System-like consolidation of olfactory memories in *Drosophila*

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Abstract

System consolidation, as opposed to cellular consolidation, is defined as the relatively slow process of reorganizing the brain circuits that maintain long-term memory. This concept is founded, in part, on observations made in mammals that recently formed memories become progressively independent on brain regions initially involved in their acquisition and retrieval, and dependent on other brain regions for their long-term storage. Here we present evidence that olfactory appetitive and aversive memories in *Drosophila* evolve using a system-like consolidation process. We show that all three classes of mushroom body neurons (MBn) are involved in the retrieval of short- and intermediate-term memory. With the passage of time, memory retrieval becomes independent of α'/β' and γ MBn, and long-term memory becomes completely dependent on α/β MBn. This shift in neuronal dependency for behavioral performance is paralleled by shifts in the activity of the relevant neurons during the retrieval of short- vs long-term memories. Moreover, transient neuron inactivation experiments show that the α'/β' MBn have a time-limited role in memory processing using flies trained to have both early and remote memories. These results argue that system consolidation is not a unique feature of the mammalian brain and memory systems, but rather a general and conserved feature of how different temporal memories are encoded from relatively simple to complex brains.

Introduction

Memories, shortly after they are acquired, are labile and susceptible to disruption by factors such as interference information, injuries, or amnesiac agents (Scoville and Milner, 1957; McGaugh and Alpern, 1966; Squire et al., 1975; Quinn and Dudai, 1976). If acquisition signals are sufficiently strong, often produced by spaced training (Carew et al., 1972; Tully et al., 1994), memories mature, become resilient to these insults, and endure in a long-lasting form. The process of progressive stabilization is referred to as memory consolidation that consists of at least two mechanistically and temporally different forms: cellular consolidation, which is relatively fast and requires cell signaling within the local nodes or individual neurons of the brain; and system consolidation, which is slow and involves communication between neurons in different regions of the brain, the reorganization of brain circuits and the relocation of the traces that represent the memory (Dudai, 2004; Frankland and Bontempi, 2005). The standard model for system consolidation in mammals (Dudai, 2004) envisions initial memories being encoded in the medial temporal lobe system and relevant areas of the neocortex, with subsequent reorganization of circuits and memory traces such that the neocortex assumes the burden of storing long-term memory (LTM).

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The concept of system consolidation was founded in several seminal observations. First, humans with lesions limited to the medial temporal lobe display temporally graded retrograde amnesia (Scoville and Milner, 1957), with recent memories being abolished and remote memories being spared (Kapur and Brooks, 1999; Manns et al., 2003). Second, selective lesions to the hippocampal formation or entorhinal cortex of laboratory animals produce a similar, temporally graded amnesia (Zola-Morgan and Squire, 1990; Cho et al., 1993; Clark et al., 2002). In contrast, pharmacological inactivation of cortical regions disrupts remote but not early memories (Frankland et al., 2004; Maviel et al., 2004). Finally, functional imaging experiments have provided a critical and defining feature of system consolidation. The retrieval of recent memories preferentially activates neurons in the hippocampus but not the cortical regions tested, while the retrieval of remote memories activates these cortical but not hippocampal regions (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004; Frankland and Bontempi, 2005).

Several lines of evidence are consistent with the hypothesis that olfactory memories generated by classical conditioning of *Drosophila* undergo system consolidation (see Discussion). Here, we offer a complete set of data consistent with the hypothesis that both aversive and appetitive olfactory memories in *Drosophila* are processed in a system consolidation-like manner. Most importantly, we have modeled our studies from the standards used to define system consolidation in mammalian organisms.

Material and Methods

Fly Lines

Flies carrying *uas* transgenes included: (1) *uas-shi[ts]*, containing one copy on the X-chromosome and two copies on the third chromosome (Kitamoto, 2001), (2) *uas-G-CaMP3.0* (Tian et al., 2009) and, (3) two lines containing a *uas-kir2.1* insertion on the second or third chromosome (Baines et al., 2001). *Gal4* driver lines included *c305a-gal4, c739-gal4, 1471-gal4* and *247-gal4*. Numerous groups have characterized the expression patterns for these lines but a single-source reference can be found in Aso et al (Aso et al., 2009). In addition the line *R28H05-gal4* was used as an alternative α/β MBn driver (Jenett et al., 2012). Finally, *tub-gal80[ts]* was used in TARGET experiments (McGuire et al., 2003).

Behavioral Conditioning

For aversive and appetitive training, flies were conditioned behaviorally using a standard, two-odor discriminative, and negatively or positively reinforced classical-conditioning paradigm (Tempel et al., 1983; Tully and Quinn, 1985). For appetitive conditioning, flies were starved 16-24 hr in vials containing 0.8% non-nutritive agar. Then, groups of ~60 flies were exposed to CS− odor with a dry filter paper, previously saturated with water, for two minutes, followed by 30 sec of air and then 2 min of CS+ odor together with a dried filter paper previously saturated with 2 M sucrose. This order in the odor presentation was performed to avoid any possible contamination of sucrose on the legs of flies that could become associated with the CS− odor. For aversive conditioning, flies were exposed to 1 min of CS+ odor together with 12 electric shocks (90 V, 1.5 s) followed by 30 s of air and 1 min of the CS− odor. Table 1 shows olfactory avoidance for all genotypes used in this study.

Functional Imaging

Functional cellular imaging was performed as previously described (Yu et al., 2005; Cervantes-Sandoval and Davis, 2012). Flies were prepared for functional imaging either 1 hr or 24 hr after conditioning. Flies were mounted in pipette tips and their exposed heads were secured to the tip opening using melted myristic acid. A small area of the cuticle was removed from the top of the head capsule and the opening was covered with a piece of

plastic wrap. The flies were then mounted beneath a 20X objective of a Leica TCS confocal microscope and imaged with a 488 nm excitation line. The emitted light was collected from 520±15 nm. Odors were delivered with pressurized air flowing at a rate of 200 ml per min. Odorants were diluted 1:10 with mineral oil and spread on a 1 cm^2 filter paper inside a scintillation vials and in line with the pressurized air. The delivery of odorants was accomplished with a three-way Teflon valve under the control of a programmable timer such that fresh air could be delivered to the animals for a determined period with an instantaneous switch to odor-laced air without altering the overall flow rate. Each fly was used only once to avoid odor habituation, adaptation, sensitization or generalization.

