1 ***Title:*** **Experimentally induced active and quiet sleep engage non-overlapping**
2 **transcriptional programs in *Drosophila***
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4 ***Short title:*** **Active and quiet sleep 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.

Introduction

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-

59 promoting neurons in the central complex (CX) – a part of the insect brain that has been found
60 to be involved in multimodal sensory processing [18]. In particular, the dorsal fan-shaped body
61 (dFB) of the CX has been proposed to serve as a discharge circuit for the insect's sleep
62 homeostat, whereby increased sleep pressure (e.g., due to extended wakefulness) alters the
63 physiological properties of dFB neurons causing them to fire more readily and thereby promote
64 decreased behavioral responsiveness [19] and thus sleep [20-22]. Crucially, activation of sleep-
65 promoting circuits including the dFB have been shown to be sleep-restorative [10, 23], but
66 confusingly, brain recordings during optogenetically-induced sleep, via electrophysiology as
67 well as whole-brain calcium imaging techniques, reveal wake-like levels of brain activity [9,
68 10]. This suggests that some approaches to optogenetically-induced sleep might be promoting
69 a form of sleep akin to the 'active' sleep stage detected during spontaneous sleep [9, 10].

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

80 In this study, we compare THIP-induced sleep with optogenetically-induced sleep in
81 *Drosophila*, using behavior, brain activity, and transcriptomics. To ensure the validity of our
82 comparisons, we performed all of our experiments in the same genetic background, employing
83 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-tetrahyrdoisoxazolopyridin-3-ol, THIP) for the duration of behavioral experiment [23].

2-photon imaging

109 2-photon imaging was performed as described previously [10] using a ThorLabs Bergamo
110 series 2 multiphoton microscope. Fluorescence was detected with a High Sensitivity GaAsP
111 photomultiplier tube (ThorLabs, PMT2000). GCaMP fluorescence was filtered through the
112 microscope with a 594 dichroic beam splitter and a 525/25nm band pass filter.
113 For imaging experiments, flies were secured to a custom-built holder [10]. Extracellular fluid
114 (ECF) containing 103 NaCl, 10.5 trehalose, 10 glucose, 26 NaHCO3, 5 C6H15NO6S, 5 MgCl2
115 (hexa-hydrate), 2 Sucrose, 3 KCl, 1.5 CaCl (dihydrate), and 1 NaH2PO4 (in mM) at room
116 temperature was used to fill a chamber over the head of the fly. The brain was accessed by
117 removing the cuticle of the fly with forceps, and the perineural sheath was removed with a
118 microlance. Flies were allowed to recover from this for one hour before commencement of
119 experiments. Imaging was performed across 18 z-slices, separated by 6µm, with two additional
120 flyback frames. The entire nlsGCaMP6f signal was located within a 256 x 256px area,
121 corresponding to 667 x 667µm. Fly behavior was recorded with a Firefly MV 0.3MP camera
122 (FMVU-03MTM-CS, FLIR Systems), which was mounted to a 75mm optical lens and an
123 infrared filter. Camera illumination was provided by a custom-built infrared array consisting
124 of 24 3mm infrared diodes. Behavioral data was collected for the duration of all experiments.
125 For THIP experiments, an initial five minutes of baseline activity was captured, followed by
126 perfusion of 0.2mg/ml THIP in ECF onto the brain at a rate of 1.25ml/minute for five minutes.
127 An additional twenty minutes of both brain and behavioral activity were recorded to allow
128 visualization of the fly falling asleep on the ball as a result of THIP exposure. All flies were
129 removed from imaging after an experiment and confirmed to have awoken by visual inpection.
130 A subset of THIP-exposed flies (n=3) remained in the imaging setup to measure brain activity
131 upon recovery.
132
133 **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

159 a custom-written MatLab (Mathworks) code, the mean fluorescent value of all pixels within a
160 given ROI were extracted for the entire time series, resulting in a n x t array for each slice of
161 each experiment, where n refers to the number of neurons in each Z-slice, and t refers to the
162 length of the experiment in time frames. These greyscale values were z-scored for each neuron,
163 and the z-scored data was transformed into a binary matrix where a value of > 3 standard
164 deviations of the mean was allocated a '1', and every value < 3 standard deviations was
165 allocated a '0'. To determine whether a neuron fired during the entire time series, a rolling sum
166 of the binary matrix was performed, where ten consecutive time frames were summed together.
167 If the value of any of these summing events was greater or equal to seven (indicating a
168 fluorescent change of > 3 standard deviations in 7/10 time frames), a neuron was deemed to be
169 active. For THIP sleep experiments, the five minutes of inactivity occurring after an initial 30
170 seconds of behavioral inactivity were used. After identifying firing neurons for each condition
171 (wake vs sleep), the percentage of active neurons was calculated in each slice by taking the
172 number of active neurons and dividing it by the total number of neurons.
173 Traces of active neurons were used to calculate the number of firing events. This was done
174 using the 'findpeaks' matlab function on the zscored fluorescent traces, with the parameters
175 'minpeakheight' of 3, and 'minpeakdistance' of 30. Data resulting from this was crosschecked
176 by taking the binary matrices of the time traces and finding the number of times each neuron
177 met the activity threshold described above. Graph-theory analyses of neural connectivity were
178 performed as described previously [10] .
179
180 **Behavioral sleep analysis**
181 Behavioral data for flies in imaging experiments was analyzed as previously [10] using a
182 custom-written MatLab code that measured the pixel change occurring over the legs of the fly
183 on the ball over the entire time series. Data was analyzed and graphed using Graphpad Prism.

184 All data was checked for Gaussian distribution using a D'Agostino-Pearson normality test prior
185 to statistical testing. Data from THIP experiments was analyzed using a non-parametric Mann-
186 Whitney test.
187 Sleep behavior in freely-walking flies was analyzed with the Drosophila ARousal Tracking
188 system (DART) as previously described [34]. Prior to analysis, 3-5 day-old females were
189 collected and loaded individually into 65 mm glass tubes (Trikinetics) that were plugged at
190 one end with our standard fly food (see above), containing either 0.1 mg/ml THIP or 0.5
191 mg/ml all-trans-retinal (ATR). Controls were placed onto normal food and housed under
192 identical conditions as the experimental groups. The tubes were placed onto platforms (6 total
193 platforms, 17 tubes per platform, up to 102 flies total) for filming. THIP-fed flies were
194 monitored for 3 days/nights, and sleep data were averaged as no significant differences were
195 detected across successive days. Optogenetically-activated were tracked for baseline day
196 without red light, after which they were exposed to ultra-bright red LED (617 nm Luxeon
197 Rebel LED, Luxeon Star LEDs, Ontario, Canada) which produce 0.1-0.2mW/mm2 at a
198 distance of 4-5 cm with the aid of 723 concentrator optics (Polymer Optics 6° 15 mm Circular
199 Beam Optic, Luxeon Star LEDs) for the duration of the experiment for optogenetic activation.
200 Significance was determined by ANOVA with Tukey's multiple comparisons test (GraphPad
201 Prism). Sleep analysis in nAchRα knockout animals was performed using Trikinetics beam-
202 crossing devices, with regular (>5min) and short sleep (1-5min) calculated as previously [10].
203

204 **Sleep deprivation**

205 Flies were sleep deprived (SD) with the use of the previously described Sleep Nullifying
206 Apparatus (SNAP), an automated sleep deprivation apparatus that has been found to keep flies
207 awake without nonspecifically activating stress responses [35]. Vials containing no greater than
208 20 flies, which contained either standard food medium or medium containing 0.1mg/ml THIP

209 were placed on the SNAP apparatus for continuous sleep deprivation. The SNAP apparatus
210 was programmed to snap the flies once every 20 seconds for the duration of the sleep
211 deprivation protocol.
212
213 **RNA-Sequencing**
214 Flies collected for RNA-sequencing analysis were first housed in vials containing either
215 0.5mg/ml all-trans retinal (ATR) or 0.1mg/ml THIP for sleep induction, along with their
216 genetically identical controls on standard food medium. Flies undergoing sleep induction by
217 optogenetic activation with ATR and their controls were placed under constant red-light from
218 8AM until 6PM to coincide with normal 12:12 light/dark cycles. Flies were collected after 1
219 hour (ZT 1) and 10 hours (ZT 10) post induction for immediate brain dissection and RNA
220 extraction. For analysis of pharmacological sleep induction, flies were placed on THIP or
221 normal food medium at 8AM (ZT 0) and collected for dissection at 6PM (ZT 10).
222 Whole fly brains were dissected in ice cold RNA*later (Sigma-Aldrich)* with 0.1% PBST as per
223 previously published protocol [36]. The dissected brains were immediately pooled into five
224 1.5-mL Eppendorf tubes containing 5 brains (n = 25) each. Total RNA was immediately
225 purified using TRIzol according to the manufacturer's protocols (Sigma-Aldrich) and stored at
226 -80°C until commencement of RNA-sequencing.
227 cDNA libraries were prepared using the Illumina TruSeq stranded mRNA library prep kit.
228 Image processing and sequence data extraction were performed using the standard Illumina
229 Genome Analyzer software and CASAVA (version 1.8.2) software. Cutadapt (version 1.8.1)
230 was used to cut the adaptor sequences as well as low quality nucleotides at both ends. When a
231 processed read is shorter than 36 bp, the read was discarded by cutadapt, with the parameter
232 setting of "-q 20,20 --minimum-length=36". Processed reads were aligned to the *Drosophila*
233 *melanogaster* reference genome (dm6) using HISAT2 (version 2.0.5) [37], with the parameter

234 setting of "--no-unal --fr --rna-strandness RF --known-splicesite-infile dm6_splicesites.txt".
235 This setting is to i) suppress SAM records for reads that failed to align ("--no-unal"), ii) specify
236 the Illumina's paired-end sequencing assay and the strand-specific information ("--fr --rna-
237 strandness RF") and iii) provide a list of known splice sites in *Drosophila melanogaster* ("--
238 known-splicesite-infile dm6_splicesites.txt"). Samtools (version 1.3) [38] was then used to
239 convert "SAM" files to "BAM" files, sort and index the "BAM" files. The "htseq-count"
240 module in the HTSeq package (v0.7.1) was used to quantitate the gene expression level by
241 generating a raw count table for each sample (i.e. counting reads in gene features for each
242 sample). Based on these raw count tables, edgeR (version 3.16.5) [39] was adopted to perform
243 the differential expression analysis between treatment groups and controls. EdgeR used a
244 trimmed mean of M-values to compute scale factors for library size normalization [40]. It used
245 the Cox-Reid profile-adjusted likelihood method to estimate dispersions [41] and the quasi-
246 likelihood F-test to determine differential expression [42]. Lowly expressed genes in both
247 groups (the mean CPM < 5 in both groups) were removed. Differentially expressed genes were
248 identified using the following criteria: i) FDR < 0.05 and ii) fold changes > 1.5 (or logfc >0.58).
249 Gene ontology enrichment analysis for differentially expressed genes was performed using the
250 functional annotation tool in DAVID Bioinformatics Resources (version 6.8) [43, 44].
251

252 **Gene expression**

253 <u>RNA and cDNA Synthesis</u>

254 A quantitative reverse transcriptase PCR assay was used to confirm expression of genes
255 enriched during THIP sleep induction. Nineteen candidate genes were selected (eight
256 negatively and eleven positively) for the gaboxadol (THIP) sleep analysis and six genes (four
257 negatively and two positively) for the dFSB activation experiments. Total RNA was isolated
258 using the Directzol RNA kit (ZymoResearch) from twenty adult brains per condition and each

259 condition was collected in triplicate. RNA quality was confirmed using a microvolume
260 spectrophotometer NanoDrop 2000 (Thermo, USA) with only those resulting samples meeting
261 optimal density ratios between 1.8 and 2.1 used. Up to 1 µg of total RNA was reverse
262 transcribed using a High-Capacity cDNA Reverse Transcription Kit (Themo, USA) as per the
263 manufacturer's protocols. The synthesis of cDNA and subsequent amplification was performed
264 in max volumes of 20 µL per reaction using the T100 Thermal Cycler (Bio-Rad, USA).
265 Thermocycle conditions were as such; 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min,
266 and held at 4 °C . All cDNA was subsequently stored at − 20 °C until used. Target genes for
267 THIP experiments included Pxt (CG7660, FBgn0261987), RpS5b (CG7014, FBgn0038277),
268 Dhd (CG4193, FBgn0011761), CG9377 (CG9377, FBgn0032507), aKHr (CG11325,
269 FBgn0025595), Acox57D-d (CG9709, FBgn0034629), FASN1 (CG3523, FBgn0283427), Pen
270 (CG4799, FBgn0287720), CG10513 (CG10513, FBgn0039311), Gasp (CG10287,
271 FBgn0026077), Act57B (CG10067, FBgn0000044), Bin1 (CG6046, FBgn0024491), verm
272 (CG8756, FBgn0261341), CG16885 (CG16885, FBgn0032538), CG16884 (CG16884,
273 FBgn0028544), CG5999 (CG5999, FBgn0038083), Fbp1 (CG17285, FBgn0000639), CG5724
274 (CG5724, FBgn0038082), Eh (CG5400, FBgn0000564). Target genes for dFSB experiments
275 included Vmat (CG33528, FBgn0260964), Dop1R1 (CG9652, FBgn0011582), Salt (CG2196,
276 FBgn0039872), Dysb (CG6856, FBgn0036819), Irk3 (CG10369, FBgn0032706), Blos1
277 (CG30077, FBgn0050077). Housekeeping genes included Rpl32 (CG7939, FBgn0002626),
278 Gapdh2 (CG8893, FBgn0001092), Actin 5C (CG4027, FBgn0000042). Primer sequences can
279 be found in Supplementary Table 8.
280
281 Quantitative real-time PCR
282 Quantitative (q) RT-PCR was carried out using the Luna Universal qPCR Master Mix (NEB)
283 in the CFX384 Real-Time system (Bio-Rad, USA). Cycling conditions were: 1. 95°C for 60 s,

284 2. 95°C for 15s, 3. 60°C for 60s with 39 cycles of steps two and three. Melt curve analysis was
285 then performed with the following conditions 1. 95°C for 15s, 2. 60°C for 60s, 3. 95°C for 15s.
286 Three biological replicates for each condition as well as three technical replicates per biological
287 sample were loaded. Each experiment was then repeated on three separate occasions. Cq values
288 and standard curves were generated using Bio Rad CFX Manager Software to ensure
289 amplification specificity. Results were normalized to the above housekeeping genes and gene
290 expression was calculated following the 2^− ΔΔCq method (Livak and Schmittgen 2001).
291

292 **Gene knockouts and knockdowns**

293 Dα1KO harboured an ends-out mediated deletion of Dα1 in a w^{1118} background with the X
294 chromosome replaced with one from the wild type line DGRP line 59 [45]. For Dα2KO, 7kb
295 of the Da2 locus was removed using the endogenously expressing Actin-Cas9 strain (BDSC
296 5490) crossed to a strain expressing two Dα2 gene locus specific sgRNAs (Dα_sgRNA_start_1
297 5'CATGTTTAGCGCTGCAATGC, Dα2_sgRNA_end_1 5'TTACAAGCCATCTGCCTAG).
298 Transgenic flies were generated using the pCFD4-U6:1_U6:3tandem gRNAs construct. This
299 was a gift from Simon Bullock. For Dα3KO (*Dα3Δ1020*), Dα4KO (*Dα4ΔBA*), Dα6KO,
300 Dα7KO (*Dα7ΔD6*), two sgRNAs were designed to target the start and the end of the coding
301 sequence and cloned into either pU6-BbsI-gRNA or pCFD4 plasmids. These plasmids were
302 then microinjected into *Drosophila* embryos to generate transgenic strains stably expressing
303 sgRNAs. These strains were crossed to another strain expressing Cas9 under Actin promoter
304 (ActinCas9). Their offspring were screened for deletion events with PCR and crossed to
305 appropriate balancer strains to isolate and generate homozygous knockout strains. Full
306 deletions were identified for all these subunit genes except for Dα3 which has two partial
307 deletions at the 3' and 5' ends; these were verified to be true knockouts by the lack of RNA
308 expression [46]. ActinCas9 strain was used as genetic control for Dα3KO and Dα7KO, while

309 this same strain with the X chromosome replaced with one from w^{1118} (w^{1118}ActinCas9) was
310 used as genetic control for Dα2KO, Dα4KO, and Dα6KO. The RNAi strain for the adipokinetic
311 hormone receptor gene knockdown experiments (UAS-*AkhR*-RNAi) was obtained from the
312 VDRC (KK109300). This RNAi construct was expressed in neurons across the fly brain by
313 crossing the UAS flies to R57C10-Gal4 [30]. RNAi strains for the nicotinic alpha receptor gene
314 knockdown experiments (UAS- nAchRα1-7-RNAi) were obtained from the BDRC. These
315 were: nAChRα1 RNAi – BL# 28688 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03103}attP2 ,
316 nAChRα2 RNAi – BL# 27493 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02643}attP2 ,
317 nAChRα3 RNAi – BL# 27671 – y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02750}attP2 ,
318 nAChRα4 RNAi – BL# 31985 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03419}attP2 ,
319 nAChRα5 RNAi – BL# 77418 - y[1] sc[*] v[1] sev[21]; P{y[+t7.7]
320 v[+t1.8]=TRiP.HMC06550}attP2 ,
321 nAChRα6 RNAi – BL# 52885 - y[1] sc[*] v[1] sev[21]; P{y[+t7.7]
322 v[+t1.8]=TRiP.HMC03623}attP40 ,
323 nAChRα7 RNAi – BL# 27251 - y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02570}attP2.
324 These RNAi constructs were expressed in the sleep-promoting neurons by crossing the UAS
325 flies to R23E10-Gal4.
326

327 **Code and data availability**

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

333 **Results**

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

336 We first compared pharmacological and optogenetic sleep (**Figure 1**) by using the traditional
337 behavioral metrics employed by most *Drosophila* sleep researchers: >5 minutes inactivity for
338 flies confined in small glass tubes over multiple days and nights [15, 16]. We found that
339 optogenetic- and THIP-induced sleep yielded almost identical effects on sleep duration, with
340 both significantly increasing total sleep duration for both the day and night, when compared to
341 controls (**Figure 2A-D**; **Supplementary Table 1**). An increase in total sleep duration can be
342 due to either an increase in the number of sleep bouts that are occurring (reflective of more
343 fragmented sleep), or an increase in the average duration of individual sleep bouts, which
344 indicates a more consolidated sleep structure [47-49]. To investigate whether both sleep
345 induction methods also had similar effects on sleep architecture, we plotted bout number as a
346 function of bout duration for optogenetic and THIP-induced sleep, for the day and night [50].
347 We found that both optogenetic activation and THIP provision produce a similar increase in
348 sleep consolidation during the day (**Figure 2E, F; Figure 3**). During the night, induced sleep
349 effects were also similar, although less clearly different to the spontaneous sleep seen in control
350 flies (**Figure 2G, H; Figure 3**). Interestingly, red light exposure decreased average night bout
351 duration in non-ATR control flies (**Figure 3A-D**), suggesting a light-induced artefact at night.
352 For THIP, we observed an increase in both bout number and duration during the day, and an
353 increase in bout duration during the night (**Figure 3E,F**). Taken together, these results show
354 that prolonged optogenetic activation and THIP provision have similar behavioral effects on
355 induced sleep duration in *Drosophila*, although some differences in night bout architecture
356 were noted (**Figure 3**). Without any further investigations, this might suggest that both sleep
357 induction methods represent similar underlying processes that uniformly increase sleep in flies.

