Science Advances
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Supplementary Materials for

Disrupting flight increases sleep and identifies a novel sleep-promoting pathway in *Drosophila*

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Published 8 May 2020, *Sci. Adv.* **6**, eaaz2166 (2020)
DOI: 10.1126/sciadv.aaz2166

This PDF file includes:

Fig. S1. Extended characterization of *burs* loss of function. (**A**) Representative photographs of flies confined pre-expansion ('Pre') and isolated flies ('isolated'). **(B)** Waking activity was not altered in flies confined pre-expansion (pre) compared to controls (n.s. p >0.05). (**C**) Representative images of brains from R58H05-GAL4 / + > CaLexA / +flies that were isolated ('isolated') and confined pre-expansion ('Pre'). (**D**) Quantification of CalexA; signal was higher in flies in brains of flies that had been confined pre-expansion ('Pre') compared to isolated controls ('isolated') (p<0.01, t-test, n=7-8 flies / condition) (**E**) The intensity of waking activity was elevated in 4-5 day old *burs GAL4* / + > *UAS burs RNAi* / + (* p <0.001) flies compared to controls. (**F**) *burs-GAL4/+>UAS-pburs^{RNAi}/+* slept more than parental controls (n=20-32 flies / genotype, Repeated measures ANOVA for Time X condition, p< 0.001). (**G** and **H**) *burs-GAL4/+>UAS-pburs^{RNAi}/+* displayed increased daytime sleep (**G**) and sleep bout duration (**H**) compared to controls (*p< .01 Tukey correction). (**I**) The intensity of waking activity was elevated in *burs-GAL4/+>UAS-pburs^{RNAi}/+* flies relative to controls (*, p<0.01, Tukey correction).(**J**) Sleep was rapidly reversible in response to a mechanical stimulus for all genotypes (n=25-30 flies / condition, *p< .01 Tukey correction). (**K**) Sleep in *burs-GAL4/+>UAS-pburs^{RNAi}/+* flies was associated with increased arousal thresholds (n=14 flies per condition; *p< .01 Tukey correction). (**L**) All genotypes displayed similar sleep rebound following 12 h of sleep deprivation (n=21-32 flies / condition). (**M**) In contrast to confinement, *burs GAL4* / + > *UAS burs RNAi* / + flies did not alter sleep on the day flies eclosed relative to parental controls (day0, n.s. p>0.44 Tukey correction). (**N**) Sleep was significantly elevated however, in *burs GAL4* / + > *UAS burs RNAi* / + flies on the day after eclosion (Day1, n=21 flies per genotype, * p< 0.001 Tukey correction). (**O**) The intensity of waking activity was elevated in the *burs GAL4* / + > *UAS burs RNAi* / + flies on day 1 (* p< 0.001, Tukey correction). (**P** to **R**) Loss of function *burs* mutants exhibited increased sleep (**P**,* p < 0.05, Tukey correction, n=16 flies per condition), increased sleep consolidation during the day (**Q**,* p <0.05), and did not decrease waking activity (**R**, n.s. p>0.05, * p<0.05). (**S**) Sleep of *burs GAL4* / + > *UAS ash RNAi* / +, *burs GAL4* / + > *UAS lark RNAi* / +, and *burs GAL4* / + > *UAS mip RNAi* / + flies was not altered compared to parental controls (n=16 flies / genotype, n.s. p> 0.44 Tukey correction). (**T**) *elav GAL4* / + > *UAS CCAP RNAi* / + and *elav GAL4* / + > *UAS CCAPR RNAi* / + flies did not alter sleep relative to controls (n=16 flies / genotype, n.s. p>0.99 Tukey correction). (**A**) Photo credit: Krishna Melnattur, Washington University School of Medicine.

Fig. S2. Manipulating the activity of *burs* neurons increases sleep. (**A**) Sleep was rapidly reversible in response to a mechanical stimulus for all genotypes (n=26-30 flies / condition, *p< .01 Tukey correction). (**B**) Sleep in *bursGAL4/+>UAS-Kir2.1/+* flies was associated with increased arousal thresholds (n=14 flies per condition; *p< .01 Tukey correction). (**C**) All genotypes displayed similar sleep rebound following 12 h of sleep deprivation (n=20-30 flies / genotype). (**D**) Waking activity was not decreased in *bursGAL4>Kir* flies relative to controls (* p<0.001). (**E** to **G**) *burs GAL4* / + > *UAS NaChBac* / + flies increased sleep (**E**, n=24-30 flies / genotype, repeated measures ANOVA for time X genotype p< 0.001) particularly in the day (**F**, * p< 0.001, Tukey correction), and increased daytime sleep consolidation (**G**, * p<0.01) relative to controls. This manipulation completely blocked wing expansion. (**H**) Waking activity was elevated in *burs GAL4* / + > *UAS NaChBac* / + flies relative to controls (*, p <0.001). (**I**) All genotypes were similarly awakened by a mechanical stimulus (n=30 flies / condition, * p< 0.001 Tukey correction). (**J** and **K**) *burs GAL4* / + > *UAS NaChBac* / + flies exhibited higher arousal thresholds during the day (**J**, n=14 flies per condition, * p<0.001), and a similar level of rebound sleep following sleep deprivation to parental controls (**K**, n=27-31flies / genotype, p>0.08). (**L**) *CCAP-16 GAL4 / +> UAS NaChBac / +* flies increased sleep relative to parental controls. (n=16 flies / genotype, repeated measures ANOVA for time X genotype,* p < 0.001). (**M** to **O**) *CCAP-16 GAL4 / +> UAS NaChBac / +* flies displayed increased daytime sleep (**M**,* p< 0.001 Tukey correction), sleep bout length (**N**,* p < 0.01), and waking activity throughout the day (**O**, * p< 0.001) relative to controls. (**P**) *CCAP-9 GAL4 / +> UAS NaChBac / +* flies increased sleep relative to parental controls. (n=16 flies / genotype, repeated measures ANOVA for time X genotype, * p < 0.001). (**Q** to **S**)**,** *CCAP-9 GAL4 / +> UAS NaChBac / +* flies displayed increased daytime sleep (**Q**,* p< 0.001 Tukey correction), sleep bout length (**R**,* p < 0.05), and waking activity throughout the day (**S**,*, p< 0.001) relative to controls. (**T**) *burs* neurons were inhibited by expressing the inward rectifying K+ channel Kir2.1 with *burs* GAL4. A temperature sensitive GAL8O, *GAL80^{ts}* was used to temporally restrict the activity of *burs GAL4*, and thus the inhibition of *burs* neurons to defined time windows (*burs GAL4* / + > *GAL80^{ts}* / + ; *UAS*

Kir / +). Flies were reared at 25°C; in these conditions they eclose 10days after egg-laying (day10). Days 9-10 represent days after egg laying, Adult Day1&2 represent days after eclosion. Sleep data is shown for the second day post eclosion. (**U**) Raising *burs GAL4* / + > *GAL80^{ts}* / + ; *UAS Kir* / + flies at the permissive temperature throughout development (No shift, n=31 flies / genotype) or shifting flies to the restrictive temperature post wing expansion (adults, n=27-30 flies/ genotype) had no effect on wing expansion or sleep. In contrast, shifting *burs GAL4* / + > *GAL80^{ts}* / + ; *UAS Kir* / +flies to the restrictive temperature one day prior to eclosion (Day 9-adults, n=28-30 flies / genotype, * p<0.001 Tukey correction) or ~12 hours prior to eclosion (Day9.5-adults) blocked wing expansion, with the flies that emerged with wing defects (~80% of progeny, n=40) exhibiting increased sleep relative to controls (n=32flies / genotype, * p <0.001). (A to U) All manipulations blocked wing expansion.

Fig. S3. Defining a minimal subset of burs/CCAP expressing neurons. (**A**) Inhibition of the Bseg partially blocked wing expansion with ~60% of Bseg>Kir progeny displaying wing defects, and increased sleep (repeated measures ANOVA for time X genotype p< 0.001). Bseg > Kir flies with wing expansion defects increased sleep during the day (**B**, * p< 0.001, Tukey correction) without changing sleep consolidation (**C**) and were more active while awake (**D**, * p <0.001). (**E**) Waking activity was unchanged in Bag>Kir flies (n.s. p> 0.64). (**F**) Summary of the FINGR method, CCAP GAL4 neurons are disrupted by expressing a Glued-DN transgene with CCAP GAL4 to disrupt the cytoskeleton and thereby neural function. A FRT flanked stop cassette blocks the expression of GAL80, permitting GAL4 activity ("W4 flies"). This manipulation blocks wing expansion and generates long-sleeping flies. The W4 flies were crossed to a number of enhancer-trap flipase lines (ET-flp). GAL80 is "flipped in" and GAL4 consequently repressed, in cells defined by the intersection of the expression pattern of the CCAP enhancer, and the enhancer driving the flipase. (**G**) Representative sleep profile of an Et-flp line (820a flp) that suppressed the wing expansion defects seen in W4 flies (n=22-30 flies/ genotype).(**H**) Sleep of *820a flp* / + > *W4* / + flies is indistinguishable from the *820a flp / +* parental control line (*, p< 0.001, n.s. p> 0.77). Similar changes were seen in day bouts (**I**, *, p< 0.001, n.s. p> 0.99) and waking activity (**J**, *, p< 0.001, n.s. p> 0.40). (**K**) Sleep data in (**H**) expressed as a percentage of change in sleep amount normalized to the W4 / + parental control line. W4 flies are long-sleeping flies, so a 'normal' fly would be expected to exhibit a dramatic decrease in sleep relative to W4 flies. (**L**) Seven different ET-flp lines that were previously shown to suppress wing expansion defects when crossed to W4 flies, were obtained and crossed to W4 flies. Sleep is expressed as the percent change in total sleep relative to W4 flies. All of the progeny with expanded (normal) wings reduced sleep compared to the W4/+ parental control (p< 0.001), to levels statistically indistinguishable from their parental Et-flp / + controls (n.s. p> 0.20). In contrast, progeny with unexpanded wings had levels of sleep significantly higher than their Et-flp / + controls (p< 0.001), but indistinguishable from the W4 / + control (p>0.10). These Et-flp lines overlapped with CCAP GAL4 in the subesophageal ganglion. n=12-30 flies per condition.

