Re-evaluation of learned information in *Drosophila*

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Animals constantly assess the reliability of learned information to optimize their behaviour. On retrieval, consolidated long-term memory can be neutralized by extinction if the learned prediction was inaccurate1. Alternatively, retrieved memory can be maintained, following a period of reconsolidation during which it is labile2. Although extinction and reconsolidation provide opportunities to alleviate problematic human memories3–5, we lack a detailed mechanistic understanding of memory updating. Here we identify neural operations underpinning the re-evaluation of memory in *Drosophila*. Reactivation of reward–reinforced olfactory memory can lead to either extinction or reconsolidation, depending on prediction accuracy. Each process recruits activity in specific parts of the mushroom body output network and distinct subsets of reinforcing dopaminergic neurons. Memory extinction requires output neurons with dendrites in the α and α′ lobes of the mushroom body, which drive negatively reinforcing dopaminergic neurons that innervate neighbouring zones. The aversive valence of these new extinction memories neutralizes previously learned odour preference. Memory reconsolidation requires the γ2α′1 mushroom body output neurons. This pathway recruits negatively reinforcing dopaminergic neurons innervating the same compartment and re-engages positively reinforcing dopaminergic neurons to reconsolidate the original reward memory. These data establish that recurrent and hierarchical connectivity between mushroom body output neurons and dopaminergic neurons enables memory re-evaluation driven by reward-prediction error.

Valence of *Drosophila* olfactory memory is coded as a skew in the mushroom body output network6–8 (Extended Data Fig. 1a–f). Reward memories are written by dopaminergic neurons (DANs) that innervate the horizontal mushroom body lobes8,9, by depressing drive evoked by learned odour to mushroom body output neuron (MBON) pathways that direct avoidance8. Aversive memories are reinforced by DANs that primarily innervate the vertical mushroom body lobes11,12 and reduce odour-evoked drive to MBONs directing approach6,13. Learned preference is weakened (or extinguished) in flies that experience the conditioned odour without reinforcement14–16, suggesting that flies reassess learned predictions (Extended Data Fig. 1g–i). We investigated mechanisms of re-evaluation of sugar-rewarded appetitive memory (Fig. 1a). Food-deprived flies were trained with one odour without reinforcer (conditioned stimulus minus, CS−), followed by air, then another odour (conditioned stimulus plus, CS+) with sugar reward17. This training establishes long-term memory that is consolidated within hours of training18. We challenged memory 3 h after training with two presentations of CS+, CS−, a novel odour (specificity control) or air (handling control), and then 3 h later tested the flies’ preference between CS+ and CS−. Flies exposed to CS−, air or novel odour displayed robust 6 h memory performance (Fig. 1a and Extended Data Fig. 2a). However, CS+ exposure, without an accompanying sugar reward, at 3 h abolished 6 h performance. Memory performance was similarly reduced following one or three CS− presentations at 3 h (Extended Data Fig. 2b) and remained depressed for at least 24 h (Extended Data Fig. 2c). Importantly, odour exposure alone in naïve flies did not change

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Figure 1 | Extinction of reward memory requires negatively reinforcing dopaminergic neurons. a, Top, schematic of trials. Bottom, only CS−-evoked memory reactivation at 3 h leads to extinction of appetitive memory (*n* ≥ 8). b, Blocking rewarding DANs in the protocerebral anterior medial (PAM) cluster during 3 h CS+ or CS− re-exposure did not alter extinction or 6 h learned approach (*n* ≥ 15). c, Blocking aversive DANs in the paired posterior lateral 1 (PPL1) cluster during CS− reactivation significantly impairs extinction, while block during CS+ reactivation leads to loss of memory (*n* ≥ 10). d, Blocking PPL1 DANs without reactivation does not alter 6 h performance (*n* ≥ 10). Unless otherwise noted, in all figures data represent the mean ± s.e.m. Asterisks denote significant difference (*P* < 0.05, ANOVA or *t*-test) between groups of same genotype treated differently; hash (#) denotes significant difference (*P* < 0.05, ANOVA) between different genotypes treated identically. A break in the x axis indicates independent experiments.
Reconsolidation of reward memory is triggered by CS– exposure and requires MB-MV1/PPL1–Ca2+1 dopaminergic neurons. a, Re-exposing trained flies to CS− odour renders reward memory sensitive to cold-shock anaesthesia (n ≥ 7). b, Memory remains sensitive 30 min after CS− reactivation but returns to a cold-shock-resistant state by 90 min (n = 10). Asterisk in b indicates a significant difference (P < 0.05) to the non-cold-shocked group. c, Blocking MB-MV1 DANs during CS− reactivation abolishes 6 h learned approach but blocking during CS+ reactivation leaves extinction intact (n ≥ 12). d, MB-MV1 block after CS− reactivation does not significantly impair 6 h performance (n ≥ 23). e, MB-MV1 output is dispensable during 3 h memory retrieval (n ≥ 14). f, Blocking MB-MV1 DANs during CS− reactivation abolishes 6 h approach towards the CS+ (n ≥ 11). NS, not significant.

