Early-life nutrition interacts with developmental genes to shape the brain and sleep behavior in *Drosophila melanogaster*

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G.H.O. and P.O. conceived and designed experiments with input from R.A.V. and T.F.C.M. F.N. and N.C. maintained fly stocks. G.H.O., F.N. F.V.-M., and N.Z. performed sleep experiments. G.H.O., F.N. F.V.-M., and N.C. performed immunohistochemistry experiments. G.H.O. and F.N. performed morphometric measurements. F.N. performed RNAi validation for sleep and morphometric analyses with input from G.H.O. G.H.O. performed statistical analyses with input from R.A.V. G.H.O. and K.O. performed gene networks analyses. G.H.O. and C.M. performed bioinformatic analyses with input from R.A.V. Figures and tables were prepared by G.H.O. The manuscript was written by G.H.O. and P.O. with input from R.A.V. and T.F.C.M. The authors declare no conflict of interest.

Abstract

The genetic variation of complex behaviors depends on the variation of brain structure and organization. The mechanisms by which the genome interacts with the nutritional environment during development to shape the brain and behaviors of adults are not well understood. Here we use the *Drosophila* Genetic Reference Panel to identify genes and pathways underlying this interaction in sleep behavior and mushroom bodies morphology.

We identify genes associated with sleep sensitivity to early nutrition, from which protein networks responsible for translation, endocytosis regulation, ubiquitination, lipid metabolism, and neural development emerge. We confirm that genes regulating neural development and insulin signaling in mushroom bodies contribute to the variable response to nutrition. We propose that natural variation in genes that control the development of the brain interact with early-life malnutrition to contribute to variation of adult sleep behavior.

Main Text

Introduction

Nutrition is an environmental factor that plays a crucial role in the maturation and functional development of the central nervous system [1-3]. In mammals, including humans, severe prenatal malnutrition negatively impacts neural development and complex behaviors such as sleep, memory, and learning [1, 4-7]. At the population level, adults that were exposed to hunger *in utero* have an increased risk to develop schizophrenia, affective disorders, addiction and decreased cognitive function [8, 9]. The origin of these disorders may be associated with defects in early brain development [10].

Little is known about the mechanisms by which individual genotypes (G) respond differently to a nutritional environment (E) during development. We define this type of genotype by environment interaction (GEI) as a genotype by early-life nutrition interaction (GENI) [11]. *Drosophila* provides exceptionally powerful tools and approaches for exploring the mechanisms underlying GENI at the single gene and genome-wide level [12]. The *D. melanogaster* Genetic Reference Panel (DGRP) [13] consists of sequenced inbred lines derived from a natural population that has been extensively used to chart the genotype-phenotype architecture of complex traits, including behaviors and brain morphology [12]. DGRP lines reared under different nutritional conditions show changes in behaviors and metabolic and transcriptional profiles, revealing a key role of GENI [14-18].

Alterations in sleep behavior are a common symptom of many neurological and psychiatric disorders, including neurodegenerative dementias and schizophrenia [19, 20]. Therefore, uncovering the genes and pathways underlying GENI's contribution to sleep behavior variation may shed light on altered neurodevelopmental mechanisms that can lead to mental illness. Few studies have focused on the role of early life nutrition in sleep behavior. Prenatally malnourished rats exhibit decreased sleep and increased waking activity [21, 22]; however, the genetic basis of such responses remains elusive. The influence of genetic variation on the effects of restrictive early life nutrition on adult sleep behavior and the extent to which this influence is mediated by changes in neural development and brain structure remains unknown.

A genome-wide association (GWA) study of sleep using the DGRP [23], allowed the identification of naturally occurring sleep behavior related genetic variants. Many of these variants were located in or near candidate genes with human orthologs that have been associated with sleep, which suggests that genes affecting variation in sleep are conserved across species [23]. Several of the candidate genes associated with natural variation in sleep affect developmental processes and neural function [23, 24] supporting the idea that variation in sleep is influenced by variation in the brain structures that control it.

The most anatomically and functionally characterized brain structures that regulate sleep behavior in *Drosophila* are the mushroom bodies (MBs), capable of both sleep and wake promotion [25-27]. Chemical ablation of the MBs showed a significant decrease in sleep [25, 26]. MBs contain two types of sleep-regulating neurons: those that promote sleep when cyclic-AMP-dependent protein kinase A (PKA) is increased

and those that inhibit sleep under such conditions [26]. Each MB consists of around 2000 Kenyon cells (KCs) whose axons are arranged in parallel arrays projecting into different lobular structures, the α and β, the α' and β' and the γ lobes [28]. KCs are sequentially generated from four neuroblasts in each hemisphere [29] that start to proliferate in embryos at stage 13 and continue uninterrupted until adult eclosion [29, 30]. Therefore, nutritional restriction during early life stages is likely to impact the development of CNS structures such as MBs [31]. Interestingly, natural genetic variation in the length and width of the α and β MB lobes has been associated with variation in aggression and sleep behaviors [32].

Here, we assessed the effect of GENI on adult sleep behavior and MB morphology and found significant effects on both. By using GWA studies we identified genetic variants and top candidate genes underlying GENI in variation of sleep and MBs morphology traits. Many proteins encoded by candidate genes are expressed in the MBs and form conserved protein-protein interaction networks required for endocytosis regulation, ubiquitination, lipid metabolism, and neural development. Finally, we confirmed that a group of genes required for neural development and insuling signaling, modifies the response of sleep behavior and MB morphology to early-life nutritional restriction. Together, our results indicate that natural variation in genes controlling nervous system development and physiology underlie variation in sleep in response to early life nutrition.

Results

GENI contributes to sleep variation in *Drosophila*

To determine the contribution of GENI to sleep variation, 73 DGRP lines were raised for one generation during larval stages on normal food (NF) or restricted food (RF) in which nutrients have been reduced to 20% those of NF. We quantified nine sleep traits: total, day and night sleep duration, day and night sleep bout number, and day and night average sleep bout length, waking activity and latency (Fig 1, S1 and S2 Figs, S1 and S2 Tables). We observed significant variation in sleep parameters among the DGRP lines reared under both nutritional conditions (Fig 1, S1 and S2 Figs, S1-S3 Tables). Cross-sex genetic correlations (r_{MF}), which represent the extent to which the same variants affect a trait in males and females, were significantly different from unity (NF, r_{MF} = 0.54-0.87; RF, r_{MF} = 0.60-0.83) (S3 Table). Thus, some polymorphisms affect sleep susceptibility to rearing diet in both sexes, while others will have sex-specific or sex-biased effects.

Fig 1. Sleep trait response to early life nutrition.

Histograms of female sleep trait mean + SEM for (A) Day time sleep, (B) Night time sleep. Reaction norms for sleep traits. (C) Day time sleep, (D) Day average bout length, (E) Day bout number, (F) Night time sleep, (G) Night average bout length, (H) Night bout number. Each DGRP line is represented by a different color. N: Normal food; R: Restricted food.

The differential responses of different genotypes to RF are evident from the complex pattern of crossing reaction norms, which is a hallmark of genotype by environment interaction (Fig 1, S1 and S2 Figs). To quantitate the contribution of GENI to the genetic variance, we estimated the interaction coefficient (i^2) across food, which is calculated by subtracting the cross-environment genetic correlations from 1 (1- r_{NR}).

Estimates for i^2 showed that GENI contributes from 5 to 17% to sleep genetic variation when flies are reared during larval stages in NF and RF (S3 Table).

These data together led us to hypothesize that the differential responses in sleep behavior of different genotypes to early-life malnutrition may depend on the variation in genes that act during development.

Genetic variants associated with GENI for sleep

To identify genetic variants underlying GENI, we performed GWA analyses for the difference in sleep between the two diets for each of the nine sleep traits, separately for males and females. All variants associated with all nine sleep variables from both sexes were pooled together for subsequent analysis as variants associated with sleep susceptibility to early life nutrition. We found a total of 1,410 variants across all sleep traits (at a nominal reporting threshold of $P \leq 10^{-5}$) (S3 and S4 Figs, S4 Table). Among these, 11% of such variants were located within coding sequences, while 47% were in introns and the 5' and 3' untranslated region (UTR), and the remaining 42% were intergenic (outside the transcribed region) (Fig S5, S4 Table).110 (15%) candidate genes are highly enriched for GO terms (FDR < 0.05) associated with the function and development of the nervous system, including nervous system development, neurogenesis, neuron differentiation and development, and axonogenesis (Fig S7, S4 Table). These results suggest that the effect of differences in early life nutrition on sleep behavior in adulthood is mediated in part through the regulation of the neurodevelopmental programs.

To identify potential cellular processes and molecular pathways underlying GENI we generated protein-protein interaction networks with proteins encoded by candidate genes using the STRING database, which considers interactions based on direct (physical) and indirect (functional) associations [33]. We found a significantly enriched network (PPI enrichment *P*-value = 2.3×10^{-6}) using a high confidence score (score ≥ 0.700) (Fig 2). These proteins are highly connected through processes that include translation, regulation of endocytosis, ubiquitination, lipid metabolism, neural development and protease activity (Fig 2).

Fig 2. Significant protein-protein interaction network of proteins encoded by candidate genes underlying GENI in sleep behavior.

Borders indicate the strength of the evidence for a human ortholog. Black: DIOPT score < 3; Blue: DIOPT score 3–6; Green: DIOPT score 7–9; Orange: DIOPT score 10–12; Red: DIOPT score 13–15. See S4 Table for the complete list of human orthologs and their DIOPT scores. Orange background indicates Gene Ontology enrichment category for Nervous System Development. Circles have effects on at least one sleep trait behavior from RNAi knockdown experiments.

Functional assessment of candidate genes associated with GENI for sleep

We found that 20% (152 out of 747) of our set of candidate genes are known to be expressed in MBs either during larval stages or in the adult (S5 Table) [34-37]. We selected 15 candidate genes, 10 of which are involved in nervous system development, to analyze if their expression in the MBs modifies the sleep behavior response to early life nutrition. We reduce their expression in all MB neuroblasts from embryonic stages

onwards by driving the expression of specific *UAS*-RNAi transgenes using the *OK107-Gal4* [35]. Next, we reared the flies expressing the RNAi and the control flies in NF or RF and quantified sleep in adults.

Reduction of gene expression in MBs has sex-specific effects on sleep (Fig 3, S8 and S9 Figs, S6 Table), with all tested genes affecting at least one of the sleep parameters in response to early life nutrition in females and males when compared with their respective control. Knockdown of *bunched* (*bun*), *Fasciclin 2* (*Fas2*), *GUK-holder* (*gukh*), *slowpoke* 2 (*SLO2*) or *Furin 1* (*Fur1*) affected at least 6 out of 9 sleep variables in response to early life nutrition in females (Fig 3, S8 Fig, S6 Table), while *bunched* (*bun*), *RNA-binding Fox protein 1* (*Rbfox1*) and *Tenascin accessory* (*Ten-a*) affected at least 5 out of 9 sleep variables in males (Fig S9, S6 Table).

Fig 3. RNAi-mediated knockdown of the sleep associated genes results in altered sleep response to early life nutrition.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of female sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day time sleep, (B) Day bout number, (C) Night time sleep, and (D) Night bout number. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

These results show that variation in the expression of genes that regulate the development of the nervous system in the MBs can affect the response of sleep behavior to differences in early life nutrition.

GENI contributes to morphological variation of the mushroom bodies

To answer to what extent GENI contributes to variation of MBs and to map variants underlying this interaction, we raised 40 DGRP lines under both dietary conditions during larval stages and examined the morphology of MBs of adult females. These lines included 36 lines that were previously used to demonstrate natural variation of MB morphology [32]. First, we assessed the gross morphology of α and β lobes [32]. We found a variety of large morphological defects at a broad range of frequencies (5–80%) (Fig 4, S7 Table), including missing or very thin structures and lobe fusions. These gross defects have been attributed to fixation of recessive mutations that affects MBs morphology [32]. Strikingly, a total of 14 (35%) and 6 (15%) DGRP lines exhibited a decrease of the α- and β- lobe defects, respectively when reared on restricted food (Fig 4, S7 Table). In turn, a total of 9 (22%) and 17 (42%) DGRP lines showed an increase of the α- and β- lobe defects, respectively, when reared on restricted food (Fig 4, S7 Table). These data indicate that early-life nutrition may be able to modify the penetrance of recessive mutations that have strong effects on the phenotype. We evaluated quantitative variation in MB morphology by measuring the length and width of the α and β lobes (Fig 5A, S8 and S9 Tables) [32] to reveal more subtle effects on the morphology of brain structures (Fig 5). Quantitative genetic analyses revealed substantial and significant genetic variation among the lines for the mean of length and width of α and β

lobes when early-life nutrition conditions were compared (S10 Table). The contribution of genetic variation to phenotypic variation ranged from low for β lobe length (H^2 = 0.07 in NF and H^2 = 0.13 in RF) to moderate for β lobe width (H^2 = 0.28 in NF and H^2 = 0.30 in RF), α lobe length (H^2 = 0.23 in NF and RF) and α lobe width (H^2 = 0.31 in NF and H^2 = 0.32 in RF) (S10 Table).

Fig 4. Gross morphological defects of MBs in the DGRP lines under prenatal nutritional restriction.

Quantification of variation in gross MB defects of the 40 DGRP lines reared under (A,C) Normal or (B,D) Restricted food. (A,B) α-lobe phenotypes, (C,D) β-lobe phenotypes. Anti-Fas2 staining visualizing the α- and β-lobes of the MBs in the adult brain of 3–7 day old females (scale bar, 50 μm). Categories of each MBs phenotypes are showed on the right side.

Fig 5. MBs morphometric variation in response to early life nutrition.

