

Aversive learning hijacks a brain sugar sensor to consolidate memory

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The formation of food-related memories involves post-ingestion nutrient sensing signals^{1–5}. Whether nutrient sensors act beyond feeding-relevant behaviour is less well understood. Here we show that an internal sugar sensor in the *Drosophila* brain⁶ is involved in memory consolidation, both in fasted flies subjected to an appetitive learning task involving a sucrose reward and in flies fed ad libitum subjected to an aversive learning task independent of food cues^{7,8}. In the latter, spaced repetition of learning sessions, a prerequisite to induce long-term memory, lures brain fructose-sensing neurons into a fasted state through a disinhibition mechanism that transiently restores their sensing ability despite satiation⁹. Post-learning sugar ingestion activates disinhibited fructose-sensing neurons, which triggers memory consolidation through the release of the glycoprotein hormone thyrostimulin^{10,11}, as in appetitive learning. The reset of fructose-sensing neurons by spaced training also results in a fasted state-like feeding behaviour, manifesting in a strong increase in sucrose preference and intake. By revealing a mechanism of non-homeostatic hunger and its critical relevance for memory consolidation, our results provide a neural circuit basis, and a cognitive value, to a behaviour akin to emotional eating.

Sensing the content of ingested food is critical for the body to evaluate energy availability and accordingly set an adequate metabolic state. In addition to peripheral taste receptors, animals and humans detect the nutritional value of food through post-ingestion mechanisms involving internal nutrient sensors in the digestive tract and the brain. Internal nutrient-sensing systems are major regulators of appetite and feeding behaviour¹². As such, they participate in memory processes that enable the assignment of value to food-related sensory cues¹³. However, the extent to which the brain's cognitive efficiency relies on its nutrient sensors, in particular beyond food-related processes, is unclear.

Laboratory experiments using genetically tractable species allow the study of targeted neural circuits in precisely defined behavioural tasks. In the fruit fly *Drosophila melanogaster*, we investigated the cognitive role of a major brain sugar sensor using a Pavlovian aversive olfactory learning task comprising the association of an odorant with mild electric shocks. After such conditioning, flies develop a learned avoidance towards this odorant⁷. After a single learning session, this memory decays within hours. Multiple sessions spaced in time (spaced training) induce the formation of long-term memory (LTM) that lasts for days⁸, is protein-synthesis dependent⁸ and is critically linked to glucose-based metabolism in neurons of the mushroom body (MB) brain area^{14–16}. The same number of immediately consecutive sessions (massed training) induces another form of consolidated memory that lasts for around 1 day, but this memory does not correspond to LTM^{8,16,17}: in addition to being less persistent⁸, it involves distinct neuronal circuits within the MB¹⁷ and relies on lipid-based rather than glucose-based neuronal energy metabolism¹⁸. The differential

impact on consolidation efficiency between massed and spaced learning paradigms is an experimental manifestation in flies of a well-documented cognitive phenomenon named the spacing effect^{8,19–21}. Experiments in the aversive learning paradigm are typically performed on fully satiated flies with ad libitum access to food before and after conditioning.

In the fly central brain, a small set of fructose-sensing neurons (four neurons per brain hemisphere, expressing the fructose-responsive gustatory receptor Gr43a, hereafter Gr43a neurons) respond to sugar ingestion and promote feeding in a satiation-dependent manner^{6,9,22}. Dietary sucrose contains both glucose and fructose, but fructose is also produced from glucose metabolism through the polyol pathway, so fructose sensors respond to carbohydrate intake in general⁶. In hungry flies, Gr43a neurons detect an increase in fructose levels after sugar intake, and their activity promotes feeding⁶. The nature of the molecular signalling from Gr43a neurons and their downstream targets are currently unclear. When flies are satiated, the sensitivity of Gr43a neurons to fructose is disabled by the inhibitory action of the neuropeptide tachykinin (Tk), released by upstream dFB neurons, which are localized in the dorsal layer of the fan-shaped body region (dFB)⁹ (Extended Data Fig. 1a).

Our experiments reveal hunger-independent sugar-sensing plasticity underlying LTM, whereby spaced training restores the sensitivity of brain fructose-sensing neurons despite satiety, enabling their activation by post-training feeding. This endows food ingestion with a cognitive value, notably as a memory consolidation signal that extends beyond food-related experiences.

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Feeding activates Gr43a neurons for LTM

We performed acute silencing of Gr43a neurons through targeted expression of the dominant-negative thermosensitive Shibire protein (Shi^{ts})²³, which is known to efficiently silence neurotransmission when flies are placed at the restrictive temperature of 33 °C (ref. 17). Genetic targeting of brain fructose-sensing neurons was achieved using a previously published transgenic line carrying a *GAL4* knock-in in the *Gr43a* locus (*Gr43a^{GAL4}*)⁶ in combination with the *Cha^{74kb}-GAL80* transgene, which prevents expression outside the brain²² (hereafter, brain *Gr43a^{GAL4}*; Extended Data Fig. 1b). Silencing brain Gr43a neurons for 3 h immediately after spaced training abolished aversive LTM performance (Fig. 1a (i) and Extended Data Fig. 1c). By contrast, shifting the silencing time windows—starting 3 h (Fig. 1a (ii)) or 6 h (Extended Data Fig. 1d) after training for 3 h—did not affect LTM. When flies were kept at the permissive temperature, no silencing was expected to occur and LTM performance was normal (Extended Data Fig. 1e). Silencing Gr43a neurons for 3 h after massed training left 24 h memory performance intact, and silencing Gr43a neurons for 1 h after a single training session had no effect on 3 h memory either (Extended Data Fig. 1f). To confirm these observations, we conducted silencing experiments using a different genetic driver line, a *Gr43a-GAL4* enhancer trap line²⁴ targeting two pairs of Gr43a neurons (Extended Data Fig. 1b), with similar results (Extended Data Fig. 1g). Thus, despite flies being satiated, signalling from brain fructose-sensing neurons is required in the aversive learning paradigm specifically for LTM formation, within a restricted time period after training. This result raises the question of what could activate these neurons.

In the previous series of experiments, flies were constantly maintained on food-containing vials after training. We conducted a similar experiment to silence Gr43a neurons, with the difference that flies were deprived of food during the 3 h while Gr43a neurons were silenced. Consequently, Gr43a neurons were not silenced when flies were put back on food after training. Notably, LTM performance was normal in that condition (Fig. 1a (iii)). However, with the same sequence of food accessibility, LTM was again fully impaired when the silencing of Gr43a neurons was shifted to 3–6 h after training, that is, when flies recovered access to food (Fig. 1a (iv)). Taken together, these experiments show that LTM is formed after spaced training, provided that, when flies start eating after learning, their brain fructose-sensing neurons are able to signal. Accordingly, when wild-type flies were continuously food deprived after spaced training, LTM formation was defective, although this condition did not affect memory formed after massed training (Fig. 1b). Notably, forced activation of Gr43a neurons for 1 h immediately after spaced training, mediated by ectopic expression in Gr43a neurons of the heat-activated cation channel TrpA1^{25,26}, rescued LTM in flies deprived of food after training (Fig. 1c). Moreover, LTM was rescued if flies were fed with sucrose or glucose, but not with coconut oil, after training (Extended Data Fig. 1h), showing a critical effect of carbohydrate-based feeding rather than energy intake in itself. This suggests that, in a regular situation, the sensitivity of brain fructose-sensing neurons is restored by spaced training, so that Gr43a neurons can be activated by the onset of food intake after spaced training, giving rise to signalling that is critical for LTM formation. These findings raise two questions that we address in subsequent parts of this study: how Gr43a neurons can be activated and what signal they release to initiate memory consolidation.

Spaced training resets Gr43a neurons

To examine the sensitivity of brain fructose-sensing neurons, we conducted *in vivo* calcium imaging experiments in response to bath application of fructose. In naive, 24-h food-deprived flies, Gr43a neurons showed a strong and long-lasting calcium increase in response to fructose stimulation. By contrast, we detected no calcium response

in satiated flies (Fig. 1d). This experiment therefore confirmed the state-dependent nutrient sensitivity of Gr43a neurons in naive flies⁹. But, given that aversive learning experiments are performed on satiated flies with free access to food before conditioning, this raises the question of how Gr43a neurons are activated by feeding after spaced training.

We performed similar calcium imaging experiments comparing flies that had received spaced training with flies that went through an unpaired protocol of similar duration (in which electric shocks and odours were delivered separately). Notably, after spaced training, fructose application resulted in a strong response in brain Gr43a neurons, of the same magnitude as in naive fasted flies, whereas it did not elicit a response in the case of an unpaired protocol, as in naive satiated animals (Fig. 1e). While these experiments involved stimulation with a relatively high fructose concentration (75 mM), similar results were obtained using a more sensitive calcium sensor (GCaMP6s), allowing a concentration of fructose stimulation at a physiologically relevant concentration (20 mM) (Extended Data Fig. 2a). The recovery of fructose sensitivity was specific to spaced training, as Gr43a neurons were not responsive to fructose after massed training (Extended Data Fig. 2b). After spaced training, even without food intake, the fructose sensitivity of Gr43a neurons extinguished within 6 h after training (Extended Data Fig. 2c,d). Accordingly, flies granted access to food only from 6 h after spaced training were unable to form LTM (Extended Data Fig. 2e). These results demonstrate that spaced associative aversive training, despite not involving food-relevant stimuli, opens a time window of a few hours when the fructose sensitivity of Gr43a neurons in the brain is restored, raising the question of how this is implemented.

Circuit of Gr43a neuron disinhibition

In naive satiated flies, Gr43a neurons are inhibited by their upstream dFB neurons⁹. Indeed, it was reported that dFB neurons are highly active in fed flies, as *in vivo* calcium imaging reported large-amplitude spontaneous calcium transients in these neurons, whereas this calcium activity was substantially reduced in fasted flies⁹. Through similar *in vivo* calcium imaging measurements performed in satiated flies, we observed that dFB neurons show large-amplitude ongoing calcium activity in flies that received an unpaired conditioning protocol. Notably, spaced training induced a substantial reduction in dFB neuron activity, manifested as a decrease in the amplitude and the power spectrum of dFB calcium signals (Fig. 2a). Spaced training therefore restores dFB neurons to a starvation-like physiological state, thereby resetting the activatability of Gr43a neurons. If this physiological shift leading to dFB neuron inhibition is critical to activate Gr43a neurons, we reasoned that forcing dFB neuron activity should be detrimental for LTM formation specifically (similar to silencing of Gr43a neurons). Notably, activating dFB neurons immediately after spaced training for 1 h using TrpA1 expression abolished LTM performance (Fig. 2b). When flies were kept at the permissive temperature, LTM remained intact (Extended Data Fig. 3a). Activation of dFB neurons after massed or single trial training had no effect on memory (Extended Data Fig. 3a). These results were replicated using three distinct driver lines targeting dFB neurons (Extended Data Fig. 3b). As would be expected, the fructose response in Gr43a neurons after spaced training was abolished after 1 h activation of dFB neurons (Fig. 2c and Extended Data Fig. 3c,d).

Glutamatergic neurons—called Janus neurons—are presynaptic to dFB neurons in a brain region called the asymmetric body (Extended Data Fig. 4a), and have been shown to induce the onset of dFB neurons calcium oscillations when food satiation is reached⁹. With the hypothesis that spaced training might silence Janus neurons, which would result in the observed dampening of dFB neuron activity, we reasoned that forcing activity of Janus neurons might impair LTM, as did dFB neuron activation. However, TrpA1-mediated activation of Janus neurons after spaced training did not impair LTM performance

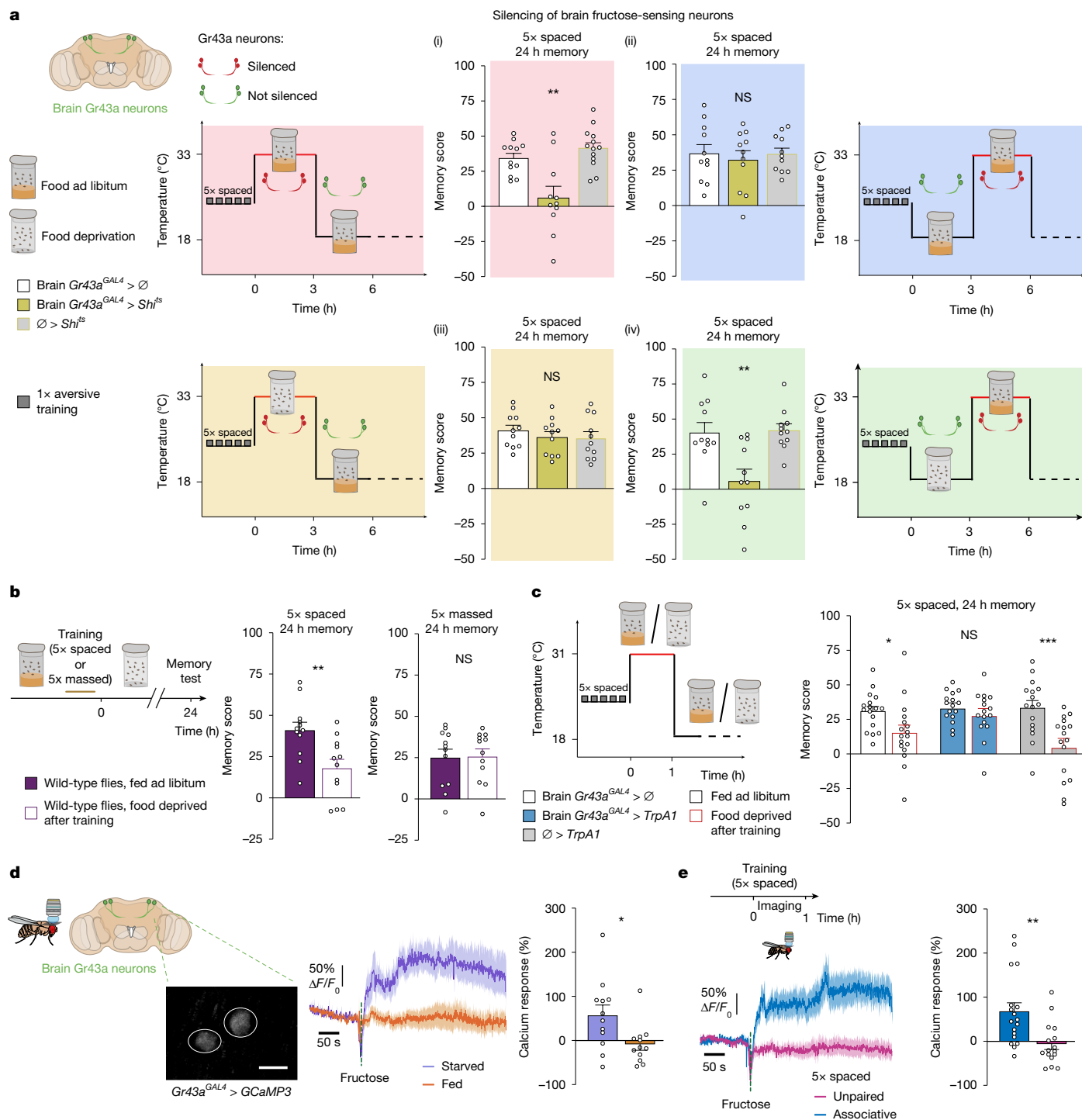


Fig. 1 | Food intake after spaced training activates brain Gr43a neurons to allow LTM formation. **a**, Schematic and results of experiments measuring LTM performance in flies subjected to different combinations of silencing of brain Gr43a neurons expressing *Shi^{ts}* and periods of restricted food access. In all cases, the flies were fed ad libitum before training ($n = 12, 11$ and 13 , $F_{2,33} = 12.18$, $P = 0.0001$ (i); $n = 11$, $F_{2,30} = 0.20$, $P = 0.82$ (ii); $n = 11$, $F_{2,30} = 0.54$, $P = 0.58$ (iii); $n = 11$, $F_{2,30} = 8.78$, $P = 0.001$ (iv); each datapoint is derived from two groups of typically 30–50 flies, as described in the Methods). **b**, The effect of continuous food deprivation on 24 h memory in wild-type flies after spaced training ($n = 12$, $t_{22} = 3.19$, $P = 0.004$) or massed training ($n = 12$, $t_{22} = 0.09$, $P = 0.92$). **c**, The combined effect of food deprivation and 1 h activation of brain Gr43a neurons after spaced training as illustrated (two-way analysis of variance (ANOVA); $n = 17, 17, 16, 16, 16$ and 16 ; $F_{\text{genotype}} = 3.55$, $P = 0.03$; $F_{\text{feeding status}} = 19.23$,

$P = 0.0003$; $F_{\text{interaction}} = 3.00$, $P = 0.05$). **d**, Time traces and quantification ($n = 12$, $t_{22} = 2.50$, $P = 0.02$) of the calcium response in starved or fed flies to bath application of fructose to brain Gr43a neurons, recorded using two-photon in vivo imaging; typical region of interest is indicated on one example. Scale bar, 10 μm . **e**, The calcium response as in **d**, comparing fed flies subjected to spaced training and to the unpaired spaced protocol ($n = 18; 16$, $t_{32} = 3.21$, $P = 0.003$). Data are mean $\pm$ s.e.m. P values were calculated using one-way ANOVA followed by Tukey's pairwise comparisons (**a** and **b**), two-way ANOVA followed by Šidák pairwise comparisons (**c**) or two-tailed unpaired t -tests (**d** and **e**); pairwise comparisons: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, not significant ($P > 0.05$). Further details are provided in the 'Quantification and statistical analysis' section of the Methods.

