

25 **Author summary**

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

36

37 **Introduction**

38 Sleep is a widely conserved and critical behavior that impacts numerous aspects of
39 nervous system function, including neuronal development [1], clearance of metabolic
40 waste [2], synaptic plasticity [3-6], and the generation of complex behaviors such as
41 courtship and aggression [1, 7]. The fruit fly, *Drosophila melanogaster*, exhibits a
42 sleep-like state characterized by periods of immobility, altered posture and elevated
43 arousal thresholds [8, 9]. Similarly to mammals, sleep in *Drosophila* is regulated by
44 circadian and homeostatic processes [10, 11]. Furthermore, just as human sleep can be
45 separated into distinct temporal stages of differing arousal thresholds (REM and non-
46 REM sleep), sleep in *Drosophila* also varies in intensity throughout the day/night
47 cycle, with night sleep associated with higher arousal thresholds relative to day sleep
48 [12].

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

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

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

70 However, such genome-wide approaches are highly laborious, requiring
71 screening of thousands of fly lines to identify a limited number of sleep mutants [15,
72 17]. Thus, targeted screening strategies of higher efficiency may represent a useful
73 complement to unbiased high-throughput, yet low yield, methodologies. Here we
74 uncover a novel sleep-relevant gene in *Drosophila* using an approach based on
75 genetic and neurobiological correlates between *Drosophila* sleep and a human
76 movement disorder, primary dystonia. From an initial screen of just five candidate
77 loci, we identify the neuronal calcium sensor Neurocalcin (NCA) as a sleep regulatory
78 factor that specifically promotes night sleep in *Drosophila*. Our results reveal a novel
79 role for NCA and demonstrate the utility of targeted mini-screens to study sleep in
80 *Drosophila*.

81

82

83 **Results**

84 **Identification of Neurocalcin as a sleep-promoting factor**

85 We identified NCA as a novel sleep regulator using a guilt-by-association strategy
86 based on phenotypes linked to mutations in *KCTDI7/insomniac*, homologous genes
87 encoding a Cullin-3 adaptor protein in humans and *Drosophila* respectively [17, 24].
88 In humans, a *KCTDI7* mutation causes myoclonus dystonia, a disorder characterised
89 by repetitive movements, contorted postures and non-epileptic myoclonic jerks in the
90 upper body [24]. In *Drosophila*, null or hypomorphic mutations in the *KCTDI7*
91 homologue *insomniac (inc)* result in profound reductions in sleep [16, 17]. A potential
92 link between these two phenotypes is dopamine signalling. Altered plasticity of
93 dopaminergic circuits within the striatum, a motor control centre, has been proposed
94 as a potential mechanism underlying dystonic movements [25]. In *Drosophila*,
95 dopamine is a pro-arousal factor. Elevated dopaminergic neurotransmission strongly
96 reduces sleep [26-28], and INC has been suggested to control sleep by negatively
97 regulating dopamine signalling [16].

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

108 (*kk108825*, termed *kk*, Fig 1A) targeting *nca* mRNA reduced night sleep, but not day
109 sleep, in adult male flies housed under 12 h light: 12 h dark conditions (12L: 12D) at
110 25°C (Fig 1A-C). Night-specific sleep loss due to neuronal *nca* knockdown was also
111 observed in virgin adult female flies (S1A, B Fig), and in male flies expressing the *kk*
112 *nca* RNAi using other pan-neuronal (*nsyb-Gal4*) or broadly expressed (*insomniac-*
113 *Gal4*) drivers (S1C Fig).

114 Sleep architecture in *Drosophila* is generally studied in 12L: 12D conditions.
115 Interestingly, we found that reduced night sleep in *nca* knockdown males appeared
116 enhanced under short photoperiod conditions (8L: 16D) (Fig 1D). Similarly to 12L:
117 12D conditions, in 8L: 16D day sleep was unaffected whilst night sleep was robustly
118 reduced (Fig 1E, F). Night sleep loss under short photoperiod was also observed in
119 flies expressing two other independent RNAi lines targeting *nca* mRNA (*hmj21533*
120 and *jf03398*, termed *hmj* and *jf* respectively) (S2A-D Fig). The *kk* and *jf* dsRNAs
121 target a partially overlapping sequence of *nca*, whereas *hmj* targets a distinct upstream
122 sequence (S2A Fig). For each RNAi line, we confirmed reduced *nca* expression using
123 qPCR (S2E Fig). Transcription of *nca* occurs from promoter regions shared with the
124 downstream locus *cg7646* (S2A Fig), yet *cg7646* transcription was not affected by
125 *nca* RNAi (S2F Fig), nor were any common off-target mRNAs predicted for the *kk*,
126 *hmj* or *jf* dsRNA hairpins (data not shown). Since pan-neuronal expression of three
127 independent RNAi lines targeting two separate regions of *nca* mRNA reduce night
128 sleep, we conclude that NCA acts in the *Drosophila* nervous system to promote night
129 sleep. For simplicity, we use the *kk nca* RNAi for all subsequent experiments, and
130 refer to flies expressing *kk nca* RNAi under *elav-Gal4* as *nca*^{KD} (*nca* knockdown). All
131 experiments below are performed under 8L: 16D conditions unless otherwise stated.