358

359 **THIP-induced sleep decreases brain activity and connectivity**

360 The brain presents an obvious place to look for any potential differences between sleep
361 induction methods. In a previous study employing whole-brain calcium imaging in tethered
362 flies we showed that optogenetic activation of the R23E10 circuit promotes wake-like sleep,
363 with neither neural activity levels nor connectivity metrics changing significantly even after
364 15min of optogenetically-induced sleep [10]. We therefore utilized the same fly strain as in
365 that study (R23E10-Gal4>UAS-Chrimson88-tdTomato;Nsyb-LexA>LexOp-nlsGCaMP6f) to
366 examine the effect of THIP-induced sleep on brain activity (**Figure 4A,B**). Since we were
367 interested in comparing acute sleep induction effects on brain activity (as opposed to
368 prolonged sleep induction effects on behavior, as in Figures 2,3), we adapted our calcium
369 imaging approach to allow a brief perfusion of THIP directly onto the exposed fly brain
370 (**Figure 4A**, see Methods). As done previously for examining optogenetically-induced sleep
371 [10], we examined calcium transients in neural soma scanning across 18 optical slices of the
372 central fly brain (**Figure 4B,** left) and identified regions of interest (ROIs) corresponding to
373 neuronal soma in this volume (**Figure 4B**, right, and see Methods). As shown previously
374 [10], optogenetically activating the R23E10 circuit renders flies asleep (albeit twitchy at
375 times) without changing the average level of neural activity measured this way (**Figure 4C**).
376 We have previously shown that even 15min of R23E10 activation fails to produce a 'quiet'
377 sleep stage that is evident after 5min in spontaneously sleeping flies [10], suggesting that this
378 manipulation promotes ongoing active sleep until the red light is turned off – although this
379 has not been investigated for longer epochs, e.g. the chronic activation experiments described
380 in Figure 2. To determine the effect of THIP on neural activity in the exact same strain, we
381 transiently perfused onto the fly brain the minimal THIP dosage required to reliably promote
382 sleep in flies within five minutes (0.2mg/ml) [9]. In contrast to optogenetic-induced sleep, we

383 observed overall decreased neural activity coincident with the flies falling asleep, and flies
384 remained asleep well after the drug was washed out (**Figure 4D**). To ensure that we were
385 actually putting flies (reversibly) to sleep in this preparation, we probed for behavioral
386 responsiveness by puffing air onto the fly once every minute (50 ms duration, 10 psi) (**Figure**
387 **5A**). Since the time when flies fell asleep following five minutes of THIP perfusion could be
388 variable [9], arousal probing during sleep was only initiated after 5 min of complete
389 quiescence (**Figure 5A**, behavioral responsiveness testing). We observed decreased
390 arousability for flies that had been induced to sleep via THIP perfusion (**Figure 5B**). Drug-
391 induced sleep was however reversible, with flies returning to baseline levels of behavioral
392 responsiveness to the air puffs ~20-30 min after sleep initiation. This confirmed that the brief
393 exposure to THIP was indeed putting flies to sleep, with an expected sleep inertia lasting the
394 length of a typical spontaneous sleep bout [15, 16]. This contrasts with optogenetic sleep
395 induction using the R23E10-Gal4 circuit, which does not show appear to show any sleep
396 inertia (**Figure 4C**) [51].
397
398 We then examined more closely neural activity in flies that had been put to sleep with THIP.
399 We found that neural activity decreased rapidly within 5 min after sleep onset (**Figure 5C**,
400 +THIP, early). Correlation analysis also revealed a decrease in connectivity among the
401 remaining active neurons (**Figure 5D**, +THIP, early). We also analyzed the next 5 min of
402 sleep and observed similar results (**Figure 5C,D**, +THIP, mid). All flies eventually woke up
403 from THIP-induced sleep, and brain activity returned to wake levels in three flies that were
404 recorded throughout (**Figure 5C**). These observations suggest that acute THIP exposure is
405 promoting rapid entry into a 'quiet' sleep stage in flies, bypassing the wake-like sleep evident
406 during the first 5 min of spontaneous sleep onset, and closely resembling spontaneous quiet
407 sleep typically seen after 5 minutes of inactivity [10]. Importantly, THIP-induced sleep

408 appears to be dissimilar from optogenetically-induced sleep in this genotype, at the level of
409 neural activity as well as connectivity [10].
410
411 In recent work we showed that rendering flies unresponsive with a general anesthetic,
412 isoflurane, in surprising contrast to THIP, does not quieten the fly brain [51]. Isoflurane also
413 decreased neural connectivity, as well as the diversity of neural activity across the fly brain,
414 whereas optogenetic-induced sleep did not show any differences in neural activity or
415 ensemble dynamics [51]. Since in our current THIP experiments we were similarly recording
416 from neural soma that we could track through time, we were able to assess the level of
417 overlap between the neurons that remained active during THIP-induced sleep and
418 wakefulness (**Figure 5E, F**). We found that ~30% of active neurons during THIP-induced
419 sleep were also active during wake (**Figure 5F, G**). We next examined whether the same
420 neurons remained active across successive 5min epochs during THIP-induced sleep
421 compared to wake. We found that there is significantly more overlap between successive
422 5min sleep epochs (41%), compared to the waking average (**Figure 5G**), suggesting less
423 neural turnover during THIP-induced sleep than during wake. This contrasts with optogenetic
424 sleep, where the rate of neural turnover was not different from wake [51]. Taken together, our
425 calcium imaging data confirm that pharmacological sleep induction promotes a different kind
426 of sleep than optogenetic sleep induction in the same strain. Henceforth, we call this 'quiet'
427 sleep, in contrast to the 'active' sleep that seems to be engaged by optogenetic activation [2,
428 10]. Notably, calcium imaging of spontaneous sleep bouts in *Drosophila* also revealed active
429 and quiet sleep stages [10], suggesting that both of our experimental approaches are
430 physiologically relevant. Whether drug perfusion to the brain is equivalent to feeding is of
431 course less clear. When feeding on 0.1mg/ml THIP-laced food, flies were continuously
432 exposed to the drug over days, with comparatively less reaching the brain. With perfusion,

433 the brain was directly exposed to 0.2mg/ml THIP for only 5 minutes. Interestingly, in both
434 cases this induces daytime sleep bouts which average around 25min (**Figure 3F, Figure 5B)**,
435 the average duration of a spontaneous night-time sleep bout (**Figure 3F; Table S1**).
436
437 **Transcriptional analysis of flies induced to sleep by THIP provision**
438 Our calcium imaging experiments suggest that different biological processes might be
439 engaged by optogenetic-induced sleep compared to THIP-induced sleep. Additionally, we
440 observed neural effects encompassing much of the fly brain (**Figure 5E**), as our recording
441 approach exploited a pan-neural driver. We therefore wondered if either sleep-induction
442 method might lead to differences in gene expression across the whole brain, and if these
443 might highlight distinct molecular pathways engaged by either kind of sleep. To address this,
444 we collected brains from flies that had been induced to sleep by either method, and compared
445 the resulting transcriptomes with identically-handled control animals that had not been
446 induced to sleep by these methods.
447
448 To control for genetic background, we again used the same R23E10-Gal4 > UAS-Chrimson
449 flies as in our multi-day behavioral experiments and fed the flies either THIP or ATR, as in
450 **Figure 2**. We only examined daytime sleep-induction effects for either method, as this is when
451 we observed the greatest increase in sleep compared to controls (**Figure 2**), and previous work
452 has shown that daytime sleep induction using either method achieves sleep functions [10, 23].
453 We present our THIP results first. Since THIP is a GABA-acting drug that probably affects a
454 variety of processes in the brain aside from sleep, we also assessed the effect of THIP on flies
455 that were prevented from sleeping (**Figure 6A**, left panel). Sleep deprivation (SD) was
456 performed by mechanically arousing flies once every 20 seconds for the duration of the
457 experiment, on a 'SNAP' apparatus [23, 35]. RNA was extracted from the brains of all groups

458 of flies (+/- THIP, +/- SD) after 10 hours of daytime (8am-6pm) THIP (or vehicle) provision.
459 Samples for RNA-sequencing were collected in replicates of 5 to ensure accuracy, and any
460 significant transcriptional effects were thresholded at a log fold change of 0.58 (see Methods).
461
462 Flies allowed to eat food containing 0.1mg/ml THIP *ad lib* over 10 daytime hours led to 129
463 significant changes in gene expression compared to vehicle-fed controls, with the large
464 majority (110) being downregulated and only 19 upregulated (**Figure 6B,C,E;**
465 **Supplementary Table 2**). In contrast, when THIP-fed flies were prevented from sleeping
466 this led to mostly upregulated genes (88 upregulated vs 21 downregulated, **Figure 6B,D,F;**
467 **Supplementary Table 3**), with many of the upregulated genes showing a high level of fold-
468 change in expression. Not surprisingly, preventing sleep in THIP-fed flies led to an almost
469 entirely non-overlapping set of gene expression changes (**Figure 6B**). This suggests that the
470 large number of down-regulated genes in +THIP / -SD flies pertain to sleep processes rather
471 than the effect of ingesting THIP, since only a few (9) genes overlapped with the +THIP /
472 +SD dataset which revealed mostly upregulated genes.
473
474 Gene Ontology analysis on genes that were downregulated as a result of THIP-induced sleep
475 highlighted a significant enrichment of metabolism pathways (**Figure 6E,F; Supplementary**
476 **Figure 1**). The top Gene Ontology biological processes included primary, organic substance,
477 cellular, biosynthetic and nitrogen compound metabolic pathways, as well as ribosomal
478 processes. Interestingly, these downregulated processes are largely consistent with a recently
479 published mouse sleep transcriptome study [52]. Among the metabolism pathways uncovered
480 in this dataset we observed over-representation of expected genes such as *bgm* (bubblegum
481 CG4501) and *Acer* (Angiotensin-converting enzyme-related CG10593). Both of these genes
482 are found in the primary metabolic and organic substance metabolic processes as well as within

483 the Sleep Gene Ontology dataset (GO:0030431). Another downregulated metabolic gene is
484 AkhR (adipokinetic hormone receptor), which has been found to be involved in starvation-
485 induced sleep loss in *Drosophila* [53]. AkhR belongs to the Class A GPCR Neuropeptide and
486 protein hormone receptors which are a gene class involved in storage fat mobilization,
487 analogous to the glucagon receptor found in mammals [54].
488
489 Although THIP-induced sleep overwhelmingly led to gene downregulation, a few genes (19)
490 were significantly upregulated. Gene Ontology analysis on these upregulated genes highlighted
491 enrichment in varying groups including developmental processes and multicellular organismal
492 processes (**Figure 6E,F; Supplementary Figure 1**). Some groups were enriched under the
493 organic substance metabolic process pathways; however, there was no overlap when
494 comparing these to the pathways enriched due to downregulation of genes. There were some
495 overlapping enriched pathways when we compared the gene sets from sleep-deprived flies
496 which had also been treated with THIP (**Figure 6F**; **Supplementary Figure 2**). However, the
497 gene sets they involve are upregulated in the SD dataset but downregulated in sleeping flies.
498 Interestingly, the non-sleeping THIP dataset uncovered a significant enrichment of pathways
499 involved in the response to stress. This might be expected for flies exposed to regular
500 mechanical stimuli over 10 hours. None of these pathways featured in the THIP sleep dataset.
501
502 To validate these findings, we conducted qRT-PCR analyses on 19 genes from our THIP sleep
503 dataset and compared these results to our original transcriptional data. The genes represented
504 a range of both up – and down-regulated genes, and we found convincing correspondence
505 between the groups (**Figure 5G**). qRT-PCR comparisons with RNAseq data and associated
506 statistics are presented in **Supplementary Table S9**.
507

508 **Transcriptional analysis of flies induced to sleep by optogenetic activation**

509 We next examined the effect of optogenetically-induced sleep on the whole-brain
510 transcriptome, to compare to our THIP-induced sleep data. Based on our earlier findings that
511 showed that optogenetic activation results in rapidly inducible sleep behavior that consolidates
512 over at least 12 daytime sleep hours (**Figure 2C,E,G**), as well as our previous study showing
513 that 10 daytime hours of optogenetic activation corrects attention defects in sleep-deprived flies
514 [10], we induced sleep in R23E10-Gal4 x UAS-Chrimson flies for 10 daytime hours and
515 collected tissue for whole-brain RNA-sequencing (**Figure 7A**). We selected two time points
516 for collection, for both the sleep-induced flies (+ATR) as well as their genetically identical
517 controls that were not fed ATR (-ATR; **Figure 7A**). Optogenetic activation was matched to the
518 normal day-time light cycle (8 AM – 8 PM). The first collection point was after 1 hour (ZT1,
519 9 AM) of red-light exposure, to control for effects of ATR provision (when compared to ZT1,
520 -ATR controls) as well as to uncover any potential short-term genetic effects of optogenetic
521 activation. We then collected flies after 10 hours of red-light exposure (ZT 10, 6 PM) to
522 examine longer-term genetic effects of optogenetic sleep induction, and to match exactly our
523 THIP sleep collection timepoint (i.e., 10 hours of induced daytime sleep by either method).
524 The combined collection points also allowed us to compare transcriptional profiles between
525 conditions (e.g., ZT10 +ATR vs. ZT10 -ATR), to identify sleep genes, as well as within
526 conditions (ZT1 vs. ZT10), to account for genetic effects potentially linked to light-dark cycles.
527 As for the THIP sleep data in the same strain, samples for RNA-sequencing were collected in
528 replicates of 5 to ensure accuracy, and any significant transcriptional effects were thresholded
529 at a log fold change of 0.58 (see Methods).

530

531 We first examined the effect of 10 hours of daytime optogenetic-induced sleep. Here, we
532 compared ATR-fed R23E10-Gal4 x UAS-Chrimson flies to genetically identical animals that

533 were also exposed to red light for 10 hours but not provided with ATR in their food (ATR-).
534 The control flies were therefore never induced to sleep by optogenetic activation, although they
535 were still able to sleep spontaneously (see **Figure 2C,E,G**). We found that 10 hours of
536 optogenetic activation led to 278 significant transcriptional changes, comprising mostly of
537 upregulated genes, with 171 upregulated compared to 107 downregulated (**Figure 7B,C,E;**
538 **Supplementary Table 4**). In contrast to the THIP-induced sleep dataset, transcriptional
539 analysis of 10hr optogenetic sleep induction uncovered a variety of different processes
540 predominantly related to the regulation of biological and cellular processes, rather than
541 metabolism specifically (**Figure 7E; Supplementary Figure 3**). For example, of the genes
542 that were overexpressed there is an enrichment of the Semaphorin-plexin signaling pathway
543 (GO:0071526, GO:1902287, GO:1902285 and GO:2000305) and the ephrin receptor signaling
544 pathway (GO:0046011), both of which are known to be involved in axonal guidance (**Figure**
545 **7F**). Interestingly, several upregulated genes code for different subunits of nicotinic
546 acetylcholine receptors (nAchRα1,3,4 &5; **Figure 7C**). Importantly, there was almost no
547 overlap with our sleep deprivation dataset (**Supplementary Table 3**), suggesting that
548 optogenetic activation is not sleep depriving (or stressing) the flies (only one upregulated gene
549 was shared, CG40198). Of the genes that were downregulated there is enrichment of pathways
550 that relate to synaptic vesicle recycling (GO:0036465 and GO:0036466) as well as
551 neurotransmitter metabolic processes (GO:0042133) (**Figure 7F; Supplementary Figure 3**).

