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CIRCUIT MECHANISM OF LONG TERM COURTSHIP MEMORY CONSOLIDATION IN *DROSOPHILA*

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ABSTRACT

Drosophila melanogaster, fruit flies, can associate either aversive or rewarding stimuli with odors to form aversive or appetitive olfactory memories, respectively. In addition to this simple form of associative learning, *Drosophila* males are able to perform a more complex learning called courtship conditioning where they learn to suppress their courtship towards unreceptive mated females after being persistently rejected by this type of females. Previously, it has been shown that activity of dopaminergic aSP13 (DAN-aSP13) neurons that innervate the tip of the mushroom body gamma neurons, is required and sufficient for acquisition of the short-term courtship memory. More recently, it was determined that activity of the same DAN-aSP13 neurons is also sufficient and necessary for long-term courtship memory consolidation after memory acquisition. Reminiscent of results in rodents, that spontaneous reactivation during sleep of neurons previously engaged in memory acquisition is sufficient to consolidate spatial memory I hypothesized that DAN-aSP13 neurons might be activated during sleep. I systemically examined requirement and sufficiency of both DAN-aSP13 and sleep for memory consolidation. Interestingly, sleep and activity of DAN-aSP13 neurons are sufficient and necessary in the same time window after learning experience suggesting that sleep activates DAN-aSP13. Accordingly, I identified a novel class of sleep promoting neurons in the ventral fan-shaped body (vFB) to be not only necessary and sufficient for long-term memory consolidation but as well providing an excitatory input onto DAN-aSP13. These data suggested that sleep after courtship experience, sufficient to induce long-term memory, triggers post-learning activation of DAN-aSP13 neurons which promotes memory consolidation.

1. INTRODUCTION

All animals exhibit an impressive repertoire of hardwired behaviors tuned to the requirement of the specific environment they live in. However, to survive in this environment they need constantly adapt these behaviors to new behavioral and environmental occurrences presented by the world around them. One of the most fascinating features of the animal behavior is its ability to be modified, as needed, based on the memories of past learning experiences. This ability is evolutionarily conserved from simple animals like *Aplysia* to the most complex organisms like humans (Kemenes, 2009). Interestingly, depending on the salience or intensity of the learning experience these memories can persist only for minutes, while animals are engaged in a specific behavior, or can last for hours, days or even a lifetime. Although studies in various animal systems over the last few decades provided a lot of knowledge about the mechanisms underlying memory processes however, the exact neuronal and molecular mechanisms underlying memory formation and persistence are still elusive.

In general, memory can be divided into two categories based on the duration of its persistence: short-term and long-term memories based on the time of their storage (Goelet et al., 1986). Short-term memory can last from minutes to hours and often reflects either changes in neuronal activity patterns or transient modifications of existing proteins to strengthen previously formed synaptic connections (Castellucci et al., 1970). Short-term memories may be transformed into long-term memories through a process called memory consolidation (McGaugh, 2000).

Long-term memories can last from days to years (Goelet et al., 1986) and require the synthesis of the protein (Kandel, 2001), which facilitates lasting morphological changes at synapses within neuronal networks and hence lasting memories (Carew et al., 1972). Long-term memory consolidation requires not only protein synthesis at the neuronal soma, but also depends on local translation at activated during learning synapses (Martin et al., 1997). Accordingly, components of the protein synthesis machinery have been identified in the neural processes (Steward and Levy, 1982), including Drosophila neurons (Hill et al., 2012) and subsets of mRNAs undergo activity dependent translation in cultured neurons (Kang and Schuman, 1996).