Preference measured 3 or 21 h later (Extended Data Fig. 2d). Therefore, sugar-rewarded memories can be extinguished by presenting the conditioned stimulus without reward after training.

Since distinct DANs reinforce aversive11,12 and appetitive memories8,9 we tested each group’s involvement in memory extinction. We blocked DAN output with the dominant temperature-sensitive UAS-shibire111 (shi111) transgene18. At temperatures > 29°C, shi111 blocks membrane recycling and thus synaptic vesicle release; at < 25°C, synaptic release is restored. All flies were trained at permissive 23°C, raised to 32°C 30 min before and during reactivation at 3 h, immediately returned to 23°C and tested for memory at 6 h. Blocking rewarding DANs did not impair extinction (Fig. 1b): R58E02-GAL4;UAS-shi111 flies retained no measurable 6 h memory following CS+ exposure at 3 h but displayed robust performance following CS− exposure. We also tested whether, without a DAN-mediated reward signal to the mushroom body, appetitive memory would be extinguished, by retraining flies at 3 h while blocking rewarding DANs. Perhaps surprisingly, R58E02-GAL4;UAS-shi111 flies retained appetitive memory performance that was indistinguishable from their controls (Extended Data Fig. 2e), consistent with attenuation of rewarding DAN signals when learned performance is maximal19.

We blocked aversively reinforcing DANs specifically during memory reactivation using the drivers TH-GAL4 and the even more restricted MB504B-GAL4 to express UAS-shi111 (Fig. 1c). Extinction driven by CS+ exposure was compromised in TH-GAL4;UAS-shi111 and MB504B-GAL4;UAS-shi111 flies. In addition, when TH-GAL4 or MB504B-GAL4 output was blocked during 3 h CS− exposure, 6 h memory was decreased. Neither disruption was evident in experiments at 23°C (Extended Data Fig. 2f). Moreover, blocking aversive DANs did not affect 6 h memory in flies exposed to no odour or a novel odour at 3 h, (Fig. 1d and Extended Data Fig. 2g) nor odour preference of untrained flies 3 h after odour exposure (Extended Data Fig. 2h). Therefore,
aversive DANs play a prominent role in appetitive memory extinction; blocking them also uncovers an unexpected role for the CS− in memory re-evaluation.

Prior work suggests that reward omission during CS+ exposure might alter the predictability of learned information and drive extinction learning. Small changes in a subsequent training trial instead trigger reconsolidation, which maintains the original memory20,21. Since CS− exposure represents part of a learning trial, we tested whether it induced reconsolidation. *Drosophila* reward memory consolidation is apparent as a time-dependent resistance to cold-shock anaesthesia17. We therefore followed 3 h CS−-driven memory reactivation with 2 min of cold-shock (Fig. 2a). Cold-shock immediately or 30 min after CS− exposure abolished 6 h appetitive memory, but not if anaesthesia was applied without reactivation or 90 min after CS− exposure (Fig. 2a, b and Extended Data Fig. 3a). Day-old memories also became sensitive to cold-shock when reactivated with CS− exposure 21 h after training (Extended Data Fig. 3b). CS− exposure therefore induces time-dependent memory reconsolidation. Blocking aversive DANs during CS− exposure (Fig. 1c) revealed a similar effect to that of cold-shock afterwards (Fig. 2a, b), suggesting reconsolidation ordinarily requires aversive DANs.

