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Sensory processing during sleep in *Drosophila melanogaster*

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Alice S. French[1], Quentin Geissmann[2], Esteban J. Beckwith[3] & Giorgio F. Gilestro[1]✉

During sleep, most animal species enter a state of reduced consciousness characterized by a marked sensory disconnect. Yet some processing of the external world must remain intact, given that a sleeping animal can be awoken by intense stimuli (for example, a loud noise or a bright light) or by soft but qualitatively salient stimuli (for example, the sound of a baby cooing or hearing one's own name[1–3]). How does a sleeping brain retain the ability to process the quality of sensory information? Here we present a paradigm to study the functional underpinnings of sensory discrimination during sleep in *Drosophila melanogaster*. We show that sleeping vinegar flies, like humans, discern the quality of sensory stimuli and are more likely to wake up in response to salient stimuli. We also show that the salience of a stimulus during sleep can be modulated by internal states. We offer a prototypical blueprint detailing a circuit involved in this process and its modulation as evidence that the system can be used to explore the cellular underpinnings of how a sleeping brain experiences the world.

It was first shown in the 1960s that sleeping humans respond to the sound of their own names being called[3] and conceptually similar observations were later made in rats[4], cats[5] and primates[6]. To explore the neuronal underpinnings of this phenomenon in a genetically amenable model, we turned to the vinegar fly, *D. melanogaster*. The reaction of a sleeping fly to a mechanical stimulus varies with the intensity of the stimulus and the internal state of the animal[7], but evidence showing whether sleeping flies can also discriminate stimuli qualitatively is lacking. To address this, we built a robotic machine[8,9] that is able to selectively probe single flies with air puffs of identical mechanical intensity but different odour saliency (Fig. 1a). To modulate saliency, we initially chose acetic acid, the main component of vinegar, because, as the name suggests, it is an ecologically relevant odour for the vinegar fly *D. melanogaster*. Acetic acid is a byproduct of fermenting fruits and at lower concentrations (1–5%) acts as a strong attractant[10], promoting gathering and oviposition[11]. When the concentration of acetic acid increases, flies lose attraction to it especially if satiated[12], possibly because its valence changes to mimic the smell of less appealing spoiled fruits[13].

Perception of qualitative stimuli during sleep

In our prototypical experiment (Extended Data Fig. 1a), male flies that were inactive for at least 5 min were challenged with a gentle puff of air bubbled either through water as mock control or through different aqueous solutions of acetic acid at increasing concentrations. To limit the confounding of habituation, each experiment was run for no more than 6 h and multiple experiments were arranged in overlap to span through the entire 24 h (Extended Data Fig. 1b). Given that air puffs were administered only after 5 min of inactivity, the number of stimuli received varied across the day, reflecting the natural sleep pattern of the animals (Extended Data Fig. 1b). In control flies (grey in Fig. 1b), stimulation with mere humidified air was sufficient to elicit a baseline response during sleep. The attractive concentrations of acetic acid induced the strongest response (shades of blue in Fig. 1b), whereas the repulsive (10%) and neutral (30%) concentrations showed a response that was largely statistically similar or inferior despite the higher odour intensity (shades of purple in Fig. 1b). The response to salient stimuli was also dependent on the time of the day, with awakenings to 1% and 5% acetic acid being more likely during late-night sleep than during early-night sleep or siesta, when sleep pressure reaches its apex[9,14] (Fig. 1b). In accordance with our previous findings, where flies were probed mechanically[9], we found that siesta sleep at Zeitgeber time (ZT) 4–6 showed the highest arousal threshold (Extended Data Fig. 1c), yet still woke to 5% acetic acid. Analysis of sleep bout distributions suggests that the probability of waking to acetic acid decreases as sleep consolidation increases; flies slept longer and had more consolidated bouts in the early phase of the night than during the siesta or during the late phase of the night (Extended Data Fig. 1d), confirming that siesta sleep is more resistant to non-salient mechanical stimuli (such as a puff of air or a tube rotation[9]) but has a greater discriminatory power towards salience than deep, early-night sleep.

Internal states modulate the saliency of a stimulus

To extend the findings beyond acetic acid, we next screened a larger panel made of 26 odour conditions (12 compounds in a range of concentrations), chosen for their ecological nature and valence (that is, food or non-food; attractive, neutral or aversive; Supplementary Table 1). In satiated flies, 11 of the 26 odourants tested elicited a waking response greater than their respective controls at ZT18–20 (Fig. 2a). We found a mild but significant inverse correlation between the ability of a stimulus to wake up a fly and its associated preference index as determined

[1]Department of Life Sciences, Imperial College London, London, UK. [2]Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada. [3]Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), UBA-CONICET, Buenos Aires, Argentina. ✉e-mail: giorgio@gilest.ro

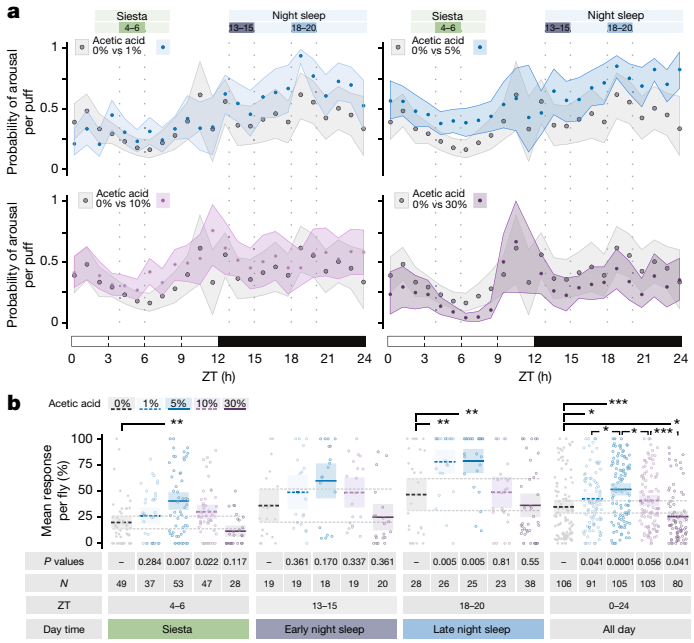

Fig. 1 | Sleeping *D. melanogaster* react to salient olfactory stimuli. **a**, Response to four different concentrations of acetic acid across 24 h. Grey indicates air control; blues indicate attractive concentrations of acetic acid (1% and 5%); and purples indicate aversive (10%) or neutral (30%) concentrations of acetic acid. The dots indicate the median response, and the shading indicates the 95% confidence interval (CI). **b**, Quantification of three time ranges from **a**: siesta (ZT4–6), early-night sleep (ZT13–15) and late-night sleep (ZT18–20). The error bars represent bootstrapped 95% CI. The horizontal dashed grey lines indicate 95% CI of the control for comparison. The number of animals (*N*) and the *P* values versus air control are shown below. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

through choice in a trap assay; odourants that were avoided in a trap assay were more likely to wake satiated flies than those that were attractive or neutral (Fig. 2b).