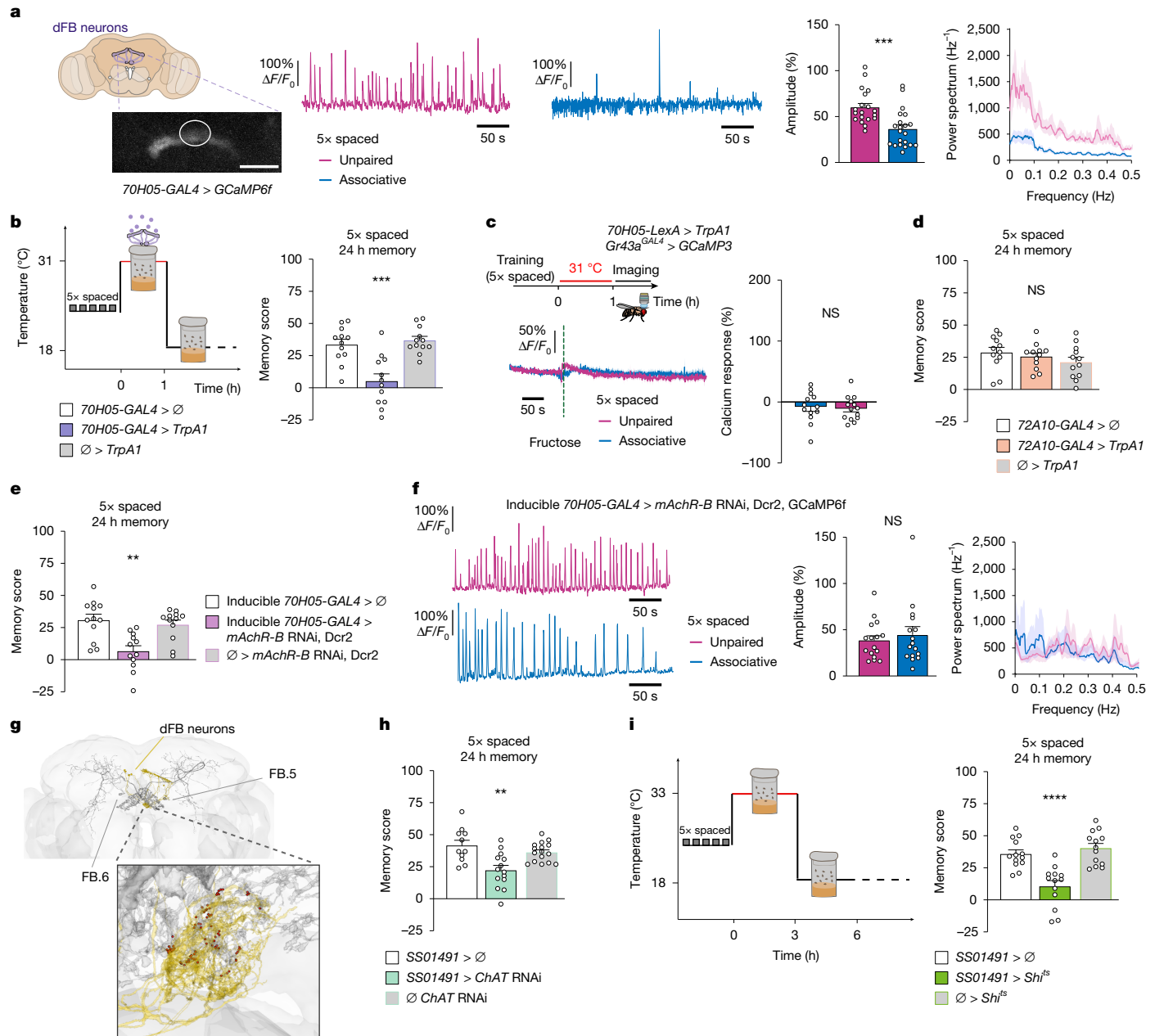


Fig. 2 | Spaced training disinhibits fructose-sensing neurons despite satiety. **a**, Spontaneous activity in dFB neurons. Calcium signals in dFB neurons were recorded using two-photon in vivo imaging within 1 h after the indicated conditioning; a typical region of interest⁹ is indicated on one example image. Scale bar, 40 μm . Shown are two individual time traces, the average power spectra across all collected flies and a comparison of signal amplitudes ($n = 19$ and 21, $t_{38} = 3.83$, $P = 0.0005$). **b**, The effect of dFB neuron activation on LTM ($n = 12$, $F_{2,33} = 15.95$, $P = 0.00001$). **c**, The effect of 1 h dFB neuron activation after conditioning on Gr43a neuron fructose response ($n = 14$ and 13, $t_{25} = 0.28$, $P = 0.77$). Genotypic controls assayed in parallel are shown in Extended Data Fig. 3d. **d**, The effect of Janus neuron activation on LTM ($n = 12$, $F_{2,33} = 1.14$, $P = 0.33$). **e**, The LTM performance of flies with *mAChR-B* knockdown (RNAi

KK107137) in dFB neurons ($n = 12$, $F_{2,33} = 10.28$, $P = 0.0003$). **f**, Power spectra and amplitude of calcium activity after conditioning in dFB neurons with *mAChR-B* knockdown ($n = 16$, $t_{30} = 0.58$, $P = 0.57$). Genotypic controls assayed in parallel are shown in Extended Data Fig. 4d. **g**, FB.5 and FB.6 neurons (grey) and a sample of 7 dFB neurons (yellow) from FAFB dataset, visualized using FlyWire^{30–32}. The 89 synapses (red dots in the inset) from FB.5/6 neurons to dFB neurons are located in the posterior part of the asymmetric body. **h**, The effect of *ChAT* knockdown (RNAi JF01877) in FB.5/6 neurons on LTM ($n = 11$, 14 and 16, $F_{2,38} = 9.60$, $P = 0.0004$). **i**, The effect of 3 h silencing of FB.5/6 neurons on LTM ($n = 13$, $F_{2,36} = 18.32$, $P = 3 \times 10^{-6}$). Data are mean $\pm$ s.e.m. P values were calculated using two-tailed unpaired t -tests (**a**, **c** and **f**) and one-way ANOVA with Tukey pairwise comparisons (**b**, **d**, **e**, **h** and **i**) (Methods).

(Fig. 2d). Searching for alternative pathways, we noticed that one of the genomic enhancer fragments that drives expression in dFB neurons (VT038216) derives from muscarinic acetylcholine receptor B (*mAChR-B*), which is unique among cholinergic receptors in that it reportedly exerts inhibitory action after activation^{27,28}. This raised the hypothesis that a cholinergic input could inhibit dFB neurons after spaced training. We therefore addressed the effect of a genetic

knockdown of *mAChR-B* in dFB neurons on LTM formation, using two previously characterized RNA interference (RNAi) constructs targeting *mAChR-B*²⁸. We used the TARGET system²⁹ to achieve inducible GAL4-mediated RNAi expression. This strategy relies on the ubiquitous expression of a thermosensitive GAL4 inhibitor under a tubulin promoter (*tub-GAL80^{ts}*). Induction of RNAi expression at the adult stage was achieved by placing flies at 30 $^{\circ}\text{C}$ for 3 days before

conditioning. *mAChR-B* knockdown in dFB neurons impaired LTM (Fig. 2e and Extended Data Fig. 4b), but did not alter naive odour or electric shock avoidance (Supplementary Table 1), indicating that the observed memory impairment was not due to impaired sensory perception. LTM performance was normal when RNAi expression was not induced, excluding the possibility that the observed memory defect could be due to leaky RNAi expression during development (Extended Data Fig. 4b). Memory measured 24 h after massed training or 3 h after single training was not affected by *mAChR-B* knockdown (Extended Data Fig. 4b). These results were reproduced with the second RNAi construct (Extended Data Fig. 4c). Last, when *mAChR-B* expression was inhibited in dFB neurons, spaced training did not decrease their calcium activity (Fig. 2f and Extended Data Fig. 4d). Using FlyWire^{30–32} to examine the reconstructed fly brain connectome derived from the full adult fly brain (FAFB) dataset³³, we identified a pair of neurons (FB.5 and FB.6 neurons, hereafter FB.5/6) predicted to be cholinergic that are presynaptic to dFB neurons in the asymmetric body³⁴ (Fig. 2g; details are provided in the Supplementary Note and Supplementary Tables 7 and 8). NeuronBridge³⁵ enabled us to identify that the split-GAL4 line SS01491 targets these neurons (Extended Data Fig. 4d). According to a previous study, this line additionally targets three pairs of female-specific PC1 neurons³⁶, but restrictively targets FB.5/6 neurons within the brain and VNC in male flies³⁶. Notably, knockdown in FB.5/6 neurons of the choline acetyltransferase (*ChAT*) enzyme involved in acetylcholine biosynthesis using an RNAi line that we previously validated³⁷ impaired LTM (Fig. 2h), both in male and female flies (Extended Data Fig. 4f). This result is in accordance with the prediction of FB.5/6 neurons being cholinergic and, importantly, reveals their specific involvement in LTM formation, as no effect was observed after massed or single training (Extended Data Fig. 4f). Moreover, flies expressing *Shi^{ts}* through SS01491 showed a memory defect after spaced training (Fig. 2i), in both male and female flies (Extended Data Fig. 4g), but not after massed or single training (Extended Data Fig. 4g). Together, our data demonstrate that resetting the nutrient sensitivity of Gr43a neurons, through the silencing of their upstream inhibitory neurons, is critical for LTM formation. This effect mobilizes a cholinergic pathway that inhibits dFB neurons in an experience-dependent manner, in parallel to feeding state-dependent glutamatergic modulation by Janus neurons. While our data identify FB.5/6 neurons as the probable final element of this new circuit, the full delineation of how spaced learning patterns translates into dFB neurons inhibition will require further investigation.

Gr43a neurons release thyrostimulin

Brain fructose-sensing neurons detect sugar intake through variations in fructose levels, which are sensed by the Gr43a receptor that they express⁶. We therefore addressed the effect of a genetic knockdown of *Gr43a* in these neurons on LTM formation. The efficiency of all RNAi constructs used in this study that had not been already characterized was verified using quantitative PCR with reverse transcription (RT-qPCR; Supplementary Tables 5 and 6). Knockdown of *Gr43a* in brain fructose-sensing neurons resulted in a defect in LTM (Fig. 3a, Extended Data Fig. 5a and Supplementary Table 2). Memory measured 24 h after massed training or 3 h after a single training was not affected by *Gr43a* knockdown (Extended Data Fig. 5a). These behavioural experiments were replicated using a distinct, non-overlapping *Gr43a* RNAi construct (Extended Data Fig. 5b). Knockdown of *Gr64a*, another sugar receptor that is reportedly expressed by Gr43a neurons but not involved in their fructose response³⁸, had no effect on LTM (Extended Data Fig. 5c and Supplementary Tables 5 and 6). The requirement of the Gr43a receptor in fructose-sensing neurons for aversive LTM is consistent with the fact that these neurons are activated by food intake after spaced training.

Regarding which molecular signalling is delivered by these neurons, brain Gr43a neurons express the neuropeptide corazonin (*Crz*)²²; however, *Crz* knockdown in Gr43a neurons did not impair LTM (Extended

Data Fig. 5d and Supplementary Tables 5 and 6), prompting a search for alternative candidates. From a publicly available single-cell transcriptomics dataset of around 60,000 fly brain cells³⁹, we identified a subset of approximately 10–12 cells with strong co-expression of both *Gr43a* and *Crz*, which we further considered as putative Gr43a neurons. In the transcript list of these candidate cells (Supplementary Data 1), we noticed a consistently strong expression of glycoprotein beta subunit 5 (*Gpb5*). *Gpb5* heterodimerizes with glycoprotein alpha subunit 2 (*Gpa2*) to form a highly conserved glycoprotein hormone, Gpa2–Gpb5⁴¹ (known as thyrostimulin in vertebrates). Gpa2–Gpb5 is the ligand of leucine-rich-repeat-containing receptor 1 (*Lgr1*)⁴⁰, which, according to the same transcriptomics dataset, is expressed in neurons of the MB, the major brain region underlying associative memory encoding. Using a previously validated antibody against *Gpb5*⁴¹, we indeed observed immunostaining in the cell bodies of brain fructose-sensing neurons, which was strongly decreased after expression of an RNAi against *Gpb5* (Fig. 3b, Supplementary Tables 5 and 6). We therefore tested whether *Gpb5* could be involved in LTM-relevant signalling by these neurons. Knockdown of *Gpb5* in brain Gr43a neurons led to a strong impairment of LTM (Fig. 3c and Extended Data Fig. 5e). Memory measured 24 h after massed training or 3 h after a single training was not affected by *Gpb5* knockdown (Extended Data Fig. 5e). This result was confirmed using a distinct non-overlapping RNAi against *Gpb5* (Extended Data Fig. 5f). We then performed a similar series of experiments using an RNAi against *Gpa2*, the molecular partner of *Gpb5* (Supplementary Tables 5 and 6). As for *Gpb5*, inducible *Gpa2* knockdown in brain fructose-sensing neurons specifically impaired LTM formation (Extended Data Fig. 5g,h). As this was the only available RNAi construct, and no *Gpa2* antibody had been characterized, we did not further investigate the role of *Gpa2*. Overall, these results reveal that Gpa2–Gpb5 signalling by Gr43a neurons supports LTM formation after spaced training.

Thyrostimulin action on the MB

The neuronal silencing experiments presented earlier show that signalling from Gr43a neurons occurs shortly after spaced training. Although genetic knockdown experiments do not offer the same acute manipulation as temperature-induced silencing, we sought to confirm that thyrostimulin signalling also operates on the same time scale. In our previous studies, in vivo imaging of the metabolic activity of MB neurons using pyruvate and glucose FRET biosensors enabled the identification of two early metabolic hallmarks required for LTM formation that are observable within 2 h after spaced training: an increased rate of mitochondrial pyruvate uptake in the axons of Kenyon cells, the MB-intrinsic neurons^{14,42}, and increased glucose consumption by the pentose phosphate pathway in the somatic compartment of these neurons¹⁵. In vivo two-photon pyruvate imaging experiments conducted in that time window in the axonal compartment of MB neurons confirmed that spaced training induces an increased pyruvate metabolic rate as compared to a spaced unpaired protocol (Fig. 3d). Notably, this effect was lost when either *Gr43a* or *Gpb5* was knocked down in Gr43a neurons (Fig. 3d). Similarly, glucose imaging in Kenyon cell somas showed that the increased glucose consumption induced by spaced training was impaired by *Gr43a* or *Gpb5* knockdown in Gr43a neurons (Extended Data Fig. 6a). These results therefore support the idea that both Gr43a-mediated fructose sensing and Gpa2–Gpb5 signalling by brain Gr43a neurons allow the metabolic activation of MB neurons in the first hours after spaced training, which was previously shown to be a critical initial step in memory consolidation^{14,42}.

