

Experimentally induced active and quiet sleep engage non-overlapping transcriptional programs in *Drosophila*

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Abstract

Sleep in mammals can be broadly classified into two different physiological categories: rapid eye movement (REM) sleep and slow wave sleep (SWS), and accordingly REM and SWS are thought to achieve a different set of functions. The fruit fly *Drosophila melanogaster* is increasingly being used as a model to understand sleep functions, although it remains unclear if the fly brain also engages in different kinds of sleep as well. Here, we compare two commonly used approaches for studying sleep experimentally in *Drosophila*: optogenetic activation of sleep-promoting neurons and provision of a sleep-promoting drug, Gaboxadol. We find that these different sleep-induction methods have similar effects on increasing sleep duration, but divergent effects on brain activity. Transcriptomic analysis reveals that drug-induced deep sleep ('quiet' sleep) mostly downregulates metabolism genes, whereas optogenetic 'active' sleep upregulates a wide range of genes relevant to normal waking functions. This suggests that optogenetics and pharmacological induction of sleep in *Drosophila* promote different features of sleep, which engage different sets of genes to achieve their respective functions.

eLife assessment

Drosophila is a powerful model organism for understanding the molecular and neural regulation of sleep. However, methodological limitations exist that would appear to limit the relevance of work done in the fly to our understanding of mammalian sleep. In this **important** work, the authors provide **solid** physiological, behavioral, and molecular evidence for the existence of two potential sleep stages in *Drosophila*. The experiments are generally well conducted and the authors' interpretations of their results are **solid** overall. Although technically innovative and conceptually provocative, there are aspects of the approaches used and results obtained that leave the central conclusions open to interpretation.

There is increasing evidence that sleep is a complex phenomenon in most animals, comprising of distinct stages that are characterized by dramatically different physiological processes and brain activity signatures [1, 2]. This suggests that different sleep stages, such as rapid eye movement (REM) and slow-wave sleep (SWS) in humans and other mammals [3] are accomplishing distinct functions that are nevertheless collectively important for adaptive behavior and survival [4]. While REM and SWS appear to be restricted to a subset of vertebrates (e.g., mammals, birds, and possibly some reptiles [5–7]) a broader range of animals, including invertebrates, demonstrate evidence of ‘active’ versus ‘quiet’ sleep [1, 2, 8]. Whether active and quiet sleep represent evolutionary antecedents of REM and SWS, respectively, remains speculative [1, 2]. However, during active sleep, although animals are less responsive, brain recordings reveal a level of neural activity that is similar to wakefulness, in contrast to quiet sleep, which is characterized by significantly decreased neural activity in invertebrates [9, 10] as well as certain fish [11], mollusks [12], and reptiles [6].

Although it is likely that even insects such as fruit flies and honeybees sleep in distinct stages [13, 14], sleep studies using the genetic model *Drosophila melanogaster* still mostly measure sleep as a single phenomenon, defined by 5 minutes (or more) of inactivity [15, 16]. As sleep studies increasingly employ *Drosophila* to investigate molecular and cellular processes underpinning potential sleep functions, this simplified approach to measuring sleep in flies carries the risk of overlooking different functions accomplished by distinct kinds of sleep. Sleep physiology and functions are increasingly being addressed in the fly model by imposing experimentally controlled sleep regimes, either pharmacologically or via transient control of sleep-promoting circuits by using opto- or thermogenetic tools [17]. Yet, there is little knowledge available on whether these different approaches are producing qualitatively similar sleep. For example, sleep can be induced genetically in flies by activating sleep-promoting neurons in the central complex (CX) – a part of the insect brain that has been found to be involved in multimodal sensory processing [18]. In particular, the dorsal fan-shaped body (dFB) of the CX has been proposed to serve as a discharge circuit for the insect’s sleep homeostat, whereby increased sleep pressure (e.g., due to extended wakefulness) alters the physiological properties of dFB neurons causing them to fire more readily and thereby promote decreased behavioral responsiveness [19] and thus sleep [20–22]. Crucially, activation of sleep-promoting circuits including the dFB have been shown to be sleep-restorative [10, 23], but confusingly, brain recordings during optogenetically-induced sleep, via electrophysiology as well as whole-brain calcium imaging techniques, reveal wake-like levels of brain activity [9, 10]. This suggests that some approaches to optogenetically-induced sleep might be promoting a form of sleep akin to the ‘active’ sleep stage detected during spontaneous sleep [9, 10].

An alternate way to induce sleep in *Drosophila* is by feeding flies drugs that have been designed to treat insomnia in humans, such as the GABA-agonist 4,5,6,7-tetrahydroisoxazopyridin-3-ol, (THIP), also known as Gaboxadol [24]. Several studies have shown that THIP-induced sleep in flies is also restorative and achieves key functions ranging from memory consolidation to cellular repair and waste clearance [23, 25–27]. This pharmacological approach centered on GABA function has a solid foundation based on better-understood sleep processes: in mammals, many sleep-inducing drugs also target GABA receptors, and this class of drugs tends to promote SWS [28]. In contrast, there are no obvious drugs that promote REM sleep, although local infusion of cholinergic agonists (e.g., carbachol) to the brainstem has been shown to induce REM-like states in cats [29].

In this study, we compare THIP-induced sleep with optogenetically-induced sleep in *Drosophila*, using behavior, brain activity, and transcriptomics. To ensure the validity of our comparisons, we performed all of our experiments in the same genetic background, employing a canonical Gal4 strain that expresses a transgenic cation channel in a sleep-promoting circuit: R23E10-Gal4 > UAS-

Chrimson [30, 31]. When these flies are fed all-trans-retinal (ATR) and then exposed to red light, they are put to sleep optogenetically. When these flies are instead fed THIP, they are put to sleep pharmacologically. By using the same genetic background, we were thus able to contrast the effects of either kind of sleep at the level of behavior, brain activity, and gene expression (Figure 1). We questioned how similar either form of induced sleep was.

Materials and Methods

Animals

Drosophila melanogaster flies were reared in vials (groups of 20 flies / vial) on standard yeast-sugar-agar based media (1.0-1.5-0.5 gram ratio) under a 12:12 light/dark (8 AM:8 PM) cycle and maintained at 25°C with 50% humidity. Adult, 3-5 day-old female, flies were used for all experiments and randomly assigned to experimental groups. Fly lines used for behavioral and RNA-sequencing experiments include R23E10-Gal4 (attp2; Bloomington 49032; Bloomington Drosophila Stock Center, Bloomington, Indiana) and UAS-CsChrimson-mVenus (attp18; Bloomington 55134; Provided by Janelia Research Campus, Ashburn, Virginia) [31]. For all 2-photon experiments, flies with the genotype 10XUAS-Chrimson88-tdTomato (attp18) / +; LexAop-nlsGCaMP6f (VIE-260b; kindly provided by Barry J. Dickson) / +; Nsyb-LexA (attP2) [32], LexAop-PAGFP (VK00005) / R23E10-Gal4 were used. Optogenetically-manipulated fly lines were maintained on food containing 0.5mg/ml all-trans retinal (ATR; Merck, Darmstadt, Germany) for 24 hours prior to assay to allow for sufficient consumption. Pharmacologically-manipulated flies were maintained on food with 0.1 mg/ml of Gaboxadol (4,5,6,7-tetrahydoisoxazopyridin-3-ol, THIP) for the duration of behavioral experiment [23].

2-photon imaging

2-photon imaging was performed as described previously [10] using a ThorLabs Bergamo series 2 multiphoton microscope. Fluorescence was detected with a High Sensitivity GaAsP photomultiplier tube (ThorLabs, PMT2000). GCaMP fluorescence was filtered through the microscope with a 594 dichroic beam splitter and a 525/25nm band pass filter.

For imaging experiments, flies were secured to a custom-built holder [10]. Extracellular fluid (ECF) containing 103 NaCl, 10.5 trehalose, 10 glucose, 26 NaHCO₃, 5 C₆H₁₅NO₆S, 5 MgCl₂ (hexahydrate), 2 Sucrose, 3 KCl, 1.5 CaCl₂ (dihydrate), and 1 NaH₂PO₄ (in mM) at room temperature was used to fill a chamber over the head of the fly. The brain was accessed by removing the cuticle of the fly with forceps, and the perineural sheath was removed with a microlance. Flies were allowed to recover from this for one hour before commencement of experiments. Imaging was performed across 18 z-slices, separated by 6µm, with two additional flyback frames. The entire nlsGCaMP6f signal was located within a 256 x 256px area, corresponding to 667 x 667µm. Fly behavior was recorded with a Firefly MV 0.3MP camera (FMVU-03MTM-CS, FLIR Systems), which was mounted to a 75mm optical lens and an infrared filter. Camera illumination was provided by a custom-built infrared array consisting of 24 3mm infrared diodes. Behavioral data was collected for the duration of all experiments. For THIP experiments, an initial five minutes of baseline activity was captured, followed by perfusion of 0.2mg/ml THIP in ECF onto the brain at a rate of 1.25ml/minute for five minutes. An additional twenty minutes of both brain and behavioral activity were recorded to allow visualization of the fly falling asleep on the ball as a result of THIP exposure. All flies were removed from imaging after an experiment and confirmed to have awoken by visual inspection. A subset of THIP-exposed flies (n=3) remained in the imaging setup to measure brain activity upon recovery.

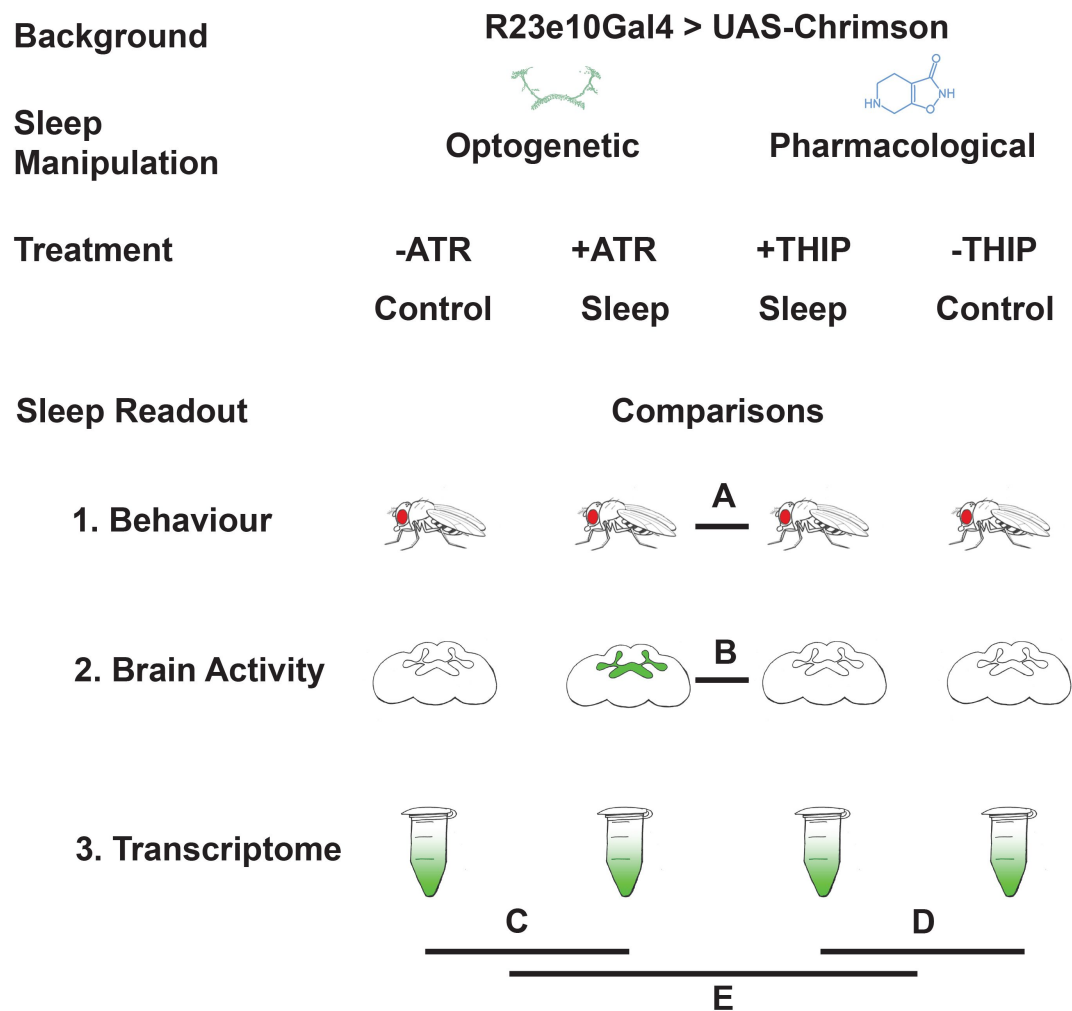


Figure 1.

Study rationale and design.

The same genetic background strain (R23E10;UAS-Chrimson) was used for optogenetic or pharmacologically-induced sleep. Flies were fed either all-trans retinal (ATR) or 4,5,6,7-tetrahydoisoxazolopyridin-3-ol (THIP) to promote either kind of sleep, which was assessed in three different ways: behavioral analysis, whole brain imaging, and gene expression changes. The comparisons made for each level of analysis are labelled A-E.

Behavioral responsiveness probing

For probing behavioral responsiveness in the brain imaging preparation, flies walking on an air-supported ball were subjected to a 50ms long, 10psi air puff stimulus, which was generated using a custom-built apparatus and delivered through a 3mm-diameter tube onto the front of the fly. Flies were subjected to 10 pre-THIP stimuli at a rate of one puff/minute, to characterize the baseline response rate. Flies were then perfused with 0.2mg/ml THIP in ECF for five minutes, followed by continuous ECF perfusion for the remaining experimental time. Five minutes after the fly had fallen asleep on the ball, a further 20 air puff stimuli were delivered, at a rate of one puff/minute. Behavioral responses to the air puff were noted as a 'yes' (1) or 'no' (0), which were characterized as the fly rapidly walking on the ball immediately following the air puff. For statistical analysis, the pre-THIP condition was compared to either the first or last 10 minutes of the post-THIP condition.

Imaging analysis

Preprocessing of images was carried out using custom written Matlab scripts and ImageJ. Motion artifacts of the images were corrected as described previously [10]. Image registration was achieved using efficient sub-pixel image registration by cross-correlation. Each z-slice in a volume (18 z-slices and 2 flyback slices) is acquired at a slightly different time point compared to the rest of the slices. Hence to perform volume (x,y,z) analysis of images, all the slices within a volume need to be adjusted for timing differences. This was achieved by using the 9th z-slice as the reference slice and temporal interpolation was performed for all the other z-slices using 'sinc' interpolation. The timing correction approach implemented here is conceptually similar to the methods using in fMRI for slice timing correction.

For each individual z-slice, a standard deviation projection of the entire time series was used for watershed segmentation with the 'Morphological segmentation' ImageJ plugin [33]. Using a custom-written MatLab (Mathworks) code, the mean fluorescent value of all pixels within a given ROI were extracted for the entire time series, resulting in a $n \times t$ array for each slice of each experiment, where n refers to the number of neurons in each Z-slice, and t refers to the length of the experiment in time frames. These grayscale values were z-scored for each neuron, and the z-scored data was transformed into a binary matrix where a value of > 3 standard deviations of the mean was allocated a '1', and every value < 3 standard deviations was allocated a '0'. To determine whether a neuron fired during the entire time series, a rolling sum of the binary matrix was performed, where ten consecutive time frames were summed together. If the value of any of these summing events was greater or equal to seven (indicating a fluorescent change of > 3 standard deviations in 7/10 time frames), a neuron was deemed to be active. For THIP sleep experiments, the five minutes of inactivity occurring after an initial 30 seconds of behavioral inactivity were used. After identifying firing neurons for each condition (wake vs sleep), the percentage of active neurons was calculated in each slice by taking the number of active neurons and dividing it by the total number of neurons.

Traces of active neurons were used to calculate the number of firing events. This was done using the 'findpeaks' matlab function on the zscored fluorescent traces, with the parameters 'minpeakheight' of 3, and 'minpeakdistance' of 30. Data resulting from this was crosschecked by taking the binary matrices of the time traces and finding the number of times each neuron met the activity threshold described above. Graph-theory analyses of neural connectivity were performed as described previously [10].

Behavioral sleep analysis

Behavioral data for flies in imaging experiments was analyzed as previously [10] using a custom-written MatLab code that measured the pixel change occurring over the legs of the fly on the ball over the entire time series. Data was analyzed and graphed using Graphpad Prism. All

data was checked for Gaussian distribution using a D'Agostino-Pearson normality test prior to statistical testing. Data from THIP experiments was analyzed using a non-parametric Mann-Whitney test.

Sleep behavior in freely-walking flies was analyzed with the *Drosophila* Arousal Tracking system (DART) as previously described [34]. Prior to analysis, 3-5 day-old females were collected and loaded individually into 65 mm glass tubes (Trikinetics) that were plugged at one end with our standard fly food (see above), containing either 0.1 mg/ml THIP or 0.5 mg/ml all-trans-retinal (ATR). Controls were placed onto normal food and housed under identical conditions as the experimental groups. The tubes were placed onto platforms (6 total platforms, 17 tubes per platform, up to 102 flies total) for filming. THIP-fed flies were monitored for 3 days/nights, and sleep data were averaged as no significant differences were detected across successive days. Optogenetically-activated were tracked for baseline day without red light, after which they were exposed to ultra-bright red LED (617 nm Luxeon Rebel LED, Luxeon Star LEDs, Ontario, Canada) which produce 0.1-0.2mW/mm² at a distance of 4-5 cm with the aid of 723 concentrator optics (Polymer Optics 6° 15 mm Circular Beam Optic, Luxeon Star LEDs) for the duration of the experiment for optogenetic activation. Significance was determined by ANOVA with Tukey's multiple comparisons test (GraphPad Prism). Sleep analysis in nAChRα knockout animals was performed using Trikinetics beam-crossing devices, with regular (>5min) and short sleep (1-5min) calculated as previously [10].

Sleep deprivation

Flies were sleep deprived (SD) with the use of the previously described Sleep Nullifying Apparatus (SNAP), an automated sleep deprivation apparatus that has been found to keep flies awake without nonspecifically activating stress responses [35]. Vials containing no greater than 20 flies, which contained either standard food medium or medium containing 0.1mg/ml THIP were placed on the SNAP apparatus for continuous sleep deprivation. The SNAP apparatus was programmed to snap the flies once every 20 seconds for the duration of the sleep deprivation protocol.

RNA-Sequencing

Flies collected for RNA-sequencing analysis were first housed in vials containing either 0.5mg/ml all-trans retinal (ATR) or 0.1mg/ml THIP for sleep induction, along with their genetically identical controls on standard food medium. Flies undergoing sleep induction by optogenetic activation with ATR and their controls were placed under constant red-light from 8AM until 6PM to coincide with normal 12:12 light/dark cycles. Flies were collected after 1 hour (ZT 1) and 10 hours (ZT 10) post induction for immediate brain dissection and RNA extraction. For analysis of pharmacological sleep induction, flies were placed on THIP or normal food medium at 8AM (ZT 0) and collected for dissection at 6PM (ZT 10).

Whole fly brains were dissected in ice cold RNeasy lysis buffer (Qiagen) with 0.1% PBST as per previously published protocol [36]. The dissected brains were immediately pooled into five 1.5-mL Eppendorf tubes containing 5 brains ($n = 25$) each. Total RNA was immediately purified using TRIzol according to the manufacturer's protocols (Sigma-Aldrich) and stored at -80°C until commencement of RNA-sequencing.

cDNA libraries were prepared using the Illumina TruSeq stranded mRNA library prep kit. Image processing and sequence data extraction were performed using the standard Illumina Genome Analyzer software and CASAVA (version 1.8.2) software. Cutadapt (version 1.8.1) was used to cut the adaptor sequences as well as low quality nucleotides at both ends. When a processed read is shorter than 36 bp, the read was discarded by cutadapt, with the parameter setting of “-q 20,20 --minimum-length=36”. Processed reads were aligned to the *Drosophila melanogaster* reference genome (dm6) using HISAT2 (version 2.0.5) [37], with the parameter setting of “--no-unal --fr --

rna-strandness RF --known-splicesite-infile dm6_splicesites.txt". This setting is to i) suppress SAM records for reads that failed to align ("--no-unal"), ii) specify the Illumina's paired-end sequencing assay and the strand-specific information ("--fr --rna-strandness RF") and iii) provide a list of known splice sites in *Drosophila melanogaster* ("--known-splicesite-infile dm6_splicesites.txt"). Samtools (version 1.3) [38] was then used to convert "SAM" files to "BAM" files, sort and index the "BAM" files. The "htseq-count" module in the HTSeq package (v0.7.1) was used to quantitate the gene expression level by generating a raw count table for each sample (i.e. counting reads in gene features for each sample). Based on these raw count tables, edgeR (version 3.16.5) [39] was adopted to perform the differential expression analysis between treatment groups and controls. EdgeR used a trimmed mean of M-values to compute scale factors for library size normalization [40]. It used the Cox-Reid profile-adjusted likelihood method to estimate dispersions [41] and the quasi-likelihood F-test to determine differential expression [42]. Lowly expressed genes in both groups (the mean CPM < 5 in both groups) were removed. Differentially expressed genes were identified using the following criteria: i) FDR < 0.05 and ii) fold changes > 1.5 (or logfc > 0.58). Gene ontology enrichment analysis for differentially expressed genes was performed using the functional annotation tool in DAVID Bioinformatics Resources (version 6.8) [43, 44].

