Neurocalcin acts in a clock- and light-modulated dopaminergic pathway to promote night sleep in *Drosophila*

Ko-Fan Chen, Angélique Lamaze, Patrick Kratschmer and James E. C. Jepson*

Department of Experimental and Clinical Epilepsy, UCL Institute of Neurology, UK

*Correspondence to: j.jepson@ucl.ac.uk

Short title: *Drosophila* Neurocalcin promotes night sleep

Abstract

Members of the neuronal calcium sensor (NCS) family act as key regulators of calcium signaling in neurons by modulating target proteins such as voltage- and ligand-gated ion channels in response to changes in intracellular calcium. In *Drosophila*, the NCS protein Neurocalcin has been shown to be broadly expressed within the nervous system, suggesting important neurobiological roles. However, whether Neurocalcin impacts complex behaviors in *Drosophila* has remained unclear. Here, using a hypothesis-based guilt-by-association strategy, we identify a novel role for Neurocalcin in promoting night sleep but not day sleep. We show that Neurocalcin acts in a common pathway with the D1-type Dop1R1 dopamine receptor, and that both the circadian clock and light-sensing pathways demarcate the temporal window during which NCA promotes sleep. Furthermore, we find that Neurocalcin functions in a multi-component wake-promoting neural network and demonstrate that the mushroom bodies, a known sleep-regulatory center, are a module within this circuit. Our results define a critical role for Neurocalcin in *Drosophila* and further our understanding of how distinct sleep periods are genetically regulated.

Author summary

Sleep in *Drosophila* occurs during both the day and night, yet the genetic pathways that selectively impact day versus night sleep are poorly understood. Here we uncover a link between the neuronal calcium sensor Neurocalcin and sleep in *Drosophila*. We show that Neurocalcin acts in a pathway involving the Dop1R1 dopamine receptor to promote sleep during the night, but not the day, and that the night-specific effect of NCA is coordinated by the circadian clock and light-sensing pathways. Furthermore, we identify a complex wake-promoting neuronal network in which NCA functions to regulate sleep, and our results suggest that NCA suppresses output from this circuit. Thus, we identify a novel role for Neurocalcin in *Drosophila* and shed light on the genetic regulation of distinct sleep stages.

Introduction

Sleep is a widely conserved and critical behavior that impacts numerous aspects of nervous system function, including neuronal development [1], clearance of metabolic waste [2], synaptic plasticity [3-6], and the generation of complex behaviors such as courtship and aggression [1, 7]. The fruit fly, *Drosophila melanogaster*, exhibits a sleep-like state characterized by periods of immobility, altered posture and elevated arousal thresholds [8, 9]. Similarly to mammals, sleep in *Drosophila* is regulated by circadian and homeostatic processes [10, 11]. Furthermore, just as human sleep can be separated into distinct temporal stages of differing arousal thresholds (REM and non-REM sleep), sleep in *Drosophila* also varies in intensity throughout the day/night cycle, with night sleep associated with higher arousal thresholds relative to day sleep [12].

Due to the vast genetic toolkit of *Drosophila*, many research groups have utilized this organism to identify sleep-relevant genes [11, 13-18], several of which have been shown to play a conserved role in regulating mammalian sleep [19, 20]. Of note, mutations in a select number of genes modulate either day or night sleep in *Drosophila*, suggesting an underlying genetic basis for the distinct characteristics of these separate sleep stages [21, 22]. Nonetheless, it is still unclear how day versus night sleep is designated to allow a given gene to specifically regulate one sleep phase or the other, and whether day and night sleep are defined by the circadian clock, the presence or absence of light, or both.

The identification of new genes selectively impacting day versus night sleep in *Drosophila* will help provide answers to such questions. Previously, unbiased large-scale screens of EMS-mutagenized [14, 17], P-element insertion [15], or transgenic RNAi knockdown lines [18] have been used to identify *Drosophila* sleep mutants. As

well as advancing our understanding of the genetic basis of sleep, such studies have yielded an additional benefit. Since distinct genes that similarly impact a given phenotype may act in a shared genetic network, identification of mutations that modulate sleep generates testable hypotheses regarding gene function. For example, the finding that Sleepless, a Ly6/neurotoxin-like protein, promotes expression of the Shaker potassium channel arose from the fact that both *sleepless* and *shaker* mutants exhibit sleep loss and ether-induced leg shaking [14, 15, 23], suggesting that these two proteins act in a common molecular pathway.

However, such genome-wide approaches are highly laborious, requiring screening of thousands of fly lines to identify a limited number of sleep mutants [15, 17]. Thus, targeted screening strategies of higher efficiency may represent a useful complement to unbiased high-throughput, yet low yield, methodologies. Here we uncover a novel sleep-relevant gene in *Drosophila* using an approach based on genetic and neurobiological correlates between *Drosophila* sleep and a human movement disorder, primary dystonia. From an initial screen of just five candidate loci, we identify the neuronal calcium sensor Neurocalcin (NCA) as a sleep regulatory factor that specifically promotes night sleep in *Drosophila*. Our results reveal a novel role for NCA and demonstrate the utility of targeted mini-screens to study sleep in *Drosophila*.

Results

Identification of Neurocalcin as a sleep-promoting factor

We identified NCA as a novel sleep regulator using a guilt-by-association strategy based on phenotypes linked to mutations in *KCTD17*/*insomniac*, homologous genes encoding a Cullin-3 adaptor protein in humans and *Drosophila* respectively [17, 24]. In humans, a *KCTD17* mutation causes myoclonus dystonia, a disorder characterised by repetitive movements, contorted postures and non-epileptic myoclonic jerks in the upper body [24]. In *Drosophila*, null or hypomorphic mutations in the *KCTD17* homologue *insomniac* (*inc*) result in profound reductions in sleep [16, 17]. A potential link between these two phenotypes is dopamine signalling. Altered plasticity of dopaminergic circuits within the striatum, a motor control centre, has been proposed as a potential mechanism underlying dystonic movements [25]. In *Drosophila*, dopamine is a pro-arousal factor. Elevated dopaminergic neurotransmission strongly reduces sleep [26-28], and INC has been suggested to control sleep by negatively regulating dopamine signalling [16].

Since genes often act in conserved modules [29], we posited the existence of a partially conserved genetic network that, in humans, is linked to dystonia, and which in *Drosophila* may influence sleep, both through dopamine signalling. To test this, we used transgenic RNAi driven by the pan-neuronal driver *elav*-Gal4 to knockdown expression of *Drosophila* homologs of the dystonia-genes *TOR1A*, *GNAL*, *ANO3*, *THAP1* and *HPCA* [24, 30-34] (*dTorsin*, *GαS*, cg6938, cg10403 and *nca* respectively) in neurons, and subsequently measured sleep in knockdown flies and respective controls. Here, sleep is defined as 5 min of inactivity as measured by the *Drosophila* Activity Monitoring (DAM) system, a common standard in the field [35]. From this targeted small-scale screen, we found that expression of a transgenic RNAi line

(*kk108825*, termed *kk*, Fig 1A) targeting *nca* mRNA reduced night sleep, but not day sleep, in adult male flies housed under 12 h light: 12 h dark conditions (12L: 12D) at 25°C (Fig 1A-C). Night-specific sleep loss due to neuronal *nca* knockdown was also observed in virgin adult female flies (S1A, B Fig), and in male flies expressing the *kk nca* RNAi using other pan-neuronal (*nsyb*-Gal4) or broadly expressed (*insomniac*-Gal4) drivers (S1C Fig).

Sleep architecture in *Drosophila* is generally studied in 12L: 12D conditions. Interestingly, we found that reduced night sleep in *nca* knockdown males appeared enhanced under short photoperiod conditions (8L: 16D) (Fig 1D). Similarly to 12L: 12D conditions, in 8L: 16D day sleep was unaffected whilst night sleep was robustly reduced (Fig 1E, F). Night sleep loss under short photoperiod was also observed in flies expressing two other independent RNAi lines targeting *nca* mRNA (*hmj21533* and *jf03398,* termed *hmj* and *jf* respectively) (S2A-D Fig). The *kk* and *jf* dsRNAs target a partially overlapping sequence of *nca*, whereas *hmj* targets a distinct upstream sequence (S2A Fig). For each RNAi line, we confirmed reduced *nca* expression using qPCR (S2E Fig). Transcription of *nca* occurs from promoter regions shared with the downstream locus *cg7646* (S2A Fig), yet *cg7646* transcription was not affected by *nca* RNAi (S2F Fig), nor were any common off-target mRNAs predicted for the *kk*, *hmj* or *jf* dsRNA hairpins (data not shown). Since pan-neuronal expression of three independent RNAi lines targeting two separate regions of *nca* mRNA reduce night sleep, we conclude that NCA acts in the *Drosophila* nervous system to promote night sleep. For simplicity, we use the *kk nca* RNAi for all subsequent experiments, and refer to flies expressing *kk nca* RNAi under *elav*-Gal4 as nca^{KD} (*nca* knockdown). All experiments below are performed under 8L: 16D conditions unless otherwise stated.

Neurocalcin promotes long sleep bouts in the early night

To analyse the impact of NCA on night sleep in more detail, we utilised a custom-made sleep analysis package (see Methods) to measure the initiation and length of individual sleep bouts in *nca*^{KD} and controls. We visualised individual sleep bouts across each population, plotting the time of onset and offset for night sleep bouts from n = 48 flies per genotype. Using this method, control flies containing either *nca kk* RNAi transgene or *elav*-Gal4 driver alone were observed to frequently initiate long sleep bouts in the early night (Fig 1G, H). In contrast, *nca*^{KD} flies did not exhibit equivalent longer sleep bouts (Fig 1I). Thus, NCA acts in neurons to promote consolidated sleep, particularly during the early phase of the night.

