Distinct dopamine neurons mediate reward signals for short- and long-term memories

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Drosophila melanogaster **can acquire a stable appetitive olfactory memory when the presentation of a sugar reward and an odor are paired. However, the neuronal mechanisms by which a single training induces long-term memory are poorly understood. Here we show that two distinct subsets of dopamine neurons in the fly brain signal reward for short-term (STM) and long-term memories (LTM). One subset induces memory that decays within several hours, whereas the other induces memory that gradually develops after training. They convey reward signals to spatially segregated synaptic domains of the mushroom body (MB), a potential site for convergence. Furthermore, we identified a single type of dopamine neuron that conveys the reward signal to restricted subdomains of the mushroom body lobes and induces long-term memory. Constant appetitive memory retention after a single training session thus comprises two memory components triggered by distinct dopamine neurons.**

dopamine | learning and memory | *Drosophila* | mushroom body

Memory of a momentous event persists for a long time. Whereas some forms of long-term memory (LTM) require repetitive training (1–3), a highly relevant stimulus such as food or poison is sufficient to induce LTM in a single training session (4–7). Recent studies have revealed aspects of the molecular and cellular mechanisms of LTM formation induced by repetitive training (8–11), but how a single training induces a stable LTM is poorly understood (12).

Appetitive olfactory learning in fruit flies is suited to address the question, as a presentation of a sugar reward paired with odor induces robust short-term memory (STM) and LTM (6, 7). Odor is represented by a sparse ensemble of the 2,000 intrinsic neurons, the Kenyon cells (13). A current working model suggests that concomitant reward signals from sugar ingestion cause associative plasticity in Kenyon cells that might underlie memory formation (14–20). A single activation session of a specific cluster of dopamine neurons (PAM neurons) by sugar ingestion can induce appetitive memory that is stable over 24 h (19), underscoring the importance of sugar reward to the fly.

The mushroom body (MB) is composed of the three different cell types, α/β, α′/β′, and γ, which have distinct roles in different phases of appetitive memories (11, 21–25). Similar to midbrain dopamine neurons in mammals (26, 27), the structure and function of PAM cluster neurons are heterogeneous, and distinct dopamine neurons intersect unique segments of the MB lobes (19, 28–34). Further circuit dissection is thus crucial to identify candidate synapses that undergo associative modulation.

By activating distinct subsets of PAM neurons for reward signaling, we found that short- and long-term memories are independently formed by two complementary subsets of PAM cluster dopamine neurons. Conditioning flies with nutritious and nonnutritious sugars revealed that the two subsets could represent different reinforcing properties: sweet taste and nutritional value of sugar. Constant appetitive memory retention after a single training session thus comprises two memory components triggered by distinct reward signals.

Results

Distinct Subsets of Dopamine Neurons Drive Appetitive STM and LTM. We activated different sets of PAM cluster neurons by thermogenetic stimulation with a temperature-sensitive cation channel *dTrpA1* (35) in the presence of an odor and evaluated reward signals behaviorally by measuring appetitive odor memory immediately (STM) or 24 h after training (LTM). As we previously showed, *R58E02-GAL4* labels the majority of the reward-signaling PAM neurons (Fig. 1*A*) (19). Thermoactivation paired with an odor presentation in *R58E02-GAL4/UAS-dTrpA1* flies after starvation resulted in the formation of robust STM and LTM (Fig. 1 *B* and *C*). Our anatomical screen identified two additional drivers, *R48B04-GAL4* (Fig. 1*D*, Fig. S1 *C* and *G*, and Movie S1) and *R15A04-GAL4* (Fig. 1*G*, Fig. S1 *D* and *H*, and Movie S2), which target transgene expression in distinct sets of PAM cluster neurons with little overlapping expression. Those neurons were mostly tyrosine hydroxylase (TH) immunoreactive (Fig. S2). Activation of a PAM subset with *R48B04-GAL4* induced STM but not LTM (Fig. 1 *E* and *F*). Strikingly, thermoactivation of the other subset of PAM cluster neurons with *R15A04-GAL4* induced robust LTM without forming STM (Fig. 1 *H* and *I*), similar to appetitive memory without a short-term component of mutants for *TβH* (36). The temporal dynamics of these two forms of memory were

