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RESEARCH ARTICLE

Figures and figure supplements

Neurocalcin regulates nighttime sleep and arousal in *Drosophila*

Ko-Fan Chen *et al*

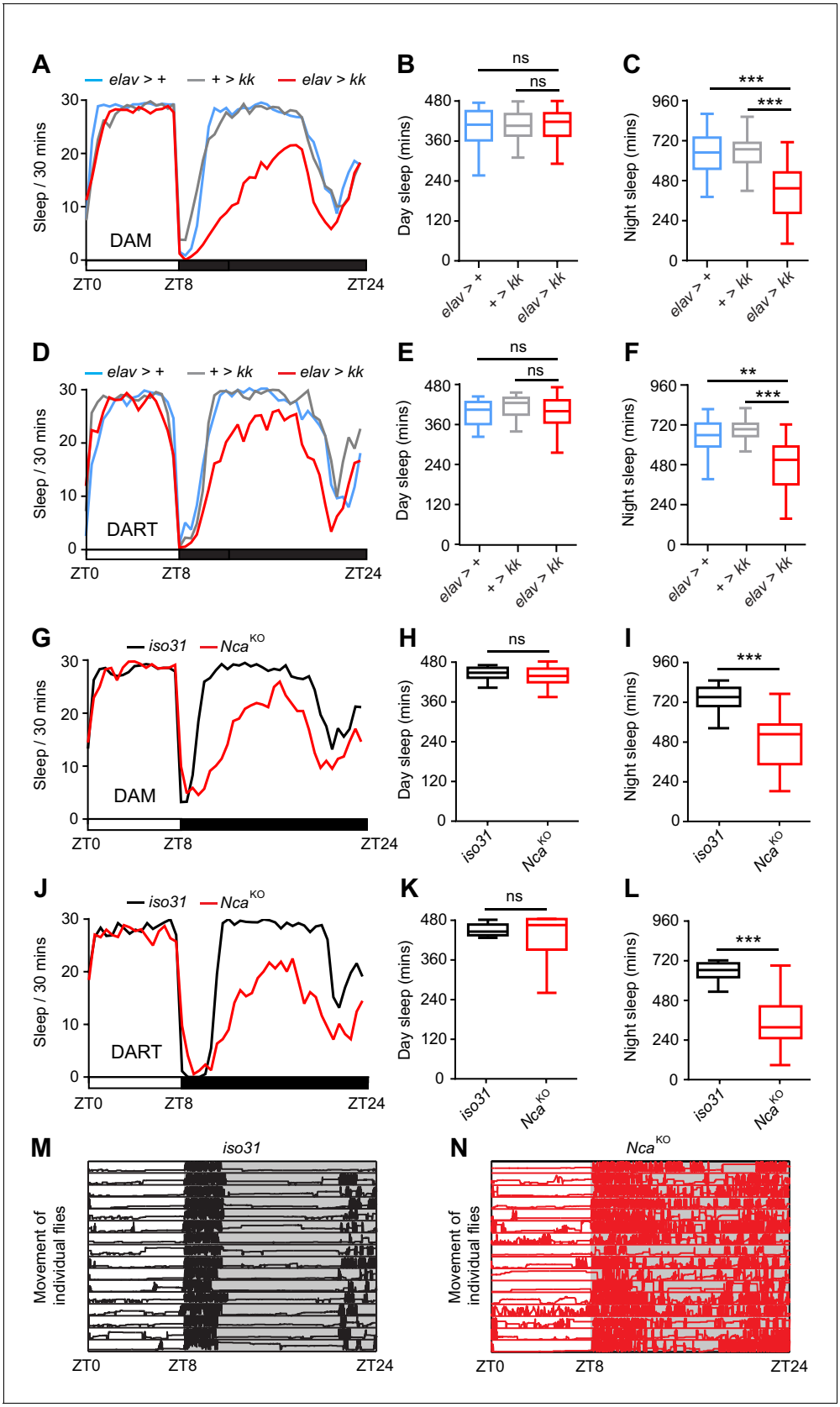

Figure 1. Neurocalcin promotes night sleep. (**A**) Mean sleep levels measured using the DAM system under 8L: 16D conditions for adult male pan-neuronal *Nca* knockdown flies (*elav > kk*) and associated controls (*elav*-Gal4 driver or *kk* RNAi transgene heterozygotes). (**B–C**) Median day (**B**) and night

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Figure 1 continued

(**C**) sleep levels in the above genotypes. n = 54–55. Data are presented as Tukey box plots. The 25th, Median, and 75th percentiles are shown. Whiskers represent 1.5 x the interquartile range. Identical representations are used in all subsequent box plots. (**D**) Mean sleep levels measured using the DART system in 8L: 16D conditions for male adult pan-neuronal *Nca* knockdown flies (*elav > kk*) and associated controls. (**E–F**) Median day (**E**) and night (**F**) sleep levels in the above genotypes. n = 20 per genotype. (**G**) Mean sleep levels in 8L: 16D conditions for *Nca*^{KO} adult males and *iso31* controls measured using the DAM. (**H–I**) Median day (**H**) and night (**I**) sleep levels in the above genotypes. n = 32 per genotype. (**J**) Mean sleep levels in 8L: 16D conditions for *Nca*^{KO} adult males and *iso31* controls measured by DART. (**K–L**) Median day (**K**) and night (**L**) sleep levels in the above genotypes. n = 16 per genotype. (**M–N**) The longitudinal movement for individual *iso31* (**M**) and *Nca*^{KO} (**N**) flies are shown as rows of traces plotting vertical position (Y-axis) over 24 hr (X-axis) under 8L: 16D condition. ns (not significant) - p>0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's post-hoc test (**B–C, E–F**) or Mann-Whitney U-test (**H–I, K–L**).

DOI: https://doi.org/10.7554/eLife.38114.002

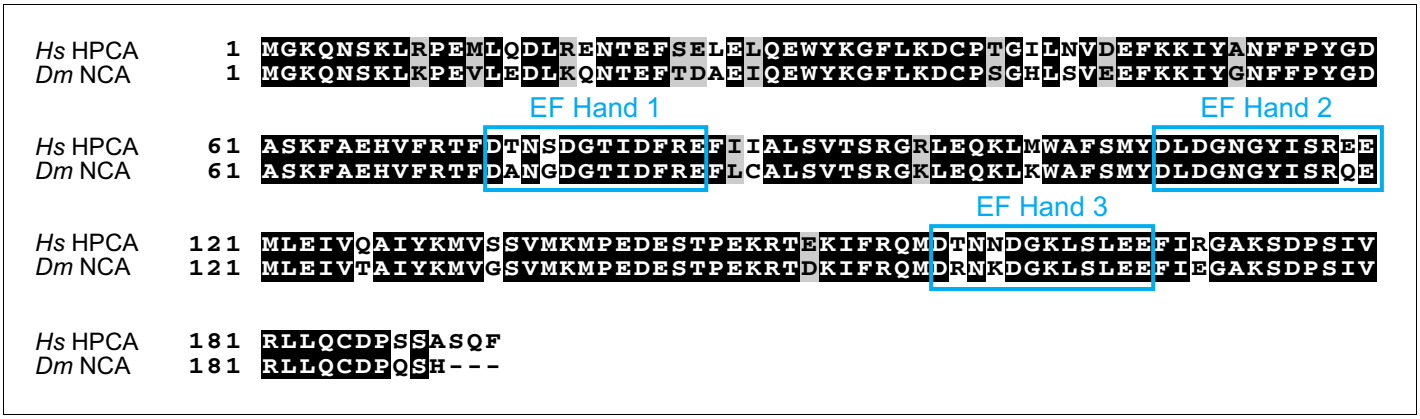

Figure 1—figure supplement 1. Human Hippocalcin and *Drosophila* Neurocalcin are highly homologous neuronal calcium sensors. Amino-acid alignment of human Hippocalcin (*Hs* HPCA) and *Drosophila* Neurocalcin (*Dm* NCA) is shown. Blue boxes: location of the calcium-binding EF-hand domains of Hippocalcin and Neurocalcin. Black boxes represent full amino-acid conservation, grey boxes represent functional conservation.
DOI: https://doi.org/10.7554/eLife.38114.003

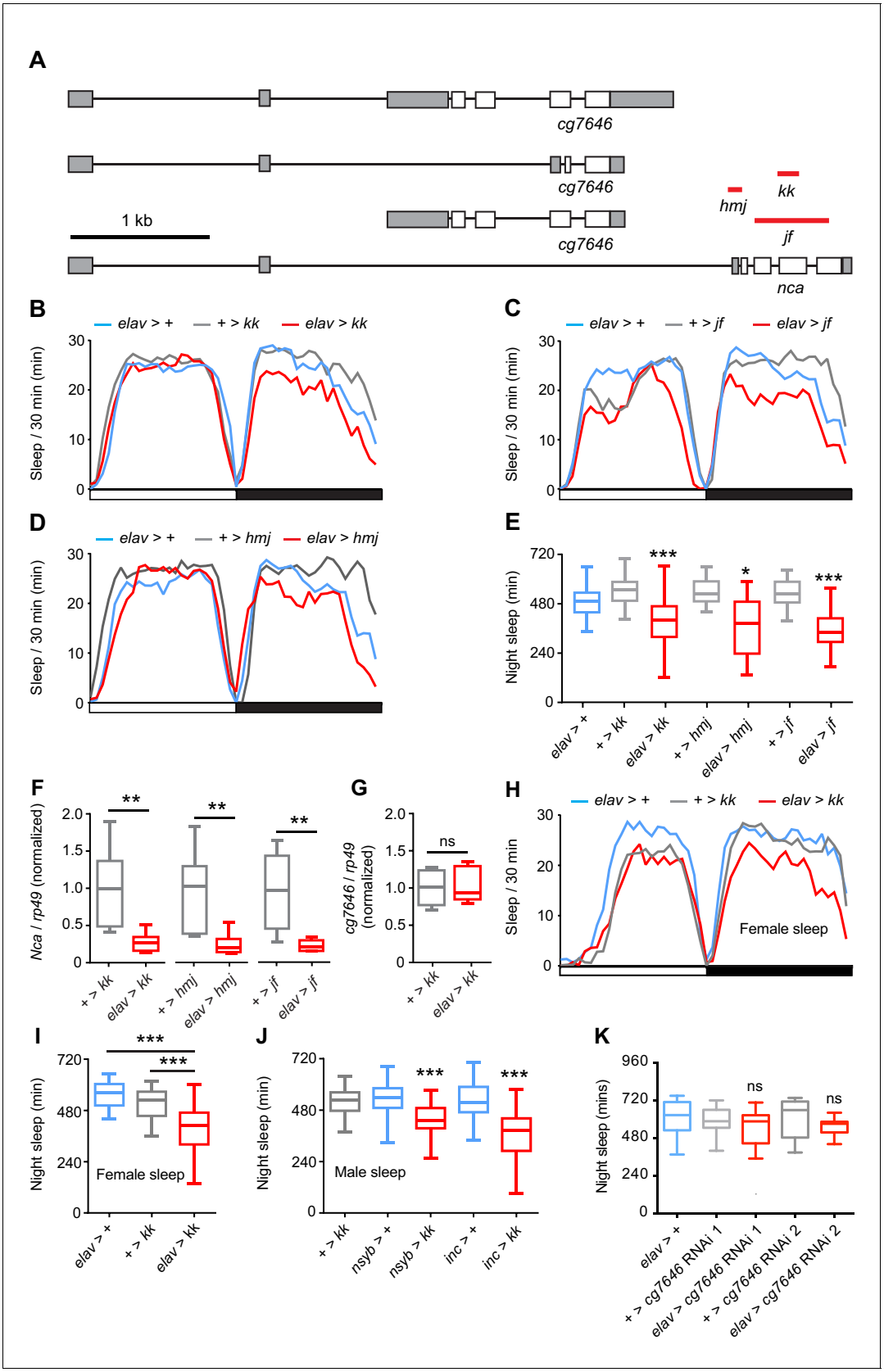

Figure 1—figure supplement 2. Pan-neuronal knockdown of *Nca* using independent RNAi lines causes night sleep loss. (**A**) Schematic showing transcripts derived from the *Nca* locus alongside transcripts derived from the *cg7646* locus, which shares common 5′ untranslated regions with *Nca*.

