Research

Requirement of the combination of mushroom body γ lobe and α/β lobes for the retrieval of both aversive and appetitive early memories in *Drosophila*

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Extensive studies of *Drosophila* mushroom body in formation and retrieval of olfactory memories allow us to delineate the functional logic for memory storage and retrieval. Currently, there is a questionable disassociation of circuits for memory storage and retrieval during *Drosophila* olfactory memory processing. Formation of the initial aversive olfactory memory involves mushroom body γ lobe, whereas α/β lobes are reported to be necessary for the retrieval of such memory. In contrast, formation and retrieval of the short-term appetitive olfactory memory appears to involve γ lobe. With the help of newly identified Gal4 lines and of focusing on 3-h memory for both aversive and appetitive conditionings, our reexamination of the retrieval of aversive and appetitive olfactory memories suggests a new view. Blocking γ lobe output led to severe deficiency of aversive early memory retrieval and partial impairment of appetitive early memory retrieval. Interrupting α/β lobe output impaired the retrieval of both aversive and appetitive early memories. The contribution of the γ lobe and α/β lobes appeared to be additive for the retrieval of appetitive early memory. Thus, these results suggest that the retrieval of aversive and appetitive olfactory early memories requires the synaptic outputs from both γ lobe and α/β lobe neurons. This discovery may help us to rethink how aversive and appetitive memories are processed from memory formation to memory retrieval.

[Supplemental material is available for this article.]

Characterizing the neural circuits involved in memory retrieval is crucial for understanding memory processing within an intact neural circuit (Silva et al. 2009; van Strien et al. 2009), as well as the pathological basis of various neurological diseases associated with memory deficiency (Kuhlmann et al. 2005; Small et al. 2011; Tye and Deisseroth 2012). *Drosophila* memory studies have allowed impressive progress in elucidating functional neural circuits and evolutionarily conserved molecular basis underlying memory processing (Davis 2005; Margulies et al. 2005; Keene and Waddell 2007; Bellen et al. 2010; Zars 2010).

Drosophila can associate odor stimulus with electric-shock punishment or sugar reward to form aversive or appetitive olfactory memory (Tempel et al. 1983; Tully and Quinn 1985). Mushroom body (MB) plays a central role in olfactory memory formation and retrieval in *Drosophila* (Davis 2005; Margulies et al. 2005; Keene and Waddell 2007). It consists of three substructures: α/β lobe neurons, α'/β' lobe neurons, and γ lobe neurons (Tanaka et al. 2008; Aso et al. 2009). Previous understanding indicates the usage of distinct MB neural ensembles to retrieve aversive and appetitive early phase memories, including short-term memory (STM) and intermediate-term memory (ITM). α/β lobes are reported to be involved in aversive memory retrieval (McGuire et al. 2001; Krashes et al. 2007), while γ lobe appears to be necessary for appetitive memory retrieval (Trannoy et al. 2011).

However, such understanding leads to a questionable disassociation of circuits between storage and retrieval of aversive memo-

ry in MB, because previous experiments suggest γ lobe, but not α/β lobes, is essential for formation of initial aversive memory (Blum et al. 2009; Qin et al. 2012). In MB, appetitive STM formation and retrieval all seem to be confined to γ lobe, while α/β lobes are dispensable (Trannoy et al. 2011).

In mammalian brain, memory can be retrieved through at least two neuronal ensembles (Takahashi et al. 2008; Goshen et al. 2011; Suzuki and Naya 2011). Since MB shares common origin with vertebrate pallidum (Tomer et al. 2010), as well as conserved molecular basis underlying memory processing (Davis 2005; Margulies et al. 2005; Keene and Waddell 2007), it will be meaningful to investigate whether the strategy of using at least two neuronal ensembles to retrieve memory is conserved in *Drosophila* MB.

To test this hypothesis, the Gal4 lines strongly and preferentially labeling γ lobe neurons are needed to examine the roles of γ lobe neurons in aversive early memory retrieval (Aso et al. 2009). Stable appetitive memory performance and additional α/β lobe Gal4 lines are required to test the role of α/β lobe neurons in appetitive early memory retrieval (Trannoy et al. 2011).

We started with screening Gal4 lines reported in the literature, which preferentially label MB γ lobe or α/β lobe neurons. Using the well-developed genetic tools in *Drosophila*, we can acutely manipulate the neuronal activity of each MB substructure with good spatial and temporal resolution. Through this approach, we examined the role of each MB substructure during the retrieval of aversive and appetitive 3-h memories. On this basis, these studies may help us to rethink how aversive and appetitive memories are processed in the example of olfactory learning and memory center in *Drosophila*.

