Re-evaluation of learned information in *Drosophila*

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Abstract

Animals constantly reassess the reliability of learned information to optimize their behavior. On retrieval, consolidated long-term memory can be neutralized by extinction if the learned prediction was inaccurate [1]. Alternatively, retrieved memory can be maintained, following a period of lability in which it is reconsolidated [2]. Although extinction and reconsolidation provide opportunities to alleviate problematic human memories [1,3-5], we lack a detailed mechanistic understanding of memory updating processes. Here we identify neural operations underpinning re-evaluation of memory in *Drosophila*. Accuracy of prediction during reactivation of sugar-reinforced olfactory memory can lead to either extinction or reconsolidation. Each process recruits activity in specific parts of the mushroom body output network and different 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 the same zones. The aversive valence of new extinction memories neutralizes previously learned odor preference. Memory reconsolidation requires the $\gamma 2 \alpha' 1$ 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 supports memory re-evaluation driven by reward prediction error.

The valence of *Drosophila* olfactory memory is coded as a skew in the mushroom body (MB) output network [6,7]. Reward memories are written by positive dopaminergic neurons (DANs) that innervate the horizontal lobes of the MB [8,9] and that appear to depress odor-drive to mushroom body output neuron (MBON) pathways that direct avoidance [10,11]. In contrast, aversive memories are reinforced by negative DANs that primarily innervate the vertical MB lobes [12-14] and reduce odor-drive to MBON pathways directing approach [6,15]. Learned behavior is weakened (or extinguished) in flies that experience the conditioned odor without reinforcement [16-18], suggesting that flies constantly reassess the reliability of learned predictions. We developed a paradigm to investigate neural mechanisms of the re-evaluation of sugar-rewarded appetitive memory (Fig. 1a). Flies were trained using a standard regimen [19]: food-deprived for 18-24 h, presented with one odor for 2 min without reinforcer (Conditioned Stimulus minus, CS-) followed by 30 s of clean air and the other odor for 2 min (Conditioned Stimulus plus, CS+) with sugar reward. This training establishes robust long-term memory that is consolidated within hours after training [19]. We next challenged the memory 3 h after training with two 2 min presentations of either the CS+, the CS-, a novel odor (to control for odor specificity) or air (a handling control), and then 3 h later tested the flies preference between the CS+ and CS- odors in a T-maze. Flies pre-exposed to the CS-, air or novel odor displayed robust 6 h memory performance (Fig. 1a). However, CS+ exposure at 3 h effectively abolished memory performance measured at 6 h. Importantly, odor exposure alone in naïve flies did not change odor preference measured 3 h later (Supplementary Fig. 1a). Therefore, sugar-rewarded memory performance can be robustly and specifically extinguished by presenting the CS+ odor 3 h after training.

Since distinct classes of DANs reinforce aversive [12-14] and reward memories [8,9] we tested DAN involvement in memory extinction by blocking their presynaptic release with uas-*shi*^{ts1} [20] during memory reactivation 3 h after training. All flies were trained at permissive 23˚C, raised to restrictive 33˚C 30 min before and during reactivation at 3 h, immediately returned to 23˚C and finally tested for 6 h appetitive memory performance. Blocking rewarding DANs

with R58E02-GAL4 driven uas-*shi*^{ts1} did not impair extinction (Fig. 1b). R58E02; uas-*shi*^{ts1} flies exhibited no measurable 6 h memory performance following memory reactivation with CS+ pre-exposure at 3 h but displayed robust performance following CS- exposure. We also tested whether the absence of a DAN-mediated reward signal to the MB is sufficient to induce extinction of appetitive memory by retraining the flies at 3 h while blocking the R58E02 DANs. Perhaps surprisingly, R58E02; uas-*shi*^{ts1} flies retained appetitive memory performance in this experiment that was indistinguishable from that of their genetic controls (Supplementary Fig. 1b). This result also supports a reward prediction model where dopaminergic neurons stop contributing positive reinforcement when learned performance has reached a maximum.

