

Two Pairs of Mushroom Body Efferent Neurons Are Required for Appetitive Long-Term Memory Retrieval in *Drosophila*

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SUMMARY

One of the challenges facing memory research is to combine network- and cellular-level descriptions of memory encoding. In this context, *Drosophila* offers the opportunity to decipher, down to single-cell resolution, memory-relevant circuits in connection with the mushroom bodies (MBs), prominent structures for olfactory learning and memory. Although the MB-afferent circuits involved in appetitive learning were recently described, the circuits underlying appetitive memory retrieval remain unknown. We identified two pairs of cholinergic neurons efferent from the MB α vertical lobes, named MB-V3, that are necessary for the retrieval of appetitive long-term memory (LTM). Furthermore, LTM retrieval was correlated to an enhanced response to the rewarded odor in these neurons. Strikingly, though, silencing the MB-V3 neurons did not affect short-term memory (STM) retrieval. This finding supports a scheme of parallel appetitive STM and LTM processing.

INTRODUCTION

Research across animal species from *Aplysia* to mammals has identified general features in memory formation, particularly at the genetic and biochemical levels (Bailey et al., 1996; Dubnau and Tully, 1998; Mayford and Kandel, 1999; Pittenger and Kandel, 2003). Biochemical processes that are modified or triggered at memory formation often result in synaptic plasticity, i.e., a modification in the strength of targeted synapses, which is thought to drive learned behavior when memory is expressed (Martin et al., 2000; Benito and Barco, 2010). Localizing the relevant synapses and describing the type of plasticity they undergo are therefore major challenges in memory research (Neves et al.,

2008). Progress hinges on precise knowledge of the neuronal networks involved at the different stages of memory processing, which obviously gets increasingly challenging with increasingly complex brains. *Drosophila* is an ideal model for studying memory circuits because the *Drosophila* brain is only modestly complex yet sophisticated enough to feature elaborate memory processes, and the model is well geared to powerful molecular genetics techniques enabling highly specific and reproducible targeting of identified neurons.

A fly can form robust aversive associative olfactory memory after pairing an odor with electric shocks (Tully and Quinn, 1985). Appetitive memory forms in a starved fly after pairing an odor with sugar delivery (Tempel et al., 1983). Both types of olfactory memory rely on the mushroom bodies (MBs) (de Belle and Heisenberg, 1994; Heisenberg, 2003; Krashes et al., 2007), a paired lobed structure of $\sim 2,000$ neurons—the Kenyon cells (KCs)—per brain hemisphere (Aso et al., 2009). KCs receive dendritic input from the antennal lobes through projection neurons (Masse et al., 2009) in the calyx area on the posterior part of the brain and send their axons anteriorly through the peduncle. Based on their axonal morphology, KCs are classed into three different subtypes: axons from α/β and α'/β' KCs branch into vertical (α and α') and medial (β and β') lobes, whereas axons from γ neurons form only a medial γ lobe (Crittenden et al., 1998). KCs are located at the junction between the circuit conveying olfactory information on MB calyces and the different subsets of dopaminergic neurons that mediate the signaling of aversive unconditioned stimuli (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Aso et al., 2010, 2012) or appetitive unconditioned stimuli (Liu et al., 2012; Burke et al., 2012) on distinct regions of MB lobes.

Looking at aversive memory, several temporally and biochemically distinct memory phases have been described depending on the conditioning protocol (Tully et al., 1994; Isabel et al., 2004; Plaçais et al., 2012). A single cycle of associative conditioning yields labile short-term memory (STM) and more persistent anesthesia-resistant memory (ARM). Long-term memory (LTM), the most persistent form of memory, requires

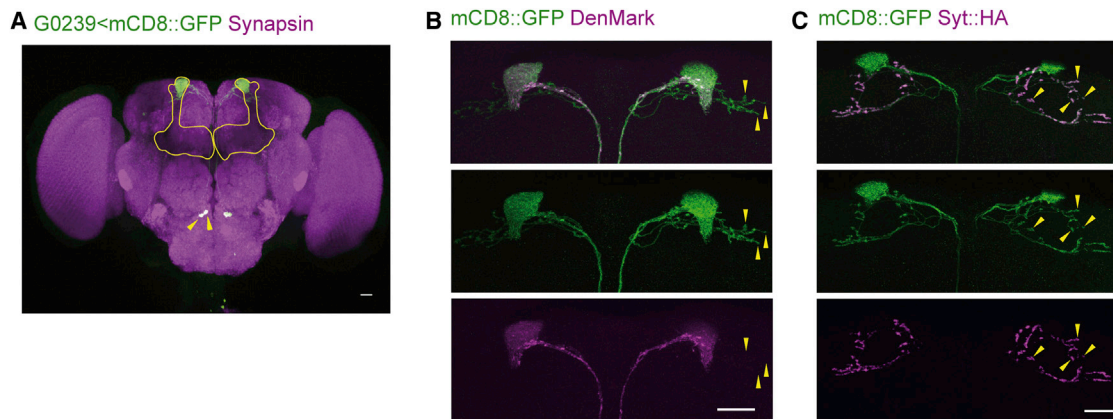


Figure 1. MB-V3 Neurons Are Efferent to the MBs

(A) Expression pattern of the *G0239* GAL4 driver line in the adult brain visualized with mCD8::GFP (green) and neuropil staining using anti-Synapsin antibody (magenta) is shown. Two neurons are labeled per brain hemisphere (cell bodies are indicated by arrowheads). MBs are outlined in yellow. (B) Arbors of the MB-V3 neurons in the MB α lobe are strongly labeled with a dendritic marker DenMark (magenta), whereas the terminals outside the MB (arrowheads) are devoid of DenMark. DenMark and mCD8::GFP were expressed through *G0239*. (C) A presynaptic marker Syt::HA (magenta) is highly enriched in the projections of MB-V3 neurons in the *asmpr*, *msmpr*, and *psmpr* (arrowheads), but not in the MB α lobe. Syt::HA and mCD8::GFP were expressed through *G0239*. Scale bars, 20 μ m.

multiple cycles of conditioning spaced by rest intervals, whereas multiple consecutive cycles induce ARM, but not LTM. MB-V2 neurons, efferent neurons from the MB vertical lobes, are required for the retrieval of aversive memory (Séjourné et al., 2011). Synapses between KCs and MB-V2 neurons are thus a potential site of aversive memory-encoding plasticity. However, alternative or additional sites of plasticity upstream of synapses between KCs and MB-V2 neurons are likely to occur because calcium-imaging studies reported changes in olfactory responses to conditioned odor following aversive conditioning in different KC subsets (Yu et al., 2006; Wang et al., 2008).