NCA does not impact circadian rhythmicity or waking activity

NCA could potentially impact sleep indirectly via an effect on the circadian clock and/or locomotor activity. Firstly, we therefore tested whether NCA was under clock control or regulated circadian rhythms. *nca* mRNA expression did not cycle throughout the day/night cycle in whole head tissue (S3A Fig). Importantly, *nca*^{KD} flies did not exhibit any change in circadian patterns of locomotor activity under constant dark conditions (S3B-C Fig). Furthermore, waking locomotor activity (defined as the number of DAM beam breaks per waking minute) was not significantly altered in *nca*^{KD} flies (Fig 1K). These results suggest that *nca* knockdown does not indirectly impact sleep/wake behavior by altering locomotor activity or impacting the circadian clock**.**

Light-sensing and circadian pathways modulate the effect of NCA on sleep

157 The night-specificity of sleep loss in *nca*^{KD} flies prompted us to test whether circadian
158 and/or light-sensing pathways restrict the effect of *nca* knockdown on sleep to the
159 night phase. Initially, we examined sleep patterns in *nca*^{KD} flies under constant dark
160 conditions, during which the circadian clock acts to distinguish subjective day from
161 night. Interestingly, robust sleep loss in *nca*^{KD} flies was restricted to the subjective
162 night (Fig 2A, B).

163 As a complementary approach, we next analysed sleep in *nca*^{KD} flies under
164 two conditions in which the circadian clock is no longer functional. Firstly, in
165 constant dark (DD) conditions and in an arrhythmic background due homozygosity
166 for a *timeless* knockout allele [36]. In DD, and without a functional clock to
167 demarcate subjective day from night, sleep loss was now observed throughout the 24
168 h dark period (Fig 2C, D). Secondly, we examined the effect of *nca* knockdown under
169 constant light conditions, in which the circadian clock becomes rapidly arrhythmic
170 due to light-dependent degradation of Timeless [37-39]. Strikingly, in constant light,
171 sleep loss in *nca*^{KD} flies was completely suppressed (Fig 2E, F). From the above data,
172 we conclude that the circadian clock is not required for NCA to promote sleep per se,
173 but instead defines when NCA is sleep promoting, with light acting as an
174 environmental signal that suppresses the impact of NCA on sleep.

175 We sought to determine which light-sensing pathways restrict the sleep-
176 promoting role of NCA to the night. We reasoned that removing relevant
177 photoreceptive molecules, cells or transduction pathways might restore sleep loss in
178 *nca*^{KD} flies during constant light conditions. Ablation of photoreceptor cells through
179 expression of the pro-apoptotic gene *hid* (*gmr* > *hid*) did not restore sleep loss to
180 *nca*^{KD} flies during constant light conditions (Fig 2G, H). In contrast, using a loss of
181 function allele of *cry* (*cry*^{02}), we found that loss of CRY in constant light resulted in a

small but significant loss of sleep in *nca*^{KD} flies (Fig 2I, J). CRY acts as a blue-light photoreceptor and has dual roles in synchronization of the circadian clock by light and light-dependent regulation of clock cell excitability [39, 40], suggesting that one or both of these pathways acts to modulate the timing of sleep loss in *nca*^{KD} flies. However, the reduction in sleep in *nca*^{KD}, *cry*^{02} flies in constant light is lower in magnitude compared to *nca*^{KD} flies in DD or 8L: 16D conditions (Fig 1B and 2A), suggesting that additional light-sensing pathways act in concert with CRY to inhibit the effect of reduced NCA levels on sleep/arousal. The restoration of clock function in *cry*^{02} homozygotes in constant light conditions may also contribute to the observed sleep loss in *nca*^{KD}, *cry*^{02} flies under constant light [39].

NCA acts in a common pathway with the Dop1R1 dopamine receptor

NCA is highly homologous to the human neuronal calcium sensor Hippocalcin (S4 Fig), and mutations in Hippocalcin cause DYT2 primary isolated dystonia [33]. One neuronal signalling mechanism frequently linked to dystonia is dopaminergic neurotransmission, with altered dopaminergic signalling within the striatum proposed to underlie forms of primary dystonia [41]. We therefore tested whether NCA modulates sleep via a dopaminergic mechanism. Under 8L: 16D conditions *nca* knockdown in dopaminergic neurons did not result in night sleep loss (Fig 3A and S1 Table). However, heterozygosity for a null or strongly hypomorphic allele of the *Dop1R1* dopamine receptor (*Dop1R1*$^{MI03085\text{-}G}$, a homozygous lethal MiMIC insertion) rescued night sleep loss in *nca*^{KD} flies (Fig 3B, C). Importantly, in both *elav*-Gal4/+ and *kk*/+ control backgrounds, heterozygosity for *Dop1R1*$^{MI03085\text{-}G}$ did not alter sleep levels (Fig 3B, C; $p > 0.05$, Kruskal-Wallis test with Dunn's post-hoc test). A similar epistatic interaction between *nca* and *Dop1R1* was observed using a second, weaker

Dop1R1 allele (*Dop1R1*^{MI004437}) (S5 Fig). From the above results, we propose that NCA acts in a common pathway with Dop1R1 to regulate night sleep, potentially downstream of dopaminergic neurons.

NCA acts in two distinct circuits to promote night sleep

We next sought to delineate the neural circuits in which NCA functions to promote night sleep. Using cell-specific *nca* knockdown, we performed an extensive screen of sleep relevant circuits defined by numerous *promoter*-Gal4 driver lines (Fig 3A and S1 Table). These include clock, neurotransmitter-specific, fan-shaped body, mushroom body (MB), and sensory neurons (Fig 3A) [11, 36, 42-46]. Furthermore, given the genetic interaction between *nca* and *Dop1R1*, we utilised genomic enhancer elements in the *Dop1R1* locus to drive *nca* knockdown in subsets of potential Dop1R1-expressing neurons (Fig 3A and S1 Table) [47, 48]. However, in contrast to broadly expressed drivers (*elav*-, *nsyb*- and *inc*-Gal4), *nca* knockdown in restricted neural subsets was insufficient to significantly reduce night sleep (Fig 3A and S1 Table).

These results suggested a complex sleep-relevant circuit requirement for NCA. Thus, we sought to reduce NCA levels in multiple sub-circuits to test for a simultaneous role of NCA in distinct anatomical regions. Through this approach, we found that *nca* knockdown using two *enhancer*-Gal4 lines (*R21G01* – an enhancer in the *TrpA1* locus, and *R72C01* – an enhancer in the *Dop1R1* locus), was sufficient to strongly phenocopy the effect of pan-neuronal *nca* knockdown on night sleep (Fig 4A-C; compare Fig 4B with Fig 1B). For simplicity we refer to these drivers as *G01* and *C01* respectively.

231 Each *enhancer*-Gal4 line drives transgenic fluorophore expression in 150-200
232 neurons in the adult male *Drosophila* brain (Fig 4A), and label neuropil regions that
233 only partially overlap between the two drivers. The *G01* enhancer drives expression in
234 a subset of MB neurons, a cluster of cell bodies adjacent to the anterior ventrolateral
235 protocerebrum (AVP), and two visual sub-circuits: optic lobe (OL) and anterior optic
236 tubercle (AoT) neurons. *C01* drives expression in the MBs, neurons projecting to the
237 MB γ-lobes, and the antennal mechanosensory and motor center (AMMC) (Fig 4A).
238 *G01* and *C01* also label dispersed cell bodies of unknown identity. The potential
239 overlap of *G01* and *C01* in the MBs raised the possibility that sleep loss in *G01/C01*
240 > *nca* RNAi flies was due to strong NCA knockdown in neurons common to both the
241 *G01* and *C01* enhancers. If so, driving *nca* RNAi with two copies of either *G01* or
242 *C01* should mimic sleep loss in *G01/C01* > *nca* RNAi flies. However, this was not the
243 case (S6 Fig). Thus, NCA is simultaneously required in non-overlapping sub-circuits
244 labelled by the *G01* and *C01* enhancers.
245 Given that *C01* is a *Dop1R1* enhancer element, that *nca* and *Dop1R1*
246 genetically interact to regulate sleep (Fig 3B), and that Dop1R1 is highly expressed in
247 the MBs [49], we tested whether the MBs were a constituent of the *C01* expression
248 domain by swapping *C01* for the MB-specific driver *ok107* and measuring sleep in
249 flies expressing *nca* RNAi in both *G01* and MB neurons. Indeed, knockdown of *nca*
250 in both *G01*- and MB-neurons also specifically reduced night sleep (Fig 4D), albeit to
251 a weaker degree compared to knockdown in *G01*- and *C01*-neurons (compare Fig 4C
252 and 4D). Thus, we conclude that the MBs are an important component of a complex
253 network defined by *C01*-Gal4 with additional, as yet undefined, neurons acting within
254 both the *C01* and *G01* domains to regulate night sleep.
255

256 **_G01_-neurons enhance _C01_-mediated arousal**

257 To confirm a sleep-relevant role for *C01*- and *G01*-neurons we tested whether
258 activation of either subpopulation was sufficient to alter sleep. To do so, we expressed
259 the temperature-sensitive channel TrpA1 in either neuronal subset or both, and shifted
260 flies from a non-activating temperature (22°C) to an activating temperature (27°C)
261 sufficient to cause hyperactive neurotransmission through TrpA1-mediated cation
262 influx [50] (Fig 5A). At the non-activating temperature, over-expression of TrpA1 in
263 either circuit or both did not alter sleep levels (Fig 5B). At the activating temperature,
264 excitation of *G01*-neurons did not alter night sleep levels relative to controls (Fig 5C).
265 In contrast, excitation of *C01*-neurons profoundly reduced night sleep (Fig 5C, D) as
266 well as day sleep (Fig 5C). Interestingly, simultaneous activation of *C01*- and *G01*-
267 neurons further reduced night sleep relative to activation of *C01*-neurons alone,
268 despite the lack of effect of *G01*-neuron activation on sleep (Fig 5C, D). These results
269 suggest that the *C01*- and *G01*-circuits interact to regulate sleep, with *C01*-neurons
270 acting as a predominant pro-arousal circuit and *G01*-neurons acting in a modulatory
271 manner to enhance the impact of *C01*-activation on night sleep. We infer from the
272 above data that NCA suppresses excitability and/or neurotransmitter release in *C01*-
273 and *G01*-neurons in a clock- and light-dependent manner. These modulatory inputs
274 are likely to be bypassed by ectopic TrpA1 activation, resulting in sleep loss during
275 both day and night, in contrast to the night-specific effect of *nca* knockdown.

Discussion

Drosophila Neurocalcin (NCA) is a member of the neuronal calcium sensor family of cytosolic proteins [51]. Previous work has shown that NCA is a calcium-binding protein and that NCA is widely expressed throughout synaptic neuropil regions of the adult fly brain [52]. Despite this, the neurobiological roles of NCA have remained unclear. Using a hypothesis-driven approach based on genetic and neurobiological correlates between *Drosophila* sleep and a human movement disorder, we have uncovered a novel role for NCA as a night sleep-promoting factor.