132

133 **Neurocalcin promotes long sleep bouts in the early night**

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

143

144 **NCA does not impact circadian rhythmicity or waking activity**

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

155

156 **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

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

192

193 **NCA acts in a common pathway with the Dop1R1 dopamine receptor**

194 NCA is highly homologous to the human neuronal calcium sensor Hippocalcin (S4
195 Fig), and mutations in Hippocalcin cause DYT2 primary isolated dystonia [33]. One
196 neuronal signalling mechanism frequently linked to dystonia is dopaminergic
197 neurotransmission, with altered dopaminergic signalling within the striatum proposed
198 to underlie forms of primary dystonia [41]. We therefore tested whether NCA
199 modulates sleep via a dopaminergic mechanism. Under 8L: 16D conditions *nca*
200 knockdown in dopaminergic neurons did not result in night sleep loss (Fig 3A and S1
201 Table). However, heterozygosity for a null or strongly hypomorphic allele of the
202 *Dop1R1* dopamine receptor (*Dop1R1*^{M103085-G}, a homozygous lethal MiMIC insertion)
203 rescued night sleep loss in *nca*^{KD} flies (Fig 3B, C). Importantly, in both *elav-Gal4/+*
204 and *kk/+* control backgrounds, heterozygosity for *Dop1R1*^{M103085-G} did not alter sleep
205 levels (Fig 3B, C; $p > 0.05$, Kruskal-Wallis test with Dunn's post-hoc test). A similar
206 epistatic interaction between *nca* and *Dop1R1* was observed using a second, weaker

207 *Dop1R1* allele (*Dop1R1*^{M1004437}) (S5 Fig). From the above results, we propose that
208 NCA acts in a common pathway with Dop1R1 to regulate night sleep, potentially
209 downstream of dopaminergic neurons.

210

211 **NCA acts in two distinct circuits to promote night sleep**

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

223 These results suggested a complex sleep-relevant circuit requirement for NCA.
224 Thus, we sought to reduce NCA levels in multiple sub-circuits to test for a
225 simultaneous role of NCA in distinct anatomical regions. Through this approach, we
226 found that *nca* knockdown using two *enhancer*-Gal4 lines (*R21G01* – an enhancer in
227 the *TrpA1* locus, and *R72C01* – an enhancer in the *Dop1R1* locus), was sufficient to
228 strongly phenocopy the effect of pan-neuronal *nca* knockdown on night sleep (Fig
229 4A-C; compare Fig 4B with Fig 1B). For simplicity we refer to these drivers as *G01*
230 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.

276 **Discussion**

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

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

295 Similarly to many sleep-promoting factors in *Drosophila* [13, 17, 18, 22, 53,
296 54], sleep-relevant NCA activity does not map onto a single cell-type within the
297 *Drosophila* nervous system. Rather, NCA acts in multiple neuropil regions, each
298 contributing to a net promotion of night sleep (Figs 3 and 4). One such region is the
299 MB, a well-defined memory and sleep-regulatory center [43, 44, 46, 55]. However,
300 further work is required to identify the full spectrum of key cell-types within the *G01*-

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

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

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

326 role in dopamine metabolism by regulating expression of GTP cyclohydrolase, a
327 component of the dopamine synthesis pathway that is also mutated in L-Dopa-
328 responsive dystonia [30, 61-63]. *Drosophila insomniac* is homologous to the
329 myoclonus dystonia-gene *KCTD17* (as well the paralogs *KCTD2* and *KCTD5*) [17,
330 24, 64], and sleep loss in *insomniac* mutants can be rescued by inhibition of dopamine
331 synthesis [16]. Correspondingly, our work suggests that NCA acts downstream of
332 dopaminergic neurons in a pathway involving the Dop1R1 dopamine receptor. Given
333 the link between Torsin, Insomniac and NCA with dopamine signalling, it will be
334 intriguing to test whether dystonia-gene homologues modulate other dopamine-related
335 behaviours in *Drosophila*, including sleep, learning, forgetting and courtship [26, 65-
336 69].
337

