Nighttime sleep modulates mechanosensory habituation in *Drosophila*

Simon A. Lowe[1*], Ko-Fan Chen[2], and James E.C. Jepson[1*]

[1]Department of Epilepsy, UCL Queen Square Institute of Neurology, London, UK

[2]Division of Genetics and Genome Biology, University of Leicester, UK

*To whom correspondence should be addressed: s.lowe@ucl.ac.uk, j.jepson@ucl.ac.uk

ABSTRACT

Organisms must focus their attention on ethologically relevant stimuli to maximise survival and reproduction. This is achieved, in part, through a process termed habituation, in which responses to repeated or constant sensory inputs are sequentially dampened, favouring robust responses to novel stimuli. Prior investigations in the fruit fly, *Drosophila*, have uncovered genes and neural mechanisms that facilitate habituation. However, these works have primarily focused on habituation to visual, olfactory, and gustatory, stimuli. In contrast, neurogenetic processes promoting habituation to mechanosensory stimuli have been less studied. Here we develop an automated system that yields long-term analogue measurements of mechanosensory habituation in adult fruit flies. Using this platform, we reveal a role for the neuronal calcium sensor Neurocalcin in habituation to mechanical stimuli. Loss of Neurocalcin disrupts nighttime but not daytime sleep, particularly under long-night conditions. Mimicking a method used to treat insomnia, we show that compressing nighttime duration restores consolidated night sleep in *Neurocalcin* mutants. Surprisingly, this manipulation also renormalises mechanosensory habituation in this background, suggesting a link between sleep and mechanosensory habituation. Indeed, we show that limiting night sleep duration in wild type flies similarly impairs habituation to mechanosensory stimuli. Collectively, our findings reveal a previously unappreciated link between nighttime sleep and mechanosensory habituation in *Drosophila*.

INTRODUCTION

Habituation is an ancient and widely conserved form of non-associative memory in which repetition of a sensory stimulus leads to diminishing behavioural responses [1].

Habituation promotes the filtering of irrelevant sensory stimuli, and is thus considered a pre-requisite for more complex forms of memory and cognition requiring active organismal attention [2]. Indeed, studies have observed impaired habituation in patients with a range of neuropsychiatric disorders [3], including Fragile-X syndrome [4-6] – the most common monogenic form of inherited intellectual disability (ID) in humans [7] – and autism spectrum disorder (ASD) [8,9].

These studies emphasise the clinical importance of delineating the genetic and neural circuit basis of habituation. The fruit fly, *Drosophila*, has provided a powerful means to study these issues. Numerous assays have been developed to quantify habituation of adult *Drosophila* to a variety of sensory inputs (reviewed in [10]), including visual [11], olfactory [12], and gustatory [13], stimuli. Combined with the powerful genetic toolkit of *Drosophila*, these paradigms have facilitated extensive efforts to identify genes that promote or inhibit habituation – including regulators of cyclic AMP (cAMP) synthesis and degradation [11,13], neurotransmitter release [14,15], neuronal excitability [16,17], and local translation of synaptic proteins [18,19] – and to define how alterations in neural plasticity contribute to behavioural habituation [20,21]. Furthermore, a recent large-scale analysis identified impaired habituation across diverse *Drosophila* models of ID, demonstrating deep conservation between humans and flies of genetic programs that influence habituation [22].

Nonetheless, limitations exist in the protocols used to study habituation in *Drosophila*. Readouts of commonly used methods such as the visually-induced startle assay or the gustatory proboscis extension response are binary (flies respond or not) and may require relatively complex equipment [22], physical tethering of flies [13], or quantification of habituation at the population level [21], limiting throughput, accessibility, and the ability to perform long-term analogue measurements of habituation in individual intact flies. Furthermore, although recently examined in *Drosophila* larvae [23], the mechanisms underlying habituation to

mechanical stimuli have been understudied in adult *Drosophila* in comparison to other sensory modalities.

To expand the range of tools to study habituation in *Drosophila*, we developed an automated protocol using available software/hardware that facilitates long-term, quantitative measurements of mechanosensory habituation in individual adult flies. We used this paradigm to examine genetic links between habituation and a neurophysiological process well-known to influence other forms of memory: sleep.

Following memory-encoding periods, sleep has been shown to promote the consolidation of episodic, procedural, and/or aversive memory in humans, mice, and *Drosophila* [24,25]. However, whether changes in sleep can influence habituation is relatively unexplored. We were particularly interested in examining whether impairing sleep during the primary period of consolidated sleep (the night in diurnal mammals and *Drosophila*) could perturb habituation. In most cases, mutations that reduce sleep in *Drosophila* (for example, *fumin*, *shaker*, *sleepless*, *taranis*, and *insomniac*) disrupt both day and night sleep in oscillating light: dark conditions [26-30], preventing investigation of a specific role for night sleep in modulating habituation. We therefore tested for links between sleep and habituation in a genetic background lacking the neuronal calcium sensor Neurocalcin.

We recently showed that loss of Neurocalcin reduces sleep in *Drosophila* solely during the night – an effect that is enhanced under winter-like long-night conditions [31]. Hence, Neurocalcin provides a genetic entry point to study how loss of night sleep affects habituation. Neuronal calcium sensors such as Neurocalcin and its mammalian homolog Hippocalcin are cytoplasmic proteins that bind calcium through EF hand domains, leading to their translocation to lipid membranes via a calcium-dependent myristoylation switch and a concomitant alteration in their interactions with membrane-associated proteins [32,33]. Hippocalcin is strongly expressed in the mammalian hippocampus [34], a brain region critical for long-term memory [35], and facilitates the slow afterhyperpolarisation (a calcium-dependent potassium current) and glutamate receptor endocytosis during long-term depression in hippocampal neurons [36-38]. Importantly, loss of function mutations in Hippocalcin disrupt

spatial and associative memory in mice [39] and cause ID in humans [40,41]. However, evidence of a habituation-promoting role for mammalian Hippocalcin, *Drosophila* Neurocalcin, or related homologs in other species, is lacking.

Applying our new habituation protocol, we found that loss of Neurocalcin indeed significantly impairs habituation to repeated mechanosensory stimuli, demonstrating a conserved role for Neurocalcin/Hippocalcin homologs in promoting memory across species. Since long night conditions exaggerate *Neurocalcin* knockout-induced night sleep loss [31], we examined the effect of shortening night length, and found that summer-like short night conditions strongly suppressed nighttime sleep loss in *Neurocalcin* mutants. This finding provided us with a means to acutely tune night sleep levels in *Neurocalcin* knockout flies and examine the resulting effects on habituation. Results from these studies, alongside complementary investigations of wild type flies, support a model in which nighttime sleep promotes mechanosensory habituation during subsequent active periods in *Drosophila*.

MATERIALS AND METHODS

Experimental model and subject details

Experimental models were fruit flies of the species *Drosophila melanogaster*. Genotypes used were iso31 (an isogenized strain of w^{1118} flies and a kind gift from Prof. Kyunghee Koh, Thomas Jefferson University), w^{1118};+; Nca^{ko1}/TM2, and w^{1118};+; Nca^{ko2}/TM2 [31] To control for genetic background, these lines were outcrossed into the iso31 background for 5 generations. Flies were raised and maintained on standard fly food under 12 h: 12 h light-dark cycles (12L: 12D) at a constant 25°C. Experiments took place at 25°C, and unless otherwise indicated, in 12L: 12D conditions. Experiments were conducted on male flies.

Habituation protocol

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

Sleep analysis

Sleep analysis was performed using the *Drosophila* Activity Monitor 5 system (DAM5H, Trikinetics inc., MA, USA), which improves on the fidelity of the earlier DAM2 system by using 15 infrared beams per tube, rather than a single one, to track movement [2]. As above, three- to five-day old male flies were loaded in behavioural tubes, and were left for two full days to acclimatise at the relevant light cycle. On the third day the number of beam breaks was measured using the *Drosophila* Activity Monitor. Sleep was defined using the standard definition of ≥ 5 mins of inactivity, which has been shown to correlate with postural changes, increased arousal threshold and altered brain activity patterns [2]. Sleep bout number and duration was analysed using the open-source software suite phaseR (https://github.com/abhilashlakshman/phaseR/tree/main) [3].

RESULTS

Induction of mechanosensory habituation in adult *Drosophila*

A common tool to quantify movement and sleep in *Drosophila* is the *Drosophila* Activity Monitor, in which individual flies traverse a glass tube and, in doing so, cross an infra-red beam, allowing activity and sleep to be measured as a function of beam breaks or their absence over time [42,43]. Recently, video-based tracking methods have allowed *Drosophila* activity to be continuously monitored with increased spatio-temporal resolution. One such method is termed the DART (*Drosophila* ARousal Tracking) system (Fig. 1A) [44], which combines continuous video-tracking of individual flies with platform-associated motors. These motors can be programmed to deliver consistent mechanosensory stimuli that elicit robust, transient, locomotor responses in control flies (Fig. 1B), the magnitude and dynamics of which can be quantified by via DART software.