Our results show that both internal and external cues are integrated by the *Drosophila* nervous system to regulate when NCA impacts sleep. Internal cues are generated by the circadian clock with light acting in parallel as an external signal (Fig 2). Together, these demarcate the temporal window within which NCA promotes sleep. Interestingly, this mode of coordinate regulation by the clock and light contrasts with *Drosophila* NMDA receptor 1 (*Nmdar1*) knockdown flies [22]. Reduced Nmdar1 similarly results in night-specific sleep loss under light-dark cycles, but sleep loss is extended to both subjective day and night during constant-dark conditions, indicating that the effect of Nmdar1 on sleep is gated solely by light [22]. Why *Drosophila* utilizes distinct mechanisms to time the effect-windows of specific sleep-promoting factors is an intriguing question for future investigations.

Similarly to many sleep-promoting factors in *Drosophila* [13, 17, 18, 22, 53, 54], sleep-relevant NCA activity does not map onto a single cell-type within the *Drosophila* nervous system. Rather, NCA acts in multiple neuropil regions, each contributing to a net promotion of night sleep (Figs 3 and 4). One such region is the MB, a well-defined memory and sleep-regulatory center [43, 44, 46, 55]. However, further work is required to identify the full spectrum of key cell-types within the *G01-*

and *C01*-Gal4 expression domains. Acute activation of *G01*- and/or *C01*-neurons demonstrates that *C01*-neurons are wake-promoting, while *G01*-neurons enhance wakefulness only in the context of *C01*-neuron activation (Fig 5). Based on our data, we therefore hypothesize that reduction of NCA in both neuronal subsets causes mild hyperexcitation, which in *C01*-neurons alone is insufficient to modulate sleep but in both populations simultaneously causes an increase in network excitability sufficient to reduce night sleep. In this model, dopamine signalling through Dop1R1 gives excitatory drive to wake-promoting *C01*-neurons (defined by a *Dop1R1* enhancer element), and thus reducing Dop1R1 expression negates the increased firing and/or synaptic release caused by *nca* knockdown (Fig 3). Alternatively, Dop1R1 may act downstream of wake-promoting *C01*-neurons. Interestingly, neither *C01*- or *G01*-population includes the fan-shaped body (FSB), a region previously shown to facilitate dopamine-sensitive arousal via Dop1R1 [27, 28].

How might NCA regulate neuronal activity? The mammalian NCA homolog Hippocalcin undertakes pleiotropic roles in mammalian neurons, including facilitating NMDA receptor endocytosis during LTD and gating the slow afterhyperpolarisation, a potassium current mediated by KCNQ channels [56-59]. Furthermore, recent data suggest that Hippocalcin negatively regulates calcium influx through N-type voltage-gated calcium channels [60]. Given the strong homology between Hippocalcin and NCA, it is possible that NCA plays similar roles in *Drosophila* neurons. However, further research is required to identify the key molecular pathways through which NCA impacts sleep.

Finally, our work adds to a growing body of evidence linking *Drosophila* homologues of primary dystonia genes to dopaminergic signalling. *Torsin* is the *Drosophila* homologue of the dystonia-gene *TOR1A* and has been shown to play a

role in dopamine metabolism by regulating expression of GTP cyclohydrolase, a component of the dopamine synthesis pathway that is also mutated in L-Dopa-responsive dystonia [30, 61-63]. *Drosophila insomniac* is homologous to the myoclonus dystonia-gene *KCTD17* (as well the paralogs *KCTD2* and *KCTD5*) [17, 24, 64], and sleep loss in *insomniac* mutants can be rescued by inhibition of dopamine synthesis [16]. Correspondingly, our work suggests that NCA acts downstream of dopaminergic neurons in a pathway involving the Dop1R1 dopamine receptor. Given the link between Torsin, Insomniac and NCA with dopamine signalling, it will be intriguing to test whether dystonia-gene homologues modulate other dopamine-related behaviours in *Drosophila*, including sleep, learning, forgetting and courtship [26, 65-69].

Materials and Methods

Fly husbandry

Fly strains and crossings are maintained on standard fly food at constant temperature 25^{o}C under 12 h:12 h light-dark cycles (12L: 12D). The following strains were obtained from Bloomington and/or VDRC stock centers: kk108825 (v100625), hmj21533 (54814), jf03398 (29461), *Dop1R1*$^{MI03085\text{-}GFSTF.2}$ (59802), *Dop1R1*^{MI04437}(43773), *ple*-Gal4 (8848), *Chat*-Gal4 (6798), *vGlut*-Gal4 (26160), *GAD*-Gal4 (51630), *Ddc*-Gal4(7010), *GMR*-Gal4 (1104), *Trh.1*-Gal4 (38388), *Tdc2*-Gal4(9313), *C5*-Gal4 (30839) and *ok107*-Gal4 (854). The remaining lines obtained from Bloomington stock center are Janelia Flylight collection with identifiable prefixes: R23E10-Gal4, R55B01-Gal4, R52H12-Gal4, *Hdc*-Gal4 (R17F12-Gal4), R21G01-Gal4, R72B05-Gal4, R72B07-Gal4, R72B08-Gal4, R72B11-Gal4, R72C01-Gal4, and R72C02-Gal4. The following lines were gifts from laboratories of Kyunghee Koh: *elav*-Gal4, *nsyb*-Gal4, *tim*-Gal4 and *TUG*-Gal4, Joerg Albert: *nompC*-Gal4 [70] and Nicolas Stavropoulos: *inc*-Gal4:2 [17]. *ppk*-Gal4, TrpA1-CD-Gal4 was described previously [71]. *GMR-hid*, *tim*^{KO} and *cry*^{02} were previously described in [36]. Except for *Ddc*-Gal4, *Trh.1*-Gal4, *Tdc2*-Gal4, *nompC*-Gal4 and *Hdc*-Gal4, all *Drosophila* strains used for sleep-wake assay were either outcrossed five times into a standard isogenic background (iso31) or insertion-free chromosomes were exchanged with the iso31 line (*hmj21533, jf03398, Dop1R1*$^{MI03085\text{-}G}$ and *Dop1R1*^{MI04437}).

RNA extraction and Quantitative PCR

10-20 fly heads per genotype were collected with liquid nitrogen and dry ice at indicated time points (S3 Fig). Total RNA was extracted using TRIzol™ reagent following manufacturer's manual (Thermo Fisher Scientific). cDNA were reversed

363 transcribed from 250 or 500 ng of DNase I (M0303S, NEB) treated RNA sample via
364 MMLV RT (M170A, Promega). A set of five or six standards across 3125 fold
365 dilution were prepared from the equally pooled cDNA of all genotypes in each
366 experiment. Triplicated PCR reactions were prepared in 96-well or 384-well plates for
367 standards and the cDNA sample of each genotype (20 to 40 fold dilution) by mixing
368 in Power SYBR Green Master Mix (Thermo Fisher Scientific) and the following
369 primer sets: ncaqF2: acagagttcacagacgctgag, ncaqR2: ttgctagcgtcaccatatggg;
370 cg7646F: gcctttcgaatgtacgatgtcg, cg7646R: cctagcatgtcataaattgcctgaac or
371 rp49F:cgatatgctaagctgtcgcaca, rp49R: cgcttgttcgatccgtaacc. The PCR reactions were
372 then performed in Applied Biosystems StepOne (96-wells module) or QuantStudio
373 6Flex instruments (384 wells module) using the standard thermocycle. Melting curve
374 analysis was also performed to evaluate the quality of the PCR product and avoiding
375 contamination. The Ct values were exported as csv files and a standard curve between
376 Ct values and logarithm of dilution were calculated using liner regression function in
377 Graphpad. The relative expression level for *nca*, *cg7646* and *rp49* of each sample
378 were estimated by interpolation and anti-logarithm. The expression levels for *nca* and
379 cg7646 of each genotype were further normalized to their respective average *rp49*
380 level. The statistical difference between the normalized expression levels of each
381 genotype were determined by Kruskal-Wallis test with Dunn's post-hoc test using
382 Graphpad software.

383 **Sleep-wake behavioral analysis**

384 Three to five days old male or virgin female flies of given genotypes were collected
385 and loaded into glass tubes containing 4% sucrose and 2% agar (w/v). The sleep-wake
386 behaviors were recorded by Drosophila Activity Monitor (DAM) system for 3 days in
387 the designated LD regime (L12:D12. L8:D16, DD or LL). The behavior recordings

388 from the third day of the given LD regime were then analyzed. All flies were
389 entrained to 12L: 12D prior to entering designed LD regimes, except for one *cry*^{02}
390 experiment that flies were entrained to 8L: 16D condition (one of the three
391 independent experiments shown in Fig 2I and J). *Drosophila* activity (or wake) is
392 measured by infra-red beam crosses and sleep is defined by 5 minutes with no activity
393 (no beam crosses). Customized Excel calculators (supplementary file 1) and R scripts
394 (https://github.com/PatrickKratsch/DAM_analysR) were developed to calculate the
395 following parameters for individual flies: *Onset and offset of each sleep bout*, *sleep*
396 *bout length*, *day and night sleep minutes*, *daily total sleep minutes,* and *daily sleep*
397 *profile* (30 minutes interval). An established MATLAB® based tool, Flytoolbox, was
398 used for circadian rhythmicity analysis [72, 73]. Briefly, the strength of rhythmicity
399 (RI) was estimated using the height of the third peak coefficient in the auto-
400 correlogram calculated for the activity time series of each fly. Rhythmic Statistics
401 values were then obtained from the ratio of the RI value to the 95% confidence
402 interval for the correlogram ($2/\sqrt{N}$, where N is the number of observations, which
403 correlatively increase with the sampling frequency), in order to determine statistical
404 significance of any identified period (RS is ≥ 1)

405 **Immunohistochemistry and confocal microscopy**

406 Adult male fly of R72C01 > CD8::*GFP* and R21G01 > CD4::*tdTomato* were
407 anesthetized in 70% ethanol before brains were dissected in PBT (0.1M phosphate
408 buffer with 0.3% TritonX100) and collected in 4% paraformaldehyde/PBT on ice.
409 The fixation was then performed at room temperature for 15 mins before washing 3
410 times with PBT. The brain samples were blocked by 5% goat serum/PBT for 1 h at
411 room temperature before incubation with mouse anti-nc82 (1:200) plus rabbit anti-
412 GFP (1:1000) or rabbit anti-dsRED (1:2000) in 5% goat serum/PBT at 4°C over 48 h.

