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**,Differential mechanisms underlie trace and delay conditioning in *Drosophila***

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**Two forms of associative learning, delay and trace conditioning, have been widely investigated in humans and higher-order animals<sup>1</sup>. In delay conditioning, an unconditioned stimulus (US) (e.g., electric shock) is introduced in the final moments of a conditioned stimulus (CS) (e.g., tone), ending with it. In trace conditioning, a “trace” interval separates the CS and US. Trace conditioning therefore relies on maintaining a CS neural representation after its termination (hence distractible<sup>2</sup>), to learn the CS-US contingency<sup>3</sup>, making it more cognitively demanding than delay conditioning<sup>4</sup>. Here, combining virtual-reality behavior with neurogenetic manipulations and *in vivo* two-photon brain imaging, we demonstrate *Drosophila* visual trace and delay conditioning mobilizing ellipsoid body R2/R4m ring neurons. In trace conditioning, calcium transients during the trace interval show increased oscillations and slower declines over repeated training, both distractions sensitive. Dopaminergic activity accompanied ring neuron signal persistence, decreased by**

**distractions solely during trace conditioning. Finally, dopamine D1- and D2-like receptor signaling in ring neurons played differential roles in delay and trace conditioning, Dop1R1 mediating both while Dop2R exclusively in sustaining ring neuron activity during the trace interval of trace conditioning. These observations recall those in mammals during arousal<sup>5</sup>, prefrontal activation<sup>6</sup>, and high-level cognitive learning<sup>7,8</sup>.**

Studies of mammalian brain circuits underlying trace and delay paradigms have pointed to significant overlaps in neural circuitry. Trace conditioning is thought additionally to recruit the hippocampus and neocortex in preserving the sustained CS neural trace across the temporal gap<sup>9,10</sup>, though little is known about what form this representation takes and how it eventually converges with the US. An ongoing debate addresses the extent to which higher brain functions such as attention<sup>2</sup>, working memory<sup>11</sup> and awareness<sup>12</sup> are necessary for bridging the temporal gap in trace conditioning. *Drosophila*'s excellent experimental and neurogenetic tractability<sup>13</sup>, and its complex cognitive repertoire<sup>14</sup>, make it an attractive model for studying both the behavioral and physiological properties of trace and delay conditioning and the purported causal link with higher brain functions.

### **Distracting trace vs. delay conditioning**

Pavlovian conditioning studies in *Drosophila* have traditionally employed aversive olfactory learning<sup>15-17</sup>, some even demonstrating olfactory trace conditioning<sup>18,19</sup>. However, in an olfactory paradigm, control of lingering odors after removal can be challenging<sup>18</sup>. Here, we develop a visual trace and delay conditioning paradigm that not only affords precise stimulus control, but also could provide a new way to use *Drosophila* in the study of higher brain functions in general. For this, we developed a panoramic spherical virtual-reality arena for

tethered flying flies and coupled it with an infra-red laser-based heat punishment, analogous to those developed previously<sup>20</sup> (Fig. 1a, Extended Data Fig. 1a-c). The flies' yaw-axis turns were quantified by analyzing the left and right wingbeat amplitude difference using real-time machine vision techniques (Fig. 1b). Conditioning involved presenting an image (upright- or inverted-T) in the tethered fly's frontal visual field (CS<sup>+</sup>), paired with heat (US). A CS<sup>-</sup> (upright- or inverted-T, whichever was not used as CS<sup>+</sup>) without heat served as control. We tested both delay (where CS and US co-terminated), and trace conditioning with variable trace intervals (5-40 s), to determine *Drosophila's* ability to learn the CS-US contingency. Post-training, both images were presented 180° apart in the fly's lateral visual field with closed-loop feedback (Fig. 1c). The fly's avoidance of CS<sup>+</sup>, and towards CS<sup>-</sup>, served as a measure of learning. Wildtype flies learn the CS<sup>+</sup> and US association in both delay and trace conditions, up to a trace interval of ~20 s (Fig. 1d, Extended Data Fig. 1e). When either the CS or US was omitted during training, or their order reversed, no learning was observed (Extended Data Fig. 2a-b). However, expectedly, the CS<sup>+</sup> and CS<sup>-</sup> presentation order had no effect on the conditioned response (Extended Data Fig. 2a, c).

With these paradigms, we next determined whether *Drosophila* trace, and not delay conditioning, is sensitive to distractions, as in mammals<sup>2</sup>. We used gentle air puffs (0.5 s) as neutral distractors, ensuring that they did not physically disturb the animal by altering flight trajectory (Extended Data Fig. 1f). Timing and number of air puffs were constant during training across all conditions tested, only the US timing varied (Fig. 1c). We found that air puffs during training interfered selectively with trace and not delay conditioning (Fig. 1d). The distracting nature of the air puff on trace conditioning was examined in the following four ways: first, confirming that the air puff remained neutral after trace conditioning, ruling out effects on learning by competing with CS<sup>+</sup> for association with US (Extended Data Fig. 1g), and supporting

its role as a true distractor; second, confirming that distractions presented during the trace interval affected trace conditioning (Extended Data Fig. 3b); third, examining whether delay (and not trace) conditioning is refractory to distractions simply due to its strength, not the learning type. By increasing the strength, duration, and frequency of distractions independently, we confirmed that delay learning remained immune to stronger distractions, while trace learning worsened (Extended Data Fig. 3c). Delay learning resisted distractions even when undertrained, while trace learning expectedly remained distraction sensitive (Extended Data Fig. 3d). Fourth, we examined whether different distractor modalities (here, auditory) could affect trace conditioning. However, as sound vibrations from an auditory stimulus might physically disturb the animal, we instead stimulated (optogenetically) the sensory neurons<sup>21</sup> that send auditory information to the central brain<sup>22</sup>. We drove expression of the *UAS-CsChrimson* optogenetic activator specifically in the A/B neural subgroups of the Johnston's organ (sensitive to sound vibrations) with red-light activating laser pulses (in lieu of the air puff distractor). Activating these neurons during conditioning, interfered with trace but not delay conditioning (Extended Data Fig. 3e). Taken together, this demonstrates *Drosophila*'s capability of visual trace conditioning and modality-invariant distractibility.

### **CS mobilizes Ellipsoid Body ring**

Most evidence for the necessary visual learning architecture in *Drosophila* points to Central Complex (CX)<sup>23–25</sup> and Mushroom Body (MB) structures<sup>26,27</sup> (Fig. 1e). To identify brain regions involved in our conditioning paradigms, we suppressed activity in distinct neural populations singly and tested resulting effects on learnt behavior, using targeted Gal4 drivers in combination with the Tub-Gal80<sup>ts</sup> conditional repressor transgene to drive expression of Kir2.1

(an inwardly rectifying, neural activity suppressing  $K^+$  channel). Following temperature induction of Kir2.1 expression in these disparate neural structures, we screened for delay and trace learning deficits. Under both delay and trace conditioning, flies with neural activity silenced specifically in the CX's Ellipsoid Body (EB) R2/R4m ring neurons displayed a significant loss of learning (Fig. 1e, Extended Data Fig. 4). Inactivation of the other rings of the EB, Fan-shaped Body layers, or MB lobe-projecting neurons had no significant effect on these behaviors.