We reasoned that the DART system could be repurposed to test for mechanosensory habituation in adult flies. Indeed, delivering multiple identical stimuli at intervals of 10 min (Fig. 1C) elicited a gradual attenuation in the magnitude of resulting locomotor responses, quantified either as the mean peak speed in the 1 min following each stimulus, or mean speed during successive 1 min bins ($p<.001$, one-way ANOVA) (Fig. 1D, E).

Several criteria must be met for a stimulus-response to be classified as ‘habituating’ to ensure response decrements do not occur due to other factors, such as structural damage or fatigue (as defined in [1] and more recently in [2]). We therefore tested whether the attenuation of locomotor responses to mechanical stimuli delivered by the DART system met these key criteria.

Firstly, following attenuation of locomotion following 20 mechanical stimuli delivered at 10 min intervals (Fig. 2A), locomotor speeds exhibited spontaneous recovery over time in response to single mechanical stimuli, with ~ 80% recovery occurring following 24 h (Fig. 2B-E). Hence, the stimulus protocol does not cause long-lasting damage to the fly’s motor system.

A second characteristic of habituation is that its rate of induction is potentiated by consecutive bouts of habituation [1,2]. To test for potentiation of habituation, the same paradigm of 20 identical mechanical stimuli was presented on three consecutive days, separated by ~ 21 h of quiescence, with individual flies remaining in glass behaviour tubes in 12 h light: 12 h dark conditions (L12: D12). By day 3, we observed a clear alteration in the dynamics of habituation compared to the initial trial, with locomotor responses initially exhibiting an elevated amplitude (perhaps reflecting increased sensitization [1]), but then reaching a lower baseline more rapidly compared to the same responses on day 1 of the habituation protocol (Fig. 3A, B).

Thirdly, to test whether attenuated locomotor responses in our paradigm were due to fatigue or a generalised sensory adaptation, we delivered the same 20 consecutive mechanical stimuli, followed by a distinct 'lights-off' visual stimulus similarly expected to induce a startle response (Fig. 3C). Notably, despite the robust decrease in locomotor response to repeated mechanical stimuli (Fig.3C,D), subsequent locomotor responses to the visual stimulus did not differ in magnitude between naïve and mechanically habituated flies (Fig.3C, E). Thus, the attenuation in response reflects habituation to a specific stimulus. These data further indicate that our protocol does not damage or desensitise the motor network of adult flies, supporting the premise that the decrease in response amplitudes to repeated mechanical stimuli represents true habituation.

Neurocalcin promotes mechanosensory habituation in *Drosophila*

As noted above, the closely-related Neurocalcin homolog Hippocalcin promotes long-term synaptic plasticity in murine hippocampal neurons, and mutations in Hippocalcin disrupt memory in mice and humans [39-41]. However, whether Hippocalcin or homologs such as Neurocalcin regulate habituation has remained unclear. We therefore used our mechanical paradigm to measure the rate and amplitude of mechanosensory habituation in control flies and flies homozygous for a null allele in *Neurocalcin* (*Nca* knockout 1, *Nca*[ko1]) [31]. Notably, loss of Neurocalcin significantly reduced the degree of mechanosensory habituation, an

effect that became particularly prominent after ~ 10 mechanical stimuli (Fig. 4A, B). Reduced mechanosensory habituation was also observed in flies homozygous for an independent *Neurocalcin* null allele (*Nca*[ko2]) (Supp Fig. 1A-B), confirming the above genotype-phenotype correlation.

Neurocalcin could potentially modulate mechanosensory habituation by modulating mechano-sensation itself – for example, altering the sensitivity to mechanical stimuli. We therefore tested locomotor responses in control and *Neurocalcin* knockout flies to a range of mechanical stimulus strengths (N = 7) ranging from 1% to 100% of the stimulus strength used in the mechanical habituation protocol. *Neurocalcin* knockout flies showed statistically similar amplitudes of locomotor responses to isogenic cotrols at 6/7 stimulus strengths tested, and a slightly enhanced response to stimuli 10% of the stimulus strength used in the habituation protocol (Supp. Fig. 2). Importantly, however, these data did not support a general increase in mechanosensory sensitivity in *Neurocalcin* knockout flies, and confirmed that the habituation protocol utilised a stimulus strength that was beyond the plateau at which there was no difference between control and *Neurocalcin* knockout flies.

Since our habituation protocol allowed long-term, quantitative recordings of individual flies, we were able to examine whether the reduction in habituation in *Neurocalcin* knockout flies was due to a change in number of flies responding to mechanical stimuli, or a decrease in the magnitude of the individual flies' responses. To separate response to a stimulus from background noise, we defined a "response" as being $\geq$10% of the response to the mean naïve response. We found that for both control and in *Neurocalcin* knockout flies, the attenuation in response came entirely from the proportion of flies responding. When only "responding" flies were included in analysis, there was no decrease in the amplitude of locomotor responses across repetitive stimuli, nor any difference between genotype (P = 0.46, main effect of Genotype & of Stimulus #, 2-way ANOVA) (Supp. Fig. 3). Hence, in our paradigm, responses to mechanical stimuli were likely all-or-nothing, with habituation consisting of an increased probability of no response.

Short nights suppress sleep loss and modulate habituation in *Neurocalcin* knockout flies

We previously documented night-specific sleep loss in *Neurocalcin* loss-of-function flies, a phenotype that was particularly prominent under long night, winter-like, conditions [31]. Interestingly, increasing sleep drive by restricting the period of consolidated night sleep is utilised in cognitive behavioural therapy for insomnia, and has similarly been shown to rescue sleep loss in a variety of sleep mutants and neurological disease models in *Drosophila* [45]. We therefore examined sleep architecture in control and *Neurocalcin* knockout flies under short and long night conditions using a multibeam monitoring system that facilitates high-resolution quantification of *Drosophila* movement and sleep [46] (see Methods) (Fig. 5A).

Control and *Neurocalcin* knockout flies were raised under standard 12 h light: 12 h dark conditions then, after 3-5 days of adulthood, were shifted to three days of long (8 h light, 16 h dark; L8: D16) or short (16 h light, 8 h dark; L16: D8) night conditions, with sleep quantified on the third day of each light: dark condition. Consistent with prior results [31], we confirmed reduced nighttime (but not daytime) sleep in *Neurocalcin* knockout flies under long night conditions (Fig. 5B-D), and found that this decrease was associated with fragmentated sleep, characterised by a significant increase in the number of sleep bouts and a concomitant reduction in sleep bout duration (Supp. Fig. 4A, B). In contrast, under short night conditions, overall night sleep (Fig. 5E, G) and the degree of sleep consolidation (Supp. Fig. 4C, D) was not significantly different between *Neurocalcin* knockout flies and controls. Day sleep was also slightly increased in *Neurocalcin* knockout flies under short night conditions, but this was likely driven by reduced locomotor activity towards lights-off as opposed to an alteration in daytime sleep properties (Fig. 5E, F). Thus, acute tuning of night-length provides a means to vary nighttime sleep quality on-demand in *Neurocalcin* knockout flies.

Taking advantage of this finding, we repeated the same protocol and then quantified mechanosensory habituation in control and *Neurocalcin* knockout flies previously entrained

to short or long night conditions (Fig. 6A). Strikingly, while mechanosensory habituation was reduced in *Neurocalcin* knockout flies compared to controls under long night conditions (Fig. 6B), this difference was abolished under short night conditions (Fig. 6C), suggesting that Neurocalcin regulates habituation through its effect on night sleep levels.

To further examine whether night sleep can modulate mechanosensory habituation in a distinct genetic background, we entrained isogenic control flies to long or short night conditions for three days, then quantified mechanosensory habituation during a period of 12 h lights-on as before (Fig. 7A). As shown in Fig. 5, control flies sleep for ~ 600 min during the night in L8: D16 conditions, compared to ~ 400 min in L16: D8 conditions (Fig. 5D, G). Strikingly, altering night sleep by increasing or decreasing nighttime duration for just three days prior to the habituation assay was sufficient to significantly alter the rate of mechanosensory habituation in otherwise identical populations of control flies, with controls entrained to L8: D16 conditions (which promote a longer of duration night sleep) habituating significantly more rapidly to mechanical stimuli relative to flies entrained to L16: D8 conditions (Fig. 7B).

Collectively, the above findings reveal a role for Neurocalcin in regulating habituation to repetitive mechanosensory stimuli, and suggest that Neurocalcin does so at least in part by promoting night time sleep, a process that also modulates mechanosensory habituation in wild type flies.

DISCUSSION

Drosophila has been extensively used to uncover the neurogenetic basis of habituation to olfactory, visual, and gustatory, stimuli [10,19,21,22]. However, habituation to mechanosensory stimuli has been comparatively less studied. Recent work utilising a custom-made device to deliver vibrational stimuli found that *Drosophila* larvae frequently respond to vibrations by reversing the direction of ongoing movement – a well-characterised response to aversive stimuli [23,47]. Importantly, the frequency of such reversals diminished with repeated vibrational stimuli, indicative of habituation [23]. Here, deploying a similar approach, we re-purpose an accessible, automated platform – the DART system [44] – to induce and quantify mechanosensory habituation in adult *Drosophila*. As well as the DART, an array of publicly and commercially generated monitoring systems and software packages have been developed to video-track and quantify locomotor activity in adult *Drosophila* [48-52]. Our approach should, in principle, be readily transferable to these platforms, only requiring the incorporation of hardware capable of delivering vibrational/mechanical stimuli to fly-containing chambers at defined frequencies and intensities.

