Report

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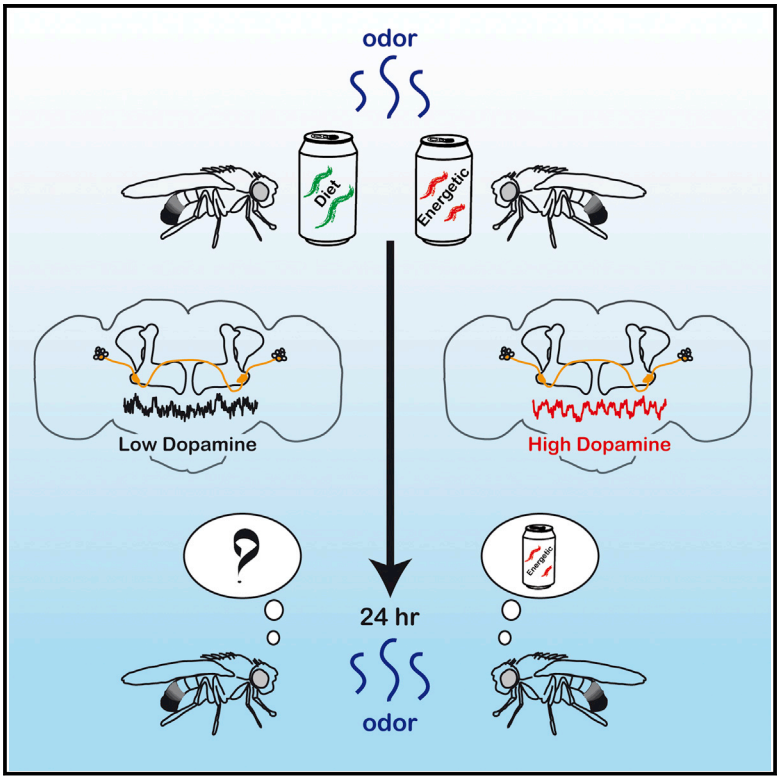
Delayed Dopamine Signaling of Energy Level Builds Appetitive Long-Term Memory in *Drosophila*

Graphical Abstract



Highlights

- Delayed energetic supply allows appetitive long-term memory formation
- MB-MP1 dopaminergic neurons control appetitive long-term memory consolidation
- MB-MP1 oscillations are enhanced after conditioning with an energetic sugar
- The DAMB dopaminergic receptor is required for appetitive long-term memory

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In Brief

Appetitive long-term memory (LTM) forms in *Drosophila* after presentation of an odorant with sugar. Musso et al. now show that, during LTM formation, a pair of dopaminergic neurons informs the olfactory memory center, the mushroom body, about the energy content of the ingested food.

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Report

Delayed Dopamine Signaling of Energy Level Builds Appetitive Long-Term Memory in *Drosophila*

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SUMMARY

Sensory cues relevant to a food source, such as odors, can be associated with post-ingestion signals related either to food energetic value or toxicity. Despite numerous behavioral studies, a global understanding of the mechanisms underlying these long delay associations remains out of reach. Here, we demonstrate in *Drosophila* that the long-term association between an odor and a nutritious sugar depends on delayed post-ingestion signaling of energy level. We show at the neural circuit level that the activity of two pairs of dopaminergic neurons is necessary and sufficient to signal energy level to the olfactory memory center. Accordingly, we have identified in these dopaminergic neurons a delayed calcium trace that correlates with appetitive long-term memory formation. Altogether, these findings demonstrate that the *Drosophila* brain remembers food quality through a two-step mechanism that consists of the integration of olfactory and gustatory sensory information and then post-ingestion energetic value.

INTRODUCTION

Visual, olfactory, and gustatory cues can drive the efficient selection of food sources. However, the true nutritive value (or toxicity) of food can only be evaluated after absorption. Accordingly, post-ingestion signals have an essential role in food source evaluation (Sclafani and Ackroff, 2004). This well-documented feature has been demonstrated by conditioned taste or odor aversion assays, in both mammals (Scott, 2011) and invertebrates (Wright et al., 2010; Simões et al., 2012). Additionally, several research groups have shown that post-ingestion signals attribute a reward value to nutritious or energetic food sources (Sclafani and Ackroff, 2004; de Araujo et al., 2008; Dus et al., 2011; Fujita and Tanimura, 2011). Because sensory cues perceived during feeding must be associated with metabolic information that is only available after a long delay, a specific form of associative memory is required to remember food source quality. Despite recent progress in the identification of molecular mechanisms and neural circuits involved in these processes (Scott, 2011; Domingos et al., 2011, 2013; Uematsu et al., 2014), the complexity of these neural networks has hindered a global view of the mechanisms underlying these associations, especially regarding reward associations.

Recently, Burke and Waddell (2011) showed that *Drosophila* forms short-term memories (STM) by associating an odor with a sweet food source, whereas long-term memory (LTM) is only formed if the food source is nutritious. The authors concluded that nutrient value is assessed within less than 2 min. In this report, we demonstrate that LTM formation relies not only on sensory processing during or immediately after feeding but also on delayed post-ingestion signaling of energy level, which can occur as much as 5 hr after training. Our two-step conditioning protocol, combined with a precise temporal inhibition/activation of neuronal circuits and in vivo brain imaging, has now enabled a comprehensive view of appetitive LTM mechanisms.

RESULTS

We investigated the post-ingestion component of appetitive LTM processing using either D- or L-glucose as a reward. L-glucose is an enantiomer of D-glucose that cannot be metabolized and therefore does not provide any energy. Because the sweetness of L-glucose approximates that of D-glucose, as measured in particular by the fly proboscis extension reflex and the labellar nerve response, it is a valuable tool for identifying energetic food-dependent processes (Fujita and Tanimura, 2011; Stafford et al., 2012). Our initial attempt to investigate whether LTM is formed with energetic D-glucose rather than L-glucose employed a classical protocol (Colomb et al., 2009) with the following sequence: 21 hr starvation followed by training with an odorant and sugar; starvation; and, finally, a memory test with the odorant 24 hr after training. However, this straightforward strategy was unsuitable because the total 45-hr fasting duration of flies trained with non-metabolizable L-glucose was likely to affect their performance during the memory test in a non-specific manner (Figure S1A). Thus, the low performance of flies trained on L-glucose (Figure S1B) cannot be interpreted as a bona fide appetitive LTM defect, as it may be a consequence of the deleterious 45-hr fasting. By contrast, control flies trained on nutritious D-glucose were re-fed during training and so were not starved for more than 24 hr; these flies thus displayed normal LTM (Figure S1B). The non-specific effect due to prolonged starvation of L-glucose-trained flies could be

