More details are provided below and described in our previous manuscript.[30]

Non-invasive Assessment of Sleep and Wake: Mice were individually housed in a sound-attenuated facility with control of light/dark, temperature, and humidity. Lights on was at 7:00AM and lights off at 7:00PM. During lights on the lux level at the base of the mouse cage was 80 lux. Temperature was regulated between 22-25°C. Humidity was 40-60%. Food and water (chlorinated tap water) were provided *ad libitum*.

Mice were acclimated for 7-10 days. Thereafter, we recorded their activity patterns for 5 days by recording in 10-second epochs the number of times a mouse crossed electronic beams that intersected the mouse cage (Micromax, Accuscan Instruments, Inc.). As we have done previously,[30,35] sleep was defined as 40 seconds or more of continuous inactivity. This has been previously validated in C57BL/6J mice.[35]

Following assessment of sleep, we investigated other sleep/circadian phenotypes, as previously described.[30] The current manuscript focuses mainly on sleep characteristics, since we obtained a quantitative trait locus for sleep amounts in the lights off period.

Circadian Period: Analyses of circadian period were performed similarly to those described previously.[30] Briefly, mice were singly housed with a running wheel and allowed 7-10 days for acclimation to the new environment. After acclimation, voluntary wheel running was recorded during 10 days with 12-hour/12-hour light/dark (L/D) and then for 18-20 days of constant darkness (D/D), during which free-running circadian period was recorded. ClockLab (Actimetrics, Wilmette, IL, USA) software was used to estimate

circadian period in hours during D/D using the least-squares line fit to the wheel running onset times.

Electroencephalogram/electromyography (EEG/EMG): We used techniques previously described from our laboratory to perform EEG/EMG recording and gold-standard determination of sleep characteristics.[35] In brief, mice were instrumented for recording of EEG/EMG signals. These signals were amplified and filtered as previously described.[35] Following surgery, animals were allowed a period of 7-10 days to recover and acclimate to their surroundings. EEG/EMG signals were recorded for 24 hours and recordings were scored in 10-second epochs as wake, non-rapid eye movement (NREM) or rapid eye movement (REM) sleep. Our quality control procedure for scoring accuracy started with a visual inspection of each recording to evaluate signal quality prior to scoring. Any mechanical or electrical artifact was identified and removed from the data pool during the inspection process. Sleep stage scoring was performed by two different scorers, blinded to genotype. These data were compared for accuracy and agreement. As a final quality control, a third scorer reviewed only the sections of the record where there was disagreement and re-scored those sections to determine a final percentage of agreement. Using this method of scoring, we generally see 88-94% agreement between scorers for all epochs.

Behavioral Phenotyping Studies in Mice—A battery of behavioral phenotyping assays were performed in *Dpyd* knockout and wildtype mice. In order, they were the elevated zero maze, open field activity, object location memory, social preference, accelerating Rotarod, and acoustic startle response with pre-pulse inhibition. At least one day of rest was allowed between each procedure.

Elevated Zero Maze: Exploration of the elevated zero maze is sensitive to anxiolytic drugs and some antidepressants; thus, it is routinely used to assess anxiety-related behavior. The maze consists of a 2.5-inch wide circular track with 2 walled and 2 open regions, elevated 12 inches above the floor. A 30-minute habituation to the testing room occurred prior to the procedure. Each mouse received a single trial. To begin a 5-minute trial, a mouse was placed in the center of a walled area. Digitally recorded trials were processed for automated analysis by ANY-maze software (Stoelting Co., Wood Dale, IL, USA) to generate the time spent in the open areas of the maze and distance traveled.

Open Field Activity: Spontaneous activity in an open field arena is commonly used to assess locomotion and rearing as part of a general health assessment. Activity data are collected with a Photobeam Activity System (San Diego Instruments, Inc., San Diego, CA, USA). The square Plexiglas arena (14 inches x 14 inches) has clear 18-inch walls fitted with IR emitters and photosensors to record rearing, center and peripheral activity as beam breaks. After 30 minutes of habituation to the procedure room, a mouse was placed in the center of the arena for a 10-minute trial. Each mouse received a single trial.

Object Location Memory: The object location memory procedure is used to assess spatial memory. With repeated exposure to an arena where unique objects are placed in specific locations, a familiarity with the location of each object is established. In a subsequent trial, when one of the objects is presented in a different location, mice will spend a greater time

exploring the displaced object. If a preference for exploration of the displaced object is not seen, a failure to recall its placement during training is assumed. The arena is 12 inches x 12 inches, with 18-inch walls. The objects used are a glass bottle, a 1-inch diameter PVC tube, and a metal tower (5H x 2W x 2L inches). Prior to the procedure, each mouse was handled for two minutes a day for three consecutive days. The actual procedure began with a 6-minute habituation phase to the empty arena with a black and white striped placard attached to one wall. Three 6-minute acquisition trials followed, where the three objects were placed at measured distances away from three of the corners of the arena. The inter-trial interval was about 6 minutes, after which the mice were returned to their home cage so that the arena and objects could be wiped with 70% EtOH. Twenty-four hours later, one object is placed catty-corner, diagonal to its position in the acquisition trials. The entire procedure was performed once on each mouse. All trials were digitally recorded for automated analysis. ANY-maze software (Stoelting Co., Wood Dale, IL, USA) was applied to the recordings to generate distance traveled, time spent exploring each object and bouts of exploration. Exploration was defined as an epoch when the snout of the mouse is within 1 cm of an object.