Fig. S4. Disrupting *rickets* signaling blocks wing expansion and increases sleep. (**A**) Knocking down *rk* with RNAi (*rk GAL4* / + > *UAS rk RNAi* / +) resulted in a partially penetrant effect on wing expansion (with ~40% of progeny displaying wing defects). Two way repeated measures ANOVA detected a significant effect of genotype on time (n=16 flies / condition, p<0.001). (**B**) Daytime sleep was elevated in *rk GAL4* / + > *UAS rk RNAi* / + flies with expanded wings and *rk GAL4* / + > *UAS rk RNAi* / + flies with unexpanded wings relative to controls (*, p< 0.05, Tukey correction). (**C** and **D**) *rk GAL4* / + > *UAS rk RNAi* / + flies with unexpanded wings showed increased sleep consolidation during the day (* p<0.01, n.s. p> 0.8) and increased waking activity (* p < 0.01, n.s. p> 0.17) compared to controls. (**E**) Sleep was elevated in two different loss of function *rickets* (*rk*) mutants – *rk^{1}* & *rk^{4}* compared to controls (n=12-14 flies / genotype *, p< 0.01 Tukey correction). (**F**) Daytime sleep consolidation was not altered in either mutant relative to controls (n.s. p>0.28). (**G**) Waking activity was not decreased in either mutant relative to controls (* p <0.05, n.s. p > 0.17, Tukey correction). (**H**) The intensity of waking activity was elevated in rk > PKA-DN flies compared to controls (*, p <0.001, Tukey correction). (**I**) Sleep was increased in *rk GAL4* / + > *UAS dnc* / + flies (n=8) compared to controls (n=12-16 flies / genotype *, p <0.01 Tukey correction). (**J** and **K**) *rk GAL4* / + > *UAS dnc* / + flies displayed sleep consolidation during the day (* p <0.05, Tukey correction) and increased waking activity (* p < 0.001) compared to controls. (**L**) A temperature sensitive dynamin (UAS Shi^{ts}) was

used to inducibly block chemical transmission from *rk*GAL4 expressing neurons. *rk GAL4* / + > *UAS Shi^{ts}* / + flies and parental controls were shifted to the restrictive temperature of 31° for 1.5 hrs just after eclosion. This manipulation was sufficient to block wing expansion. Flies eclose ~10 days after egg-laying in these conditions (day10). Sleep data is shown for the second day after eclosion (Adult day2-3) for flies subjected to the temperature shift (**M** to **O**, n=12-16 flies / genotype) and sibling flies maintained at the permissive temperature (**P** to **R**, n=14-16 flies / genotype). Maintaining *rk GAL4* / + > *UAS Shi^{ts}* / + flies at the permissive temperature did not affect wing expansion. (**M** to **O**) *rk GAL4* / + > *UAS Shi^{ts}* / + flies subjected to the temperature shift increased sleep (* p< 0.001 Tukey correction), sleep consolidation during the day (* p<0.01), and intensity of waking activity * p<0.001)) compared to parental controls. (**P** to **R**) In contrast, sleep architecture of *rk GAL4* / + > *UAS Shi^{ts}* / + flies maintained at the permissive temperature were not altered relative to controls.

Fig. S5. Activating *rk*+ neurons increases sleep. (**A**) *rk GAL4* / + > *UAS NaChBac* / + flies increased sleep relative to controls (n=18-30 flies / genotype, Two-way repeated measures ANOVA for time X genotype (p<0.001). This manipulation completely blocked wing expansion. (**B** and **C**) Daytime sleep amount (* p< 0.001, Tukey correction) and sleep consolidation (* p< 0.001), were elevated in *rk GAL4* / + > *UAS NaChBac* / + flies compared to both controls. (**D**) Waking activity was elevated in *rk GAL4* / + > *UAS NaChBac* / + flies compared to both controls (* p< 0.001). (**E**) Sleep plot of *rk GAL4* / +> *UAS dTRPA1* / + flies and parental controls at the permissive temperature (23°) on the baseline day (n-12-16 flies / genotype). (**F**) Sleep plot of *rk GAL4* / +> *UAS dTRPA1* / + flies and parental controls for the day of the temperature shift. *rk* neurons were acutely activated on the day following the baseline day, by shifting flies to the restrictive temperature (28.5°) for 6 hrs (orange box). (**G**) *rk GAL4* / +> *UAS dTRPA1* / + flies acutely increased sleep for the 6 hours of the temperature shift compared to parental controls (*, p < 0.001 Tukey correction). Sleep is quantified as 6 hr totals of sleep at restrictive temperature – 6 hr totals at permissive temperature for each genotype. (**H**) A dominant negative PKA was expressed in subsets of PI neurons with different drivers. One of the lines decreased (R65C11) decreased sleep relative to controls (*, p<0.005 Tukey correction). None of the other lines significantly altered sleep.

Fig. S6. Manipulating R64F01 neurons increases sleep. (**A**) Driving rk RNAi with seven different GAL4 lines (hits from Fig. 3H) disrupted wing expansion and increased sleep (*, P<0.01 Tukey correction, n=16 flies per genotype). (**B** to **E**) Driving *rk* RNAi with R64F01 GAL4 had a partially penetrant effect on wing expansion with ~48% of *R64F01 GAL4 / +* > *UAS rk RNAi* / + flies exhibiting wing expansion defects. (**B**) Sleep plot of *R64F01 GAL4 / +* > *UAS rk RNAi* / + flies and parental controls. Two-way repeated measures ANOVA detected a significant genotype X time interaction (p<0.001). (**C** to **E**) *R64F01 GAL4 / +* > *UAS rk RNAi* / + flies with unexpanded wings (n=8) increased daytime sleep (**C**, * p <0.01 n.s. p > 0.10), sleep consolidation (**D**, * p< 0.01), and waking activity (**E**, * p<0.001) compared to controls (n=13-16 flies per genotype). (**F** to **H**) The *rk* receptor is known to signal through the Gβ subunit Gβ13F. Knocking down Gβ13F with RNAi in *R64F01 GAL4* neurons (*R64F01 GAL4* / + > *UAS Gβ13F RNAi /* +) resulted in a partially penetrant effect on wing expansion with ~68% of progeny displaying wing defects (n=53). (**F**) *R64F01 GAL4* / + > *UAS Gβ13F RNAi /* + flies with wing expansion defects increased sleep compared to parental controls (n=32 flies / genotype, * p<0.001 Tukey correction). (**G** and **H**) Sleep consolidation (**G**) and waking activity (**H**) were not altered in *R64F01 GAL4* / + > *UAS Gβ13F RNAi /* + flies relative to controls. (**I** to **L**) Chronic activation of *R64F01 GAL4* expressing neurons with NaChBac blocks wing expansion and increases sleep. (**M** to **O**) Acute activation of *R64F01 GAL4* expressing neurons with dTRPA1 in 4-5day old adult flies increases sleep. (**I**) *R64F01 GAL4* / + > *UAS NaChBac* / + flies increased sleep relative to controls (n=20-30 flies / genotype, two-way repeated measures ANOVA for time X genotype p<0.001). (**J** to **L**) Daytime sleep amount (**J**, * p< 0.001 Tukey correction), sleep consolidation (**K**, * p< 0.001), and waking activity (**L**, * p< 0.001) were all elevated in *R64F01 GAL4* / + > *UAS NaChBac* / + flies compared to both controls. (**M**) Sleep plot of *rk GAL4* / +> *UAS dTRPA1* / + flies and parental controls at the permissive temperature (23°) on the baseline day (n-12-16 flies / genotype). (**N**) Sleep plot of *R64F01 GAL4* / +> *UAS dTRPA1* / + flies and parental controls for the day of the temperature shift (n=14-16 flies / genotype). *R64F01 GAL4* neurons were acutely activated on the day following the baseline day, by shifting flies to the restrictive temperature (31°) for 6 hours

(orange box). (**O**) *R64F01 GAL4* / +> *UAS dTRPA1* / + flies acutely increased sleep for the 6 hours of the temperature shift compared to parental controls (*, $p < 0.05$ Tukey correction). Sleep is quantified as 6 hr totals of sleep at restrictive temperature – 6 hr totals at permissive temperature for each genotype.

Fig. S7. Detailed characterization of wing-disruption induced sleep. (**A**) Sleep plot of *dvGlut GAL80; R64F01 GAL4 / +* > *UAS rk RNAi* / + flies and parental controls (n=15-16 flies/ genotype). This manipulation did not affect wing expansion. (**B** and **C**) *dvGlut GAL80; R64F01 GAL4 / +* > *UAS rk RNAi* / + flies did not change sleep amount (n.s. $p > 0.3$) or consolidation (n.s. $p > 0.88$). (**D**) Waking activity of *dvGlut GAL80; R64F01 GAL4 / +* > *UAS rk RNAi* / + flies was not altered relative to controls (n.s. $p > 0.7$). (**E**) Comparison of GFP expression driven by *dvGlut GAL80*; *64F01 GAL4* with that of sibling controls lacking the *dvGlut GAL80* transgene reveals that *dvGlut GAL80* suppresses expression in most *R64F01* GAL4 neurons, including in the prominent subesophageal ganglion neurons labelled by this line (Green, GFP). (**F** and **G**) Wing-cut increased sleep amount and consolidation at night compared to intact wing controls (*, $p < 0.05$, ttest). (**H**) Waking activity of flies with cut wings was unchanged compared to intact wing controls (n.s. $p > 0.88$ Student's t-test). (**I**) Both flies with cut wings and their sibling controls with cut wings were equally aroused by a mechanical stimulus delivered at ZT15, on the second day following wing cut (*, $p < 0.001$ Tukey correction). (**J**) Flies with cut wings displayed a greater arousal threshold during the day compared to their siblings with intact wings (* $p < 0.001$ Student's t-test). (**K**) CantonS wild type flies and *per* mutant flies increased sleep following wing-cut even when maintained in the dark. (**L** to **O**) Flies with wings cut on the day of eclosion slept more than siblings with intact wings on the first (**L**), second (**M**), third (**N**), and fourth (**O**) days of adult life ($p < 0.01$, n=16-30 flies/ condition). (**P**) Mutations in immune system genes did not impair the ability of wing cut to induce sleep. Wing-cut dependent sleep is quantified for flies homozygous mutant for immune deficiency (*imd*^{1}) gene and the NFκB *relish* (*rel*^{e20}) as the change in sleep (cut-intact) normalized to the appropriate heterozygous allele (n.s. $p > 0.1$ Student's t-test). (**Q**) Protocol for wing glue. Wings of 3 day old adult flies were glued, and sleep assessed on the day following wing glue. (**R** to **T**) Flies with wings glued slept more (**R**, n=19-36 flies/ condition, * $p < 0.001$ Tukey correction), with more consolidated bouts during the day (**S**, * $p < 0.001$) without changing activity while awake (**T**, n.s. $p > 0.06$) compared to controls with intact wings and with an equivalent amount of glue applied to the abdomen (glue on body). (**U** to **W**) The cell-death activator reaper (rpr) was expressed in the wing disc with the Bx^{MS1096} GAL4 driver. This manipulation generated flies with unexpanded wings. *Bx^{MS1096} GAL4 / +* > *UAS rpr* / + flies increased sleep (**U**, n=14-28 flies / genotype * $p < 0.001$, Tukey correction), sleep consolidation (**V**, * $p < 0.001$) and were more active while awake compared to parental controls (**W**, * $p < 0.01$).

