

RESEARCH ARTICLE

# The differential requirement of mushroom body $\alpha/\beta$ subdivisions in long-term memory retrieval in *Drosophila*

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## ABSTRACT

The mushroom body (MB), a bilateral brain structure possessing about 2000–2500 neurons per hemisphere, plays a central role in olfactory learning and memory in *Drosophila melanogaster*. Extensive studies have demonstrated that three major types of MB neurons ( $\alpha/\beta$ ,  $\alpha'/\beta'$  and  $\gamma$ ) exhibit distinct functions in memory processing, including the critical role of approximately 1000 MB  $\alpha/\beta$  neurons in retrieving long-term memory. Inspired by recent findings that MB  $\alpha/\beta$  neurons can be further divided into three subdivisions (surface, posterior and core) and wherein the  $\alpha/\beta$  core neurons play an permissive role in long-term memory consolidation, we examined the functional differences of all the three morphological subdivisions of MB  $\alpha/\beta$  by temporally precise manipulation of their synaptic outputs during long-term memory retrieval. We found the normal neurotransmission from a combination of MB  $\alpha/\beta$  surface and posterior neurons is necessary for retrieving both aversive and appetitive long-term memory, whereas output from MB  $\alpha/\beta$  posterior or core subdivision alone is dispensable. These results imply a specific requirement of about 500 MB  $\alpha/\beta$  neurons in supporting long-term memory retrieval and a further functional partitioning for memory processing within the MB  $\alpha/\beta$  region.

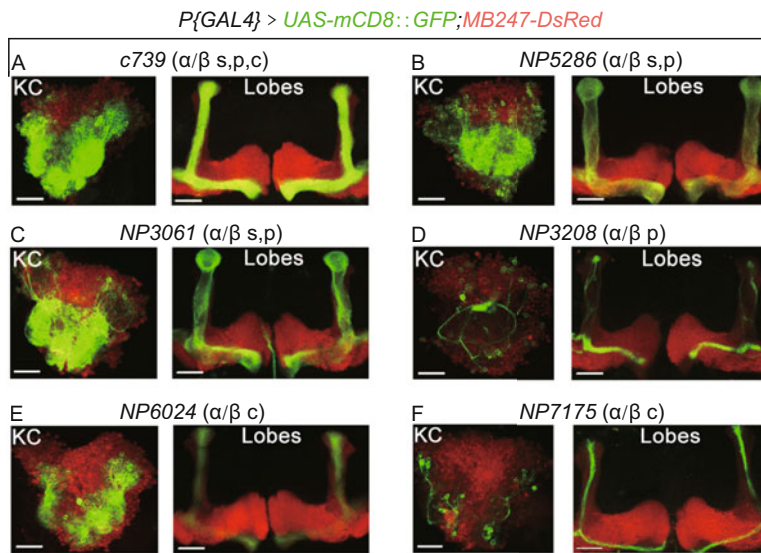
**KEYWORDS** memory retrieval, neural circuits, aversive olfactory conditioning, appetitive olfactory conditioning, mushroom body

## INTRODUCTION

Memory is of a complex and dynamic nature, requiring the co-

ordination of different neuron ensembles to support its distinct stages (i.e. formation, consolidation and retrieval), so dissecting the neural circuits underlying is crucial for mechanistically understanding the memory processing and the pathological basis of neurological diseases associated with memory deficits (Silva et al., 2009; van Strien et al., 2009; Small et al., 2011; Tye and Deisseroth, 2012). In *Drosophila*, with the power of sophisticated genetic toolbox and behavioral paradigm, extensive studies suggest a dynamic requirement for the three major subtypes of mushroom body (MB) neurons in olfactory conditioning:  $\gamma$  neurons serve as a gateway to support memory formation by dopaminergic signal-mediated CS-US association (Blum et al., 2009; Qin et al., 2012),  $\alpha'/\beta'$  neurons were transiently required to stabilize memory after training (Krashes et al., 2007) and to retrieve the immediate memory (Wang et al., 2008), and the outputs of  $\alpha/\beta$  neurons are necessary for retrieving both aversive and appetitive long-term memory (LTM) (Isabel et al., 2004; Krashes et al., 2008; Trannoy et al., 2011).

Through detailed characterization of enhancer trap lines,  $\alpha/\beta$ ,  $\alpha'/\beta'$  and  $\gamma$  can be further divided into more specific subdivisions. For instance, the  $\alpha/\beta$  neurons consist of surface, posterior and core subdivisions (Aso et al., 2009; Tanaka et al., 2008). In addition, recent literatures also reported that different expression patterns of genes within  $\alpha/\beta$  subdivisions, such as DmGluRA (the only *Drosophila* mGluR) and Ago-3 (an Argonaute protein) are selectively expressed in  $\alpha/\beta$  core neurons (Sinakevitch et al., 2010; Perrat et al., 2013), whereas the NMDA receptor subunit dNR1 and short NPF are exclusively expressed in non-core  $\alpha/\beta$  neurons (Sinakevitch et al., 2010; Johard et al., 2008). Given the well-known postulation “form follows function”, the observations above may imply the existence of functional differences among subdivisions of MB  $\alpha/\beta$