We next tested aversively reinforcing DANs using the broadly expressing TH-GAL4 and more restricted MB504B-GAL4 drivers to express uas-*shi*^{ts1} (Fig. 1c). DANs were again blocked during memory reactivation by raising flies to restrictive 33˚C only during this period. Memory extinction driven by CS+ exposure was significantly compromised in TH-GAL4; uas-*shi*^{ts1} and MB504B; uas-*shi*^{ts1} flies as compared to their relevant controls. In addition, blocking TH-GAL4 or MB504B output during CS- exposure at 3 h led to a surprising decrease in 6 h memory performance. Neither disruptive effect of blocking TH-GAL4 or MB504B neurons was evident when experiments were performed at permissive 23˚C (Supplementary Fig. 1c). Moreover, blocking PPL1 DANs without memory reactivation, or while exposing flies to a novel odor at 3 h did not impact 6 h memory performance (Fig. 1d and Supplementary Fig. 1d). In addition, combining PPL1 DAN block and repeated odor exposure in naïve flies did not change odor preference behavior 3 h later (Supplementary Fig.1e). Therefore, aversively reinforcing PPL1 DANs play a prominent role in appetitive memory extinction and blocking them also uncovers an unexpected and specific role for the CS- odor in memory re-evaluation.

Prior work suggests that whereas reward omission during CS+ exposure would alter the predictability/reliability of learned information and drive extinction learning, small changes in a subsequent training trial trigger reconsolidation, resulting in the maintenance of the original memory [21,22]. Since CS- exposure is equivalent to part of a new learning trial, we tested whether it induced reconsolidation of the original CS-/CS+ memory. Fruit fly memory consolidation is evident as a time-dependent resistance to cold-shock anesthesia [19,23]. We therefore followed 3 h CS- driven memory reactivation with a 2 min period of cold-shock anesthesia (Fig. 2a). Cold-shock immediately or 30 min after CS- exposure abolished 6 h appetitive memory whereas no statistical difference in behavior was apparent if anesthesia was applied in the absence of reactivation or 90 min after CS- exposure (Fig. 2a-b). These results suggest that CS- exposure induces a time-dependent memory reconsolidation. Since blocking the PPL1 DANs during CS- exposure (Fig. 1c) revealed a similar effect to that of cold-shock afterwards (Fig. 2a-b), we conclude that reconsolidation ordinarily requires the function of PPL1 DANs.

We next used more restricted GAL4 drivers to express uas-*shi*^{ts1} and refine the PPL1 DANs responsible for reconsolidation and extinction. We individually blocked output from MP1, V1, α3/α´3, MV1 DANs during memory reactivation with either the CS+ or CS- odors and measured 6 h memory performance (Fig. 2c and Supplementary Fig. 2a-d). None of these manipulations impaired CS+ driven memory extinction suggesting that formation of extinction memory does not rely on individual aversive DANs. In contrast, blocking the MV1 DANs in MB296B-GAL4; uas-*shi*^{ts1} or R73F07-Gal4; uas-*shi*^{ts1} flies during CS- memory reactivation significantly reduced 6 h memory performance (Fig. 2c and Supplementary Fig. 2d). No defect was apparent when MV1 DANs were blocked with MB296B; uas-*shi*^{ts1} immediately or 1.5 h after the CS- exposure (Fig. 2d and Supplementary Fig. 2e) or when experiments were performed at permissive temperature (Supplementary Fig. 2f). Furthermore, the MV1 DANs are not required for learning or expression of 3 h appetitive memory (Fig. 2e and Supplementary Fig. 2g). We next tested whether blocking the MV1

DANs during CS- driven memory reactivation disrupted the memory of the CS+ odor by allowing the flies at 6 h to choose between the CS+ and a novel odor, rather than the CS+ and the CS-. Whereas control flies exhibited robust preference for the CS+ over the novel odor, flies in which the MV1 DANs were blocked during CS- re-exposure displayed no measurable CS+ memory (Fig. 2f). We therefore conclude that synaptic output from the MV1 DANs throughout, but not after, CS- driven memory reactivation is necessary to reconsolidate memory of the specific association between the CS- and CS+ odors.

