Supplemental Information

Intrinsic maturation of sleep output neurons

regulates sleep ontogeny in *Drosophila*

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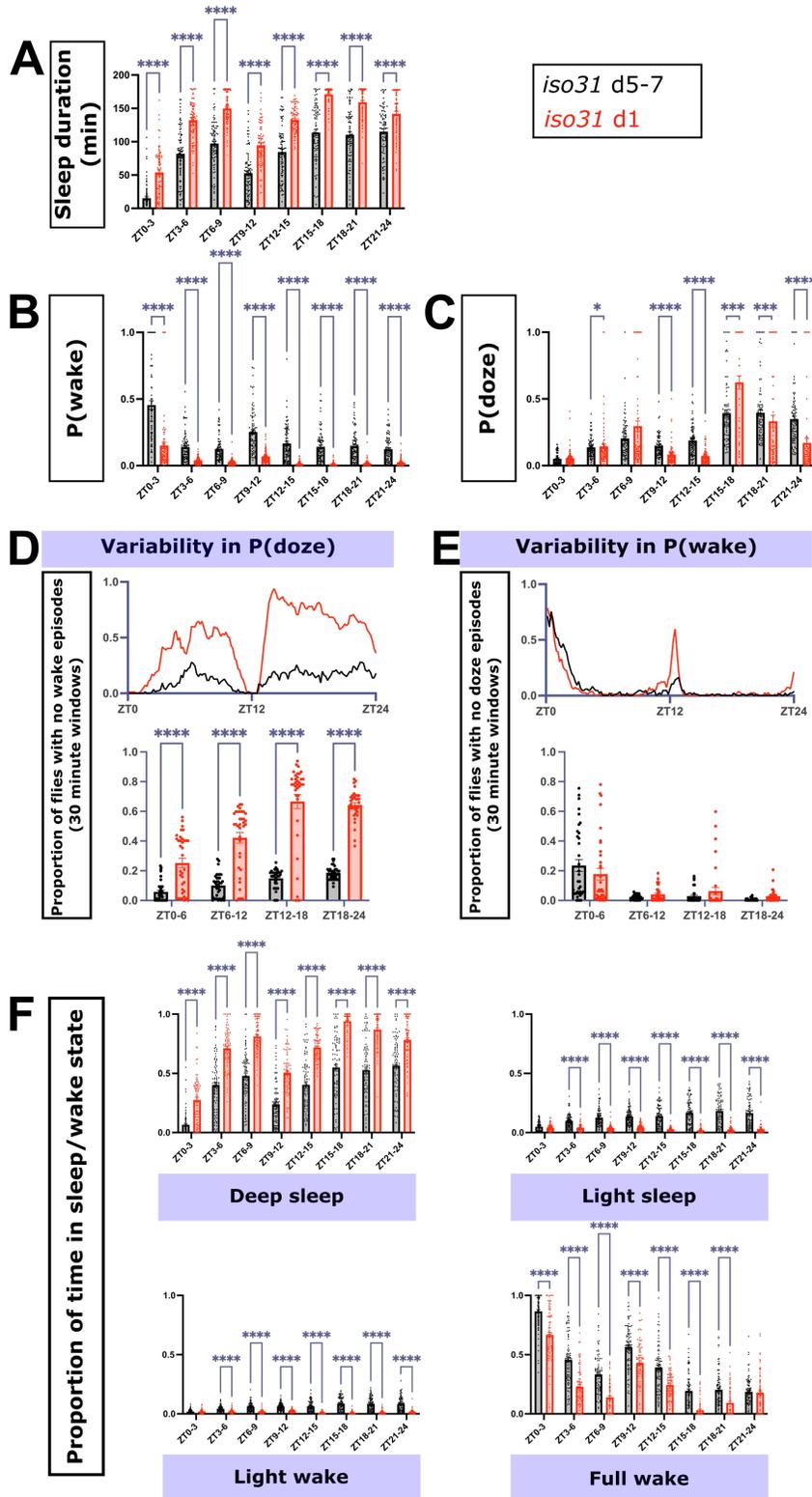
Figure S1: Sleep metrics of *iso31* flies binned in 3-hour intervals and P(doze) variability. Related to Figure 1. A) Sleep duration, B) P(wake), and C) P(doze) in mature (black, n = 87) and juvenile (red, n = 82) *iso31* flies binned into 3-hour intervals across 24 hours. Proportion of undefined D) P(doze) and E) P(wake) values across 24 hours in mature and juvenile flies shown in Figure 1 Top traces are a rolling 30-minute window sampled every 10 minutes, Bottom graphs show the average proportion of undefined values per 30-minute window across 6-hour intervals. (F) Proportion of time spent in deep sleep, light sleep, light wake, and full wake (Mann-Whitney U tests).