**Figure 1. Expression patterns in MB  $\alpha/\beta$  subdivisions of six GAL4 enhancer trap lines.** Indicated GAL4 lines were crossed to flies carrying both *UAS-mCD8::GFP* and *MB247-DsRed* transgenes. Confocal imaging of whole-mount adult central brain reveals the MB structure (displayed by *MB247-DsRed*, red signal) and GAL4 expression (indicated by GFP, green signal) in both Kenyon Cell region (KC) (left panel) and MB Lobes region (right panel). (A) *c739* had a strong GFP expression in the whole MB  $\alpha/\beta$  region, including all three subdivisions (s: surface, p: posterior and c: core neurons). Scale bar is 20  $\mu\text{m}$ . (B and C) Both *NP5286* and *NP3061* labeled MB  $\alpha/\beta$  surface and posterior neurons, but not core subdivision. Scale bar is 20  $\mu\text{m}$ . (D) *NP3208* exhibited a specific expression in MB  $\alpha/\beta$  posterior subdivision. Scale bar is 20  $\mu\text{m}$ . (E) *NP6024* labeled both the inner and outer neurons of MB  $\alpha/\beta$  core subdivision. Scale bar is 20  $\mu\text{m}$ . (F) *NP7175* specifically labeled the inner MB  $\alpha/\beta$  core subdivision. Scale bar is 20  $\mu\text{m}$ .

neurons.

In the current work, we tested necessity of the synaptic output from different MB  $\alpha/\beta$  subdivisions during LTM retrieval. Here we show that a subgroup of about 500 MB  $\alpha/\beta$  neurons, instead of the whole MB  $\alpha/\beta$  region (~1000 neurons) suggested before (Isabel et al., 2004; Krashes et al., 2008; Trannoy et al., 2011), is critical for successfully retrieving both aversive and appetitive LTM. Combined with the previous finding that  $\alpha/\beta$  core neurons play a distinct role in gating LTM consolidation (Huang et al., 2012), we propose that a further functional partitioning within MB  $\alpha/\beta$  region in LTM processing.

## RESULTS

### A panel of GAL4 enhancer trap lines marks MB $\alpha/\beta$ subdivisions

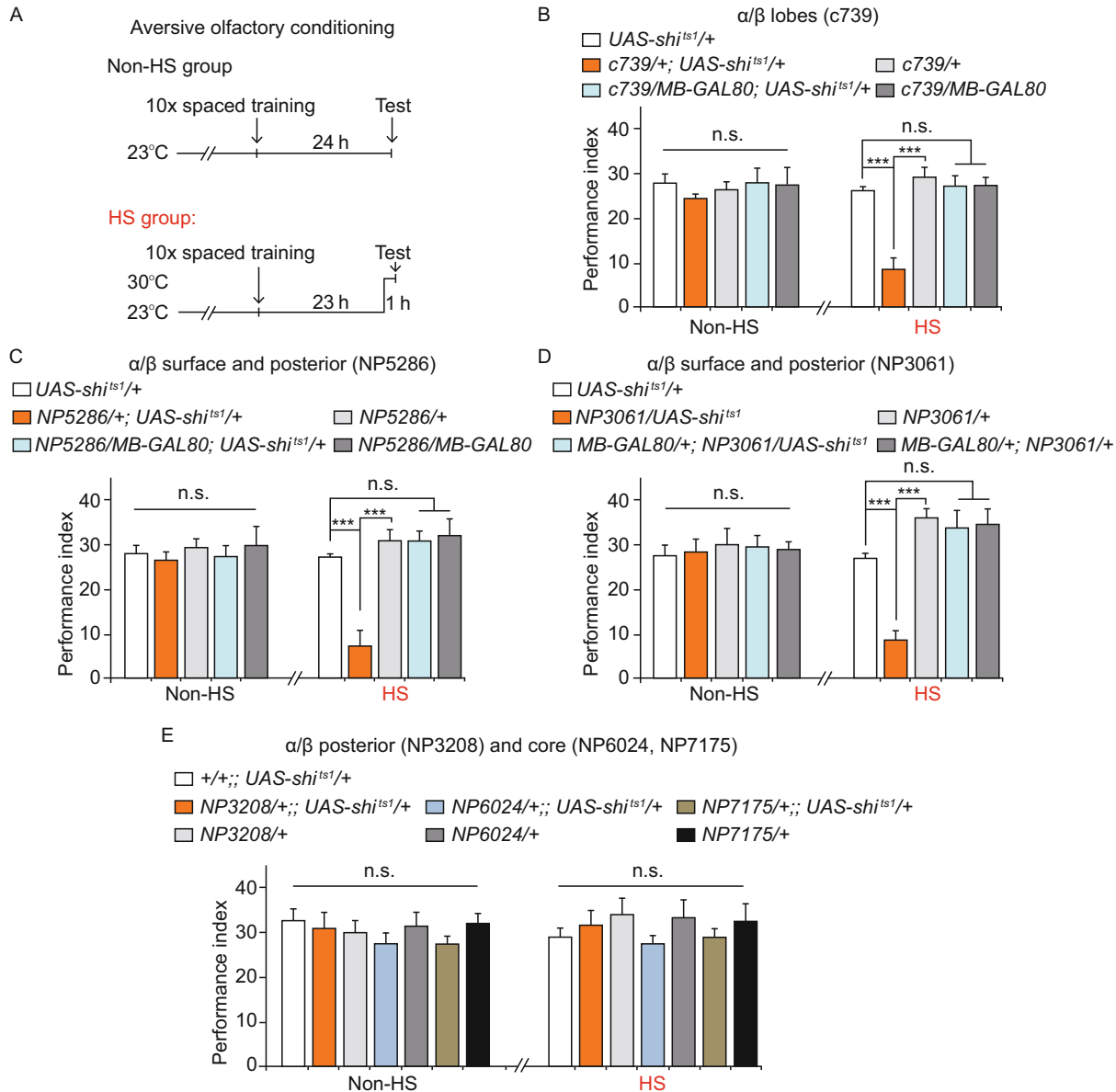
According to gene expression patterns and morphological structures, about 2000–2500 intrinsic MB Kenyon cells (Aso et al., 2009; Heisenberg, 2003) can be classified into three major subtypes- $\alpha/\beta$ ,  $\alpha'/\beta'$  and  $\gamma$  neurons (Crittenden et al., 1998; Lee et al., 1999) and each subtype can be further divided into smaller subdivisions such as the posterior, surface, and core neurons in MB  $\alpha/\beta$  region (Tanaka et al., 2008; Aso et al., 2009). Even though a wealth of evidence suggests that MB  $\alpha/\beta$  neurons are critical in retrieving both aversive and appetitive LTM (Isabel et al., 2004; Krashes et al., 2008; Trannoy et al., 2011), our recent finding revealed the  $\alpha/\beta$  core subdivision permits LTM consolidation, which is functionally distinct from the other two  $\alpha/\beta$  subdivisions (Huang et al., 2012). In order to investigate the functional heterogeneity of MB  $\alpha/\beta$  subdivisions, we chose six established GAL4 lines that are reported with the expression patterns covered all three  $\alpha/\beta$  subdivisions. A close examination of confocal images from this panel of GAL4 lines with co-expressing *mCD8::GFP* and *MB247-DsRed* (outlines the MB structure) indicated *c739* strongly expressed