Gene expression

RNA and cDNA Synthesis

A quantitative reverse transcriptase PCR assay was used to confirm expression of genes enriched during THIP sleep induction. Nineteen candidate genes were selected (eight negatively and eleven positively) for the gaboxadol (THIP) sleep analysis and six genes (four negatively and two positively) for the dFSB activation experiments. Total RNA was isolated using the Directzol RNA kit (ZymoResearch) from twenty adult brains per condition and each condition was collected in triplicate. RNA quality was confirmed using a microvolume spectrophotometer NanoDrop 2000 (Thermo, USA) with only those resulting samples meeting optimal density ratios between 1.8 and 2.1 used. Up to 1 µg of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo, USA) as per the manufacturer's protocols. The synthesis of cDNA and subsequent amplification was performed in max volumes of 20 µL per reaction using the T100 Thermal Cycler (Bio-Rad, USA). Thermocycle conditions were as such; 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and held at 4 °C. All cDNA was subsequently stored at - 20 °C until used. Target genes for THIP experiments included Pxt (CG7660, FBgn0261987), RpS5b (CG7014, FBgn0038277), Dhhd (CG4193, FBgn0011761), CG9377 (CG9377, FBgn0032507), aKHr (CG11325, FBgn0025595), Acox57D-d (CG9709, FBgn0034629), FASN1 (CG3523, FBgn0283427), Pen (CG4799, FBgn0287720), CG10513 (CG10513, FBgn0039311), Gasp (CG10287, FBgn0026077), Act57B (CG10067, FBgn0000044), Bin1 (CG6046, FBgn0024491), verm (CG8756, FBgn0261341), CG16885 (CG16885, FBgn0032538), CG16884 (CG16884, FBgn0028544), CG5999 (CG5999, FBgn0038083), Fbp1 (CG17285, FBgn0000639), CG5724 (CG5724, FBgn0038082), Eh (CG5400, FBgn0000564). Target genes for dFSB experiments included Vmat (CG33528, FBgn0260964), Dop1R1 (CG9652, FBgn0011582), Salt (CG2196, FBgn0039872), Dysb (CG6856, FBgn0036819), Irk3 (CG10369, FBgn0032706), Blos1 (CG30077, FBgn0050077). Housekeeping genes included Rpl32 (CG7939, FBgn0002626), Gapdh2 (CG8893, FBgn0001092), Actin 5C (CG4027, FBgn0000042). Primer sequences can be found in Supplementary Table 8.

Quantitative real-time PCR

Quantitative (q) RT-PCR was carried out using the Luna Universal qPCR Master Mix (NEB) in the CFX384 Real-Time system (Bio-Rad, USA). Cycling conditions were: 1. 95°C for 60 s, 2. 95°C for 15s, 3. 60°C for 60s with 39 cycles of steps two and three. Melt curve analysis was then performed with the following conditions 1. 95°C for 15s, 2. 60°C for 60s, 3. 95°C for 15s. Three biological replicates for each condition as well as three technical replicates per biological sample were loaded. Each experiment was then repeated on three separate occasions. Cq values and standard curves were

generated using Bio Rad CFX Manager Software to ensure amplification specificity. Results were normalized to the above housekeeping genes and gene expression was calculated following the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001).

Gene knockouts and knockdowns

Da1KO harboured an ends-out mediated deletion of Da1 in a w^{1118} background with the X chromosome replaced with one from the wild type line DGRP line 59 [45]. For Da2KO, 7kb of the Da2 locus was removed using the endogenously expressing Actin-Cas9 strain (BDSC 5490) crossed to a strain expressing two Da2 gene locus specific sgRNAs (Da_sgRNA_start_1 5'CATGTTTAGCGCTGCAATGC, Da2_sgRNA_end_1 5'TTACAAGCCATCTGCCTAG).

Transgenic flies were generated using the pCFD4-U6:1_U6:3tandem gRNAs construct. This was a gift from Simon Bullock. For Da3KO (*Da3111020*), Da4KO (*Da411BA*), Da6KO, Da7KO (*Da711D6*), two sgRNAs were designed to target the start and the end of the coding sequence and cloned into either pU6-BbsI-gRNA or pCFD4 plasmids. These plasmids were then microinjected into *Drosophila* embryos to generate transgenic strains stably expressing sgRNAs. These strains were crossed to another strain expressing Cas9 under Actin promoter (ActinCas9). Their offspring were screened for deletion events with PCR and crossed to appropriate balancer strains to isolate and generate homozygous knockout strains. Full deletions were identified for all these subunit genes except for Da3 which has two partial deletions at the 3' and 5' ends; these were verified to be true knockouts by the lack of RNA expression [46]. ActinCas9 strain was used as genetic control for Da3KO and Da7KO, while this same strain with the X chromosome replaced with one from w^{1118} (w^{1118} ActinCas9) was used as genetic control for Da2KO, Da4KO, and Da6KO. The RNAi strain for the adipokinetic hormone receptor gene knockdown experiments (UAS-*AkhR*-RNAi) was obtained from the VDRC (KK109300). This RNAi construct was expressed in neurons across the fly brain by crossing the UAS flies to R57C10-Gal4 [30]. RNAi strains for the nicotinic alpha receptor gene knockdown experiments (UAS-nAChR α 1-7-RNAi) were obtained from the BDRC. These were: nAChR α 1 RNAi – BL# 28688 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03103}attP2, nAChR α 2 RNAi – BL# 27493 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02643}attP2, nAChR α 3 RNAi – BL# 27671 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02750}attP2, nAChR α 4 RNAi – BL# 31985 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03419}attP2, nAChR α 5 RNAi – BL# 77418 - y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC06550}attP2, nAChR α 6 RNAi – BL# 52885 - y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03623}attP40, nAChR α 7 RNAi – BL# 27251 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02570}attP2. These RNAi constructs were expressed in the sleep-promoting neurons by crossing the UAS flies to R23E10-Gal4.

Code and data availability

The data and analysis tools underpinning this study is stored in The University of Queensland Research Data Management (UQRDM) via UQ eSpace, the institutional data storage repository of the University of Queensland (UQ). Datasets in this study are also available from the Lead Contact upon request. All Matlab analysis code for brain imaging have been made available in a previous publication [10], and are also available from the Lead Contact upon request.

Results

Prolonged optogenetic and THIP-induced sleep have near identical effects on sleep duration

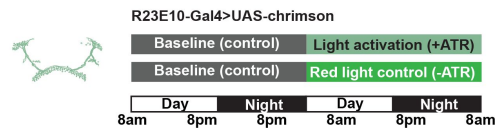
We first compared pharmacological and optogenetic sleep (Figure 1) by using the traditional behavioral metrics employed by most *Drosophila* sleep researchers: >5 minutes inactivity for flies confined in small glass tubes over multiple days and nights [15, 16]. We found that optogenetic- and THIP-induced sleep yielded almost identical effects on sleep duration, with both

significantly increasing total sleep duration for both the day and night, when compared to controls (**Figure 2A-D**; **Supplementary Table 1**). An increase in total sleep duration can be due to either an increase in the number of sleep bouts that are occurring (reflective of more fragmented sleep), or an increase in the average duration of individual sleep bouts, which indicates a more consolidated sleep structure [47–49]. To investigate whether both sleep induction methods also had similar effects on sleep architecture, we plotted bout number as a function of bout duration for optogenetic and THIP-induced sleep, for the day and night [50]. We found that both optogenetic activation and THIP provision produce a similar increase in sleep consolidation during the day (**Figure 2E, F**; **Figure 3**). During the night, induced sleep effects were also similar, although less clearly different to the spontaneous sleep seen in control flies (**Figure 2G, H**; **Figure 3**). Interestingly, red light exposure decreased average night bout duration in non-ATR control flies (**Figure 3A-D**), suggesting a light-induced artefact at night. For THIP, we observed an increase in both bout number and duration during the day, and an increase in bout duration during the night (**Figure 3E,F**). Taken together, these results show that prolonged optogenetic activation and THIP provision have similar behavioral effects on induced sleep duration in *Drosophila*, although some differences in night bout architecture were noted (**Figure 3**). Without any further investigations, this might suggest that both sleep induction methods represent similar underlying processes that uniformly increase sleep in flies.

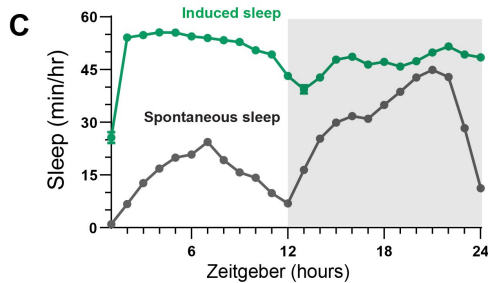
THIP-induced sleep decreases brain activity and connectivity

The brain presents an obvious place to look for any potential differences between sleep induction methods. In a previous study employing whole-brain calcium imaging in tethered flies we showed that optogenetic activation of the R23E10 circuit promotes wake-like sleep, with neither neural activity levels nor connectivity metrics changing significantly even after 15min of optogenetically-induced sleep [10]. We therefore utilized the same fly strain as in that study (R23E10-Gal4>UAS-Chrimson88-tdTomato;Nsyb-LexA>LexOp-nlsGCaMP6f) to examine the effect of THIP-induced sleep on brain activity (**Figure 4A,B**). Since we were interested in comparing acute sleep induction effects on brain activity (as opposed to prolonged sleep induction effects on behavior, as in **Figures 2,3**), we adapted our calcium imaging approach to allow a brief perfusion of THIP directly onto the exposed fly brain (**Figure 4A**, see Methods). As done previously for examining optogenetically-induced sleep [10], we examined calcium transients in neural soma scanning across 18 optical slices of the central fly brain (**Figure 4B**, left) and identified regions of interest (ROIs) corresponding to neuronal soma in this volume (**Figure 4B**, right, and see Methods). As shown previously [10], optogenetically activating the R23E10 circuit renders flies asleep (albeit twitchy at times) without changing the average level of neural activity measured this way (**Figure 4C**). We have previously shown that even 15min of R23E10 activation fails to produce a ‘quiet’ sleep stage that is evident after 5min in spontaneously sleeping flies [10], suggesting that this manipulation promotes ongoing active sleep until the red light is turned off – although this has not been investigated for longer epochs, e.g. the chronic activation experiments described in **Figure 2**. To determine the effect of THIP on neural activity in the exact same strain, we transiently perfused onto the fly brain the minimal THIP dosage required to reliably promote sleep in flies within five minutes (0.2mg/ml) [9]. In contrast to optogenetic-induced sleep, we observed overall decreased neural activity coincident with the flies falling asleep, and flies remained asleep well after the drug was washed out (**Figure 4D**). To ensure that we were actually putting flies (reversibly) to sleep in this preparation, we probed for behavioral responsiveness by puffing air onto the fly once every minute (50 ms duration, 10 psi) (**Figure 5A**). Since the time when flies fell asleep following five minutes of THIP perfusion could be variable [9], arousal probing during sleep was only initiated after 5 min of complete quiescence (**Figure 5A**, behavioral responsiveness testing). We observed decreased arousability for flies that had been induced to sleep via THIP perfusion (**Figure 5B**). Drug-induced sleep was however reversible, with flies returning to baseline levels of behavioral responsiveness to the air puffs ~20-30 min after sleep initiation. This confirmed that the brief exposure to THIP was indeed putting flies to sleep, with an

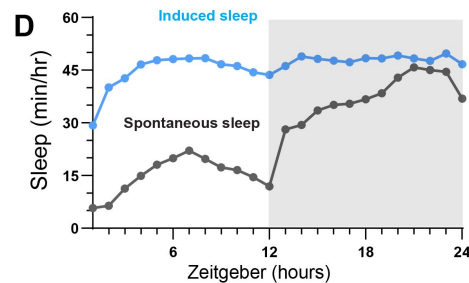
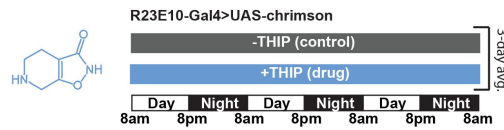
A Optogenetically induced sleep



Sleep duration



B Drug (THIP) induced Sleep



Sleep architecture

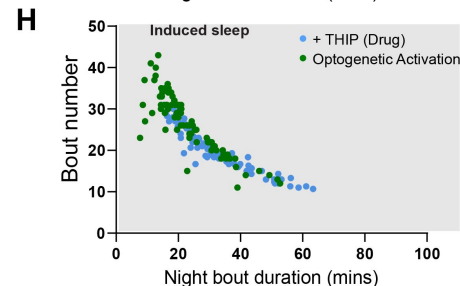
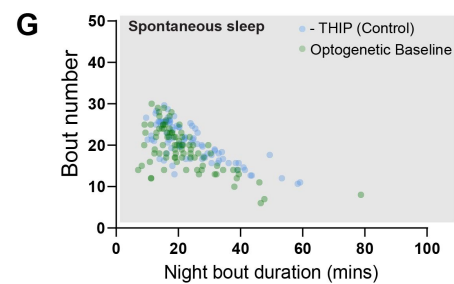
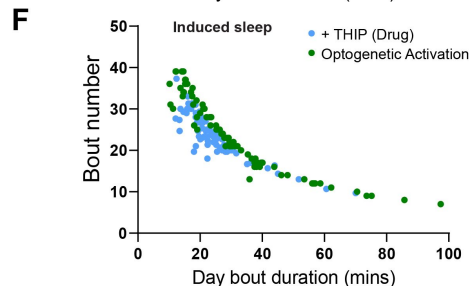
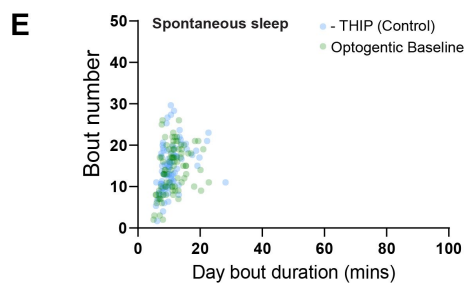


Figure 2.

Optogenetic- and THIP-induced sleep have similar effects on sleep duration and consolidation.

A) Experimental regime for observing the effects of optogenetic activation and THIP provision **(B).** **C)** Sleep profile across 24 hours in the baseline condition (grey) and optogenetic activation condition (green). **D)** 3-day average of the 24-hour sleep profile of control (grey) and THIP fed (blue) flies. **E)** Daytime sleep consolidation scatterplot for optogenetic baseline and THIP control flies. **F)** Daytime sleep consolidation scatterplot for optogenetic- and THIP-induced sleep. **G)** Night-time sleep consolidation scatterplot for optogenetic baseline and THIP control flies. **H)** Night-time sleep consolidation scatterplot for optogenetic- and THIP-induced sleep. $n = 87$ for optogenetic activation across three replicates; $n = 88$ for -THIP, $n = 85$ for +THIP, across three replicates. Maximum bout duration possible is 720 minutes, or 12 hours. See [Supplementary Figure 1](#) for summary histograms and Supplementary Table 1 for statistics.

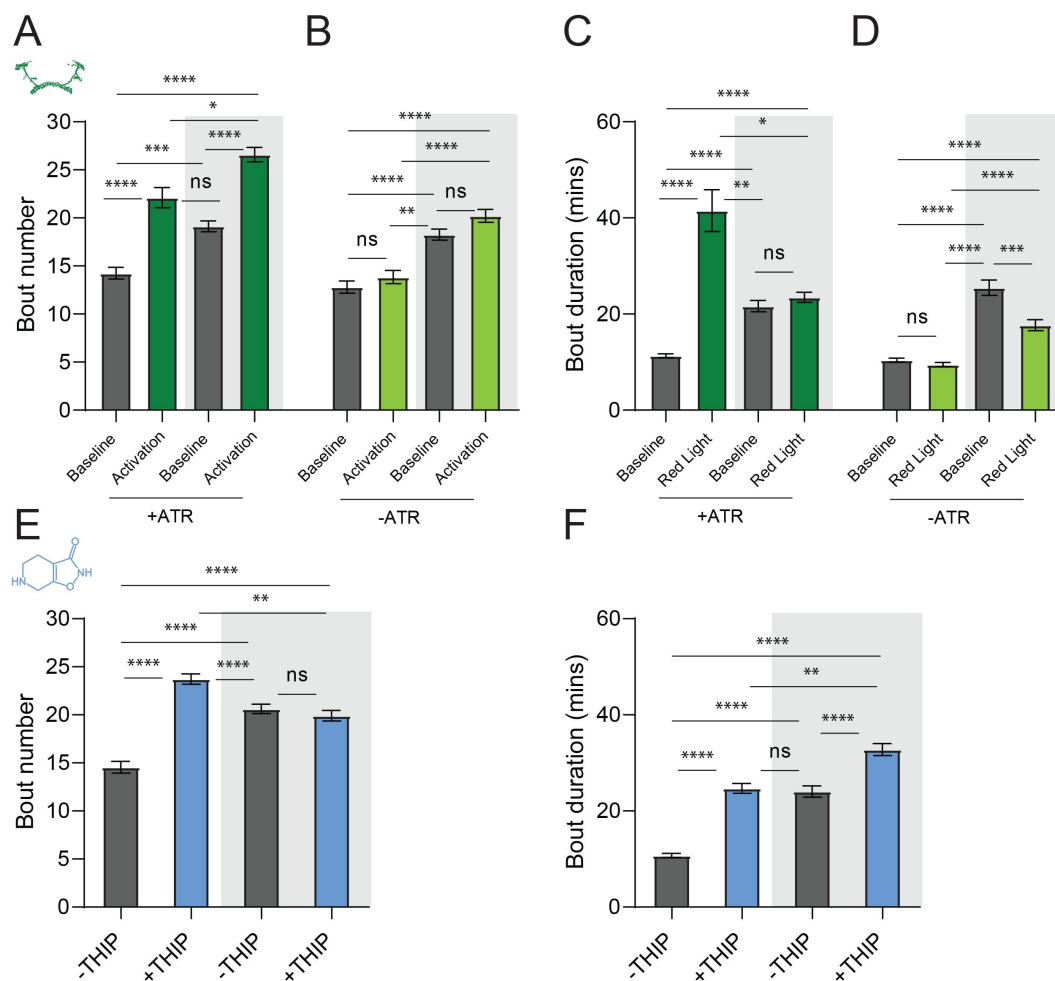


Figure 3.

Sleep architecture in optogenetic and THIP induced sleep.

A-B. Average number of sleep bouts in control (grey) and optogenetic activation (green) conditions in the day and night for both +ATR (**A**) and -ATR (**B**) fed flies. optogenetic-induced sleep results in an increase in the number of sleep bouts both during the day and the night, whereas red light alone has no effect. **C-D.** optogenetic activation (green) increases the average sleep duration during the day, but not the night when compared to controls (grey) in +ATR flies (**C**). **D.** -ATR flies show no difference in mean sleep bout duration during the day, but show a decrease in average bout duration during the night. THIP (blue) increases both the average number of sleep bouts (**E**) and the average duration of sleep bouts (**F**) during the day, but not the night, when compared to controls (grey). Analysis for a and b = Kruskal-Wallis test with Dunn's multiple comparison correction. * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$. For e and f, analysis = Ordinary one-way ANOVA with Tukey correction for multiple comparisons. *** = $p < 0.001$, **** = $p < 0.0001$.

expected sleep inertia lasting the length of a typical spontaneous sleep bout [15, 16]. This contrasts with optogenetic sleep induction using the R23E10-Gal4 circuit, which does not show appear to show any sleep inertia (Figure 4C) [51].

We then examined more closely neural activity in flies that had been put to sleep with THIP. We found that neural activity decreased rapidly within 5 min after sleep onset (Figure 5C, +THIP, early). Correlation analysis also revealed a decrease in connectivity among the remaining active neurons (Figure 5D, +THIP, early). We also analyzed the next 5 min of sleep and observed similar results (Figure 5C,D, +THIP, mid). All flies eventually woke up from THIP-induced sleep, and brain activity returned to wake levels in three flies that were recorded throughout (Figure 5C). These observations suggest that acute THIP exposure is promoting rapid entry into a ‘quiet’ sleep stage in flies, bypassing the wake-like sleep evident during the first 5 min of spontaneous sleep onset, and closely resembling spontaneous quiet sleep typically seen after 5 minutes of inactivity [10]. Importantly, THIP-induced sleep appears to be dissimilar from optogenetically-induced sleep in this genotype, at the level of neural activity as well as connectivity [10].

In recent work we showed that rendering flies unresponsive with a general anesthetic, isoflurane, in surprising contrast to THIP, does not quieten the fly brain [51]. Isoflurane also decreased neural connectivity, as well as the diversity of neural activity across the fly brain, whereas optogenetic-induced sleep did not show any differences in neural activity or ensemble dynamics [51]. Since in our current THIP experiments we were similarly recording from neural soma that we could track through time, we were able to assess the level of overlap between the neurons that remained active during THIP-induced sleep and wakefulness (Figure 5E, F). We found that ~30% of active neurons during THIP-induced sleep were also active during wake (Figure 5F, G). We next examined whether the same neurons remained active across successive 5min epochs during THIP-induced sleep compared to wake. We found that there is significantly more overlap between successive 5min sleep epochs (41%), compared to the waking average (Figure 5G), suggesting less neural turnover during THIP-induced sleep than during wake. This contrasts with optogenetic sleep, where the rate of neural turnover was not different from wake [51]. Taken together, our calcium imaging data confirm that pharmacological sleep induction promotes a different kind of sleep than optogenetic sleep induction in the same strain. Henceforth, we call this ‘quiet’ sleep, in contrast to the ‘active’ sleep that seems to be engaged by optogenetic activation [2, 10]. Notably, calcium imaging of spontaneous sleep bouts in *Drosophila* also revealed active and quiet sleep stages [10], suggesting that both of our experimental approaches are physiologically relevant. Whether drug perfusion to the brain is equivalent to feeding is of course less clear. When feeding on 0.1mg/ml THIP-laced food, flies were continuously exposed to the drug over days, with comparatively less reaching the brain. With perfusion, the brain was directly exposed to 0.2mg/ml THIP for only 5 minutes. Interestingly, in both cases this induces daytime sleep bouts which average around 25min (Figure 3F, Figure 5B), the average duration of a spontaneous nighttime sleep bout (Figure 3F; Table S1).