338 **Materials and Methods**

339 **Fly husbandry**

340 Fly strains and crossings are maintained on standard fly food at constant temperature
341 25°C under 12 h:12 h light-dark cycles (12L: 12D). The following strains were
342 obtained from Bloomington and/or VDRC stock centers: *kk108825* (v100625),
343 *hmj21533* (54814), *jf03398* (29461), *Dop1RI*^{MI03085-GFSTF.2} (59802),
344 *Dop1RI*^{MI04437} (43773), *ple-Gal4* (8848), *Chat-Gal4* (6798), *vGlut-Gal4* (26160),
345 *GAD-Gal4* (51630), *Ddc-Gal4*(7010), *GMR-Gal4* (1104), *Trh.1-Gal4* (38388), *Tdc2-*
346 *Gal4*(9313), *C5-Gal4* (30839) and *ok107-Gal4* (854). The remaining lines obtained
347 from Bloomington stock center are Janelia Flylight collection with identifiable
348 prefixes: R23E10-Gal4, R55B01-Gal4, R52H12-Gal4, *Hdc-Gal4* (R17F12-Gal4),
349 R21G01-Gal4, R72B05-Gal4, R72B07-Gal4, R72B08-Gal4, R72B11-Gal4, R72C01-
350 Gal4, and R72C02-Gal4. The following lines were gifts from laboratories of
351 Kyunghee Koh: *elav-Gal4*, *nsyb-Gal4*, *tim-Gal4* and *TUG-Gal4*, Joerg Albert:
352 *nompC-Gal4* [70] and Nicolas Stavropoulos: *inc-Gal4:2* [17]. *ppk-Gal4*, *TrpA1-CD-*
353 *Gal4* was described previously [71]. *GMR-hid*, *tim*^{KO} and *cry*⁰² were previously
354 described in [36]. Except for *Ddc-Gal4*, *Trh.1-Gal4*, *Tdc2-Gal4*, *nompC-Gal4* and
355 *Hdc-Gal4*, all *Drosophila* strains used for sleep-wake assay were either outcrossed
356 five times into a standard isogenic background (iso31) or insertion-free chromosomes
357 were exchanged with the iso31 line (*hmj21533*, *jf03398*, *Dop1RI*^{MI03085-G} and
358 *Dop1RI*^{MI04437}).

359 **RNA extraction and Quantitative PCR**

360 10-20 fly heads per genotype were collected with liquid nitrogen and dry ice at
361 indicated time points (S3 Fig). Total RNA was extracted using TRIzol™ reagent
362 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: ttgctagcgtcaccatattggg;
370 cg7646F: gccttcgaatgtacgatgtcg, cg7646R: cctagcatgtcataaattgcctgaac or
371 rp49F:cgatatgctaagctgtcgaca, rp49R: cgcttgctgatccgtaacc. 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

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430

431 **Author Contributions**

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

438

439

440 **Figure Legends**

441

442 **Fig. 1 Neurocalcin knockdown results in a loss of consolidated sleep during the**
443 **early night in *Drosophila*. (A)** Mean sleep levels in 12L: 12D conditions for nca^{KD}
444 adult males and associated controls (*elav*-Gal4 driver or RNAi transgene
445 heterozygotes). **(B-C)** Median day **(B)** and night **(C)** sleep levels in the above
446 genotypes. **(D)** Mean sleep levels in 8L: 16D conditions for nca^{KD} adult males and
447 associated controls. **(E-F)** Median day and night sleep levels in the above genotypes
448 during 8L: 16D conditions. Data are presented as Tukey box plots. The 25th, Median,
449 75th percentiles are shown. Whiskers represent 1.5 x the interquartile range. Identical
450 representations are used in all subsequent box plots. **A-C:** n = 48 for each genotype;
451 **D-F:** 55 > n > 54. **(G-I)** Individual sleep bout durations were measured using a
452 custom-made R program and visualised by plotting sleep bout onset against offset for
453 sleep bouts in control and nca^{KD} adult males under 8L: 16D conditions. In control
454 flies (*elav* > + and + > *kk*), longer sleep bouts initiated early during the night are
455 highlighted in red **(G, H)**, which are largely absent in nca^{KD} adult males **(I)**. n = 48
456 for each genotype. **(J)** Distribution of sleep bout lengths in nca^{KD} and control adult
457 males. Note the significant shift towards shorter sleep bout lengths in nca^{KD} flies
458 (nca^{KD} vs. driver: χ^2 , *df*: 142.0, 4, p < 0.0001; nca^{KD} vs. RNAi: χ^2 , *df*: 2112.0, 4, p <
459 0.0001). **(K)** Number of DAM beam breaks per waking minute for nca^{KD} and control
460 adult males during night phase of 8L: 16D cycles. 55 > n > 54. ***p < 0.0005, ns - p
461 > 0.05 as compared to driver and RNAi alone controls via Kruskal-Wallis test with
462 Dunn's post-hoc test.