Brain Gr43a neurons do not anatomically project to the MB region⁶. However, as hormones and neuropeptides can mediate long-range signalling, Gpa2–Gpb5 released by Gr43a neurons could still act on MB neurons. Three major populations of neurons— α/β , α'/β' and γ —have been defined within Kenyon cells. Single-cell transcriptomics

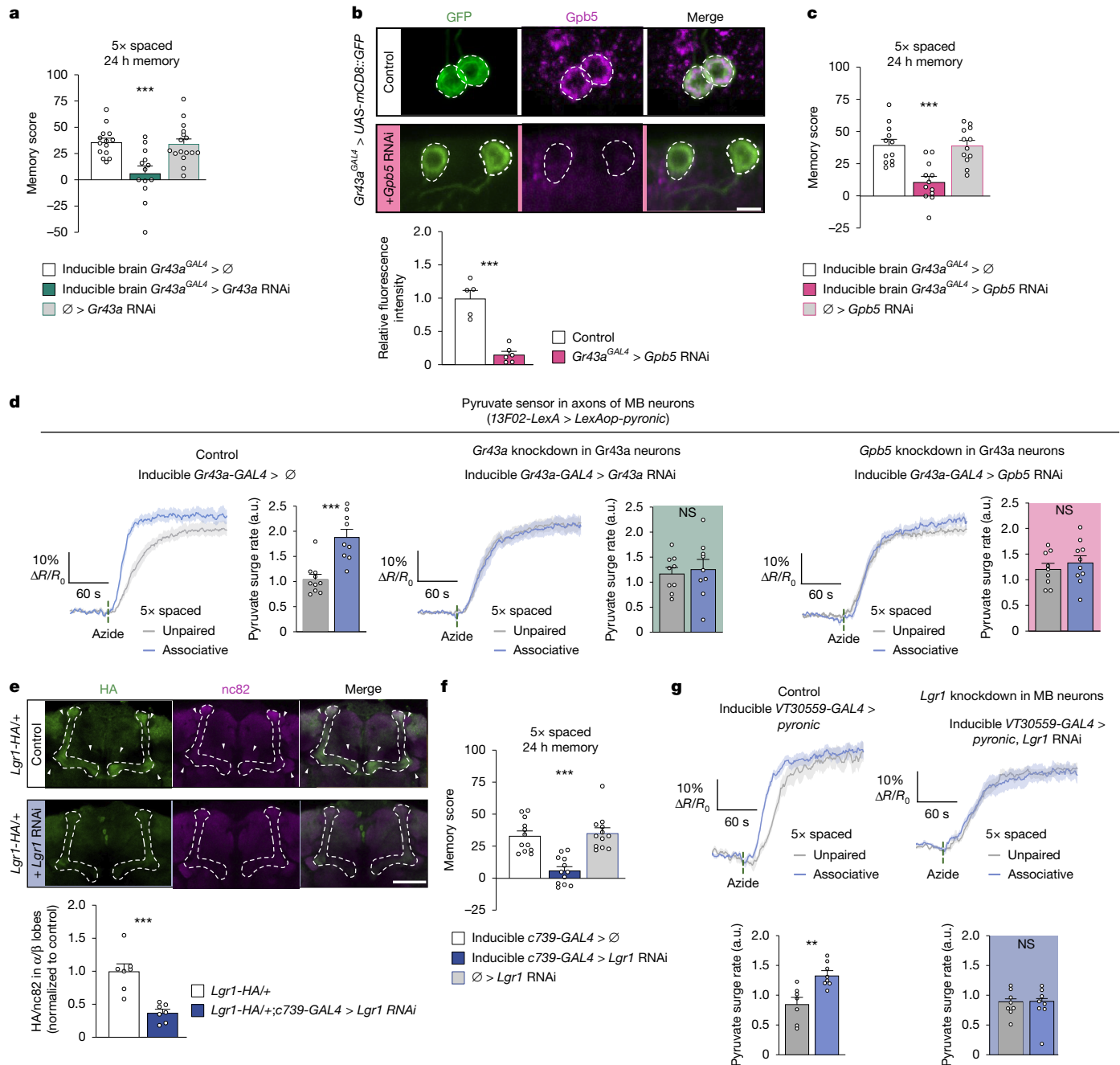


Fig. 3 | Thyrostimulin from fructose-sensing neurons targets the MB.

a, The effect of *Gr43a* knockdown (RNAi HMC05754) in brain fructose-sensing *Gr43a* neurons on LTM ($n = 14, 13$ and 16 , $F_{2,40} = 10.11$, $P = 0.0003$). **b**, *Gpb5* immunostaining in *Gr43a* neurons expressing GFP, and the effect of *Gpb5* knockdown (RNAi line from ref. 41; $n = 5$ and 6 , $t_9 = 7.06$, $P = 0.00006$); images from two single brains are shown for illustration. Scale bar, $5 \mu\text{m}$. **c**, The effect of *Gpb5* knockdown (RNAi vsh330796) in brain *Gr43a* neurons on LTM ($n = 12$, $F_{2,33} = 14.7$, $P = 0.00002$). **d**, Pyruvate imaging in MB vertical lobes within 2 h after conditioning, showing time traces of pyruvate accumulation after sodium azide application and bar plots of pyruvate surge rate (reflecting pyruvate consumption) in the control condition (no RNAi ($n = 10$ and 9 , $t_{17} = 4.70$, $P = 0.0002$), *Gr43a* knockdown (RNAi HMC05754) in *Gr43a* neurons

($n = 9$, $t_{16} = 0.39$, $P = 0.70$) and *Gpb5* knockdown (RNAi vsh330796) in *Gr43a* neurons ($n = 9$ and 10 , $t_{17} = 0.73$, $P = 0.47$). a.u., arbitrary units. **e**, *Lgr1-HA* immunostaining and nc82 neuropil counterstaining with or without *Lgr1* RNAi (JF02659) expressed in MB α/β neurons using *c739-GAL4* driver ($n = 7$, $t_{12} = 5.04$, $P = 0.0003$); images from two single brains are shown for illustration. Scale bar, $50 \mu\text{m}$. The dashed lines show α/β lobes; the arrowheads point to other MB lobes. **f**, The effect of *Lgr1* knockdown (RNAi JF02659) in α/β MB neurons on LTM ($n = 12$, $F_{2,33} = 20.55$, $P = 2 \times 10^{-6}$). **g**, Pyruvate imaging in MB neurons as in **d** for control (no RNAi; $n = 7$; $t_{13} = 3.68$, $P = 0.002$) and *Lgr1* knockdown (RNAi JF02659) in MB neurons ($n = 8$, $t_{14} = 0.05$, $P = 0.96$). Data are mean $\pm$ s.e.m. P values were calculated using two-tailed unpaired t -tests (**b**, **d**, **e** and **g**) and one-way ANOVA with Tukey pairwise comparisons (**a**, **c** and **f**) (Methods).

analysis has revealed that the *Gpa2*–*Gpb5* receptor *Lgr1* is a specific marker of the cluster of α/β neurons³⁹, the subpopulation that is known to be pivotal in LTM encoding⁴³. To confirm that *Lgr1* is expressed in α/β neurons, we generated a fly line in which a HA-tag coding sequence was knocked in at the C-terminal end of the *Lgr1* gene. In the MB region,

these flies showed strong HA-positive staining in the α and β lobes, corresponding to the axons of α/β neurons, exclusively. This staining was significantly decreased after expression of an RNAi targeting *Lgr1* in α/β neurons (Fig. 3e and Supplementary Tables 5 and 6), confirming the strong and preferential expression of *Lgr1* in α/β neurons as

compared to subsets of other Kenyon cells. This prompted us to question the role of *Lgr1* in α/β Kenyon cells in LTM formation. Inducible knockdown of *Lgr1* in α/β Kenyon cells led to a strong impairment in LTM (Fig. 3f and Extended Data Fig. 6b). Memory measured 24 h after massed training or 3 h after a single training was not affected by *Lgr1* knockdown (Extended Data Fig. 6b). We replicated these results using a distinct non-overlapping RNAi against *Lgr1* (Extended Data Fig. 6c). Moreover, *Lgr1* knockdown in α/β MB neurons prevented the increased pyruvate metabolism and glucose consumption in the vertical lobes of MB neurons (Fig. 3g and Extended Data Fig. 6d). Together, these results argue for a direct hormonal action mediated by thyrostimulin from brain fructose-sensing neurons on α/β neurons of the MB, enabling their metabolic activation.

Spaced training makes flies hungry

In fasted flies, Gr43a neurons activation promotes food intake⁶. As spaced training resets these neurons in a fasted-like state, we sought for possible consequences on feeding behaviour, using a two-choice feeding assay (FlyPad⁴⁴; Fig. 4a) to assess the preference of single flies for sucrose versus agarose—a substrate with low energy value. As expected, naive fasted flies in this assay displayed strong preference for sucrose, in contrast to satiated flies (Extended Data Fig. 7a). After spaced associative training, flies developed a strong preference for sucrose, compared with the unpaired controls, an effect that did not occur after massed training (Fig. 4b and Extended Data Fig. 7b,c). Knockdown of *Gr43a* or *Gpb5* in fructose-sensing neurons or knockdown of *Lgr1* in α/β MB neurons strongly impaired the effect of spaced training on feeding behaviour (Fig. 4d–f), showing that a common signalling from Gr43a neurons supports memory consolidation and bias feeding behaviour after spaced training.

Commonality of aversive and reward LTM

It is well established that flies form appetitive LTM after a single trial of associative learning consisting in pairing of an odorant with sucrose ingestion^{45,46}. In this paradigm, flies need to be fasted before learning (Extended Data Fig. 8a), a state in which dFB neurons are silenced and the sensing ability of Gr43a neurons is switched on. As appetitive LTM requires post-ingestive signalling of the caloric value of the sugar reward^{5,47}, we questioned whether brain fructose sensors were also involved in appetitive LTM. Silencing Gr43a neurons after appetitive learning impaired LTM specifically, leaving short-term memory intact (Extended Data Fig. 8b,c). Notably, knockdown of *Gr43a*, *Gpb5* or *Gpa2* in Gr43a neurons, as well as knockdown of *Lgr1* in α/β MB neurons all specifically impaired appetitive LTM (Extended Data Fig. 8d–j and Supplementary Table 3), indicating that thyrostimulin signalling from fructose-sensing neurons to MB is a general memory consolidation signal that is shared between aversive and appetitive learning.

Bypassing the spacing effect

The fact that spaced training can be more efficient than intensive (or massed) training in lifting the default inhibition of LTM is a well-documented cognitive effect known as the spacing effect, and is conserved from invertebrates to humans¹⁹. Comparing the contexts of appetitive and aversive LTM formation, we wondered whether the disinhibition of fructose sensors after spaced training in satiated flies could be integral to the spacing effect; if so, we reasoned that mimicking the silencing of dFB neurons that is normally achieved by spaced training should be sufficient to switch the brain to an LTM-ready state, and therefore facilitate memory consolidation.

dFB neurons inhibit Gr43a neurons through Tk signalling acting on the Tk receptor Tk99D in Gr43a neurons⁹. Knockdown of *Tkr99D* in brain Gr43a neurons had no detectable effect on 24 h

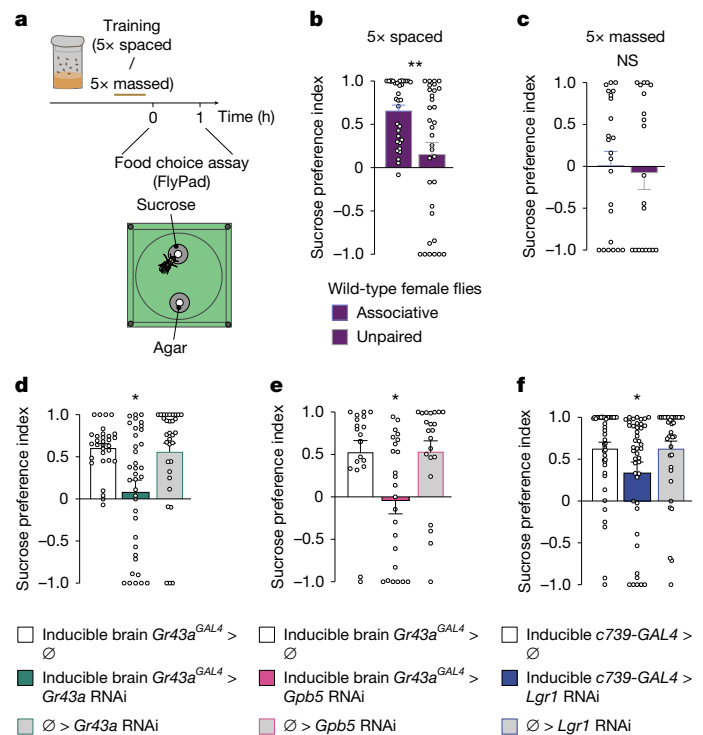


Fig. 4 | Brain fructose-sensing neurons mediate a fasted-like feeding behaviour after spaced training. **a**, Illustration of sucrose intake measurement using FlyPad. **b**, The sucrose preference index in female flies after 5x spaced associative or unpaired training ($n = 32$ and 33 , $U = 236$, $P = 0.007$). **c**, The sucrose preference in female flies after 5x massed associative or unpaired training ($n = 23$ and 20 , $U = 220$, $P = 0.81$). **d**, The effect of Gr43a receptor knockdown (RNAi HMC05754) in Gr43a brain neurons on sucrose preference after 5x spaced training ($n = 31$, 37 and 32 , $H = 12.50$, $P = 0.0019$). **e**, The effect of *Gpb5* knockdown (RNAi vsh330796) in Gr43a brain neurons on sucrose preference after 5x spaced training ($n = 19$, 22 and 24 , $H = 11.90$, $P = 0.0026$). **f**, The effect of *Lgr1* knockdown (RNAi JF02659) in MB α/β neurons on sucrose preference after 5x spaced training ($n = 43$, 44 and 36 , $H = 8.46$, $P = 0.01$). Data are mean $\pm$ s.e.m. P values were calculated using two-tailed Mann–Whitney U -tests (**b,c**) or Kruskal–Wallis tests followed by Dunn’s multiple-comparison correction (**d–f**) (Methods).

memory performance after either spaced or massed training (Fig. 5a and Extended Data Fig. 9a), showing that the magnitude of the memory was not increased. We next subjected flies to a spaced training with fewer learning sessions. Notably, only two spaced trials induced a 24 h persistent memory in flies expressing the *Tkr99D* RNAi in Gr43a neurons, whereas this protocol did not induce significant memory in genotypic control groups (Fig. 5a and Supplementary Table 4). The memory performance of *Tkr99D*-knockdown flies after two spaced training sessions (2x spaced training) was comparable to that of wild-type flies after a regular 5x spaced training (Fig. 5a). No increase occurred in the absence of induction (Extended Data Fig. 9a). These results were replicated with a second non-overlapping RNAi against the Tk receptor (Extended Data Fig. 9b). In accordance with our hypothesis, imaging experiments revealed that two spaced training sessions were sufficient to restore fructose sensitivity in *Tkr99D*-knockdown flies, as opposed to genotypic control flies (Fig. 5b). To further confirm this facilitative effect, we aimed to silence dFB neurons using Shi¹⁵. Similar to Tk receptor knockdown in Gr43a neurons, silencing dFB neurons for 3 h immediately after 2x spaced training induced an increase in 24 h memory (Fig. 5c and Extended Data Fig. 9c,d), while delayed silencing had no effect (Extended Data Fig. 9c). Notably, this facilitative effect did not occur when flies had no access to food during the silencing period (Fig. 5c and Extended Data Fig. 9d), consistent with food intake being necessary to activate Gr43a neurons once they are disinhibited.

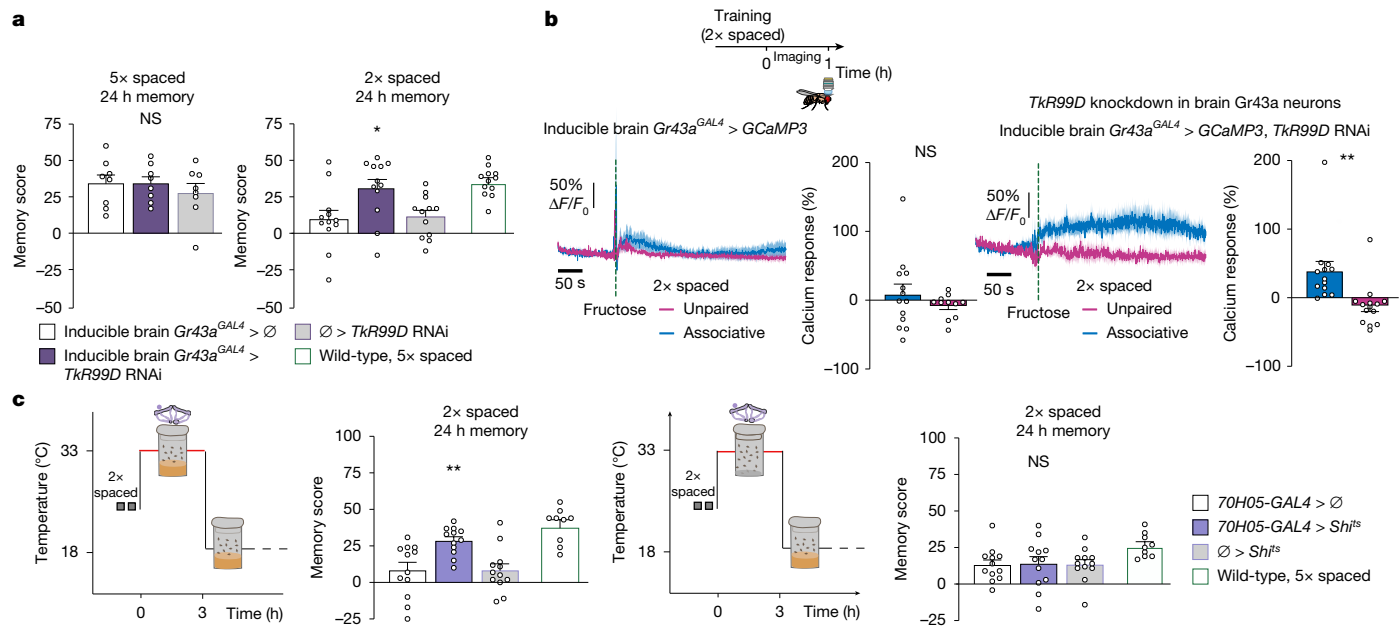


Fig. 5 | Disinhibition of brain fructose-sensing neurons facilitates aversive LTM. **a**, The memory performance of flies with *TkR99D* knockdown (RNAi HMC003749) in Gr43a brain neurons and of genotypic controls, measured 24 h after 5× spaced training ($n = 8$, $F_{2,21} = 0.43$, $P = 0.65$) or only 2× spaced training ($n = 12$, $F_{2,33} = 4.54$, $P = 0.018$). **b**, The fructose response of brain Gr43a neurons after 2× spaced associative or unpaired training without ($n = 13$ and 11 , $t_{22} = 0.99$, $P = 0.33$) or with ($n = 13$ and 14 , $t_{25} = 3.11$, $P = 0.004$) *TkR99D* knockdown (RNAi HMC003749) in these neurons. **c**, Memory performance was measured 24 h after 2× spaced training in flies expressing *Shi^{ts}* in dFB neurons and in genotypic controls. Flies were placed at 33 °C for the first 3 h after the end of the

conditioning protocol, as illustrated by the schematics accompanying the bar plots. When flies had continuous food access, silencing dFB neurons allowed LTM formation, which did not occur in the genotypic control groups ($n = 12$, $F_{2,33} = 7.46$, $P = 0.002$). When flies did not have access to food while dFB neurons were silenced, the LTM facilitation effect did not occur ($n = 12$, $F_{2,33} = 0.01$, $P = 0.98$). Wild-type flies were trained in parallel with 5× spaced training as a reference for regular LTM performance but were not included in the statistical analysis. Data are mean $\pm$ s.e.m. P values were calculated using two-tailed unpaired t -tests (**b**) and one-way ANOVA with Tukey pairwise comparisons (**a** and **c**) (Methods).