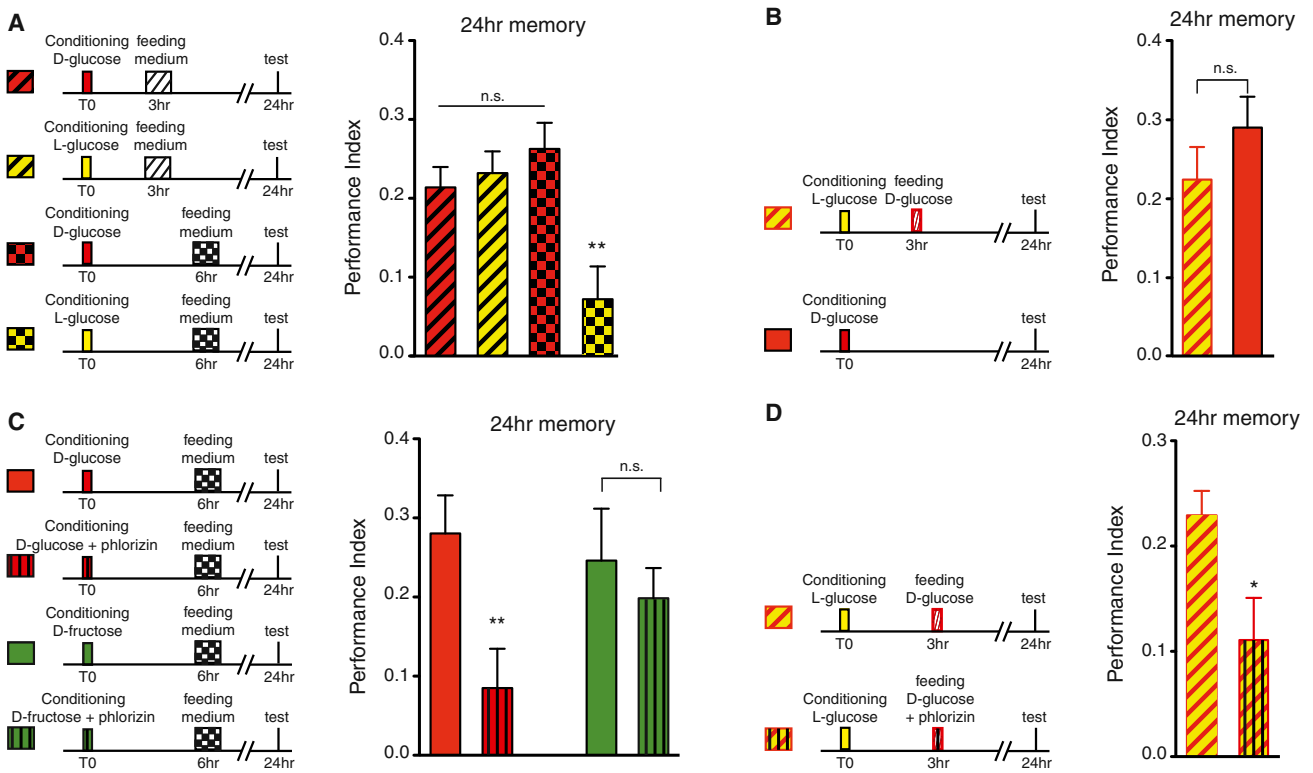

Figure 1. Appetitive LTM Is Formed when Energy Is Delivered 3 hr after Training by a Post-ingestion Mechanism

(A) L-glucose-conditioned flies fed with food (for 30 min) 6 hr after acquisition did not form LTM (score is not statistically different from 0, t test, $t_{26} = 1.684$, p = 0.104 and is significantly lower than all other groups $F_{(3,92)} = 6.904$, p = 0.0003; n ≥ 21). L-glucose-conditioned flies fed with food at 3 hr (for 30 min) did form LTM, and their score is not significantly different from D-glucose-conditioned flies ($F_{(3,92)} = 6.904$; p = 0.0003). n ≥ 21 for all data.

(B) L-glucose-conditioned flies fed with D-glucose (for 1 min) 3 hr after acquisition performed as well as D-glucose-conditioned flies in the LTM test (t test; $t_{25} = 1.134$; p = 0.267; n ≥ 12).

(C) Flies conditioned with a mixture of D-glucose and phlorizin did not form LTM (score statistically not different from 0; t test; $t_{15} = 1.708$; p = 0.1083) and presented a significantly lower score than D-glucose-conditioned flies (t test; $t_{31} = 2.283$; p = 0.008). Flies conditioned with a mixture of D-fructose and phlorizin exhibited a non-statistically different score from D-fructose-conditioned flies (t test; $t_{30} = 0.661$; p = 0.513). n ≥ 14 for all data. All groups were placed on fly medium 6 hr after training for 30 min.

(D) L-glucose-conditioned flies fed a mixture of D-glucose and phlorizin (for 1 min) 3 hr after acquisition exhibited a significantly lower memory as compared to D-glucose re-fed flies (t test; $t_{22} = 2.442$; p = 0.023; n ≥ 11).

See also Figure S1. Means ± SEM; statistical test: t test and one-way ANOVA; n.s.: p ≥ 0.05; *p < 0.05; **p < 0.01 in comparison between two groups for t test and in post hoc comparisons with both parental controls for ANOVA.

suppressed by re-feeding with fly medium after training. We therefore reasoned that these non-specific effects could be rescued with the proper delay between training and re-feeding. Indeed, this delay must be long enough to not interfere with any potential post-feeding signaling that could be associated with the odorant but short enough to maintain fly health. We found that a 6-hr delay between training and re-feeding on standard fly medium (30-min duration) met these requirements. Accordingly, interrupting the 45-hr starvation session after 27 hr with a 30 min re-feeding rescued the non-specific defect previously observed for L-glucose training after 45-hr starvation (Figure S1C). When flies were starved for 21 hr, trained, re-fed 6 hr later, and finally tested after 18 hr of additional starvation, we observed that flies trained with L-glucose exhibited an LTM score that was significantly lower than the score of flies trained on D-glucose (Figure 1A). Thus, by using glucose enantiomers, we confirmed that appetitive LTM formation depends on the nutritive value of the sugar reward (cf. Burke and Waddell, 2011). These results suggest that it is the energetic value of food that triggers appetitive LTM formation. However, this does not resolve whether the energetic value is associated with the odor during training or whether there is a delayed mechanism that allows the brain to use post-feeding signals to integrate the value of the food, as in mammals (Sclafani and Ackroff, 2004; de Araujo et al., 2008).