in the whole MB  $\alpha/\beta$  region including all the three subdivisions (Fig. 1A), while *NP5286* (Fig. 1B) and *NP3061* (Fig. 1C) both have a strong expression in  $\alpha/\beta$  surface neurons and labeled posterior subdivision more weakly as reported (Tanaka et al., 2008). Also, posterior neurons can be marked by *NP3208* specifically (Fig. 1D), while core neurons are labeled by *NP6024* (Fig. 1E) or *NP7175* (Fig. 1F). It is worth noting that no specific  $\alpha/\beta$  surface driver is available to our knowledge, but the combinational expression of this panel of GAL4 lines still provides us a great opportunity to probe the different functions of MB  $\alpha/\beta$  subdivisions.

### A combination of MB $\alpha/\beta$ surface and posterior neurons is necessary for retrieving aversive LTM

In order to dissect the requirement of different MB  $\alpha/\beta$  subdivisions in aversive LTM retrieval, we measured the memory performance at 24 h after spaced aversive training (reinforced by electric shock punishment) (Tully et al., 1985, 1994) and used *UAS-shi<sup>ts1</sup>*, the temperature-sensitive *shibire* mutant transgene, to manipulate synaptic output reversibly and temporal precisely (Kitamoto, 2001). At the permissive temperature (below 23°C), the mutated Dynamin encoded by *shi<sup>ts1</sup>* gene allows normal neurotransmitter release, whereas, at the restrictive temperatures (above 30°C), it blocks synaptic transmission (Kitamoto, 2001).

All flies were subjected to two training procedures (Fig. 2A). For non-HS group, flies were trained and tested at the permissive temperature (23°C) and it turned out that they could perform normal LTM at this circumstance (Fig. 2B–F). For HS group, flies were tested at the restrictive temperatures (30°C). As shown in Fig. 2B, blockage of the synaptic output from *c739* labeled neurons during testing disrupted the normal LTM performance, and the involvement of non-MB neurons in *c739* could be ruled out by introducing *MB-Gal80* to eliminate the



**Figure 2. The synaptic output from a combination of MB  $\alpha/\beta$  surface and posterior subdivisions is required for retrieving aversive LTM.**

(A) Temperature-shift protocols for non-HS and HS groups. For non-HS group, flies were trained by ten-session spaced aversive training and tested in 23°C. For HS group, flies were trained and rested for 23 h at 23°C, then transferred to 30°C for 1 h resting followed by testing. (B) c739 labeled MB  $\alpha/\beta$  region is necessary for aversive LTM retrieval. In HS group, blocking synaptic output from c739 labeled neurons disrupted aversive LTM retrieval ( $P < 0.001$ ,  $n = 8-10$ , ANOVA), while blocking non-MB c739 neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.99$ ,  $n = 8-10$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.71$ ,  $n = 8-10$ , ANOVA and Kruskal-Wallis ANOVA). (C) *NP5286* labeled neurons (MB  $\alpha/\beta$  surface and posterior) are required for aversive LTM retrieval. In HS group, blocking synaptic output from *NP5286* labeled neurons disrupted aversive LTM retrieval ( $P < 0.001$ ,  $n = 8-10$ , ANOVA), while blocking non-MB *NP5286* neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.49$ ,  $n = 8-10$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.93$ ,  $n = 8-10$ , ANOVA and Kruskal-Wallis ANOVA). (D) *NP3061* labeled neurons (MB  $\alpha/\beta$  surface and posterior) are required for aversive LTM retrieval. In HS group, blocking synaptic output from *NP3061* labeled neurons disrupted aversive LTM retrieval ( $P < 0.001$ ,  $n = 8-10$ , ANOVA), while blocking non-MB *NP3061* neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.23$ ,  $n = 8-10$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.99$ ,  $n = 8$ , ANOVA). (E) Output from MB  $\alpha/\beta$  posterior or core neurons is dispensable for aversive LTM retrieval. In HS group, blocking synaptic output from MB  $\alpha/\beta$  posterior subdivision (labeled by *NP3208*) and core subdivision (labeled by *NP6024* or *NP7175*) alone had no effect on LTM retrieval ( $P > 0.40$ ,  $n = 8-10$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.29$ ,  $n = 8-10$ , ANOVA). For the X chromosome-located *NP3208*, *NP6024* and *NP7175*, only female results are shown. Average data are presented as means  $\pm$  SEM, \*\*\*  $P < 0.001$ , n.s. for non-significance.