Utilising this method, we demonstrate that adult flies respond to repeated mechanical stimuli with decreasing locomotor responses. This behavioural change meets established criteria for habituation [1,2], since the diminution in responses exhibits spontaneous recovery (Fig. 2), is potentiated over multiple cycles of habituation (Fig. 3A,B), and is stimulus-specific (Fig. 3C-F). Furthermore, the all-or-nothing nature of the diminished responses (Supp. Fig. 2) supports the premise that this represents a learning event i.e an altered “decision” of whether to respond to a given mechanical stimulus, as opposed to (for example) a motor defect affecting the ability to sustain robust reactions over multiple stimuli.

Our findings reveal three key aspects of mechanosensory habituation in adult *Drosophila*. Firstly, as noted above, the effect of mechanosensory habituation does not generalise to visual stimuli; hence, this form of habituation is unlikely to involve motoneuron inhibition or a generalised sensory adaptation. Instead, we hypothesize that cellular events driving habituation may occur within mechanosensory neurons – for example, chordotonal

neurons in the antennae and/or legs that sense gravity and mediate proprioception [53] – or downstream circuits that link mechanosensory organs with pre-motor and motoneurons [54-57].

Secondly, we identify the neuronal calcium sensor Neurocalcin – a nighttime sleep-promoting factor in *Drosophila* [31] – as a molecular regulator of mechanosensory habituation. Prior studies in mice have shown that the Neurocalcin homolog Hippocalcin regulates long-term depression (LTD) in hippocampal synapses by driving internalisation of glutamate receptors [36], and defined a role for Hippocalcin in spatial and associative memory [39]. Interestingly, we find that normal levels of consolidated night sleep can be acutely restored in *Neurocalcin* knockout flies by exposure to short night conditions, and that this environmental manipulation is sufficient to renormalise the rate of mechanosensory habituation relative to isogenic controls. Thus, our data suggests that Neurocalcin may influence habituation (at least in part) by promoting consolidated night sleep rather than via molecular cascades involved in plasticity-associated processes such as LTD [36].

Thirdly, we show that in isogenic control flies, the rate of mechanosensory habituation is also influenced by the degree of sleep during prior nights, providing independent support for a link between night sleep and habituation to mechanical stimuli across genotypes.

We posit that the interaction between night sleep loss and habituation is consistent with the synaptic homeostasis hypothesis (SHY), which stipulates that sleep facilitates downscaling of synapses that have been potentiated during waking experience, thus reducing neuronal energy consumption and enhancing signal-to-noise ratio within memory-associated circuits [58]. Interpreting our data in the context of SHY suggests a model in which the elevation in nighttime locomotor activity in *Neurocalcin* knockout flies under longer night conditions ($\geq$ 12 h) [31] – and similarly, the increase in overall activity of control flies entrained in short night conditions – potentiates synaptic connections in mechanosensory and/or downstream motor circuits relative to flies experiencing robust night sleep. This potentiation could in turn impair the ability of habituation-promoting mechanisms (such as enhanced

synaptic transmission from local inhibitory neurons [21]) to dampen the functional connectivity between mechanosensory neurons and downstream motoneuron outputs.

Further studies will be required to add additional support to this model – in particular, identifying mechanosensory and/or pre-motor circuits in which synaptic output is modulated by habituation-promoting mechanisms. Limitations also exist with our method to induce mechanosensory habituation. The protocol requires a low level of baseline locomotor activity for stimulus-induced increases in movement to be accurately measured. Therefore, it is constrained to occur during periods of low activity during the day/night cycle, such as during the daytime siesta. Correspondingly, we found that mechanosensory habituation was difficult to quantify in genetic backgrounds exhibiting a high degree of baseline locomotor activity, such as mutants in the *insomniac* gene[28] (data not shown).

Nonetheless, the platform described herein represents an accessible system to perform long-term analogue measurements of mechanosensory habituation in freely behaving flies. Applying this method in concert with spatio-temporal manipulations of gene expression and neural circuit activity [59] promises to advance the understanding of the genetic and neural basis of mechanosensory habituation in *Drosophila*, and given the deep conservation of habituation-promoting genes between flies and humans [22], potentially across species. Recent work has also shown that continuous or repetitive mechanosensory stimulation, akin to the gentle rocking of a human baby, increases daytime sleep in *Drosophila* through a habituation-like mechanism [60]. In concert with our findings, these data suggest an intertwined and potentially bi-directional relationship between mechano-sensation, sleep, and habituation, across the day-night cycle.

ACKNOWLEDGEMENTS

We thank Dr. Abigail Wilson and Dr. Gabriel Aughey for comments on the manuscript, and members of BKFLab for technical assistance with the DART system. This study was funded by a MRC Senior Non-Clinical Fellowship (MR/V03118X/1) and a BBSRC Research Grant (BB/R02281X/1) to J.E.C.J; a BBSRC Research Grant (BB/X00094X/1) to S.L and J.E.C.J; and a BBSRC Research Grant (BB/W014939/1), and a Startup and CRE fund from the Division of Genetics and Genome Biology of University of Leicester (RM33077K), to K-F.C.

AUTHOR CONTRIBUTIONS

S.A.L – Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing – Review & Editing, Visualization, Funding acquisition; 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.

FIGURE LEGENDS

Fig. 1. Adult *Drosophila* locomotor responses attenuate in response to repeated mechanical stimulations. (**A**) Schematic illustrating components of the *Drosophila* ARousal Tracking (DART) system. Mechanical stimuli are delivered to the bottom of a plastic base holding individual flies housed within glass tubes. (**B**) Quantification of locomotor speed over time in response to a single mechanical stimulus. Mean speed of N = 11 control (iso31) male flies are shown. Line: mean; dots: upper and lower values of standard error of the mean (SEM). (**C**) Protocol utilised to induce mechanosensory habituation. (**D**) Mean peak speed of locomotion over time in N = 24 control adult male flies, in response to 20 consecutive mechanosensory stimuli as described in (C). Error bars, SEM. (**E**) Mean speed of locomotion over time in the same population of flies but quantified as the mean speed during successive 1 min bins. Line: mean; dots: upper and lower values of SEM. Data in (D) and (E) were normalised to the peak of the initial stimulus.

Fig. 2. Spontaneous recovery from mechanosensory habituation. (**A**) Mean peak speed of locomotion over time in N = 87 control adult male flies, in response to 20 consecutive mechanosensory stimuli. (**B-D**) Mean peak speed of locomotion over time in response to a single mechanical stimulus occurring 1 h (B), 4 h (C), or 24 h (D) following the initial 20 consecutive mechanosensory stimuli. (**E**) Quantification of peak locomotor speed in response to single mechanical stimuli occurring 0, 1, 4, and 24 h following induction of mechanosensory habituation. Speeds are normalised to the peak speed of the initial response during the habituation protocol. Error bars, SEM.

Fig. 3. Potentiation of mechanosensory habituation and dishabituation via a visual stimulus. (**A-B**) Mean speed of locomotion over time (A) and mean peak response speed (B) in N = 24 control adult male flies, in response to 20 consecutive mechanosensory stimuli applied repeatedly at the same time over consecutive days. Data are shown from Day 1 versus Day 3. Repeated induction of habituation over 3 days significantly increased the tempo of

habituation (B: ***p<0.0005, two-way ANOVA). Data in A and B are normalised to the peak of the first mechanical stimulus. (**C**) Mean speed of locomotion over time in response to 20 consecutive mechanosensory stimuli followed directly by a light-off visual stimulus. Also shown are the responses to the visual stimulus of flies recorded in parallel, but which did not receive mechanical stimuli. (**D**) Normalised locomotor responses of control adult male flies to the 1st and 20th mechanical stimulus. N = 17. ***p<0.0005, Mann-Whitney U-test. (**D**) Normalised locomotor responses of naïve (N = 24) or mechanically habituated (N = 17) adult male flies to the lights-off visual stimulus. ns: p>0.05, Mann-Whitney U-test. Error bars, SEM.

Fig. 4. *Neurocalcin* knockout flies exhibit impaired mechanosensory habituation. (**A-B**) Mean speed of locomotion over time (A) and mean peak response speed (B) in control (N = 87) and *Nca* knockout (*Nca*[ko1]) (N = 58) adult male flies, in response to 20 consecutive mechanosensory stimuli. Data are normalised within each genotype to the peak of the first mechanical stimulus. ***p<0.0005, two-way ANOVA. Error bars, SEM.

Fig. 5. Restricting sleep opportunity restores night sleep in *Neurocalcin* mutant flies. **A.** Schematic of the *Drosophila* Activity Monitor multibeam system. **B**. Mean sleep levels over 24 h under short day, long night (L8: D16) conditions in control (N = 32) and *Nca* knockout (*Nca*[ko]) (N = 32) adult male flies. **C-D**. Mean day (C) and night (D) sleep duration in control and *Nca* knockout (*Nca*[ko]) adult male flies under L8: D16 conditions. **E**. Mean sleep levels over 24 h under long day, short night (L16: D8) conditions in control (N = 32) and *Nca* knockout (*Nca*[ko]) adult male flies (N = 20). **F-G**. Mean day (F) and night (G) sleep duration in control and *Nca* knockout (*Nca*[ko]) adult male flies under L16: D8 conditions. *p<0.05, ***p<0.0005, ns: p>0.05, Mann-Whitney U-test (C, D, G) or Welch's t-test (F). Error bars, SEM.