463

464 **Fig. 2 The circadian clock and light-sensing pathways regulate sleep promotion**
465 **by NCA. (A-B)** Mean sleep levels in *nca*^{KD} and control adult males across 24 h in
466 constant-dark (DD) conditions **(A)**, and total median sleep levels in the above
467 genotypes **(B)**. 47 > n > 44. Note the reduced sleep in the subjective night in *nca*^{KD}
468 relative to control adult males, but not the day. **(C-D)** Mean sleep levels in *nca*^{KD} and
469 control adult males across 24 h in constant-dark (DD) conditions in a *timeless*
470 knockout (*tim*^{KO}) background **(C)**, and total median sleep levels **(D)**. 39 > n > 32. **(E-**
471 **F)** Mean sleep levels in *nca*^{KD} and control adult males across 24 h in constant-light
472 (LL) conditions **(E)**, and total median sleep levels in the above genotypes **(F)**. 47 > n
473 > 44. **(G-H)** Mean sleep levels in *nca*^{KD} and control adult males across 24 h in
474 constant-light (LL) conditions in a *gmr > hid* background **(G)**, and total median sleep
475 levels in the above genotypes **(H)**. *elav > kk*, *gmr-hid/+*: n = 51; + > *kk*, *gmr-hid/+*: n
476 = 48; *elav > +*, *gmr-hid/+*: n = 24. **(I-J)** Mean sleep levels in *nca*^{KD} and control adult
477 males across 24 h in constant-light (LL) conditions in a *cryptochrome* null (*cry*⁰²)
478 background **(I)**, and total median sleep levels in the above genotypes **(J)**. 72 > n > 61.
479 Note the small but consistent reduction in sleep in *nca*^{KD}, *cry*⁰² males **(I)**, leading to a
480 significant decrease in total median sleep levels relative to controls **(J)**. ***p <
481 0.0005, ns - p > 0.05, as compared to driver and RNAi alone controls via Kruskal-
482 Wallis test with Dunn's post-hoc test.

483

484 **Fig. 3 NCA and Dop1R1 genetically interact to regulate sleep. (A)** Transgenic
485 RNAi-based mini-screen to identify key NCA-expressing neurons. NCA knockdown
486 with broadly expressed drivers results in reduced night sleep in adult males under 8L:
487 16D conditions. In contrast, NCA knockdown in previously defined sleep-regulatory
488 centers, clock neurons, the visual system or subsets of Dop1R1-expressing neurons

489 did not impact night sleep. FSB: fan-shaped body. MB: mushroom body. Grey and
490 blue box plots: control lines. Red box plots: experimental lines showing reduced night
491 sleep relative to both controls. Green box plots: experimental lines failing to show
492 reduced night sleep relative to one or both controls. See S1 Table for n-values and
493 statistical comparisons. **(B-C)** heterozygosity for the null or strongly hypomorphic
494 *Dop1R1* allele *Dop1R1*^{MI03085-G} (*DIR1/+*) suppressed sleep loss in *nca*^{KD} adult males,
495 but did not alter sleep in control males. Mean sleep patterns in 8L: 16D conditions are
496 shown in **(B)**. Median night sleep levels are shown in **(C)**. **p < 0.005, ***p <
497 0.0005, as compared to driver and RNAi alone controls via Kruskal-Wallis test with
498 Dunn's post-hoc test. *elav>kk, DIR1/+*: n = 32; *elav > +, DIR1/+*: n = 15; *+ > kk,*
499 *DIR1/+*: n = 32; *elav > kk*: n = 48; *elav > +*: n = 47; *+ > kk*: n = 48.

500

501 **Fig. 4 NCA acts in a distributed neural network to regulate night sleep. (A)**

502 Confocal z-stacks of adult male brains expressing genetically-encoded fluorophores
503 under the *G01* or *C01*-Gal4 drivers. Neuropil regions are labelled with anti-Bruchpilot
504 (BRP, nc82). Scale bar = 100 μ m. Arrows point to neuropil centers. AoT: anterior
505 optic tubercle. MBs: mushroom body neurons. OL: optic lobe. AMMC: antennal
506 mechanosensory and motor center. **(B-C)** *nca* knockdown in both *G01* and *C01*-
507 neurons recapitulates the effect of pan-neuronal *nca* knockdown, whereas *nca*
508 knockdown in either neuronal subpopulation alone does not reduce sleep relative to
509 controls. Mean sleep patterns in 8L: 16D conditions are shown in **(B)**. Median night
510 sleep levels are shown in **(C)**. *+ > kk*: n = 80; *C01 > +*: n = 64, *C01 > kk*: n = 80; *G01*
511 *> +*: n = 31; *G01 > kk*: n = 31; *C01/G01 > +*: n = 42; *C01/G01 > kk*: n = 71. **(D-E)**
512 *nca* knockdown in both *G01* and MB-neurons (defined by *ok107*-Gal4; *ok107*) also
513 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