We then coupled an *in vivo* two-photon calcium imaging preparation with our projector-based panoramic display, expressing GcaMP6f and tdTomato specifically in EB R2/R4m ring neurons (*EB1-Gal4* >> *UAS-GcaMP6f.myr-tdTomato*), and performing ratiometric neural activity imaging during conditioning (Fig. 2a-d). In trace conditioning, calcium transients recorded during the trace interval revealed a progressively increasing oscillatory component (from 0.5 to 2 Hz) with repeated training (Fig. 2e, f). This gradual frequency increase was specific to trace conditioning, distraction sensitive (Fig. 2e, f, Extended Data Fig. 5a, g, h), and correlated strongly with learning (Fig. 2g). Though responsive, no gradual frequency increase was observed on repeated CS-only presentation (without US, Fig. 2f, Extended Data Fig. 7a-f), and no notable calcium activity was observed in the US-only control (without CS, Extended Data Fig. 7g-i). We also observed a gradual trial-by-trial frequency increase during CS presentation in trace conditioning (Extended Data Fig. 5a, e, f). While such an increase was also observed in delay conditioning (Extended Data Fig. 6a, e-g), distractions slowed that increase exclusively in trace conditioning. Also, during trace conditioning, we observed an increase in calcium signal persistence (slower decay) following CS termination over repeated training, a persistence that was susceptible (faster decay) to distractions (Fig. 2e, f, Extended Data Fig. 5a, d,

Supplementary Video 1, 2), and strongly correlated with learnt behavior (Fig. 2h). Interestingly, no such signal persistence was found during delay conditioning (Extended Data Fig. 6a, d, Supplementary Video 3, 4), suggesting that the increased CS signal persistence after its termination is specific to trace conditioning.

### **Dopamine encodes negative valence**

Dopaminergic neurons (DANs) in flies are known to specify the association between the CS and valence of approach (appetitive) or avoidance (punishment)<sup>17,28–30</sup>. We examined their relevance to visual trace and delay conditioning using Kir2.1-based silencing of most DANs across the brain (using the *TH-Gal4* driver, excluding the PAM cluster, Extended Data Fig. 8a), resulting in loss of both forms of learning (Fig. 3a). To localize the DANs involved, we targeted the PPL1 MB-projecting cluster, known to mediate aversive olfactory learning (Extended Data Fig. 9b). Kir2.1 silencing of these neurons affected neither visual trace nor delay learning (Fig. 3a). Involvement of CX-projecting DANs, where the PPM3 cluster dopaminergic terminals have been identified<sup>31</sup>, was then tested. However, the existing Gal4 drivers that label this PPM3 cluster of DANs (here, *c346-Gal4*, Extended Data Fig. 9c) also target other parts of the brain, necessitating the use of intersectional *TH-FLP* to restrict expression to PPM3 DANs from the broader labeling (Extended Data Fig. 9d). We expressed Kir2.1 in those isolated neurons (using *UAS-FRT-stop-FRT-Kir2.1*) and found that silencing them affected both forms of learning (Fig. 3a). To test sufficiency, we optogenetically drove expression of CsChrimson in PPM3 DANs (using *UAS-FRT-stop-FRT-CsChrimson*). Activating these DANs during training with a red-light pulse as US (in-lieu of heat punishment), generated successful trace and delay aversive learning (Fig. 3b, left) to the visual CS. In contrast, optogenetic activation (using *UAS-CsChrimson*) of

PPL1 MB-projecting DANs as a US (in-lieu of heat punishment), did not lead to delay or trace learning (Fig. 3b, right).

*In vivo* calcium imaging of the PPM3 EB-projecting DANs (expressing GCaMP6m and tdTomato transgenes) revealed that these neurons responded to the US (Fig. 3c, d, Extended Data Fig. 8, 9). Strikingly, after repeated training and selectively in trace conditioning, we observed transient, yet distraction sensitive, dopaminergic activity not only to the US, but also during the trace interval (Fig. 3c, d), prior to US onset (Supplementary Video 5, 6). Dopaminergic responses during the trace interval also preceded activity during CS presentation in later trials, potentially an early event in the general learning process, and were expectedly diminished by distractions in trace (Fig. 3c-e) but not delay conditioning (Extended Data Fig. 8e-f, Supplementary Video 7, 8).

### **Dop2R mediates sustained CS neural trace**

A closer look at PPM3 DAN and corresponding EB R2/R4m ring neuron activity during trace conditioning revealed transient dopaminergic activity during the trace interval (Fig. 3c-e) coinciding with increased signal persistence in EB ring neurons, in both oscillations and decay rate (Fig. 2f). To test whether the observed dopaminergic activity might be linked to the trial-by-trial increase in EB ring activity, we selectively targeted the dopamine family receptors (D1-, D2-like, and EcR) expressed in the ring neurons of the CX<sup>32,33</sup>. An RNAi construct selectively impaired D1 (*UAS-Dop1R1<sup>RNAi</sup>* and *UAS-Dop1R2<sup>RNAi</sup>*), D2 (*UAS-Dop2R<sup>RNAi</sup>*) and EcR (*UAS-DopEcR<sup>RNAi</sup>*) signaling in the EB R2/R4m ring neurons in assays for learning deficits (Fig. 4a) showing that Dop1R1 impairment affected both delay and trace learning, whereas Dop2R impairment selectively diminished trace learning, leaving delay learning intact.

To better understand the differential involvement of dopamine receptor signaling, we expressed GCaMP6f and tdTomato specifically in EB R2/R4m ring neurons while also silencing each of the four dopamine receptors in those neurons. Ratiometric calcium imaging during delay and trace conditioning revealed each dopamine receptor's effect on the oscillatory component of the calcium transients. As expected, frequency profiles were altered in flies with impaired Dop1R1 and Dop2R signaling (Fig. 4b-c), whereas Dop1R2 and DopEcR impaired flies exhibited expected increases in ring neuron frequency (Extended Data Fig. 10). During delay conditioning, Dop1R1 impairment significantly diminished the trial-by-trial increase in frequency during CS presentation (Fig. 4b, top-left, c, top), a frequency unaffected by Dop2R impairment (Fig. 4b, bottom-left, c, top). During trace conditioning, both Dop1R1 and Dop2R impairments significantly affected the frequency increase during CS presentation (Fig. 4b, right, c, middle). However, during the trace interval, Dop1R1 and Dop2R impairments differentially affected ring neuron calcium dynamics: Dop1R1-impaired flies exhibited an increase in frequency during the initial trials, but that increase plateaued and never reached the levels expected for trace learning (Fig. 4b, top-right, c, bottom). Dop2R impairment, however, abolished any increase in signal persistence and frequency (Fig. 4b, bottom-right, c, bottom), suggesting its critical role in maintaining the CS neural representation during the trace interval.

## **Discussion**

In conclusion, we present behavioral and neurophysiological evidence for visual trace and delay conditioning and the selective interference with trace conditioning by distractions in *Drosophila*. Intriguingly, the mushroom body (lobe-projecting neurons and DANs), heavily studied as a site for associative learning in *Drosophila*<sup>34</sup>, is not involved in this response;

implying that it does not necessarily serve as a site for general-purpose “conditioning”. Notably in trace conditioning is a necessary occurrence of sustained, yet distraction sensitive, CS neural activity in the EB ring (during the trace interval), informative towards understanding the purported link with and neural basis of attention<sup>35</sup>. Inextricably linked to attention is working memory<sup>36</sup>, where strong inhibitory signaling has been suggested as a critical neural component that slows down temporal dynamics and enables working memory maintenance<sup>37</sup>, a mechanism that may even subserve our findings here, since these ring neurons are GABAergic (i.e., inhibitory)<sup>38</sup>.

Accompanying the ring neuron signal persistence was a surprising anticipatory role of dopamine in that structure, that became gradually less responsive to the US over training and conversely, more responsive to the CS. These two key response properties<sup>39</sup> appear to resemble those of a phasic dopamine response signaling a reward prediction error-(RPE)-like activity, albeit negative in this case, studied extensively in non-human primates and rodents<sup>40,41</sup>. Though similar, our fly responses appear after only seven training trials, striking given that primates and rodents go through days and weeks of training before RPE signals are examined<sup>42,43</sup>.

Consequently, the entire RPE signal development could be traced in the fly (along with the effect of distractions) unlike mammalian studies that only examine responses before and after training. Finally, Dop2R signaling played a critical role in sustaining ring neuron CS neural activity during the trace interval, and could begin to shine a spotlight on the molecular mechanisms of working memory. Intriguingly, Dop2R signaling was recently reported as required for forgetting in olfactory learning<sup>44</sup>, which taken together, suggests perhaps its role in prioritizing information and adaptability.