*sh<sup>1</sup>* expression in Kenyon cells (Krashes et al., 2007). This observation confirmed the conclusion that MB  $\alpha/\beta$  region is critical for retrieving aversive LTM as reported before (Isabel et al., 2004). Next, we demonstrated that blocking neural outputs from either *NP5286* or *NP3061* labeled neurons could impair aversive LTM retrieval, while LTM was normal by co-expression of *MB-Gal80* (Fig. 2C and 2D), implying a necessity of the combination of MB  $\alpha/\beta$  surface and posterior neurons in retrieving LTM. Importantly, those LTM impairments observed in *c739*, *NP5286* and *NP3061* flies were not the result of any change in sensory responses that are essential for the memory task (Table 1). Surprisingly, disrupting neurotransmission of *NP3208* labeled MB  $\alpha/\beta$  posterior neurons alone has no effect on retrieving aversive LTM (Fig. 2E). Moreover, in consistence with our previous report (Huang et al., 2012), outputs from  $\alpha/\beta$  core neurons are dispensable for the read-out of aversive LTM (Fig. 2E).

#### A combination of MB $\alpha/\beta$ surface and posterior neurons is also required for appetitive LTM retrieval

In the appetitive memory task, we measured memory performance at 24 h after single appetitive training (reinforced by sugar rewarding) (Krashes et al., 2008). Since appetitive memory is controlled by fly's motivational states and can only be formed or retrieved when flies are hungry (Krashes et al., 2008 and 2009), all flies were food-deprived for 36 h before and 24 h after training (Fig. 3A). Control experiments (non-HS group) showed that all genotypes tested exhibited a normal appetitive LTM performance (Fig. 3B–F). We then demonstrated that, in HS-group, disrupting synaptic output from *c739* labeled MB  $\alpha/\beta$  neurons impaired appetitive LTM retrieval (Fig. 3B), which is in consistent with previous reports (Isabel et al., 2004). Likewise, blocking of MB  $\alpha/\beta$  surface and posterior neurons, labeled by *NP5286* and *NP3061*, during testing could induce LTM deficits, which can be reversed by the existence of *MB-Gal80* in those GAL4 lines (Fig. 3C and 3D). The task-relevant sensori-motor responses of *c739*, *NP5286* and *NP3061* flies were not signifi-

cantly altered (Table 1). However, appetitive LTM was normal in flies when synaptic transmission was blocked in the *NP3208* labeled  $\alpha/\beta$  posterior neurons and *NP6024* or *NP7175* labeled  $\alpha/\beta$  core region (Fig. 3E).

#### Rutabaga adenylyl cyclase is sufficient in *NP5286* and *NP3061* labeled MB subdivisions for supporting appetitive LTM

*rutabaga* (*rut*) gene encoded Calcium/calmodulin-dependent adenylyl cyclase is necessary to both aversive and appetitive LTM formation (Blum et al, 2009; Trannoy et al, 2011). Interestingly, restoring *rut* signaling in MB  $\alpha/\beta$  neurons is sufficient to fully rescue appetitive LTM defect (Trannoy et al, 2011), suggesting a predominant role of those neurons in *rut*-dependent LTM formation. Because *shibire* blocking experiments indicating MB  $\alpha/\beta$  subdivisions involve differently in LTM processing, we investigated if *rut* signaling in MB  $\alpha/\beta$  subdivision could be sufficient to support appetitive LTM. We found appetitive LTM defect in *rut<sup>2080</sup>* mutant could be fully rescued by expressing *UAS-rut* into *NP5286* and *NP3061* labeled MB subdivisions as well as restoring *rut* signaling in *c739* labeled neurons (Fig. 4).

Taken together, our results showed that, for both aversive and appetitive LTM retrieval, the normal neurotransmission from a combination of MB  $\alpha/\beta$  surface and posterior neurons is necessary, whereas output from  $\alpha/\beta$  posterior or core subdivisions alone is totally dispensable. Moreover, *rut*-dependent signaling in this combination of MB  $\alpha/\beta$  neurons is sufficient to support LTM formation.