552

553 In contrast to the 10hr timepoint, 1 hr of optogenetically-induced sleep had far fewer
554 transcriptomic consequences, with only 17 genes upregulated and 10 downregulated (**Figure**
555 **7B,D**). This small number of transcriptomic changes (see **Supplementary Table 5**) may
556 reflect the effect of ATR feeding, rather than any genes relevant to optogenetic sleep. That 9

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

560

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

581 expression changes between optogenetic-induced sleep and THIP-induced sleep
582 (**Supplementary Tables 2 & 4**), and the respective GO pathways analyses of biological
583 processes are also largely non-overlapping (**Supplementary Figure 5**).

584

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

591

592 **Nicotinic acetylcholine receptors regulate sleep architecture**

593 While THIP-induced sleep caused a systemic downregulation of metabolism-related genes,
594 the effect of optogenetic-induced sleep on gene expression was not dominated by a single
595 category. This may be consistent with our earlier observation that brain activity looks similar
596 to wake during optogenetic-induced sleep [10], so we could essentially be highlighting
597 biological processes relevant to an awake fly brain, such as dopamine function [57].
598 However, optogenetic activation of the R23E10 circuit is not like wake, in that flies are
599 rendered highly unresponsive to external stimuli, so perhaps like REM sleep in mammals a
600 different category of molecular processes could be involved. In mammals, acetylcholine
601 generally promotes wakefulness and alertness, but activity of cholinergic neurons is also high
602 during REM sleep [58]. Neurotransmission in the insect brain is largely cholinergic, with 7
603 different nicotinic 'alpha' receptor subunits expressed in neural tissue [59]. Interestingly, four

604 of these subunits were significantly upregulated in our optogenetic sleep dataset: nAchRα1,
605 nAchRα3, nAchRα4, and nAchRα5 (with nAchRα6 and nAchRα7 approaching
606 significance, **Supplementary Table 4**). For comparison, none of these were upregulated in
607 our sleep deprivation dataset, suggesting a sleep-relevant role. Previous studies have
608 demonstrated a role for some of these same receptor subunits in sleep regulation, in particular
609 nAchRα4 (also called *redeye*) which is upregulated in short-sleeping mutants [60] and
610 nAchRα3 which has been reported to regulates arousal levels in flies [61]. Together, these
611 studies suggest processes that might be reconsidered in the context of active sleep, as
612 highlighted by our gene expression findings. We therefore sought to examine the role of
613 cholinergic signalling in sleep more closely, by knocking out each nAchRα subunit and
614 examining effects of each subunit knockout on sleep architecture. Since our transcriptomic
615 analysis encompassed effects of active sleep on whole-brain gene expression, we first
616 eliminated each nAchRα subunit across the brain, by testing confirmed genetic deletions [46,
617 62].

618 We first examined the effect of each nAchRα subunit deletion on sleep duration, using the 5-
619 minute criterion for quantifying sleep in *Drosophila* [15]. We found that the nAchRα mutants
620 fell into two different categories: removal of nAchRα1 and nAchRα2 significantly decreased
621 sleep, day and night; whereas removal of nAchRα3, nAchRα4, nAchRα6 and nAchRα7
622 significantly increased sleep, day and night (**Figure 8A**). The nAchRα5 knockout was found
623 to be homozygous lethal, so was not included. To examine sleep architecture in these
624 mutants, we quantified sleep bout number and duration and plotted these together as done
625 previously for our sleep induction experiments (**Figure 2**). Examining the data this way, it is
626 clear to see how nAchRα1 and nAchRα2 are different: most sleep bouts are very short, day
627 and night (**Figure 8B**, top 2 rows, left panels, green dots). In contrast, knocking out the other

628 alpha subunits seems to consolidate sleep, especially at night (**Figure 8B**, bottom 4 rows, left
629 panels). nAchRα3 knockouts were most striking in this regard, with these flies sleeping
630 uninterrupted for an average of 156.53 minutes (± 18.06) during the day and 160.76 minutes
631 (± 17.92) at night. Increased sleep consolidation in these mutants was however not due to
632 lack of activity. While awake, nAchRα3 animals were just as active as controls (activity per
633 waking minute = 2.69 ± 0.18 versus 2.5 ± 0.06, respectively). However, some knockouts did
634 increase waking activity levels; waking activity data for all of the knockout strains and their
635 genetic controls are presented in **Supplementary Figure 6**.

636 We next questioned what kind of sleep the nAchRα knockout flies might be getting. In
637 previous work we have shown that flies can be asleep already after the first minute of
638 spontaneous inactivity, and that during the first five minutes of sleep the fly brain displays
639 wake-like levels of neural activity [10]. We have termed this early sleep stage 'active sleep'
640 to distinguish it from 'quiet sleep' that typically follows after 5-10 minutes [63]. One way of
641 estimating the amount of 'active sleep' in *Drosophila* flies is to sum all short sleep epochs
642 lasting between 1-5 minutes and expressing this as a percentage of total sleep [10]. When we
643 re-examined our nAchRα knockouts in this way, we found that this behavioral readout for
644 'active sleep' was significantly affected by the loss of select nAchRα subunits. Short sleep
645 increased significantly during both the day and the night in nAchRα1 and nAchRα2 (**Figure**
646 **8B**, top 2 rows, right panel, green dots). In contrast, and consistent with our sleep architecture
647 analyses (above), nAchRα3 displayed almost no short sleep (**Figure 8B**, row 3, right panel).
648 Finally, in nAchRα4 and nAchRα6 short sleep was significantly decreased at night, while in
649 nAchRα7 short sleep was significantly decreased day and night (**Figure 8B**, rows 4-6, right
650 panel). In conclusion, every one of the nAchRα knockouts we tested affect short sleep in
651 some way, either increasing (nAchRα1 and nAchRα2) or decreasing it (nAchRα3,

652 nAchRα4, nAchRα6, nAchRα7). Additionally, tallying the proportion of short sleep bouts
653 provides valuable insight into altered sleep architecture in mutant strains.

654 We nevertheless questioned whether these systemic effects of nicotinic receptors on short
655 sleep were perhaps a trivial consequence of altered >5min sleep duration in these mutants,
656 especially regarding the striking differences between nAchRα1&2 and the other subunit
657 knockouts. We therefore returned to our 'quiet' sleep (THIP) dataset to contrast a gene
658 derived from that study. We had found that several of the THIP-induced sleep genes are
659 involved in metabolic processes, which are mostly downregulated (**Supplementary Table 2**).
660 This included the adipokinetic hormone receptor (AkhR), which has previously been
661 associated with starvation-induced sleep regulation [53]. We employed an RNAi strategy to
662 downregulate this metabolic gene's expression across the fly brain in AkhR-RNAi / R57C10-
663 Gal4 flies (see Methods). We found that downregulating AkhR significantly decreased sleep
664 duration during the day as well as night, compared to genetic control strains (**Figure 9A,B**).
665 Accordingly, sleep bout duration and number decreased, especially during the day (**Figure**
666 **9C**). However, in contrast to knocking out nAchRα1 and nAchRα2, which also significantly
667 decreased sleep duration day and night, short sleep was not significantly altered in AkhR
668 knockdown animals compared to genetic controls (**Figure 9D**). This suggests that short (1-
669 5min) sleep might be under separate regulatory control than >5min sleep.

670 We then returned to the nAchRα subunits and employed an RNAi strategy to knock them
671 down in the R23E10 sleep-promoting neurons specifically. While it is unlikely that these
672 cholinergic receptor subunits are restricted to just these neurons, we were curious if removing
673 any of them from this specific circuit had similar effect on sleep as in the knockout strains.
674 We found that nAchRα1 knockdown in the R23E10 neurons significantly decreased total
675 sleep during both day and night (**Figure 10A,B**), which was also true for the nAchRα1

676 knockouts (**Figure 8A**). Also consistent with the knockouts, the proportion of short sleep
677 bouts was increased in this this specific knockdown (**Figure 10C**). Among all of the other
678 alpha subunits, knockdown of nAchRα5 and nAchRα6 in the R23E10 neurons significantly
679 increased daytime and night-time sleep, respectively (**Figure 10A,B**), while short sleep was
680 correspondingly decreased only in nAchRα5 (**Figure 10C**). None of the other alpha subunits
681 had any effect in these sleep-promoting neurons. This suggests that their sleep-regulatory role
682 lies outside of this circuit, while nAchRα1, nAchRα5, and nAchRα6 might regulate sleep-
683 related functions within this circuit.

684

685 **Discussion**

686 One of the key advantages of studying sleep in *Drosophila* is that this versatile model
687 provides a variety of reliable approaches for inducing and controlling sleep. By being able to
688 induce sleep on demand, either genetically or pharmacologically, researchers have been able
689 to manipulate sleep as an experimental variable, and in this way be better able to assess
690 causality when probing potential sleep functions. However, this approach has often
691 sidestepped the question of whether different sleep induction methods are equivalent, or
692 whether distinct forms of sleep might be engaged by different genetic or pharmacological
693 treatments. In mammals, GABA agonists typically promote slow-wave sleep (SWS), which
694 has been associated with cellular homeostasis and repair process in the brain [4, 64]. In
695 contrast, drugs targeting acetylcholine receptors, such as carbachol, have been found to
696 promote brain states more reminiscent of REM sleep [65, 66]. Although these drugs all
697 induce sedative (or dissociative) states, they are clearly producing dissimilar forms of sleep in
698 mammals, with likely different functions or consequences for the brain. In *Drosophila*,
699 evidence suggests that the GABA agonist THIP promotes a form of deep or 'quiet' sleep,

700 which may be functionally analogous to mammalian SWS [9, 27, 67]. In contrast,
701 optogenetic activation of the dFB as well as other neurons may promote a form of active
702 sleep, which we have suggested could be achieving some similar sleep functions as REM [2].
703 THIP-induced sleep in flies has been associated with waste clearance from the brain [27], just
704 as SWS has been associated with clearance of waste metabolites via the mammalian brain's
705 glymphatic system [64]. Such functional homology suggests that the transcriptional changes
706 we uncovered for THIP-induced sleep in *Drosophila* might also be relevant for mammalian
707 SWS, with these largely centered on reduced metabolic processes and stress regulation [52].
708 In contrast, except for the upregulation of cholinergic signaling [68], there is little to compare
709 to test hypotheses potentially linking active sleep in flies with REM sleep in mammals,
710 except for the potential upregulation of cholinergic signaling. Even this cholinergic
711 connection seems odd, seeing that the predominant excitatory neurotransmitters are reversed
712 in the brains of insects and mammals: glutamate in mammals and acetylcholine in insects
713 [69]. Additionally, only nicotinic receptor subunits were identified in our analyses, whereas
714 muscarinic receptors have been more commonly associated with REM sleep in mammals [70,
715 71]. Nevertheless, it is clear from our results that optogenetic-induced active sleep
716 upregulates the expression of multiple nicotinic acetylcholine subunits, and that knocking
717 these out individually has profound (and opposing) effects on sleep architecture in flies, and
718 that a subset might function intrinsically within sleep promoting neurons. This supports other
719 studies showing a role for nAchRα's in sleep regulation [60, 61]. It will be especially
720 interesting in future brain imaging studies to see whether a knockout such as nAchRα3 is
721 eliminating one kind of sleep (e.g., active sleep) as predicted by our behavioral data, and
722 whether this is associated with any functional consequences. Similarly, it will be telling to
723 see whether the opposite sleep phenotypes observed in nAchRα1 for example result in a
724 distinct class of functional consequences. A previous study has shown that nAchRα1

725 knockout animals have significantly shorter survivorship compared to controls, with flies
726 dying almost 20 days earlier [45]. One reason could be because of impaired or insufficient
727 deep sleep functions (e.g., brain waste clearance [27]). The nAchRα knockouts provide an
728 opportunity to further examine mutant animals potentially lacking either kind of sleep,
729 although this will have to be confirmed by brain imaging or electrophysiology.

730 We found little similarity between two different approaches to inducing sleep in flies, at the
731 level of gene expression as well as acute effects on brain activity. It may however not be
732 surprising that these entirely different sleep induction methods produce dissimilar
733 physiological effects. After all, one method requires flies to ingest a drug which then must
734 make its way to the brain, while the other method directly activates a subset of neurons in the
735 central brain, as well as in the ventral nerve cord [72]. Yet both methods yield similarly
736 increased sleep duration profiles and consolidated sleep architecture (**Figure 2**). One
737 underlying assumption with focusing on sleep duration as the most relevant metric for
738 understanding sleep function in *Drosophila* is that sleep is a unitary phenomenon in the fly
739 model, meaning that primarily one set of functions and one form of brain activity are
740 occurring when flies sleep. There is now substantial evidence that this is unlikely to be true,
741 and that like other animals flies probably also experience distinct sleep stages that accomplish
742 different functions [9, 10, 27, 63, 73, 74]. This does not mean that these functions are
743 mutually exclusive; for example, both THIP provision and neuronal activation of central
744 brain circuits have been found to promote memory consolidation in *Drosophila* [23]. Indeed,
745 it seems reasonable to propose that different sleep stages could be synergistic, accomplishing
746 a variety of homeostatic functions that might be required for adaptive behaviors in an animal.
747 Our results suggest that THIP provision promotes a 'quiet sleep' stage in flies, which induces
748 a brain-wide downregulation of metabolism-related genes. This is consistent with studies in
749 flies showing that metabolic rate is decreased in longer sleep bouts, especially at night, and

750 that this is recapitulated by THIP-induced sleep [75]. Our findings also align with a previous
751 study showing that mitochondrial metabolic processes are upregulated in the absence of sleep
752 [76]. One argument for why metabolism-related genes are downregulated during THIP-
753 induced sleep might be that flies are starved (because they are sleeping more, and thus
754 perhaps not eating). However, flies induced to sleep by optogenetic activation are also
755 sleeping more (indeed, their average sleep bouts are longer), yet these did not reveal a similar
756 downregulation of metabolic processes. Another view might be that our sampling was done
757 after flies had achieved 10 hours of induced sleep, so sleep functions might have already been
758 achieved by that time. Thus, we might not be uncovering genes required for achieving 'quiet'
759 sleep functions as much as identifying exactly the opposite: genetic pathways that have been
760 satisfied by 10 hours of induced quiet sleep. Other studies using THIP to induce sleep have
761 examined longer timeframes (e.g., 2 days [23]), so it remains unclear whether changes in
762 gene regulation relate to sleep functions that have been achieved or that are still being
763 engaged. For our transcriptomic study, both sleep induction protocols were conducted during
764 the day (thus in presumably well-rested flies) to deliberately avoid any sleep pressure
765 confounds. Since the dFB has been proposed as a regulator of sleep homeostasis [20-22], one
766 could question why its prolonged activation did not incur a deep sleep debt – which might
767 have been detectable at a transcriptomic level by some overlap with our sleep deprivation
768 controls. That we did not see this suggests induced daytime active sleep might not accrue any
769 sleep debt. We have shown previously that daytime sleep is qualitatively different than night-
770 time sleep in flies, which is 'deeper' [63]. It is for example possible that most quiet sleep
771 happens at night in flies, so a daytime regime of induced active or quiet sleep might not incur
772 any sleep debt for either. Following this logic, our RNA analyses simply revealed the
773 transcriptomic changes resulting from either manipulation in well-rested flies, with the key
774 result being that there are zero shared genes after 10 daytime hours of either manipulation.

