Nighttime sleep promotes mechanosensory habituation in *Drosophila*

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ABSTRACT

Sleep is a highly conserved behaviour that facilitates episodic, procedural, and/or associative modes of memory across species [1-3], a process hypothesised to involve structural downscaling of synapses potentiated during waking episodes [4-6]. Habituation, an ancient form of non-associative memory in which repetition of a stimulus leads to diminishing behavioural responses, is considered a prerequisite for such complex forms of learning [7,8]. Yet whether sleep also influences the capacity of organisms to habituate is poorly understood. Here we examine this question using the fruit fly, *Drosophila*. We describe an automated system that yields long-term analogue measurements of habituation to mechanosensory stimuli in adult flies. Using this platform, we find that *Drosophila* lacking neuronal calcium sensor Neurocalcin – which exhibit reduced sleep specifically during the night [9] – display impaired mechanosensory habituation during the day. Mimicking a method used to treat insomnia [10], we show that compressing nighttime duration both restores consolidated night sleep and renormalises habituation in *Neurocalcin* mutants. Conversely, in wild type flies without sleep deficits, decreasing night length reduces total night sleep and disrupts daytime habituation. In wild type flies, exposure to longer nights – which increases overall night sleep – enhances synaptic downscaling in sensory circuits. Strikingly, this effect is abolished in *Neurocalcin* mutants, which do not exhibit increased sleep under these conditions. Collectively, our findings reveal a role for nighttime sleep in facilitating habituation and suggest that this may occur by promoting synaptic downscaling.

RESULTS AND DISCUSSION

Induction of mechanosensory habituation in adult *Drosophila*

Numerous assays have been developed to induce habituation of adult *Drosophila* to visual, olfactory, and gustatory, stimuli [11-14]. However, while recently examined in *Drosophila* larvae [15], habituation to mechanical stimuli has been comparatively understudied in adult fruit flies. More broadly, whether sleep quality modulates the ability of adult flies (or other organisms) to habituate to environmental stimuli remains an open question.

We therefore sought to investigate potential links between sleep and habituation to mechanosensory stimuli in adult *Drosophila*. The synaptic homeostasis hypothesis stipulates that sleep facilitates downscaling of synapses potentiated during waking experience, thus enabling memory by preventing neuronal hyper-metabolism [16] and enhancing signal-to-noise ratios across circuits [5]. Ongoing locomotor activity occurring during wakefulness could induce an opposite change, potentiating synaptic connections in circuits mediating movement [17,18] and mechanosensory feedback [19-21]. We therefore hypothesised that enhancing this potentiation by limiting sleep (thus prolonging locomotion) might reduce sleep-induced synaptic downscaling and impair the ability of habituation-promoting inhibitory mechanisms [22,23] to suppress motor circuit activity in response to repeated mechanical stimuli.

To test this hypothesis, we developed a simple, high-throughput habituation assay using the DART (*Drosophila* ARousal Tracking) system (Fig. 1A) [24], which combines continuous video-tracking of individual flies with mechanical stimuli delivered via platform-associated motors. DART can be programmed to deliver consistent mechanosensory stimuli that elicit robust, transient, locomotor responses in control flies (Figure S1A), the magnitude and dynamics of which can be quantified. We repurposed the DART system to test for mechanosensory habituation in adult flies by delivering multiple identical stimuli at intervals of 10 min (Fig. 1B). Flies responded to repeated mechanical stimuli with locomotor responses that gradually attenuated in magnitude (Fig. 1C), quantified as the mean peak speed in the 1 min following each stimulus (Fig. 1D).

57 If this decrement represents true habituation rather than generalised sensory
58 adaptation, fatigue, or structural damage, it should exhibit key properties that define
59 habituation: spontaneous recovery, potentiation of habituation over repeated bouts, and
60 stimulus-specificity [7,8]. We sought to validate the assay by testing for these characteristics.
61 After attenuation of locomotion following 20 mechanical stimuli, locomotor speeds
62 exhibited spontaneous recovery over time in response to single mechanical stimuli, with ~
63 80% recovery occurring following 24 h (Figure S1B-F). We found that the rate of
64 mechanosensory habituation could be significantly enhanced by applying a habituation-
65 inducing paradigm of 20 identical mechanical stimuli on three consecutive days (Fig. 1E, F).
66 Finally, after 20 consecutive mechanical stimuli, we delivered a distinct 'lights-off' visual
67 stimulus expected to similarly induce a startle response (Fig. 1G). Despite the robust
68 decrease in locomotor response to repeated mechanical stimuli (Fig. 1G, H), locomotor
69 responses to the visual stimulus did not differ in magnitude between naïve and mechanically
70 habituated flies (Fig. 1G, I). Hence, progressive reductions in locomotor responses in our
71 paradigm are consistent with well-defined characteristics of habituation [7,8].
72 A number of platforms analogous to DART can track locomotor activity of populations
73 of flies using video- or infrared beam-based analysis, and can deliver mechanosensory
74 stimuli or be modified to do so. These include the *Drosophila* Activity Monitor [25], Ethoscope,
75 [26] and Zantiks [27] systems. We designed a similar habituation protocol using the Zantiks
76 automated-tracking system (Figure S1F; see Methods). The Zantiks system could detect and
77 quantify robust locomotor responses of adult flies to single motor-driven stimuli (Figure
78 S1G), as well as a progressive decline in locomotor response to repeated, identical
79 mechanosensory stimuli that was qualitatively similar to that observed using the DART
80 system (Figure S1H, I and Fig. 1D). These data provide cross-platform validation of our
81 findings using the DART.
82
83 **Neurocalcin promotes mechanosensory habituation in *Drosophila***

84 Similarly to humans, deep sleep in *Drosophila* primarily occurs during the night [28]. We
85 therefore sought to test whether night sleep influences mechanosensory habituation induced
86 during the day. Mechanically disrupting sleep provides a potential means to do so. However,
87 homeostatic rebound sleep induced by sleep deprivation [29] could potentially cause
88 confounding changes in stimulus-induced movement during the day. Hence, we utilised a
89 genetic manipulation that selectively disrupts night sleep whilst leaving daytime sleep intact:
90 loss of the highly conserved neuronal calcium sensor Neurocalcin [9].