Transcriptional analysis of flies induced to sleep by THIP provision

Our calcium imaging experiments suggest that different biological processes might be engaged by optogenetic-induced sleep compared to THIP-induced sleep. Additionally, we observed neural effects encompassing much of the fly brain (Figure 5E), as our recording approach exploited a pan-neural driver. We therefore wondered if either sleep-induction method might lead to differences in gene expression across the whole brain, and if these might highlight distinct molecular pathways engaged by either kind of sleep. To address this, we collected brains from flies that had been induced to sleep by either method, and compared the resulting transcriptomes with identically-handled control animals that had not been induced to sleep by these methods.

To control for genetic background, we again used the same R23E10-Gal4 > UAS-Chrimson flies as in our multi-day behavioral experiments and fed the flies either THIP or ATR, as in Figure 2. We only examined daytime sleep-induction effects for either method, as this is when we observed the

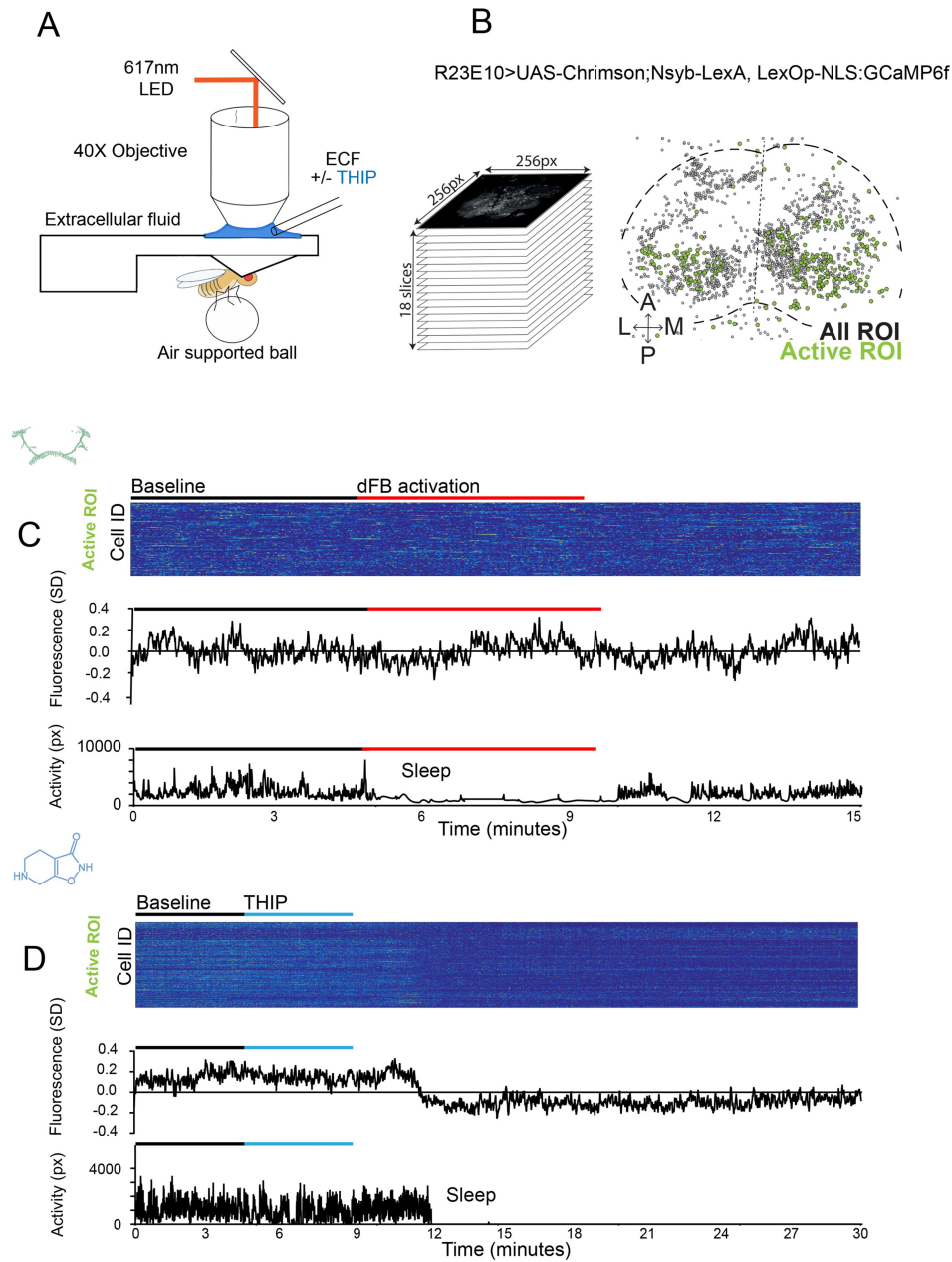


Figure 4.

Brain imaging during optogenetic and THIP-induced sleep

A) Flies were mounted onto a custom-built holder that allowed a coronal visualization of the brain through the posterior side of the head. Perfusion of extracellular fluid (ECF) occurred throughout all experiments. A 617nm LED was delivered to the brain through the imaging objective during optogenetic experiments. During THIP experiments, 4% THIP in ECF was perfused onto the brain through a custom perfusion system. Behavior was recorded as the movement of flies on an air suspended ball.

B) Left: Imaging was carried out across 18 z-slices, with a z-step of 6 μ m. Each z-plane spanned 667 μ m x 667 μ m, which was captured across 256 x 256 pixels. Right: A collapsed mask from one fly of neurons found to be active (green) in C alongside all identified regions of interest (ROIs, gray).

C) Neural activity in an example fly brain, represented across cells (top) and as the population mean (middle) did not change following optogenetic-induced sleep (bottom).

D) Neural activity in an example fly brain, represented across cells (top) and as the population mean (middle) showed an initial high level of activity in the baseline condition, which decreased when the fly fell asleep (bottom) following THIP exposure. The Y axis scale is standard deviation of the experiment mean, so the baseline is not absolute but rather reflects any difference with the overall experiment mean.

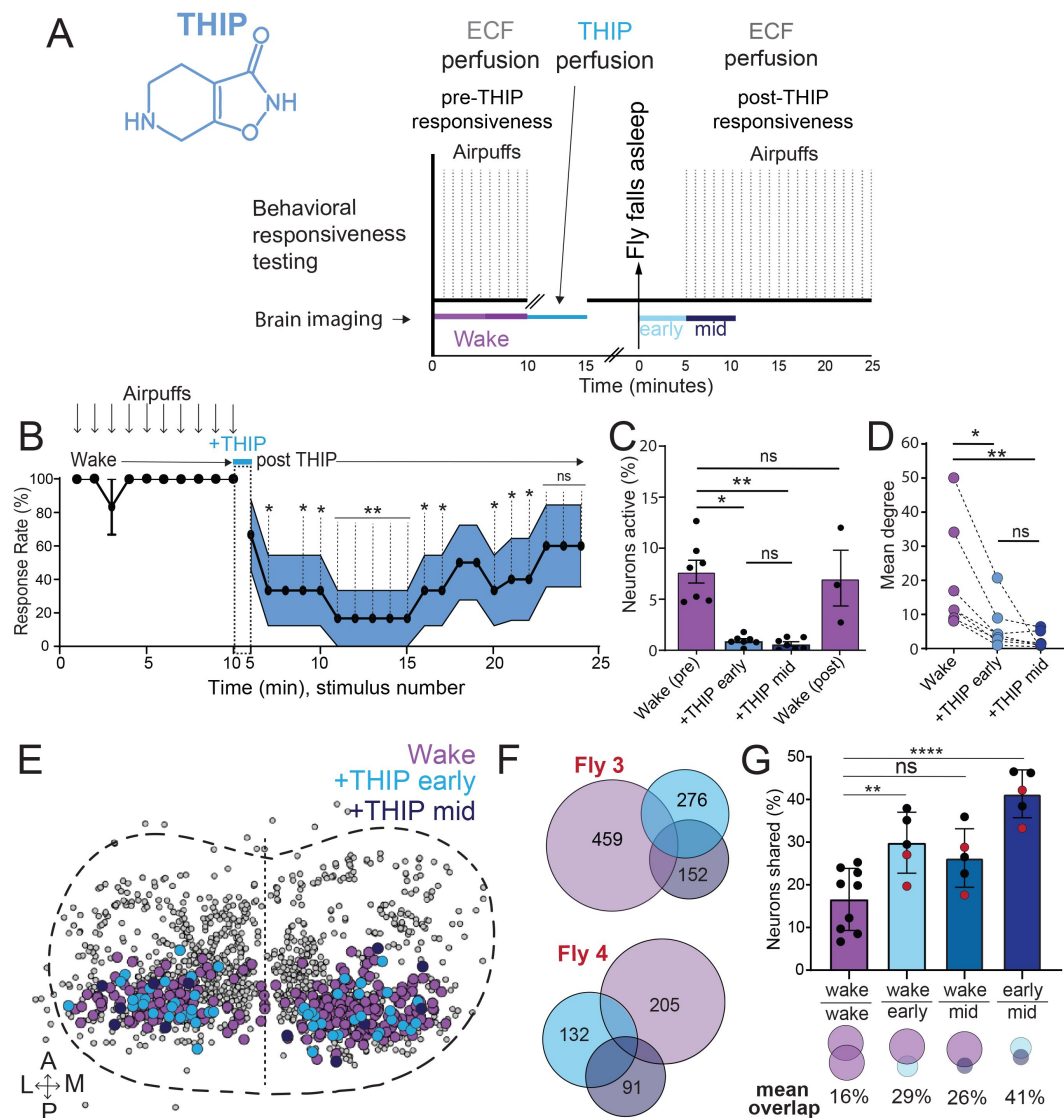


Figure 5.

Brain activity and connectivity decreases during THIP-induced sleep.

A) Experimental protocol for behavioral responsiveness and brain imaging experiments. 5 mins of baseline condition were recorded, during which the exposed brain was perfused with extracellular fluid (ECF), followed by 5 mins of THIP perfusion. Following sleep induction, an additional 10 minutes of calcium activity was recorded, which was separated into 'Early' and 'Mid' sleep for analysis. Air puff stimuli were delivered to test for behavioral responsiveness. **B**) Mean behavioral response rate (% $\pm$ sem) to air puff stimuli over the course of an experiment ($n = 6$). Air puff delivery times are indicated by the solid dots. **C**) Percent neurons active ($\pm$ sem) in non ATR-fed UAS:Chrimson / X; Nsyb:LexA/+; LexOp:nlsGCaMP6f / R23E10:Gal4 flies during wake, THIP-induced sleep, and recovery ($n = 9$; 3 flies were recorded post-waking). **D**) Correlation analysis (mean degree $\pm$ sem) of active neurons in (C). **E**) Collapsed mask of neurons active during wake, and both early and mid THIP sleep. **F**) Overlap in neural identities between wake and THIP-induced sleep in two example flies. Number indicates active neurons within each condition; same color code as in (E). **G**) Quantification of neural overlap data. Red dots indicate the flies shown in (F). $n = 9$ flies. All tests are one-way ANOVA with Dunnett's multiple comparison test. ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.001$.

greatest increase in sleep compared to controls (**Figure 2**), and previous work has shown that daytime sleep induction using either method achieves sleep functions [10, 23]. We present our THIP results first. Since THIP is a GABA-acting drug that probably affects a variety of processes in the brain aside from sleep, we also assessed the effect of THIP on flies that were prevented from sleeping (**Figure 6A**, left panel). Sleep deprivation (SD) was performed by mechanically arousing flies once every 20 seconds for the duration of the experiment, on a ‘SNAP’ apparatus [23, 35]. RNA was extracted from the brains of all groups of flies (+/-THIP, +/-SD) after 10 hours of daytime (8am-6pm) THIP (or vehicle) provision. Samples for RNA-sequencing were collected in replicates of 5 to ensure accuracy, and any significant transcriptional effects were thresholded at a log fold change of 0.58 (see Methods).

Flies allowed to eat food containing 0.1mg/ml THIP *ad lib* over 10 daytime hours led to 129 significant changes in gene expression compared to vehicle-fed controls, with the large majority (110) being downregulated and only 19 upregulated (**Figure 6B,C,E; Supplementary Table 2**). In contrast, when THIP-fed flies were prevented from sleeping this led to mostly upregulated genes (88 upregulated vs 21 downregulated, **Figure 6B,D,F; Supplementary Table 3**), with many of the upregulated genes showing a high level of fold-change in expression. Not surprisingly, preventing sleep in THIP-fed flies led to an almost entirely non-overlapping set of gene expression changes (**Figure 6B**). This suggests that the large number of down-regulated genes in +THIP / -SD flies pertain to sleep processes rather than the effect of ingesting THIP, since only a few (9) genes overlapped with the +THIP / +SD dataset which revealed mostly upregulated genes.

Gene Ontology analysis on genes that were downregulated as a result of THIP-induced sleep highlighted a significant enrichment of metabolism pathways (**Figure 6E,F; Supplementary Figure 1**). The top Gene Ontology biological processes included primary, organic substance, cellular, biosynthetic and nitrogen compound metabolic pathways, as well as ribosomal processes. Interestingly, these downregulated processes are largely consistent with a recently published mouse sleep transcriptome study [52]. Among the metabolism pathways uncovered in this dataset we observed over-representation of expected genes such as *bgm* (bubblegum CG4501) and *Acer* (Angiotensin-converting enzyme-related CG10593). Both of these genes are found in the primary metabolic and organic substance metabolic processes as well as within the Sleep Gene Ontology dataset (GO:0030431). Another downregulated metabolic gene is *AkhR* (adipokinetic hormone receptor), which has been found to be involved in starvation-induced sleep loss in *Drosophila* [53]. *AkhR* belongs to the Class A GPCR Neuropeptide and protein hormone receptors which are a gene class involved in storage fat mobilization, analogous to the glucagon receptor found in mammals [54].

Although THIP-induced sleep overwhelmingly led to gene downregulation, a few genes (19) were significantly upregulated. Gene Ontology analysis on these upregulated genes highlighted enrichment in varying groups including developmental processes and multicellular organismal processes (**Figure 6E,F; Supplementary Figure 1**). Some groups were enriched under the organic substance metabolic process pathways; however, there was no overlap when comparing these to the pathways enriched due to downregulation of genes. There were some overlapping enriched pathways when we compared the gene sets from sleep-deprived flies which had also been treated with THIP (**Figure 6F; Supplementary Figure 2**). However, the gene sets they involve are upregulated in the SD dataset but downregulated in sleeping flies. Interestingly, the non-sleeping THIP dataset uncovered a significant enrichment of pathways involved in the response to stress. This might be expected for flies exposed to regular mechanical stimuli over 10 hours. None of these pathways featured in the THIP sleep dataset.

To validate these findings, we conducted qRT-PCR analyses on 19 genes from our THIP sleep dataset and compared these results to our original transcriptional data. The genes represented a range of both up – and down-regulated genes, and we found convincing correspondence between

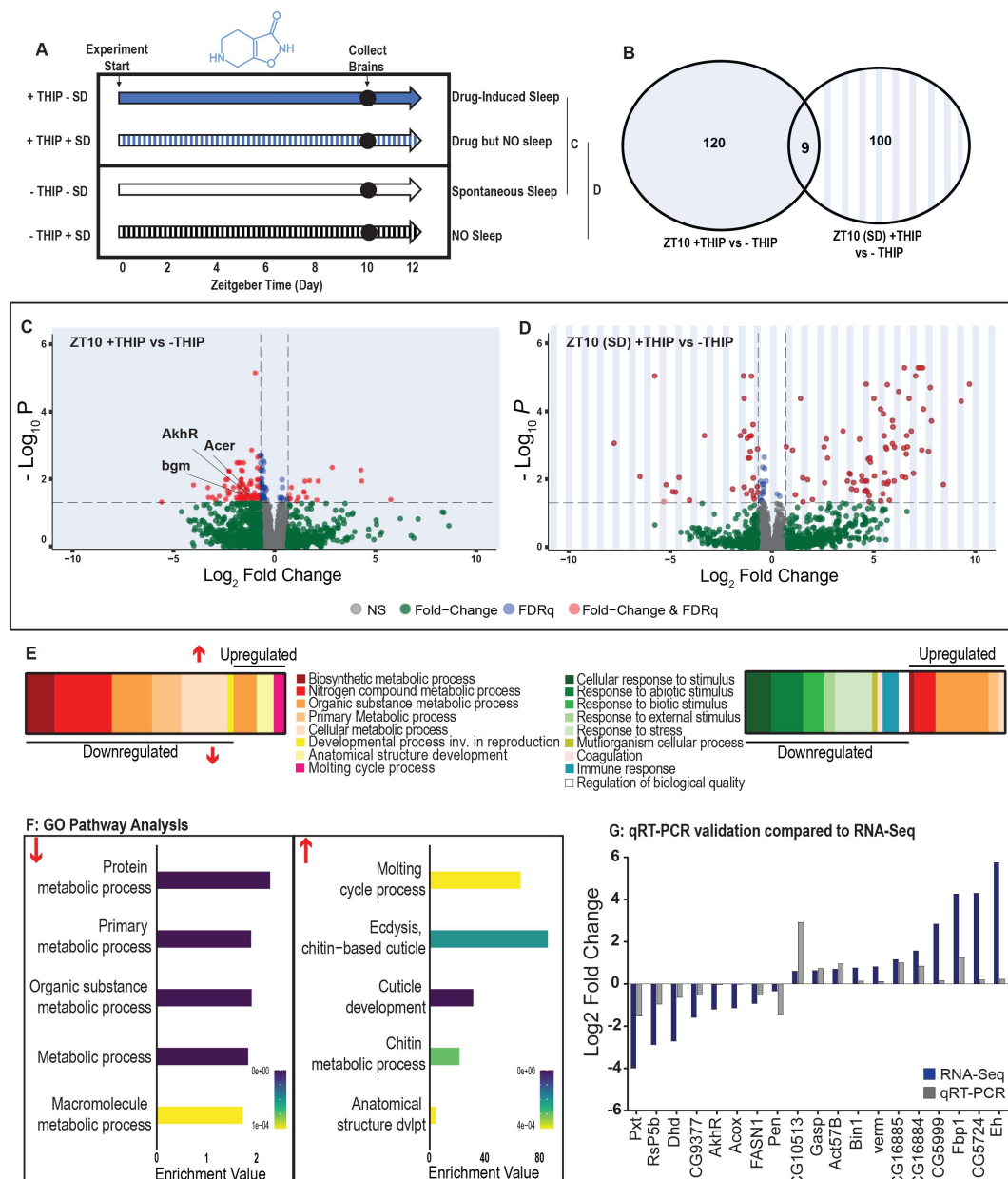


Figure 6.

Metabolic processes are downregulated during THIP-induced sleep.

A) Schematic representation of the experimental set-up and samples processed using RNA-Sequencing. **B**) Venn diagram showing the gene expression overlap between flies that had been treated with THIP versus their control (shaded blue) and flies that had been treated with THIP in a sleep deprived background versus their control (shaded blue bars). The number of significant differentially-expressed genes in each category is indicated. **C**) Volcano plot representing the distribution of differentially expressed genes in the presence or absence of THIP. Genes that are significantly up/down regulated meeting a Log2Fold change of 0.58 and FDRq value of 0.05 are shown in red. Genes meeting the threshold for FDRq value only are shown in blue. Fold change only is shown in green. Those genes not meeting any predetermined criteria are shown in grey. **D**) Volcano plot representing the distribution of differentially expressed genes in the presence or absence of THIP in a sleep deprived background. Criteria as above (C). **E**) Schematic representation of Gene Ontology (GO) enrichment of biological process results. Colour coded to indicate parent and child terms for comparisons between groups highlighted above (C) - Left and (D) - Right. **F**) Bar chart representation of a subset of interesting significant GO pathway terms originating from the organic substance and primary metabolic processes for the dataset shown in (C). **G**) Comparison between significant gene hits obtained via RNA-Sequencing (Blue) and qRT-PCR (Grey) in response to THIP, represented by Log2Fold change values. See Supplemental Tables 2&3 and **Supplementary Figures 1** & **2**.

Transcriptional analysis of flies induced to sleep by optogenetic activation

We next examined the effect of optogenetically-induced sleep on the whole-brain transcriptome, to compare to our THIP-induced sleep data. Based on our earlier findings that showed that optogenetic activation results in rapidly inducible sleep behavior that consolidates over at least 12 daytime sleep hours (**Figure 2C,E,G**), as well as our previous study showing that 10 daytime hours of optogenetic activation corrects attention defects in sleep-deprived flies [10], we induced sleep in R23E10-Gal4 x UAS-Chrimson flies for 10 daytime hours and collected tissue for whole-brain RNA-sequencing (**Figure 7A**). We selected two time points for collection, for both the sleep-induced flies (+ATR) as well as their genetically identical controls that were not fed ATR (-ATR; **Figure 7A**). Optogenetic activation was matched to the normal day-time light cycle (8 AM – 8 PM). The first collection point was after 1 hour (ZT1, 9 AM) of red-light exposure, to control for effects of ATR provision (when compared to ZT1, -ATR controls) as well as to uncover any potential short-term genetic effects of optogenetic activation. We then collected flies after 10 hours of red-light exposure (ZT 10, 6 PM) to examine longer-term genetic effects of optogenetic sleep induction, and to match exactly our THIP sleep collection timepoint (i.e., 10 hours of induced daytime sleep by either method). The combined collection points also allowed us to compare transcriptional profiles between conditions (e.g., ZT10 +ATR vs. ZT10 -ATR), to identify sleep genes, as well as within conditions (ZT1 vs. ZT10), to account for genetic effects potentially linked to light-dark cycles. As for the THIP sleep data in the same strain, samples for RNA-sequencing were collected in replicates of 5 to ensure accuracy, and any significant transcriptional effects were thresholded at a log fold change of 0.58 (see Methods).