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

791

792 A compelling argument could nevertheless be made for two kinds of sleep in most animals,
793 with two distinct sets of functions [1, 67]. Most animals have been shown to require a form of
794 'quiet' sleep to ensure survival, suggesting that these might encompass an evolutionarily
795 conserved set of cellular processes that promote neural health and development [79], and that
796 operate best during periods of behavioral quiescence. Nematode worms thus experience a
797 form of quiet sleep when they pause to molt ('lethargus') into a different life stages during
798 their development [80], or when cellular repair processes are needed following environmental
799 stress [81]. In flies, quiet sleep seems to be similarly required for neuronal repair [26] or

800 waste clearance [27], and there is evidence that glia might play a key role in these cellular
801 homeostatic processes in flies [26] as well as other animals [82]. Thus, SWS in mammals and
802 birds might present a narrow neocortical view of a more ancient set of sleep functions
803 centered on quiescence and decreased metabolic rate. Indeed, neural quiescence is also a
804 feature of SWS, both at the level of pulsed inhibition (down-states), as well as in other parts
805 of the brain beyond the cortex [1]. Similar to findings in flies that are induced into a quiet
806 sleep stage with THIP [75], metabolic rate also decreases during SWS in mammals [83]. In
807 contrast, metabolic rate is similar to waking during REM sleep in mammals [84], suggesting
808 an alternate set of functions not linked to cellular homeostasis. What might these functions
809 be, and could some of these be conserved between active sleep in invertebrates and REM
810 sleep in mammals? A REM-like sleep stage has now been identified in a variety of
811 invertebrate species, including cephalopods [12] and jumping spiders [85]. In humans, REM
812 sleep has been implicated in emotion regulation [86], and cognitive disorders where emotions
813 are dysregulated, such as depression, are often associated with REM sleep dysfunction [87].
814 While it is not evident how to study emotions in insects (but see [88]), it could be argued that
815 arousal systems more generally are employed to detect prediction errors and thereby promote
816 learning [89]. Thus, we and others have suggested that REM sleep might be crucial for
817 optimizing prediction, and attention, and learning [2, 67, 90], and this may involve different
818 kinds of homeostatic mechanisms centered on brain circuits rather than cells. Whether active
819 sleep in flies plays a similar homeostatic role remains to be determined. Our finding that
820 optogenetically -induced sleep in *Drosophila* upregulates different nAchRα subunits is
821 consistent with new findings that these subunits regulate appetitive memories in flies [91] and
822 that cholinergic systems more generally underpin learning and memory in this animal [92].
823 Yet learning and memory in flies clearly also benefits from quiet sleep, as evidenced by
824 multiple studies using THIP as a sleep-promoting agent [23, 25, 93]. One view consistent

825 with our findings and previous studies is that both kinds of sleep are crucial for optimal
826 behavior: quiet sleep for cellular homeostasis and active sleep for circuit-level homeostasis.
827 Manipulating these separately, alongside the non-overlapping pathways engaged by either
828 kind of sleep, should help further disambiguate the functions potentially associated with these
829 distinct sleep stages.

830

831 **Author contributions:** Designed experiments, collected and analyzed data: NA, LALT-H,
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833 PJS. Designed experiments, analyzed data, and wrote the paper: BvS.

834

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837

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839

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1058 **Figure Legends**

1059 **Figure 1. Study rationale and design.** The same genetic background strain (R23E10;UAS-
1060 Chrimson) was used for optogenetic or pharmacologically-induced sleep. Flies were fed
1061 either all-trans retinal (ATR) or 4,5,6,7-tetrahyrdoisoxazolopyridin-3-ol (THIP) to promote
1062 either kind of sleep, which was assessed in three different ways: behavioral analysis, whole
1063 brain imaging, and gene expression changes. The comparisons made for each level of
1064 analysis are labelled A-E.

1065

1066 **Figure 2. Optogenetic- and THIP-induced sleep have similar effects on sleep duration**
1067 **and consolidation. A)** Experimental regime for observing the effects of optogenetic
1068 activation and THIP provision **(B)**. **C)** Sleep profile across 24 hours in the baseline condition
1069 (grey) and optogenetic activation condition (green). **D)** 3-day average of the 24-hour sleep
1070 profile of control (grey) and THIP fed (blue) flies. **E)** Daytime sleep consolidation scatterplot
1071 for optogenetic baseline and THIP control flies. **F)** Daytime sleep consolidation scatterplot
1072 for optogenetic- and THIP-induced sleep. **G)** Night-time sleep consolidation scatterplot for
1073 optogenetic baseline and THIP control flies. **H)** Night-time sleep consolidation scatterplot for
1074 optogenetic- and THIP-induced sleep. n = 87 for optogenetic activation across three
1075 replicates; n = 88 for –THIP, n = 85 for +THIP, across three replicates. Maximum bout
1076 duration possible is 720 minutes, or 12 hours. See Supplementary Figure 1 for summary
1077 histograms and Supplementary Table 1 for statistics.

1078

1079 **Figure 3. Sleep architecture in optogenetic and THIP induced sleep.**
1080 **A-B.** Average number of sleep bouts in control (grey) and optogenetic activation (green)
1081 conditions in the day and night for both +ATR **(A)** and –ATR **(B)** fed flies. optogenetic-
1082 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. *** = $p<0.001$, **** = $p <0.0001$.

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 6µm. Each z-plane spanned 667µm x 667µm, 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 (% ± 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 (± 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 ± 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, * = $p<0.05$, ** = $p<0.01$, **** = $p < 0.001$.

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

1132 THIP in a sleep deprived background versus their control (shaded blue bars). The number of
1133 significant differentially-expressed genes in each category is indicated. **C)** Volcano plot
1134 representing the distribution of differentially expressed genes in the presence or absence of
1135 THIP. Genes that are significantly up/down regulated meeting a Log2Fold change of 0.58
1136 and FDRq value of 0.05 are shown in red. Genes meeting the threshold for FDRq value only
1137 are shown in blue. Fold change only is shown in green. Those genes not meeting any
1138 predetermined criteria are shown in grey. **D)** Volcano plot representing the distribution of
1139 differentially expressed genes in the presence or absence of THIP in a sleep deprived
1140 background. Criteria as above (C). **E)** Schematic representation of Gene Ontology (GO)
1141 enrichment of biological process results. Colour coded to indicate parent and child terms for
1142 comparisons between groups highlighted above (C) - Left and (D) - Right). **F)** Bar chart
1143 representation of a subset of interesting significant GO pathway terms originating from the
1144 organic substance and primary metabolic processes for the dataset shown in (C). **G)**
1145 Comparison between significant gene hits obtained via RNA-Sequencing (Blue) and qRT-
1146 PCR (Grey) in response to THIP, represented by Log2Fold change values. See Supplemental
1147 Tables 2&3 and Supplementary Figures 1&2.
1148
1149 **Figure 7. A variety of biological processes including axon guidance are upregulated**
1150 **during optogenetic-induced sleep.**
1151 **A)** Schematic representation of the experimental set-up and samples processed using RNA-
1152 Sequencing. **B)** Venn diagram showing the gene expression overlap between flies that
1153 experienced 10 hours of optogenetic-induced sleep (ZT10) compared to -ATR controls
1154 (ZT10) and those flies where optogenetic activation was restricted to 1 hour (ZT1) and
1155 compared to -ATR controls (ZT1). **C)** Volcano plot representing the distribution of
1156 differentially expressed genes resulting from optogenetic optogenetic activation for 10 hours

1157 versus control flies which were allowed to sleep spontaneously for 10 hours. Genes that are
1158 significantly up/down regulated meeting a Log2Fold change of 0.58 and FDRq value of 0.05
1159 are shown in red. Genes meeting the threshold for FDRq value only are shown in blue. Fold
1160 change only in green. Those genes not meeting any predetermined criteria are shown in grey.
1161 **D)** Volcano plot representing the distribution of differentially expressed genes resulting from
1162 optogenetic activation for 1 hour versus control flies which were allowed to sleep
1163 spontaneously for 1 hour. Criteria as above (C). **E)** Schematic representation of Gene
1164 Ontology (GO) enrichment of biological process results. Color coded to indicate parent and
1165 child terms comparing flies that had been activated optogenetically for 10 hours versus flies
1166 which had been allowed to spontaneously sleep for the same duration. **F)** Bar chart
1167 representation of a subset of interesting significant GO pathway terms originating from the
1168 regulation of cellular processes and signalling biological processes. **G)** Comparison between
1169 significant gene hits obtained via RNA-Sequencing (Green) and qRT-PCR (Grey) in response
1170 to optogenetic sleep, represented by Log2Fold change values. See Supplemental Tables 4-7
1171 and Supplementary Figures 3&4.
1172
1173 **Figure 8. nAchRα subunit knockouts bidirectionally regulate >5min sleep as well as**
1174 **short sleep. A.** Average total day and night sleep duration (minutes±95% confidence
1175 intervals) in nAchRα knockout mutants, expressed as difference to their respective
1176 background controls (see Methods). α1, N=91; contro1 ($X^{59}w^{1118}$) = 93; α2, N=70; control
1177 (w^{1118}ActinCas9) = 65; α3, N=43; (ActinCas9) =9; α4, N=87; (w^{1118}ActinCas9) =98; α6,
1178 N=91; (w^{1118}ActinCas9) =91; α7, N=94; (ActinCas9) =95. **P*<0.05, ****P*<0.001,
1179 *****P*<0.0001 by t-test adjusted for multiple comparisons. **B.** Left two panels: sleep
1180 architecture for the same six knockout strains as in A (green), shown against their respective
1181 controls (black). Each datapoint is a fly. Right panels: cumulative short sleep (1-5min)

1182 expressed as a percentage of total sleep duration. Data are the from the same experiment as in
1183 A&B. Each datapoint is a fly. ***P*<0.01, ****P*<0.001, *****P*<0.0001 Man-Whitney U Test.
1184 All data were collected over three days and three nights and averaged.
1185
1186 **Figure 9. AkhR knockdown decreases >5min sleep but not short sleep. A,B.** Total sleep
1187 (>5min) in UAS-AkhR:RNAi / R57C10-Gal4 flies (blue, N=126) compared to genetic
1188 controls (light grey: UAS-AkhR:RNAi / + , N= 124; dark grey: R57C10-Gal4 / + , N= 120).
1189 **C.** Sleep architecture (average bout duration versus bout number per fly) in data from A,B. **D.**
1190 Cumulative short sleep (1-5min, expressed as a % of total sleep) in UAS-AkhR:RNAi /
1191 R57C10-Gal4 flies (blue) compared to genetic controls (light grey: UAS-AkhR:RNAi / + ;
1192 dark grey: R57C10-Gal4 / +). Wild-type background (+) is Canton-S(w^{1118}). Each datapoint
1193 is a fly. ****P*<0.001, *****P*<0.0001 Man-Whitney U Test. ns, not significant. All data were
1194 collected over two days and two nights and averaged.
1195
1196 **Figure 10. nAchRα subunit knockdowns in sleep-promoting neurons. A.** Average total
1197 day and night sleep duration (minutes±95% confidence intervals) in UAS-nAchRα RNAi /
1198 R23E10-Gal4 flies, expressed as difference to RNAi / + controls (all were also compared
1199 Gal4 / + controls, not shown). α1 N=60, Gal4 N=45, RNAi N=37; α2 N=21, Gal4 N=24,
1200 RNAi N=50; α3 N=33, Gal4 N=50, RNAi N=59; α4 N=92, Gal4 N=90, RNAi N=80; α5
1201 N=94, Gal4 N=47, RNAi N=74; α6 N=44, Gal4 N=47, RNAi N=43; α7 N=32, Gal4 N=57,
1202 RNAi N=44. ***P*<0.01, ****P*<0.001 by t-test adjusted for multiple comparisons. **B.** Total
1203 >5min sleep duration data for significant knoockdowns in (A). **C.** Cumulative short sleep (1-
1204 5min) expressed as a percentage of total sleep duration. Data are the from the same
1205 experiment as in A&B. Each datapoint is a fly. ****P*<0.001, *****P*<0.0001 Man-Whitney U
1206 Test. All data were collected over two days and two nights and averaged.

1207 **Supplementary Figure Legends**

1208

1209 **Figure S1. Gene Ontology (GO) enrichment analysis for THIP-induced sleep (related to**
1210 **Figure 6).** Significantly downregulated and upregulated GO categories for THIP-sleep (Table
1211 S2), listed from most enriched at the top. Broad GO categories are identified below.

1212

1213 **Figure S2. Gene Ontology (GO) enrichment analysis for THIP-provisioned flies that**
1214 **were sleep deprived (related to Figure 6).** Significantly downregulated and upregulated GO
1215 categories for sleep deprived flies (Table S3), listed from most enriched at the top. Broad GO
1216 categories are identified below.

1217

1218 **Figure S3. Gene Ontology (GO) enrichment analysis for optogenetic-induced sleep**
1219 **(related to Figure 7).** Significantly downregulated and upregulated GO categories for
1220 optogenetic-sleep (Table S4), listed from most enriched at the top. Broad GO categories are
1221 identified below.

1222

1223 **Figure S4. Circadian-related genes uncovered in optogenetic-sleep dataset (related to**
1224 **Figure 7). A.** Zeitgeber (ZT) 10 timepoint was compared with ZT in to uncover potential
1225 circadian-regulated genes, in two separate datasets (-ATR and +ATR). 98 genes were shared
1226 between these datasets. **B.** Of the 98 shared genes, circadian-related processes were highly
1227 enriched. **C.** Expression levels of 7 circadian genes drawn from the two different datasets in
1228 A.

1229

1230 **Figure S5. Summary of different Gene Ontogeny pathways engaged by optogenetic-**
1231 **induced sleep and THIP-induced sleep. A.** Either sleep induction method produces

1232 different levels of activity in the fly brain. We term optogenetic-induced sleep 'active sleep'
1233 because brain activity levels are not different than during wake. We term THIP-induced sleep
1234 'quiet sleep' because neural activity is significant decreased already in the first 5 minutes.
1235 Both of these induced forms of sleep resemble sleep stages seen during spontaneous sleep in
1236 flies. **B.** Number of GO pathways engaged by either induced active or quiet sleep, separated
1237 by upregulated versus downregulated biological processes.
1238
1239 **Figure S6. Waking activity levels of nAchRα knockout mutants.** Top: mutants are
1240 compared to their genetic background strain. Da1-7 = nAchRα1-7. Bottom: statistical tests
1241 for waking activity levels in each knockout compared to its genetic control, during bith day
1242 and night.

1243 **Supplementary Tables**

1244 **Table S1, related to Figure 2. A comparison of sleep duration profiles (min/hr) during**
1245 **optogenetic and THIP induced sleep.** Tested with 2way ANOVA with Tukey's multiple
1246 comparison test.

1247

1248 **Table S2, related to Figure 6.** List of significant THIP-sleep genes. Raw data for all genes.

1249

1250 **Table S3, related to Figure 6.** List of significant sleep-deprivation genes in THIP-fed flies.
1251 Raw data for all genes.

1252

1253 **Table S4, related to Figure 7.** List of significant optogenetic-sleep genes after 10 hours
1254 activation. Raw data for all genes.

1255

1256 **Table S5, related to Figure 7.** List of significant optogenetic-sleep genes after 1 hour of
1257 activation. Raw data for all genes.

1258

1259 **Table S6, related to Figure 7.** List of significant ZT10 vs ZT1 genes in ATR+ dataset. Raw
1260 data for all genes.

1261

1262 **Table S7, related to Figure 7.** List of significant ZT10 vs ZT1 genes in ATR- dataset. Raw
1263 data for all genes.

1264

1265 **Table S8, related to Figures 6 & 7.** Primer list for RT qPCR validation experiments.

1266

1267 **Table S9, related to Figures 6&7.** Raw data and statistics for RT qPCR experiments.