413 The samples were washed 6 times with PBT before incubated with Alexa Fluor 647
414 goat anti-mouse IgG (1:500) plus Alexa Fluor 488 goat anti-rabbit IgG (1:2000) or
415 Alexa Fluor 555 goat anti-rabbit IgG (1:2000) in 5% goat serum/PBT at 4°C over 24
416 h. After washing 6 times with PBT, the samples were mounted in SlowFade Gold
417 antifade reagent (S36936, Thermo Fisher Scientific) on microscope slides and stored
418 at 4°C until imaged by inverted confocal microscope Zeiss LSM 710.

419 **Bioinformatics**

420 Conservation of amino acid residues between *Drosophila* Neurocalcin and human
421 Hippocalcin was determined using ClustalW2 software for multiple sequence
422 alignment. Amino-acid identity and similarity was visualised using BOXSHADE.
423

Acknowledgements

We thank Jack Humphrey for performing initial work on *neurocalcin* knockdown flies, and Kyunghee Koh for helpful comments on the manuscript. This study was supported by the Wellcome Trust (Synaptopathies strategic award (104033)), and by the MRC (New Investigator Grant MR/P012256/1). P.K is supported by a Wellcome Trust Neuroscience PhD studentship.

Author Contributions

Conceptualization, K-F.C and J.E.C.J; Data curation, P.K; Formal Analysis, K-F.C, A.L and P.K; Methodology, K-F.C and P.K; Investigation, K-F.C, A.L and J.E.C.J; Resources, J.E.C.J; Software, P.K; Writing – Original Draft, K-F.C and J.E.C.J; Writing – Review & Editing, K-F.C, A.L. P.K and J.E.C.J; Visualisation, K-F.C and J.E.C.J; Supervision, J.E.C.J; Project Administration, J.E.C.J; Funding Acquisition, J.E.C.J.

Figure Legends

Fig. 1 Neurocalcin knockdown results in a loss of consolidated sleep during the early night in *Drosophila*. **(A)** Mean sleep levels in 12L: 12D conditions for nca^{KD} adult males and associated controls (*elav*-Gal4 driver or RNAi transgene heterozygotes). **(B-C)** Median day **(B)** and night **(C)** sleep levels in the above genotypes. **(D)** Mean sleep levels in 8L: 16D conditions for nca^{KD} adult males and associated controls. **(E-F)** Median day and night sleep levels in the above genotypes during 8L: 16D conditions. Data are presented as Tukey box plots. The 25th, Median, 75th percentiles are shown. Whiskers represent 1.5 x the interquartile range. Identical representations are used in all subsequent box plots. **A-C**: n = 48 for each genotype; **D-F**: 55 > n > 54. **(G-I)** 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 under 8L: 16D conditions. In control flies (*elav* > + and + > *kk*), longer sleep bouts initiated early during the night are highlighted in red **(G, H)**, which are largely absent in nca^{KD} adult males **(I)**. n = 48 for each genotype. **(J)** 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: χ^2, *df*: 142.0, 4, $p < 0.0001$; nca^{KD} vs. RNAi: χ^2, *df*: 2112.0, 4, $p < 0.0001$). **(K)** Number of DAM beam breaks per waking minute for nca^{KD} and control adult males during night phase of 8L: 16D cycles. 55 > n > 54. ***$p < 0.0005$, ns - $p > 0.05$ as compared to driver and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test.

Fig. 2 The circadian clock and light-sensing pathways regulate sleep promotion by NCA. (A-B) Mean sleep levels in nca^{KD} and control adult males across 24 h in constant-dark (DD) conditions **(A),** and total median sleep levels in the above genotypes **(B)**. 47 > n > 44. 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 h in constant-dark (DD) conditions in a *timeless* knockout (tim^{KO}) background **(C),** and total median sleep levels **(D)**. 39 > n > 32. **(E-F)** Mean sleep levels in nca^{KD} and control adult males across 24 h in constant-light (LL) conditions **(E),** and total median sleep levels in the above genotypes **(F)**. 47 > n > 44. **(G-H)** Mean sleep levels in nca^{KD} and control adult males across 24 h in constant-light (LL) conditions in a *gmr > hid* background **(G),** and total median sleep levels in the above genotypes **(H)**. *elav > kk, gmr-hid*/+: n = 51; + > *kk, gmr-hid*/+: n = 48; *elav* > +, *gmr-hid*/+: n = 24. **(I-J)** Mean sleep levels in nca^{KD} and control adult males across 24 h in constant-light (LL) conditions in a *cryptochrome* null (cry^{02}) background **(I),** and total median sleep levels in the above genotypes **(J)**. 72 > n > 61. Note the small but consistent reduction in sleep in nca^{KD}, cry^{02} males **(I)**, leading to a significant decrease in total median sleep levels relative to controls **(J)**. ***p < 0.0005, ns - p > 0.05, as compared to driver and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test.

Fig. 3 NCA and Dop1R1 genetically interact to regulate sleep. (A) Transgenic RNAi-based mini-screen to identify key NCA-expressing neurons. NCA knockdown with broadly expressed drivers results 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. Red box plots: experimental lines showing reduced night sleep relative to both controls. Green box plots: experimental lines failing to show reduced night sleep relative to one or both controls. See S1 Table for n-values and statistical comparisons. **(B-C)** heterozygosity for the null or strongly hypomorphic Dop1R1 allele *Dop1R1*$^{MI03085\text{-}G}$ (*D1R1*/+) suppressed sleep loss in *nca*^{KD} adult males, but did not alter sleep in control males. Mean sleep patterns in 8L: 16D conditions are shown in **(B)**. Median night sleep levels are shown in **(C)**. **p < 0.005, ***p < 0.0005, as compared to driver and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test. *elav>kk, D1R1/+*: n = 32; *elav > +, D1R1/+*: n = 15; *+ > kk, D1R1/+*: n = 32; *elav > kk*: n = 48*; elav > +*: n = 47; *+ > kk*: n = 48.

Fig. 4 NCA acts in a distributed neural network to regulate night sleep. (A) Confocal z-stacks of adult male brains expressing genetically-encoded fluorophores under the *G01* or *C01*-Gal4 drivers. Neuropil regions are labelled with anti-Bruchpilot (BRP, nc82). Scale bar = 100 µm. Arrows point to neuropil centers. AoT: anterior optic tubercle. MBs: mushroom body neurons. OL: optic lobe. AMMC: antennal mechanosensory and motor center. **(B-C)** *nca* knockdown in both *G01* and *C01*-neurons recapitulates the effect of pan-neuronal *nca* knockdown, whereas *nca* knockdown in either neuronal subpopulation alone does not reduce sleep relative to controls. Mean sleep patterns in 8L: 16D conditions are shown in **(B)**. Median night sleep levels are shown in **(C)**. *+ > kk*: n = 80; *C01 > +*: n = 64, *C01 > kk*: n = 80; *G01 > +*: n = 31; *G01 > kk*: n = 31; *C01/G01 > +*: n = 42; *C01/G01 > kk*: n = 71. **(D-E)** *nca* knockdown in both *G01* and MB-neurons (defined by *ok107*-Gal4; *ok107*) also results in reduced night sleep (also see Fig. 3A showing *ok107 > kk* does not cause

514 sleep loss). Mean sleep patterns in 8L: 16D conditions are shown in **(D)**. Median night
515 sleep levels are shown in **(E)**. + > *kk*: n = 31; *G01/ok107* > +: n = 33; *G01/ok107* > *kk*:
516 n = 42. *p < 0.05, **p < 0.005, ***p < 0.0005, ns – p > 0.05, as compared to driver
517 and RNAi alone controls via Kruskal-Wallis test with Dunn's post-hoc test.
518
519 **Fig. 5 *C01*-neurons are wake-promoting and are modulated by *G01*-neurons. (A)**
520 Experimental paradigm for acute activation of *G01* or *C01*-neurons. 22°C: non-
521 activating temperature for TrpA1. 27°C: activating temperature. Sleep measurements
522 were measured over two days in 8L: 16D conditions. **(B-C)** Mean sleep levels across
523 8L: 16D following expression of TrpA1 in *G01*-, *C01*- or *G01*- and *C01*-neurons (and
524 associated controls) at 22°C **(B)** or 27°C **(C)**. **(D)** Median change in night sleep levels
525 (Δ night sleep) following the shift from 22°C on day 1 to 27°C on day 2. + > *TrpA1*: n
526 = 53; *G01* > +: n = 23; *G01* > *TrpA1*: n = 68; *C01* > +: n = 24; *C01* > *TrpA1*: n = 40;
527 *C01/G01* > +: n = 33; *C01/G01* > *TrpA1*: n = 40. ***p < 0.0005, ns – p > 0.05, as
528 compared to *TrpA1* or driver alone controls by Kruskal-Wallis test with Dunn's post-
529 hoc test (for *C01, G01* or *C01/G01* > *TrpA1* compared to controls) or Mann-Whitney
530 test (for *C01/G01* > *TrpA1* compared to *C01* > *TrpA1*).
531

References

1. Kayser MS, Yue Z, Sehgal A. A critical period of sleep for development of courtship circuitry and behavior in *Drosophila*. Science. 2014;344(6181):269-74. doi: 10.1126/science.1250553.

2. Xie L, Kang H, Xu Q, Chen MJ, Liao Y, Thiyagarajan M, et al. Sleep drives metabolite clearance from the adult brain. Science. 2013;342(6156):373-7. doi: 10.1126/science.1241224.

3. Havekes R, Park AJ, Tudor JC, Luczak VG, Hansen RT, Ferri SL, et al. Sleep deprivation causes memory deficits by negatively impacting neuronal connectivity in hippocampal area CA1. Elife. 2016;5. doi: 10.7554/eLife.13424.

4. Kuhn M, Wolf E, Maier JG, Mainberger F, Feige B, Schmid H, et al. Sleep recalibrates homeostatic and associative synaptic plasticity in the human cortex. Nat Commun. 2016;7:12455. doi: 10.1038/ncomms12455.

5. Li W, Ma L, Yang G, Gan WB. REM sleep selectively prunes and maintains new synapses in development and learning. Nat Neurosci. 2017;20(3):427-37. doi: 10.1038/nn.4479.