Fig. S8. Sleep in flightless mutants and additional characterization of signaling components in R64F01 GAL4 neurons. (**A**) Sleep of three different mutations that are known to impair flight was evaluated compared to appropriate controls. The canonical *wingless* mutation (*wg*^{1}) mutation has a partially penetrant effect on wing development with some homozygous mutant *wg*^{1} flies emerging with two wings and some with one wing. *wg*^{1} mutant flies with one wing slept more than their siblings with two wings (n=12-20 flies / genotype * $p < 0.001$ Student's t test). Similarly, flies carrying the dominant marker CyO slept more than their wild-type siblings (n=19-26 flies/ genotype, *, $p < 0.05$ Student's t test). Flies homozygous mutant for protein kinase c Δ (pkcΔ) also slept more than their heterozygous controls (n=7-23 flies / genotype, * $p < 0.001$ Student's t-test). (**B**) The intensity of waking activity was not decreased in any of the mutants relative to controls (*, $p < 0.05$, n.s. $p > 0.05$). (**C**) Different G protein subunits were knocked down in R64F01 neurons with RNAi. The extent of wing-cut induced sleep was significantly reduced in *UAS Dcr2;; R64F01 GAL4 /* + > *UAS Gαi RNAi* / + flies relative to controls (n=24-52 flies / condition, *, $p < 0.05$, n.s. $p > 0.10$). (**D**) Signalling through the Store Operated Calcium Entry (SOCE) in dispensable in R64F01 neurons for wing-cut mediated sleep increase. The extent of wing-cut induced sleep was not different in *UAS Dcr2;; R64F01 GAL4 /* + > *UAS plc RNAi* / + , *UAS Dcr2;; R64F01 GAL4 /* + > *UAS ItpR RNAi* / +, or *UAS Dcr2;; R64F01 GAL4 /* + > *UAS Stim RNAi* / + flies relative to controls (n=16-32 flies / condition, n.s. $p > 0.54$). (**E**) Signalling through inward rectifying potassium channels is dispensable in R64F01 neurons for wing cut mediate sleep. The extent of wing-cut induced sleep was not different in *UAS Dcr2;; R64F01 GAL4 /* + > *UAS Irk1 RNAi* / +, *UAS Dcr2;; R64F01 GAL4 /* + > *UAS Irk2 RNAi* / +, or *UAS Dcr2;; R64F01 GAL4 /* + >

UAS Irk3 RNAi / + flies relative to controls (n=26-32 flies / condition, n.s. p >0.98). (**F**) *R64F01 GAL4 / +* > *UAS mCD8GFP / +* neural processes were detected in close proximity to dopaminergic neural processes (labeled by anti-TH) in the SEG. (**G**) The extent of wing-cut induced sleep was not different *in dvGlut GAL80; 64F01 GAL4* / + > *UAS Dop2R RNAi* / + flies relative to controls (n=21-30 flies / genotype n.s. p >0.15). (**F**) Single confocal slice, green – GFP, magenta – anti TH (tyrosine hydroxylase). Scale bar - 20μm.

Fig. S9. Additional characterization of wing circuitry regulating wing cut. (**A**) Total sleep was increased in *Ir76b*, *Ir52a GAL4*/+ > *UASKir*/+ flies compared to controls (n=34-46 flies/ condition) under baseline conditions (in flies with intact wings). (**B** and **C**) *Ir76b*, *Ir52a GAL4*/+ > *UASKir*/+ flies displayed increased daytime sleep consolidation and waking activity compared to controls. (**D-F**) *Ir52a GAL4*/+ > *UASKir*/+ flies with intact wings did not change sleep amount (**D**), consolidation (**E**) or waking activity (**F**) compared to controls. (**G-I**) *Ir76b GAL4*/+ > *UASKir*/+ flies with intact wings did not change sleep amount (**G**), consolidation (**H**) or waking activity (**I**) compared to controls. (**J**) Like *Ir52a GAL4* (Fig. 5B), GFP driven by *Ir76b GAL4* (green) also labelled sensory neurons in the wing (right), and sensory afferents from legs into the VNC (middle). In addition, *Ir76b GAL4* also drove expression in classes of olfactory sensory neurons and putative gustatory neurons in the labellum that project into the brain (left). CNS tissues were counterstained with nc82, a neuropil marker (magenta). (**K**) Consistent with the expression of *Ir76b GAL4* in many different classes of sensory neurons (green, left), *Ir76b GAL4 > trans-tango* (magenta, middle) labelled a broad population of second-order neurons, with strong labelling in the SEG and antennal lobes, including olfactory projection neurons. (**L**) A sub-stack of optical slices in (**J**), revealed I*r76b GAL4* > *trans-tango* expression in a projection neuron tract that resembled the medial tract seen in Fig. 5e (orange arrows). (**M**) As a negative control, trans-tango components were crossed to a control yw strain, faint signal was detected in the ventral medial SEG (grey arrows). (**N**) 3*1C06 GAL4* / + > *GFP* / + labelled projection neuronal tracts that connect the VNC to higher order brain centres along a medial (orange arrow) and a lateral tract (yellow arrow) with extensive arborization in the SEG and VLP Green – GFP, magenta – nc82. (**J** to **N**) Images are maximal intensity z-projections of confocal stacks. Scale bar for all images = 20μm. VLP – ventro-lateral protocerebrum, SEG – subesophageal ganglion.

Fig. S10. Additional anatomical and behavioral characterization of R31C06 GAL4 projection neurons. (**A**) Sleep plot of *R31C06 GAL4* / +> *UAS dTRPA1* / + flies and parental controls at the permissive temperature (22°) on the baseline day (n-20-30 flies / genotype). (**B**) Sleep plot of *R31C06 GAL4* / +> *UAS dTRPA1* / + flies and parental controls for the day of the temperature shift. *R31C06 GAL4* neurons were acutely activated on the day following the baseline day, by shifting flies to the restrictive temperature (31°) for 24 hrs. (**C**) *R31C06 GAL4* / +> *UAS dTRPA1* / + flies acutely increased sleep compared to parental controls (*, p < 0.001 Tukey correction). Sleep is quantified as total sleep at restrictive temperature – total sleep at permissive temperature for each genotype. (**D**) In region 3, the intensity of BRP puncta was increased in cut *R31C06GAL4/+>STaR* flies and *R31C06GAL4/+>CyO/+;STaR* flies compared to intact controls. (*p <0.001, Tukey correction). The number of puncta was not altered, however. (**E**) The intensity of BRP puncta was also increased in region 4 in cut *R31C06GAL4/+>STaR* flies and *R31C06GAL4/+>CyO/+;STaR* flies compared to intact controls without changing the number of BRP puncta (*p <0.001, Tukey correction). (**F** and **G**) The number of BRP puncta and intensity per punctum were elevated in 31C06-GAL4 / + > STaR / + brains in region 1 (**F**) and region 2 (**G**) one day following wing-cut (**H** and **I**) *31C06GAL4/+>UAS-Denmark,UAS syt:EGFP/+* (**H**) and *R64F01GAL4/+>UAS-Denmark*, ,*UAS syt:EGFP/+* (**I**) staining patterns in the brain. *UAS-Denmark* (magenta) labels dendrites, syt:EGFP (green) labels presynaptic sites. SEG-subesophageal ganglion. (**J**) Representative images of Gcamp6.0s fluorescence in *64F01GAL4* cell bodies in the SEG before and after activation of 31C06LeXa projection neurons by ATP mediated stimulation of the P2X2 cation channel. The region shown correspond to cells labeled with yellow arrows in (Fig. 6L). (**K**) P2X2 mediated stimulation of 31C06LexA projection neurons by bath application of ATP, increases Gcamp fluorescence in 64F01 GAL4 cell bodies (*31C06LexA/+, 64F01GAL4/+>LeXaop P2X2/+*,*UAS Gcamp6.0s/+)*. Control animals lacking the 31C06 LexA transgene did not exhibit this response (*64F01GAL4/+>LeXaop P2X2/+*,*UAS Gcamp6.0s/+*)(n=6cells/condition). (**L**) Maximum change in fluorescence in experimental ('exp', *31C06LexA/+, 64F01GAL4/+>LeXaop P2X2/+*,*UAS Gcamp6.0s/+)*, and control

('ctrl',*64F01GAL4/+>LeXaop P2X2/+,UAS Gcamp6.0s/+*) animals.(*, p<0.01, ttest). (**H** and **I**) Images are maximal intensity z-projections of confocal stacks. Scale bar for all images = 20μm.