A *iso31* D5-7
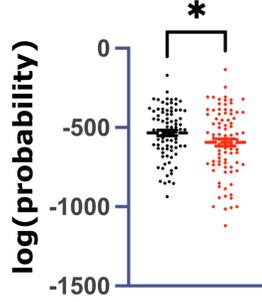
B *iso31* D1
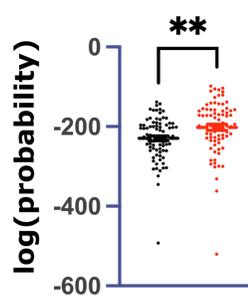
HMM-mature
HMM-juvenile

Applying HMM-mature:

C ZT0-12

D ZT12-24
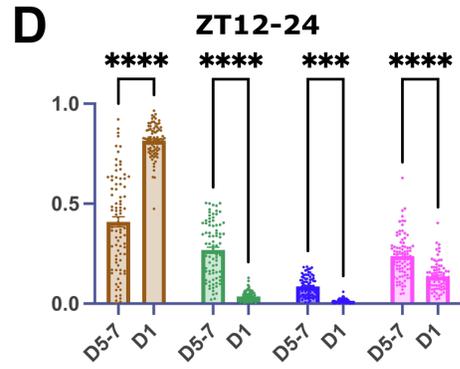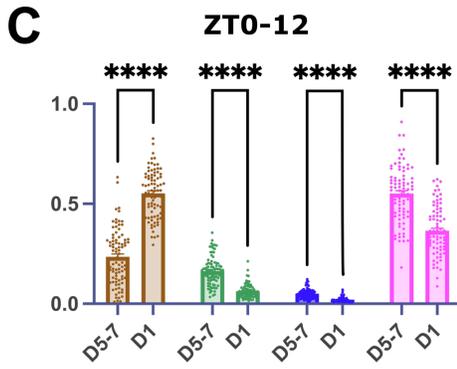
Applying HMM-juvenile:

E ZT0-12

F ZT12-24
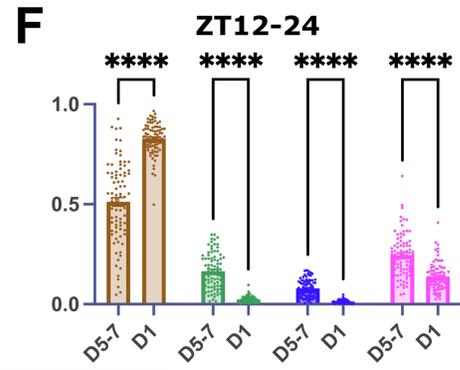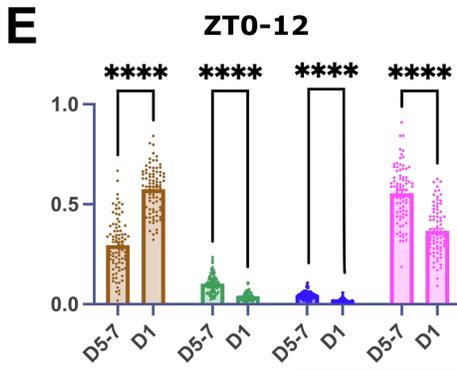
Proportion of time in sleep/wake state

Deep sleep
Light sleep
Light wake
Full wake

Figure S2: HMMs trained on mature vs juvenile *iso31* fly locomotor datasets.