To address this issue, we trained flies with non-energetic L-glucose and re-fed them 3 hr after training, in order to generate potential post-feeding reinforced signaling. Strikingly, flies trained on L-glucose performed as well at 24 hr as those trained on D-glucose, when they were re-fed 3 hr after training for 30 min on standard fly medium (Figure 1A). A similar effect was observed when flies were re-fed for 1 min with D-glucose 3 hr after training (Figure 1B), indicating that the food energetic value is the critical parameter to generate LTM. We analyzed the temporal window

where energetic post-training re-feeding allows LTM formation for L-glucose-trained flies. LTM score remained high for re-feeding delays as long as 4 hr, after which it decayed and vanished for a 6-hr delay (Figure S1D). The possibility to delay the energetic food supply with respect to training suggested that a post-ingestion mechanism was involved. To demonstrate this, we utilized phlorizin, a specific blocker of the intestinal glucose transporter, which does not affect the intestine-to-hemolymph transport of other sugars like fructose (Dus et al., 2013). Recently, phlorizin was successfully used to block glucose entry into hemolymph in *Drosophila* (Dus et al., 2013). First, we checked that phlorizin did not interfere with LTM formation despite its slightly bitter taste. For this, we conditioned flies with a mixture of the energetic fructose and phlorizin. These flies performed LTM as well as fructose-conditioned flies (Figure 1C). Then, flies were conditioned with a mixture of D-glucose and phlorizin. Interestingly, when glucose no longer entered into the hemolymph, flies failed to form LTM (Figure 1C). This indicates that hemolymph sugar level controlled LTM formation. Accordingly, re-feeding flies with a mixture of D-glucose and phlorizin 3 hr after training with L-glucose failed to rescue LTM (Figure 1D). Together, these results demonstrate that sugar energetic value was detected by a post-ingestion mechanism and not through external or digestive-tract sensory receptors. Our two-step protocol therefore enabled investigating how the *Drosophila* brain uses post-ingestion signals to remember the energetic value of food.

Appetitive olfactory memory in *Drosophila* is encoded in the mushroom bodies (MBs), a symmetrical structure comprising 2,000 neurons (Aso et al., 2009). The main inputs to the MBs are cholinergic projection neurons from the antennal lobes, which encode olfactory information, as well as various dopaminergic (DA) neurons that encode aversive or appetitive signals (Waddell, 2010). We demonstrate here that post-ingestion signaling involves neurotransmission from the PPL1 DA cluster. Expression of the thermo-sensitive shibire protein (Shi^{ts}) in a given set of neurons allows the blockade of their neurotransmission at the restrictive temperature (33°C), which is released at the permissive temperature (25°C or below). We expressed Shi^{ts} with the *TH-GAL4* driver (Aso et al., 2010) in a large population of DA neurons, including within the PPL1 cluster and a few neurons of the PAM cluster, and subsequently trained flies at the restrictive temperature. Consequently, LTM in flies trained on sucrose (a nutritious sugar) was impaired following this blockade (Figure 2A), but not STM (Figure S2A). We checked that LTM was normal in flies trained at the permissive temperature (Figure S2B) and that sugar response (Figure S2C) and olfactory acuity (Figure S2D) were normal at the restrictive temperature. This LTM impairment was unexpected, because it was previously established that PAM DA neurons not labeled by *TH-GAL4* are responsible for delivering the reward signal to the MBs during appetitive training for both STM and LTM (Liu et al., 2012). Our results thus potentially suggest that PPL1 neurons are involved during training or early consolidation for LTM formation, although PPL1 neurons are not involved in appetitive STM formation (Schwaerzel et al., 2003). Here, the spatial resolution of the thermo-sensitive Shi^{ts} is provided by the GAL4 driver specificity. However, it is not possible to achieve the same temporal resolution as with other techniques (e.g., optogenetics): when training occurs at 33°C, the transition to the permissive temperature is generally progressive, and neurotransmission may be inhibited during the first 20–30 min of the consolidation phase. To separate the contribution of PPL1 DA neurotransmission during learning from the early consolidation phase, we designed protocols with a sharp temperature transition immediately after training (see Experimental Procedures). In the sharp version of the training blockade, flies were maintained at the restrictive temperature for 30 min before and during the training but were then returned to the permissive temperature within 3 min of odorant and sugar presentation. The sharp training blockade of *TH-GAL4* neurons did not impair LTM (Figure 2B). We verified that the sharp training blockade of the PAM cluster with the *58E02-GAL4* driver did affect LTM (Figure S2E), as previously reported using a classical protocol (Liu et al., 2012). In the sharp version of the consolidation blockade, flies were trained at the permissive temperature and transferred to the restrictive temperature within 3 min of training; finally, they were maintained at the restrictive temperature for 3 hr. Sharp blockade of *TH-GAL4* neurons after training impaired LTM (Figure 2C), demonstrating that only post-training activity of *TH-GAL4* neurons was required for appetitive LTM.

Next, we identified which PPL1 DA neurons are involved in LTM formation after training. LTM impairment was reproduced by blocking *NP0047-GAL4* neurons, which include only three types of DA neurons shared with *TH-GAL4*: MB-V1; MB-MV1; and MB-MP1 (Figures S2F–S2K; Tanaka et al., 2008; Plaçais et al., 2012). We confirmed that the LTM defect could be attributed specifically to DA neurons within the *NP0047-GAL4* expression pattern, by using the *TH-GAL80* construct that inhibits GAL4 transcriptional activity in DA neurons. Indeed, the LTM defect was abolished when Shi^{ts} was expressed in non-DA *NP0047* neurons (Figure S2I). There are two MB-MP1 neurons per brain hemisphere (labeled by the *NP2758-GAL4* and *C061-GAL4* drivers; Tanaka et al., 2008; Krashes et al., 2009), a single MP-MV1 neuron (labeled by the *MZ604-GAL4* driver; Tanaka et al., 2008), and a single MB-V1 neuron (labeled with the *15E10-GAL4* and *50B03-GAL4* drivers; Jenett et al., 2012). By blocking individual subtypes of *NP0047* DA neurons using these more spatially refined drivers, we have established that MB-MP1 neurons specifically control appetitive LTM formation (Figures S2L–S2O; see Figures S2P–S2U for controls). More precisely, the *NP2758-GAL4* driver allowed us to determine that LTM is impaired by sharp blockade of MB-MP1 after training, but not during training (Figures 2D and 2E).