We first examined the effect of 10 hours of daytime optogenetic-induced sleep. Here, we compared ATR-fed R23E10-Gal4 x UAS-Chrimson flies to genetically identical animals that were also exposed to red light for 10 hours but not provided with ATR in their food (ATR-). The control flies were therefore never induced to sleep by optogenetic activation, although they were still able to sleep spontaneously (see **Figure 2C,E,G**). We found that 10 hours of optogenetic activation led to 278 significant transcriptional changes, comprising mostly of upregulated genes, with 171 upregulated compared to 107 downregulated (**Figure 7B,C,E; Supplementary Table 4**). In contrast to the THIP-induced sleep dataset, transcriptional analysis of 10hr optogenetic sleep induction uncovered a variety of different processes predominantly related to the regulation of biological and cellular processes, rather than metabolism specifically (**Figure 7E; Supplementary Figure 3**). For example, of the genes that were overexpressed there is an enrichment of the Semaphorin-plexin signaling pathway (GO:0071526, GO:1902287, GO:1902285 and GO:2000305) and the ephrin receptor signaling pathway (GO:0046011), both of which are known to be involved in axonal guidance (**Figure 7F**). Interestingly, several upregulated genes code for different subunits of nicotinic acetylcholine receptors (nAChR α 1,3,4 & 5; **Figure 7C**). Importantly, there was almost no overlap with our sleep deprivation dataset (**Supplementary Table 3**), suggesting that optogenetic activation is not sleep depriving (or stressing) the flies (only one upregulated gene was shared, CG40198). Of the genes that were downregulated there is enrichment of pathways that relate to synaptic vesicle recycling (GO:0036465 and GO:0036466) as well as neurotransmitter metabolic processes (GO:0042133) (**Figure 7F; Supplementary Figure 3**).

In contrast to the 10hr timepoint, 1 hr of optogenetically-induced sleep had far fewer transcriptomic consequences, with only 17 genes upregulated and 10 downregulated (**Figure 7B,D**). This small number of transcriptomic changes (see **Supplementary Table 5**) may reflect the effect of ATR feeding, rather than any genes relevant to optogenetic sleep. That 9 hours of

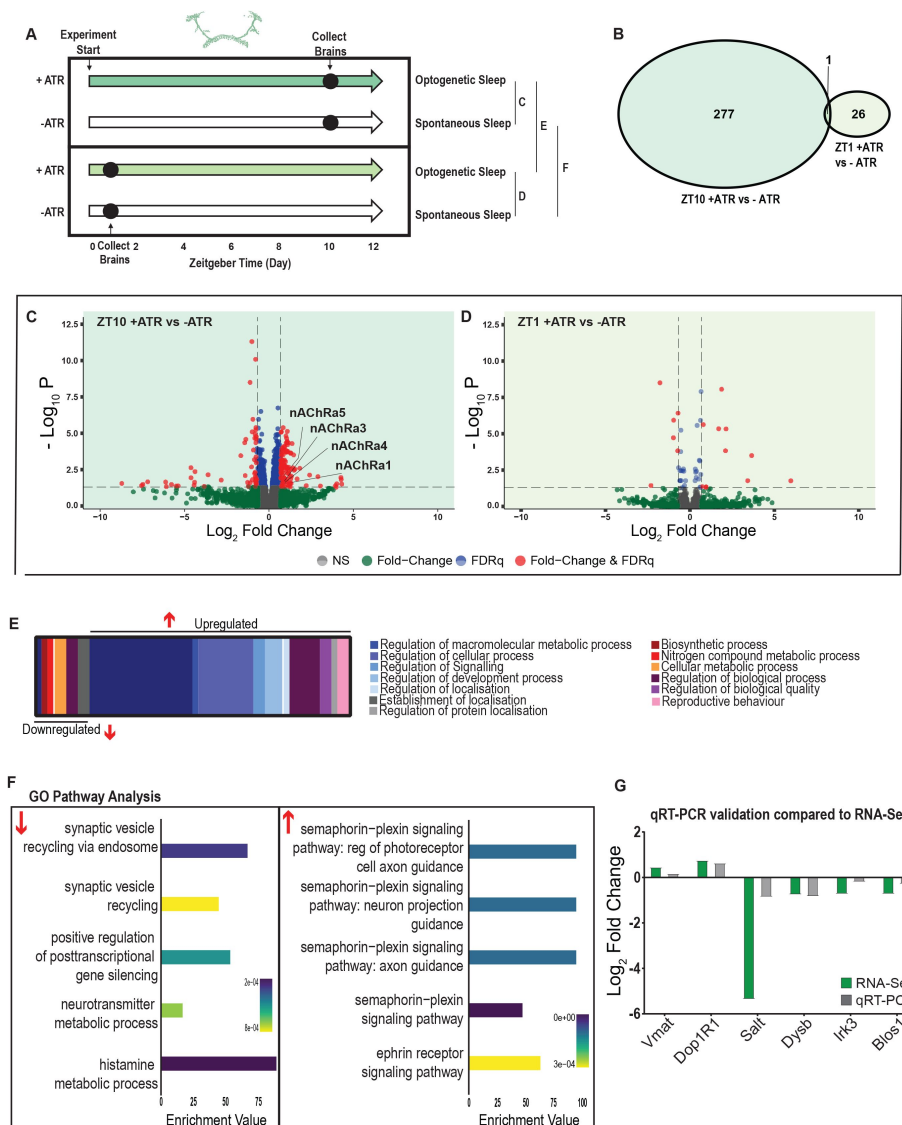


Figure 7.

A variety of biological processes including axon guidance are upregulated during optogenetic-induced sleep.

A) Schematic representation of the experimental set-up and samples processed using RNA-Sequencing. **B)** Venn diagram showing the gene expression overlap between flies that experienced 10 hours of optogenetic-induced sleep (ZT10) compared to -ATR controls (ZT10) and those flies where optogenetic activation was restricted to 1 hour (ZT1) and compared to -ATR controls (ZT1). **C)** Volcano plot representing the distribution of differentially expressed genes resulting from optogenetic activation for 10 hours versus control flies which were allowed to sleep spontaneously for 10 hours. Genes that are significantly up/down regulated meeting a Log_2Fold change of 0.58 and FDRq value of 0.05 are shown in red. Genes meeting the threshold for FDRq value only are shown in blue. Fold change only in green. Those genes not meeting any predetermined criteria are shown in grey. **D)** Volcano plot representing the distribution of differentially expressed genes resulting from optogenetic activation for 1 hour versus control flies which were allowed to sleep spontaneously for 1 hour. Criteria as above (C). **E)** Schematic representation of Gene Ontology (GO) enrichment of biological process results. Color coded to indicate parent and child terms comparing flies that had been activated optogenetically for 10 hours versus flies which had been allowed to spontaneously sleep for the same duration. **F)** Bar chart representation of a subset of interesting significant GO pathway terms originating from the regulation of cellular processes and signalling biological processes. **G)** Comparison between significant gene hits obtained via RNA-Sequencing (Green) and qRT-PCR (Grey) in response to optogenetic sleep, represented by Log_2Fold change values. See Supplemental Tables 4-7 and **Supplementary Figures 3** & **4**.

additional optogenetic sleep increased transcriptomic changes by an order of magnitude lends confidence to the interpretation that relevant genes linked to prolonged optogenetic activation are being engaged.

To account for potential genetic effects linked to light-dark expression cycles, we compared transcriptional profiles between 10 hours of optogenetic-induced sleep to 1 hour of induced sleep. Here, we found 220 differentially regulated genes (119 upregulated and 101 downregulated) when comparing ATR-fed flies at both time points (ZT10 vs ZT1, **Supplementary Table 6**). Since the 1-hour group was collected in the morning and the 10-hour group was collected in the evening, we expected this dataset to expose a number of circadian-regulatory genes, and this is indeed what we found (**Supplementary Figure 4A,B**). We then compared these results with a parallel ZT10 vs ZT1 experiment where flies were not fed ATR. Here we uncovered 503 differentially expressed genes (252 upregulated and 251 downregulated) when comparing flies that had not been fed ATR at both timepoints (**Supplementary Table 7**). Importantly, there were 98 genes that overlapped between these independent Z10 vs ZT1 datasets, suggesting commonalities linked to circadian processes. Indeed, GO Pathway analysis of Biological Processes revealed a number of genes involved in the regulation of the circadian rhythm among these 98 overlapping genes, including the well-known circadian genes *period*, *timeless*, *clockwork-orange*, *clock* and *vriille*. Notably, co-factors *period* and *timeless* are both upregulated whereas *clk* is downregulated, and this is replicated in both independent datasets (**Supplementary Figure 4C**). This correspondence with expectations for zeitgeber or light-dark effects provides a level of confidence that our respective sleep datasets are highlighting transcriptomic changes and biological pathways relevant to either sleep induction approach. Notably, there was no overlap at all in gene expression changes between optogenetic-induced sleep and THIP-induced sleep (**Supplementary Tables 2 & 4**), and the respective GO pathways analyses of biological processes are also largely non-overlapping (**Supplementary Figure 5**).

To validate these findings, we compared our transcriptional results with qRT-PCR on six genes. This included the dopamine receptor Dop1R1, which regulates arousal levels [55], as well as the schizophrenia susceptibility gene dysbindin (*Dysb*), which has been shown to regulate dopaminergic function [56]. We found convincing correspondence between our qRT-PCR data and our transcriptomic data (**Figure 7G**). qRT-PCR comparisons with RNAseq data and associated statistics are presented in **Supplementary Table S9**.

Nicotinic acetylcholine receptors regulate sleep architecture

While THIP-induced sleep caused a systemic downregulation of metabolism-related genes, the effect of optogenetic-induced sleep on gene expression was not dominated by a single category. This may be consistent with our earlier observation that brain activity looks similar to wake during optogenetic-induced sleep [10], so we could essentially be highlighting biological processes relevant to an awake fly brain, such as dopamine function [57].

However, optogenetic activation of the R23E10 circuit is not like wake, in that flies are rendered highly unresponsive to external stimuli, so perhaps like REM sleep in mammals a different category of molecular processes could be involved. In mammals, acetylcholine generally promotes wakefulness and alertness, but activity of cholinergic neurons is also high during REM sleep [58]. Neurotransmission in the insect brain is largely cholinergic, with 7 different nicotinic ‘alpha’ receptor subunits expressed in neural tissue [59]. Interestingly, four of these subunits were significantly upregulated in our optogenetic sleep dataset: nAChRa1, nAChRa3, nAChRa4, and nAChRa5 (with nAChRa6 and nAChRa7 approaching significance, **Supplementary Table 4**). For comparison, none of these were upregulated in our sleep deprivation dataset, suggesting a sleep-relevant role. Previous studies have demonstrated a role for some of these same receptor subunits in sleep regulation, in particular nAChRa4 (also called *redeye*) which is upregulated in short-sleeping mutants [60] and nAChRa3 which has been reported to regulate arousal levels in flies [61]. Together, these studies suggest processes that might be reconsidered in the context of

active sleep, as highlighted by our gene expression findings. We therefore sought to examine the role of cholinergic signalling in sleep more closely, by knocking out each nAChR α subunit and examining effects of each subunit knockout on sleep architecture. Since our transcriptomic analysis encompassed effects of active sleep on whole-brain gene expression, we first eliminated each nAChR α subunit across the brain, by testing confirmed genetic deletions [46, 62].

We first examined the effect of each nAChR α subunit deletion on sleep duration, using the 5-minute criterion for quantifying sleep in *Drosophila* [15]. We found that the nAChR α mutants fell into two different categories: removal of nAChR α 1 and nAChR α 2 significantly decreased sleep, day and night; whereas removal of nAChR α 3, nAChR α 4, nAChR α 6 and nAChR α 7 significantly increased sleep, day and night (Figure 8A). The nAChR α 5 knockout was found to be homozygous lethal, so was not included. To examine sleep architecture in these mutants, we quantified sleep bout number and duration and plotted these together as done previously for our sleep induction experiments (Figure 2). Examining the data this way, it is clear to see how nAChR α 1 and nAChR α 2 are different: most sleep bouts are very short, day and night (Figure 8B, top 2 rows, left panels, green dots). In contrast, knocking out the other alpha subunits seems to consolidate sleep, especially at night (Figure 8B, bottom 4 rows, left panels). nAChR α 3 knockouts were most striking in this regard, with these flies sleeping uninterrupted for an average of 156.53 minutes ($\pm$ 18.06) during the day and 160.76 minutes ($\pm$ 17.92) at night. Increased sleep consolidation in these mutants was however not due to lack of activity. While awake, nAChR α 3 animals were just as active as controls (activity per waking minute = 2.69 ± 0.18 versus 2.5 ± 0.06 , respectively). However, some knockouts did increase waking activity levels; waking activity data for all of the knockout strains and their genetic controls are presented in Supplementary Figure 6.

We next questioned what kind of sleep the nAChR α knockout flies might be getting. In previous work we have shown that flies can be asleep already after the first minute of spontaneous inactivity, and that during the first five minutes of sleep the fly brain displays wake-like levels of neural activity [10]. We have termed this early sleep stage ‘active sleep’ to distinguish it from ‘quiet sleep’ that typically follows after 5-10 minutes [63]. One way of estimating the amount of ‘active sleep’ in *Drosophila* flies is to sum all short sleep epochs lasting between 1-5 minutes and expressing this as a percentage of total sleep [10]. When we re-examined our nAChR α knockouts in this way, we found that this behavioral readout for ‘active sleep’ was significantly affected by the loss of select nAChR α subunits. Short sleep increased significantly during both the day and the night in nAChR α 1 and nAChR α 2 (Figure 8B, top 2 rows, right panel, green dots). In contrast, and consistent with our sleep architecture analyses (above), nAChR α 3 displayed almost no short sleep (Figure 8B, row 3, right panel). Finally, in nAChR α 4 and nAChR α 6 short sleep was significantly decreased at night, while in nAChR α 7 short sleep was significantly decreased day and night (Figure 8B, rows 4-6, right panel). In conclusion, every one of the nAChR α knockouts we tested affect short sleep in some way, either increasing (nAChR α 1 and nAChR α 2) or decreasing it (nAChR α 3, nAChR α 4, nAChR α 6, nAChR α 7). Additionally, tallying the proportion of short sleep bouts provides valuable insight into altered sleep architecture in mutant strains.

We nevertheless questioned whether these systemic effects of nicotinic receptors on short sleep were perhaps a trivial consequence of altered >5min sleep duration in these mutants, especially regarding the striking differences between nAChR α 1&2 and the other subunit knockouts. We therefore returned to our ‘quiet’ sleep (THIP) dataset to contrast a gene derived from that study. We had found that several of the THIP-induced sleep genes are involved in metabolic processes, which are mostly downregulated (Supplementary Table 2). This included the adipokinetic hormone receptor (AkhR), which has previously been associated with starvation-induced sleep regulation [53]. We employed an RNAi strategy to downregulate this metabolic gene’s expression across the fly brain in AkhR-RNAi / R57C10-Gal4 flies (see Methods). We found that downregulating AkhR significantly decreased sleep duration during the day as well as night, compared to genetic control strains (Figure 9A,B). Accordingly, sleep bout duration and number decreased, especially during the day (Figure 9C). However, in contrast to knocking out nAChR α 1

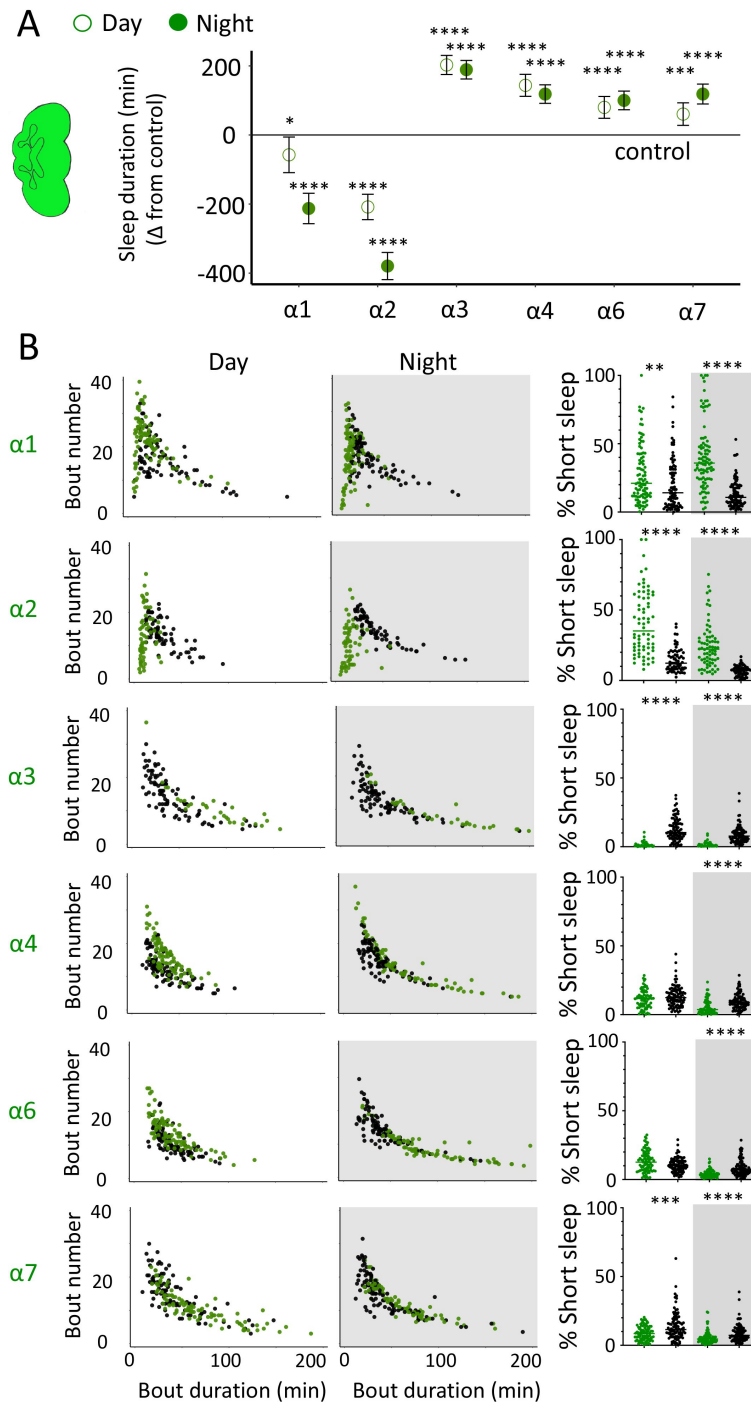


Figure 8.

nAChRα subunit knockouts bidirectionally regulate >5min sleep as well as short sleep.

A. Average total day and night sleep duration (minutes±95% confidence intervals) in nAChRα knockout mutants, expressed as difference to their respective background controls (see Methods). α1, N=91; control1 ($X^{59}w^{1118}$) = 93; α2, N=70; control ($w^{1118}ActinCas9$) = 65; α3, N=43; (ActinCas9) = 9; α4, N=87; ($w^{1118}ActinCas9$) = 98; α6, N=91; ($w^{1118}ActinCas9$) = 91; α7, N=94; (ActinCas9) = 95. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$ by t-test adjusted for multiple comparisons. **B.** Left two panels: sleep architecture for the same six knockout strains as in A (green), shown against their respective controls (black). Each datapoint is a fly. Right panels: cumulative short sleep (1-5min) expressed as a percentage of total sleep duration. Data are the from the same experiment as in A&B. Each datapoint is a fly. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ Man-Whitney U Test. All data were collected over three days and three nights and averaged.

and nAChR α 2, which also significantly decreased sleep duration day and night, short sleep was not significantly altered in AkhR knockdown animals compared to genetic controls (**Figure 9D**). This suggests that short (1-5min) sleep might be under separate regulatory control than >5min sleep.

We then returned to the nAChR α subunits and employed an RNAi strategy to knock them down in the R23E10 sleep-promoting neurons specifically. While it is unlikely that these cholinergic receptor subunits are restricted to just these neurons, we were curious if removing any of them from this specific circuit had similar effect on sleep as in the knockout strains.

We found that nAChR α 1 knockdown in the R23E10 neurons significantly decreased total sleep during both day and night (**Figure 10A,B**), which was also true for the nAChR α 1 knockouts (**Figure 8A**). Also consistent with the knockouts, the proportion of short sleep bouts was increased in this specific knockdown (**Figure 10C**). Among all of the other alpha subunits, knockdown of nAChR α 5 and nAChR α 6 in the R23E10 neurons significantly increased daytime and night-time sleep, respectively (**Figure 10A,B**), while short sleep was correspondingly decreased only in nAChR α 5 (**Figure 10C**). None of the other alpha subunits had any effect in these sleep-promoting neurons. This suggests that their sleep-regulatory role lies outside of this circuit, while nAChR α 1, nAChR α 5, and nAChR α 6 might regulate sleep-related functions within this circuit.