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Results

The Gal4 lines used in labeling MB substructures

MB α/β lobe neurons and olfactory projection neurons can affect the retrieval of olfactory memories (McGuire et al. 2001; Krashes et al. 2007) or the transmission of olfactory information (Masse et al. 2009). So in order to explicitly investigate the roles of γ lobe neurons in olfactory conditionings, the Gal4 used to label γ lobe should satisfy the following requirements: (1) it has no expression in other MB substructures and olfactory projection neurons; (2) its expression in γ lobe is strong; and (3) inhibiting its Gal4 activity specifically in MB should reverse the phenotypes driven by it. Here, we identified two Gal4 lines strongly and preferentially labeling MB γ lobe, *5-HT1B-Gal4* (Yuan et al. 2005) and *R92F10-Gal4* (Pfeiffer et al. 2008; Jenett et al. 2012), which have not been used in learning and memory studies yet.

We crossed *uas-mCD8::GFP;MB247-DsRed* flies with each Gal4 line we screened to visualize its expression pattern and its colocalization with MB. *MB247-DsRed* induces DsRed expression specifically in MB neurons (Lee and Luo 1999; Riemensperger et al. 2005). The expression pattern of *mCD8::GFP* demonstrated that *5-HT1B-Gal4* strongly and specifically labeled the horizontally located γ lobe in MB, without any expression in α/β lobes and α'/β' lobes (Fig. 1A) (Yuan et al. 2005). In *MB-Gal80;5-HT1B-Gal4* flies, the expression of Gal80 in MB (Lee and Luo 1999; Krashes et al. 2007) specifically inhibited the Gal4 activity of *5-HT1B-Gal4* in γ lobe in MB, while the limited expression of *5-HT1B-Gal4* in neurons outside MB was not affected (Fig. 1B). Anti-GFP labeling in previous experiments also supports the intensity and preference of *5-HT1B-Gal4* expression in γ lobe neurons in MB (Yuan et al. 2005). *R92F10-Gal4* is a new Gal4 line strongly and preferentially labeling MB γ lobe neurons (Supplemental Fig. S1; Pfeiffer et al. 2008; Jenett et al. 2012).

C739 and *C305a* are commonly used MB Gal4 lines preferentially expressed in α/β lobes and α'/β' lobes in MB, respectively (McGuire et al. 2001; Yu et al. 2006; Krashes et al. 2007; Aso et al. 2009; Blum et al. 2009; Buchanan and Davis 2010; Lee et al. 2011; Huang et al. 2012). *NP3061* and *NP6024* are another two Gal4 lines preferentially labeling MB α/β lobe neurons (Tanaka et al. 2008; Aso et al. 2009; Huang et al. 2012).

Figure 1. *5-HT1B-Gal4* strongly and preferentially labels MB γ lobe neurons. The expression of each Gal4 driver is denoted by the pattern of *mCD8::GFP* and its colocalization with MB specifically expressed DsRed protein (MB-DsRed). (A) *5-HT1B-Gal4* strongly and restrictively drives the expression of *mCD8::GFP* in the horizontally located γ lobe, without any expression in α/β and α'/β' lobes. (B) *MB-Gal80;5-HT1B-Gal4* drives no *mCD8::GFP* expression in γ lobe. The limited expression of *5-HT1B-Gal4* in the neurons outside MB is not inhibited by *MB-Gal80*. Scale bar, 50 μ m.

Figure 2. Blocking the output from γ lobe or α/β lobe neurons impairs the retrieval of aversive 3-h memory. (A) When constantly under the permissive temperature of 23°C, all genotypes showed normal aversive 3-h memory performances. (B) Blocking the output from *5-HT1B-Gal4* labeled γ lobe neurons under the restrictive temperature of 33°C severely impaired aversive 3-h memory retrieval. Blocking the output from *C739* labeled α/β lobe neurons also impaired memory retrieval. Output from *C305a* labeled α'/β' lobe neurons was dispensable for aversive 3-h memory retrieval. (C) Under the permissive temperature, aversive 3-h memory performance was normal in all genotypes. (D) Inhibiting the Gal4 activity of *5-HT1B-Gal4* or *C739-Gal4* in MB by *MB-Gal80* reversed the deficiencies of aversive 3-h memory retrieval. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

Blocking the synaptic output from γ lobe or α/β lobe neurons impairs the retrieval of aversive 3-h memory