The AND gate of LTM

Together, these experiments show that the spacing effect in flies amounts to luring the brain fructose sensor into a fasted state. As a result, the perception and signalling of sugar intake act as a gating signal that launches memory consolidation, the same way as rewarding sugar ingestion in fasted flies induces appetitive LTM. Formally, the initiation of memory consolidation is therefore controlled by an AND logic gate with ‘sensing ability’ and ‘sugar ingestion’ as inputs, establishing a hierarchical organization whereby memory consolidation is subordinated to food intake, even though energy availability is not limited (Extended Data Fig. 10a–c). LTM formation represents a high metabolic cost⁴⁸ that, in starving flies, can jeopardize survival⁴⁹. Therefore, in a wild context in which food scarcity and a fasted state are the norm, it seems logical that memory consolidation would be subordinated to securing an adequate energy supply. However, the fact that the spacing effect in satiated flies amounts to mimicking the fasted state, at least at the nutrient sensor level, suggests that the sugar-sensing AND gate could be a general mechanism controlling LTM formation. This could be true in more than just flies, given the large range of species in which the spacing effect has been reported¹⁹. Considering the marked similarities between aversive and appetitive LTM, it is tempting to speculate that aversive consolidation mechanisms might have evolved from food-reward memory schemes.

An experience-dependent feeding drive

Our study reveals that the sensitivity of a brain nutrient sensor can be tuned in an experience-dependent manner, as opposed to a nutritional status-dependent manner^{6,9}, as an integral part of the cognitive process leading to the storage of a learned experience as LTM. Notably, hunger- and experience-dependent modulation of fructose sensors

recruit distinct circuits and neurotransmitter systems, converging on upstream inhibitory dFB neurons at the level of the asymmetric body. This small brain region, which is unilateral in over 90% of wild-type flies^{34,50}, may therefore be a critical site underlying appetite modulation. Our behavioural and imaging data suggest that cholinergic inhibition, probably from FB.5/6 neurons, overrules excitatory glutamatergic drive delivered by Janus neuron in a satiation state⁹. Although the complete circuit that toggles inhibitory cholinergic input on dFB neurons after spaced training, and perhaps during other experiences, remains to be delineated, this architecture provides a basis for the occurrence of a feeding drive independent of organismal need for food intake. Indeed, spaced training leads to increased sucrose consumption¹⁴, which we confirmed here in addition to showing that it involves fructose-sensing neurons. In contrast to upstream regulation, the downstream consequences of fructose-sensing neurons activation on memory and food intake are mediated by the same signalling pathway, resulting in an intricate relationship between increased feeding drive and memory consolidation. Together, this study starts delineating a neuronal circuit substrate, as well as a cognitive function, for a behavioural trait resembling what is often designated in humans as emotional eating.

Online content

Any methods, additional references, Nature Portfolio 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/s41586-026-10306-z>.

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Drosophila strains and culture

Flies (*D. melanogaster*) were raised on standard medium (inactivated yeast 6% (w/v); corn flour 6.66% (w/v); agar 0.9% (w/v); methyl-4-hydroxybenzoate 22 mM) under a 12 h–12 h light–dark cycle at 18 °C with 60% humidity (unless mentioned otherwise). All of the experiments were performed on young (aged <5 days) adult flies. For behaviour experiments, groups of mixed-sex flies were used unless indicated otherwise. For imaging experiments involving surgery, female flies were used because of their larger size. All flies obtained from libraries or received after the injection of transgenes were outcrossed for five generations to a reference strain. In general, this reference line carried the *w¹¹¹⁸* mutation in an otherwise Canton-S genetic background; as an exception, and because TriP RNAi transgenes are labelled by a *y⁺* marker, lines from this collection were outcrossed to a *y⁺w^{67c23}* strain in an otherwise Canton-S background. To restrict UAS/GAL4-mediated expression to the adult stage, we used the TARGET system involving the ubiquitous expression of the thermosensitive GAL4 inhibitor under a tubulin promoter (*tub-Gal80^{ts}*)²⁹. In general, for crosses involving binary expression control systems (GAL4/UAS and/or LexA/LexAop), female flies carrying the driver transgene(s) were crossed to male flies carrying the effector transgene(s). A list of all of the single-transgene strains used in this study is provided in Supplementary Table 9. When needed, fly lines carrying combinations of multiple transgenes were obtained from these lines through routine crossing schemes.

Classical aversive or appetitive olfactory conditioning

To induce RNAi expression using the TARGET system, adult flies (aged 0 to 2 days) were kept at 30.5 °C for 3 days before conditioning. In the case of appetitive conditioning, flies were transferred to starvation vials (containing only a mineral-water-soaked cotton disk) for the last 16 h of the induction time. For experiments that did not involve thermal induction of transgene expression, experimental flies (aged 0–3 days) were transferred to fresh bottles containing standard medium 24 h before conditioning in the case of aversive conditioning experiments. For appetitive conditioning, flies (aged 0–2 days) were transferred to fresh food vials 1 day before being transferred to starvation vials for 21 h at 25 °C.

The aversive behaviour experiments, including sample sizes, were conducted similarly to other studies from our research group. Groups of 20–50 flies were subjected to one of the following olfactory conditioning protocols: a single cycle (1× training; duration of around 4 min), five consecutive associative training cycles (5× massed training; duration of around 20 min) or five associative cycles spaced by 15 min intertrial intervals (5× spaced training; duration of around 1 h 30 min). Non-associative control protocols (unpaired protocols) were also used for imaging experiments. Conditioning was performed using previously described barrel-type machines that allow parallel training of up to six groups. Throughout the conditioning protocol, each barrel was plugged into a constant air flow at 2 l min^{−1}. For a single cycle of associative training, flies were first exposed to an odorant (the CS+) for 1 min while 12 pulses of 5-s-long 60 V electric shocks were delivered; flies were then exposed 45 s later to a second odorant without shocks (the CS−) for 1 min. The odorants 3-octanol and 4-methylcyclohexanol, diluted in paraffin oil to a final concentration of 2.79 × 10^{−1} g l^{−1}, were alternately used as conditioned stimuli. In all of the behaviour experiments, two conditioning protocols were conducted sequentially on two batches of genotypically identical flies: a first set of flies was conditioned with 3-octanol as CS+ and 4-methylcyclohexanol as CS−, and a second reciprocal set with 4-methylcyclohexanol as CS+ and 3-octanol as CS−. This enabled us to balance for potential systematic choice bias during memory retrieval test (see below), as is commonly done in *Drosophila* memory experiments. During unpaired conditionings, the odour and shock stimuli were delivered separately in time, with the onset of

electric shock delivery occurring 3 min and terminating 2 min before the first odorant.

The appetitive behaviour experiments, including the sample sizes, were conducted similarly to other studies from our research group. During appetitive conditioning, flies were first exposed to an odorant (the CS+) for 1 min paired with a dried sugar reward, followed 45 s later by a second odorant (the CS−) presented without reward for 1 min, as described previously⁴⁶. The odorants were the same, and were used at the same concentration, as for the aversive conditioning experiments. Plexiglas tubes covered with dried sugar were prepared the day before conditioning: a 1.5 M sucrose solution in mineral water was spread on the tube surface and tubes were placed in front of a ventilator overnight at room temperature.

Test of memory retrieval

For aversive memory behaviour experiments, unless indicated otherwise in the figures, flies were kept on standard medium between conditioning and the memory test, at either 25 °C for flies tested 3 h after 1× training, or at 18 °C for flies tested 24 h after training. After 1× training, to assess separately the two components of memory formed after 1× training (middle-term memory, which is anaesthesia sensitive, and anaesthesia-resistant memory), a subset of the trained groups were subjected to a 4 °C cold treatment for 2 min, 2 h after training (that is, 1 h before the memory test). For appetitive memory behaviour experiments, flies were kept in starvation vials between conditioning and the memory test. The memory test was performed in a T-maze apparatus, typically 3 h after single-cycle training or 24 h after massed or spaced training. Each arm of the T-maze was connected to a bottle containing 3-octanol or 4-methylcyclohexanol, diluted in paraffin oil to a final concentration identical to the one used for conditioning. Flies were given 1 min in complete darkness to choose between either arm of the T-maze. A score was calculated as the number of flies in the CS− arm of the T-maze minus the number of flies in the CS+ arm, divided by the total number of flies in both arms. To balance for potential systematic choice bias due to a preference for either side of the maze, we combined the performance of flies conditioned with either 3-octanol or 4-methylcyclohexanol as the CS+ condition. Thus, a single performance index value reported on graphs is the average of two scores obtained from two groups of genotypically identical flies conditioned sequentially using either odorant (3-octanol or 4-methylcyclohexanol) as the CS+ condition. In particular, for cases that required counting a subset of the assayed flies after the memory test (for example, disaggregated evaluation of female and male flies, or the presence of a balancer chromosome in a parental line), scores involving fewer than six flies in total were discarded to avoid giving disproportionate statistical importance to a small number of flies. The indicated *n* is the number of independent performance index values for each genotype.

Innate shock avoidance assessment

Shock-response tests were performed at 25 °C by placing flies in two connected chambers identical to those used for olfactory conditioning. Electric shocks were delivered in only one of the compartments. Flies were given 1 min to move freely in these compartments, after which they were trapped, collected and counted. The compartment where the electric shocks were delivered was alternated between two consecutive groups. Shock avoidance was calculated as for the memory performance.

Innate olfactory acuity assessment

As the delivery of electric shocks can modify olfactory acuity, our olfactory avoidance tests were performed on flies that had first been presented with another odour paired with electric shocks. Innate odour avoidance was measured in a T-maze similar to those used for memory tests, in which one arm of the T-maze was connected to a bottle with

odour diluted in paraffin oil at the same concentration as that used for olfactory conditioning, and the other arm was connected to a bottle with paraffin oil only. Naive flies were given the choice between the two arms during 1 min. The odour-interlaced side was alternated for successive tested groups. At these concentrations, both odorants (octanol and methylcyclohexanol) are innately repulsive.

Sugar preference assessment

Innate sugar preference assessment was measured at 25 °C in a T-maze similar to those used for memory tests, in which only one of the two arms of the T-maze was covered with dried sugar. Naive flies were given the choice between the two arms during 1 min. The tests were performed on starved flies submitted to the thermal RNAi induction protocol described above. The arm with sugar was placed alternately on the right or left between two consecutive groups. Sugar response was calculated as for the memory performance.

In vivo pyruvate and glucose imaging

Pyruvate imaging experiments were performed on flies expressing the pyruvate sensor Pyronic in MB neurons through the VT30559-GAL4 driver or 13F02-LexA driver in combination with either the UAS-Pyronic or LexAop-Pyronic line, which were previously described^{14,16}. Glucose imaging experiments were performed on flies expressing the glucose sensor FLII12Pglu-700μδ6 in MB neurons through the 13F02-LexA driver, in combination with LexAop-FLII12Pglu-700μδ6. RNAis were expressed in MB neurons using the inducible tub-GAL80¹⁸; VT30559-GAL4 driver. Crosses for imaging experiments were raised at 23 °C. To achieve the induction of RNAi expression, adult flies were kept at 30.5 °C for 3 days before conditioning. Data were collected indiscriminately from 30 min to 1.5 h after 5× spaced training. A single fly was picked after few-second immobilization in a prechilled tube, and prepared for imaging using an established method¹⁴. The head capsule was opened, and the brain exposed by gently removing the superior tracheae. The head capsule was bathed in artificial haemolymph solution for the duration of the preparation. The composition of this solution was as follows: NaCl 130 mM (Sigma-Aldrich, S9625), KCl 5 mM (Sigma-Aldrich, P3911), MgCl₂ 2 mM (Sigma-Aldrich, M9272), CaCl₂ 2 mM (Sigma-Aldrich, C3881), D-trehalose 5 mM (Sigma-Aldrich, 9531), sucrose 30 mM (Sigma-Aldrich, S9378) and HEPES hemisodium salt 5 mM (Sigma-Aldrich, H7637). At the end of surgery, any remaining solution was wicked away and a fresh 90 μl droplet of this solution was applied on top of the brain. Two-photon imaging was performed using the Leica TCS-SP5 upright microscope equipped with a ×25/0.95 NA water-immersion objective. Two-photon excitation was achieved using a Mai Tai DeepSee laser tuned to 825 nm (for pyruvate sensor) or 820 nm (glucose sensor). Light emission was collected on external PMTs in the spectral ranges 468–500 nm and 529–556 nm for the blue and yellow fluorophores, respectively. Images were acquired using Leica LAS-AF (v.2.7.3).

Measurements of mitochondrial pyruvate consumption were performed as previously described^{14,16,42}. This consists in pharmacological blocking of the mitochondrial respiratory chain by sodium azide, which induces an acute increase in cytosolic pyruvate, at a rate that reflects the pyruvate consumption rate before blockade. Two-photon images were acquired in both channels at a frame rate of two images per second. After 1 min of baseline acquisition, 10 μl of a 50 mM sodium azide solution (Sigma-Aldrich, 71289; prepared in the same artificial haemolymph solution) was injected into the 90 μl droplet bathing the fly's brain, bringing the sodium azide to a final concentration of 5 mM. To analyse the pyruvate imaging experiments, regions of interest (ROIs) were delimited by hand around each visible MB vertical lobe and the average intensity of both mTFP and Venus channels over each ROI were calculated over time after background subtraction. The Pyronic sensor was designed so that FRET from mTFP to Venus decreases when pyruvate concentration increases. To obtain a signal that positively

correlates with pyruvate concentration, the inverse FRET ratio was computed as the mTFP intensity divided by the Venus intensity. This ratio was normalized to the baseline value calculated over the 30 s before drug injection. The rate of azide-induced pyruvate surge was calculated as the slope between 10 and 70% of the plateau.

Cellular glucose consumption measurements were performed as previously described¹⁵. This consisted of pharmacological inhibition of the enzyme trehalase by application of validamycin A, resulting in a decrease in the cytosolic glucose concentration. The kinetics of the glucose decrease were reflective of the rate of glucose consumption. Two-photon images were acquired in both channels at a frame rate of 1 image per second. Validamycin A (Sigma-Aldrich, 32347) was directly diluted into the artificial haemolymph solution at a final concentration of 40 mM, aliquoted and stored at –20 °C. A freshly thawed aliquot was used for every fly. After 2 min of baseline acquisition, 10 μl of the solution was added to the 90 μl saline droplet on top of the brain, bringing validamycin A to a final concentration of 4 mM. The signal was then acquired for another 14 min. ROIs were delimited by hand around the labelled ROIs (somas of MB neurons). The average intensity of the YFP and CFP channels over each ROI was calculated over time after background subtraction. The FRET ratio (YFP/CFP) of the FLII12Pglu-700μδ6 glucose sensor was computed to obtain a signal that positively correlates with glucose concentration. This ratio was normalized to the baseline value calculated over the 30 s before drug injection. As the decrease in FRET signal was not linear, the area over the curve (AOC) was calculated as a metric of the decrease that was positively correlated with glucose consumption. The AOC was calculated as the integral between 200 s and 900 s of the acquisition. The indicated *n* value is the number of flies that were assayed in each condition.

In vivo calcium imaging

Calcium imaging experiments were performed by expressing the genetically encoded calcium reporters GCaMP3 or GCaMP6s in Gr43a neurons, and GCaMP6f in dFB neurons. GCaMP3 was initially chosen to image Gr43a neurons as its higher baseline fluorescence enabled localization of the neurons, which we could not achieve with the GCaMP6f sensor. Using GCaMP6s, another GCaMP6 variant with higher baseline fluorescence, we could verify that a more-sensitive sensor and a lower fructose concentration yielded similar results to GCaMP3. Fly preparation was performed as described above for in vivo pyruvate and glucose imaging. Two-photon imaging was performed on the Leica SP8 DIVE microscope equipped with spectrally tunable hybrid detectors and a ×25/1.0 NA water-immersion objective, coupled to an Insight X3 dual-emission IR laser (Spectra Physics). Two-photon excitation was tuned at 920 nm. Emitted light was collected in the 492–570 nm spectral range. Images were acquired with Leica LAS-X software v.3.5.7. Images were acquired at a rate of one frame every 385 ms for a total of 7 min. For experiments performed on starved flies, flies were starved for 24 h at 25 °C before imaging. The composition of the physiological solution for starved flies (Fig. 1d) was the same as used for glucose imaging, except that it contained 36 mM ribose (Sigma-Aldrich, W379301) instead of sucrose and trehalose, as in ref. 37. For fructose-response experiments, fructose was added at a final concentration of 75 mM (or 20 mM when using GCaMP6s) after 120 s. This fructose concentration was previously shown to elicit Gr43a responses in the fly brain²³. Image alignment was performed using the Template Matching plugin in Fiji to correct for motion across frames and ensure accurate measurement of calcium responses. After alignment, ROIs for data analysis were delimited by hand and the signal was calculated over time after background subtraction and normalized to the baseline value calculated over the 30 s before fructose injection. Calcium response was calculated as the average from the injection time to the end of the recording. For experiments measuring the spontaneous calcium activity in dFB neurons, signals were analysed as reported previously²⁶.