In the fly, recently identified navigational pre-motor CX circuits in EB columnar neurons<sup>45</sup> and protocerebral bridge<sup>46</sup> showed persisting activity in stimulus-void environments. In all, this suggests a motif of convergence and hierarchical integration<sup>47</sup> in the anatomical connectomics of the ellipsoid body and other structures of the central complex<sup>48,49</sup>, one that is evocative of the long-range top-down organization of the global neuronal workspace engaged in decision making and conscious processing in vertebrates<sup>50</sup>. In sum, we think this to be an important step in the study of the role of higher cognitive processes in the “mindful” behavior of *Drosophila*.

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## Figure legends

**Figure 1. *Drosophila* visual trace and not delay conditioning is distraction sensitive, both requiring ellipsoid body R2/R4m ring neurons.** (a) Tethered-flight conditioning behavior assay illustrating upright-T (CS<sup>+</sup>) paired with heat (US), inverted-T (CS<sup>-</sup>) without heat. Post-training, fly's closed-loop orientation towards CS<sup>+</sup> or CS<sup>-</sup> is scored. (b) Wing-beat amplitude flying straight (left), yaw-turning clockwise (center), counter-clockwise (right). (c) Delay or trace conditioning, with or without (air-puff) distractors, following single 20 s test. (d) Delay and trace conditioning performance index (PI) with distractors (gray), without distractors (white) (mean with s.e.m., n = 40 flies per group). Scatters represent single-fly PI. Groups compared using two-factor ART-ANOVA. \*\* indicates p-value < 0.01, exact p-values in Supplementary Information. (e) Implicated fly brain learning structures, ellipsoid body (yellow), fan-shaped body (purple), mushroom body (red). PI heatmap (mean, n = 20 flies) for delay and trace conditioning in specific neural silenced ellipsoid body ring neurons, fan-shaped body columnar layers, mushroom body projecting lobes, driver- and effector-less controls. Flies with intact Gal80 repression of Kir2.1 (18 °C), warm-induced Kir2.1 (30 °C).

**Figure 2. EB ring neuron ratiometric calcium imaging during trace conditioning reveals increasing, distraction sensitive, oscillatory component and persistence.** (a) Conditioning assay coupled with two-photon *in vivo* brain imaging - tethered flying fly under an objective (inset) shown an upright-T paired with heat. (b) Fly wing-beat amplitude computed from camera below. (c) EB R2/R4m expression (female, *EBI-Gal4*>>*UAS-myr-EGFP*, scale 50 μm). (d) Sample ratiometric two-photon image of *EBI-Gal4*>>*UAS-GCaMP6f.myr-tdTomato* female. Left-right; tdTomato, GCaMP6f, tdTomato channel with hot pseudocolor  $F_{\text{ratio}} =$

$F_{\text{GCaMP6f}}/F_{\text{tdTomato}}$  GCaMP6f signal. Scale 20  $\mu\text{m}$ . (e) Ratiometric imaging of *E1-Gal4*>>*UAS-GCaMP6f.myr-tdTomato* female during trace conditioning without (air-puff) distractors (top), with distractors (bottom). Shown,  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity (trials 1, 4, 7). Single-term exponential model curve fits (red) through  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity starting at CS offset. (f) Top, frequency (see methods, ratiometric fluorescence quantification), bottom, exponential curve fit decay, of EB1 activity during TI without distractors (blue,  $n = 16$  flies), with distractors (red,  $n = 16$  flies), CS-only trials (black,  $n = 17$  flies). Trace learning as a function of frequency (g,  $R^2$  0.7 (blue), 0.48 (red)), decay rate (h,  $R^2$  0.51 (blue), 0.42 (red)) (see methods, correlating physiological and behavioral metrics). Second-degree polynomial curve fits through median activity without distractors (blue), with distractors (red). Boxplot center (median), edges (IQR), whiskers (1.5x IQR). Scatters represent single-fly metrics. Groups compared using two-factor ART-ANOVA. \*\* indicates  $p$ -value  $< 0.01$ , exact  $p$ -values in Supplementary Information.

**Figure 3. EB-projecting dopaminergic neurons required for trace and delay conditioning, selectively active during the trace interval and negatively impacted by distractions in trace conditioning.** (a) Delay and trace conditioning PI (mean with s.e.m.,  $n = 20$  flies per group) with neural silencing in most DANs (*TH-Gal4*>>*UAS-Kir2.1*), PPL1 DANs (*MB504B-Gal4*>>*UAS-Kir2.1*), PPM3 DANs (*c346-Gal4, TH-FLP*>>*UAS-FRT-stop-FRT-Kir2.1*). Experimental genotypes (gray) compared to parentals using Kruskal-Wallis and post-hoc bonferroni-corrected unpaired two-sided Mann-Whitney U tests. (b) Delay and trace learning PI (mean with s.e.m.,  $n = 20$  flies per group, on standard food (white) and all-trans-retinal supplemented food (gray)), where PPM3 DANs (left), PPL1 DANs (right), are optogenetically stimulated as US. Groups compared using unpaired two-sided Mann-Whitney U tests. Scatters represent single-fly PI. (c)

Ratiometric imaging of *c346-Gal4>>UAS-GCaMP6m.myr-tdTomato* female during trace conditioning without (air-puff) distractors (left), with distractors (right). Shown,  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity (trials 1, 3, 5, 7). (d) Peak  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity during CS (left), during TI (center), post-US (right) without distractors (blue, n = 5 flies), with distractors (red, n = 5 flies). Boxplot center (median), edges (IQR), whiskers (1.5x IQR). Scatters represent single-fly activity. Groups compared using two-factor ART-ANOVA. (e) First  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity peak (see methods, ratiometric fluorescence quantification), without distractors (blue), with distractors (red). In (d) and (e), single-term exponential model curve fits through median activity without distractors (blue), with distractors (red). \*\* indicates p-value < 0.01, exact p-values in Supplementary Information.

**Figure 4. EB ring dopamine receptor signaling during delay and trace conditioning.** (a) Delay and trace learning (5 s TI) PI (mean with s.e.m., n = 20 flies per group) with impaired Dop1R1, Dop1R2, Dop2R, DopEcR receptor signaling in EB R2/R4m neurons. Experimental genotypes (gray) compared to parentals using Kruskal-Wallis and post-hoc bonferroni-corrected unpaired two-sided Mann–Whitney U tests. (b) Ratiometric imaging of *EB1-Gal4>>UAS-GCaMP6f.myr-tdTomato,UAS-Dop1R1<sup>RNAi</sup>* (top) or *EB1-Gal4>>UAS-GCaMP6f.myr-tdTomato,UAS-Dop2R<sup>RNAi</sup>* females (bottom) during delay (left), trace conditioning (right). Shown,  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity (trials 1, 4, 7). Single-term exponential model curve fits (red) through  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity at CS offset. (c) Frequency (see methods, ratiometric fluorescence quantification) of EB1 activity for delay conditioning (during CS, top), trace conditioning (during CS, middle; during TI, bottom) for *EB1-Gal4>>UAS-GCaMP6f.myr-tdTomato* (black), *EB1-Gal4>>UAS-GCaMP6f.myr-tdTomato,UAS-Dop1R1<sup>RNAi</sup>* (red), *EB1-Gal4>>UAS-*

*GCaMP6f.myr-tdTomato,UAS-Dop2R<sup>RNAi</sup>* (blue). Top, n = 9 (black), n = 5 (red), n = 5 (blue) flies. Middle and bottom, n = 16 (black), n = 5 (red), n = 5 (blue) flies. Boxplot center (median), edges (IQR), whiskers (1.5x IQR). Scatters represent single-fly activity. Groups compared using two-factor ART-ANOVA. \*\* indicates p-value < 0.01, exact p-values in Supplementary Information.