Individual DANs tile the MB lobes into 15 discrete compartments, each one of which also contains the dendritic field of a corresponding mushroom body output neuron (MBON) [24]. Moreover, anatomy suggests MBONs may connect to the dendrites of DANs, forming recurrent MB-MBON-DAN-MB feedback loops [24,25], which could serve an odor re-evaluation process. Since sugar-reward learning skews the relative CS+ odor drive towards vertical lobe MBONs that direct behavioral approach [10,11], we tested the role of these MBONs in extinction and reconsolidation. We again restricted neural blockade to the period of CS+ or CS- driven memory reactivation by elevating the temperature to 33˚C, returned the flies to 23˚C and tested 6 h memory. MB052B-GAL4 labels a collection of cholinergic V2 MBONs whose dendrites tile the vertical lobes and overlap with the PPL1 DANs required for extinction. Blocking the V2 MBONs also impaired CS+ driven memory extinction and did not impact CS- driven reconsolidation (Fig. 3a). No defect was apparent when the neighboring approach directing GABAergic MVP2 MBONs were blocked during memory reactivation (Supplementary Fig. 3a) or when the V2 MBON experiments were performed at permissive temperature (Supplementary Fig. 3b). Moreover, the V2 MBONs are not necessary for expression of 3 h memory performance (Fig. 3b), suggesting their output serves a function distinct from behavioral direction.

To test for functional V2-PPL1 DAN connectivity we stimulated V2 MBONs by expressing the red-light sensitive channelrhodopsin lexAop-CsChrimson [26] with either R65B09-LexA,

R71D08-LexA or R24H08-LexA control, while recording activity in MB504B-GAL4 labeled PPL1 DANs expressing UAS-GCaMP6f. Light-triggered activation of V2 MBONs produced a consistent and robust calcium response in the cell bodies of PPL1 DANs (Fig. 3c and Supplementary Fig. 3c-d). Considering the functional connectivity and behavioral data, we propose that CS+ odor exposure in trained flies drives V2 MBON-DAN activity that leads to the formation of a new parallel and competing CS+ specific aversive memory. This manifests as extinction of the original reward memory.

We next investigated the cholinergic γ2α′1 MBONs whose dendrites occupy the same MB compartment as the MV1 DANs required for reconsolidation. These MBONs have to potentially accommodate two functions: explain how the CS- becomes represented after training and how the CS- can drive reconsolidation of the original CS+ reward memory. Like the MV1 DANs, blocking the γ2α′1 MBONs with uas-*shi*^{ts1} during, but not after, CS- driven memory reactivation impaired memory reconsolidation (Fig. 4a and Supplementary Fig. 4a). In addition, blocking γ2α′1 MBONs during CS+ driven reactivation did not disrupt memory extinction (Fig. 4a). However, unlike the MV1 DANs, or the V2 MBONs, blocking the γ2α′1 MBONs abolished 3 h appetitive memory performance (Fig. 4b and Supplementary Fig. 4b) demonstrating that the γ2α′1 MBONs direct memory-guided behavior as well as odor re-evaluation.