91 In contrast to mutations such as *shaker*, *sleepless*, *fumin*, and *insomniac* – which
92 disrupt both day and night sleep in *Drosophila* [30-34] – loss of Neurocalcin specifically impairs
93 sleep during the night in oscillating light: dark conditions [9], providing a means to examine the
94 relationship between night sleep and daytime habituation. Indeed, using our paradigm to
95 measure the rate and amplitude of mechanosensory habituation in control flies and flies
96 homozygous for a *Neurocalcin* null allele (Nca^{KO1}) [9], we found that loss of Neurocalcin
97 significantly reduced the degree of mechanosensory habituation, an effect that was
98 particularly prominent after ~ 10 mechanical stimuli (Fig. 2A, B). We observed similarly
99 reduced mechanosensory habituation in flies homozygous for an independent *Neurocalcin*
100 null allele (Nca^{KO2}) (Figure S2A-B), confirming the above genotype-phenotype correlation.

101 Neurocalcin could potentially modulate mechanosensory habituation by enhancing
102 sensitivity to mechanical stimuli. We therefore tested locomotor responses in control and
103 *Neurocalcin* knockout flies to mechanical stimulus strengths ranging from 1% to 100% of the
104 strength used in the habituation protocol. *Neurocalcin* knockout flies showed statistically
105 similar amplitudes of locomotor responses to isogenic controls at 6 out of 7 stimulus
106 strengths tested, and a slightly enhanced response to stimuli 10% of the strength used in the
107 habituation protocol (Figure S2C). These data therefore did not support a general increase in
108 mechanosensory sensitivity in *Neurocalcin* knockout flies, and confirmed that the habituation
109 protocol utilised a stimulus strength that was beyond the plateau at which there was no
110 difference between control and *Neurocalcin* knockout flies.

111 Since our habituation protocol yielded long-term quantitative recordings of individual
112 flies, we were able to examine whether the reduction in habituation in *Neurocalcin* knockout
113 flies was due to a change in number of flies responding to mechanical stimuli, or a decrease
114 in the magnitude of the individual flies' responses. To separate response to a stimulus from
115 background noise, we defined a threshold for a "response" to each stimulus as $\geq$10% of the
116 response to the mean naïve response. We found that for both control and *Neurocalcin*
117 knockout flies, the attenuation in response came entirely from the proportion of flies
118 responding (Fig. 2C). When only "responding" flies were included in analysis, there was no
119 decrease in the amplitude of locomotor responses across repetitive stimuli, nor any
120 difference between genotype ($p = 0.46$, main effect of Genotype & of Stimulus #, 2-way
121 ANOVA) (Fig. 2D). The all-or-nothing nature of locomotor responses in our paradigm
122 supports the premise that the decrease in mean amplitude across repeated stimuli
123 represents a learning event i.e an altered "decision" of whether to respond or not to a given
124 stimulus, as opposed to a motor defect affecting the ability to sustain robust reactions over
125 multiple stimuli.
126
127 **Short nights restore consolidated sleep and mechanosensory habituation in**
128 ***Neurocalcin* knockout flies**
129 Hippocalcin, the mammalian homologue of Neurocalcin, promotes synaptic plasticity and
130 memory in mice [35-39], and cognitive development in humans [40,41]. Thus, we sought to test
131 whether Neurocalcin facilitates habituation through its effect on night sleep, or via sleep-
132 independent processes potentially linked to memory.
133 We previously documented that night sleep loss in *Neurocalcin* mutant flies was
134 particularly prominent under long night, winter-like, conditions [9], suggesting that night length
135 can modulate sleep loss in *Neurocalcin* mutant flies. Interestingly, increasing sleep drive by
136 restricting the period of consolidated night sleep is utilised in cognitive behavioural therapy
137 for insomnia, and has similarly been shown to rescue sleep loss in a variety of *Drosophila*
138 sleep mutants [10]. We therefore examined sleep architecture in control and *Neurocalcin*

139 knockout flies under short and long night conditions using a multibeam monitoring system
140 that facilitates high-resolution quantification of sleep in *Drosophila* [42] (Figure S3A).
141 Control and *Neurocalcin* knockout flies were raised under standard 12 h light: 12 h
142 dark conditions then, after 3-5 days of adulthood, were shifted to three days of long (8 h
143 light: 16 h dark) or short (16 h light: 8 h dark) night conditions, with sleep quantified on the
144 third day of each light: dark cycle. Consistent with prior results [9], we confirmed reduced
145 nighttime but not daytime sleep in *Neurocalcin* knockout flies under long night conditions
146 (Fig. 3A, C and Figure S3B-C), and found that this decrease was associated with
147 fragmentated sleep, characterised by a significant increase in the number of sleep bouts and
148 a concomitant reduction in sleep bout duration (Fig. 3D, E). In contrast, under short night
149 conditions, overall night sleep (Fig. 3B, C) and the degree of sleep consolidation (Fig. 3D, E)
150 were not significantly different between *Neurocalcin* knockout flies and controls. Day sleep
151 was also slightly increased in *Neurocalcin* knockout flies under short night conditions, likely
152 due to reduced locomotor activity towards lights-off (Fig. 3B, Figure S3B-C). Thus, changing
153 night length provides a means to tune night sleep quality on-demand in *Neurocalcin*
154 knockout flies, allowing us to disentangle sleep-dependent versus -independent functions of
155 Neurocalcin.
156 Taking advantage of this finding, we quantified mechanosensory habituation in
157 control and *Neurocalcin* knockout flies entrained to short or long night conditions (Fig. 3F).
158 Strikingly, while mechanosensory habituation was reduced in *Neurocalcin* knockout flies
159 compared to controls under long night conditions (Fig. 3F), this difference was abolished
160 under short night conditions (Fig. 3G). These data strongly suggest that Neurocalcin
161 regulates habituation directly through its effect on night sleep.
162
163 **Loss of Neurocalcin decorrelates night length and synaptic protein expression**
164 Intriguingly, within-genotype comparisons revealed that control flies exposed to short nights
165 exhibited a reduced rate of mechanosensory habituation compared to control flies exposed
166 to long nights (Figure S4A), indicating that limiting night sleep (Fig. 3C) disrupts

167 mechanosensory habituation during the following day. Conversely, *Neurocalcin* knockout
168 flies, which exhibit improved night sleep in short versus long nights (Fig. 3C), exhibited the
169 opposite correlation: an enhanced rate of habituation in short versus long night conditions
170 (Figure S4B).