(A) Scheme showing morphometric measurements (scale bar, 50 μm). Morphometric measurements of (B-B') α-lobe length, (C-C') α-lobe width, (D-D') β-lobe length, (E-E') β-lobe width. Histograms of 40 DGRP female lines reared under Normal (blue bars) or Restricted (red bars) food (B, C, D and E). Reaction norms for (B') α-lobe length, (C') α-lobe width, (D') β-lobe length, (E') β-lobe width. Each DGRP line is represented by a different color. N: Normal food; R: Restricted food.

We found a significant line by food interaction term (*L x F*) for all four traits, indicating that flies with different genotypes respond differently to RF (Fig 5A'-D', S10 Table). The contribution of GENI for the different traits, measured as i^2, was low for in α lobe width (i^2 = 0.06), moderate for α lobe length (i^2 = 0.27) and β lobe width (i^2 = 0.18),

and high for β lobe length (i^2 = 0.94) (S10 Table), indicating that the contribution of GENI to genetic variation highly depends on the trait.

In summary, we found that GENI contributes considerably to variation of MBs morphology, suggesting an important role in the development of these structures.

Genetic variants associated with GENI for MB morphology

To identify variants associated with MB morphology changes in response to early life nutrition, we performed GWA analyses on the difference between the two diets for each of the morphometric parameters evaluated (Fig S10). At a lenient significance threshold of $P \leq 1 \times 10^{-4}$, we identified 755 variants across all four MB morphology traits that mapped in or near to 437 candidate genes (Fig S11, S11 Table). A total of 10 candidate genes were shared with a previous report on natural variation of MB morphology (S12 Table) [32] and 22% (97 out of 437) are known to be expressed in MBs (S13 Table) [34-37]. These 437 candidate genes were enriched (FDR < 0.05) for molecular function GO categories related to nervous system development (*e.g.*, neurogenesis and neuron differentiation) (Fig S12, S11 Table) and their protein products form a significantly enriched Protein-Protein interaction network (PPI enrichment *P*-value = 1.14×10^{-4}) (Fig S13). A total of 78% (340 of 437) candidate genes have human homologs, and 9% of them (30 of 340) have been associated with brain morphology and sleep traits in human population studies (Fig S14, S11 Table) [38].

We found 78 candidate genes (7.0% of all genes compared) shared between MB morphology and sleep traits (Fig S15, S14 Table), including *axotactin* (*axo*), *slowpoke* 2 (*SLO2*), *Protein tyrosine phosphatase 61F* (*Ptp61F*) and *RNA-binding Fox protein 1*

(*Rbfox1*) whose MB-specific loss of function affects the response of sleep behavior to differences in early life nutrition (Fig 3, S8 and S9 Figs, S6 Table). However, no variants were shared between MB morphology and sleep (S4 and S11 Tables).

Candidate genes associated with GENI for MB morphology affect sleep behavior

To determine if there is a causal relationship between the function of candidate genes associated with genotype by early-life nutrition interaction in MB morphology, and sleep behavior in response to early-life nutrition, we used RNAi to reduce the expression of 13 candidate genes found associated with MB morphology in the MBs as described above, and assessed sleep and MB morphology of the RNAi and control genotypes on both diets (Fig 6, S16 and S17 Figs, S6 Table).

Fig 6. RNAi-mediated knockdown of MBs morphology associated genes results in altered sleep response to early life nutrition.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of female sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day time sleep, (B) Day bout number, (C) Night time sleep, and (D) Night bout number. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

MB-specific RNAi of all candidate genes affected at least one of the sleep traits in response to early life nutrition, in both sexes (Fig 6, S16 and S17 Figs, S6 Table). *Ccn*, *scribbler* (*sbb*), *Suppressor of cytokine signaling at 36E* (*Socs36E*) and *Src oncogene at 64B* (*Src64B*) affected at least 6 out of 9 sleep traits in response to early life nutrition in females (Fig 6, S16 Fig, S6 Table); while *dunce* (*dnc*), *enabled* (*ena*), *jing* and *Suppressor of cytokine signaling at 36E* (*Socs36E*) affected at least 6 out of 9 sleep phenotypes in males (Fig S17, S6 Table). We then evaluated the effect of MB-specific RNAi of shared genes between sleep and MB morphology associations (Fig S15, S14 Table) on MB morphology. We found that *axotactin* (*axo*), *slowpoke* 2 (*SLO2*), *Protein tyrosine phosphatase 61F* (*Ptp61F*) and *RNA-binding Fox protein 1* (*Rbfox1*) affected at least one morphometric parameter (Fig 7, S15 Table). Together, these results implicate changes in expression of genes associated with changes in MB morphology as a key mechanism underlying GENI in sleep behavior variation.

Fig 7. RNAi-mediated knockdown of the sleep and MBs morphology associated genes results in altered MBs morphometric measurements in response to early life nutrition.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of female MBs traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) α-lobe length, (B) α-lobe width, (C) β-lobe length, (D) β-lobe width. Histograms of RNAi crosses reared under Normal (blue bars) or Restricted (red bars) food (A, B, C and D). Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*p≤

0.05; **p≤0.01; ***p≤0.001; ****p<0.0001; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

Discussion

Normal development and function of the central nervous system in animals, including humans, rely on proper nutrition during the prenatal period [21, 39]. Here, we show that adult flies that developed under control and restricted nutritional conditions are genetically variable in the sensitivity of sleep MB morphology phenotypes to early life nutrition. Our analyses indicated that the variation in sleep parameters upon nutritional restriction exhibit an important genetic contribution with broad sense heritability ranging from 16 up to 53%, depending on sex and trait (S3 Table). In humans, sleep has been found to be highly heritable, although the reported heritability (twin- and family-based heritability) varies depending on the sleep parameter from 9 up to 45% [40-44]. At the same time, we showed that GENI is an important factor that contributed to shape the complex trait variability observed and we identified key genes that support this natural variation in response to the environment. Importantly, candidate genes associated with GENI for sleep were used to create a protein network that allowed us to identify a subset of cellular processes involved in nervous system development and function, indicating that perturbations in the proper expression of such genes may have impact on central nervous system function later on. A total of 141 of the 747 candidate genes (19%) associated with GENI for sleep phenotypes were also identified in a previous analysis of sleep variation in the DGRP under standard nutritional conditions [23] (S16

Table) and only 19 out of the 747 have been previously linked to sleep phenotypes [45]. Therefore, the contribution of exposure to disadvantageous nutritional conditions during development to variation in sleep behavior involves a new set of genes.

Recent GWA studies on sleep traits and insomnia in humans provide insight into the genetic basis for variation in sleep [42-44, 46]. We found that 46 genes identified in these GWA are human orthologs of *Drosophila* genes associated with different sleep phenotypes in response to early life nutrition. For example, Furin convertase is an enzyme that processes the precursor of endothelin-1 (ET-1) [47] and is associated with insomnia [43]. Furin convertase also cleaves the precursor form of brain-derived neurotrophic factor (BDNF) to generate mature BDNF [48]. BDNF plays a role in the homeostatic regulation of sleep [49] and an important role during brain development and synaptic plasticity [50]. We found that RNAi mediated knockdown of *Furin 1* (*Fur1*) expression in MBs showed different sensitivity to early life nutrition for seven sleep traits in females and four in males. Therefore, we propose that variation in *Fur1* activity could trigger neural perturbations during development that can impact behaviors dependent on proper brain function.

Another gene associated with sleep and MBs morphology sensitivity to early life nutrition also found in human studies is *RNA-binding Fox protein 1* (*Rbfox1*) [42-44, 46]. *Rbfox1* encodes a conserved RNA-binding protein with nuclear isoforms that regulate tissue-specific alternative splicing [51], while cytoplasmic isoforms regulate mRNA translation [52, 53]. Disruption of *Rbfox1* in the central nervous system leads to neuronal hyperactivity, while the deletion of *Rbfox2* results in cerebellum development defects in mice [54, 55]. Cytoplasmic *Rbfox1* regulates the expression of synaptic genes

by binding to the 3'UTR of mRNAs that are targets of microRNAs, independent of its effect on splicing [53]. *Rbfox1* target alternative splicing mRNA TSC2 [56] has been demonstrated to antagonize cell growth and cell proliferation induced by insulin signaling in *Drosophila* [57]. Based on these results, we suggest that sensitivity to early life nutrition is mediated by regulation of mRNA metabolism, including alternative splicing, stability, and translation of tissue-specific isoforms that will impact complex behaviors associated with the central nervous system development and function.

We identified *bunched* (*bun*) and *Nedd4*, two negative regulators of Notch signaling [58, 59]. Knockdown of these genes in the MBs affects sleep sensitivity to early life nutrition. Homeostatic response to sleep deprivation requires *bun* function [58]. Therefore, modifying the activity of Notch signaling regulators will impact the developmental program of the nervous system that in the end will have consequences on adult behaviors such as sleep.

DGRP lines reared under different nutritional conditions showed changes in the metabolic phenotype and transcriptional profiles that rely on genotype by environment interactions [14-18, 60]. Additionally, these metabolic changes are correlated with previously reported phenotypes on the DGRP [14]. We found that knockdown of *forkhead box* transcription factor, *foxo*, affects MB morphology and sleep behavior sensitivity to early life nutrition. FOXO is the transcriptional effector of insulin/insulin-like growth factor (IGF) signaling (IIS) involved in cell metabolism, growth, differentiation, oxidative stress, senescence, autophagy, resistance to starvation and aging [61-63]. Under normal conditions, loss of function of FOXO in *Drosophila* did not induce any sleep phenotypes; however, when IIS is diminished, day sleep phenotypes are

observed [64]. Therefore, *foxo* and *Rbfox1* occurring polymorphisms can have a great impact on the response to environmental cues that will impact on nervous system development and function together with complex traits such as sleep.

Most of the candidate genes identified by our GWA studies are novel and have not previously been associated with either sleep or MB morphology. This highlights the value of testing the effects of natural variants represented in the DGRP to understand the genetic architecture of such quantitative traits under multiple environmental conditions. Future studies will be needed to determine the specific mechanisms by which these candidate genes interact with early-life nutrition to shape the MBs, ultimately affecting adult sleep behavior.

Materials and Methods

Drosophila stocks and husbandry

We used 74 DGRP lines [13]. The DGRP lines and *GAL4* driver were obtained from the Bloomington *Drosophila* stock center (http://flystocks.bio.indiana.edu/). *UAS*-RNAi transgenic flies were obtained from Vienna *Drosophila* RNAi Center (VDRC) (https://stockcenter.vdrc.at), and the Transgenic RNAi Project (TRiP) at Harvard Medical School (http://www.flyrnai.org). All fly lines used are listed in S17 Table. All flies were reared under standard culture conditions (25 °C, 60–70% humidity,12-hour light:dark cycle).

Drosophila culture media

The Normal Food (NF) diet for stock maintenance contains: 10% (w/v) Brewer's yeast, 5% (w/v) sucrose, 1.2% (w/v) agar, 0.6% (v/v) propionic acid, 3% (v/v) nipagin [65]. The Restricted Food (RF) medium contains 20% of Brewer's yeast and sucrose of NF : 2% (w/v) Brewer's yeast, 1% (w/v) sucrose, 1.2% (w/v) agar, 0.6% (v/v) propionic acid, 3% (v/v) nipagin [65]. Adult flies were discarded after five days of egg-laying on NF or RF. Newly eclosed adults were transferred to new NF vials for three days before any behavioral assay.

Sleep phenotypes

We evaluated sleep traits in 73 DGRP lines (S1 Table). We randomly picked groups of 3 to 10 DGRP lines to grow them in the two diet conditions. After eclosion, adult flies were transferred to vials with NF for 3 days until the sleep behavior was assessed. Sleep measurements were replicated three times for each line. Eight flies of each sex and each diet (NF and RF) were measured in one monitor per replicate, resulting in sleep measurements for 24 flies per sex per line per diet. To mitigate the effects of both social exposure and mating on sleep behavior, males and virgin females were collected from each line and retained at 30 flies per same-sex same-diet vial. Individual flies were loaded into *Drosophila* Activity Monitors (DAM2, Trikinetics, Waltham, MA) and sleep and activity parameters were recorded for five continuous days. The monitors use an infra-red beam to detect activity counts in individual flies as they move past it; five minutes without an activity count is defined as sleep [66, 67]. To mitigate the effects of CO_2 anesthesia or any other potential acclimation effects, the first two days of data recording were discarded. Flies were visually examined after the sleep and activity

recordings were completed; data from any flies that did not survive the recording period was discarded. All behavior analysis was done with data collected from days 3 to 4 after flies were placed into the DAMs. PySolo [68] software was used to calculate night and day sleep duration in minutes, night and day sleep bout number, and night and day average sleep bout length; it also calculated sleep latency, the time in minutes to the first sleep bout after lights are turned off, and waking index, the average number of beam crossings within an active bout.

Immunohistochemistry

We used 40 DGRP lines based on lines previously tested for MB morphology (36 lines in common) [32], which were also used to evaluate sleep behavior (39 lines were contains in the 73 lines evaluated) (S17 Table). Adult brains from female flies reared on NF or RF were dissected and processed for immunohistochemistry as described previously [32]. All flies were between 3 and 7 days old at the time of dissection. *Drosophila* brains were fixed in phosphate-buffered saline (PBS)-4% formaldehyde for 25 min at room temperature, washed 3 times with PBS-0.3% Triton X (PBST), and blocked in PBST containing 5% Normal Donkey Serum for 30 min at room temperature. Brains were incubated overnight at 4ºC with mouse monoclonal anti-Fasciclin 2 antibody (1D4) (1:10; Developmental Studies Hybridoma Bank, University of Iowa, IO, USA) to visualize mushroom body α and β lobes. After washing 3 times with PBST, brains were incubated with a 100-fold dilution of Rhodamine (TRITC) AffiniPure donkey anti-mouse IgG for 2 h at room temperature, followed by washing 3 times with PBST. Brain samples were mounted in Vectashield mounting medium (Vector Laboratories,

Burlingame, CA). Immunostaining was documented with an Olympus Fluoview FV1000 confocal microscope. To avoid any effects of variation in Fas2 expression between DGRP lines, we adjusted fluorescence intensities so that unambiguous measurements could be made [32].