Images were acquired at 5 frames per second at a resolution of 256×256 pixels. The image data were analyzed as already described (Yu et al., 2005). In general, xyt imaging stacks were obtained and regions of interest circumscribed. The value of F_o was calculated for each pixel within the region of interest as the fluorescence prior to odor application as averaged over five successive frames. The value of ΔF was calculated for each pixel within the region of interest as the difference between the maximum average intensity during 3 sec odor application for five successive frames and F_o (Yu et al., 2005; Yu et al., 2006). Imaging data is presented as the ratio of the response to the CS+ ($\Delta F/F_o$) to the CS− ($\Delta F/F_o$) (Cervantes-Sandoval and Davis, 2012). Statistical significance was determined using two-way ANOVA followed by pairwise non-parametric Mann-Whitney comparisons.

Results

Disrupting different MB neurons impairs retrieval of different temporal phases of appetitive and aversive olfactory memories

To determine which candidate regions of the *Drosophila* brain are required for retrieval of recent vs remote memories, we first probed whether retrieval processes required intact synaptic function of the three classes of MBn at six different times after appetitive olfactory conditioning. Prior characterization of appetitive memory in *Drosophila* revealed that a single training session generates memory that lasts for days, with the memory becoming dependent on protein synthesis by 6 hr after conditioning (Krashes and Waddell, 2008; Colomb et al., 2009). We used *shi^{ts}*, a temperature-sensitive and dominant-negative version of dynamin, to impose a synaptic blockade during the test for memory performance. Flies containing a specific MBn *gal4* driver (*c305a-gal4, 1471-gal4, c739-gal4* or *247-gal4* for α′β′, γ, αβ, and αβγ, respectively) and the *uas-shi^{ts}* transgene where trained at 24°C and shifted to 32°C, 10 min before and during testing at different post-training time points. Control flies containing only the *gal4* driver or the *uas-shi^{ts}* transgene showed no decrease in memory performance, but combining the two elements to block synaptic output from α′/β′ MBn completely impaired memory retrieval from 15 min to 3 hr after appetitive conditioning (Figure 1A). A prior study suggested that appetitive memory retrieval was independent of α′/β′ MBn output tested at 2 hr (Trannoy et al., 2011). This conflicting result may be explained by the use of different versions of *uas-shi^{ts}* transgene, and/or assay insensitivity in the prior study revealed by low performance indices for control flies (~0.2) compared to ~0.4 obtained here. Moreover, testing at four sequential time points, as performed here, offers greater reliability than the single time point tested previously (Trannoy et al., 2011). Sugar responsiveness has been previously shown to be normal in the genotypes tested here (Krashes and Waddell, 2008; Trannoy et al, 2011).

Memory expression was also blocked after aversive conditioning from 15 min to 1.5 hr after training, with expression becoming independent of α′/β′ MBn blockade by 3 hr (Figure 2A), consistent with other studies (Krashes et al., 2007). Aversive olfactory memory generated by a single training session decays to near zero by 24 hr, and is independent of protein synthesis. Therefore, spaced training was utilized to generate protein synthesis-

dependent 24 h LTM after aversive training (Tully et al., 1994). Blocking the output of α′/β′ MBn had no effect on the retrieval of 24 h protein synthesis-dependent LTM (Figure 2A). These data indicate that memory retrieval is dependent on the synaptic output of the α′/β′ MBn to at least 3 hr for appetitive memories and at least 1.5 hr for aversive memories. It then becomes independent of neurotransmission from these neurons.

Blocking neurotransmission from γ MBn partially impaired (~30-50%) retrieval of appetitive olfactory STM and ITM (15 min-3 hr) but left LTM (9-24 hr) retrieval intact (Figure 1B). Similar results were obtained upon blocking the output of γ MBn after aversive training (Figure 2B). These results reveal as for the α′/β′ MBn, that olfactory memories in *Drosophila* are initially dependent on the output of γ MBn and that they lose this dependency with time.

Finally, blocking the output of α/β MBn impaired (~30%) the performance of STM and ITM and completely abrogated the retrieval of LTM (Figure 1C). Once again, similar results were obtained when we evaluated the output requirement of α/β MBn for the retrieval of aversive memories (Figure 2C). Our results with aversively conditioned flies are in agreement with data from more limited studies (McGuire et al., 2001; Akalal et al., 2006).

A small decrement in expression of aversive memory was observed during retrieval at early time points at restrictive temperature in control flies carrying only the *gal4* or *uas-shi^{ts}* elements due to heat stress (Figure 2A-D). This non-specific effect of heat was observed by two independent laboratory investigators collecting data for this study, occurs only at early time points (<1.5 hr) after conditioning, is not observed after appetitive conditioning (Figure 1), and has been observed in numerous prior studies employing aversive olfactory conditioning (Waddell et al., 2000; Dubnau et al., 2001; McGuire et al., 2001; Zhang et al., 2008; Berry et al., 2012). Nevertheless, this decrement in performance was much smaller than the decrement observed in experimental flies carrying both the *gal4* and *uas-shi^{ts}* transgenes.

The partial effect of blocking neurotransmission of α/β and γ MB cells in the retrieval of STM/ITM led us to ask whether the partial contribution of these neuron subsets for STM/ITM retrieval are summative and therefore involve parallel pathways, or whether they are serial in the same pathway. For this, we imposed synaptic blockade during retrieval using the *247-gal4* transgene which drives expression in both subsets of MBn. Complete impairment of appetitive STM/ITM and LTM was observed when α/β and γ MBn were blocked during retrieval (Figure 1D), suggesting that these subsets contribute to memory expression in parallel pathways for the normal retrieval of STM/ITM. Shock avoidance has been shown to be normal in the genotypes tested here (Isabel et al, 2004; Krashes et al, 2007).

MB neurons' requirement for retrieval is independent of odor selection

Due to reports that odors are sparsely represented in the MBn (Wang et al., 2003; Turner et al., 2008), with different odors perhaps represented unequally by the three subtypes of MBn, there was the possibility that the memory retrieval dependency results obtained above could be dependent on the odor combination used for conditioning [3-octanol (Oct) and 4-methylcyclohexanol (Mch)]. To test this possibility, we evaluated the dependency on MBn subsets for retrieval of 1h and 24 hr appetitive memory using a different odor combination of benzaldehyde (Ben) and methylsalicylate (Mes). Our results showed a similar dependency on synaptic transmission from the various classes of MBn for the retrieval of olfactory memories. Synaptic blockade of α′/β′ and γ MBn disrupted 1 hr but not 24 hr memory, whereas blocking α/β MBn compromised 1 hr memory and completely abolished LTM at 24 hr (Figure 3).