To define aversive DANs responsible for reconsolidation and extinction we blocked MB-MP1 (paired posterior lateral 1 (PPL1)-α′ pedc), MB-MV1 (PPL1-α′), MB-V1 (PPL1-α′,2) or PPL1-α 3 DANs of the mushroom body during memory reactivation with either CS− or CS+ odours and measured 6 h memory (Fig. 2c and Extended Data Fig. 2f). None of these manipulations significantly impaired CS−-driven memory extinction, suggesting that extinction does not rely on individual aversive DANs. By contrast, blocking MB-MV1 DANs significantly reduced 6 h memory when done during CS− memory reactivation (Fig. 2c and Extended Data Fig. 2f), but not immediately or 90 min after CS− exposure (Fig. 2d and Extended Data Fig. 3g) or in experiments at 23°C (Extended Data Fig. 3 h). Furthermore, MB-MV1 DANs are dispensable for learning and expression of 3 h appetitive memory (Fig. 2e and Extended Data Fig. 3i). We tested whether the CS− odour memory was affected after presenting the CS− while blocking MB-MV1 DANs, by testing the flies’ preference between CS− and a novel odour, rather than CS− and CS+. Whereas controls robustly chose CS− over the novel odour, flies in which MB-MV1 DANs were blocked during CS− re-exposure displayed no CS− memory (Fig. 2f). Therefore, output from MB-MV1 DANs throughout, but not after, CS−-driven memory...
reactivation is necessary to reconsolidate memory of the CS⁻ and CS⁺ relationship.

Individual DANs tile the mushroom body lobes into 15 discrete compartments, each containing the dendrites of a corresponding MBON. Anatomy suggests that MBONs then connect to DAN dendrites, forming recurrent mushroom body→MBON→DAN→mushroom body feedback loops (Extended Data Fig. 1c). Since sugar-reward learning inhibits relative CS⁻ odour drive to MBONs at the tip of the horizontal lobe, we tested MBON involvement in extinction and reconsolidation. We again restricted disruption to CS⁺- or CS⁻-driven memory reactivation at 3 h and tested 6 h memory. MB502B-GAL4 labels cholinergic V2 cluster MBONs (α2sc, α', α2p3p, α3ap and α3m') whose dendrites overlap with aversive MBONs required for extinction. Blocking V2 MBONs impaired CS⁻-driven memory extinction and not CS⁺-driven reconsolidation (Fig. 3a). No defect was apparent when adjacent MVP2 MBONs were blocked during memory reactivation (Extended Data Fig. 4a) or in V2 MBON experiments at 23°C (Extended Data Fig. 4b). Moreover, V2 MBONs are dispensable for 3 h memory expression (Fig. 3b), suggesting that they serve a function distinct from behavioural direction.

To test the functional connectivity between V2 MBONs and aversive PPL1 DANs, we used light to stimulate V2 MBONs expressing lexAop-CsChrimson, while recording activity in PPL1 DANs with UAS-GCaMP6f. V2 MBON activity produced a robust calcium response in two of three PPL1 DAN cell bodies (Fig. 3c–e and Extended Data Fig. 4c, d), with one activated and the other inhibited. Dopaminergic plasticity in γ and α2 mushroom body zones has been implicated in aversive odour memory, and DANs respond differently to odours after aversive training. However, we detected no obvious change in odour-drive of V2 MBONs or PPL1 DANs after reward training (Extended Data Fig. 4e, f). Considering connectivity and behavioural data along with the prior demonstration of reduced drive to horizontal-lobe MBONs after reward training, we propose that CS⁻ exposure in trained flies drives an MBON network skewed towards V2 MBONs. This in turn preferentially activates MB-MP1, and perhaps MB-MV1 and PPL1-α3 DANs, which write a new, parallel and competing CS⁻-specific aversive memory. This manifests behaviourally as extinction of the original reward memory (Extended Data Fig. 1g–i).

We next investigated cholinergic γ2α1 MBONs whose dendrites overlap with MB-MV1 DANs required for reconsolidation. These MBONs have to potentially perform two roles: they must represent the CS⁻ after training and provide a means to link a CS⁻ response to reconsolidation of the CS⁺ memory. Like MB-MV1 DANs, blocking γ2α1 MBONs during, but not after, CS⁻-driven memory reactivation impaired memory reconsolidation (Fig. 4a and Extended Data Fig. 5a), while blocking γ2α1 MBONs during CS⁺ reactivation did not disrupt extinction (Fig. 4a). However, unlike MB-MV1 DANs, or V2 MBONs, blocking the γ2α1 MBONs abolished the learned approach at 3 h (Fig. 4b and Extended Data Fig. 5b), demonstrating that γ2α1 MBONs direct memory-guided behaviour as well as odour re-evaluation.