Morphometric measurements

The length and width of the MB α and β lobes were measured by using Fiji software and expressed as values relative to the distance between the α lobe heels as described previously [32]. This internal calibration controls for differences in brain size when assessing variation in morphometric parameters among genotypes. MBs were scored individually (*i.e.*, per hemisphere). Values were obtained for 10-12 brains for all genotypes, thus allowing the analysis of 20-24 hemispheres. Images, diagrams, and figures were assembled using Adobe Photoshop CS6 and Illustrator CS6.

Quantitative genetic analyses of sleep in the DGRP

We partitioned the variance in each sleep parameter in the DGRP using mixed model analyses of variance (ANOVA): $Y = \mu + L + F + S + (L \times F) + (L \times S) + (F \times S) + (L \times F \times S) + \varepsilon$, where Y is the sleep parameter; μ is the overall mean; L is the random effect of line; F and S are the fixed effects of food (control, restricted), and sex (males, females), respectively; and ε is the error variance. In addition, we performed reduced analyses within: (i) each food condition using mixed model ANOVAs of form $Y = \mu + L + S + (L \times S) + \varepsilon$; and (ii) each sex condition using mixed model ANOVAs of form $Y = \mu + L + F + (L \times F) + \varepsilon$. All ANOVAs were performed using the PROC GLM function in SAS.

We calculated broad-sense heritability by sex across food as $H^2 = (\sigma^2_L + \sigma^2_{LF})/(\sigma^2_L + \sigma^2_{LF} + \sigma^2_E)$, where σ^2_L is the variance component among lines, σ^2_{LF} is the line-by-food variance component, and σ^2_E is the sum of all other sources of variation. In addition, we calculated broad-sense heritability by food across sex as $H^2 = (\sigma^2_L + \sigma^2_{LS})/(\sigma^2_L + \sigma^2_{LS} + \sigma^2_E)$, where σ^2_{LS} is the line-by-sex variance component. We calculated broad-sense heritability by food and sex as $H^2 = (\sigma^2_L)/(\sigma^2_L + \sigma^2_E)$. We calculated genetic correlations by sex across food $r_{NR} = (\sigma^2_L)/(\sigma^2_L + \sigma^2_{LF})$, and by food across sex $r_{MF} = (\sigma^2_L)/(\sigma^2_L + \sigma^2_{LS})$. We defined the interaction coefficient across food (i^2) as a measurement of the genotype by early-life nutrition interaction (GENI) contribution to the genetic variation, $i^2 = 1 - r_{NR}$.

Quantitative genetic analyses of morphometric measurements in the DGRP

We partitioned the variation in the length and width of the α and β MB lobes in the DGRP using mixed model ANOVA: $Y = \mu + L + F + (L \times F) + \varepsilon$, where Y is the morphometric parameter; μ is the overall mean; L is the random effect of line; F is the fixed effect of food; and ε is the error variance. We estimated broad sense heritability (H^2) by food as $H^2 = (\sigma^2_L)/(\sigma^2_L + \sigma^2_E)$, where σ^2_L is the variance component among lines, and σ^2_E is the sum of all other sources of variation. In addition, we calculated broad sense heritability across food as $H^2 = (\sigma^2_L + \sigma^2_{LF})/(\sigma^2_L + \sigma^2_{LF} + \sigma^2_E)$, where σ^2_{LF} is the line-by-food variance component. We calculated genetic correlations across food $r_{NR} = (\sigma^2_L)/(\sigma^2_L + \sigma^2_{LF})$.

Sensitivity to early life nutrition

To identify which lines are more sensitive or resistant to early life nutrition, we computed the measure of sensitivity for a behavioral and morphometric trait as $[(\bar{x}_{NF\ Line\ i} - \bar{x}_{RF\ Line\ i})/(\bar{x}_{NF\ Pop} - \bar{x}_{RF\ Pop})]$, which is the difference in individual line means of flies reared under NF and RF conditions divided by the difference in overall population mean in both NF and RF conditions [69].

Genotype-phenotype associations

We performed GWA analyses using line means for all sleep parameters and morphometric measurements using the DGRP pipeline (http://dgrp2.gnets.ncsu.edu/). This pipeline accounts for the effects of *Wolbachia* infection status, major polymorphic inversions and polygenic relatedness [70] and implements single-variant tests of association for additive effects of variants with minor allele frequencies ≥ 0.05. We tested the effects of 1,901,174 DNA sequence variants on each trait. We focus our GWA analysis on the associations that represents the interaction of both diets, that is, the difference of phenotypic means between restricted and control food. All annotations the map within or nearby (<± 5,000 bp from gene body) are based on FlyBase release 5.57 (http://www.flybase.org).

Network analysis

We annotated candidate genes identified by the GWA analyses using FlyBase release 5.57 and mapped gene-gene networks through the genetic interaction database

downloaded from FlyBase. We then constructed gene networks using STRING [33] where candidate genes directly interact with each other. We used the following STRING settings: (i) Experiments and Databases as active interaction sources, (ii) high confidence (0.700) as the minimum required interaction score. For network visualization we used the igraph R package to plot gene networks, where nodes correspond to genes and edges indicate the interaction between genes.

Gene Ontology analysis

We performed gene ontology (GO) enrichment analysis using PANTHER 14.1 (http://www.pantherdb.org/) [71] and STRING 11.0 (https://string-db.org/) [33]. We used the DIOPT–*Drosophila* RNAi Screening Center (DRSC) Integrative Ortholog Predictive Tool, with all available prediction tools and only retrieving the best match when there is more than one match per input gene or protein, to identify human orthologs [38].

Functional analyses

We performed MB-specific RNAi-mediated knockdown of 28 candidate genes implicated by the GWA analyses using *OK107-Gal4* (Bloomington, IN, USA) (S15 Table). We crossed males from *UAS*–RNAi lines with virgin females with the *OK107-Gal4* driver and reared them at 25ºC in the two diet conditions. After eclosion, adult flies were transferred to vials with NF for 3 days until the sleep behavior or MB morphology was assessed as described above. Sleep measurements were replicated three times for each line. We performed by two-way ANOVA followed by Bonferroni's post hoc test to

compare the effect of RNAi knockdown in each food condition considering their appropriate control.

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Figures

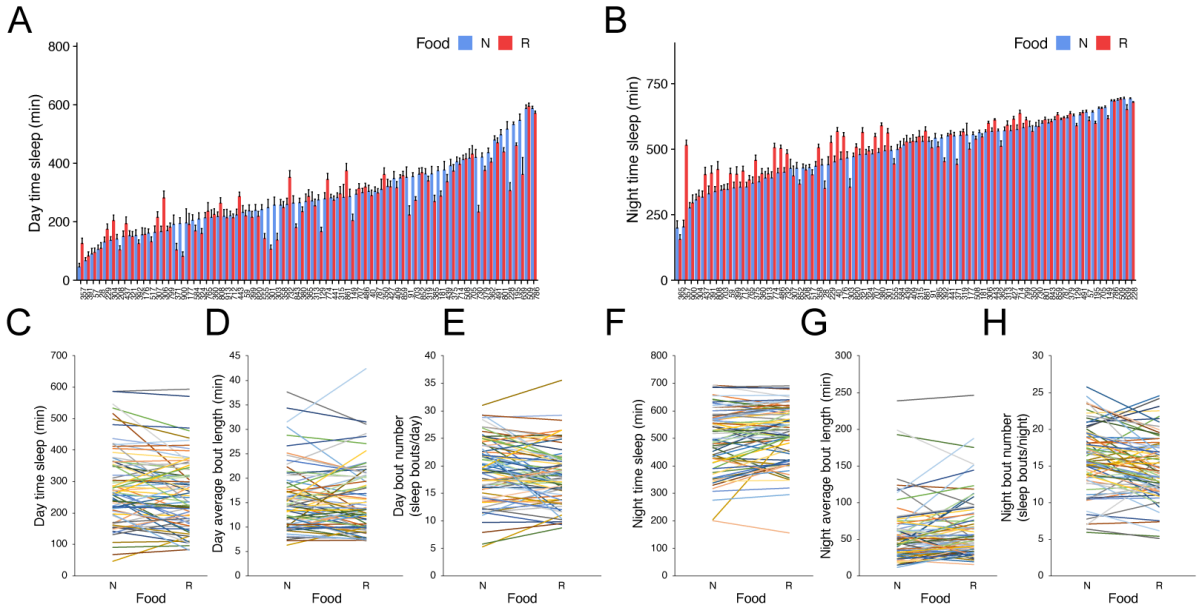
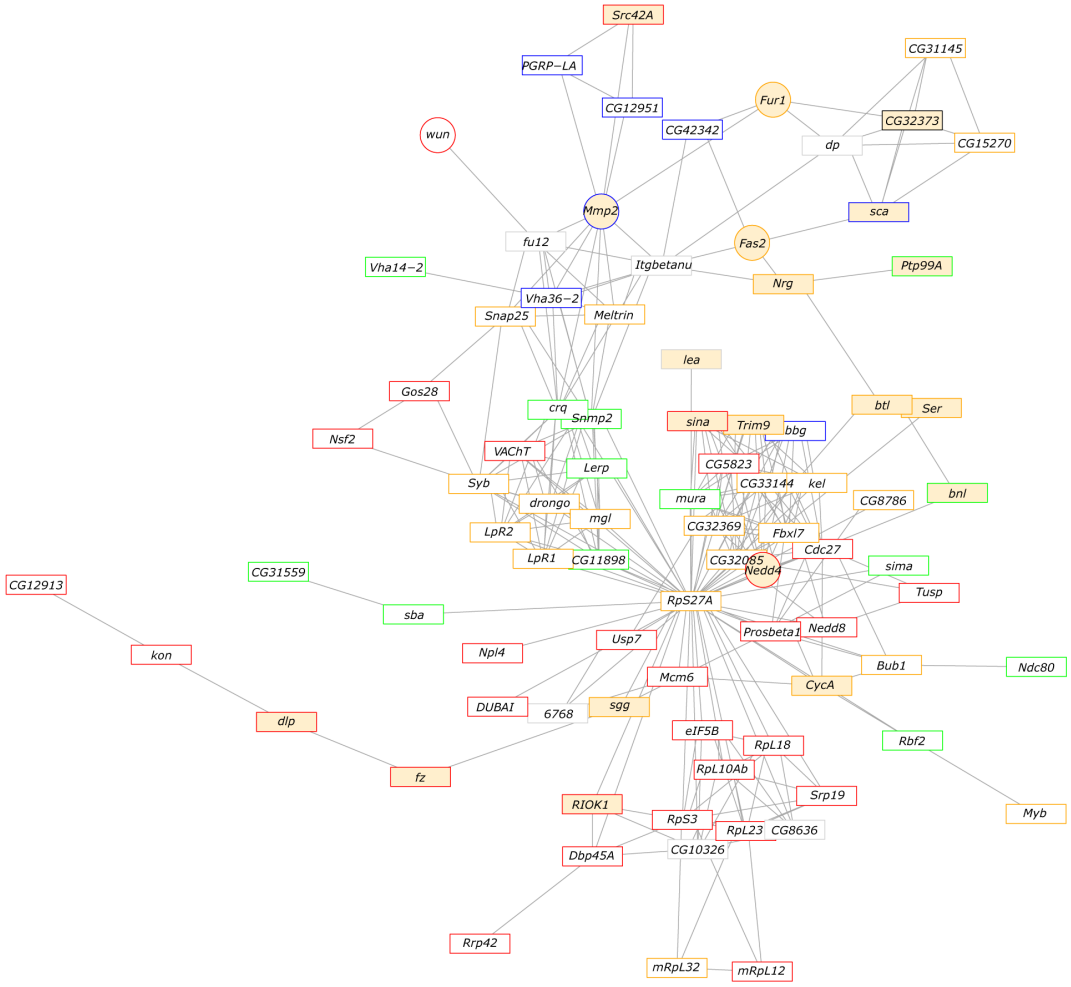


Fig 1. Olivares et al., 2020.