6. Yang G, Lai CS, Cichon J, Ma L, Li W, Gan WB. Sleep promotes branch-specific formation of dendritic spines after learning. Science. 2014;344(6188):1173-8. doi: 10.1126/science.1249098.

7. Kayser MS, Mainwaring B, Yue Z, Sehgal A. Sleep deprivation suppresses aggression in *Drosophila*. Elife. 2015;4:e07643. doi: 10.7554/eLife.07643.

8. Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, et al. Rest in *Drosophila* is a sleep-like state. Neuron. 2000;25(1):129-38.

9. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in *Drosophila melanogaster*. Science. 2000;287(5459):1834-7.

557 10. Huber R, Hill SL, Holladay C, Biesiadecki M, Tononi G, Cirelli C. Sleep
558 homeostasis in *Drosophila melanogaster*. Sleep. 2004;27(4):628-39.
559 11. Liu S, Lamaze A, Liu Q, Tabuchi M, Yang Y, Fowler M, et al. WIDE
560 AWAKE mediates the circadian timing of sleep onset. Neuron. 2014;82(1):151-66.
561 doi: 10.1016/j.neuron.2014.01.040.
562 12. van Alphen B, Yap MH, Kirszenblat L, Kottler B, van Swinderen B. A
563 dynamic deep sleep stage in *Drosophila*. J Neurosci. 2013;33(16):6917-27. doi:
564 10.1523/JNEUROSCI.0061-13.2013.
565 13. Afonso DJ, Liu D, Machado DR, Pan H, Jepson JE, Rogulja D, et al.
566 TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control
567 Sleep in *Drosophila*. Curr Biol. 2015;25(13):1717-26. doi:
568 10.1016/j.cub.2015.05.037.
569 14. Cirelli C, Bushey D, Hill S, Huber R, Kreber R, Ganetzky B, et al. Reduced
570 sleep in *Drosophila Shaker* mutants. Nature. 2005;434(7037):1087-92. doi:
571 10.1038/nature03486.
572 15. Koh K, Joiner WJ, Wu MN, Yue Z, Smith CJ, Sehgal A. Identification of
573 SLEEPLESS, a sleep-promoting factor. Science. 2008;321(5887):372-6. doi:
574 10.1126/science.1155942.
575 16. Pfeiffenberger C, Allada R. *Cul3* and the BTB adaptor *insomniac* are key
576 regulators of sleep homeostasis and a dopamine arousal pathway in *Drosophila*. PLoS
577 Genet. 2012;8(10):e1003003. doi: 10.1371/journal.pgen.1003003.
578 17. Stavropoulos N, Young MW. *insomniac* and *Cullin-3* regulate sleep and
579 wakefulness in *Drosophila*. Neuron. 2011;72(6):964-76. doi:
580 10.1016/j.neuron.2011.12.003.

581 18. Rogulja D, Young MW. Control of sleep by cyclin A and its regulator.
582 Science. 2012;335(6076):1617-21. doi: 10.1126/science.1212476.
583 19. Douglas CL, Vyazovskiy V, Southard T, Chiu SY, Messing A, Tononi G, et
584 al. Sleep in Kcna2 knockout mice. BMC Biol. 2007;5:42. doi: 10.1186/1741-7007-5-
585 42.
586 20. Graves LA, Hellman K, Veasey S, Blendy JA, Pack AI, Abel T. Genetic
587 evidence for a role of CREB in sustained cortical arousal. J Neurophysiol.
588 2003;90(2):1152-9. doi: 10.1152/jn.00882.2002.
589 21. Ishimoto H, Lark A, Kitamoto T. Factors that Differentially Affect Daytime
590 and Nighttime Sleep in *Drosophila melanogaster*. Front Neurol. 2012;3:24. doi:
591 10.3389/fneur.2012.00024.
592 22. Tomita J, Ueno T, Mitsuyoshi M, Kume S, Kume K. The NMDA Receptor
593 Promotes Sleep in the Fruit Fly, *Drosophila melanogaster*. PLoS One.
594 2015;10(5):e0128101. doi: 10.1371/journal.pone.0128101.
595 23. Wu MN, Joiner WJ, Dean T, Yue Z, Smith CJ, Chen D, et al. SLEEPLESS, a
596 Ly-6/neurotoxin family member, regulates the levels, localization and activity of
597 Shaker. Nat Neurosci. 2010;13(1):69-75. doi: 10.1038/nn.2454.
598 24. Mencacci NE, Rubio-Agusti I, Zdebik A, Asmus F, Ludtmann MH, Ryten M,
599 et al. A missense mutation in KCTD17 causes autosomal dominant myoclonus-
600 dystonia. Am J Hum Genet. 2015;96(6):938-47. doi: 10.1016/j.ajhg.2015.04.008.
601 25. Peterson DA, Sejnowski TJ, Poizner H. Convergent evidence for abnormal
602 striatal synaptic plasticity in dystonia. Neurobiol Dis. 2010;37(3):558-73. doi:
603 10.1016/j.nbd.2009.12.003.

604 26. Kume K, Kume S, Park SK, Hirsh J, Jackson FR. Dopamine is a regulator of
605 arousal in the fruit fly. J Neurosci. 2005;25(32):7377-84. doi:
606 10.1523/JNEUROSCI.2048-05.2005.
607 27. Ueno T, Tomita J, Tanimoto H, Endo K, Ito K, Kume S, et al. Identification of
608 a dopamine pathway that regulates sleep and arousal in *Drosophila*. Nat Neurosci.
609 2012;15(11):1516-23. doi: 10.1038/nn.3238.
610 28. Liu Q, Liu S, Kodama L, Driscoll MR, Wu MN. Two dopaminergic neurons
611 signal to the dorsal fan-shaped body to promote wakefulness in *Drosophila*. Curr
612 Biol. 2012;22(22):2114-23. doi: 10.1016/j.cub.2012.09.008.
613 29. Lehner B. Genotype to phenotype: lessons from model organisms for human
614 genetics. Nat Rev Genet. 2013;14(3):168-78. doi: 10.1038/nrg3404.
615 30. Ozelius LJ, Hewett JW, Page CE, Bressman SB, Kramer PL, Shalish C, et al.
616 The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. Nat
617 Genet. 1997;17(1):40-8. doi: 10.1038/ng0997-40.
618 31. Fuchs T, Saunders-Pullman R, Masuho I, Luciano MS, Raymond D, Factor S,
619 et al. Mutations in GNAL cause primary torsion dystonia. Nat Genet. 2013;45(1):88-
620 92. doi: 10.1038/ng.2496.
621 32. Charlesworth G, Plagnol V, Holmstrom KM, Bras J, Sheerin UM, Preza E, et
622 al. Mutations in ANO3 cause dominant craniocervical dystonia: ion channel
623 implicated in pathogenesis. Am J Hum Genet. 2012;91(6):1041-50. doi:
624 10.1016/j.ajhg.2012.10.024.
625 33. Charlesworth G, Angelova PR, Bartolome-Robledo F, Ryten M, Trabzuni D,
626 Stamelou M, et al. Mutations in HPCA cause autosomal-recessive primary isolated
627 dystonia. Am J Hum Genet. 2015;96(4):657-65. doi: 10.1016/j.ajhg.2015.02.007.

628 34. Fuchs T, Gavarini S, Saunders-Pullman R, Raymond D, Ehrlich ME,
629 Bressman SB, et al. Mutations in the THAP1 gene are responsible for DYT6 primary
630 torsion dystonia. Nat Genet. 2009;41(3):286-8. doi: 10.1038/ng.304.
631 35. Pfeiffenberger C, Lear BC, Keegan KP, Allada R. Processing sleep data
632 created with the *Drosophila* Activity Monitoring (DAM) System. Cold Spring Harb
633 Protoc. 2010;2010(11):pdb prot5520. doi: 10.1101/pdb.prot5520.
634 36. Lamaze A, Ozturk-Colak A, Fischer R, Peschel N, Koh K, Jepson JE.
635 Regulation of sleep plasticity by a thermo-sensitive circuit in *Drosophila*. Sci Rep.
636 2017;7:40304. doi: 10.1038/srep40304.
637 37. Koh K, Zheng X, Sehgal A. JETLAG resets the *Drosophila* circadian clock by
638 promoting light-induced degradation of TIMELESS. Science. 2006;312(5781):1809-
639 12. doi: 10.1126/science.1124951.
640 38. Peschel N, Veleri S, Stanewsky R. Veela defines a molecular link between
641 Cryptochrome and Timeless in the light-input pathway to *Drosophila*'s circadian
642 clock. Proc Natl Acad Sci U S A. 2006;103(46):17313-8. doi:
643 10.1073/pnas.0606675103.
644 39. Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, et al.
645 The *cryb* mutation identifies cryptochrome as a circadian photoreceptor in
646 *Drosophila*. Cell. 1998;95(5):681-92. PubMed PMID: 9845370.
647 40. Fogle KJ, Parson KG, Dahm NA, Holmes TC. CRYPTOCHROME is a blue-
648 light sensor that regulates neuronal firing rate. Science. 2011;331(6023):1409-13. doi:
649 10.1126/science.1199702.
650 41. Breakefield XO, Blood AJ, Li Y, Hallett M, Hanson PI, Standaert DG. The
651 pathophysiological basis of dystonias. Nat Rev Neurosci. 2008;9(3):222-34. doi:
652 10.1038/nrn2337.