These results implicate the activity of specific mechanisms involving DA signaling to the MBs, following training to form appetitive LTM. To further investigate these mechanisms, we inhibited D1-type DA receptors in adult MBs neurons with RNAi and the thermo-inducible TARGET system (which uses the *238Y-GAL4*;*tub-GAL80^{ts}* driver; McGuire et al., 2003). Inhibition of the dumb DA receptor in adult MBs induced a 2-hr STM defect (Figure 3A; see Figures S3A–S3C for controls), as previously reported elsewhere for *dumb* constitutive mutants (Kim et al., 2007; Liu et al., 2012). Here, we confirmed that the *dumb^3* mutant displays an STM defect (Figure S3D). Strikingly, flies with reduced dumb expression in adult MBs and *dumb^3* mutants displayed normal LTM (Figure 3B; see Figures S3E and S3F for

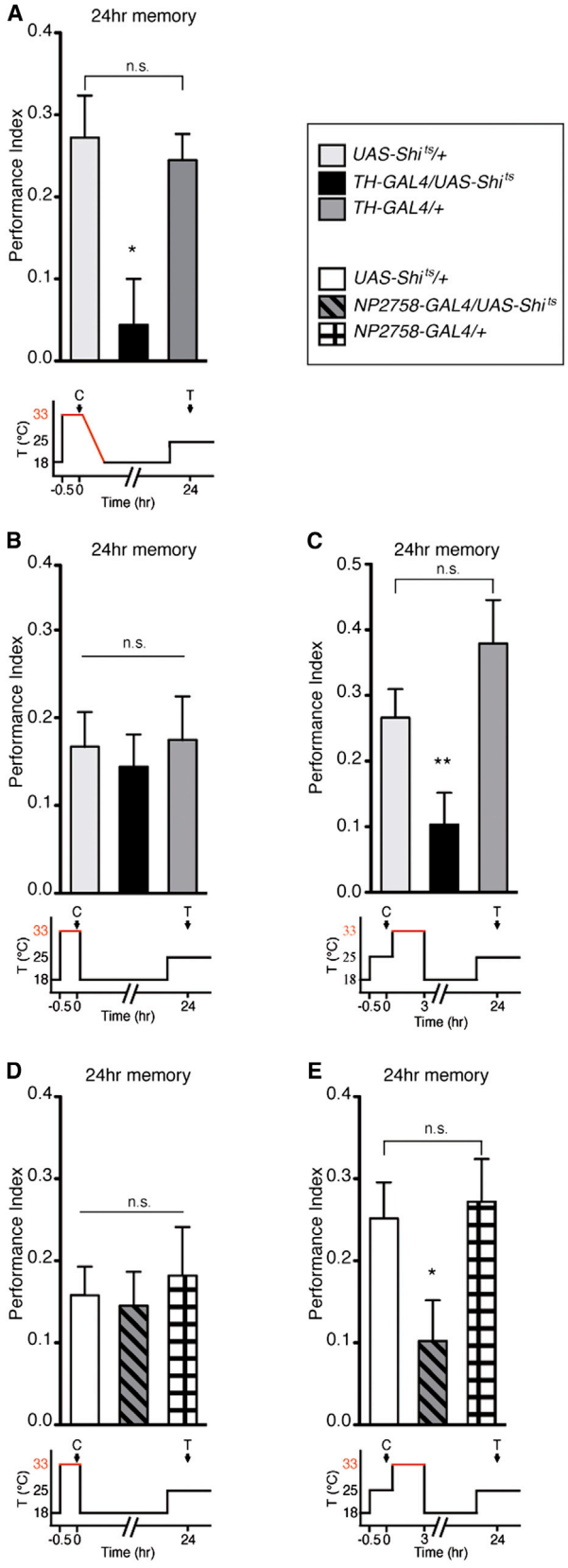

Figure 2. Specific Requirement of MB-MP1 Dopaminergic Neurons during LTM Consolidation

(A) Blocking *TH-GAL4* neurons during training and early consolidation (approximately 30 min) impaired LTM ($F_{(2,34)}$ = 6.842; p = 0.003; n ≥ 11).

(B) Blocking *TH-GAL4* neurons strictly during training did not affect LTM ($F_{(2,36)}$ = 0.127; p = 0.880; n = 13).

(C) Strict blockade of *TH-GAL4* neurons during the first 3 hr of consolidation impaired LTM ($F_{(2,44)}$ = 6.450; p = 0.003; n = 15).

(D) MB-MP1 neurons (*NP2758-GAL4*) were not required during LTM acquisition ($F_{(2,53)}$ = 0.1621; p = 0.8508; n = 18).

(E) The same MB-MP1 neurons were required during consolidation ($F_{(2,52)}$ = 3.686; p = 0.0321; n ≥ 17).

See also Figure S2. Means ± SEM; statistical test: one-way ANOVA; n.s.: p ≥ 0.05; *p < 0.05; **p < 0.01 in post hoc comparisons with both parental controls.

control), suggesting that another DA receptor is active downstream of MB-MP1 neurons. We then expressed an RNAi construct that targets the DAMB DA receptor ($DAMB^{RNAi1}$) in adult MBs (Han et al., 1996) and observed normal appetitive STM, whereas LTM was strongly affected (Figures 3C and 3D). LTM at the permissive temperature as well as sugar response and olfactory acuity controls were all normal in the $DAMB^{RNAi1}$ mutant (Figures S3G–S3I). A specific LTM defect was also observed for flies expressing an alternative non-overlapping RNAi construct that targets *DAMB* (Figures S3J–S3O). Thus, the DAMB receptor is specifically required for appetitive LTM and the dumb receptor is specifically required for appetitive STM. This suggests a post-training role for DAMB during MB-MP1 DA signaling, although the time resolution for RNAi experiments is too low to independently analyze the role of DAMB during the training and post-training phases.