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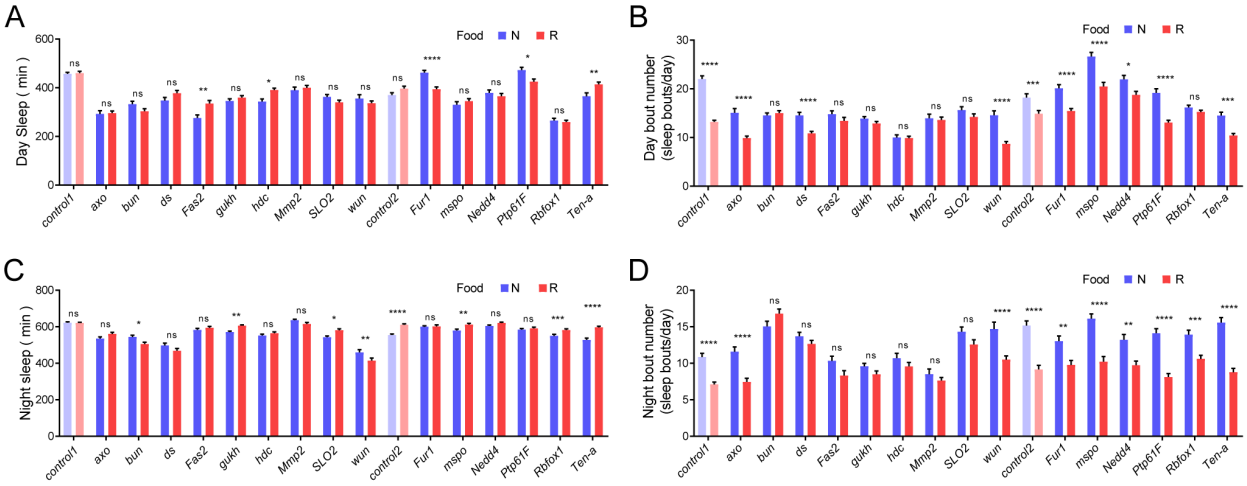

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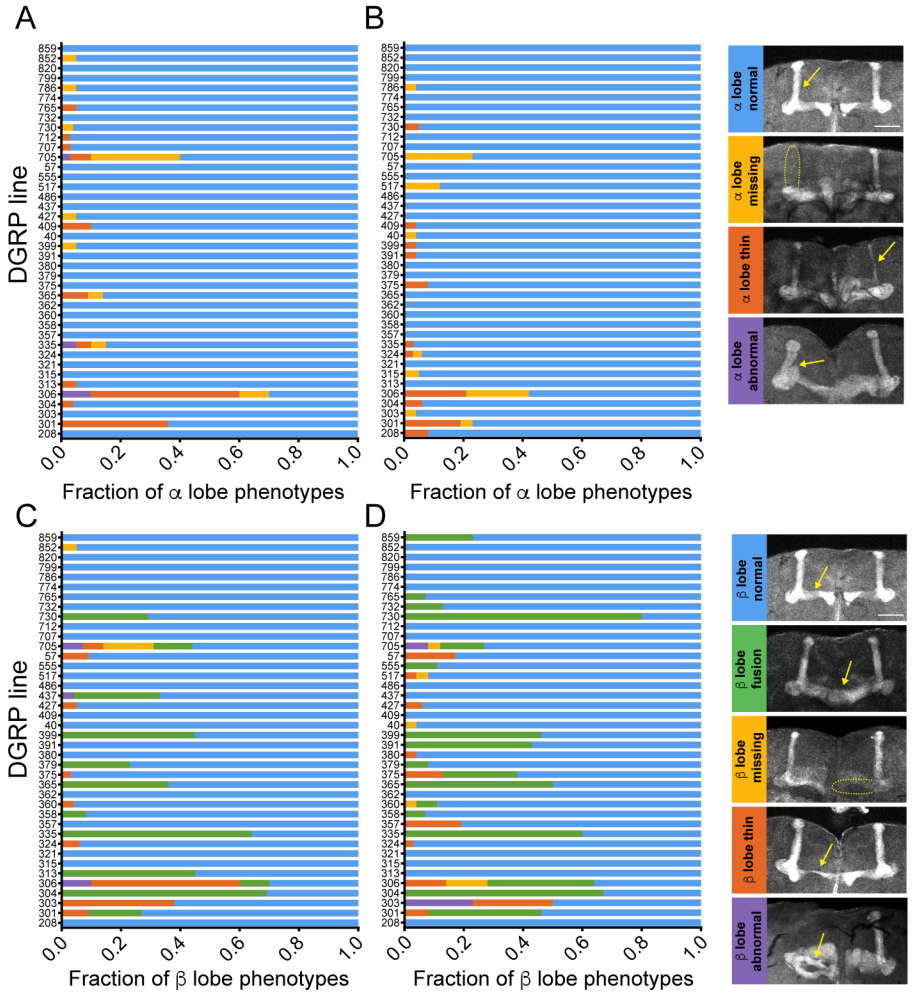

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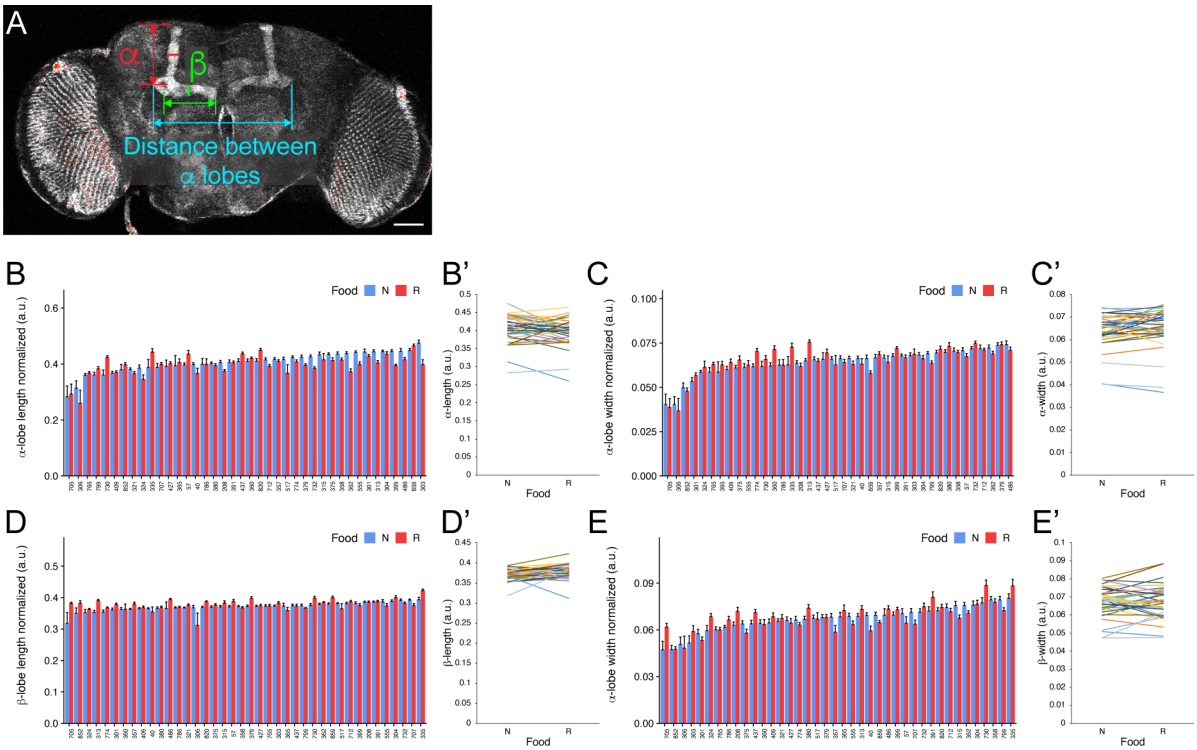

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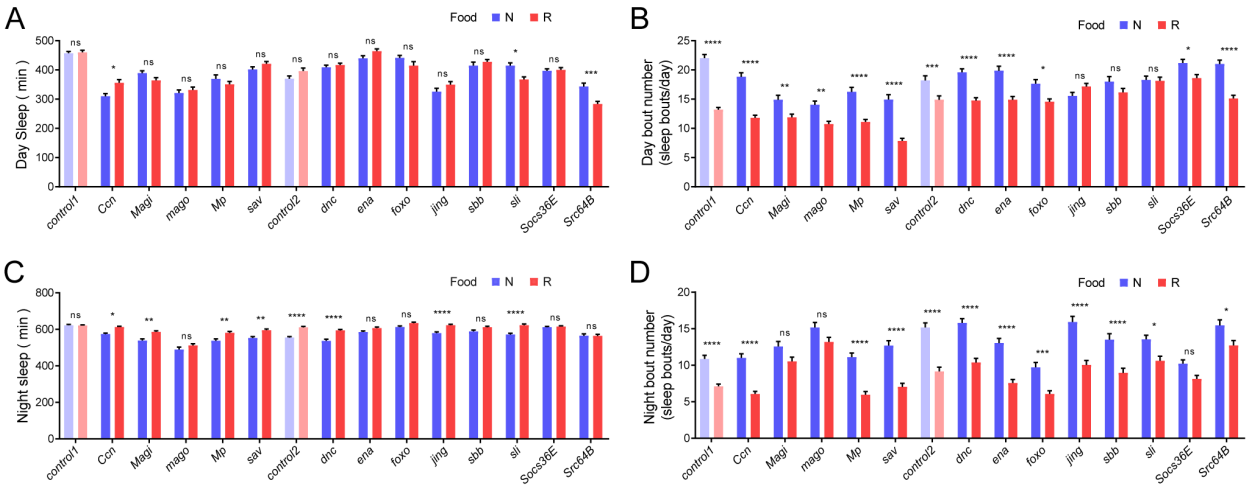


Fig 6. Olivares et al., 2020.

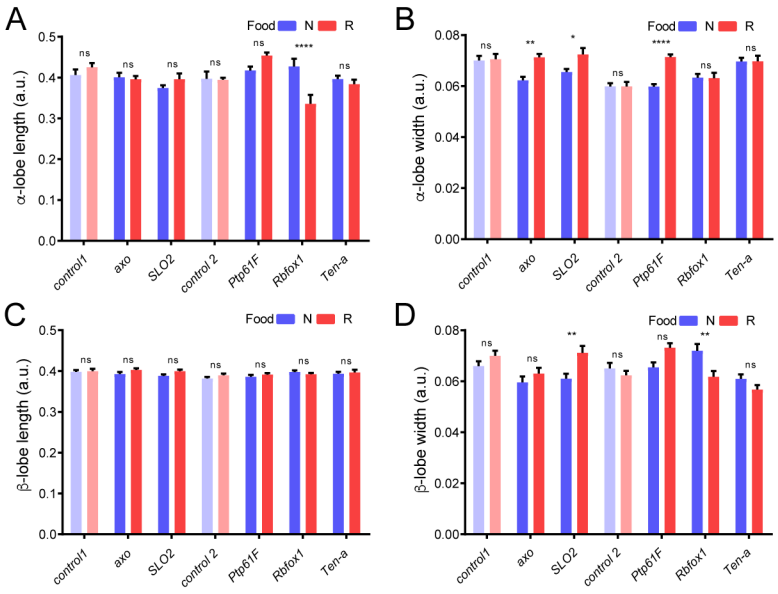


Fig 7. Olivares et al., 2020.

Supporting Information

S1 Fig. Sleep trait response to early life nutrition.

Histograms of female sleep trait mean + SEM for (A) Day average bout length, (B) Day bout number, (C) Night average bout length, (D) Night bout number, (E) Total sleep, (F) Waking activity, (G) Latency. Reaction norms for sleep traits. (E') Total sleep, (F') Waking activity, and (G') Latency. Each DGRP line is represented by a different color. N: Normal food; R: Restricted food.

S2 Fig. Sleep trait response to early life nutrition.

Histograms of male sleep trait mean + SEM for (A) Day time sleep, (B) Day average bout length, (C) Day bout number, (D) Night time sleep, (E) Night average bout length, (F) Night bout number, (G) Total sleep, (H) Waking activity, and (I) Latency. Reaction norms for sleep traits. (A') Day time sleep, (B') Day average bout length, (C') Day bout number, (D') Night time sleep, (E') Night average bout length, (F') Night bout number, (G') Total sleep, (H') Waking activity, and (I') Latency. Each DGRP line is represented by a different color. N: Normal food; R: Restricted food.

S3 Fig. Genome-wide association analyses for sleep traits in response to early life nutrition.

Manhattan plots of all SNPs associated with female sleep traits difference between diets. (A) Day time sleep, (B) Day average bout length, (C) Day bout number, (D) Night time sleep, (E) Night average bout length, (F) Night bout number, (G) Total sleep, (H) Waking activity, and (I) Latency. SNPs with *P*-value ≤ 10^{-3} on either Normal food only,

Restricted food only or both are highlighted (blue, red or green respectively). The green line indicates the nominal *P*-value ≤ 10^{-5} reporting threshold. *P*-values are plotted as $-\log_{10}$(*P*-value).

S4 Fig. Genome-wide association analyses for sleep traits in response to early life nutrition.

Manhattan plots of all SNPs associated with male sleep traits difference between diets. (A) Day time sleep, (B) Day average bout length, (C) Day bout number, (D) Night time sleep, (E) Night average bout length, (F) Night bout number, (G) Total sleep, (H) Waking activity, and (I) Latency. SNPs with *P*-value ≤ 10^{-3} on either Normal food only, Restricted food only or both are highlighted (blue, red or green respectively). The green line indicates the nominal *P*-value ≤ 10^{-5} reporting threshold. *P*-values are plotted as $-\log_{10}$(*P*-value).

S5 Fig. Genomic locations of variants associated with sleep traits.

Variants were annotated as intergenic (outside the transcribed region of an annotated gene), UTR (5' and 3'), coding or intron according to the site class. (A) Genomic localization of variants as percentage (%) of sleep associated variants in females for each trait. (B) Genomic localization of variants as percentage (%) of sleep associated variants in males for each trait. The total number of associated variants is indicated at the right of each graph.

S6 Fig. Human orthologs of sleep associated genes and their role in complex traits and disease.

(A) Human orthologs associated to sleep phenotypes. (B) Human orthologs associated to brain structure phenotypes.

S7 Fig. Gene Ontology enrichment analysis of candidate genes associated with sleep traits.

Biological process enrichment analysis of candidate sleep genes. Fisher's exact test, Bonferroni's correction for multiple testing *P*-value. *P*-values are $-\log_{10}$ transformed.

S8 Fig. RNAi-mediated knockdown of the sleep associated genes results in altered sleep response to early life nutrition.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of female sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day average bout length, (B) Night average bout length, (C) Total sleep, (D) Waking activity, and (F) Latency. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

S9 Fig. RNAi-mediated knockdown of the sleep associated genes results in altered sleep response to early life nutrition in males.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of male sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day time sleep, (B) Day average bout length, (C) Day bout number, (D) Night time sleep, (E) Night average bout length, (F) Night bout number, (G) Total sleep, (H) Waking activity, and (I) Latency. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

S10 Fig. Genome-wide association analyses for MBs morphometric traits in response to early life nutrition.