Anatomy suggests that γ2α1 MBONs connect with dendrites of MB-MV1 and rewarding DANs that innervate the horizontal lobes of the mushroom body. We therefore assessed functional connectivity by stimulating γ2α1 MBONs while monitoring activity in either MB-MV1 or rewarding DANs. Light-triggered activation of γ2α1 MBONs evoked robust calcium responses in MB-MV1 (Fig. 4c) and rewarding DANs (Fig. 4d). To determine whether γ2α1 MBONs contained a trace of reward learning, we expressed UAS-GCaMP6f in γ2α1 MBONs and performed two-photon functional calcium imaging of CS⁻ and CS⁺ odour responses (normalized to those of a novel odour) 3 h after sugar reward or mock training (Fig. 4e). Responses evoked after training in γ2α1 MBONs by either of two CS⁻ odours were significantly increased over those in mock-trained flies. CS⁻-evoked responses were not statistically different between groups. Therefore, reward learning produces a CS⁻-specific increased response in γ2α1 MBONs.

Since γ2α1 MBONs also connect to rewarding DANs (Fig. 4d), we tested whether reconsolidation involved signalling from rewarding DANs after memory reactivation. Flies were trained and 3 h memory was reactivated with CS⁻ odour. Blocking rewarding DANs immediately, but not 90 min, after memory reactivation abolished 6 h memory (Fig. 4f). Therefore, reconsolidation results from increased CS⁻-drive to γ2α1 MBONs after training that recruits recurrent MB-MV1 DANs and also re-engages rewarding DANs to reinitiate the original CS⁻ reward memory. We cannot yet establish what generates the different temporal requirements for MB-MV1 and rewarding DANs, or the nature of updating memory.

Our data demonstrate that flies re-evaluate memory at retrieval using discrete modules of their mushroom-body-directed dopaminergic system. Different pathways are engaged depending on whether the difference between the learned expectation and the actual experience is large or small, yielding either memory extinction or reconsolidation, respectively. It will be important to determine whether dopamine-based error prediction and reassessment of learned information in mammals utilizes similar neural network motifs and operating logic.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions J.F. and S.W. conceived the project and designed all experiments. S.L. performed initial extinction experiments. J.F. performed and analysed all behavioural experiments with help from P.C. O.B. performed imaging experiments assisted by J.F. Live imaging data were analysed by O.B. and P.C. The manuscript was written by S.W. and J.F. with comments from P.C. and O.B.

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METHODS

Data reporting. Experimental conditions, odours and genotypes were alternated and interleaved to balance out biases in order, timing, equipment and environmental factors.

Fly strains. All fly strains (Drosophila melanogaster) were raised on standard cornmeal-agar food with additional molasses and active dried yeast at 25°C in 40–50% humidity. The wild-type strain was Canton-S. GAL4 lines (Extended Data Fig. 6) have been described previously: TH-GAL4, R5802-GAL4, MB052B, MB055B, MB077C, MB083C, MB112C, MB298B, MB308B and MB549B-GAL4, R7307-GAL4, 061-GAL4, MB168B30 and UAS-shits (ref. 18). LexA lines have been described: R24H08, R25D01, R65B09 and R71D08-LexA32 and R5802-LexA43. The reporter stocks UAS-GCaMP6f and lexAop-GCaMP6m44 and optogenetic trigger flies UAS-CsChrimson::mVenus35 and lexAop-CsChrimson::tdTomato,UAS-GCaMP6m44 have also been described previously.

Behaviour. For behavioural experiments, males from GAL4 lines were crossed to UAS-shits females, except in the case of the 061-GAL4;MB168B flies where UAS-shits flies were crossed to 061-GAL4;MB168B females. For heterozygous controls GAL4 or UAS-shits flies were crossed to Canton-S. All flies were raised at 25°C and mixed sex populations of 4–9-day-old flies were used in all experiments. Flies were starved for 18–24 h on 1% agar before training and were kept starved for the entire experiment. Appetitive training was performed and quantified as described31. In brief, groups of 50–100 flies were exposed to the CS− for 2 min followed by 30 s of room air and then 2 min of the CS+ in the presence of dry sucrose. For memory reactivation experiments flies were given two 2-min exposures (spaced by 15 min) of the CS− odour, the CS+ odour (without reward), a novel odour, or room air. During the final memory test flies were given 2 min to choose between two odours in a T-maze: either the CS− versus the CS+, or the CS− versus a novel odour. 3-octanol (OCT, 7 μl in 8 ml mineral oil) and 4-methylcyclohexanol (MCH, 7–12 μl in 8 ml mineral oil) were used as trained odours and isoamyl alcohol (IAA, 7 μl in 8 ml mineral oil) and ethyl butyrate (EB, 7 μl in 8 ml mineral oil) were used as novel odours in all experiments, except Fig. 2f and Extended Data Figs 2d and 3a. In Fig. 2f ethyl butyrate was used as CS+ and 3-octanol and 4-methylcyclohexanol were used as CS− or as novel odour. In Extended Data Figs 2d and 3a, IA and EB were used as trained odours. For cold-shock anesthesia flies were transferred into pre-chilled plastic vials and kept on ice for 2 min as described previously31,37. For neural inactivation experiments with UAS-shits, all flies were shifted to restrictive 33°C. Restrictive temperature was imposed immediately after reactivation by transferring flies to pre-heated vials containing 1% agar.