Looking at appetitive memory, both STM and LTM are formed in starved flies after a single pairing of odor perception with sugar delivery (Krashes and Waddell, 2008; Colomb et al., 2009). Appetitive STM and LTM are processed independently in γ and α/β KCs, respectively (Trannoy et al., 2011). The plasticity that sustains appetitive memory may occur between KCs and MB output neurons, but no efferent neurons involved in the retrieval of appetitive memory have yet been characterized in *Drosophila*. Identifying the output neurons for appetitive memory is therefore an essential first step in attempts to study the synaptic plasticity underlying appetitive memory. Because appetitive STM and LTM are formed in different subsets of KCs (Trannoy et al., 2011), we hypothesized that they could be retrieved through distinct neuronal routes. We therefore looked for efferent neurons from α/β KCs, the site of appetitive LTM, that would be required specifically for LTM retrieval. We identified two pairs of neurons, named MB-V3 neurons (Tanaka et al., 2008), that are efferent from the tip of MB α lobes. Blocking synaptic transmission from these cholinergic neurons, or disrupting their neurotransmitter synthesis, abolished appetitive LTM retrieval specifically, but it had no effect on appetitive STM. By contrast, blocking these neurons failed to disrupt aversive STM or LTM, but it disrupted the recently described aversive “fasting LTM” (fLTM) that forms

when starved flies are put back on food immediately after training and that shares molecular features with appetitive LTM (Hirano et al., 2013). In addition, the response to the conditioned odor was enhanced in MB-V3 neurons in the day range after appetitive training, but not in the hour range. This memory trace was abolished under conditions where appetitive LTM is disturbed.

RESULTS

MB-V3 Neurons Are Efferent from the MB α Lobes

The *G0239* GAL4 line (Chiang et al., 2011) is a very specific GAL4 driver whose expression pattern in the adult brain corresponds to only two symmetrical pairs of neurons (Figure 1A; also see Chiang et al., 2011) with a morphology corresponding to the already-described MB-V3 neurons (Tanaka et al., 2008). In each hemisphere, two MB-V3 cell bodies are located over the subesophageal ganglion (SOG). Each neuron sends a fiber vertically to the dorsal part of the brain, where it branches into dense arborization in the tip of MB α lobes, and sparse terminals spread in anterior-, middle-, and posterior-superior-medial protocerebra (*asmpr*, *msmpr*, and *psmpr*, respectively; Tanaka et al., 2008). The expression pattern of the *G0239* GAL4 line in the CNS is thus very specific to MB-V3 neurons (see Experimental Procedures).

A dendrite marker, DenMark (Nicolai et al., 2010), expressed through *G0239*, labeled the processes of MB-V3 neurons in the MB α lobes (Figure 1B). Conversely, the expression of HA-tagged synaptotagmin, a specific presynaptic marker (Robinson et al., 2002), labeled the terminals in *asmpr*, *msmpr*, and *psmpr*, whereas no signal was detectable in the MB lobes (Figure 1C). These data are consistent with an independent report by Pai et al. (2013) that MB-V3 neurons are efferent from the MB α/β KCs and that they convey information from the tip of MB α lobes to *smpr* regions.

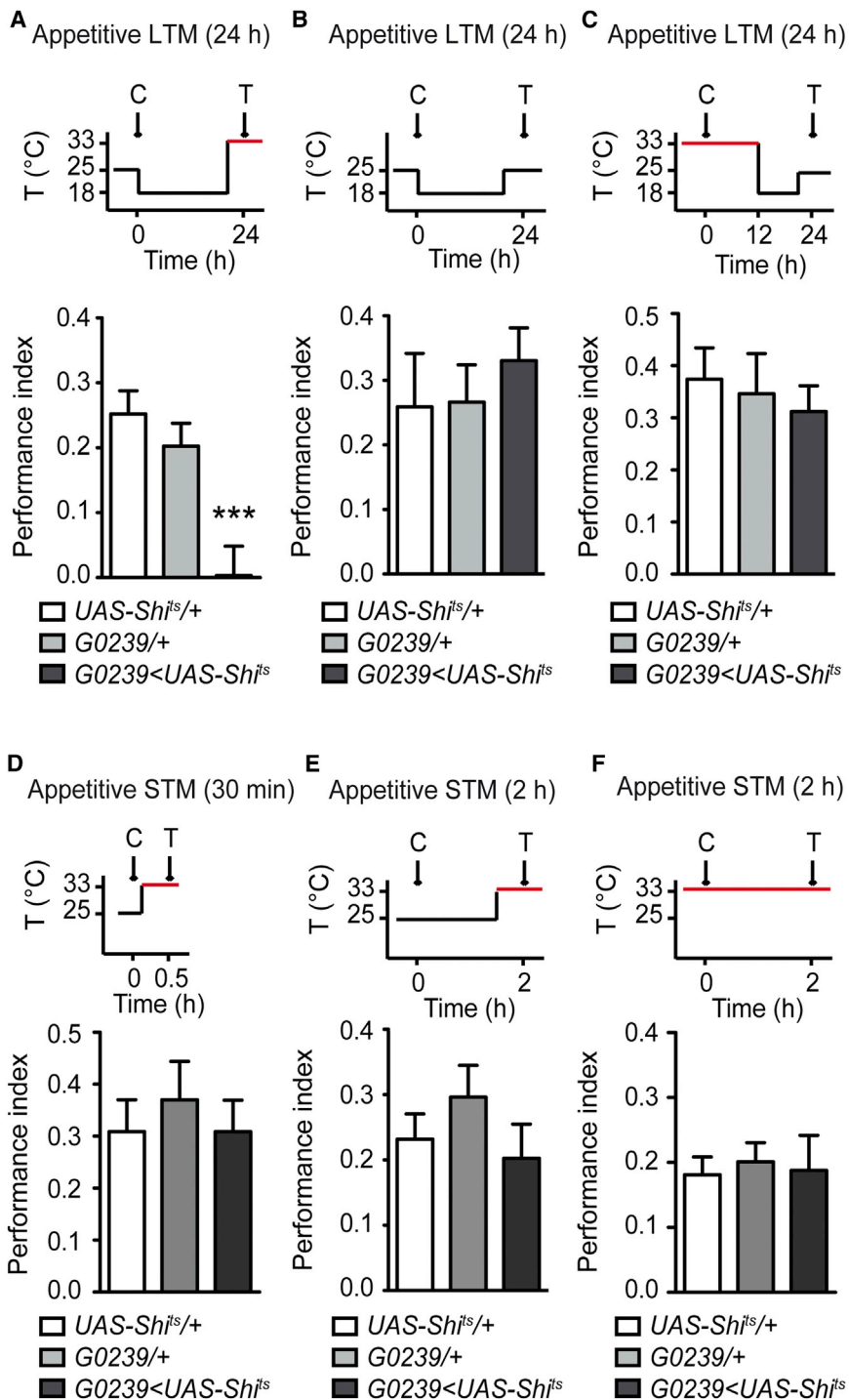


Figure 2. Output from MB-V3 Neurons Is Specifically Required for the Retrieval of Appetitive LTM

For each experiment, the time course of temperature shifts is displayed above the performance index histogram (C, one cycle of appetitive conditioning; T, memory test). Periods when MB-V3 neurons were blocked in *G0239 < UAS-Shi^{ts}* flies are shown in red.

(A) Blocking MB-V3 neurons during LTM retrieval fully abolished memory performance ($F_{(2,44)} = 11.30$, $p = 0.0001$; $n = 14-16$).

(B) *G0239 < UAS-Shi^{ts}* flies showed normal memory performance when tested at permissive temperature (25°C) 24 hr after training ($F_{(2,29)} = 0.37$, $p = 0.70$; $n = 10$).