Manhattan plots of all SNPs associated with female MBs morphology traits difference between diets. (A) α-lobe length, (B) α-lobe width, (C) β-lobe length, and (D) β-lobe width. SNPs with *P*-value $\leq 10^{-3}$ on either Normal food only, Restricted food only or both are highlighted (blue, red or green respectively). The green line indicates the nominal *P*-value $\leq 10^{-4}$ reporting threshold. *P*-values are plotted as $-\log_{10}$(*P*-value).

S11 Fig. Genomic locations of variants associated with MB morphology.

Variants were annotated as intergenic (outside the transcribed region of an annotated gene), UTR (5' and 3'), coding or intron according to the site class. Genomic localization of variants as percentage (%) of MB morphology associated variants in females for each trait. The total number of associated variants is indicated at the right of the graph.

S12 Fig. Gene Ontology enrichment analysis of candidate genes associated with MB morphometric traits.

Biological process enrichment analysis of candidate MBs morphology genes. Fisher's exact test, Bonferroni's correction for multiple testing *P*-value. *P*-values are $-\log_{10}$ transformed.

S13 Fig. Significant genetic interaction network of candidate MBs genes identified in the GWA analyses for all MB morphology related traits combined.

Borders indicate the strength of the evidence for a human ortholog. Black: DIOPT score < 3; Blue: DIOPT score 3–6; Green: DIOPT score 7–9; Orange: DIOPT score 10–12; Red: DIOPT score 13–15. See S11 Table for the complete list of human orthologs and their DIOPT scores. Orange background indicates Gene Ontology enrichment category for Nervous System Development. Circles have effects on at least one sleep trait behavior from RNAi knockdown experiments.

S14 Fig. Human orthologs of MBs morphology associated genes and their role in complex traits and disease.

(A) Human orthologs associated to sleep phenotypes. (B) Human orthologs associated to brain structure phenotypes.

S15 Fig. Common genes associated with sleep and MBs morphometric traits in response to early life nutrition.

Venn diagram showing common candidate genes (orange) between sleep (yellow) and sleep (red) traits. Values shown represent number of genes and percentage from total in parenthesis.

S16 Fig. RNAi-mediated knockdown of the MBs morphology associated genes results in altered sleep response to early life nutrition.

Sleep analyses in response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of female sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day average bout length, (B) Night average bout length, (C) Total sleep, (D) Waking activity, and (F) Latency. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

S17 Fig. RNAi-mediated knockdown of the MB morphology associated genes results in altered sleep response to early life nutrition in males. Sleep analyses in

response to early life nutrition when specific genes are knockdown at the MBs by *OK107-Gal4* driver. Results showing mean of male sleep traits + SEM comparing two nutrition conditions: Normal (blue bars) and Restricted (red bars) for (A) Day average bout length, (B) Night average bout length, (C) Total sleep, (D) Waking activity, and (F) Latency. Control RNAi results for Normal (light blue bars) or Restricted diet (light red bars) are indicated. We performed by two-way ANOVA followed by Bonferroni's post hoc test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$; ns. non-significant) to compare the effect of RNAi knockdown in each food condition considering their appropriate control. N: Normal food; R: Restricted food.

S1 Table. DGRP raw sleep data.

F: female; M: male; N: normal; R: restricted. (XLSX).

S2 Table. DGRP line means for all sleep traits.

(A) Sleep traits from females reared on Normal food. (B) Sleep traits from females reared on Restricted food. (C) Sleep traits from males reared on Normal food. (D) Sleep traits from males reared on Restricted food. F: female; M: male; N: normal; R: restricted. (XLSX).

S3 Table. Analyses of variance of sleep traits.

Food, Sex, and their interaction are fixed effects, the rest are random. Mixed model, two-way factorial ANOVAs are given for males and females as well as reduced models by Sex and Food. L: DGRP Line; S: Sex; F: Food; df: degrees of freedom; MS: Type III

mean squares; F: F-ratio test; *P*: *P*-value; σ^2: variance component estimate; *SE*: standard error; H^2: Broad-sense heritability; *r*: cross-food, cross-sex genetic correlations; i^2: interaction coefficient across food. (A) Day time sleep, (B) Day average bout length, (C) Day bout number, (D) Night time sleep, (E) Night average bout length, (F) Night bout number, (G) Total sleep, (H) Waking activity, (I) Latency. (XLSX).

S4 Table. Results of genome wide association (GWA) analyses for sleep behavior.

(A) Top variants ($P \leq 1 \times 10^{-5}$) and associated genes for each sleep trait in females. (B) Variants and genes for the sleep traits in females. (C) Top variants ($P \leq 1 \times 10^{-5}$) and associated genes for each sleep trait in males. (D) Variants and genes for the sleep traits in males. (E) Genes for the sleep traits in females and males. (F) GWAS summary for sleep behavior. (G) Gene ontology enrichment analysis for the sleep GWA analyses (PANTHER). (H) Human orthologs of sleep associated genes, indicating relevant disease and traits (DIOPT-Disease). (XLSX).

S5 Table. Comparison of sleep associated genes with genes expressed in MBs from previous reports.

(A) A single-cell atlas of adult fly brains [34] Table S3 - Marker genes and statistics. (B) Adult brain MB expressed GAL4 lines [35] FlyLight web server. (C) Third instar larva brain MB expressed GAL4 lines [36] FlyLight web server. (D) Genes enriched in individual MB classes [37] Table S2. (XLSX).

S6 Table. Sleep data for RNAi and control genotypes.

(A) Raw sleep data of RNAi knockdown. (B) Summary data with mean and SEM for each RNAi in each sleep parameter. RNAi lines were driven to MBs with OK107-Gal4. F: female; M: male; N: normal; R: restricted. (XLSX).

S7 Table. Percentage of gross MB phenotypes observed in the 40 DGRP lines.

(A) Phenotypes in Normal food. (B) Phenotypes in Restricted Food. (XLSX).

S8 Table. DGRP raw MBs data.

F: female; N: normal; R: restricted. Normalized means are given in arbitrary units (a.u.). (XLSX).

S9 Table. DGRP line means for all MBs traits.

(A) MBs traits from females reared on Normal food. (B) MBs traits from females reared on Restricted food. F: female; M: male; N: normal; R: restricted. (XLSX).

S10 Table. Analyses of variance of MBs traits.

Food, and its interaction are fixed effects, the rest are random. Mixed model, one-way factorial ANOVAs are given females as well as reduced models by Food. L: DGRP Line; F: Food; df: degrees of freedom; MS: Type III mean squares; F: F-ratio test; *P*: *P*-value; σ^2: variance component estimate; *SE*: standard error; H^2: Broad-sense heritability; *r*: cross-food genetic correlation; i^2: interaction coefficient across food. (A) α-lobe length, (B) α-lobe width, (C) β-lobe length, (D) β-lobe width. (XLSX).

S11 Table. Results of genome wide association (GWA) analyses for MBs traits.

(A) Top variants ($P \leq 1 \times 10^{-5}$) and associated genes for each MB trait in females. (B) Variants and genes for the MBs traits in females. (C) Genes for the MBs traits in females. (D) GWAS summary for MBs traits. (E) Gene ontology enrichment analysis for the MBs GWA analyses (PANTHER). (F) Human orthologs of MBs associated genes, indicating relevant disease and traits (DIOPT-Disease). (XLSX).

S12 Table. Comparison of genes associated with MBs behavior from previous reports.

(A) Genes associated with natural variation of MBs [32] Supplementary Data 5. (XLSX).

S13 Table. Comparison of MB morphology associated genes with genes expressed in MBs from previous reports.

(A) A single-cell atlas of adult fly brains [34] Table S3 - Marker genes and statistics. (B) Adult brain MB expressed GAL4 lines [35] FlyLight web server. (C) Third instar larva brain MB expressed GAL4 lines [36] FlyLight web server. (D) Genes enriched in individual MB classes [37] Table S2. (XLSX).

S14 Table. Sleep and MB common genes.

Shared genes between sleep and MB morphology candidate gene lists. (XLSX).

S15 Table. MB morphometric data for RNAi and control genotypes.

(A) Raw normalized MB morphometric data of RNAi knockdown. (B) Summary data with mean and SEM for each RNAi in each morphology parameter. RNAi lines were driven to MBs with OK107-Gal4. F: female; M: male; N: normal; R: restricted. (XLSX).

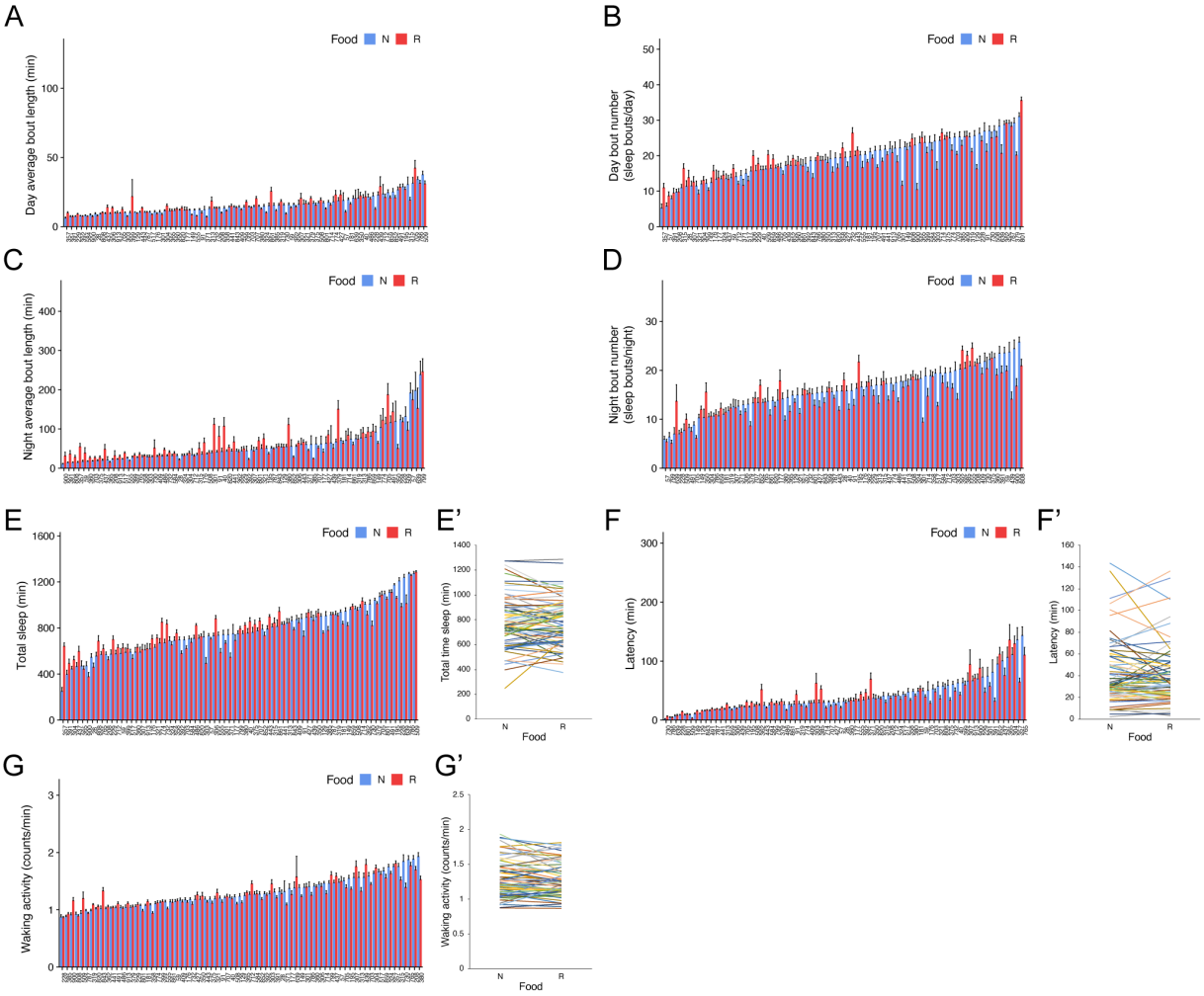
S16 Table. Comparison of genes associated with sleep behavior from previous reports.

(A) Genes associated with natural variation of sleep[23] Additional file 9. (B) Genes associated with natural variation of sleep[23] Additional file 10. (C) Genes associated with natural variation of sleep[24] Supplementary Table 2a. (XLSX).