Explant brain two-photon calcium imaging. Prior to all optogenetic experiments flies were housed for 1–3 days on standard cornmeal food supplemented with 1 mM retinal. Combined optogenetic and calcium imaging experiments were conducted using a two-photon microscope (Scientifica). Explant brains were placed on a polylysine-coated glass cover slip bathed in carbogenated (95% O2, 5% CO2) buffer solution (103 mM NaCl, 3 mM KCl, 5 mM N-Tris, 10 mM trehalose, 10 mM glucose, 7 mM sucrose, 26 mM NaHCO3, 1 mM Na2HPO4, 1.5 mM CaCl2, 4 mM MgCl2, osmolarity 275 mM, pH 7.3) following dissolving in cold calcium-free buffer. For light stimulation a high-power LED (Multicomp OSW-6338, 630 nm) was relayed onto the specimen via a 0.5 mm diameter lens with focal length 60 μm filtered through a 632.10 bandpass filter (Edmund Optics). Power at the specimen was measured to be 0.85 mW mm−2. The LED was triggered using a microcontroller (Arduino MEGA). After rapid identification of focus on the respective field of view, brains were left to settle for 5 min before being imaged. Following 10 s of baseline recording, a light pulse was delivered at 40 Hz, with 10 ms duration for a total of 200 ms (Fig. 4d) or 500 ms (all other experiments). Fluorescence (F) was excited using 140-fs pulses, 800 nm repetition rate, centred on 910 nm generated by a Ti: Sapphire laser (Chameleon Ultra II, Coherent). Images of 256 × 256 pixels were acquired at 5.92 Hz, controlled by ScanImage 3.8 software34. PPL.1 DANs were imaged at the level of the cell body in order to avoid inadvertent CsChrimson stimulation from the two-photon imaging laser. PAM DANs were imaged at the level of the β5 compartment. In general, light exposure to the brain was kept at a minimum. Two-photon fluorescence images were manually segmented using ImageJ and further analysed using custom-written MATLAB scripts. For quantification, F0 was defined as the mean F from the 9 s prior until 1 s before stimulation. ΔF/F0 was compared between 1 s before stimulation with 1 s after stimulation onset, using a paired t-test.

In vivo two-photon calcium imaging. Two-photon imaging of odour-evoked calcium responses was performed as described30. 3–8-day-old flies were briefly (5–10 s) immobilized on ice and mounted in a custom chamber 2.5–3.5 h after appetitive training using MCH or OCT (see ‘Behaviour’ section) or mock treatment (odour presentation without sugar). Legs and proboscis were immobilized with wax to reduce movement artefacts. The head capsule was opened under room temperature carbogenated buffer (see above). Odours were delivered on a clean air carrier stream using a custom-designed system35, which also synchronizes timing of odour delivery and two-photon image acquisition. Two-photon fluorescence images were acquired as described above and manually segmented using ImageJ. Movement of the animal was small enough such that images did not require registration. Two-photon image acquisition was performed using custom-written MATLAB routines. After 40 s of clean air, flies were exposed to 5 s of either trained odour (MCH or OCT; air stream passing over 100× odour dilution in mineral oil, and then further blended 1:9 with a clean air stream), then 15 s clean air, followed by 5 s of the other odour (MCH or OCT) pulse, then 15 s clean air, followed by 5 s of a novel odour (IAA). The protocol was repeated five times but only the first presentation is shown in the figures. We excluded flies from further analysis if they did not show any visible odour responses. Trained and mock-trained flies were constantly alternated during training and testing. In Fig. 4e, 15 out of 73 flies were excluded (mock group: 9, trained group: 6). In Extended Data Fig. 4e, 9 out of 56 flies were excluded (mock: 5, trained: 4). In Extended Data Fig. 4f, 3 out of 34 flies were excluded (mock MP1: 1, trained MP1: 3). For quantification, baseline fluorescence F0 was defined for each stimulus response as the mean F from 1 s before up to the point of stimulation. F/F0 accordingly describes the fluorescence relative to this baseline. The area under the curve (AUC) was measured as the integral of F/F0 during the 5 s between odour stimulation onset and offset. In order to reduce the level of variance between flies, CS− and CS+ were further normalized by dividing each AUC by the novel odour AUC from the respective trial and the same fly, thus obtaining a normalized AUC.