(C) Blocking MB-V3 neurons during training and consolidation, but not retrieval, had no effect on LTM performance ($F_{(2,27)} = 0.23$, $p = 0.80$; $n \geq 8$). Neurons were blocked for the first 12 hr of the consolidation period because prolonging the blockade at 33°C for 24 hr had deleterious effects on the flies' fitness, and LTM is already formed 12 hr after conditioning (Colomb et al., 2009).

(D) Blocking MB-V3 neuron output during STM retrieval 30 min after training had no effect on memory scores ($F_{(2,27)} = 0.29$, $p = 0.75$; $n = 9-10$).

(E) Blocking MB-V3 neuron output during STM retrieval 2 hr after training had no effect on memory scores ($F_{(2,41)} = 1.05$, $p = 0.36$; $n = 14$).

(F) Continuous blockade of MB-V3 neurons from training through to STM retrieval 2 hr after training had no effect on memory scores ($F_{(2,29)} = 0.068$, $p = 0.93$; $n = 10$).

Mean \pm SEM. Statistical test was performed with one-way ANOVA: *** $p < 0.001$ in post hoc comparisons with each parental control.

See also Figure S1 and Table S1 for controls of sugar perception and olfactory acuity.

MB-V3 Neurons Are Required for Appetitive LTM Retrieval

The *G0239* line's specificity makes it a very efficient tool for the functional study of MB-V3 neurons. To transiently block neurotransmission from MB-V3 neurons by a temperature shift, we expressed the *Shibire^{ts}* (*Shi^{ts}*) transgene encoding a thermosensitive, dominant-negative mutant form of dynamin

silencing because both were normal at the restrictive temperature (Table S1). The same mutant flies tested at permissive temperature showed normal LTM performance (Figure 2B), which unambiguously shows that MB-V3 silencing was responsible for the loss of appetitive LTM. LTM was normal when MB-V3 neurons were blocked during conditioning and for the first 12 hr of consolidation after training, but not during retrieval (Figure 2C).

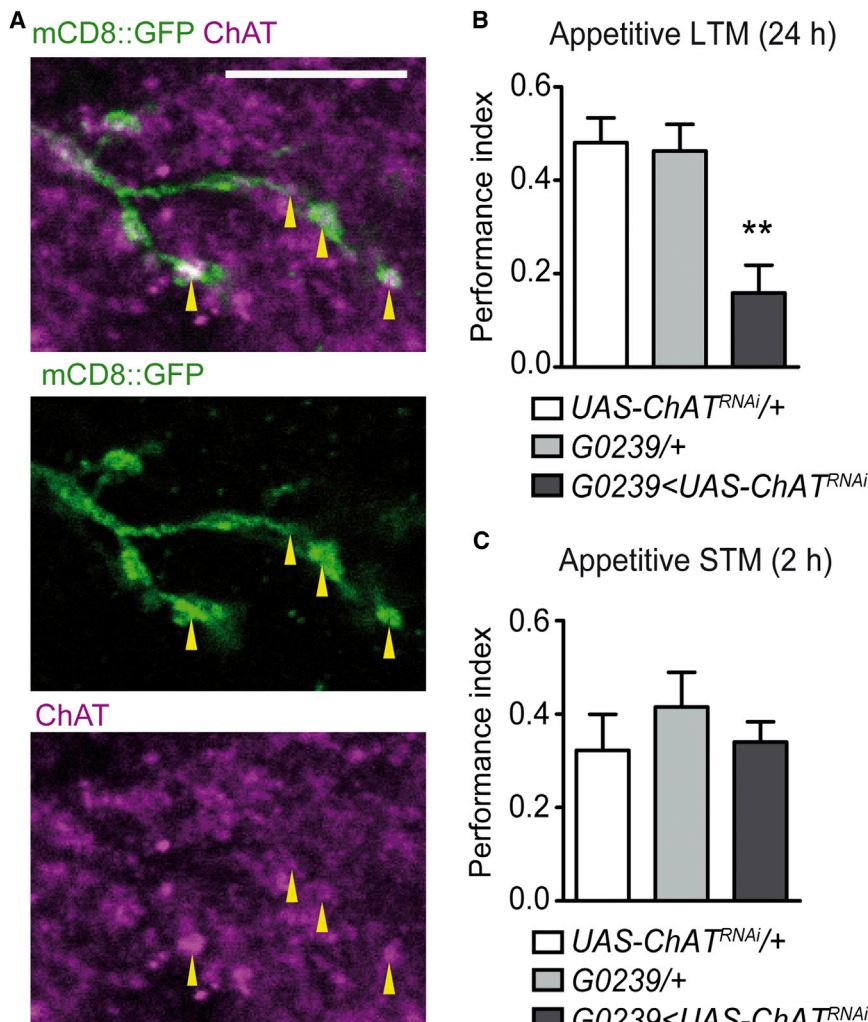


Figure 3. Cholinergic Signaling from MB-V3 Neurons Is Necessary for Appetitive LTM

(A) Single confocal slice of a brain preparation shows colocalization between immunolabeling for MB-V3 neurons' presynaptic terminals (green) and for ChAT (magenta). Scale bar, 20 μ m. mCD8::GFP was expressed in MB-V3 neurons through *G0239*.

(B and C) Disrupting *ChAT* expression in MB-V3 neurons with RNAi strongly impaired appetitive LTM (B, $F_{(2,29)} = 10.31$, $p = 0.0005$; $n = 10$), but not appetitive STM (C, $F_{(2,22)} = 0.557$, $p = 0.58$; $n = 7-8$). Mean \pm SEM. Statistical test was performed with one-way ANOVA: ** $p < 0.01$ in post hoc comparisons with each parental control.

See also Figure S2 and Table S1 for controls of sugar perception and olfactory acuity.

Therefore, MB-V3 neurons are required for the retrieval of LTM, which points to a role as MB output neurons. Strikingly, silencing MB-V3 neurons during the retrieval of appetitive STM, either 30 min (Figure 2D) or 2 hr (Figure 2E) after training, or continuously for 2 hr from training through to retrieval (Figure 2F), had no effect on memory performance. This shows that appetitive STM and LTM are retrieved through separate pathways and that MB-V3 neurons are specifically required for LTM retrieval. Although the *G0239* driver very specifically targeted MB-V3 neurons, we sought to confirm these behavioral data with another MB-V3-targeting driver. The less-specific *NP7125* GAL4 driver (Tanaka et al., 2008) yielded consistent results (Figure S1; Table S1).

Cholinergic Signaling from MB-V3 Neurons Is Necessary for Appetitive LTM

We next investigated which neurotransmitter was at work in MB-V3 neurons. We first examined immunoreactivity for choline acetyltransferase (ChAT) in the presynaptic terminals of MB-V3 neurons because the antibody often does not label cell bodies of cholinergic neurons (Yasuyama et al., 2002).

immunohistochemistry data provided strong evidence that MB-V3 neurons are cholinergic.