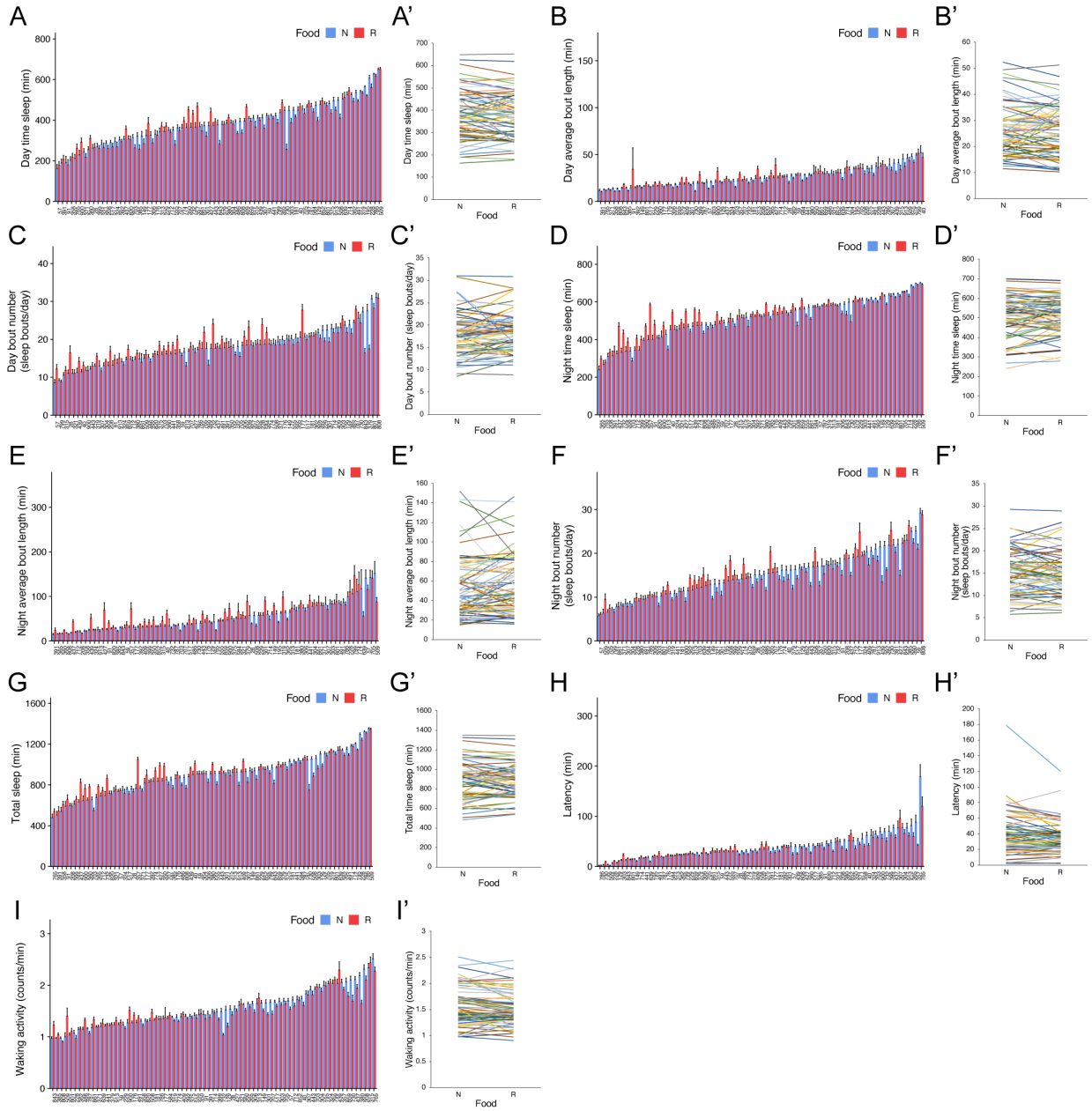
S17 Table. *Drosophila* lines used in this study.

(A) DGRP lines for sleep behavior. (B) DGRP lines for MBs morphology. (C) RNAi lines, control genotypes and GAL4 driver line. (XLSX).

Supplementary figures



S1 Fig. Olivares et al., 2020.



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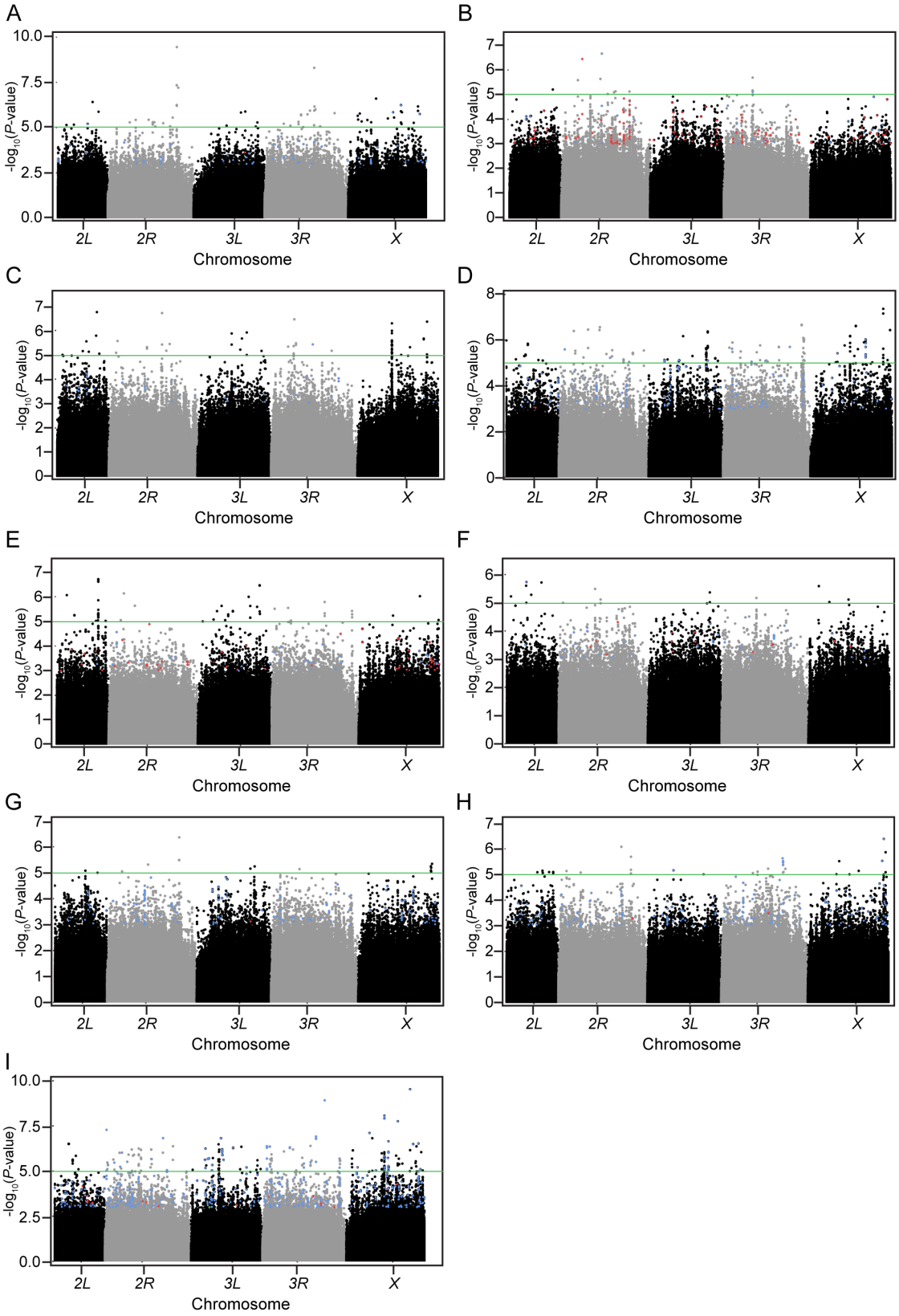

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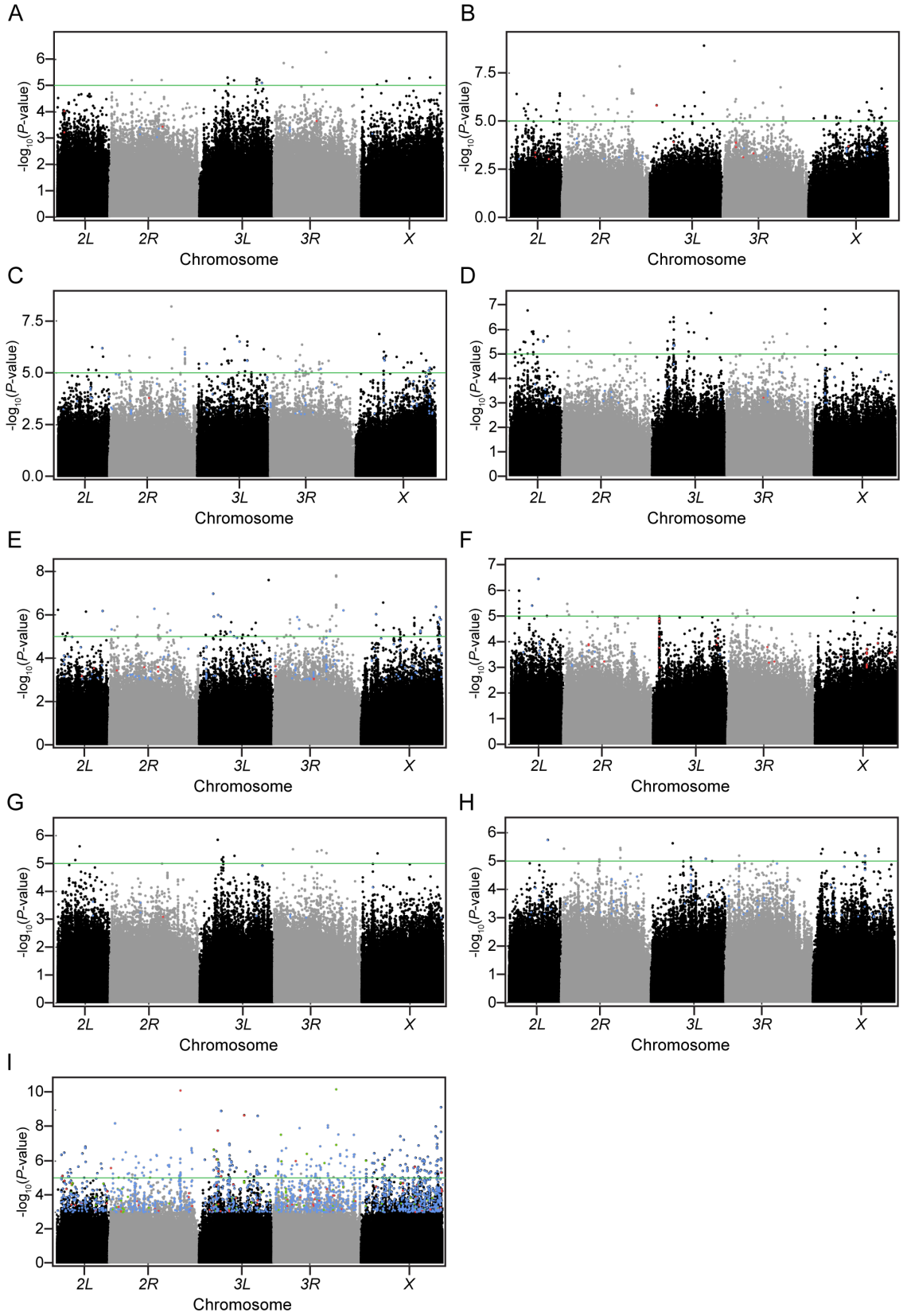

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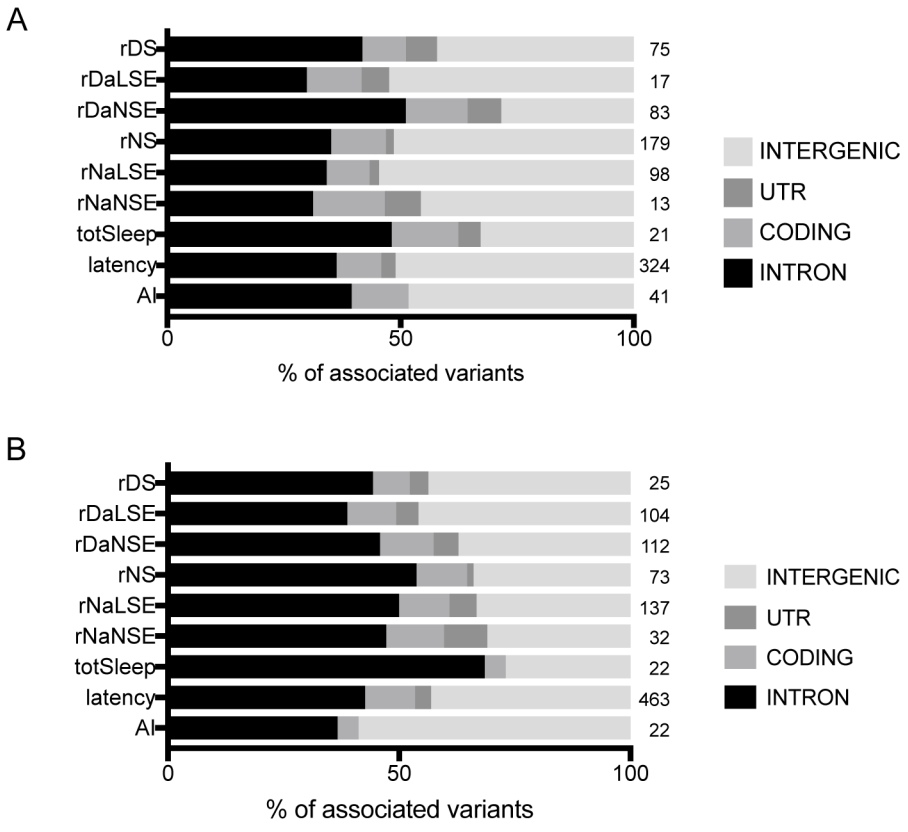


S5 Fig. Olivares et al., 2020.

