

Mushroom body efferent neurons responsible for aversive olfactory memory retrieval in *Drosophila*

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Aversive olfactory memory is formed in the mushroom bodies in *Drosophila melanogaster*. Memory retrieval requires mushroom body output, but the manner in which a memory trace in the mushroom body drives conditioned avoidance of a learned odor remains unknown. To identify neurons that are involved in olfactory memory retrieval, we performed an anatomical and functional screen of defined sets of mushroom body output neurons. We found that MB-V2 neurons were essential for retrieval of both short- and long-lasting memory, but not for memory formation or memory consolidation. MB-V2 neurons are cholinergic efferent neurons that project from the mushroom body vertical lobes to the middle superior-medial protocerebrum and the lateral horn. Notably, the odor response of MB-V2 neurons was modified after conditioning. As the lateral horn has been implicated in innate responses to repellent odorants, we propose that MB-V2 neurons recruit the olfactory pathway involved in innate odor avoidance during memory retrieval.

Different odors induce innate approach or avoidance behaviors in *Drosophila*. Innate odor responses can be modulated by experience, such as associative learning. After simultaneous exposure to an electric shock and an odorant, flies form aversive memory and show robust conditioned odor avoidance that lasts for hours to days, depending on the training protocol^{1–3}. The neural pathways for odor or shock processing and signal integration in the fly brain have been intensively studied in recent years. Odor information is first represented in the antennal lobes in the form of activity in olfactory receptor neurons⁴. Projection neurons then convey this information to higher-order processing centers⁴: the mushroom bodies and the lateral horn. By contrast, aversive reinforcement signals that occur in response to electric shock are relayed to the mushroom bodies through dopaminergic neurons^{5–7}. The olfactory and electric shock signals are integrated in the mushroom bodies to form aversive olfactory memories^{1,2}. However, the mushroom bodies are not required for innate avoidance of repellent odors^{8,9}.

In adult *Drosophila*, each mushroom body consists of about 2,000 Kenyon cells, which can be classified into three major types on the basis of their axonal projections: γ neurons, which form only a medial lobe; α/β neurons, whose axons branch to form a vertical (α) and a medial (β) lobe; and α'/β' neurons, which also form a vertical (α') and a medial (β') lobe¹⁰. Functional brain imaging has revealed localized activation of cAMP-PKA signaling in the mushroom body α -lobe in response to simultaneous cholinergic and dopaminergic stimulation^{11,12}, which represent, respectively, the odorant and electric shock pathways. Calcium imaging studies have shown that, after

associative conditioning, a short-term memory trace is formed in the α'/β' neurons¹³ and a long-term one in α -lobes¹⁴. The output of the α/β neurons is necessary for the retrieval of all phases of olfactory memory^{15,16}, but the neural circuits that translate the associative memory trace in the mushroom body into conditioned odor avoidance remain unknown.

To understand the neuronal basis of memory retrieval, we conducted a functional screen based on collections of GAL4 driver lines for mushroom body extrinsic neurons^{17,18} (data not shown). We blocked the neurotransmission of GAL4-expressing cells during the retrieval of different forms of short- and long-lasting memory using Shi^{ts} (ref. 19), a dominant-negative, temperature-sensitive variant of dynamin that can reversibly block synaptic vesicle endocytosis at elevated temperatures. Because neurons that are responsible for memory retrieval should receive synaptic inputs from the mushroom bodies and project to other parts of the brain, we characterized the polarity of mushroom body extrinsic neurons by expressing a pre-synaptic marker²⁰. We further characterized the neurotransmitter and physiological response properties of the identified mushroom body output neurons using immunohistochemistry and functional calcium imaging.

RESULTS

MB-V2 neurons are efferent to the mushroom body

In a preliminary screen, we functionally analyzed 58 enhancer-trap GAL4 lines that were expressed in putative mushroom body extrinsic neurons for a defect in aversive memory retrieval (data not shown).

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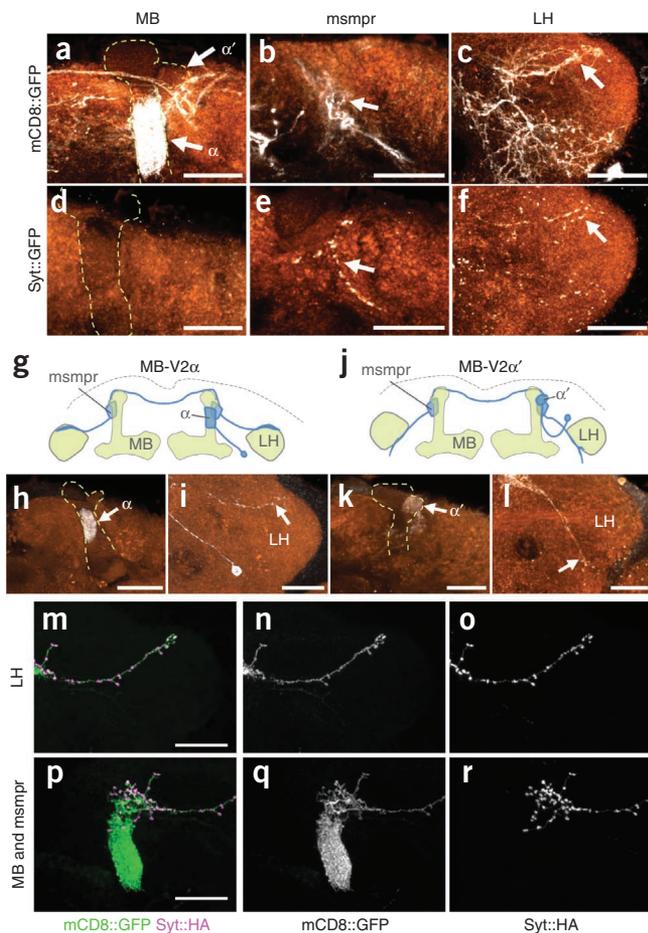


Figure 1 MB-V2 neurons connect the mushroom body to the lateral horn and middle superior medial protocerebrum. (**a–f**) Distribution of mCD8::GFP (white, **a–c**) and Syt::GFP (white, **d–f**) in the MB-V2 neurons driven by *NP2492*. Neuropils were labeled with an antibody to synapsin (orange). Mushroom body (MB) vertical lobes are outlined (dashed lines in **a, d, h** and **k**). (**a–c**) A membrane reporter mCD8::GFP was localized in the terminals of MB-V2 neurons (arrows) in the α - and α' -lobes of the mushroom body (**a**), msmpr (**b**) and lateral horn (LH, **c**). (**d–f**) The presynaptic marker Syt::GFP was highly enriched in the terminals of MB-V2 neurons (arrows) in the msmpr (**e**) and in the lateral horn (**f**) but not in the mushroom body vertical lobes (**d**). (**g–l**) Two subtypes of MB-V2 neurons, MB-V2 α (**g–i**) and MB-V2 α' (**j–l**), were identified by single-cell analyses. (**g, j**) Schematic diagrams show the two types of MB-V2 neurons (blue) relative to the mushroom body and lateral horn (light green). Dashed lines outline the brain surface. MB-V2 α neurons formed arbors in the mushroom body α lobes (**h**) and projected to the msmpr and the dorsal part of the lateral horn (**i**, arrow; $n = 87$). MB-V2 α' neurons formed arbors in the α' lobes (**k**) and projected to the msmpr, the ventral part of the lateral horn (arrow) and to a region ventromedial to the lateral horn (**l**; $n = 44$). (**m–r**) The polarity of the single MB-V2 α neuron visualized by a flip-out clone driving mCD8::GFP and Syt::HA using *NP2492*. The terminals of a single MB-V2 α neuron in the lateral horn (**m–o**) and the mushroom body and msmpr (**p–r**) are magnified. Scale bars represent 20 μ m.

tip of the α' lobe of the ipsilateral mushroom body and projected to the msmpr and ventral area of the lateral horns (**Fig. 1j–l**) of both sides. The collaterals of MB-V2 α' neurons also terminated in an area ventral and medial to the lateral horn. MB-V2 α and MB-V2 α' neurons were labeled in *NP2492*, whereas only the MB-V2 α neurons appeared to be labeled in *MZ160* (**Supplementary Videos 1 and 2**). Neurons in the MB-V2 cluster are the only GAL4-expressing cells that are clearly shared by *NP2492* and *MZ160*.