Immunohistochemistry. Brains were dissected in ice-cold PBS and fixed for 20 min in 4% paraformaldehyde in PBS, then stained as described36. Brains were incubated for 1–2 days with primary antibodies against GFP (chicken, Abcam 13970, diluted 1:2000) and Bruchpilot (mouse, DSHB nc82, diluted 1:50) followed by 1–2 days with secondary antibodies (anti-chicken Alexa 488 diluted 1:1000 and anti-mouse Alexa 633 diluted 1:200, Life Technologies), interspersed with washes in PBS with 0.3% Triton. Brains were mounted in Vectashield medium (Vector Labs), then the immunostained fluorescent signal and (when present) native RFP fluorescence were imaged using a Leica SP5 confocal microscope at 25× magnification, using manually adjusted laser and gain settings.

Statistics. Statistical analyses were performed in GraphPad Prism 6. All behavioural data was tested for normality using the D’Agostino and Pearson omnibus test. Normally distributed data were analysed with one-way ANOVA followed by Tukey’s honest significant difference (HSD) post hoc test. For non-Gaussian distributed data, a Kruskal–Wallis test was performed following Dunn’s multiple comparison test. Odour response normalized AUC was compared between groups using multiple t-tests with Holm–Sidak correction. No statistical methods were used to predetermined sample size.

Data availability. The data supporting the findings of this study are available from the corresponding author upon reasonable request.
Network structure

a) Dopaminergic neurons (DANs)

b) Mushroom body output neurons (MBONs)

c) Recurrent networks

CS⁺ triggered response

d) Naive

During reward conditioning

During extinction

h) After extinction

CS⁻ triggered response

j) After reward conditioning

During reactivation

After reactivation

Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Extinction and reconsolidation of reward memory requires distinct subsets of dopaminergic neurons that are driven by recurrent and hierarchical connections within the mushroom body output network (related to Figs 1–4). a, Aversively reinforcing DANs in the paired posterior lateral 1 (PPL1) cluster innervate discrete regions of the vertical mushroom body lobe whereas rewarding DANs in the protocerebral anterior medial (PAM) cluster innervate unique zones on the horizontal lobe. b, Each zone innervated by a particular DAN houses the dendritic field of a corresponding MBON. Aversive DANs overlap with the dendrites of MBONs directing behavioural approach whereas rewarding DANs overlay the dendrites of MBONs driving avoidance. c, The presynaptic fields of many MBONs overlap with the dendrites of DANs that innervate the same mushroom body zones, suggesting the presence of local recurrent feedback loops. d, The weight of behavioural drive to approach- and avoidance-directing MBONs is balanced in naive flies (orange and blue circles of equal size). e, Sugar conditioning engages rewarding DANs that innervate the tips of the horizontal lobes of the mushroom body and that drive depression of synaptic connections between odour-activated mushroom body Kenyon cells and MBONs. f, Following reward conditioning the CS⁻ drive to avoidance-directing MBONs is reduced (smaller orange circle) thereby favouring activation of odour-driven behavioural approach pathways. g, The reward-learning-induced skew in the MBON network is expressed when flies re-encounter the CS⁻ odour. Preferential CS⁺ drive of approach-directing MBONs in turn activates aversively reinforcing PPL1 DANs which feed back to encode a competing aversive odour memory. h, We propose that, after this extinction process, reduced CS⁺ drive to approach-directing MBONs (smaller blue circle) equals, and so neutralizes, the previously coded approach memory (smaller orange circle). i, Example of fly behaviour. Naïve flies approach odours equally as a result of balanced drive to avoidance and approach MBON pathways. Reward-conditioned flies exhibit odour preference as a result of reduced CS⁻ drive to avoidance MBONs. j, Extinction restores the balance by reducing the CS⁻ drive to the approach MBONs. k, Sugar conditioning establishes enhanced CS⁻ odour drive to γ2α′1 MBONs. l, During memory reactivation the CS⁻ odour drives the γ2α′1 MBON which activates the MB-MV1 DAN that feeds back and releases dopamine within the same mushroom body compartment. This activity is required at the time of odour re-exposure to induce memory reconsolidation. m, CS⁻ memory reactivation of the γ2α′1 MBON also activates rewarding DANs that innervate the tips of the horizontal lobes of the mushroom body, and that were earlier required for the formation of the original reward memory. The output of these rewarding DANs is required for a restricted period of time after odour exposure to reconsolidate memory.
Extended Data Figure 2 | Extinction of reward memory requires negatively reinforcing dopaminergic neurons (related to Fig. 1).