Figure 1—figure supplement 2 continued on next page

Figure 1—figure supplement 2 continued

Regions of *Nca* mRNA targeted by the *kk108825*, *hmj21533* and *jf03398* RNAi lines (termed *kk*, *hmj* and *jf* respectively) are shown as red bars. (**B–D**) Mean sleep levels measured using the DAM system under 12L: 12D conditions for male adult pan-neuronal *Nca* knockdown flies (B: *elav* > *kk,* C: *elav* > *jf;* D: *elav* > *hmj*) and associated controls (*elav*-Gal4 driver or RNAi transgene heterozygotes). n = 17–48. (**E**) Median sleep levels in the above genotypes. Night sleep is significantly reduced in all knockdown backgrounds compared to both transgene and driver alone controls. (**F**) qPCR verification of *Nca* knockdown by the *kk*, *hmj* and *jf* RNAi constructs. Transgene insertions lacking the *elav*-Gal4 driver were used as controls. (**G**) Knockdown of *Nca* had no effect on expression of *cg7646*. Expression levels of *Nca* or *cg7646* were normalised to the *ribosomal protein 49* (*rp49*) control transcript and are displayed as the ratio to the mean level of the respective RNAi alone controls (+ > *kk*, + > *hmj* or + > *jf*). n = 6–9 for all qPCRs (2–3 independent biological repetitions of RNA extraction with triplicated qPCR reactions for each genotype). (**H–I**) Pan-neuronal *Nca* knockdown in adult *Drosophila* females reduces night sleep. Mean sleep patterns of Nca^{KD} females and associated controls in 12L: 12D conditions are shown in (**H**). Median night sleep levels are shown in (**I**). Day sleep levels are unaffected relative to heterozygous *kk* RNAi insertion controls (**H**). n = 31–32. (**J**) Pan-neuronal or broad *Nca* knockdown in adult males using either *nsyb*- or *insomniac* (*inc*)-Gal4 also reduced total night sleep levels in 12L: 12D conditions compared to both transgene and driver alone controls. n = 38–53. (**K**) Pan-neuronal expression of RNAi targeting *cg7646* mRNA did not alter night sleep in *Drosophila* males compared to both transgene and driver alone controls. Two different chromosomal insertions of the same RNAi hairpin were used (RNAi one and RNAi 2). n = 12–15. ns - p>0.05, *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's post-hoc test (**E, I, J, K**) or Mann-Whitney U-test (**F, G**).

DOI: https://doi.org/10.7554/eLife.38114.004

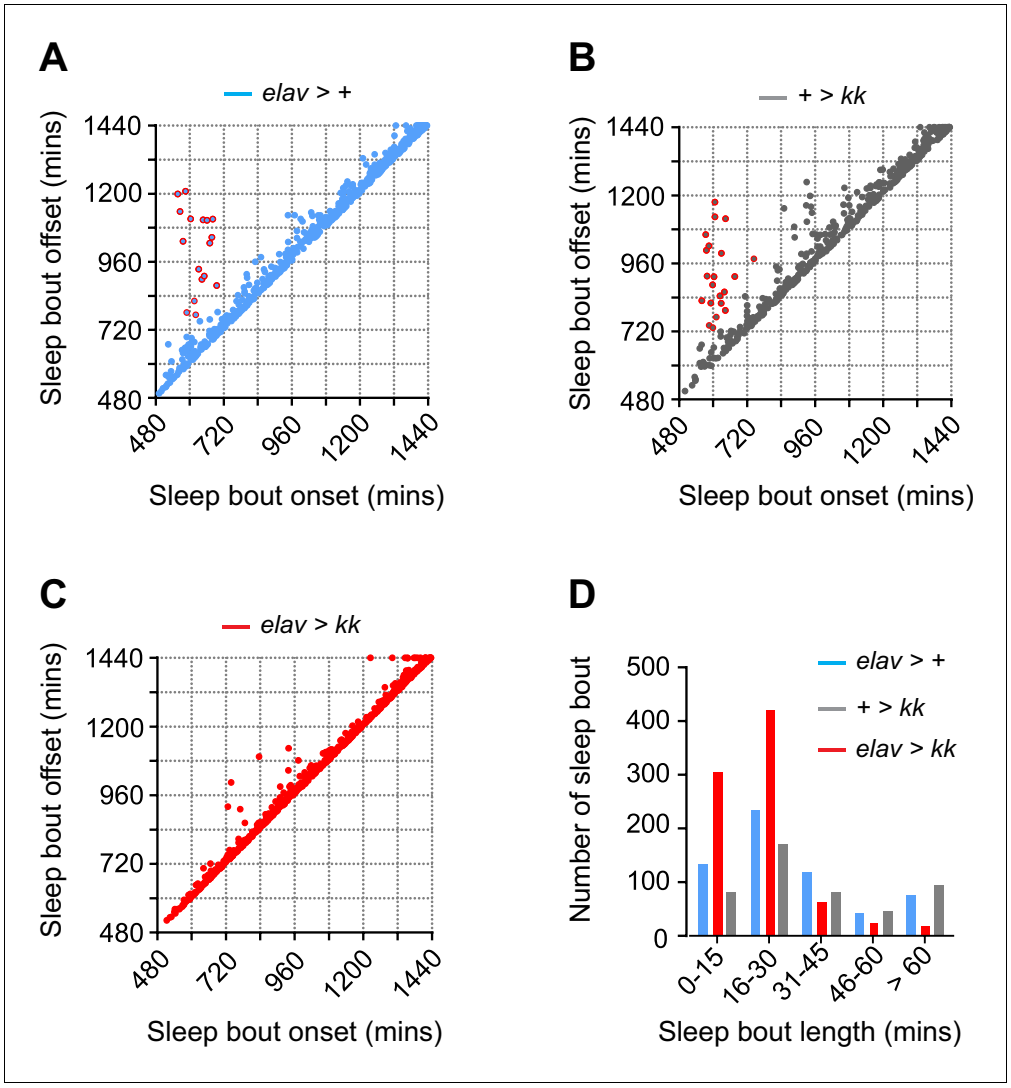

Figure 1—figure supplement 3. Reduced consolidated sleep in Nca^{KD} flies. (**A–C**) Individual sleep bout durations were measured using a custom-made R program and visualised by plotting sleep bout onset against offset for sleep bouts in control and Nca^{KD} adult males. In control flies (*elav* > + and + > *kk*), longer sleep bouts initiated early during the night are highlighted in red (**A, B**), which are largely absent in Nca^{KD} adult males (**C**). n = 48 for each genotype. (**D**) Distribution of sleep bout lengths in Nca^{KD} and control adult males. Note the significant shift towards shorter sleep bout lengths in Nca^{KD} flies (Nca^{KD} vs. driver alone control, χ^2, *df*: 142.0, 4, p<0.0001; vs. RNAi alone control, χ^2, *df*: 2112.0, 4, p<0.0001).

DOI: https://doi.org/10.7554/eLife.38114.005

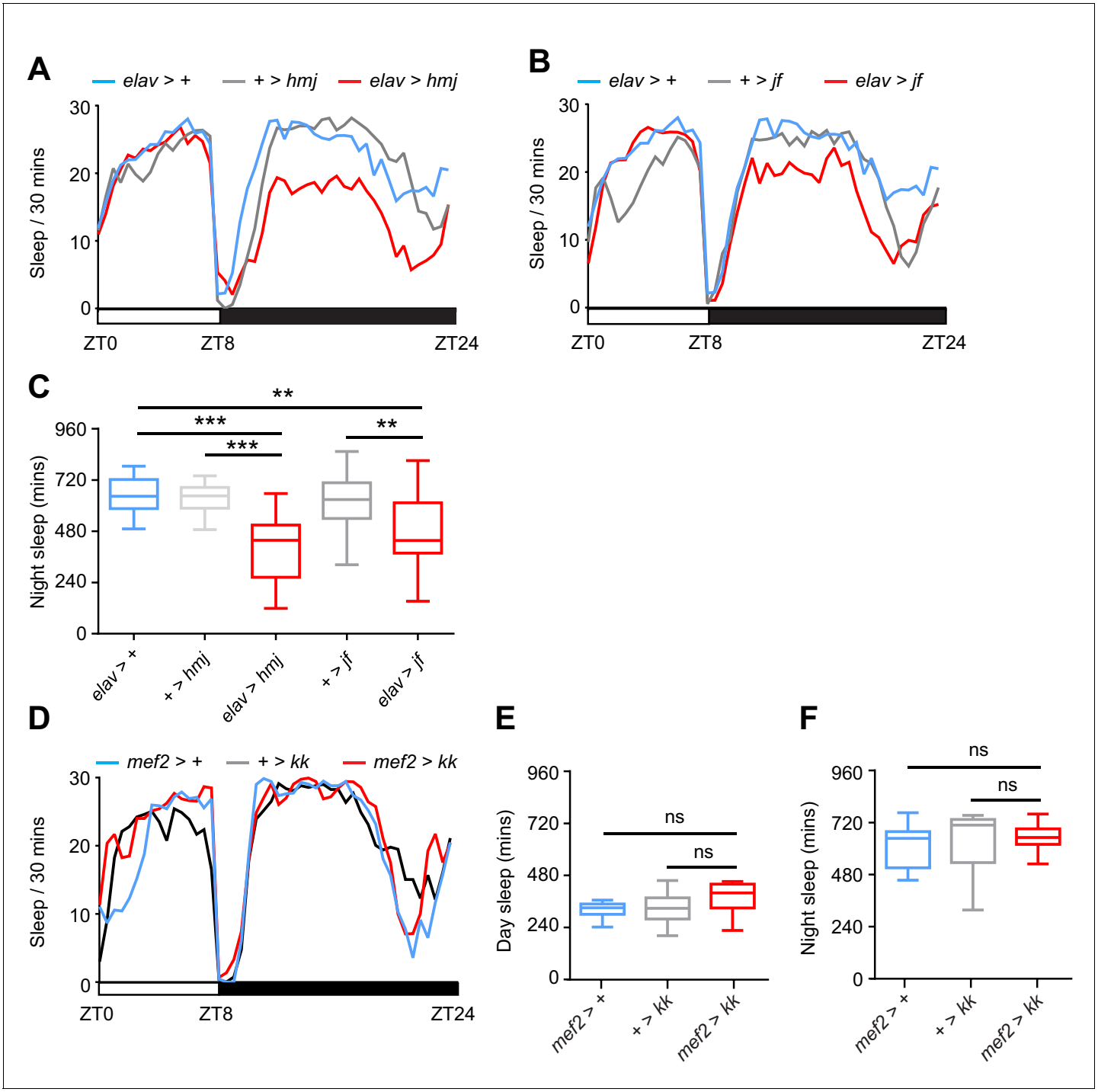

Figure 1—figure supplement 4. Pan-neuronal expression of independent *Nca* RNAi lines results in night sleep loss in 8L: 16D conditions. (**A–B**) Mean sleep profiles under 8L: 16D conditions for *elav*-Gal4 driven *hmj* (**A**) or *jf* (**B**) *Nca* RNAi. (**C**) Median night sleep amounts for genotypes shown in (**A–B**). *elav > +*: n = 32; *+ > hmj*: n = 26; *elav > hmj*: n = 17; *+ > jf*: n = 32; *elav > jf*: n = 32. (**D–F**) *Nca* knockdown in muscle cells does not affect sleep in *Drosophila*. (**D**) Mean sleep patterns of adult male flies with muscle-specific *Nca* knockdown via *mef2*-Gal4 (*mef2 > kk*) and associated controls under 8L: 16D. (**E–F**) Median day (**E**) and night (**F**) sleep levels are unaffected relative to controls. n = 16 per genotype. ns – p>0.05, Kruskal-Wallis test with Dunn's post-hoc test. **p<0.01, ***p<0.001, ns – p>0.05, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.006