171 How might night duration modulate mechanosensory habituation occurring after
172 nighttime sleep? Prior studies have shown that sleep deprivation (SD) in adult flies impairs
173 synaptic downscaling, resulting in increased levels of the presynaptic active zone protein
174 Bruchpilot [43] that can be visualised through immuno-fluorescent microscopy. Prolonged (16
175 h) SD was initially shown to increase presynaptic Bruchpilot expression by ~ 75% across
176 *Drosophila* brain neuropil regions [44]. More recently, 12 h night-specific SD was shown to
177 modulate Bruchpilot to a more limited degree, enhancing Bruchpilot expression in cholinergic
178 neurons by ~ 25%, and in GABAergic neurons by ~ 10% [45]. Loss of the *Drosophila*
179 Insomniac protein, which reduces overall day and night sleep by ~ 65% in male flies [32], also
180 leads to a 40-50% increase in presynaptic Bruchpilot expression [46].

181 Extending our analysis in the context of the synaptic homeostasis hypothesis [5] and
182 the above studies, we investigated whether altering night length was sufficient to modify
183 presynaptic Bruchpilot expression in control flies, and if so, whether loss of Neurocalcin
184 affected this relationship. We immuno-labelled synaptic Bruchpilot in isolated brains of
185 control and *Neurocalcin* knockout flies following three days of entrainment to short or long
186 night conditions, then quantified fluorescent intensity of synaptic Bruchpilot through confocal
187 microscopy, normalising fluorescence to a control DNA marker (DAPI, see Methods).

188 Consistent with the relative subtlety of this manipulation compared to mechanical
189 sleep deprivation [44,45] or loss of Insomniac [46], we observed a trend towards a small increase
190 (~ 16%) in Bruchpilot throughout the brain neuropil region of control flies subjected to short
191 versus long nights, which was non-significant (Figure S4C). However, in the neuropil-rich
192 antennal lobe regions, which process olfactory information in insects, we observed a
193 significant increase (~ 40%) in synaptic Bruchpilot levels in short versus long night
194 conditions (Fig. 4A, B).. Notably, we did not observe a similar increase in antennal lobe

195 Bruchpilot expression in *Neurocalcin* mutants exposed to short versus long nights (Fig. 4C,
196 D), nor in whole-brain Bruchpilot immuno-fluorescence (Figure S4D), suggesting that the
197 impaired sleep quality of *Neurocalcin* exposed to long nights (Fig. 3A, C) blocks enhanced
198 synaptic downscaling that occurs in wild types flies under this condition.

199 Collectively, the above data support a model in which reduced night sleep in control
200 flies exposed to short nights perturbs synaptic downscaling in specific neuropil domains
201 compared to flies experiencing long nights. We hypothesise that such domains may include
202 circuits relevant to mechano-sensation and movement, where impaired downscaling could
203 limit the ability of habituation-promoting mechanisms (such as enhanced synaptic
204 transmission from local inhibitory neurons [23]) to dampen functional connectivity between
205 mechanosensory neurons and downstream motoneuron outputs. The improvement of sleep
206 quality in *Neurocalcin* mutants exposed to short nights (Fig. 3A, C-E) breaks the normal
207 correlation between night length and synaptic Bruchpilot expression (Fig. 4), leading to
208 improved rather than impaired mechanosensory habituation in short compared to long night
209 conditions (Figure S4).

210 Further studies will be required to test this model – in particular, by identifying
211 mechanosensory and/or pre-motor circuits in which synaptic output is modulated by
212 habituation-promoting mechanisms. Intriguingly, recent work has shown that continuous or
213 repetitive mechanosensory stimulation, akin to the gentle rocking of a human baby,
214 increases daytime sleep in *Drosophila* through a habituation-like mechanism [47]. In concert
215 with our findings, these data suggest an intertwined and potentially bi-directional relationship
216 between mechano-sensation, sleep, and habituation, across both day-night and seasonal
217 cycles. Finally, we note that sleep and habituation are dysregulated in a variety of human
218 neurodevelopmental disorders [48-52] and corresponding animal models [49,53-57]. Since
219 habituation promotes attention to environmental novelties that form the substrates of
220 memory-related coincidence detection mechanisms [58], our work has implications for
221 understanding the common co-morbidity of sleep, habituation, and memory defects in these
222 clinically important conditions.

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AUTHOR CONTRIBUTIONS

S.A.L – Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing – Review & Editing, Visualization, Funding acquisition; A.D.W – Methodology, Investigation, Writing - Original Draft, Writing – Review & Editing, Visualization. K-F.C – Investigation, Writing – Review & Editing. J.E.C.J – Conceptualization, Investigation, Visualization, Writing - Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

DECLARATION OF INTERESTS

The authors declare no competing interests.

243 **MATERIALS AND METHODS**

244 **Experimental model and subject details**

245 Experimental models were fruit flies of the species *Drosophila melanogaster*. Genotypes
246 used were iso31 (an isogenized strain of w^{1118} flies and a kind gift from Prof. Kyunghee Koh,
247 Thomas Jefferson University), w^{1118};+; Nca^{KO1}/TM2, and w^{1118};+; Nca^{KO2}/TM2 [9] To control for
248 genetic background, these lines were outcrossed into the iso31 background for 5
249 generations. Flies were raised and maintained on standard fly food under 12 h: 12 h light-
250 dark cycles (12L: 12D) at a constant 25°C. Experiments took place at 25°C, and unless
251 otherwise indicated, in 12L: 12D conditions. Experiments were conducted on male flies.