MB-V2 output is required for olfactory memory retrieval

To address the role of the MB-V2 neurons in innate odor responses, we first measured avoidance of 4-methylcyclohexanol and 3-octanol while blocking the output of MB-V2 neurons. Consistent with previous reports that mushroom bodies are not essential for innate odorant avoidance^{8,9}, we found that the odor avoidance of both *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* flies at restrictive temperature was normal (**Table 1**). We then examined the effect of the transient blockade of these neurons during the test on memory retrieval 2 h after a single cycle of conditioning. Both *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* flies showed strong memory impairment (**Fig. 2a**).

To assess whether MB-V2 output is also required for memory formation, we next blocked these neurons transiently during training and consolidation and tested the memory 2 h later at the

We identified a particular set of mushroom body extrinsic neurons, the MB-V2 neurons (**Fig. 1**), as candidates for the memory retrieval pathway. MB-V2 neurons were labeled in the GAL4 driver lines *NP2492* (ref. 18; **Fig. 1a–c** and **Supplementary Fig. 1a**) and *MZ160* (refs. 17,18; **Supplementary Fig. 1d**). The cell bodies of the MB-V2 neurons were posterior to the lateral horn (**Supplementary Fig. 1b,e**), and formed arbors in the vertical lobes of the mushroom bodies, the middle superiormedial protocerebrum (msmpr) just posterior to the vertical lobes, and the lateral horn (**Fig. 1a–c**). Membrane-targeted markers (mCD8::GFP or myr::RFP) uniformly labeled MB-V2 processes in all three target neuropils (**Fig. 1a–c** and **Supplementary Fig. 2**), whereas the signals of the presynaptic marker Syt::GFP (a fusion of eGFP and the synaptic vesicle protein synaptotagmin²⁰) were highly enriched in the msmpr and lateral horn but not detectable in the mushroom body vertical lobes (**Fig. 1d–f**). We observed similar results with other presynaptic markers (Syt::HA (ref. 21) and nSyb::GFP (ref. 17); **Fig. 1m–r** and **Supplementary Fig. 2d–i**). Quantification of Syt::HA and mCD8::GFP signal intensity in the MB-V2 terminals confirmed that Syt::HA was highly enriched in the msmpr and lateral horn, but not in the mushroom bodies (**Supplementary Fig. 2j**). Thus, MB-V2 neurons are likely to relay information from the mushroom body vertical lobes to the msmpr and lateral horn.

Flp-out clones²² of single MB-V2 neurons revealed two stereotyped neuron classes: MB-V2 α and MB-V2 α' . MB-V2 α neurons innervated across the shaft of the α lobe of the ipsilateral mushroom body and terminated in the msmpr and dorsal area of the lateral horns of both hemispheres (**Fig. 1g–i**), whereas MB-V2 α' neurons arborized in the

Table 1 Sensory acuity controls

Genotype	Odor avoidance	
	Octanol	Methylcyclohexanol
Wild type	0.54 ± 0.12	0.53 ± 0.10
<i>UAS-shi^{ts}</i>	0.51 ± 0.08	0.70 ± 0.06
<i>NP2492</i>	0.45 ± 0.10	0.80 ± 0.04
<i>NP2492/+; UAS-shi^{ts}/+</i>	0.49 ± 0.10	0.78 ± 0.07
Wild type	0.50 ± 0.11	0.56 ± 0.10
<i>UAS-shi^{ts}</i>	0.49 ± 0.08	0.70 ± 0.08
<i>MZ160</i>	0.59 ± 0.09	0.59 ± 0.08
<i>MZ160/UAS-shi^{ts}</i>	0.59 ± 0.06	0.59 ± 0.06

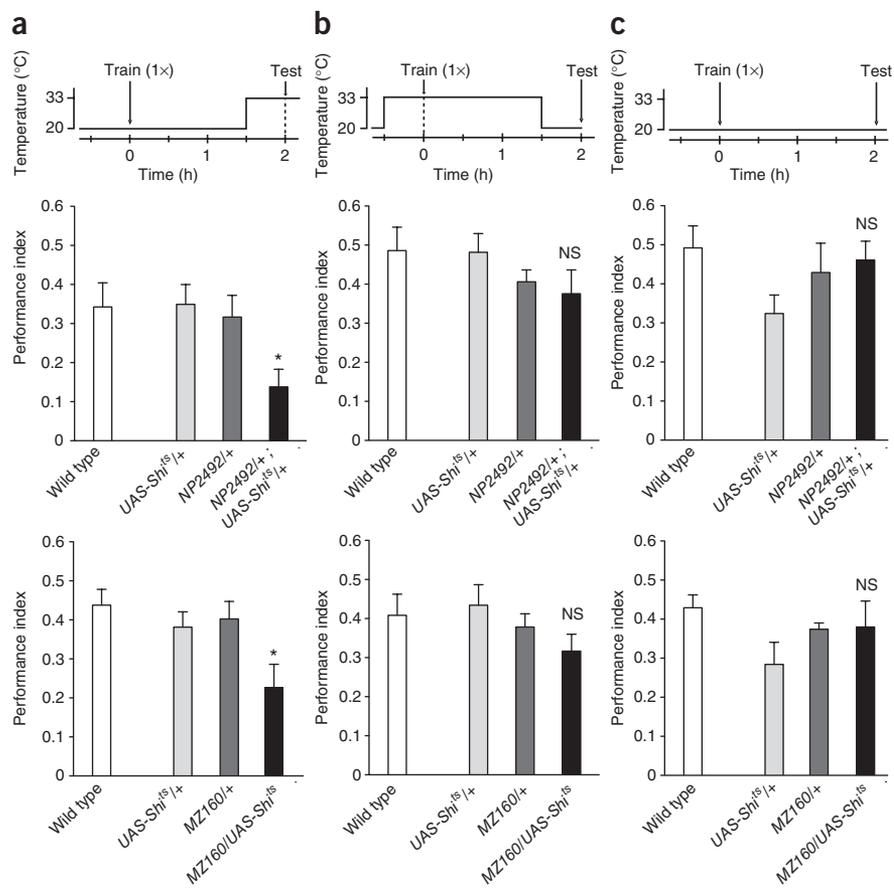
Response of naive flies to the odorants used in the conditioning experiments at the restrictive temperature (33 °C). Temperature was shifted 30 min before the measurement of odorant avoidance. Flies had 1 min to choose between the aversive odor or air bubbled through paraffin oil. For 3-octanol and 4-methylcyclohexanol, one-way ANOVA revealed no significant differences between the genotypes (wild type, *UAS-shi^{ts}*, GAL4 and *UAS-shi^{ts}/GAL4*; $P > 0.05$). $n \geq 8$ groups. Data are mean performance indices ± s.e.m.

Figure 2 Output of the MB-V2 neurons is specifically required for the retrieval of short-lasting memory. Temperature shift protocols are shown above the graphs. (a) Blocking MB-V2 neuron output during retrieval impaired 2-h memory (top graph, *NP2492*, $F_{2,39} = 4.937$, $P = 0.0123$; bottom graph, *MZ160*, $F_{2,36} = 3.935$, $P = 0.0185$; ANOVA, $n \geq 12$). *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* flies were significantly different from their respective genetic controls. (b) Blocking MB-V2 neuron output during training and consolidation did not affect 2-h memory (top graph, *NP2492*, $F_{2,32} = 1.337$, $P = 0.28$; bottom graph, *MZ160*, $F_{2,33} = 1.796$, $P = 0.18$; ANOVA, $n \geq 10$). (c) Expression of *Shi^{ts}* in MB-V2 neurons did not affect 2-h memory when flies were conditioned and tested at the permissive temperature (top, *NP2492*, $F_{2,27} = 1.508$, $P = 0.24$; bottom, *MZ160*, $F_{2,27} = 1.101$, $P = 0.35$; ANOVA, $n \geq 10$). Data are mean performance indices \pm s.e.m. NS, not significant ($P \geq 0.05$); * $P < 0.05$.

permissive temperature. Blocking the MB-V2 during training and consolidation did not impair memory performance (Fig. 2b). Moreover, there was no memory defect when flies were conditioned and tested at the permissive temperature (Fig. 2c).