a, 6 h reward memories for other odours (IAA and EB) can also be extinguished with two CS+ odour exposures 3 h after training \((n \geq 4)\).
b, One or three odour exposures 3 h after training, varying the memory reactivation regimen of Fig. 1, abolish odour preference behaviour of trained flies measured 3 h later \((n \geq 6)\). c, Memory extinguished with two odour exposures at 3 h remains low 24 h after training \((n = 8)\). Spontaneous recovery of the initial reward memory is not obvious with our current training and extinction protocols\(^5\). d, Two odour exposures, matching the memory reactivation regimen of Fig. 1, do not change the odour preference behaviour of naive flies measured 3 or 21 h later \((n = 7)\). e, Blocking a reinforcement signal from rewarding RS8E02-GAL4 DANs during retraining does not induce memory extinction \((n \geq 9)\). f, Permissive temperature control for Fig. 1c. No differences in CS+-directed extinction or approach behaviour following CS− exposure are apparent when the experiment in Fig. 1c is performed at permissive 23 °C throughout \((n \geq 7)\). g, Exposing flies to novel odours, IAA or EB, while MB504B-GAL4 PPL1 aversive DANs are blocked does not significantly impact 6 h memory performance \((n \geq 7)\). h, Blocking aversive PPL1 DANs during odour pre-exposure in naive flies does not attach a value to the pre-exposed odour \((n \geq 9)\).
Extended Data Figure 3 | Reconsolidation of reward memory is triggered by CS⁻ exposure and requires MB-MV1 dopaminergic neurons (related to Fig. 2). a, Reward memories formed with other odours (IAA and EB) can also be rendered sensitive to cold-shock by reactivating them with CS⁻ exposure 3 h after training (n = 9). b, Reward memories can also be made labile by reactivation 21 h after training (n = 10). c–e, Extinction of reward memory is insensitive to blocking small groups (<3 neurons per hemisphere) or individual classes of aversive PPL1 DANs during CS⁻-driven memory reactivation. Blocking MB-MP1 (n ≥ 10, c); MB-V1 (n ≥ 9, d) or PPL1-α3 and PPL1-α’3 (n ≥ 6, e) during CS⁻ reactivation leaves 6 h memory performance unaltered.