Significance

A biologically relevant event such as finding food under starvation conditions or being poisoned can drive long-term memory (LTM) in a single training session. Neuronal mechanisms by which such a strong reward or punishment induces stable memory are poorly understood. Here we show that distinct subsets of dopamine neurons signal reward for short- and long-term appetitive memories in *Drosophila*. The temporal dynamics of memory components triggered by the distinct reward signals are complementary, and together contribute to a temporally stable memory retention. Two subsets of dopamine neurons could signal different reward properties: sweet taste and nutritional value of sugar. Sugar reward is thus intricately encoded in the fly brain, given the importance of long-lasting food-related memory in survival.

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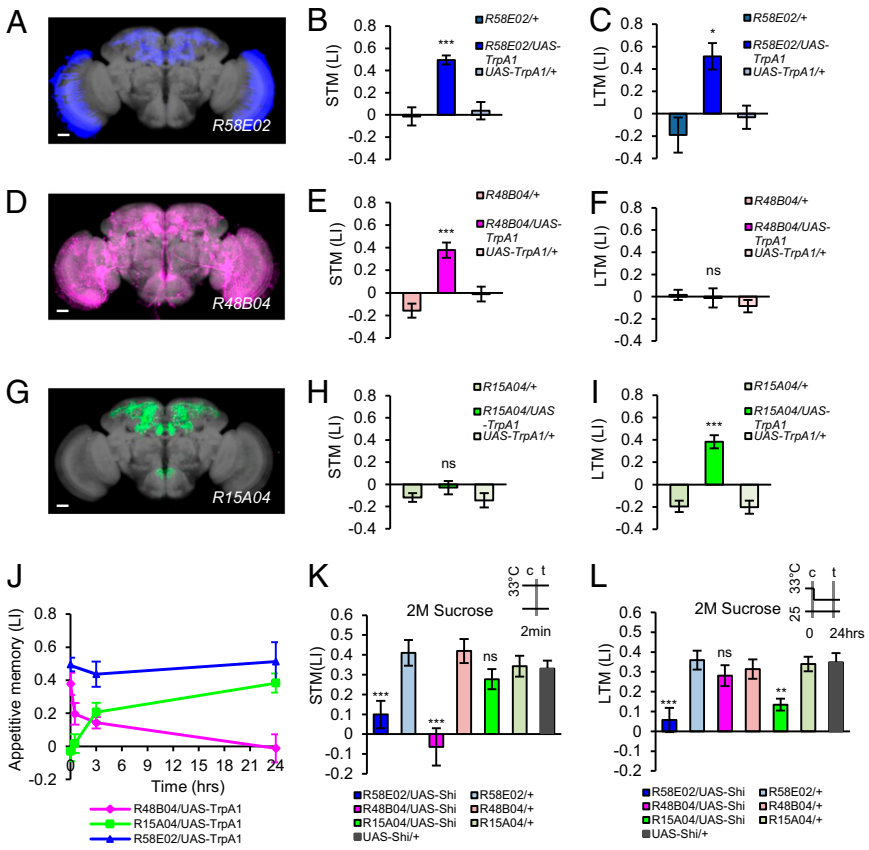

Fig. 1. Two dopamine subsets induce appetitive STM and LTM independently. (*A*) Expression pattern of *R58E02-GAL4* shown in a standardized brain. (*B* and *C*) Thermoactivation with *58E02-GAL4* tested immediately (*B*) or 24 h later (*C*). LI, learning index; STM, short-term memory; LTM, long-term memory; $n = 10–12$. (*D*) Expression pattern of *R48B04-GAL4* shown in a standardized brain. (*E* and *F*) Thermoactivation with *48B04-GAL4* tested immediately (*E*) or 24 h later (*F*). $n = 12$. (*G*) Expression pattern of *R15A04-GAL4* shown in a standardized brain. (*H* and *I*) Thermoactivation with *15A04-GAL4* tested immediately (*H*) or 24 h later (*I*). $n = 12$. (*J*) Retention of induced memory. $n = 10–19$. (*K* and *L*) Blockade of the PAM neurons in *R58E02-GAL4*, *stm-PAM-GAL4* (*R48B04-GAL4*), and *ltm-PAM-GAL4* (*R15A04-GAL4*) with *UAS-shi^{ts1}* during memory acquisition with sucrose tested immediately (*K*) or 24 h later (*L*). $n = 11–36$. c, conditioning; t, training. Results are means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.