Light microscope-level anatomy suggests that the γ2α′1 V2 MBONs might connect with the dendrites of MV1 and those of rewarding DANs that innervate the horizontal MB lobes [24]. We therefore again assessed functional connectivity using CsChrimson to stimulate γ2α′1 MBONs while monitoring activity with GCaMP6f/m expressed in either MB296B-GAL4 labeled aversive MV1 DANs or in R58E02-LexA labeled rewarding DANs. Light-triggered activation of γ2α′1 MBONs evoked robust calcium responses in both MV1 (Fig. 4c) and rewarding DANs (Fig. 4d). These results are consistent with CS- driven reconsolidation

169 involving the γ2α′1 MBONs driving recurrently connected aversive MV1 DANs and also
170 triggering activity in other parts of the ensemble of rewarding DANs.
171
172 We next determined whether γ2α′1 MBONs contained a trace of reward learning by
173 measuring their odor-driven activity. We expressed GCaMP6f in γ2α′1 MBONs with
174 MB077C-GAL4 and performed two-photon functional calcium imaging of odor responses 3 h
175 after sugar-reward, or mock training (Fig. 4e). Responses to the CS- and the CS+ were
176 normalized to the response generated by a novel odor. The responses evoked after training
177 in γ2α′1 MBONs by either of two CS- odors was statistically increased over those in mock-
178 trained flies. In contrast, the CS+ evoked responses were not statistically different between
179 the groups. Therefore reward learning specifically enhances responses evoked by CS-
180 odors in γ2α′1 MBONs.
181
182 Since γ2α′1 MBONs are functionally connected to R58E02-labeled rewarding DANs (Fig.
183 4d), we tested whether rewarding DANs contribute a delayed signal that is necessary for
184 reconsolidation. Flies were trained and memory was reactivated with either the CS+ or CS-
185 odors at 3 h. Blocking rewarding DANs with uas-*shi*^{ts1} immediately, but not 90 min, after
186 memory reactivation abolished 6 h memory performance (Fig. 4f). We therefore conclude
187 that reconsolidation results from increased CS- drive to γ2α′1 MBONs after training that
188 recruits the recurrent MV1 DAN and also results in the re-engagement of rewarding DANs to
189 update the original CS+ reward memory.
190
191 Our data demonstrate that flies re-evaluate memory at the time of retrieval using discrete
192 functional modules of their dopaminergic system, that acquire differential odor-drive via a
193 skew in the MBON network encoded by learning. The findings are consistent with a model
194 wherein a large negative prediction error between learned expectation of reward and the
195 actual experience of reward being absent triggers memory extinction. In contrast, a small

196 difference from expectation, such as when the flies only experience part of the previous
197 training trial, evokes memory reconsolidation [27,28]. It will be important to determine whether
198 dopamine based error-prediction [29] and reassessment of learned information in mammals
199 [30,31,32] utilize similar neural network motifs and an operating logic.
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201

Material and Methods

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 used have been described: TH-GAL4 [33], R58E02-GAL4 [8], MB052B-, MB058B-, MB077C-, MB083C-, MB112C-, MB296B-, MB308B- and MB504B-GAL4 [24], R73F07-GAL4 [34] and c061-GAL4;MBGAL80 [35]. UAS-*shi*^{ts1} [20]. LexA lines are described: R24H08-, R25D01-, R65B09- and R71D08-LexA [34], R58E02-LexA [8]. The reporter UAS-GCaMP6f, lexAop-GCaMP6m [36] and optogenetic trigger flies UAS-CsChrimson::mVenus [26], lexAop-CsChrimson::tdTomato,uas-GCaMP6f [37] are described.

Behavior

For behavior experiments males from GAL4 lines were crossed to UAS-*shi*^{ts1} females, except in the case of the c061-GAL4;MBGAL80 crosses where UAS-*shi*^{ts1} males were crossed to c061-GAL4;MBGAL80 females. For heterozygous controls GAL4 or UAS-*shi*^{ts1} 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 prior to training and were kept starved for the entire experiment. Appetitive training was performed and quantified as described [19]. In brief, 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 2X 2 min exposures (spaced by 15 min) of the CS- odor, the CS+ odor (without reward), a novel odor, or room air. During the final memory test flies were given 2 min to choose between two odors in a T-maze: either the CS+ versus the CS-, or the CS+ versus a novel odor. 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 odors and isoamyl acetate (IAA, 7 µl in 8 ml mineral oil) and ethyl butyrate (EB, 7 µl in 8 ml mineral oil) were used as novel odors in all experiments, except Fig. 2f. In Fig. 2f ethyl butyrate was