Figure S1

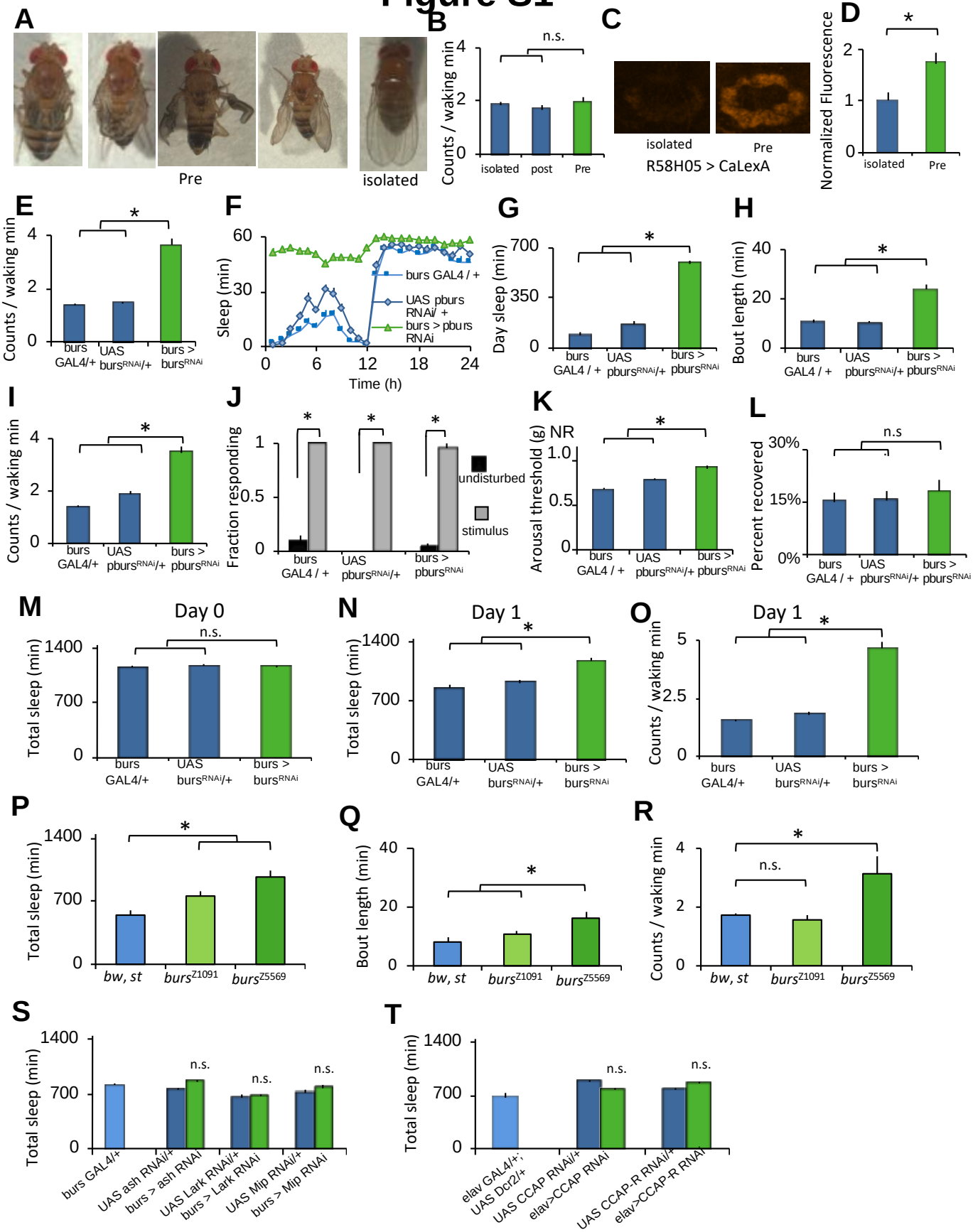

A
Pre
isolated
B
Counts / waking min
n.s.
isolated
post
Pre
C
isolated
Pre
R58H05 > CaLexA
D
Normalized Fluorescence
*
isolated
Pre
E
Counts / waking min
burs GAL4/+
UAS burs^RNAi/+
burs > burs^RNAi
F
Sleep (min)
Time (h)
burs GAL4 / +
UAS pburs RNAi/ +
burs > pburs RNAi
G
Day sleep (min)
burs GAL4 / +
UAS pburs^RNAi/+
burs > pburs^RNAi
H
Bout length (min)
burs GAL4 / +
UAS pburs^RNAi/+
burs > pburs^RNAi
I
Counts / waking min
burs GAL4/+
UAS pburs^RNAi/+
burs > pburs^RNAi
J
Fraction responding
undisturbed
stimulus
burs GAL4 / +
UAS pburs^RNAi/+
burs > pburs^RNAi
K
NR
Arousal threshold (g)
burs GAL4 / +
UAS pburs^RNAi/+
burs > pburs^RNAi
L
Percent recovered
n.s
burs GAL4 / +
UAS pburs^RNAi/+
burs > pburs^RNAi
M
Day 0
Total sleep (min)
n.s.
burs GAL4/+
UAS burs^RNAi/+
burs > burs^RNAi
N
Day 1
Total sleep (min)
burs GAL4/+
UAS burs^RNAi/+
burs > burs^RNAi
O
Day 1
Counts / waking min
burs GAL4/+
UAS burs^RNAi/+
burs > burs^RNAi
P
Total sleep (min)
bw, st
burs^Z1091
burs^Z5569
Q
Bout length (min)
bw, st
burs^Z1091
burs^Z5569
R
Counts / waking min
n.s.
bw, st
burs^Z1091
burs^Z5569
S
Total sleep (min)
n.s.
n.s.
n.s.
burs GAL4/+
UAS ash RNAi/+
burs > ash RNAi
UAS Lark RNAi/+
burs > Lark RNAi
UAS Mip RNAi/+
burs > Mip RNAi
T
Total sleep (min)
n.s.
n.s.
elav GAL4/+; UAS Dcr2/+
UAS CCAP RNAi/+
elav>CCAP RNAi
UAS CCAP-R RNAi/+
elav>CCAP-R RNAi

Figure S2

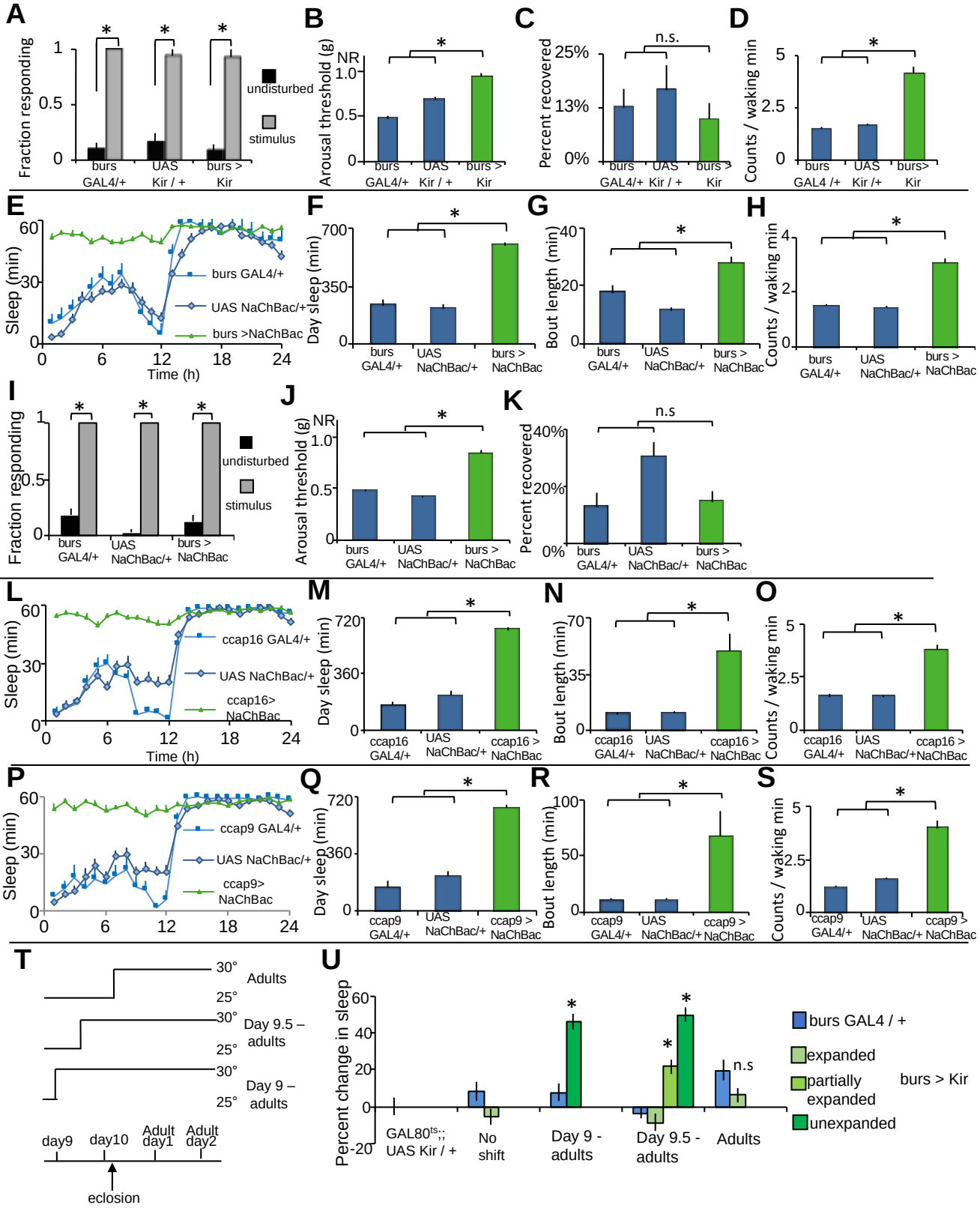

A
Fraction responding
undisturbed
stimulus
burs GAL4/+
UAS Kir / +
burs > Kir
B
Arousal threshold (g)
NR
C
Percent recovered
n.s.
D
Counts / waking min
E
Sleep (min)
burs GAL4/+
UAS NaChBac/+
burs >NaChBac
Time (h)
F
Day sleep (min)
G
Bout length (min)
H
Counts / waking min
I
Fraction responding
undisturbed
stimulus
burs GAL4/+
UAS NaChBac/+
burs > NaChBac
J
Arousal threshold (g)
NR
K
Percent recovered
n.s
L
Sleep (min)
ccap16 GAL4/+
UAS NaChBac/+
ccap16> NaChBac
Time (h)
M
Day sleep (min)
N
Bout length (min)
O
Counts / waking min
P
Sleep (min)
ccap9 GAL4/+
UAS NaChBac/+
ccap9> NaChBac
Time (h)
Q
Day sleep (min)
R
Bout length (min)
S
Counts / waking min
T
30°
25°
Adults
Day 9.5 – adults
Day 9 – adults
day9
day10
Adult day1
Adult day2
eclosion
U
Percent change in sleep
GAL80ts;; UAS Kir / +
No shift
Day 9 - adults
Day 9.5 - adults
Adults
n.s
burs GAL4 / +
expanded
partially expanded
unexpanded
burs > Kir