Figure 1

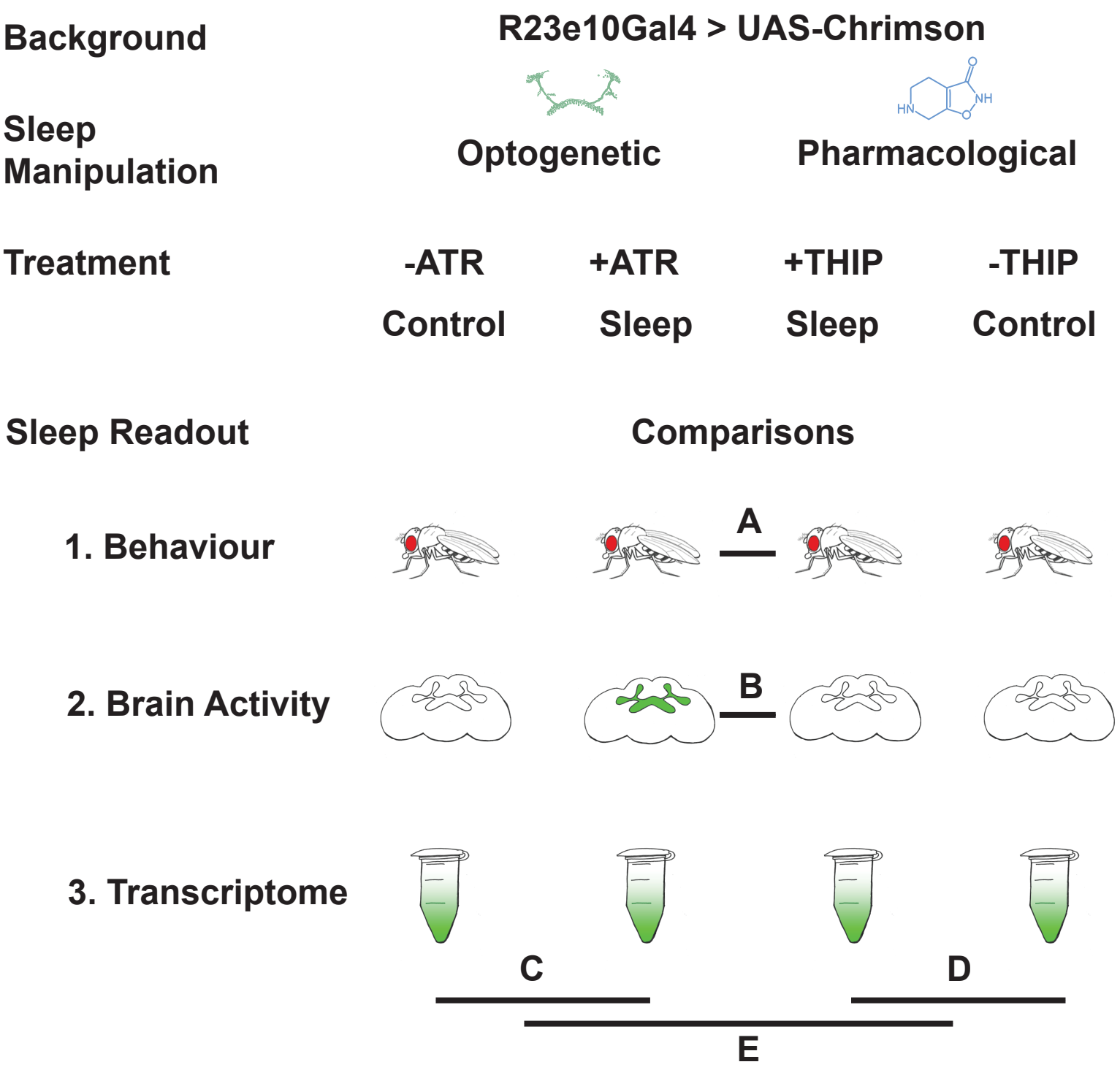

Background
R23e10Gal4 > UAS-Chrimson
Sleep Manipulation
Optogenetic
Pharmacological
HN
NH
O
Treatment
-ATR
+ATR
+THIP
-THIP
Control
Sleep
Sleep
Control
Sleep Readout
Comparisons
1. Behaviour
A
2. Brain Activity
B
3. Transcriptome
C
D
E

Figure 2

A Optogenetically induced sleep

R23E10-Gal4>UAS-chrimson

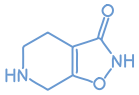
Baseline (control) | Light activation (+ATR)

Baseline (control) | Red light control (-ATR)

Day | Night | Day | Night

8am 8pm 8pm 8am 8pm 8am

B Drug (THIP) induced Sleep



R23E10-Gal4>UAS-chrimson

-THIP (control)

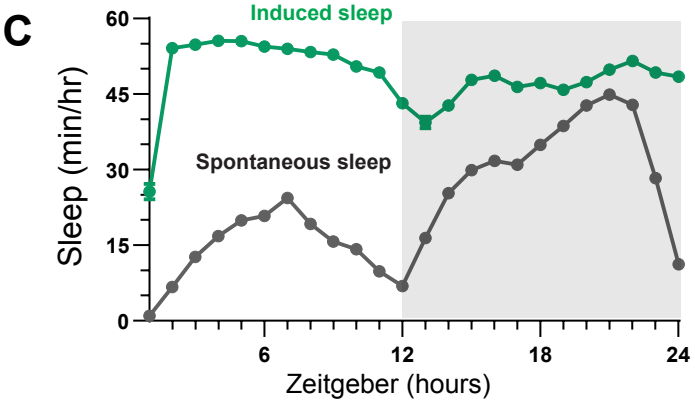
+THIP (drug)

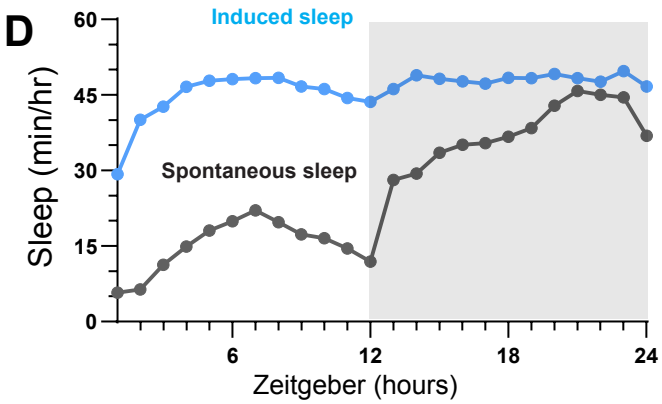
3-day avg.

Day | Night | Day | Night | Day | Night

8am 8pm 8am 8pm 8am 8pm 8am

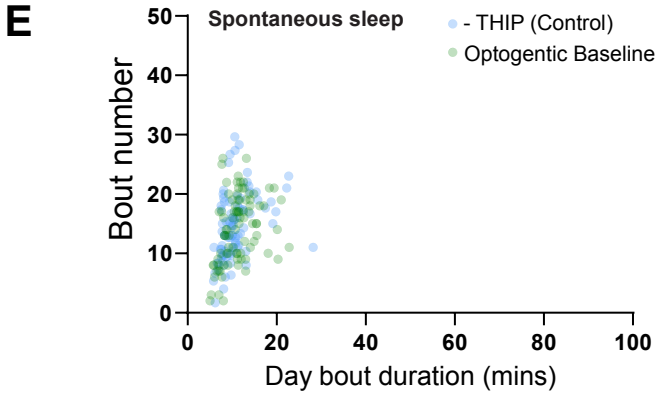
Sleep duration

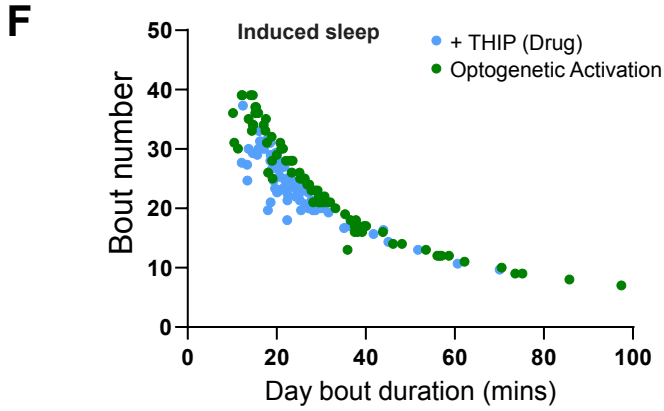




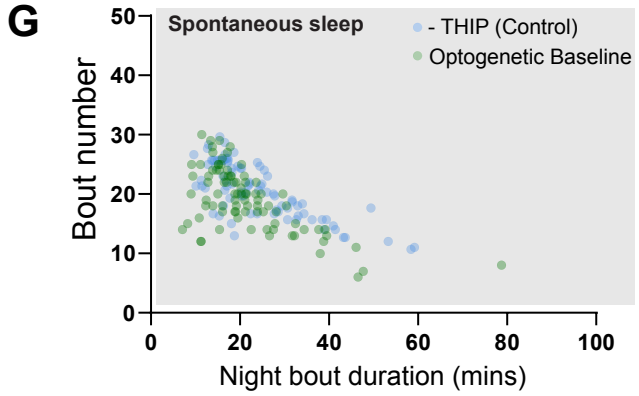
Sleep architecture

Day





Night



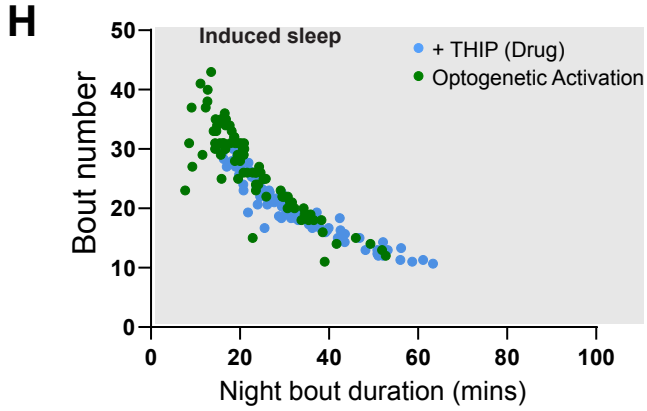


Figure 3

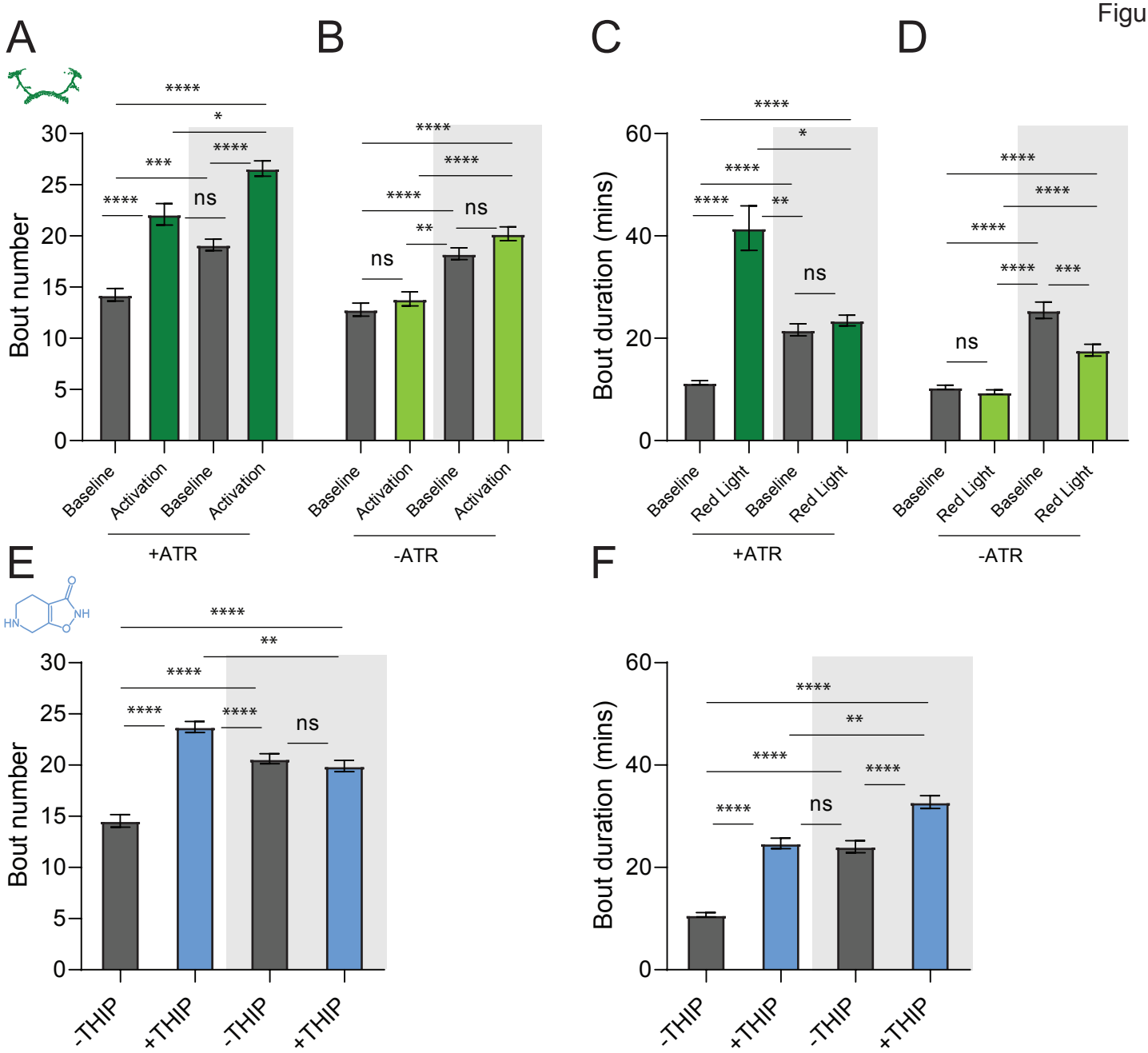

A
B
C
D
E
F
Bout number
Bout duration (mins)
Baseline
Activation
Red Light
+ATR
-ATR
-THIP
+THIP
ns
HN
NH

Figure 4

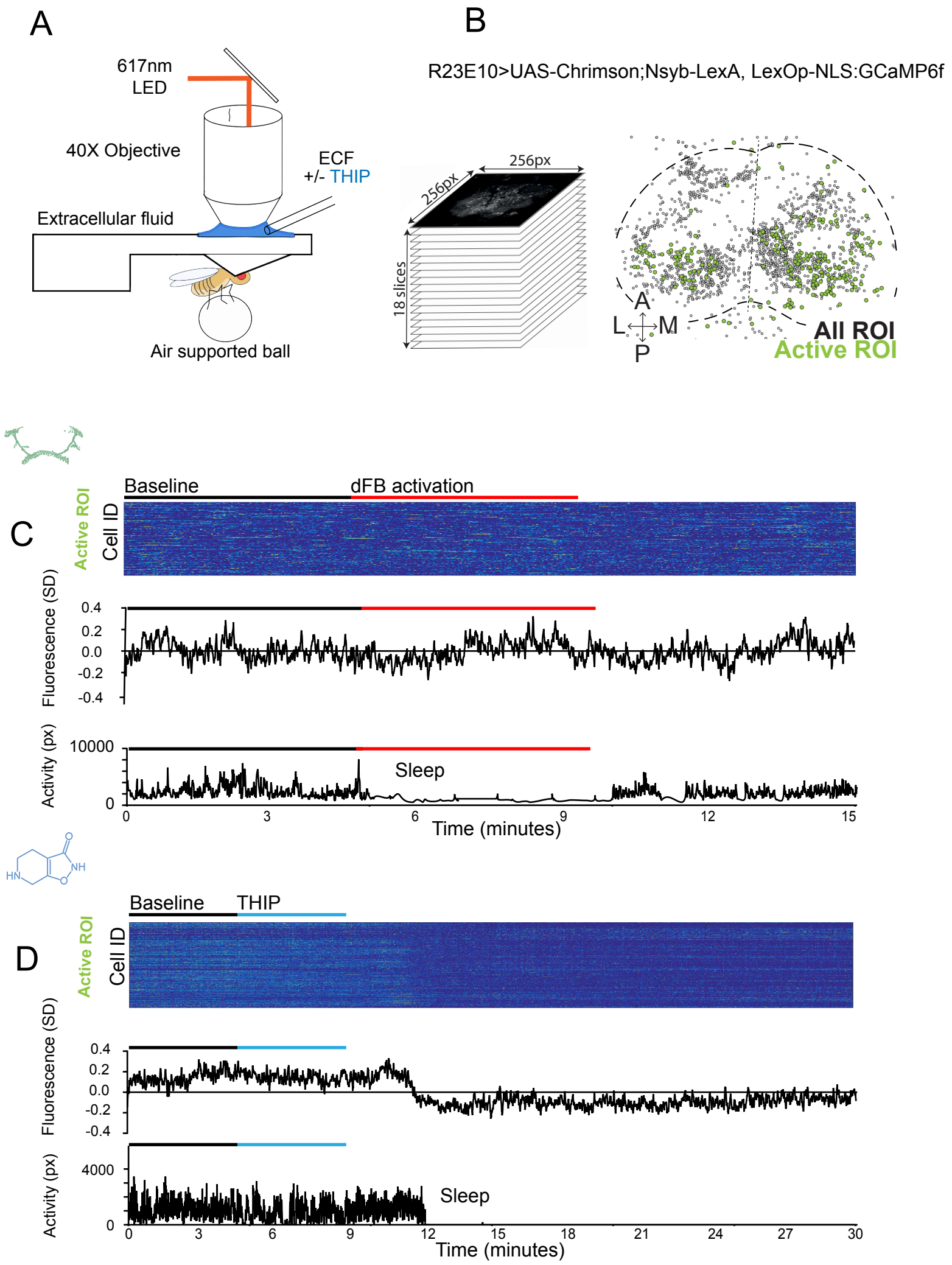

A
617nm
LED
40X Objective
ECF
+/- THIP
Extracellular fluid
Air supported ball
B
R23E10>UAS-Chrimson;Nsyb-LexA, LexOp-NLS:GCaMP6f
256px
256px
18 slices
A
L
M
P
All ROI
Active ROI
C
Baseline
dFB activation
Active ROI
Cell ID
Fluorescence (SD)
0.4
0.2
0.0
-0.2
-0.4
Activity (px)
10000
0
Sleep
0
3
6
9
12
15
Time (minutes)
D
Baseline
THIP
Active ROI
Cell ID
Fluorescence (SD)
0.4
0.2
0.0
-0.2
-0.4
Activity (px)
4000
0
Sleep
0
3
6
9
12
15
18
21
24
27
30
Time (minutes)

