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21. Electrophysiological recordings were performed from LTRPC2 transfectants by means of a voltage-clamp technique [O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, Pflugers Arch. **391**, 85 (1981)]. Recordings were made with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) by means of patch electrodes with a resistance of 3 to 10 megohms. Single-channel recording data were filtered at 2 kHz and sampled at 20 kHz. The internal pipette solution contained 150 mM CsCl, 5 mM MgCl$_2$, and 10 mM Hepes-Cs (pH 7.2). The external solution contained 145 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM Hepes-Na (pH 7.4). The Na$^+$,Ca^{2+}-free solution contained 150 mM N-methyl-D-glucamine, 2 mM MgCl$_2$, and 10 mM Hepes [pH 7.4 (adjusted with HCl)]. For the inside-out patch experiments, the recording pipette contained the normal external solution, and the bath solution contained 150 mM KCl, 2 mM MgCl$_2$, and 10 mM Hepes-K (pH 7.4). For the calcium permeability experiments, the bath solution was changed to 117 mM CaCl$_2$ and 5 mM Hepes [pH 7.4 (adjusted with Ca(OH)$_2$)] after the establishment of a gigohm seal with the recording pipette containing 135 mM CsCl, 4.5 mM EGTA, and 9 mM Hepes-Cs (pH 7.2). The low Cl$^-$ solution contained 145 mM Na gluconate, 5 mM K gluconate, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM Hepes-Na (pH 7.4). Ca permeability was calculated relative to Cs by the Goldman-Hodgkin-Katz modified constant field equation [C. A. Lewis, J. Physiol. **286**, 417 (1979)]. All recordings were performed at room temperature (25°C). Analysis was carried out on a personal computer with pCLAMP software (Axon Instruments). All values are expressed as means ± SEM. Statistical significance was tested with Student's t test. No corrections were made for junction potentials. All reagents were purchased from Sigma. The ATP was 2 Na$^+$ salt.
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The Role of *Drosophila* Mushroom Body Signaling in Olfactory Memory

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The mushroom bodies of the *Drosophila* brain are important for olfactory learning and memory. To investigate the requirement for mushroom body signaling during the different phases of memory processing, we transiently inactivated neurotransmission through this region of the brain by expressing a temperature-sensitive allele of the *shibire* dynamin guanosine triphosphatase, which is required for synaptic transmission. Inactivation of mushroom body signaling through α/β neurons during different phases of memory processing revealed a requirement for mushroom body signaling during memory retrieval, but not during acquisition or consolidation.

In the insect, distinct brain structures termed the mushroom bodies (MBs) play a central role in associative learning of olfactory information (*1, 2*). The MBs of *Drosophila melanogaster* comprise about 2500 neurons per brain hemisphere (Fig. 1). The cell bodies of these neurons are situated in the dorsal posterior brain region and extend axons anteriorly and ventrally through the peduncle to give rise to the α/β, α'/β', and γ lobes (*3–6*). These lobes are neuropil regions that contain the MB cell axons and other processes that synapse with MB neurons.

Genetic and chemical disruption of the MBs produces flies that are normal for general behaviors but are defective in olfactory learning (*7, 8*). Many genes involved in olfactory learning and memory show enriched expression in the MBs, particularly those encoding components of the cyclic adenosine monophosphate signaling pathway (*2*). Targeting of a constitutively active G-protein α subunit to the MBs disrupts olfactory learning (*9*), and restoring the *rutabaga*-encoded adenylyl cyclase specifically to the MBs of *rutabaga* mutants is sufficient to restore

short-term memory in these flies (*10*). The model that has emerged from these experiments posits the MBs as important centers in olfactory associative learning and the likely site of convergence of the conditional (CS) and unconditioned (US) stimuli in classical conditioning (*1, 2, 11*).

A limitation of the previous experiments is that they all involve permanent alterations to the fly's brain throughout development, leading to the possibility that some of the effects on learning might reflect developmental perturbations rather than modifications of the physiology of these neurons that subserve learning and memory processes. Additionally, the irreversible nature of these interventions has made it impossible to dissect the roles of the MBs at the different stages of memory acquisition, consolidation, and retrieval.