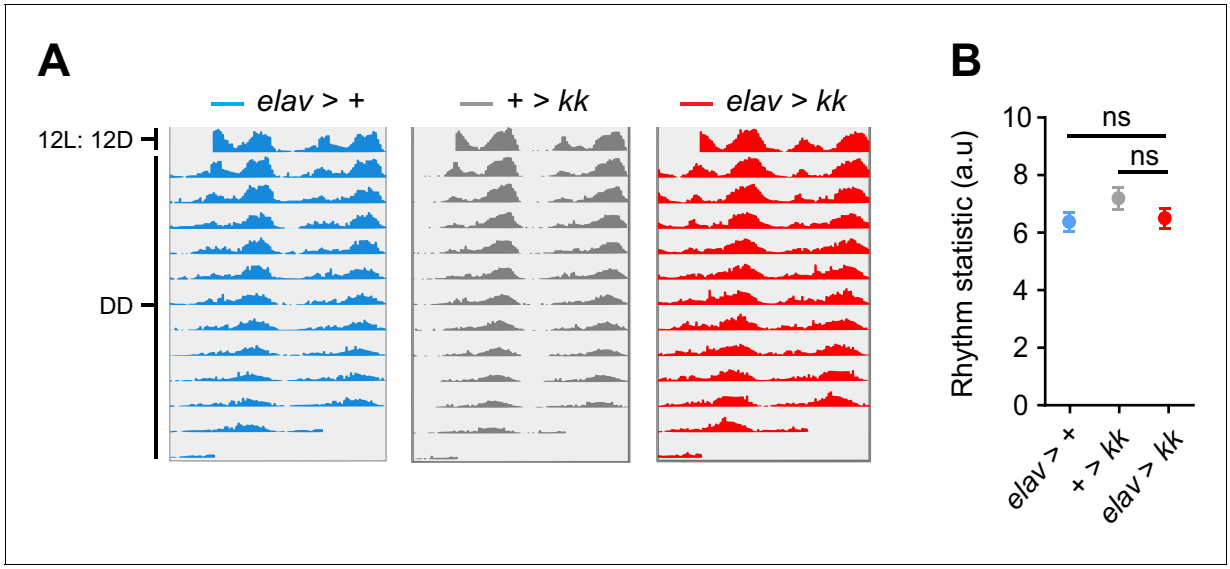

Figure 1—figure supplement 5. *Nca* knockdown does not alter circadian rhythmicity. (**A**) Actograms showing representative individual patterns of locomotor activity in one day of 12L: 12D conditions followed by 11 days of free-running activity in constant dark (DD) conditions. (**B**) Mean locomotor rhythm strength in Nca^{KD} adult males and controls. Robust circadian patterns of locomotor activity were still observed following in adult males expressing *Nca* RNAi (*kk*) under *elav*-Gal4 relative to controls. Error bars represent standard error of the mean. n = 14–15.

DOI: https://doi.org/10.7554/eLife.38114.007

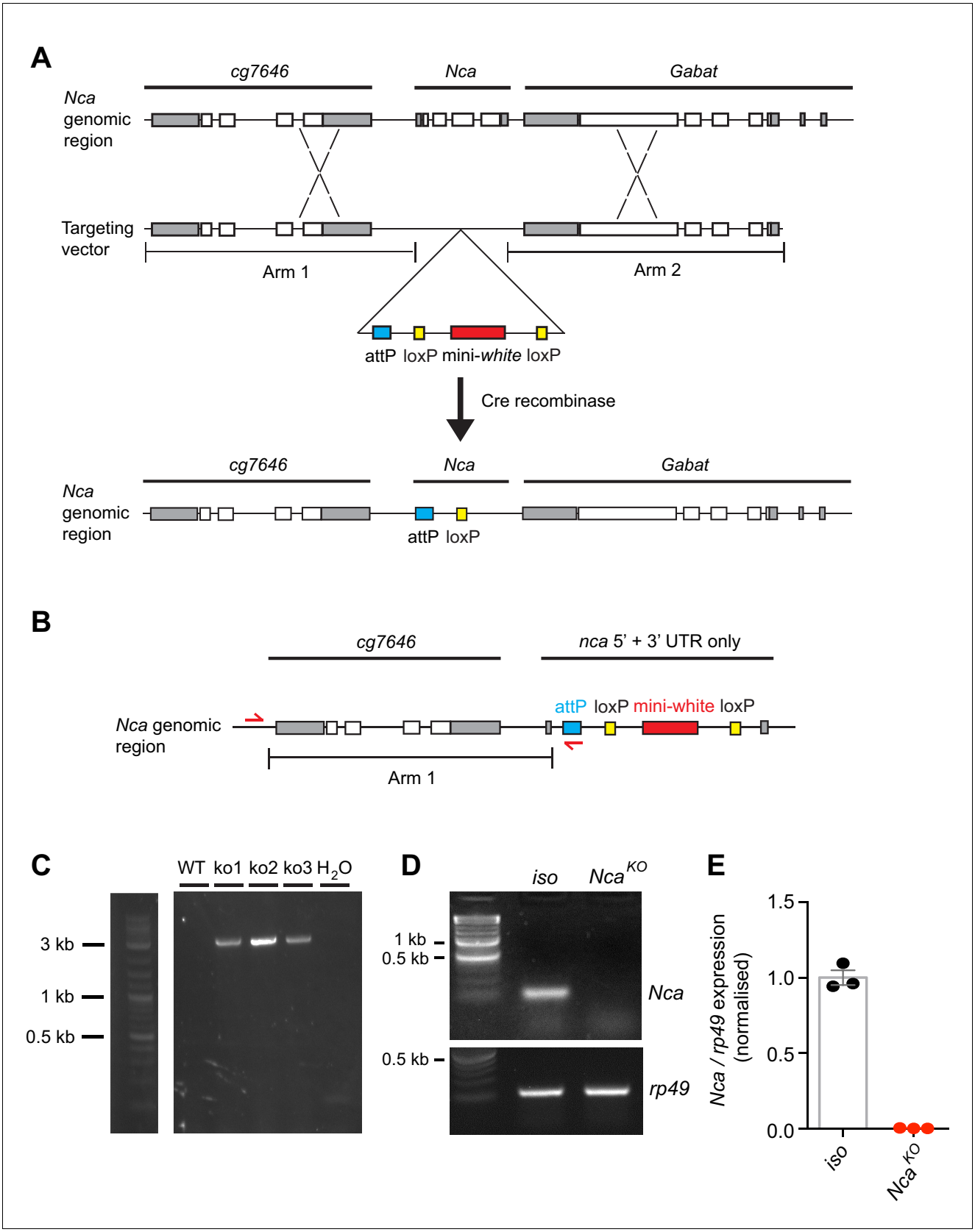

Figure 1—figure supplement 6. Generation of *Nca* null alleles using ends-out homologous recombination. (**A**) Schematic illustration of the procedure used to generate *Nca* knockout alleles. Homologous arms upstream (Arm 1) and downstream (Arm 2) of the *Nca* locus are indicated. Upstream 5'

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Figure 1—figure supplement 6 continued

promoter regions shared by the *cg7646* and *Nca* loci (see ***Figure 1—figure supplement 2A***) are external to the homologous arm sequences and are not shown. Following homologous recombination, the endogenous *Nca* locus is replaced by a cassette containing the mini-*white* selection marker (red bar), and attP (blue bar) and loxP sites (yellow bars). The mini-*white* cassette was subsequently removed via Cre-loxP recombination. (**B–C**) PCR validation of homologous recombination events. Correct recombination was verified using primers designed to the attP site and upstream of the *cg7646* coding regions (**B**), which will only generate a ~ 3 kb product following homologous recombination between the targeting vector and the *Nca* locus (**C**). Three independent targeting events (ko1-3) were validated by genomic PCR. WT: wild-type genome lacking an attP site 3'of *cg7646*. (**D–E**) No *Nca* mRNA was detected in Nca^{KO1} using either standard RT-PCR (**D**) or quantitative RT-PCR (E; n = 3 qPCR reactions for *iso31* control and Nca^{KO1} flies).

DOI: https://doi.org/10.7554/eLife.38114.008

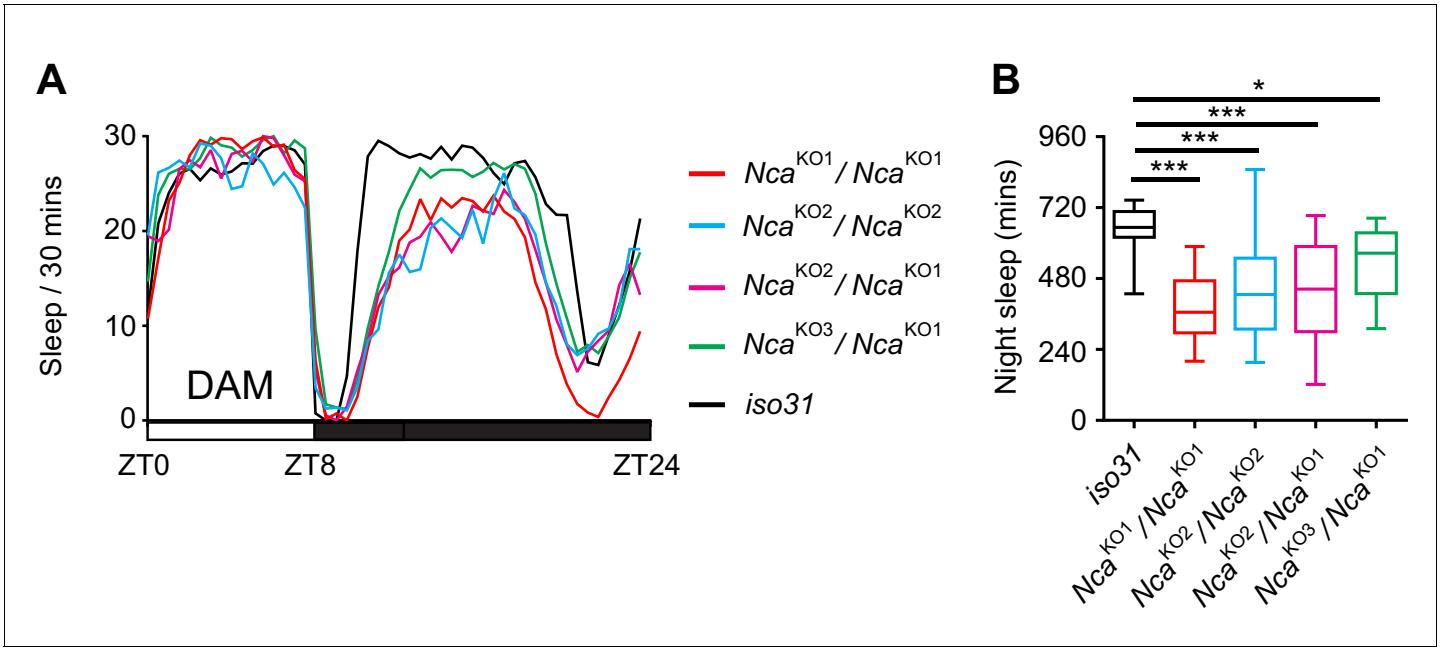

Figure 1—figure supplement 7. Independent combinations of *Nca* knockout alleles exhibit night sleep loss. (**A–B**) Trans-heterozygotic combinations of the Nca^{KO1-3} alleles, as well as homozygotes for the Nca^{KO2} allele, all result in significant night sleep loss compared to *iso31* controls. Night sleep levels in Nca^{KO1} homozygotes are also shown. (**A**) Mean sleep levels in the above genotypes in 8L: 16D conditions. (**B**) Median night sleep. n = 15–26. *p<0.05, **p<0.01, ***p<0.001 compared to iso31 controls, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.009