Discussion

One of the key advantages of studying sleep in *Drosophila* is that this versatile model provides a variety of reliable approaches for inducing and controlling sleep. By being able to induce sleep on demand, either genetically or pharmacologically, researchers have been able to manipulate sleep as an experimental variable, and in this way be better able to assess causality when probing potential sleep functions. However, this approach has often sidestepped the question of whether different sleep induction methods are equivalent, or whether distinct forms of sleep might be engaged by different genetic or pharmacological treatments. In mammals, GABA agonists typically promote slow-wave sleep (SWS), which has been associated with cellular homeostasis and repair process in the brain [4, 64]. In contrast, drugs targeting acetylcholine receptors, such as carbachol, have been found to promote brain states more reminiscent of REM sleep [65, 66]. Although these drugs all induce sedative (or dissociative) states, they are clearly producing dissimilar forms of sleep in mammals, with likely different functions or consequences for the brain. In *Drosophila*, evidence suggests that the GABA agonist THIP promotes a form of deep or ‘quiet’ sleep, which may be functionally analogous to mammalian SWS [9, 27, 67]. In contrast, optogenetic activation of the dFB as well as other neurons may promote a form of active sleep, which we have suggested could be achieving some similar sleep functions as REM [2]. THIP-induced sleep in flies has been associated with waste clearance from the brain [27], just as SWS has been associated with clearance of waste metabolites via the mammalian brain’s glymphatic system [64]. Such functional homology suggests that the transcriptional changes we uncovered for THIP-induced sleep in *Drosophila* might also be relevant for mammalian SWS, with these largely centered on reduced metabolic processes and stress regulation [52]. In contrast, except for the upregulation of cholinergic signaling [68], there is little to compare to test hypotheses potentially linking active sleep in flies with REM sleep in mammals, except for the potential upregulation of cholinergic signaling. Even this cholinergic connection seems odd, seeing that the predominant excitatory neurotransmitters are reversed in the brains of insects and mammals: glutamate in mammals and acetylcholine in insects [69]. Additionally, only nicotinic receptor subunits were identified in our analyses, whereas muscarinic receptors have been more commonly associated with REM sleep in mammals [70, 71]. Nevertheless, it is clear from our results that optogenetic-induced active sleep upregulates the expression of multiple nicotinic acetylcholine subunits, and that knocking these out individually has profound (and opposing) effects on sleep architecture in flies, and that a subset might function intrinsically within sleep

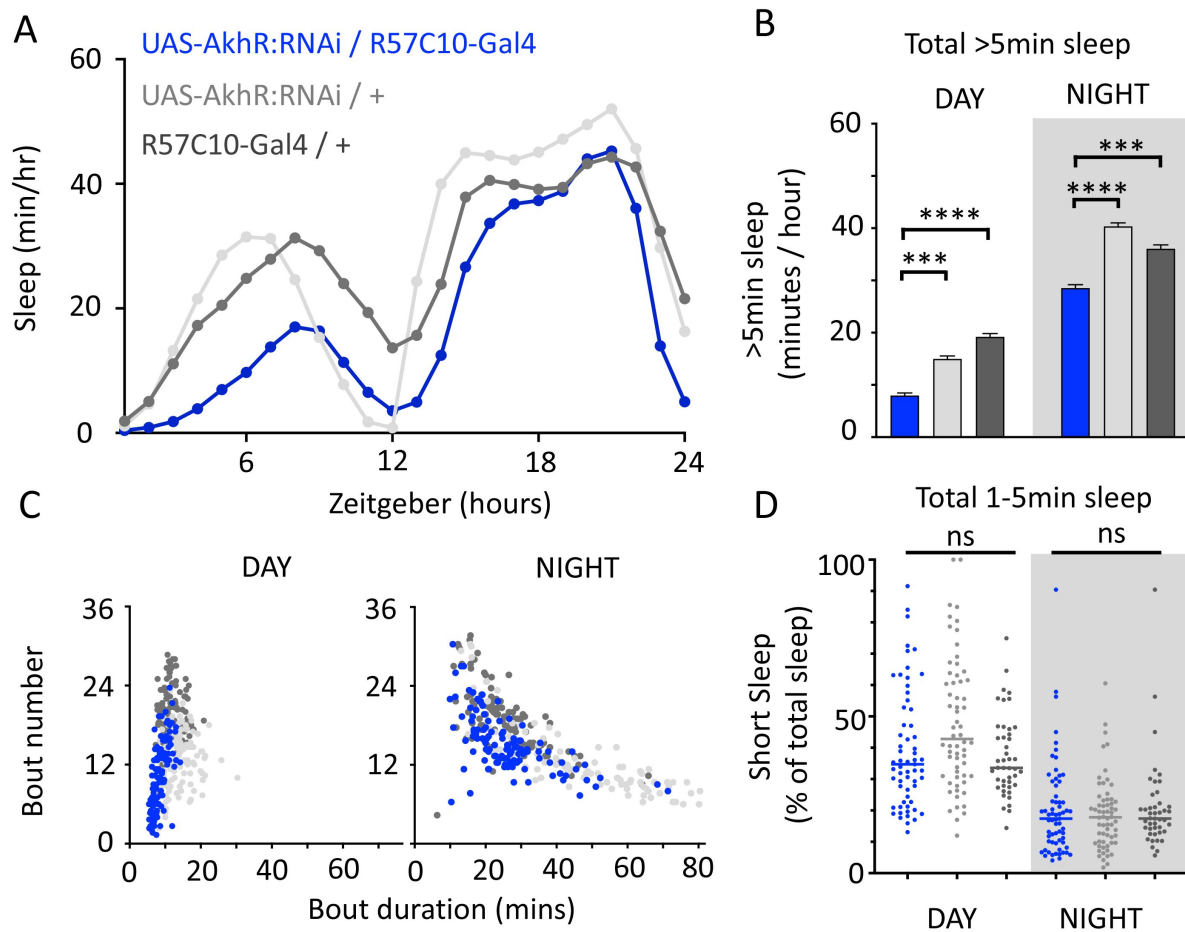


Figure 9.

AkhR knockdown decreases >5min sleep but not short sleep.

A,B. Total sleep (>5min) in UAS-AkhR:RNAi / R57C10-Gal4 flies (blue, N=126) compared to genetic controls (light grey: UAS-AkhR:RNAi / +, N= 124; dark grey: R57C10-Gal4 / +, N= 120). **C.** Sleep architecture (average bout duration versus bout number per fly) in data from A,B. **D.** Cumulative short sleep (1-5min, expressed as a % of total sleep) in UAS-AkhR:RNAi / R57C10-Gal4 flies (blue) compared to genetic controls (light grey: UAS-AkhR:RNAi / +; dark grey: R57C10-Gal4 / +). Wild-type background (+) is Canton-S(w¹¹¹⁸). Each datapoint is a fly. *** $P < 0.001$, **** $P < 0.0001$ Man-Whitney U Test. ns, not significant. All data were collected over two days and two nights and averaged.

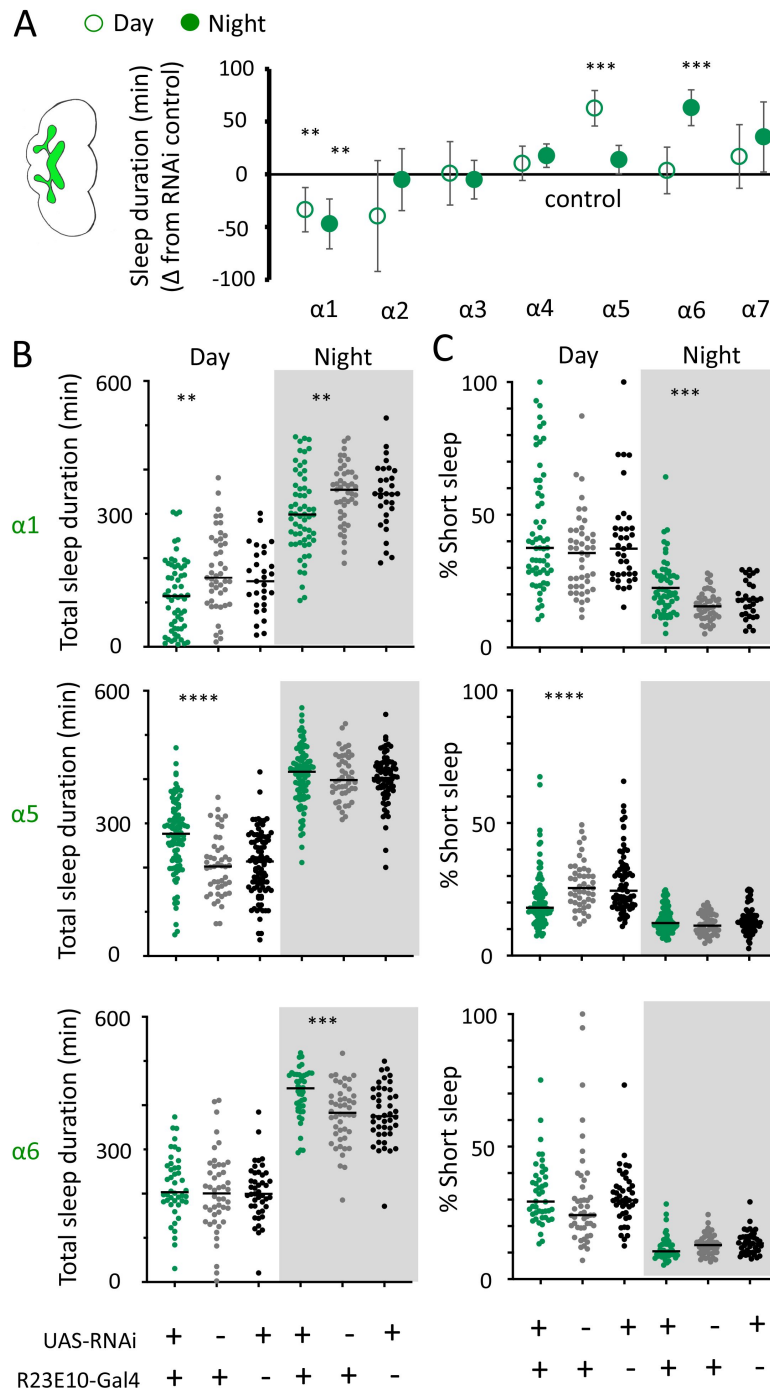


Figure 10.

nAChRa subunit knockdowns in sleep-promoting neurons.

A. Average total day and night sleep duration (minutes $\pm$ 95% confidence intervals) in UAS-nAChRa RNAi / R23E10-Gal4 flies, expressed as difference to RNAi / + controls (all were also compared Gal4 / + controls, not shown). $\alpha 1$ N=60, Gal4 N=45, RNAi N=37; $\alpha 2$ N=21, Gal4 N=24, RNAi N=50; $\alpha 3$ N=33, Gal4 N=50, RNAi N=59; $\alpha 4$ N=92, Gal4 N=90, RNAi N=80; $\alpha 5$ N=94, Gal4 N=47, RNAi N=74; $\alpha 6$ N=44, Gal4 N=47, RNAi N=43; $\alpha 7$ N=32, Gal4 N=57, RNAi N=44. ** P <0.01, *** P <0.001 by t-test adjusted for multiple comparisons. **B.** Total >5min sleep duration data for significant knockdowns in (A). **C.** Cumulative short sleep (1-5min) expressed as a percentage of total sleep duration. Data are the from the same experiment as in A&B. Each datapoint is a fly. *** P <0.001, **** P <0.0001 Man-Whitney U Test. All data were collected over two days and two nights and averaged.

promoting neurons. This supports other studies showing a role for nAChR α 's in sleep regulation [60, 61]. It will be especially interesting in future brain imaging studies to see whether a knockout such as nAChR α 3 is eliminating one kind of sleep (e.g., active sleep) as predicted by our behavioral data, and whether this is associated with any functional consequences. Similarly, it will be telling to see whether the opposite sleep phenotypes observed in nAChR α 1 for example result in a distinct class of functional consequences. A previous study has shown that nAChR α 1 knockout animals have significantly shorter survivorship compared to controls, with flies dying almost 20 days earlier [45]. One reason could be because of impaired or insufficient deep sleep functions (e.g., brain waste clearance [27]). The nAChR knockouts provide an opportunity to further examine mutant animals potentially lacking either kind of sleep, although this will have to be confirmed by brain imaging or electrophysiology.

We found little similarity between two different approaches to inducing sleep in flies, at the level of gene expression as well as acute effects on brain activity. It may however not be surprising that these entirely different sleep induction methods produce dissimilar physiological effects. After all, one method requires flies to ingest a drug which then must make its way to the brain, while the other method directly activates a subset of neurons in the central brain, as well as in the ventral nerve cord [72]. Yet both methods yield similarly increased sleep duration profiles and consolidated sleep architecture (**Figure 2**). One underlying assumption with focusing on sleep duration as the most relevant metric for understanding sleep function in *Drosophila* is that sleep is a unitary phenomenon in the fly model, meaning that primarily one set of functions and one form of brain activity are occurring when flies sleep. There is now substantial evidence that this is unlikely to be true, and that like other animals flies probably also experience distinct sleep stages that accomplish different functions [9, 10, 27, 63, 73, 74]. This does not mean that these functions are mutually exclusive; for example, both THIP provision and neuronal activation of central brain circuits have been found to promote memory consolidation in *Drosophila* [23]. Indeed, it seems reasonable to propose that different sleep stages could be synergistic, accomplishing a variety of homeostatic functions that might be required for adaptive behaviors in an animal. Our results suggest that THIP provision promotes a 'quiet sleep' stage in flies, which induces a brain-wide downregulation of metabolism-related genes. This is consistent with studies in flies showing that metabolic rate is decreased in longer sleep bouts, especially at night, and that this is recapitulated by THIP-induced sleep [75]. Our findings also align with a previous study showing that mitochondrial metabolic processes are upregulated in the absence of sleep [76]. One argument for why metabolism-related genes are downregulated during THIP-induced sleep might be that flies are starved (because they are sleeping more, and thus perhaps not eating). However, flies induced to sleep by optogenetic activation are also sleeping more (indeed, their average sleep bouts are longer), yet these did not reveal a similar downregulation of metabolic processes. Another view might be that our sampling was done after flies had achieved 10 hours of induced sleep, so sleep functions might have already been achieved by that time. Thus, we might not be uncovering genes required for achieving 'quiet' sleep functions as much as identifying exactly the opposite: genetic pathways that have been satisfied by 10 hours of induced quiet sleep. Other studies using THIP to induce sleep have examined longer timeframes (e.g., 2 days [23]), so it remains unclear whether changes in gene regulation relate to sleep functions that have been achieved or that are still being engaged. For our transcriptomic study, both sleep induction protocols were conducted during the day (thus in presumably well-rested flies) to deliberately avoid any sleep pressure confounds. Since the dFB has been proposed as a regulator of sleep homeostasis [20–22], one could question why its prolonged activation did not incur a deep sleep debt – which might have been detectable at a transcriptomic level by some overlap with our sleep deprivation controls. That we did not see this suggests induced daytime active sleep might not accrue any sleep debt. We have shown previously that daytime sleep is qualitatively different than night-time sleep in flies, which is 'deeper' [63]. It is for example possible that most quiet sleep happens at night in flies, so a daytime regime of induced active or quiet sleep might not

incur any sleep debt for either. Following this logic, our RNA analyses simply revealed the transcriptomic changes resulting from either manipulation in well-rested flies, with the key result being that there are zero shared genes after 10 daytime hours of either manipulation.

In contrast to THIP, our optogenetic manipulation induced a brain-wide upregulation of a variety of neural mechanisms unrelated to metabolic processes, suggesting a different kind of sleep was engaged. Although many studies have shown that the R23E10-Gal4 circuit is sleep promoting (e.g. [19], [21], [74]), and we have shown that acute activation of these neurons produces a form of active sleep [10], it seems unlikely that active sleep regulation is limited to the dFB neurons alone [72]. Other circuits in the fly central brain are also sleep-promoting, including in the ellipsoid body [77] and the ventral fan-shaped body (vFB) [78], although it remains unknown if activation of these other circuits also promotes an active sleep stage, or whether a similar transcriptome might be engaged by these alternate approaches to optogenetic sleep induction in flies. This again highlights a variant of the same problem we have uncovered in the current study comparing pharmacology with optogenetics: different circuit-based approaches could all be increasing sleep duration but achieving entirely different functions by engaging distinct transcriptomes and thus different sleep functions. How many different kinds of functions are engaged by sleep remains unclear: is it roughly two functional categories linked to quiet and active sleep, or is it a broader range of sub-categories that are not so tightly linked to these obviously different brain activity states?

A compelling argument could nevertheless be made for two kinds of sleep in most animals, with two distinct sets of functions [1], [67]. Most animals have been shown to require a form of ‘quiet’ sleep to ensure survival, suggesting that these might encompass an evolutionarily conserved set of cellular processes that promote neural health and development [79], and that operate best during periods of behavioral quiescence. Nematode worms thus experience a form of quiet sleep when they pause to molt (‘lethargus’) into a different life stages during their development [80], or when cellular repair processes are needed following environmental stress [81]. In flies, quiet sleep seems to be similarly required for neuronal repair [26] or waste clearance [27], and there is evidence that glia might play a key role in these cellular homeostatic processes in flies [26] as well as other animals [82]. Thus, SWS in mammals and birds might present a narrow neocortical view of a more ancient set of sleep functions centered on quiescence and decreased metabolic rate. Indeed, neural quiescence is also a feature of SWS, both at the level of pulsed inhibition (down-states), as well as in other parts of the brain beyond the cortex [1]. Similar to findings in flies that are induced into a quiet sleep stage with THIP [75], metabolic rate also decreases during SWS in mammals [83]. In contrast, metabolic rate is similar to waking during REM sleep in mammals [84], suggesting an alternate set of functions not linked to cellular homeostasis. What might these functions be, and could some of these be conserved between active sleep in invertebrates and REM sleep in mammals? A REM-like sleep stage has now been identified in a variety of invertebrate species, including cephalopods [12] and jumping spiders [85]. In humans, REM sleep has been implicated in emotion regulation [86], and cognitive disorders where emotions are dysregulated, such as depression, are often associated with REM sleep dysfunction [87]. While it is not evident how to study emotions in insects (but see [88]), it could be argued that arousal systems more generally are employed to detect prediction errors and thereby promote learning [89]. Thus, we and others have suggested that REM sleep might be crucial for optimizing prediction, and attention, and learning [2], [67], [90], and this may involve different kinds of homeostatic mechanisms centered on brain circuits rather than cells. Whether active sleep in flies plays a similar homeostatic role remains to be determined. Our finding that optogenetically -induced sleep in *Drosophila* upregulates different nAChR subunits is consistent with new findings that these subunits regulate appetitive memories in flies [91] and that cholinergic systems more generally underpin learning and memory in this animal [92]. Yet learning and memory in flies clearly also benefits from quiet sleep, as evidenced by multiple studies using THIP as a sleep-promoting agent [23], [25], [93]. One view consistent with our findings and previous studies is that both kinds of sleep are crucial for optimal

behavior: quiet sleep for cellular homeostasis and active sleep for circuit-level homeostasis. Manipulating these separately, alongside the non-overlapping pathways engaged by either kind of sleep, should help further disambiguate the functions potentially associated with these distinct sleep stages.

Author contributions

Designed experiments, collected and analyzed data: NA, LALT-H, HL, EN, AK. Designed experiments and analyzed data: QZ. Designed experiments: TP, PB, PJS. Designed experiments, analyzed data, and wrote the paper: BvS.

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Declaration of Interests

The authors declare no conflicts of interest.

Supplementary Figure Legends

Supplementary Tables

Table S1, related to Figure 2 [↗](#). A comparison of sleep duration profiles (min/hr) during optogenetic and THIP induced sleep. Tested with 2way ANOVA with Tukey's multiple comparison test.

Table S2, related to Figure 6 [↗](#). List of significant THIP-sleep genes. Raw data for all genes.

Table S3, related to Figure 6 [↗](#). List of significant sleep-deprivation genes in THIP-fed flies. Raw data for all genes.

Table S4, related to Figure 7 [↗](#). List of significant optogenetic-sleep genes after 10 hours activation. Raw data for all genes.

Table S5, related to Figure 7 [↗](#). List of significant optogenetic-sleep genes after 1 hour of activation. Raw data for all genes.

Table S6, related to Figure 7 [↗](#). List of significant ZT10 vs ZT1 genes in ATR+ dataset. Raw data for all genes.

Table S7, related to Figure 7 [↗](#). List of significant ZT10 vs ZT1 genes in ATR-dataset. Raw data for all genes.

Table S8, related to Figures 6 [↗](#) **& 7** [↗](#). Primer list for RT qPCR validation experiments.

	GO Term	Pvalue	Enrichment value
Downregulated	GO:0046460 neutral lipid biosynthetic process	0.0006	52.92
	GO:0046463 acylglycerol biosynthetic process	0.0006	52.92
	GO:0002181 cytoplasmic translation	0.0000	33.41
	GO:0009059 macromolecule biosynthetic process	0.0000	6.44
	GO:0009058 biosynthetic process	0.0000	4.53
	GO:0009064 glutamine family amino acid metabolic process	0.0008	16.54
	GO:0006412 translation	0.0000	11.7
	GO:0043043 peptide biosynthetic process	0.0000	11.54
	GO:0043604 amide biosynthetic process	0.0000	10.48
	GO:0006518 peptide metabolic process	0.0000	8.89
	GO:1901566 organonitrogen compound biosynthetic process	0.0000	6.38
	GO:0044271 cellular nitrogen compound biosynthetic process	0.0000	5.07
	GO:0034641 cellular nitrogen compound metabolic process	0.0000	2.31
	GO:1901564 organonitrogen compound metabolic process	0.0000	2.06
	GO:0006807 nitrogen compound metabolic process	0.0002	1.6
	GO:1901576 organic substance biosynthetic process	0.0000	4.53
	GO:0019752 carboxylic acid metabolic process	0.0008	3.36
	GO:0043436 oxoacid metabolic process	0.0010	3.26
	GO:0006082 organic acid metabolic process	0.0010	3.25
	GO:0019538 protein metabolic process	0.0000	2.27
	GO:0071704 organic substance metabolic process	0.0000	1.9
	GO:0043170 macromolecule metabolic process	0.0001	1.72
	GO:0006591 ornithine metabolic process	0.0006	52.92
	GO:0006525 arginine metabolic process	0.0008	44.1
	GO:1901605 alpha-amino acid metabolic process	0.0003	6.73
	GO:0008152 metabolic process	0.0000	1.83
	GO:0044238 primary metabolic process	0.0000	1.89
	GO:0019432 triglyceride biosynthetic process	0.0003	66.15
	GO:0006414 translational elongation	0.0000	34.51
	GO:0034645 cellular macromolecule biosynthetic process	0.0000	7.55
	GO:0043603 cellular amide metabolic process	0.0000	7.7
	GO:0044249 cellular biosynthetic process	0.0000	4.69
	GO:0044267 cellular protein metabolic process	0.0000	2.58
	GO:0044260 cellular macromolecule metabolic process	0.0000	2.02
	GO:0044237 cellular metabolic process	0.0000	1.67
	GO:0007548 sex differentiation	0.0003	22.05
Upregulated	GO:0006030 chitin metabolic process	0.0003	21.44
	GO:1901071 glucosamine-containing compound metabolic process	0.0004	19.78
	GO:0006040 amino sugar metabolic process	0.0004	19.33
	GO:0006022 aminoglycan metabolic process	0.0006	17.48
	GO:0018990 ecdysis, chitin-based cuticle	0.0002	85.05
	GO:0022404 molting cycle process	0.0004	65.42
	GO:0040003 chitin-based cuticle development	0.0000	32.89
	GO:0042335 cuticle development	0.0000	31.33
	GO:0048856 anatomical structure development	0.0004	4.39

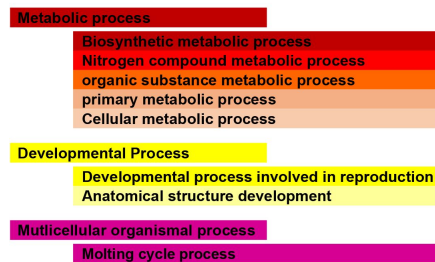


Figure S1.