Memory directly after training was also reduced when MB-V2 output was blocked continuously (Supplementary Fig. 3a). As MB-V2 output is not required for memory acquisition (Fig. 2b), this result suggests that immediate retrieval of memories also depends on MB-V2 output.

The output of α/β neurons is required for appetitive memory retrieval²³. To determine whether MB-V2 output is required for all types of memory retrieval, we performed appetitive conditioning, in



which electric shocks are replaced by sugar reward²⁴. There was a sugar response defect in *NP2492/UAS-shi^{ts}* flies at the restrictive temperature (Supplementary Fig. 3b) that may correspond to a motivation defect, and therefore we could not assess appetitive memory in these flies. There was no sugar defect in *MZ160/UAS-shi^{ts}* flies at the restrictive temperature (Supplementary Fig. 3b) and no appetitive memory defect in these flies when MB-V2 neurons were blocked during retrieval (Supplementary Fig. 3c). These results show that MB-V2 α output is not required for the retrieval of 2-h appetitive memory.

The odorants are highly repellent to naive flies at the concentration we typically use for conditioning (Table 1). To address whether the requirement of MB-V2 for memory retrieval depends on how aversive the odor is, we next used odorants that are neutral to naive flies

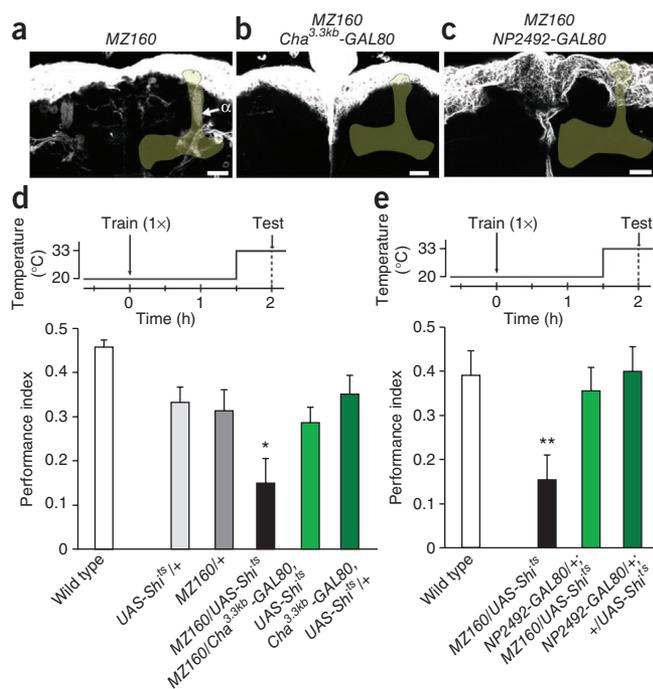
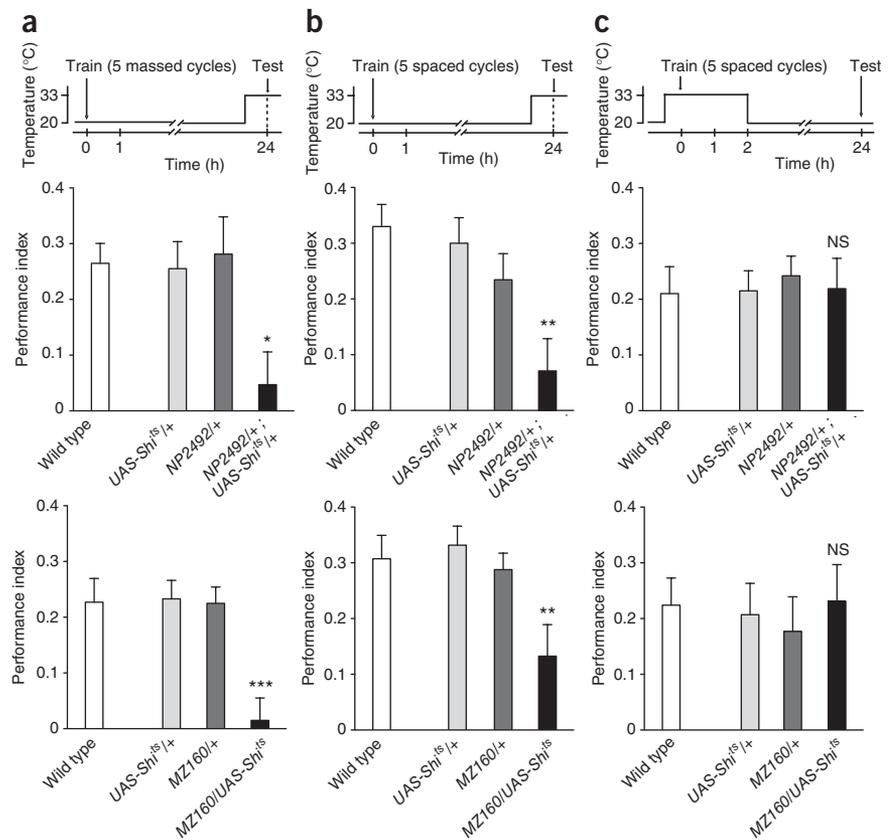


Figure 3 Suppressing MB-V2 neurons in MZ160 rescues the memory defect. (a–c) Projection of the brain region including the mushroom body lobes (light green). In *UAS-mCD8GFP;MZ160* flies (a), terminals of MB-V2 in the α lobe were visualized (arrow), whereas the signal was strongly reduced in combination with *Cha^{3.3kb}-GAL80* (b) and *NP2492-GAL80* (c). Scale bars represent 20 μ m. (d) Suppressing GAL4 expression in MB-V2 neurons with *Cha^{3.3kb}-GAL80* restored wild-type 2-h memory ($F_{4,45} = 3.305$, $P = 0.0186$). *MZ160/UAS-shi^{ts}* flies differed significantly from *UAS-shi^{ts}*, *MZ160/+* and *Cha^{3.3kb}-GAL80/+;MZ160/UAS-shi^{ts}* flies. *Cha^{3.3kb}-GAL80/+;MZ160/UAS-shi^{ts}* flies did not differ significantly from *UAS-shi^{ts}*, *MZ160/+* and *Cha^{3.3kb}-GAL80/+;UAS-shi^{ts}* flies. $n \geq 10$. (e) Suppressing GAL4 expression in MB-V2 neurons with *NP2492-GAL80* restored wild-type 2-h memory ($F_{2,36} = 5.774$, $P = 0.0067$). *MZ160/UAS-shi^{ts}* flies differed significantly from *NP2492-GAL80/+;MZ160/UAS-shi^{ts}* flies. *NP2492-GAL80/+;MZ160/UAS-shi^{ts}* flies did not differ significantly from *NP2492-GAL80/+;UAS-shi^{ts}* flies. Only females were used in this experiment. $n \geq 12$. Data are mean performance indices \pm s.e.m. ANOVA; * $P < 0.05$; ** $P < 0.01$.

Figure 4 Output of the MB-V2 neurons is required for retrieval of consolidated long-lasting memories. Temperature shift protocols are shown above each graph. **(a)** Blocking MB-V2 neuron output during retrieval impaired 24-h memory after massed conditioning (top graph, *NP2492*, $F_{2,39} = 4.822$, $P = 0.0134$; bottom graph, *MZ160*, $F_{2,27} = 12.95$, $P = 0.0001$; ANOVA). *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* were significantly different from their respective genetic controls and did not differ significantly from 0 (one-sample *t* test, $P = 0.44$ and $P = 0.72$, respectively). $n \geq 10$. **(b)** Blocking MB-V2 neuron output during retrieval impaired 24-h memory after spaced conditioning (top graph, *NP2492*, $F_{2,30} = 5.469$, $P = 0.0094$; bottom graph, *MZ160*, $F_{2,38} = 6.247$, $P = 0.0037$; ANOVA). *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* were significantly different from their respective genetic controls and *NP2492/UAS-shi^{ts}* did not differ significantly from 0 (one-sample *t* test, $P = 0.25$). $n \geq 10$. **(c)** Blocking MB-V2 neuron output during spaced training and 2 h after training did not affect 24-h memory. ANOVA revealed no significant difference among the groups with *NP2492* (top, $F_{2,27} = 0.1157$, $P = 0.89$) and *MZ160* (bottom, $F_{2,27} = 0.1936$, $P = 0.83$). $n \geq 10$. Data show mean performance indices \pm s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; NS, not significant.



for conditioning. We found that 3-octanol and 4-methylcyclohexanol were no longer aversive when diluted 300 times compared to the usual concentration (1.2×10^{-6} M and 1.08×10^{-6} M, respectively; data not shown). When we carried out conditioning and testing with the diluted odors, both *NP2492/UAS-shi^{ts}* and *MZ160/UAS-shi^{ts}* flies showed a robust memory impairment after 2 h (Supplementary Fig. 2d), suggesting that MB-V2 output is required for aversive memory retrieval irrespective of the repulsiveness of odorants.