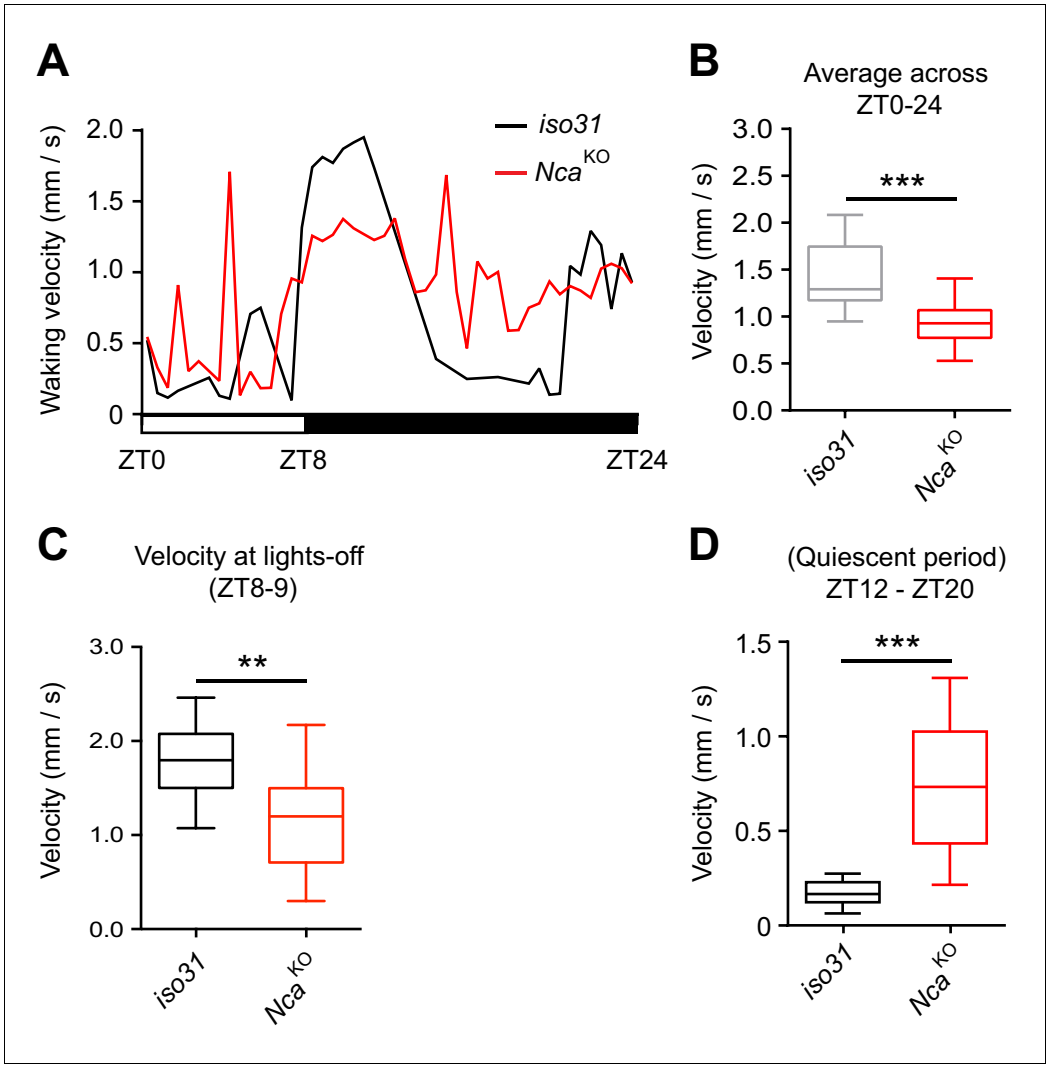

Figure 1—figure supplement 8. Locomotor velocities in *Nca* knockout flies. (**A**) Mean locomotor velocities across a 24 hr period in 8L: 16D conditions in Nca^{KO1} adult males and iso31 controls, measured via the DART system, reveals increased locomotor velocity during the night and reduced velocity during the evening activity peak Nca^{KO1} flies. (**B**) Median locomotor velocities across 24 hr in 8L: 16D conditions in Nca^{KO1} adult males and iso31 controls. (**C**) Median locomotor velocity is significantly reduced in Nca^{KO1} flies during the evening activity peak (ZT8-ZT9). (**D**) Nca^{KO1} flies exhibit a significant increase in locomotor velocity between ZT12-ZT20 compared to controls – a normally quiescent period. n = 16 per genotype. **p<0.01, ***p<0.001, Mann-Whitney U-test.
DOI: https://doi.org/10.7554/eLife.38114.010

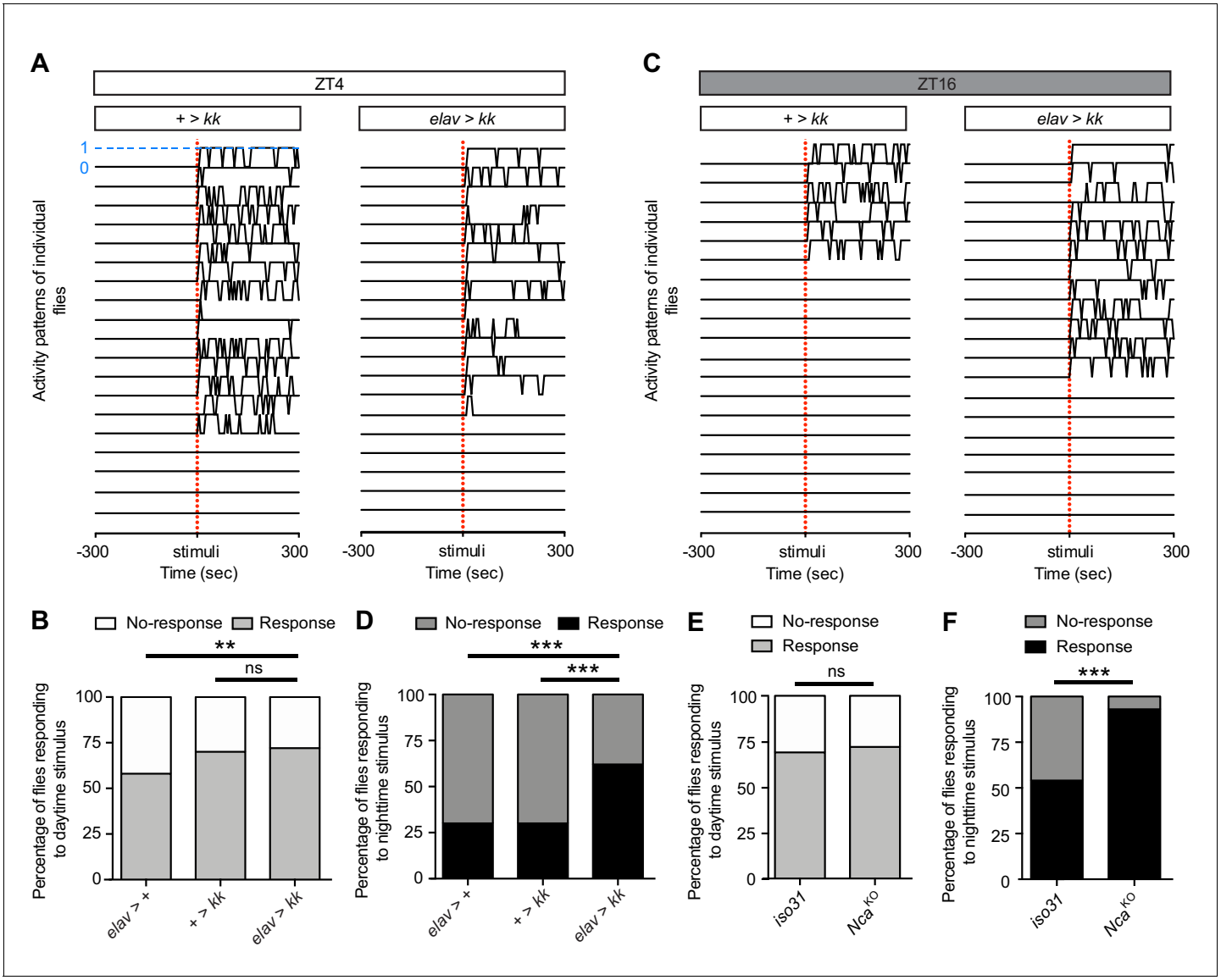

Figure 2. NCA reduces responsiveness to stimuli at night under 8L: 16D conditions. (**A, C**) Locomotor activity in twenty representative control (+ > *kk*) and *Nca*^{KD} (*elav* > *kk*) adult male flies at either ZT4 (**A**) or ZT16 (**C**), as measured using the DART system. X-axis denotes 300 s before and after a vibration stimulus (red dotted line). Y-axis represents movement of individual flies in a binary manner (1 = movement, marked by blue dotted line for one fly; 0 = immobility). Only flies that were immobile for five mins preceding the stimulus were selected for analysis. (**B, D**) Percentage of *Nca*^{KD} and control flies responding or not responding to vibration stimulus at either ZT4 (**B**) or ZT16 (**D**). ZT4: *elav* > +: n = 24, + > *kk*: n = 33, *elav* > *kk*: n = 33. ZT16: *elav* > +: n = 23, + > *kk*: n = 30, *elav* > *kk*: n = 29. (**E, F**) Percentage of *Nca*^{KO} and *iso31* control flies responding or not responding to vibration stimulus at either ZT4 (**E**) or ZT16 (**F**). ZT4: *iso31*: n = 48, *Nca*^{KO}: n = 53. ZT16: *iso31*: n = 48, *Nca*^{KO}: n = 44. ns – p>0.05, **p<0.01, ***p<0.001, Binomial test with Bonferonni correction for multiple comparisons.