Together, these results indicate that olfactory memory retrieval is initially dependent on synaptic transmission of all three subsets of MBn. With maturation of the memory engram, the dependence on α'/β' and γ MBn wanes and retrieval becomes completely dependent on synaptic transmission from the α/β MBn. This shift in dependence suggests that olfactory memory processing in *Drosophila* is analogous to how memories are processed in mammals. Spatial and declarative memories are initially encoded in both hippocampal and cortical systems, which then undergo circuit reorganization leading to the loss of dependency on the hippocampal system and the complete dependence on the cortical network (Dudai, 2004).

Retrieval of recent and remote memories activates different subsets of MB neurons

Imaging studies in mammals, utilizing 2-deoxyglucose mapping or immediate-early gene activation, have revealed that the retrieval of recent and remote memories activates different brain regions. The recall of recent spatial or contextual fear memories is associated with activation of the hippocampus and entorhinal cortex. In contrast, the recall of remote spatial memories is predominantly associated with the activation of cortical regions (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004). We have previously utilized functional cellular imaging in living flies to show that odor evoked calcium responses in the α/β MBn to the learned odor increase at 9 hr after aversive conditioning. This enhancement persists through 24 hr and requires normal protein synthesis, spaced conditioning, the activity of the transcription factor Creb, and the normal function of 26 different long-term memory genes (Yu et al., 2006; Akalal et al., 2011). A similar enhanced response to the learned odor occurs in the α'/β' MBn at 1 hr after single cycle, aversive conditioning (Wang et al., 2008). We therefore quantitated the Ca^{2+} responses using G-CaMP 3.0 (Tian et al., 2009) in the vertical lobes of α'/β' and α/β MBn upon retrieval of appetitive STM/ITM (1 hr) and LTM (24 hr). We used the ratio of the response of the CS+ and CS− odor within each individual fly to quantitate the change in response to the learned odor (Cervantes-Sandoval and Davis, 2012).

As with aversive conditioning, we detected an increased response in the vertical branch of α'/β' MBn upon presenting the retrieval cue 1 hr after appetitive conditioning but not at 24 hr (Figure 4A). Naïve flies and flies trained with unpaired appetitive conditioning (Figure 4A and data not shown) exhibited no selective increase in the response to the CS+. This is an important observation in itself, showing that the α'/β' MBn form a time-limited, memory trace with both aversive and appetitive conditioning shortly after conditioning. We also detected an increased response in the vertical branch of the α/β MBn upon presenting the retrieval cue at 24 hr after appetitive conditioning but not at 1 hr (Figure 4B). Naïve flies and flies trained with unpaired appetitive conditioning (Figure 4B and data not shown) exhibited no increase in the response to the CS+. This illustrates that a single session of appetitive conditioning generates a late-forming, memory trace in the α/β MBn, similar to that observed when flies were trained with spaced, aversive conditioning (Figure 4C, D). These results are strikingly similar to results obtained using mammalian systems (Frankland et al., 2004), where the retrieval of recent memories preferentially activates the hippocampal region of the brain whereas no activation of this region is observed when a remote memory is retrieved (Figure 4E). In contrast, the retrieval of remote but not recent memories is accompanied by an activation of cortical regions (Figure 4F).

Disrupting α′/β′ MB neurons impairs recent but spares remote memories

The data above indicate a time-limited role for the α'/β' MBn in olfactory memory processing (Figure 1-4), whereas the α/β MBn have a clear role in retrieval across all time points (Figure 1-4). To definitively test the time-limited role for the α'/β' MBn, we employed a novel training paradigm in which flies were trained to have both early and remote memories using two different odor pairs. This experiment is analogous to hippocampal lesioning experiments in mammals, where animals are trained to bear both

recent and remote memories and then the impact of hippocampal lesions is evaluated in the retrieval of both memories. We used the Kir2.1 inward-rectifying potassium channel (Baines et al., 2001) to conditionally silence the activity of α′/β′ MBn or α/β MBn and then measured the capacity of these flies to retrieve a memory learned before the disruption ("remote memory") and a memory learned shortly after disruption ("recent memory") (Figure 5A). To control the expression of *kir2.1* we used the TARGET system: a ubiquitously expressed *gal80[ts]* transgene that represses the expression of the channel at 18°C but permits expression at 30°C (McGuire et al., 2003). We used the Kir2.1 channel for these inactivation experiments along with the TARGET system in order to produce an inactivation effect that is more permanent than can be achieved with the rapid inactivation and reversal obtained using *shi[ts]*.

We first determined the minimum time required for Kir2.1 under the dual control of *gal4/gal80[ts]* to exert effects using the pan-neuronal driver *c155-gal4*. Flies containing one of two versions of *uas-kir2.1, tubP-gal80[ts]* and *c155-gal4* were reared at 18°C and two days after hatching flies were transferred to 30°C and monitored every hour for complete paralysis. Flies carrying *uas-kir2.1* on the second chromosome became completely paralyzed within 6 hr after the temperature switch whereas flies carrying the third chromosomal insertion required 12 hr for complete paralysis (data not shown). The effect of expressing Kir2.1 under these conditions was irreversible. Subsequent experiments were performed using the second chromosomal *uas-Kir2.1* insertion given its greater potency.

Experimental flies were constructed to contain *uas-kir2.1, tubP-gal80[ts]* and *c305a-gal4*. They were starved at 18°C for 28 hr and then trained using olfactory appetitive conditioning with Oct and Mch as the CS+ and CS− odors for measuring LTM (Figure 5A; Training A - "remote memory"). They were then re-fed, re-starved, and trained again with Ben and Mes as the CS+ and CS− odors for measuring STM (Figure 5A; training B -"recent memory"). The experimental group was transferred to 30°C 6 hr prior to the second training experience whereas the control group was kept at 18°C throughout the experiment. Silencing the α′/β′ MBn 6 hr prior the second training episode completely disrupted the expression of the recent memory, whereas this silencing had no detectable effect on the remote memory (Figure 5B,C). The control flies containing only the *uas-kir2.1* or *gal4, tubP-gal80[ts]* genetic elements were able to display both remote and recent memories, with no apparent retroactive or proactive interference detected (Figure 5B,C). These data indicate that newly formed memories require the function of the α′/β′ MBn, but that remote memories are independent of this function.