Aversive and appetitive 3-h memories formed after one-session training are typical memory phases (intermediate-term memory) for studying different processes of olfactory memory processing, including memory acquisition, consolidation, and retrieval (McGuire et al. 2001; Keene et al. 2006; Krashes et al. 2007; Pitman et al. 2011; Berry et al. 2012).

shibire^{ts1} is a dominant temperature-sensitive allele of dynamin (Kim and Wu 1990; Kitamoto 2001; Kasuya et al. 2009). Under restrictive temperature, synaptic outputs are acutely blocked in those neurons expressing *Shibire^{ts1}* (Kim and Wu 1990; Kitamoto 2001; Kasuya et al. 2009). Here, we expressed *Shibire^{ts1}* in each MB substructure driven by different Gal4 lines. Under the permissive temperature of 23°C, all the genotypes showed normal aversive 3-h memory performances (Fig. 2A,C). When the output from *5-HT1B-Gal4* labeled neurons was blocked under the restrictive temperature of 33°C, aversive 3-h memory retrieval was severely impaired (Fig. 2B). Besides, inhibiting the Gal4 activity in MB by *MB-Gal80* rescued this memory deficiency (Fig. 2D), which further attested to the role of γ lobe in aversive memory retrieval. In addition, *5-HT1B-Gal4/uas-shi* and *MB-Gal80/+;5-HT1B-Gal4/uas-shi* flies showed normal odor acuity and shock acuity under both permissive and restrictive temperatures (Supplemental Table S1).

Figure 3. Interrupting the neuronal transmission from the neurons labeled by three other γ lobe Gal4s also impairs aversive 3-h memory. (A) Aversive 3-h memory performance was normal in all genotypes under the permissive temperature. (B) Blocking the output from *R92F10-Gal4* labeled neurons severely inhibited the retrieval of aversive 3-h memory. Aversive 3-h memory performance was reduced to near zero. Inhibiting the Gal4 activity of *R92F10-Gal4* in MB by *MB-Gal80* rescued this memory retrieval deficiency. (C) All genotypes had normal aversive 3-h memory performances under the permissive temperature. (D) Aversive 3-h memory was impaired when the neuronal transmission was interrupted in those neurons labeled by another two γ lobe Gal4s: *NP1131*, *1471*. *NP1131/+;uas-shi/+* and *1471/+;uas-shi/+* flies showed significantly lower memory performance than control groups. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

Consistent with previous studies, blocking the output from *C739* labeled α/β lobe neurons also impaired aversive 3-h memory retrieval (Fig. 2B,D; McGuire et al. 2001; Krashes et al. 2007). Output from α'/β' lobe neurons was dispensable for aversive 3-h memory retrieval (Fig. 2B; Krashes et al. 2007). In accordance with aversive 3-h memory, blocking the γ lobe or α/β lobe output also impaired the aversive immediate memory (Supplemental Fig. S2A) and aversive 1-h memory retrieval (Supplemental Fig. S3).

To further testify the role of γ lobe in aversive memory retrieval, we also examined another three γ lobe Gal4s: *R92F10-Gal4*, *NP1131*, and *1471*. *R92F10-Gal4* is strongly and preferentially expressed in MB γ lobe neurons (Supplemental Fig. S1; Pfeiffer et al. 2008; Jenett et al. 2012). *NP1131* and *1471* are preferentially expressed in γ lobe, although their Gal4 expression in MB is not strong enough (Aso et al. 2009). Blocking the output from *R92F10-Gal4* labeled neurons also severely interrupted the retrieval of aversive 3-h memory, while inhibiting its Gal4 activity in MB rescued this memory retrieval deficiency (Fig. 3B). Interrupting the neuronal transmission from *NP1131* or *1471* labeled neurons also impaired aversive 3-h memory retrieval (Fig. 3D).

In conclusion, in MB, aversive 3-h memory is retrieved through the combination of γ lobe and α/β lobe neurons. The crucial role of γ lobe in aversive early memory retrieval is confirmed.

Only γ lobe neurons can support the Rutabaga-dependent aversive 3-h memory

Rutabaga is suggested to be the coincidence detector mediating olfactory associative memory formation (Tempel et al. 1983; Tully and Quinn 1985; Han et al. 1992; Levin et al. 1992; Zhong and Wu 1993; Zars et al. 2000a,b; McGuire et al. 2003; Mao et al.