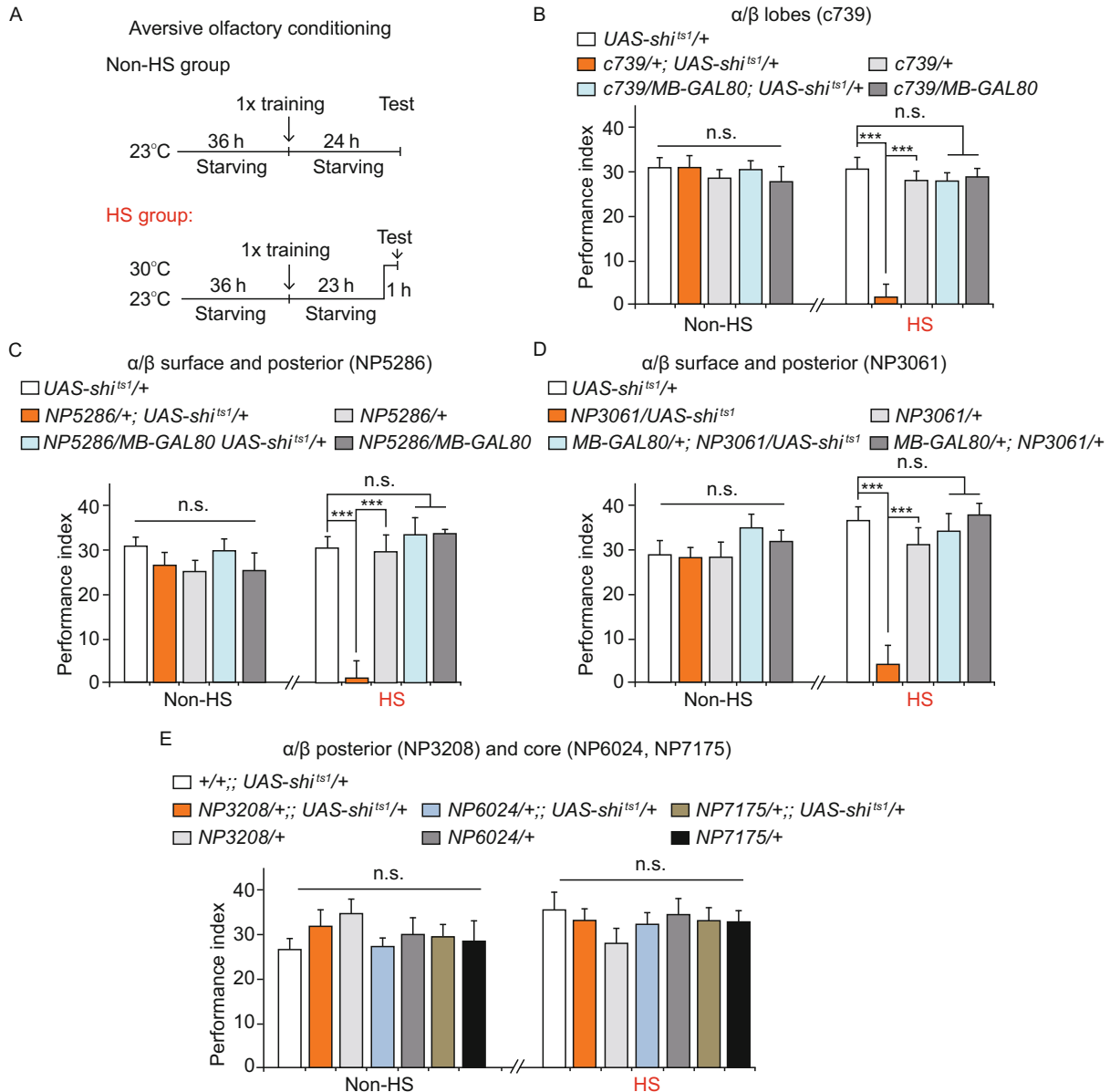
## DISCUSSION

The current work revisited the role of  $\alpha/\beta$  neurons in LTM retrieval and tested if any functional difference exists in the individual morphological subdivision of MB  $\alpha/\beta$  neurons. To do so, we used a panel of established GAL4 enhancer trap lines that faithfully label the  $\alpha/\beta$  subdivisions in a combinational manner and target neurons of interest with *UAS-sh<sup>1</sup>* to manipulate their synaptic transmission temporally. Since all of GAL4 lines

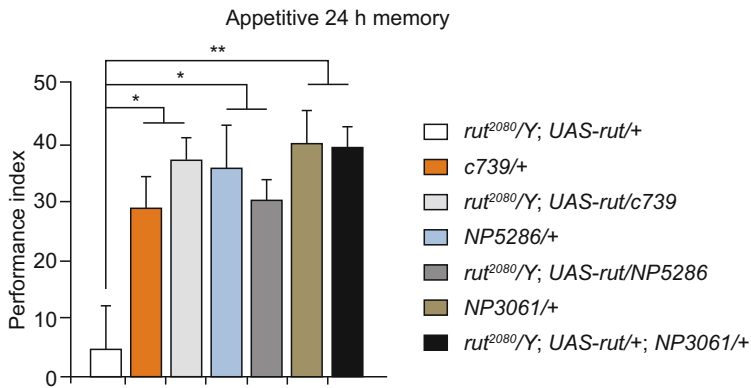
**Table 1. Task-relevant sensori-motor abilities**