We next used in vivo imaging to further investigate the post-training implication of MB-MP1 during LTM processing. Previously, we demonstrated that MB-MP1 neurons display spontaneous calcium oscillations (Plaçais et al., 2012) that are sensitive to satiety state (Plaçais and Preat, 2013). Here, MB-MP1 activity was monitored for 90 min post-training with a reinforcing stimulus that was either energetic (D-glucose) or non-energetic (L-glucose; Figures 4B and S4C). We observed 30 min after conditioning that the frequency and quality factor of MB-MP1 neuron oscillations were significantly higher (Figures 4C and S4D) in nutritious D-glucose-trained flies with respect to those in L-glucose-trained flies, whereas the oscillation amplitude did not differ (Figure S4E). At longer time points (60 and 90 min), flies from both groups displayed an equivalent oscillation frequency (Figure 4C), amplitude, and quality factor (Figures S4D and S4E). These results demonstrate an early post-training activity of MB-MP1 neurons that correlated with the energetic value of the sugar reward. Accordingly, L-glucose-conditioned flies re-fed 3 hr after training with D-glucose (Figures 4E and S4F), which exhibited normal LTM scores (cf. Figure 1B), displayed a higher oscillation frequency within MB-MP1 neurons 30 min after the re-feeding than flies re-fed with L-glucose (Figure 4F). Quality factor and amplitude oscillations were equivalent in both groups (Figures S4G and S4H). Together, these results confirm MB-MP1 implication in the processing of energetic level information in appetitive LTM.

Here, we showed that MB-MP1 activity is required for energetic level signaling during appetitive LTM consolidation. We

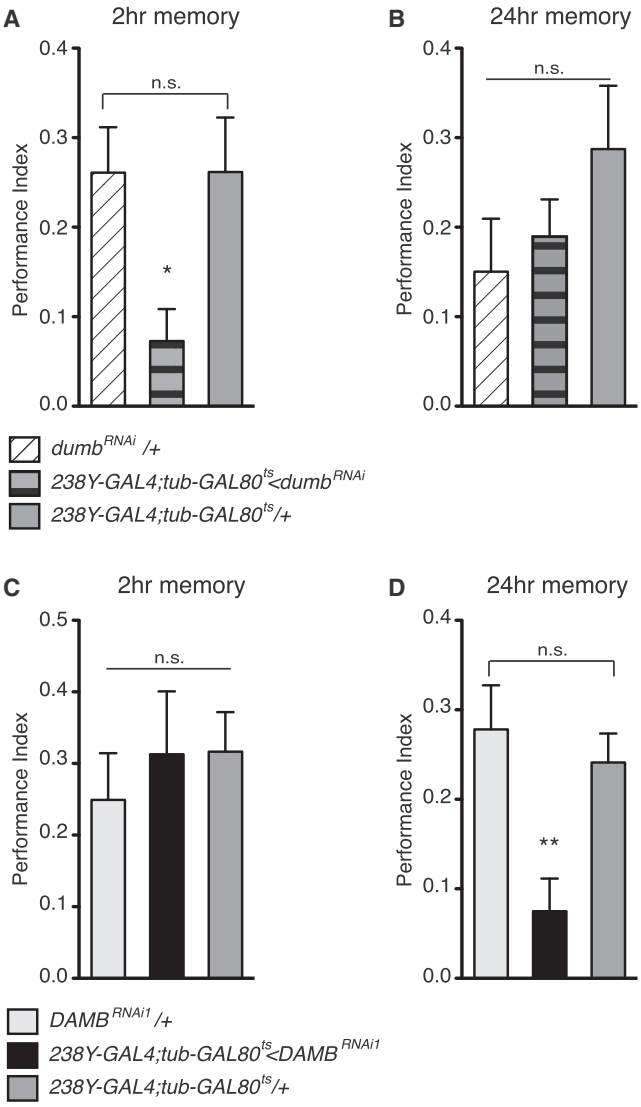

Figure 3. DAMB Is Required Specifically for Appetitive LTM Formation

(A) STM was impaired by RNAi inhibition of *dumb* in MBs of adult flies ($F_{(2,48)}$ = 4.909; p = 0.011; n ≥ 16).

(B) LTM was not impaired by RNAi inhibition of *dumb* in MBs of adult flies ($F_{(2,40)}$ = 1.463; p = 0.244; n ≥ 13).

(C) STM was not impaired by RNAi inhibition of *DAMB* in MBs of adult flies ($F_{(2,34)}$ = 0.299; p = 0.743; n ≥ 11).

(D) LTM was impaired by RNAi inhibition of *DAMB* in MBs of adult flies ($F_{(2,48)}$ = 6.87; p = 0.002; n ≥ 15).

See also Figure S3. Means ± SEM; statistical test: one-way ANOVA; n.s.: p ≥ 0.05; *p < 0.05; **p < 0.01 in post hoc comparisons with both parental controls.

next asked whether MB-MP1 activity was sufficient to drive LTM consolidation in the absence of energy supply during the appropriate time window. To address this issue, we used the thermosensitive tool *UAS-TrpA1*, which allows transient neuronal activation at high (30°C) temperature (Rosenzweig et al., 2008). We conditioned flies with L-glucose at 20°C and activated MB-MP1 for 30 min, 3 hr after training. Strikingly, these flies formed LTM (Figure 5), unlike the genotypic control groups (Figure 5) or flies kept at the permissive (20°C) temperature (Figure S5). Thus, MB-MP1 activation mimicked energetic post-training refeeding and was sufficient for LTM formation in the absence of any energy supply during the 6 hr following training.

DISCUSSION

Our behavioral results with enantiomeric sugars confirm that appetitive LTM can be formed only if the sugar reward is nutritious (Burke and Waddell, 2011). Moreover, our results uncover fundamental aspects of appetitive LTM formation, as we demonstrate that the processing of nutritive value depends on post-ingestion signaling of energy level. Thus, we established that hemolymph energetic sugar level controls appetitive LTM consolidation by taking advantage of the specific blocking of glucose intestinal transport to the hemolymph via phlorizin.