To confirm that cholinergic transmission is needed for appetitive LTM retrieval, we sought to knock down the expression of *ChAT* using an RNAi targeting this gene. We first checked by a qPCR experiment that the RNAi line we used efficiently targeted *ChAT* mRNA (*elav-GAL4/+*; 1; *elav-GAL4 < UAS-ChAT*^{RNAi}: 0.17). We then expressed this RNAi in MB-V3 neurons. This strongly impaired appetitive LTM (Figure 3B), whereas leaving appetitive STM unaffected (Figure 3C), in accordance with our thermal-blocking experiments (Figure 2). We also confirmed that the RNAi expression in MB-V3 neurons did not alter odor perception or sugar motivation (Table S1). Therefore, cholinergic transmission from MB-V3 neurons is functionally required for the retrieval of appetitive LTM, specifically.

Appetitive LTM Retrieval Requires Patterned Activity in MB-V3 Neurons

What is the functional role of MB-V3 neurons during LTM retrieval? MB-V3 neurons could carry an odor-specific message,

or alternatively, activity in MB-V3 neurons could have a permissive role, odor independent, in the behavioral expression of LTM (for example, by mediating the satiation state of the fly, as it was shown for other neurons in appetitive STM; Krashes et al., 2009). As a first insight into this point, we performed an experiment where MB-V3 neurons were artificially activated during memory retrieval, through the thermosensitive cation channel *dTrpA1* (Hamada et al., 2008; Plaçais et al., 2012). Because the two odors are present during the memory test, the artificial activation of MB-V3 neurons is likely to override any odor-specific information carried by these neurons. We found that appetitive LTM retrieval was severely impaired by MB-V3 neuron activation, whereas appetitive STM was unaffected (Figure 4A). The observed impairment was not due to deficient odor perception or deficient sugar motivation (Table S1). This result suggests that MB-V3 neurons might encode odor-specific information during appetitive LTM retrieval. To further investigate this possibility, we expressed the fluorescent calcium reporter GCaMP3 (Tian et al., 2009) in MB-V3 neurons through the *G0239* driver and measured response to the odorants used for appetitive conditioning by in vivo confocal imaging of intracellular calcium concentration in MB-V3 projections in the MB (Figure 4B). In naive starved flies, octanol (Oct) and methyl-cyclohexanol (Mch) yielded robust responses (Movie S1) that did not differ in magnitude (Figures 4B and 4C). In flies trained with one cycle of appetitive conditioning, the response to the odor used as conditioned stimulus (CS+) was significantly enhanced the day after training (Figures 4D, 4E, S3A, and S3B). No difference between Oct and Mch responses was observed in flies conditioned with an unpaired protocol temporally separating odor and sugar deliveries (Figures 4F, S3A, and S3B) that does not result in memory formation (Colomb et al., 2009). The enhancement of CS+ response was not observed in flies fed with the protein synthesis inhibitor cycloheximide (CXM) prior to training (Figures 4D, S3A, and S3B), a treatment known to inhibit the formation of LTM (Krashes and Waddell, 2008; Colomb et al., 2009). When flies were put back on food after training instead of being kept on starvation, the CS+ enhancement was also absent (Figures 4D, S3A, and S3B). Because being satiated after training prevents LTM from being retrieved but not from being formed (Krashes and Waddell, 2008), this latter result strongly correlates the enhancement of CS+ response to the retrieval of appetitive LTM. Finally, the enhancement of CS+ responses was not observed 2 hr after training, a time point corresponding to STM expression (Figures 4D, S3A, and S3B). Overall, these results show that the retrieval of appetitive LTM is functionally correlated to a specific enhancement of the response to the conditioned odorant in MB-V3 neurons.

We wondered whether this physiological trace of appetitive LTM retrieval was a readout of a similar calcium trace located in the KCs or resulted from a potentiation of the synapses between KCs and MB-V3 neurons. We therefore performed similar imaging experiments of responses to olfactory stimulations but looking at the response in the α branch of α/β KCs, at the level of MB-V3 dendrites. We targeted GCaMP3 expression in α/β KCs by means of the well-documented *c739* GAL4 driver (e.g., Aso et al., 2009; Trannoy et al., 2011). In the same conditions under which we observed an enhancement of the response

to CS+ in MB-V3 neurons (Figure 4D), we detected no significant difference between Oct and Mch responses in the α branch of KCs (Figure S3C). The comparison of Oct-trained flies with flies trained with an unpaired protocol did not reveal any difference either (Figure S3C). This suggests that the synapses between KCs and MB-V3 are an important spot for appetitive LTM-relevant plasticity.

MB-V3 Neurons Are Not Involved in the Retrieval of Aversive STM, ARM, or LTM

We next investigated whether MB-V3 neurons are specifically required for appetitive memory or if they are also involved in the retrieval of some aversive forms of memory. Contrary to the appetitive paradigm, the various forms of aversive memory require different conditioning protocols. A single cycle of associative conditioning yields labile STM and more persistent ARM. Aversive LTM, the most persistent form of memory, forms only after multiple conditioning cycles spaced by rest intervals, whereas multiple consecutive cycles (massed training) induce ARM. We blocked neurotransmission from MB-V3 neurons during memory retrieval after the three different protocols, and we observed no effect on any type of memory (Figure 5A). A recent work addressed the involvement of MB-V3 neurons in the retrieval of aversive memory, using the *Shi^{ts}* protein expressed through the *G0239* driver (Pai et al., 2013). Pai et al. (2013) reported that blocking MB-V3 neurons during the retrieval of aversive LTM 24 hr after a spaced training resulted in a mild but significant memory defect, whereas it did not affect the memory after single-cycle or massed training. Surprisingly, despite a high number of repeats ($n \geq 30$, Figure 5A), we failed to observe such a defect. The expression of anti-*ChAT* RNAi had also no effect on aversive STM, ARM, or LTM (Figure 5B). Finally, we activated MB-V3 neurons, using *dTrpA1*, during LTM retrieval after spaced training, but once again, this had no effect on memory performance (Figure 5C).

We then performed imaging experiments of odor responses in MB-V3 neurons after spaced training. Contrary to the results reported in the same study by Pai et al. (2013), we observed no increase in CS+ response in spaced-trained flies. Moreover, no difference could be detected between spaced-trained flies and flies conditioned with a spaced unpaired protocol (Figure 5D). We propose below several hypotheses that could explain the discrepancies between our results and the results of this other group (Pai et al., 2013) (see Discussion).

MB-V3 Neurons Are Required for the Retrieval of Aversive fLTM

Recent studies have shown that the satiety state of the fly influences the dynamics of aversive memory consolidation in several ways. Severe starvation before and after conditioning inhibits the formation of aversive LTM (Plaçais and Preat, 2013). By contrast, flies can form aversive protein synthesis-dependent LTM after a single cycle of aversive conditioning, provided that they are submitted to a very specific feeding procedure (Hirano et al., 2013): flies must be fasted for ~12–14 hr before training and put back on food immediately after training (“fLTM regime,” Figure 6A). This new form of aversive LTM was named “fasting LTM” (fLTM) because it differs from the standard aversive LTM