Gene Ontology (GO) enrichment analysis for THIP-induced sleep (related to Figure 6).

Significantly downregulated and upregulated GO categories for THIP-sleep (Table S2), listed from most enriched at the top. Broad GO categories are identified below.

	GO Term	Pvalue	Enrichment value
Downregulated	GO:0104004 cellular response to environmental stimulus	0.0005	19.04
	GO:0050896 response to stimulus	0.0000	3.79
	GO:0034644 cellular response to UV	0.0000	85.05
	GO:0009411 response to UV	0.0001	41.15
	GO:0071482 cellular response to light stimulus	0.0001	32.71
	GO:0071478 cellular response to radiation	0.0003	22.78
	GO:0071214 cellular response to abiotic stimulus	0.0005	19.04
	GO:0009266 response to temperature stimulus	0.0000	16.88
	GO: 0009617 response to bacterium	0.0000	21.07
	GO: 0051707 response to other organism	0.0000	15.39
	GO:0043207 response to external biotic stimulus	0.0000	15.14
	GO: 0009607 response to biotic stimulus	0.0000	15.09
	GO:0050830 defense response to Gram-positive bacterium	0.0000	36.98
	GO:0009605 response to external stimulus	0.0000	9.31
	GO:0034605 cellular response to heat	0.0000	60.75
	GO:0009408 response to heat	0.0000	26.58
	GO:0006979 response to oxidative stress	0.0002	13.83
	GO:0042742 defense response to bacterium	0.0000	12.89
	GO:0098542 defense response to other organism	0.0000	11.63
	GO:0006952 defense response	0.0000	10.12
	GO:0006950 response to stress	0.0000	5.79
	GO:0042381 hemolymph coagulation	0.0001	141.75
	GO:0006955 immune response	0.0007	9.83
	GO:0002376 immune system process	0.0002	9.01
	GO:0007599 hemostasis	0.0001	141.75
	GO:0050878 regulation of body fluid levels	0.0005	56.7
	GO:0051704 multi-organism process	0.0000	12.34
	GO:0050817 coagulation	0.0001	141.75
Upregulated	GO:0008152 metabolic process	0.0000	1.86
	GO:0006032 chitin catabolic process	0.0001	36.6
	GO:1901072 glucosamine-containing compound catabolic process	0.0001	32.53
	GO:0006022 aminoglycan metabolic process	0.0000	13.37
	GO:0006807 nitrogen compound metabolic process	0.0000	1.94
	GO:0046348 amino sugar catabolic process	0.0001	32.53
	GO:0006026 aminoglycan catabolic process	0.0003	21.69
	GO:0006030 chitin metabolic process	0.0000	16.4
	GO:1901071 glucosamine-containing compound metabolic process	0.0000	15.13
	GO:0006040 amino sugar metabolic process	0.0000	14.79
	GO:0006508 proteolysis	0.0000	5.13
	GO:1901135 carbohydrate derivative metabolic process	0.0002	3.94
	GO:1901564 organonitrogen compound metabolic process	0.0000	2.31
	GO:0043170 macromolecule metabolic process	0.0000	2.1
	GO:0071704 organic substance metabolic process	0.0000	1.92
	GO:0019538 protein metabolic process	0.0008	2.01
	GO:0044238 primary metabolic process	0.0004	1.67
	GO:0017144 drug metabolic process	0.0000	6.24

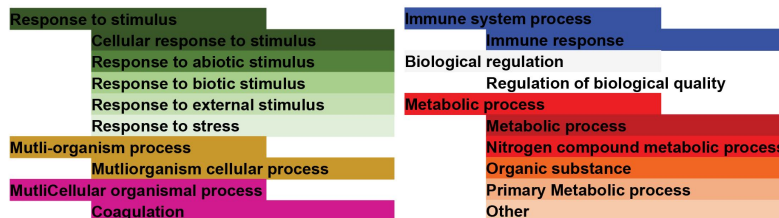


Figure S2.

Gene Ontology (GO) enrichment analysis for THIP-provisioned flies that were sleep deprived (related to Figure 6 [↗](#)).

Significantly downregulated and upregulated GO categories for sleep deprived flies (Table S3), listed from most enriched at the top. Broad GO categories are identified below.

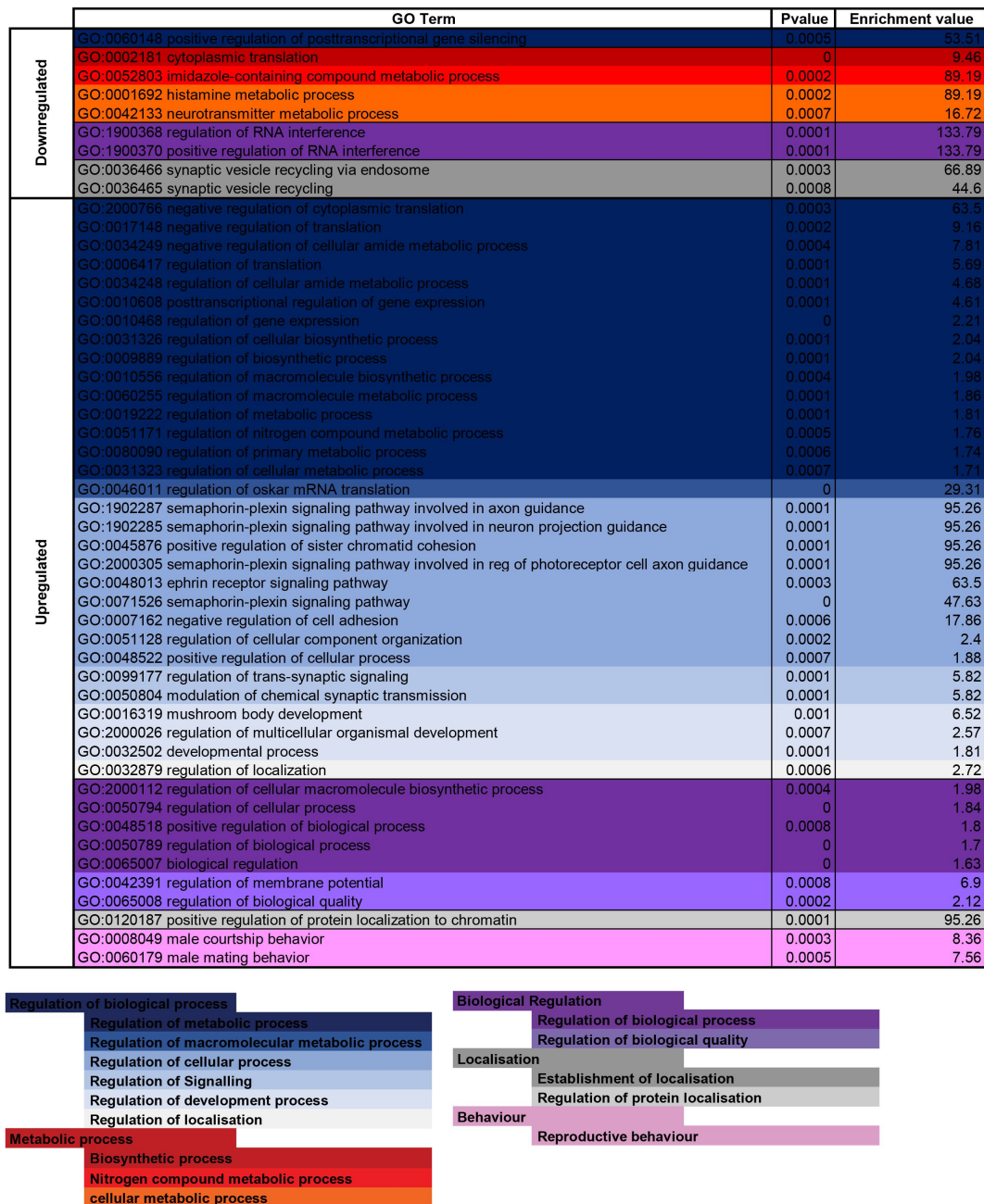


Figure S3.

Gene Ontology (GO) enrichment analysis for optogenetic-induced sleep (related to Figure 7 [↗](#)).

Significantly downregulated and upregulated GO categories for optogenetic-sleep (Table S4), listed from most enriched at the top. Broad GO categories are identified below.

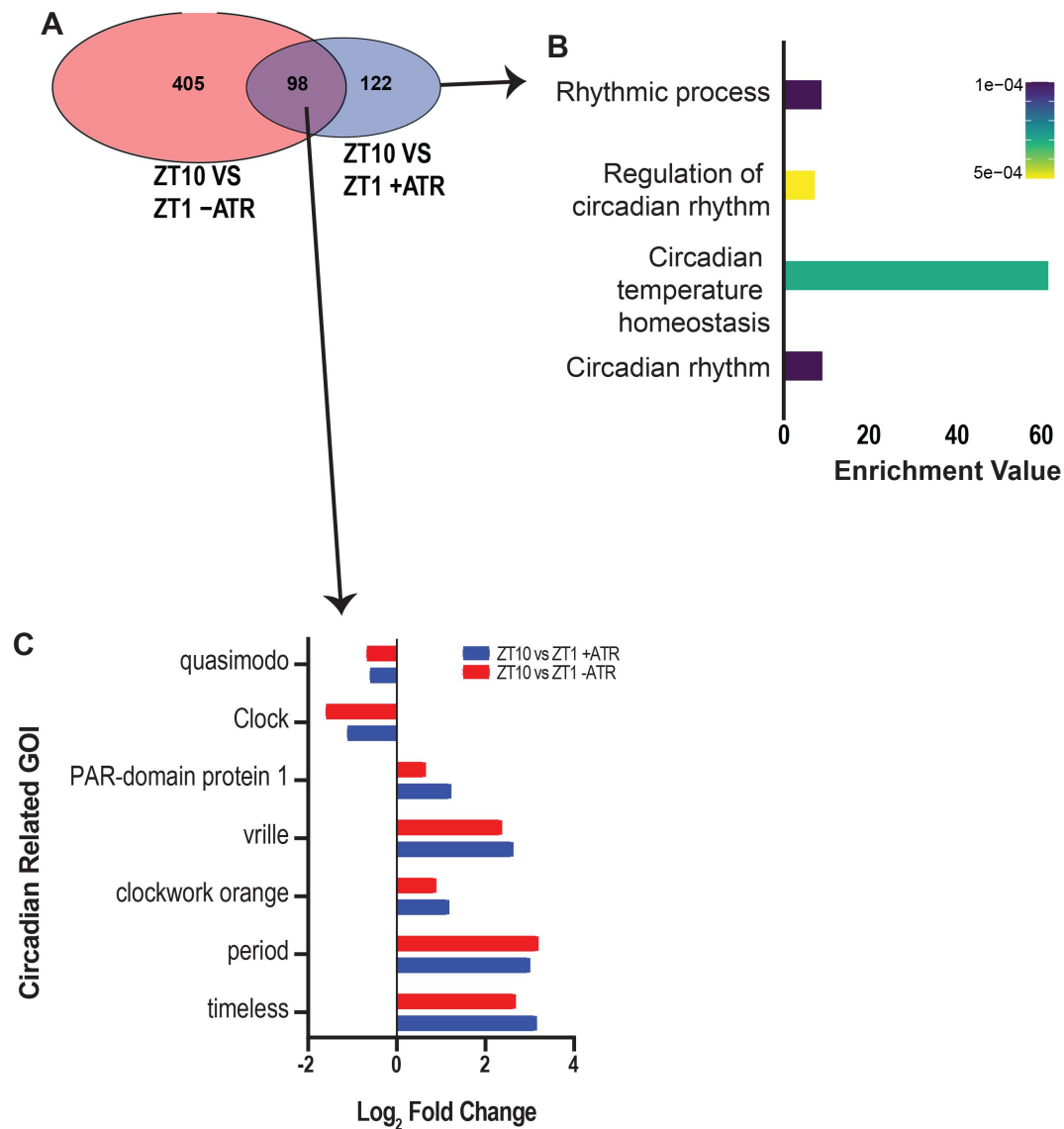
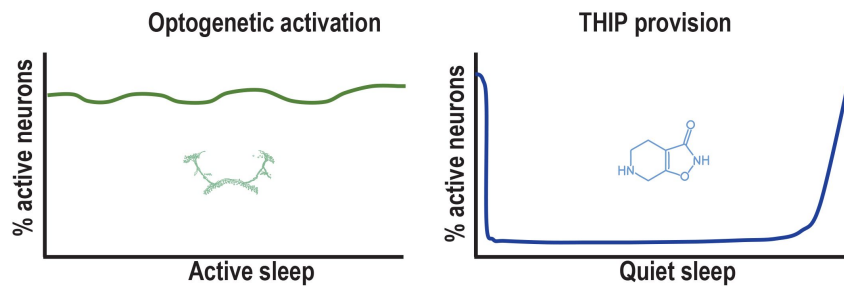


Figure S4.

Circadian-related genes uncovered in optogenetic-sleep dataset (related to Figure 7).

A. Zeitgeber (ZT) 10 timepoint was compared with ZT in to uncover potential circadian-regulated genes, in two separate datasets (-ATR and +ATR). 98 genes were shared between these datasets. **B.** Of the 98 shared genes, circadian-related processes were highly enriched. **C.** Expression levels of 7 circadian genes drawn from the two different datasets in A.

A: Sleep induction method



B: Induced Sleep Transcriptome: GO Pathways of Biological Processes

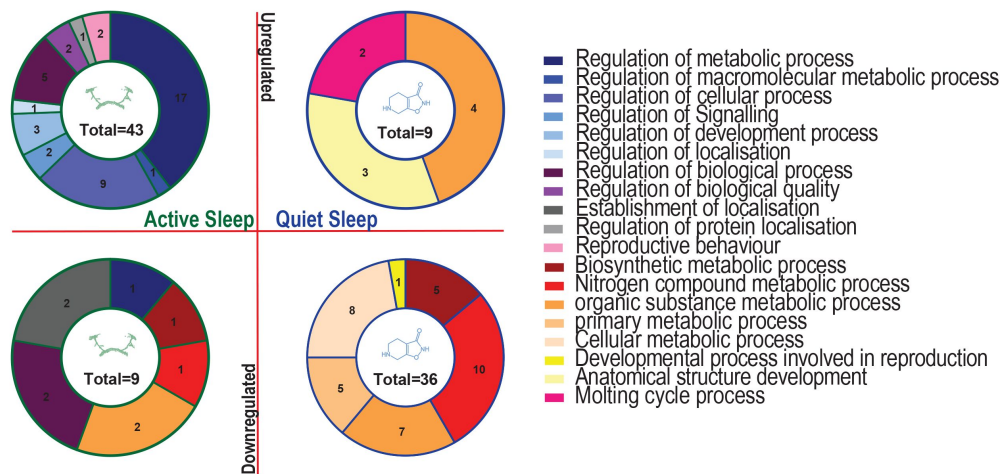
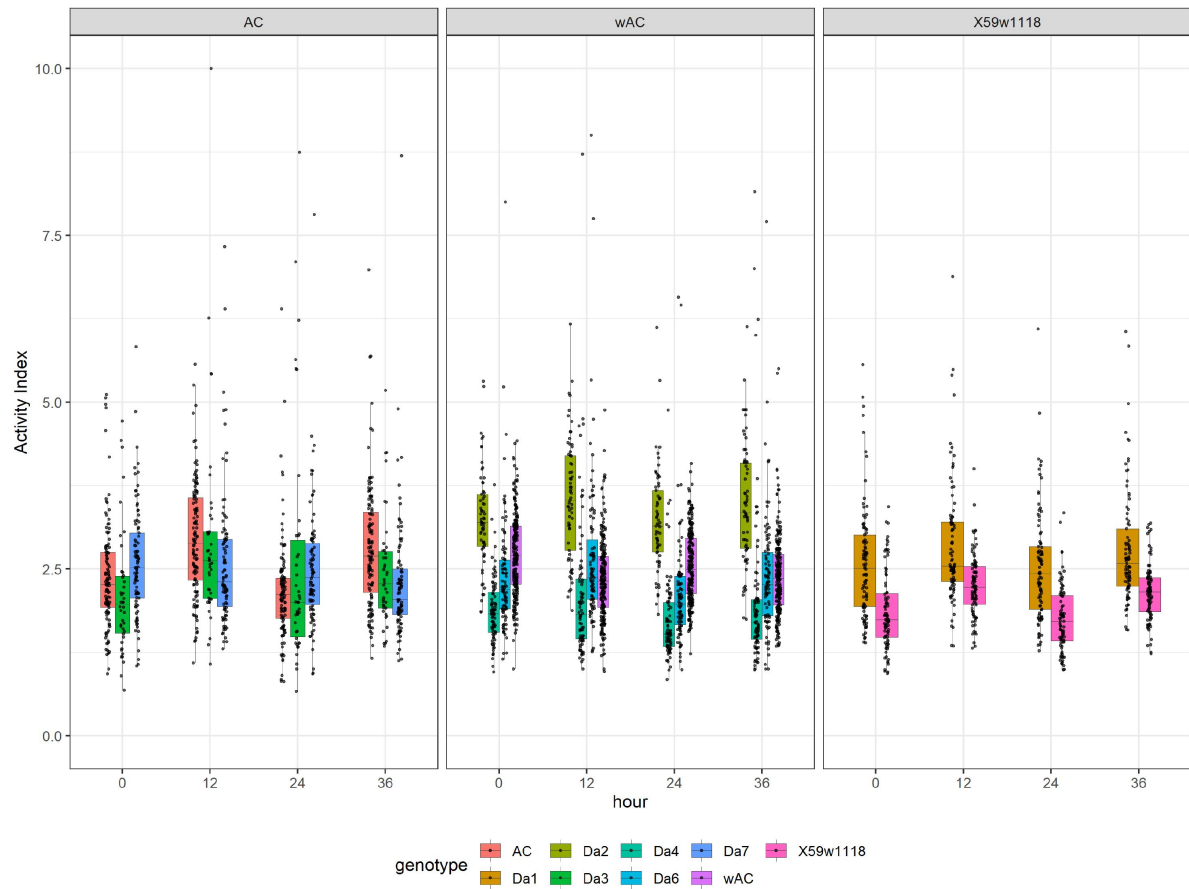


Figure S5.

Summary of different Gene Ontology pathways engaged by optogenetic-induced sleep and THIP-induced sleep.

A. Either sleep induction method produces different levels of activity in the fly brain. We term optogenetic-induced sleep 'active sleep' because brain activity levels are not different than during wake. We term THIP-induced sleep 'quiet sleep' because neural activity is significantly decreased already in the first 5 minutes. Both of these induced forms of sleep resemble sleep stages seen during spontaneous sleep in flies. **B.** Number of GO pathways engaged by either induced active or quiet sleep, separated by upregulated versus downregulated biological processes.



ttest_AI															
hour	gene	estimate	estimate1	estimate2	.y.	group1	group2	n1	n2	statistic	p	df	conf.low	conf.high	method
Day	Da1	0.74701194797923	1.8108506902361	2.55786201700353	activity_index	control	knockout	91	93	-7.68674248159984	1.63e-12	154	732513840803	0.938986358845125	T-test
Night	Da1	0.821402556761431	2.20459173404308	2.82599429080452	activity_index	control	knockout	91	93	-6.523944305852639	1.39e-09	129	928135878636	0.809843317756889	T-test
Day	Da2	0.802741622150127	2.43199942296601	3.23474104511614	activity_index	control	knockout	70	65	-7.4427805261935	2.03e-11	113	734263925721	1.01640704197762	T-test
Night	Da2	1.23901539806897	2.26258176444676	3.50159716251373	activity_index	control	knockout	70	65	-10.1194970663198	8.52e-17	95	688788875377	1.48206392653957	T-test
Day	Da3	0.415534948767109	2.16037312206754	2.57590807083465	activity_index	control	knockout	91	43	-1.34323807941607	0.186	45	4132347049576	1.03844775713935	T-test
Night	Da3	-0.0227331901857819	2.83159939569465	2.80886620550887	activity_index	control	knockout	91	43	0.0990781987694288	0.921	55	2553143772094	0.43704101471215	T-test
Day	Da4	-0.61837129538045	2.42331822907521	1.80484693369476	activity_index	control	knockout	98	87	8.66914228246151	2.56e-15	178	965595681112	-0.47761492132397	T-test
Night	Da4	-0.110300614309892	2.26586151382992	2.15556089952023	activity_index	control	knockout	98	87	0.83573550651872	0.405	115	736113589999	0.151109873134961	T-test
Day	Da6	-0.62368327284424	2.8853437323253	2.26167514594811	activity_index	control	knockout	91	91	6.44405236041315	5.29e-10	150	02407366578	-0.43819297313673	T-test
Night	Da6	0.0488610995058534	2.54148490606688	2.590359600557274	activity_index	control	knockout	91	91	-0.47619750862764	0.635	153	272240702089	0.251667217217754	T-test
Day	Da7	0.326181724510013	2.22741317915403	2.55359490366404	activity_index	control	knockout	95	94	-3.12088657353367	0.00211	176	4794317341	0.53244523747717	T-test
Night	Da7	-0.236825775153197	2.70854101277	2.4717152376168	activity_index	control	knockout	95	94	1.93009604618506	0.0552	181	874501771687	0.00527760128503382	T-test

Figure S6.