In humans, the ability to process information during sleep can be modulated by experience or internal states: humans will react more promptly when sleeping in an unfamiliar location[15] and first-time parents unlock the ability to recognize their baby's cry during the night[16]. Here we wanted to investigate whether flies could also modulate their response to odour during sleep. To address this, we subjected animals to different treatments aimed at changing their internal states (Fig. 2, Extended Data Fig. 2) and measured their sensory perception during the following sleep. We started with a simplistic alteration: ethanol. In humans, ethanol is one of the most commonly used sedatives and its acute consumption is well known to modify sleep pattern, increasing sleep depth and reducing arousal threshold[17]. We exposed flies to ethanol vapours for 1 h and then, after a 90-min recovery, we probed their response to 5% acetic acid during sleep. In line with what has been observed in mammals, we found that flies became less responsive to a stimulus that would have woken them when sober (Extended Data Fig. 2b). We next manipulated sleep pressure by mechanically forcing flies to stay awake for the entire night, and investigated their response during rebound sleep the following morning. After forced wakefulness, flies became less responsive to 5% acetic acid than their mock rested control counterparts (Extended Data Fig. 2c). These experiments establish that the arousal threshold of a sleeping fly can be modulated by internal states and speaks in favour of an evolutionary conservation of the underpinnings. Nothing, however, can yet be extrapolated about the specificity of this phenomenon, which is arguably the most interesting aspect: whether internal states can modulate saliency recognition during sleep (for instance, whether a sleeping fly previously starved will increase its sensory acumen towards food-related odours only). To address this, we briefly deprived flies of nutrients and probed them with our panel of odours during sleep, focusing once more on the late-night window (Fig. 2c, d). Starved flies were woken more readily by some (16) but not

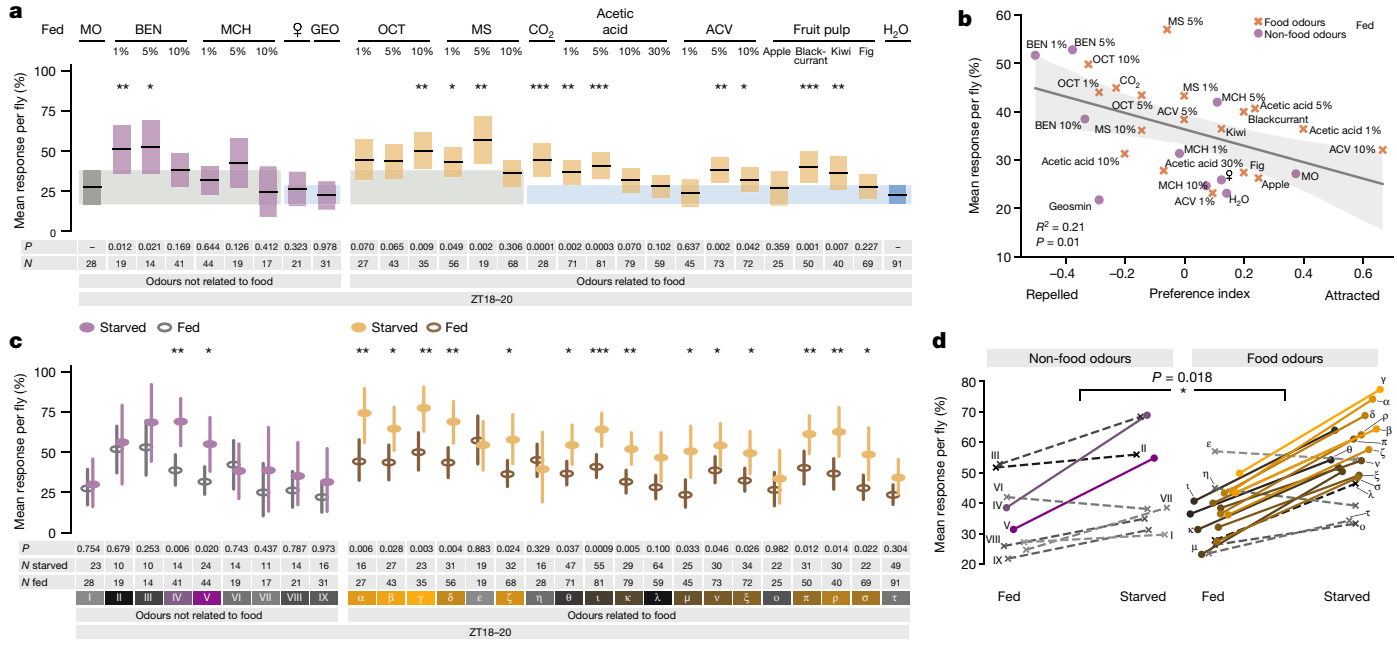

Fig. 2 | Subconscious processing of information can be modulated by internal states. **a**, Mean response per fly to a panel of odourants between ZT18 and ZT20 under fed conditions. The shaded horizontal bars indicate the diluent used (blue: water; grey: mineral oil). The graph shows mean and 95% CI. Odorants are separated into non-food-related (left) and food-related (right). **b**, Regression analysis between the preference index determined in a trap assay and the response to odour in the sleep arousal assay. The shaded grey area indicates the 95% CI. Abbreviations in panels **a** and **b** are included in Supplementary Table 1. **c**, Mean response per fly between ZT18 and ZT20 under fed (filled ellipse) or starved (empty ellipse) conditions. *N* and *P* values are shown below, with a legend for **d**. The chart indicates mean and 95% CI. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **d**, Summary of responses to non-food-related (left) and food-related odours (right) under fed and starved conditions. The dashed lines indicate *P* > 0.05.

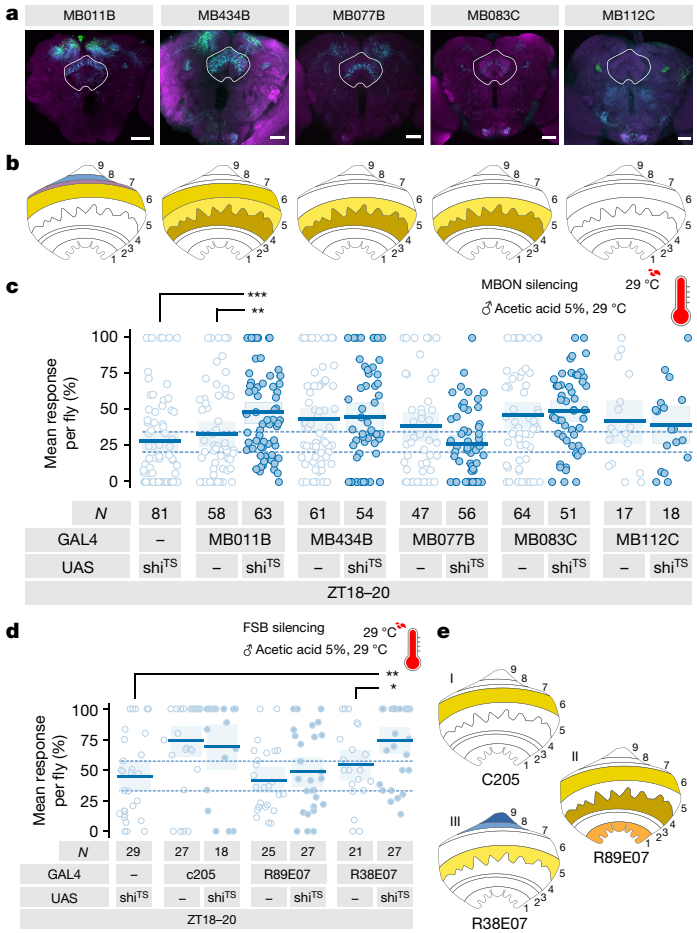

Fig. 3 | Genetic manipulation of MBONs and their targets in the FSB modulates waking. a, Maximum intensity projections showing trans-synaptic mapping of five MBONs using trans-Tango. MBONs are shown in green and postsynaptic neurons in cyan. Scale bars, 50 µm. **b**, Schematic of the FSB target layers of each MBON. **c**, Waking responses at 29 °C in parental control flies and flies with thermo-inhibited MBON neurons to 5% acetic acid between ZT18 and ZT20.The horizontal hashed blue line indicate 95% CI of the UAS-Shi^{TS} control for comparison. **d**, Waking responses at 29 °C in parental control flies and flies with thermo-inhibited FSB neurons to 5% acetic acid between ZT18 and ZT20. **e**, Expression pattern of the GAL4 lines tested in **d**. *P < 0.05, **P < 0.01, ***P < 0.001.