Figure S3

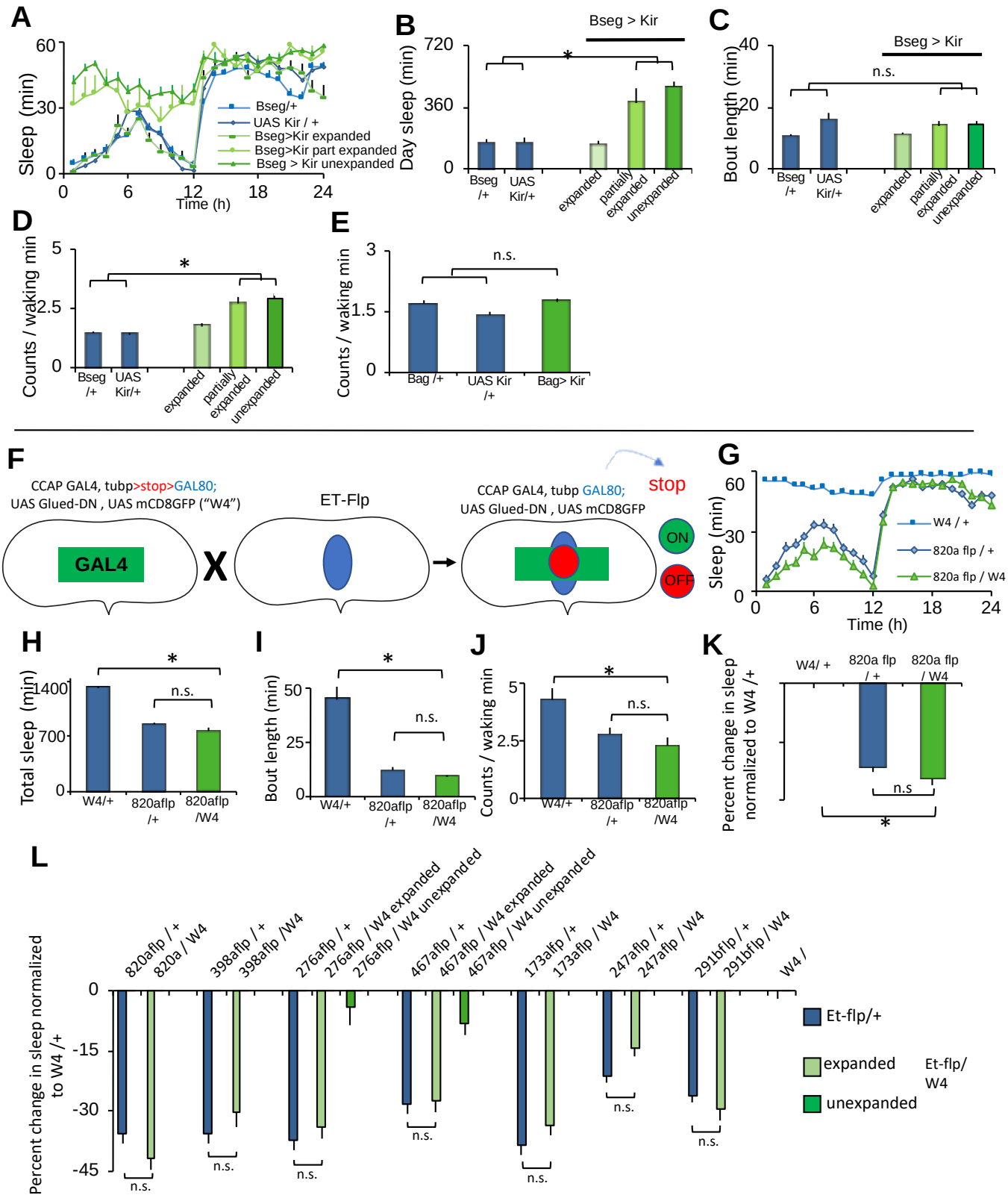

A
Sleep (min)
Time (h)
Bseg/+
UAS Kir / +
Bseg>Kir expanded
Bseg>Kir part expanded
Bseg > Kir unexpanded
B
Day sleep (min)
Bseg > Kir
Bseg /+
UAS Kir/+
expanded
partially expanded
unexpanded
C
Bout length (min)
Bseg > Kir
n.s.
D
Counts / waking min
E
Counts / waking min
Bag /+
UAS Kir /+
Bag> Kir
F
CCAP GAL4, tubp>stop>GAL80;
UAS Glued-DN , UAS mCD8GFP ("W4")
GAL4
X
ET-Flp
CCAP GAL4, tubp GAL80;
UAS Glued-DN , UAS mCD8GFP
stop
ON
OFF
G
Sleep (min)
Time (h)
W4 / +
820a flp / +
820a flp / W4
H
Total sleep (min)
W4/+
820aflp /+
820aflp /W4
I
Bout length (min)
J
Counts / waking min
K
Percent change in sleep normalized to W4 /+
W4/ +
820a flp / +
820a flp / W4
n.s
L
Percent change in sleep normalized to W4 /+
820aflp / + / 820a / W4
398aflp / + / 398aflp /W4
276aflp / + / 276aflp / W4 expanded / 276aflp / W4 unexpanded
467aflp / + / 467aflp / W4 expanded / 467aflp / W4 unexpanded
173alfp /+ / 173aflp / W4
247aflp / + / 247aflp / W4
291bflp / + / 291bflp / W4
W4 /
Et-flp/+
expanded
unexpanded
Et-flp/ W4
n.s.

Figure S4

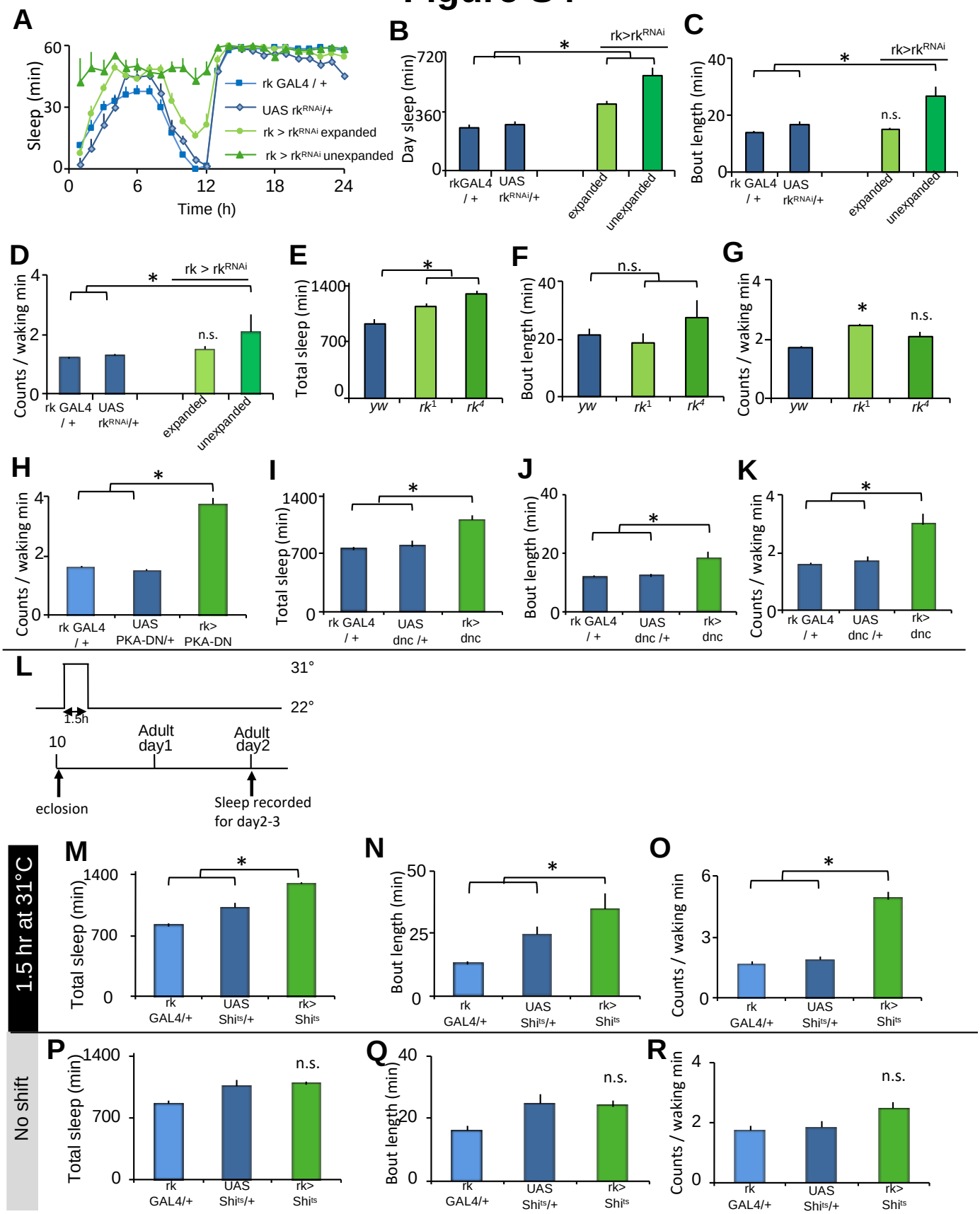

A
Sleep (min)
Time (h)
rk GAL4 / +
UAS rkRNAi/+
rk > rkRNAi expanded
rk > rkRNAi unexpanded
B
Day sleep (min)
rk>rkRNAi
rkGAL4 / +
UAS rkRNAi/+
expanded
unexpanded
C
Bout length (min)
n.s.
D
Counts / waking min
E
Total sleep (min)
yw
rk1
rk4
F
Bout length (min)
G
Counts / waking min
H
rk GAL4 / +
UAS PKA-DN/+
rk> PKA-DN
I
Total sleep (min)
UAS dnc /+
rk> dnc
J
Bout length (min)
K
Counts / waking min
L
31°
22°
1.5h
10
Adult day1
Adult day2
eclosion
Sleep recorded for day2-3
1.5 hr at 31°C
M
Total sleep (min)
rk GAL4/+
UAS Shits/+
rk> Shits
N
Bout length (min)
O
Counts / waking min
No shift
P
Total sleep (min)
Q
Bout length (min)
R
Counts / waking min