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Figure 1

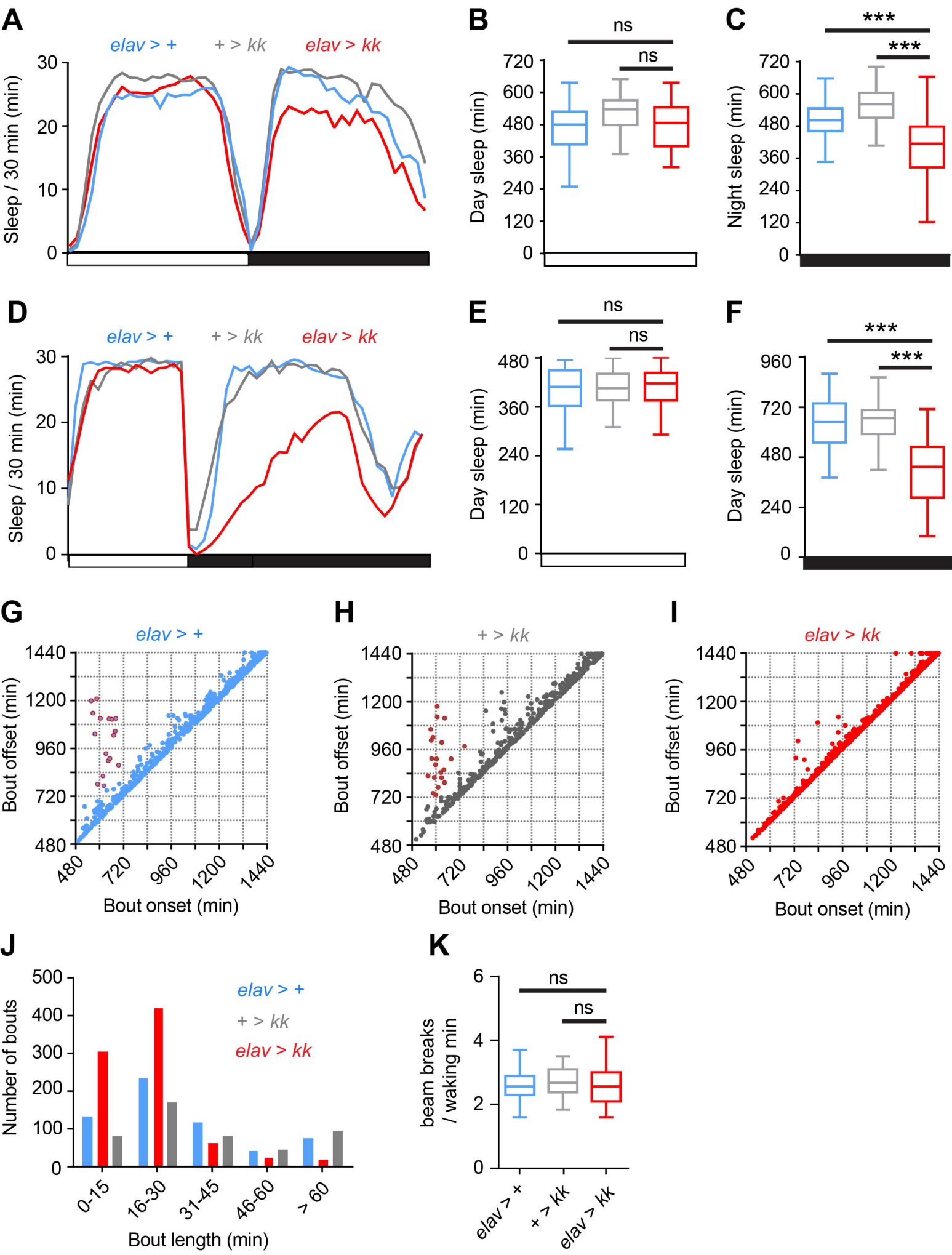


Figure 2