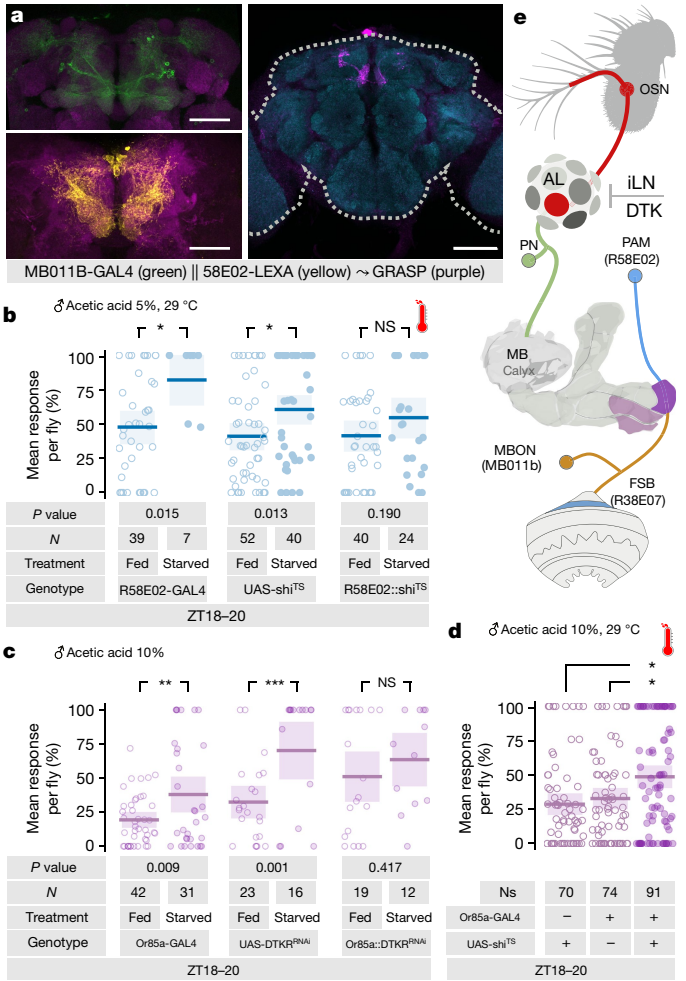

Fig. 4 | At least two check points gate waking responses to odours. **a**, Expression pattern of R58E02-LEXA (bottom left, yellow), MB011B-GAL4 (top left, green) and their GRASP intersection (right, purple). Scale bars, 50 µm. **b**, Waking responses of parental controls and PAM-inhibited flies to 5% acetic acid under fed and starved conditions. **c**, Waking responses of parental control and experimental flies with DTK signalling knocked down in Or85a OSNs to 10% acetic acid under fed (left) and starved (right, dashed box) conditions. NS, not significant. **d**, Waking responses at 29 °C in parental control flies and with thermo-inhibited Or85a OSN. **e**, Summary of the neuronal circuit. *P < 0.05, **P < 0.01, ***P < 0.001.

all (28) odourants tested and, notably, this effect was largely confined to food-related odours (14 out of 18) and less to non-food-related odours (2 out of 8; Fig. 2c, d). The observation that not all odourants elicited a higher waking response under starved compared to fed conditions is an important one and points to a mechanism that allows for specific modulation of sensory discrimination rather than a global adjustment in arousal: starved flies are not merely more aroused by just any stimulus, they are woken more easily specifically by food-related odours and continue to sleep when challenged with other odours. The fact that we can now study this phenomenon in a genetically amenable organism prompted us to delve into the underlying neuronal circuitry.

Neurons regulating valence perception during sleep

In *Drosophila*, the antennal lobe (AL) receives and collates olfactory signals from the peripheral olfactory sensory neurons (OSNs) and relays organized information via projection neurons (PNs) towards the calyx of the mushroom bodies (MBs)[18], where deeper information processing takes place. Neurons in the calyx interact with a sparse group of mushroom body output neurons (MBONs) that are known to modulate odour valence[19] and novelty[20]. When trying to identify neurons that could convey odour information to a sleep centre, MBONs are therefore excellent candidates. We used a trans-synaptic labelling technique to screen for MBONs that make anatomical connections to known sleep regulatory centres, finding four that synapsed with neurons located on different layers of the fan-shaped body (FSB; Fig. 3a, b), a known sleep regulatory area in the fly brain[21]. This anatomical connection suggested that the ability of a fly to encode valence during sleep could rely on MBON signalling to the FSB. We screened our four candidates and a negative control using thermogenetic neuronal inactivation (Fig. 3c, Extended Data Fig. 3), seeking to identify neurons that would change arousal responses when silenced. We found one such neuron, MB011B, whose inactivation led to a specific increase in arousal to 5% acetic acid (Fig. 3c). Downstream, we selectively inhibited different layers of the FSB[22] and found a specific GAL4 line (R38E07) that, when silenced, led to an increase in arousal to 5% acetic acid (Fig. 3d). R38E07 neurons were previously shown to innervate layers five, eight and nine of the FSB[22], the same area that appears to be targeted by MB011B (Fig. 3a, b versus Fig. 3e).

Inactivation of MB011B makes flies more responsive to salient stimuli, and this may happen through specific modulation of sensory perception or, simply, by making flies hyperaroused. To address this, we monitored how thermogenetic manipulations of MBONs affected baseline sleep and found that inhibition of MB011B had, if anything, the opposite effect, leading to an increase in night sleep (Extended Data Fig. 4). We also investigated the role of the dorsal FSB neurons (another important sleep-regulating cluster of the central complex[23]) labelled by 23E10 GAL4 and found no role for those neurons in olfactory processing during sleep (Extended Data Fig. 5). These data suggest that processing of information during sleep is a neuronal feature naturally modulated through a specific circuit and neurons that control the amount of sleep are not necessarily controlling salient arousal, and vice versa. Internal states and previous experience can gate this circuit in different directions; to explore how this may happen, we first looked at a cluster of dopaminergic neurons (PAM) known to target the γ-lobe of the mushroom bodies, including the γ5 area in which MB011B dendrites are found. Previous work has shown that PAM neurons are important for assigning positive valence to odours during associative memory formation and work as modulators of MBON activity[24]. We first explored the anatomical connectivity between MB011B and the PAM cluster using a green fluorescent protein (GFP) reconstitution assay (GRASP). Synapses were observed in an area compatible with γ5, confirming that MB011B receives input from the PAM neurons (Fig. 4a). In the simplest hypothesis of a gate model, silencing of PAM neurons during sleep should modulate the response of the fly by counteracting the normally arousing properties of starvation. This was precisely what we observed (Fig. 4b).