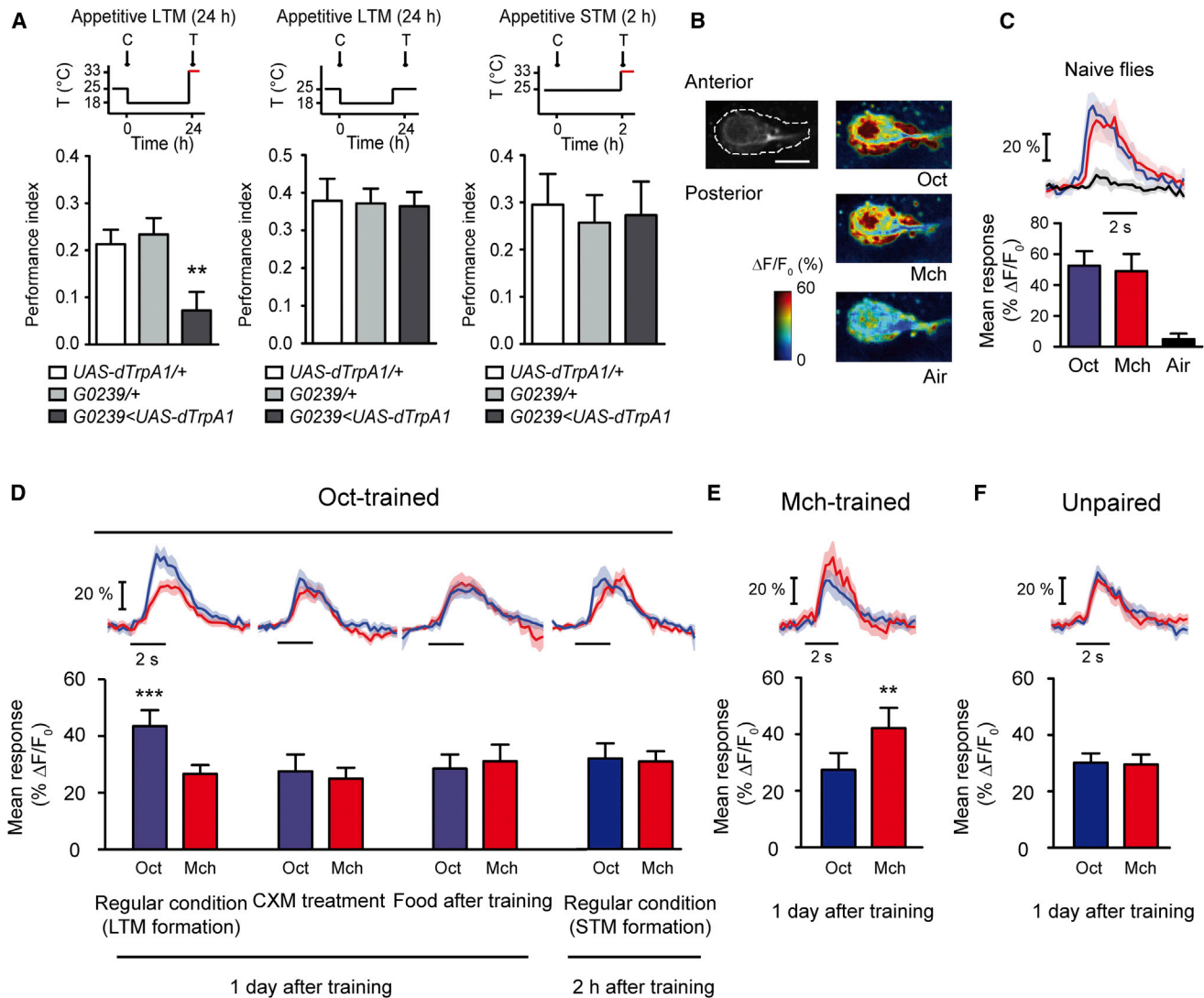


Figure 4. Appetitive LTM Retrieval Is Associated with an Increased Response to the Conditioned Odor in MB-V3 Neurons

(A) Artificial activation of MB-V3 neurons during memory retrieval strongly impaired appetitive LTM ($F_{(2,42)} = 6.338$, $p = 0.0041$; $n = 13-16$). Similar flies tested without activation showed no defect ($F_{(2,31)} = 0.0254$, $p = 0.98$; $n = 10-11$). Activation during the retrieval of appetitive STM showed no effect either ($F_{(2,33)} = 0.086$, $p = 0.92$; $n = 11-12$). Mean \pm SEM. Statistical test was performed with one-way ANOVA: ** $p < 0.01$ in post hoc comparisons with each parental control. Periods when MB-V3 neurons were activated in *G0239<UAS-dTrpA1* flies are shown in red on temperature sketches.

(B) Grayscale image and color-coded olfactory responses in an illustrative naive starved fly expressing the fluorescent GCaMP3 calcium reporter through *G0239* are shown. Images were acquired in a plane where dendritic projections from MB-V3 neurons on MB α lobes were visible, and the selected ROI included all visible projections of MB-V3 neurons in that plane, in and around MB vertical lobes. Scale bar, 10 μ m. See [Movie S1](#) for another example.

(C) Average time traces and mean responses obtained in naive starved flies ($n = 6$) are shown. The time scale bar also indicates stimulus duration. The delay between the trigger of the valve and onset of the response is due to the time required for the odor-interlaced air to reach the fly. MB-V3 neurons showed the same patterns of response to the two odors used in our behavioral experiments. Non-odorized air elicited very little response ($n = 6$).