Figure 5

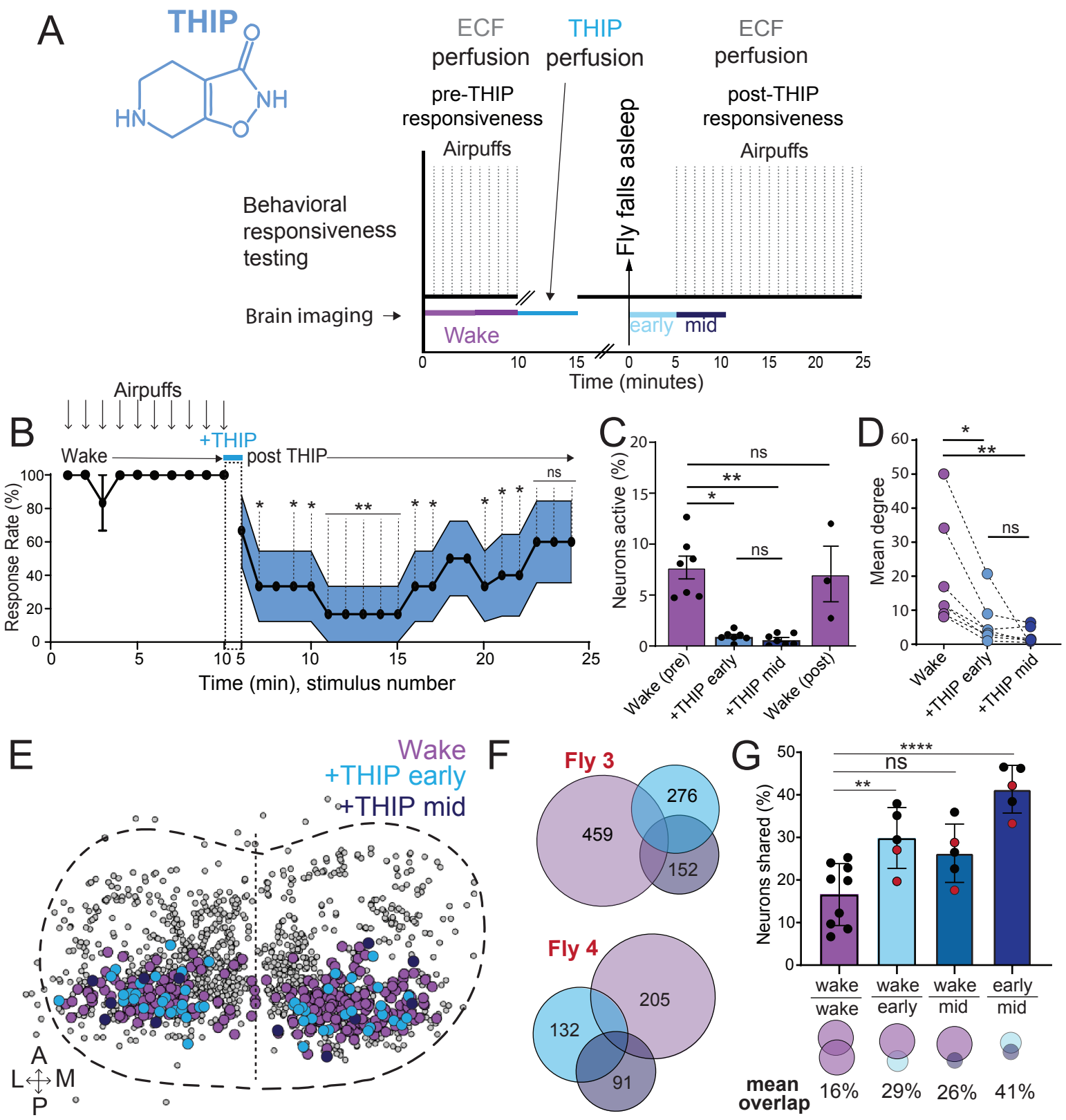
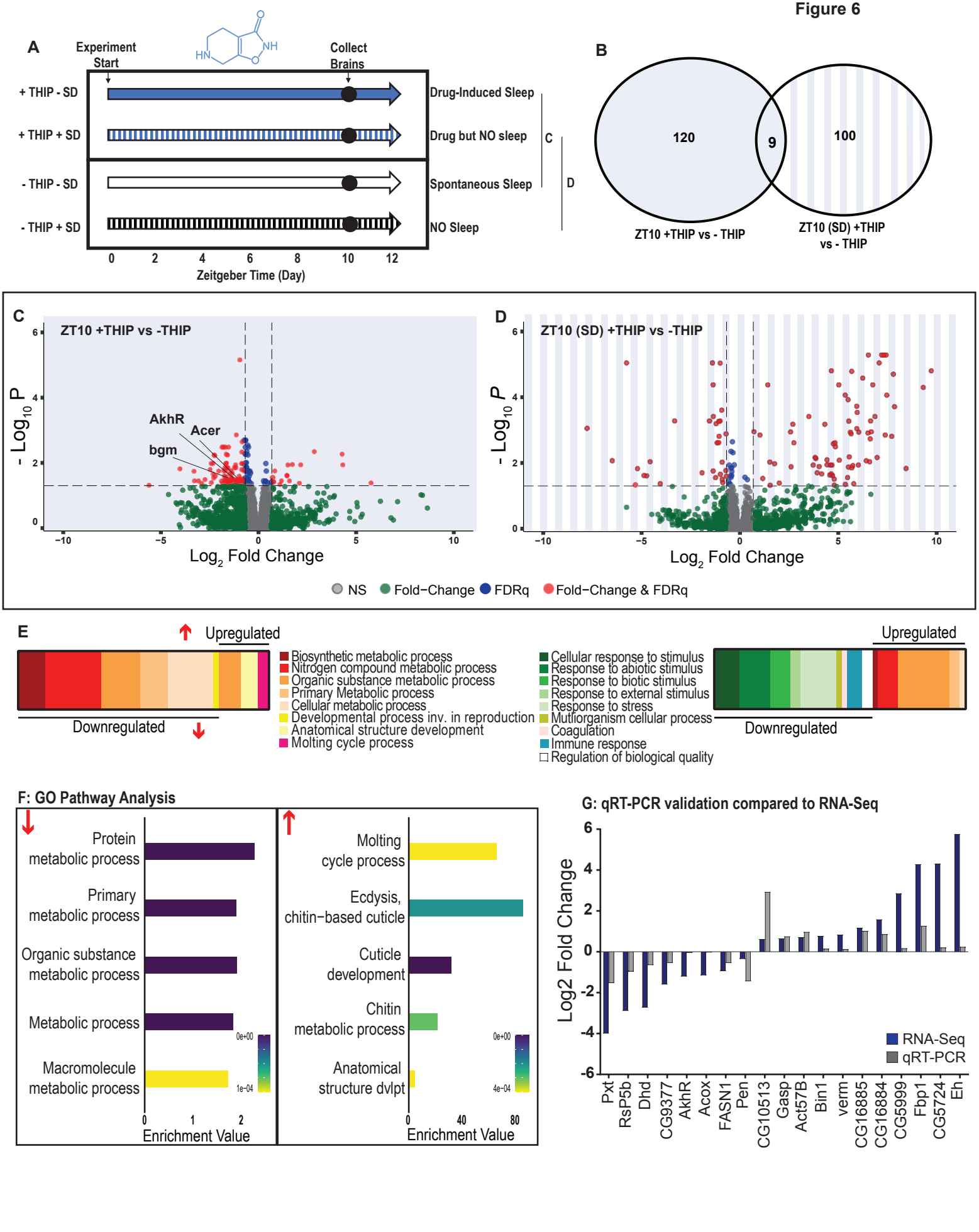
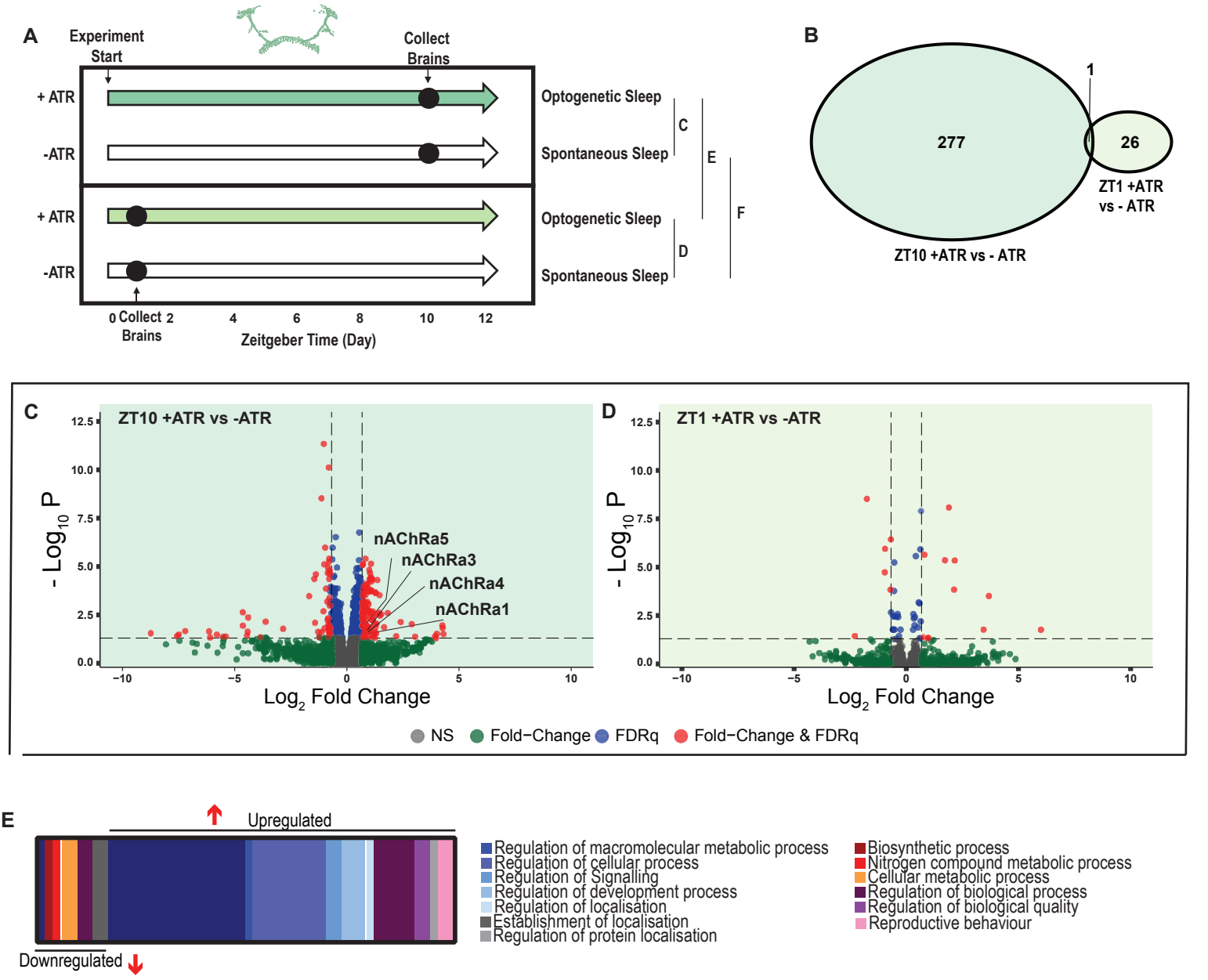
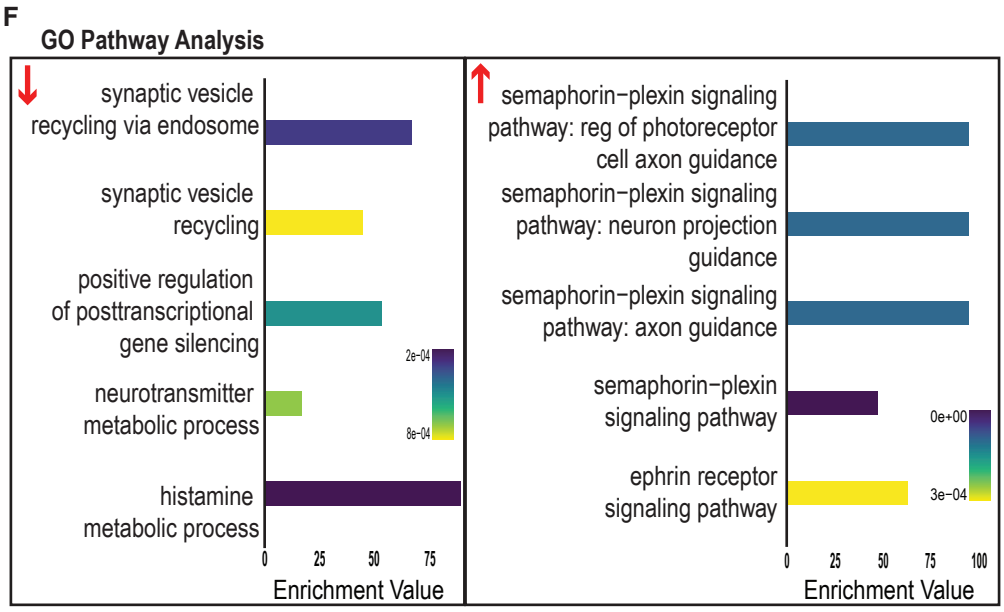

A
THIP
HN
NH
O
ECF perfusion
THIP perfusion
ECF perfusion
pre-THIP responsiveness
post-THIP responsiveness
Airpuffs
Airpuffs
Behavioral responsiveness testing
Fly falls asleep
Brain imaging
Wake
early
mid
0
10
15
0
5
10
15
20
25
Time (minutes)
B
Airpuffs
+THIP
Wake
post THIP
Response Rate (%)
100
80
60
40
20
0
ns
0
5
10 5
10
15
20
25
Time (min), stimulus number
C
Neurons active (%)
20
15
10
5
0
ns
**
*
ns
Wake (pre)
+THIP early
+THIP mid
Wake (post)
D
Mean degree
60
50
40
30
20
10
0
*
**
ns
Wake
+THIP early
+THIP mid
E
Wake
+THIP early
+THIP mid
A
L
M
P
F
Fly 3
459
276
152
Fly 4
205
132
91
G
Neurons shared (%)
50
40
30
20
10
0
****
ns
**
wake
wake
wake
early
wake
mid
mid
mean overlap
16%
29%
26%
41%

Figure 6


A
Experiment Start
Collect Brains
+ THIP - SD
+ THIP + SD
- THIP - SD
- THIP + SD
Drug-Induced Sleep
Drug but NO sleep
Spontaneous Sleep
NO Sleep
C
D
0 2 4 6 8 10 12
Zeitgeber Time (Day)
B
120
9
100
ZT10 +THIP vs - THIP
ZT10 (SD) +THIP vs - THIP
C
ZT10 +THIP vs -THIP
AkhR
Acer
bgm
- Log10 P
Log2 Fold Change
D
ZT10 (SD) +THIP vs -THIP
NS
Fold−Change
FDRq
Fold−Change & FDRq
E
Upregulated
Downregulated
Biosynthetic metabolic process
Nitrogen compound metabolic process
Organic substance metabolic process
Primary Metabolic process
Cellular metabolic process
Developmental process inv. in reproduction
Anatomical structure development
Molting cycle process
Cellular response to stimulus
Response to abiotic stimulus
Response to biotic stimulus
Response to external stimulus
Response to stress
Mutliorganism cellular process
Coagulation
Immune response
Regulation of biological quality
F: GO Pathway Analysis
Protein metabolic process
Primary metabolic process
Organic substance metabolic process
Metabolic process
Macromolecule metabolic process
Molting cycle process
Ecdysis, chitin−based cuticle
Cuticle development
Chitin metabolic process
Anatomical structure dvlpt
Enrichment Value
G: qRT-PCR validation compared to RNA-Seq
Log2 Fold Change
RNA-Seq
qRT-PCR
Pxt
RsP5b
Dhd
CG9377
AkhR
Acox
FASN1
Pen
CG10513
Gasp
Act57B
Bin1
verm
CG16885
CG16884
CG5999
Fbp1
CG5724
Eh


A
Experiment Start
Collect Brains
+ ATR
-ATR
+ ATR
-ATR
Optogenetic Sleep
Spontaneous Sleep
Optogenetic Sleep
Spontaneous Sleep
C
D
E
F
0 Collect Brains 2
4
6
8
10
12
Zeitgeber Time (Day)
B
277
26
1
ZT1 +ATR vs - ATR
ZT10 +ATR vs - ATR
C
ZT10 +ATR vs -ATR
- Log10 P
nAChRa5
nAChRa3
nAChRa4
nAChRa1
Log2 Fold Change
D
ZT1 +ATR vs -ATR
- Log10 P
Log2 Fold Change
NS
Fold−Change
FDRq
Fold−Change & FDRq

E
Upregulated
Downregulated
Regulation of macromolecular metabolic process
Regulation of cellular process
Regulation of Signalling
Regulation of development process
Regulation of localisation
Establishment of localisation
Regulation of protein localisation
Biosynthetic process
Nitrogen compound metabolic process
Cellular metabolic process
Regulation of biological process
Regulation of biological quality
Reproductive behaviour


F
GO Pathway Analysis
synaptic vesicle recycling via endosome
synaptic vesicle recycling
positive regulation of posttranscriptional gene silencing
neurotransmitter metabolic process
histamine metabolic process
2e-04
8e-04
Enrichment Value
semaphorin−plexin signaling pathway: reg of photoreceptor cell axon guidance
semaphorin−plexin signaling pathway: neuron projection guidance
semaphorin−plexin signaling pathway: axon guidance
semaphorin−plexin signaling pathway
ephrin receptor signaling pathway
0e+00
3e−04
Enrichment Value