Pilot experiments performed for the complementary experiment demonstrated that expressing *uas-kir2.1* using the *c739-gal4* driver resulted in paralysis/lethality, presumably due to expression of the *c739-gal4* driver in regions other than the α/β MBn. To circumvent this problem, we employed a more specific *gal4* driver, *R28H05-gal4*, that exhibits expression in most but not all α/β MBn (Jenett et al, 2012). As expected, silencing the α/β MBn 6 hr prior the second training episode impaired the expression of recent memories (Figure 5D). Similarly, silencing α/β MBn with Kir2.1 significantly impaired the retrieval of remote memories (Figure 5E). The impairment of both recent and remote memory retrieval was expected, given this durable disruption, but the incomplete impairment was not anticipated. The incomplete impairment is presumably the result of differences in α/β MBn coverage by the *c739-gal4* versus the *R28H05-gal4* drivers (Jenett et al, 2012). Control flies carrying only the *uas-kir2.1* or *gal4, tubP-gal80[ts]* genetic elements exhibited robust retrieval of both remote and recent memories (Figure 5D, E). These data indicate that the retrieval of both newly formed and remote memories require the function of the α/β MBn.

Discussion

We present here, an extensive set of experiments utilizing transient synaptic blockade, functional cellular imaging, and cellular inactivation to probe the role of different sets of MBn on memory expression at multiple time points after the acquisition of appetitive or aversive olfactory memories. We found that the expression of appetitive and aversive olfactory memories follow the same general rules: they are dependent on synaptic transmission from the α'/β', α/β, and γ MBn for a few hours after conditioning but become independent of α'/β' and γ MBn and completely dependent on α/β MBn for the expression of memories at 24 hr. These observations led us to offer the hypothesis that olfactory memories in *Drosophila* are processed in system consolidation manner.

At least three lines of evidence prior to this study were consistent with the hypothesis that olfactory memories generated by classical conditioning of *Drosophila* undergo system consolidation: (1) Disrupting the synaptic function of γ MBn during retrieval of appetitive memory at 2 hr after training impairs performance (recent memory), but is without effect on retrieval when the disruption is applied at 24 hr. In contrast, disrupting synaptic function of the α/β MBn during retrieval at 2 hr after training was reported to be without apparent effect on performance at this time point, but impairs retrieval of remote memory when the disruption is applied at 24 hr (Trannoy et al., 2011). These observations suggest that the γ MBn participate in processes underlying recent appetitive memories, while the α/β MBn participate in processes underlying remote appetitive memories. The role of other possible participants – the α'/β' neurons in particular – was not investigated in this study nor were retrieval disruptions tested that were earlier than 2 hr after conditioning. A possible role of the α'/β' neurons in system consolidation is important to consider since similar disruptions in synaptic transmission during or after conditioning disrupts recent (3 hr) memory (Krashes et al., 2007). (2) The short-term memory (STM) deficit of the *rutabaga* (*rut*, calcium-dependent adenylyl cyclase) mutant in aversive memory can be partially rescued by expression of a *rut* wild-type transgene in γ MBn, whereas the long-term memory (LTM) deficit is partially rescued with expression in α/β MB cells (Zars et al., 2000; Akalal et al., 2006; Blum et al., 2009; Trannoy et al., 2011; Qin et al., 2012). These observations indicate that *rut* function is required in one set of neurons for recent memories, and in another set for remote memories. (3) Imaging studies indicate that retrieval of STM and LTM activates different regions of the *Drosophila* brain. For instance, the retrieval of LTM after aversive training activates the α/β MB (Yu et al., 2006; Akalal et al., 2011) whereas retrieval of STM activates the α'/β' MBn (Wang et al., 2008) and antennal lobe projection neurons (Yu et al., 2004). Functional imaging experiments have also revealed a LTM trace in the γ lobes that is exposed by retrieval (Akalal et al, 2010), but no STM traces have been discovered in these neurons despite their consistent implication in STM processes.

Despite these hints, there exist discrepancies in the data between some studies and the complete set of data necessary to draw firm conclusions have been lacking. For instance, *rut* rescue experiments (Akalal et al., 2006; Blum et al., 2009) reveal a requirement for *rut* function for STM and LTM in different sets of neurons, showing that this genetic function is somehow important for processing memory in these two sets of neurons, but they do not directly address whether the responsibility for storing a particular memory shifts between the two regions. In addition, experiments to acutely disrupt synaptic function are usually performed during or early after conditioning, with retrieval measured hours after the acute disruption is removed. Such experiments reveal a role for synaptic activity of the disrupted neurons for the formation of the memory tested much later, but do not clearly define the temporal window for when neuronal activity is required, nor reveal where memories are stored, which is best assessed through disruption experiments at the time of retrieval.

Three lines of evidence presented here argue that both aversive and appetitive olfactory memories in *Drosophila* are processed using system consolidation processing. First, we showed that memories that are initially dependent on synaptic transmission of α'/β' and γ MBn become independent of these neurons with time. The time course for gaining independence for the two sets of neurons is similar (Figure 1, 2), consistent with the possibility that they cooperate in the formation and storage of early memories (Figure 5D). The α/β MBn are also partially required for the retrieval of STM/ITM, with LTM becoming completely dependent on this subset of MBn. These observations are consistent with the system consolidation of memories from the $\alpha'/\beta'/\gamma$ MBn to the α/β MBn (Figure 5D). Second, we showed by functional imaging that α'/β' MBn express an increased calcium response to a retrieval cue for both aversive and appetitive olfactory STM/ITM, but not LTM. In contrast, the retrieval of LTM but not STM/ITM produces an increased response in the α/β MBn. These results echo the transfer of altered responses observed between the hippocampus and neocortex (Figure 4) due to the system consolidation reorganization of the brain circuitry underlying different phases of memories (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004). Finally, the most definitive demonstration of system consolidation was performed by the measuring "early" and "remote" memories in the same genotype of flies after inactivation of specific brain regions. We demonstrated that STM and LTM to two different odor pairs can coexist in the brain of the fly without any obvious interference, and that the α'/β' MBn perform a transient role in the formation and retrieval of new memories and are apparently without a function for retrieval of LTM. Together, these results support the idea that olfactory memories in *Drosophila* are processed by system consolidation.

