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Supplementary Materials for

Developmental emergence of sleep rhythms enables long-term memory in Drosophila

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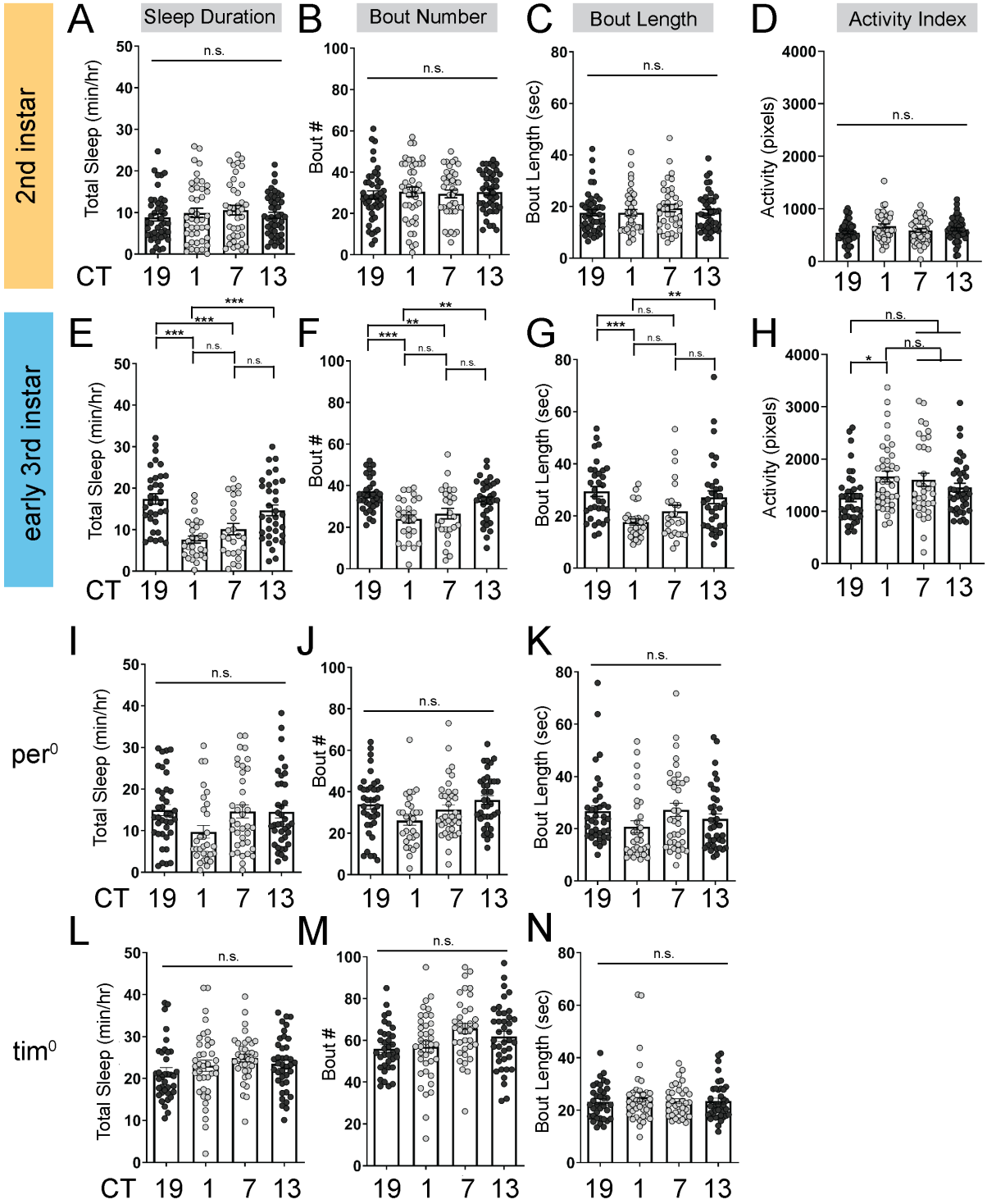
This PDF file includes:

Figs. S1 to S12

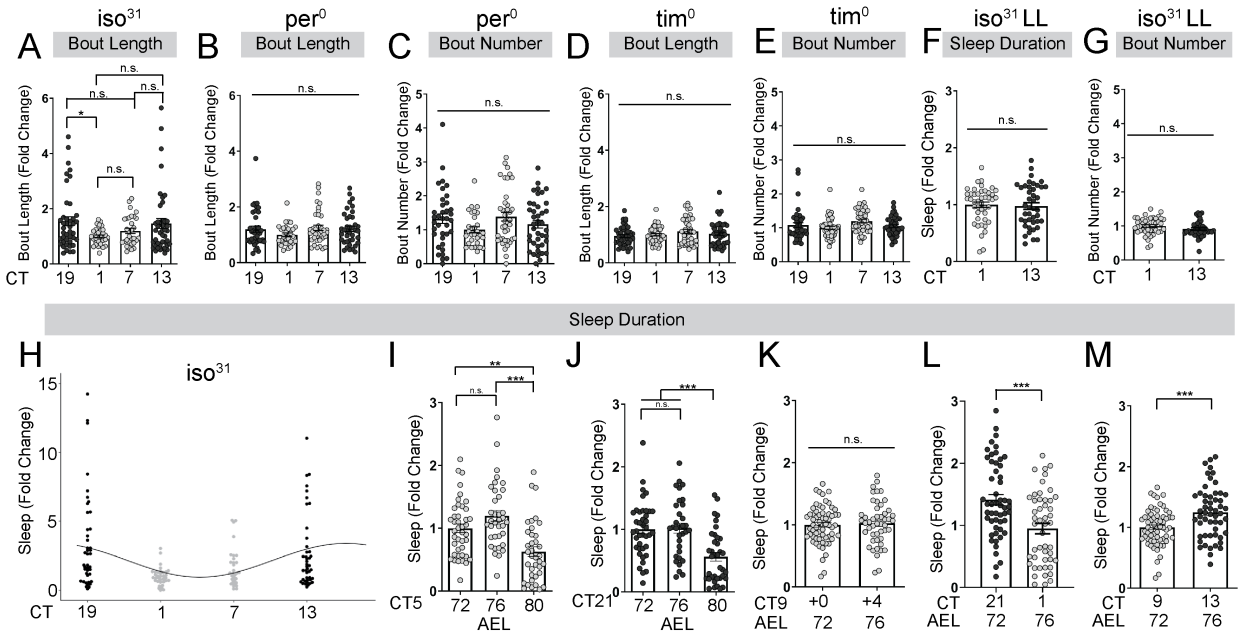
Fig. S1.