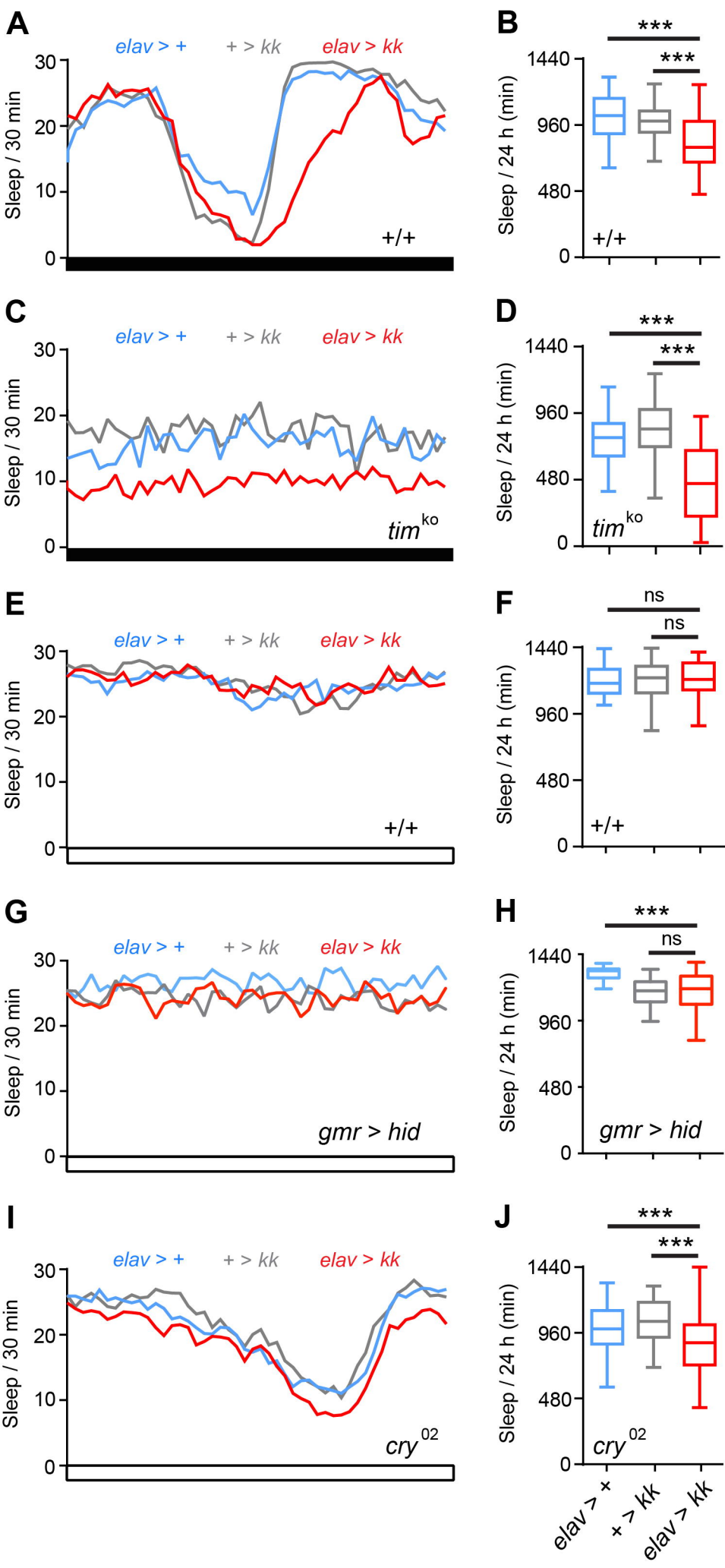
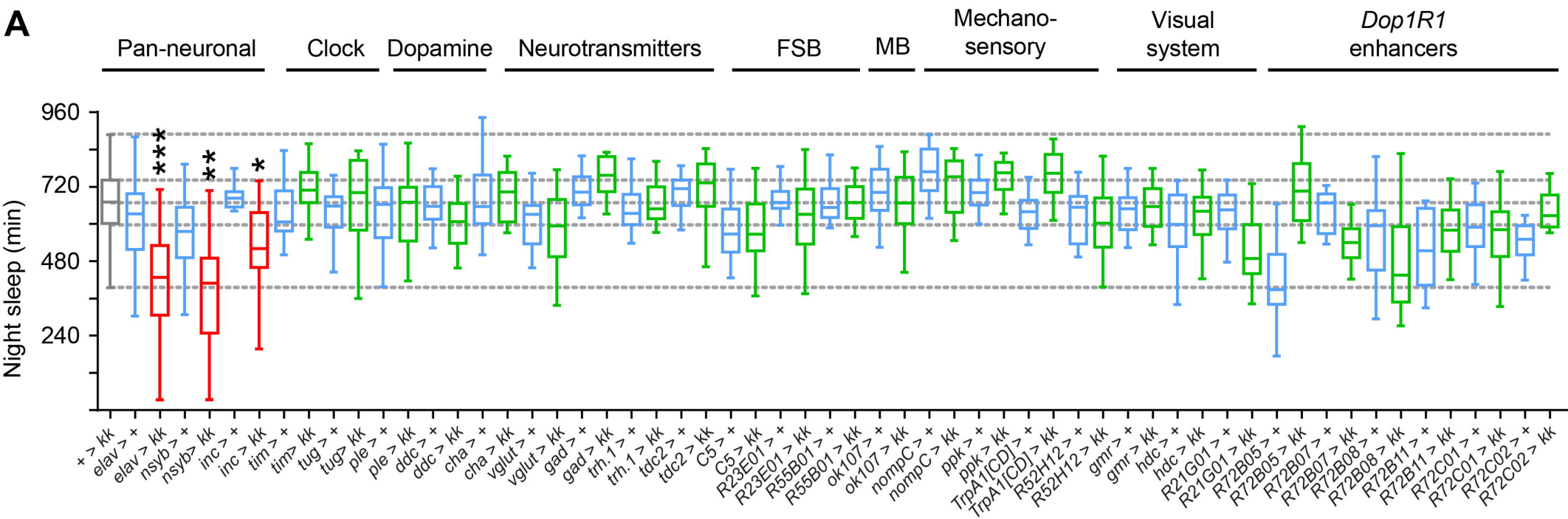
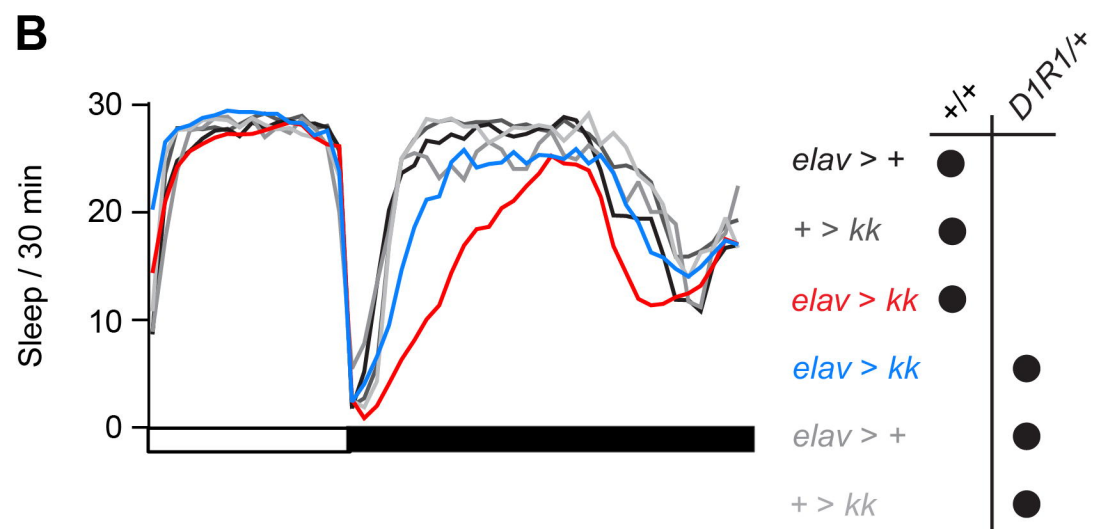