252

253 **Habituation protocol**

254 Drosophila ARousal Tracking (DART) analysis was performed as described previously [1].
255 Briefly, five- to seven-day old male flies were individually loaded into glass behavioural tubes
256 (Trikinetics inc., MA, USA) containing 4% sucrose and 2% agar, and left for 24 h to
257 acclimatise in the indicated L:D conditions. The mechanical stimulus consisted of a
258 mechanical vibration in the form of a train of five 200 ms pulses separated by 800 ms
259 intervals, set at the DART system's maximum intensity, which elicited a robust movement
260 response. The habituation protocol consisted of 20 mechanical stimuli delivered at ten-
261 minute intervals, beginning at Zeitgeber time 3 (ZT3). Videos were taken using a USB
262 webcam (Logitech). Speed (mm / s) was analysed by the DART system from absolute
263 position, and binned in one min intervals. Flies were excluded from analysis if they exceeded
264 an average of 1 mm / s speed in the five one-minute bins preceding the first stimulus.
265 Response to the stimulus was defined as the mean speed (mm / s) in the one-minute bin
266 following the stimulus. Speed was normalised within genotype to the mean of the response
267 to the first stimulus. To derive the % of flies responding to stimuli, a "response" was
268 distinguished from background movement by the (arbitrary) threshold of a change in mean
269 speed between the one-minute bins preceding and following a stimulus of $\geq$10% of the mean
270 response to the first stimulus.

271 A distinct automated *Drosophila* tracking system, the Zantiks MWP unit (Zantiks Ltd.,
272 Cambridge, UK) was used to generate an equivalent habituation protocol. 5-7 day old male
273 iso31 control flies were loaded into individual wells filled with 4% sucrose and 2% agar in a
274 48-well plate, and left to acclimatise for 24 h in a 12 h light: 12 h dark cycle at 25°C.
275 *Drosophila* were loaded into the Zantiks unit at ZT0, and the habituation protocol
276 commenced at ZT3. The habituation protocol again consisted of 20 mechanical stimuli
277 delivered at 10 min intervals, each stimulus consisting of a single 1 s pulse set at medium
278 strength, generated by the Zantiks mechanical stimulation stand. Distance travelled is
279 automatically tracked by the Zantiks system, and total distance travelled was binned in 1 min
280 intervals. Scripts used for the habituation protocol on the Zantiks MWP are available at
281 github.com/abigaildwilson/zanscripts.
282
283 **Sleep analysis**
284 Sleep analysis was performed using the *Drosophila* Activity Monitor 5 system (DAM5H,
285 Trikinetics inc., MA, USA), which improves on the fidelity of the earlier DAM2 system by
286 using 15 infrared beams per tube, rather than a single one, to track movement [2]. As above,
287 three- to five-day old male flies were loaded in behavioural tubes, and were left for two full
288 days to acclimatise at the relevant light cycle. On the third day the number of beam breaks
289 was measured using the *Drosophila* Activity Monitor. Sleep was defined using the standard
290 definition of > 5 mins of inactivity, which has been shown to correlate with postural changes,
291 increased arousal threshold and altered brain activity patterns [2]. Sleep bout number and
292 duration was analysed using the open-source software suite phaseR
293 (https://github.com/abhilashlakshman/phaseR/tree/main) [3].
294
295 **Immuno-histochemistry and confocal microscopy**
296 Brains were dissected in phosphate-buffered saline (PBS) (Sigma Aldrich) between ZT1-2,
297 fixed by 20 min room temperature incubation in 4% paraformaldehyde (MP biomedicals)
298 diluted in PBS + 0.3% Triton-X (Sigma-Aldrich) (PBT), washed three times in PBT, and

299 blocked for 1 h in 5% normal goat serum in PBT. Brains were incubated for 48 hours at 4°C
300 in primary antibody (mouse anti-BRP [nc82], Developmental Studies Hybridoma Bank, at a
301 concentration of 1:200); then washed three times in PBT and incubated overnight at 4°C in
302 secondary antibody (goat anti-mouse AlexaFluor 647, ThermoFisher Scientific, at a
303 concentration of 1:500) and counterstain (DAPI, ThermoFisher Scientific, at a concentration
304 of 1:500). Brains were washed three final times before mounting in SlowFade Gold anti-fade
305 mountant (ThermoFisher). Images were taken with a Zeiss LSM 980 confocal microscope
306 with an EC 'Plan-Neofluar' 20x/0.50 M27 air objective, taking z-stacks through the entire
307 brain with step sizes of 1 µm. Images were visualised and analysed using ImageJ; maximum
308 intensity z-stack projections were generated, and following background subtraction, regions
309 of interest (ROIs) were manually drawn and mean pixel intensity calculated. BRP intensity
310 readings from both the whole central brain and limited to the antennal lobes were normalised
311 to DAPI readings from the whole brain. Data is pooled from three separate experiments, with
312 brains within experiments dissected, immuno-stained and imaged in parallel. Values are
313 normalised to the mean values of the long night (L8: D16) condition within an experiment.
314

315 **FIGURE LEGENDS**

316

317 **Fig. 1.** Mechanosensory habituation in adult *Drosophila*. (**A**) Schematic illustrating the
318 *Drosophila* ARousal Tracking (DART) system. (**B**) Protocol used to induce mechanosensory
319 habituation. (**C**) Mean speed over time in one-minute bins, and (**D**) mean speed in the bin
320 following each stimulus, of N = 24 control (iso31) male flies, exposed to 20 successive
321 mechanical stimuli. Data are normalised to the mean in the bin following the first stimulus,
322 i.e. the naïve response. (**E-F**) Mean speed of locomotion over time (E) and mean response
323 in the one-minute bin following each stimulus (F) in N = 25 control adult male flies, in
324 response to an identical habituation protocol applied on three consecutive days. Data from
325 Day 1 and Day 3 are shown. Data are normalised to the response to the first stimulus within
326 each day. Interaction between stimulus number and day: ***$p<0.0005$, two-way ANOVA. (**G**)
327 Mean speed of locomotion over time as a "lights-off" stimulus is delivered to naïve flies
328 (black, N = 24) and immediately following a train of 20 mechanosensory stimuli (blue, N =
329 17). (**H**) Normalised locomotor responses of control adult male flies to the 1st and 20th
330 mechanical stimulus shows a clear decline in response to repeated stimulus: N = 17.
331 ***$p<0.0005$, Mann-Whitney U-test. (**I**) Response to lights-off does not differ between naïve
332 flies (N = 24) and those habituated to the distinct mechanosensory stimuli (N = 17). $p =$
333 0.7187, Mann-Whitney U-test. Error bars: standard error of the mean (SEM).