Supplemental Fig. 1: Early L3 show rhythmic differences in raw sleep measures. **(A-H)** Sleep duration, sleep bout number, sleep bout length, and activity index across the day under constant dark conditions in L2 (A-D) and L3 (E-H). **(I-N)** Sleep duration, sleep bout number, and sleep bout length across the day in L3 clock mutants.

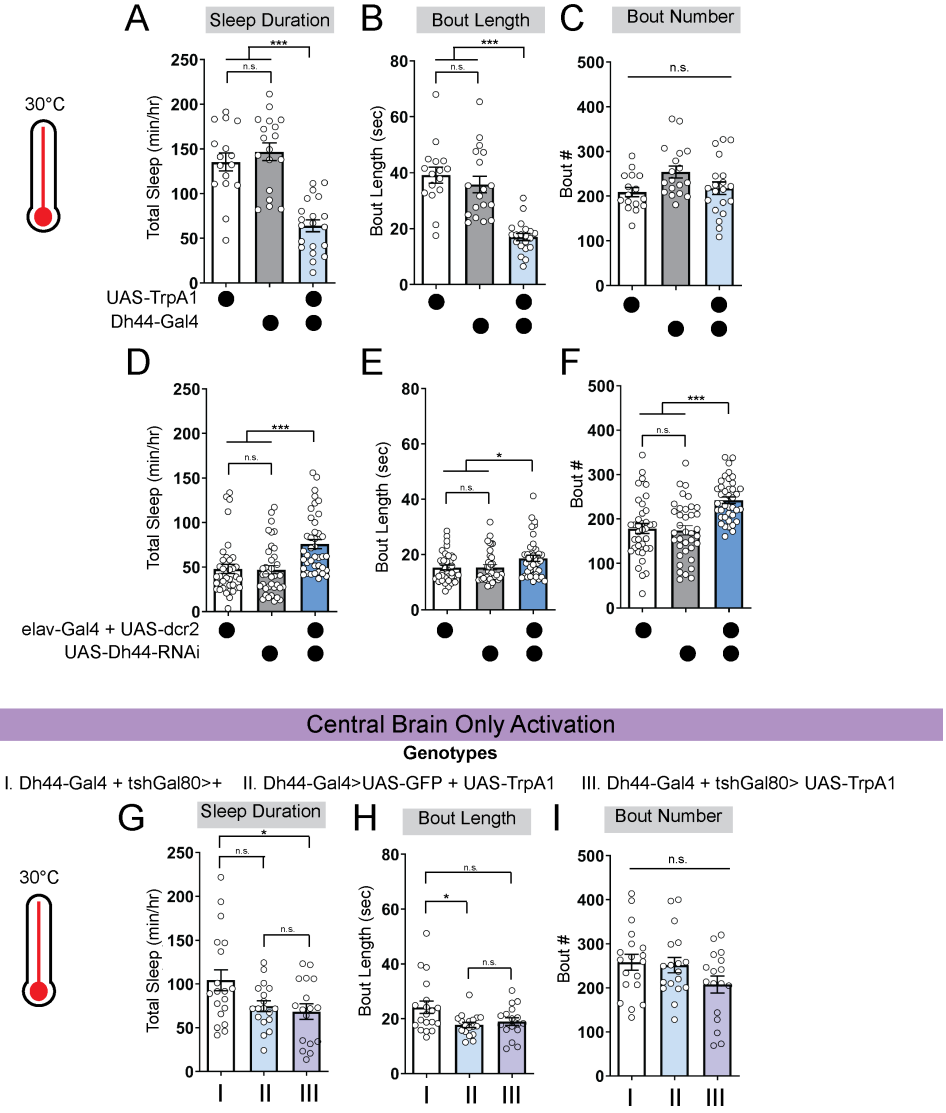
Subjective day is shown in light gray circles, subjective night in black. A-N, n=31-44 larvae. One-way ANOVA followed by Tukey's multiple comparison test (A-N).

Fig. S2.



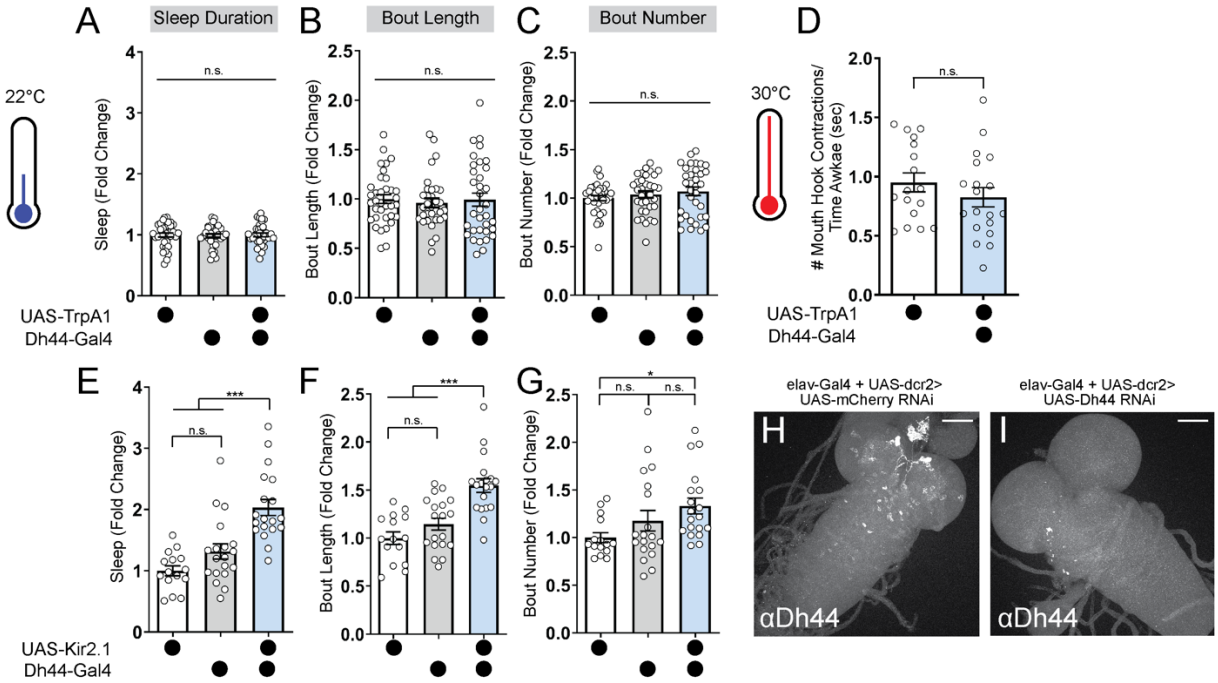
Supplemental Fig. 2: Early L3 show rhythmic differences in sleep. (A) Bout length of L3 controls across the day. **(B-E)** Sleep bout length and number of L3 clock mutants. **(F-G)** Sleep duration and bout number in L3 reared in constant light (LL). **(H)** Cycling analysis of L3 sleep pattern. **(I-K)** Sleep duration at CT5 (I), CT21 (J), and CT9 (K) in newly molted L3 (72 AEL), larvae aged 4 hrs (76 AEL), and larvae aged 8 hrs (80 AEL). Circadian time is held constant while development proceeds. **(L,M)** Sleep duration in L3 during the CT21 to CT1 transition (L) and CT9 to CT13 transition (M) as development proceeds. Subjective day is shown in light gray circles, subjective night in black. All sleep metrics represent fold change (normalized to avg. value of control). A-E, n=31-44 larvae; F-G, n=42-44 larvae; L-M, n=40-60 larvae. One-way ANOVA followed by Tukey's multiple comparison test [(A-E) and (I-J)] and unpaired two-tailed Student's *t*-test [(F-G) and (K-M)].