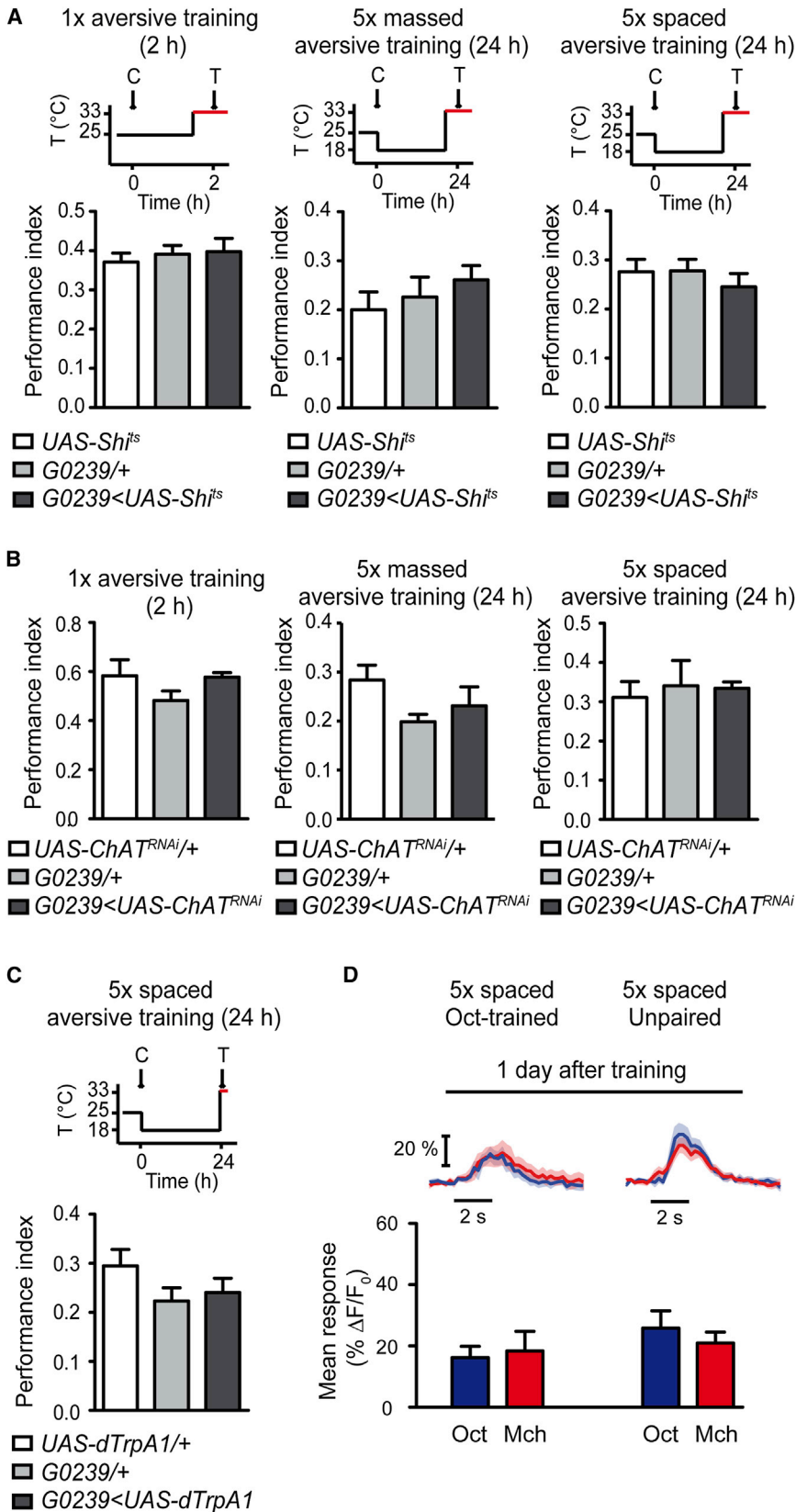
(D) Flies were trained with Oct as CS+. MB-V3 neurons showed an enhanced response to the conditioned odor 1 day after an appetitive training ($n = 17$, $p < 0.0001$, $t_{(16)} = 6.584$, paired t test). This enhancement was not observed if flies had been treated with CXM prior to training ($n = 8$, $p = 0.52$, $t_{(7)} = 0.67$, paired t test), if flies were put back on food after training ($n = 10$, $p = 0.58$, $t_{(9)} = 0.58$, paired t test), or if the response was measured 2 hr after training ($n = 8$, $p = 0.74$, $t_{(7)} = 0.34$, paired t test).

(E) The same enhancement was measured in flies trained with Mch as CS+ ($n = 9$, $p = 0.006$, $t_{(8)} = 3.696$, paired t test).

(F) There was no difference between responses to the two odors in flies that underwent an unpaired protocol ($n = 9$, $p = 0.904$, $t_{(8)} = 0.125$, paired t test).

Data are presented as mean \pm SEM. ** $p < 0.01$ and *** $p < 0.001$, according to a paired t test between Oct and Mch responses.

See also [Figure S3](#).



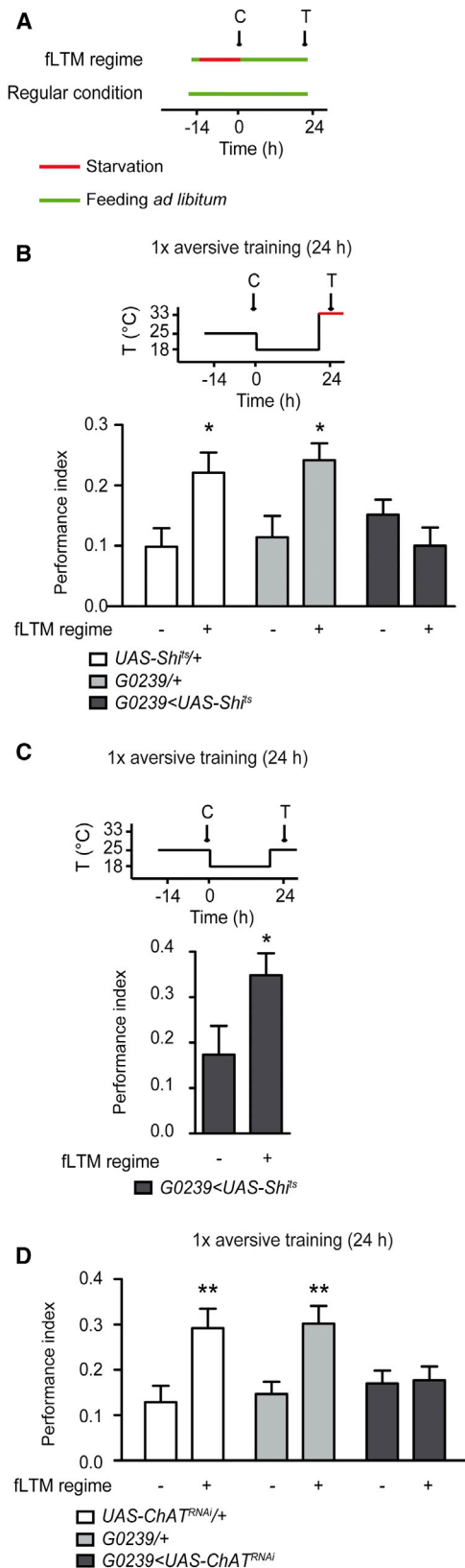


Figure 6. MB-V3 Neurons Are Required for the Retrieval of Aversive fLTM

(A) Sketch illustrates the feeding protocol that results in fLTM formation ("fLTM regime").

(B) Blocking MB-V3 neurons during the memory test prevents the retrieval of fLTM (two-way ANOVA, $F_{\text{REGIME}}^{(1,66)} = 7.005$, $p = 0.0102$; $F_{\text{REGIME} \times \text{GENOTYPE}}^{(2,66)} = 5.561$, $p = 0.0059$; $n = 12$).

(C) $G0239 < UAS-Shi^{ts}$ flies could form fLTM when tested at permissive temperature (25°C) 24 hr after single-cycle training (unpaired t test, $t_{(25)} = 2.237$, $p = 0.0344$; $n = 12-14$).

(D) Disrupting *ChAT* expression in MB-V3 neurons also blocked fLTM (two-way ANOVA, $F_{\text{REGIME}}^{(1,94)} = 14.58$, $p = 0.0002$; $F_{\text{REGIME} \times \text{GENOTYPE}}^{(2,94)} = 3.22$, $p = 0.0444$; $n = 16-18$).

Mean \pm SEM. Statistical tests in (B) and (D) were performed with two-way ANOVA ($*p < 0.05$ and $**p < 0.01$, respectively, in Bonferroni pairwise comparisons between flies of the same genotype that went through different regimes) and in (C) with an unpaired t test ($*p < 0.05$).

in its molecular support. Indeed, standard LTM relies on the CREB cofactor CREB-binding protein (CBP), whereas fLTM, as well as appetitive LTM, relies on cAMP-regulated transcriptional coactivator (CRTC), that is activated by fasting (Hirano et al., 2013). Because fLTM shares molecular features with appetitive LTM, we wondered whether the two forms of memory also recruited the same neuronal circuit. We trained flies, submitted or not to an fLTM regime, with a single cycle of aversive conditioning and tested their memory 24 hr after training at the restrictive temperature to assess an effect of blocking neurotransmission from MB-V3 neurons. As expected, the fLTM regime indeed increased the memory in the heterozygous genotypic controls (Figure 6B). Strikingly, though, flies expressing *Shi^{ts}* in MB-V3 neurons did not show such an increase (Figure 6B), although flies of the same genotype tested at permissive temperature actually displayed fLTM (Figure 6C). Similar results were obtained by expressing the RNAi against *ChAT* in MB-V3 neurons (Figure 6D). Therefore, MB-V3 neurons are necessary for aversive fLTM retrieval. These results support the idea that fLTM, as an aversive avatar of appetitive LTM due to fasting, not only shares its molecular basis but also involves similar circuitry.