## Methods

### Fly stocks

All fly strains were maintained on standard cornmeal-agar medium at 25 °C and 60 % humidity throughout development on a 12-h light, 12-h dark cycle. All experimental flies were non-mated females, 4-10 days old, reared at 25 °C post-eclosion unless otherwise stated, and were tested 0-3 hours before the onset of their subjective night. Canton-S flies served as the wildtype *Drosophila* strain in this study. Unless otherwise stated, stocks with initials BDSC and VDRC (and corresponding stock numbers) were obtained from the Bloomington Drosophila Stock Center and Vienna Drosophila Resource Center respectively.

Ellipsoid body drivers for expression in ring neurons were: R1 - *c105-Gal4* (BDSC 30822), R3/R4d - *c232-Gal4* (BDSC 30828), and R2/R4m - *E1-Gal4* (BDSC 44409). Fan-shaped body drivers for expression in columnar layers were: dorsal and ventral layers - *104y-Gal4* (gift from W. Joiner at UCSD), dorsal layers - *c205-Gal4* (BDSC 30826), and dorsal and central layers - *R38E07-Gal4* (BDSC 50007). Mushroom body lobe projecting drivers were:  $\alpha$  and  $\beta$  lobes - *17d-Gal4* (BDSC 51631),  $\alpha'$  and  $\beta'$  lobes - *c305a-Gal4* (BDSC 30829), and  $\gamma$  lobes - *MB009B-Gal4* (BDSC 68292). Gal4 drivers for dopaminergic experiments were *TH-Gal4* (BDSC 8848), *c346-Gal4* (BDSC 30831) and *MB504B-Gal4* (BDSC 68329). Auditory sensory neuron driver used was *JO-AB-Gal4* (BDSC 6753). Empty-Gal4 driver used was *pBDP-Gal4* (BDSC 68384). Dopamine neurons were isolated using TH-FLP recombinase<sup>51</sup> (gift from M. Wu at JHMI).

Neural silencing experiments were performed using either *UAS-Kir2.1*<sup>52</sup> or *UAS-Kir2.1, Tub-Gal80<sup>ts</sup>* (BDSC 6595, 6596, 7017)<sup>53</sup> or *UAS-FRT-stop-FRT-Kir2.1* (BDSC 67686). For Gal80-based conditional repression experiments, flies were raised at 18 °C and temperature

shifted to 30 °C for 48 hours and then returned to 18 °C for 2 hours prior to testing. Controls were flies maintained at the permissive temperature that had intact Gal80 repression of Kir2.1 expression but were otherwise genetically identical to experimental genotypes.

Optogenetic activation experiments were performed using either *UAS-CsChrimson* (BDSC 55136)<sup>54</sup> or *UAS-FRT-stop-FRT-CsChrimson::mVenus* (gift from V. Jayaraman at JFRC). For these experiments, newly eclosed female flies expressing *CsChrimson* in targeted neurons were collected and reared in constant darkness (25 °C, 60 % humidity) on standard cornmeal medium supplemented with 200 µM all-trans-retinal (Sigma-Aldrich). Control flies were siblings of experimental groups and reared in the darkness with standard cornmeal medium only.

*In vivo* calcium imaging experiments used *20XUAS-GCaMP6m.myr-tdTomato* and *20XUAS-GCaMP6f.myr-tdTomato* flies<sup>55</sup> (gifts from A. Calhoun at Princeton). Immunostaining experiments used *UAS-myr-EGFP* (BDSC 32197) or *UAS-FRT-stop-FRT-mCD8-GFP* (BDSC 30125) flies.

Dopamine receptor RNAi lines used were *UAS-Dop1R1<sup>RNAi</sup>* (VDRC KK107058), *UAS-Dop1R2<sup>RNAi</sup>* (VDRC KK105324), *Dop2R<sup>RNAi</sup>* (BDSC 78804) and *UAS-DopEcR<sup>RNAi</sup>* (VDRC KK103494).

### **Tethered flight behavior assay**

For tethered-flight behavior experiments, flies were cold-anesthetized (4 °C) and tethered to a stainless steel minuten pin (0.2 mm rod diameter, Fine Science Tools) with UV-cured glue. After at least one hour of recovery, tethered flying flies were suspended within a custom-built high-speed projector based spherical virtual-reality flight setup (sphere diameter, 2 in). A rear-

projection coating (Screen Goo, Goo Systems Global) was applied to the outer wall of the transparent sphere to enable presentation of computer-generated imagery (Extended Data Fig. 1a).

The tethered-flight setup employed a display system composed of a high-speed projector (Texas Instruments, LightCrafter 4500 EVM) and two mirrors (diameter, 2 in, Edmund Optics) placed laterally to the sphere, to immerse the fly in a panoramic virtual-reality environment, covering  $\sim 330$  deg of azimuth and  $\sim 85$  deg of elevation. Custom software was written in Microsoft Visual C++ using a 3D graphics programming library OpenSceneGraph, to warp and display images on a curved spherical projection screen (Extended Data Fig. 1b). The projector was set to display 6-bit images at a resolution of  $912 \times 1140$  at 300 Hz. Blue light emitted from the projector was filtered through a 450 nm long-pass emission filter (62-982, Edmund Optics). Light intensity in the sphere was controlled by modulating the current of projector LED via the supplied software and set to 10% of the maximum power. Calibration of light intensity was performed using a SpectraScan PR-701S spectroradiometer. The overall radiance inside the spherical arena for an all-ON stimulus was set to be  $0.4 \text{ Watts m}^{-2} \text{ sr}^{-1}$ , to match the light intensity in the natural environment during crepuscular sunset conditions.

A custom infra-red diffused backlight of 49 LEDs (Vishay, 2 in x 2 in, 880 nm) was positioned below the sphere to illuminate the fly and enable measurement of the fly's wing beat amplitude. A high-speed camera (FL3-U3-13Y3M-C, Point Grey Research) with attached lens (Tamron macro lens,  $f/2$ , 60 mm) and an IR-pass only filter (850 nm, Edmund Optics) was positioned above the sphere. The amplitude of the fly's left- and right-wing beats were computed in real-time at 200 Hz using machine vision techniques, allowing us to present visual stimuli in either open- or closed-loop mode.

Heat punishment was delivered with a focusable dot infra-red laser module (808 nm, maximum power 350 mW, Roithner LaserTechnik), the power level of which was calibrated to raise the fly's body temperature to 35 °C from ambient room temperature (25 °C) within 0.5 s (Extended Data Fig. 1c). The relationship between laser power and the fly's internal body temperature was determined by inserting the tip of a hypodermic needle thermocouple probe (HYP1-30-1/2-T-G-SMPW-M, Omega) in the thorax of a live fly (positioned in the virtual reality environment) and recording the temperature with a thermocouple data acquisition module (TC-08, Omega). An 850 nm long-pass dichroic beamsplitter (Edmund Optics) was placed in between the display sphere and the high-speed camera above it to direct the punishment laser light beam down on to the fly without interfering with the wing-beat image.

To create a distractive environment during delay and trace conditioning and enable direct comparison of the effects of distractions on each paradigm, we presented distractors at all stages of the conditioning process, prior to and during CS, during the trace interval, and after US (Fig. 1c). Gentle air puffs (0.5 s pulse, regulated at a rate of 50 ml min<sup>-1</sup>) were used as distractors, controlled to ensure that it did not physically disturb the animal (Extended Data Fig. 1f), nor affect conditioning by competing with the CS after trace conditioning (Extended Data Fig. 1g). For these distractor response experiments, air-puffs (0.5 s pulse) were delivered randomly (across experiments) from one of two air-puff nozzles that were placed laterally (45 deg), on each side of the fly. Change in flight orientation response, as a tendency to move towards, away (greater than 20 deg) or no change in orientation (less than or equal to 20 deg) was measured in the 5 s interval after air-puff delivery.