Fig. S3.



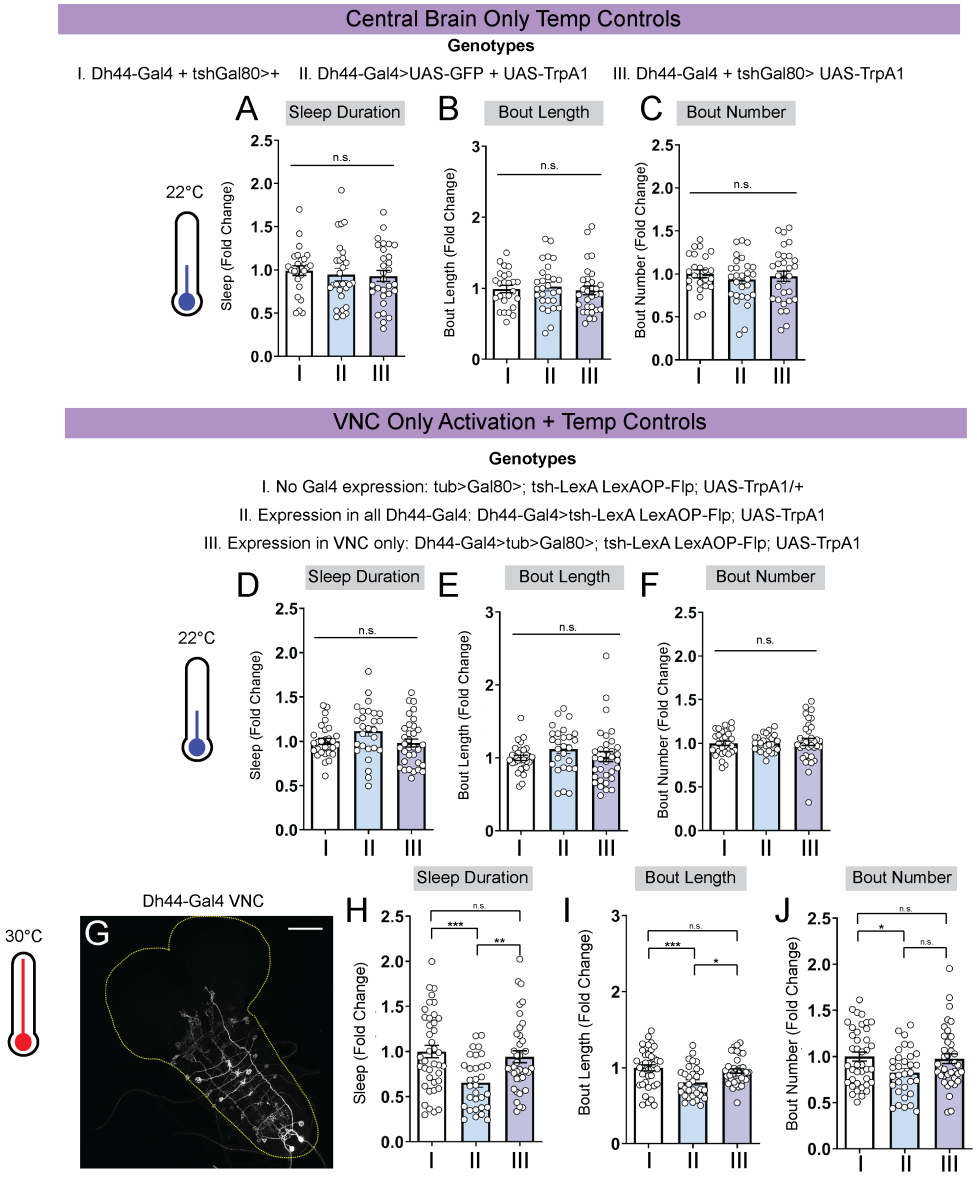
Supplemental Fig. 3: Dh44 manipulations affect raw sleep measures in L2. (A-C) Sleep duration, bout length, and bout number in L2 expressing *UAS-TrpA1* with *Dh44-Gal4* and genetic controls at 30°C. **(D-F)** Sleep duration, bout length, and bout number in L2 expressing *UAS-Dh44-RNAi* with *elav-Gal4* + *UAS-dcr2* and genetic controls. **(G-I)** Sleep duration, bout length, and bout number in L2 expressing *Dh44*-Gal4 in presence of *tshGal80* and *UAS-TrpA1* with genetic controls. Sleep metrics represent raw values. A-C, n=16-20 larvae; D-F, n=37-40 larvae; G-I, n=30-36 larvae. One-way ANOVA followed by Tukey's multiple comparison test.

Fig. S4.