Genotype	OCT ( $1.5 \times 10^{-3}$ )		MCH ( $1.0 \times 10^{-3}$ )		Sucrose acuity		Shock reactivity
	23°C	30°C	23°C	30°C	23°C	30°C	23°C
<i>UAS-sh<sup>1</sup>/+</i>	47.7 ± 5.8	47.7 ± 5.9	44.0 ± 5.1	48.3 ± 4.7	34.8 ± 4.5	40.5 ± 5.4	70.4 ± 3.7
<i>c739/+</i>	52.7 ± 3.1	48.7 ± 5.7	45.2 ± 3.7	41.1 ± 5.2	33.4 ± 3.8	42.7 ± 4.9	73.4 ± 3.8
<i>c739/+; UAS-sh<sup>1</sup>/+</i>	47.1 ± 3.2	49.0 ± 6.4	40.5 ± 3.7	48.1 ± 6.2	39.3 ± 4.5	45.2 ± 6.2	68.7 ± 3.4
<i>NP5286/+</i>	55.2 ± 3.6	49.7 ± 5.1	44.9 ± 4.8	49.6 ± 6.4	35.2 ± 4.7	35.6 ± 5.8	70.4 ± 5.6
<i>NP5286/+; UAS-sh<sup>1</sup>/+</i>	52.4 ± 4.7	48.1 ± 4.8	39.2 ± 4.0	47.9 ± 7.4	39.8 ± 5.3	36.6 ± 5.4	68.9 ± 3.0
<i>NP3061/+</i>	47.8 ± 2.0	45.0 ± 7.0	39.2 ± 5.2	43.3 ± 5.4	33.4 ± 3.9	34.0 ± 4.7	68.9 ± 3.5
<i>NP3061/UAS-sh<sup>1</sup></i>	49.6 ± 5.8	53.4 ± 9.1	37.7 ± 4.3	48.9 ± 7.1	35.1 ± 6.8	40.0 ± 6.2	72.3 ± 3.0
<i>NP6024/+</i>	48.2 ± 3.7	58.6 ± 6.1	45.2 ± 4.3	49.2 ± 6.2	39.2 ± 3.1	38.4 ± 4.9	71.6 ± 4.0
<i>NP6024/+; UAS-sh<sup>1</sup>/+</i>	53.5 ± 3.2	55.4 ± 5.9	41.7 ± 6.2	47.6 ± 7.4	39.5 ± 7.2	43.6 ± 6.3	74.1 ± 4.3

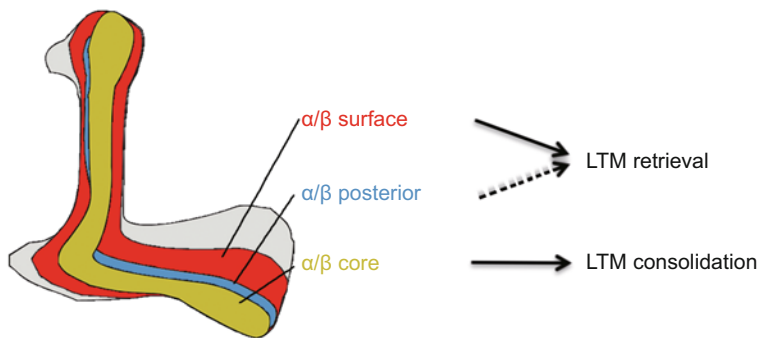
No statistical difference was detected between the relevant groups ( $n = 8$ , ANOVA). Average data are presented as means ± SEM.



**Figure 3. The synaptic output from a combination of MB  $\alpha/\beta$  surface and posterior subdivisions is also necessary for retrieving appetitive LTM.** (A) Temperature-shift protocols for non-HS and HS groups. Flies were food-deprived for 36 h before single-session appetitive training and for another 24 h after training. For non-HS group, flies were starved, trained and tested at 23°C. For HS group, temperature was shifted from 23°C to 30°C at 1 h before testing. (B) *c739* labeled MB  $\alpha/\beta$  region is necessary for appetitive LTM retrieval. In HS group, blocking synaptic output from *c739* labeled neurons disrupted LTM retrieval ( $P < 0.001$ ,  $n = 9-10$ , ANOVA), while blocking non-MB *c739* neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.99$ ,  $n = 8-9$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.53$ ,  $n = 8-10$ , ANOVA and Kruskal-Wallis ANOVA). (C) *NP5286* labeled neurons (MB  $\alpha/\beta$  surface and posterior) are required for appetitive LTM retrieval. In HS group, blocking synaptic output from *NP5286* labeled neurons disrupted appetitive LTM retrieval ( $P < 0.001$ ,  $n = 9-10$ , Kruskal-Wallis ANOVA), while blocking non-MB *NP5286* neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.99$ ,  $n = 8-9$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.46$ ,  $n = 8-10$ , ANOVA). (D) *NP3061* labeled neurons (MB  $\alpha/\beta$  surface and posterior) are required for appetitive LTM retrieval. In HS group, blocking synaptic output from *NP3061* labeled neurons disrupted appetitive LTM retrieval ( $P < 0.001$ ,  $n = 9-10$ , ANOVA), while blocking non-MB *NP3061* neurons (by co-expressing *MB-Gal80* and *UAS-shi<sup>ts1</sup>*) had no effect ( $P > 0.99$ ,  $n = 8-9$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.22$ ,  $n = 8-10$ , Kruskal-Wallis ANOVA). (E) Output from either MB  $\alpha/\beta$  posterior or core neurons are dispensable for appetitive LTM retrieval. In HS group, blocking synaptic output from MB  $\alpha/\beta$  posterior subdivision (labeled by *NP3208*) or core subdivision (labeled by *NP6024* and *NP7175*) alone had no effect on appetitive LTM retrieval ( $P > 0.50$ ,  $n = 8-10$ , ANOVA). In non-HS group, all genotypes showed normal LTM performance ( $P > 0.36$ ,  $n = 8-10$ , ANOVA). For the X chromosome-located *NP3208*, *NP6024* and *NP7175*, only female results are shown. Average data are presented as means  $\pm$  SEM, \*\*\*  $P < 0.001$ , n.s. for non-significance.