PAM neurons may act deep in the signalling cascade to modulate state-dependent salient arousal, but we wanted to investigate whether a gate point could act even earlier in the pathway. In *D. melanogaster*, acetic acid is sensed by two distinct olfactory receptors responding to different concentrations of odorant and carrying symmetrical information: Or42b responds to low concentrations of acetic acid and encapsulates attraction, whereas Or85a is engaged by higher concentrations and modulates aversion[25]. Upon starvation, the activity of Or85a is suppressed by tachykinin (DTK) (released from inhibitory lateral neurons (iLNs)) and its receptor (DTKR), meaning that starved flies will gain an attractive response also towards a high concentration of acetic acid[26]. We used RNA interference to knock down the expression levels of DTKR in the Or85a-expressing neurons and then measured the arousal responses during sleep in starved versus fed flies (Fig. 4c). Starved DTKR-knockout flies retain their metabolic impairment due to lack of feeding, and this manipulation allows us to specifically test the role of the gate point while keeping intact the metabolic alterations of starvation. Knock-down of DTKR in Or85a neurons was sufficient to mask the effect of starvation and restored the aversion to higher concentrations of acetic acid (Fig. 4c). Conversely, thermogenetic silencing of Or85a neurons tipped the circuit towards attraction, causing sleeping flies to wake even to a higher concentration of acetic acid (Fig. 4d).

Discussion

Together, these results highlight a circuit that specifically modulates sensory perception during sleep, altering the behavioural response of the animal according to its ecological needs, and is modulated through at least two gate points: a peripheral gate point that takes action in the OSNs and a central gate point in the depth of the mushroom bodies (Fig. 4e). A sleeping brain is one that can still process sensory information and discriminate between relevant and irrelevant stimuli. We can remain sound asleep in front of a TV playing an action movie, and yet wake upon the perception of a quieter but relevant stimulus, such as the sound of our own name being called or a baby cooing. We described a reductionist model of this phenomenon, providing a behavioural paradigm that works in the vinegar fly and drafting a functional circuit of sensory processing that connects peripheral olfactory input to sleep regulatory neurons. While the anatomical description of this circuit is certainly different from anything described in humans, its functional properties are possibly overlapping. In this age of renaissance in *Drosophila* neurobiology, flies could provide a convenient model to study how information processing changes during sleep: a reductionist playground to study the basics of consciousness.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03954-w.

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Methods

Fly strains

The following strains were used in this study: Or85a-GAL4 (#23133), MBON-GAL4s (#68294, 68325, 68287, 68283 and 68263), R23E10-GAL4 (#49032), R58E02-GAL4 (#41347), R58E02-LexA (#52740), 89E07-GAL4 (#40553), 38E07-GAL4 (#50007), C205-GAL4 (#30826) and GRASP (#79039) were obtained from Bloomington Drosophila Stock Centre; the trans-Tango lines (CS; UAS-myrGFP; QUAS-mtdTomatoX3HA), UAS-Shi^{TS} and UAS-TRPA1 from J. Jepson (University College London, UK); UAS-DTKR2-RNAi (#55732) from M. Alenius (University of Umeåm, Sweden); and the CantonS strain originally from R. Stanewsky (Münster University, Germany). Flies were raised on polenta and yeast-based fly media (agar 96 g, polenta 240 g, fructose 960 g and Brewer's yeast 1,200 g in 12 litres of water). CS; UAS-myrGFP; QUAS-mtdTomatoX3HA; trans-Tango < MBON-GAL4, UAS-Shi^{TS} < MBON-GAL4, UAS-TRPA1 < R58E02-GAL4 and UAS-Shi^{TS} < FSB-GAL4 were raised at 18 °C. Otherwise flies were raised at 25 °C, 65% humidity and under a regime of 12 h:12 h light:dark. With the exception of CS; UAS-myrGFP; QUAS-mtdTomatoX3HA; trans-Tango < MBON-GAL4, which were aged for 25–30 days, flies were 2–3 days old at the start of the experiment.

Behavioural experiments

Odourants. Mineral oil (30779, Sigma), acetic acid (A6283, Honeywell), benzaldehyde (418099, Sigma), methyl salicylate (M6752, Sigma), 4-methylcyclohexanol (153095, Sigma), 1-octanol (Alfa Aesar), gesomin (G5908) and Aspall Organic Cyder vinegar (Waitrose) were used. Fruits were bought from Sainsbury's and liquified in a blender. Blackcurrants were hand-picked from Crockford Bridge farm, Weybridge, UK.

Arousal experiments. Male flies were cold anaesthetized and placed in 140-mm-long glass tubes with food, traversed by a 5-µl capillary, at one end and a 30-mm-long piece of hollow silicone tubing serving as a plug at the other. Tubes were placed into customized arenas, which were inserted into ethoscopes and connected to air/gas/odour (AGO) modules. In all behavioural experiments, with the exception of those involving starvation (where individuals were placed in food or agar tubes 9 or 18 h before receiving puffs), flies were allowed to acclimatize to their environment for at least 2 days. After this baseline period, 5-s puffs of air or odour were delivered to flies immediately following 5 min of immobility at a flow rate of 0.4 l per min. Whether a fly moved in the 10 s following the onset of a stimulus delivery was recorded. If it moved above a predefined velocity threshold (which was validated using human-generated ground truth), it was deemed to have woken. Because each fly could receive multiple puffs within its stimulus window, a mean response proportion per time bin, per fly was calculated. Flies received puffs at different times of the day or night according to the experiment. All regular behavioural experiments were conducted at 25 °C, whereas thermogenetic activations were performed at 29 °C.

Ethanol exposure. On experimental day, CS male flies received 5-s puffs of 20% ethanol (VWR 20821) or humidified air each time they were immobile for 2 min between ZT14.5 and ZT15.5. Flies were then allowed 90 min of recovery time before each of the two groups received puffs of 5% acetic acid following each 5-min immobility bout between ZT18 and ZT20.

Sleep disturbance. CS male flies received puffs of humidified air each time they crossed the midline of the tube between ZT12 and ZT24. Typically this induced periods of increased activity and reduced sleep particularly during active phases. Control flies were undisturbed. Between ZT0 and ZT6, flies received puffs of 5% acetic acid following each 5-min immobility bout.

Starvation. CS, R23E10-GAL4<UAS-Shi^{TS}, UAS-Shi^{TS}<+, R23E10<UAS-TRPA1, UAS-TRPA1<+, Or85a-GAL4<UAS-DTKR-RNAi, Or85a-GAL4<+ and UAS-DTKR-RNAi<+ flies were placed in food or agar tubes 9 h before receiving puffs of odour at 25 °C or 29 °C (in the case of thermogenetic experiments). A 9-h window of starvation was chosen because it did not drastically influence baseline sleep[27]. For starvation experiments involving GMR58E02-GAL4, 18 h of starvation was used. Our own and previous work has shown that raising flies at lower temperatures increases tolerance to starvation. Note that the puff stimulation period lasts 2 h, during which flies are not provided with food, so the experiment starts with 9 h of starvation and ends with 11 h of starvation.

Trap assays for calculating preference index. Flies were collected and placed in fresh food vials in groups of 20 males at least 24 h before the trap assay. At ZT18, flies were introduced into the trap assay without anaesthesia through a hole in top of the assay (under infrared light conditions as it was during dark phase). Traps were sealed and placed in a dark incubator at 25 °C. The number of flies in the odour and control vial was counted after 2 h (ZT20). The preference index (PI) was calculated as follows: PI = (odour_vial − control_vial)/(odour_vial + control_vial). Traps were made by placing 50 µl of odourant solution (in water or mineral oil) inside a 2-ml glass vial (Chromacol VAGK, CERT5000-79, Thermo Scientific), which was then placed together with the flies inside a 100-ml academy low-form beakers (A/2218/100, Rapid), covered by a custom 3D-printed element (stand, lid and funnel; available at http://lab.gilest.ro/ethoscope).