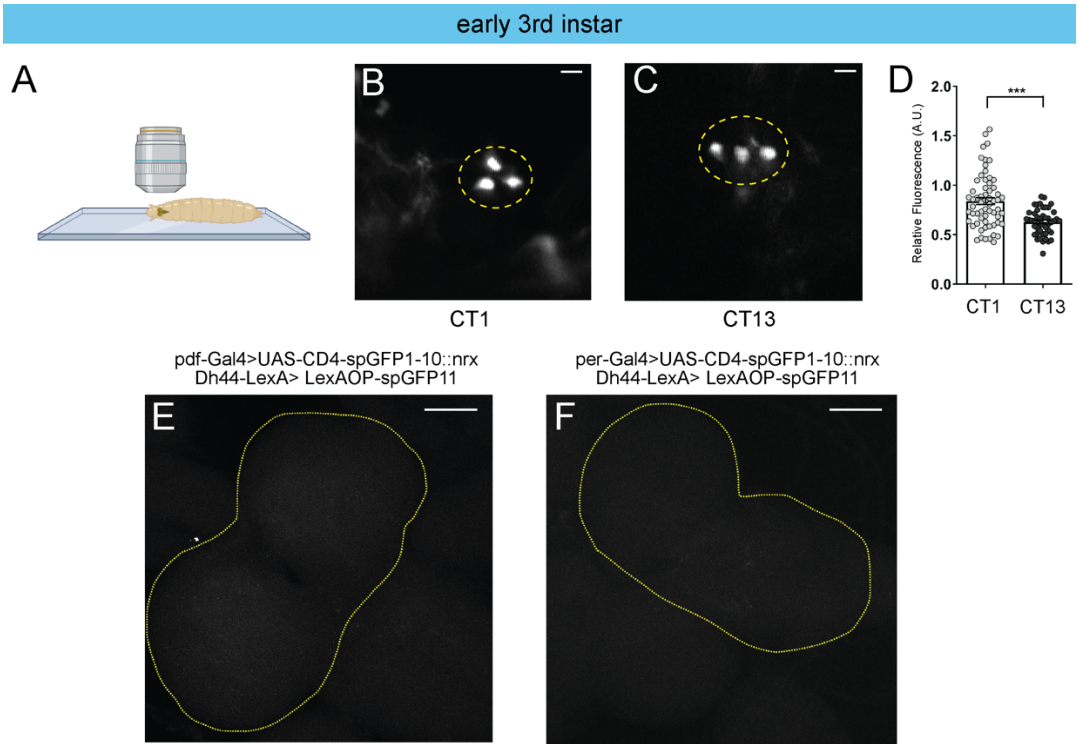
Supplemental Fig. 4: Dh44 modulates L2 waking. (A-C) Sleep duration, bout length, and bout number in L2 expressing *UAS-TrpA1* with *Dh44-Gal4* and genetic controls at 22°C (temperature controls). **(D)** Normalized feeding (# mouth hook contractions/time awake [sec]) in L2 expressing *UAS-TrpA1* with *Dh44-Gal4* and controls at 30°C. **(E-G)** Sleep duration, bout length, and bout number in L2 expressing *UAS-Kir2.1* with *Dh44-Gal4* and genetic controls. **(H-I)** Images of *elav-Gal4>UAS-dcr2 + UAS-mCherry RNAi* (H) and *elav-Gal4>UAS-dcr2 + UAS-Dh44 RNAi* (I) L2 brains immunostained for Dh44. n=5 brains. All sleep metrics represent fold change (normalized to avg. value of control). A-C, n=30 larvae; D, n=18-20 larvae; E-G, n=14-18 larvae. One-way ANOVA followed by Tukey's multiple comparison test [(A-C) and (E-G)] and unpaired two-tailed Student's *t*-test (D). Scale bars=50 microns.

Fig. S5.



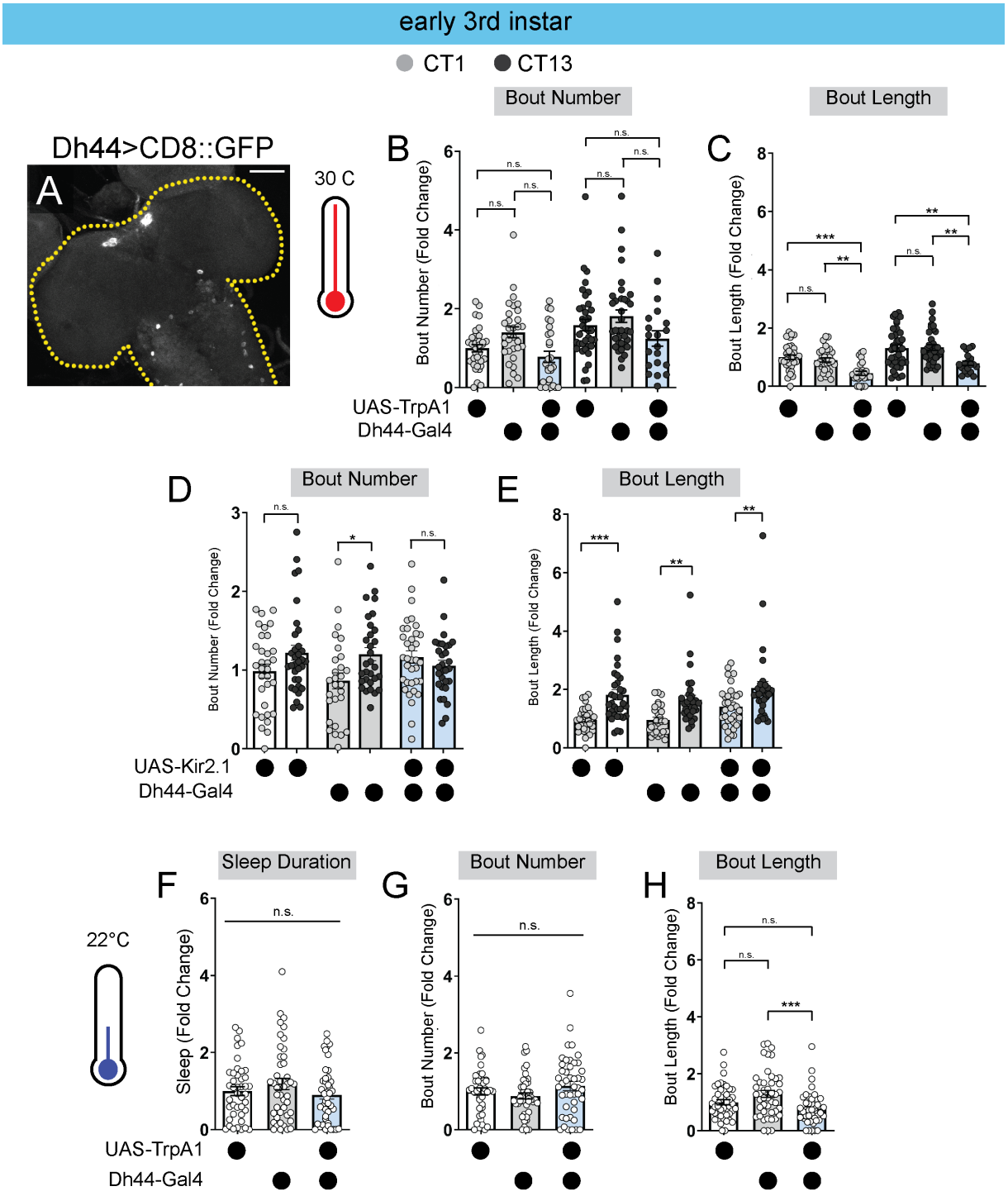
Supplemental Fig. 5: Intersectional analysis of Dh44 neurons in L2. (A-C) Sleep duration, bout length, and bout number in L2 expressing *Dh44*-Gal4 in presence of *tshGal80* and *UAS-TrpA1* with genetic controls at 22°C (temperature controls). **(D-F)** Sleep duration, bout length, and bout number of VNC-only Dh44 neurons and genetic controls at 22°C (temperature controls). **(G)** L2 brain and ventral nerve cord showing GFP expression in larvae expressing *Dh44*-Gal4 only in VNC. **(H-J)** Sleep duration, bout length, and bout number with thermogenetic activation of VNC-only Dh44 neurons and genetic controls at 30°C. All sleep metrics represent fold change (normalized to avg. value of control). A-C, D-F, n=25-30 larvae; H-J, n=30-40 larvae. One-way ANOVA followed by Tukey's multiple comparison test. Scale bars=50 μm.