Related to Figure 1. Log(probability) of observing a given sequence of locomotor behavior in A) mature or B) juvenile *iso31* flies by applying HMM-mature (black, n = 87) or HMM-juvenile (red, n = 82) (two-tailed T-tests). Proportion of time spent in each sleep/wake hidden state in mature vs juvenile flies from ZT0-12 and ZT12-24 when applying C, D) HMM-mature or E, F) HMM-juvenile (two-way ANOVA with post-hoc Sidak's multiple comparison test).
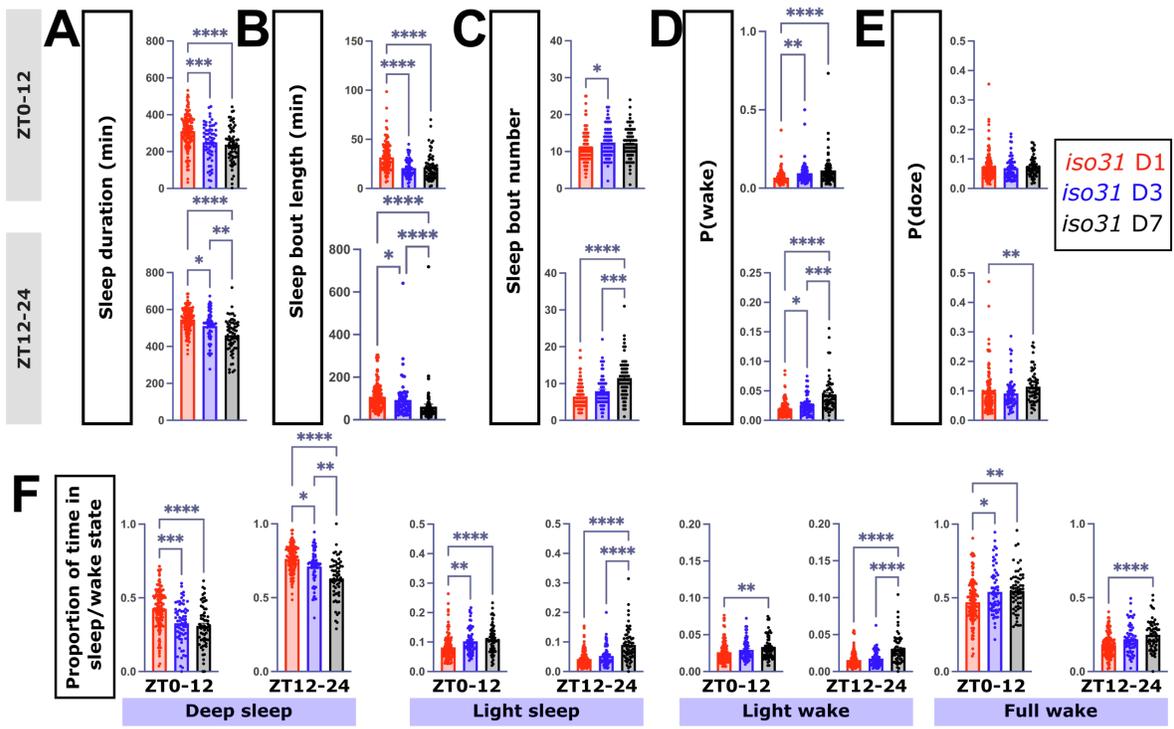
Figure S3: Developmental progression of sleep architecture and substates.

Related to Figure 1. A) Sleep duration, B) bout length, C) bout number, D) P(wake), and E) P(doze) in *iso31* flies at post-eclosion day 1 (red, n = 127), 3 (blue, n = 64), and 7 (black, n = 64) from ZT0-12 (top) and ZT12-24 (bottom). Proportion of time spent in F) deep sleep, light sleep, light wake, and full wake across the three age groups from ZT0-12 (left) and ZT12-24 (right) (Kruskall-Wallis with post-hoc Dunn's multiple comparison test).