In our experiments, during the training phase, we presented either an upright-T or inverted-T shaped bright image over a dark background. The Ts were displayed in the frontal

visual field of the fly and were held fixed during presentation. The relative luminance values of the dark and bright pixels were 0 and 1 (Extended Data Fig. 1b). The T-shape measured 40 deg vertically and horizontally, with the bars of the Ts being 14 deg wide. We randomized which image was used as CS<sup>+</sup> (paired with heat), vs. the control CS<sup>-</sup> condition (not paired with heat) between experiments. Each training trial lasted 60 s which included presentation of stimuli in the order shown (Fig. 1c). A 30 s inter-trial-interval was included. We empirically determined 7 training trials as optimal for our conditioning experiments (Extended Data Fig. 1d). Post-training, during the test phase, both upright-T and inverted-T shapes were presented simultaneously in closed-loop mode. The Ts were presented lateral to the fly and 180 deg apart, however which shape was displayed on the left vs. right of the fly was randomized between experiments. This procedure eliminated the spontaneous vs non-associatively induced pattern preferences. A 60 s gap was introduced between the end of the last training trial and start of the test sequence. Naïve flies, not exposed to any conditioning, were used in all experiments to ensure the CS had no prior valence associated with it. Since the goal of our assay is to study the conditioned behavior (and the effect of distractions on that behavior), a single test of learning was performed for each conditioned fly as subsequent rounds of testing could impact the efficacy of the conditioning.

During the test phase, if  $t_a$  was the time the fly spent orienting towards the CS<sup>-</sup> image quadrant, and  $t_b$  was the time the fly spent orienting towards the CS<sup>+</sup> image quadrant, we calculated the performance index score (PI) as  $(t_a - t_b)/(t_a + t_b)$ . Therefore,  $PI > 0$  indicates successful learning as the fly fixates more on CS<sup>-</sup> than CS<sup>+</sup>, with  $PI < 0$  indicating the opposite.  $PI = 0$  indicates equal probability of fixating on CS<sup>+</sup> and CS<sup>-</sup>. Only experiments where the fly

fixated (on either pattern quadrant) for at least 50 % of the total experiment time (10 out of 20 s) were considered in our analysis.

For optogenetic experiments, experimental procedures were the same as previous tethering tests except that the heat punishment was replaced by red light stimulation (635 nm, Roithner LaserTechnik) set at  $0.8 \text{ mW cm}^{-2}$  (Fig. 3b). Some experiments required both optogenetic stimulation (red light pulse) to distract the animal, and US heat punishment (IR laser). An additional 735 nm long-pass dichroic beamsplitter (Edmund Optics) was placed in the laser path for this purpose (Extended Data Fig. 3e).

### **Fly brain window surgery**

The fly brain window surgery is largely similar to a previous study<sup>56</sup>, wherein a custom flyholder was used to immobilize the fly head (while allowing its wings to move freely) under an imaging device and expose its brain for optical recordings. The flyholder consisted of two parts – a 3D printed plastic frame, and a soft annealed stainless-steel shim (with a fly brain sized hole) folded and glued with epoxy to fit the contour of the frame. After gluing the metal shim to the holder, charcoal primer and paint were applied to the bottom side of the shim to minimize reflections during wing-beat tracking.

Briefly, our fly mounting and surgery procedure was as follows - cold-anesthetized flies ( $4 \text{ }^{\circ}\text{C}$ ) were positioned ventral side up in a fly-sized divot machined in a custom-made brass block. The first pair of legs (T1) were cut at the first segment and the middle and rear pairs (T2 and T3) were removed completely. The proboscis was gently pushed into the head capsule and a small drop of UV-cured glue was applied to fix it in place. The fly was then flipped over, dorsal side up, and a small drop of UV-cured glue was applied in the gap between the head and thorax,

thereby tilting the fly head slightly upwards. The flyholder was then positioned and glued to the head, following which, the holder and the fly were removed from cold-anesthesia and allowed to recover.

After recovery (determined by the fly exhibiting flight), the flyholder was filled with saline to fully cover the head. 1x saline containing 103 mM NaCl, 5 mM TES, 8 mM Trehalose, 10 mM Glucose, 26 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, and 3mM CaCl<sub>2</sub>, with a pH = 7.3 was prepared and used. With the head covered in saline, the cuticle, air sacs and fat bodies were removed by hand dissection using fine forceps (Dumont 5SF, Fine Science Tools, or tip-size A, re-sharpened at Corte Instruments). An incision was made first along the posterior side of the head, then along both lateral edges, and finally along the anterior side to remove the cuticle.

### **Two-photon calcium imaging**

The tethered flight behavior assay described above was coupled with a two-photon microscopy setup with some modifications. First, a cylindrical acrylic display (4 in diameter, 3.5 in height, ~330 deg of azimuth and ~85 deg of elevation) with an adhesive rear-projection film was used for visual stimulus presentation. The display was tilted downwards (pitch angle of ~20 deg) relative to the flyholder positioned in it (Fig. 2a). The projector used for visual stimulus presentation was identical to the tethered flight assay with the exception of a 447/60 bandpass filter (Chroma) that was placed in the light path to avoid any bleed through of visual stimulus light into the imaging recordings. Two 8x10 cm first-surface mirrors (Edmund Optics) were positioned laterally for panoramic display coverage.

Two IR leds (880 nm), placed directly under the flyholder and behind the fly, were used to illuminate the wings and enable tracking of wingbeat amplitude in a high-speed camera placed

under the fly (Fig. 2b). An identical high-speed camera setup to that of the tethered flight assay was used for wingbeat tracking. The punishment and distractor delivery apparatus were also identical to the tethered flight assay and delivered from underneath the fly.

We performed ratiometric imaging by recording simultaneously calcium-dependent *GCaMP6f* or *GCaMP6m* fluorescence and calcium-independent *tdTomato* fluorescence in the same neural populations. Our imaging experiments were performed using a Bruker Ultima Investigator multiphoton microscope with a Nikon 40x NIR Apo objective water-immersion lens (0.8 N.A., pixel size 0.27 x 0.27  $\mu\text{m}$ , FOV 69.1 x 69.1  $\mu\text{m}$ ). GaAsP photomultiplier tubes (H10770, Hamamatsu) band-passed with either et525/70m-2p or et595/50m-2p emission filters (Chroma) and a t565lpxr dichroic beam-splitter (Chroma) were used for simultaneous acquisition. A 920 nm mode-locked Ti:Sapphire laser (Mai Tai, Spectra Physics) provided a maximum power of 15 mW on the sample. Bi-directional resonant scanning galvos and a high-speed Z-piezo (Bruker, max range, 400  $\mu\text{m}$ ) were used for imaging a 6-plane volume of the EB at a rate of  $\sim 6$  Hz (image resolution of 256x256 px, 5  $\mu\text{m}$  spacing between imaging planes).

### **Ratiometric fluorescence quantification**

We performed all post-acquisition image analyses using Matlab R2019b. Our raw *in vivo* imaging experiment data consisted of multi-plane 3D volumes of both *GCaMP* and *tdTomato* channels from the same neural populations, simultaneously captured with dual GaAsP PMTs. A maximum intensity projection (MIP) sequence was generated for each pixel in the 3D volume for both *GCaMP* and *tdTomato* channels. The *tdTomato* channel video was then spatially aligned using a fast-normalized cross-correlation template matching algorithm based on the first frame in

the MIP video. The same transformations were then applied to the MIP video of the GCaMP channel to ensure pixel-pixel correspondence between frames of the two videos.

Next, we applied a 3x3 median spatial filter followed by 2D gaussian smoothing with a square gaussian kernel standard deviation of 1, to smooth acquisition noise. A binary mask of labeled neurons was then created by applying adaptive thresholding to each frame of the tdTomato channel. The calcium transient was measured as a ratio, of green calcium-dependent fluorescence (GCaMP) over red calcium-independent fluorescence (tdTomato) within the masked region and shown in a red-hot pseudocolor (Fig. 2d).