Olfactometer. Chemotaxis experiments were carried out in a trap assay with the exception of 2% CO_2. Flies were collected and placed in fresh food vials in groups of 40 males at least 24 h before the olfactometer assay. At ZT18, flies were introduced into the olfactometer arenas without anaesthesia through a hole in the top of the assay (under IR light conditions as it was during dark phase). Flies were allowed to acclimatize to the arena for 30 min. From this point, flies were filmed using an ethoscope. Flies were initially exposed to air in all four corners for 5 min and then 2% CO_2 was introduced into one corner of the arena for a further 5 min. Videos were then scored manually. The number of flies in the CO_2 corner pre-stimulus and during stimulus was recorded every 30 s. The number of flies observed in the odour corner during each time bin was used to calculate an average value for pre-stimulus and post-stimulus. Using these average values, a PI was calculated as follows: PI = (*N* in odour_corner (during stimulus) − *N* in odour_corner (pre-stimulus))/(*N* in odour_corner (during stimulus) + *N* in odour_corner (pre-stimulus)). Olfactometers were custom built following descriptions from Lin et al[28]. Detailed instructions are available at https://lab.gilest.ro/ethoscope.

Trans-tango. CS; UAS-myrGFP; QUAS-mtdTomatoX3HA; Trans-Tango < MBON-GAL4 flies were raised at 18 °C for 25–30 days before dissection. Flies were cold immobilized on ice and their brains were dissected in 0.01 M PBS. Brains were then fixed in 4% paraformaldehyde for 20 min and washed 3× 10 mins in 0.3% PBST (PBS with Triton-X). Brains were then blocked for 1 h in 5% normal goat serum (NGS; Ab7481, Abcam) and incubated for 2 nights in 1:10 mouse anti-nc82 (Ab 2314866, DSHB), 1:200 rat anti-HA (ROAHAHA, Merck), 1:200 rabbit anti-GFP (ab6556, Abcam) in 5% NGS in PBST at 4 °C. The following, day brains were washed 3× 10 min in PBST and incubated of 2 days in 1:200 anti-mouse Alexa Fluor 568 (Ab175473, Abcam), 1:200 goat anti-rat Alexa Fluor 647 and 1:200 goat anti-rabbit Alexa Fluor 488 (Ab150077, Abcam) at 4 °C. Brains were mounted on microscope slides in vectashield (Vector Laboratories) and imaged using a Leica SPF inverted confocal microscope. A ×20 lens was used to capture confocal Z stacks of dorsal and ventral brain regions. Four images were averaged at acquisition and Z stacks were analysed using LASX v3.52.18963 and imageJ v1.8.0.172[29]. Stacks were converted into maximum intensity projections. The protocol was performed as previously described[30].

GRASP. GMRR58E02-GAL4/UAS-GFP, MB011B-GAL4/UAS-GFP and GMRR58E02-lexA/UAS-post-t-GRASP, LexAop2-pre-t-GRASP; MB011B-GAL4 male flies were raised at 25 °C and dissected 5 days after eclosion. Immunohistochemistry procedure, image capture and processing were performed as described for the trans-tango experiments. The following antibodies were used: 1:10 mouse anti-nc82 (Ab 2314866, DSHB), 1:200 rabbit anti-GFP (ab6556, Abcam), 1:200 anti-mouse Alexa Fluor 568 (Ab175473, Abcam) and 1:200 goat anti-rabbit Alexa Fluor 488 (Ab150077, Abcam). The protocol was performed as previously described[31].

Statistics, data availability and reproducibility. All ethoscope data were analysed using rethomics[32]. Statistical comparisons were performed as indicated in the text and figure legends, mostly using Wilcoxon rank-sum test with false rate discovery correction, with the only exception of a generalized linear model for Fig. 2d. In all summary plots, the intermediate reference mark indicates the mathematical mean and the surrounding error estimates always indicate the bootstrapped 95% confidence intervals. Whenever possible, the entire dataset is shown as a dot plot. All figures explicitly mention the biological *N*, that is, the number of biologically independent animals for each data point. Each conclusion relies on multiple independent experiments and never fewer than three independent experiments; sample size was estimated based on previous experience and it is above power in all cases; in all experiments, randomization was used to scatter experimental lines throughout different ethoscopes or conditions. The actual number of experiments for each panel can be found in the metadata descriptions that are supplied along with the R and Python scripts. Unless differently stated in the legend, all *P* values arise from Wilcoxon rank-sum tests. *P* values are intended to be supportive and indicate where statistical significance occurs in the presence of slight confidence interval limit overlap. In all figures, the asterisks are used to indicate customary thresholds of statistical significance: $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$. The actual numerical *P* value is shown in each figure whenever possible and full statistical comparisons among all combinations are available as Supplementary Information in a dedicated file. Moreover, all the scripts (in R and Python3) used to generate the figures in this paper as well the related statistical analysis and the original behavioural raw data as obtained with ethoscopes are publicly available through the Zenodo repository[33]. All the hardware and software created in the laboratory is open source and can be explored at http://lab.gilest.ro/ethoscope[1] and http://lab.gilest.ro/rethomics[32]. Rethomics versions used to analyse the data were as follows: behavr: 0.3.2; sleepr: 0.3.0; zeitgebr: 0.3.3; ggetho: 0.3.4; scopr: 0.3.3.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All raw data used for analysis are made available through the Supplementary Information and through a Zenodo repository (https://doi.org/10.5281/zenodo.5109970). Source data are provided with this paper.

Code availability

All scripts used for analysis are made available through the Supplementary Information and through a Zenodo repository (https://doi.org/10.5281/zenodo.5109970).

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Author contributions A.F. and E.J.B. performed all of the experiments. Q.G. wrote the software for the collection and analysis of the data. A.F. and G.F.G. analysed the data. A.F. and G.F.G. devised and planned all of the experiments. All authors contributed to the preparation of the manuscript.

Competing interests The authors declare no competing interests.