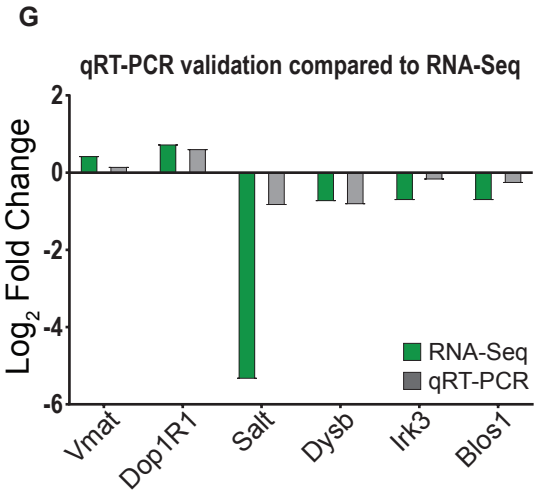
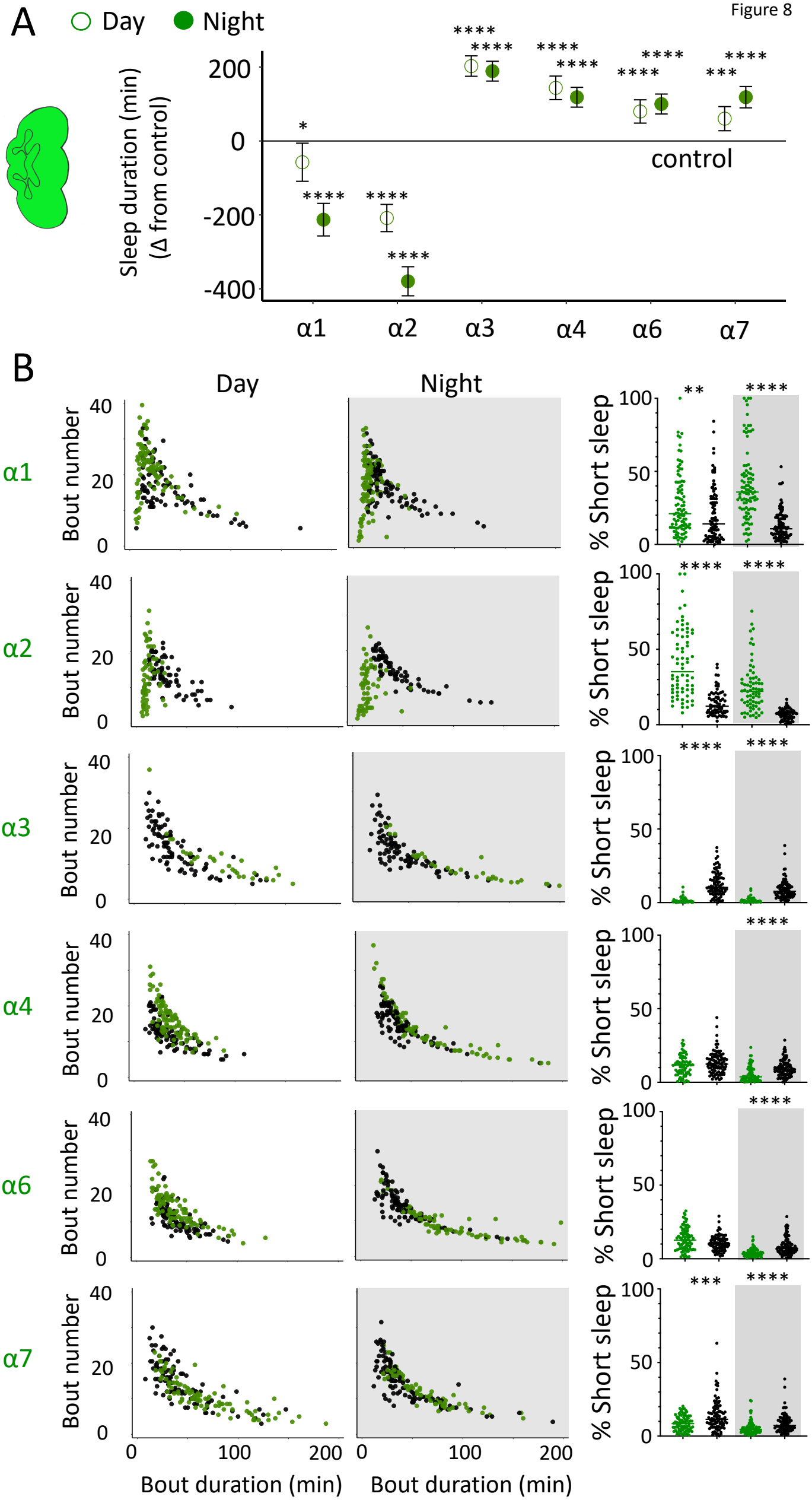

G
qRT-PCR validation compared to RNA-Seq
Log2 Fold Change
RNA-Seq
qRT-PCR
Vmat
Dop1R1
Salt
Dysb
Irk3
Blos1

Figure 8


A
Day
Night
Sleep duration (min)
(Δ from control)
200
0
-200
-400
control
α1
α2
α3
α4
α6
α7
B
Day
Night
Bout number
% Short sleep
Bout duration (min)

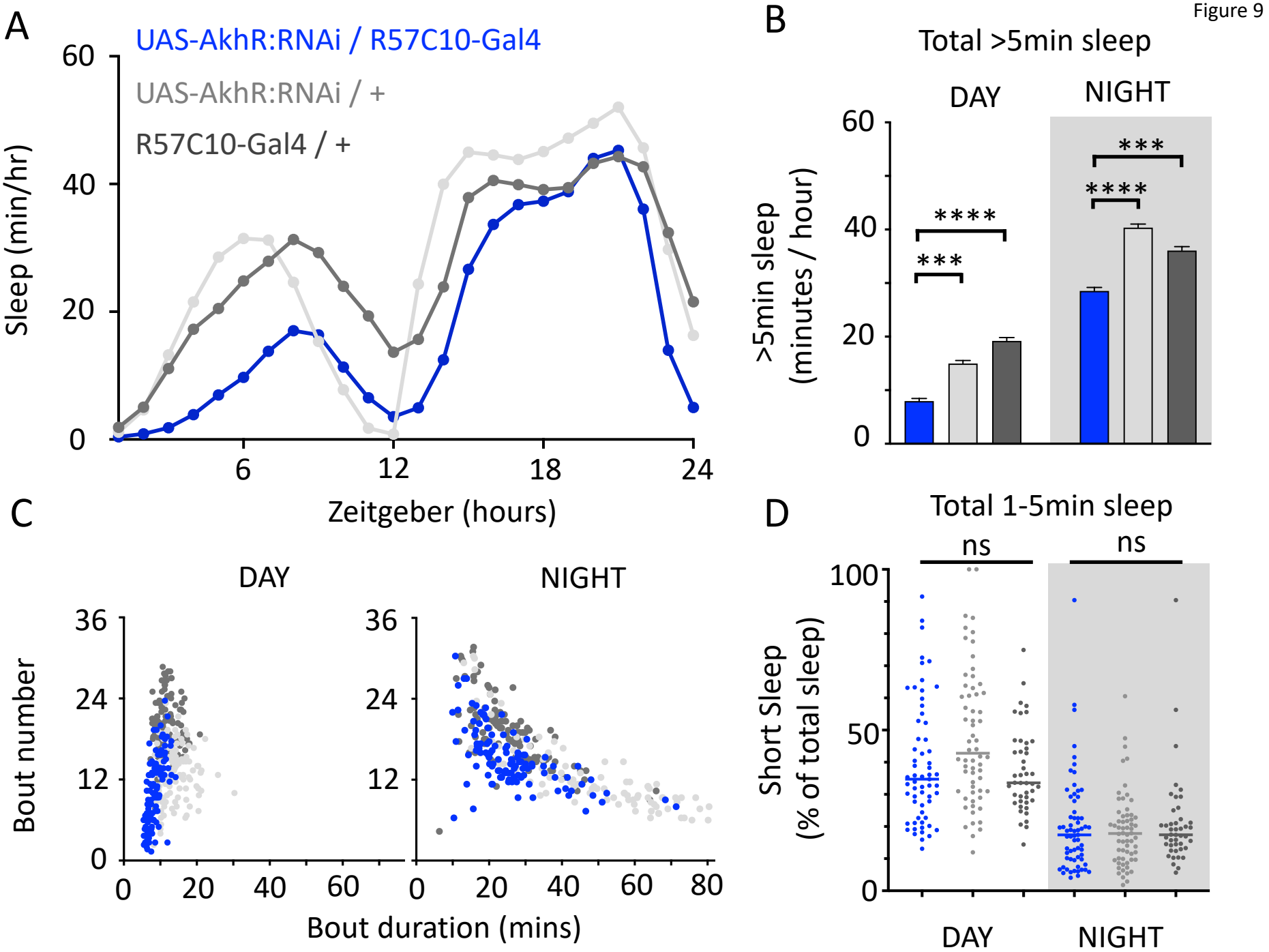
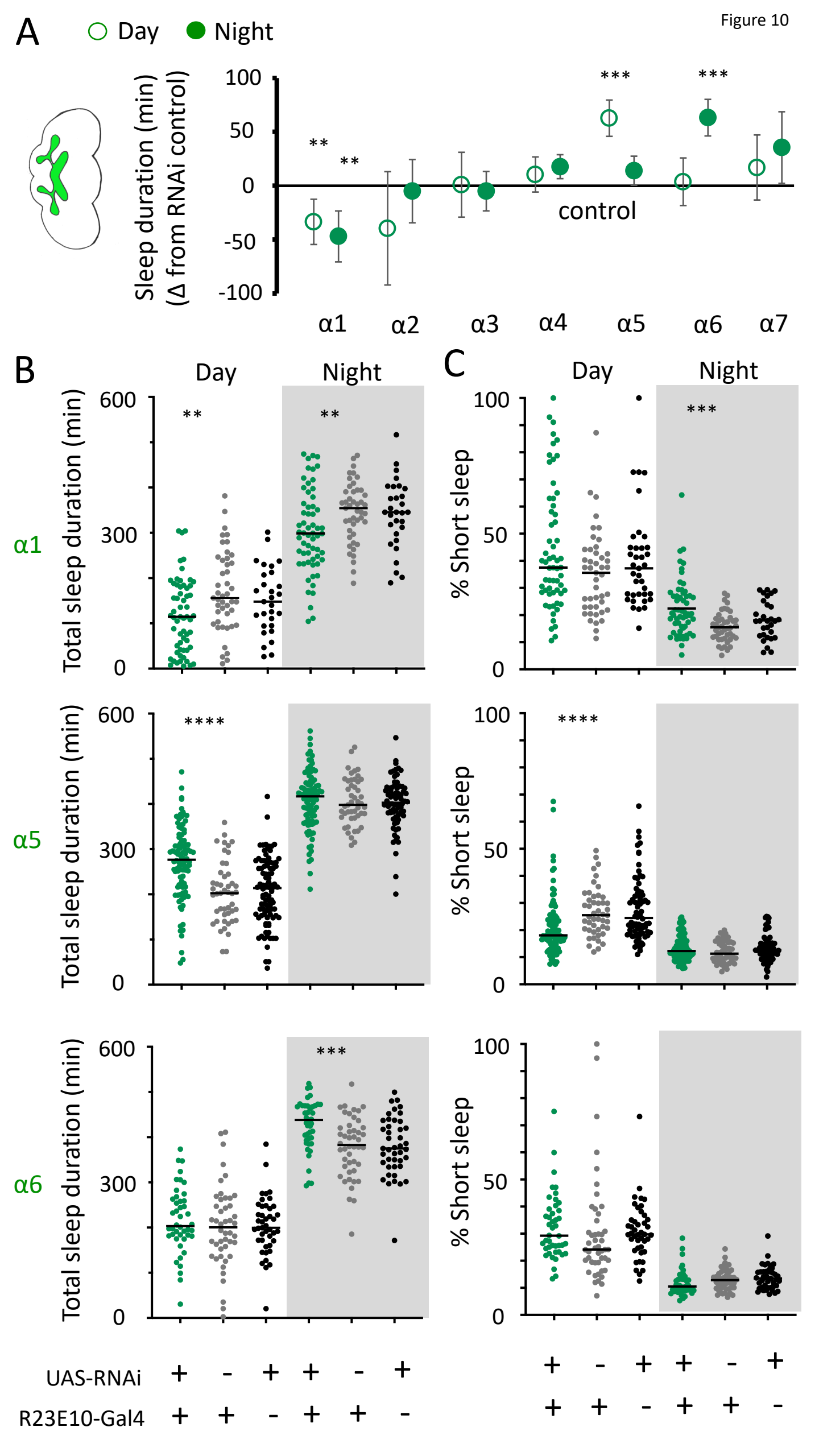

A
UAS-AkhR:RNAi / R57C10-Gal4
UAS-AkhR:RNAi / +
R57C10-Gal4 / +
Sleep (min/hr)
60
40
20
0
6
12
18
24
Zeitgeber (hours)
B
Total >5min sleep
DAY
NIGHT
>5min sleep (minutes / hour)
60
40
20
0
****
***
****
***
C
DAY
NIGHT
Bout number
36
24
12
0
20
40
60
80
Bout duration (mins)
D
Total 1-5min sleep
ns
ns
Short Sleep (% of total sleep)
100
50
0
DAY
NIGHT

Figure 10


A
Day
Night
Sleep duration (min)
(Δ from RNAi control)
100
50
0
-50
-100
**
**
***
***
control
α1
α2
α3
α4
α5
α6
α7
B
Day
Night
Total sleep duration (min)
600
300
0
**
**
α1
****
α5
***
α6
C
% Short sleep
100
50
0
***
****
UAS-RNAi
R23E10-Gal4

Supplementary Figure 1

	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

Metabolic process

- **Biosynthetic metabolic process**
- **Nitrogen compound metabolic process**
- **organic substance metabolic process**
- **primary metabolic process**
- **Cellular metabolic process**

Developmental Process

- **Developmental process involved in reproduction**
- **Anatomical structure development**

Mutlicellular organismal process

- **Molting cycle process**

	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

Response to stimulus
- Cellular response to stimulus
- Response to abiotic stimulus
- Response to biotic stimulus
- Response to external stimulus
- Response to stress

Mutli-organism process
- Mutliorganism cellular process

MutliCellular organismal process
- Coagulation

Immune system process
- Immune response

Biological regulation
- Regulation of biological quality

Metabolic process
- Metabolic process
- Nitrogen compound metabolic process
- Organic substance
- Primary Metabolic process
- Other

Supplementary Figure 3

	GO Term	Pvalue	Enrichment value
Downregulated	GO:0060148 positive regulation of posttranscriptional gene silencing	0.0005	53.51
	GO:0002181 cytoplasmic translation	0	9.46
	GO:0052803 imidazole-containing compound metabolic process	0.0002	89.19
	GO:0001692 histamine metabolic process	0.0002	89.19
	GO:0042133 neurotransmitter metabolic process	0.0007	16.72
	GO:1900368 regulation of RNA interference	0.0001	133.79
	GO:1900370 positive regulation of RNA interference	0.0001	133.79
	GO:0036466 synaptic vesicle recycling via endosome	0.0003	66.89
	GO:0036465 synaptic vesicle recycling	0.0008	44.6
Upregulated	GO:2000766 negative regulation of cytoplasmic translation	0.0003	63.5
	GO:0017148 negative regulation of translation	0.0002	9.16
	GO:0034249 negative regulation of cellular amide metabolic process	0.0004	7.81
	GO:0006417 regulation of translation	0.0001	5.69
	GO:0034248 regulation of cellular amide metabolic process	0.0001	4.68
	GO:0010608 posttranscriptional regulation of gene expression	0.0001	4.61
	GO:0010468 regulation of gene expression	0	2.21
	GO:0031326 regulation of cellular biosynthetic process	0.0001	2.04
	GO:0009889 regulation of biosynthetic process	0.0001	2.04
	GO:0010556 regulation of macromolecule biosynthetic process	0.0004	1.98
	GO:0060255 regulation of macromolecule metabolic process	0.0001	1.86
	GO:0019222 regulation of metabolic process	0.0001	1.81
	GO:0051171 regulation of nitrogen compound metabolic process	0.0005	1.76
	GO:0080090 regulation of primary metabolic process	0.0006	1.74
	GO:0031323 regulation of cellular metabolic process	0.0007	1.71
	GO:0046011 regulation of oskar mRNA translation	0	29.31
	GO:1902287 semaphorin-plexin signaling pathway involved in axon guidance	0.0001	95.26
	GO:1902285 semaphorin-plexin signaling pathway involved in neuron projection guidance	0.0001	95.26
	GO:0045876 positive regulation of sister chromatid cohesion	0.0001	95.26
	GO:2000305 semaphorin-plexin signaling pathway involved in reg of photoreceptor cell axon guidance	0.0001	95.26
	GO:0048013 ephrin receptor signaling pathway	0.0003	63.5
	GO:0071526 semaphorin-plexin signaling pathway	0	47.63
	GO:0007162 negative regulation of cell adhesion	0.0006	17.86
	GO:0051128 regulation of cellular component organization	0.0002	2.4
	GO:0048522 positive regulation of cellular process	0.0007	1.88
	GO:0099177 regulation of trans-synaptic signaling	0.0001	5.82
	GO:0050804 modulation of chemical synaptic transmission	0.0001	5.82
	GO:0016319 mushroom body development	0.001	6.52
	GO:2000026 regulation of multicellular organismal development	0.0007	2.57
	GO:0032502 developmental process	0.0001	1.81
	GO:0032879 regulation of localization	0.0006	2.72
	GO:2000112 regulation of cellular macromolecule biosynthetic process	0.0004	1.98
	GO:0050794 regulation of cellular process	0	1.84
	GO:0048518 positive regulation of biological process	0.0008	1.8
	GO:0050789 regulation of biological process	0	1.7
	GO:0065007 biological regulation	0	1.63
	GO:0042391 regulation of membrane potential	0.0008	6.9
	GO:0065008 regulation of biological quality	0.0002	2.12
	GO:0120187 positive regulation of protein localization to chromatin	0.0001	95.26
	GO:0008049 male courtship behavior	0.0003	8.36
	GO:0060179 male mating behavior	0.0005	7.56