Specifically, for the EB ring neuron imaging data (including those with dopamine receptor impairment), we applied (to the smoothed raw data) a Principal Component Analysis (PCA) to decorrelate the data and uncover unknown, independent components of the observed features across time. We assume that the most discriminative information is captured by the largest variance in the feature space, an assumption likely true since the direction of the largest variance encodes the most information (Extended Data Fig. 5b, 6b). We empirically determine the number of eigenvectors to consider and reduce the dimensionality of the raw data across all experiments to be 12, based on computing the total variance of the data represented by each ordered principal component (Extended Data Fig. 5c, 6c).

We calculated GCaMP activity levels by first applying a Savitzky-Golay filtering (order of 3, frame length of 7)<sup>57</sup> to the ratio-metric intensity time-series data, then computing the difference of maximum Fratio values from a baseline level:  $dF/F = (F_{ratio} - F_{ratio\_baseline}) / F_{ratio\_baseline}$ . Baseline levels were computed by averaging the lowest 25 % intensity frames during the baseline period (5 s) of each trial before CS stimulus is presented.

For EB ring neuron data, we performed a frequency analysis on the reduced dimensionality data as well as determined the decay rate in ratio-metric activity post-CS presentation. Underlying frequencies were extracted by computing a discrete Fourier transform using the fast Fourier transform (FFT) algorithm. First, the raw data were low pass filtered to exclude frequencies greater than 75 % of the Nyquist rate, which in our experiments was ~3 Hz. This detectable frequency range is well within that of GCaMP6f intermediate kinetics which offers a temporal resolution (median  $\pm$  s.e.m.) of  $26 \pm 2$  ms (1 action-potential dF/F half-rise time) and  $140 \pm 20$  ms (1 action-potential dF/F half-decay time)<sup>55</sup>.

The frequencies with maximum power were then obtained for the following time bins and compared across the different learning conditions – pre-CS baseline, CS presentation, trace interval (only for trace learning) and post-US. Computing the decay rate involved fitting an exponential curve to the raw data starting at the end of CS presentation. The exponential decay constant ( $\tau$ ), defined as the amount of time the activity signal would take to decay by a factor of  $1/e$ , was used as a measure of signal decay and compared across learning conditions.

For EB DAN imaging experiments, we determined peaks in activity by computing the local maxima with a minimum peak prominence of 5s greater than the mean response. The first peak activity location was then extracted and compared across trials.

### **Correlating physiological and behavioral metrics**

The following approach was used to correlate per-trial physiological metrics (frequency and decay rate) with per-trial behavioral performance metrics (Fig. 2g, h, Extended Data Fig. 6g). Since learnt behavior can only be tested once per fly (after training), per-trial behavioral performance necessitated testing learnt behavior on different populations of flies exposed to

training trials ranging from 1 to 7 ( $n = 20$  flies were used for each sample population). As a result, physiology and behavior metrics were collected from different subject populations (with different sample sizes), and there was no apparent direct pairing between the two sets of data. We therefore created random pairings by sampling without replacement from each group (behavior vs. frequency or behavior vs. decay rate). Since both physiological and behavioral measurements were categorized by trial number, we randomly picked at minimum 8 samples per trial to generate a paired set of at least 56 data points (across seven trials). Spearman correlation coefficient was then computed on these paired data points ( $p$ -value  $< 0.01$  was verified for the correlation). This random sampling procedure was then repeated 10,000 times to generate the same number of correlation coefficients, the mean of which was reported as the final  $R^2$ .

### **Two-factor statistical comparisons**

Both behavioral and neurophysiological recordings consisted of certain data with a two-factor structure. These data were organized by groups (e.g., no distractor, distractor, CS only) across trials (or conditioning variants). Since the objective was to assess the difference not only across trials, but also across groups, a two-factor aligned-rank-transform analysis of variance (ART ANOVA) was used<sup>58</sup>. Two-way interaction post-hoc comparisons were then performed using the Tukey method.

### **Immunostaining**

The following procedure was used for immunostaining whole-mount brains. First, brains dissected from 3-7 day old of female flies were fixed with 4 % paraformaldehyde for 2 hours at room temperature and followed by extensive washes with washing buffer (0.3 % PBT) for 4 x 20

min. After washes, brains were incubated in blocking buffer (5 % normal goat serum) for 1.5 hours. Next, the brains were incubated in primary and secondary antibodies at 4 °C for 48 hours each, with extensive washes (0.3 % PBT, 4 X 20 min) between steps. Primary antibodies used here included Rabbit anti-GFP (1:1000, Invitrogen) and mouse anti-nc82 (1:30, Developmental Studies Hybridoma Bank). Secondary antibodies used here were AF488 goat  $\alpha$ -rabbit (1:400, Invitrogen), AF568 goat  $\alpha$ -mouse (1:400, Invitrogen), and AF647 goat  $\alpha$ -mouse (1:400, Invitrogen). The brains were then mounted on microscope slides with Vectashield antifade mounting medium for fluorescence (Vector Laboratories). Confocal laser scanning micrographs were obtained using a Zeiss LSM880 confocal microscope with a 20X (1.0 N.A.) water immersion objective. Spacing between individual planes was 1  $\mu$ m and the pixel resolution was set to 1024 x 1024.

## **Method references**

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### **Author contributions**

DG designed the behavior and imaging assay hardware and software. DG and J-YC performed behavior, neural and receptor silencing and optogenetic activation experiments. JX, JL, and DG acquired *in vivo* imaging data. J-YC performed immunostaining experiments. DG, J-YC, JX, and JL, analyzed the data. DG, J-PC, J-YC and RJG drafted the manuscript. All authors reviewed and approved the final manuscript.

### **Competing interests**

The authors declare no competing interests.

### **Additional information**

Correspondence and requests for materials should be addressed to RJG or J-PC.

### **Code availability**

The source code for the different assays developed are available for download from the public repository at <https://github.com/dgrover/flyCAVE>.

### **Data availability**

Datasets generated as part of this study are available from the corresponding author on reasonable request.

## Extended Data legends

### Extended Data Figure 1. *Drosophila* behavior assay for visual trace and delay aversive

**conditioning.** (a) Panoramic virtual-reality system components with real-time machine-vision based wing-beat amplitude tracking for tethered flying flies. 1) Projector, 2) 880 nm IR diffused backlight, 3) Rear-projection coated acrylic sphere, 4) High-speed camera for wing tracking, 5) 850 nm long-pass optical filter, 6) photographic macro lens, 7) xyz-translation micro-manipulator, 8) Fly tether-rod, 9-10) Mirrors for re-directing projection patterns for panoramic visual stimulus presentation, 11) 808 nm IR laser for delivery of heat punishment, 12) 850 nm long-pass dichroic filter (see Methods for optical configurations used in optogenetic experiments). (b) Sample display frame consisting of three orthogonal views of a virtually created sphere that is projected onto the curved spherical projection screen from frontal and two lateral sides. The virtual sphere with warped T's is programmatically calibrated to fit the dimensions and curvature of the physical display screen. Top, inverted-T displayed 45 deg from front-center in CW direction with upright-T 180 deg away. Bottom, inverted-T displayed 45 deg from front-center in CCW direction with upright-T 180 deg away. (c) Internal body temperature of a tethered fly when exposed to IR laser-based heat punishment. Red bars indicate 0.5 s of IR laser exposure, power-level of which was optimized to cause an immediate and robust rise in temperature from ambient room temperature of 25 °C to 35 °C. (d) Delay learning PI (n = 20 flies per group) measured as a factor of CS duration and number of training trials.  $PI > 0$  indicates successful learning as the fly spends more time fixating on  $CS^-$  than  $CS^+$ . Peak learning (indicated with black arrow) occurred with CS duration of 10 s and 7 training trials. (e) Change in flight orientation in the 5 s after air-puff (0.5 s). See Methods section on tethered flight behavior assay for experimental details. Binned is the fraction of time each fly (n = 40 flies)

spent orienting towards or away from the stimulus (orientation change greater than 20 deg), or no change in flight orientation (less than or equal to 20 deg). (f) Test of conditioning to distractor stimulus. Flies are subjected to full trace conditioning protocol (5 s TI) with distractors (n = 40 flies). Following training, same experimental protocol as (e) was followed to test distractor-only conditioning response. Binned is the fraction of time each fly spent orienting towards (attraction, positive conditioning) or away (aversion, negative conditioning) from the stimulus, or no change in flight orientation (neutral). Boxplot whiskers are 1.5x interquartile range. Groups were compared using a Kruskal-Wallis and post-hoc Mann-Whitney U tests with bonferroni corrected multiple comparisons. \*\* indicates p-value < 0.01. Scatters represent individual fly PI scores.