2004; Tomchik and Davis 2009; Gervasi et al. 2010). Although the unconditional stimulus relayed by dopaminergic neurons is mediated by γ lobe in MB (Aso et al. 2012; Burke et al. 2012; Liu et al. 2012; Qin et al. 2012; Waddell 2013), it has not been reported whether γ lobe neurons alone could support the Rutabaga-dependent aversive 3-h memory (Blum et al. 2009). Since output from γ lobe neurons is crucial for the retrieval of aversive 3-h memory (Figs. 2, 3), it is still possible that γ lobe neurons can support the Rutabaga-dependent aversive 3-h memory, besides aversive immediate memory (Zars et al. 2000a; Akalal et al. 2006; Blum et al. 2009) and appetitive STM (Trannoy et al. 2011).

rut²⁰⁸⁰ is a loss of function allele of the *rutabaga* gene (Han et al. 1992; Thum et al. 2007; Blum et al. 2009). Here, we restored the Rutabaga protein expression in *rut²⁰⁸⁰* mutants driven by different MB Gal4s. Consistent with our speculation, restoring Rutabaga expression in *5-HT1B-Gal4* labeled neurons could sufficiently rescue the deficiency of aversive 3-h memory in *rut²⁰⁸⁰* mutants (Fig. 4). Meanwhile, inhibiting its Gal4 activity in MB reversed this rescue phenotype (Fig. 4), which further testified the role of γ lobe neurons in supporting aversive memory formation. In addition, MB α'/β' lobe and α/β lobe neurons could not support the Rutabaga-dependent aversive 3-h memory (Fig. 4; Thum et al. 2007; Blum et al. 2009).

Therefore, γ lobe neurons are the only MB substructure that could support the Rutabaga-dependent aversive 3-h memory. We revealed the consistency between aversive memory formation and retrieval. The Rutabaga-dependent aversive memory is originally formed or stored in MB γ lobe neurons. The retrieval of this memory requires the synaptic output from MB γ lobe besides recruiting the output from α/β lobes.

Blocking the synaptic output from γ lobe or α/β lobe neurons partially impairs the retrieval of appetitive 3-h memory

We then tested whether the retrieval of appetitive 3-h memory also required the output from both γ lobe and α/β lobe neurons. Under the permissive temperature, all genotypes showed normal appetitive 3-h memory performances (Fig. 5A,C). Both *5-HT1B-Gal4/uas-shi* and *C739/+;uas-shi/+* flies showed partial deficiencies of appetitive 3-h memory retrieval under restrictive temperature (Fig. 5B). Besides, *MB-Gal80* expression inhibited these memory retrieval deficiencies (Fig. 5D) which further testified the role of γ lobe and α/β lobe neurons in appetitive early memory retrieval. In addition, *5-HT1B-Gal4/uas-shi* and *C739/+;uas-shi/+* flies showed normal odor acuity and sugar response under both permissive and restrictive temperatures (Supplemental Table S1). Consistent with aversive 3-h memory retrieval (Figs. 2, 3; Krashes et al. 2007), blocking the output from α'/β' lobe

Figure 4. Only γ lobe neurons could support the Rutabaga-dependent aversive 3-h memory in MB. The memory deficiency in *rut²⁰⁸⁰* mutants could only be rescued by expressing *uas-rut* in *5-HT1B-Gal4* labeled γ lobe neurons, but not in α/β lobe and α'/β' lobe neurons labeled by *C739-Gal4* and *C305a-Gal4*. Inhibiting the Gal4 activity in MB by *MB-Gal80* reversed the rescue phenotype driven by *5-HT1B-Gal4*. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

Figure 5. γ lobe and α/β lobe neurons are both required for the retrieval of appetitive 3-h memory. (A) When constantly under the permissive temperature, all genotypes showed normal appetitive 3-h memory performances. (B) Only blocking the output from *5-HT1B-Gal4* labeled γ lobe or *C739-Gal4* labeled α/β lobes impaired appetitive 3-h memory retrieval. (C) Appetitive 3-h memory performance was normal in all genotypes when constantly under the permissive temperature. (D) Inhibiting the Gal4 activity of *5-HT1B-Gal4* or *C739-Gal4* in MB by MB-Gal80 rescued the deficiencies of appetitive 3-h memory retrieval. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

neurons did not affect appetitive 3-h memory retrieval (Fig. 5B; Trannoy et al. 2011). Interrupting the γ lobe or α/β lobe output also impaired the retrieval of appetitive immediate memory (Supplemental Fig. S2B) and appetitive 1-h memory (Supplemental Fig. S4).