Figure 3

A



B



C

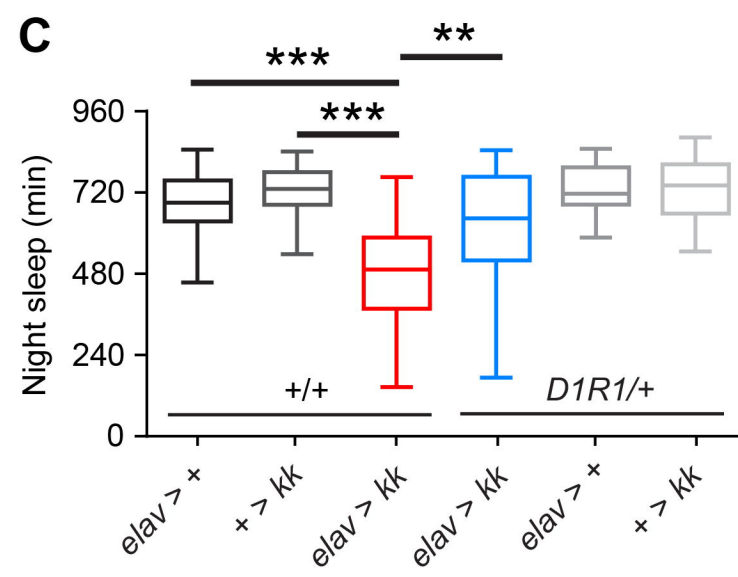
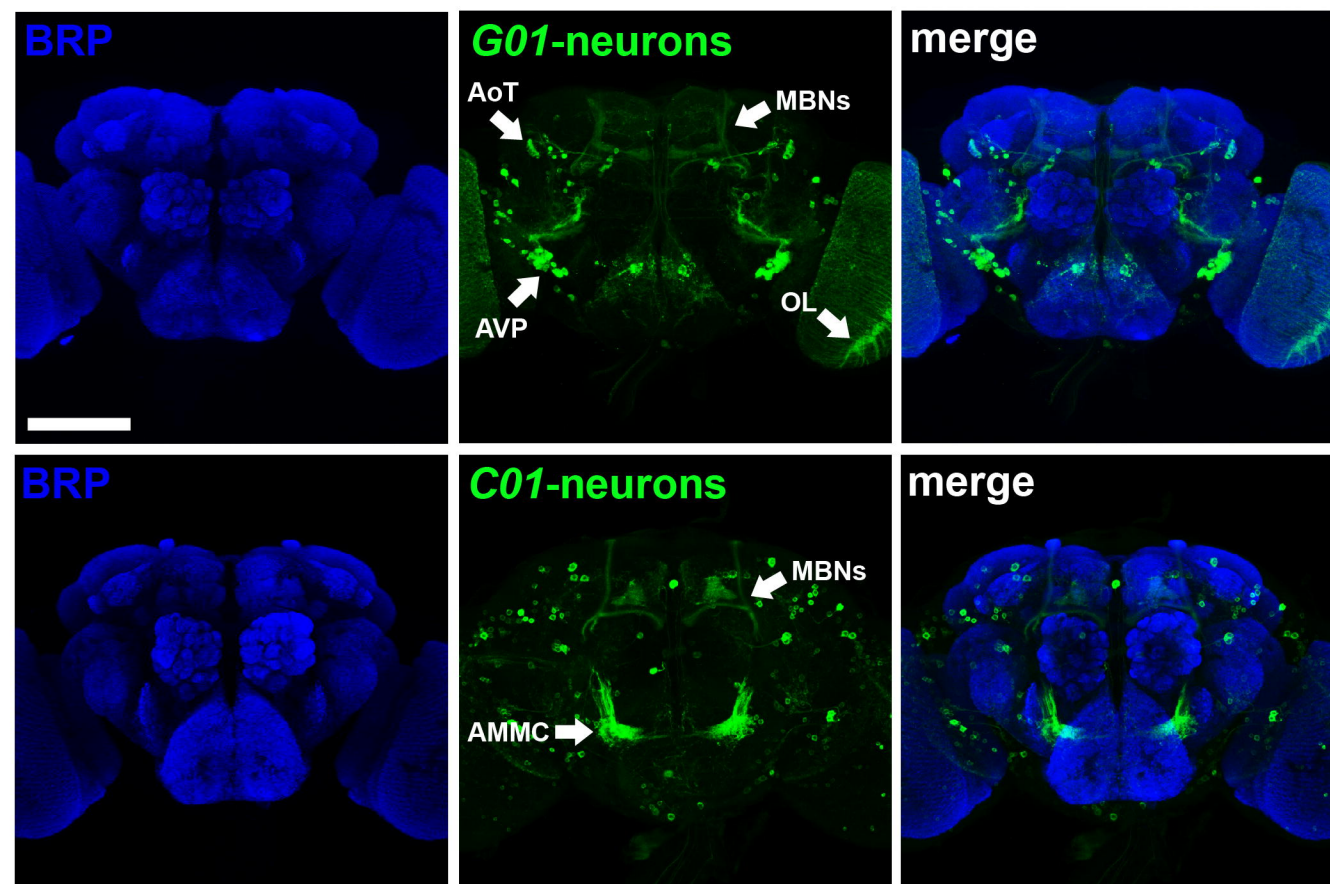
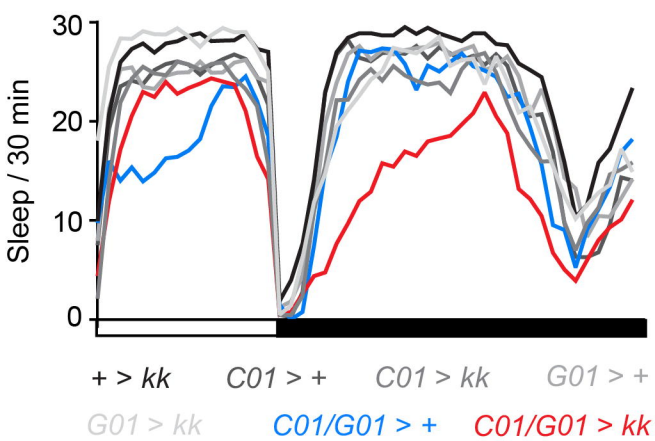