Figure S5

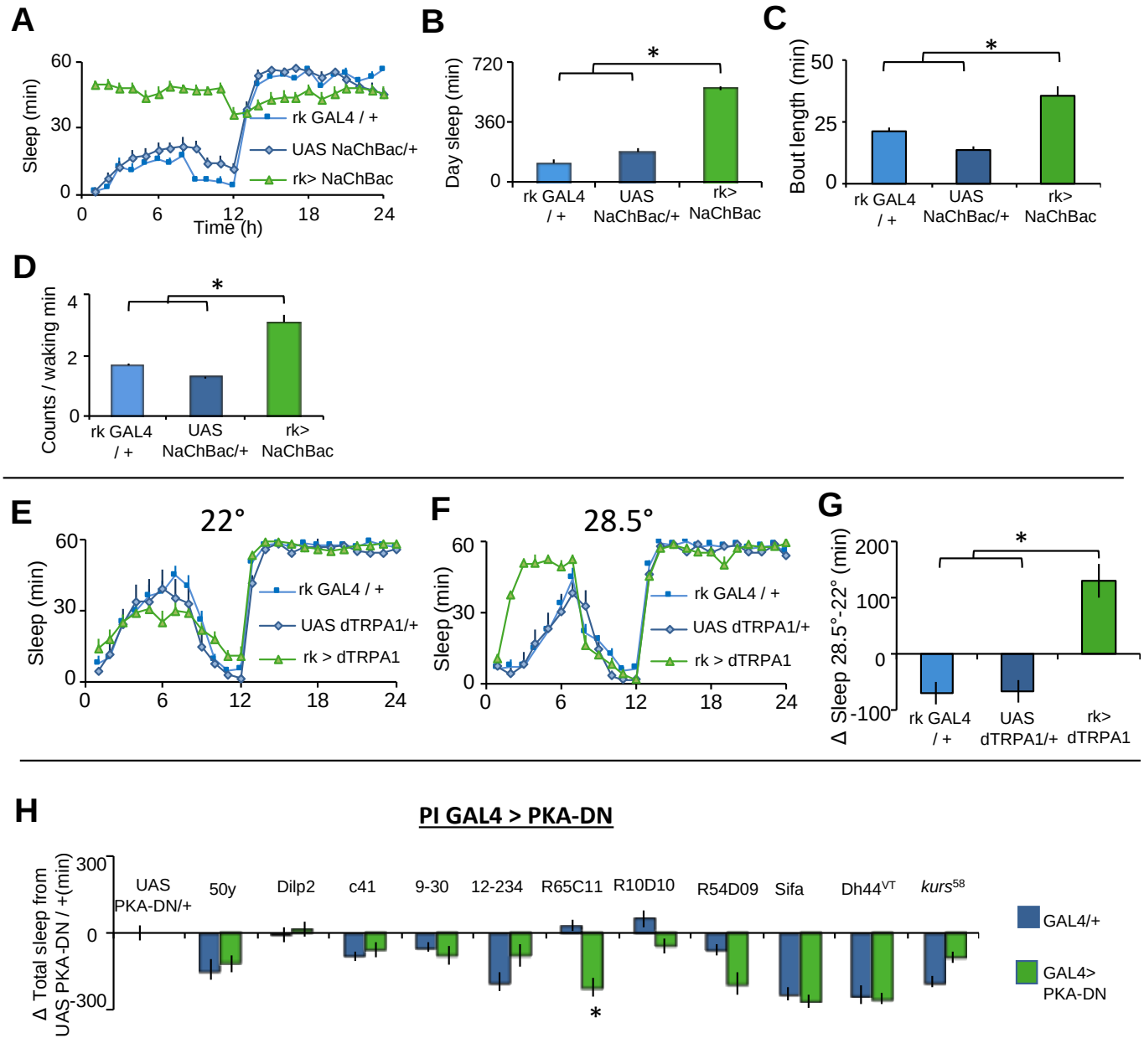

A
Sleep (min)
60
30
0
0
6
12
18
24
Time (h)
rk GAL4 / +
UAS NaChBac/+
rk> NaChBac
B
Day sleep (min)
720
360
0
*
rk GAL4 / +
UAS NaChBac/+
rk> NaChBac
C
Bout length (min)
50
25
0
*
rk GAL4 / +
UAS NaChBac/+
rk> NaChBac
D
Counts / waking min
4
2
0
*
rk GAL4 / +
UAS NaChBac/+
rk> NaChBac
E
22°
Sleep (min)
60
30
0
0
6
12
18
24
rk GAL4 / +
UAS dTRPA1/+
rk > dTRPA1
F
28.5°
Sleep (min)
60
30
0
0
6
12
18
24
rk GAL4 / +
UAS dTRPA1/+
rk > dTRPA1
G
Δ Sleep 28.5°-22° (min)
200
100
0
-100
*
rk GAL4 / +
UAS dTRPA1/+
rk> dTRPA1
H
PI GAL4 > PKA-DN
Δ Total sleep from UAS PKA-DN / +(min)
300
0
-300
UAS PKA-DN/+
50y
Dilp2
c41
9-30
12-234
R65C11
R10D10
R54D09
Sifa
Dh44VT
kurs58
*
GAL4/+
GAL4> PKA-DN

Figure S6

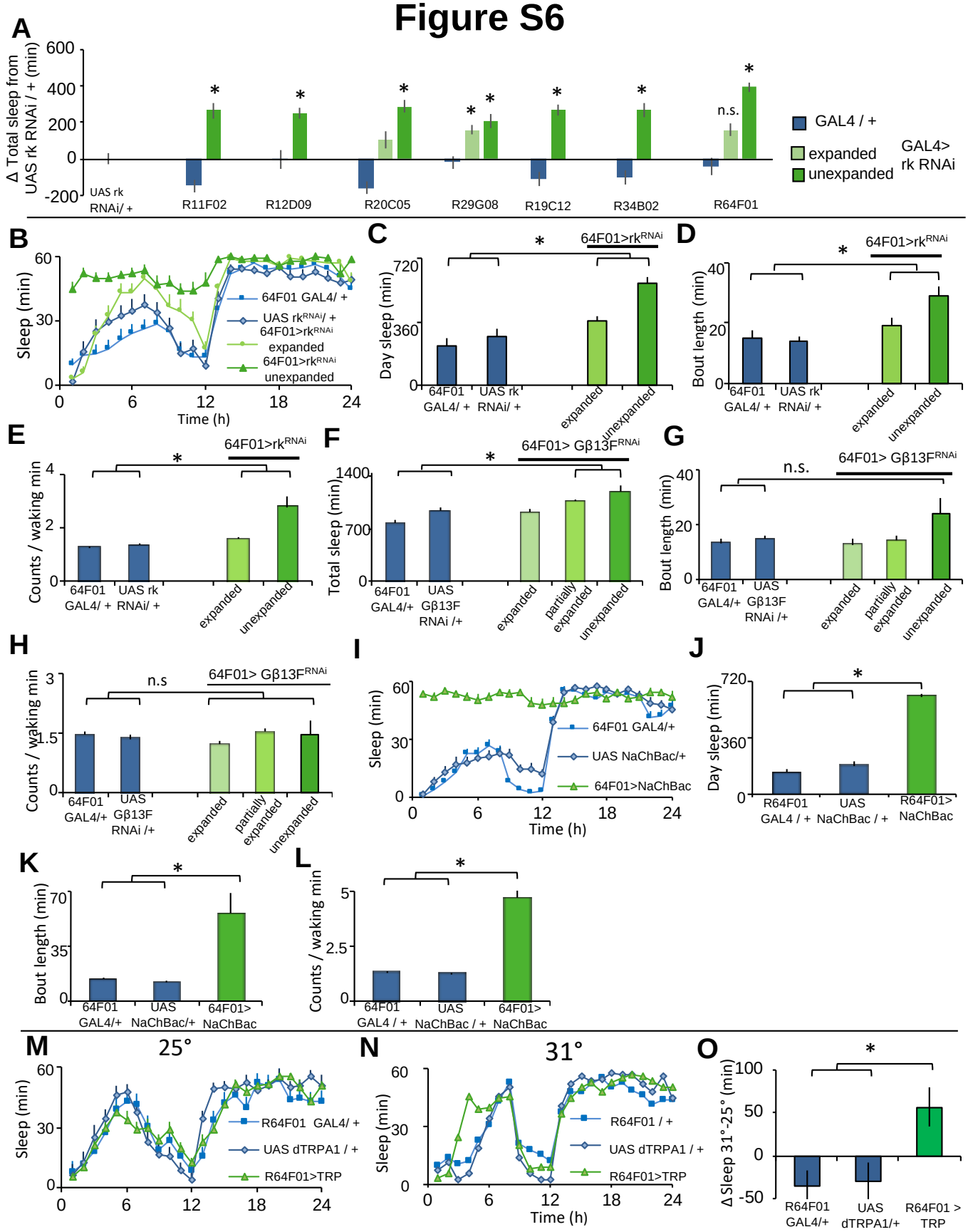

A
Δ Total sleep from UAS rk RNAi / + (min)
600
400
200
0
-200
UAS rk RNAi/ +
R11F02
R12D09
R20C05
R29G08
R19C12
R34B02
R64F01
n.s.
GAL4 / +
expanded
unexpanded
GAL4> rk RNAi
B
Sleep (min)
60
30
0
0
6
12
18
24
Time (h)
64F01 GAL4/ +
UAS rkRNAi/ +
64F01>rkRNAi
expanded
64F01>rkRNAi
unexpanded
C
64F01>rkRNAi
Day sleep (min)
720
360
0
64F01 GAL4/ +
UAS rk RNAi/ +
expanded
unexpanded
D
64F01>rkRNAi
Bout length (min)
40
20
0
64F01 GAL4/ +
UAS rk RNAi/ +
expanded
unexpanded
E
64F01>rkRNAi
Counts / waking min
4
2
0
64F01 GAL4/ +
UAS rk RNAi/ +
expanded
unexpanded
F
64F01> Gβ13FRNAi
Total sleep (min)
1400
700
0
64F01 GAL4/+
UAS Gβ13F RNAi /+
expanded
partially expanded
unexpanded
G
n.s.
64F01> Gβ13FRNAi
Bout length (min)
40
20
0
64F01 GAL4/+
UAS Gβ13F RNAi /+
expanded
partially expanded
unexpanded
H
n.s
64F01> Gβ13FRNAi
Counts / waking min
3
1.5
0
64F01 GAL4/+
UAS Gβ13F RNAi /+
expanded
partially expanded
unexpanded
I
Sleep (min)
60
30
0
0
6
12
18
24
Time (h)
64F01 GAL4/+
UAS NaChBac/+
64F01>NaChBac
J
Day sleep (min)
720
360
0
R64F01 GAL4 / +
UAS NaChBac / +
R64F01> NaChBac
K
Bout length (min)
70
35
0
64F01 GAL4/+
UAS NaChBac/+
64F01> NaChBac
L
Counts / waking min
5
2.5
0
64F01 GAL4 / +
UAS NaChBac / +
64F01> NaChBac
M
25°
Sleep (min)
60
30
0
0
6
12
18
24
R64F01 GAL4/ +
UAS dTRPA1 / +
R64F01>TRP
N
31°
Sleep (min)
60
30
0
0
6
12
18
24
Time (h)
R64F01 / +
UAS dTRPA1 / +
R64F01>TRP
O
Δ Sleep 31°-25° (min)
100
50
0
-50
R64F01 GAL4/+
UAS dTRPA1/+
R64F01 > TRP