To explore the roles of the MBs in the different phases of memory processing, we used an approach that allows us to transiently inactivate synaptic transmission from the MBs by targeting expression of a temperature-sensitive *shibire*[ts1] transgene to the MBs by the GAL4/UAS system (*12, 13*). The *shibire* gene encodes a dynamin guanosine triphosphatase (GTPase) that is essential for synaptic vesicle recycling (*14, 15*) and maintenance of the readily releasable pool of synaptic vesicles (*16*). The temperature-sensitive

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allele *shibire*[ts1] bears a mutation in the GTPase domain, which renders the protein inactive at restrictive temperatures (>29°C) and causes a rapid inactivation of synaptic transmission and subsequent paralysis (*17–19*). Restricted expression of the *shibire*[ts1] transgene in specific cells produces blindness and paralysis at restrictive temperatures (*12*). Recently, the transgene was used to demonstrate the role of the dorsal paired medial neurons in memory formation (*20*).

We screened a number of GAL4 lines that exhibited enriched MB expression patterns for 3-min memory performance when driving the UAS-shi[ts1] transgene at both permissive (25°C) and restrictive (32°C) temperatures in an olfactory classical conditioning paradigm (*21, 22*). In this assay, flies are conditioned by exposure to one odor paired with electric shock (CS+) and subsequent exposure to a second odor in the absence of electric shock (CS−). Memory is then assayed at predetermined time points after training by forcing the flies to choose between the CS+ and CS−. Several MB GAL4 lines demonstrated significant memory impairment at 3 min when tested at the restrictive temperature. These lines were next analyzed for sensorimotor functions required for the conditioning assay, including locomotion, odor avoidance, and electric shock avoidance, at both the permissive and restrictive temperatures (*22*). Subsequently, we focused on the GAL4 lines, c739 and 247, which demonstrated intact sensorimotor functions when driving the UAS-shi[ts1] at both permissive and restrictive temperatures (Table 1). For these MB GAL4

lines, memory at 3 min at the permissive temperature was indistinguishable among flies bearing both the GAL4 element and the UAS-shi[ts1] transgene in combination and control flies bearing the GAL4 element or the UAS-shi[ts1] element alone (Fig. 2A). At the restrictive temperature, however, the combination of c739 or 247 with the UAS-shi[ts1] transgene resulted in a significant impairment of performance. The line 201Y; UAS-shi[ts1] showed a slight but nonsignificant decrease in memory performance under these conditions (Fig. 2B). These data indicate that the

Fig. 1. The mushroom bodies of *Drosophila* and associated neural pathways. The olfactory system is illustrated in shades of yellow and green. The MBs and their processes are in shades of gray and blue. Information about odors is conveyed from about 1500 olfactory sensory neurons on each antenna through the antennal nerves (AN) to 43 glomeruli in each antennal lobe (AL), where these axons synapse upon the processes of antennal lobe relay neurons. Relay neurons with cell bodies in the antennal lobe transmit olfactory information through the antennal cerebral tract (ACT) to synapse on the dendrites of mushroom body cells (MBC). These dendrites are located in a neuropil structure known as the calyx (C). The antennal cerebral tract continues laterally to synapse with the processes of other neurons in the lateral protocerebrum (LPC). The MB axons are bundled to form the peduncle (P), which projects toward the anterior face of the brain to synapse on the dendrites of follower neurons in neuropil regions termed the lobes. Some lobes are oriented vertically and some horizontally with respect to the fly head. The α/β MB neurons project axon branches into the vertically oriented α lobe and the horizontally oriented β lobe (blue structures). The α'/β' neurons project axon branches into similarly oriented lobes termed α' and β' (gray structures). The γ MB neurons do not branch and project only into the horizontally oriented γ lobe (purple-blue structures). Several different classes of neurons receive information from MB neurons and convey this to diverse brain regions (*6*). [Figure adapted from (*23*)]

inactivation of MB neurotransmission disrupts the processes underlying the encoding, storage, or retrieval of memory tested 3 min after training.