Sucrose preference assay using FlyPad

The food-choice assay was done using FlyPAD⁴⁴ according to the manufacturer's instructions. One channel of the arena was loaded with 3 µl of 1% agar mixed with sucrose 0.6 M (Sigma-Aldrich), while the other channel was loaded with 3 µl of 1% agar. Single flies were captured by mouth aspiration and gently blown into each arena. Data were acquired for 60 min. Interaction with either channel (number of sips) was extracted for each arena. Flies showing zero interaction with both electrodes were not taken into account for further analysis. A sucrose preference index (PI) for each fly was calculated as: (interactions with sucrose – interactions with agar)/(interactions with sucrose + interactions with agar).

Lgr1-HA line generation

The Lgr1-HA line was generated using CRISPR–Cas9 (outsourced to Rainbow Transgenic Flies). The 3×HA sequence (TACCCATACG ATGTTCTGACTATGCGGGCTATCCCTATGACGTCCCGGACTATGCAG GATCCTATCCATATGACGTTCCAGATTACGCT) was preceded by a GS linker (GGTGGCGGCGGAAGCGGAGGTGGAGGCTCG) and inserted at the end of the *Lgr1* coding sequence using a guide RNA (5'-ATTA TGTTTAAACCGAACCCGGG-3'). A loxP-flanked sequence of the mini-white gene was added after the *Lgr1* coding sequence to provide a phenotypic marker of the presence of the HA-tagged construct.

Immunohistochemistry

Before dissection, 2- to 4-day-old female flies from appropriate crosses were fixed in 4% paraformaldehyde in PBST (PBS containing 1% Triton X-100) at 4 °C overnight. Brains were dissected on ice in PBS solution and rinsed three times for 20 min in PBST, then blocked with 2% BSA in PBST for 2 h. Next, brains were incubated with primary antibodies. For primary antibodies, this study used 1:400 rabbit anti-GFP (Invitrogen, A11122), 1:200 rat anti-Gpb5⁴¹ (provided by P. Herrero), 1:200 rat anti-HA (Roche, 11867423001) and 1:100 mouse anti-nc82 (DSHB, nc82). Primary antibodies were incubated in the blocking solution (2% BSA in PBST) at 4 °C overnight. The next day, brains were rinsed three times for 20 min with PBST and then incubated for 3 h at room temperature with secondary antibodies diluted in blocking solution. For secondary antibodies, we used 1:400 anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen, A11034), 1:400 anti-rat conjugated to Alexa Fluor 594 (Invitrogen, A11007), 1:400 anti-mouse conjugated to Alexa Fluor 594 (Invitrogen, A11005) and 1:400 anti-rat conjugated to Alexa Fluor 488 (Invitrogen, A11006). Brains were then rinsed once in PBST for 20 min, and twice in PBS for 20 min. After rinsing, brains were mounted in Prolong Mounting Medium. Images were acquired with a Nikon A1R confocal microscope using Nikon NIS-Element v.4.40 software or with an Olympus BX61-FluoView FV1000 confocal microscope using FV10-ASW v.4.2 software, as z-stack slices in 1 µm steps. For expression patterns of genetic driver lines, maximum projections are shown, obtained using Fiji (ImageJ1.52p).

RT-qPCR analyses

To validate the efficiency of the knockdowns used in this study, the mRNA of the target gene was measured using RT-qPCR. Female flies carrying the elav-GAL4 pan-neuronal driver were crossed with either male flies carrying the specified UAS-RNAi or with CS male flies, and the resulting crosses were reared at 25 °C. Adult fly progeny (aged 0–1 days) were transferred to fresh food for 1 day before RNA extraction. RNA extraction and cDNA synthesis were performed as described previously¹⁶ using the same reagents: the RNeasy Plant Mini Kit (Qiagen), the RNA MinElute Cleanup kit (Qiagen), 614 oligo(dT)20 primers and the SuperScript III First-Strand kit (Life Technologies). The cDNA level for each gene of interest was compared against the level of the *αTub84B* (CG1913) reference cDNA. Amplifications were performed using a Light-Cycler 480 (Roche) and the SYBR Green I Master mix (Roche). Reactions

were carried out in triplicate. The specificity and size of amplification products were assessed by melting curve analyses. Expression relative to the reference was expressed as a ratio ($2^{-\Delta C_p}$, where C_p is the crossing point). RT-qPCR results are presented in Supplementary Table 5. The primers used in this study are described in Supplementary Table 6.

Quantification and statistical analysis

Sample sizes in this study were similar to previous studies from our or other groups for aversive memory assays¹⁴, appetitive memory assays³⁷, metabolic imaging^{15,16} and calcium imaging⁹, rather than being predetermined by statistical power estimation. As group assignment was based on the fly genotype, random assignment of the experimental animals could not be performed, and experimenters were not blinded to the group assignment during experiments. For memory assays, a single datapoint is the mean of two scores from two groups of flies of the relevant genotype conditioned with octanol or methycyclohexanol as the odorant paired with electric shocks, as described above, which represents an experimental replicate. For in vivo imaging experiments and immunohistochemistry, one replicate corresponds to one fly brain. For RT-qPCR experiments, one replicate corresponds to the average value of three separate measurements (triplicate) done in parallel on the same biological sample. For FlyPad experiments, one replicate corresponds to one fly. All behavioural experiments were run at least twice and in vivo imaging experiments were run at least three times, on different days and with different batches of flies. All replicates are included in figures and extended data figures. Memory analyses of Gr43a neurons and dFB neurons were replicated in independent series of experiments, using different genetic drivers targeting Gr43a neurons or dFB neurons. Memory analyses involving gene knockdown in Gr43a neurons were replicated in independent series of experiments with two different RNAi constructs, except *Gpa2* for which only one RNAi line was publicly available. All replicates were successful. In general, comparisons of the data series between two conditions were achieved by a two-tailed unpaired *t*-test. Comparisons between more than two distinct groups were made using a one-way ANOVA test, followed by Tukey pairwise comparisons between the experimental group and its controls, or using a two-way ANOVA followed by Sidak pairwise comparison (Fig. 1c). ANOVA results are presented as the value of the Fisher distribution $F(x,y)$ obtained from the data, where x is the number of degrees of freedom between groups and y is the residual number of degrees of freedom for the distribution. For the analysis of sucrose preference indices obtained with FlyPAD, data series were intrinsically non-normally distributed, therefore nonparametric statistical tests were applied, that is, Mann–Whitney test for pairwise comparisons between two conditions and Kruskal–Wallis test for comparisons across multiple groups, followed by Dunn's multiple-comparison test between the experimental group and its controls. Statistical tests were performed using the GraphPad Prism v.9.0. On the figures, asterisks illustrate the significance level of the *t*-test or Mann–Whitney *U*-test, or of the least significant pairwise comparison after an ANOVA or – Kruskal–Wallis test.

Reporting summary

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

Data availability

No dataset requiring mandatory deposition into a public database was generated during the current study. Unprocessed images, which represent a large volume, are available on request by email to the corresponding authors, and will be shared without restriction. This study made use of the Flywire website to browse the publicly available FAFB connectomics dataset (<https://flywire.ai/>), and the Scope web interface to browse publicly available single-cell transcriptomics

dataset (https://scope.aertslab.org/#/Davie_et_al_Cell_2018/Davie_et_al_Cell_2018%2FAerts_Fly_AdultBrain_Filtered_57k.loom/gene). Source data are provided with this paper.

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Author contributions R.F., T.P. and P.-Y.P. conceived the project. R.F. performed most of the experiments, with the following exceptions: T.C. did pyruvate imaging experiments. A.P. carried out the design and construction of the *Lgr1-HA* line. F.B. performed immunostainings of genetic driver line expression patterns and a subset of sensory acuity control experiments. C.D. performed a part of appetitive memory experiments involving *Gr43a* and *Gpb5* knockdowns and set up FlyPad in the laboratory. P.-Y.P. performed behaviour experiments involving FB.5/6 neurons and carried out the routine genetic constructions. P.-Y.P. designed and supervised experiments. R.F. and P.-Y.P. visualized data and designed the figures. The manuscript was written by P.-Y.P., edited by R.F., A.P. and T.P., and reviewed and approved by all of the authors. T.P. and P.-Y.P. acquired funding.

Competing interests The authors declare no competing interests.

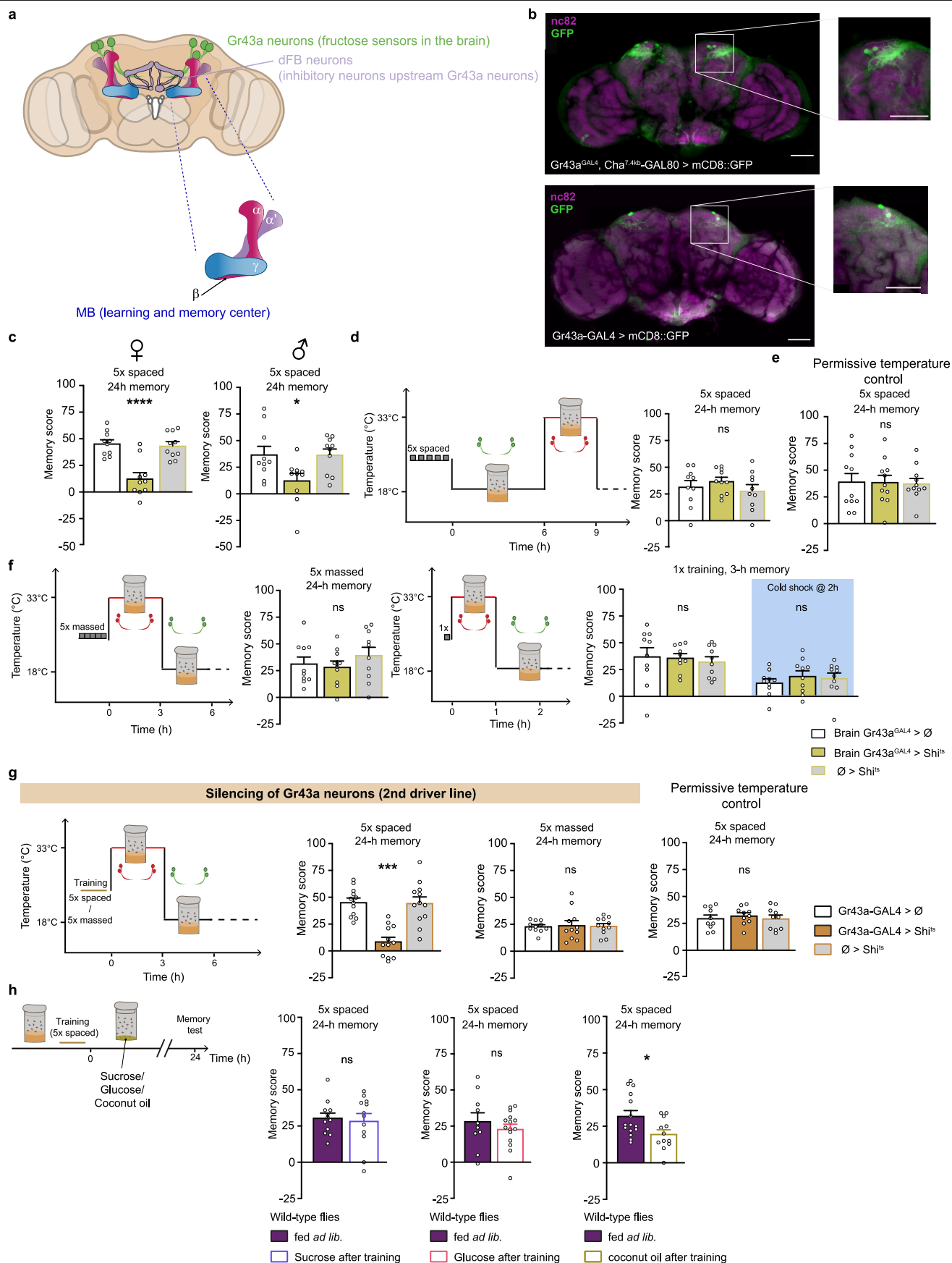
Additional information

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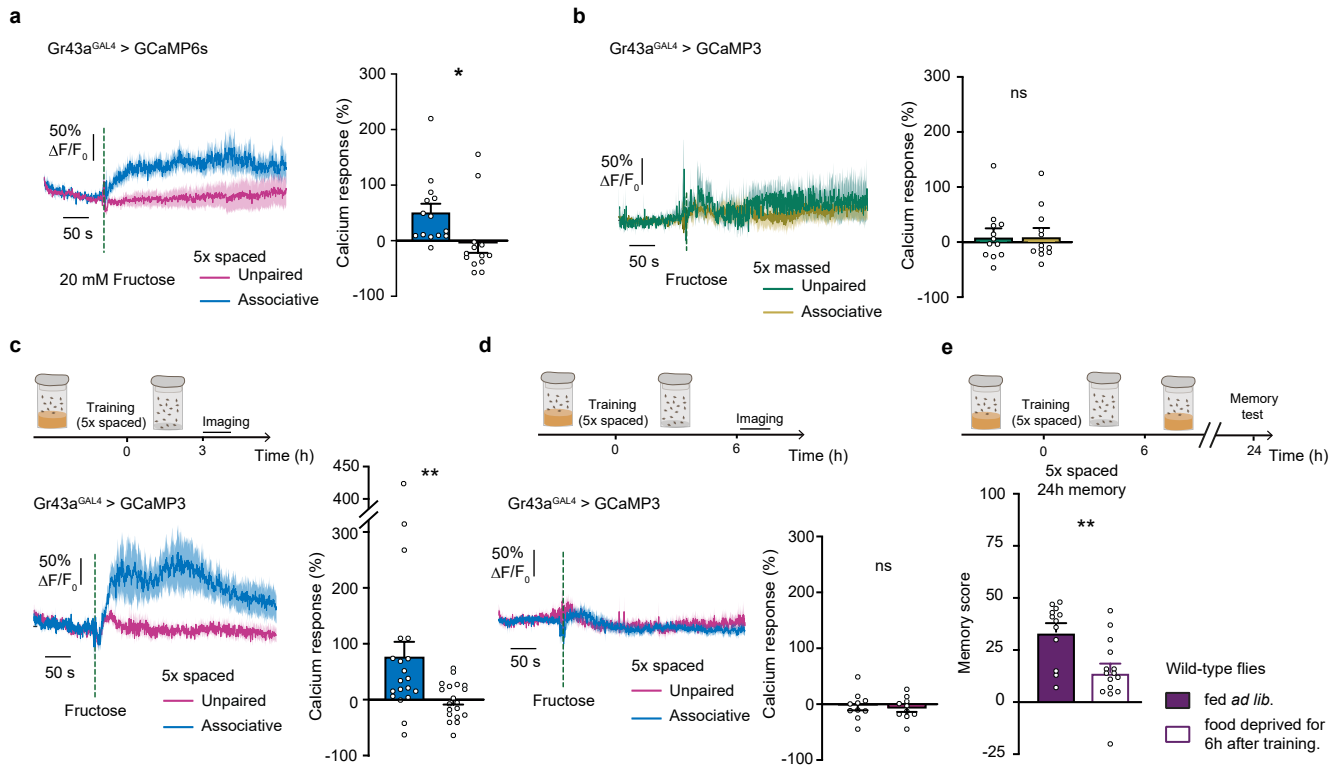
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Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Effect of Gr43a neuron silencing on other memory phases. **a**, Schematics of a fly central brain depicting the neuronal structures of interest in the study. The zoom focuses on the region of the MB lobes, formed by the bundled axons of Kenyon cells (KCs), the MB intrinsic neurons. Three categories of KCs are commonly defined (α/β , α'/β' , γ), based on the arborization and branching pattern of their axons within the different lobes. **b**, Brain expression pattern of the two driver lines used to target Gr43a neurons in this study (scale bars = 50 μ m; for each line, one example out of 5 collected brains is shown). **c**, A distinct experiment performed according to the same protocol as in Figure 1a, with female and male performance disaggregated (females: $n = 10$, $F_{2,27} = 17.74$, $p = 0.00001$; males: $n = 10$, $F_{2,27} = 4.46$, $p = 0.021$). **d**, LTM performance after spaced training when Gr43a brain neurons were silenced 6 h after training for 3 h ($n = 10$, $F_{2,27} = 0.76$, $p = 0.47$). **e**, LTM performance of flies expressing Shi^{ts} in brain fructose-sensing neurons and genotypic controls, with flies kept at

permissive temperature after training ($n = 11$, $F_{2,30} = 0.018$, $p = 0.98$). **f**, Effect of silencing brain fructose-sensing neurons with Shi^{ts} after massed training on 24-h memory ($n = 10$, $F_{2,27} = 0.75$, $p = 0.47$) or after 1x training on 3-h memory ($n = 10$, $F_{2,27} = 0.17$, $p = 0.84$; after cold shock: $n = 10$, $F_{2,27} = 0.45$, $p = 0.64$). **g**, Effect of silencing fructose-sensing neurons on memory using a second driver line targeting Gr43a neurons (spaced training: $n = 12$, $F_{2,33} = 20.72$, $p = 1.10^{-6}$; massed training: $n = 11$, $F_{2,30} = 0.03$, $p = 0.96$); LTM performance was also measured when flies were kept at the permissive temperature after spaced training ($n = 10$, $F_{2,27} = 0.25$, $p = 0.77$). **h**, LTM performance in wild flies subjected to continuous diet of sucrose 1 M ($n = 12$, $t_{22} = 0.32$, $p = 0.74$), glucose 1 M ($n = 10; 15$, $t_{23} = 0.83$, $p = 0.41$), or coconut oil ($n = 15; 12$, $t_{25} = 2.46$, $p = 0.02$) for 24-h after spaced training. Data are presented as mean $\pm$ SEM. P-values are derived from one-way ANOVA. Asterisks illustrate the outcome of Tukey pairwise comparisons as detailed in Methods–Quantification and statistical analysis.