GAL80 expression rescues the memory retrieval impairment

Neurons in the MB-V2 cluster are the only GAL4-expressing cells that are clearly shared by *NP2492* and *MZ160*, but each line shows expression in additional brain regions, such as the fan-shaped body in *NP2492* (Supplementary Fig. 1a and Supplementary Video 1) and the optic lobes in *MZ160* (Supplementary Fig. 1c,g and Supplementary Video 2). Even though the behavioral phenotype is common and very specific for the retrieval of aversive memory (Fig. 2 and Supplementary Fig. 3), we could not formally exclude the possibility that different sets of neurons outside MB-V2 happened to induce the same memory phenotype. To confirm that blocking MB-V2 neurons was the cause of the memory impairment, we aimed to use the intersectional approach and searched for GAL80 lines that could preferentially silence GAL4 activity in MB-V2. *Cha^{3.3kb}-GAL80* (ref. 25) reduced the reporter expression in MB-V2 in *NP2492* and *MZ160* (Fig. 3a,b, Supplementary Fig. 1a,c,d,f and Supplementary Video 3). To improve the specificity of the intersectional manipulation to MB-V2, we also replaced the *GAL4* gene in *NP2492-GAL4* with *GAL80* (ref. 26; see Online Methods). The resulting line, *NP2492-GAL80*, appeared to largely recapitulate the expression of *NP2492-GAL4*, as it silenced the majority of reporter expression in *NP2492-GAL4* (Supplementary Fig. 1h). In combination with *MZ160*, *NP2492-GAL80* markedly reduced the reporter expression in MB-V2 (Fig. 3c, Supplementary Fig. 1i,j and Supplementary Video 4).

Consistent with the anatomical data, the impaired memory of *MZ160/UAS-shi^{ts}* flies 2 h after conditioning was fully rescued by combination to *Cha^{3.3kb}-GAL80* (Fig. 3d) and *NP2492-GAL80* (Fig. 3e). As both *Cha^{3.3kb}-GAL80* and *NP2492-GAL80* suppress most of the GAL4-expressing cells in the *NP2492* driver (Supplementary Fig. 1c,h), we did not perform rescue experiments with these genotypes. These results suggest that the memory retrieval defect in *MZ160/UAS-shi^{ts}* flies is due to MB-V2 blockade.

MB-V2 output is required to retrieve consolidated memories

Aversive odor¹⁻³ memory in *Drosophila* consists of various phases¹⁻³. Short-term memory (STM) can be formed by a single cycle of training and is labile. Several cycles of massed or spaced training (without or with rest intervals between conditioning cycles, respectively) induce distinct forms of long-lasting memory³: anesthesia-resistant memory (ARM) and long-term memory (LTM), respectively¹⁵.

Memory 24 h after both massed and spaced conditioning was strongly impaired in both genotypes when MB-V2 output was blocked during testing (Fig. 4a,b). Consistent with the fact that MB-V2 output is not required during training or consolidation after a single conditioning cycle (Fig. 2b), blockade of MB-V2 output during spaced training and the following 2 h did not affect 24-h memory (Fig. 4c). Together, these results show that the retrieval of all phases of aversive olfactory memory (STM, LTM and ARM) requires MB-V2 output whereas training and consolidation do not.

MB-V2 neurons are cholinergic

To characterize the neurotransmitter used by MB-V2 neurons, we examined colocalization of MB-V2 neurons and markers for various transmitters. The cell bodies of MB-V2 neurons were not colocalized with *Drosophila* vesicular glutamate transporter (DVGLUT),

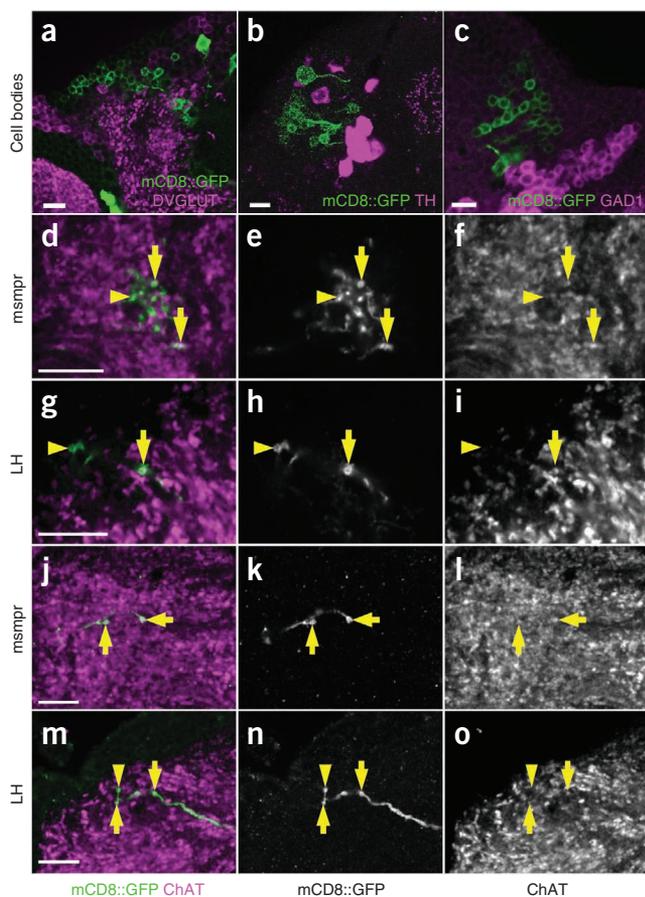


Figure 5 MB-V2 neurons are cholinergic. (a–c) Cell bodies, including those of MB-V2 neurons in *NP2492* flies, did not overlap with the markers for glutamate (DVGLUT, a), dopamine (tyrosine hydroxylase (TH), b) and GABA (GAD1, c). See **Supplementary Figure 6** for consistent results with *R71D08*. (d–o) Terminals of the MB-V2 neurons in the lateral horn and msmpr colocalized with ChAT (arrows), whereas the signal was occasionally undetectable in some terminals (arrowheads). The processes of MB-V2 were visualized with mCD8::GFP driven by *R71D08*. Terminals of a population of MB-V2 neurons (d–i) or a single MB-V2 α neuron (j–o) were labeled with mCD8::GFP. See **Supplementary Figure 5** for consistent results with *NP2492*. Scale bars represent 10 μ m.

NP2492 and *MZ160* drivers, memory was impaired 2 h after conditioning when we blocked MB-V2 output during the test using the *R71D08* driver (**Supplementary Fig. 5a**). Moreover, we found no memory defect when *R71D08/UAS-shi^{ts}* flies were conditioned and tested at the permissive temperature (**Supplementary Fig. 5b**) and the odor avoidance of *R71D08/UAS-Shi^{ts}* flies was normal at the restrictive temperature (**Supplementary Fig. 5c**).

We next examined immunoreactivity for choline acetyltransferase (ChAT) in the presynaptic terminals of MB-V2, as the antibody does not notably label cell bodies²⁸. We found that a fraction, but not all, of the terminals of MB-V2 neurons colocalized with ChAT (**Fig. 5d–i** and **Supplementary Figs. 6** and **7**), indicating that at least some of the MB-V2 neurons were cholinergic. We also confirmed the colocalization of ChAT with the terminals of single MB-V2 neurons (**Fig. 5j–o**). The presynaptic terminals of MB-V2 were not immunoreactive to DVGLUT or GAD1 (**Supplementary Figs. 8** and **9**), consistent with the observations of the cell bodies (**Fig. 5a–c** and **Supplementary Fig. 4**). The conclusion that MB-V2 neurons are cholinergic is in line with the suppression of transgene expression in MB-V2 by *Cha^{3.3kb}-GAL80* (**Fig. 3b**).