Fig. 6. Neurocalcin modulates mechanosensory habituation through a sleep-dependent mechanism. **A.** Schematic illustrating the protocol for varying exposure to short or long night

conditions in control and *Nca* knockout (*Nca*[ko]) adult male flies prior to the habituation assay. **B-C.** Mean peak response speed in control and *Nca* knockout (*Nca*[ko]) adult male flies, in response to 20 consecutive mechanosensory stimuli, re-entrained to long night (B) or short night (C) conditions. N = 60-66 per genotype and condition. Data are normalised within each genotype to the peak of the first mechanical stimulus. ***p<0.0005, ns – p>0.05, two-way ANOVA. Error bars, SEM.

Fig. 7. Nighttime duration modulates mechanosensory habituation in control adult flies. **A.** Schematic illustrating the protocol for exposure to short or long night conditions in control adult male flies prior to the habituation assay. Quantification of mechanosensory habituation in flies previously exposed to short or long nights was performed simultaneously within the same recording apparatus. **B.** Mean peak response speed in control adult male flies entrained to short or long night conditions, in response to 20 consecutive mechanosensory stimuli. N = 38 flies entrained to short nights (L16: D8) and N = 43 flies entrained to long nights (L8: D16). Data are normalised within each genotype to the peak of the first mechanical stimulus. **p<0.005, two-way ANOVA. Error bars, SEM.

Supp. Fig. 1. A-B. Mean speed of locomotion over time (A) and mean peak response speed (B) in control (N = 41) and *Nca* knockout (*Nca*[ko2]) (N = 31) adult male flies, in response to 20 consecutive mechanosensory stimuli. Data are normalised within each genotype to the peak of the first mechanical stimulus. ***p<0.0005, two-way ANOVA. Error bars, SEM.

Supp. Fig. 2. Mean peak response speed in control and *Nca* knockout (*Nca*[ko1]) adult male flies to a range of mechanical stimulus strengths (values are normalised to the strength used in the habituation assay). Control: N = 22-52; *Nca*[ko1]: N = 15-41. *p<0.05, ns: p>0.05, Mann-Whitney U-test.

Supp. Fig. 3. **A**. Proportion of control (N = 87) and *Nca* knockout (*Nca*[ko1]) (N = 58) adult male flies responding to 20 consecutive mechanosensory stimuli. See Methods for definition of a locomotor response. **B.** Mean peak response speed in control and *Nca* knockout adult male flies over 20 consecutive mechanosensory stimuli, analyses solely for flies that exhibit a locomotor response to each mechanical stimulus. Error bars, SEM. ns: $p>0.05$, two-way ANOVA.

Supp. Fig. 4. A-B. Number (A) and duration (B) of sleep bouts in control (N = 32) and *Nca* knockout (*Nca*[ko1]) (N = 32) adult male flies in under short day, long night (L8: D16) conditions. C-D. Number (C) and duration (D) of sleep bouts in control (N = 32) and *Nca* knockout (N = 20) adult male flies in under long day, short night (L16: D8) conditions. Error bars, SEM. **$p<0.005$, ***$p<0.0005$, ns: $p>0.05$, Mann-Whitney U-test.

REFERENCES

1 Thompson, R. F. & Spencer, W. A. Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychol Rev* **73**, 16-43 (1966). https://doi.org:10.1037/h0022681

2 Rankin, C. H. *et al.* Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. *Neurobiol Learn Mem* **92**, 135-138 (2009). https://doi.org:10.1016/j.nlm.2008.09.012

3 McDiarmid, T. A., Bernardos, A. C. & Rankin, C. H. Habituation is altered in neuropsychiatric disorders-A comprehensive review with recommendations for experimental design and analysis. *Neurosci Biobehav Rev* **80**, 286-305 (2017). https://doi.org:10.1016/j.neubiorev.2017.05.028

4 Miller, L. J. *et al.* Electrodermal responses to sensory stimuli in individuals with fragile X syndrome: a preliminary report. *Am J Med Genet* **83**, 268-279 (1999).

5 Bruno, J. L., Garrett, A. S., Quintin, E. M., Mazaika, P. K. & Reiss, A. L. Aberrant face and gaze habituation in fragile x syndrome. *Am J Psychiatry* **171**, 1099-1106 (2014). https://doi.org:10.1176/appi.ajp.2014.13111464

6 Rigoulot, S. *et al.* Altered visual repetition suppression in Fragile X Syndrome: New evidence from ERPs and oscillatory activity. *Int J Dev Neurosci* **59**, 52-59 (2017). https://doi.org:10.1016/j.ijdevneu.2017.03.008

7 Hagerman, R. J. *et al.* Fragile X syndrome. *Nat Rev Dis Primers* **3**, 17065 (2017). https://doi.org:10.1038/nrdp.2017.65

8 Ewbank, M. P. *et al.* Repetition Suppression in Ventral Visual Cortex Is Diminished as a Function of Increasing Autistic Traits. *Cereb Cortex* **25**, 3381-3393 (2015). https://doi.org:10.1093/cercor/bhu149

9 Swartz, J. R., Wiggins, J. L., Carrasco, M., Lord, C. & Monk, C. S. Amygdala habituation and prefrontal functional connectivity in youth with autism spectrum disorders. *J Am Acad Child Adolesc Psychiatry* **52**, 84-93 (2013). https://doi.org:10.1016/j.jaac.2012.10.012

10 Engel, J. E. & Wu, C. F. Neurogenetic approaches to habituation and dishabituation in Drosophila. *Neurobiol Learn Mem* **92**, 166-175 (2009). https://doi.org:10.1016/j.nlm.2008.08.003

11 Engel, J. E. & Wu, C. F. Altered habituation of an identified escape circuit in Drosophila memory mutants. *J Neurosci* **16**, 3486-3499 (1996). https://doi.org:10.1523/JNEUROSCI.16-10-03486.1996

12 Asztalos, Z., Arora, N. & Tully, T. Olfactory jump reflex habituation in Drosophila and effects of classical conditioning mutations. *J Neurogenet* **21**, 1-18 (2007). https://doi.org:10.1080/01677060701247508

13 Duerr, J. S. & Quinn, W. G. Three Drosophila mutations that block associative learning also affect habituation and sensitization. *Proc Natl Acad Sci U S A* **79**, 3646-3650 (1982). https://doi.org:10.1073/pnas.79.11.3646

14 Godenschwege, T. A. *et al.* Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *Eur J Neurosci* **20**, 611-622 (2004). https://doi.org:10.1111/j.1460-9568.2004.03527.x

15 Sadanandappa, M. K. *et al.* Synapsin function in GABA-ergic interneurons is required for short-term olfactory habituation. *J Neurosci* **33**, 16576-16585 (2013). https://doi.org:10.1523/JNEUROSCI.3142-13.2013

16 Engel, J. E. & Wu, C. F. Genetic dissection of functional contributions of specific potassium channel subunits in habituation of an escape circuit in Drosophila. *J Neurosci* **18**, 2254-2267 (1998). https://doi.org:10.1523/JNEUROSCI.18-06-02254.1998

17 Joiner, M. A., Asztalos, Z., Jones, C. J., Tully, T. & Wu, C. F. Effects of mutant Drosophila K+ channel subunits on habituation of the olfactory jump response. *J Neurogenet* **21**, 45-58 (2007). https://doi.org:10.1080/01677060701247375

18 Bakthavachalu, B. *et al.* RNP-Granule Assembly via Ataxin-2 Disordered Domains Is Required for Long-Term Memory and Neurodegeneration. *Neuron* **98**, 754-766 e754 (2018). https://doi.org:10.1016/j.neuron.2018.04.032

19 Sudhakaran, I. P. *et al.* FMRP and Ataxin-2 function together in long-term olfactory habituation and neuronal translational control. *Proc Natl Acad Sci U S A* **111**, E99-E108 (2014). https://doi.org:10.1073/pnas.1309543111

20 Larkin, A. *et al.* Central synaptic mechanisms underlie short-term olfactory habituation in Drosophila larvae. *Learn Mem* **17**, 645-653 (2010). https://doi.org:10.1101/lm.1839010

21 Das, S. *et al.* Plasticity of local GABAergic interneurons drives olfactory habituation. *Proc Natl Acad Sci U S A* **108**, E646-654 (2011). https://doi.org:10.1073/pnas.1106411108

22 Fenckova, M. *et al.* Habituation Learning Is a Widely Affected Mechanism in Drosophila Models of Intellectual Disability and Autism Spectrum Disorders. *Biol Psychiatry* **86**, 294-305 (2019). https://doi.org:10.1016/j.biopsych.2019.04.029

23 Berne, A. *et al.* Mechanical vibration patterns elicit behavioral transitions and habituation in crawling Drosophila larvae. *Elife* **12** (2023). https://doi.org:10.7554/eLife.69205