We next analyzed these data relative to the expression patterns of the three GAL4 lines to gain insights into possible functional subdivisions of the MBs. The GAL4 line 247, in which GAL4 is under the control of a 247–base pair (bp) enhancer fragment isolated from the *D-mef2* gene, drives reporter gene expression in all lobes of the MB (*10*). In the line 201Y, the γ lobe is preferentially marked, along with a
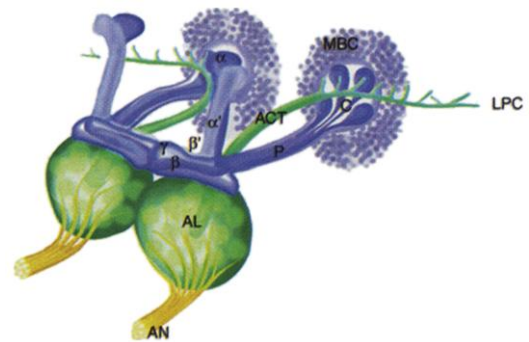
Table 1. Task-relevant sensory functions. Odor avoidance was assayed by measuring the choice of naïve flies between odor and fresh air at odor concentrations used during training and testing as described (*30*). In the first four rows (wild type, UAS-shi[ts1], c739, and c739;UAS-shi[ts1]), air was bubbled through an odorant:mineral oil mixture, whereas for 247 and 247;UAS-shi[ts1], evaporated odors were delivered. For each odorant, a two-way ANOVA with genotype and temperature as main effects was performed for groups tested under similar conditions. For octanol (OCT) avoidance in wild type, UAS-shi[ts1], c739, and c739;UAS-shi[ts1], c739;UAS-shi[ts1] was compared with UAS-shi[ts1] and c739 at each of the temperatures, producing four pairwise planned comparisons. To maintain an error rate of $\alpha = 0.05$, we adjusted the critical P value to $\alpha = 0.013$ (*29*). No significant differences were detected. In 247 and 247;UAS-shi[ts1], 247;UAS-shi[ts1] was compared with 247 at both of the temperatures, producing two planned comparisons. To maintain an error rate at $\alpha = 0.05$, we adjusted the critical P value to $\alpha = 0.025$. No significant differences were detected. Additionally, no overall effect of temperature was observed (wild type, UAS-shi[ts1], c739, and c739;UAS-shi[ts1]; $n = 6$ per group;

247 and 247;UAS-shi[ts1], $n = 5$ per group). For benzaldehyde (BEN) avoidance in wild type, UAS-shi[ts1], c739, and c739;UAS-shi[ts1], c739;UAS-shi[ts1] was compared with UAS-shi[ts1] and c739 at each of the temperatures, producing four pairwise planned comparisons. To maintain an error rate of $\alpha = 0.05$, we adjusted the critical P value to $\alpha = 0.013$. No significant differences were detected between these groups at either 25°C or 32°C (25°C, $n = 12$ per group; 32°C, $n = 5$, except for c739, $n = 6$). All lines in wild type, UAS-shi[ts1], c739, and c739;UAS-shi[ts1] showed higher avoidance of benzaldehyde at 32°C, demonstrating intact odor perception at the restrictive temperature [$F(1,62) = 38.48, P < 0.0001$]. In 247 and 247;UAS-shi[ts1], 247;UAS-shi[ts1] was compared with 247 at both of the temperatures, producing two planned comparisons. To maintain an error rate at $\alpha = 0.05$, we adjusted the critical P value to $\alpha = 0.025$. No significant differences were detected ($n = 5$ per group). Electric shock avoidance was assayed by measuring the avoidance responses of naïve flies to 90-V dc shock as described in (*30*). A two-way ANOVA with genotype and temperature as main effects did not detect any significant differences ($n = 6$ per group).

Genotype	Odor avoidance				Shock avoidance	
	OCT		BEN			
	25°C	32°C	25°C	32°C	25°C	32°C
Wild type	0.70 ± 0.06	0.61 ± 0.05	0.79 ± 0.03	0.94 ± 0.03	0.66 ± 0.05	0.47 ± 0.05
UAS-shi[ts1]	0.66 ± 0.03	0.82 ± 0.04	0.63 ± 0.04	0.96 ± 0.02	0.64 ± 0.13	0.64 ± 0.05
c739	0.79 ± 0.02	0.61 ± 0.12	0.88 ± 0.03	0.97 ± 0.01	0.59 ± 0.08	0.69 ± 0.06
c739;UAS-shi[ts1]	0.80 ± 0.04	0.66 ± 0.05	0.75 ± 0.05	0.96 ± 0.02	0.73 ± 0.14	0.66 ± 0.04
247	0.76 ± 0.09	0.59 ± 0.06	0.77 ± 0.05	0.67 ± 0.10	0.70 ± 0.05	0.46 ± 0.04
247;UAS-shi[ts1]	0.79 ± 0.06	0.72 ± 0.05	0.69 ± 0.06	0.58 ± 0.09	0.46 ± 0.08	0.61 ± 0.04