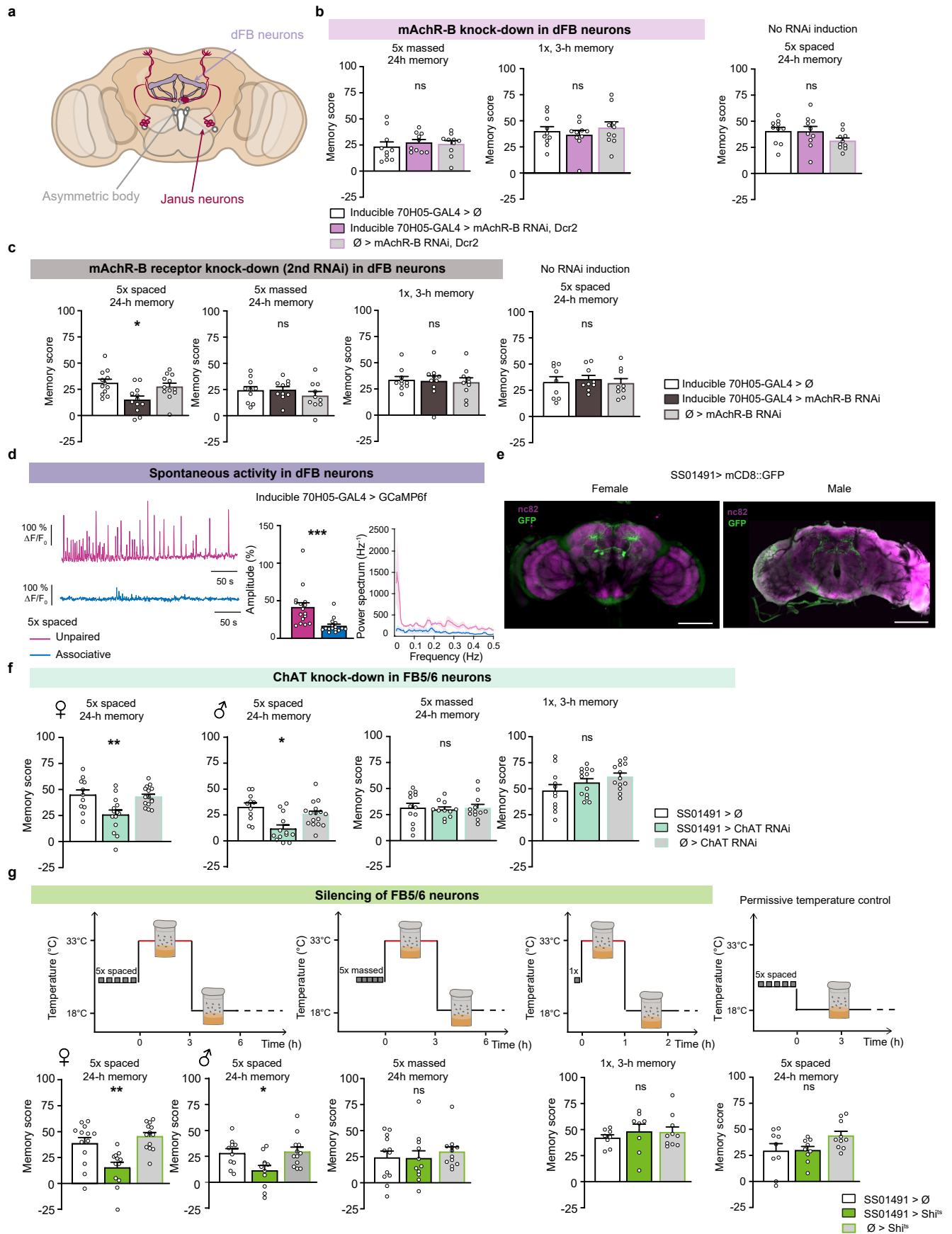


Extended Data Fig. 2 | Timecourse analysis of Gr43a neurons responsiveness after conditioning. **a**, 2-photon in vivo imaging of brain Gr43a neurons expressing the GCaMP6s calcium reporter. Time traces show the response of Gr43a neurons to bath application of 20 mM fructose (green dashed line) comparing fed flies subjected to spaced associative or unpaired protocol, quantified on the barplot ($n = 14; 13$, $t_{25} = 2.24$, $p = 0.03$). **b**, 2-photon in vivo imaging of brain Gr43a neurons expressing the GCaMP3 calcium reporter. Time traces and barplot show fructose response of Gr43a neurons in flies subjected

to massed training and to the unpaired protocol ($n = 12$, $t_{22} = 0.02$, $p = 0.97$). **c**, Fructose response of Gr43a neurons 3 h after training, quantified on the barplot ($n = 20; 19$, $t_{37} = 2.71$, $p = 0.01$). **d**, Fructose response of Gr43a neurons 6 h after training, quantified on the barplot ($n = 10$, $t_{18} = 0.40$, $p = 0.69$). **e**, Effect on LTM of food deprivation within the first 6 h after spaced training ($n = 12; 15$, $t_{25} = 3.34$, $p = 0.002$). Data are presented as mean $\pm$ SEM. P-values are derived from two-tailed unpaired t-test. Asterisks illustrate the outcome of t-test as detailed in Methods—Quantification and statistical analysis.

Extended Data Fig. 3 | Additional experiments confirming the effect of dFB neuron activation. **a**, Brain (scale bar: 100 μm) and ventral nerve chord (VNC, scale bar: 50 μm) expression pattern of the 70H05-Gal4 line (with nc82 synaptic counterstaining). Effect on memory performance of a 1-h activation of dFB neurons after massed training ($n = 10$, $F_{2,27} = 2.31$, $p = 0.11$), or single-trial training ($n = 12$, $F_{2,33} = 0.07$, $p = 0.92$). LTM performance was measured when flies were kept at permissive temperature after spaced training ($n = 11$, $F_{2,30} = 1.20$, $p = 0.31$). **b**, Experiments involving dFB neurons activation through 70H05-GAL4 were reproduced using three additional genetic drivers: a custom-built split-GAL4 line (dFB split-GAL4, Supplementary Table 9), the SS00266 Split-GAL4 line⁵¹, and the VT005528 GAL4 line⁹. Brain and VNC expression pattern of the three lines are shown additional dFB driver lines used for the experiments (with nc82 synaptic counterstaining) (scale bars: 100 μm (brain) and 50 μm (VNC)). For each line barplots show the effect on memory performance of a 1-h activation after spaced training (dFB-split: $n = 11$, $F_{2,30} = 9.14$, $p = 0.0007$; SS00266: $n = 12$, $F_{2,33} = 11.03$, $p = 0.0002$; VT005528: $n = 11$, $F_{2,30} = 8.72$, $p = 0.001$), massed training (dFB-split: $n = 10$, $F_{2,27} = 0.04$, $p = 0.95$; SS00266: $n = 11$, $F_{2,30} = 0.48$, $p = 0.62$;

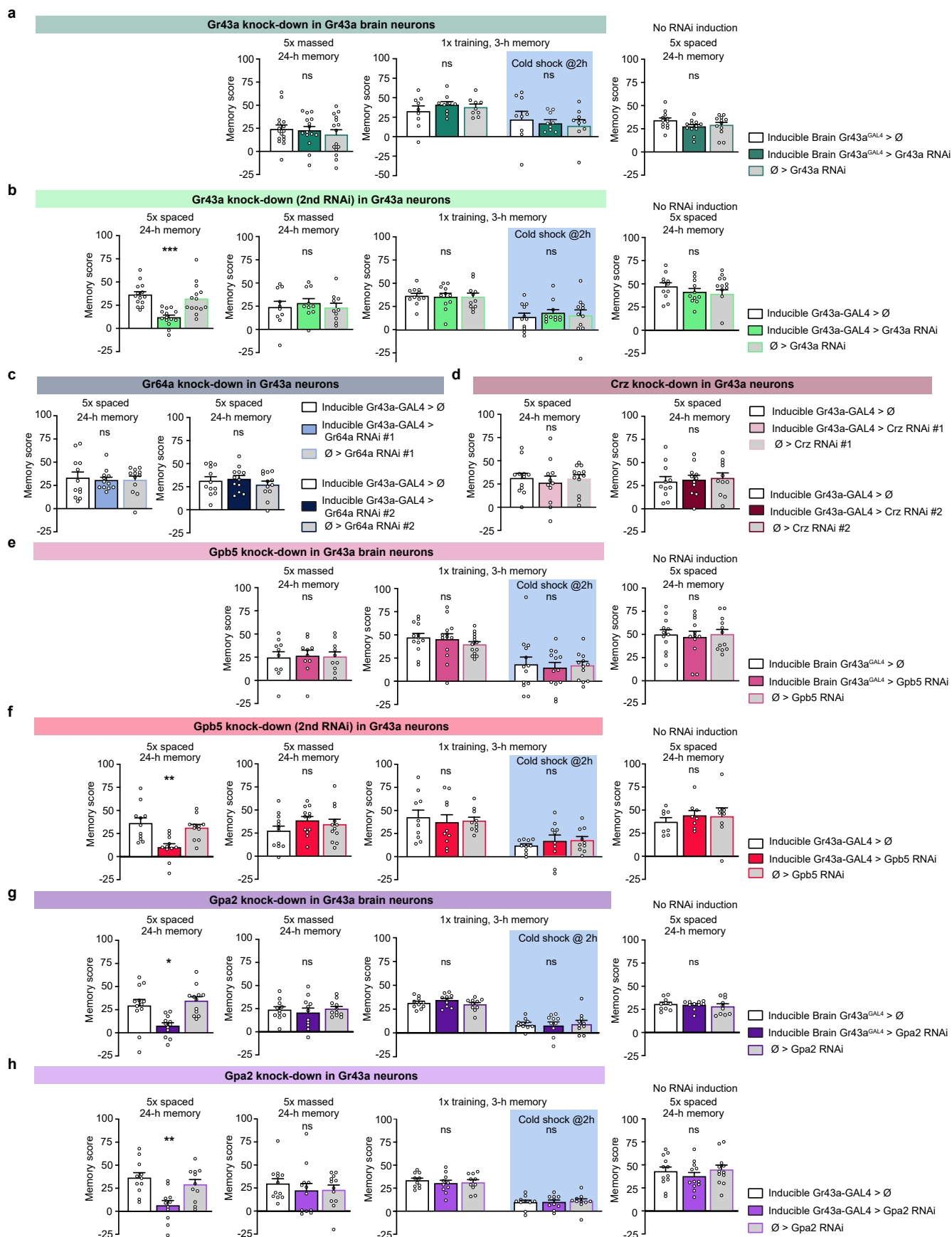
VT005528: $n = 9$, $F_{2,24} = 0.007$, $p = 0.99$), single-trial training (dFB-split: $n = 10$, $F_{2,27} = 0.59$, $p = 0.56$; SS00266: $n = 10$, $F_{2,27} = 0.27$, $p = 0.76$; VT005528: $n = 9$, $F_{2,24} = 0.44$, $p = 0.64$) and LTM performance in a permissive temperature experiment (dFB-split: $n = 10$, $F_{2,27} = 0.40$, $p = 0.66$; SS00266: $n = 10$, $F_{2,27} = 0.94$, $p = 0.40$; VT005528: $n = 11$, $F_{2,30} = 0.26$, $p = 0.76$). **c**, Brain expression pattern of the 70H05-LexA driver line used in the imaging experiment displayed on Fig. 2c. Effect on memory performance of a 1-h activation of dFB neurons using this genetic driver line after spaced training ($n = 12$, $F_{2,33} = 12.58$, $p = 0.00008$) and spaced training at permissive temperature ($n = 12$, $F_{2,33} = 0.07$, $p = 0.92$). **d**, Positive control for Fig. 3c. Flies carrying no LexA driver but undergoing the same thermal induction procedure were assayed in parallel for Gr43a neurons fructose response after the indicated conditioning ($n = 15$, $t_{28} = 2.74$, $p = 0.01$). Data are presented as mean $\pm$ SEM. P-values are derived from two-tailed unpaired t-test (**d**) or one-way ANOVA (**a-c**). Asterisks illustrate the outcome of t-test (**d**) or Tukey pairwise comparisons (**a-c**) as detailed in Methods–Quantification and statistical analysis.



Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Multiple driver lines confirm the effect of dFB neuron activation on LTM. **a**, Schematics of a fly central brain depicting Janus neurons, presynaptic to dFB neurons in the asymmetric body. **b**, Memory performance of flies with mAChR-B knock-down in dFB neurons after massed training ($n = 10$, $F_{2,27} = 0.28$, $p = 0.75$) or 1x training ($n = 10$, $F_{2,27} = 0.51$, $p = 0.60$); LTM was also tested when the expression of the RNAi against mAChR-B (KK107137) was not induced ($n = 10$, $F_{2,27} = 1.84$, $p = 0.17$). **c**, Effect on memory performance of mAChR-B knock-down in dFB neurons using a second RNAi (HMS05691; spaced training: $n = 12$, $F_{2,33} = 5.93$, $p = 0.006$; massed training: $n = 10$, $F_{2,27} = 0.71$, $p = 0.49$; 1x training: $n = 10$, $F_{2,27} = 0.07$, $p = 0.92$); LTM was also tested when the expression of the RNAi against mAChR-B receptor was not induced ($n = 9$, $F_{2,24} = 0.17$, $p = 0.83$). **d**, Positive control for Fig. 4f. Flies carrying no RNAi but undergoing the same thermal induction procedure were assayed in parallel for calcium activity of dFB neurons following spaced training. Graphs show the quantification of signal amplitudes ($n = 16$, $t_{30} = 3.69$, $p = 0.0009$) as well as the average power spectra. **e**, Expression pattern in a female or male brain of the SS01491 driver line (with nc82 synaptic counterstaining), confirming the reported expression

pattern (see 'PC1-SSI' line on Extended Data Fig. 1 of ref. 36). FB.5/6 neurons are labelled in both male and female brains, while PC1 neurons are only labelled in female (Scale bars = 100 μm ; for each sex, one example out of 4 collected brains is shown). **f**, Memory performance with ChAT knock-down (RNAiJF01877) in FB.5/6 neurons after spaced training (same data as Fig. 2h, with males and females evaluated separately; females: $n = 11;14;16$, $F_{2,38} = 7.31$, $p = 0.002$; males: $n = 11;13;16$, $F_{2,37} = 8.85$, $p = 0.0007$), massed ($n = 12;11;12$, $F_{2,32} = 0.06$, $p = 0.93$) or 1x training ($n = 11;13;13$, $F_{2,34} = 2.30$, $p = 0.11$). **g**, Effect of FB.5/6 neuron silencing after spaced training (same data as Fig. 2i, with males and females evaluated separately; females: $n = 13;12;13$, $F_{2,35} = 10.65$, $p = 0.0002$; males: $n = 10;11;12$, $F_{2,30} = 4.97$, $p = 0.01$), massed training ($n = 12;11;12$, $F_{2,32} = 0.32$, $p = 0.72$) or 1x training ($n = 8;8;10$, $F_{2,23} = 0.39$, $p = 0.67$). LTM performance was also measured when flies were kept at the permissive temperature after spaced training ($n = 9;9;10$, $F_{2,25} = 2.72$, $p = 0.08$). Data are presented as mean $\pm$ SEM. P-values are derived from two-tailed unpaired t-test (**d**) or one-way ANOVA (**a-g**). Asterisks illustrate the outcome of t-test (**d**) or Tukey pairwise comparisons (**a-g**) as detailed in Methods—Quantification and statistical analysis.