Social Preference: The three-chamber social choice preference procedure is used to quantify interactions with a novel mouse relative to an inanimate cue. It is routinely applied to mouse models related to Autism as a measure of social behavior. Mice preferentially explore the social cue versus an inanimate cue; reduced exploration of the social cue suggests reduced social interaction. The equipment consists of an arena and cylinders that contain the cues explored. The rectangular arena is approximately 10 x 18 inches with 18-inch high walls; internal walls jut perpendicularly into the arena to create three contiguous chambers. Clear acrylic cylinders (3-inch diameter x 5 inches tall) with multiple 0.25 inch holes around their perimeter are placed at opposite ends of the arena. A ten-minute habituation phase was performed first, where the experimental mouse was allowed to freely explore the arena with the empty cylinders in place. Immediately after habituation, the choice phase began upon loading an inanimate cue (rock) in one cylinder and a social cue (same sex gonadectomized A/J mouse) in the opposite cylinder. The procedure was performed in dim light, illuminated from below with infrared (IR) lights. IR-capable cameras were used to digitally record the trails for automated analysis. Each mouse was tested once with this procedure. ANY-maze software (Stoelting Co., Wood Dale, IL, USA) was applied to the recordings to generate distance traveled, time spent exploring each cue and bouts of exploration. Exploration is defined as an epoch when the snout of the mouse is within 1 cm of a cylinder.

Accelerating Rotarod: The Rotarod (IITC Life Science, Inc., Woodland Hills, CA, USA) is a 1-inch diameter, horizontal rod with a rough surface that is set to rotate and/or accelerate at different rates. As the rod speed increases, the mouse must adjust its stride to remain walking on the rod. An inability to adjust cadence will cause the mouse to fall or grip onto the rotating rod. A decrease in the latency to stop walking on the Rotarod suggests a coordination or balance impairment. The complete procedure consisted of three trials per day over three consecutive days with the Rotarod programmed to accelerate from 4 to 40 rotations per minute (rpm) during a 5-minute trial. On the first day only, mice were placed

on the stationary rod to allow for habituation before rotation starts. All inter-trial intervals were about 30 minutes. The equipment was wiped with 70% EtOH between all trials. Every test day, mice were habituated to the procedure room for 30 minutes. Latency to fail was defined as the time to drop from the rod or time to make a full rotation while gripping on to the rod. Each mouse was tested on this procedure once.

Acoustic Startle Response and Pre-pulse Inhibition: The acoustic startle response (ASR) is a standard test of motor responsiveness to a strong sensory stimulus. Pre-pulse inhibition (PPI) is included in the procedure. PPI naturally occurs when a weaker, non-startle evoking stimulus precedes a stronger startle-evoking stimulus. The weak pre-pulse predicts the higher intensity stimuli to attenuate the response and is considered a measure of sensory-motor gating. Deficits in ASR and PPI are seen in post-traumatic stress disorder and schizophrenia.[82-85] A rudimentary assessment of habituation was also obtained by comparing responses late in the train of stimuli delivered to responses early in the procedure. Mice were tested for ASR and PPI with 4 SR-Lab systems chambers (San Diego Instruments, Inc., San Diego, CA, USA). The chambers are sound attenuating, ventilated boxes illuminated with a 15-watt light bulb. A 5-inch x 1.75-inch diameter Plexiglas tube was mounted on a platform with a stabilimeter. The stabilimeter transduces motor activity, which is digitized and recorded. Acoustic stimuli were delivered through small speakers mounted inside each chamber. A continuous 70-dB white noise was presented throughout the session. An ASR session consisted of blocks of trials to assess responses to different intensity stimuli, pre-pulse inhibition and habituation. Each mouse received one ASR session. After a 5-minute acclimation to the background noise, the mice received a string of six 120dB white noise bursts to collect baseline response. This was immediately followed by 40 presentations of 4 different white noise intensities (100-dB, 110-dB, 120-dB and background noise) spaced about 15 seconds apart. The white noise bursts have 40 millisecond durations. Each of the intensities were presented 6 times in the session in a pseudo-randomized fashion. The movement of the mouse in response to the acoustic stimulus was transduced to a digital format stored on a computer. Data were collected as peak amplitude of the startle response, average startle response, total movement during the trial and latency to peak startle response. The data were used to calculate an intensity-response curve.

The PPI block of stimuli began immediately after startle testing was completed. The PPI session consists of forty 120-dB, 40-millisecond white noise bursts spaced 15 seconds apart. The 120-dB stimuli were delivered either alone or preceded by a 20-millisecond pre-pulse stimulus delivered at three different intensities (78-dB, 81-dB or 85-dB). The percent decrease in the startle response with the pre-pulse compared to the startle response without pre-pulse is a measure of PPI.