To further confirm the role of α/β lobe neurons in appetitive memory retrieval, we examined another two α/β lobe Gal4 lines: *NP3061* and *NP6024* (Tanaka et al. 2008; Aso et al. 2009). These two Gal4s specifically label α/β lobe neurons in MB (Tanaka et al. 2008; Aso et al. 2009). Acutely blocking the output from these two Gal4 labeled neurons also impaired the retrieval of appetitive 3-h memories (Fig. 6B).

In conclusion, the retrieval of appetitive 3-h memory also requires the synaptic outputs from both γ lobe and α/β lobe neurons. The role of α/β lobe neurons in appetitive early memory retrieval is confirmed.

The retrieval of appetitive and aversive 3-h memories is completely abolished when the outputs from γ lobe and α/β lobe neurons are blocked simultaneously

Blocking the output from γ lobe neurons severely impaired the retrieval of aversive 3-h memory (Figs. 2, 3), but from either γ lobe or α/β lobes just partially impaired the retrieval of appetitive 3-h memory (Figs. 5, 6). This may due to the possibility that the contribution of γ lobe and α/β lobe neurons to appetitive early memory retrieval is additive so that γ lobe or α/β lobe neurons alone

could partially support the retrieval of appetitive memory. We therefore generated a driver line *C739-Gal4; 5-HT1B-Gal4*, which labeled both γ lobe and α/β lobe neurons (Fig. 7A). As speculated, the retrieval of appetitive 3-h memory was completely abolished when the outputs from both γ lobe and α/β lobe neurons were blocked simultaneously (Fig. 7B,C,F), which was statistically different with the phenotype of blocking the output from either γ lobe or α/β lobe neurons (Fig. 7F). So did appetitive 1-h memory retrieval (Supplemental Figs. S5, S6). The abolishment of memory performance was also observed in aversive 3-h memory retrieval (Fig. 7D,E) and olfactory immediate memory tests (Supplemental Fig. S2).

Discussion

Using at least two neuronal ensembles to retrieve aversive and appetitive memories

Previous experiments found a single but different neuronal ensemble to retrieve aversive versus appetitive early memory in MB. α/β lobe neurons are reported to be necessary for aversive memory retrieval (McGuire et al. 2001; Krashes et al. 2007), whereas γ lobe neurons appear to be indispensable for appetitive memory retrieval (Trannoy et al. 2011).

However, some experimental conditions may restrict our understanding to such a view. To examine the roles of γ lobe in aversive memory retrieval, a strong γ lobe-specific Gal4 line is still needed (Tanaka et al. 2008; Aso et al. 2009). For example, *201y-Gal4* only labels a limited portion of γ lobe neurons and has non-specific expression in MB α/β lobe neurons (Aso et al. 2009). *NP21-Gal4* has strong expression in olfactory projection neurons and many other brain areas outside MB (Kimura et al. 2005). If the γ lobe Gal4 used labels MB α/β lobe neurons and olfactory projection neurons, the retrieval of olfactory memories (McGuire et al. 2001; Krashes et al. 2007) or the transmission of olfactory information (Masse et al. 2009) can be affected.

Some differences in experimental conditions might make us reveal the crucial role of α/β lobe neurons in appetitive early memory retrieval. In the previous study, the association time of CS+ and sucrose reward was 1 min, which may lead to the relatively low performance index of appetitive early memory in experiments containing *C739/uas-shi* group (generally near or below 20%) in the previous study. The association duration of CS+ and sucrose is 2 min in our study. We produced relatively higher and more stable appetitive early memory performances (near or above 30%) so that the statistical difference between the memory performances of *C739/uas-shi* group and control groups can be

Figure 6. Interrupting the synaptic output from α/β lobe neurons driven by another two α/β lobe Gal4s also impairs the retrieval of appetitive 3-h memory. (A) Under permissive temperature, all genotypes showed normal appetitive 3-h memory performances. (B) When blocking the output from *NP3061* or *NP6024* labeled α/β lobe neurons, the retrieval of appetitive 3-h memory was also impaired. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