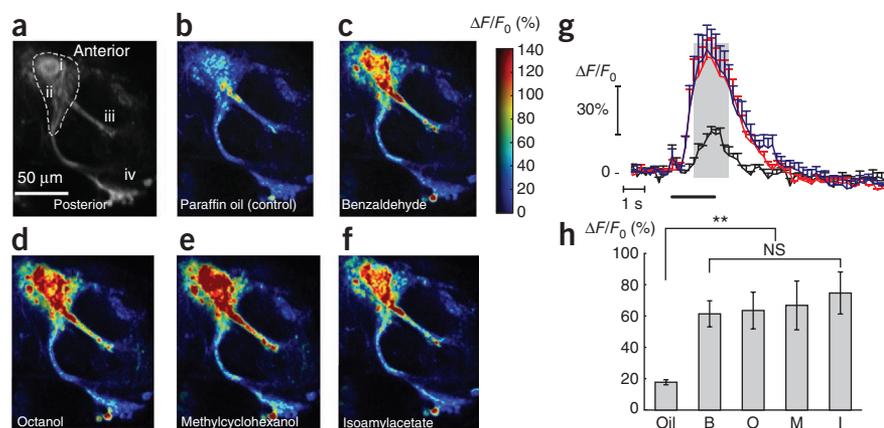
MB-V2 neurons show a reduced response to trained odorants

To understand the physiological role of the MB-V2 neurons during aversive olfactory memory retrieval, we analyzed the MB-V2 response to odors using *in vivo* calcium imaging. We expressed GCaMP 3.0, a calcium-sensitive fluorescent protein²⁹, using the *NP2492* driver and recorded the fluorescence of GCaMP3 from the MB-V2 neurons in the region of the mushroom body vertical lobes and msmpr in naive flies

tyrosine hydroxylase, glutamate decarboxylase 1 (GAD1; **Fig. 5a–c**), octopamine, tyramine or serotonin (data not shown). We confirmed the results obtained with *NP2492* with another driver line, *R71D08* (**Supplementary Fig. 4**), constructed as described²⁷ (see Online Methods). The *R71D08* driver strongly labels a smaller number of MB-V2 cluster neurons (**Supplementary Fig. 1k** and **Supplementary Video 5**). Consistent with the behavioral results obtained with the

Figure 6 MB-V2 neurons in naive flies respond to olfactory stimuli. (a) Illustrative mean grayscale image of a time series acquisition showing a horizontal section of the right hemisphere of *NP2492/+;UAS-GCaMP3/+* fly brain. MB-V2 neurons showed basal GCaMP3 fluorescence in their projections on the mushroom body vertical lobes (i), msmpr (ii), lateral horn (iii) and in cell bodies (iv). Dashed line encloses mushroom body and msmpr regions and was used to quantify odorant responses, here and in **Figure 7**.

(b–f) Color-coded variations of fluorescence in the same fly, after 2-s exposure to benzaldehyde (c), octanol (d), methylcyclohexanol (e) and isoamylacetate (f). MB-V2 neurons responded with calcium increase to all four odorants. Strong activation was observed in the mushroom body and msmpr area, as delimited in a. The calcium influx was much lower when no odorant was diluted in the paraffin oil (f). (g) Time course, averaged across all animals, of responses to octanol (red, $n = 7$), methylcyclohexanol (blue, $n = 7$) and oil (black, $n = 5$). The solid black bar indicates the delivery of the stimulus. (h) Peak responses for all odorants, calculated as the temporal mean over the time window shaded in gray (traces for benzaldehyde and isoamylacetate are not shown). There were no significant differences among the mean responses to isoamylacetate (I, $n = 3$), benzaldehyde (B, $n = 3$), octanol (O) and methylcyclohexanol (M), whereas the response to pure oil was significantly lower (one-way ANOVA, $F_{4,220} = 26.56$, $P < 0.01$). ** $P < 0.01$; NS, not significant.



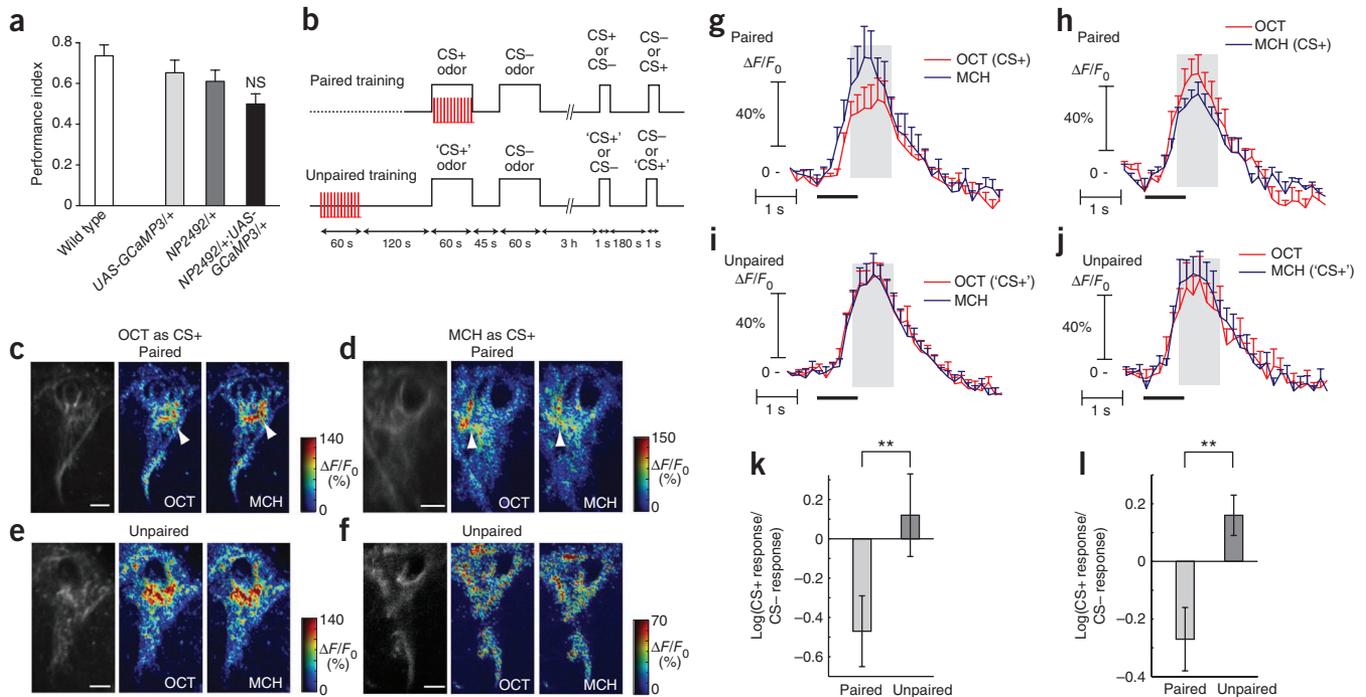


Figure 7 MB-V2 neurons show a reduced response to the trained odor after conditioning. (a) Overexpression of the GCaMP3 driven by *NP2492-GAL4* did not significantly alter 3-h memory performance ($n = 13$, one-way ANOVA, $F_{2,36} = 2$, $P = 0.15$). (b) Sketch illustrating delivery of odors (black line) and electric shocks (red spikes) during the paired and unpaired training. (c–l) Two sets of reciprocal experiments, performed on flies trained with either 3-octanol (OCT) (c, e, g, i, k) or 4-methylcyclohexanol (MCH) (d, f, h, j, l) as the CS+ odor ('CS+' in unpaired training). (c–f) Mean grayscale images (scale bars represent 15 μm) of MB-V2 projections to msmpr, and color-coded patterns of the responses obtained to both odors in representative paired (c, d) and unpaired (e, f) flies. Paired flies specifically show lower activation after CS+ than after CS– (arrowheads). Average time courses of odor responses are shown for paired (g, h) and unpaired (i, j) flies. Black bars indicate the delivery of the stimulus. In paired flies, during the time window shaded in gray, the time course of CS+ response was decreased compared to the CS– response (g, $n = 7$, $P = 0.0035$; h, $n = 6$, $P = 4.5 \times 10^{-4}$), whereas 'CS+' and CS– responses remain undistinguishable in unpaired flies (i, $n = 7$, $P = 0.27$; j, $n = 6$, $P = 0.13$). The CS+ to CS– relative response, quantified as the natural logarithm of the CS+/CS– response, was lower in paired than in unpaired flies (k, $P = 0.0024$; l, $P = 0.0043$). ** $P < 0.01$; NS, not significant.