334

335 **Fig. 2**. *Neurocalcin* knockout flies exhibit impaired mechanosensory habituation. (**A**) Mean
336 speed of locomotion over time, and (**B**) mean speed in one-minute bin following each
337 stimulus of control (N = 87) and *Nca* knockout (Nca^{KO1}) (N = 58) adult male flies exposed to
338 20 consecutive mechanosensory stimuli. Data are normalised within each genotype to the
339 naïve response. *Nca* knockout flies habituate more slowly than controls (interaction between
340 stimulus number and genotype: $p<0.0001$, two-way ANOVA). Error bars: SEM. (**C**)
341 Proportion of flies "responding" decreased over multiple stimuli, with the rate of decrease
342 slower for Nca^{KO1} flies. (**B**) Mean speed of flies "responding" did not decrease in response to

343 repeated stimuli, nor did it differ between genotype (main effect of stimulus number:
344 p=0.2407, and of genotype: p=0.3761; interaction between stimulus and genotype:
345 p=0.4639, two-way ANOVA). Error bars: SEM.
346
347 **Fig. 3.** *Neurocalcin* modulates mechanosensory habituation through a sleep-dependent
348 mechanism. (**A-B**) Mean sleep levels of adult male flies over 24 h under (A) short day, long
349 night (L8: D16) conditions (control: N = 32; *Nca*^{KO1}: N = 32), and (B) long day, short night
350 (L16: D8) conditions (control: N = 31; *Nca*^{KO1}: N = 20). (**C-E**) Total minutes of sleep during
351 subjective night (C), number of individual sleep bouts occurring during the night normalised
352 to hours of night (D), and mean length of sleep bouts occurring during the night (E). Kruskal
353 Wallis test with Dunn's multiple comparison test. (**F**) Protocol for exposure to short or long
354 night conditions prior to habituation assay. (**G-H**) *Nca*^{KO1} flies habituate more slowly than
355 controls in (G) long night conditions: N = 60 (control), 66 (*Nca*^{KO1}), interaction between
356 stimulus and genotype: p = 0.0472. Performance does not differ between the genotypes in
357 (H) short night conditions: N = 61 (control), 61 (*Nca*^{KO1}), interaction between stimulus and
358 genotype: p = 0.2395. Two-way ANOVA with Dunn's multiple comparison tests. Error bars:
359 SEM.
360
361 **Fig. 4.** Night sleep duration modulates Bruchpilot immunofluorescence in sensory circuits.
362 (**A, C**) Representative images of (A) iso31 and (C) *Nca*^{KO1} whole brains immuno-stained for
363 anti-BRP (top), with a DAPI counterstain (bottom), in long night (L8: D16) vs short night (L16:
364 D8) conditions. Scale bars: 100μM. (**B, D**). Mean BRP immunofluorescence within antennal
365 lobe neuropil regions (two data points per brain), normalised to whole-brain DAPI
366 immunofluorescence, is increased in short- versus long-night conditions in controls (p =
367 0.0018, unpaired t-test with Welch's correction), but unchanged in *Nca*^{KO1} brains (p = 0.81,
368 Mann-Whitney U-test). Values are normalised to the mean value in long night conditions.
369 Error bars: SEM.
370

371 **SUPPLEMENTARY FIGURE LEGENDS**

372 **Supp. Fig. 1.** (**A**) Quantification of mean locomotor speed over time in response to a single
373 mechanical stimulus. Mean speed of N = 11 control (iso31) male flies are shown. (**B-D**)
374 Mean speed over time as a further single mechanosensory stimulus is delivered following an
375 interval of (B) 1 h (N = 87), (C) 4 h (N = 36), or (D) 24 h (N = 27). (**E**) Mean speed in the 1
376 min bin following a single stimulus at the indicated interval after the initial habituation
377 protocol. Data are normalised to the naïve response to the first stimulus in the 20-stimulus
378 train (N = 87). Responses progressively return towards the baseline level. (**F**) Schematic of
379 the Zantiks system. Individual flies are loaded into a 48-well plate containing a solidified agar
380 and sucrose food surface. Mechanical stimuli are delivered via a motor beneath the plate,
381 with locomotor responses recorded via videography. (**G**) Mean locomotor responses over
382 time of N = 24 flies to a single mechanical stimulus (solid line), quantified as distance
383 travelled in a 1 min interval. (**H-I**) Mean distance travelled per 1 min (**H**; line represents mean
384 value over time), and mean distance travelled in the 1 min following each mechanical
385 stimulus (**I**), in N = 48 control (iso31) male flies exposed to 20 successive mechanical stimuli
386 in the Zantiks system. Error bars in E, I: SEM

387

388 **Supp. Fig. 2.** (**A**) Mean speed of locomotion over time, and (**B**) mean speed in one-minute
389 bin following each stimulus of control (iso31) (N = 41) and a second independently
390 generated *Nca* knockout line (Nca^{KO2}) (N = 30) adult male flies exposed to 20 consecutive
391 mechanosensory stimuli. Data are normalised within each genotype to the naïve response.
392 Nca^{KO2} flies habituate more slowly than controls (interaction between stimulus number and
393 genotype: $p < 0.0001$, two-way ANOVA). Error bars: SEM. (**C**) Mean speed of control and
394 Nca^{KO1} adult male flies in the one-minute bin following a single mechanosensory stimulus of
395 varying strength (values are normalised to the strength used in the habituation assay).
396 Control: N = 22-52; Nca^{KO1}: N = 15-41. *$p < 0.05$, ns: $p > 0.05$, Mann-Whitney U-test.

397

Supp. Fig. 3. (**A**) Schematic illustrating components of the *Drosophila* Activity Monitor 5 (DAM5) system. Flies are individually housed within glass behavioural tubes. Locomotion is tracked via breaking of 15 infrared beams across the tubes. Sleep is derived from periods without locomotion (see Methods). (**B-D**) Total minutes of sleep during subjective day (B), number of individual sleep bouts during subjective day normalised to hours of day (C), and mean length of sleep bouts occurring during the day (D), for populations presented in Fig. 3A-B. Kruskal Wallis test with Dunn's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars: SEM.