small subset of the α/β neurons (*9, 10*). In contrast, the GAL4 c739 element drives reporter gene expression preferentially in the α/β

lobes (*23*). The expression overlap between the two GAL4 lines that disrupted 3-min memory when combined with UAS-shi[ts1] at the restric-

tive conditions is within the α/β lobes, suggesting the importance of this subset of MB neurons for the expression of memory. At the restrictive temperature, the UAS-shi[ts1] in combination with 201Y, which preferentially drives reporter gene expression principally in the γ lobes, did not significantly impair 3-min memory. The mild memory impairment in this line could be due to insufficient levels of expression of the UAS-shi[ts1] transgene or rather it could reflect the possibility that the neurons in which this line drives the UAS-shi[ts1] are not necessary for memory expression at this time point.

Fig. 2. Disruption of memory at 3 min when MB signaling is blocked. Canton-S flies carrying the w1118 mutation (wCS10) served as the wild-type control for calibration of the training and testing apparatuses. (**A**) Three-minute memory performance with training and testing under permissive conditions (25°C). Groups 1 to 4 were tested in a single experiment. A Performance Index (P.I.) was calculated by subtracting the number of flies making an incorrect choice from the number of flies making a correct choice, divided by the total number of flies. P.I.'s from the four groups were subjected to a one-way analysis of variance (ANOVA) with genotype as the main effect. For these groups, c739;UAS-shi[ts1] was compared with the control group c739 and UAS-shi[ts1], producing two pairwise planned comparisons. To maintain an error rate of α = 0.05, we adjusted the critical P value to α = 0.025 (*29*). No significant differences were detected (*n* = 6 per group). In separate experiments, groups 5 and 6 and groups 7 and 8, were tested under permissive conditions. A *t* test demonstrated a marginal difference between 247;UAS-shi[ts1] and 247 (*P* = 0.049, *n* = 6 per group). No significant difference was observed between 201Y;UAS-shi[ts1] and 201Y (*n* = 6 for line 7 and *n* = 4 for line 8). (**B**) Three-minute memory performance with training and testing under restrictive conditions (32°C). Groups 1 to 4 were tested in a single experiment. P.I.'s from each of the four groups were subjected to a one-way ANOVA with genotype as a main effect. For these groups, c739;UAS-shi[ts1] was compared with the control groups c739 and UAS-shi[ts1], producing two pairwise planned comparisons. To maintain an error rate of α = 0.05, we adjusted the critical P value to α = 0.025. At 32°C, the line c739;UAS-shi[ts1] was significantly different from both UAS-shi[ts1] and c739 (*P* = 0.012 and *P* = 0.0003, respectively; *n* = 6 per group, except *n* = 5 for c739). In separate experiments, groups 5 and 6 and groups 7 and 8 were tested under restrictive conditions. A *t* test demonstrated a significant difference between 247;UAS-shi[ts1] and 247 (*P* < 0.0001, *n* = 6 per group). No significant difference was observed between 201Y;UAS-shi[ts1] and 201Y (*n* = 6 for group 7 and *n* = 3 for group 8).

To determine whether the deficient performance of these flies arose from a defect in memory acquisition, consolidation, or retrieval, we examined memory at a later time point (3 hours). Prior research has shown that most of the memory measured at this time point has been consolidated into an anesthesia-resistant form (*24*). The separation of training and testing also allowed us to reversibly inactivate MB signaling separately during each phase and then ask whether memory performance was affected. We first examined 3-hour memory at the permissive temperature throughout the experiment. Under these conditions, the performance of the c739;UAS-shi[ts1] flies was indistinguishable from flies bearing either the c739 element or the UAS-shi[ts1] element (Fig. 3A). The lines 247 and 201Y in combination with UAS-shi[ts1] disrupted 3-hour memory at the permissive temperature and were not analyzed further (*25*).
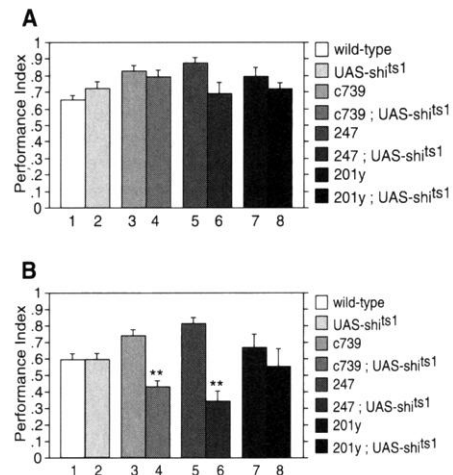
To examine the requirement for signaling