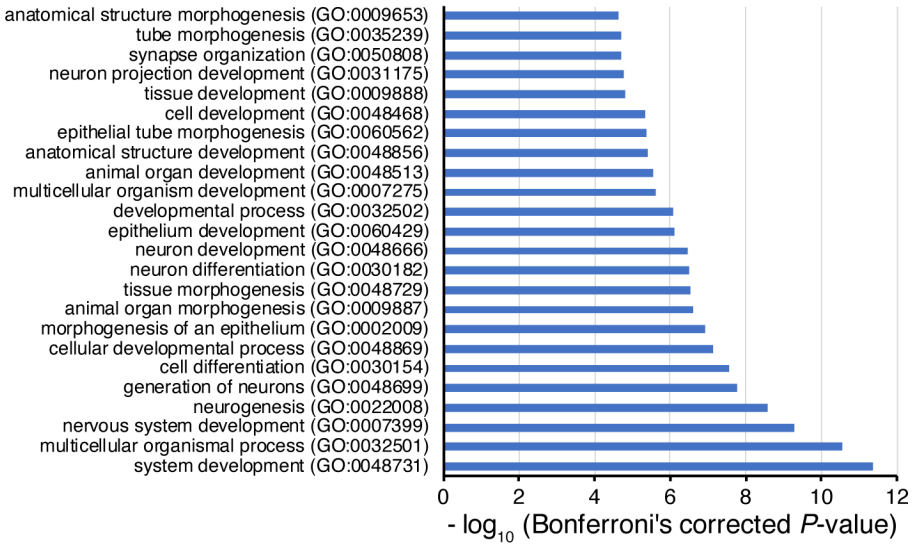
A

Fly	Human	Disease/Trait
CG44153	*CNTN4*	Daytime sleep phenotypes
NimC4	*STAB2*	
shot	*DST*	
norpA	*PLCB4*	
mamo	*ZNF311*	
Fur1	*FURIN*	
Ptp99A	*PTPRG*	
PsGEF	*FRMPD2*	
shn	*HIVEP2*	
brp	*ERC2*	
stops	*ASB17*	
tou	*BAZ2B*	Night sleep phenotypes
dpy	*FBN2*	
CG8834	*SLC27A6*	
CG44153	*CNTN4*	
pyd	*TJP2*	
rgr	*ZNF500*	
JMJD5	*KDM8*	
Shawl	*KCNC1*	
CG11050	*HDDC2*	
CG30116	*WDSUB1*	
vg	*VGLL2*	
CG7368	*HIVEP3*	
shep	*RBMS3*	
CG32373	*FBN2*	
Snmp2	*CD36*	
PsGEF	*USH1C*	
PsGEF	*GOPC*	
PsGEF	*LNX2*	
CG5921	*USH1C*	
Ca-Ma2d	*CACNA2D3*	
beat-VI	*F11R*	
CG18480	*LRTM1*	
brp	*ERC2*	
CG7016	*PSORS1C2*	
dsx	*DMRT1*	
ERR	*ESRRG*	Sleep duration
dpr3	*OPCML*	Sleep-related phenotypes
dpr2	*OPCML*	
dpr17	*OPCML*	
DIP-alpha	*OPCML*	
dpr9	*OPCML*	
dpr13	*OPCML*	
dpr10	*OPCML*	
dpr5	*OPCML*	

B

Fly	Human	Disease/Trait
PsGEF	*PARD3B*	Hippocampal volume
tau	*MAPT*	Intracranial volume
CG8786	*RNF146*	
Svil	*SVIL*	Normalized brain volume
tau	*MAPT*	Subcortical brain region volumes
shg	*FAT3*	
CG8786	*RNF146*	
CG44153	*CNTN4*	Total ventricular volume
CG1902	*CLVS1*	Whole-brain volume
CG12926	*CLVS1*	

S6 Fig. Olivares et al., 2020.



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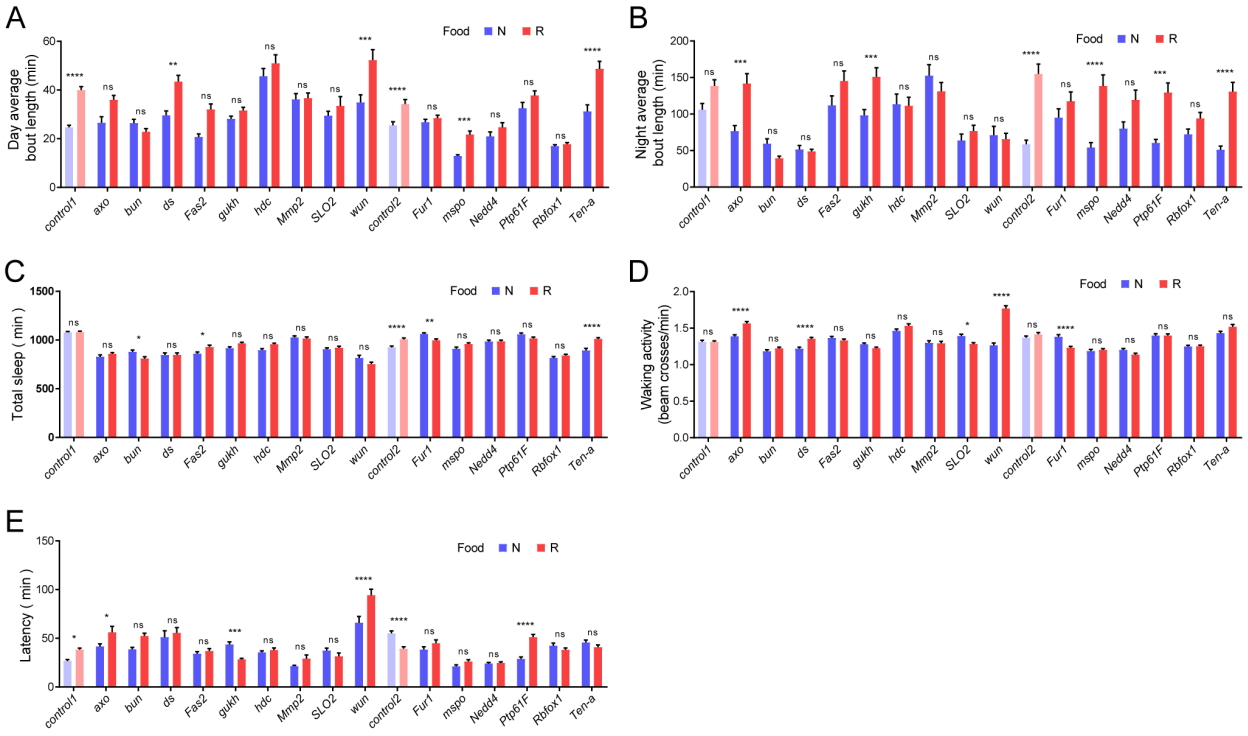

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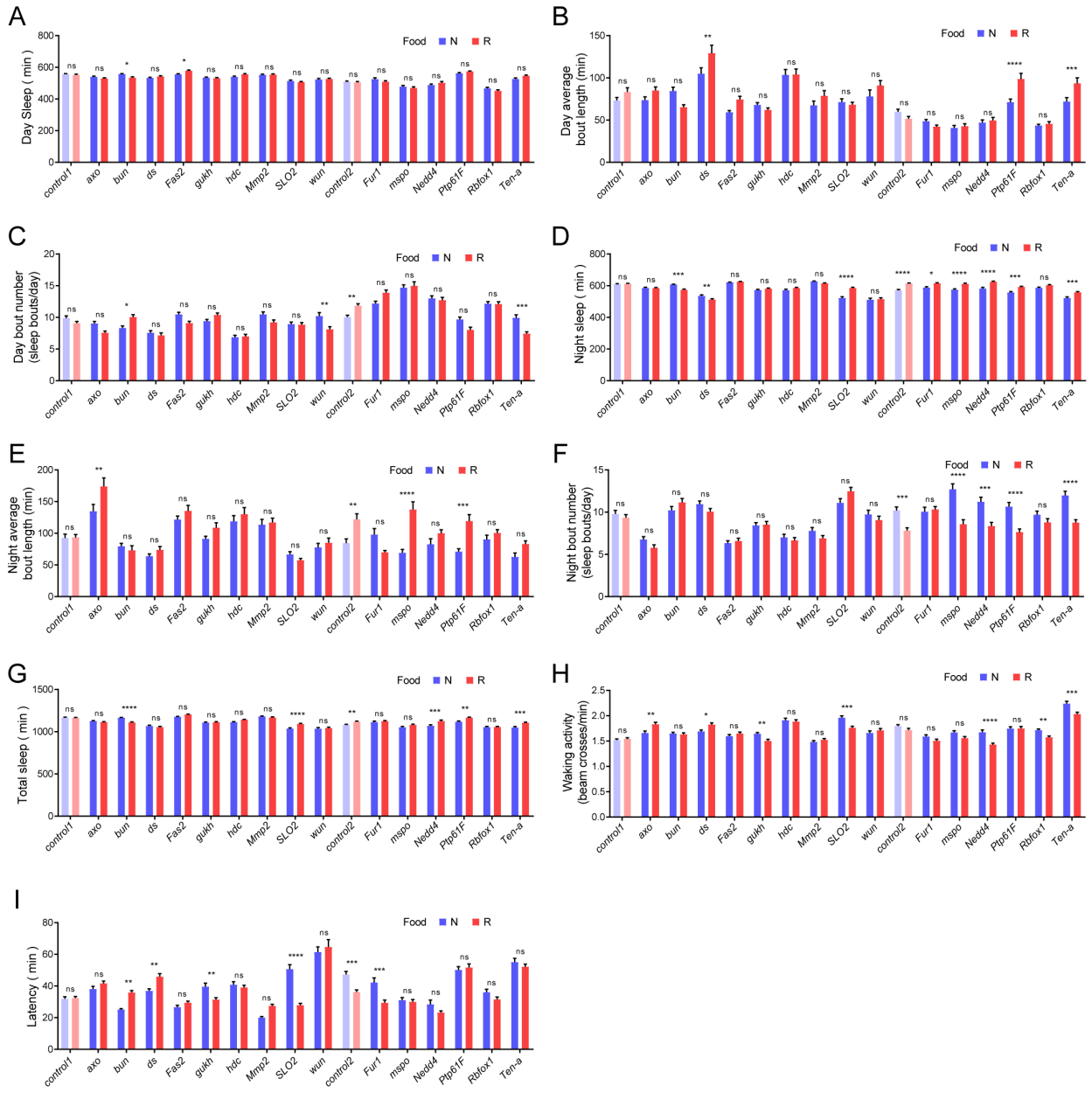
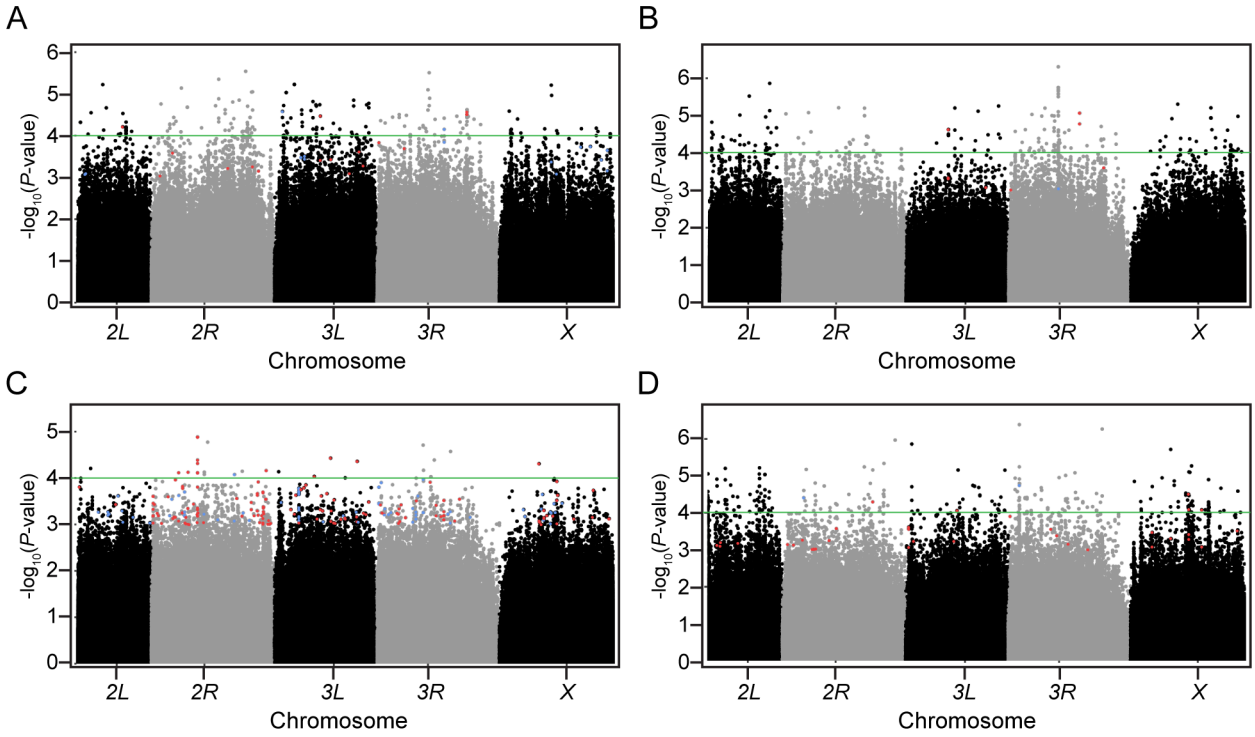
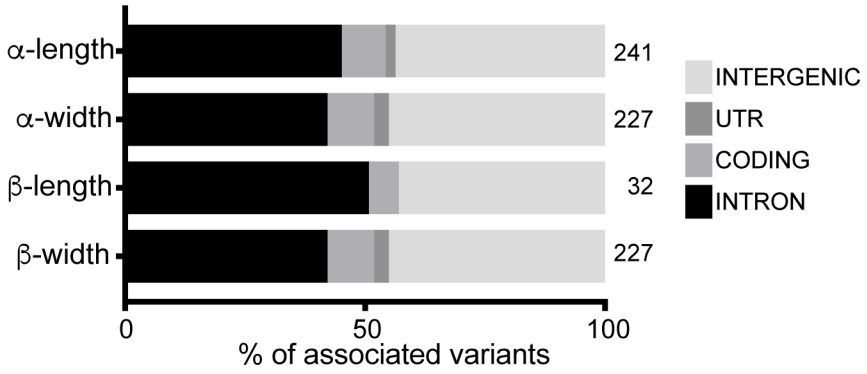


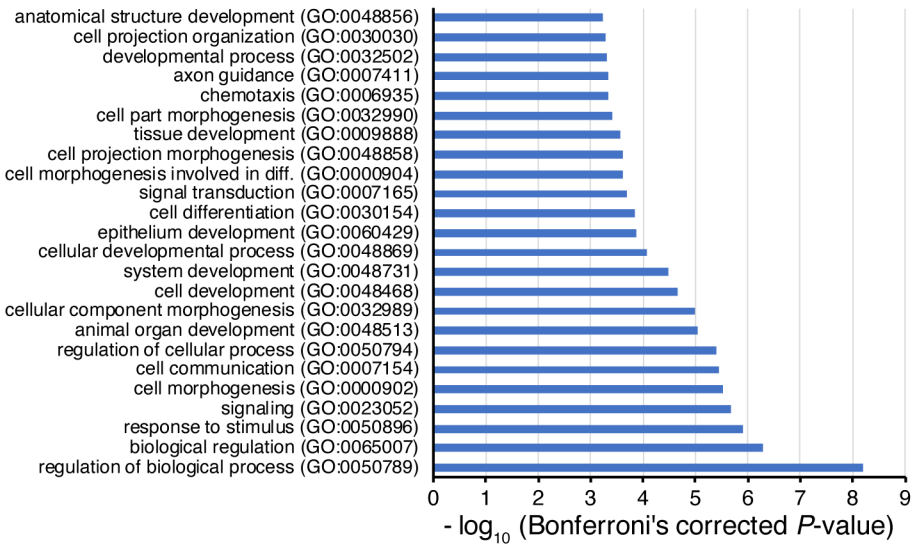
S9 Fig. Olivares et al., 2020.