Fig. S6.



Supplemental Fig. 6: DN1as regulate sleep rhythms in early L3. (A) Schematic of two-photon *in vivo* imaging of Dh44 neuronal activity. **(B-C)** *Dh44*-Gal4>*UAS-GCaMP7f* expression in brains of intact L3 at CT1 and CT13. **(D)** GCaMP intensity in Dh44 neurons in L3 at CT1 and CT13 *in vivo*. **(E-F)** Absence of neurexin-based GFP reconstitution (GRASP) between s-LNvs (*pdf*-Gal4) (E) or DN2s (*per*-Gal4) (F) and Dh44 neurons (*Dh44*-LexA) in L3 brains. Yellow dotted lines indicate central brain region. D, n=47-62 cells, 12-23 brains; E-F, n=8-10 brains. Unpaired two-tailed Student's *t*-test (D). Scale bars=10 μm for B-C. Scale bars=50 μm for E-F.

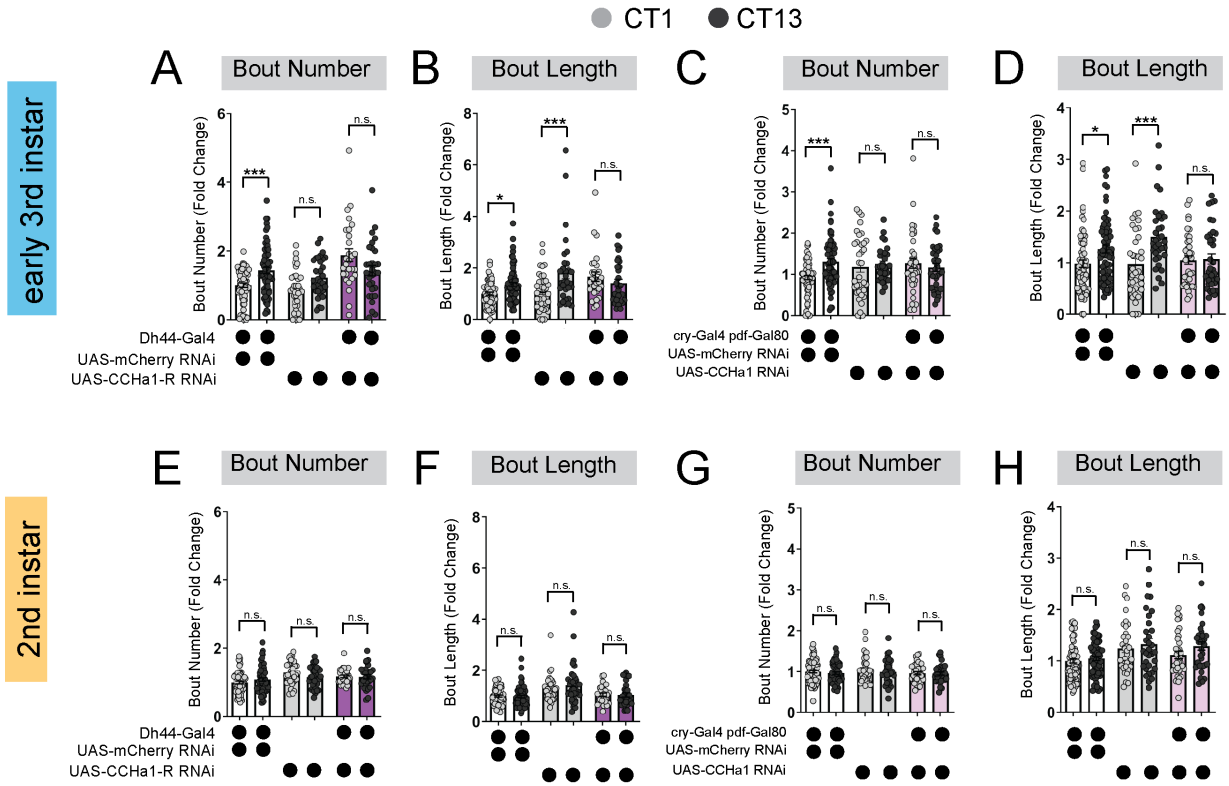
Fig. S7.