**Extended Data Figure 2. Conditioning-related experimental controls.** (a) Delay and trace conditioning experimental control protocols tested in (b) and (c). (b) PI (mean with s.e.m., n = 20 flies per group) for flies tested with US-only presentation (no CS), CS-only presentation (no US), reverse trace conditioning wherein US precedes CS<sup>+</sup> presentation with a gap of 2 s. PI = 0 indicates no learning as the fly spends an equal amount of time fixating on CS<sup>-</sup> and CS<sup>+</sup> during the test trial. Groups were compared using Kruskal-Wallis and post-hoc Mann-Whitney U tests with bonferroni corrected multiple comparisons. No significant differences were observed between groups. (c) PI (mean with s.e.m., n = 40 flies per group) for delay and trace conditioning (5-40 s TI) where CS<sup>+</sup> precedes CS<sup>-</sup> presentation (blue) and CS<sup>-</sup> precedes CS<sup>+</sup> (red). Group comparisons were performed using a two-factor ART-ANOVA test. No significant difference was observed between groups. Scatters represent individual fly PI scores.

**Extended Data Figure 3. Testing the effects of multi-modality distractors on conditioning.**

(a) Delay and trace conditioning protocols under different sets of (air-puff and optogenetic) distractor conditions tested in (b-e). (b) PI (mean with s.e.m., n = 40 flies per group) for flies tested under delay and trace conditioning (5-40 s TI) without (air-puff) distractors (black), multiple distractors including during the TI (distractor set #1, blue), single distractor during the TI (distractor set #2, red), multiple distractors except during the TI (distractor set #3, gray). Groups were compared using a two-factor ART-ANOVA test. \*\* indicates p-value < 0.01, comparing no distractor conditions with corresponding distractor set #1 conditions (blue), and corresponding distractor set #2 conditions (red). (c) PI (mean with s.e.m., n = 40 flies per group) for flies tested under delay and trace conditioning (5 s TI) testing the effect of amplified (air-puff) distractions - distractor set #1 (white, control), distractor set #4 with double the normal air flow rate (regulated at a rate of  $100 \text{ ml min}^{-1}$ ) (gray), distractor set #5 with double the normal air puff duration (1 s) (blue), and distractor set #6 with double the number of air puffs with an inter-pulse interval of 0.5 s, starting at times shown in (a). Groups were compared using a Kruskal-Wallis and post-hoc Mann-Whitney U tests with bonferroni corrected multiple comparisons. (d) PI (mean with s.e.m., n = 20 flies per group) delay and trace conditioning (5 s TI), with (distractor set #1, gray) and without distractors (white) by varying the number of training trials used for conditioning. Group comparisons were performed using the Mann-Whitney U test. (e) Delay and trace learning (5 s TI) in flies where optogenetic activation (red-light pulse) of auditory sensory neurons (*JO\_AB-Gal4*>>*UAS-CsChrimson*) was used as distractors in-lieu of the air-puff, as shown in (a). PI (mean with s.e.m., n = 20 flies per group) raised on standard food (white bars), and food supplemented with all-trans-retinal (gray). Gal4 control activation (*pBDPGal4*>>*UAS-CsChrimson*) with no brain expression included as a secondary control.

Group comparisons were performed using the Mann–Whitney U test. \*\* indicates p-value < 0.01. Scatters in all panels represent either individual fly probability or PI scores.

**Extended Data Figure 4. Effect of neural silencing of central complex and mushroom body**

**structures on delay and trace conditioning.** (a) Individual flies were tested under delay and trace conditioning (5 and 10 s TI). (b) Top-left, illustration of implicated learning and memory structures in the fly brain, ellipsoid body (yellow), fan-shaped body (purple) and mushroom body (red). PI for delay conditioning, and trace conditioning with (c) 5 s TI, and (d) 10 s TI. Shown are flies with neural activity silenced (*UAS-Kir2.1,Tub-Gal80ts*) in the ellipsoid body ring neurons – R1 (*c105-Gal4*), R3/R4d (*c232-Gal4*) and R2/R4m (*Eb1-Gal4*), fan-shaped body columnar neurons – dorsal layers (*c205-Gal4*), dorsal and central layers (*R38E07-Gal4*), and dorsal and ventral layers (*104y-Gal4*), and neurons projecting to the mushroom body lobes –  $\alpha$  and  $\beta$  lobes (*17d-Gal4*),  $\alpha'$  and  $\beta'$  lobes (*c305a-Gal4*) and  $\gamma$  lobes (*MB009B-Gal4*), along with respective driver-less and effector-less controls. PI (mean with s.e.m., n = 20 flies per group) for flies with intact Gal80 repression of Kir2.1 (white), and warm-induction of Kir2.1 (gray).

Scatters represent individual fly PI scores. Group comparisons were performed using the Mann–Whitney U test. \*\* indicates p-value < 0.01.

**Extended Data Figure 5. Ratiometric calcium imaging of ellipsoid body ring neurons**

**during trace conditioning reveals increased oscillatory component and slower decline with repeated training that is susceptible to distractions.** (a) Ratiometric imaging of an *Eb1-Gal4, UAS-GCaMP6f.myr-tdTomato* female during a single trace conditioning (5 s TI) experiment without (air-puff) distractors (left), and with distractors (right). Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity

traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6f}}/F_{\text{tdTomato}}$ ) for trials 1, 4, and 7. Red curve fits are single-term exponential model fits through the raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces starting at the end of CS presentation. (b) Scree plots of cumulative variance explained by each of the top 50 principal components for all trace conditioning trials without distractors (blue), and with distractors (red). (c) Cumulative variance explained by the top 12 components per trial for trace conditioning without distractors (blue), and with distractors (red). (d) Exponential curve fit decay rates of EB1 neural activity post-CS for trials without distractors (blue), and with distractors (red). (e) Normalized power spectral density estimates of EB1 neural activity for trials 1, 4 and 7 (left to right) during CS. Shown are means (solid line) with s.e.m. (shaded region) for trials without distractors (blue), and with distractors (red). (f) Frequency (with maximum power, see Methods section on ratiometric fluorescence quantification) of EB1 neural activity during CS for trials without distractors (blue), and with distractors (red). (g) Normalized power spectral density estimates of EB1 neural activity for trials 1, 4 and 7 (left to right) during the 5 s TI. Shown are means (solid line) with s.e.m. (shaded region) for trials without distractors (blue), and with distractors (red). (h) Frequency (with maximum power, see Methods section on ratiometric fluorescence quantification) of EB1 neural activity across trials during the 5 s TI for trials without distractors (blue), and with distractors (red). No distractor trials ( $n = 16$  flies), distractor trials ( $n = 16$  flies). Boxplot whiskers are 1.5x interquartile range. Scatters represent individual fly activity scores. Group comparisons were performed using a two-factor ART-ANOVA test. \*\* indicates  $p\text{-value} < 0.01$ .