complementary and sum roughly to the memory induced by activation of the PAM cluster neurons with *R58E02-GAL4* (Fig. 1*J*).

We controlled GAL4 expression outside the PAM neurons in *R48B04-GAL4* and *R15A04-GAL4* using *R58E02-GAL80*, which suppresses transgene expression in the PAM cluster neurons (Fig. S1) (19). The addition of *R58E02-GAL80* abrogated memory induction in both cases (Fig. S3 *A* and *B*) and thus localized the reward signal to the respective PAM neurons. These results suggest that appetitive reinforcement can be functionally broken down into two complementary dopamine signals within the PAM cluster. We here refer to these two neuronal subsets as stm-PAM and ltm-PAM, respectively.

Stm- and ltm-PAM Neurons Are Differentially Required for Sugar-Induced STM and LTM. To examine whether STM and LTM of the sugar reward respectively require the stm- and ltm-PAM neurons, we targeted the expression of *shibire^{ts1}* (*shi^{ts1}*) (37) to the respective subsets and blocked their outputs during associative training. Inactivation of a majority of the PAM neurons with *R58E02-GAL4* impaired both STM and LTM, whereas inactivation with *R48B04-GAL4* and *R15A04-GAL4* caused selective defects in STM and LTM, respectively (Fig. 1 *K* and *L*). The impaired memory caused by inactivating *R48B04-GAL4* and *R15A04-GAL4* was fully restored by blocking GAL4 activity in the respective PAM neurons using *R58E02-GAL80* (Fig. S4). These data demonstrate that a single presentation of sugar triggers parallel reward signals of stm-PAM and ltm-PAM and suggest that constant appetitive memory of sugar is a composite of two separable elements.

Input and Output Sites of stm- and ltm-PAM Neurons Are Spatially Segregated. The PAM cluster neurons have presynaptic terminals in the MB (18, 19). The *R48B04-GAL4* and *R15A04-GAL4* target largely distinct dopamine neurons with the exception of overlapping expression in the neurons projecting to the γ5 subdomain (Fig. 2 *A* and *B*). To compare the detailed projections of the stm- and ltm-PAM neurons, we applied a nonrigid transformation to individual confocal stacks and computationally registered them onto a standardized average brain. The target regions of the stm-PAM are enriched in the medial region of the MB, whereas the dendrites of these two dopamine subsets in the protocerebrum are also largely segregated (Fig. 2 *E–G*). Interestingly, computational subtraction of one expression pattern from the other revealed that dendritic processes unique to stm- and ltm-PAM neurons are differentially clustered to the medial and lateral protocerebrum, respectively (Fig. 2 *H* and *I*). These results suggest that the stm- and ltm-PAM neurons receive distinct synaptic inputs and differentially modulate the local circuits of the MB.

Stm- and ltm-PAM Neurons Are Differentially Required for Appetitive Memories of Sweetness and Nutritious Sugars. The nonnutritious sugar arabinose reportedly induces only STM but not LTM when used alone, but induces LTM when combined with sorbitol,