653 42. Donlea JM, Thimgan MS, Suzuki Y, Gottschalk L, Shaw PJ. Inducing sleep
654 by remote control facilitates memory consolidation in *Drosophila*. Science.
655 2011;332(6037):1571-6. doi: 10.1126/science.1202249.
656 43. Joiner WJ, Crocker A, White BH, Sehgal A. Sleep in *Drosophila* is regulated
657 by adult mushroom bodies. Nature. 2006;441(7094):757-60. doi:
658 10.1038/nature04811.
659 44. Pitman JL, McGill JJ, Keegan KP, Allada R. A dynamic role for the
660 mushroom bodies in promoting sleep in *Drosophila*. Nature. 2006;441(7094):753-6.
661 doi: 10.1038/nature04739.
662 45. Seidner G, Robinson JE, Wu M, Worden K, Masek P, Roberts SW, et al.
663 Identification of Neurons with a Privileged Role in Sleep Homeostasis in *Drosophila*
664 *melanogaster*. Curr Biol. 2015;25(22):2928-38. doi: 10.1016/j.cub.2015.10.006.
665 46. Sitaraman D, Aso Y, Jin X, Chen N, Felix M, Rubin GM, et al. Propagation of
666 Homeostatic Sleep Signals by Segregated Synaptic Microcircuits of the *Drosophila*
667 Mushroom Body. Curr Biol. 2015;25(22):2915-27. doi: 10.1016/j.cub.2015.09.017.
668 47. Jenett A, Rubin GM, Ngo TT, Shepherd D, Murphy C, Dionne H, et al. A
669 GAL4-driver line resource for *Drosophila* neurobiology. Cell Rep. 2012;2(4):991-
670 1001. doi: 10.1016/j.celrep.2012.09.011.
671 48. Jiang Y, Pitmon E, Berry J, Wolf FW, McKenzie Z, Lebestky TJ. A Genetic
672 Screen To Assess Dopamine Receptor (DopR1) Dependent Sleep Regulation in
673 *Drosophila*. G3 (Bethesda). 2016;6(12):4217-26. doi: 10.1534/g3.116.032136.
674 49. Lebestky T, Chang JS, Dankert H, Zelnik L, Kim YC, Han KA, et al. Two
675 different forms of arousal in *Drosophila* are oppositely regulated by the dopamine D1
676 receptor ortholog DopR via distinct neural circuits. Neuron. 2009;64(4):522-36. doi:
677 10.1016/j.neuron.2009.09.031.

678 50. Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, et al.
679 An internal thermal sensor controlling temperature preference in *Drosophila*. Nature.
680 2008;454(7201):217-20. doi: 10.1038/nature07001.
681 51. Burgoyne RD, Haynes LP. Understanding the physiological roles of the
682 neuronal calcium sensor proteins. Mol Brain. 2012;5(1):2. doi: 10.1186/1756-6606-5-
683 2.
684 52. Teng DH, Chen CK, Hurley JB. A highly conserved homologue of bovine
685 neurocalcin in *Drosophila melanogaster* is a Ca(2+)-binding protein expressed in
686 neuronal tissues. J Biol Chem. 1994;269(50):31900-7.
687 53. Wu M, Robinson JE, Joiner WJ. SLEEPLESS is a bifunctional regulator of
688 excitability and cholinergic synaptic transmission. Curr Biol. 2014;24(6):621-9. doi:
689 10.1016/j.cub.2014.02.026.
690 54. Shi M, Yue Z, Kuryatov A, Lindstrom JM, Sehgal A. Identification of Redeye,
691 a new sleep-regulating protein whose expression is modulated by sleep amount. Elife.
692 2014;3:e01473. doi: 10.7554/eLife.01473.
693 55. Guven-Ozkan T, Davis RL. Functional neuroanatomy of *Drosophila* olfactory
694 memory formation. Learn Mem. 2014;21(10):519-26. doi: 10.1101/lm.034363.114.
695 56. Jo J, Son GH, Winters BL, Kim MJ, Whitcomb DJ, Dickinson BA, et al.
696 Muscarinic receptors induce LTD of NMDAR EPSCs via a mechanism involving
697 hippocalcin, AP2 and PSD-95. Nat Neurosci. 2010;13(10):1216-24. doi:
698 10.1038/nn.2636.
699 57. Kim KS, Kobayashi M, Takamatsu K, Tzingounis AV. Hippocalcin and
700 KCNQ channels contribute to the kinetics of the slow afterhyperpolarization. Biophys
701 J. 2012;103(12):2446-54. doi: 10.1016/j.bpj.2012.11.002.

58. Tzingounis AV, Kobayashi M, Takamatsu K, Nicoll RA. Hippocalcin gates the calcium activation of the slow afterhyperpolarization in hippocampal pyramidal cells. Neuron. 2007;53(4):487-93. doi: 10.1016/j.neuron.2007.01.011.

59. Tzingounis AV, Nicoll RA. Contribution of KCNQ2 and KCNQ3 to the medium and slow afterhyperpolarization currents. Proc Natl Acad Sci U S A. 2008;105(50):19974-9. doi: 10.1073/pnas.0810535105.

60. Helassa N, Antonyuk SV, Lian LY, Haynes LP, Burgoyne RD. Biophysical and functional characterization of hippocalcin mutants responsible for human dystonia. Hum Mol Genet. 2017. doi: 10.1093/hmg/ddx133.

61. Wakabayashi-Ito N, Ajjuri RR, Henderson BW, Doherty OM, Breakefield XO, O'Donnell JM, et al. Mutant human torsinA, responsible for early-onset dystonia, dominantly suppresses GTPCH expression, dopamine levels and locomotion in Drosophila melanogaster. Biol Open. 2015;4(5):585-95. doi: 10.1242/bio.201411080.

62. Wakabayashi-Ito N, Doherty OM, Moriyama H, Breakefield XO, Gusella JF, O'Donnell JM, et al. Dtorsin, the *Drosophila* ortholog of the early-onset dystonia TOR1A (DYT1), plays a novel role in dopamine metabolism. PLoS One. 2011;6(10):e26183. doi: 10.1371/journal.pone.0026183.

63. Ichinose H, Ohye T, Takahashi E, Seki N, Hori T, Segawa M, et al. Hereditary progressive dystonia with marked diurnal fluctuation caused by mutations in the GTP cyclohydrolase I gene. Nat Genet. 1994;8(3):236-42. doi: 10.1038/ng1194-236..

64. Li Q, Kellner DA, Hatch HAM, Yumita T, Sanchez S, Machold RP, et al. Conserved properties of *Drosophila insomniac* link sleep regulation and synaptic function. PLoS Genet. 2017;13(5):e1006815. doi: 10.1371/journal.pgen.1006815.

725 65. Kuo SY, Wu CL, Hsieh MY, Lin CT, Wen RK, Chen LC, et al. PPL2ab
726 neurons restore sexual responses in aged *Drosophila* males through dopamine. Nat
727 Commun. 2015;6:7490. doi: 10.1038/ncomms8490.
728 66. Zhang SX, Rogulja D, Crickmore MA. Dopaminergic Circuitry Underlying
729 Mating Drive. Neuron. 2016;91(1):168-81. doi: 10.1016/j.neuron.2016.05.020.
730 67. Berry JA, Cervantes-Sandoval I, Chakraborty M, Davis RL. Sleep Facilitates
731 Memory by Blocking Dopamine Neuron-Mediated Forgetting. Cell.
732 2015;161(7):1656-67. doi: 10.1016/j.cell.2015.05.027.
733 68. Berry JA, Cervantes-Sandoval I, Nicholas EP, Davis RL. Dopamine is
734 required for learning and forgetting in *Drosophila*. Neuron. 2012;74(3):530-42. doi:
735 10.1016/j.neuron.2012.04.007.
736 69. Kaun KR, Rothenfluh A. Dopaminergic rules of engagement for memory in
737 *Drosophila*. Curr Opin Neurobiol. 2017;43:56-62. doi: 10.1016/j.conb.2016.12.011.
738 70. Kamikouchi A, Inagaki HK, Effertz T, Hendrich O, Fiala A, Göpfert MC, et
739 al. The neural basis of *Drosophila* gravity-sensing and hearing. Nature.
740 2009;458(7235):165-71.
741 71. Zhong L, Bellemer A, Yan H, Ken H, Jessica R, Hwang RY, et al.
742 Thermosensory and nonthermosensory isoforms of *Drosophila melanogaster* TRPA1
743 reveal heat-sensor domains of a thermoTRP Channel. Cell Rep. 2012;1(1):43-55. doi:
744 10.1016/j.celrep.2011.11.002.
745 72. Levine JD, Funes P, Dowse HB, Hall JC. Signal analysis of behavioral and
746 molecular cycles. BMC Neuroscience. 2002;3(1):1.
747 73. Levine JD, Funes P, Dowse HB, Hall JC. Advanced analysis of a
748 cryptochrome mutation's effects on the robustness and phase of molecular cycles in
749 isolated peripheral tissues of *Drosophila*. BMC Neuroscience. 2002;3(1):5.