Waking activity levels of nAChRα knockout mutants.

Top: mutants are compared to their genetic background strain. Da1-7 = nAChRα1-7. Bottom: statistical tests for waking activity levels in each knockout compared to its genetic control, during both day and night.

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Editors

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Joint Public Review:

Anthony et al. provide an honorable attempt at furthering our understanding of the different sleep stages that may exist in *Drosophila*. The establishment of definable sleep stages/state in the fly model should be seen as a central goal in the field and this study represents an important step toward that goal. In particular, managing to draw parallels between sleep stages in flies and humans would make it relevant to use the power of fly genetics to better understand the molecular and cellular basis of these sleep stages. The authors use behavioral, physiological and transcriptomics approaches to describe the differences that exist between sleep triggered by optogenetic stimulation of dFB neurons and sleep induced by consumption of the sleep-promoting drug Gaboxadol (THIP). While there are still concerns regarding the interpretation of the major results, the authors have, in general, adequately responded to the reviewers' concerns.

The strengths of this work are:

- 1- The article is easy to read, and the figures are mostly informative.
- 2- The authors employ state-of-the-art techniques to measure neuronal activity and locomotion in a single assay.
- 3- The analysis of transcriptomic data is appropriate.
- 4- The authors identify many new genes regulated in response to specific methods for sleep induction. These are all potentially interesting candidates for further studies investigating the molecular basis of sleep. It would be interesting to know which of these genes are already known to display circadian expression patterns.

Concerns:

- 1- The fact that flies with dFB activation seem to keep a basal level of locomotor activity whereas THIP-treated ones don't is quite striking. Is it possible that there is an error with Figure 4C-D? Based on this, it is hard to believe that dFB stimulation and THIP consumption have similar behavioral effects on sleep.
- 2- The authors seem satisfied with the 'good correspondence' between their RNA-seq and qPCR results, this is true for only ~9/19 genes in Fig 6G and 2/6 genes in Fig 7G. The variability between the three biological replicates is not represented in the figure.

Comments

- 1- The fact that THIP-induced sleep persists long after THIP removal (Fig 3D) is intriguing. This suggests that the drug might trigger a sleep-inducing pathway that, once activated, can

continue on its own without the drug.

2- The claim that induction of the two forms (active/quiet) of sleep produce distinct transcriptomic and physiological effects while producing highly similar behavioral effects makes it difficult to understand the relationships between the former changes with sleep state. In their response the authors argue that sleep behavior, as currently measured using duration of inactivity, is not necessarily expected to allow for a differentiation between active and quiet sleep. This further argues for a need for better physiological/molecular correlates of sleep state in the fly (a laudable goal of this very study). However, until clear behavioral correlates can be strongly associated with physiological/molecular correlates, we will be limited to speculation about this important issue.

3- The authors suggest that the duration of the periods of inactivity may not be particularly useful for defining sleep states/stages in the fly. If this is the case, it is certainly an important issue as this measurement is the behavioral criteria for defining sleep in the field. In this regard, it raises the question of the relationship between the active and quiet sleep states examined in this study and the growing evidence for a deep sleep state (characterized by, among other things, a lowered metabolism).

4- Although the methodological concerns regarding the dose of Gaboxadol and the controls for the optogenetic/transcriptomic experiments remain, the authors have explained the rationale for the experimental design they used.

5- Overall, the authors have managed to clearly illustrate the differences between dFB stimulation and THIP consumption on behavior, neuronal activity, and gene expression. In this regard, they have achieved what they claim in the title of the article. Overall, the results support the conclusions, however the main point to consider is that the methods employed here are artificial, and there is no guarantee at this stage that 'spontaneous' sleep has the same effects on the transcriptome than what is presented here.

5- This article represents an interesting attempt at addressing very important questions, and some of the data presented here, especially the RNA-seq, can be very useful for others. However, because of the lack of definitive conclusion about whether the results presented are applicable to 'natural' sleep, the impact of this article may remain limited beyond a relatively small field.

Comments to authors

The authors have suitably addressed all comments from this section.

Author Response

The following is the authors' response to the original reviews.

This important study shows that two methods of sleep induction in the fly, optogenetically activation of the dorsal fan-shaped body (which is rapidly reversible and maintains a neuronal activity signature similar to wakefulness), and Gaboxadol-induced sleep (which shuts down neuronal activity), produce distinct forms of sleep and have different effects on brain-wide neural activity. The majority of the conclusions of the paper are supported by compelling data, but the evidence supporting the claim that the two interventions trigger distinct transcriptional responses is incomplete.

Thank you for the helpful and detailed reviews. We feel that these have improved the manuscript considerably, and hopefully the additional figures in this Reply letter will help further convince our readers.

Public Review

*In this study, Anthoney and coworkers continue an important, unique, and technologically innovative line of inquiry from the van Swinderen lab aimed at furthering our understanding of the different sleep stages that may exist in *Drosophila*. Here, they compare the physiological and transcriptional hallmarks of sleep that have been induced by two distinct means, a pharmacological block of GABA signaling and optogenetic activation of dorsal fan-shaped-body neurons. They first employ an incredibly impressive fly-on-the-ball 2-photon functional imaging setup to monitor neural activity during these interventions, and then perform bulk RNA sequencing of fly brains at different stages. These transcriptomic analyses leads them to (a) knocking out nicotinic acetyl-choline receptor subunits and (b) knocking down AkhR throughout the fly brain testing the impact of these genetic interventions on sleep behaviors in flies. Based on this work, the authors present evidence that optogenetically and pharmacologically induced sleep produces highly distinct brain-wide effects on physiology and transcription. The study is of significant interest, is easy to read, and the figures are mostly informative. However there are features of the experimental design and the interpretation of results that diminish enthusiasm.*

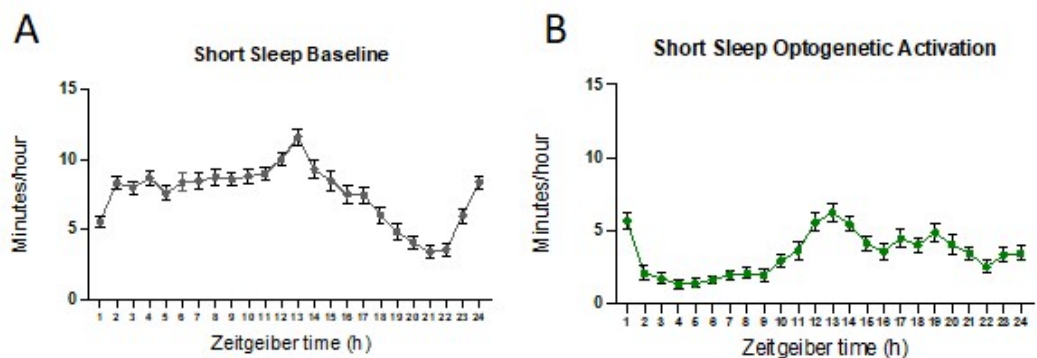
a- Conditions under which sleep is induced for behavioral vs neural and transcriptional studies

1- There is a major conceptual concern regarding the relationships between the physiological and transcriptomic effects of optogenetic and pharmacological sleep promotion, and the effects that these manipulations have on sleep behavior. The authors show that these two means of sleep-induction produce remarkably distinct physiological and transcriptional responses, however, they also show that they produce highly similar effects on sleep behavior, causing an increase in sleep through increases in the duration of sleep bouts. If dFB neurons were promoting active sleep, the sleep it produces should be more fragmented than the sleep induced by the drug, because the latter is supposed to produce quiet sleep. Yet both manipulations seem to be biasing behavior toward quiet sleep.

This is a correct observation, which is already evident in our sleep architecture data (Figure 2E-H): chronic optogenetic sleep induction promotes longer sleep bouts that are similar in structure (bout number vs bout duration) to those produced by THIP feeding. Since our plots in Figure 2E-H follow the 5min sleep criterion cutoff, upon the Reviewer's advice we re-analyzed our optogenetic experiments for short (1-5min) sleep. These are graphed below in Author response image 1. As can be seen, and as suspected by the Reviewer, the optogenetic manipulation does not increase the total amount of short sleep; indeed, it decreases it compared to baseline (these are for the exact same data as in Figure 2). Optogenetic sleep induction does not create a bunch of short sleep bouts.

Author response image 1.

Short sleep in optogenetic experiments. A. Average baseline ($\pm$ SEM) 1-5min sleep across a day and night. B. Average ($\pm$ SEM) 1-5min sleep in optogenetically-activated flies, across a day and night.



We agree with the reviewer that this observation might seem inconsistent with the idea that optogenetic activation promotes active sleep, and that short sleep is active sleep. However, it does not necessarily follow that optogenetic activation has to produce short sleep. Indeed, we know from our brain imaging data (and the associated behavioral analysis) that active sleep will persist for as long as we induce it with red light. While we have not induced it for longer than 15 minutes (Tainton-Heap et al, Current Biology, 2021; Troup et al, J. of Neuroscience, 2023), this is already clearly longer than a <5min sleep bout. So our interpretation is that the longer sleep bouts induced by optogenetic activation are prolonged active sleep, rather than quiet sleep. In other words, this artificial sleep manipulation induces prolonged active sleep, rather than many short sleep bouts. This is of course different than what happens during spontaneous sleep. We have tried to be clearer about sleep bout durations in the revised manuscript (e.g., the new Figure 3), and we now admit early in the results (lines 376-380) that that we don't know what optogenetic activation looks like in the fly brain beyond 15 minutes.

2- The authors show that the pharmacological block of GABA signaling and the optogenetic activation of dorsal fan-shaped-body neurons cause different responses on brain activity. Based on these recordings and the behavioral and brain transcriptomic data they then claim that these responses correspond to different sleep states and are associated with the expression and repression of a different constellation of genes. Nevertheless, neural activity in animals was recorded following short stimulations whereas behavioral and transcriptomic data were obtained following chronic stimulation. In this regard, it would be interesting to determine how the 12-hour pharmacological intervention they employed for their transcriptomic analysis changes neural activity throughout the brain - 12 hours will likely be too long for the open-cuticle preps, but an in-between time-point (e.g. 1h) would probably be equally informative.

The longest we've imaged brain activity for optogenetic sleep induction is 15 minutes, as discussed above. We see no changes in activity across this time, which would normally have led to a quiet sleep stage in spontaneous sleep recordings. Whole-brain imaging after 10 hours of optogenetic sleep induction (our RNA collection timepoint) is not realistic, and even 1 hour is difficult. We have however conducted overnight electrophysiological recordings (with multichannel silicon probes), where we activated the same R23E10 neurons for successive 20-minute bouts (alternating with 20min of no red light). We are preparing this work for publication (Van De Poll, et al). We see no evidence of optogenetic activation of this circuit ever producing anything resembling quiet sleep. Since we are not in a position to provide this new electrophysiological data in the current study, we are careful to clarify that we have not investigated what brain imaging looks like after chronic optogenetic activation (lines 376-380). We are showing through diverse lines of evidence that what is called sleep can look different in flies.

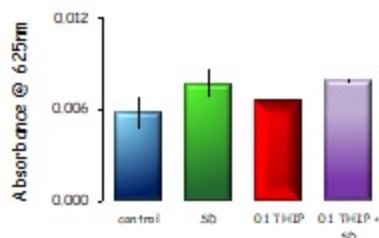
b- Efficiency of THIP treatment under different conditions

1- There are no data to quantify how THIP alters food consumption. It is evident that flies consume it otherwise they would not show increased sleep. However, they may consume different amounts of food overall than the minus THIP controls. This might have an influence on the animal's metabolism, which could at least explain the fact that metabolism-related genes are regulated (Figure 5). Therefore, in the current state, it is not possible to be certain that gene regulation events measured in this experiment are solely due to THIP effects on sleep.

We have two arguments against this reasonable criticism. First, as discussed above, the optogenetic flies are sleeping at least as much as the THIP-fed flies, so in principle they also might be feeding less. But we see no metabolic gene downregulation in the optogenetic dataset. We include this counterargument in the discussion (lines 752-756). Then, together with our co-author Paul Shaw we have shown that THIP-fed flies are not eating less compared to controls (Dissel et al, Current Biology, 2015), by tracking dye consumption. We show those results again below in Author response image 2 to support our reasoning that feeding is not an issue.

Author response image 2.

Flies were fed blue dye in their food while being sleep deprived (SD), or while being induced to sleep with 0.1mg/ml THIP in their food, or both. Dye consumption was measured in triplicate for pooled groups of 16 flies. Average absorbance at 625nm ($\pm$ stan dev) is shown. Experiments were not significantly different (ANOVA of means).



2- A similar problem exists in the sleep deprivation experiments. If flies are snapped every 20 seconds, they may not have the freedom to consume appropriate amounts of food, and therefore their consumption of THIP or ATR may be smaller than in non-sleep deprived controls. Thus, it would be crucial to know whether the flies that are sleep-deprived (i.e. shaken every 20 seconds for 12 hours) actually consume comparable amounts of food (and therefore THIP) as those that are undisturbed. If not, then perhaps the transcriptional differences between the two groups are not sleep-specific, but instead reflect varying degrees of exposure to THIP.

Please see our response to the similar critique above, and how Figure R2 addresses this concern.

3- The authors should further discuss the slow action of THIP perfusion vs dFB activation, especially as flies only seem to fall asleep several minutes after THIP is being washed away. Is it a technical artifact? If not, it may not be unreasonable to hypothesize that THIP, at the concentration used, could prevent flies from falling asleep, and that its removal may lower the concentration to a point that allows its sleep-promoting action. The authors could easily test this by extending THIP treatment for another 4-5 minutes.

The reviewer is partially correct in suggesting a technical artifact: THIP does not get washed away immediately after 5min of perfusion. The drip system we employ means that THIP

concentration will slowly increase to the maximum concentration of 0.2mg/ml, and then slowly get diluted away at a rate of 1.25ml/minute (this is all in the Methods). In a previous study (Yap et al, Nature Communications, 2017) we used this exact same perfusion procedure to test a range of THIP concentrations, and settled on 0.2mg/ml as the lowest that reliably induced quiet sleep within 5 minutes. Higher concentrations induced quiet sleep faster, so the alternate explanation proposed by the Reviewer is not supported. We feel that our previous electrophysiological study provided the necessary groundwork for using the same approach and dosage here for our whole-brain imaging readout.

c- Comments regarding the behavioral assays

1- L319-322: the authors conclude that dFB stimulation and THIP consumption have similar behavioral effects on sleep. However, this is inaccurate as in Figure S1 they explain that one increases bout number in both day and night and the other one only during the day.

We have now added a caveat about night bout architecture being different (lines 353-356). Figure S1 is now Figure 3.

2- The behavioral definitions used for active and quiet sleep do not fit well with strong evidence that deep sleep (defined by lowered metabolic rates) is probably most closely associated with bouts of inactivity that are much longer than the >5min duration used here, i.e., probably 30min and longer (Stahl et al. 2017 Sleep 40: zsx084). Given that the authors are providing evidence that quiet sleep is correlated with changes in the expression of metabolism related genes, they should at least discuss the fact that reductions in metabolism have been shown to occur after relatively long bouts of inactivity and might reconsider their behavioral sleep analysis (i.e., their criteria for sleep state) with this in mind.

Interestingly, induced sleep bout durations are on average longer for the optogenetic manipulation (40min vs 25min); this was evident in Figure S1C vs S1F (now Figure 3). So as discussed above, this provides a counterargument for sleep bout duration alone being indicative of metabolic processes associated with quiet sleep: the optogenetic dataset did not uncover metabolic-related pathways as relevant to that sleep manipulation. We refer to Stahl et al, Sleep, 2017, in our discussion (lines 748-750), making exactly this point about metabolic rates being decreased in longer sleep bouts, and flowing up with our observation that optogenetic flies sleep just as much, and their bouts are actually longer. So clearly different processes must be involved.

d- Comments regarding the recordings of neuronal activity

1- There is an additional concern regarding the proposed active and quiet sleep states that rest at the heart of this study. Here these two states in the fly are compared to the REM and NREM sleep states observed in mammals and the parallels between active fly sleep and REM and quiet fly sleep and NREM provide the framework for the study. The establishment of such parallel sleep states in the fly is highly significant and identifying the physiological and molecular correlates of distinct sleep stages in the fly is of critical importance to the field. However, the proposal that the dorsal fan shaped body (dFB) neurons promote active sleep runs counter to the prevailing model that these neurons act as a major site of sleep homeostasis. If quiet sleep were akin to NREM, wouldn't we expect the major site of sleep homeostasis in the brain to promote it? Furthermore, the authors state that the effects of dFB neuron excitation on transcription have "almost no overlap" (line 500) with the transcriptomic effects of sleep deprivation (Supplementary Table 3), which is not what would be expected if dFB neurons are tracking sleep pressure and promoting sleep, as suggested by a growing body of convergent work summarized

on page four of the manuscript. Wouldn't the 10h excitation of the dFB neurons be predicted to mimic the effects of sleep deprivation if these neurons "...serve as the discharge circuit for the insect's sleep homeostat..." (line 60)? Shouldn't their prolonged excitation produce an artificial increase in sleep drive (even during sleep) that would favor deep, restorative sleep? How do the authors interpret their results with regard to the current prevailing model that dFB neurons act as a major site of sleep homeostasis? This study could be seen as evidence against it, but the authors do not discuss this in their Discussion.

These are all excellent and thoughtful points, which have made us re-think parts of our discussion. First off, the potential comparison with REM and NREM is entirely speculative, and we have tried to make that more obvious in introduction) and the discussion (e.g, see lines 43, 708, 818). The evidence that the FB neurons (and maybe others) are involved in the homeostatic regulation of sleep is well-supported in the literature, so that part of the discussion holds. However, we concede that the timing of our sleep manipulations could benefit from more explanation. We conducted these during the flies' subjective day, after the animals had presumably had a good night's sleep. This means that we induced either kind of sleep for 10 daytime hours, which presumably replaced whatever behavioural states would 'naturally' be happening during the day. Female flies sleep less during the day than at night, and we have shown in previous work that daytime sleep quality is different than night-time sleep (van Alphen et al, Journal of Neuroscience, 2013), leading us to suggest that most 'deep' or quiet sleep happens at night, for flies. Following this reasoning, daytime optogenetic activation might not be depriving flies of much quiet sleep, or accumulating a deep sleep drive as the Reviewer proposes. Rather, both induced sleep manipulations could be providing 10 hours of either kind of sleep that the flies don't really 'need'. Why did we design it this way? Firstly, we were interested in simply asking what these chronic sleep manipulations do to gene expression in rested flies, and how they might be similar or different. We focussed on daytime manipulations to avoid precisely the confound of sleep pressure, and also because we observed red-light artifacts at night for our optogenetic experiments (which we reported). Our sleep deprivation strategy was designed specifically as a control for the THIP (Gaboxadol) experiments, to control for non-sleep related effects of the drug (see below our rationale for why this was less crucial for the optogenetic experiments). In conclusion, we had a logical rationale for how the experiments were done, centred on the straightforward question of whether these two different approaches to sleep induction were having similar effects in well-rested flies. In retrospect, we were not anticipating the Reviewer's thoughtful logic regarding the dFB's potential role in also regulating deep sleep homeostasis. We now provide some discussion along these lines to make readers aware of this line of reasoning, as well as our rationale for why prolonged optogenetic sleep induction was not sleep-depriving (lines 768-777).

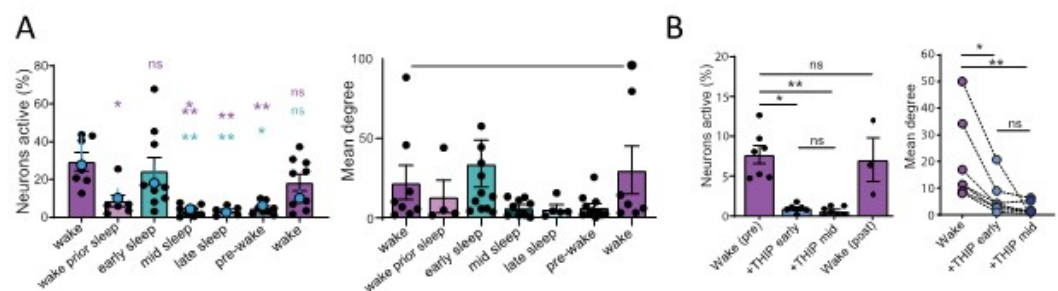
2- Regarding the physiological effects of Gaboxadol, to what extent is the quieting induced by this drug reminiscent of physiology of the brains of flies spontaneously meeting the behavioral criterion for quiet sleep? Given the relatively high dose of the drug being delivered to the de-sheathed brain in the imaging experiments (at least when compared to the dose used in the fly food), one worries that the authors may be inducing a highly abnormal brain state that might bear very little resemblance to the deeply sleeping brain under normal conditions. As the authors acknowledge, it is difficult to compare these two situations. Comparing the physiological state of brains put to sleep by Gaboxadol and brains that have spontaneously entered a deep sleep state therefore seems critical.