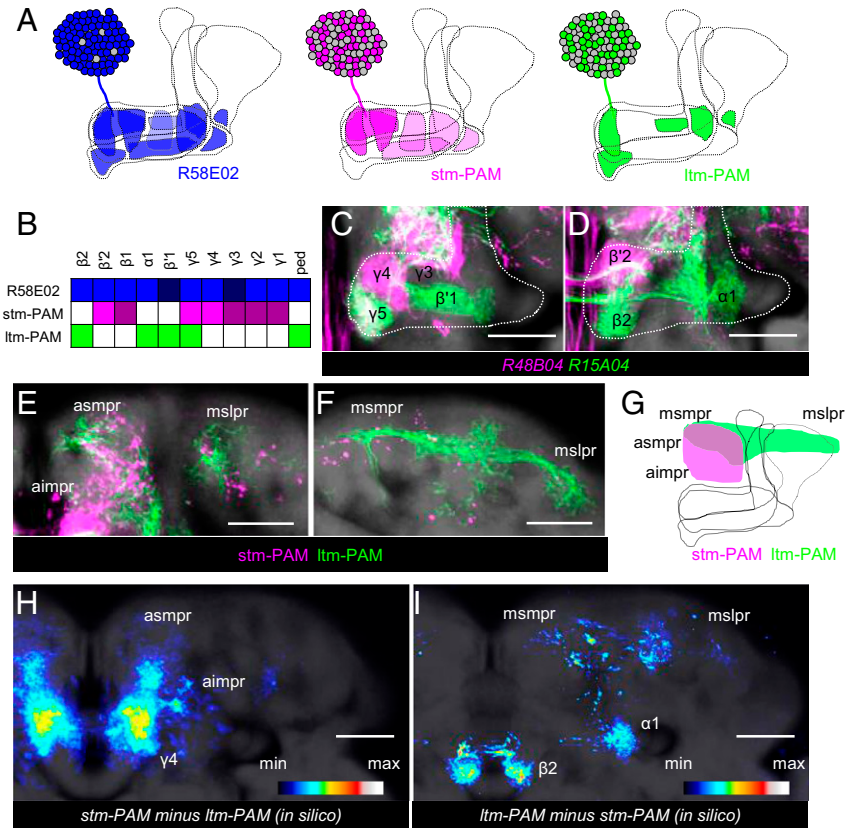

Fig. 2. Complementary expression patterns of stm- and ltm-PAM neurons. (*A*) Schematic drawings of expression patterns of *R58E02-GAL4*, stm-PAM, and ltm-PAM neurons (Fig. S5) in the MB. (*B*) The MB subdomains innervated by the three lines. Dark blue in *R58E02* and dark magenta in stm-PAM indicates weak GFP expression. (*C* and *D*) Expression patterns of *R48B04-GAL4* (magenta) and *R15A04-GAL4* (green) neurons visualized in different MB subdomains of a standardized brain. Outline, MB. (*E* and *F*) Stm-PAM (magenta) and ltm-PAM (green) neurons visualized in a standardized brain at different protocerebrum regions. The *R48B04-GAL4* and *R15A04-GAL4* expression patterns were refined with *R58E02-LexA* GRASP (Fig. S5). In the course of this processing, expression pattern of some cell types got weaker (e.g., β′1 neurons in the ltm-PAM). (*G*) Schematic drawing of innervation areas by the two PAM groups in the protocerebrum. (*H* and *I*) Image subtraction of ltm-PAM from stm-PAM (*H*) and stm-PAM from ltm-PAM (*I*). To improve the signal-to-noise ratio of the signals, averaged expression patterns (n = 14 for stm-PAM, 9 for ltm-PAM) are used. (Scale bars, 20 μm.) Aimpr, anterior inferiormedial protocerebrum; asmpr, anterior superiormedial protocerebrum; mslpr, middle superiorlateral protocerebrum; and msmpr, middle superiormedial protocerebrum (27).

a tasteless nutritious sugar (38, 39). This prompted us to examine whether the stm- and ltm-PAM neurons mediate different properties of the sugar reward, such as sweetness and nutrition. We separately blocked the stm- and ltm-PAM neurons while training flies with arabinose with or without additional sorbitol, and measured the flies' STM and LTM. Blocking the stm- but not ltm-PAM neurons impaired arabinose STM, whereas LTM of the combined sugars selectively required ltm-PAM neurons (Fig. 3 *A* and *B*). Consistent with this result, the genomic DNA fragment in *R48B04-GAL4* is derived from the *Drosophila* octopamine receptor gene (40), which mediates the sweet taste and water reward but not nutrition (18, 41).