Finally, a string of six, 120-dB white noise bursts were presented to be compared to the block of 120-dB bursts that initiate the session. The latter block was compared to the former to calculate habituation.

Diversity Outbred Mouse Genotyping—Diversity Outbred mice were euthanized by cervical dislocation and tail samples were collected, frozen in dry ice and stored at

−80°C. Tail samples from all mice were delivered to the Jackson Laboratory, where DNA was extracted for genotyping carried out using the high-density Mega Mouse Universal Genotyping Array (MegaMUGA) at GeneSeek (Lincoln, NE). A hidden Markov model analysis was utilized to infer the haplotype blocks in each DO genome from the array intensities. A total of 325 DO mice met quality criteria and were included in the specific QTL analyses presented here. Given 8 Founder mouse strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, WSB/EiJ, PWK/PhJ) each mouse has one of 36 possible diplotypes at each locus (8 homozygous and 28 heterozygous). The diplotype probability at each locus was derived for each mouse using a hidden Markov model applied to genotype data,[26] which was then utilized in genetic analyses (described in more detail below).

Expression Profiling Data in Hippocampus—To examine differences in gene expression associated with identified QTLs, we leveraged publicly available gene expression profiling data in the mouse hippocampus generated using RNA sequencing in an independent sample of 258 Diversity Outbred mice.[32] Details on data generation, including RNA isolation, sample preparation, sequencing depth, alignment and normalization, are provided in Skelly *et al.*[32] Genotyping on these DO mice was performed using the Mouse Universal Genotyping Array (MUGA)[36] and haplotypes blocks inferred using a hidden Markov model.[26] Data were visualized and LOD peaks of associations between genotypes and expression data identified via the *DO Hippocampus QTL Viewer* created by the Churchill Lab and available at https://churchilllab.jax.org/qtlviewer/DO/hippocampus. Raw data are also available as described by Skelly *et al.*[32]

QUANTIFICATION AND STATISTICAL ANALYSIS

Genetic Association Analysis to Identify Quantitative Trait Loci (QTLs)—To identify quantitative trait loci (QTLs), we utilized established methods for Diversity Outbred mice that account for the multi-parental nature of the outbreeding[26,37] and available software packages and functions in R (www.r-project.org), primarily *qtl2*.[37] Specifically, genome scans were performed using an additive model implemented via a linear mixed model, with founder genotype probability as a random effect[26] and accounting for relatedness among DO mice. This model was used to derive 8 Founder-specific β-coefficients describing the additive effect of each founder haplotype on the outcome. Relatedness was quantified based on a chromosome-specific genetic kinship matrix generated among all DO mice using the leave-one-chromosome-out method via the *calc_kinship* function in *qtl2*.[37] Association scans to determine genetic loci associated with phenotypes of interest were performed using the *scan1* function in *qtl2*.[37] Significance at each locus was based on a likelihood-ratio test with 7 degrees of freedom, with LOD scores estimated as the difference in $\log_{10}$ likelihoods of models with and without founder genotypes. Genome-wide plots of LOD scores were used to identify associated loci, with suggestive or significant loci determined based on permutation testing, using 1,000 permutations and the *scan1perm* function in *qtl2*.[37] Suggestive loci were identified using a 90% threshold and statistically significant loci based on a 95% threshold. For any loci of interest, 95% Bayesian Credible Intervals were estimated to determine the linkage region and identify the set of genes most likely causative for the observed association.

Comparisons of Phenotypes in Collaborative Cross or Knockout Mice— Additional analyses were performed within selected Collaborative Cross and knockout mice based on the results of the QTL association analyses. As detailed above, phenotypes of interest included sleep traits from high-throughput phenotyping in multiple CC strains, EEG/EMG-based sleep characteristics in CC strains and knockout/wildtype mice, and circadian and behavioral phenotypes in knockout mice and wildtype littermate controls. Data are summarized using averages and appropriate measures of uncertainty, including standard deviations, standard errors or 95% confidence intervals, as noted. In addition, to provide context with respect to the magnitude of the observed differences between mice, we calculated standardized mean differences (Cohen's d); absolute values of these differences equal to 0.2, 0.5 or >0.8 can be interpreted as small, medium and large effects, respectively.[86]

Comparisons of high-throughput sleep phenotypes among multiple CC strains were performed using analysis of variance (ANOVA) to test the global null hypothesis of no phenotypic differences among strains. If this global hypothesis was rejected, pairwise comparisons between pairs of CC strains were subsequently performed. Primary pairwise comparisons of interest were between CC strains carrying the PWK/PhJ *Dpyd* haplotypes and other non-PWK carrying lines. In addition to high-throughput phenotyping, gold-standard EEG/EMG was performed in one CC strain with the PWK/PhJ *Dpyd* haplotype (CC003/Unc) and one with the NOD/LtJ *Dpyd* haplotype (CC011/Unc). Given the relatively low number of mice studied, comparisons of EEG/EMG phenotypes between these two CC strains were based on non-parametric Wilcoxon rank-sum tests. Similar analyses were performed when comparing sleep and circadian traits in *Dpyd* knockout and wildtype mice, utilizing Wilcoxon rank-sum tests in analyses performed both pooled and stratified by sex (where indicated). For sleep traits, statistical interaction tests were used to assess for any evidence of effect modification by sex by evaluating the significance of a product term ([Genotype] x [Sex]) in the context of a regression model that included both main effects.