Figure 1

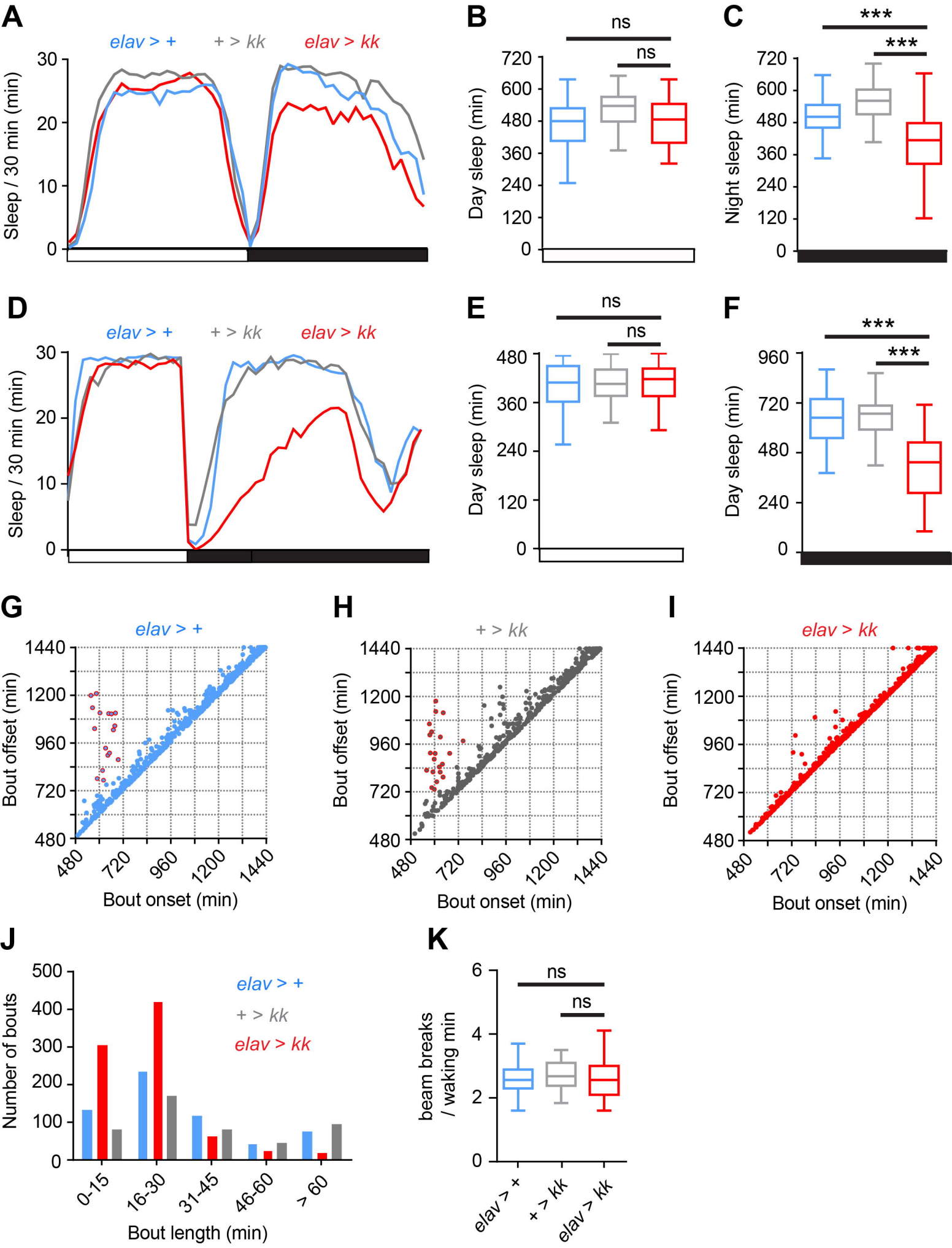

A
elav > +
+ > kk
elav > kk
Sleep / 30 min (min)
B
ns
ns
Day sleep (min)
C
***
***
Night sleep (min)
D
E
F
G
elav > +
H
+ > kk
I
elav > kk
Bout offset (min)
Bout onset (min)
J
Number of bouts
Bout length (min)
0-15
16-30
31-45
46-60
> 60
K
beam breaks / waking min

Figure 2

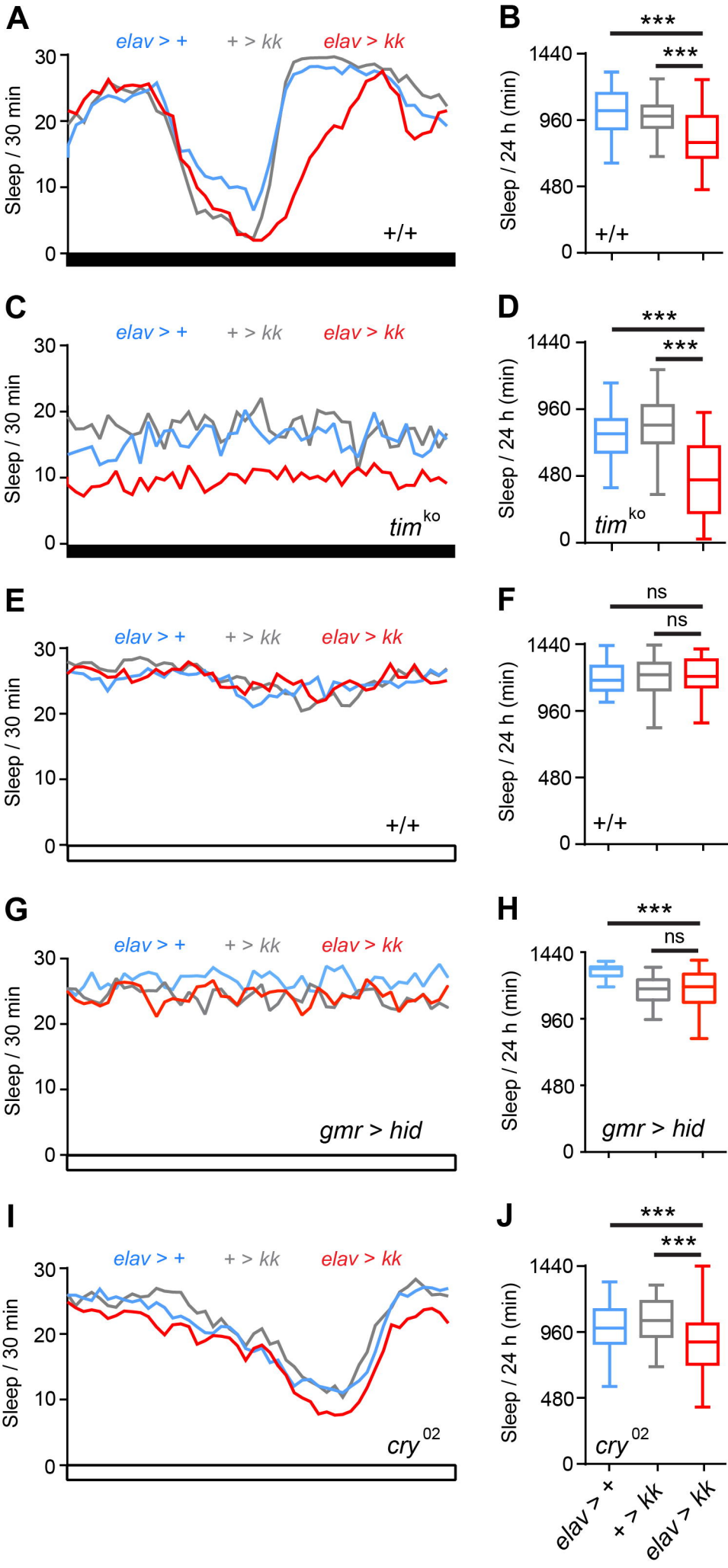

A
elav > +
+ > kk
elav > kk
Sleep / 30 min
30
20
10
0
+/+
B
***
***
Sleep / 24 h (min)
1440
960
480
0
+/+
C
elav > +
+ > kk
elav > kk
Sleep / 30 min
tim ko
D
***
***
Sleep / 24 h (min)
tim ko
E
elav > +
+ > kk
elav > kk
Sleep / 30 min
+/+
F
ns
ns
Sleep / 24 h (min)
+/+
G
elav > +
+ > kk
elav > kk
Sleep / 30 min
gmr > hid
H
***
ns
Sleep / 24 h (min)
gmr > hid
I
elav > +
+ > kk
elav > kk
Sleep / 30 min
cry 02
J
***
***
Sleep / 24 h (min)
cry 02
elav > +
+ > kk
elav > kk

Figure 3

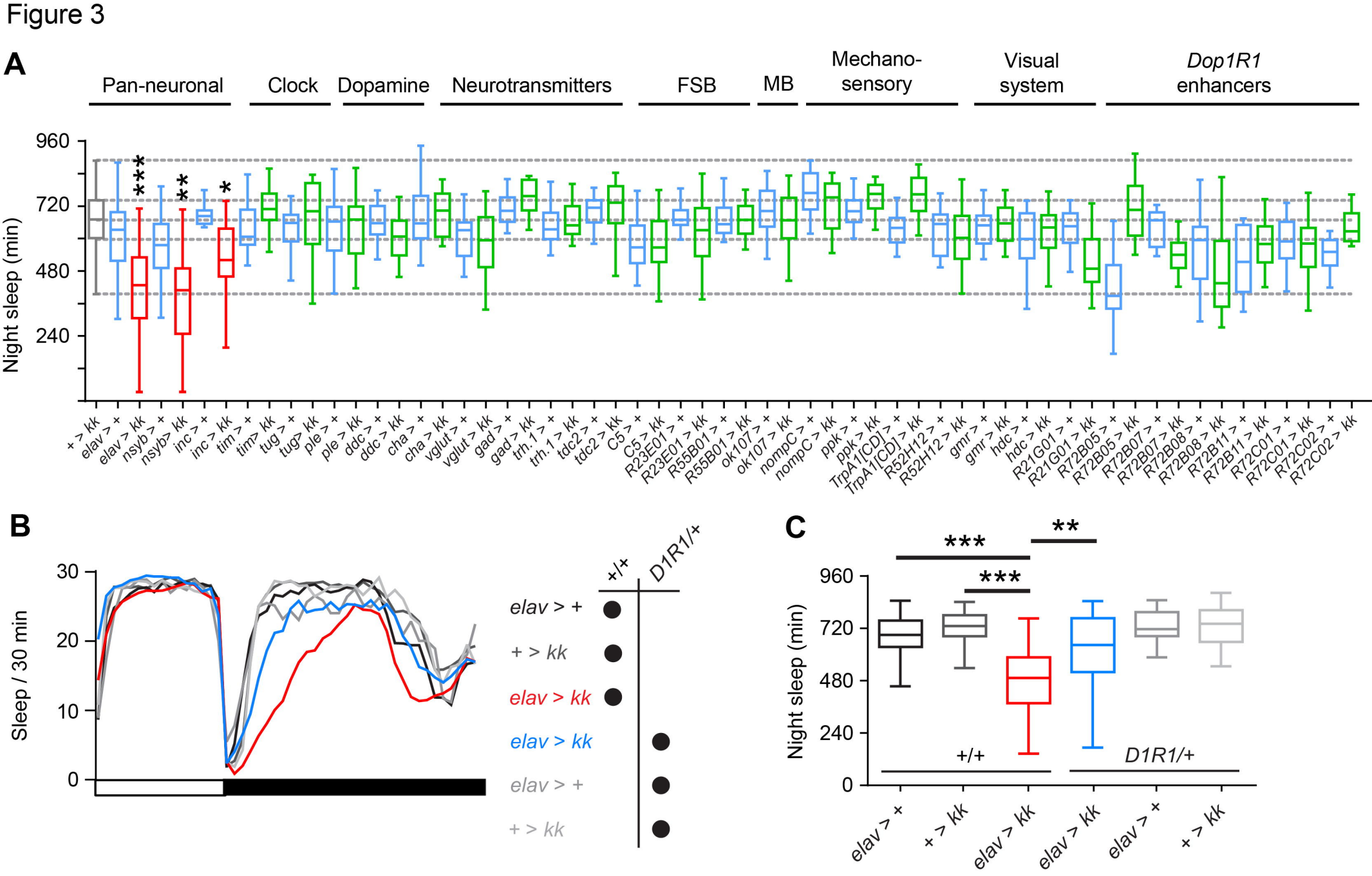

A
Pan-neuronal
Clock
Dopamine
Neurotransmitters
FSB
MB
Mechano-sensory
Visual system
Dop1R1 enhancers
Night sleep (min)
960
720
480
240
***
**
*
+ > kk
elav > +
elav > kk
nsyb > +
nsyb > kk
inc > +
inc > kk
tim > +
tim > kk
tug > +
tug > kk
ple > +
ple > kk
ddc > +
ddc > kk
cha > +
cha > kk
vglut > +
vglut > kk
gad > +
gad > kk
trh.1 > +
trh.1 > kk
tdc2 > +
tdc2 > kk
C5 > +
C5 > kk
R23E01 > +
R23E01 > kk
R55B01 > +
R55B01 > kk
ok107 > +
ok107 > kk
nompC > +
nompC > kk
ppk > +
ppk > kk
TrpA1[CD] > +
TrpA1[CD] > kk
R52H12 > +
R52H12 > kk
gmr > +
gmr > kk
hdc > +
hdc > kk
R21G01 > +
R21G01 > kk
R72B05 > +
R72B05 > kk
R72B07 > +
R72B07 > kk
R72B08 > +
R72B08 > kk
R72B11 > +
R72B11 > kk
R72C01 > +
R72C01 > kk
R72C02 > +
R72C02 > kk
B
Sleep / 30 min
30
20
10
0
+/+
D1R1/+
elav > +
+ > kk
elav > kk
elav > kk
elav > +
+ > kk
C
***
**
***
Night sleep (min)
960
720
480
240
0
+/+
D1R1/+
elav > +
+ > kk
elav > kk
elav > kk
elav > +
+ > kk