One proposition for how different sets of neurons in the olfactory pathway underlie different temporal forms of memory is that parallel and fully independent traces form for STM and LTM after conditioning, with a trace for STM forming in the γ MBn and a LTM trace forming in the α/β MBn (Trannoy et al., 2011). In this proposition, cellular consolidation within the α/β MBn would play an exclusive role for solidifying LTM, with no need for interactions between the γ MBn and α/β MBn. But other neurons need to be considered beyond these two types, since it has been reported that α'/β' and DPM neuron function is required during the time window of consolidation for the expression of ITM and LTM (Yu et al., 2006; Krashes et al., 2007; Krashes and Waddell, 2008). Similarly, it was recently shown that blocking synaptic output of α/β MBn 6 hr after training also impairs LTM expression (Huang et al., 2012). The latter data are inconsistent with a model whereby olfactory memories are processed by completely independent parallel traces. The data we provide here along with selected prior studies point to system consolidation: STM is guided by memory traces that form in the α'/β', α/β, and γ MBn with synaptic interactions occurring between α'/β' MBn (and perhaps γ MBn) with α/β MBn that guides the evolution of a LTM trace in the α/β MBn.

Our *Drosophila*-adapted version for the standard model of system consolidation (Dudai, 2004) is presented in Figure 5F. For simplicity, this model emphasizes the roles for the three different, well-established subsets of MBn, although further subdivision of function is conceivable (Huang et al., 2012) and other neuron types may be added with future studies (Chen et al., 2012). Initial encoding occurs in all three subsets of MBn. With time, consolidation reinforces the α/β memory trace while STM/ITM traces in the α'/β' and γ MBn decay, perhaps partly through dopamine-dependent forgetting mechanisms (Berry et al., 2012). The α'/β'-γ memory traces contribute to the retrieval of STM/ITM. With time, the retrieval of LTM becomes completely independent of α'/β' and γ, with α/β emerging as the major player in driving the learned behavior.

There are differences to be noted between the standard model for system consolidation in mammals (Dudai, 2004) and our *Drosophila* adapted version (Figure 5D). The most apparent is that the mammalian model is based on recent and remote memories that are both protein-synthesis dependent, whereas the STM in *Drosophila* assayed here is protein-synthesis independent. Nevertheless, the model for system consolidation processing of olfactory memories in *Drosophila* employs the same principles of interaction and reorganization between brain circuits, with early and late memories being supported by different brain regions.

Acknowledgments

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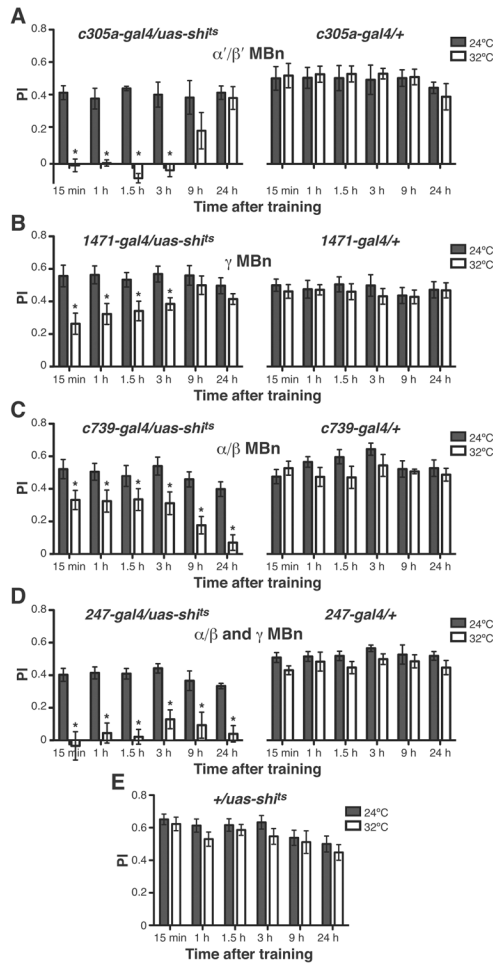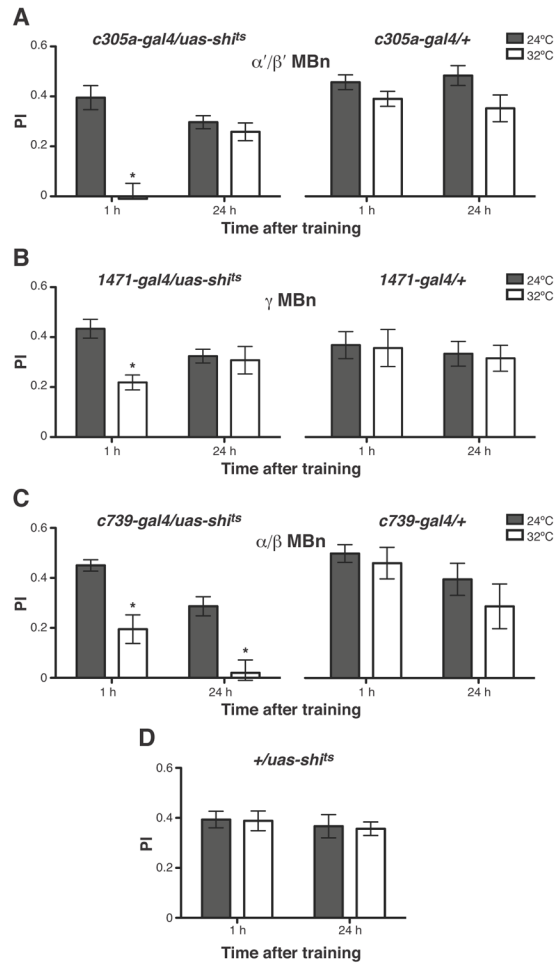
Figure 1.
Time requirements for synaptic output from MBn subsets for the retrieval of appetitive olfactory memory. (A) All flies were conditioned using an appetitive US at 24°C and shifted to 32°C ten minutes prior to a retrieval test. A synaptic blockade imposed by the expression and activation of shi^{ts} in the α'/β' MBn significantly reduced memory expression from 15 min to 3 hr after training (Mann-Whitney pairwise comparisons, p 0.0022). No significant difference was observed during retrieval of memory from 9 to 24 hr after training (Mann-Whitney pairwise comparisons, p 0.2403). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *c305a-gal4* element (Mann-Whitney pairwise comparisons, p 0.5887). (B) A synaptic blockade of γ MBn significantly reduced appetitive olfactory memory expression from 15 min to 3 hr after training (Mann-Whitney pairwise comparisons, p 0.0411). No significant difference was observed during retrieval of memory from 9 to 24 hr after training (Mann-Whitney pairwise comparisons, p 0.3315). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *1471-gal4* element (Mann-Whitney pairwise comparisons, p 0.4696). (C) A synaptic blockade α/β MBn significantly reduced appetitive olfactory memory expression from 15 min to 24 hr after training (Mann-Whitney pairwise comparisons, p 0.045). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *c739-gal4* element (Mann-Whitney pairwise comparisons, p 0.1727). (D) A synaptic blockade using shi^{ts} of α/β and γ MBn eliminated appetitive olfactory memory expression from 15 min to 24 hr after training (Mann-Whitney pairwise comparisons, p 0.0304). The