Sugar ingestion leads to fast elevation of hemolymph fructose level, which may represent nutritional value. Because fructose is sensed by its receptor Gr43a in the brain (42), we blocked Gr43a-expressing neurons (Fig. S6*A*) during sucrose training and examined STM and LTM. Indeed, this blockade specifically impaired LTM but not STM formation (Fig. 3 *C* and *D* and Fig. S6*B*). Intriguingly, the projections of the Gr43a neurons are confined to the lateral protocerebrum, in close proximity to the dendrites of ltm-PAM (Fig. 3*E*, Fig. S6 *C* and *D*, and Movie S3) but not stm-PAM neurons (Fig. 3*F*). We imaged calcium responses of these two subsets to sucrose, arabinose, and the mixture of arabinose and sorbitol. Interestingly, the representation of arabinose in the PAM neurons was similar to that of sucrose (Fig. S7 *A*–*D*), although the intensity was generally lower and off responses were more visible for sucrose. There was no obvious difference in response profiles of two subsets of PAM neurons. As dopaminergic modulation of cAMP drives nonlinear plasticity across the MB lobes (43), it is perhaps not surprising that sugar responses are widely distributed beyond the PAM subsets.

Prediction of Individual Dopamine Neurons for LTM Formation by Combined Behavioral and Anatomical Screens. We next sought to identify individual reward neurons by examining the correlation between induced memory performances and GAL4 expression in all cell types of PAM cluster neurons. To this end, we used a collection of 20 Split-GAL4 driver lines with specific expression in different PAM cluster neurons; the construction and detailed anatomical analysis of these lines are described in Aso et al. (33, 34). We performed a behavioral screen of thermoactivation in starved flies with this driver collection and measured the formation of STM and LTM. The performances of STM and LTM among these 20 drivers did not show any significant correlation (Fig. 4*A*), providing further evidence for independent reward neurons for STM and LTM. For each cell type, we performed a linear regression analysis between induced memory performances and GAL4 expression levels, estimated by GFP fluorescence in corresponding MB subdomains, of the 20 drivers (Fig. S8). Although some GAL4 lines induced robust STM, no cell type was significantly correlated with STM performances, implying