Figure S7

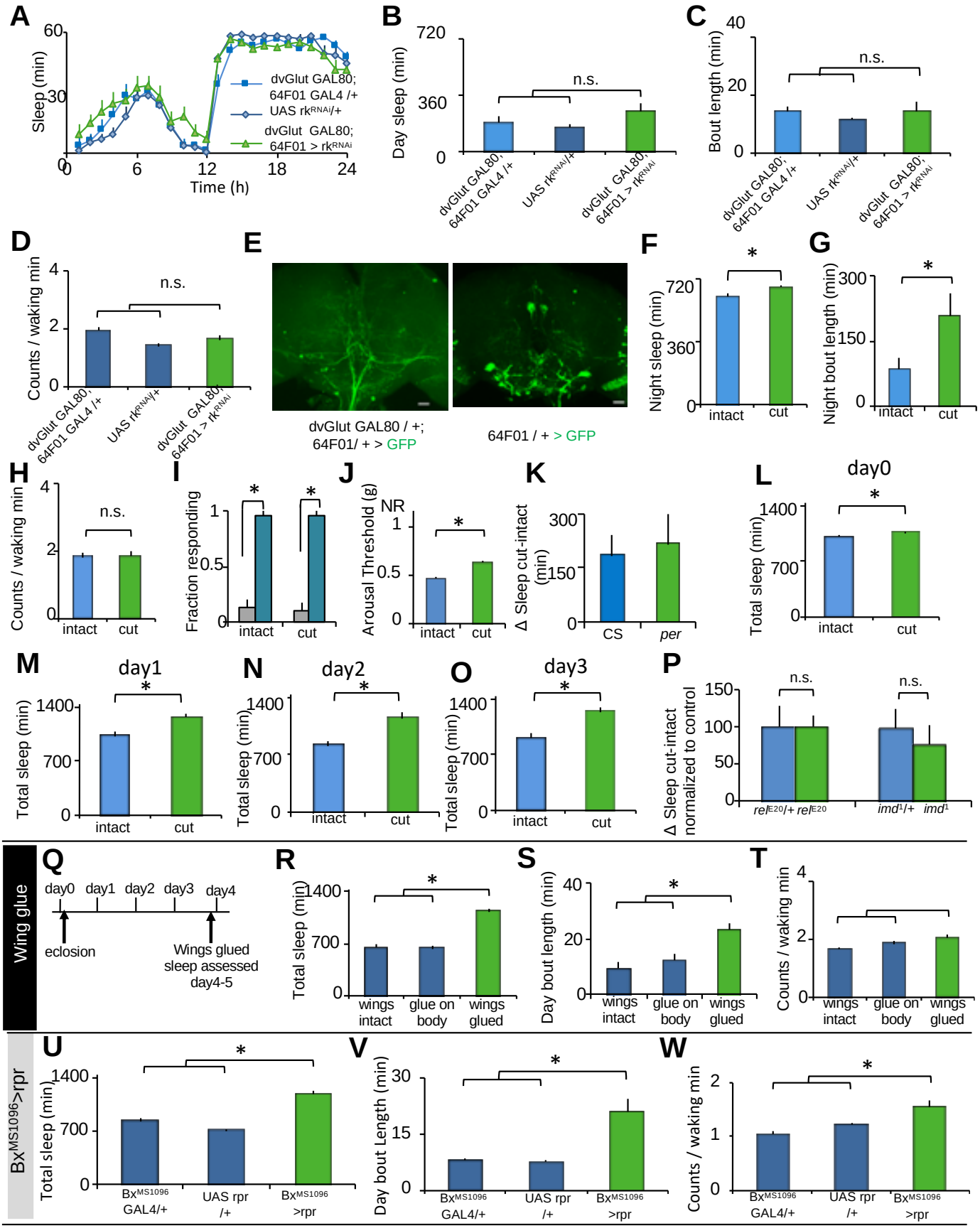
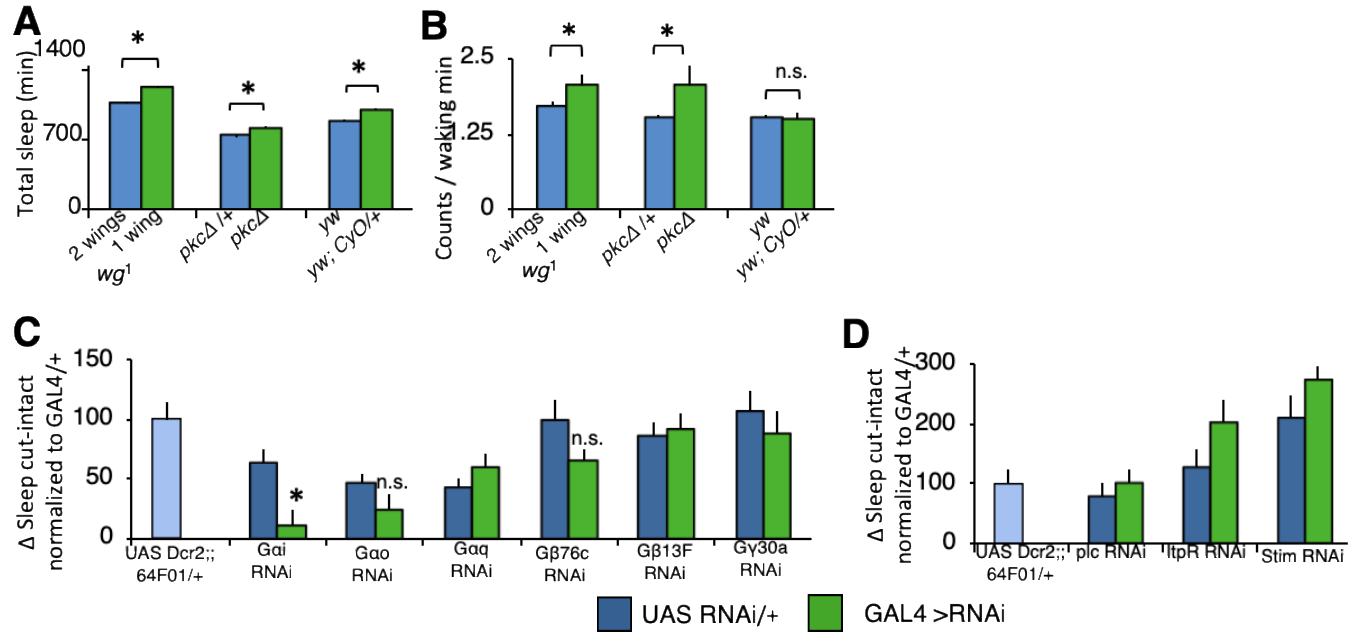

A
Sleep (min)
Time (h)
dvGlut GAL80; 64F01 GAL4 /+
UAS rkRNAi/+
dvGlut GAL80; 64F01 > rkRNAi
B
Day sleep (min)
n.s.
C
Bout length (min)
n.s.
D
Counts / waking min
n.s.
E
dvGlut GAL80 / +; 64F01/ + > GFP
64F01 / + > GFP
F
Night sleep (min)
intact
cut
G
Night bout length (min)
intact
cut
H
Counts / waking min
n.s.
intact
cut
I
Fraction responding
intact
cut
J
Arousal Threshold (g)
NR
intact
cut
K
Δ Sleep cut-intact (min)
CS
per
L
day0
Total sleep (min)
intact
cut
M
day1
Total sleep (min)
intact
cut
N
day2
Total sleep (min)
intact
cut
O
day3
Total sleep (min)
intact
cut
P
Δ Sleep cut-intact normalized to control
n.s.
n.s.
relE20/+
relE20
imd1/+
imd1
Wing glue
Q
day0
day1
day2
day3
day4
eclosion
Wings glued
sleep assessed
day4-5
R
Total sleep (min)
wings intact
glue on body
wings glued
S
Day bout length (min)
wings intact
glue on body
wings glued
T
Counts / waking min
wings intact
glue on body
wings glued
BxMS1096>rpr
U
Total sleep (min)
BxMS1096 GAL4/+
UAS rpr /+
BxMS1096 >rpr
V
Day bout Length (min)
BxMS1096 GAL4/+
UAS rpr /+
BxMS1096 >rpr
W
Counts / waking min
BxMS1096 GAL4/+
UAS rpr /+
BxMS1096 >rpr

Figure S8


A
Total sleep (min)
1400
700
0
2 wings
1 wing
wg1
pkcΔ /+
pkcΔ
yw
yw; CyO/+
B
Counts / waking min
2.5
1.25
0
n.s.
C
Δ Sleep cut-intact normalized to GAL4/+
150
100
50
0
UAS Dcr2;; 64F01/+
Gαi RNAi
Gαo RNAi
Gαq RNAi
Gβ76c RNAi
Gβ13F RNAi
Gγ30a RNAi
D
300
200
100
plc RNAi
ItpR RNAi
Stim RNAi
UAS RNAi/+
GAL4 >RNAi