To explain how local protein synthesis might be restricted to specific synapses the "synaptic tagging" hypothesis has been proposed (Frey and Morris, 1997). According to this hypothesis, synapses that underwent specific learning and thus activation are marked for subsequent long-term memory consolidation. One of the candidates for such a "synaptic tag" is the family RNA-binding translational regulators, cytoplasmic polyadenylation element binding proteins (CPEB) (Si et al., 2003). CPEB proteins are evolutionarily conserved among species and might be divided into two subfamilies. CPEB-I proteins, including Drosophila CPEB, Orb1, are known to regulate protein synthesis during development, particularly oogenesis (Hake and Richter, 1994). On the other hand, proteins in the CPEB-II subfamily, were shown to be involved in synaptic plasticity (Richter, 2001). Drosophila CPEB-II protein, Orb2, was implicated in long-term memory consolidation (Hervas et al., 2016; Keleman et al., 2007; Kruttner et al., 2012; Kruttner et al., 2015; Li et al., 2016; Majumdar et al., 2012; McGinnis et al., 2016)

Despite the essential role of consolidation for long-term memory persistence the exact mechanisms of how is this memory processing regulated are not fully known. Post-learning, off-line, reactivation during sleep of quiescent period of the learning activity pattern in hippocampal neurons, a neuropil central to memory formation in mammals, has been implicated in spatial long-term memory consolidation in rats (Buzsaki, 1998; Wilson and McNaughton, 1994). However, the casual link between sleep and long-term memory is still elusive; as neuronal, circuits and molecular mechanisms of sleep in memory consolidation are not fully understood yet.

1.1. Study of memory in flies

Drosophila melanogaster with its numerically simple brain and sophisticated behavioral repertoire has become a very powerful model organism to study learning and memory. Combination of the genetic tools which allow to manipulate gene expression and reproducibly access specific neurons to perturb their function with behavioral analyses enables to dissect the neuronal and circuit mechanisms of various behaviors including memory (Owald et al., 2015).

Especially, the Gal4-UAS system has been one of the most useful techniques in Drosophila toolkit. Gal4 is a yeast transcription factor and it binds upstream recognizing sequence (UAS), which when combined with the effectors, such as neuronal activators or silencers can reproductively disrupt the neuronal function in the spatial and temporal manner (Brand and Perrimon, 1993).

It is also well established that flies exhibit a wide range of learning behaviors. They can form associative memories by associating conditioned (CS) and unconditioned stimuli (US) (Spatz et al., 1974). If the US is rewarding, for example sweet taste of sugar, flies form appetitive memories (Tempel et al., 1983), but if CS is paired with a punishing mild electrical shock, then aversive memory is induced (Quinn et al., 1974). One of the most well-studied memory paradigms in flies is olfactory memory. In this paradigm, an odor (CS^+) is paired with an electric shock (US) during training sessions while another odor (CS^-) is delivered without any US. After training, odor preferences of flies to either CS^+ or CS^- are tested on a T-maze (Tully and Quinn 1985). The same logic applies when odor is paired with a sweet and nutritious sugar, flies specifically approach the odor during the test which was earlier paired with sugar (Krashes and Waddell, 2008).

Flies can also form associative visual and place memory. In a visual memory, different visual patterns are paired with aversive cue, usually heat. After conditioning, flies are less likely to approach the visual pattern associated with punishment (van Swinderen et al., 2009). Similarly, flies can exhibit either a preference or avoidance of a specific place which was previously associated with an appetitive or aversive cues, respectively (Putz and Heisenberg, 2002).

1.2. Courtship conditioning in flies

Although associative learning paradigms mentioned above have been successfully used to reveal basic genetic and neuronal mechanisms of memory in *Drosophila*, they are unlikely to occur in the nature as performed in the laboratory conditions. A naturally occurring and robust form of

memory in *Drosophila* is courtship conditioning whereby *Drosophila* males associate the outcome of their own behavior with multisensory cues received from females (Sokolowski, 2001).