Figure 7. Blocking the outputs from γ lobe and α/β lobe neurons simultaneously completely abolishes the retrieval of appetitive and aversive 3-h memories. (A) The expression pattern of mCD8::GFP denoted that *C739;5-HT1B-Gal4* specifically labeled MB γ lobe and α/β lobes, but not α'/β' lobes. (B) When constantly under the permissive temperature, all genotypes showed normal appetitive 3-h memory performances. (C) Appetitive 3-h memory performance was reduced to near zero when the neuronal transmissions from γ lobe and α/β lobes were disrupted simultaneously in *C739/+;5-HT1B-Gal4/uas-shi* flies. (D) All genotypes showed normal aversive 3-h memory performances under the permissive temperature. (E) Aversive 3-h memory retrieval was completely abolished when blocking the outputs from γ lobe and α/β lobes simultaneously in *C739/+;5-HT1B-Gal4/uas-shi* flies. (F) Appetitive 3-h memory performance was statistically lower when inhibiting the outputs from γ lobe and α/β lobe neurons simultaneously, compared with blocking the output from either γ lobe or α/β lobe neurons. Scale bar, 50 μ m. In all groups, $N \geq 8$. All error bars indicate SEM. (*) $P < 0.05$, (PI) performance index.

uncovered. Besides, our study used the *uas-shi* fly strain with two copy insertions rather than the one with only one copy insertion in the previous study (Trannoy et al. 2011). The two copy insertion *uas-shi* line generally has a better effect on blocking the neuronal transmission than the one copy insertion *uas-shi* line (Schwaerzel et al. 2002) so that the necessity of α/β lobe neurons in appetitive memory retrieval could be revealed using the two copy insertion *uas-shi* line. In addition, we used another two α/β lobe Gal4 lines to exam and confirm the role of α/β lobe neurons in appetitive memory retrieval (Fig. 6) even though it was not detected by just using *C739* in the previous study. Blocking the synaptic outputs from these two Gal4 labeled neurons also impaired appetitive early memory retrieval (Fig. 6).

In our study, using Gal4 lines preferentially labeling γ lobe or α/β lobes, we revealed a crucial role of MB γ lobe neurons in aversive intermediate-term memory retrieval (Figs. 2, 3) and α/β lobe neurons in appetitive intermediate-term memory retrieval (Figs. 5–7). Inhibiting the Gal4 activity in MB by expressing MB-Gal80 further testified the role of γ lobe and α/β lobes in olfactory memory retrieval. Besides intermediate-term memory, γ lobe and α/β lobes are both necessary for retrieving aversive and appetitive STMs (Supplemental Figs. S2–S4). These discoveries are consistent with a recent study (Cervantes-Sandoval et al. 2013). In accordance with previous studies, α'/β' lobe neurons are dispensable for the retrieval of aversive 3-h memory (Krashes et al. 2007; Cervantes-Sandoval et al. 2013) and appetitive early memory (Trannoy et al. 2011). A variance on the observation of α'/β' lobe neurons in appetitive early memory retrieval in another study can be explained by some differences in experimental conditions discussed in that study (Cervantes-Sandoval et al. 2013), as well as the odor concentrations and training parameters.

The functions of different MB substructures are further confirmed now. Thus, our study and previous experiments together suggest a new view of how memory is retrieved in MB, the olfactory learning and memory center in *Drosophila*. Both aversive and appetitive early memories are retrieved through at least two neuronal ensembles in MB: the combination of γ lobe and α/β lobe neurons.

It has been shown that MB may have common origins with vertebrate pallium (Tomer et al. 2010). Recently, accumulating studies on mammalian neural circuits began to reveal that mammalian brain also uses two neuronal ensembles to retrieve certain memories (Sepulcre et al. 2008; Takahashi et al. 2008; Goshen et al. 2011; Izumi et al. 2011; Suzuki and Naya 2011). Therefore, our study implies that using at least two neuronal ensembles to retrieve memory may be an evolutionarily conserved strategy across various species during memory processing.

In the anterior part of MB, the axons of MB γ lobe and β lobe neurons are horizontally located, while the axons of MB α lobe neurons are vertically located (Tanaka et al. 2008; Aso et al. 2009). Several evidences indicate that there may be some functional dissociation between these spatially differentiated lobes (Pascual and Preat 2001; Hu et al. 2010). These complexities imply that MB γ lobe and α/β lobes may connect to diverse downstream circuits and regulatory circuits during memory retrieval.

Olfactory early memories: from formation to retrieval

During the formation of aversive olfactory memory, the unconditional dopaminergic input is mediated by γ lobe in MB (Qin et al. 2012). Rutabaga serves as a coincidence detector mediating the formation of olfactory associative memory by associating the olfactory information with unconditional stimuli (Tempel et al. 1983; Tully and Quinn 1985; Han et al. 1992; Levin et al. 1992; Zhong and Wu 1993; Zars et al. 2000a,b; Mao et al. 2004; Tomchik and Davis 2009; Gervasi et al. 2010). The Rutabaga rescue experiments indicate that aversive STM is formed in MB γ lobe (Fig. 3; Zars et al. 2000a; Blum et al. 2009), while the appetitive early memory is formed in both MB γ lobe and olfactory projection neurons (Thum et al. 2007; Trannoy et al. 2011). Our study further confirmed that aversive ITM is formed and stored in MB γ lobe neurons (Fig. 4). Subsequently, these olfactory memories may be consolidated by the recurrent activity of MB α'/β' neuron-DPM/APL neuron loop (Keene et al. 2006; Krashes et al. 2007; Lee et al. 2011; Pitman et al. 2011; Wu et al. 2011; Cervantes-Sandoval and Davis 2012; Qin et al. 2012).