Supp. Fig. 4. (**A-B**) Within-genotype comparisons of the effect of light cycle on habituation. (A) Iso31 flies habituate more quickly in long-night conditions (interaction between genotype and light cycle: $p = 0.0027$). In contrast, *Nca*^{KO1} flies (B) habituate more quickly in short-night conditions (interaction between genotype and light cycle: $p = 0.0494$). (**C-D**) Mean Bruchpilot (BRP) immunofluorescence within the whole central brain, normalised to DAPI, is not significantly changed between short- versus long-night conditions in control (C: $p = 0.1648$), or *Nca*^{KO1} brains (D: $p = 0.5461$). Unpaired t-test with Welch's correction (C) or Mann Whitney U-test (D). Values are normalised to the mean value in long night conditions. Error bars: SEM.

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Figure 1

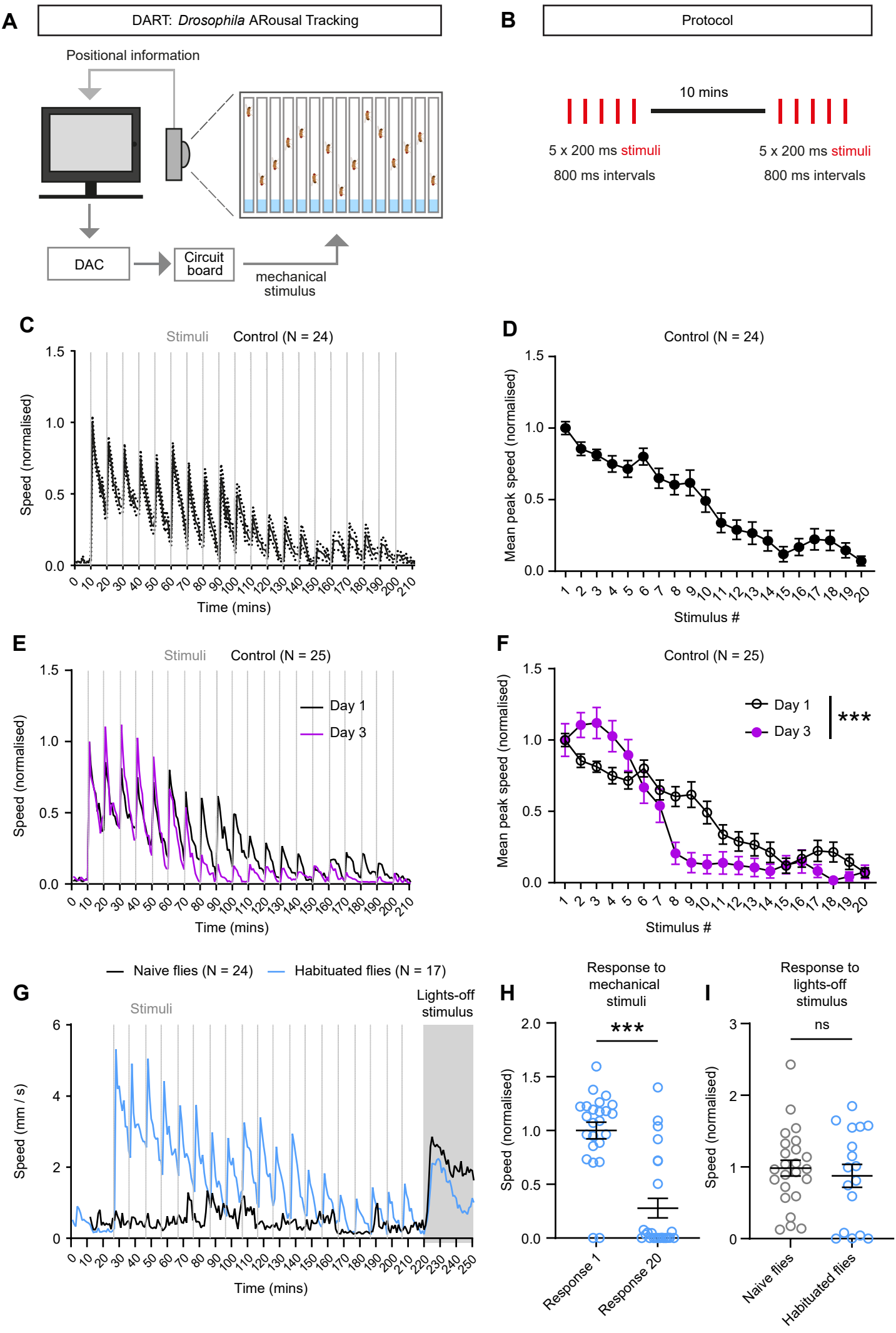

A
DART: *Drosophila* ARousal Tracking
Positional information
DAC
Circuit board
mechanical stimulus
B
Protocol
10 mins
5 x 200 ms stimuli
800 ms intervals
5 x 200 ms stimuli
800 ms intervals
C
Stimuli
Control (N = 24)
Speed (normalised)
Time (mins)
D
Control (N = 24)
Mean peak speed (normalised)
Stimulus #
E
Stimuli
Control (N = 25)
Day 1
Day 3
Speed (normalised)
Time (mins)
F
Control (N = 25)
Day 1
Day 3
***
Mean peak speed (normalised)
Stimulus #
G
Naive flies (N = 24)
Habituated flies (N = 17)
Stimuli
Lights-off stimulus
Speed (mm / s)
Time (mins)
H
Response to mechanical stimuli
***
Speed (normalised)
Response 1
Response 20
I
Response to lights-off stimulus
ns
Speed (normalised)
Naive flies
Habituated flies