(Fig. 6). There was a strong calcium increase in response to benzaldehyde, 3-octanol, methylcyclohexanol and isoamylacetate compared to that obtained with pure paraffin oil (Fig. 6a–f, h). Responses to all odors were similar in MB-V2 neurons (Fig. 6h) and decreased rapidly after the end of the stimulation (3-octanol, 4-methylcyclohexanol, Fig. 6g; isoamylacetate and benzaldehyde, data not shown). These results show that the MB-V2 neurons respond to odors by increasing intracellular calcium concentration.

As the MB-V2 neurons respond to odorants, we tested whether they show training-induced changes in their responses to the conditioned stimulus after aversive training (Fig. 7). *NP2492/+; UAS-GCaMP3/+* flies showed wild-type memory (Fig. 7a) suggesting that expression of the GCaMP3 reporter in the MB-V2 neurons does not affect their physiology. Three hours after the flies had been trained with 3-octanol as the conditioned stimulus (paired training; Fig. 7b), the calcium response to 3-octanol in MB-V2 neurons was decreased compared to the response to 4-methylcyclohexanol (Fig. 7c, g). This decrease is the result of the temporal pairing of the odorant and the shock stimuli, as there was no such decrease (Fig. 7e, i) when 3-octanol and electric shocks were delivered 120 s apart (unpaired training, Fig. 7b). Consistently, the response of 3-octanol to MCH in paired flies was reduced by 45% compared to unpaired flies (Fig. 7k). We obtained similar results when we used 4-methylcyclohexanol as the conditioned odorant (Fig. 7d, f, h, j, l). Thus, the MB-V2 neurons transmit a memory trace in the mushroom body by decreasing their calcium response to the trained odorant.

DISCUSSION

Here, we have described the function of a class of mushroom body efferent neurons in *Drosophila*, MB-V2. We found two distinct populations of MB-V2 neurons, MB-V2 α and MB-V2 α' , which innervate the shaft of the mushroom body α lobe and the tip of the mushroom body α' lobe, respectively. The mushroom body α lobe has been implicated as a site of olfactory learning that uses an associative synergy of cholinergic and dopaminergic inputs to activate cAMP-PKA signaling^{11,12}. In addition to the mushroom body, both MB-V2 α and MB-V2 α' form arbors in the neuropil just posterior to the mushroom body vertical lobes and in the lateral horn, the other major secondary olfactory center. By transiently blocking synaptic transmission, we found that MB-V2 output is required specifically to retrieve aversive olfactory memory.

Although the molecular pathways that underlie STM, LTM and ARM differ^{1,2,15}, our results suggest that a common neuronal network is used to translate the memory trace in the Kenyon cells that project to the mushroom body vertical lobes into avoidance behavior. Other mushroom body output neurons may also be involved in the retrieval of short-lasting memories, as blockade of MB-V2 output, while fully abolishing consolidated memories, only partially impairs short-lasting memories. MB-V2 neurons are not required for the retrieval of sugar-associated memory and thus other neurons must provide the output for appetitive memory. Finally, we found no significant behavioral differences with the aversive protocol when both V2 α and α' (*NP2492*) or only V2 α (*MZ160*) were blocked, and the role of MB-V2 α' neurons remains to be elucidated.

Functional optical imaging revealed that MB-V2 neurons responded to odorants and decreased their responses to the trained odorant. Thus, aversive olfactory conditioning represses the activity of MB-V2 cholinergic neurons. How can an increased calcium response in the mushroom body lobes to the learned odor (CS⁺)^{13,14} induce a decreased calcium response in the MB-V2 neurons? Inhibitory neurons that project onto MB-V2 may be recruited during conditioning. Notably, the PE1 neuron in *Apis mellifera* relays information from the mushroom body to the lateral horn and shows a reduced response to CS⁺ after appetitive training³⁰. It has been proposed that this reduced response could be due to inhibitory GABAergic inputs onto PE1³¹. The conservation between bees and flies suggests that this circuit is particularly important in both insects. Alternatively, the decreased calcium response of MB-V2 to the CS⁺ may correspond to synaptic depression. As shown in mammals, long-term potentiation or depression can recruit differential molecular mechanisms, such as NMDA receptor activation^{32,33} and retrograde signaling by endocannabinoids^{32–34}. Such depression can occur in the mushroom body-efferent synapses during memory acquisition³⁵, although its underlying mechanisms have yet to be identified in *Drosophila*. In *Drosophila*, the GABAergic APL neuron that projects onto the mushroom body shows a reduced response to the trained odor³⁶. Therefore, the mechanism of plasticity at the mushroom body synapse could be shared between APL and MB-V2 neurons.

How is the reduced calcium response to the conditioned odorant in MB-V2 neurons translated into avoidance behavior? We found that MB-V2 neurons project to the lateral horn, which receives direct olfactory inputs and is involved in the innate avoidance of repellent odorants^{8,9}. Thus, in *Drosophila*, two olfactory pathways converge on the lateral horn: one innate odorant avoidance pathway that goes directly from antennal lobes to the lateral horn, and an associative learning-dependant one that requires integration of the negative value of shock by the mushroom bodies. The presynaptic cholinergic terminals of MB-V2 neurons were close to GABAergic neurons in the lateral horn (Supplementary Fig. 7). Thus, reduced calcium responses in MB-V2 neurons upon exposure to the conditioned odorant could lead to enhanced avoidance of the aversive odor by reducing GABAergic inhibitory input onto projection or lateral horn neurons. Alternatively, in mammals, activation of muscarinic receptors by acetylcholine can directly induce LTD³⁷. Thus, the reduced calcium response to CS⁺ in MB-V2 neurons could directly result in lower inhibition of olfactory signaling mediated by the lateral horn.

MB-V2 neurons also have presynaptic terminals in the msmpr area. As there are interneurons that connect the dorsal lateral horn and msmpr³⁸, the learned odor may induce avoidance behavior at the msmpr upon the stimulation of the lateral horn interneurons by MB-V2 neurons. The functional relationship between the lateral horn and the msmpr, both targets of the MB-V2 neurons, remains to be resolved.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Y.A. and H.T. designed and I.S., S.R.T. and V.T. carried out anatomical experiments. I.S., Y.A. and H.T. analyzed the microscopic data and assembled figures. K.I. and G.M.R. provided Gal4 lines with their expression patterns. J.S., G.I. and T.P. designed and J.S., S.T. and P.-Y.P. carried out behavior experiments. J.S. generated the NP2492-Gal80 mutant. P.-Y.P., T.P. and P.T. designed the *in vivo* imaging experiments and P.-Y.P. carried them out. J.S., P.-Y.P., I.S., Y.A., H.T. and T.P. wrote the paper. T.P. and H.T. supervised the work.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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- Keene, A.C. & Waddell, S. *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat. Rev. Neurosci.* **8**, 341–354 (2007).
- McGuire, S.E., Deshazer, M. & Davis, R.L. Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. *Prog. Neurobiol.* **76**, 328–347 (2005).
- Tully, T., Preat, T., Boynton, S.C. & Del Vecchio, M. Genetic dissection of consolidated memory in *Drosophila*. *Cell* **79**, 35–47 (1994).
- Masse, N.Y., Turner, G.C. & Jefferis, G.S. Olfactory information processing in *Drosophila*. *Curr. Biol.* **19**, R700–R713 (2009).
- Aso, Y. *et al.* Specific dopaminergic neurons for the formation of labile aversive memory. *Curr. Biol.* **20**, 1445–1451 (2010).
- Claridge-Chang, A. *et al.* Writing memories with light-addressable reinforcement circuitry. *Cell* **139**, 405–415 (2009).
- Schwaerzel, M. *et al.* Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* **23**, 10495–10502 (2003).
- de Belle, J.S. & Heisenberg, M. Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* **263**, 692–695 (1994).
- Wang, Y. *et al.* Blockade of neurotransmission in *Drosophila* mushroom bodies impairs odor attraction, but not repulsion. *Curr. Biol.* **13**, 1900–1904 (2003).
- Crittenden, J.R., Skoulakis, E.M., Han, K.A., Kalderon, D. & Davis, R.L. Tripartite mushroom body architecture revealed by antigenic markers. *Learn. Mem.* **5**, 38–51 (1998).
- Gervasi, N., Tchenio, P. & Preat, T. PKA dynamics in *Drosophila* olfactory learning and memory center: coincidence detection by Rutabaga adenylyl cyclase and regulation by Dunce phosphodiesterase. *Neuron* **65**, 516–529 (2010).
- Tomchik, S.M. & Davis, R.L. Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. *Neuron* **64**, 510–521 (2009).
- Wang, Y., Mamiya, A., Chiang, A.S. & Zhong, Y. Imaging of an early memory trace in the *Drosophila* mushroom body. *J. Neurosci.* **28**, 4368–4376 (2008).
- Yu, D., Akalal, D.B. & Davis, R.L. *Drosophila* alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron* **52**, 845–855 (2006).
- Isabel, G., Pascual, A. & Preat, T. Exclusive consolidated memory phases in *Drosophila*. *Science* **304**, 1024–1027 (2004).
- McGuire, S.E., Le, P.T. & Davis, R.L. The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* **293**, 1330–1333 (2001).
- Ito, K. *et al.* The organization of intrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn. Mem.* **5**, 52–77 (1998).
- Tanaka, N.K., Tanimoto, H. & Ito, K. Neuronal assemblies of the *Drosophila* mushroom body. *J. Comp. Neurol.* **508**, 711–755 (2008).
- Kitamoto, T. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81–92 (2001).
- Zhang, Y.Q., Rodesch, C.K. & Broadie, K. Living synaptic vesicle marker: synaptotagmin-GFP. *Genesis* **34**, 142–145 (2002).
- Robinson, I.M., Ranjan, R. & Schwarz, T.L. Synaptotagmins I and IV promote transmitter release independently of Ca²⁺ binding in the C(2)A domain. *Nature* **418**, 336–340 (2002).
- Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451–461 (1999).