24 Brodt, S., Inostroza, M., Niethard, N. & Born, J. Sleep-A brain-state serving systems memory consolidation. *Neuron* **111**, 1050-1075 (2023). https://doi.org:10.1016/j.neuron.2023.03.005

25 Dissel, S. Drosophila as a Model to Study the Relationship Between Sleep, Plasticity, and Memory. *Front Physiol* **11**, 533 (2020). https://doi.org:10.3389/fphys.2020.00533

26 Kume, K., Kume, S., Park, S. K., Hirsh, J. & Jackson, F. R. Dopamine is a regulator of arousal in the fruit fly. *J Neurosci* **25**, 7377-7384 (2005). https://doi.org:10.1523/JNEUROSCI.2048-05.2005

27 Cirelli, C. *et al.* Reduced sleep in Drosophila Shaker mutants. *Nature* **434**, 1087-1092 (2005). https://doi.org:10.1038/nature03486

28 Stavropoulos, N. & Young, M. W. insomniac and Cullin-3 regulate sleep and wakefulness in Drosophila. *Neuron* **72**, 964-976 (2011). https://doi.org:10.1016/j.neuron.2011.12.003

29 Koh, K. *et al.* Identification of SLEEPLESS, a sleep-promoting factor. *Science* **321**, 372-376 (2008). https://doi.org:10.1126/science.1155942

30 Afonso, D. J. *et al.* TARANIS Functions with Cyclin A and Cdk1 in a Novel Arousal Center to Control Sleep in Drosophila. *Curr Biol* **25**, 1717-1726 (2015). https://doi.org:10.1016/j.cub.2015.05.037

31 Chen, K. F., Lowe, S., Lamaze, A., Kratschmer, P. & Jepson, J. Neurocalcin regulates nighttime sleep and arousal in Drosophila. *Elife* **8** (2019). https://doi.org:10.7554/eLife.38114

32 Braunewell, K. H. & Klein-Szanto, A. J. Visinin-like proteins (VSNLs): interaction partners and emerging functions in signal transduction of a subfamily of neuronal Ca2+ -sensor proteins. *Cell Tissue Res* **335**, 301-316 (2009). https://doi.org:10.1007/s00441-008-0716-3

33 Burgoyne, R. D. & Haynes, L. P. Understanding the physiological roles of the neuronal calcium sensor proteins. *Mol Brain* **5**, 2 (2012). https://doi.org:10.1186/1756-6606-5-2

34 Saitoh, S., Takamatsu, K., Kobayashi, M. & Noguchi, T. Expression of hippocalcin in the developing rat brain. *Brain Res Dev Brain Res* **80**, 199-208 (1994). https://doi.org:10.1016/0165-3806(94)90105-8

35 Bird, C. M. & Burgess, N. The hippocampus and memory: insights from spatial processing. *Nat Rev Neurosci* **9**, 182-194 (2008). https://doi.org:10.1038/nrn2335

36 Jo, J. *et al.* Muscarinic receptors induce LTD of NMDAR EPSCs via a mechanism involving hippocalcin, AP2 and PSD-95. *Nat Neurosci* **13**, 1216-1224 (2010). https://doi.org:10.1038/nn.2636

37 Tzingounis, A. V., Kobayashi, M., Takamatsu, K. & Nicoll, R. A. Hippocalcin gates the calcium activation of the slow afterhyperpolarization in hippocampal pyramidal cells. *Neuron* **53**, 487-493 (2007). https://doi.org:10.1016/j.neuron.2007.01.011

38 Palmer, C. L. *et al.* Hippocalcin functions as a calcium sensor in hippocampal LTD. *Neuron* **47**, 487-494 (2005). https://doi.org:10.1016/j.neuron.2005.06.014

39 Kobayashi, M. *et al.* Hippocalcin-deficient mice display a defect in cAMP response element-binding protein activation associated with impaired spatial and associative memory. *Neuroscience* **133**, 471-484 (2005). https://doi.org:10.1016/j.neuroscience.2005.02.034

40 Atasu, B. *et al.* HPCA confirmed as a genetic cause of DYT2-like dystonia phenotype. *Mov Disord* **33**, 1354-1358 (2018). https://doi.org:10.1002/mds.27442

41 Magrinelli, F. *et al.* Childhood-Onset Choreo-Dystonia Due to a Recurrent Novel Homozygous Nonsense HPCA Variant: Case Series and Literature Review. *Mov Disord Clin Pract* **10**, 101-108 (2023). https://doi.org:10.1002/mdc3.13529

42 Pfeiffenberger, C., Lear, B. C., Keegan, K. P. & Allada, R. Processing sleep data created with the Drosophila Activity Monitoring (DAM) System. *Cold Spring Harb Protoc* **2010**, pdb prot5520 (2010). https://doi.org:10.1101/pdb.prot5520

43 Pfeiffenberger, C., Lear, B. C., Keegan, K. P. & Allada, R. Locomotor activity level monitoring using the Drosophila Activity Monitoring (DAM) System. *Cold Spring Harb Protoc* **2010**, pdb prot5518 (2010). https://doi.org:10.1101/pdb.prot5518

44 Faville, R., Kottler, B., Goodhill, G. J., Shaw, P. J. & van Swinderen, B. How deeply does your mutant sleep? Probing arousal to better understand sleep defects in Drosophila. *Sci Rep* **5**, 8454 (2015). https://doi.org:10.1038/srep08454

45 Belfer, S. J., Bashaw, A. G., Perlis, M. L. & Kayser, M. S. A Drosophila model of sleep restriction therapy for insomnia. *Mol Psychiatry* **26**, 492-507 (2021). https://doi.org:10.1038/s41380-019-0376-6

46 Sitaraman, D., Vecsey, C. G. & Koochagian, C. Activity Monitoring for Analysis of Sleep in Drosophila melanogaster. *Cold Spring Harb Protoc* **2024**, pdb top108095 (2024). https://doi.org:10.1101/pdb.top108095

47 Kernan, M., Cowan, D. & Zuker, C. Genetic dissection of mechanosensory transduction: mechanoreception-defective mutations of Drosophila. *Neuron* **12**, 1195-1206 (1994). https://doi.org:10.1016/0896-6273(94)90437-5

48 Revel M, Nagoshi E & RK, M. Drosophila Video-assisted Activity Monitor (DrosoVAM): a versatile method for behavior monitoring. *bioRxiv* (2025). https://doi.org:https://doi.org/10.1101/2025.07.29.667363

49 Omelchenko A.A, H. A., Castaneda A.N, Vaden T.J, Ni L. Using TrackMate to Analyze Drosophila Larval and Adult Locomotion. *bioRxiv* (2021). https://doi.org:doi: https://doi.org/10.1101/2021.09.28.462241

50 Slawson, J. B., Kim, E. Z. & Griffith, L. C. High-resolution video tracking of locomotion in adult Drosophila melanogaster. *J Vis Exp* (2009). https://doi.org:10.3791/1096

51 Geissmann, Q. *et al.* Ethoscopes: An open platform for high-throughput ethomics. *PLoS Biol* **15**, e2003026 (2017). https://doi.org:10.1371/journal.pbio.2003026

52 Allen, T., Budenberg, W. J. Replicating Light-Off Startle Responses in Drosophila melanogaster. *bioRxiv* (2021). https://doi.org:10.1101/2021.02.24.432669

53 Tuthill, J. C. & Wilson, R. I. Mechanosensation and Adaptive Motor Control in Insects. *Curr Biol* **26**, R1022-R1038 (2016). https://doi.org:10.1016/j.cub.2016.06.070

54 Azevedo, A. *et al.* Connectomic reconstruction of a female Drosophila ventral nerve cord. *Nature* **631**, 360-368 (2024). https://doi.org:10.1038/s41586-024-07389-x

55 Kamikouchi, A. *et al.* The neural basis of Drosophila gravity-sensing and hearing. *Nature* **458**, 165-171 (2009). https://doi.org:10.1038/nature07810

56 Chen, C. *et al.* Functional architecture of neural circuits for leg proprioception in Drosophila. *Curr Biol* **31**, 5163-5175 e5167 (2021). https://doi.org:10.1016/j.cub.2021.09.035

57 Tuthill, J. C. & Wilson, R. I. Parallel Transformation of Tactile Signals in Central Circuits of Drosophila. *Cell* **164**, 1046-1059 (2016). https://doi.org:10.1016/j.cell.2016.01.014

58 Tononi, G. & Cirelli, C. Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. *Neuron* **81**, 12-34 (2014). https://doi.org:10.1016/j.neuron.2013.12.025

59 Venken, K. J., Simpson, J. H. & Bellen, H. J. Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron* **72**, 202-230 (2011). https://doi.org:10.1016/j.neuron.2011.09.021

60 Ozturk-Colak, A. *et al.* Sleep Induction by Mechanosensory Stimulation in Drosophila. *Cell Rep* **33**, 108462 (2020). https://doi.org:10.1016/j.celrep.2020.108462