Figure 4

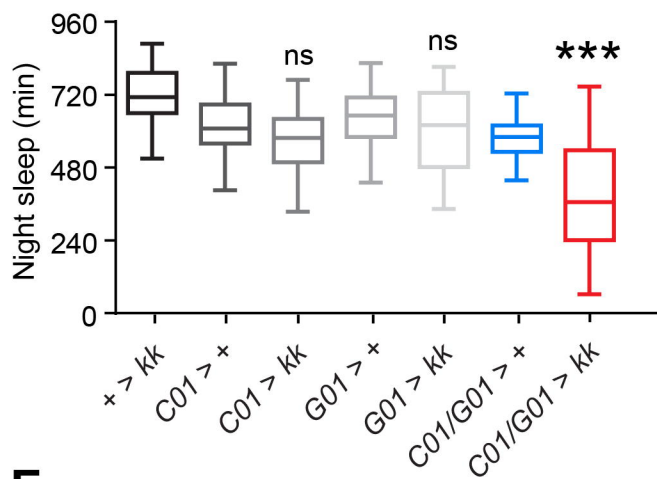
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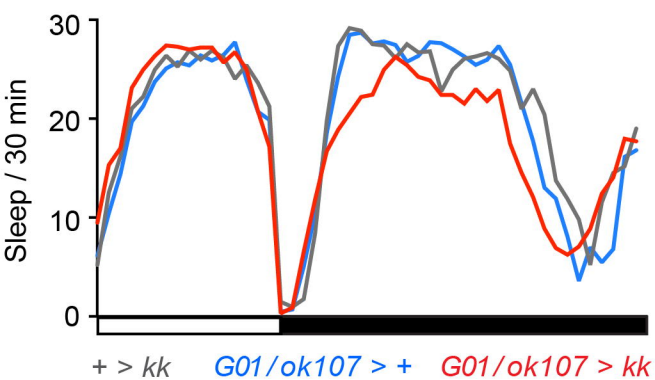
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C



D



E

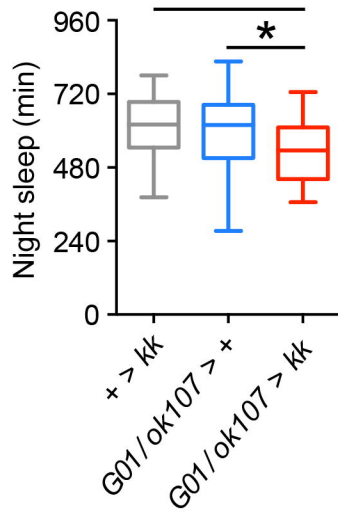


Figure 5