Thus, the current study leads us to rethink the information flow of memory processing in *Drosophila* from memory formation

to memory retrieval. Before memory retrieval, aversive memory formed in γ lobe neurons is at least not fully transferred to α/β lobe neurons, since γ lobe output is still needed during aversive memory retrieval (Fig. 2) and γ lobe neurons can support the Rutabaga-dependent aversive 3-h memory (Fig. 4). Appetitive memory formed in MB γ lobe and olfactory projection neurons is not completely retrieved through γ lobe, but through the combination of γ lobe and α/β lobes (Figs. 5–7).

Although aversive and appetitive STMs are mainly not formed in α/β lobes, α/β lobes are recruited during the process of memory retrieval. It is possible that there may be some communication between γ lobe neurons and α/β lobe neurons before or during memory retrieval. This communication can be mediated by MB intrinsic neurons and extrinsic neurons (Krashes et al. 2007; Lee et al. 2011; Qin et al. 2012), or achieved by direct interaction (Christiansen et al. 2011).

Differentiated usage of γ lobe output during the retrieval of aversive and appetitive 3-h memories

Blocking the synaptic output from γ lobe during retrieval severely impaired aversive 3-h memory, but just partially impaired appetitive 3-h memory. Two possible reasons may account for the differentiated usage of γ lobe output: multiple memory traces and downstream neural circuits.

Aversive early memory is mainly formed in MB γ lobe (Fig. 4; Blum et al. 2009; Qin et al. 2012). Thus, γ lobe output is indispensable for retrieving aversive memory (Fig. 2). But γ lobe output is just partially dispensable for retrieving appetitive memory. This may be because the appetitive memory stored in olfactory projection neurons can still be retrieved through the output from α/β lobes when the memory stored in MB γ lobe neurons cannot be retrieved through the γ lobe output. Therefore, only simultaneously blocking the outputs from γ lobe and α/β lobes could completely interrupt the retrieval of appetitive 3-h memory (Fig. 7).

Another possibility is that the downstream neurons of MB may use different strategies to integrate the information from γ lobe and α/β lobes. Compared with the appetitive memory downstream, the activation of aversive memory downstream may rely more on the information from γ lobe output. In addition, the activation thresholds may differ in these two downstream circuits. The activation threshold of aversive memory downstream circuits may be higher than appetitive memory downstream circuits so that more signals from MB are needed to activate the aversive memory downstream circuits efficiently. Unfortunately, the exact downstream circuits of MB remain unclear, although some neurons affecting the retrieval of certain olfactory memories were identified recently (Krashes et al. 2009; Liu and Davis 2009; Waddell 2010; Sejourne et al. 2011; Chen et al. 2012; Pai et al. 2013). With the progress on neural circuit studies in *Drosophila* (Pfeiffer et al. 2008; Tanaka et al. 2008; Aso et al. 2009; Chiang et al. 2011; del Valle Rodriguez et al. 2012), this possibility may be tested in the future.

In conclusion, our studies characterized the MB substructures involved in the retrieval of aversive and appetitive early memories. On this basis, our study and previous experiments together suggest a new view of how olfactory memories are retrieved when considering the MB substructures involved in memory formation together. In MB, the *Drosophila* learning and memory center, aversive and appetitive early memories are retrieved through the outputs from both γ lobe and α/β lobes. The discovery of using at least two neuronal ensembles to retrieve memory leads us to rethink how olfactory memories are processed from memory formation to retrieval. More broadly, this discovery implies that the manner of using two neuronal ensembles to retrieve memory

may serve as an evolutionarily conserved strategy during memory processing across various species.