As discussed above, our Gaboxadol (THIP) perfusion concentration (0.2mg/ml) was the minimal dosage that effectively induced sleep within 5 minutes, based upon previously published work (Yap et al, Nature Communications, 2017). Lower concentrations were

unreliable, with some never inducing sleep at all. Comparisons with feeding THIP are tenuous, and we make that clear in our discussion (lines 731-735). Nevertheless, the Reviewer makes an excellent point about comparisons with spontaneous ‘quiet’ sleep. Here, we feel well supported (please see Author response image 3 below, comparing THIP-induced sleep (this work, B) and spontaneous sleep (A) from previous study). In our previous study (Tainton-Heap et al, 2021) we showed that neural activity and connectivity decreases during spontaneous quiet sleep. This is what we also see with THIP perfusion. In contrast, in Troup et al, J. of Neuroscience (2023) we confirm that neither neural activity nor connectivity changes during optogenetic R23E10 activation, and general anesthesia – unlike THIP – does NOT produce a quiet brain state. Our finding that THIP effects are nothing like general anesthesia (at the level of brain activity levels) suggests a physiological sleep state closer to spontaneous quiet sleep. We elaborate on this important observation in our results, also pointing to crucial differences with general anesthesia (lines 411-415).

Author response image 3.

THIP-induced sleep resembles quiet spontaneous sleep. A. Calcium imaging data from spontaneously sleeping flies, taken from Tainton-Heap et al, 2021. Left, percent neurons active; right, mean degree, a measure connectivity among active neurons. Both measures decrease during later stages of sleep. B. Calcium imaging data from flies induced to sleep with 5min of 0.2mg/ml THIP perfusion (this study). Left, percent neurons active; right, mean degree. Both measures are significantly decreased, resembling the later stages of spontaneous sleep, which we have termed ‘quiet sleep’. Hence THIP-induced sleep resembles quiet sleep. Note that the genetic background is different in A and B, hence the different baseline activity levels.



3- There are some issues with Figure 3, in particular 3C-D. It is not clear whether these panels show representative traces or an average, however both the baseline activity and fluorescence are different between C and D, in particular in their amplitude. Therefore, it is difficult to attribute the differences between C and D to the stimulation itself or to the previously different baseline. In addition, the fact that flies with dFB activation seem to keep a basal level of locomotor activity whereas THIP-treated ones don't is quite striking, however it is not being discussed. Finally, the authors claim that the flies eventually wake up from THIP-induced sleep (L360-361), however there are no data to support this statement.

These are representative traces, which is a way of showing the raw calcium data (Cell ID) so readers can see for themselves that one manipulation silences whereas the other does not – even though flies become inactive for both. The Y-axis scale is standard deviation of the experiment mean. Since THIP decreases neural activity, then the baseline is comparatively higher. Since optogenetic activation does not change average neural activity levels, the baseline is centered on zero. This is an outcome of our analysis method and does not reflect any ‘true’ baseline. We have now clarified this in our figure legend. We now also confess that flies rendered asleep optogenetically can be ‘twitchy’ (line 374). Finally, we show data for 3

flies that were recorded until they woke up. The rest were verified behaviorally, after the experiment. This is now explained in the Methods.

4- In Figure 4C, it is strange that the SEM is always exactly the same across the whole experiment. Readers should be aware that there might have been an issue when plotting the figure.

This is not a mistake, the standard errors are just all quite close (between 0.17 and 0.22). This is because of the way we did the analysis, asking how many flies responded to each stimulus event, with incremental levels of responsiveness. This is explained in the Methods. The figure makes the important point of sleep and recovery.

e- Comments regarding the transcript analyses

1- General comment: the title of this manuscript is inaccurate - the "transcriptome" commonly refers to the entirety of all transcripts in a cell/tissue/organ/animal (including genes that are not differentially expressed following their interventions), and it is therefore impossible to "engage two non-overlapping transcriptomes" in the same tissue. Perhaps the word "transcriptional programs" or transcriptional profiles" would be more accurate here?

We thank the Reviewer for this advice and have changed the title as proposed.

2- Given the sensitivity of transcriptomic methods, there is a significant concern that the optogenetic experiments are not as well controlled as they could be. Given the need for supplemental all-trans retinal (ATR) for functional light gating of channelrhodopsins in the fly, it is convenient to use flies with Gal4-driven opsin that have not been given supplemental ATR as a negative control, particularly as a control for the effects of light. However, there is another critical control to do here. Flies bearing the UAS-opsin responder element but lacking the GAL4 driver and that have been fed ATR are critical for confirming that the observed effects of optogenetic stimulation are indeed caused by the specific excitation of the targeted neurons and not due to leaky opsin expression, or the effect of ATR feeding under light stimulation or some combination of these factors. Given the sensitivity of transcriptomic methods, it would be good to see that the candidate transcripts identified by comparing ATR+ and ATR- R23E10GAL4/UAS-Chrimson flies are also apparent when comparing R23E10GAL4/UAS-Chrimson (ATR+) with UAS-Chrimson (ATR+) alone.

We have not done these experiments on UAS-Chrimson/+ controls. Like many others in our field, we viewed non-ATR flies as the best controls, because this involves identical genotypes. Since we were however aware that ATR feeding itself could be affect gene expression, we specifically checked for this with our early (1hour) collection timepoint. We only found 26 gene expression differences between ATR and -ATR flies at this early timepoint, compared with 277 for the 10-hour timepoint. We detail this rationale in our results, explaining why this is a convincing control for ATR feeding. If there was leaky opsin expression / activity, this would have been evident in our design. Regarding the cumulative effect of light, this would also have been accounted in our design, as only 1 hour would have elapsed in our first timepoint compared to 10 hours in our second. While the Reviewer is correct in saying that parental controls are called for in many Drosophila experiments, this becomes quickly unmanageable in transcriptomic studies, which is exactly why well-designed +ATR vs -ATR comparisons in the exact same strain are most appropriate. We feel that our 1-hr timepoint mostly addresses this concern.

3- Figures about qPCR experiments (5G and 6G) are problematic. First, whereas the authors seem satisfied with the 'good correspondence' between their RNA-seq and qPCR results, this is true for only ~9/19 genes in 5G and 2/6 genes in 6G. Whereas discrepancies are not rare between RNA-seq and qPCR, the text in L460-461 and 540-541 is misleading. In addition, it is unclear whether the n=19 in L458 refers to the number of genes tested or the number of replicates. If the qPCR includes replicates, this should be more clearly mentioned, and error bars should be added to the corresponding figures.

We consider that our qPCR validations were convincing, as they were all mostly changed in the 'right' direction. We agree that there are some discrepancies, so have modified our language to reflect this. We have also clarified that 19 refers to the number of genes validated by qPCR in that THIP dataset. All qPCRs involved three technical replicates. We prefer to keep these histograms the way they are to convey these simple trends. For complete transparency, we now provide a supplemental Excel worksheet with all of the qPCR data, alongside corresponding RNAseq data and stats for the selected genes (Supplementary Table 9).

4- There is a lack of error bars for all their RNAseq and qPCR comparisons, which is particularly surprising because the authors went to great lengths and analyzed an applaudably large amount of independent biological replicates, yet the variability observed in the corresponding molecular data is not reported.

The genes reported in each of our datasets and associated supplemental figures and tables were all significant, as determined by criteria outlined in the Methods. However, we appreciate that readers might want to get a sense of the values and variances involved, as well as access to the entire gene datasets. We now provide all of these as additional 'sheets' in our existing supplemental tables (S2-S7), so this should be very easy to navigate and evaluate. In addition to the previously provided lists for significant genes, in the second Excel sheet ('All genes') readers will be able to see the data for all 5 replicates, for the significant genes as well as all other ~15,000 genes (listed in alphabetical order). We feel that this will be a helpful resource, because admittedly significance thresholds can still be a little arbitrary and some readers might want to look up 'their' genes of interest.

Comments to authors

Other comments

1- Text in L441 & 606 is misleading. According to ref 52, AkhR is involved specifically in starvation-induced sleep loss, and not in general sleep regulation.

Corrected.

2- The language used in L568-570 and 573-574 is confusing. The authors should specify that the knock down of cholinergic subunits, rather than the subunits themselves is what causes sleep to increase or decrease.

Corrected.

3- The authors' investigation of cholinergic receptor subunits function is very preliminary, and it is difficult to draw any conclusion from what is presented here. In particular, their behavioral data is difficult to reconcile with the RNA-seq data showing overexpression of both short sleep increasing and short sleep decreasing subunits. Without knowing where in the brain these subunits are required for controlling sleep, the data in Figure 7 is difficult to appreciate.

We have now conducted additional experiments where we specifically knocked down these alpha receptor subunits (all 7 of them) in the R23E10 neurons. This seemed an obvious knockdown location, to determine if any of these subunits regulated activity in the same sleep promoting neurons that were the focus of this study. We found that alpha1 knockdown in these neurons had similar sleep phenotypes, which we believe is an important result. Since this functional localisation is a logical ending for the paper, we have now made it the final figure.

Suggestions & comments

1- It would be interesting if the authors could discuss their findings that metabolism genes are downregulated in THIP flies in the context of recent work that showed upregulation of mitochondrial ROS after sleep deprivation (Kempf et al, 2019).

We now add the Kempf 2019 reference and allude to how those findings could be consistent with ours.

2- The fact that THIP-induced sleep persists long after THIP removal (Fig 3D) is very intriguing and interesting. This suggests that the drug might trigger a sleep-inducing pathway that can continue on its own without the drug, once activated.

This is correct, and in stark contrast to the optogenetic manipulation we employ, which does not appear to show such sleep inertia. We have now added a sentence highlighting this interesting difference (lines 394-396).

3- The authors identify many new genes regulated in response to specific methods for sleep induction. These are all potentially interesting candidates for further studies investigating the molecular basis of sleep. It would be interesting to know which of these genes are already known to display circadian expression patterns.

By providing all of the gene lists, these are now available to ask questions such as these. We hesitate however to delve into this domain for this work, as our main goal was to compare these two kinds of sleep in flies.

4- The brain-wide monitoring of neural activity invites a number of very exciting follow-up experiments - most importantly, it would be fascinating to establish, which neurons are active in the different phases the authors describe! Are these neurons that are involved in transmitting external visual stimuli to the central brain? Do they also project into the central complex? They could make use of the large collection of existing driver lines in the fly and they could also exploit the extraordinary knowledge of the connectome and transcriptome of the fly brain.

Thank you for sharing our enthusiasm for these likely future directions.

5- The Dalpha2,3,4,6 and 7 Knock-out strains they generate will be a useful reagent for the Drosophila neuroscience community once the efficiency/success of the knock-out has been confirmed by qPCR.

These knockout strains have all been confirmed by our co-authors Hang Luong, Trent Perry, and Philip Batterham. These knockout confirmations are outlined in publications that we reference (Perry et al, 2021).

Materials and methods:

1- This study has employed custom-built apparatus and custom-written code/scripts, but these do not appear to be available to the reader. For the sake of replicability, the authors should make these available.

The code/scripts are available via the University of Queensland research data management system as described in the Methods, and can be sent by the Lead Contact. The imaging hardware and analysis code are identical to what was described in a previous publication, and available as directed therein (Tainton-Heap et al, 2021).

2- Also, the authors should give details on the food used to rear their flies. Fly media comes in several common forms and sleep is sensitive to diet.

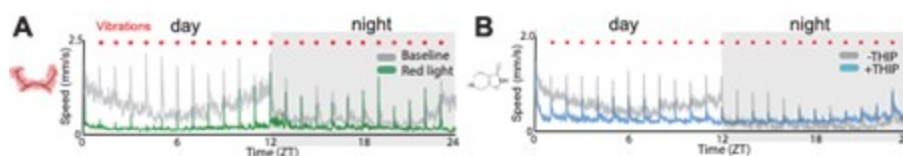
This has now been elaborated in the beginning of the Methods.

3- The light regime used for optogenetic excitation of dFB neurons consists of 12h of uninterrupted bright red LED light. Most optogenetic stimulations consist of pulsed high frequency flashes interlaced with pauses in illumination. Can dFB neurons be driven constitutively with 12 hours of bright light?

We showed in Tainton-Heap (2021) that 7Hz pulsed red light had exactly the same effect on R23E10/Chrimson readouts as continuous red light, which is why we opted here to provide continuous red light. That optogenetic sleep induction can be driven continuously for 12 hours is evident by our 24-hour sleep profiles. However, we agree that one could question whether sleep quality is similar after 12 hours. To address this, we did an additional experiment where we stimulated the flies hourly, to determine if their behavioural responsiveness to mechanical stimuli changed over the course of continued sleep induction, for both optogenetic and THIP-induced sleep. We present the data below in Author response image 4. As can be seen in these new analyses, while optogenetic sleep induction persists across 12 daytime hours (speed is close to zero throughout), flies do indeed become more responsive later in the day. This could have two different interpretations: either some sleep functions are being satisfied over time, or the activation regime is becoming less effective over time. Either way, these data show that at our 10-hour daytime timepoint, unstimulated flies are still largely inactive, even though their arousal thresholds might have gradually changed; so the uninterrupted red-light regime is still effective. The comparison with THIP is interesting: here there does not seem to be a change in responsiveness over time; the drug just decreases behavioral responsiveness throughout. Together, these experiments support our view that both approaches are sleep-promoting throughout the 12-hour day, although we appreciate that sleep quality is not identical.

Author response image 4.

A) The average speed of baseline (grey) and optogenetically-activated flies (green) across 24 hours. Red dots indicate vibration stimulus times. B) The average speed of control (grey) and THIP-fed flies (blue) across 24 hours. Flies are all R23E10/Chrimson. N= 87 for optogenetic, n=88 for -THIP, n=85 for +THIP.



4- The authors use the SNAP apparatus to prevent THIP-treated flies from sleeping to tease out possible sleep-independent effects. This is an excellent control. Why have the

authors not done the same with the optogenetic treatment? It's surprising not to see this control given the concern the authors express (lines 501 - 502) that the dFB manipulation might be paralyzing awake flies, which certainly seems possible given the light regimes used. Why not test this directly with SNAP?

We appreciate that this may have been a valuable additional control. However, we designed this control for the THIP experiments specifically because of concerns about THIP's (yet unknown) mechanism of action in flies. THIP is a gabaergic drug with most likely many off-target effects that have little to do with sleep, hence the need for a control where we compare to flies that ingested THIP but have been prevented from sleeping. In contrast, R23E10-driven sleep induction is exactly that, a circuit when activated that induces sleep. Whatever specific neurons might really be involved, the Gal4 circuit is sleep-inducing. This is well supported by multiple publications. The most appropriate control for assessing transcriptomic effects during optogenetic sleep here is not preventing sleep, but rather no increased sleep in flies that have not ingested ATR, and comparing that to effects of ATR alone, which is what we have done. Adding a sleep-deprivation layer onto both of these analyses may have been interesting, but a lot more analyses and not strictly required to identify relevant sleep-related genes. We have rephrased the misleading sentence about paralyzing flies, to instead clarify that lack of overlap with the SD dataset suggests that optogenetic activation is not preventing sleep functions from being engaged.

5- A pairwise comparison of ZT01 and ZT10 does not address circadian expression cycles in a meaningful way. There will be strong effects of the LD cycle here. I suggest toning this down. (Though it is gratifying to see the expected changes in the core clock genes.)

We have changed the language from 'circadian' to 'light-dark' to address this, although have kept the word 'circadian' when referring specifically to genes such as *per*, *clock*, *timeless*, etc.

6- Line 109: There is a reference missing.

We now provide the relevant reference.

Results

1- General comment regarding the figures: a general effort could be made to improve the design and quality of the figures and make them more readable. There are a lot of issues such as stretched or misaligned text, badly drawn frames, etc.

We think we know which figures this might relate to (e.g., Figures 3,4B), so we have adjusted where appropriate.

2- Instead of 'dFB-induced' (e.g., L77) it would be more accurate to use 'optogenetically-induced'

Thank you for this helpful advice. We have changed our language throughout to say 'optogenetically-induced'

3- Figure S1 should be integrated in the main figure to make the quantification more easily accessible.

We have integrated Figure S1 into the main figures. It is now Figure 3.

5- It would be good to include red light controls in Figure 2C, E, G.

Making Figure S1 a main figure has better highlighted the fact that we have done red light controls ('baseline').

6- line 313: Fig2E-H - these graphs would benefit if the authors made it more obvious where the maximum sleep amount would fall - i.e. the combination of bouts and minutes that add up to 12 hours (and therefore the entire day/night)

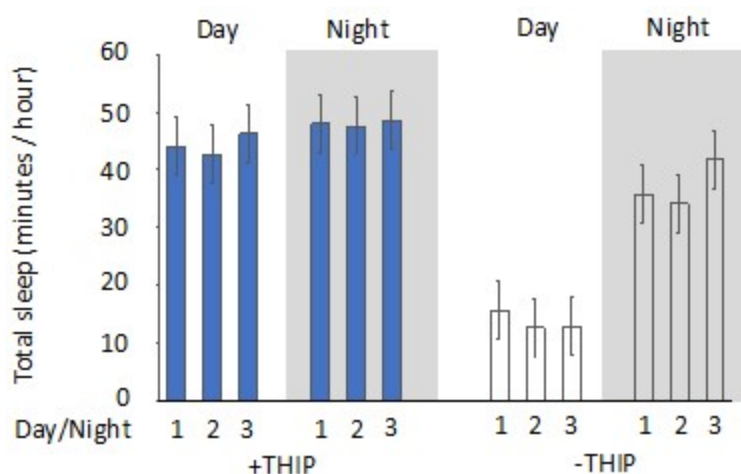
If a fly were to sleep uninterrupted for all 12 hours of a day or night, that would amount to a sleep bout 720 minutes long. We do not feel that identifying this maximum on these graphs would be helpful. It should be clear from the data that a floor is reached with very few sleep bouts exceeding 60 minutes in our paradigm. To help orient the reader though, we now clarify in the figure legend that the maximum is 720 minutes or 12 hours.

7- Fig. 2B, D: It was not clear why the authors took the 3-day average here. Doesn't that lead to a whole range of very different behaviors? I could, perhaps naively, imagine that a fly's behavior changes after 2 days of almost-permanent sleep?

We took the 3-day average because the effect of THIP on each successive day was not significantly different (see Author response image 5, below). Flies wake up enough to have a good feed (see Author response image 2) and then go back to sleep. Since this is however an important point raised by the reviewer, we now mention in the Methods that sleep duration was not different among the 3 averaged days and nights (lines 193-195).

Author response image 5.

Data from THIP feeding experiment (Figure 2B) in manuscript, separated into 3 successive days and nights, with THIP-fed flies (blue) compared to controls (white). Averages $\pm$ SD are shown, samples sizes are the same as in Figure 2D. No THIP data was significantly different across days and nights (ANOVA of means).



8- In Figure 2C the authors compare optogenetically induced to "spontaneous sleep," which I think refers to baseline sleep before stimulation, according to the figure. I think the proper comparison would be to the red light control (ATR-); though see the comment above regarding optogenetic controls).

This information was provided in Figure S1. We now provide it as a main Figure 3, as requested above.

We also made a point about red light having an effect at night, which is why we focussed on daytime effects for our transcriptomic comparisons. We feel that the ATR-fed flies (minus red light) are an appropriate control here for optogenetically-induced sleep: same exact genotype and ATR feeding, just no optogenetic activation. We therefor would prefer to keep these graphs as they are, especially since we show -ATR data subsequently.

9- Figures 3A and 4A are redundant; Figure 3B has some active ROIs that are outside of the brain. I am not sure how this is possible?

We have removed the redundant 4A and replaced it with the THIP molecule to clearly signal what this figure is focussed on. In Figure 3B (now 4B), the brain mask is a visual estimate made from the middle of the image stack. Some neurons in other layers are outside this single-layer estimate. All neurons were all accounted for.

10- Figure 4B is confusing. It took me a while to understand and so it can do with re-drawing in a more accessible way.

We agree that this was confusing, e.g. there were too many arrows. We have redrawn and simplified (Now 5A).

11- The authors state that flies wake up from THIP-induced sleep on the ball, but in Figure 4D there appears to be fewer samples for flies who have woken up from THIP (3) compared to those observed before THIP administration. Are flies dying?

None of the flies died. Most flies were removed from imaging to confirm recovery, while 3 were left in our imaging setup to measure brain activity upon recovery. These results are in Figure 5C and now clarified in the Methods.

12- Fig5C,D: I'm surprised that by far the most significant changes (in terms of log2-FC and p-val) occur in the sleep-deprived flies? It is not clear to me what the authors mean by effects that "relate waking process"? Perhaps they could elaborate on this?

We have removed the phrase 'relates to waking processes'. We now also remark on the high level of fold-change in many of these genes but refrain from discussing this further in the results. It is interesting though.

13- The sentence in L425-428 is unclear - it would be good to rephrase this.

We have rephrased this sentence, hopefully it's clearer now.

14- Text in L544-545 is confusing. What do you mean by 'less clear'?

We have replaced 'less clear' with 'not dominated by a single category'.

15- It is unclear what is the control in Fig 7A. It would be good to mention what strain was used.

Different knockout strains had different controls. These are identified in the figure legend and Methods.

16- L579-581: it would be helpful to include this data in a supplementary figure.

We now provide this as a supplementary figure as requested (Supplementary Figure 6).

17- *There is no information about R57C10 in the methods - it would be good to explain which neurons this line labels, and why you chose it.*

We now clarify in the methods that R57C10-Gal4 is a pan-neural driver, and provide a reference.

18- *Table S5 - If I'm not mistaken then the first line should say 1h, not 10h.*

Corrected