DOI: https://doi.org/10.7554/eLife.38114.012

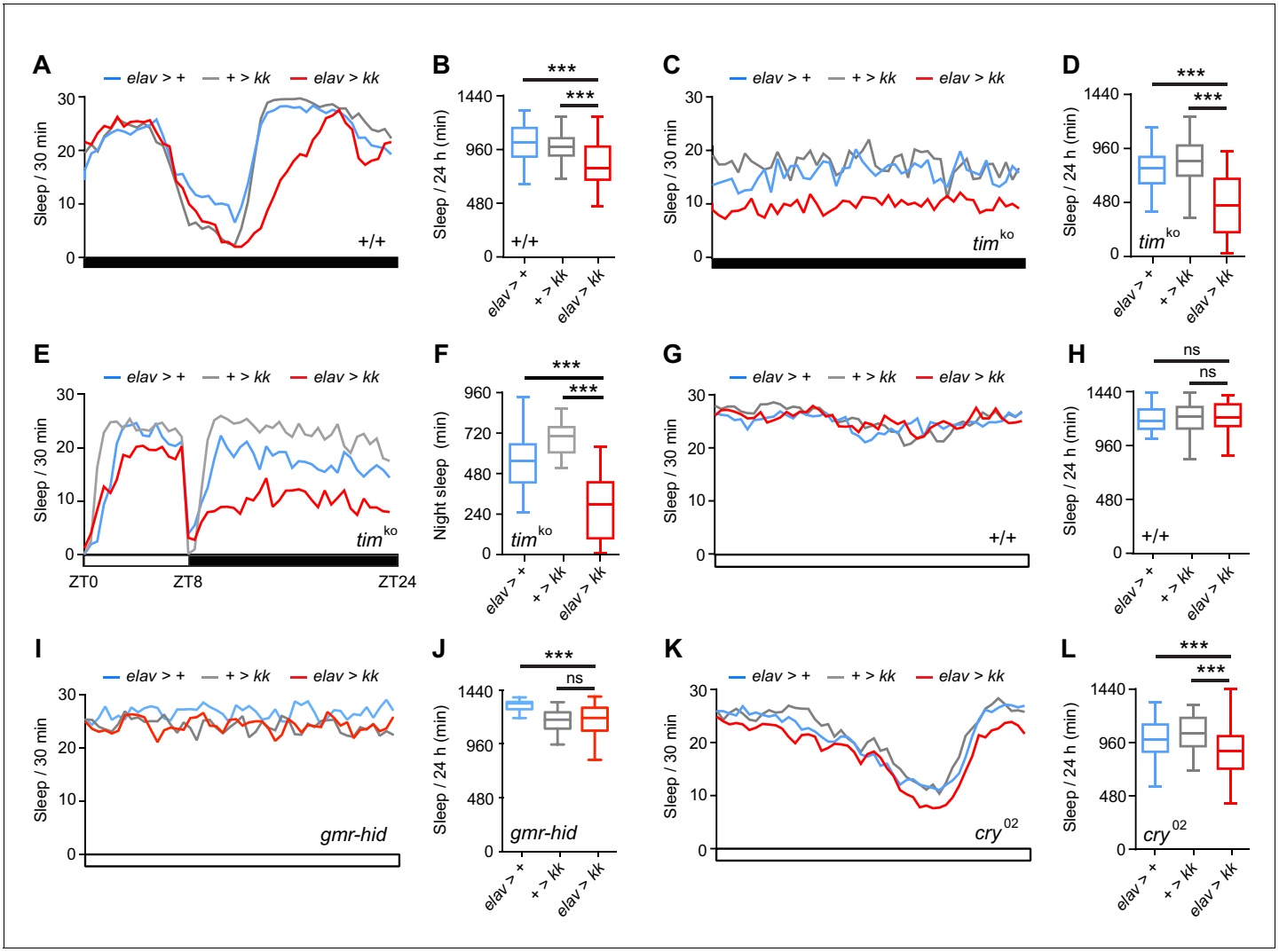

Figure 3. Circadian clock and light-sensing pathways define when NCA promotes sleep. (**A–B**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in constant-dark (DD) conditions (**A**), and total median sleep levels in the above genotypes (**B**). n = 44–47. Note the reduced sleep in the subjective night in *Nca*^{KD} relative to control adult males, but not the day. (**C–D**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in DD conditions in a *timeless* knockout (*tim*^{KO}) background (**C**), and total median sleep levels (**D**). n = 32–39. (**E–F**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in 8L: 16D conditions in a *tim*^{KO} background (**E**), median night sleep levels (**F**). n = 22–26. (**G–H**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in constant-light (LL) conditions (**G**), and total median sleep levels (**H**). n = 44–47. (**I–J**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in LL conditions in a *gmr-hid* background (**I**), and total median sleep levels (**J**). *elav > kk, gmr-hid/+*: n = 51; *+ > kk, gmr-hid/+*: n = 48; *elav > +, gmr-hid/+*: n = 24. (**K–L**) Mean sleep levels in *Nca*^{KD} and control adult males across 24 hr in LL conditions in a *cryptochrome* null (*cry*^{02}) background (**K**), and total median sleep levels in the above genotypes (**L**). n = 61–72. Note the small but consistent reduction in sleep in *Nca*^{KD}, *cry*^{02} males (**K**), leading to a significant decrease in total median sleep levels relative to controls (**L**). ns - p>0.05, ***p<0.001, as compared to driver and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.014

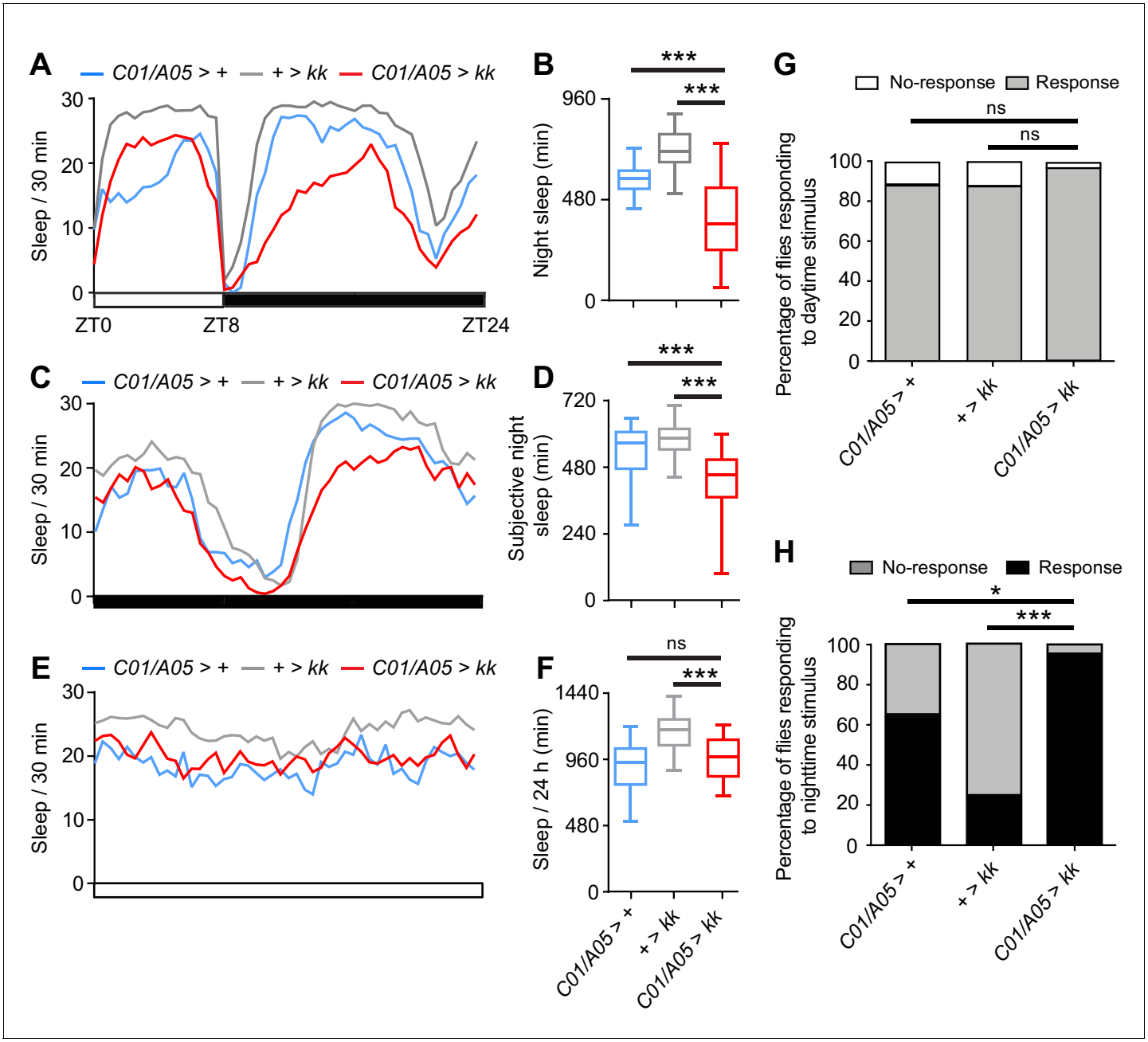

Figure 4. NCA acts in a two distinct neural subpopulations to regulate night sleep. (**A–F**) Sleep patterns in adult male flies with *Nca* knockdown (using the *kk Nca* RNAi) in two neural domains defined by the *A05*- and *C01*-Gal4 drivers in varying light/dark regimes, compared to controls. (**A–B**) A: mean sleep patterns in 8L: 16D conditions. B: median night sleep in *Nca* knockdown flies compared to heterozygote drivers and transgene alone controls. + > *kk*: n = 80; *C01/A05* > +: n = 42; *C01/A05* > *kk*: n = 71. (**C–D**) Mean sleep patterns (**C**) and median subjective night sleep (**D**) in constant dark (DD) conditions. + > *kk*: n = 64; *C01/A05* > +: n = 47; *C01/A05* > *kk*: n = 51. (**E–F**) Mean sleep patterns (**E**) and median total sleep (**F**) in constant light (LL) conditions. + > *kk*: n = 76; *C01/A05* > +: n = 26; *C01/A05* > *kk*: n = 28. (**G–H**) Percentage of *C01/A05* > *kk* and control flies responding or not responding to vibration stimuli at either ZT4 (G; *C01/A05* > *kk*, n = 38, + > *kk*, n = 61 and *C01/A05* > +, n = 26) or ZT16 (H; *C01/A05* > *kk*, n = 24, + > *kk*, n = 54 and *C01/A05* > +, n = 28) under 8L: 16D conditions. ns – p>0.05, *p<0.05, **p<0.01, ***p<0.001, compared to driver and RNAi alone controls, Kruskal-Wallis test with Dunn's post-hoc test (**B, D, F**) or Binomial test with Bonferonni correction for multiple comparisons (**G–H**).