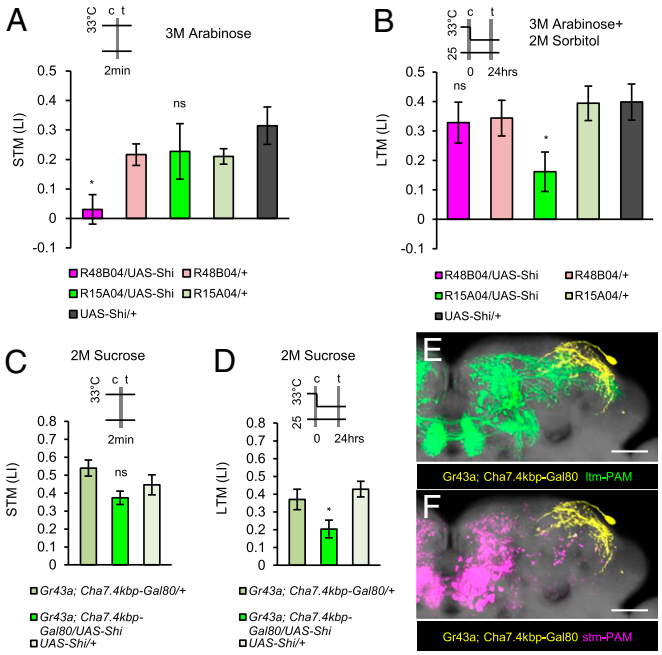

Fig. 3. Differential processing of reinforcing properties of sugar sweetness and nutrition. (*A*) Blocking stm-PAM but not ltm-PAM neurons during conditioning with arabinose significantly impairs appetitive STM. $n = 6–18$. (*B*) Blocking ltm-PAM but not stm-PAM neurons during conditioning with arabinose supplemented with sorbitol significantly impairs appetitive LTM. $n = 11–20$. c, conditioning; t, training. (*C* and *D*) Blockade of Gr43a; $Cha^{7.4kbp}$-GAL80 neurons during memory acquisition with sucrose tested immediately (*C*) or 24 h later (*D*). $n = 11–20$. (*E* and *F*) Overlapped expression patterns of Gr43a; $Cha^{7.4kbp}$-GAL80 neurons (yellow) with ltm-PAM neurons (green, *E*) but not with stm-PAM neurons (magenta, *F*) in the standardized brain. (Scale bars, 20 μm.) Results are means ± SEM. $*P < 0.05$; ns, not significant.

collective reward signals for STM (Fig. 4 *B*, *D*, and *E* and Fig. S8*A*). This also implies undefined anatomical connectivity of some PAM neurons and the input neurons, as in the connectivity between the projection neurons and the Kenyon cells (44, 45). In contrast, the regression analysis with LTM performances identified significant positive association with the dopamine neurons projecting to the base of the α-lobe (PAM-α1) (Fig. 4 *C*, *D*, and *F* and Fig. S8*B*). PAM-α1 was consistently labeled in the top 5 drivers that induced significant LTM (Fig. S8*B*). The PAM neurons terminating in the tip and shaft of the β-lobe (Fig. 4 *D* and *F*) also appeared to be correlated with LTM, suggesting the contributions of other cell types to the reward signal for LTM (18). Thus, PAM-α1 represents an essential component for the reward signal for LTM, whereas different and partially redundant dopamine neurons might collectively signal reward for STM.

PAM-α1 Neurons Are Necessary and Sufficient for LTM Formation. Using one of the Split-GAL4 drivers labeling PAM-α1 (MB299B; Fig. 4*G*), we analyzed morphology and reward function. This class of dopamine neurons consists of approximately five cells and has the lateral dendrites that are characteristic of the ltm-PAM neurons (Fig. 4*G*). The blockade of the PAM-α1 neurons during the formation of appetitive memory revealed a specific impairment of LTM but not STM (Fig. 4 *H* and *I*). Furthermore, the blockade did not significantly impair STM of arabinose conditioning (Fig. 4*J*), whereas these dopamine neurons were absolutely necessary for the acquisition of LTM of arabinose supplemented with sorbitol (Fig. 4*K*). In addition, thermoactivation of MB299B in starved flies revealed sufficiency of these neurons for LTM (Fig. S9). We thus conclude that a specific class of dopamine neurons that project to the base of the α-lobe is necessary and sufficient to signal reward for LTM, potentially upon the input of nutrition (Fig. 4*L*).

Discussion

Distinct Reward Signals for STM and LTM. In *Drosophila*, sugar ingestion in a single training session induces stable appetitive odor memory. Our results showed that observed memory represents the composite of STM and LTM that are induced by the distinct and complementary reward signals of dopamine. These separate reward signals very well corroborate parallel processing of STM and LTM in the MB (22). The stm-PAM neurons induce appetitive memory that decays within hours, whereas memory by ltm-PAM gradually develops after training. These dopamine neurons convey reward signals to spatially segregated synaptic domains of the MB, whereas the other PAM cluster neurons may also contribute to LTM reward. Furthermore, we identified a single type of dopamine neurons (PAM-α1) encoding a reward signal for LTM. The PAM-α1 targets a spatially restricted subdomain in the α-lobe of the MB, suggesting local associative modulation in the α-lobe. These results indicate that sugar ingestion activates multiple reward signals of different qualities to form complementary memories, rather than a single reward system forming STM that later transforms into LTM.

In the defensive siphon and tail withdrawal reflex of *Aplysia*, different modes of 5-HT application to the sensory neurons in the pedal and pleural ganglion differentially induce short-term and long-term sensitization memories (46, 47). As both ganglia are innervated by a single identified serotonergic neuron (48), it is likely that the tail shock activates the same cell to induce both forms of sensitization independently. This cellular configuration is in strong contrast to the reward system in *Drosophila* appetitive memory, despite parallel formation of STM and LTM in both systems. The sugar reward may be more intricately encoded in the fly, given the importance of long-lasting food-related memory in survival.