performance of *247-gal4/uas-shi^ts* flies at restrictive temperature at all times tested was not significant different from zero (Wilcoxon signed rank tests, p 0.0938). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *247-gal4* element (Mann-Whitney pairwise comparisons, p 0.1087). (E) No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *uas-shi^ts* element (Mann-Whitney pairwise comparisons, p 0.0916).
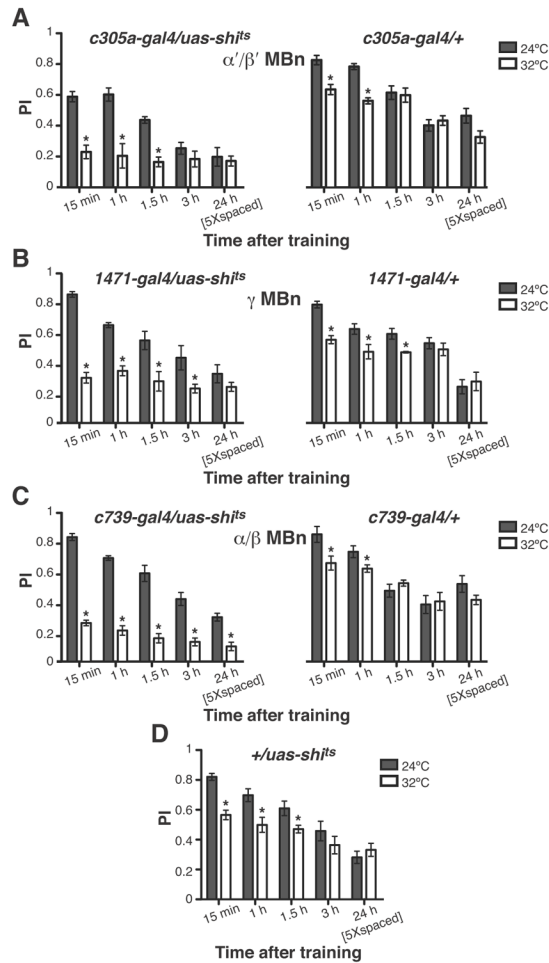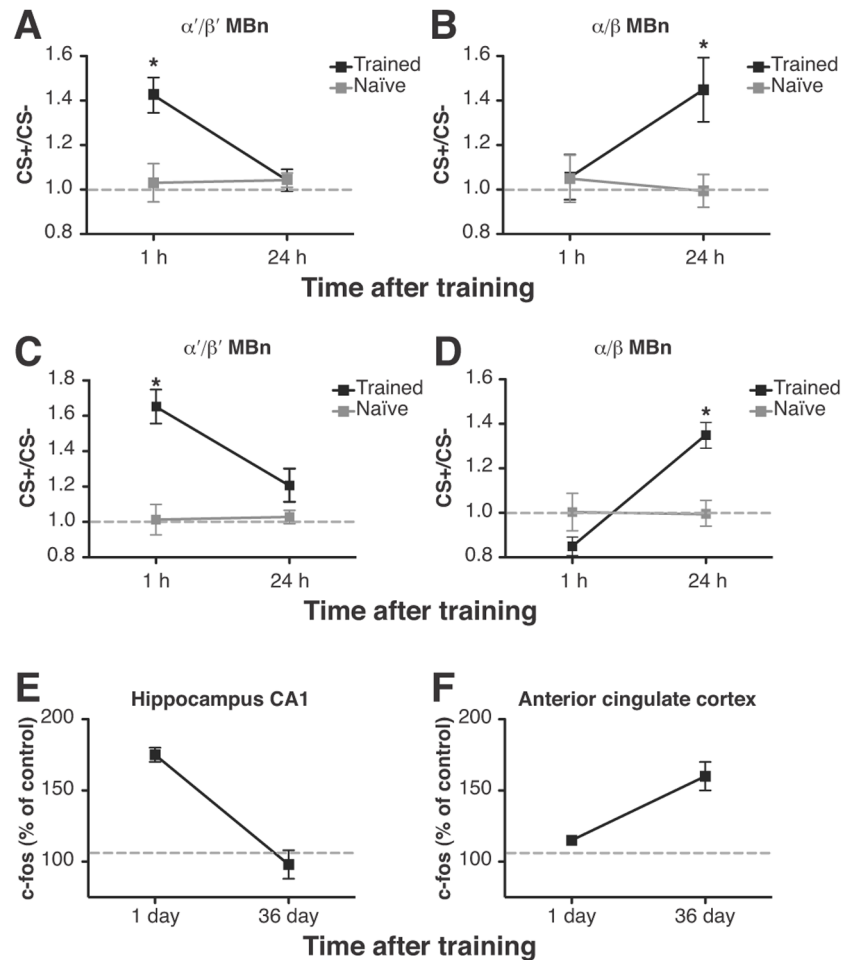
Figure 2.
Time requirements for synaptic output from MBn subsets for the retrieval of aversive olfactory memory. (A) All flies were trained using an aversive US at 24°C and shifted to 32°C ten minutes prior to a retrieval test. A synaptic blockade imposed by the expression and activation of shi^{ts} in the α′/β′ MBn significantly reduced memory expression from 15 min to 1.5 hr after training (Mann-Whitney pairwise comparisons, p ≤ 0.0043). No significant difference was observed during retrieval of memory at 3 or 24 hr after training (Mann-Whitney pairwise comparisons, p ≥ 0.4452). A small decrement in expression of aversive memory was observed during retrieval at early time points at restrictive temperature in control flies carrying only the *c305a-gal4* element (Mann-Whitney pairwise comparisons, p ≤ 0.0022) due to heat stress. This decrement was not observed at subsequent time points (Mann-Whitney pairwise comparisons, p ≥ 0.0931) and is discussed further in the text. (B) A synaptic blockade of γ MBn significantly reduced aversive olfactory memory expression from 15 min to 3 hr after training (Mann-Whitney pairwise comparisons, p ≤ 0.0260). No significant difference was observed during retrieval of memory at 24 hr after training (Mann-Whitney pairwise comparison, p = 0.4286). A small decrement in performance of aversive memory was observed during retrieval of 15 min to 1.5 hr memory at restrictive temperature in control flies carrying only the *1471-gal4* element (Mann-Whitney pairwise comparisons, p ≤ 0.0152). This impairment was not observed at subsequent time points (Mann-Whitney pairwise comparisons, p ≥ 0.6623). (C) A synaptic blockade of α/β MBn significantly reduced aversive olfactory memory expression from 15 min to 24 hr after training (Mann-Whitney pairwise comparisons, p ≤ 0.0095). A small