Additional information

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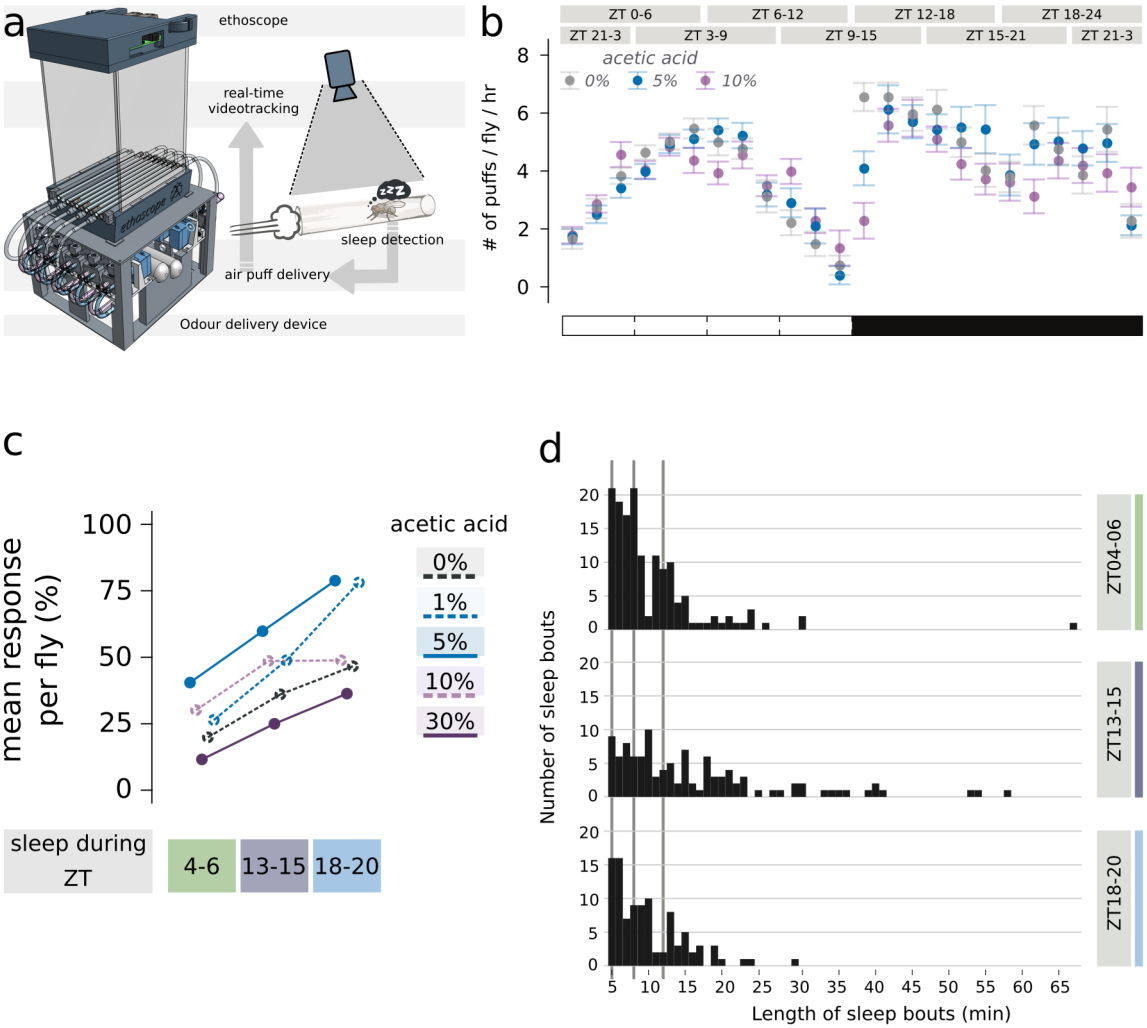
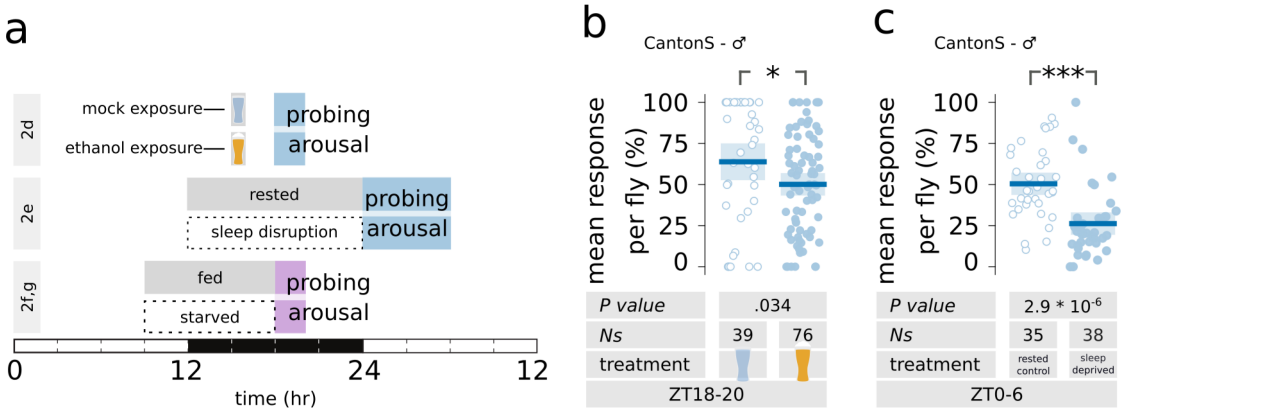
Correspondence and requests for materials should be addressed to Giorgio F. Gilestro.

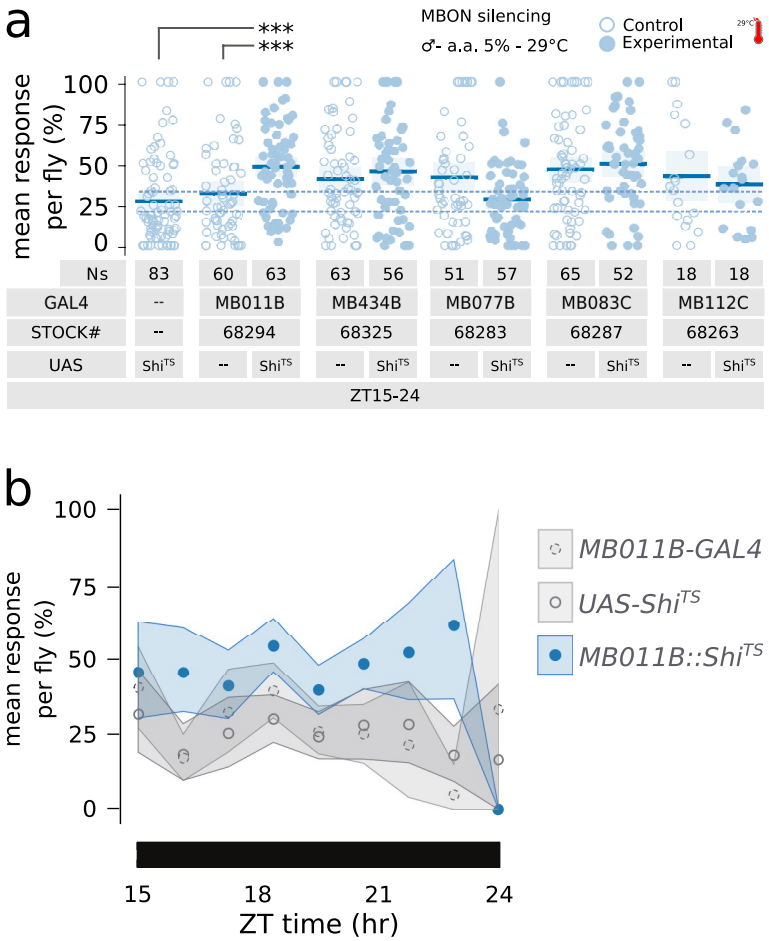
Peer review information *Nature* thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.