iso31 d5-7
iso31 d1

| ZT0-12 | ZT12-24 |

A

$m = 1.443 \times 10^{-3} \pm 2.84 \times 10^{-5}$
$r^2 = 0.9681$

$m = 1.407 \times 10^{-3} \pm 1.225 \times 10^{-5}$
$r^2 = 0.9940$

$m = 1.605 \times 10^{-3} \pm 2.65 \times 10^{-5}$
$r^2 = 0.9834$

$m = 1.397 \times 10^{-3} \pm 1.162 \times 10^{-5}$
$r^2 = 0.9945$

Deep sleep

B

$m = -1.383 \times 10^{-3} \pm 7.970 \times 10^{-5}$
$r^2 = 0.03422$

$m = 4.298 \times 10^{-5} \pm 4.564 \times 10^{-5}$
$r^2 = 0.01096$

$m = -7.824 \times 10^{-4} \pm 5.592 \times 10^{-5}$
$r^2 = 0.6973$

$m = -2.143 \times 10^{-4} \pm 4.157 \times 10^{-5}$
$r^2 = 0.2493$

Light sleep

C

$m = -5.365 \times 10^{-5} \pm 2.691 \times 10^{-5}$
$r^2 = 0.04467$

$m = 1.062 \times 10^{-8} \pm 1.617 \times 10^{-5}$
$r^2 = 5.389 \times 10^{-9}$

$m = -2.839 \times 10^{-4} \pm 1.752 \times 10^{-5}$
$r^2 = 0.7555$

$m = -8.299 \times 10^{-5} \pm 1.648 \times 10^{-5}$
$r^2 = 0.2408$

Light wake

D

$m = -1.251 \times 10^{-3} \pm 8.340 \times 10^{-5}$
$r^2 = 0.7259$

$m = -1.364 \times 10^{-3} \pm 5.263 \times 10^{-5}$
$r^2 = 0.8936$

$m = -5.388 \times 10^{-4} \pm 5.219 \times 10^{-5}$
$r^2 = 0.5563$

$m = -1.1 \times 10^{-3} \pm 5.11 \times 10^{-5}$
$r^2 = 0.8528$

Full wake

Proportion of time in sleep/wake state

Sleep duration (min)

Figure S4: Correlation between sleep duration and proportion of time spent in a given sleep state. Related to Figure 1. Sleep duration vs proportion of time spent in A) deep sleep, B) light sleep, C) light wake, and D) full wake in individual mature (black) and juvenile *iso31* flies (red). The slope of the linear regressions are shown with ± SEM, along with r^2 values, and color-coded according to the dataset used for the regression (mature or juvenile).

A ZT0-12, ZT12-24 — Sleep duration (min)

B — Sleep bout length (min)

C — Sleep bout number

D — P(wake)

E — P(doze)