Figure 4

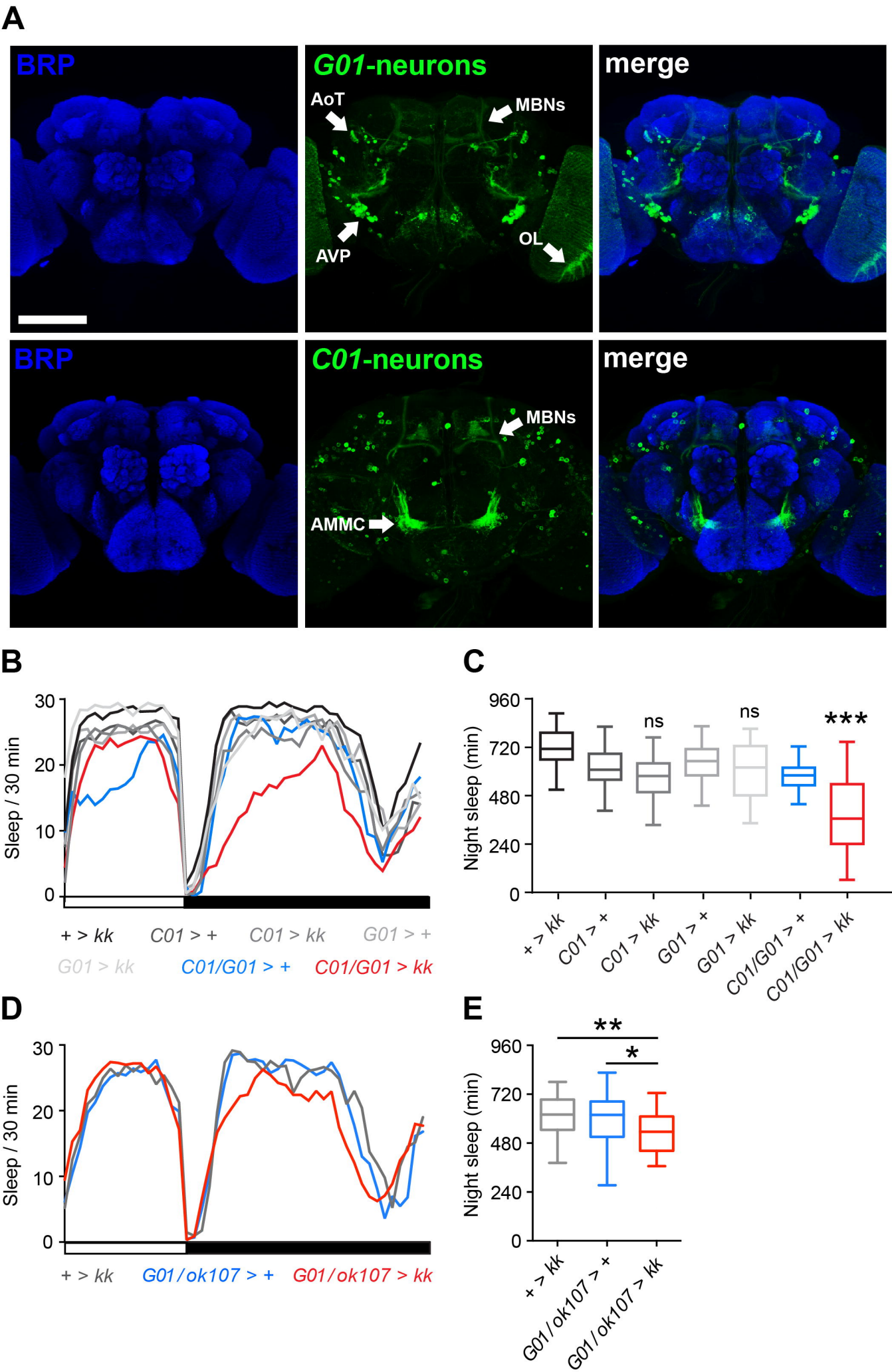
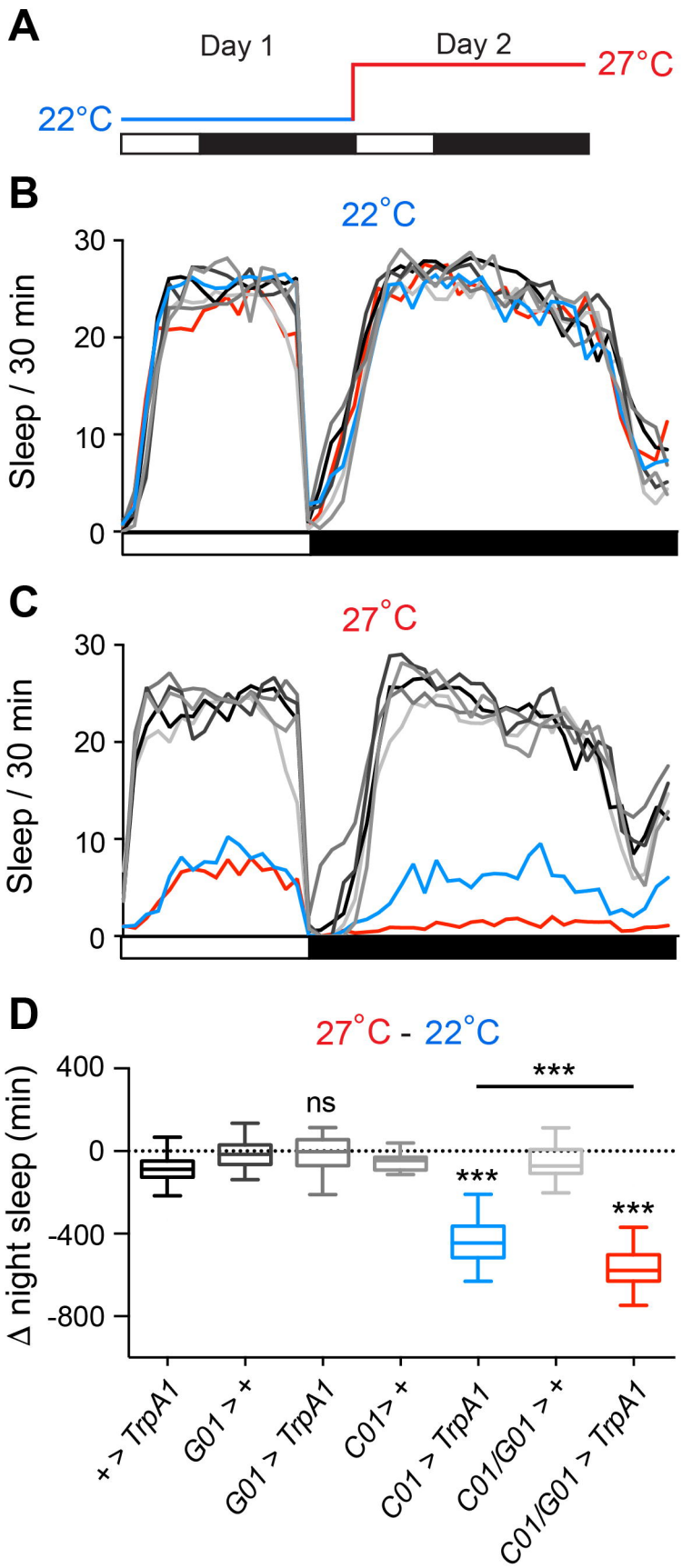

A
BRP
G01-neurons
AoT
MBNs
AVP
OL
merge
BRP
C01-neurons
MBNs
AMMC
merge
B
Sleep / 30 min
30
20
10
0
+ > kk
C01 > +
C01 > kk
G01 > +
G01 > kk
C01/G01 > +
C01/G01 > kk
C
Night sleep (min)
960
720
480
240
0
ns
ns
***
+ > kk
C01 > +
C01 > kk
G01 > +
G01 > kk
C01/G01 > +
C01/G01 > kk
D
Sleep / 30 min
30
20
10
0
+ > kk
G01/ok107 > +
G01/ok107 > kk
E
Night sleep (min)
960
720
480
240
0
**
*
+ > kk
G01/ok107 > +
G01/ok107 > kk

Figure 5


A
Day 1
Day 2
27°C
22°C
B
22°C
Sleep / 30 min
30
20
10
0
+ > TrpA1
G01 > +
G01 > TrpA1
C01 > +
C01 > TrpA1
C01/G01 > +
C01/G01 > TrpA1
C
27°C
Sleep / 30 min
30
20
10
0
+ > TrpA1
G01 > +
G01 > TrpA1
C01 > +
C01 > TrpA1
C01/G01 > +
C01/G01 > TrpA1
D
27°C - 22°C
Δ night sleep (min)
400
0
-400
-800
ns
***
***
***
+ > TrpA1
G01 > +
G01 > TrpA1
C01 > +
C01 > TrpA1
C01/G01 > +
C01/G01 > TrpA1