used as CS- and 3-octanol and 4-methylcyclohexanol were used as CS+ or as novel odor. For cold-shock anesthesia flies were transferred into pre-chilled plastic vials and kept on ice for 2 min as described previously [19,38]. For neural inactivation experiments with UAS-*shi*^{ts1} all flies were shifted together 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% O_2, 5% CO_2) buffer solution (103 mM NaCl, 3 mM KCl, 5 mM N-Tris, 10 mM trehalose, 10 mM glucose, 7 mM sucrose, 26 mM $NaHCO_3$,1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, osmolarity 275 mOsm [pH 7.3]) following dissection in cold calcium-free buffer. For light stimulation a high-power LED (Multicomp OSW-6338, 630 nm) was relayed onto the specimen via a 50 mm diameter lens with focal length 60 mm 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 sec 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, 80 MHz repetition rate, centered on 910 nm generated by a Ti-Sapphire laser (Chameleon Ultra II, Coherent). Images of 256 X 256 pixels were acquired at 5.92 Hz, controlled by ScanImage 3.8 software [39]. PPL1 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 γ5 compartment. In general, light exposure to the brain was kept at a minimum. Two-photon fluorescence images were manually segmented using ImageJ and further analyzed using

custom-written MATLAB scripts. For quantification, F_0 was defined as the mean F from the 9 s prior until 1 s before stimulation. $\Delta F/F_0$ 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 odor-evoked calcium responses was performed as described [10]. 3-8 day old flies were briefly (5-10 sec) immobilized on ice and mounted in a custom chamber 2.5-3.5 h after appetitive training (see Behavior section) or mock treatment (odor presentation without sugar). Legs and proboscis were immobilized with wax to reduce movement artifacts. The head capsule was opened under room temperature carbogenated buffer (see above). Odors were delivered on a clean air carrier stream using a custom-designed system [40], which also synchronizes timing of odor 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. All subsequent analyses utilized custom-written Matlab routines. After 40 s of clean air, flies were exposed to 5 s CS- (either MCH or OCT; air stream passing over 10^2 odor dilution in mineral oil, and then further blended 1:9 with a clean air stream), then 15 s clean air, followed by a 5 s CS+ (either MCH or OCT) pulse, then 15 s clean air, followed by 5 s of a novel odor (IAA). We excluded a total of 15 (mock group: 9, trained group: 6) flies from further analysis for not showing any visible odor responses (out of 73 flies in total). For quantification, baseline fluorescence F_0 was defined for each stimulus response as the mean F from 1 s before up to the point of stimulation. F/F_0 accordingly describes the fluorescence relative to this baseline. The area under the curve (a.u.c.) was measured as the integral of F/F_0 during the 5 s between odor stimulation onset and offset. In order to reduce the level of fly-by-fly variance, CS+ and CS- were further normalized by dividing each a.u.c. by the novel odor a.u.c. from the respective trial and the same fly, thus obtaining a normalized a.u.c.

Statistics

Statistical analyses were performed in GraphPad Prism 6. All behavioral data was tested for normality using the D'Agostino and Pearson omnibus test. Normally distributed data were analyzed with one-way ANOVA followed by Tukey's honest significant difference (HSD) post hoc test. For non-Gaussian distributed data, Kruskall-Wallis test was performed followed by Dunn's multiple comparison test. Odor response normalized a.u.c. was compared between groups using multiple t-tests with Holm-Sidak correction.

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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 analyzed all behavioral experiments with help from P.C. O.B. performed imaging experiments assisted by J.F. Live-imaging data were analyzed by O.B. and P.C. The manuscript was written by S.W. and J.F with comments from P.C. and O.B.