DISCUSSION

In this study, we identified two pairs of cholinergic neurons, MB-V3 neurons, that are efferent from the tip of the MB α lobes and are required for the retrieval of appetitive LTM, but not STM. It was previously established that appetitive STM and LTM are formed and retrieved through different sets of KCs (Trannoy et al., 2011). STM formation involves the *rutabaga* adenylyl-cyclase, probably as a coincidence detector, in γ KCs, and STM retrieval requires output from the same γ KCs. Conversely, LTM formation requires the same cyclase in α/β KCs, and LTM retrieval requires the output from α/β KCs (Trannoy et al., 2011). The fact that MB-V3 cholinergic neurons are specifically recruited for the retrieval of appetitive LTM, but not STM, and that they are efferent from the tip of α lobes is fully consistent with this scheme of parallel processing of STM and LTM. On this basis, we anticipate that other as yet unidentified

γ lobe-efferent neurons could mediate the transmission of the STM trace to relevant downstream areas.

In this work, we also addressed the question of the specific requirement of MB-V3 neurons for the retrieval of appetitive LTM, as opposed to aversive forms of memory. We found that MB-V3 neurons are dispensable for the retrieval of aversive STM and ARM (Figures 5A and 5B), in accordance with a recently published study by Pai et al. (2013). However, our results diverge from theirs on the retrieval of aversive LTM because they found that blocking MB-V3 neurons, with the same *G0239* GAL4 driver we used and *Shi^{ts}*-thermosensitive neural blocker, yielded a mild but significant defect after spaced training. In addition, we performed calcium-imaging experiments with the MB-V3-specific driver *G0239* that revealed no alteration of MB-V3 neuron olfactory responses after spaced training, consistent with our behavioral results. In contrast, Pai et al. (2013) reported an increase of the response to CS+ compared to CS– after spaced training, but not massed training. It should be noted that they used for their imaging experiments a GAL4 driver that is not specific for MB-V3 neurons and especially labels other MB-extrinsic neurons that have projections on the vertical lobes close to MB-V3 dendrites (MB-V4 neurons according to the nomenclature of Tanaka et al. [2008]). Our conclusions are drawn from a lack of effect, which as a negative result, should be interpreted with caution. However, our imaging data are consistent with behavior experiments using three different effectors (*Shi^{ts}*, *ChAT^{RNAi}*, and *dTrpA1*) that were all strong enough to completely abolish (*Shi^{ts}*, Figure 2A) or very strongly affect (*ChAT^{RNAi}*, *dTrpA1*, Figures 2B and 3A) appetitive LTM retrieval. Thus, it seems unlikely that the effects of all these manipulations could have been below our detection limits. We propose two hypotheses that would explain the discrepancy between the two reports. First, we showed that MB-V3 neurons are required for the retrieval of aversive fLTM (Figures 5C–5F). This form of aversive LTM shares molecular features (Hirano et al., 2013) and retrieval circuit (present study) with appetitive LTM. The formation of fLTM requires that flies are mildly fasted before training and put back on food immediately after training. Longer fasting periods before training and/or prolonged starvation after training prevents fLTM formation (Hirano et al., 2013). Aversive LTM in the study of Pai et al. (2013) might have contained fLTM because their spaced-training protocol takes twice as long as ours (ten versus five cycles), during which flies may be mildly fasted. Being put back on food after training, flies could form some fLTM, which would result in the mild memory impairment they observed. In addition, it could be that other neurons (for example, the MB-V4 neurons, labeled in two other GAL4 drivers they used for behavior experiments and for imaging) are actually involved in aversive LTM retrieval, by themselves or maybe in combination with MB-V3 neurons. Testing this latter hypothesis would require another GAL4 driver that would target MB-V4 neurons independently of MB-V3 neurons. Unfortunately, no such tool has been reported so far.

Retrieval of appetitive LTM was functionally correlated to an increased response of MB-V3 neurons to the odor that was associated with sugar ingestion during training (Figures 4 and S3), an increase that did not occur in the hour range after training when only STM is formed, and that was abolished under two

conditions that disrupt appetitive LTM formation or retrieval. In order to know if this increased response in MB-V3 neurons was the direct consequence of a similar phenomenon occurring upstream in the circuit, we performed similar calcium-imaging experiments in the α branch of α/β KCs, which are directly presynaptic to MB-V3 neurons. We failed to detect any trace of LTM formation in these neurons, either by comparing responses to CS+ and CS– within a fly or by comparing flies that are trained to make LTM and flies that undergo an unpaired protocol. These data contradict the conclusion from a recent study claiming that appetitive training induces an increase in the CS+ response in the MB α lobes 24 hr after training (Cervantes-Sandoval et al., 2013). However, what is shown in this latter study is that the average of the CS+/CS– ratio is higher than one, but this effect may in fact be a bias toward high ratio values due to an inappropriate mathematical method of data analysis: the correct way for analyzing the ratio of experimental measurements is to average the logarithm of the ratio (Figure S3D), as performed by several groups for similar imaging experiments (Wang et al., 2008; Séjourné et al., 2011; Pai et al., 2013). Furthermore, Cervantes-Sandoval et al. (2013) did not show in their article the most relevant control data: comparing trained flies with flies that underwent an unpaired protocol would have been more accurate than statistical comparison of KC response between naive and trained flies. Of course, one cannot exclude that a calcium trace might eventually be described in KCs that we would have missed. However, in the current situation, it seems likely that the LTM retrieval trace we observed in MB-V3 neurons is not a simple readout of a similar calcium trace already present in upstream KC axons.

In this scheme, appetitive LTM formation likely results in plasticity located at the level of the synapses between α/β KCs and MB-V3 neurons. When the conditioned odor is perceived, potentiation of these synapses would result in an increased response in MB-V3 neurons, which in turn could alter olfactory information to subsequent brain structures (Ito et al., 1998). Our data show that blocking the output of MB-V3 neurons is sufficient to fully abolish appetitive LTM retrieval. This of course does not exclude that other MB-extrinsic neurons may also be necessary, but it remains striking that appetitive LTM retrieval depends on signaling from as few as two neurons per brain hemisphere, which represents a huge convergence from the $\sim 1,000$ α/β KCs (Aso et al., 2009). This drastic convergence is consistent with proposed models of memory encoding in insects (Laurent, 2002; Turner et al., 2008), where the specificity of memory toward a given odor is conferred by the sparse representation of olfactory stimuli in the KCs (Wang et al., 2004; Turner et al., 2008; Honegger et al., 2011), whereas an altered—in this case increased—response in the restricted number of output neurons is sufficient to encode an alteration of the valence of an odorant. The specificity of memory expression toward the conditioned odor is preserved provided that synaptic plasticity occurs only in the conditioned odor-responsive KCs; in the present case, at the synapse with MB-V3 neurons. At present, there is no straightforward way to target the KCs specifically responding to a given odor. The identification of MB-V3 should prove a key step in unraveling the precise mechanisms of synaptic plasticity underlying appetitive LTM.