Finally, analyses of behavioral phenotypes within *Dpyd* knockout and wildtype mice were performed in collaboration with the Neurobehavior Testing Core at the University of Pennsylvania to comprehensively evaluate differences based on genotype and sex, as well as test-specific characteristics for multiple behavioral phenotypes. Analyses utilized a two-way ANOVA to simultaneously evaluate the presence of main effects of genotype and sex and potential statistical interaction between genotype and sex. To appropriately account for repeated measures within animals for certain behavioral tests, analyses were performed using linear mixed models to evaluate the evidence of interaction between genotype and test-specific factors (e.g., trial, social vs. inanimate cue, or dB level) in a model including the product term, both main effects, and sex (in analyses combining males and females). Main effects of sex, genotype and test-specific factors were evaluated simultaneously in a model without the interaction term.

As our primary goal was to validate identified associations with total sleep during the lights off period, total sleep was considered our primary phenotype and statistical significance was based on a $p<0.05$ for activity-based sleep or determined using the Hochberg step-down method[87] for total amount of sleep, NREM, REM and wake based on EEG/EMG.

Other sleep-related traits (e.g., bout numbers and average bout durations) were considered secondary. Statistical significance in these secondary analyses was evaluated using the Hochberg step-down method,[87] maintaining an overall type I error rate of 5% across different comparisons within a given time period (e.g., 24 hours, lights on and lights off). If significant differences were observed in ANOVA, a Bonferroni correction was used to determine statistical significance of pairwise comparisons. For behavioral traits, a Bonferroni-corrected level of significance was used to determine statistical significance of genotype or sex differences, adjusted for the number of traits evaluated within a given test. This includes a $p<0.05$ for acoustic startle response and habituation, a $p<0.025$ for elevated zero maze, social preference and social choice, a $p<0.0167$ for open field, Rotarod and PPI, and a $p<0.01$ for object location memory. A $p<0.05$ was used for significance in comparisons of circadian period and considered nominal evidence in all analyses. All statistical analyses were performed using Stata/SE 14.2 (StataCorp LLC, College Station, TX).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Dihydropyrimidine dehydrogenase (Dpyd)* affects sleep in Diversity Outbred mice.
- Knockout of *Dpyd* resulted in 78.4 minutes less sleep in lights off period.
- No differences in other measured behaviors with knockout of *Dpyd*.
- Results support β-alanine as a neurotransmitter that promotes sleep.