Supplemental Fig. 7: Dh44 manipulations affect L3 sleep. (A) L3 central brain showing GFP expression in Dh44 neurons (*Dh44*-Gal4>*UAS-CD8::GFP*). **(B-C)** Sleep bout number and bout length in L3 expressing *Dh44*-Gal4>*UAS-TrpA1* and genetic controls at CT1 and CT13. **(D-E)** Sleep bout number and bout length in L3 expressing *Dh44-Gal4>UAS-Kir2.1* and genetic controls at CT1 and CT13. **(F-H)** Sleep duration,

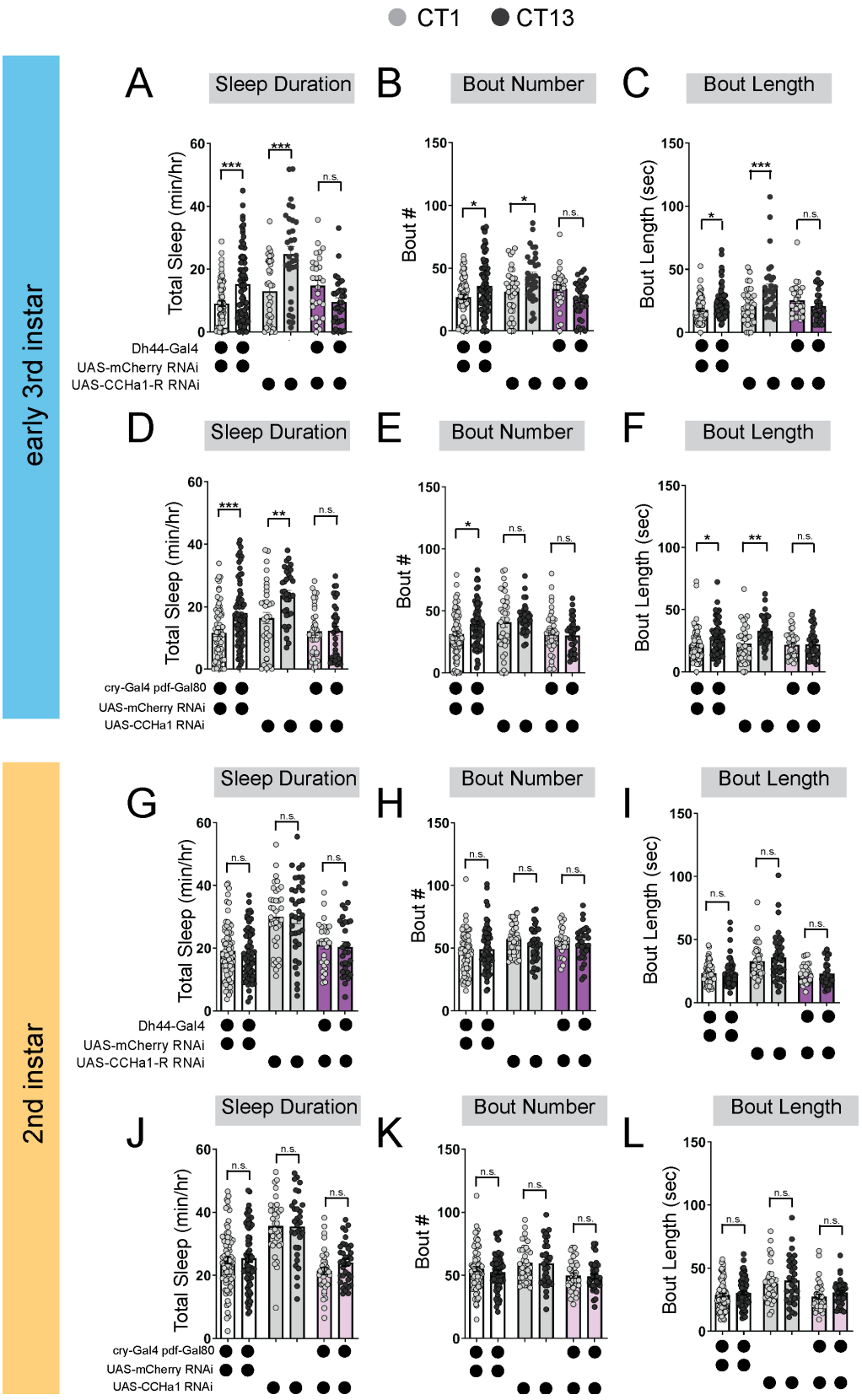
bout number, and bout length in L3 expressing *UAS-TrpA1* with *Dh44-Gal4* and genetic controls at 22°C (temperature controls). Subjective day is shown in light gray circles, subjective night in black. All sleep metrics represent fold change (normalized to avg. value of control). B-C, n=20-36 larvae; D-E, n=28-35 larvae; F-H, n=39-41 larvae. One-way ANOVA followed by Tukey's multiple comparison test [(B-C) and (F-H)]; two-way ANOVAs followed by Sidak's multiple comparisons tests [(D-E)]. Scale bars=50 μm for A.

Fig. S8.