Figure 2

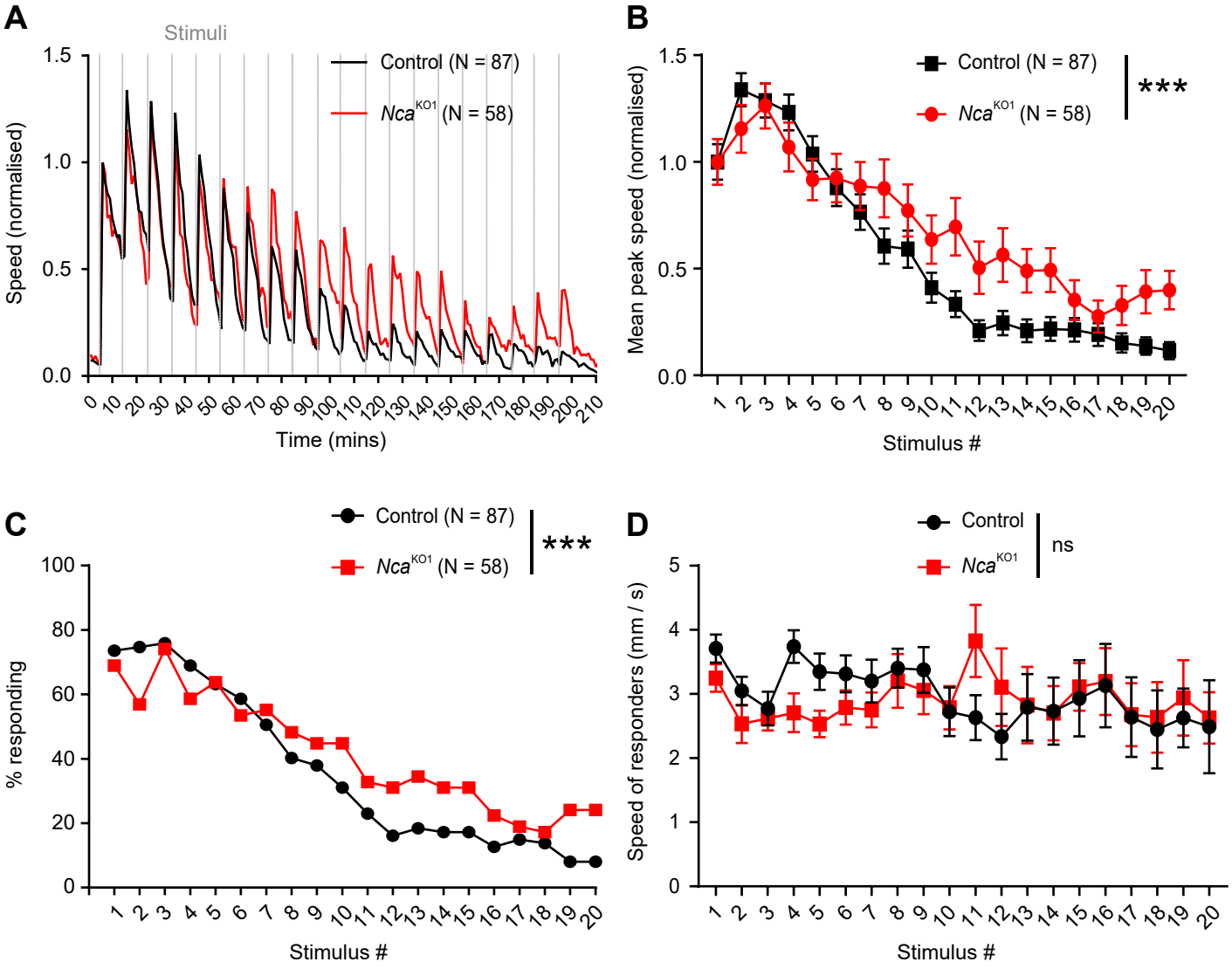

A
Stimuli
Control (N = 87)
Nca^KO1 (N = 58)
Speed (normalised)
Time (mins)
B
Control (N = 87)
Nca^KO1 (N = 58)
***
Mean peak speed (normalised)
Stimulus #
C
Control (N = 87)
Nca^KO1 (N = 58)
***
% responding
Stimulus #
D
Control
Nca^KO1
ns
Speed of responders (mm / s)
Stimulus #