D5 virgin
D5 mated

F — Proportion of time in sleep/wake state: Deep sleep, Light sleep, Light wake, Full wake (ZT0-12, ZT12-24)
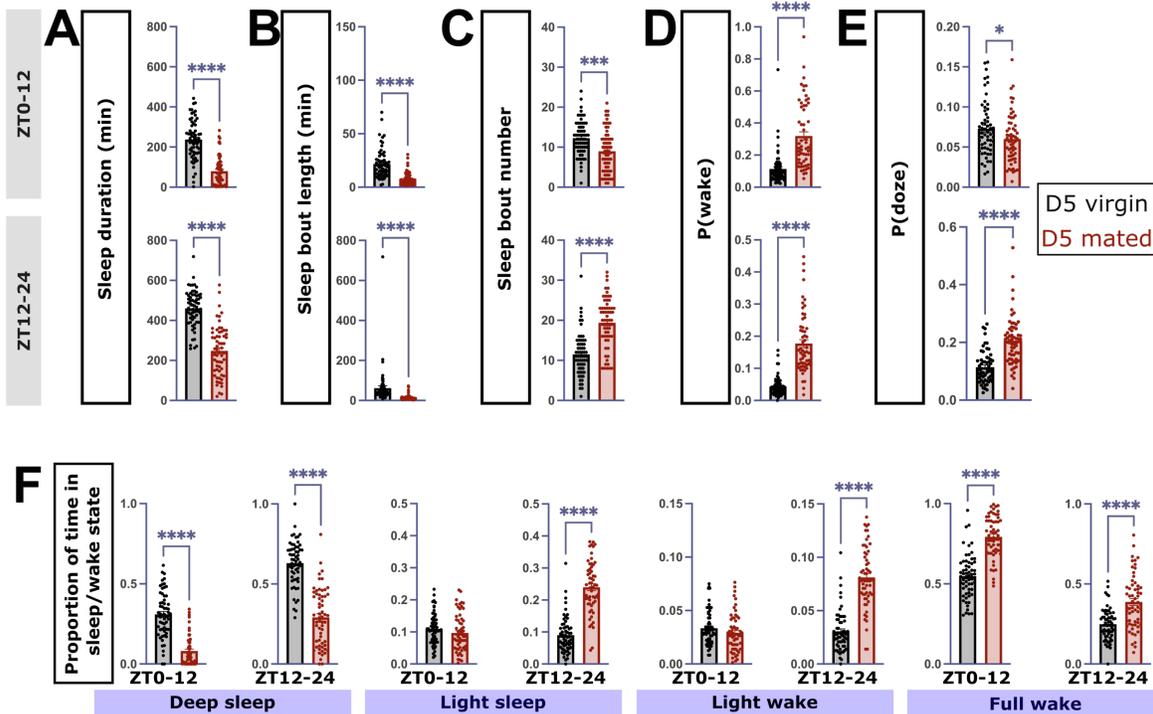
Figure S5: Mating status affects sleep architecture. Related to Figure 1. A) Sleep duration, B) bout length, C) bout number, D) P(wake), and E) P(doze) in unmated (black, n = 64) and mated (maroon, n = 63) mature *iso31* flies from ZT0-12 (top) and ZT12-24 (bottom). Proportion of time spent in F) deep sleep, light sleep, light wake, and full wake in unmated vs mated flies from ZT0-12 (left) and ZT12-24 (right) (Mann-Whitney tests).
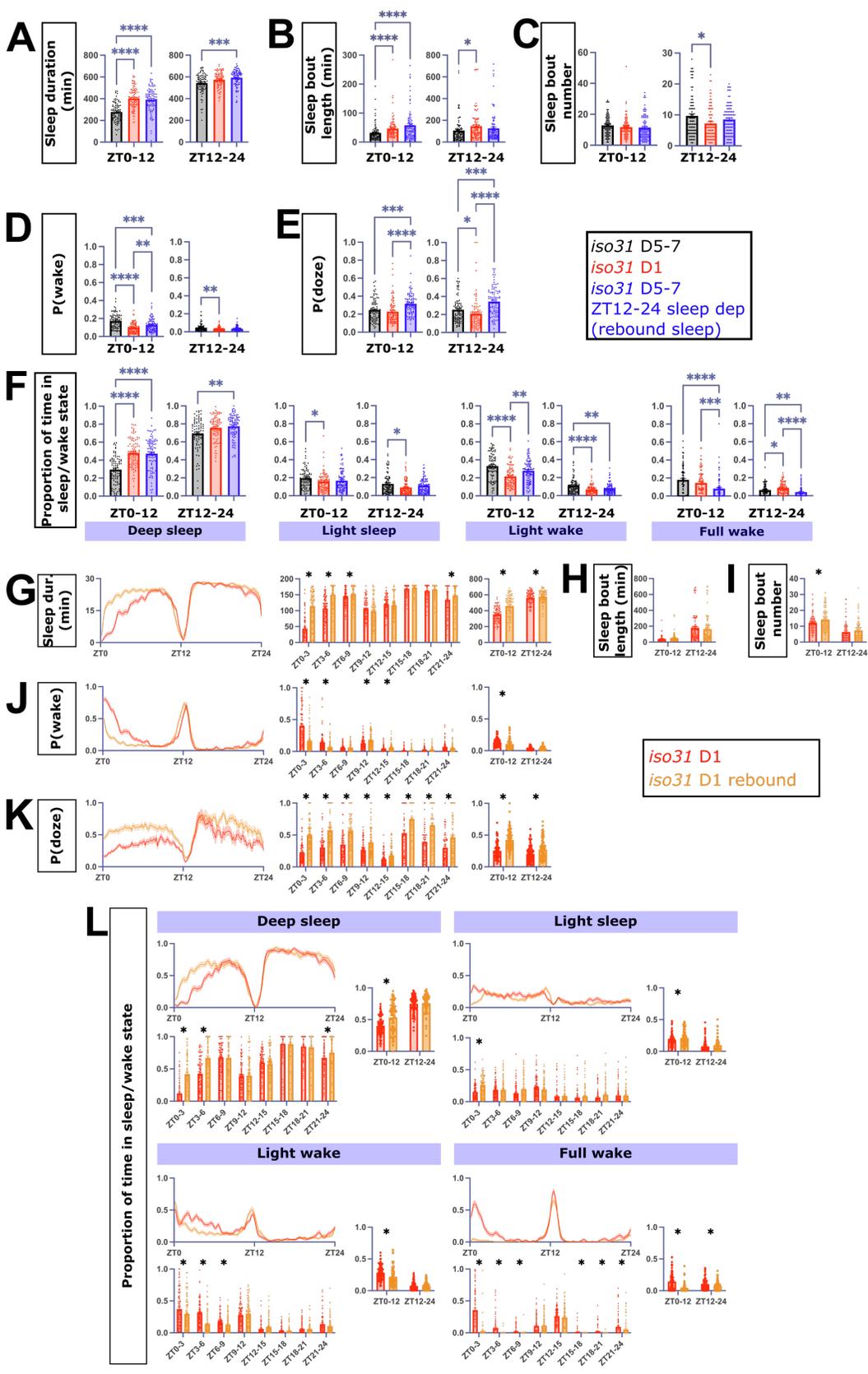
Figure S6: Extended sleep metrics after sleep deprivation in mature and juvenile flies. Related to Figure 3. A) Sleep duration, B) bout length, C) bout number, D) P(wake), and E) P(doze) in juvenile (red, n = 90), non-deprived control (black, n = 85), and mechanically sleep-deprived mature *iso31* flies (blue, n = 90) from ZT0-12 (left) and ZT12-24 (right). Proportion of time spent in F) deep sleep, light sleep, light wake, and full wake across the three conditions from ZT0-12 (left) and ZT12-24 (right) (Kruskall-Wallis with post-hoc Dunn's multiple comparison test for A-F). G) Sleep duration, H) bout length, I) bout number, J) P(wake), and K) P(doze) in non-deprived juvenile (orange, n = 92) and rebounding juvenile flies (light orange, n = 93). Proportion of time spent in L) deep sleep, light sleep, light wake, and full wake for all groups (Mann-Whitney tests for G-L).