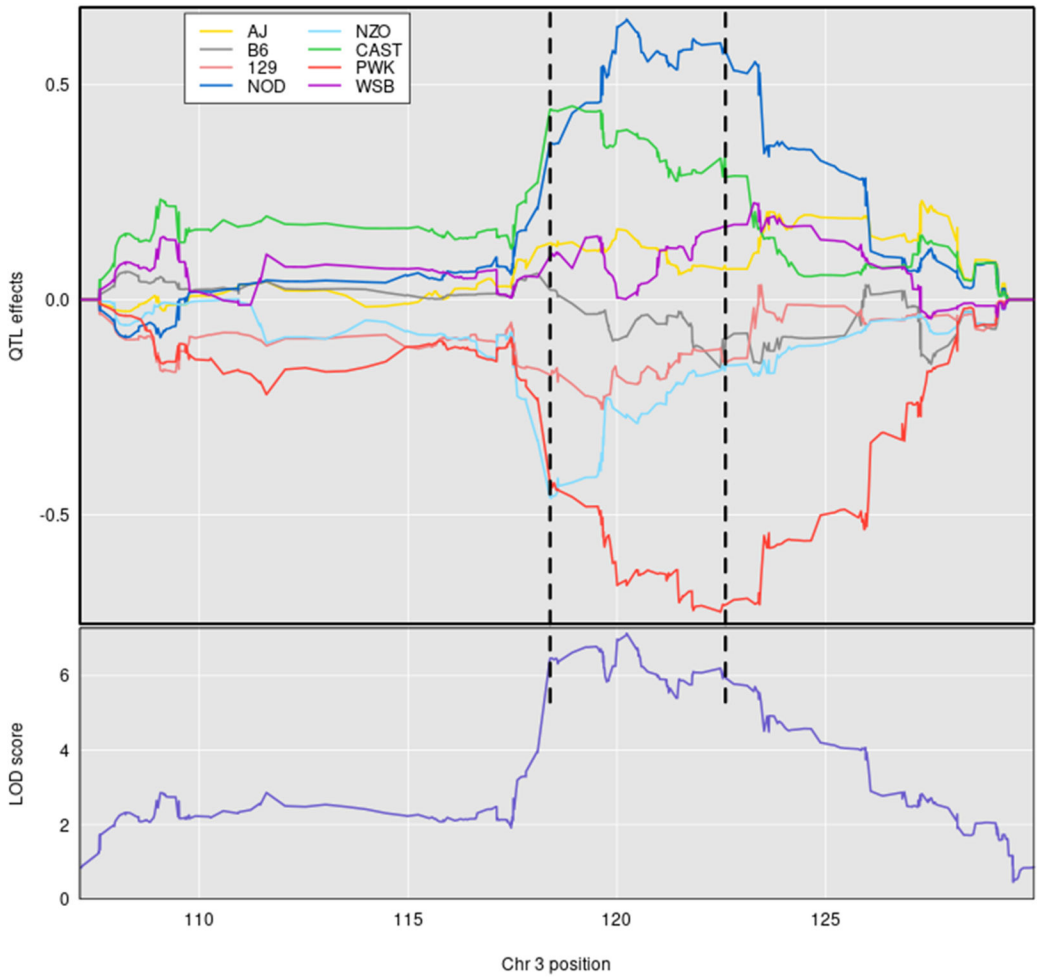

Figure 1. Allele effects at the LOD peak for total sleep during lights off in DO mice.

Founder-specific additive genetic effects on total sleep during lights off are shown in the linkage region identified on chromosome 3 (top panel). The 95% Bayesian credible interval (118.4-122.6 Mb) is denoted with black dashed lines. Colored lines illustrate the relative change in total sleep duration attributable to each of the founder haplotypes, with lines below zero indicating a reduction in total sleep and lines above zero indicating increased total sleep. A large relative reduction in sleep is observed for the PWK/PhJ haplotype (red) in this region, with the NOD haplotype associated with increased sleep (dark blue). LOD scores across the region are shown in the bottom panel. See Figure S1 for candidate genes under LOD peak.

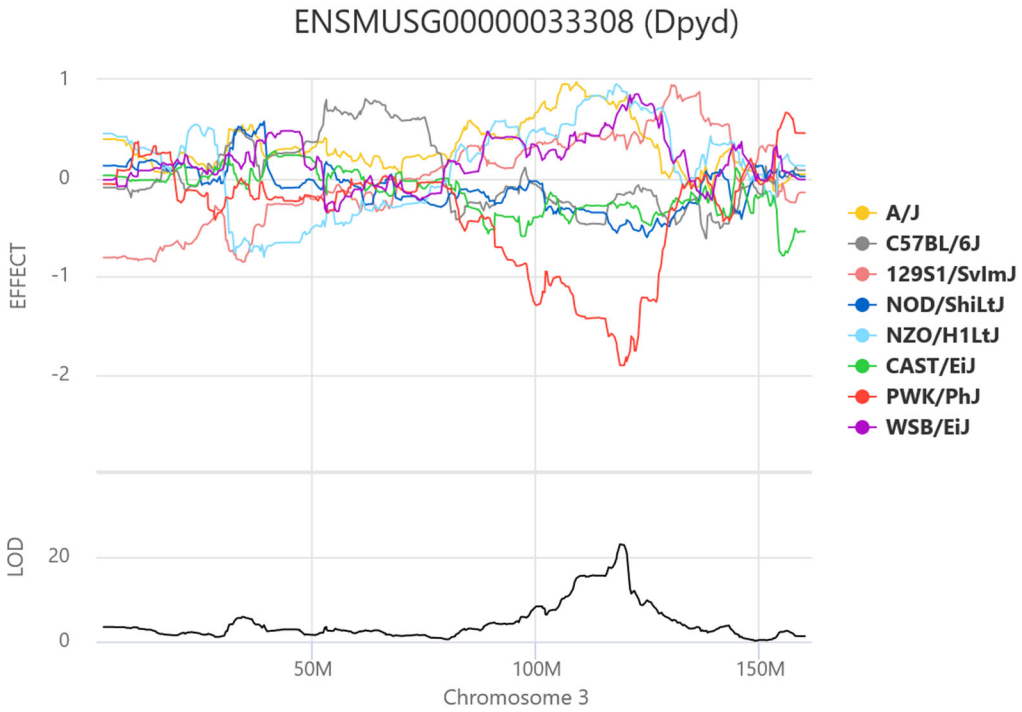

Figure 2. Expression of Dpyd in the hippocampus of DO mice.

Founder-specific additive genetic effects on *Dpyd* expression from RNA sequencing of the hippocampus in an independent sample of DO mice are shown in the top panel. There is a marked reduction in *Dpyd* expression associated with the PWK/PhJ haplotype at our linkage region of interest, with a LOD score >20. This PWK/PhJ-specific reduction in *Dpyd* expression is similar to the association seen for total sleep duration during lights off, leading to the selection of *Dpyd* as the likely causal gene under the linkage peak. Figure and data are available through the online *DO Hippocampus QTL Viewer* (https://churchilllab.jax.org/qtlviewer/DO/hippocampus). See Figure S1 for founder effects for expression of all genes within the linkage region.