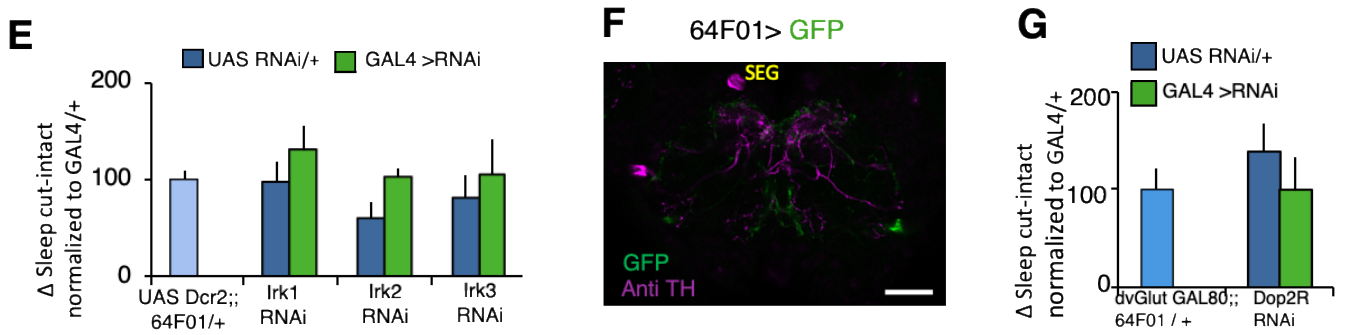
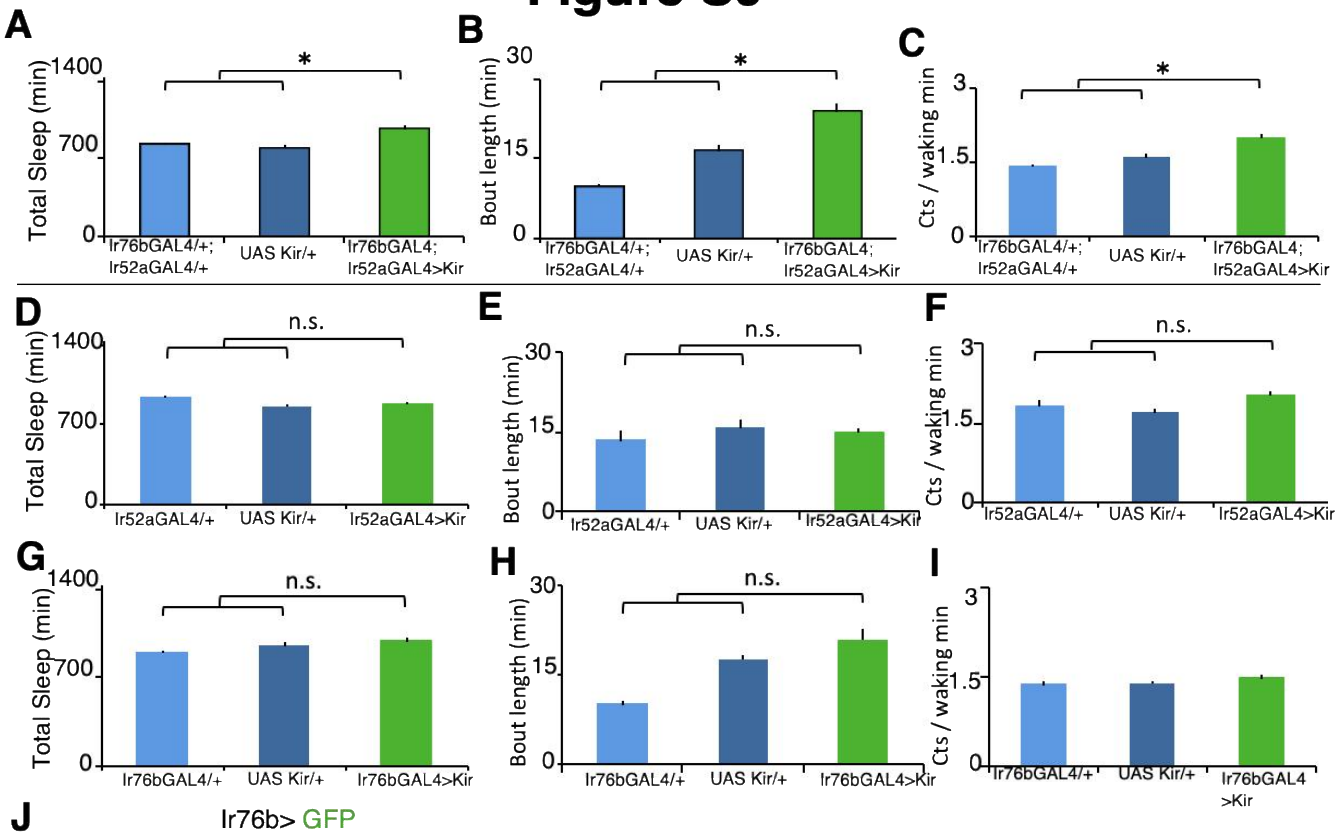
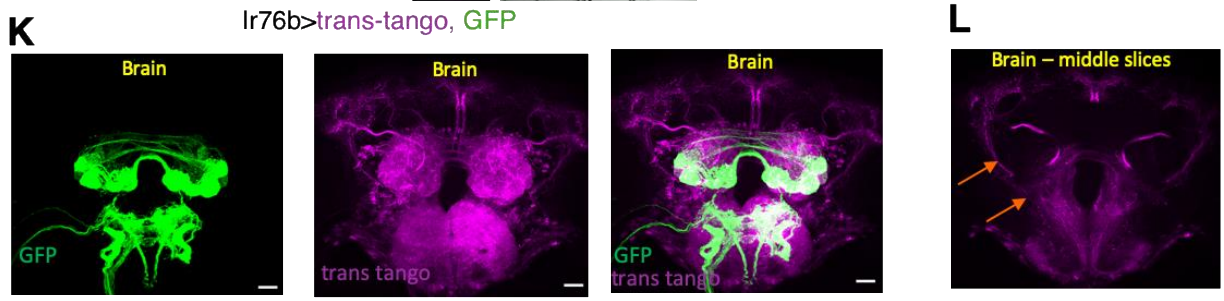
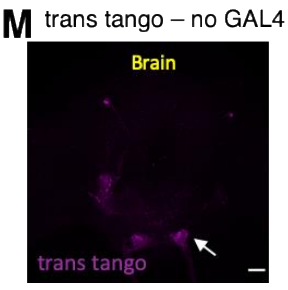

E
UAS RNAi/+
GAL4 >RNAi
Δ Sleep cut-intact normalized to GAL4/+
200
100
0
UAS Dcr2;; 64F01/+
Irk1 RNAi
Irk2 RNAi
Irk3 RNAi
F
64F01> GFP
SEG
GFP
Anti TH
G
UAS RNAi/+
GAL4 >RNAi
Δ Sleep cut-intact normalized to GAL4/+
200
100
0
dvGlut GAL80;; 64F01 / +
Dop2R RNAi

Figure S9


A
Total Sleep (min)
1400
700
0
*
Ir76bGAL4/+; Ir52aGAL4/+
UAS Kir/+
Ir76bGAL4; Ir52aGAL4>Kir
B
Bout length (min)
30
15
0
*
C
Cts / waking min
3
1.5
0
*
D
n.s.
Ir52aGAL4/+
UAS Kir/+
Ir52aGAL4>Kir
E
F
G
Ir76bGAL4/+
Ir76bGAL4>Kir
H
I
Ir76bGAL4 >Kir


J
Ir76b> GFP
Brain
GFP
nc82
VNC
wing margin


K
Ir76b>trans-tango, GFP
Brain
GFP
trans tango
L
Brain – middle slices


M
trans tango – no GAL4
Brain
trans tango

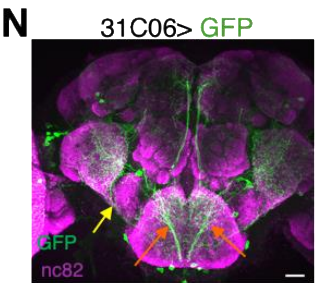
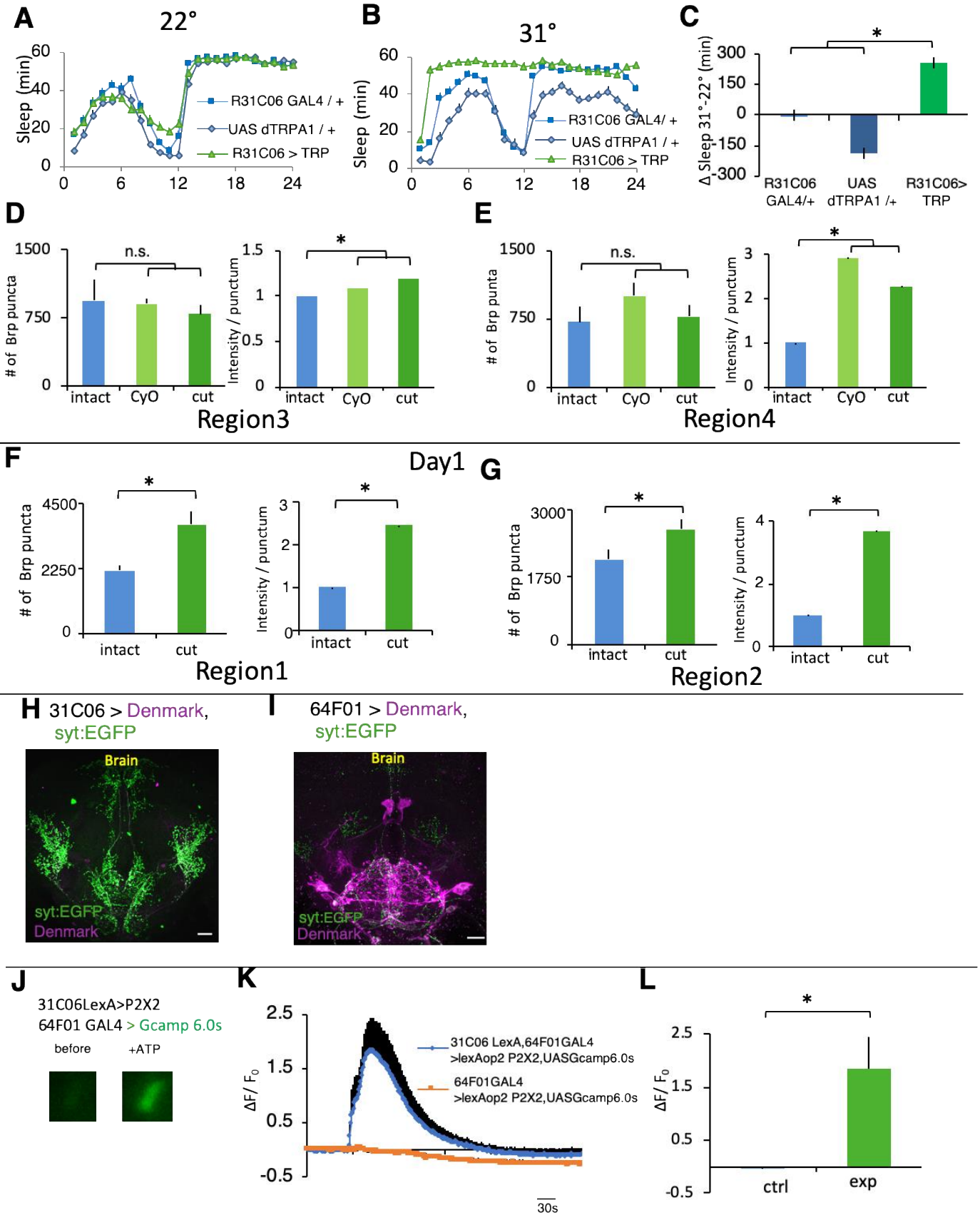

N
31C06> GFP
GFP
nc82

Figure S10


A
22°
Sleep (min)
R31C06 GAL4 / +
UAS dTRPA1 / +
R31C06 > TRP
B
31°
Sleep (min)
R31C06 GAL4/ +
UAS dTRPA1 / +
R31C06 > TRP
C
Δ Sleep 31°-22° (min)
R31C06 GAL4/+
UAS dTRPA1 /+
R31C06> TRP
D
# of Brp puncta
n.s.
Intensity / punctum
intact
CyO
cut
Region3
E
# of Brp punta
n.s.
Intensity / punctum
intact
CyO
cut
Region4
F
Day1
# of Brp puncta
Intensity / punctum
intact
cut
Region1
G
# of Brp puncta
Intensity / punctum
intact
cut
Region2
H 31C06 > Denmark, syt:EGFP
Brain
syt:EGFP
Denmark
I 64F01 > Denmark, syt:EGFP
Brain
syt:EGFP
Denmark
J
31C06LexA>P2X2
64F01 GAL4 > Gcamp 6.0s
before
+ATP
K
ΔF/ F0
31C06 LexA,64F01GAL4 >lexAop2 P2X2,UASGcamp6.0s
64F01GAL4 >lexAop2 P2X2,UASGcamp6.0s
30s
L
ΔF/ F0
ctrl
exp