DOI: https://doi.org/10.7554/eLife.38114.016

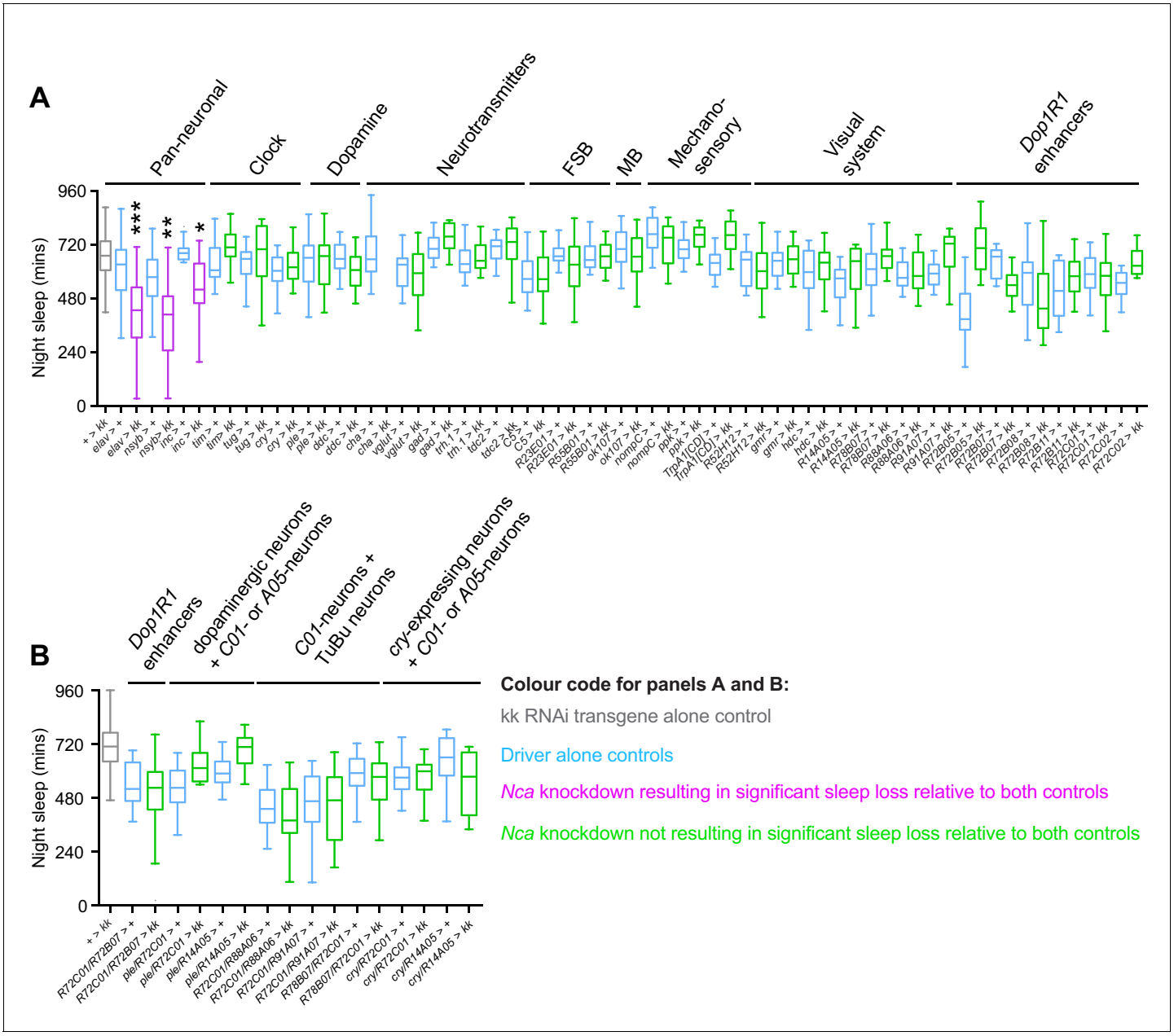

Figure 4—figure supplement 1. Transgenic RNAi-based mini-screen to identify key NCA-expressing neurons. (**A**) *Nca* knockdown with broadly expressed drivers resulted in reduced night sleep in adult males under 8L: 16D conditions. In contrast, *Nca* knockdown in previously defined sleep-regulatory centers, clock neurons, the visual system or subsets of *Dop1R1*-expressing neurons did not impact night sleep. FSB: fan-shaped body. MB: mushroom body. Grey and blue box plots: control lines. Magenta box plots: experimental lines showing reduced night sleep relative to controls. Green box plots: experimental lines failing to show reduced night sleep relative to one or both controls. Grey box plot: *kk Nca* RNAi alone (+ > *kk*) controls. Blue box plots: Gal4 driver heterozygotes. (**B**) *Nca* knockdown using combinations of driver lines labelling *C01*- and *A05*-neurons in addition to neurons in the dopaminergic pathway (dopamine-release and Dop1R1-expressing neurons), the anterior visual pathway (tubercular-bulbar (TuBu) neurons), and *cryptochrome* (*cry*)-expressing neurons. See ***Figure 4*** Source Data for n-values and additional statistical comparisons. *p<0.05, **p<0.01, ***p<0.001, as compared to driver and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.017

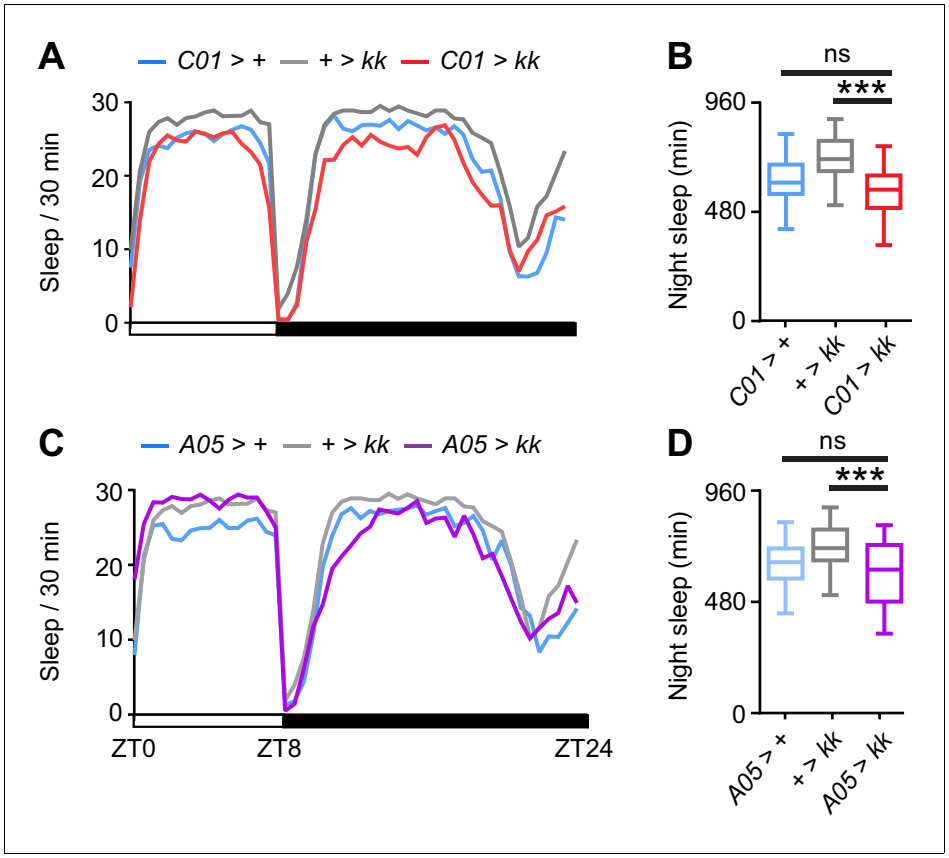

Figure 4—figure supplement 2. *Nca* knockdown in *C01*- or *A05*-neurons alone does not significantly alter sleep. (**A–B**) Mean sleep patterns (**A**) and median night sleep (**B**) of adult males with expressing *Nca* RNAi (*kk*) in *C01*-neurons compared to heterozygote driver and transgene alone controls in 8L: 16D conditions. + > *kk*: n = 80, *C01* > +: n = 64, *C01* > *kk*: n = 80. (**C–D**) Mean sleep patterns (**A**) and median night sleep (**B**) of adult males with expressing *Nca* RNAi (*kk*) in *A05*-neurons compared to controls in 8L: 16D conditions. + > *kk*: n = 80, *A05* > +: n = 31, *A05* > *kk*: n = 31. Note that the same population of + > *kk* control males was used in ***Figure 4A–B***, as the combined *C01*- and *A05*-Gal4 experiments were performed in parallel. ns – p>0.05, ***p<0.001 compared to driver and RNAi alone controls, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.018

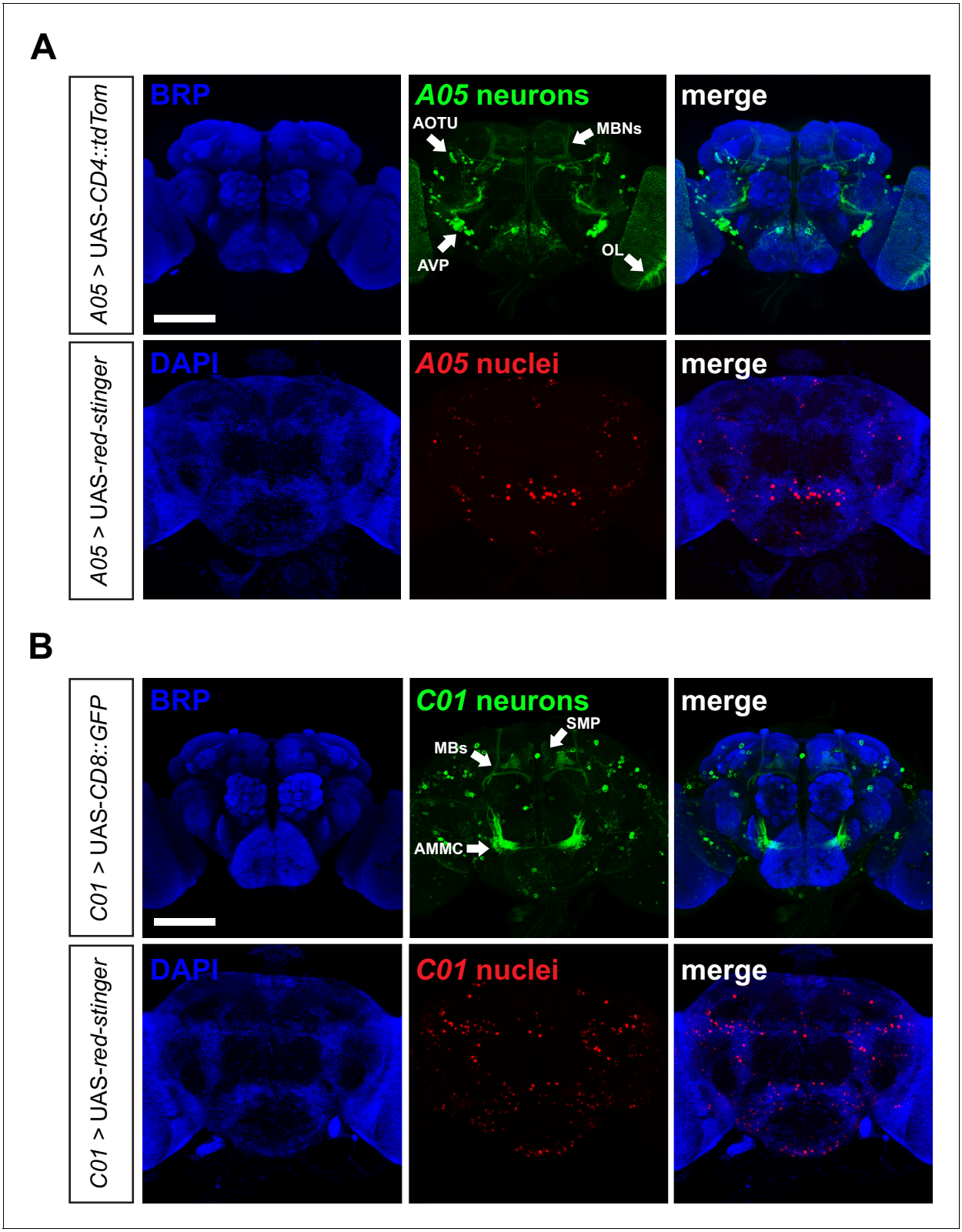

Figure 5. Distribution of *A05*- and *C01*-neurons in the adult *Drosophila* brain. (**A–B**) Confocal z-stacks of adult male brains expressing genetically-encoded fluorophores labelling either neuronal processes (CD4::TdTom or CD8::GFP) or nuclei (Red-stinger) under the *A05*- (**A**) or *C01*-Gal4 (**B**) drivers. Neuropil regions are labelled with anti-Bruchpilot (BRP). Nuclei are co-labelled with DAPI. Scale bars, 100 μm. Arrows point to neuropil centers. AOTU:

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anterior optic tubercle. MBNs: mushroom body neurons. OL: optic lobe. AMMC: antennal mechanosensory and motor center. AVP: anterior ventrolateral protocerebrum. SMP: superior medial protocerebrum.