Materials and Methods

Fly stocks and culture

Flies were raised at 23°C, 50% humidity on standard medium. *5-HT1B-Gal4* and *R92F10-Gal4* were obtained from Bloomington *Drosophila* Stock Center. *C739*, *C305a*, and *1471* lines were kindly provided by K. Ito. *uas-shi* (with two copy insertions [Kitamoto 2001]) and *uas-mCD8::GFP;MB247-DsRed* (Lee and Luo 1999; Riemensperger et al. 2005) lines were gifts from Cold Spring Harbor Laboratory. *rut²⁰⁸⁰*; *uas-rut* flies were a gift from J. Dubnau. *MB-Gal80* flies were kindly provided by S. Waddell. *NP1131*, *NP6024*, and *NP3061* were provided by *Drosophila* Genetic Resource Center (DGRC). All the *Gal4* and *Gal80* lines were outcrossed with the wild-type strain *w¹¹¹⁸ (isoCJ1)* for five generations. We generated *MB-Gal80;uas-shi*, *MB-Gal80;5-HT1B-Gal4*, and *C739-Gal4;5-HT1B-Gal4* lines ourselves. Unless specified, all the flies tested were a mixed population of sexes. *rut²⁰⁸⁰* and *NP6024* alleles both locate on the first chromosome. In *rut²⁰⁸⁰* rescue experiments, only male hemizygotes were counted. Only females were counted in experiments with *NP6024/+;uas-shi/+* flies.

Behavioral analysis

Olfactory aversive conditioning was performed as described previously (Tully and Quinn 1985). Briefly, about 100 flies successively experienced 90-sec air, 1-min CS+ odor and electric shock, 45-sec air, 1-min CS− odor without electric shock, and 45-sec air, in a training tube covered internally with copper grid. The two odors used for behavior analysis were 3-octanol (0.15% v/v) and 4-methylcyclohexanol (0.10% v/v; Sigma-Aldrich Co. LLC). The 1-min electric shock was delivered as 12 cycles (60 V for 1.5 sec and subsequent rest for 3.5 sec). 60 V is a commonly used voltage for electric shock (Yin et al. 1994; Pagani et al. 2009; Shuai et al. 2010; Qin et al. 2012). All the flies were trained, kept, or tested at 23°C or 33°C with 70% relative humidity in dark dimly red light. If temperature shift was needed, flies were accustomed to the new temperature 15 min ahead. A half performance index (PI) value is calculated as follows using the fly numbers choosing CS+ or CS− odors: $(CS− - CS+)/ (CS− + CS+) \times 100\%$. A single PI value is the average of two half PIs with opposite CS+ odors.

Olfactory appetitive conditioning was performed similar to the previously described protocol (Keene et al. 2006). Briefly, about 100 flies successively experienced 20-sec air, 2-min CS− odor, 1-min air in a training tube without sugar, and then 2-min CS+ odor, 20-sec air in a training tube covered internally with dried sucrose paper. All the flies were transferred to new food vials 24 h before starvation, and then starved for ~36 h at 23°C before training. A half PI value is calculated as $(CS+ - CS−) / (CS+ + CS−) \times 100\%$. Other conditions were the same as for aversive conditioning.

Sensory acuity tests

Odor acuity

Odors were the same as for olfactory conditioning. Flies were allowed to choose between each odor and air in a T-maze for 2 min.

Shock avoidance

Electric shocks were delivered the same as in aversive conditioning. Flies were allowed to choose between the arm with electric shock and the arm without electric shock in the T-maze for 1 min.

Sugar acuity

Flies were allowed to choose between the arm covered with dried sucrose paper and the arm without sugar in the T-maze for 2 min.

Gal4 expression pattern analysis

We crossed *uas-mCD8::GFP;MB247-dsRed* virgin female flies with the male flies of each Gal4 line to generate the genotypes for Gal4 expression pattern analysis. The first generation female offspring were dissected at 3- to 5-d old. The dissected central brains were fixed in 4% paraformaldehyde (in PBS) for 15 min, and then rinsed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH = 7.4) three times. They were then incubated in VECTASHIELD Mounting Medium (Vector Laboratories, Inc.). Confocal analysis was performed on a Zeiss LSM 710 confocal microscope. Confocal stacks were then processed using Imaris (Bitplane AG).

Statistics

In all sensory acuity tests, $N \geq 6$. In all other behavioral experiments, $N \geq 8$. Statistical analyses were performed using Tukey's post hoc tests following one-way ANOVA (set significance level as 0.05). If not specified, asterisks denote statistically significant differences between the experimental groups and their unmarked control groups ($P < 0.05$). All error bars indicate standard error of the mean (SEM).

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Requirement of the combination of mushroom body γ lobe and α/β lobes for the retrieval of both aversive and appetitive early memories in *Drosophila*

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