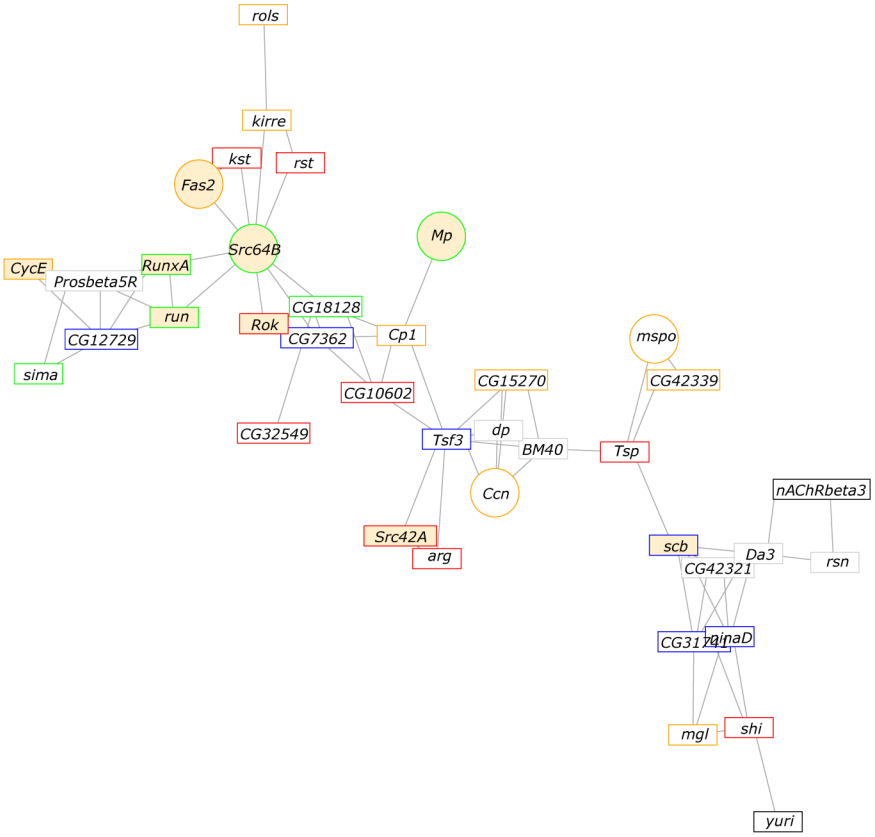
S10 Fig. Olivares et al., 2020.



S11 Fig. Olivares et al., 2020.



S12 Fig. Olivares et al., 2020.



S13 Fig. Olivares et al., 2020.

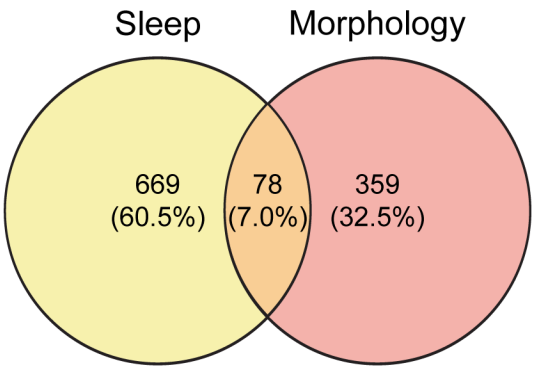
A

Fly	Human	Disease/Trait
CG32264	*PHACTR1*	Chronotype
mamo	*ZNF311*	Daytime sleep phenotypes
CG6126	*SLC22A16*	
Mur2B	*MUC19*	
CG34383	*PLEKHA5*	
CG44153	*CNTN4*	
CG45002	*DPP10*	
CG44005	*FBXL3*	Morning vs. evening chronotype
CG8641	*RASD1*	
CG13375	*RASD1*	
dpy	*FBN2*	Night sleep phenotypes
l(2)01289	*DNAJC10*	
tou	*BAZ2B*	
CG15021	*MMP20*	
CG15021	*MMP27*	
CG15021	*MMP7*	
Ir68a	*GRIN2A*	
shep	*RBMS3*	
pyd	*TJP2*	
Mur2B	*OTOG*	
Magi	*MAGI2*	
CG44153	*CNTN4*	
Kank	*KANK1*	
dpr11	*OPCML*	Sleep-related phenotypes
dpr19	*OPCML*	
dpr10	*OPCML*	
dpr15	*OPCML*	
DIP-alpha	*OPCML*	

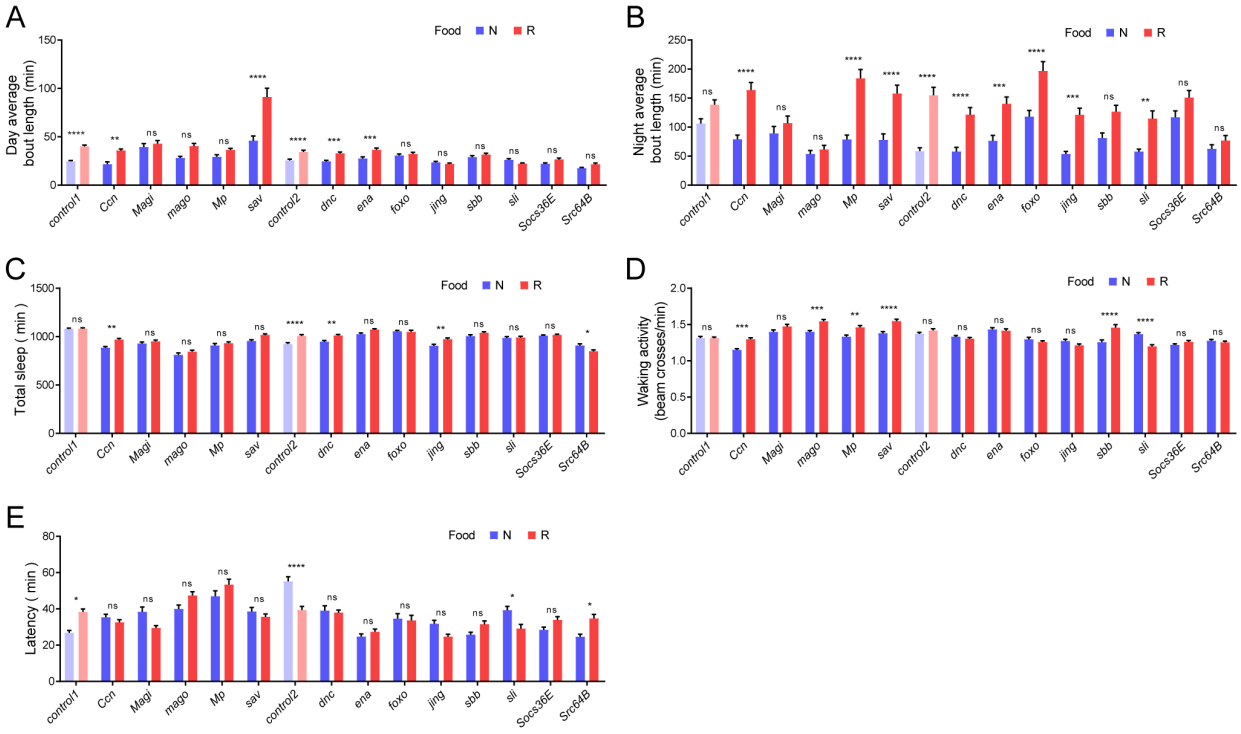
B

Fly	Human	Disease/Trait
CG44153	*CNTN4*	Brain connectivity
Ir68a	*GRIN2B*	Brain structure (temporal lobe volume)
Epac	*RAPGEF4*	Hippocampal volume
tau	*MAPT*	Intracranial volume
mamo	*SP5*	Lobe size
mthl1	*ADGRG6*	
Svil	*SVIL*	Normalized brain volume
foxo	*FOXO3*	
tau	*MAPT*	Subcortical brain region volumes
Ir68a	*GRIN2B*	Total ventricular volume
CG44153	*CNTN4*	
Cubn	*CUBN*	Whole-brain volume

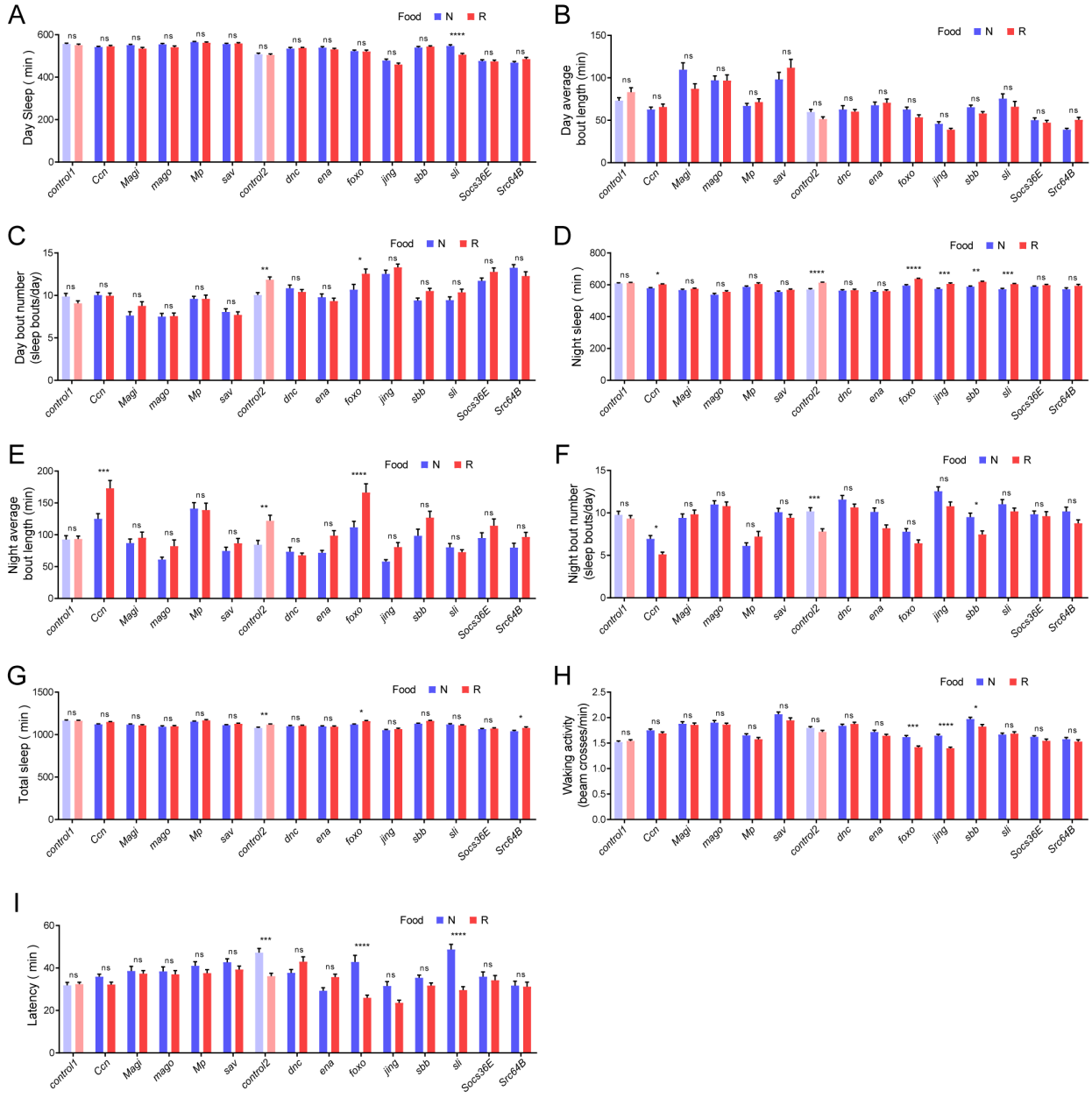
S14 Fig. Olivares et al., 2020.



S15 Fig. Olivares et al., 2020.



S16 Fig. Olivares et al., 2020.



S17 Fig. Olivares et al., 2020.