Figure 3

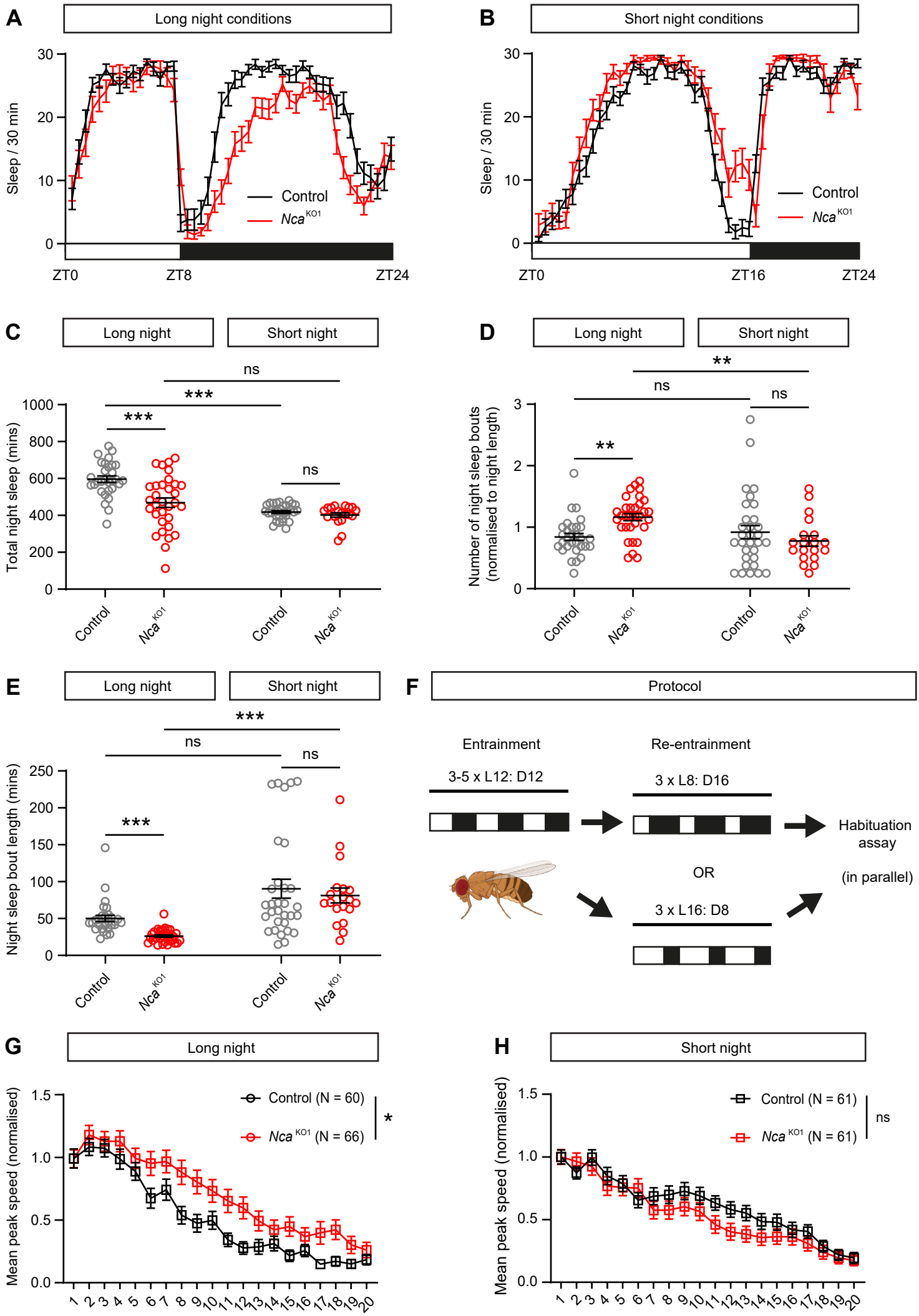
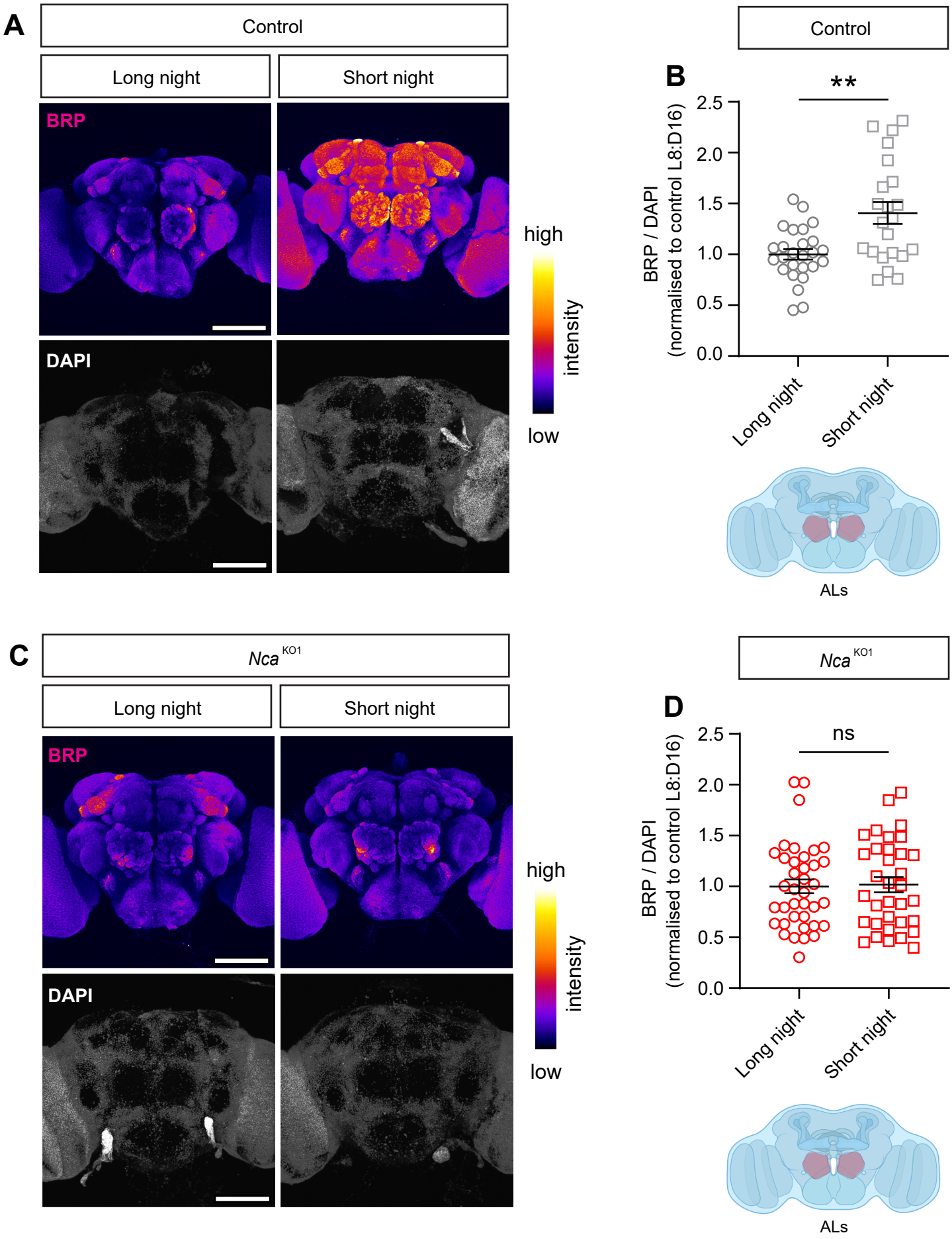

A
Long night conditions
Sleep / 30 min
Control
Nca KO1
ZT0
ZT8
ZT24
B
Short night conditions
Sleep / 30 min
Control
Nca KO1
ZT0
ZT16
ZT24
C
Long night
Short night
Total night sleep (mins)
ns
***
***
ns
Control
Nca KO1
Control
Nca KO1
D
Long night
Short night
Number of night sleep bouts (normalised to night length)
**
ns
ns
**
Control
Nca KO1
Control
Nca KO1
E
Long night
Short night
Night sleep bout length (mins)
***
ns
ns
***
Control
Nca KO1
Control
Nca KO1
F
Protocol
Entrainment
3-5 x L12: D12
Re-entrainment
3 x L8: D16
Habituation assay
(in parallel)
OR
3 x L16: D8
G
Long night
Mean peak speed (normalised)
Control (N = 60)
Nca KO1 (N = 66)
*
H
Short night
Mean peak speed (normalised)
Control (N = 61)
Nca KO1 (N = 61)
ns

Figure 4


A
Control
Long night
Short night
BRP
DAPI
high
intensity
low
B
Control
**
BRP / DAPI
(normalised to control L8:D16)
2.5
2.0
1.5
1.0
0.5
0.0
Long night
Short night
ALs
C
Nca KO1
Long night
Short night
BRP
DAPI
high
intensity
low
D
Nca KO1
ns
BRP / DAPI
(normalised to control L8:D16)
2.5
2.0
1.5
1.0
0.5
0.0
Long night
Short night
ALs