406 **Figure Legends**

407

408 **Figure 1 | Extinction of reward memory requires negatively reinforcing dopaminergic**
409 **neurons.**

410 **a**, Only CS+ evoked memory reactivation at 3h leads to extinction of appetitive memory
411 (n≥8). **b**, Blocking rewarding R58E02-GAL4 DANs in the protocerebral anterior medial
412 (PAM) cluster during 3h CS- or CS+ re-exposure did not alter extinction or 6h learned
413 approach (n≥15). **c**, Blocking TH-GAL4 or MB504B-GAL4 aversive DANs in the paired
414 posterior lateral 1 (PPL1) cluster during CS+ reactivation significantly impairs extinction,
415 while block during CS- reactivation leads to loss of memory performance (n≥9). **d**, Blocking
416 PPL1 DANs in the absence of reactivation does not alter 6h performance (n≥10). Data
417 represent the mean ± standard error of the mean (s.e.m.). Asterisks (*) denote significant
418 difference ($p<0.05$) between groups of same genotype treated differently. Hash (#) denotes
419 significant difference ($p<0.05$) between different genotypes treated identically. A break in the
420 x-axis indicates independent experiments.

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422 **Figure 2 | Reconsolidation of reward memory is triggered by CS- exposure and**
423 **requires MV1/PPL1-γ2α′1 dopaminergic neurons.**

424 **a**, Re-exposing trained flies to CS- odor renders reward memory sensitive to cold-shock
425 anesthesia (n≥7) **b**, Memory can still be disrupted 30min later but returns to a cold-shock
426 resistant consolidated state by 90 min after CS- reactivation. **c**, Blocking MB296-GAL4
427 MV1/PPL1-γ2α′1 DANs during CS- reactivation abolishes 6h learned approach but blocking
428 during CS+ reactivation leaves extinction intact (n≥12). **d**, MV1/PPL1-γ2α′1 block after CS-
429 reactivation does not significantly impair 6h performance (n≥23). **e**, MV1/PPL1-γ2α′1 output
430 is dispensable during 3h memory retrieval (n≥14). **f**, Blocking MV1/PPL1-γ2α′1 DANs during
431 CS- reactivation abolishes 6h approach towards the CS+ (n≥11).

432

Figure 3 | Reward memory extinction requires V2 MBONs that drive negatively reinforcing dopaminergic neurons.

a, Blocking MB052B-GAL4 V2 MBONs significantly impairs CS+ driven extinction but spares CS- induced reconsolidation (n≥9). **b**, V2 MBONs are not essential to express 3h memory performance (n≥9). **c**, Light-triggered (red bar) activation of R65B09-LexA V2 MBONs evoked calcium transients in MB504B-GAL4 PPL1 aversive DANs. Arrows mark time points quantified before and after LED. Asterisks in **a** denote significant difference ($p<0.05$) between groups of the same genotype treated differently and in **c** significant difference between pre- and post activation responses. Hash denotes significant difference ($p<0.05$) between groups with different genotypes treated identically.

Figure 4 | The γ2α´1 MBONs orchestrate CS- triggered reconsolidation.

a, Blocking MB077c-GAL4 γ2α´1 MBONs during CS- reactivation significantly impairs reconsolidation of 6 h memory but spares CS+ driven extinction (n≥12). **b**, γ2α´1 MBON output is required for 3 h memory expression (n≥15). **c**, Light-triggered activation (red bar) of R25D01-LexA or MB077C-GAL4 γ2α´1 MBONs evokes calcium responses in aversive MB296B-GAL4 MV1/PPL1-γ2α´1 DANs and in **d**, rewarding R58E02-LexA DANs. Arrows, time points quantified before and after LED. **e**, Sugar-reward training specifically enhances CS- odor-evoked calcium responses in γ2α´1 MBONs (n≥15). Responses to CS-, CS+ and novel odor were measured in projections outside the MB (example traces, lower left panel). Calcium transients during CS- and CS+ re-exposure were normalized to responses recorded in the same preparation to novel odor (isoamylacetate, IAA). Traces and quantification following training with MCH or OCT are shown. Asterisks, significant difference ($p<0.05$) between trained and mock responses. **f**, Blocking rewarding 58E02-GAL4 DANs immediately, but not 1.5 h, after CS- re-exposure abolishes reconsolidation of 6h memory (n≥9). Hash, significant difference ($p<0.05$) between different genotypes treated identically.