decrement in performance of aversive memory was observed during retrieval at 15 min and 1 hr at restrictive temperature in control flies carrying only the *c739-gal4* element (Mann-Whitney pairwise comparisons, p 0.0411). This impairment was not observed at subsequent time points (Mann-Whitney pairwise comparison, p 0.1320). (D) A slight decrement in performance of aversive memory expression was observed during retrieval from 15 min to 1.5 hr at restrictive temperature in control flies carrying only the *uas-shi^{ts}* genetic element (Mann-Whitney pairwise comparison, p 0.0303). This impairment was not observed at subsequent time points (Mann-Whitney pairwise comparison, p 0.3095)

Figure 3.
Time requirements for synaptic output from MBn subsets for the retrieval of appetitive olfactory memory is odor independent. (A) All flies were conditioned using an appetitive US at 24°C and shifted to 32°C ten minutes prior to a retrieval test. Conditioned odors used were benzaldehyde and methylsalicylate. A synaptic blockade imposed by the expression and activation of shi^{ts} in the α'/β' MBn significantly reduced memory expression at 1 hr but not 24 hr after training (Mann-Whitney pairwise comparisons, p = 0.0022 and p = 0.3776, respectively). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *c305a-gal4* element (Mann-Whitney pairwise comparisons, p 0.0649). (B) A synaptic blockade of γ MBn significantly reduced 1 hr but not 24 hr appetitive olfactory memory expression (Mann-Whitney pairwise comparison, p = 0.0135 and p = 0.6304, respectively). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *1471-gal4* element (Mann-Whitney pairwise comparisons, p 0.7483). (C) A synaptic blockade of α/β MBn significantly reduced appetitive olfactory memory expression during retrieval at 1 and 24 hr after training (Mann-Whitney pairwise comparisons, p 0.0081). No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *c739-gal4* element (Mann-Whitney pairwise comparisons, p 0.4848). (D) No significant difference was observed between permissive and restrictive temperatures for control flies carrying only the *uas-shi^{ts}* element (Mann-Whitney pairwise comparisons, p 0.8182). Asterisks indicate a statistically significant difference assessed by Kluskall-Wallis analysis followed by Mann-Whitney pairwise comparison. n = 6 for each group.

Figure 4.
Retrieval of short and long-term appetitive and aversive memories induces an increased retrieval signal to the learned odor in different MBn subsets. (A) Response ratio of the CS$^+$/CS$^-$ as a function of time measured in the vertical branch of the α'/β' MBn after appetitive olfactory conditioning and in naïve flies. A robust increase in calcium influx was detected in the vertical branch during the retrieval of 1 hr appetitive memory (Mann-Whitney pairwise comparison, p = 0.0041). This increase was not detected during the retrieval of the appetitive memory 24 hr after training (Mann-Whitney pairwise comparison, p = 0.8361). No change was observed at either time point in naïve animals. Asterisks indicate a statistically significant difference when compared to the naïve group. n = 10-16 for each group. (B) Response ratio of the CS$^+$/CS$^-$ as a function of time measured in the vertical branch of the α/β MBn after appetitive olfactory conditioning and in naïve flies. A robust increase in calcium influx was detected in the vertical branch during the retrieval of 24 hr appetitive memory (Mann-Whitney pairwise comparison, p = 0.0183). This increase was not detected during the retrieval of the appetitive memory 1 hr after training (Mann-Whitney pairwise comparison, p = 1). No change was observed at either time point in naïve animals. Asterisks indicate a statistically significant difference when compared to the naïve group. n = 10-16 for each group. (C) Response ratio of the CS$^+$/CS$^-$ as a function of time measured in the vertical branch of the α'/β' MBn after aversive olfactory conditioning and in naïve flies. A robust increase in calcium influx was detected in the vertical branch during the retrieval of 1 hr aversive memory (Mann-Whitney pairwise comparison, p = 0.0011). This increase was not detected during the retrieval of the aversive memory 24 hr after training (Mann-Whitney