**Extended Data Figure 6. Ratiometric calcium imaging of ellipsoid body ring neurons during delay conditioning reveals an increased oscillatory component and steady decline**

**with repeated training that is not susceptible to distractions.** (a) Ratiometric imaging of an *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato* female during a single delay conditioning experiment without (air-puff) distractors (left), and with distractors (right). Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6f}}/F_{\text{tdTomato}}$ ) for trials 1, 4, and 7. Red curve fits are single-term exponential model fits through the raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces starting at the end of CS. (b) Scree plots of cumulative variance explained by each of the top 50 principal components for all delay conditioning trials without distractors (blue), and with distractors (red). (c) Cumulative variance explained by the top 12 components per trial for delay conditioning without distractors (blue), and with distractors (red). (d) Exponential curve fit decay rates of EB1 neural activity post-CS for trials without distractors (blue), and with distractors (red). (e) Normalized power spectral density estimates of EB1 neural activity for delay conditioning trials 1, 4 and 7 (left to right) during CS. Shown are means (solid line) with s.e.m. (shaded region) for trials without distractors (blue) and with distractors (red). (f) Frequency (with maximum power, see Methods section on ratiometric fluorescence quantification) of EB1 neural activity across delay conditioning trials during CS for trials without distractors (blue), and with distractors (red). No distractor trials (n = 9 flies), distractor trials (n = 9 flies). (g) Frequency (from f) as a function of learning performance (see Methods section on correlating physiological and behavioral metrics) for trace conditioning with (red,  $R^2$  0.72) and without (blue,  $R^2$  0.77) distractors. Curve fits are second degree polynomials through the median activity for each of the no distractor (blue) and distractor (red) trials. Boxplot whiskers are 1.5x interquartile range. Scatters represent individual fly activity scores. Group comparisons were performed using a two-factor ART-ANOVA test. No significant difference was observed between groups.

**Extended Data Figure 7. Ratiometric calcium imaging of ellipsoid body ring neuron**

**activity in CS-only and US-only conditions.** (a) Ratiometric imaging of *EB1-Gal4, UAS-*

*GCaMP6f.myr-tdTomato* female during a single CS-only presentation (no US) experiment.

Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6f}}/F_{\text{tdTomato}}$ ) for trials 1, 4, and 7. Red

curve fits are single-term exponential model fits through the raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces

starting at the end of CS. (b) Exponential curve fit decay rates of EB1 neural activity post-CS. (c)

Normalized power spectral density estimates of EB1 neural activity for CS-only presentation

trials 1, 4 and 7 (left to right) during CS presentation. Shown are means (solid line) with s.e.m.

(shaded region). (d) Frequency (with maximum power, see Methods section on ratiometric

fluorescence quantification) of EB1 neural activity during CS. (e) Normalized power spectral

density estimates of EB1 neural activity for trials 1, 4 and 7 (left to right) during the 5 s post-CS

period. Shown are means (solid line) with s.e.m. (shaded region). (f) Frequency (with maximum

power, see Methods section on ratiometric fluorescence quantification) of EB1 neural activity

during the 5 s post-CS period. Trials,  $n = 17$  flies. Boxplot whiskers are 1.5x interquartile range.

Scatters represent individual fly activity scores. Friedman's repeated measure ANOVA test was

used for comparisons between trials. No significant difference was observed. (g) Ratiometric

imaging of an *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato* female during a single US-only

presentation (no CS) experiment. Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} =$

$F_{\text{GCaMP6f}}/F_{\text{tdTomato}}$ ) for trials 1, 4, and 7. (b) Normalized power spectral density estimates of EB1

neural activity for trials 1, 4 and 7 (left to right) during the 10 s CS fictitious presentation time

period (5 – 15 s). Shown are means (solid line) with s.e.m. (shaded region). (c) Normalized

power spectral density estimates of EB1 neural activity for trials 1, 4 and 7 (left to right) during

the 5 s post fictitious CS presentation period (15 – 20 s). Shown are means (solid line) with s.e.m. (shaded region). Trials, n = 8 flies.

**Extended Data Figure 8. Ratiometric calcium imaging of ellipsoid body projecting**

**dopaminergic neurons during delay conditioning.** Confocal fluorescence images of (a) *TH-Gal4*>>*UAS-myr-EGFP*, (b) *MB504b-Gal4*>>*UAS-myr-EGFP*, (c) *c346-Gal4*>>*UAS-myr-EGFP*, and (d) *c346-Gal4, TH-FLP*>>*UAS-FRT-stop-FRT-mCD8-GFP* expression in female

*Drosophila* brains staining the pattern of dopaminergic neurons in most of the brain (does not include the PAM cluster), PPL1 dopaminergic cluster projecting to the mushroom bodies, and PPM3 dopaminergic cluster projecting to the ellipsoid body respectively. In (a), left and right images correspond to different z-planes across the brain highlighting dopaminergic neuron subsets targeting the central complex and mushroom body structures. Scale bar is 50  $\mu\text{m}$ . (e)

Ratiometric imaging of a *c346-Gal4, UAS-GCaMP6m.myr-tdTomato* female during a single delay conditioning experiment without (air-puff) distractors (left), and with distractors (right).

Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6m}}/F_{\text{tdTomato}}$ ) for each training trial. (f) Peak  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity of EB DANs during CS (left), and post-US (right), across trials, without distractors (blue), and with distractors (red). Boxplot whiskers are 1.5x interquartile range.

Scatters represent individual fly activity scores. Group comparisons were performed using a two-factor ART-ANOVA test. No significant difference was observed between groups.

**Extended Data Figure 9. Ratiometric calcium imaging of ellipsoid body projecting**

**dopaminergic neurons in CS-only and US-only conditions.** (a) Ratiometric imaging of a *c346-Gal4, UAS-GCaMP6m.myr-tdTomato* female during a single CS-only presentation (no US)

experiment without (air-puff) distractors (left), and with distractors (right). Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6m}}/F_{\text{tdTomato}}$ ) for each training trial. (b) Peak  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity of EB DANs during CS (top), and post-CS (bottom), across trials, without distractors (blue), and with distractors (red). (c) Ratiometric imaging of a *c346-Gal4, UAS-GCaMP6m.myr-tdTomato* female during a single US-only (no CS) experiment without (air-puff) distractors (left), distractors with US in a simulated delay conditioning setting (middle), and distractors with US in a simulated trace conditioning (5 s TI) setting (right). Shown, raw  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity traces (where  $F_{\text{ratio}} = F_{\text{GCaMP6m}}/F_{\text{tdTomato}}$ ) for each training trial. (d) Peak  $dF_{\text{ratio}}/F_{\text{ratio}}$  activity of EB DANs prior to US (left), and post-US (right), across trials, without distractors (black), distractors with US in simulated delay conditioning setting (blue), and distractors with US in simulated trace conditioning (5 s TI) setting (red). Boxplot whiskers are 1.5x interquartile range. Scatters represent individual fly activity scores. Group comparisons were performed using a two-factor ART-ANOVA test. No significant difference was observed between groups.

**Extended Data Figure 10. Role of Dop1R2 and DopEcR dopamine receptor signaling in ellipsoid body ring neurons during delay and trace conditioning.**

(a) Frequency (with maximum power, see Methods section on ratiometric fluorescence quantification) of EB1 neural activity with either Dop1R2 or DopEcR dopamine receptor signaling impairment across trials for delay conditioning during CS for *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato* flies (black), *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato, UAS-Dop1R2<sup>RNAi</sup>* flies (red), and *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato, UAS-DopEcR<sup>RNAi</sup>* flies (blue). n = 9 flies (black), n = 5 flies (red), n = 5 flies (blue). (b) Frequency (with maximum power, see Methods section on ratiometric

fluorescence quantification) of EB1 neural activity with either Dop1R2 or DopEcR signaling impairment across trials for trace conditioning (5 s TI) (during CS, left; during TI, right) for *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato* flies (black), *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato, UAS-Dop1R2<sup>RNAi</sup>* flies (red), and *EB1-Gal4, UAS-GCaMP6f.myr-tdTomato, UAS-DopEcR<sup>RNAi</sup>* flies (blue). n = 16 flies (black), n = 5 flies (red), n = 5 flies (blue). Boxplot whiskers are 1.5x interquartile range. Scatters represent individual fly metrics. Group comparisons were performed using a two-factor ART-ANOVA test. No significant difference was observed between groups.