Figure 1

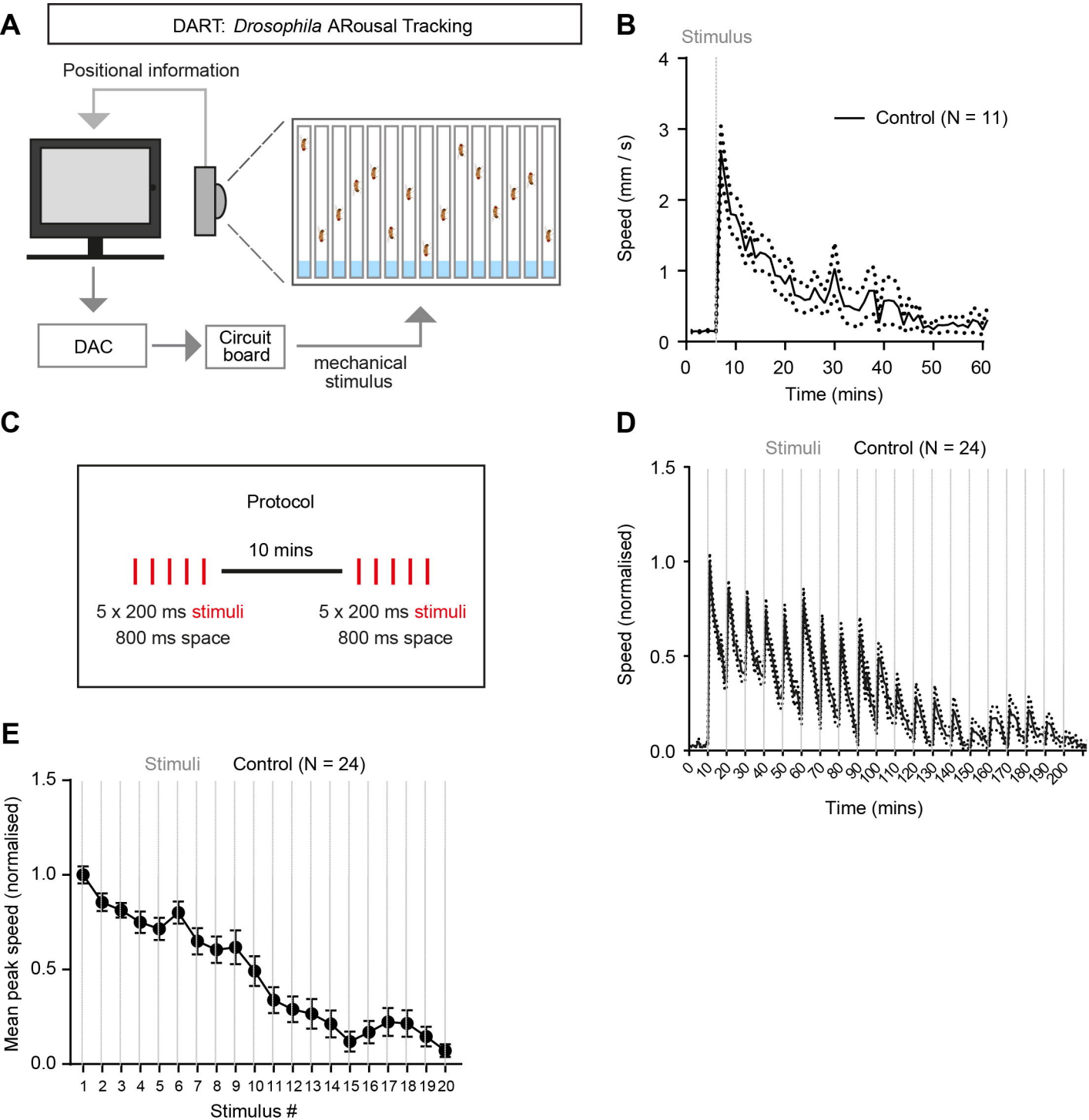

A
DART: *Drosophila* ARousal Tracking
Positional information
DAC
Circuit board
mechanical stimulus
B
Stimulus
Control (N = 11)
Speed (mm / s)
Time (mins)
C
Protocol
10 mins
5 x 200 ms stimuli
800 ms space
5 x 200 ms stimuli
800 ms space
D
Stimuli
Control (N = 24)
Speed (normalised)
Time (mins)
E
Stimuli
Control (N = 24)
Mean peak speed (normalised)
Stimulus #

Figure 2

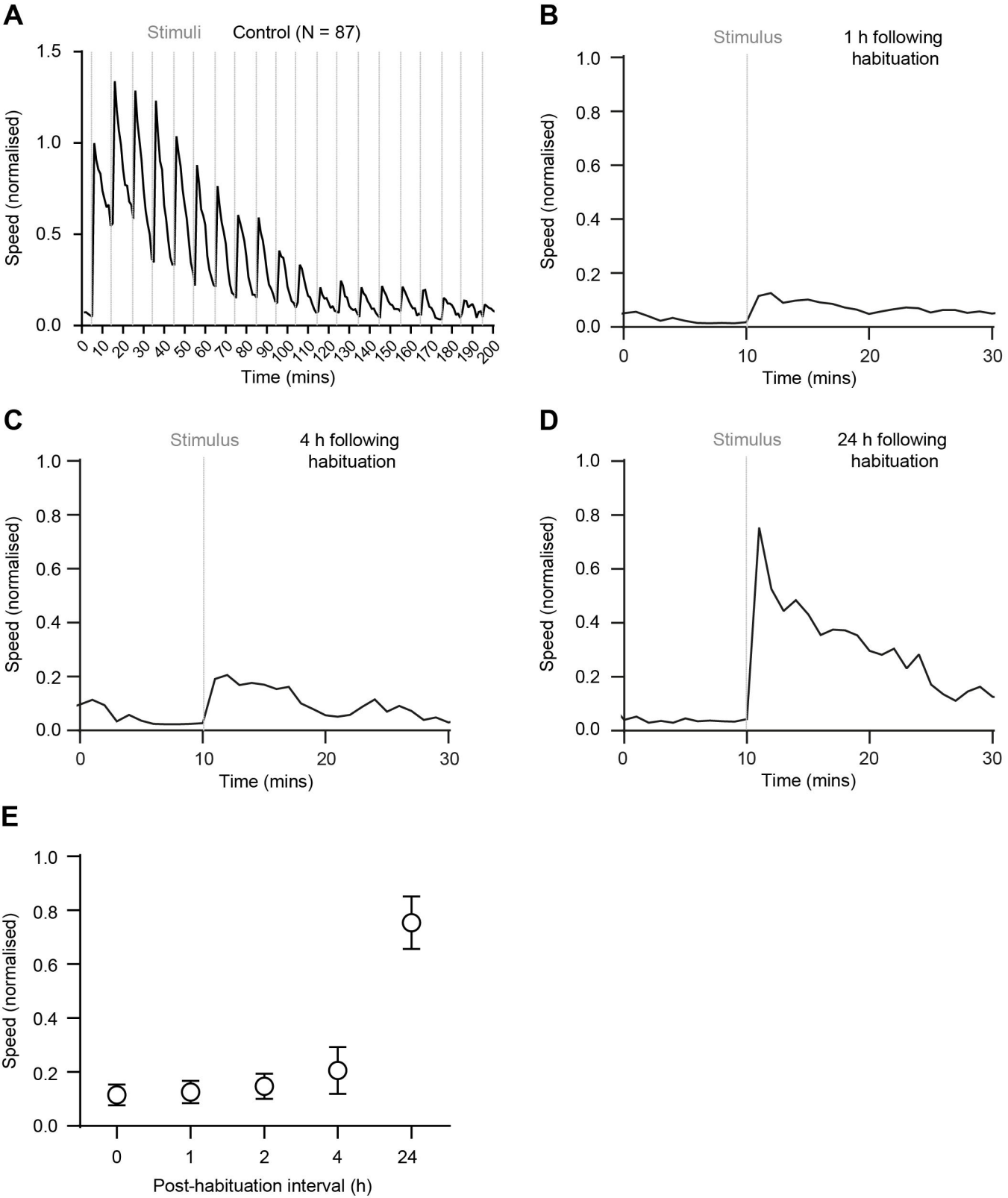

A
Stimuli
Control (N = 87)
Speed (normalised)
Time (mins)
B
Stimulus
1 h following habituation
Speed (normalised)
Time (mins)
C
Stimulus
4 h following habituation
Speed (normalised)
Time (mins)
D
Stimulus
24 h following habituation
Speed (normalised)
Time (mins)
E
Speed (normalised)
Post-habituation interval (h)