Representations of different reinforcing stimuli by the same transmitter seems to be variable. In *Drosophila* aversive memory, reinforcing signals of electric shock and heat punishment converge to the same dopamine neurons (49), whereas distinct dopamine neurons are recruited for different aversive stimuli in mammals (50). Appetitive memory of *Drosophila* is closer to the latter case. However, a critical difference is that stm- and ltm-PAM neurons seem to signal different properties of the same reward, again pointing to more complex representation of the sugar reward. It would be interesting to compare neuronal representations of different rewarding stimuli, such as ethanol (51) and water (41, 52). An important future question would be to understand physiological mechanisms by which the MB computes the distinct dopamine inputs to control approach behavior.

Sequential Regulation by Taste and Nutrition. Memory induced by ltm-PAM develops gradually after training (Fig. 1). This gradual increase may reflect the time to implement learning-dependent molecular changes. Many molecules that are specifically required for LTM have been identified, and some of these molecules are involved in learning-dependent gene transcription/translation (3, 53–57). For instance, fasting-dependent LTM that is formed without training repetition requires CREB-regulated transcription coactivator (CRTC)-mediated cAMP response element binding protein (CREB) transcription in the MB (12). Such a transcription-dependent mechanism takes time and may thus underlie gradual formation of memory induced by the ltm-PAM.

The complementary memory dynamics as a consequence of the distinct reward signals suggest that the "intent" of appetitive memory undergoes a transition from palatability to caloric content. Similar temporal transitions have been found in feeding choice (58), where flies initially choose sugars according to sweet taste, but