23. Krashes, M.J. & Waddell, S. Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in *Drosophila*. *J. Neurosci.* **28**, 3103–3113 (2008).
24. Colomb, J., Kaiser, L., Chabaud, M.A. & Preat, T. Parametric and genetic analysis of *Drosophila* appetitive long-term memory and sugar motivation. *Genes Brain Behav.* **8**, 407–415 (2009).
25. Kitamoto, T. Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **99**, 13232–13237 (2002).
26. Sepp, K.J. & Auld, V.J. Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in *Drosophila melanogaster*. *Genetics* **151**, 1093–1101 (1999).
27. Pfeiffer, B.D. *et al.* Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 9715–9720 (2008).
28. Yasuyama, K., Meinertzhagen, I.A. & Schurmann, F.W. Synaptic organization of the mushroom body calyx in *Drosophila melanogaster*. *J. Comp. Neurol.* **445**, 211–226 (2002).
29. Tian, L. *et al.* Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* **6**, 875–881 (2009).
30. Mauelshagen, J. Neural correlates of olfactory learning paradigms in an identified neuron in the honeybee brain. *J. Neurophysiol.* **69**, 609–625 (1993).
31. Okada, R., Rybak, J., Manz, G. & Menzel, R. Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain. *J. Neurosci.* **27**, 11736–11747 (2007).
32. Caporale, N. & Dan, Y. Spike timing-dependent plasticity: a Hebbian learning rule. *Annu. Rev. Neurosci.* **31**, 25–46 (2008).
33. Bender, V.A., Bender, K.J., Brasier, D.J. & Feldman, D.E. Two coincidence detectors for spike timing-dependent plasticity in somatosensory cortex. *J. Neurosci.* **26**, 4166–4177 (2006).
34. Hashimoto, Y. *et al.* Phospholipase C β serves as a coincidence detector through its Ca²⁺ dependency for triggering retrograde endocannabinoid signal. *Neuron* **45**, 257–268 (2005).
35. Menzel, R. & Manz, G. Neural plasticity of mushroom body-extrinsic neurons in the honeybee brain. *J. Exp. Biol.* **208**, 4317–4332 (2005).
36. Liu, X. & Davis, R.L. The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. *Nat. Neurosci.* **12**, 53–59 (2009).
37. Bashir, Z.I. On long-term depression induced by activation of G protein-coupled receptors. *Neurosci. Res.* **45**, 363–367 (2003).
38. Tanaka, N.K., Awasaki, T., Shimada, T. & Ito, K. Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr. Biol.* **14**, 449–457 (2004).

ONLINE METHODS

Stocks. Flies were raised at 18 °C, 60% humidity in a 12:12 h light:dark cycle. All strains were outcrossed to flies with a wild-type strain Canton-Special (Canton-S). The enhancer trap lines *NP2492* [X] and *MZ160* [III] were identified through a large-scale screen for mushroom body extrinsic neurons^{17,18}. *UAS-shi^{ts1}* [III] was used for behavioral experiments¹⁹. The transformation vector for *R71D08* was generated using the 3.2-kb DNA fragment of the *division abnormally delayed* locus, which was amplified with PCR and cloned into the transformation vector pBPGUw (ref. 27) (primer sequences: 5'-GAAGTGCATGGCAAGGAAGCAG AG-3' and 5'-GCCAACCCTTATTCTCGCCGTGT-3'). For experiments with *NP2492*, which is inserted on the X chromosome, only females of *NP2492/+;UAS-shi^{ts1}/+* as well as the respective control genotypes were taken into account to calculate the memory index. The *NP2492-GAL80* line was obtained by crossing *y¹, w¹¹¹⁸* females to *y¹, w¹¹¹⁸, NP2492-GAL4 [w⁺]; ΔHop,CyO; GAL80[y⁺], Sb* males. Positive events were detected by screening for *y¹, w¹¹¹⁸, GAL80[y⁺]* females in the next generation and verified on confocal microscopy by crossing to *NP2492-GAL4; UAS-mCD8::GFP*.

Olfactory conditioning. For aversive training, flies were conditioned by an odor paired with electric shocks and subsequent exposure to a second odor in the absence of shock, as described³⁹. Conditioning was performed on samples of 25–35 flies aged 2–3 d with 3-octanol (>95% purity; Fluka 74878, Sigma-Aldrich) and 4-methylcyclohexanol (99% purity; Fluka 66360, Sigma-Aldrich) at 0.360 mM and 0.325 mM, respectively. Odors were diluted in paraffin oil (VWR international, Sigma-Aldrich). Memory tests were performed with a T-maze apparatus⁴⁰. Flies could choose for 1 min between 2 arms, each delivering a distinct odor. An index was calculated as the difference between the numbers of flies in each arm divided by the sum of flies in both arms. A performance index (PI) results from the average of two reciprocal experiments. For appetitive training, electric shocks were replaced by 1-min sugar exposure, as described²⁴.

Statistical analysis. We performed behavioral statistical analysis using GraphPad Prism 5.0 (GraphPad Software, Inc.). One-way ANOVA was performed on each set of data followed by pairwise planned comparisons between relevant groups with a Student-Newman-Keuls test, except when specified. Wild-type flies were not taken into account for statistical analysis. For data that violated the assumption of normal distribution (Supplementary Fig. 2j), non-parametric statistics were applied (Kruskal-Wallis test followed by Dunn's multiple comparisons). Asterisks denote significant differences with the *post hoc* pair-wise comparisons.

Immunohistochemistry. We examined female F1 progenies (5–8 d after eclosion at 25 °C) between males of corresponding Gal4 lines and females carrying a single copy of *UAS-mCD8::GFP* [II] (ref. 22), *-sy1::GFP* [II] (ref. 20), *-sy1::HA* [X], *-nSyb::GFP* [III], *-myr::RFP* [III] or their combination. For single-cell clonal analyses, *y w hsp70-flp; UAS>CD2^y>mCD8::GFP/CyO; TM2/TM6b* (ref. 41) females were crossed to *NP2492, MZ160* or *R71D08* males. One day after eclosion, adult flies received a heat shock at 37 °C for 30–45 min to remove the Flp-out cassette (*CD2^y*), and were examined 5–7 d after heat shock. We analyzed 131 single cell clones (87 for MB-V2 α and 44 for MB-V2 α') with the three drivers. For single-cell analyses driving the membrane and presynaptic markers (*mCD8::GFP* and *Syt::HA*), *UAS-mCD8::GFP UAS-Syt::HA hsp70-flp* females were crossed to *NP2492; Tub>GAL80>* (II) males (ref. 42). Late 3rd-instar larvae or early pupae of the progeny were heat-shocked at 34 °C for 20 min to remove the Flp-out cassette, and dissected 10–12 d after eclosion.