Figure 3

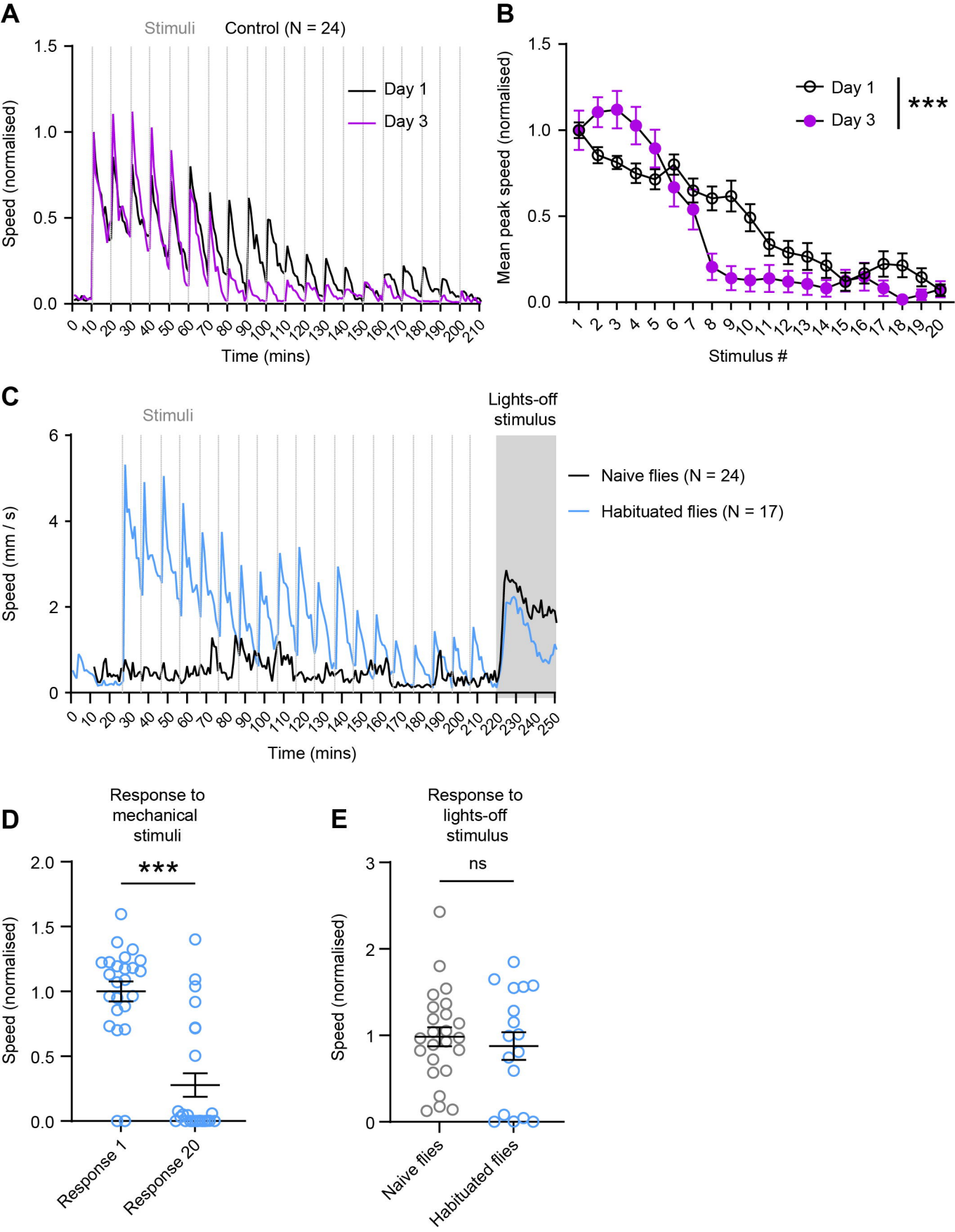

A
Stimuli
Control (N = 24)
Day 1
Day 3
Speed (normalised)
Time (mins)
B
Day 1
Day 3
***
Mean peak speed (normalised)
Stimulus #
C
Stimuli
Lights-off stimulus
Naive flies (N = 24)
Habituated flies (N = 17)
Speed (mm / s)
Time (mins)
D
Response to mechanical stimuli
***
Speed (normalised)
Response 1
Response 20
E
Response to lights-off stimulus
ns
Speed (normalised)
Naive flies
Habituated flies

Figure 4

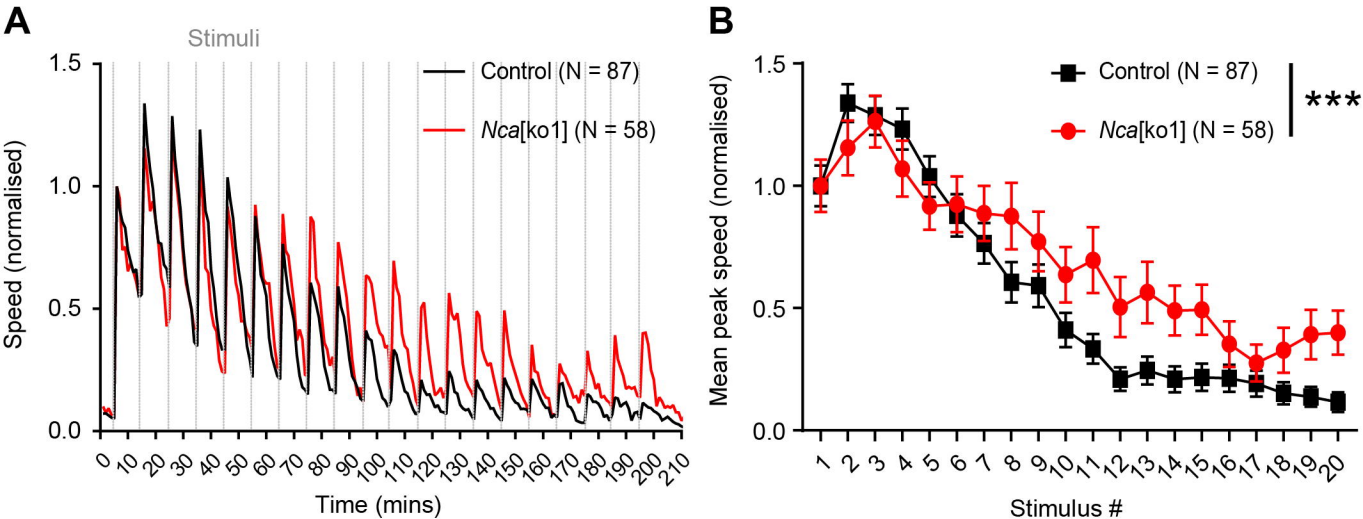

A
Stimuli
Control (N = 87)
Nca[ko1] (N = 58)
Speed (normalised)
1.5
1.0
0.5
0.0
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210
Time (mins)
B
Control (N = 87)
Nca[ko1] (N = 58)
***
Mean peak speed (normalised)
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Stimulus #