DOI: https://doi.org/10.7554/eLife.38114.020

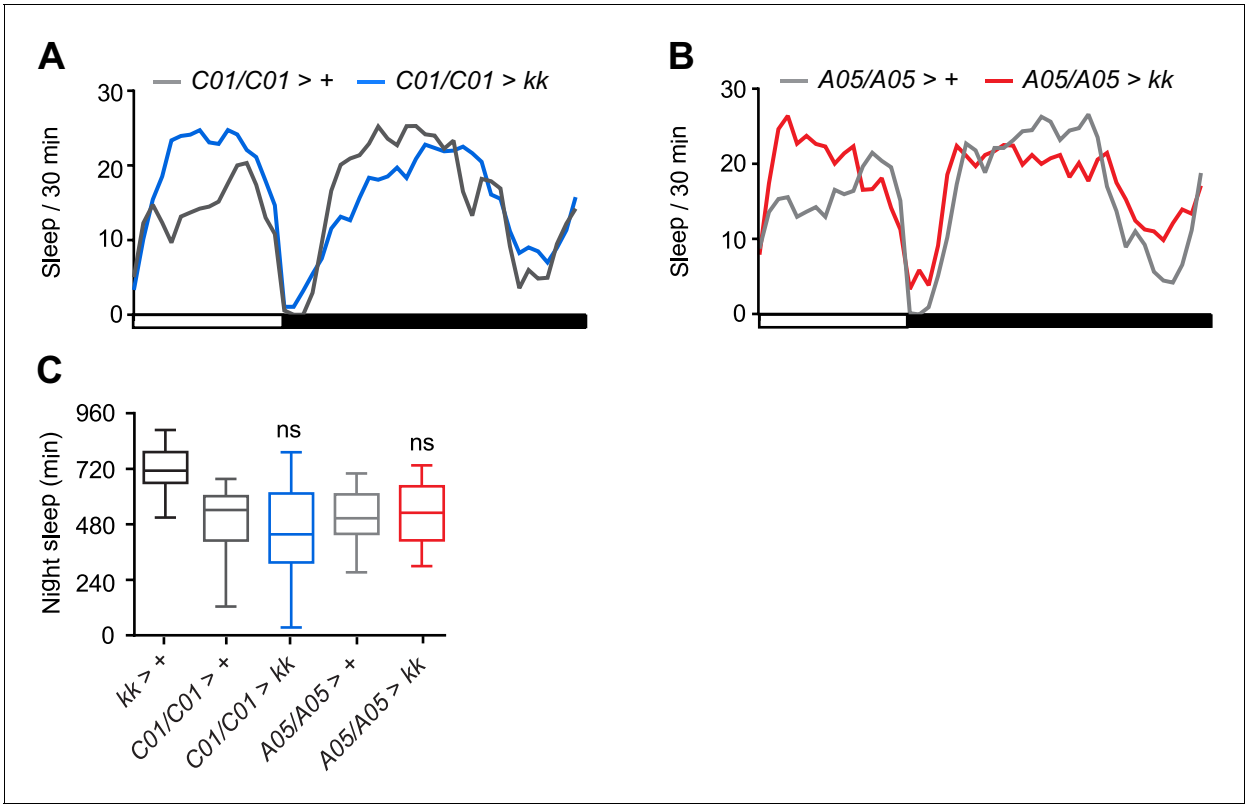

Figure 5—figure supplement 1. *Nca* knockdown using homozygous *C01*- or *A05*-Gal4 drivers does not affect night sleep. (**A**) Mean sleep patterns of adult males homozygous for the *C01*-Gal4 driver with and without the *kk Nca* RNAi insertion. (**B**) Mean sleep patterns of adult males homozygous for the *A05*-Gal4 driver with and without the *kk Nca* RNAi insertion. (**C**) Median night sleep levels for heterozygous RNAi transgene and homozygous driver controls, and males expressing *Nca* RNAi with two Gal4 driver copies. No night sleep loss was observed using two copies of either driver relative to controls. + > *kk*: n = 80 (the same population was used in ***Figure 4A–B***, as the experiments were performed in parallel), *C01/C01* > +: n = 24, *C01/C01* > *kk*: n = 39; *A05/A05* > +: n = 22, *A05/A05* > *kk*: n = 23. ns – p>0.05, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.021

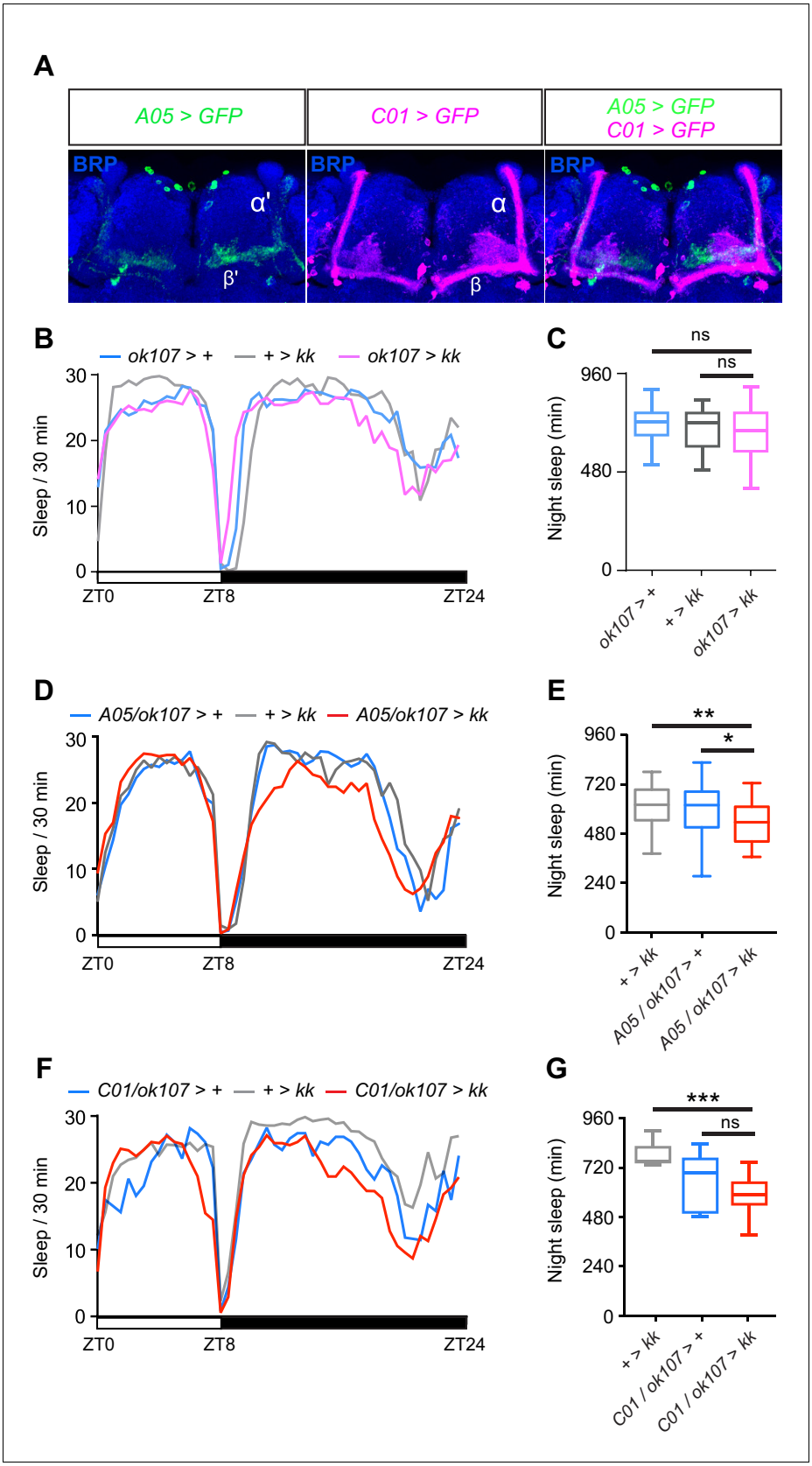

Figure 5—figure supplement 2. The mushroom bodies are a sleep-relevant subdomain within *C01*-neurons. (**A**) Standardised confocal stacks labelling *R14A05* (*A05* > GFP, green) and *R72C01* (*C01* > GFP, magenta) positive neurons. Images from ***Jenett et al. (2012)***, deposited at the Virtual Fly Brain,

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Figure 5—figure supplement 2 continued

www.virtualflybrain.org), were downloaded and digitally superimposed (right panel) onto the universal fly brain (the image source data is distributed under a CC BY-NC-SA 4.0 license). BRP, Bruchpilot. (**B–C**) Mean sleep pattern (**B**) and median night sleep levels (**C**) in adult male flies expressing *Nca* RNAi (*kk*) in MB-KCs using *ok107*-gal4 in 8L: 16D. *ok107* > +, n = 55, + > *kk*, n = 47, *ok107* > *kk*, n = 65. (**D–E**) *Nca* knockdown in both *A05* and MB-KC neurons results in reduced night sleep. Mean sleep patterns in 8L: 16D are shown in (**D**), median night sleep levels are shown in (**E**). *A05/ok107* > +: n = 33, + > *kk*: n = 31, *A05/ok107* > *kk*: n = 42. (**F–G**) Knocking down *Nca* in MB-KC and the *C01*-neurons does not result in significant night sleep loss in 8L: 16D. Mean sleep patterns are shown in (**F**), median night sleep levels are shown in (**G**). *C01/ok107* > +: n = 11, + > *kk*: n = 15, *C01/ok107* > *kk*: n = 21. ns – p>0.05, *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.022

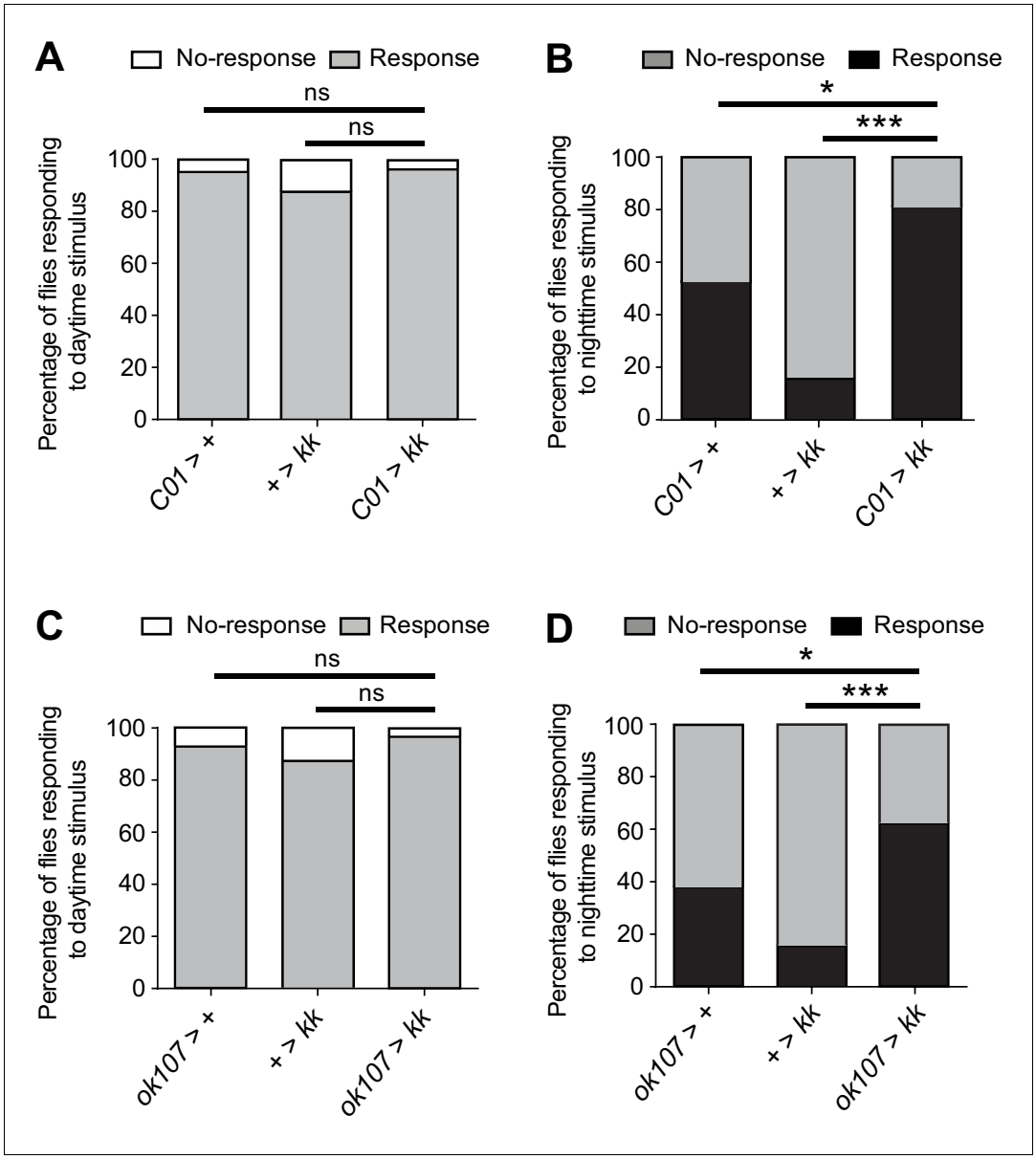

Figure 6. NCA acts in the mushroom bodies to regulate nocturnal arousal. (**A–B**) Percentage of adult male flies expressing *Nca* RNAi (*kk*) in *C01*-neurons (*C01 > kk*) and control flies responding or not responding to vibration stimulus at either ZT4 (day; **A**) or ZT16 (night; **B**). ZT4: *C01 > +*, n = 22, *+ > kk*, n = 61, *C01 > kk*, n = 27. ZT16: *C01 > +*, n = 19, *+ > kk*, n = 54, *C01 > kk*, n = 21. (**C–D**) Percentage of adult male flies expressing *Nca* RNAi (*kk*) in MB-KCs (*ok107 > kk*) and control flies responding or not responding to vibration stimulus at either ZT4 (day; **C**) or ZT16 (night; **D**). ZT4: *ok107 > +*, n = 26, *+ > kk*, n = 47, *ok107 > kk*, n = 28. ZT16: *ok107 > +*, n = 26, *+ > kk*, n = 44, *ok107 > kk*, n = 27. ns – p>0.05, *p<0.05, ***p<0.001, Binomial test with Bonferonni correction for multiple comparisons.