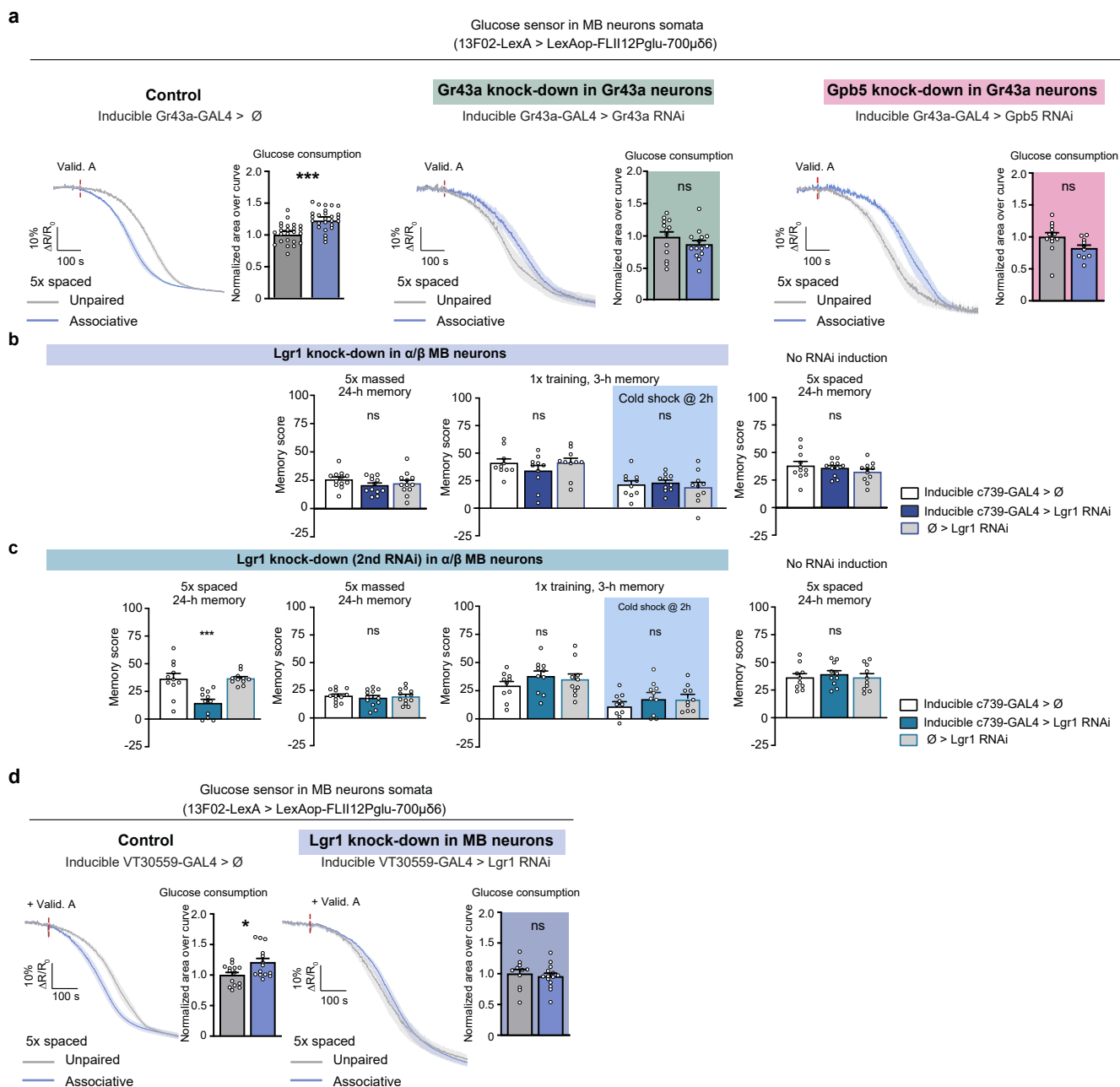


Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Extended characterization of Gr43a neurons

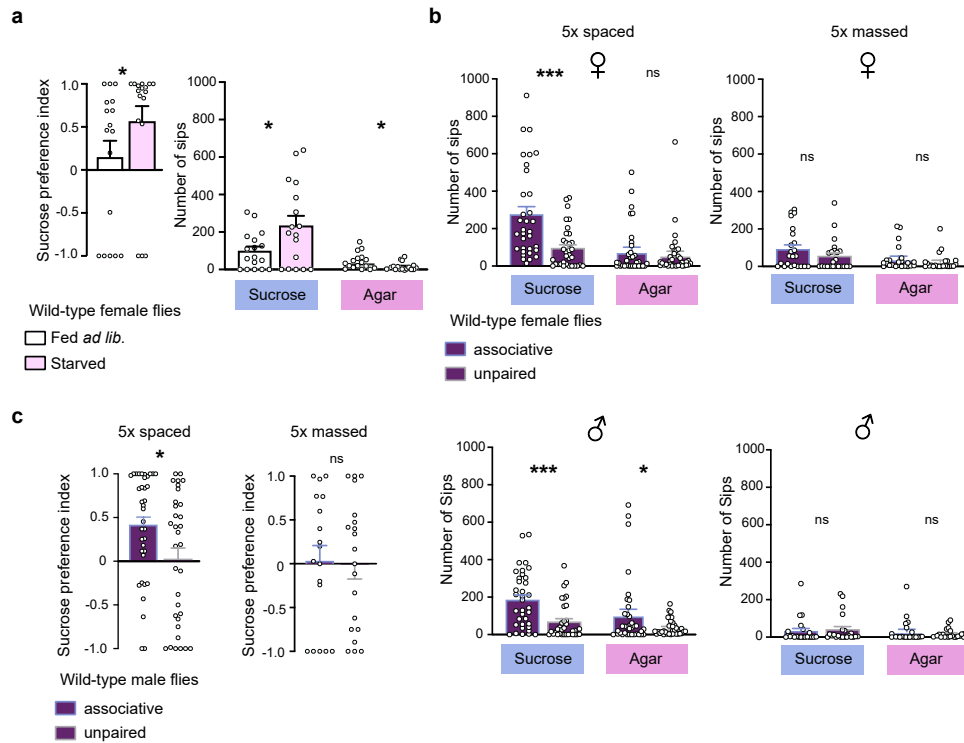
molecular signalling for aversive LTM. **a**, Memory performance of flies with Gr43a receptor knock-down (RNAi HMC05754) in brain fructose-sensing neurons after massed training ($n = 16$; 15 ; 16 , $F_{2,43} = 0.43$, $p = 0.65$) or 1x training ($n = 9$, $F_{2,24} = 0.69$, $p = 0.50$; with cold shock: $n = 9$, $F_{2,24} = 0.30$, $p = 0.73$); LTM after spaced training was tested without RNAi induction ($n = 12$, $F_{2,33} = 1.62$, $p = 0.21$). **b**, Effect on memory performance of Gr43a knock-down in fructose-sensing neurons using a second RNAi line (KK113182) and a distinct driver line (spaced training: $n = 14$, $F_{2,39} = 13.81$, $p = 0.00002$; massed training: $n = 10$, $F_{2,27} = 0.22$, $p = 0.80$; 1x training: $n = 11$, $F_{2,30} = 0.02$, $p = 0.97$, with cold shock: $n = 11$, $F_{2,30} = 0.22$, $p = 0.80$); LTM after spaced training was tested without RNAi induction ($n = 11$, $F_{2,30} = 0.97$, $p = 0.39$). **c**, LTM performance of flies with Gr64a receptor knock-down in Gr43a neurons using two different RNAi lines (RNAi#1 (vsh3300092): $n = 12$, $F_{2,33} = 0.08$, $p = 0.91$; RNAi#2 (KK112930): $n = 12$, $F_{2,33} = 0.61$, $p = 0.54$). **d**, LTM performance of flies with Crz knock-down in Gr43a neurons using two different RNAi lines (RNAi#1 (JF02023): $n = 12$; 11 ; 12 , $F_{2,32} = 0.23$, $p = 0.79$; RNAi#2 (KK110968): $n = 11$, $F_{2,30} = 0.13$, $p = 0.87$). **e**, Memory performance of flies with Gpb5 knock-down (RNAi vsh330796) in brain fructose-sensing neurons after massed training ($n = 10$, $F_{2,27} = 0.02$, $p = 0.97$) or 1x training ($n = 13$, $F_{2,36} = 0.65$, $p = 0.52$; with cold shock: $n = 13$; 13 ; 12 , $F_{2,35} = 0.07$, $p = 0.92$); LTM after spaced

training was tested without RNAi induction ($n = 12$, $F_{2,33} = 0.08$, $p = 0.92$). **f**, Effect on memory performance of Gpb5 knock-down in fructose-sensing neurons using a second RNAi construct (line published in ref. 41) and a distinct driver line (spaced training: $n = 11$, $F_{2,30} = 8.87$, $p = 0.0009$; massed training: $n = 12$, $F_{2,33} = 1.22$, $p = 0.31$; 1x training: $n = 10$, $F_{2,27} = 0.15$, $p = 0.86$, with cold shock: $n = 10$, $F_{2,27} = 0.44$, $p = 0.64$); LTM after spaced training was tested without RNAi induction ($n = 8$, $F_{2,21} = 0.31$, $p = 0.73$). **g**, Memory performance of flies with Gpa2 knock-down (RNAi vsh330752) in brain fructose-sensing neurons after spaced training ($n = 12$, $F_{2,33} = 8.23$, $p = 0.001$), massed training ($n = 11$, $F_{2,30} = 0.32$, $p = 0.72$) or 1x training ($n = 10$, $F_{2,27} = 1.28$, $p = 0.29$; with cold shock: $n = 10$, $F_{2,27} = 0.05$, $p = 0.94$); LTM after spaced training was tested without RNAi induction ($n = 10$, $F_{2,27} = 0.32$, $p = 0.72$). **h**, Memory performance of flies with Gpa2 knock-down (RNAi vsh330752) in fructose-sensing neurons using a distinct driver line and genotypic control groups after spaced ($n = 11$, $F_{2,30} = 8.30$, $p = 0.001$), massed ($n = 12$, $F_{2,33} = 0.45$, $p = 0.63$), or 1x training ($n = 10$, $F_{2,27} = 0.30$, $p = 0.73$, Cold shock: $n = 10$, $F_{2,27} = 0.04$, $p = 0.95$). LTM after spaced training was tested without RNAi induction ($n = 12$, $F_{2,33} = 0.68$, $p = 0.51$). Data are presented as mean $\pm$ SEM. P-values are derived from one-way ANOVA. Asterisks illustrate the outcome of Tukey pairwise comparisons as detailed in Methods-Quantification and statistical analysis.



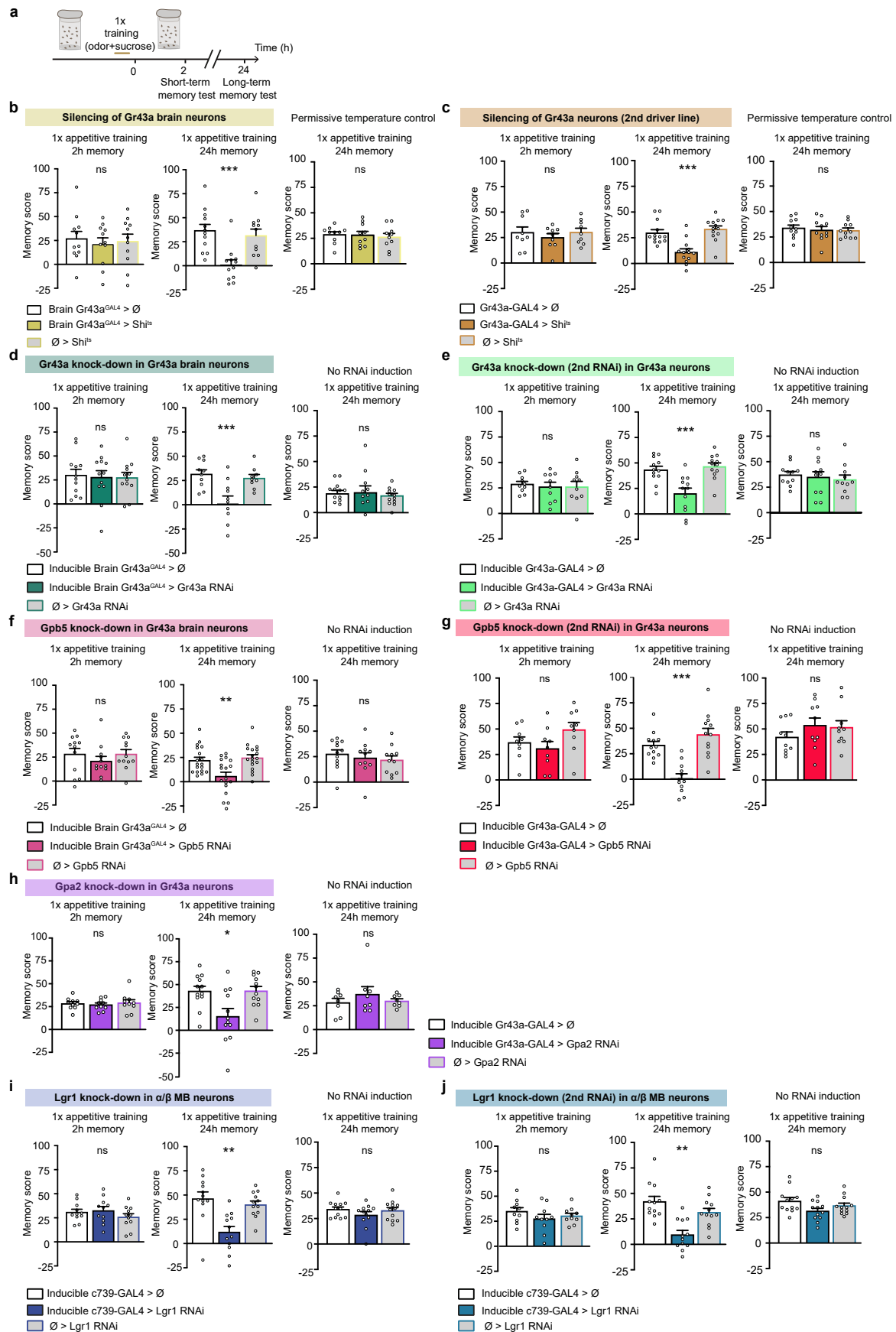
Extended Data Fig. 6 | Impact of Gr43a neuron signalling on MB glucose uptake after spaced training. **a**, Glucose imaging in MB neurons. Time traces show glucose decrease in neuronal somas following validamycin A application (red dashed line), and barplots quantify glucose consumption in flies subjected to either spaced associative or unpaired training. The three conditions shown are: no RNAi expression ($n = 24; 26$, $t_{48} = 4.81$, $p = 0.0001$); Gr43a knock-down (RNAi HMC05754) in Gr43a neurons ($n = 13; 14$, $t_{25} = 1.13$, $p = 0.26$); and Gpb5 knock-down (vsh330796) in Gr43a neurons ($n = 12; 10$, $t_{20} = 2.06$, $p = 0.05$). **b**, Memory performance of flies with Lgr1 receptor knock-down (RNAi JF02659) in α/β MB neurons and genotypic control groups after massed training ($n = 12$, $F_{2,33} = 1.18$, $p = 0.31$) or 1x training ($n = 10$, $F_{2,27} = 0.99$, $p = 0.38$; with cold shock: $n = 10$, $F_{2,27} = 0.32$, $p = 0.72$); LTM after spaced training was tested without RNAi

induction ($n = 11$, $F_{2,30} = 0.87$, $p = 0.42$). **c**, Effect on memory performance of Lgr1 knock-down in α/β MB neurons using a second RNAi construct (KK104877: spaced training: $n = 11$, $F_{2,30} = 12.97$, $p = 0.000087$; massed training: $n = 12$, $F_{2,33} = 0.25$, $p = 0.78$; 1x training ($n = 10$, $F_{2,27} = 0.92$, $p = 0.40$; with cold shock: $n = 10$, $F_{2,27} = 0.94$, $p = 0.40$); LTM after spaced training was tested without RNAi induction ($n = 10$, $F_{2,27} = 0.22$, $p = 0.79$). **d**, Glucose imaging in MB neurons, in flies subjected to either spaced associative or unpaired training. The conditions shown are no RNAi expression ($n = 14$, $t_{26} = 2.56$, $p = 0.01$) and Lgr1 knock-down (RNAi JF02659) in MB neurons ($n = 11; 14$, $t_{23} = 0.51$, $p = 0.61$). Data are presented as mean ± SEM. P-values are derived from two-tailed unpaired t-test (**a, d**) or one-way ANOVA (**b, c**). Asterisks illustrate the outcome of t-test (**a, d**) or Tukey pairwise comparisons (**b, c**) as detailed in Methods—Quantification and statistical analysis.



Extended Data Fig. 7 | Sucrose preference in females and males after massed or spaced conditioning. **a**, Sucrose preference and number of sips measured in 24h-starved or satiated wild-type female flies (Preference index: $n = 17$, two-tailed Mann-Whitney test, $U = 90$, $p = 0.035$; Number of sips: sucrose: $n = 17$, $t_{32} = 2.30$, $p = 0.028$; agar: $n = 17$, $t_{32} = 2.38$, $p = 0.023$). **b**, Number of sips in female flies subjected to an associative or unpaired spaced training (sucrose: $n = 33$; 34 , $t_{64} = 3.96$, $p = 0.00018$; agar: $n = 33$ - 34 , $t_{65} = 0.62$, $p = 0.53$) or massed training (sucrose: $n = 23$, $t_{44} = 1.32$, $p = 0.19$; agar: $n = 23$, $t_{44} = 1.08$, $p = 0.28$). **c**, The same experiment was performed on male flies: number of sips in flies

subjected to an associative or unpaired spaced training (sucrose: $n = 36$; 34 , $t_{68} = 3.86$, $p = 0.0002$; agar: $n = 36$; 34 , $t_{68} = 2.18$, $p = 0.03$) or massed training (sucrose: $n = 22$, $t_{42} = 0.39$, $p = 0.69$; agar: $n = 22$, $t_{42} = 0.78$, $p = 0.43$). Sucrose preference in male flies subjected to an associative or unpaired spaced training ($n = 36$; 33 , $U = 403$, $p = 0.02$) or to an associative or unpaired massed training ($n = 18$; 20 , $U = 179.5$, $p = 0.99$). Data are presented as mean $\pm$ SEM. P-values are derived from two-tailed unpaired t-test, unless specified otherwise in panel **a** and **c**. P-values are illustrated on graphs by asterisks as detailed in Methods-Quantification and statistical analysis.