Fig. 3. MB signaling is required for retrieval, but not acquisition or consolidation of olfactory memory. The experimental design is indicated above each graph. For each experiment, c739; UAS-shi[ts1] was compared with both c739 and UAS-shi[ts1], producing two pairwise planned comparisons. To maintain an error rate of α = 0.05, we adjusted the critical P value to α = 0.025 (*29*). (**A**) Three-hour memory assay performed under permissive conditions. A one-way ANOVA of P.I.'s with genotype as the main effect showed no significant difference between any of the groups (*n* = 6 per group). (**B**) Inactivation of MB signaling abolishes memory retrieval. Flies were trained and subsequently maintained under permissive conditions until 15 min before testing, at which point they were shifted to the restrictive temperature until tested. One-way ANOVA of P.I.'s with genotype as the main effect revealed a significant difference [*F*(3,23) = 18.437, *P* < 0.0001, *n* = 7 per group, except for *n* = 6 for c739;UAS-shi[ts1]]. Pairwise planned comparisons revealed that c739;UAS-shi[ts1] differed significantly from c739 and UAS-shi[ts1] (*P* < 0.0001 and *P* = 0.01, respectively). (**C**) MB signaling is not required during memory acquisition. Flies were trained under restrictive conditions and subsequently shifted to permissive conditions for testing. One-way ANOVA of P.I.'s detected a significant difference. Pairwise planned comparisons revealed a significant difference between c739; UAS-shi[ts1] and the c739 control (*P* = 0.001), but no difference between c739;UAS-shi[ts1] and the control line UAS-shi[ts1] (*P* = 0.71), indicating a general effect of heat on lines carrying the UAS-shi[ts1] transgene (*n* = 29 per group except *n* = 30 for UAS-shi[ts1]). (**D**) MB signaling is not required
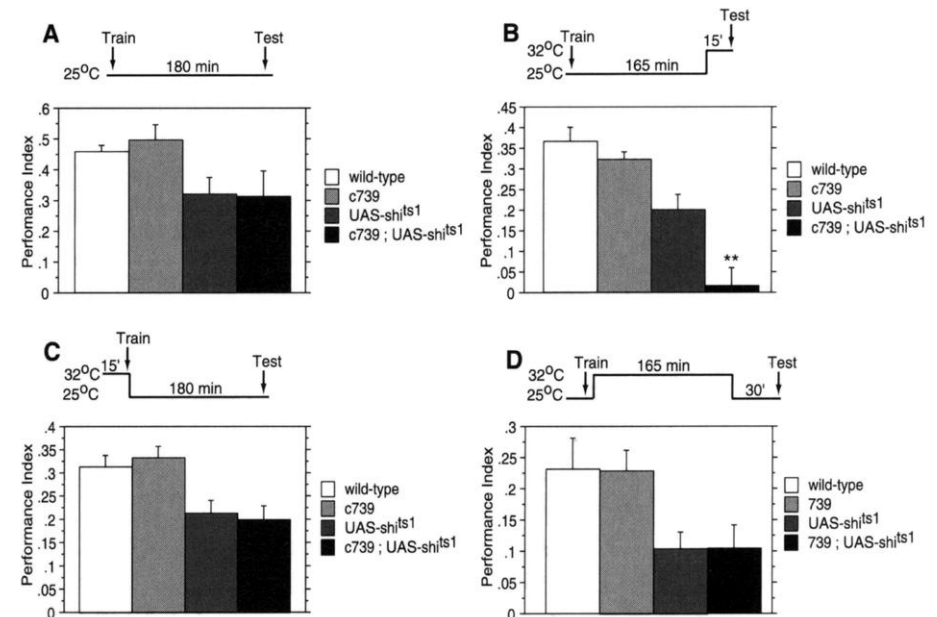
for memory consolidation. Flies were trained and tested under permissive conditions and given a shift to restrictive conditions immediately after training and a shift back to permissive conditions 30 min before testing. One-way ANOVA of P.I.'s from all groups demonstrated no significant difference (*n* = 15 per group). Memory scores for UAS-shi[ts1] and c739;UAS-shi[ts1] were significantly different from zero (95% confidence interval for UAS-shi[ts1] = 0.103 ± 0.057, *P* < 0.025; 95% confidence interval for c739;UAS-shi[ts1] = 0.104 ± 0.079, *P* < 0.025).