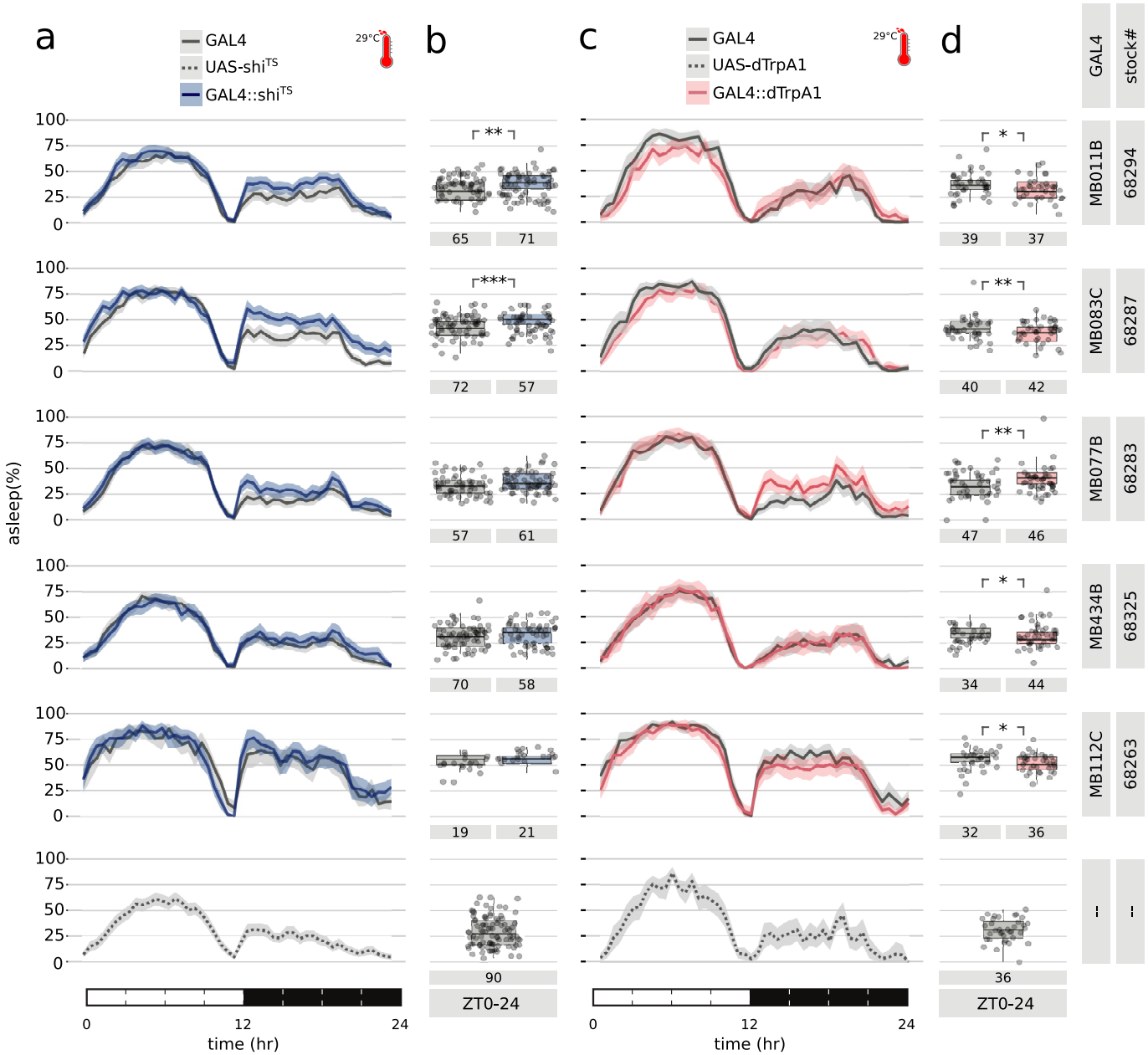
Extended Data Fig. 1 | Occurrence and length of sleep bouts vary during the day. a, Schematics of the experimental setup. **b**, Average numbers of puffs per fly per hour in three conditions (0%; 5%; 10% acetic acid. Ns 106, 105, 103 respectively). The top grey bars show how experiments were conducted at eight independent, overlapping intervals. **c**, Mean response per fly at different time point during the day, grouped by concentration of acetic acid. Same dataset as in Fig. 1d. **d**, Total distribution of sleep bouts by length, binned at 1-minute intervals during three time points representing different types of sleep (ZT 4-6, ZT 13-15 and ZT18-20). The three grey vertical lines indicate the bins for 5-, 8-, and 12-minutes sleep respectively as chosen for the analysis in Fig. 1c–e. In all panels, errors are shown as bootstrapped 95% CI.



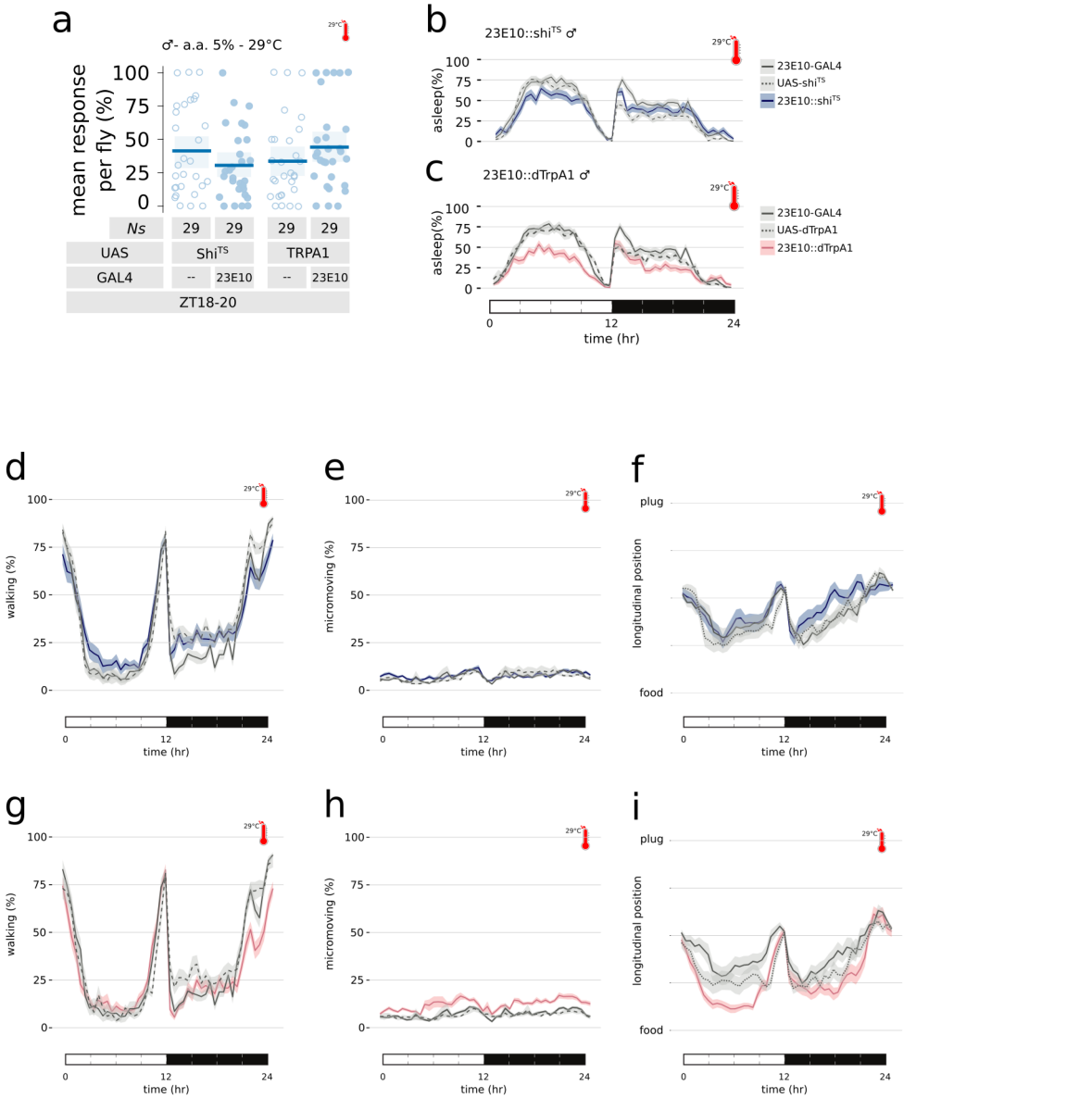
Extended Data Fig. 2 | Changes in internal states affects arousal threshold during sleep. a, Schematic showing experimental procedures used in **b**, **c** and main Fig. 2a, b. **b**, Responses to 5% acetic acid in mock control flies (left) and flies previously intoxicated with vapours of 20% ethanol (right). Ethanol or mock exposure were limited to a 1-hour window between ZT15.5-16.5 and arousal was quantified and shown for ZT18-20. **c**, Quantification of the stimulus-evoked response in rested control flies (left) or flies that were previously deprived of sleep for 12h (right). Sleep deprivation was performed during the subjective night (ZT12-24) and waking to an olfactory stimulus (5% acetic acid) was measured during the subsequent day (ZT0-6). In all panels, errors are shown as bootstrapped 95% CI.