Figure 5

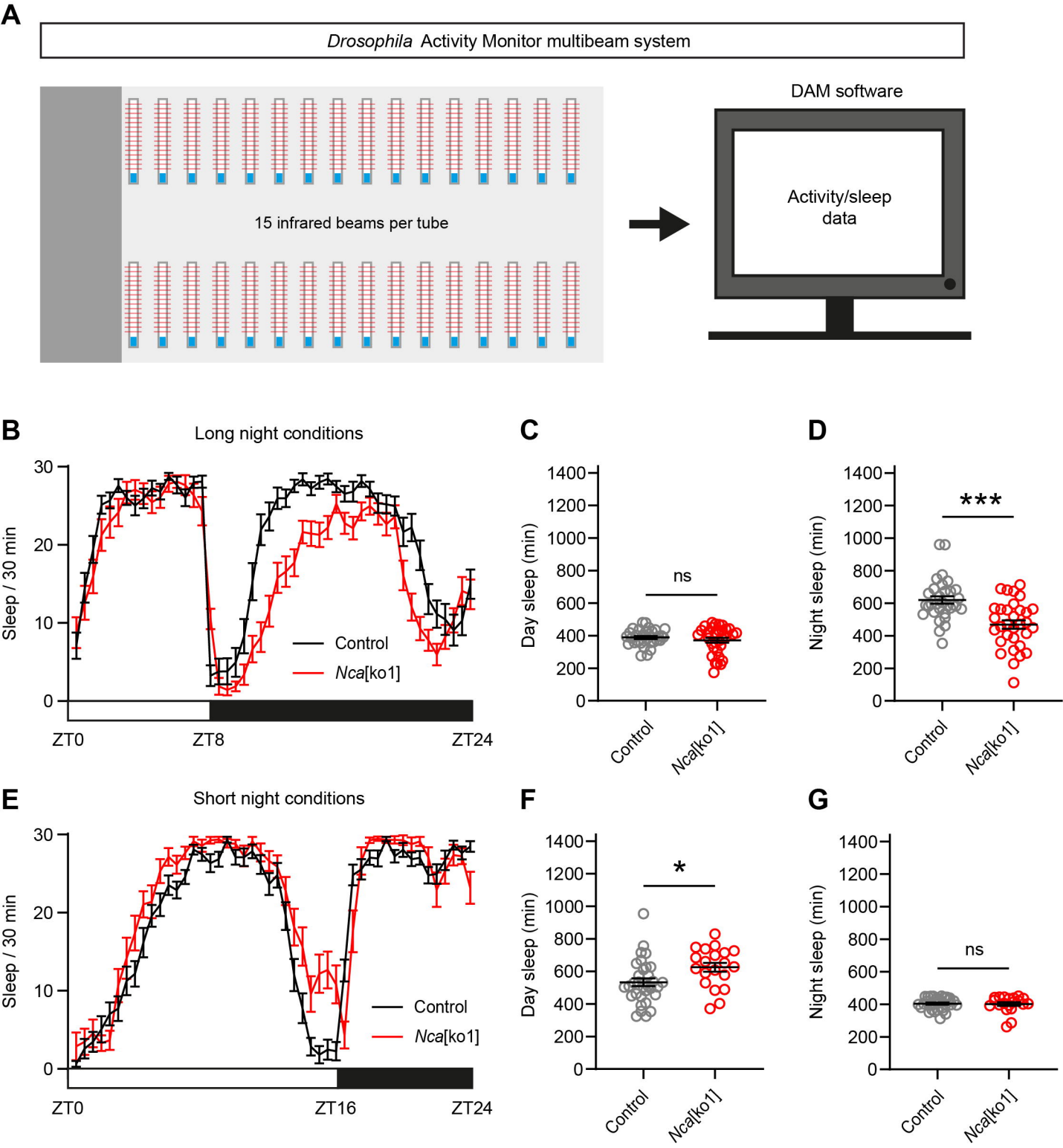

A
Drosophila Activity Monitor multibeam system
15 infrared beams per tube
DAM software
Activity/sleep data
B
Long night conditions
Sleep / 30 min
Control
Nca[ko1]
ZT0
ZT8
ZT24
C
Day sleep (min)
ns
Control
Nca[ko1]
D
Night sleep (min)
***
Control
Nca[ko1]
E
Short night conditions
Sleep / 30 min
Control
Nca[ko1]
ZT0
ZT16
ZT24
F
Day sleep (min)
*
Control
Nca[ko1]
G
Night sleep (min)
ns
Control
Nca[ko1]

Figure 6

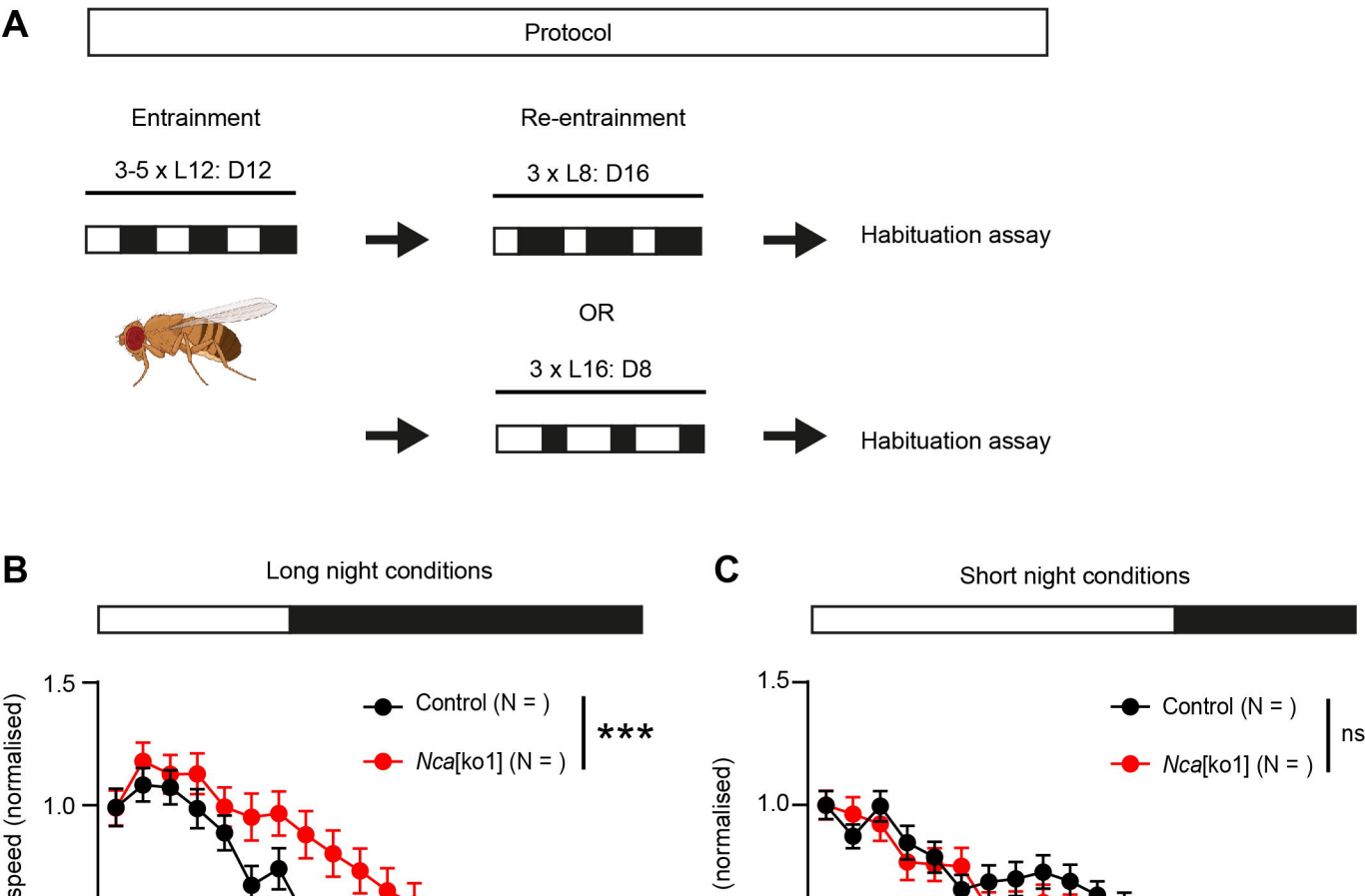
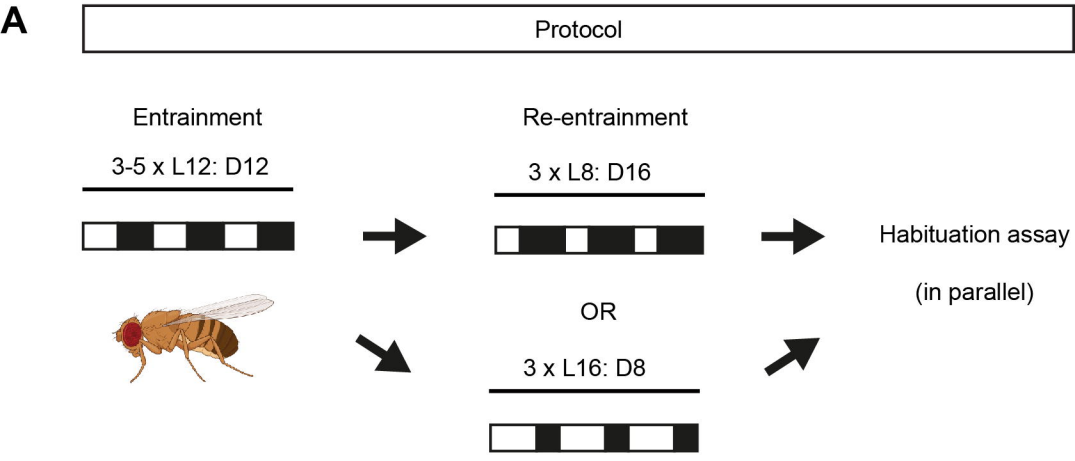
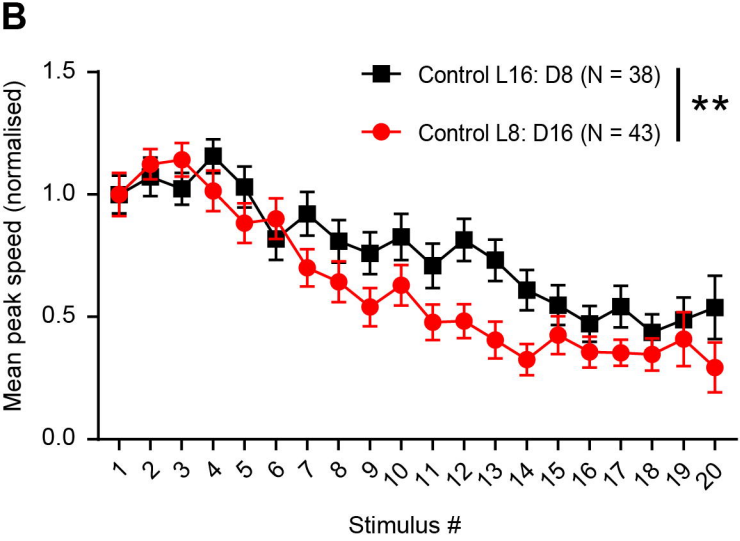

A
Protocol
Entrainment
3-5 x L12: D12
Re-entrainment
3 x L8: D16
Habituation assay
OR
3 x L16: D8
Habituation assay
B
Long night conditions
Control (N =)
Nca[ko1] (N =)
***
Mean peak speed (normalised)
Stimulus #
C
Short night conditions
Control (N =)
Nca[ko1] (N =)
ns
Speed (normalised)
Stimulus #

Figure 7


A
Protocol
Entrainment
3-5 x L12: D12
Re-entrainment
3 x L8: D16
OR
3 x L16: D8
Habituation assay
(in parallel)


B
Control L16: D8 (N = 38)
Control L8: D16 (N = 43)
**
Mean peak speed (normalised)
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Stimulus #