Regulation of biological process
- **Regulation of metabolic process**
- **Regulation of macromolecular metabolic process**
- **Regulation of cellular process**
- **Regulation of Signalling**
- **Regulation of development process**
- **Regulation of localisation**

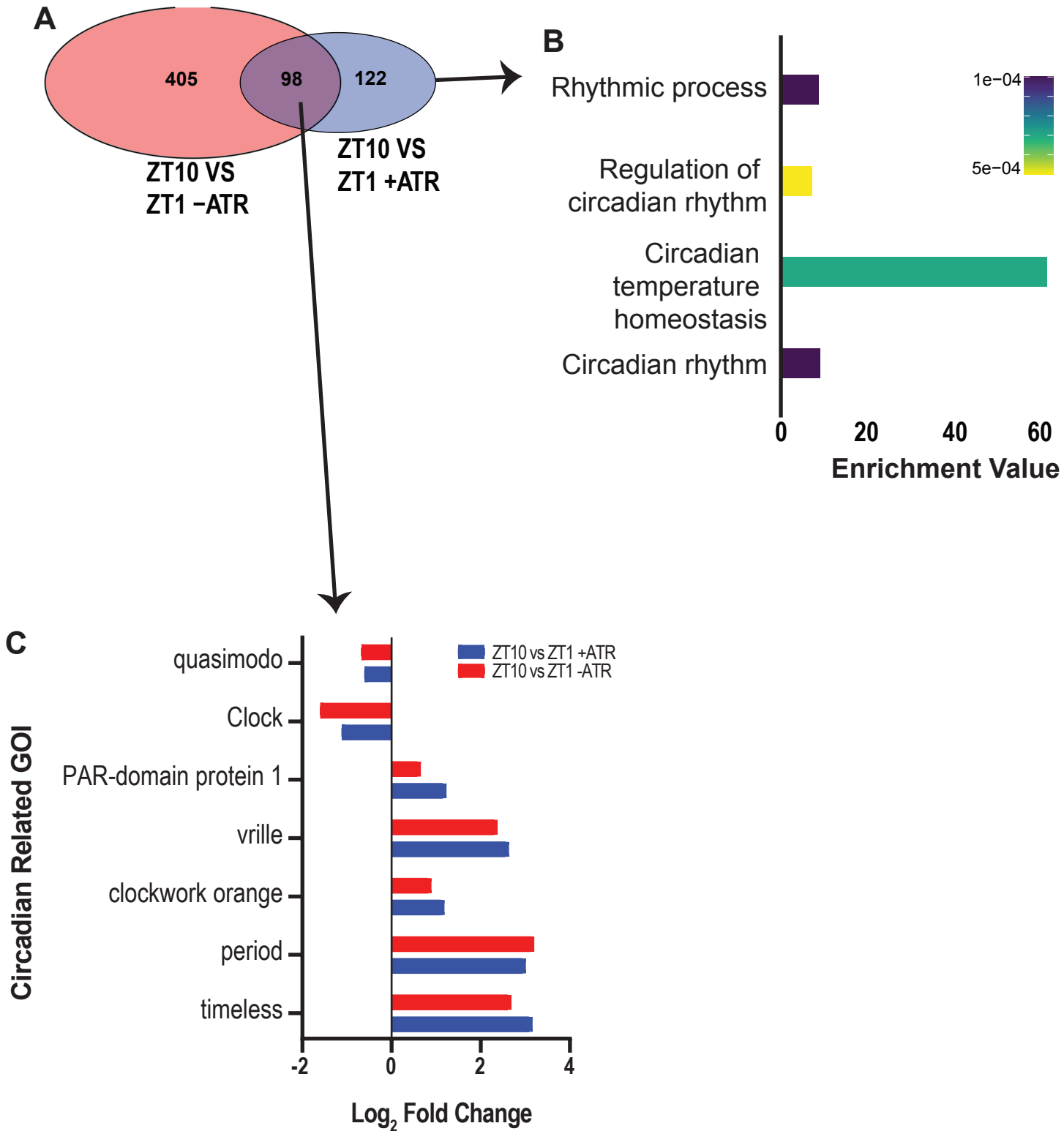
Metabolic process
- **Biosynthetic process**
- **Nitrogen compound metabolic process**
- **cellular metabolic process**

Biological Regulation
- **Regulation of biological process**
- **Regulation of biological quality**

Localisation
- **Establishment of localisation**
- **Regulation of protein localisation**

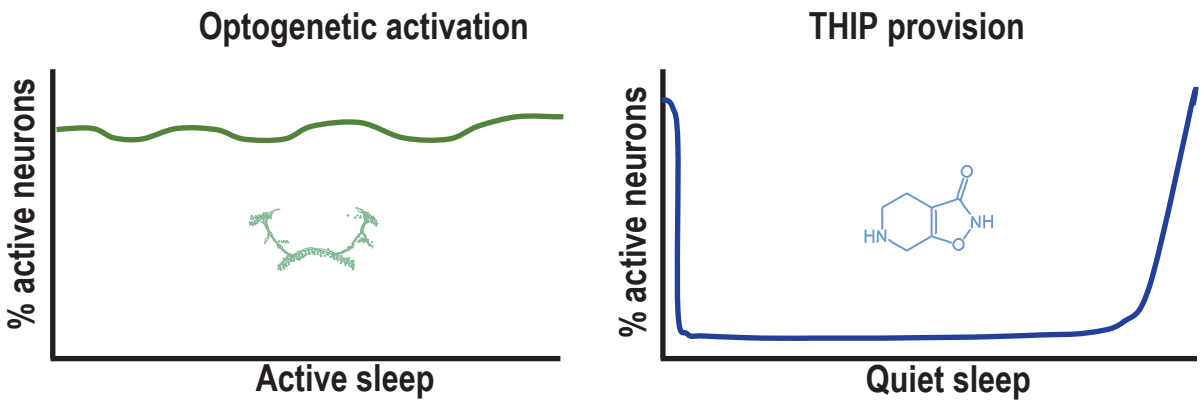
Behaviour
- **Reproductive behaviour**

Supplementary Figure 4

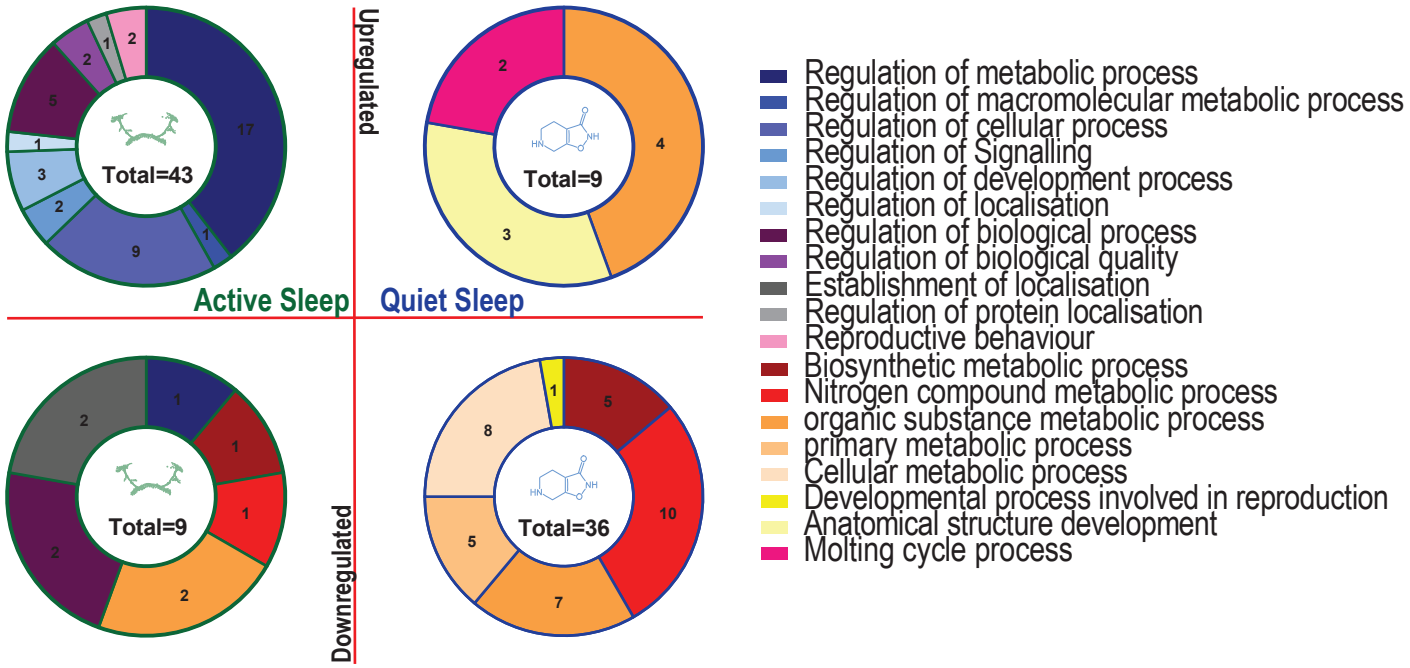


Supplementary Figure 5

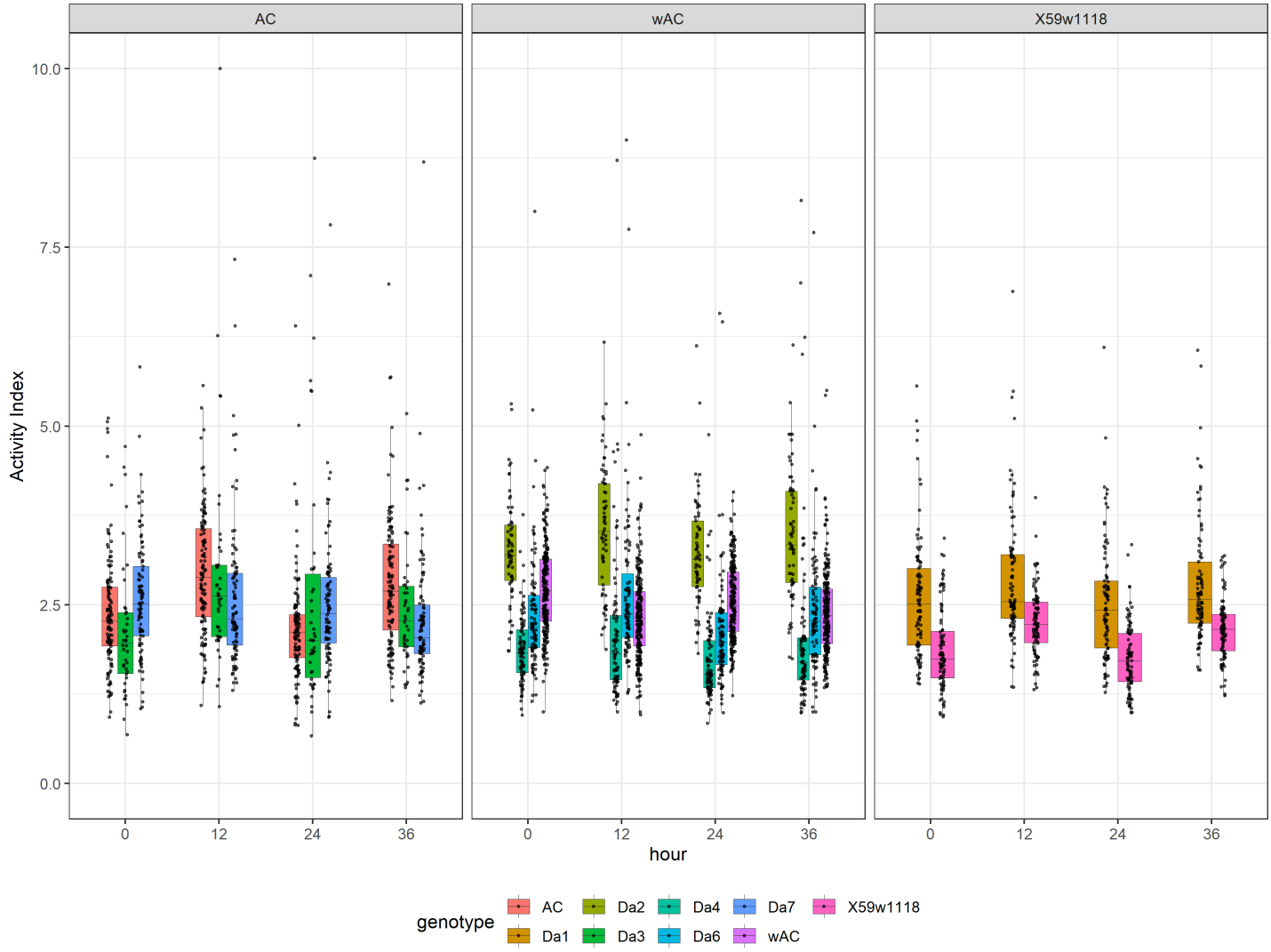
A: Sleep induction method



B: Induced Sleep Transcriptome: GO Pathways of Biological Processes



Supplementary Figure 6



ttest_AI

hour	gene	estimate	estimate1	estimate2	.y.	group1	group2	n1	n2	statistic	p	df	conf.low	conf.high	method	alternative	p.adj	p.adj.signif
Day	Da1	0.747011947979923	1.81085006902361	2.55786201700353	activity_index	control	knockout	91	93	-7.68674248159984	1.63e-12	154.732513840803	0.938986358845125	0.555037537114721	T-test	two.sided	1.63e-11	****
Night	Da1	0.621402556761431	2.20459173404308	2.82599429080452	activity_index	control	knockout	91	93	-6.52394430852639	1.39e-09	129.928135878636	0.809843317756889	0.432961795765974	T-test	two.sided	9.73e-09	****
Day	Da2	0.802741622150127	2.43199942296601	3.23474104511614	activity_index	control	knockout	70	65	-7.4427805261935	2.03e-11	113.734263925721	1.01640704197762	0.589076202322632	T-test	two.sided	1.827e-10	****
Night	Da2	1.23901539806697	2.26258176444676	3.50159716251373	activity_index	control	knockout	70	65	-10.1194970663198	8.52e-17	95.6867888875377	1.48206392653957	0.995966869594365	T-test	two.sided	1.1076e-15	****
Day	Da3	0.415534948767109	2.16037312206754	2.57590807083465	activity_index	control	knockout	91	43	-1.34323807941607	0.186	45.4132347049576	1.03844775713935	-0.207377859605138	T-test	two.sided	0.744	ns
Night	Da3	-0.0227331901857819	2.83159939569465	2.80886620550887	activity_index	control	knockout	91	43	0.0990781987694288	0.921	55.2553143772094	0.43704101471215	-0.482507395083714	T-test	two.sided	1	ns
Day	Da4	-0.61837129538045	2.42331822907521	1.80494693369476	activity_index	control	knockout	98	87	8.66914228246151	2.56e-15	178.965595681112	-0.47761492132397	-0.75912766943693	T-test	two.sided	2.816e-14	****
Night	Da4	-0.110300614309692	2.26586151382992	2.15556089952023	activity_index	control	knockout	98	87	0.835733550651872	0.405	115.736113589999	0.151109873134961	-0.371711101754344	T-test	two.sided	1	ns
Day	Da6	-0.623668327284424	2.88534347323253	2.26167514594811	activity_index	control	knockout	91	91	6.64405236041315	5.29e-10	150.02407366578	-0.43819297313673	-0.809143681432117	T-test	two.sided	4.232e-09	****
Night	Da6	0.0488610995058534	2.54149490606688	2.59035600557274	activity_index	control	knockout	91	91	-0.476197508862764	0.635	153.272240702089	0.251567217217754	-0.153845018206047	T-test	two.sided	1	ns
Day	Da7	0.326181724510013	2.22741317915403	2.55359490366404	activity_index	control	knockout	95	94	-3.12088657353367	0.00211	176.4794317341	0.532443223747717	0.119920225272308	T-test	two.sided	0.01266	*
Night	Da7	-0.236825775153197	2.70854101277	2.4717152376168	activity_index	control	knockout	95	94	1.93009604618506	0.0552	181.674501771687	0.00527760128503382	-0.478929151591428	T-test	two.sided	0.276	ns