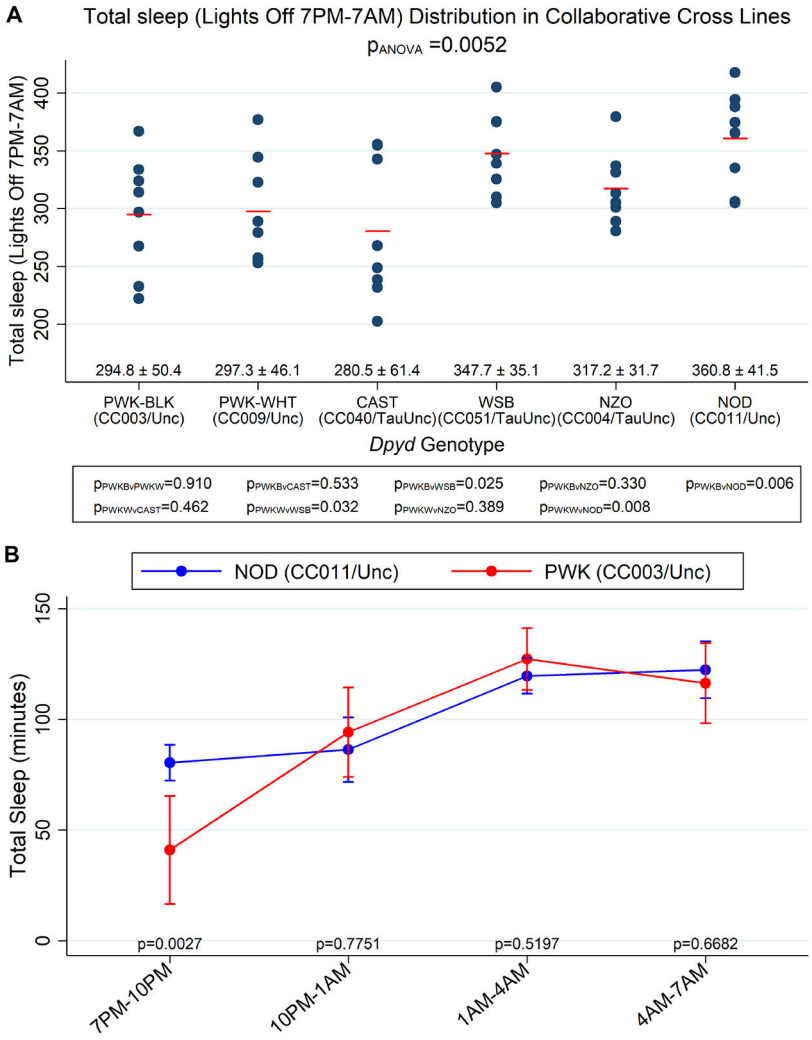

Figure 3. Comparison of total sleep during lights off in Collaborative Cross strains with relevant founder Dpyd alleles.

Figure 3A illustrates total sleep during lights off based on high-throughput phenotyping using beam breaks in Collaborative Cross strains with founder *Dpyd* alleles. There was a significant difference among strains (p=0.005), including differences between the black (CC003/Unc) and white (CC009/Unc) PWK/PhJ *Dpyd* strains and both the NOD/ShiLtJ (p=0.006 and 0.008, respectively) and WSB/EiJ (p=0.025 and 0.032, respectively) strains. See Tables S1 **and** S2 for additional phenotype comparisons. Figure 3B shows total sleep from EEG/EMG recording in 3-hour windows during lights off in the CC strains with PWK/PhJ (CC003/Unc) and NOD/ShiLtJ (CC004/TauUnc) *Dpyd* alleles. A moderately large difference on average was seen in total sleep over the entire lights off period, but this result did not reach statistical significance (p=0.240; Cohen's d = −0.71). When examining the lights off period in more detail, significantly less sleep was observed in the PWK/PhJ *Dpyd* strain during the first 3 hours (7PM-10PM; 41.0±26.4 vs. 80.4±7.7 minutes; p=0.003; Cohen's d = −2.03). See Tables S3 **and** S4 for additional phenotype comparisons. Results presented as Mean (95% CI)