**Figure 4. Restoring *rut* in MB  $\alpha/\beta$  subdivisions is sufficient for appetitive LTM formation.** *c739*-driven *rut* expression fully rescues *rut*<sup>2080</sup> LTM defect ( $P < 0.05$ ,  $n = 6-10$ , ANOVA); *NP5286*-driven *rut* expression fully rescues *rut*<sup>2080</sup> LTM defect ( $P < 0.05$ ,  $n = 6-10$ , ANOVA); *NP3061*-driven *rut* expression fully rescues *rut*<sup>2080</sup> LTM defect ( $P < 0.01$ ,  $n = 6-10$ , ANOVA). Average data are presented as means  $\pm$  SEM, \*  $P < 0.05$ , \*\*  $P < 0.001$ .



**Figure 5. Schematic of differential function of MB  $\alpha/\beta$  subdivisions in LTM regulation.** A combination of MB  $\alpha/\beta$  surface and posterior neurons supports the LTM retrieval, whereas MB  $\alpha/\beta$  core neurons permit LTM consolidation.

used have the additional non-MB expression, we also used the *MB-Gal80* to rigorously examine the requirement for MB activity in these GAL4 lines. Our results confirmed the requirement of *c739* labeled  $\alpha/\beta$  neurons in both aversive and appetitive LTM as reported previously (Isabel et al., 2004; Krashes et al., 2008; Trannoy et al., 2011) and further demonstrated output from a combination of  $\alpha/\beta$  surface and posterior neurons is required for retrieving LTM, whereas the output from  $\alpha/\beta$  posterior neurons or core neurons alone is clearly dispensable. Moreover, *rut* rescue data indicate the sufficiency of this combination of MB  $\alpha/\beta$  neurons in *rut*-dependent LTM formation. Due to the lack of specific GAL4 line for targeting  $\alpha/\beta$  surface neurons, we cannot specifically test the requirement of  $\alpha/\beta$  surface region in LTM. Despite this caveat, it is still likely that MB  $\alpha/\beta$  surface neurons serve a more important role than posterior neurons in LTM retrieval because of two lines of evidence: both *NP5286* and *NP3061* only have a weak expression in posterior subdivision (Tanaka et al., 2008), and the specific blockage of posterior neurons labeled by *NP3208* had no effect on LTM.

We believe our data, taken with the previous report (Huang et al., 2012), have two significant implications. First, successfully retrieving both aversive and appetitive stable memory requires the support from a subgroup of about 500 MB  $\alpha/\beta$  neurons; Second, a further functional partitioning exists within different anatomical subdivisions of MB  $\alpha/\beta$  neuron in LTM processing: MB  $\alpha/\beta$  core neurons permit LTM consolidation, whereas a combination of MB  $\alpha/\beta$  surface and posterior neurons supports the LTM retrieval (Fig. 5). These two implications could potentially shift our focus to a more restricted region for

localizing the LTM engram in MB and add new circuitry components to the information processing loop underlying system consolidation of LTM (Dubnau and Chiang, 2013).

An immediate question raised here is, how do the different  $\alpha/\beta$  neuron ensembles coordinate with each other to support LTM regulation. One possibility is one MB  $\alpha/\beta$  subdivision can interact with others during LTM processing. Although, to our knowledge, no evidence indicates different MB neurons can form any synaptic connection at MB lobe region, one recent study does show the output from Kenyon cells transmits back to other compartments of MB through the presynaptic sites in calyx (Christiansen et al., 2011). Also, MB  $\alpha/\beta$  subdivisions might communicate with each other through feedback loops constructed by various MB extrinsic neurons, such as DPM neurons and APL neurons (Lee et al., 1999; Keene et al., 2006; Wu et al., 2011; Pitman et al., 2011). Another possibility is that MB  $\alpha/\beta$  subdivisions, being parallel with each other, release neurotransmitters to their common downstream neurons suggested for LTM storage, such as the DAL neurons (Chen et al., 2012) MB-V2 neurons (Séjourné et al., 2011), MB-V3 neurons (Pai et al., 2013) and the ellipsoid body (Wu et al., 2007), to regulate formation, consolidation and retrieval of LTM separately.