Brains were prepared by a standard immunolabeling procedure^{18,43} using antibodies against *Drosophila* GAD1 (refs. 44,45; 1:200), DVGLUT (ref. 46; 1:10,000), ChAT (ref. 47; 1:100), tyrosine hydroxylase⁴⁸ (1:1,000), synapsin⁴⁹ (1:100), GFP (rabbit polyclonal, 1:1,000, Invitrogen; or rat monoclonal, 1:200, Chromotek), hemagglutinin (1:200, Covance) and DsRed (1:100, Clontech). Coronal optical sections of whole-mount brains taken by confocal microscopy (Olympus FV1000) were analyzed with ImageJ (US National Institutes of Health). In most figures, stained fibers and counterstaining are presented in white (RGB ratio; 255:255:255) and orange (255:85:0), respectively. Nomenclature of brain regions follows reference 43.

In vivo calcium imaging of odor responses. We targeted genetically the expression of the GCaMP3.0 calcium reporter to the MB-V2 neurons by combining a

UAS-GCaMP3 [II] construct²⁹ with the *NP2492-GAL4* driver. Measurements on naive animals were performed on 1–2-d-old flies homozygous for both *NP2492* and *UAS-GCaMP3* transgenes. Female flies were caught without anaesthesia, then glued and operated as described for *in vivo* imaging⁵⁰. The proboscis was also glued to the thorax to limit motion artifacts during image acquisition. The recording chamber was then placed beneath the water immersion 20 \times objective (NA = 1; Leica) of a TCS-SP5 laser-scanning confocal microscope (Leica), the brain of the fly being thus observed from the top. Experiments were performed at 20 °C, and the aperture on the top of the fly head was bathed in a continuously flowing perfusion of *Drosophila* Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 36 mM sucrose, 5 mM HEPES-NaOH; pH 7.3; 305 mOsm) oxygenated by air bubbling.

For experiments on trained or unpaired flies, *NP2492/+;UAS-GCaMP3/+* female flies with more moderate levels of reporter expression were used, to minimize physiological side effects of GCaMP3 expression. Consequently, GCaMP3-labeled areas and odor-evoked activation patterns may slightly differ from experiments on naive flies. One fly was captured 2.5 h after training and prepared with the same procedure, so that imaging actually took place approximately 3 h after the end of the training protocol.

Each odorant was diluted 250-fold (vol/vol) in 100 ml paraffin oil in a glass bottle. Control bottles contained pure oil. A constant air stream, bubbling through pure oil, was directed at the fly throughout the recording. Following a trigger, a set of solenoid-driven valves redirected 20% of the air stream to the appropriate odor or control bottle for the aimed duration. The odor stream (200 ml min⁻¹) rejoined the primary stream (800 ml min⁻¹) 23 cm from the end of the delivery tube, which was 4 mm in diameter. The end of the delivery tube was positioned ~7–8 mm from the fly's antennae. All odorant concentrations were therefore ~2,000-fold diluted (5.3×10^{-4} M), not including the additional dilution factor between the end of the tube and the antennae.

The GCaMP3 probe was excited by the 488 nm line of an argon laser, scanning at a line rate of 400 Hz. Fluorescence-induced light emission was collected by a photomultiplier in the 505–555 nm wavelength range. The pinhole was wide open and the collected light originated from thick horizontal sections of either of the two hemispheres of the brain.

Naive flies were exposed to each olfactory stimulus for 2 s, and odorant presentations were spaced 3 min apart, in a random order. On each trained fly, a pair of responses to 1 s of CS+ and CS- odorants was recorded twice, to ensure that the fly's olfactory ability was preserved throughout the experiment, but only the first presentation of each odor was kept for data analysis, to avoid desensitization effects. Within each pair, the odorants were pseudo-randomly ordered. Overall, 12 flies received CS+ first, and 14 flies received CS- first. During an experimental session, special care was taken to sequentially image, with minimal delay (55 min on average), on the same hemisphere and with the same odor delivered first, one paired and one unpaired fly grown in the same culture bottle, so that we could treat paired and unpaired flies as paired data for statistical analysis. For this experiment, we focused on the *msmpr* as the GCaMP signal was stronger in this region. All measurements were performed ~3 h after training.

Image analysis was performed offline with Matlab software using a custom-written program. To obtain the time course of the relative variation of fluorescence, the light intensity was averaged over a region of interest delimited by hand and surrounding the projections of the MB-V2 neurons in the area of the *msmpr* and mushroom bodies. The resulting time trace was normalized to a percent change in fluorescence $100(F-F_0)/F_0$, using a baseline value of the fluorescence F_0 that was estimated as the temporal mean over the 1-s period before the valve was switched. The peak response was calculated as the mean of this trace over a time window shown in gray in Figures 6g and 7e,f. The same procedure was applied on a pixel-by-pixel basis, yielding a color-coded image of the spatial pattern of the peak response. The brightness of each pixel was weighted by its mean fluorescence intensity over the duration of the whole acquisition, resulting in a shaded color-coded image that highlights responses in regions that were labeled by the calcium reporter.

In the course of experiments on trained flies, comparison of responses to a given odorant was clouded by significant variability among different animals. To overcome this, a normalized value was calculated for each fly as the ratio of CS+ over CS- peak responses. To accurately estimate the ratio of the mean responses, we averaged the natural logarithm of this value over all flies that had

been submitted to the same training¹³. For statistical comparisons of two series of data, we used two-tailed *t*-tests except when Lilliefors test indicated significant ($P < 0.05$) shift from normal distribution for any of the two series, in which case we used a Wilcoxon rank sum test (Fig. 7j).

39. Pascual, A. & Preat, T. Localization of long-term memory within the *Drosophila* mushroom body. *Science* **294**, 1115–1117 (2001).
40. Tully, T. & Quinn, W.G. Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J. Comp. Physiol. [A]* **157**, 263–277 (1985).
41. Wong, A.M., Wang, J.W. & Axel, R. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* **109**, 229–241 (2002).
42. Gordon, M.D. & Scott, K. Motor control in a *Drosophila* taste circuit. *Neuron* **61**, 373–384 (2009).
43. Otsuna, H. & Ito, K. Systematic analysis of the visual projection neurons of *Drosophila melanogaster*. I. Lobula-specific pathways. *J. Comp. Neurol.* **497**, 928–958 (2006).
44. Featherstone, D.E. *et al.* Presynaptic glutamic acid decarboxylase is required for induction of the postsynaptic receptor field at a glutamatergic synapse. *Neuron* **27**, 71–84 (2000).
45. Sakai, T., Kasuya, J., Kitamoto, T. & Aigaki, T. The *Drosophila* TRPA channel, Painless, regulates sexual receptivity in virgin females. *Genes Brain Behav.* **8**, 546–557 (2009).
46. Daniels, R.W., Gelfand, M.V., Collins, C.A. & DiAntonio, A. Visualizing glutamatergic cell bodies and synapses in *Drosophila* larval and adult CNS. *J. Comp. Neurol.* **508**, 131–152 (2008).
47. Takagawa, K. & Salvaterra, P. Analysis of choline acetyltransferase protein in temperature sensitive mutant flies using newly generated monoclonal antibody. *Neurosci. Res.* **24**, 237–243 (1996).
48. Neckameyer, W.S., Woodrome, S., Holt, B. & Mayer, A. Dopamine and senescence in *Drosophila melanogaster*. *Neurobiol. Aging* **21**, 145–152 (2000).
49. Klagges, B.R. *et al.* Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* **16**, 3154–3165 (1996).
50. Fiala, A. & Spall, T. *In vivo* calcium imaging of brain activity in *Drosophila* by transgenic cameleon expression. *Sci. STKE* **2003**, PL6 (2003).