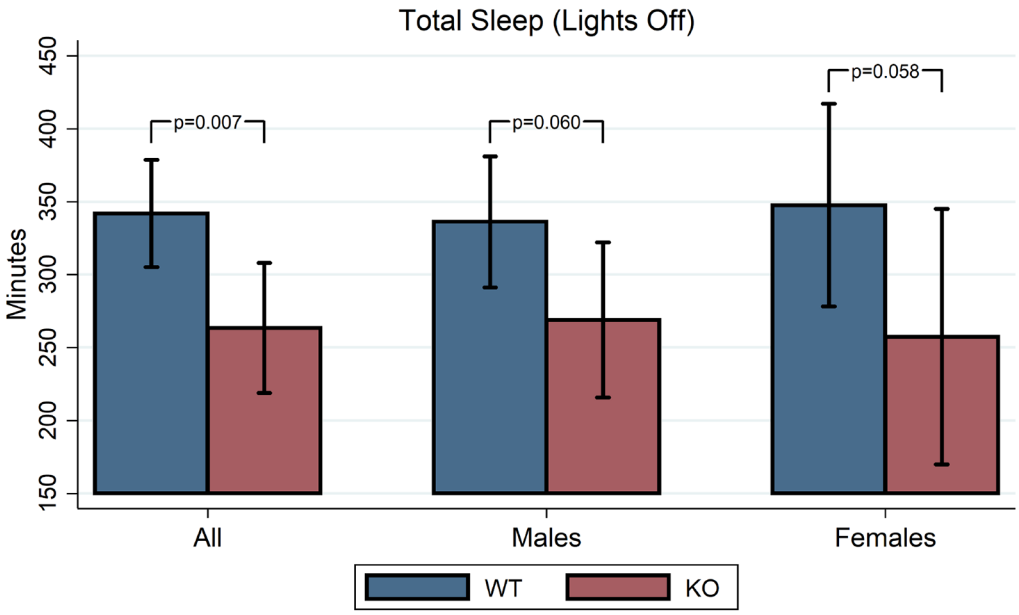

Figure 4. EEG/EMG total sleep in lights off in male and female Dpyd knockout mice.

Total sleep during the lights off period is shown in *Dpyd* knockout (KO) mice and wildtype (WT) littermate controls, overall and stratified by sex. Among all mice, there was significantly reduced sleep in the knockout mouse (263.5±92.6 minutes) compared to the wildtype control (341.9±74.0 minutes; p=0.007). The impact of the knockout was similar in male and female mice (interaction p=0.689); statistical significance is impacted by lower sample size for sex-specific analyses. See Tables S6 **and** S7 for additional phenotype comparisons.

Sex x Genotype Interaction p=0.689 | Data Presented as Mean (95% CI)

Table 1:

Sleep and wake architecture in *Dpyd* knockout and wildtype mice based on EEG/EMG recording (see also Table S5).

Measure	State	Lights On					Lights Off				
		Mean ± SD		SMD[a]	p[b]	p_{GxS}[c]	Mean ± SD		SMD[a]	p[b]	p_{GxS}[c]
		Wildtype (N=18)	*Knockout (N=19)*				*Wildtype (N=18)*	*Knockout (N=19)*			
Total Amount (minutes)	Sleep	469.7 ± 47.3	484.9 ± 33.5	0.37	0.386	0.793	341.9 ± 74.0	263.5 ± 92.6	−0.94	**0.007**	0.689
	NREM	412.8 ± 42.4	424.2 ± 33.2	0.30	0.504	0.941	310.6 ± 69.8	240.0 ± 83.8	−0.92	**0.008**	0.668
	REM	56.9 ± 14.3	60.7 ± 9.1	0.32	0.338	0.525	31.3 ± 11.0	23.4 ± 10.9	−0.72	**0.042**	0.962
	Wake	250.3 ± 47.3	235.1 ± 33.5	−0.37	0.386	0.793	378.1 ± 74.0	456.5 ± 92.6	0.94	**0.007**	0.689
Number of bouts	Sleep	132.8 ± 56.0	171.1 ± 36.2	0.81	**0.005**	0.931	87.1 ± 36.6	81.1 ± 35.3	−0.17	0.879	0.493
	NREM	146.1 ± 48.3	178.7 ± 30.0	0.81	**0.005**	0.899	101.3 ± 32.4	87.7 ± 34.6	−0.41	0.584	0.442
	REM	49.6 ± 17.0	57.8 ± 9.5	0.60	0.063	0.827	34.2 ± 12.0	26.6 ± 11.4	−0.65	0.153	0.716
	Wake	133.1 ± 56.0	171.4 ± 36.3	0.81	**0.005**	0.909	87.1 ± 36.6	81.6 ± 35.4	−0.15	0.855	0.490
Average Bout Duration (minutes)	Sleep	4.07 ± 1.53	2.97 ± 0.71	−0.92	**0.013**	0.475	4.34 ± 1.46	3.49 ± 1.13	−0.65	0.019	0.151
	NREM	3.04 ± 0.75	2.44 ± 0.48	−0.95	**0.008**	0.950	3.22 ± 0.86	2.86 ± 0.72	−0.46	0.064	0.051
	REM	1.19 ± 0.23	1.06 ± 0.16	−0.62	0.091	0.690	0.93 ± 0.17	0.87 ± 0.17	−0.36	0.395	0.182
	Wake	2.19 ± 1.06	1.45 ± 0.49	−0.90	**0.003**	0.947	5.11 ± 2.70	9.38 ± 11.61	0.51	0.316	0.826

Statistically significant p-values after Hochberg correction for total sleep or secondary sleep traits shown in **bold**.

[a] Standardized Mean Difference (Cohen's d) comparing wildtype and knockout mice

[b] p-value from Wilcoxon rank-sum test

[c] Interaction p-value testing whether genotype effect differs by sex.