through the MBs during the retrieval of olfactory memory, we performed training under permissive conditions and maintained the flies under these conditions until just before testing, at which point they were shifted to the restrictive temperature. When we examined the performance of these flies at 3 hours under these conditions, memory was abolished in the c739; UAS-shi^{ts1} flies, whereas the memory of the control groups was intact (Fig. 3B). We next examined whether the acquisition of olfactory memory shared a similar requirement for MB signaling. Here we performed training under the restrictive conditions and immediately cooled the flies to the permissive temperature. When we examined the performance of these flies at 3 hours under these conditions, we observed a difference between the c739; UAS-shi^{ts1} flies and the control line c739 but no difference between c739;UAS-shi^{ts1} and the UAS-shi^{ts1} control, indicating a general effect of heat on lines carrying the UAS-shi^{ts1} element (Fig. 3C), but no specific disruption of memory when UAS-shi^{ts1} is combined with c739. We subsequently investigated whether the interval between training and testing, during which memories are consolidated and stored, would require signaling through the MBs to observe normal memory performance at 3 hours. Flies were trained and tested under permissive conditions and given a temperature shift to restrictive conditions during the interval between these events. Under these conditions, we observed a general effect of heat on the performance of all of the lines, but we failed to detect a significant difference between any of the groups.

By transiently blocking synaptic transmission from the MBs during memory formation, consolidation, and retrieval, we were able to dissect the temporal requirements of MB signaling during the different phases of memory processing. Our results suggest quite unexpectedly that signaling through the MB α/β neurons is required during olfactory memory retrieval, but not during memory acquisition or storage. We propose that, in *Drosophila*, olfactory memory retrieval requires signaling through the α/β lobes to downstream neurons for expression. This does not preclude, however, a role for other MB lobes in memory formation, consolida-

tion, or retrieval. A recent study demonstrating the sufficiency of *rutabaga* expression in the MBs for rescue of the short-term memory defect in *rutabaga* mutants has suggested that the γ lobes might be of particular importance in the formation of short-term memories (*10*). Recent studies have also demonstrated that *fasciclinII* mutants are defective in memory acquisition and this protein is predominantly expressed in the α/β neurons, although it is expressed at lower levels in the γ lobe (*26*). One hypothesis to explain the combined observations is that memory formation occurs in the γ neurons, or in both γ and α/β neurons simultaneously, but that memory retrieval occurs principally through the output of the α/β neurons. Indeed, such a scenario involving a partial redundancy of function can explain why a subset of neurons might be sufficient, but not necessary, for memory expression. However, the observation that *rutabaga* and *fasciclinII* flies are only partially impaired in short-term memory indicates the likelihood that other mechanisms and perhaps locations of signal convergence, such as the antennal lobe or the lateral protocerebrum, may additionally mediate memory acquisition or storage. Taken together, these data suggest that acquisition and consolidation occur upstream of the MB synapse upon follower neurons, either in the MB neurons themselves or in upstream circuits. Retrieval of these memories within 3 hours would then engage signaling through a subset of the MB neurons, involving the α/β lobes. It remains to be determined whether long-term memories (>24 hours) are dependent on the MBs.

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22. We analyzed a total of nine MB GAL4 lines in our experiments. Two (238Y and 30Y) showed locomotor defects at the restrictive temperature and were discarded from further analysis. Four additional lines showed diminished odor avoidance under restrictive conditions (c309, OK107, c772, and c747) and were not analyzed further. Two lines (c739 and 247) showed disrupted memory but normal odor and shock avoidance in combination with the UAS-shi^{ts1} transgene at the restrictive temperature. The line 201Y showed normal 3-min memory in combination with the UAS-shi^{ts1} transgene under both permissive and restrictive conditions.
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