## MATERIALS AND METHODS

### Fly stocks and maintenance

GAL4 enhancer trap lines *NP3061*, *NP5286*, *NP3208*, *NP6024* and *NP7175* were kindly provided by Dr. Kei Ito. *MB247-dsRed* was ob-

tained from Dr. André Fiala. *MB-Gal80* was a gift from Dr. Scott Waddell. *c739*, *UAS-mCD8::GFP*, *UAS-sh<sup>ts1</sup>* and *rut<sup>2080</sup>;UAS-rut* were obtained from Bloomington Stock Center (Bloomington, IN). To minimize the potential “leaky” effects of *UAS-sh<sup>ts1</sup>* expression during developmental stage, all flies were reared at 18°C with 60% humidity in a 12 h light-dark cycle on standard medium. All strains were outcrossed with *w<sup>1118</sup>* (*isoCJ1*) wild-type flies for five generations to minimize differences in genetic background.

### Confocal imaging

3-day-old adult flies were anesthetized with CO<sub>2</sub> and dissected in phosphate-buffered saline (PBS). The dissected brains were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature followed by being washed in PBS for three times and then mounted in Focus-Clear (Pacgen Biopharmaceuticals). All images were acquired from a Zeiss 710 laser-scanning confocal microscope. Image stacks were obtained with Imaris 6.0 software (Bitplane, Zurich, Switzerland).

### Pavlovian olfactory aversive conditioning

To generate aversive LTM, we used Pavlovian olfactory conditioning reinforced by electric shock. The training and testing procedures was performed in a room with 70% relative humidity and desired temperature as described previously (Tully et al., 1985, 1994). Two aversive odors, 3-octanol (OCT) (Fluka,  $1.5 \times 10^{-3}$  dilution in heavy mineral oil), 4-methylcyclohexanol (MCH) (Fluka,  $1 \times 10^{-3}$  dilution in heavy mineral oil) were used in all experiments.

For one training session, about 100 flies were exposed sequentially to the two odors (OCT and MCH) for 60 s with a flush of fresh air for 45 s after each odor. Meanwhile, electric foot shock (twelve 1.5 s pulses with 3.5 s intervals; 60 V) was delivered to flies during their exposure to the first odor (CS+), but not during exposure to the second odor (CS−). This training procedure was repeated for ten times with 15 min intervals between each session to generate a normal protein synthesis-dependent aversive LTM. After that, trained flies were transferred to food vials for a 24-h resting at 18°C before testing.

To test memory, trained flies were transferred into the T-maze and allowed to choose between CS+ and CS− for 120 s at either 23°C or 30°C accordingly. The performance index (PI) was calculated from the distribution of flies in the two T-maze arms (Tully et al., 1994). A PI of 0 indicates a distribution of 50:50 (no memory), while a PI of 100 indicates all the flies made a right choice (avoided the negatively reinforced odor). To eliminate any potential effect on the PI by odor bias, the experiments had a balanced design: each experiment ( $n = 1$ ) includes two reciprocal groups with one used OCT as CS+ and the other one used MCH as CS+. The final PI was the average of PIs from the two groups.

### Pavlovian olfactory appetitive conditioning

The olfactory sugar rewarding appetitive-conditioning paradigm was performed as described previously (Krashes et al., 2008). The training tube (CS+) contained filter paper with dried sucrose that covered the entire wall of the training tube. The filter paper was made by applying a saturated sucrose solution and allowing it to dry before use. Another tube (CS−) was prepared with a dry filter paper. Before training, flies were food deprived for 36 h in vials containing a 2 × 2 cm Watmann

3MM filter paper soaked with 0.5 mL distilled water. During training, flies were transferred to the CS− tube and exposed to the CS− odor for 60 s followed by a 60 s flush of fresh air. Next, flies were moved to the CS+ tube and exposed to the CS+ odor for another 60 s. After conditioning, flies were food-deprived again for another 24 h. LTM was measured by the same method used for aversive memory testing.

### Sensori-motor responses

To rule out the effect on sensori-motor abilities by any genetic manipulation, we also tested fly's olfactory acuity, shock reactivity and sucrose acuity as previously described (Tully et al., 1994; Kim et al., 2007). For olfactory acuity testing, odor avoidance responses were calculated by exposing about 100 naïve flies to the test odor (either OCT or MCH,  $1.5 \times 10^{-3}$  and  $1 \times 10^{-3}$  dilution, respectively) versus fresh air in the T-maze. For shock reactivity testing, groups of about 100 untrained flies were exposed to two T-maze arms with 60 V electric foot shock delivered to one of the arms but not to the other. For sucrose acuity, about 100 untrained flies were subjected to two T-maze arms with a filter paper with dried sucrose in one of the arms and a dry filter paper in another arm. In all the three kinds of sensori-motor testing, flies were allowed to make choices between the two arms for 120 s, after which they were trapped, anesthetized, and counted. A performance index (PI) was then quantified from the distribution of flies in two arms as that described in the memory test.

### Statistical analysis

We used Shapiro-Wilk for the normality testing. Normally distributed data were subjected to one-way ANOVA followed by Bonferroni test (Origin version 8; OriginLab Corporation), while the non-normally distributed data were analyzed by Kruskal-Wallis ANOVA. Statistical results are presented as means ± SEM. Asterisks indicate critical values (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ ). n.s. indicates no significant difference ( $P > 0.05$ ).

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### COMPLIANCE WITH ETHICS GUIDELINES

Cheng Huang, Pengzhi Wang, Zhiyong Xie, Lianzhang Wang, and Yi Zhong declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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