Legend (top box):
R23E10-GAL4> UAS-mCherry RNAi
+; UAS-DEG RNAi
R23E10-GAL4 > UAS-DEG RNAi
R23E10-GAL4 > UAS-ringer RNAi

Column headers: ZT0-12 | ZT12-24

A — Sleep dur (min)
B — Bout length
C — Bout #
D — P(wake)
E — P(doze)
F — Deep sleep
G — Light sleep
H — Light wake
I — Full wake

RNAi: mCherry, 14-3-3ζ (1, 2, 3), ATPsynC, CG32700, CG3662, CG7582, Jdp, porin (1, 2), ringer, twz (1, 2), Vha13, Vha16-1, Vha55
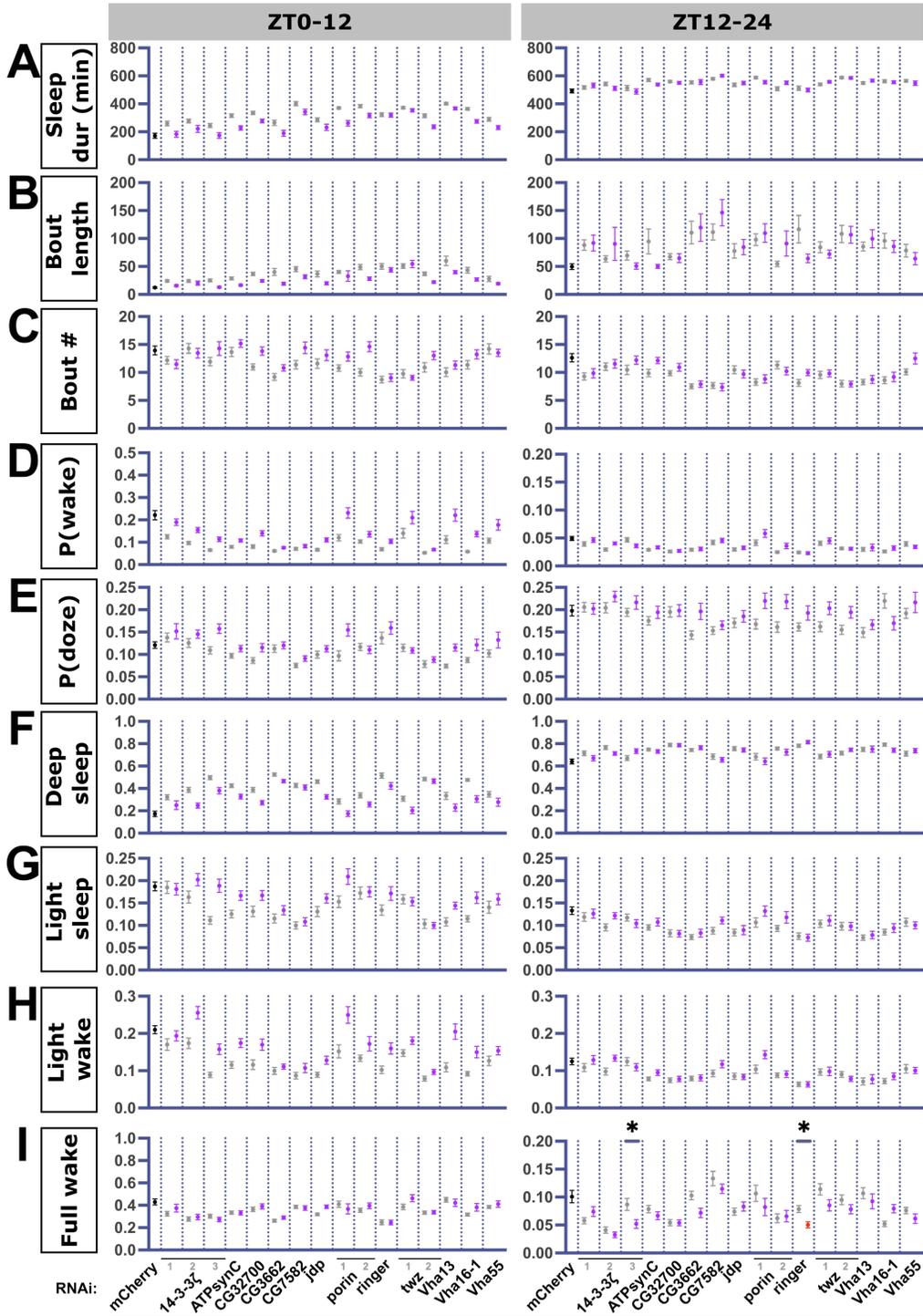
R23E10-GAL4 > UAS-RNAi