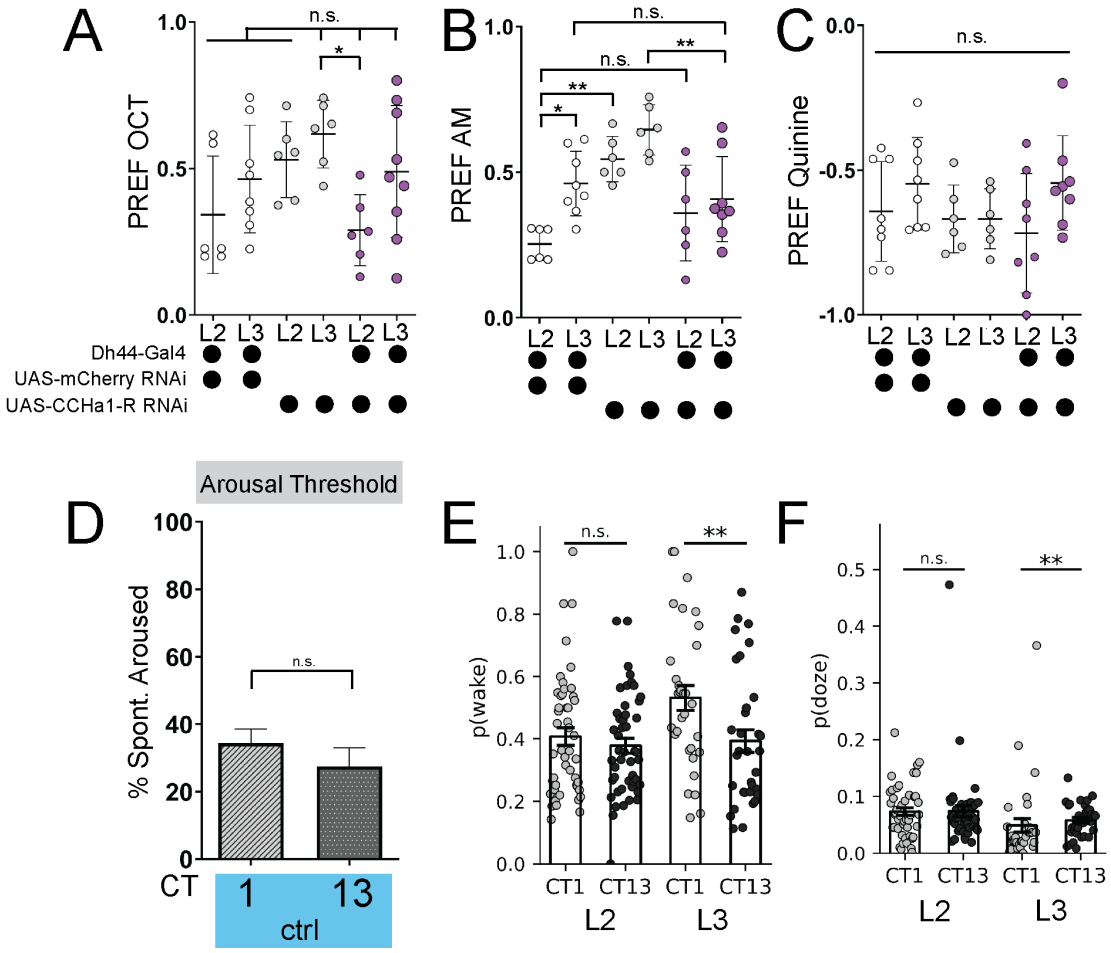
Supplemental Fig. 8: DN1as regulate sleep rhythms in early L3 through CCHa1 signaling. (A-H) Sleep bout number and bout length in L3 (A-D) or L2 (E-H) expressing *UAS-CCHa1-R-RNAi* with *Dh44-Gal4* or *UAS-CCHa1-RNAi* with *cry-Gal4 pdf-Gal80* (DN1as) and genetic controls at CT1 and CT13. Subjective day is shown in light gray circles, subjective night in black. All sleep metrics represent fold change (normalized to avg. value of control). A-D, n=64-68 mCherry larvae, 29-37 other genotypes. Two-way ANOVAs followed by Sidak's multiple comparisons tests (A-H).

Fig. S9.


CT1
CT13
early 3rd instar
2nd instar
A
Sleep Duration
B
Bout Number
C
Bout Length
D
Sleep Duration
E
Bout Number
F
Bout Length
G
Sleep Duration
H
Bout Number
I
Bout Length
J
Sleep Duration
K
Bout Number
L
Bout Length
Total Sleep (min/hr)
Bout #
Bout Length (sec)
Dh44-Gal4
UAS-mCherry RNAi
UAS-CCHa1-R RNAi
cry-Gal4 pdf-Gal80
UAS-mCherry RNAi
UAS-CCHa1 RNAi
***
**
*
n.s.

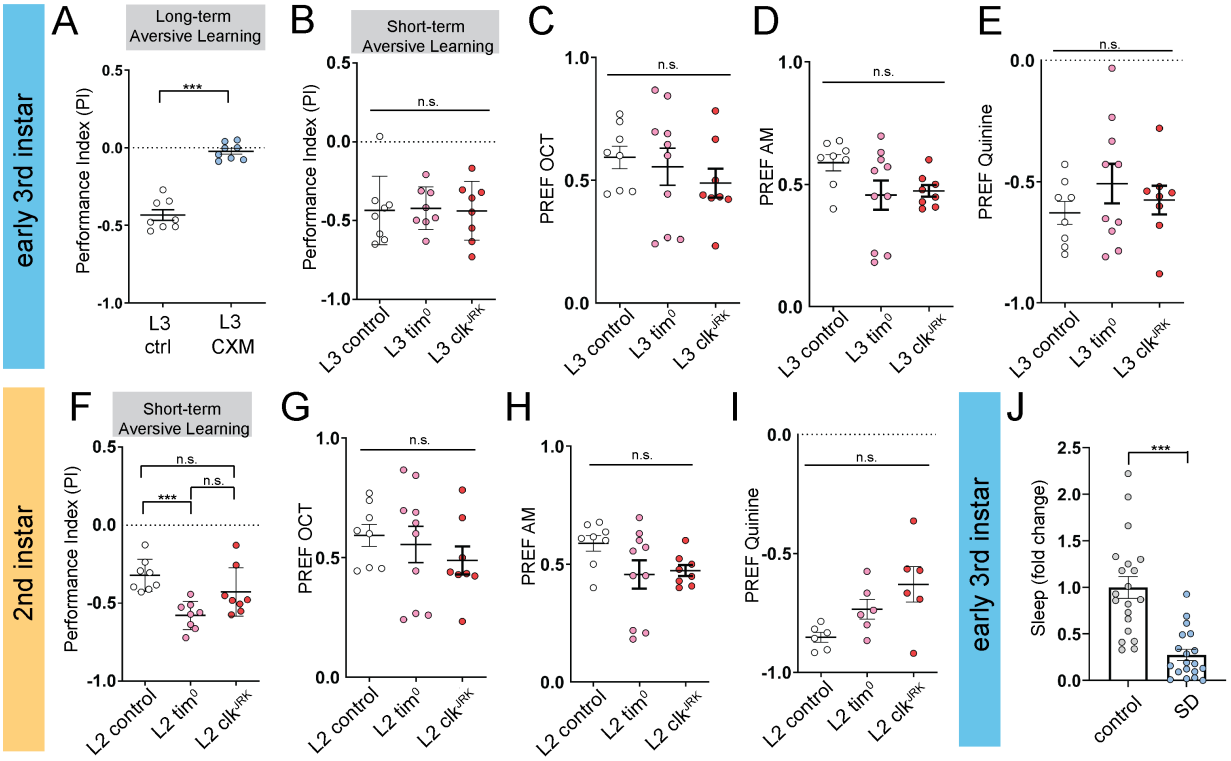
Supplemental Fig. 9: **CCHa1-signaling manipulations affect raw sleep measures in L3.** (A-L) Sleep duration, sleep bout number, and bout length in L3 (A-F) or L2 (G-L) expressing *UAS-CCHa1-R-RNAi* with *Dh44-Gal4* or *UAS-CCHa1-RNAi* with *cry-Gal4 pdf-Gal80* (DN1as) and genetic controls at CT1 and CT13. Subjective day is shown in light gray circles, subjective night in black. All sleep metrics represent raw values. A-L, n=64-68 mCherry larvae, 29-37 other genotypes. Two-way ANOVAs followed by Sidak's multiple comparisons tests (A-L).