Extended Data Fig. 3 | Inhibition of MB011B reduces arousal threshold. **a**, Waking responses of control flies (MBON-GAL4/+) and those with temperature inhibited MBONs (MBON-GAL4/Shi^{TS}) to 5% acetic acid between ZT15-24. **b**, 9 h response profile for MB011B Shi^{TS} siliencing (light blue) compared to its appropriate parental controls (shades of grey). In all panels, errors are shown as bootstrapped 95% CI.



Extended Data Fig. 4 | Activation or inhibition of some MBONs modulates sleep. a, **b**, 24h sleep profiles (**a**) and mean sleep amount (**b**) exhibited by flies with inhibited MBON neurons (MBON/Shi^{TS}: blue) or those carrying only the MBON-GAL4 (MBON-GAL4/+: grey solid) or only UAS-Shi^{TS} (UAS-Shi^{TS}/+: grey hashed) transgene over a 24hr baseline day. Ns underneath indicate the number of individual flies and refer to **b** and **a**. **c**, **d**, 24hr sleep profiles (**c**) and mean sleep amount (**d**) exhibited by flies with activated MBON neurons (MBON/dTRPA1:red) or those carrying only the MBON-GAL4 (MBON-GAL4/+: grey solid) or only UAS-dTRPA1 (UAS-dTRPA1: grey hashed) transgene over a 24hr baseline day. Ns underneath indicate the number of individual flies and refer to **c**, **d**. In all panels, errors are shown as bootstrapped 95% CI.



Extended Data Fig. 5 | Activation or inhibition R23E10 neurons alters behavior. a, Waking responses of control flies (UAS-Shi^{TS} and UAS-TRPA1) and those with temperature manipulated dFSB neurons (23E10-GAL4/Shi^{TS} and 23E10-GAL4/dTrpa1) to 5% acetic acid, between ZT18-20. Experiment performed at 29 °C. **b**, 24h sleep profile of dFSB inactivation through Shi^{TS} (blue) compared to parental controls (grey). **c**, 24h sleep profile of dFSB forced activation through dTRPA1 (red) compared to parental controls (grey). **d**–**f**, Walking (**d**), micromovements (**e**) and X-position profiles (**f**) of control flies (UAS-Shi^{TS}: grey hashed, R23E10-GAL4: grey) and those with inhibited dFSB neurons (R23E210-GAL4/UAS-Shi^{TS}) over a 24 h baseline day. **g**–**i**, Walking (**g**), micromovements (**h**) and X-position profiles (**i**) of control flies (UAS-TRPA1: grey hashed, R23E10-GAL4: grey) and those with activated dFSB neurons (R23E210-GAL4/UAS-TRPA1) over a 24 h baseline day. Experiments preformed at 29 °C on 2-3 day old male flies in 12h:12h L:D cycle. In all panels, errors are shown as bootstrapped 95% CI.

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Corresponding author(s): Giorgio Gilestro

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☒	☐	A description of all covariates tested
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☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	All behavioural data were collected using Ethoscopes, an open source software and hardware platform created by our lab and freely available (see Geissmann et al PLoS Biol 2017).
Data analysis	Behavioural data were analysed using rethomics, an open-source R based package for analysis of high throughput behavioural data (see Geissmann et al PLoS ONE 2019). The versions of R packages used are as follows: behavr (0.3.2) , sleepr (0.3.0), zeitgebr(0.3.3), ggetho (0.3.4), scopr (0.3.3). Confocal images were collected using LSM confocal and their proprietary software (Leica Application Suite 3.52.18963) and analysed using ImageJ in the FiJi distribution (Image J bundled with 64-bit Java 1.8.0_172).

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All the scripts (in R and Python3) used to generate the figures in this manuscript as well the related statistical analysis and the original behavioural raw data as obtained with ethoscopes are publicly available through the Zenodo repository doi:10.5281/zenodo.5109970

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	In all cases, sample sizes were chosen so to satisfy and exceed thresholds for statistical power. Generally, sample sizes were chosen based on the number of each condition/genotype being tested vs the experimental limitations (e.g. throughput of the experiment in the behavioural modules).
Data exclusions	Some data point had to be excluded a priori from the analysis, mostly due to hardware failures. This was always indicated in the appropriate metadata files which are integral part of the raw data publicly available through the zenodo repository. In all the relevant metadata files there is a column called "status". Data was included if the status was marked "ok". "not_ok" would indicate when there was a hardware failure on AGOs, for example if a servo motor controlling the delivery of odour to an individual fly was broken. Other examples of "not_ok" would be if the experiment or lighting was disrupted by power cut or malfunctioning of light times.
Replication	All data were reproduced. At least 2 replicates were performed for each experiment.
Randomization	Flies were spread out randomly through the machines. Each delivery machine could stimulate up to 10 flies in each experiment and when multiple genotypes were compared, they were evenly distributed in each machine (e.g. 5 genotypes per machine, 2 flies per genotype)
Blinding	Experimenters were not blinded to condition/genotype however data collection was done in non biased manner. i.e data were collected by Ethoscope software and analyzed using rethomics.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
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☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
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Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	anti-nc82 (Ab 2314866, DSHB), rat anti-HA (ROAHAHA, Merck), rabbit anti-GFP (ab6556, abcam),Anti-mouse Alexa Fluor 568 (Ab175473, Abcam), Goat anti-rat Alexa Fluor 647 (Ab150167), Goat anti-rabbit Alexa Fluor 488 (Ab150077, Abcam) were the primary antibodies used in this study. The concentrations are indicated in the methods section for each antibody used.
Validation	All antibodies used in this study were commercially developed. Validation statement, detailed instruction, and multiple published references of each antibody used in this study are available on the manufacturers' websites

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Laboratory animals	Various wildtype and transgeneic strains of Drosophila melanogaster were used in this study. These were obtained mostly from Bloomington Drosophila Stock centre or members of the Drosophila scientific community. Origin of strains used are indicated in the methods section under "Fly strains" .
Wild animals	No wild animals were used, only laboratory strains of Drosophila melanogaster.

Field-collected samples	N/A
Ethics oversight	No ethical approval or guidance was required because the use of Drosophila melanogaster is not regulated by The Animals (Scientific Procedures) Act 1986.

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