Figure S7: Sleep architecture metrics for *R23E10-GAL4>UAS-DEG RNAi* screen. Related to Figure 3. A) Sleep duration, B) sleep bout duration, C) sleep bout number, D) P(wake), E) P(doze), proportion of time spent in F) deep sleep, G) light sleep, H) light wake, and I) full wake in screened lines (*R23E10-GAL4>UAS-RNAi,* purple; *R23E10-GAL4>UAS-ringer RNAi,* red) vs *R23E10-GAL4>UAS-mCherry RNAi* (black) and *+; UAS-RNAi* (gray) controls. For all groups, n ≥ 30 (Kruskall-Wallis with post-hoc Dunn's multiple comparison test). See **Table S3** for specific RNAi lines used. Related to Figure 7

A — HMM fit to *iso31* D5-7 fly locomotor activity in the DAM5H system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.96	0.00	0.04	0.00		Deep sleep	1.00	0.00
Light sleep	0.07	0.72	0.21	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.43	0.24	0.32		Light wake	0.01	0.99
Full wake	0.00	0.07	0.00	0.93		Full wake	0.04	0.96

B — HMM fit to *iso31* D1 fly locomotor activity in the DAM5H system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.99	0.00	0.01	0.00		Deep sleep	1.00	0.00
Light sleep	0.31	0.51	0.18	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.28	0.18	0.54		Light wake	0.00	1.00
Full wake	0.00	0.03	0.00	0.97		Full wake	0.02	0.98

C — HMM fit to *CS* D5-7 fly locomotor activity in the DAM5H system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.98	0.00	0.02	0.00		Deep sleep	1.00	0.00
Light sleep	0.04	0.73	0.23	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.21	0.77	0.02		Light wake	0.17	0.83
Full wake	0.00	0.01	0.00	0.99		Full wake	0.04	0.96

D — HMM fit to *w1118* D5-7 fly locomotor activity in the DAM5H system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.99	0.00	0.01	0.00		Deep sleep	1.00	0.00
Light sleep	0.31	0.51	0.18	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.28	0.18	0.54		Light wake	0.00	1.00
Full wake	0.00	0.03	0.00	0.97		Full wake	0.02	0.98

E — HMM fit to *iso31* D5-7 fly locomotor activity from single-beam DAM system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.96	0.00	0.04	0.00		Deep sleep	1.00	0.00
Light sleep	0.07	0.72	0.21	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.43	0.24	0.32		Light wake	0.01	0.99
Full wake	0.00	0.07	0.00	0.93		Full wake	0.04	0.96

F — HMM fit to *elav-GAL4; + (attP40)* D5-7 fly activity from single-beam DAM system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.98	0.00	0.02	0.00		Deep sleep	1.00	0.00
Light sleep	0.08	0.67	0.25	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.17	0.80	0.03		Light wake	0.29	0.71
Full wake	0.00	0.02	0.00	0.98		Full wake	0.16	0.84

G — HMM fit to *R23E10-GAL4 > UAS-mCherry RNAi* D5-7 fly activity from DAM5H system:

Transition from: / State	Deep sleep	Light sleep	Light wake	Full wake		State:	Inactivity	Activity
Deep sleep	0.98	0.00	0.02	0.00		Deep sleep	1.00	0.00
Light sleep	0.06	0.78	0.16	0.00		Light sleep	1.00	0.00
Light wake	0.00	0.17	0.80	0.02		Light wake	0.13	0.87
Full wake	0.00	0.01	0.00	0.99		Full wake	0.03	0.97

Table S1. All HMM parameters: Related to Figures 1-7. HMM parameters used in this paper, described as transition probabilities between hidden states and emission probabilities from each hidden state to observed states for HMMs trained on locomotor data from A) mature *iso31* flies (n = 87 flies), B) juvenile *iso31* flies (n = 82 flies), C) mature *Canton-S* flies (n = 95 flies), and D) mature *w1118* flies (n = 96 flies) collected using the DAM5H multibeam system. HMMs trained on locomotor data from E) mature *iso31* flies (n = 90 flies) and F) mature *elav-GAL4;+ (attP40)* flies (n = 46 flies) collected using the single beam DAM system. G) HMM trained on locomotor data from mature *R23E10-GAL4 > UAS-mCherry RNAi* flies (n = 31 flies) collected using the multibeam DAM system. A total of 1439 transitions per fly per 24 hours were used to train each model.

A

Gene set name	# genes in gene set	Enrichment score	Normalized enrichment score	Nominal p-value	FDR q-val
RIBONUCLEOPROTEIN COMPLEX	25	0.8334325	3.3576221	0	0
STRUCTURAL CONSTITUENT OF RIBOSOME	23	0.7740875	3.0646384	0	0
RIBOSOME	23	0.7740875	3.0391607	0	0
CYTOSOLIC PART	23	0.7740875	3.0359354	0	0
STRUCTURAL MOLECULE ACTIVITY	23	0.7740875	3.0340197	0	0
RIBOSOMAL SUBUNIT	23	0.7740875	3.0132124	0	0
CYTOSOLIC RIBOSOME	23	0.7740875	3.0076745	0	0
MITOTIC CELL CYCLE PROCESS	15	0.5640415	2.0024745	0.00466201	0.00371607
MICROTUBULE CYTOSKELETON ORGANIZATION	15	0.5558247	1.9938301	0.00943396	0.00354415
MICROTUBULE BASED PROCESS	15	0.5558247	1.9627372	0.00485437	0.00352023
CYTOSKELETON ORGANIZATION	15	0.5558247	1.9592851	0	0.00320021
MITOTIC CELL CYCLE	16	0.5014893	1.7472951	0.01678657	0.01589365
PROTEIN COMPLEX SUBUNIT ORGANIZATION	15	0.46264157	1.6513298	0.02849741	0.02640818

B

Gene	Neurodevelopment	Synaptic transmission	Ion homeostasis
Cbp53E	■		
ringer	■		
CG45263	■		
miple1	■		
14-3-3zeta	■	■	
Syx1A	■	■	
Cam	■	■	
nSyb		■	
twz			■
Vha14-1			■
Vha36-1			■
VhaM8.9			■
Vha13			■
Vha16-1			■
Vha55			■
porin			■
ATPsynC			■
jdp			
CG7582			
CG8974			
TM4SF			
CG32700			
CG31808			
CG3662			
RNASEK			
RpL15			
Drat			

Table S2. GSEA and related GO terms for genes with differential expression in juvenile vs mature flies: Related to Figure 7. A) GSEA results for DEGs with increased expression in mature compared to juvenile dFB cells. B) Associated GO terms for DEGs with increased expression in juvenile fly dFB cells. Blue boxes indicate a given gene is associated with the listed GO term, while gray indicates the gene is not associated with a GO term.

Gene	TRiP hairpin	BDSC #
14-3-3zeta	JF02962	28327
	HM04002	31498
	GL01310	41878
ATPsynC	GL00390	35464
CG32700	HMC04908	57719
CG3662	HMC05026	60033
CG7582	HMJ21718	53002
jdp	HMC06433	67329
porin	JF03251	29572
	HMS05687	67873
ringer	HMS01740	38287
twz	JF01867	25846
	HMC04701	57397
Vha13	CG6213	38233
Vha16-1	HMS02171	40923
Vha55	CG17369	40884

Table S3, all RNAi lines used for screen: Related to Figure 7. Screened RNAi

lines for DEGs with higher expression in juvenile dFB cells.