Supplementary Figure 1 related to Figure 1 | Extinction of reward memory requires negatively reinforcing dopaminergic neurons.

a, Two odor exposures, matching the memory reactivation regimen of Fig. 1, does not change the odor preference behaviour of naïve flies measured 3h later (n=8). **b**, Blocking a reinforcement signal from rewarding R58E02-GAL4 DANs during CS+ exposure does not induce memory extinction (n≥9). **c**, Permissive temperature control for Fig. 1c. No differences in CS+ directed extinction or approach behavior following CS- exposure are apparent when the experiment in Fig. 1c is performed at permissive 23°C throughout (n≥7). **d**, Exposing flies to novel odors, isoamyl acetate or ethyl butyrate, while MB504B-GAL4 PPL1 negative DANs are blocked does not significantly impact 6h memory performance (n≥7). **e**, Blocking aversive PPL1 DANs during odor pre-exposure in naïve flies does not attach a value to the pre-exposed odor (n≥9). Data represent mean ± s.e.m. Asterisks denote significant difference ($p<0.05$) between differently treated flies of the same genotype. Break in the abscissa indicates independent experiments

Supplementary Figure 2 related to Figure 2 | Reconsolidation of reward memory is triggered by CS- exposure and requires MV1/PPL1-γ2α´1 dopaminergic neurons.

a-c, 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 **a**, MP1/PPL1-γ1pedc (n≥10); **b**, V1/PPL1-α2α´2 (b, n≥9) or **c**, PPL1-α3 and PPL1-α´3 (n≥6) during CS- reactivation leaves 6h memory performance unaltered. **d**, Manipulating the MV1/PPL1-γ2α´1 DANs with the additional R73F07-GAL4 driver, 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 did not affect reward memory extinction (n≥14). **e**, Blocking MV1/PPL1-γ2α´1 DANs (MB296B-GAL4) 1.5 h after CS- exposure did not impair reconsolidation (n≥12). **f**, Permissive temperature control for Fig. 2c and Supplementary Fig. 2d. CS- reactivation at permissive

temperature did not change 6h approach memory performance (n≥8). **g**, MV1/PPL1-γ2α′1 neurons are not required to form a 3h sugar-rewarded memory (n≥8).

Supplementary Figure 3 related to Figure 3 | Reward memory extinction requires V2 MBONs that drive negatively reinforcing dopaminergic neurons.

a, Blocking the GABAergic MVP2 MBONs (MB112C-GAL4) during CS- or CS+ triggered memory reactivation does not significantly impact 6h conditioned approach behavior or CS+ driven extinction (n≥8). **b**, Permissive temperature control for Fig. 3a. Presenting the CS+ exposure at 25˚C does not change the extinction of reward memory in V2 MBON MB052B GAL4; uas-*shi*^{ts1} flies (n≥10). **c**, Light-triggered activation (red bar) of R71D08-LexA V2 MBONs or **d**, R24H08-LexA V2 MBONs evokes calcium responses in PPL1 DANs. Bars represent mean response ± s.e.m. Asterisks denote significant difference ($p<0.05$) between pre- and post activation responses.

Supplementary Figure 4 related to Figure 4 | The γ2α′1 MBONs orchestrate CS-triggered reconsolidation.

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 3h memory performance was apparent when the entire experiment was conducted at permissive 25˚C (n≥11).