Remarkably, we were able to create artificial olfactory LTM by utilizing a non-energetic sugar during training and by feeding flies with energetic food in a critical period following training that lasted about 5 hr. These findings suggest that, after odor/sugar association, there is an open time window of 5 hr in which an energetic supply allows the consolidation of the initial association into LTM. This two-step protocol allowed us to differentiate training signaling related to initial association from energy-level signaling. We established that MB-MP1 DA neurons play a critical role in signaling energy level to the MBs during LTM-consolidation phase. It is worth noting that the same neurons were previously identified as controlling motivation during appetitive STM recall (Krashes et al., 2009). The functions of these neurons during LTM formation and recall are compatible. Indeed, if MB-MP1 neurons are active when energy level is high, the recall of appetitive STM (which normally requires that flies are starved to be motivated) occurs when MB-MP1 activity is low, because there is no available energy. Because energy level appears low to MBs when they are "misled" by MB-MP1 blockade (whatever the real energy level of the fly), STM can be recalled in fed flies. Conversely, activation of MB-MP1 impairs STM recall in starved flies (Krashes et al., 2009).

Appetitive LTM is consolidated when energy level is high, and we observed MB-MP1 to be more active during LTM consolidation in a specific time window of 30 min after training. Interestingly, LTM is formed in flies trained with a non-energetic sugar but re-fed 3 hr later with energetic food; accordingly, we observed an increase in MB-MP1 activity within a 30-min time window delayed by 3 hr with respect to training. The fact that MB-MP1 activity can be delayed by several hours with respect to LTM formation training raises the question of how the post-ingestion signal can associate specifically with the olfactory cue paired with sugar during training. This is a particularly critical question, given that two odorants are presented to flies during the learning phase, only one of which is associated with sugar. We propose here that associative LTM formation in *Drosophila* is a two-step process that involves two properties of the sugar unconditioned stimulus. First, PAM DA neurons are necessary during training to associate the sweetness stimulus with the odorant in the MB (Liu et al., 2012; Figure S2E). The initial step that

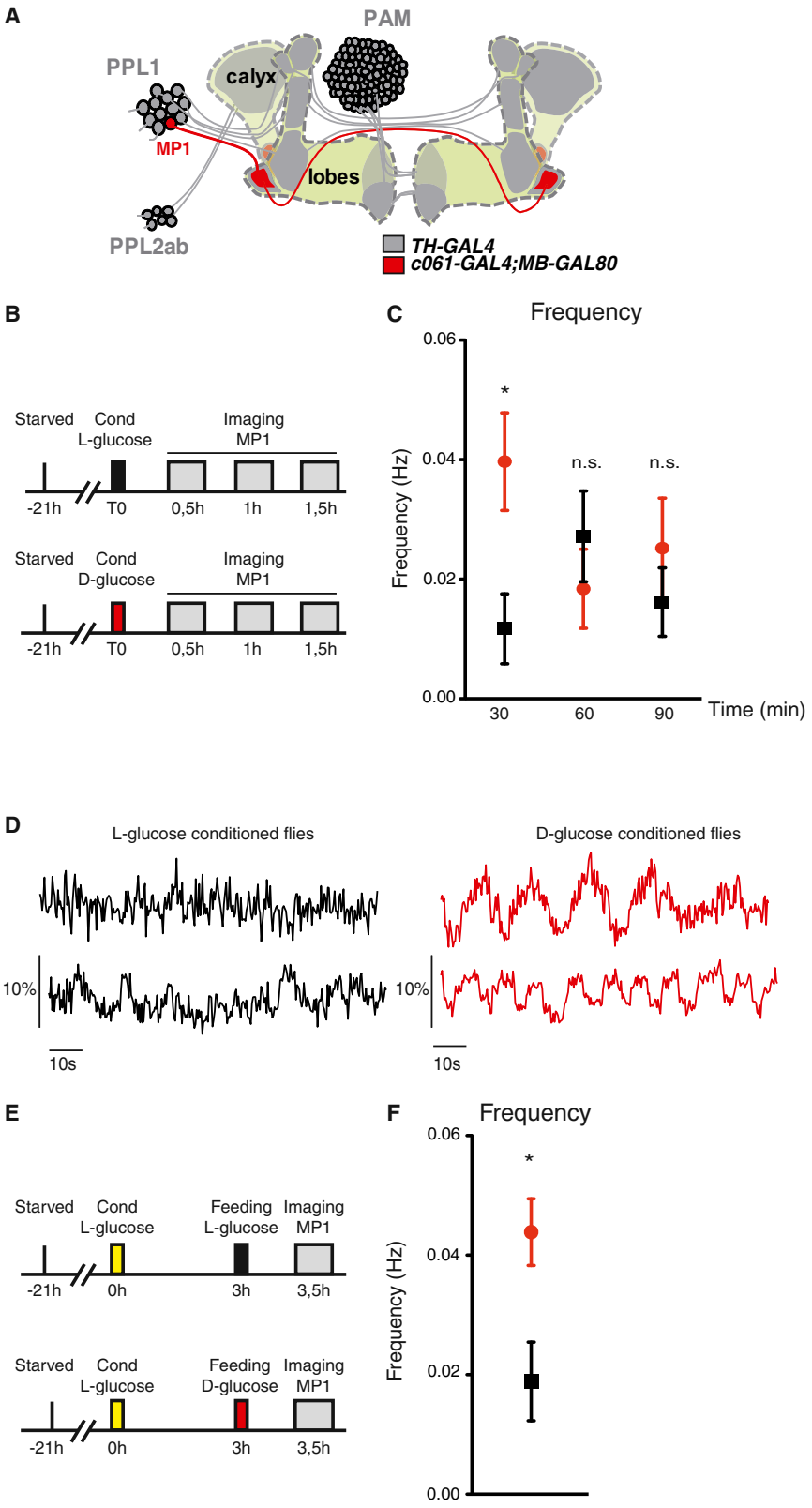

Figure 4. MB-MP1 Oscillatory Activity Is Enhanced during Appetitive LTM Formation

(A) Schematic drawing of mushroom-body-connected neurons. *c061-GAL4;MB-GAL80* expression corresponds to a single DA in the PPL1 cluster, MB-MP1 (red).

(B) Training protocols used before imaging experiments: flies were conditioned on either L-glucose or D-glucose and imaged at three time points (30, 60, and 90 min after conditioning).

(C) A higher oscillatory frequency is observed for D-glucose-conditioned flies (red) as compared to L-glucose-conditioned flies (black) during early consolidation (30 min after training; Mann-Whitney: $p = 0.0229$), although oscillation frequencies become equivalent for both groups at later time points (60 and 90 min after training; Mann-Whitney: $p = 0.423$; $p = 0.45$, respectively). $n \geq 7$ for all data.