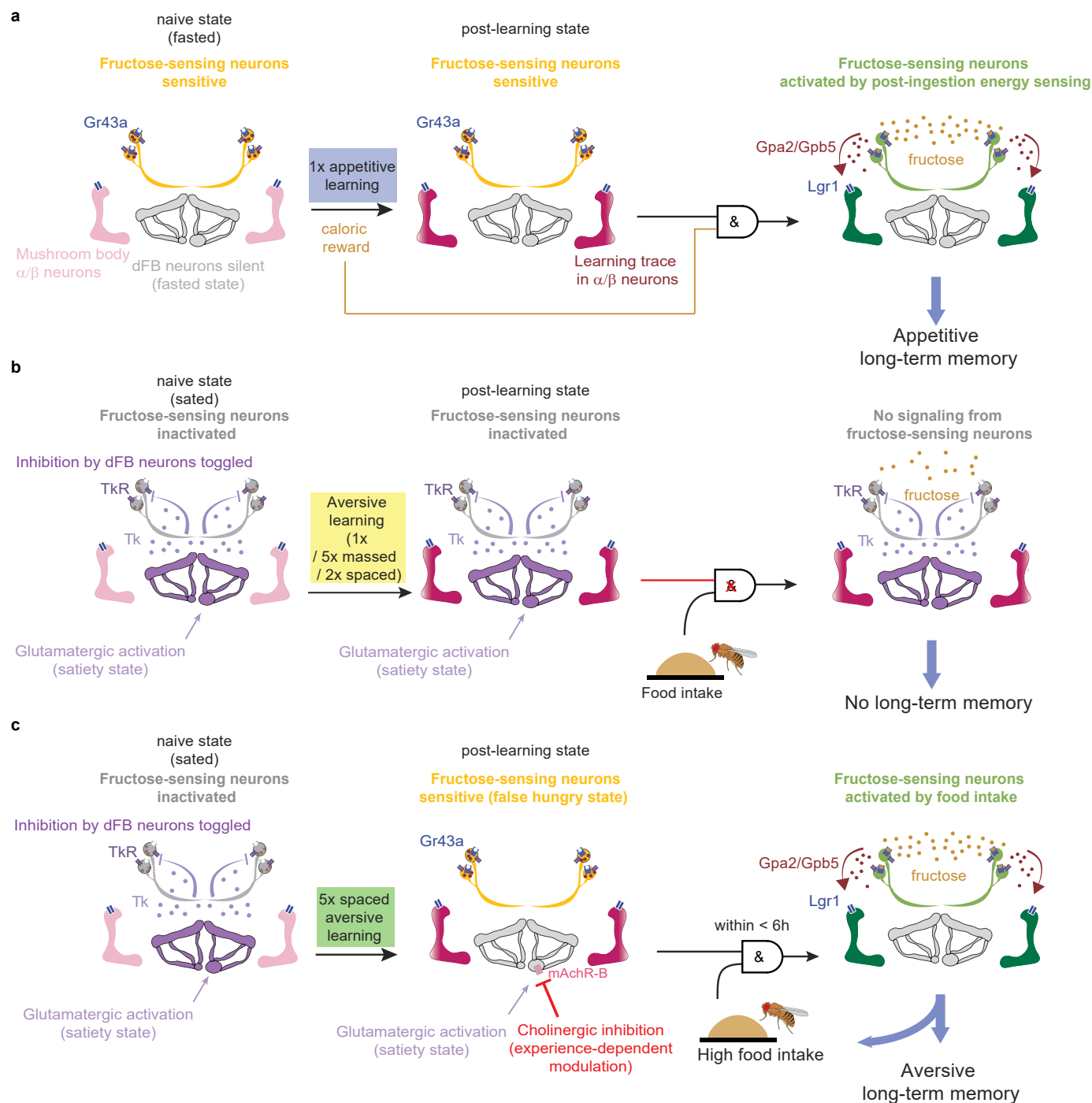
Extended Data Fig. 8 | See next page for caption.

Extended Data Fig. 8 | Thyrostimulin signalling from fructose-sensing neurons to MB is required for appetitive LTM. **a**, Schematic representation of the appetitive memory protocol. Flies are continuously food-deprived before and after learning, but receive a sucrose reward during conditioning. **b**, Effect of brain Gr43a neurons silencing for 1 h on appetitive STM ($n = 11$, $F_{2,30} = 0.16$, $p = 0.85$) and for 3 h on appetitive LTM ($n = 12; 12; 11$, $F_{2,32} = 10.25$, $p = 0.0004$), and appetitive LTM performance of flies expressing Shi^{ts} in brain fructose-sensing neurons and genotypic controls kept at the permissive temperature after training ($n = 10$, $F_{2,27} = 0.16$, $p = 0.84$). **c**, Effect on appetitive memory performance of silencing fructose-sensing neurons using a distinct driver line (2 h memory: $n = 9$, $F_{2,24} = 0.36$, $p = 0.69$; 24 h memory: $n = 13$, $F_{2,36} = 14.59$, $p = 0.00002$). LTM tested on flies kept at the permissive temperature after training ($n = 11$, $F_{2,30} = 0.20$, $p = 0.81$). **d**, Appetitive STM ($n = 12$, $F_{2,33} = 0.35$, $p = 0.71$) and appetitive LTM ($n = 10; 10; 9$, $F_{2,26} = 8.52$, $p = 0.0014$) of flies with Gr43a knock-down (RNAi HMC05754) in brain fructose-sensing neurons, and appetitive LTM tested when the expression of the RNAi was not induced ($n = 12; 10; 12$, $F_{2,31} = 1.03$, $p = 0.36$). **e**, Effect on appetitive memory performance of Gr43a knock-down in fructose-sensing neurons using a second RNAi construct (KK113182) and a distinct driver line (2 h memory: $n = 10$, $F_{2,27} = 0.13$, $p = 0.87$; 24 h memory: $n = 12$, $F_{2,33} = 12.12$, $p = 0.0001$), and LTM tested without induction procedure ($n = 11$, $F_{2,30} = 0.29$, $p = 0.74$). **f**, Appetitive STM ($n = 12$, $F_{2,33} = 0.68$, $p = 0.51$) and appetitive LTM ($n = 18$,

$F_{2,51} = 8.51$, $p = 0.0006$) of flies with Gpb5 knock-down (RNAi vsh330796) in brain fructose-sensing neurons, and appetitive LTM tested when the expression of the RNAi was not induced ($n = 12; 11; 11$, $F_{2,31} = 0.48$, $p = 0.61$). **g**, Effect on appetitive memory performance of Gpb5 knock-down in fructose-sensing neurons using a second RNAi construct (published in ref. 41) and a distinct driver line (2 h memory: $n = 9$, $F_{2,24} = 2.11$, $p = 0.14$; 24 h memory: $n = 12$, $F_{2,33} = 21.21$, $p = 1.10^{-6}$), and LTM tested without induction procedure ($n = 10$, $F_{2,27} = 0.96$, $p = 0.39$). **h**, Effect on appetitive memory performance of Gpa2 knock-down (RNAi vsh330752) in fructose-sensing neurons and genotypic controls (2 h memory: $n = 10$, $F_{2,27} = 0.17$, $p = 0.83$; 24 h memory: $n = 12$, $F_{2,33} = 6.25$, $p = 0.004$), and LTM tested without induction procedure ($n = 8$, $F_{2,21} = 0.72$, $p = 0.49$). **i**, Appetitive STM ($n = 11$, $F_{2,30} = 0.99$, $p = 0.38$) and appetitive LTM ($n = 12$, $F_{2,33} = 10.65$, $p = 0.0003$) of flies with Lgr1 receptor knock-down (RNAi JF02659) in MB α/β neurons, and appetitive LTM tested when the expression of the RNAi was not induced ($n = 12$, $F_{2,33} = 0.89$, $p = 0.41$). **j**, Effect on appetitive memory performance of Lgr1 receptor knock-down in α/β MB neurons using a second RNAi construct (KK104877; 2 h memory: $n = 10$, $F_{2,27} = 1.06$, $p = 0.36$; 24 h memory: $n = 12$, $F_{2,33} = 13.81$, $p = 0.00004$), and LTM tested without induction procedure ($n = 12$, $F_{2,33} = 2.86$, $p = 0.07$). Data are presented as mean $\pm$ SEM. P-values are derived from one-way ANOVA. Asterisks illustrate the outcome of Tukey pairwise comparisons as detailed in Methods—Quantification and statistical analysis.

Extended Data Fig. 9 | Complementary experiments on the impact of experimental disinhibition of Gr43a neurons on aversive LTM. **a**, Memory performance of flies with Tkr99D knock-down (RNAi HMC03749) in Gr43a neurons measured 24 h after 5x massed training ($n = 10$, $F_{2,27} = 0.38$, $p = 0.68$). 24-h memory after 2x spaced training was also measured without RNAi induction ($n = 11$, $F_{2,30} = 0.15$, $p = 0.85$). **b**, Effect on memory performance of Tkr99D knock-down in Gr43a neurons using a different RNAi construct (GD375) and a different driver line (5x spaced training: $n = 9$, $F_{2,24} = 0.85$, $p = 0.43$; 5x massed training: $n = 9$, $F_{2,24} = 0.19$, $p = 0.82$; 2x spaced training: $n = 14$, $F_{2,39} = 6.18$, $p = 0.004$; wild-type flies were trained in parallel with 5x spaced training as a reference for regular LTM performance, but were not included in the statistical analysis). 24-h memory after 2x spaced training was also tested without RNAi induction ($n = 12$; 12; 11, $F_{2,32} = 0.83$, $p = 0.44$). **c**, Memory performance after 2x spaced training of flies expressing Shi^{ts} in dFB neurons kept at the permissive temperature after training ($n = 11$, $F_{2,30} = 0.10$, $p = 0.90$), or when dFB neurons were silenced 6 h after training for 3 h ($n = 12$, $F_{2,33} = 0.81$, $p = 0.45$). **d**, Experiments involving dFB neurons

silencing were replicated using three additional genetic drivers presented on Extended Data Fig. 3. When relevant, wild-type flies were trained in parallel with 5x spaced training as a reference for regular LTM performance but were not included in the statistical analysis. For each line barplots show the effect on memory performance measured 24 h after 2x spaced training of dFB neuron silencing for 3 h after conditioning, with continuous access to food (dFB-split: $n = 11$, $F_{2,30} = 9.03$, $p = 0.0008$; SS00266: $n = 11$, $F_{2,30} = 10.04$, $p = 0.0004$; VT005528: $n = 12$, $F_{2,33} = 8.88$, $p = 0.0008$) or without food access while dFB neurons were silenced (dFB-split: $n = 11$, $F_{2,30} = 0.24$, $p = 0.78$; SS00266: $n = 11$, $F_{2,30} = 0.0001$, $p = 0.99$; VT005528: $n = 11$, $F_{2,30} = 0.25$, $p = 0.77$), and memory performance after 2x spaced training of flies kept at the permissive temperature (dFB-split: $n = 10$, $F_{2,27} = 0.09$, $p = 0.91$; SS00266: $n = 10$, $F_{2,27} = 0.48$, $p = 0.61$; VT005528: $n = 10$, $F_{2,27} = 0.21$, $p = 0.80$). Data are presented as mean $\pm$ SEM. P-values are derived from one-way ANOVA. Asterisks illustrate the outcome of Tukey pairwise comparisons as detailed in Methods–Quantification and statistical analysis.



Extended Data Fig. 10 | Proposed unified model of memory consolidation mediated by brain fructose-sensing system.

a, In a fasted state, dFB neurons are silent and the sensitivity of Gr43a neurons to fructose is switched on⁹ (red dots represent Gpa2/Gpb5 neuropeptide stored inside Gr43a neurons). The post-ingestive sensing of the sucrose reward presented during appetitive learning induces a transient increase in circulating fructose⁶, resulting in Gpa2/Gpb5 release that activates Lgr1 receptor in MB α/β neurons. This allows the consolidation of the associative information detected by these neurons during learning into long-term memory. **b**, In a food-sated state, ongoing activity in dFB neurons, set by glutamatergic input from Janus neurons in the asymmetric

body, induces Tk release, which through activation of its receptor (TkR) inhibits the sensitivity of brain Gr43a neurons to fructose and subsequent signalling from these neurons. As a consequence, the memory consolidation signalling from Gr43a neurons cannot occur. **c**, Multiple (typically 5) spaced aversive learning events restore the sensing ability of Gr43a neurons by silencing dFB neurons, via an inhibitory cholinergic input from FB.5/6 neurons in the asymmetric body. In this fasting-like configuration, post-learning food intake and the resulting Gpa2/Gpb5 signalling from Gr43a neurons create a self-sustained loop, while Lgr1 activation in MB α/β neurons triggers the consolidation of the labile memory trace encoded by these neurons following learning.

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed
☐	☒ The exact sample size (<i>n</i>) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i>), 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	2-photon imaging: LAS-AF software v2.7.3 and LAS-X software v3.5.7 (Leica Microsystems); Immunostainings: NIS-Element v4.40 (Nikon) or FV10-ASW v4.2 (Olympus)
Data analysis	Immunostainings: Fiji (Image J 1.52p); Statistical testing: Prism 9.0 (Graphpad); 2-photon imaging analysis: LAS-X software v3.5.7 (Leica Microsystems)

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- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

No datasets that require mandatory deposition into a public database were generated during the current study. Processed data from live imaging experiments and raw data of behavioral assays are provided as Source Data files. Unprocessed images, which represent a large volume, are available upon e-mail request to the

corresponding authors, and will be shared without restriction.

This study made use of the Flywire website to browse the publicly available FAFB connectomics dataset (<https://flywire.ai/>), and of Scope web interface to browse publicly available single-cell transcriptomics dataset https://scope.aertslab.org/#/Davie_et_al_Cell_2018/Davie_et_al_Cell_2018%2FAerts_Fly_AdultBrain_Filtered_57k.loom/gene).

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Population characteristics N/A (research not involving human participants)

Recruitment N/A (research not involving human participants)

Ethics oversight N/A (research not involving human participants)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

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

Sample size Sample sizes in this study were similar to previous studies from our or other groups for aversive memory assays¹⁴, appetitive memory assays³⁷, metabolic imaging^{15,16}, calcium imaging⁹, rather than being predetermined by statistical power estimation.

Data exclusions In memory behavior experiments, a single performance index value reported on graphs is the average of two scores obtained from two groups of genotypically identical flies conditioned sequentially using either odorant (3-octanol or 4-methylcyclohexanol) as the CS+. In particular cases that required counting a subset of the assayed flies after the memory test (e.g. disaggregated evaluation of females and males, or presence of a balancer chromosome in a parental line), scores involving less than 6 flies in total were discarded to avoid giving disproportionate statistical importance to a small number of flies.
For experiments involving the FlyPad assay, flies showing zero interaction with both electrodes during the whole course of the recording were not taken into account for further analysis.

Replication All behavioural experiments were run at least twice and in vivo imaging experiments were run at least three times, on different days and with different batches of flies. All replicates are included in figures and extended data figures. Memory analyses of Gr43a neurons and dFB neurons were replicated in independent series of experiments, using different Gr43a neurons- or dFB neurons- targeting genetic drivers. Memory analyses involving gene knock-down in Gr43a neurons were replicated in independent series of experiments with two different RNAi constructs, except Gpa2 for each only one RNAi line was publicly available. All replicates were successful.

Randomization Flies were assigned to experimental groups based on their genotypes.

Blinding Investigators were not blinded, as for each experiment the same investigator performed crosses, data collection and analysis.

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
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

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

Antibodies

Antibodies used

anti-GFP 1:400 (Molecular Probes Cat# A-11122, RRID:AB_1221569)
 anti-HA 1:200 (Roche Cat# 11867431001, RRID:AB_390919)
 anti-nc82 1:100(DSHB Cat# nc82, RRID:AB_2314866)
 anti-Gpb5 1:200 (Sellami A; Gen Comp Endocrinol. 2011 Cat# Gpb5, RRID:AB_2569510)
 The following secondary antibodies were used: 1:400 anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen, A11034), 1:400 anti-rat conjugated to Alexa Fluor 594 (Invitrogen, A11007), 1:400 anti-mouse conjugated to Alexa Fluor 594 (Invitrogen, A11005), and 1:400 anti-rat conjugated to Alexa Fluor 488 (Invitrogen, A11006)

Validation

anti-Gpb5: https://www.antibodyregistry.org/AB_2569510
 anti-GFP: https://www.antibodyregistry.org/AB_221569
 anti-nc82: https://www.antibodyregistry.org/AB_2314866
 anti-HA: https://www.antibodyregistry.org/AB_390919

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Laboratory animals

Experiments involved *Drosophila melanogaster*. The reference wild-type strain was Canton S. The primary transgenic lines used in the study are listed in Supplementary Table 9. Experiments were performed on 1-4 day old adult flies for all strains.

Wild animals

No wild animal was used in the study

Reporting on sex

In general, mixed male and female flies were used for memory behaviour experiments, except explicitly indicated cases where male and female performances were disaggregated. For in vivo imaging and immunostaining experiments, which involve dissection or surgery, female flies were assayed because of their larger size.

Field-collected samples

No field-collected samples were used in this study

Ethics oversight

No ethical approval was required for experiments done on *Drosophila melanogaster*.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks

N/A

Novel plant genotypes

N/A

Authentication

N/A