DOI: https://doi.org/10.7554/eLife.38114.024

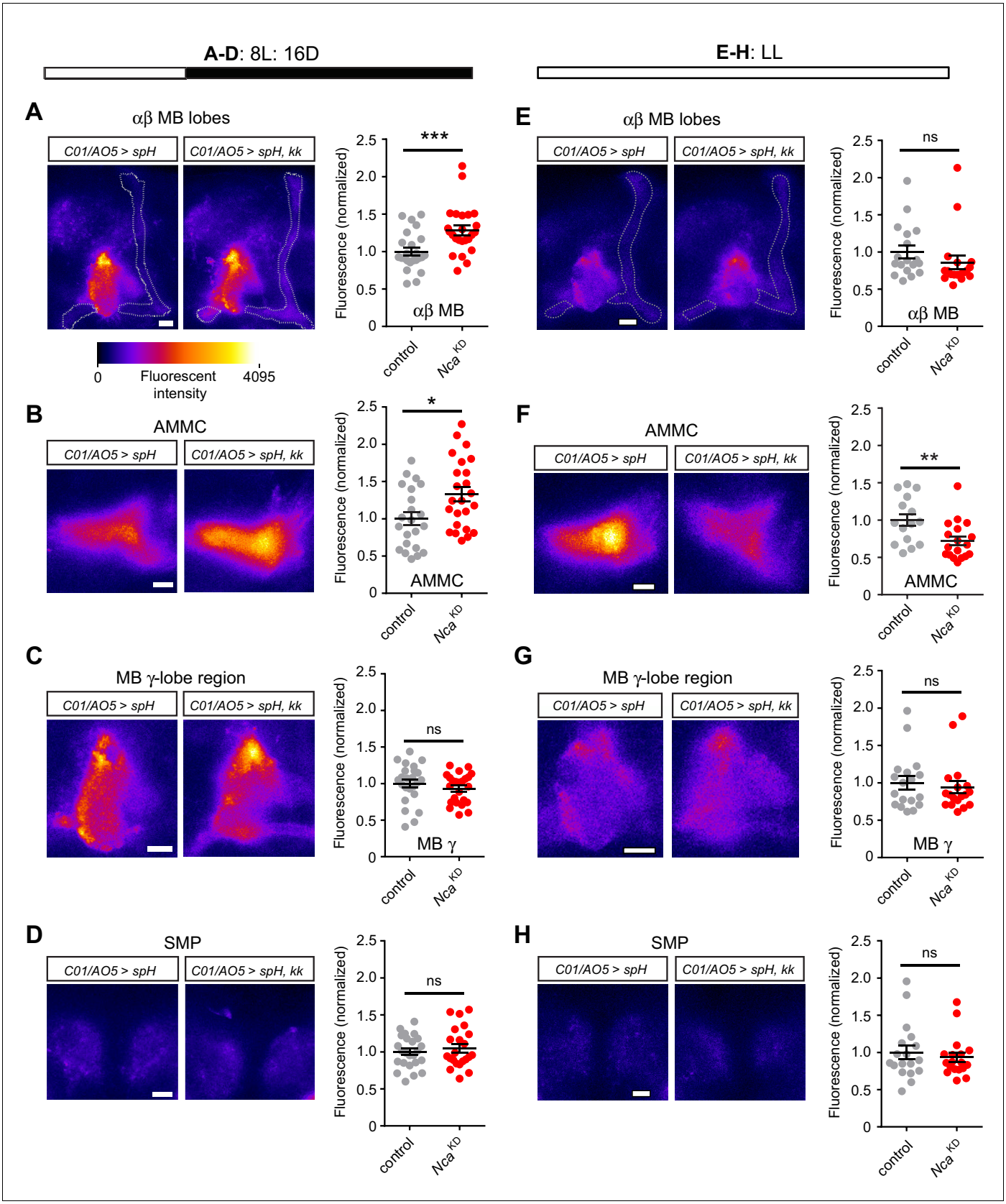

Figure 7. NCA suppresses synaptic release in subsets of *C01/A05*-neurons during darkness. (**A–D**) Fluorescence of an optical reporter of synaptic release (synapto-pHluorin, spH) in neuropil regions labelled by the *C01*- and *A05*-drivers, in control adult males (*C01/A05 > spH*) or following *Nca*

Figure 7 continued on next page

Figure 7 continued

knockdown in *C01*- and *A05*-neurons (*C01/A05 > spH, kk*). Flies were housed under 8L: 16D conditions, in which *Nca* knockdown in *C01*- and *A05*-neurons causes robust nighttime sleep loss. (**E–G**) spH fluorescence in control adult males or following *Nca* knockdown in *C01*- and *A05*-neurons (*C01/A05 > spH, kk*). Flies were housed in LL conditions, in which *Nca* knockdown in *C01*- and *A05*-neurons has no effect on sleep levels. In each panel, representative confocal images of spH fluorescence (left) and mean fluorescent intensity (right, normalized to the mean of *C01/A05 > spH* controls) are shown. Dots within dot plots represent individual brain hemisphere measurements. A-D: n = 22–24. E-H: n = 15–18. Neuropil regions are noted. MB: mushroom body. AMMC: antennal mechanosensory motor center. SMP: superior medial protocerebrum. ns – $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, Mann-Whitney U-test.

DOI: https://doi.org/10.7554/eLife.38114.026

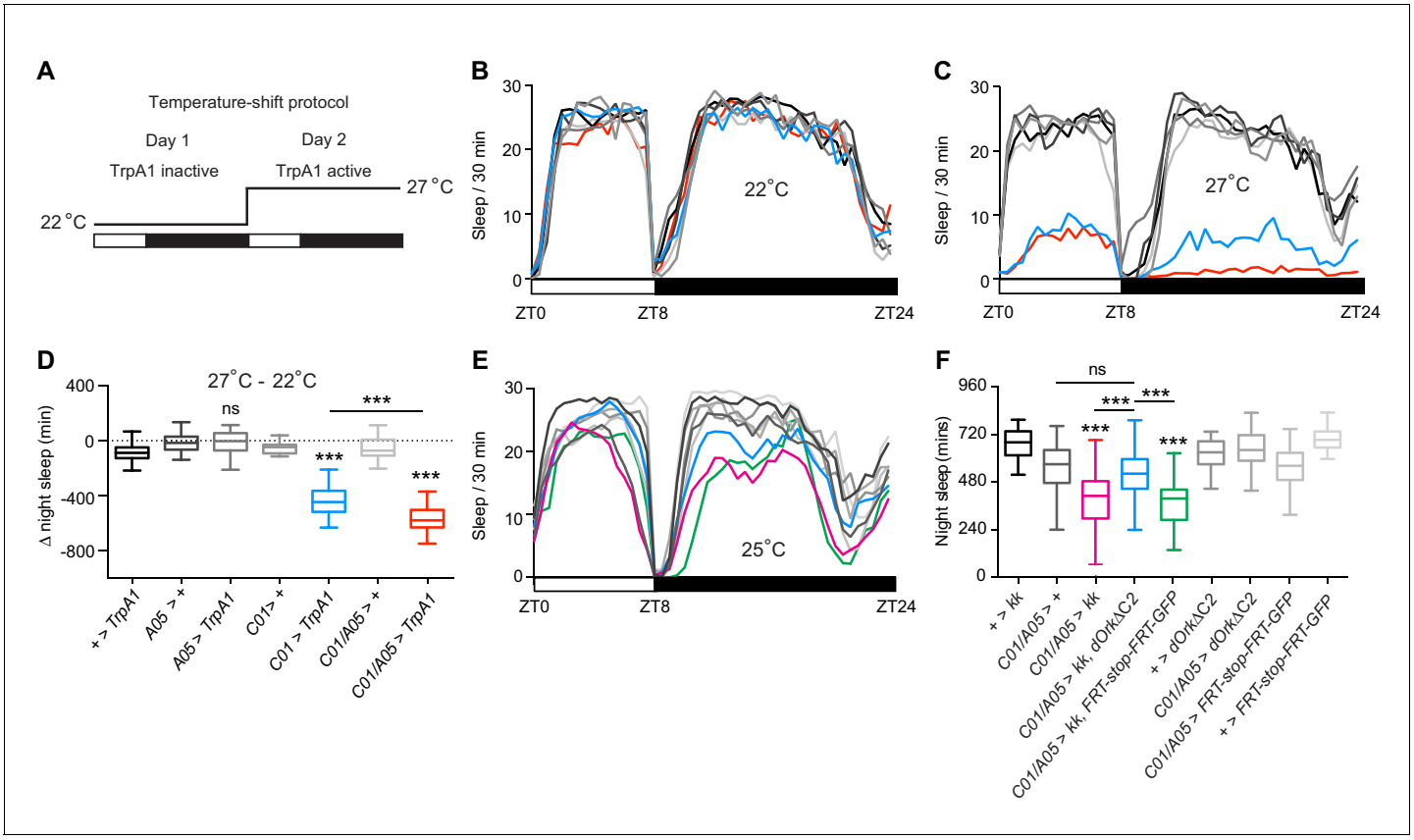

Figure 8. Sleep loss in *Nca* knockdown flies is caused by enhanced excitability of *C01/A05*-neurons. (**A**) Experimental paradigm for acute activation of *A05* or *C01*-neurons. 22°C: non-activating temperature for TrpA1. 27°C: activating temperature. Sleep levels were recorded over two days in 8L: 16D conditions. (**B–C**) Mean sleep levels across 8L: 16D following expression of TrpA1 in *A05*-, *C01*- or *A05*- and *C01*-neurons (and associated controls) at 22°C (**B**) or 27°C (**C**). (**D**) Median change in night sleep levels (Δ night sleep) following the shift from 22°C on day 1°C to 27°C on day 2. + > *TrpA1*: n = 53, *A05* > +: n = 23, *A05* > *TrpA1*: n = 68, *C01* > +: n = 24, *C01* > *TrpA1*: n = 40, *C01/A05* > +: n = 33, *C01/A05* > *TrpA1*: n = 40. ns – p>0.05, ***p<0.001, as compared to *TrpA1* or driver alone controls by Kruskal-Wallis test with Dunn's post-hoc test (for *C01* > *TrpA1*, *A05* > *TrpA1*, or *C01/A05* > *TrpA1* compared to controls) or Mann-Whitney U-test (for *C01/A05* > *TrpA1* compared to *C01* > *TrpA1*). (**E–F**) Inhibition of *C01/A05*-neurons by expressing dORKΔC2 rescues sleep loss due to *Nca* knockdown, while expression of dORKΔC2 does not change baseline sleep. Mean sleep patterns in 8L: 16D conditions are shown in (**E**). Median night sleep levels are shown in (**F**).+ > *kk*: n = 72, *C01/A05* > +: n = 85, *C01/A05* > *kk*: n = 95, *C01/A05* > *dORKΔC2, kk*: n = 77, *C01/A05* > *kk, FRT-stop-FRT-GFP*: n = 39, + > *dORKΔC2*: n = 57, *C01/A05* > *dORKΔC2*: n = 73, *C01/A05* > *FRT-stop-FRT-GFP*: n = 49, + > *FRT-stop-FRT-GFP*: n = 36. ns – p>0.05, ***p<0.001, Kruskal-Wallis test with Dunn's post-hoc test.

DOI: https://doi.org/10.7554/eLife.38114.028