EXPERIMENTAL PROCEDURES

Fly Strains

All transgenic lines were outcrossed for five generations to a w^{1118} strain (or $y^- w^{1118}$ for the *UAS-ChAT^{RNAi}* line) in a wild-type *Canton-S* background. For behavioral experiments, flies were raised on standard medium containing yeast cornmeal and agar at 18°C and 60% humidity under a 12 hr:12 hr light-dark cycle. For imaging and immunohistochemistry experiments, flies were raised at 25°C. *G0239* (inserted in chromosome II; Chiang et al., 2011) was obtained from Ann-Shyn Chiang (Tsing Hua University), *UAS-IVS-GCaMP3-p10* (inserted in the VK00005 insertion site on chromosome III; also described as *pJFRC97*; Pfeiffer et al., 2012) was from Barret Pfeiffer and Gerald Rubin (Janelia Farm Research Campus), *UAS-ChAT^{RNAi}* (TRIP collection) from Bloomington Stock Center, and *NP7125* (inserted in chromosome X; Tanaka et al., 2008) from the *Drosophila* Genetic Resource Center. To obtain the list of the putative off-target genes for the RNAi against *ChAT*, we followed the procedure described on the TRIP collection website (<http://www.flymai.org/TRIP-QNA.html>). The RNAi we used has no predicted off-target genes.

We looked for putative additional expression of the *G0239-GAL4* line in the CNS and the ventral nerve cord. In the SOG, under stronger illumination conditions, we found sparse additional neurons, showing very little expression compared to MB-V3 neurons (data not shown). Therefore, the *G0239* line is indeed quite specific for MB-V3 neurons in the brain, as confirmed by Pai et al. (2013). In the ventral nerve cord, only faint GFP expression could be detected in a few scattered neurons (data not shown).

Behavior Experiments

For all behavior experiments, 0- to 2-day-old flies were transferred in fresh food vials the day before conditioning. For appetitive conditioning, flies were then starved for 21 hr at a temperature of 25°C with only mineral water provided and trained with one cycle of appetitive conditioning as previously described (Colomb et al., 2009; Trannoy et al., 2011). For aversive conditioning, flies were trained with one cycle of aversive training, five consecutive cycles (massed training), or five cycles spaced by 15 min intertrial intervals (spaced conditioning).

The memory test was performed as described in Trannoy et al. (2011). For experiments with *Shi^{ts}*, flies were switched to the restrictive temperature (33°C) 20–30 min before the targeted time so that they could acclimatize to the new temperature. For experiments with *dTrpA1*, flies were transferred to a preheated tube at 33°C for 1 min before memory test and then tested at the same temperature. This was to avoid possible nonspecific effects due to an exceedingly long artificial activation.

Naive odor avoidance and sugar preference were assessed as described in (Trannoy et al., 2011). For the sugar preference assay, the initial sucrose solution was diluted ten times compared to the one used for conditioning. This provides higher sensitivity in detecting putative sugar preference defects (Colomb et al., 2009).

Memory scores are displayed as mean \pm SEM. Unless stated otherwise (Figure 6), statistical analyses were performed by one-way ANOVA followed by Newman-Keuls pairwise comparisons. ANOVA results are given as the value of the Fisher distribution $F_{(x,y)}$ obtained from the data, where x is the number of degrees of freedom for groups and y the total number of degrees of freedom for the distribution. More details on behavior experiments can be found in the Supplemental Experimental Procedures.

Immunohistochemistry

GAL4 lines were crossed to $y,w;UAS-mCD8::GFP/CyO$ (Lee and Luo, 1999), $w,UAS-Syt::HA/FM7a;UAS-mCD8::GFP/CyO$ (Robinson et al., 2002), *UAS-DenMark* (Nicolai et al., 2010), or $w;MB-GAL80,UAS-mCD8::GFP/CyO$. We prepared the brains of female F1 progenies (5–8 days after eclosion at 25°C) and fixed for 45 min at 22°C–23°C in 2% formaldehyde in PBT (PBS containing 0.1% Triton X-100). For GABA and DVGLUT staining, brains were fixed in 4% paraformaldehyde in PBT for 2 hr at room temperature (22°C–23°C). For serotonin labeling, brains were fixed in 5% formaldehyde in PBT for 1 hr. Samples were then rinsed twice for 10 min in PBT, blocked with 3% normal goat serum in PBT for 30 min, then incubated with primary antibodies in the blocking solution at 4°C overnight. Brains were washed for 20 min three times in PBT

then incubated with secondary antibodies in the blocking solution overnight at 4°C. After washing the brains three times for 20 min and then for 1 hr, they were mounted in VECTASHIELD (Vector Laboratories) for microscopy analysis. The antibodies used can be found in the Supplemental Experimental Procedures.

In Vivo Calcium Imaging

To monitor the activity of MB-V3 neurons, the genetically encoded GCaMP3 calcium reporter was targeted to MB-V3 neurons with *G0239*. To monitor the activity of KCs (Figure S4C), GCaMP3 was targeted to α/β KCs with the *c739 GAL4* driver. Reporter expression was enhanced using an improved transgenic construct with translational enhancer *UAS-IVS-GCaMP3-p10* (*pJFRC97* in Pfeiffer et al. [2012]). In general, flies were starved for 21 hr at 25°C before imaging, then trained with one cycle of appetitive conditioning or an unpaired protocol and kept on starvation for 16–20 hr at 18°C. Specific conditions were performed as follows: for CXM treatment, flies were provided a solution of 35 mM CXM in mineral water (instead of mineral water alone) for all the starvation period preceding the training; for the “satiated” condition, flies were transferred in food-containing vials from 30 min after training until the imaging experiment; for imaging experiments 2 hr after training, flies were kept on starvation at 25°C after training until the start of the imaging experiment. Naive flies were imaged after the first 21 hr starvation period. For experiments shown in Figure 5, nonstarved flies were trained with a spaced aversive conditioning or a spaced unpaired protocol, then kept in regular food vials for 16–20 hr at 18°C. A female fly was prepared for in vivo imaging essentially as described previously by Séjourné et al. (2011), except that the proboscis was only glued on its lateral sides to limit glue ingestion and that sucrose was replaced with ribose at the same concentration in the solution bathing the brain (see Supplemental Experimental Procedures for more details).

Data sets of Oct and Mch responses are intrinsically paired because a single fly gives a value for each of the two odors. We therefore used paired *t* tests to compare the magnitude of the responses to the two odors. To compare flies that went through different protocols or conditions, the decimal logarithms of the ratio of Oct over Mch responses were averaged across all flies of a given condition. The different conditions were then statistically compared using unpaired statistics (unpaired *t* tests or one-way ANOVA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.09.032>.

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