Fig. S10.



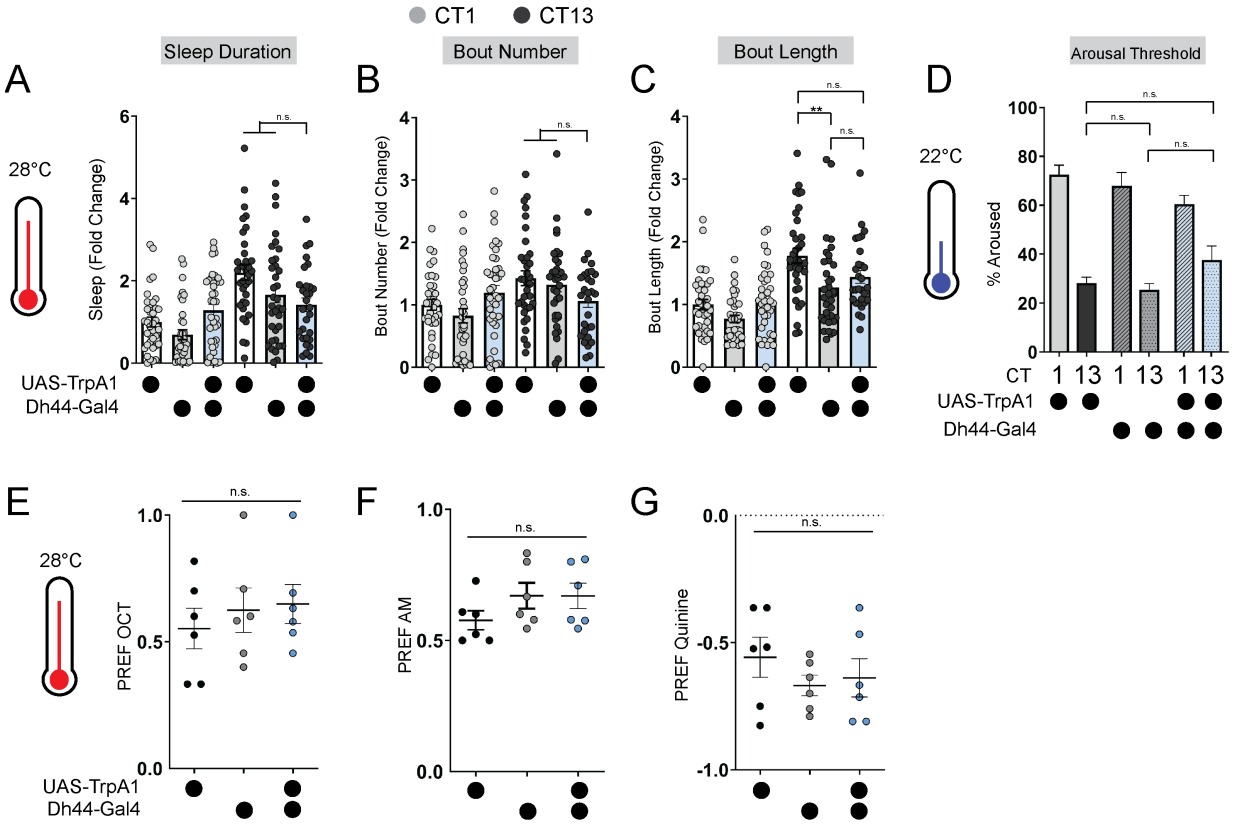
Supplemental Fig. 10: Naïve preferences for CCHa1 manipulations and sleep measures in L2 and L3. (A-C) Naïve OCT, AM, and quinine preference in L2 and L3 expressing *Dh44*-Gal4>*CCHa1-R-RNAi* and genetic controls. **(D)** Percentage of L3 controls at CT1 and CT13 spontaneously waking in absence of a light stimulus. **(E-F)** Probability of transitioning from a sleep state to wake [p(wake)] (E) and probability of transitioning from a wake state to sleep [p(doze)] (F) in L2 and L3 at CT1 and CT13. A-C, n=6 PREFs (180 larvae); D, n=240-260 sleep episodes, 36 larvae per time point. One-way ANOVA followed by Tukey's multiple comparison test [(A-C]; unpaired two-tailed Student's *t*-test [(D)]; two-sided Wilcoxon Rank-Sum test [(E-F)].

Fig. S11.



Supplemental Fig. 11: Naïve preferences for odor/aversive stimuli in L2 and L3. **(A)** Long-term aversive memory performance in L3 fed sucrose (control) or cycloheximide (CXM). **(B)** Short-term aversive memory performance in L3 controls, *tim*^{0}, and *clk*^{JRK} mutants. **(C-E)** Naïve OCT, AM, and quinine preference in L3 controls, *tim*^{0}, and *clk*^{JRK} mutants. **(F)** Short-term aversive memory performance in L2 controls, *tim*^{0}, and *clk*^{JRK} mutants. **(G-I)** Naïve OCT, AM, and quinine preference in L2 controls, *tim*^{0}, and *clk*^{JRK} mutants. **(J)** Quantification of sleep loss during sleep deprivation (SD) over 1 hr (fold change). A-B, F, n=8 PIs (240 larvae) per genotype; C-E, G-I, n=6 PREFs (180 larvae) per genotype; J, n=20-22 larvae.

Fig. S12.



Supplemental Fig. 12: Mild Dh44 activation disrupts LTM. (A-C) Sleep duration, bout number, and bout length at 28°C of L3 expressing *Dh44*-Gal4>*UAS-TrpA1* and genetic controls at CT1 and CT13. **(D)** Arousal threshold in L3 expressing *Dh44*-Gal4>*UAS-TrpA1* and genetic controls at CT1 and at CT13 at 22°C (temperature controls). **(E-G)** Naïve OCT, AM, and quinine preference in L3 expressing *Dh44*-Gal4>*UAS-TrpA1* and genetic controls at 28°C. A-C, n=32-38 larvae; D, n=120-200 sleep episodes, 18 larvae per genotype; E-G, n=6 PREFs (180 larvae) per genotype; One-way ANOVA followed by Tukey's multiple comparison test (A-G).