f, Manipulating the MB-MV1 DANs with the alternative driver R73F07-GAL4 during reactivation confirms a specific role in CS⁻-driven memory reconsolidation as seen with MB296B-GAL4 in Fig. 2c. Blocking R73F07-GAL4 neurons during CS⁺ reactivation does not affect reward memory extinction (n ≥ 14). g, Blocking MB-MV1 DANs (MB296B-GAL4) 90 min after CS⁻ exposure does not impair reconsolidation (n ≥ 12). h, Permissive temperature control for f and Fig. 2c. CS⁻ reactivation at permissive temperature does not change 6 h approach memory performance (n ≥ 8).
i, MB-MV1 neurons are not required to form a 3 h sugar-rewarded memory (n ≥ 8).
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | Reward memory extinction requires V2 cluster MBONs that drive negatively reinforcing dopaminergic neurons (related to Fig. 3). a, Blocking the GABAergic MVP2 MBONs (MB112C-GAL4) during CS- or CS+-triggered memory reactivation does not significantly impact 6 h conditioned approach behaviour or CS+-driven extinction (n ≥ 8). b, Permissive temperature control for Fig. 3a. Presenting the CS+ exposure at 23 °C does not change the extinction of reward memory in V2 MBON MB052B-GAL4;UAS-shi ts1 flies (n ≥ 8). c, d, Light-triggered activation (red bar) of R65B09-LexA V2 MBONs (c) or R24H08-LexA V2 MBONs (d) evokes calcium responses in PPL1 DANs. For c and d, asterisks denote significant differences (P < 0.05) between pre- and post-activation responses. e, Sugar-reward training does not alter CS+ or CS- odour-evoked calcium responses in V2 cluster MBONs MB052B-GAL4 (n ≥ 11). Responses to CS-, CS+ and novel odour were measured in a section through the α2 region of the vertical mushroom body lobe (example traces, lower left panel). Calcium transients during CS- and CS+ re-exposure were normalized to responses recorded in the same preparation to novel odour (IAA). f, Sugar-reward training does not alter CS- or CS+ odour-evoked calcium responses in MB-V1 or MB-MP1 MB052B-GAL4 DANs (n ≥ 7). Responses to CS-, CS+ and novel odour were measured in a section through the α2 or γ1 region of the mushroom body (example traces, lower left panel). Calcium transients during CS- and CS+ re-exposure were normalized to responses recorded to novel odour (IAA) in the same preparation. Note that the order of CS+ and CS- odour presentation is reversed for MB-V1 and MB-MP1 experiments.
Extended Data Figure 5 | The γ2α'/1 MBONs orchestrate CS^-triggered reconsolidation (related to Fig. 4). a, Blocking the cholinergic MBON-γ2α'/1 (MB077C-GAL4) after CS^- exposure does not impair memory reconsolidation (n ≥ 10). b, Permissive temperature control for Fig. 4b. No defect in 3 h memory performance is apparent when the entire experiment is conducted at permissive 23 °C (n ≥ 11).
Extended Data Figure 6 | The expression patterns of all GAL4 and LexA lines used in this study (related to Figs 1–4). Panels a–k show GFP expression driven by the relevant GAL4 (green), LexA-driven RFP expression in mushroom body Kenyon cells (red) and general neuropil stained with an antibody to the Bruchpilot presynaptic marker (blue). a, R58E02-GAL4 broadly labels rewarding DANs in the PAM cluster including PAM-α1, PAM-β1 (MVP1), PAM-β1ped, PAM-β1ap, PAM-β1p3m, PAM-β2a, PAM-β2m, PAM-β2p, PAM-γ3<α<1<β2, PAM-γ4 and PAM-γ5. b, TH-GAL4 broadly labels DANs throughout the brain including all six mushroom-body-innervating PPL1 DANs: PPL1-γ1pedc (MB-MP1), PPL1-γ1, PPL1-2α1/M (MB-MV1), PPL1-2α2 (MB-V1), PPL1-α3 and PPL1-α3. c, MB504B-GAL4 labels PPL1-γ1pedc (MB-MP1), PPL1-γ1, PPL1-2α1/M (MB-MV1), PPL1-2α2 (MB-V1) and PPL1-α3. d, e, MB296B-GAL4 (d) and R73F07-GAL4 (e) label PPL1-2α1/M (MB-MV1) neurons. f, c061-GAL4;MB-GAL80 labels PPL1-γ1pedc (MB-MP1). g, MB058B-GAL4 labels PPL1-α2<2 (MB-V1). h, MB308B-GAL4 labels PPL1-α3 and displays weak expression in PPL1-α3. i, MB122C-GAL4 labels MBON-γ1pedc<α/β (MVP2). j, MB502B-GAL4 labels MBON-α1, MBON-α2sc (V2α), MBON-α2p3p, MBON-α3ap (V2α') and MBON-α3m (V2α'). k, MB077C-GAL4 labels MBON-2α1. Panels l–p show GFP expression driven by the relevant LexA (green) and general neuropil stained with an antibody to the Bruchpilot presynaptic marker (blue). l, R65809-LexA labels MBON-α1, MBON-α2sc (V2α), MBON-α2p3p, MBON-α2 (V4), MBON-α3ap (V2α) and MBON-α3m (V2α'). m, R71D08-LexA labels MBON-α2sc (V2α), MBON-α3ap (V2α') and MBON-α3m (V2α'). n, R24H08-LexA labels MBON-α1, MBON-α3ap (V2α') and MBON-α3m (V2α'). o, R58E02-LexA labels PAM-α1, PAM-β1 (MVP1), PAM-β1ped, PAM-β1ap, PAM-β1p3m, PAM-β2a, PAM-β2m, PAM-β2p, PAM-γ3, PAM-γ4<α<1<β2, PAM-γ4 and PAM-γ5. p, R25D01-LexA labels MBON-2α1. 

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