Table 2:

Comparison of Behavioral Phenotypes in *Dpyd* Knockout and Wildtype Littermate Control Mice (see also Figures S2-S5)

Phenotype	Male				Female				ANOVA p-values[a]		
	Wildtype		Knockout		Wildtype		Knockout		Geno-type	Sex	Inter action
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD			
EZM Time in Open Areas (secs.)	6	71.37 ± 57.10	8	60.91 ± 35.87	11	92.43 ± 39.61	11	62.77 ± 27.34	0.145	0.396	0.476
EZM Distance Traveled (ins.)	6	564.0 ± 198.6	8	622.1 ± 90.3	11	878.0 ± 281.3	11	727.4 ± 140.4	0.500	**0.004**	0.134
OF Horizontal Activity (X,Y-axis Beam Breaks)	7	1216 ± 212	8	1386 ± 284	11	1500 ± 340	13	1649 ± 273	0.101	**0.007**	0.910
OF Center Activity (% Center Beam Breaks)	7	17.73 ± 6.30	8	32.47 ± 8.68	11	28.52 ± 14.92	13	30.08 ± 14.80	0.059	0.322	0.124
OF Rearing Activity (Z-axis Beam Breaks)	7	41.43 ± 33.51	8	53.38 ± 13.62	11	40.36 ± 16.11	13	44.15 ± 16.22	0.238	0.438	0.538
OLM Habituation Distance Traveled (m)	7	9.70 ± 2.38	8	9.85 ± 2.78	11	13.43 ± 2.55	13	12.19 ± 1.90	0.489	**0.0004**	0.377
OLM Displaced Object Visits (%)	7	39.18 ± 13.55	8	35.40 ± 14.34	11	39.77 ± 15.74	13	39.63 ± 13.10	0.679	0.611	0.700
OLM Displaced Object Exploration (%)	7	38.83 ± 15.59	8	36.90 ± 22.80	11	41.11 ± 15.96	13	41.71 ± 16.64	0.911	0.548	0.830
SP Habituation Distance Traveled (m)	7	17.60 ± 6.11	8	23.74 ± 5.57	11	31.95 ± 8.34	13	32.09 ± 10.65	0.267	**0.0003**	0.289
SP Total Empty Cylinder Exploration Time (secs)	7	48.59 ± 17.05	8	47.36 ± 17.87	11	48.04 ± 13.10	13	48.87 ± 19.84	0.973	0.933	0.858
PPI % of No Prestim Trials 78 dB	7	28.89 ± 49.05	8	36.45 ± 19.38	11	29.33 ± 34.19	13	35.69 ± 25.36	0.518	0.988	0.956
PPI % of No Prestim Trials 81 dB	7	46.27 ± 30.45	8	52.95 ± 15.44	11	45.67 ± 23.60	13	51.97 ± 29.19	0.448	0.926	0.982
PPI % of No Prestim Trials 87 dB	7	53.00 ± 30.67	8	72.01 ± 9.58	11	46.60 ± 33.20	13	60.74 ± 22.40	0.060	0.307	0.777
ASR Habituation % of Initial Responses	7	72.70 ± 50.35	8	103.70 ± 57.90	11	106.70 ± 24.15	13	106.87 ± 42.03	0.283	0.202	0.288

Statistically significant p-values shown in **bold**

[a] p-values from two-way ANOVA evaluating the effects of genotype, sex and the interaction between genotype and sex; *Abbreviations:* EZM: elevated zero maze; OF: open field; OLM: object location memory; SP: social preference; PPI: pre-pulse inhibition; ASR: acoustic startle response; SD: standard deviation

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
RNA Sequencing Data of Hippocampus	https://churchilllab.jax.org/qtlviewer/DO/hippocampus	See Skelly *et al.*[32]
Analysis Datasets	This paper	fig**share** repository **
Diversity Outbred Mouse Genotypes	https://www.jax.org/research-and-faculty/genetic-diversity-initiative/tools-data/diversity-outbred-database	Project Symbol: 209_DO_Pack_Sleep
Experimental Models: Organisms/Strains		
Diversity Outbred Mice	The Jackson Laboratory	J:DO Stock No: 009376
Collaborative Cross PWK/PhJ *Dpyd* Allele Mouse (Black)	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC003/Unc
Collaborative Cross PWK/PhJ *Dpyd* Allele Mouse (White)	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC009/Unc
Collaborative Cross NOD/LtJ *Dpyd* Allele Mouse	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC011/Unc
Collaborative Cross CAST/EiJ *Dpyd* Allele Mouse	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC040/TauUnc
Collaborative Cross WSB/EiJ *Dpyd* Allele Mouse	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC051/TauUnc
Collaborative Cross NZO/HILtJ *Dpyd* Allele Mouse	Mutant Mouse Resource and Research Center (MMRRC) at the University of North Carolina (http://csbio.unc.edu/CCstatus/index.py)	CC004/TauUnc
Dpyd Knockout Mouse	Toronto Center for Phenogenomics through the International Mouse Phenotyping Consortium (http://www.mousephenotype.org)	C57BL/6N-Dpyd$^{tm1b(KOMP)Wtsi>}$/Tcp
Software and Algorithms		
Stata/SE 14.2	StataCorp LLC, College Station, TX	n/a
R	www.r-project.org	n/a
R/qtl2	https://cran.r-project.org/package=qtl2	See Broman KW, et al.[37]
Example Analysis Programs	This Paper	fig**share** repository **

** https://figshare.com/projects/The_dihydropyrimidine_dehydrogenase_gene_contributes_to_heritable_differences_in_sleep_in_mice/122094