(D) Two examples of MB-MP1 recording, 30 min after L-glucose conditioning (black) or D-glucose conditioning (red). Black traces correspond to irregular non-oscillating activity with no peak.

(E) Training protocols used before imaging experiments: flies were conditioned on L-glucose and re-fed 3 hr after training on either L-glucose or D-glucose for 1 min and imaged 30 min after re-feeding.

(F) A higher oscillatory frequency is observed for D-glucose re-fed flies (red) as compared to L-glucose re-fed flies (black) (Mann-Whitney: $p = 0.011$; $n = 12$).

See also Figure S4. Means ± SEM; statistical test: Mann-Whitney; **$p < 0.01$ in comparison between two groups.

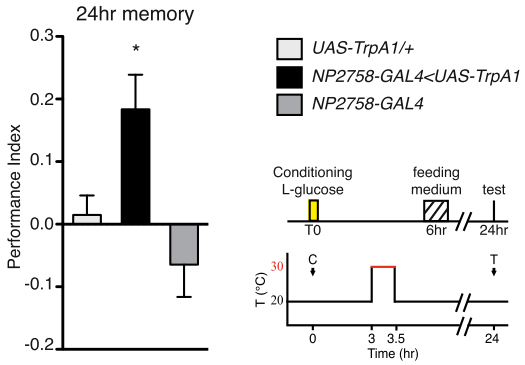

Figure 5. MB-MP1 Activation Allows LTM Formation in Absence of Energy

We trained starved flies with L-glucose, activated MB-MP1 neurons for 30 min with TrpA1 3 hr after conditioning, and finally fed flies for 30 min 6 hr after training. This protocol led to LTM formation (score significantly different from 0, t test, $t_9 = 3.311$, p = 0.009, and significantly higher than that of both parental groups, $F_{(2,27)} = 7.201$, p = 0.003). n = 10 for all data. See also Figure S5. Means ± SEM; statistical test: t test and one-way ANOVA; n.s.: $p \geq 0.05$; *$p < 0.05$ in comparison between two groups for t test and in post hoc comparisons with both parental controls for ANOVA.

associates gustatory information with odor can be considered as a priming process for LTM. It does not depend on sugar energetic value and does not involve MB-MP1 neurons. In a second step, the association formed during training is consolidated into LTM only if a post-ingestion signal that reflects the presence of energy is sent to the MBs by MB-MP1 DA neurons. We showed that MB-MP1 activity was necessary and sufficient to engage LTM consolidation, even in the absence of ingestion of energetic food. MB-MP1 activity is thus a "checkpoint" for appetitive LTM processing. The 5-hr temporal window in which the energy level signaling can occur probably reflects the lifetime of the priming process. Such a two-step mechanism for appetitive LTM formation provides additional security to associate true nutritive value with odor, rather than an association based on taste alone. Indeed, *Drosophila* innate food preference strongly correlates with the activity of the "sweetness" taste receptor response (Slone et al., 2007; Gordesky-Gold et al., 2008), which is plausible because generally sweet food is energetic. We identified post-feeding signals that can modulate this innate behavior through associative learning, to take into account the real nutritious value. Altogether, these findings explain how the *Drosophila* brain evaluates and remembers food energetic value.

Interestingly, a gating mechanism also exists for the consolidation of aversive LTM that relies on the activity of a group of three DA neurons that includes MB-MP1 (Plaçais et al., 2012). This gating of aversive LTM allows preventing aversive LTM formation when flies are starved (Plaçais and Preat, 2013). This is crucial because aversive LTM formation presents a non-negligible cost for flies (Mery and Kawecki, 2005), and flies that form aversive LTM while they are food-deprived exhibit decreased survival (Plaçais and Preat, 2013). Similarly, when ingested food is non-energetic, the two-step mechanism prevents appetitive LTM formation and thus avoids a possible waste of energy.

EXPERIMENTAL PROCEDURES

Fly Strains

Fly stocks were raised on standard food at 18°C and 60% relative humidity under a 12 hr:12 hr light:dark cycle. The Canton-Special (CS) strain was selected as the wild-type *Drosophila melanogaster* strain. All transgenes were used in a CS background. For DAMB experiments, we used lines expressing two non-overlapping RNAi constructs targeted against *DAMB*: the JF02043 line from the Transgenic RNAi Project (TRiP) and the KK110947 line from the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). For *Dumb* experiments, we used the KK102341 RNAi line from the VDRC and the hypomorphic mutant allele $dumb^3$ PL00420 (Seugnet et al., 2008). We used *50B03-GAL4* and *15E10-GAL4* drivers (Jenett et al., 2012) to manipulate MV1 DA neurons. *TH-GAL4*, *NP0047-GAL4*, *NP2758-GAL4*, *MZ604-GAL4*, *UAS-TrpA1* and $UAS\text{-}shi^{ts}$ (Plaçais et al., 2012), *c061-GAL4;MB-GAL80*, and *TH-GAL80*; $UAS\text{-}shi^{ts}$ were described in a previous study (Aso et al., 2010). We used $tub\text{-}GAL80^{ts}$ for adult transgene expression (McGuire et al., 2003). For imaging experiments, flies were raised on standard food at 25°C and 60% relative humidity under a 12 hr:12 hr light:dark cycle. For calcium reporting, we used the *20XUAS-IVS-GCaMP3* line (in the VK00005 insertion site) from the Janelia Farm Research Center (Akerboom et al., 2012).

Training Protocol

After eclosion, adult flies were kept overnight in fresh bottles containing standard medium and then transferred at 25°C into starvation bottles containing a cotton wool disk soaked with 6.8 ml of mineral water for 16–21 hr. Flies were starved for 24 hr at 20°C specifically for the activation experiment, to avoid activating TrpA1-expressing neurons during the starvation period. The conditioning apparatus and protocol were previously described (Colomb et al., 2009). Briefly, groups of 30–40 flies of a given genotype were conditioned in a barrel by exposure to one odor paired with a sugar reward, with subsequent exposure to a second odor in the absence of sucrose. The sequence of a single training session consisted of an initial 90-s period of non-odorized airflow, 60 s of one odor, 52 s of non-odorized airflow, 60 s of the second odor, and 52 s of non-odorized airflow. Odors were produced using 3-octanol (>95% purity; Fluka 74878, Sigma-Aldrich) at 3.60×10^{-4} M and 4-methylcyclohexanol (99% purity; Fluka 66360) at 3.25×10^{-4} M diluted in paraffin oil. For D-glucose, L-glucose, and D-fructose experiments, sucrose was replaced by L-glucose, D-glucose, or D-fructose as the sugar reward, with respect to the protocol described in Colomb et al. (2009). All sugars and phlorizin were obtained from Sigma-Aldrich. For phlorizin experiments, sucrose was replaced by a mixture of D-glucose and phlorizin or D-fructose and phlorizin.