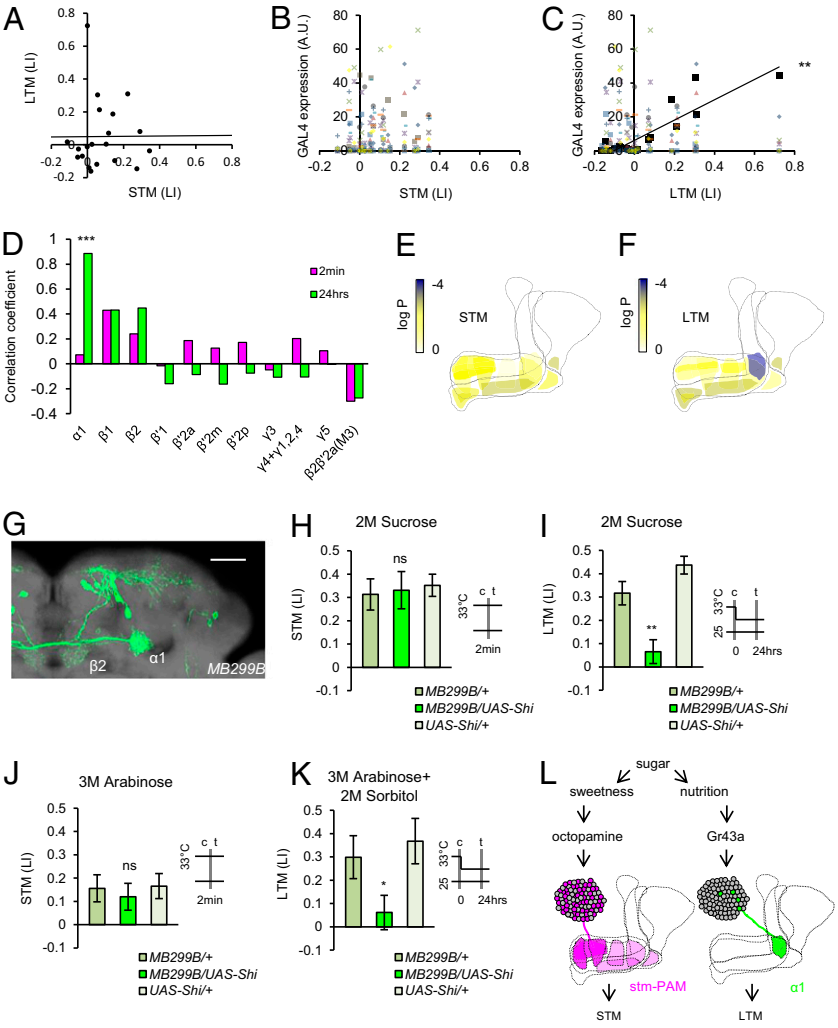

Fig. 4. PAM-α1 neurons are necessary and sufficient for appetitive LTM formation. (*A*) Correlation of STM and LTM scores. For each SplitGAL4 line, LTM score induced by thermoactivation is plotted against STM score. Linear regression is overlaid. Significance of the correlation was tested by bootstrap, revealing no association between STM and LTM ($n = 20$). (*B* and *C*) GAL4 expression levels in each PAM cell type plotted against learning indices of appetitive STM (*B*) or LTM (*C*) of all 20 SplitGAL4 lines. Different colors and markers indicate different cell types. Linear regression is shown together for PAM-α1, which marked significant positive association with LTM (*C*). (*D*) Correlation coefficient of GAL4 expression level versus STM and LTM in each cell type. (*E* and *F*) Color map of logarithm of *P* values calculated from correlation analyses of GAL4 expression and learning indices of STM (*E*) or LTM (*F*). (*G*) Expression pattern of *MB299B-GAL4* shown in a standardized brain. (Scale bar, 20 μm.) (*H* and *I*) Blockade of the PAM-α1 neurons by *MB299B-GAL4* during memory acquisition with sucrose tested immediately (*H*) or 24 h later (*I*). $n = 6$–12. c, conditioning; t, training. Results with error bars are means ± SEM. $^{**}P < 0.01$; ns, not significant. (*J* and *K*) Blockade of the PAM-α1 neurons has no significant effects during acquisition of arabinose memory tested immediately (*J*) but impairs formation of 24-h memory induced by arabinose supplemented with sorbitol (*K*). (*L*) Model of a reward circuit in the fly brain. Reinforcement properties of sugar sweetness and nutrition are independently conveyed by octopamine or Gr43a neurons to distinct subclusters of the PAM cluster. The stm-PAM neurons collectively mediate reinforcement signal of STM. In contrast, the reinforcement signal of LTM is mainly conveyed by the PAM-α1 neurons.

later prioritize caloric contents. Similarly, ethanol exposure initially acts as an aversive reinforcer, but eventually turns into reward and induces LTM (51). The sequential regulation of appetitive behavior by the same stimulus may be conserved across relevant appetitive stimuli. As palatability is not always a faithful predictor of its nutritional value, it may be a general design of reward systems to balance short-term benefit and long-term fitness.

Materials and Methods

The two GAL4 drivers, *R48B04* and *R15A04*, were identified by screening our confocal image database for PAM cluster neurons (40). Appetitive conditioning and reward substitution with thermoactivation were performed according to previously described protocols of differential conditioning (19, 29, 30). A group of flies received 1 min of odor exposure with sugar reward or temperature-sensitive cation channel (dTrpA1)-mediated thermoactivation of GAL4-expressing neurons, followed by a 1-min presentation of another odor in the absence of reward or temperature elevation. Most of the data did not violate the assumption of the normal distribution and the homogeneity of variance and were therefore subjected to parametric statistics. For data that significantly deviated from the normal distribution (Figs. 3*B* and 4*I*), nonparametric statistics were applied. Fluorescent immunohistochemistry was performed as described previously (19, 29, 30). All of the confocal images in the main figures underwent landmark matching-based image registration using BrainAligner (59). For in vivo calcium imaging, female flies expressing GCaMP3 (60) with *R48B04-GAL4* and *R15A04-GAL4* were prepared essentially as described (19, 61). A droplet of 2 M sucrose, 3 M arabinose, or a mixture of 3 M arabinose and 2 M sorbitol solution in mineral water was delivered on a plastic plate controlled by a micromanipulator (19). See *SI Materials and Methods* for more detailed experimental procedures.

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