pairwise comparison, p = 0.2618). No change was observed at either time point in naïve animals. Asterisks indicate a statistically significant difference when compared to the naïve group. n = 10-16 for each group. (D) Response ratio of the CS^+/CS^- as a function of time measured in the vertical branch of the α/β MBn after aversive olfactory conditioning and in naïve flies. A robust increase in calcium influx was detected in the vertical branch during the retrieval of 24 hr aversive memory (Mann-Whitney pairwise comparison, p = 0.0027). This increase was not detected during the retrieval of the aversive memory 1 hr after training (Mann-Whitney pairwise comparison, p = 0.2225). No change was observed at either time point in naïve animals. Asterisks indicate a statistically significant difference when compared to the naïve group. n = 10-16 for each group. (E) Expression of the activity-dependent gene *c-fos* in the hippocampus CA1 region after recent and remote memory tests for contextual fear conditioning in mice. *c-fos* expression is shown as a percentage relative to controls that were not given footshock. Adapted, with permission, from (Frankland et al., 2004). (F) Expression of the activity-dependent gene *c-fos* in the anterior cingulate cortex after recent and remote memory tests for contextual fear conditioning in mice. *c-fos* expression is shown as a percentage relative to controls that were not given footshock. Adapted, with permission, from (Frankland et al., 2004).
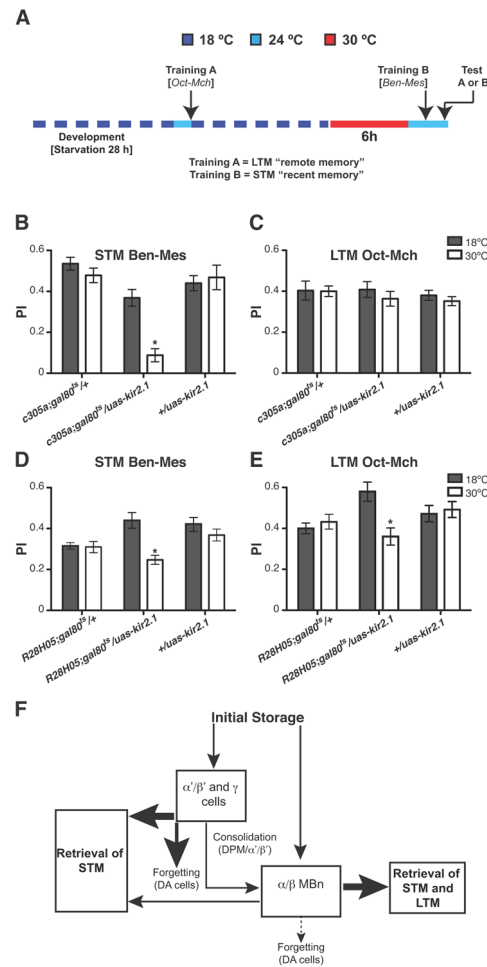
Figure 5.
Disruption of α′/β′ MBn impairs new memories while leaving remote memories in the same animal intact. (A) Diagram illustrating the appetitive conditioning protocol used in this experiment. Flies carrying one copy of *c305a-gal4, tub-gal80^ts* and *uas-kir2.1* were raised and then starved at 18°C for 28 hr. Flies were then trained using forward appetitive olfactory conditioning with Oct and Mch at 24°C (Training A). They were fed immediately after training for 12 hr, and then starved again for 12 hr prior to Training B at 24°C with a second odor combination, benzaldehyde (Ben) and methylsalicylate (Mes). They were then tested for memory of the first odor combination or the second. The experimental group was incubated at 30°C for 6 hr prior to the second training experience to allow the expression of Kir2.1 and the silencing of the α′/β′ MBn. The control group was kept at 18°C during this 6 hr incubation period. (B) Inactivating the α′/β′ MBn severely impaired expression of "recent" memory (Training B) (Mann-Whitney pairwise comparison, p 0.0022). Flies containing only *c305a-gal4;gal80^ts* or *uas-kir2.1* exhibited unaltered expression of memory from Training B (Mann-Whitney pairwise comparisons, p 0.6304). Asterisks indicate a statistically significant difference. n = 6 for each group. (C) Inactivating the α′/β′ MBn had no significant effect on the expression of "remote" memory (Training A) (Mann-Whitney pairwise comparison, p = 0.6298). Flies containing only *c305a-gal4;gal80^ts* or *uas-kir2.1* exhibited unaltered expression of memory from Training A (Mann-Whitney pairwise comparisons, p 0.2607). Asterisks indicate a statistically significant difference. n = 6 for each group. (D) Inactivating the α/β MBn impaired expression of "recent" memory (Training B) (Mann-Whitney pairwise comparison, p = 0.0159). Flies containing only

R28H05-gal4;gal80^ts or *uas-kir2.1* exhibited unaltered expression of memory from Training B (Mann-Whitney pairwise comparisons, p 0.2971). Asterisks indicate a statistically significant difference. n = 6 for each group. (E) Inactivating the α/β MBn significantly impaired the expression of "remote" memory (Training A) (Mann-Whitney pairwise comparison, p = 0.0129). Flies containing only *R28H05-gal4;gal80^ts* or *uas-kir2.1* exhibited unaltered expression of memory from Training A (Mann-Whitney pairwise comparisons, p 0.2403). Asterisks indicate a statistically significant difference. n = 6 for each group. (F) *Drosophila*-adapted, standard model of system consolidation. Short-term memory (STM) is encoded in all three classes of MBn. During system consolidation, memory traces are shifted from the three classes of MBn so that long-term memory (LTM) becomes the exclusive property of the α/β MBn. The consolidation process is mediated in part by the DPM (dorsal paired medial) neurons. Dopaminergic neurons (DA) carry out active forgetting from all sets of neurons.

Table 1

Olfactory avoidance for all genotypes used in this study

	OCT		MCH		BEN		MES	
	24°C	32°C	24°C	32°C	24°C	32°C	24°C	32°C
c305a-gal4/uas-shi^ts	0.63±0.08	0.67±0.06	0.82±0.07	0.79±0.09	0.89±0.04	0.84±0.12	0.56±0.05	0.60±0.04
1471-gal4/uas-shi^ts	0.73±0.09	0.71±0.07	0.90±0.04	0.92±0.1	0.81±0.04	0.81±0.03	0.55±0.04	0.60±0.1
c739-gal4/uas-shi^ts	0.71±0.04	0.70±0.03	0.97±0.07	0.95±0.03	0.76±0.05	0.74±0.06	0.69±0.08	0.71±0.09
247-gal4/uas-shi^ts	0.54±0.08	0.57±0.04	0.69±0.07	0.71±0.11	ND	ND	ND	ND
	OCT		MCH		BEN		MES	
	24°C	30°C	24°C	30°C	24°C	30°C	24°C	30°C
c305a-gal4/uas-kir2.1;tub-gal80^s	0.65±0.04	0.69±0.08	0.79±0.04	0.77±0.05	0.93±0.03	0.87±0.1	0.71±0.05	0.69±0.08
R28H05-gal4/uas-kir2.1;tub-gal80^s	0.69±0.03	0.71±0.1	0.65±0.08	0.62±0.12	0.84±0.08	0.81±0.11	0.57±0.09	0.60±0.08

No significant difference was found at different temperatures for any genotype tested. For flies of the genotype c305a-gal4/uas-kir2.1;tub-gal80ts, the avoidance was tested at 24°C and compared to avoidance of flies subjected to a pre-treatment of 6 hr at 30°C.