For re-feeding experiments, flies were either re-fed on standard food medium, nutritious D-glucose, or a mixture of D-glucose and phlorizin. When re-fed on standard medium, flies were transferred into normal food bottles for 30 min, either 3 or 6 hr after conditioning, and then returned to starvation bottles. For sugar re-feeding experiments, flies were placed for 1 min in a test tube. The tube walls were covered with either D-glucose or a mixture of D-glucose and phlorizin (3 hr after conditioning). Flies were then returned to starvation bottles. For imaging, flies were conditioned with either L- or D-glucose and imaged 30 min after training (see Figures 4B–4D). For the imaging experiment with re-feeding, flies were conditioned on L-glucose and then re-fed with either L- or D-glucose for 1 min, 3 hr after training (see Figures 4E and 4F).

All sugars were used at 2 M concentration in mineral water. Phlorizin was pre-dissolved in mineral water and used at a 200 mM final concentration.

Test of Memory Performance

During the memory performance test, flies were exposed to both odors simultaneously in a T-maze for 1 min. The performance index (PI) was calculated as the number of flies attracted to the conditioned odor minus the number of flies attracted to the unconditioned odor, divided by the total number of flies in the experiment. A single PI value represents the average of the scores from two groups of flies of identical genotype trained with either octanol or methylcyclohexanol as the CS+ (i.e., an odor paired with the sugar presentation).

Temperature-Shift Protocols

To block synaptic transmission during training and early consolidation, flies expressing Shi^{ts} were placed at the restrictive temperature (33°C) 30 min before training, transferred into bottles at the restrictive temperature, and put back into an incubator at the permissive temperature (18°C for LTM and 25°C for 2-hr memory experiments) approximately 10–15 min after acquisition, where they were progressively returned to the incubator temperature. For sharp blockade during training, flies were placed at the restrictive temperature 30 min before training and then conditioned on the second odor; immediately after training, an airflow at the permissive temperature (25°C) was injected through the barrels. For sharp blockade after training, flies were conditioned on the second odor at the permissive temperature, and immediately after training, an airflow at the restrictive temperature was passed through the barrels for 3 hr. Permissive-temperature control experiments were performed at 25°C. Time courses of the temperature shifts employed in each experiment have been provided alongside the graph of memory performance in each relevant figure. For RNAi expression in adult MBs, flies were maintained at 30.5°C for 2 days prior to training, after which experiments were performed at 25°C. For non-induced experiments, flies were placed at 18°C for 2 days prior to training and the experiments were then performed at 25°C. For LTM experiments, flies were stored at 18°C after acquisition, prior to testing.

To activate synaptic transmission during consolidation, flies expressing TrpA1 were trained at a permissive temperature (20°C), stored for 3 hr, and placed in a room at 30°C for 30 min and then placed back at 20°C. Memory tests were performed at 20°C. In the control experiment, starvation, conditioning, resting, and testing were all performed at the permissive temperature (20°C).

Sugar Response Tests

Tests were performed on starved flies in a T-maze apparatus as previously described (Colomb et al., 2009) but without airflow. Flies were trapped in either maze arm after 1 min. The arm with sugar was placed alternately on the right or left. Sugar response was calculated as for the memory test and then used as a score. The sugar response tests were performed at the restrictive temperature for flies carrying the *UAS-shi^{ts}* transgene (33°C) and at 25°C (following 2 days of induction at 30.5°C) for flies carrying the *tub-GAL80^{ts}* transgene.

Olfactory Acuity

Tests were performed as previously described (Colomb et al., 2009) at the restrictive temperature for flies carrying the *UAS-shi^{ts}* transgene (33°C) and at 25°C (following 2 days of induction at 30.5°C) for flies carrying the *tub-GAL80^{ts}* transgene. Flies were starved for 21 hr before the olfactory test. One odor was tested for 1 min against its solvent (paraffin oil). The response index was calculated as for the memory response test and then used as a score. The odor was delivered alternately through the right or left arm of the maze. A PI of 1 indicates complete behavioral repulsion.

In Vivo Calcium Imaging

In vivo confocal imaging and subsequent data analysis of spontaneous activity were performed following a previously described protocol (Plaçais et al., 2012; Plaçais and Preat, 2013). Images were acquired at a rate of one image every 410 ms. Only female flies of the genotype w^{1118}/w^{1118}, *c061-GAL4/+*; *UAS-IVS-GCaMP3/MB-GAL80* were used in imaging experiments. MB-MP1 neuron activity was reported from the normalized fluorescence variations (ΔF/F0) in MB projections, as previously described (Plaçais et al., 2012). Amplitude, frequency, and quality factor were calculated for each fly.

Data Analysis and Statistics

All data are presented as means ± SEM. A two-tailed unpaired t test was used to compare the data series between the two conditions. Results of t tests are given as the value t_x of the t distribution with x degrees of freedom obtained from the data. Comparisons between more than two distinct groups were analyzed by one-way ANOVA. ANOVA results are given as the value of the Fisher distribution $F_{(x,y)}$ obtained from the data, where x is the number of degrees of freedom between groups (one-way ANOVA) and *y* is the residual number of degrees of freedom. ANOVA was followed by pairwise planned comparisons between relevant groups with a Student-Newman-Keuls test. Asterisks denote the smallest significant difference between the relevant group and its genotypic controls, using post hoc pairwise comparisons (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; NS, not significant). For imaging, data frequency and quality factor were considered as null (0) in non-oscillating flies; for amplitude calculation, we retained the complete signal, including noise. Left and right MB-MP1 recordings in each fly were recorded independently, whereas a single value for amplitude, quality factor, and frequency was derived for each fly by averaging the values from the two hemispheres. Frequency and quality factor comparisons between groups were performed using the Mann-Whitney non-parametric test, whereas a two-tailed unpaired t test was used for amplitude.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.036.

AUTHOR CONTRIBUTIONS

P.-Y.M. performed experiments. P.T. and T.P. supervised the work. P.-Y.M., P.T., and T.P. designed experiments, discussed the results, and wrote the manuscript.

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