Drosophila males can innately distinguish females from males, so they only target females as courtship partners. However, in *Drosophila* natural environment majority of the females are already mated and therefore unreceptive. Therefore, to maximize their mating strategy, males need to learn how to distinguish receptive from unreceptive females (Dickson, 2008). Given the short lifespan of flies, the ability to find receptive females is a key feature for the male reproductive success. Indeed, it has been observed that males that have been rejected by already mated females suppress their courtship towards subsequent mated but not virgin females. This biological phenomenon has been called courtship conditioning (Siegel and Hall, 1979)

In this paradigm, a totally naïve male that has never encountered any type of female before, is paired with a recently mated, unreceptive female. The naïve male fly will vigorously court the recently mated female while she repetitively rejects him. During courtship towards a mated female is subjected to mechanosensory cues as female kicks, visual cues such as the female extrudes her ovipositor to reject copulation attempts and olfactory input, sex pheromones (Ejima et al., 2007). After this courting attempt and failure, males are less likely to court this mated females in the future (Dukas, 2005).

Courtship memory is quantified as Courtship Suppression Index (SI) which measures the extent of the courtship suppression of the experienced male in comparison to the naïve male (Figure

1.1). This courtship suppression is mediated, at least partially, by enhanced sensitivity to the sex pheromone, cis-Vaccenyl-Acetate, cVA (Keleman et al., 2012). cVA is a volatile male pheromone, produced in the accessory gland of the male (Butterworth, 1969). cVA is not present on virgin females and it is transferred to female during copulation and thus marks them as mated females (Ejima et al., 2007). Although the presence of cVA might be predictive of the unreceptive status of the female (Kurtovic et al., 2007), males have to constantly test the local environment as the amount of cVA changes with time and location.

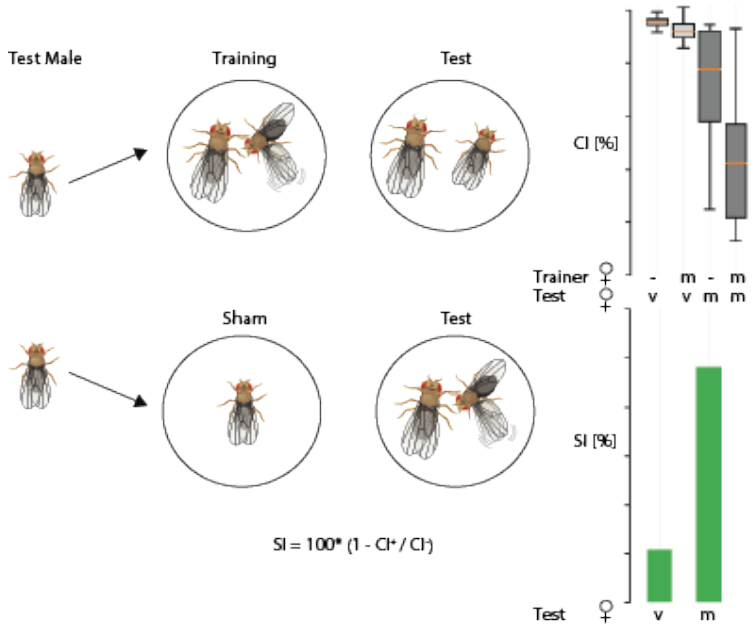

Figure 1.1: Schematic of the courtship conditioning paradigm. Test male is paired with a mated female (m) while sham male stays alone. Both groups are paired with either receptive virgin (v) or unreceptive mated females during 10-minute test periods. Courtship index (CI) is calculated as a percentage of time that male spends courting during the test period and Suppression index (SI) the extent of the courtship suppression of the experienced male in comparison to the naïve male. Trained males suppress their courtship towards subsequent mated females significantly more than virgin females

Depending on the duration of the time naïve male spends with a mated female, it is possible to induce a short-term or long-term courtship memory (Figure 1.2). 1-hour training is sufficient to induce short-term memory which can last for 2-3 hours. On the other hand, training males longer, for 6-7 hours, induces courtship suppression that can persist for at least for 24 hours (Keleman et al., 2007; McBride et al., 1999; Siegel and Hall, 1979).

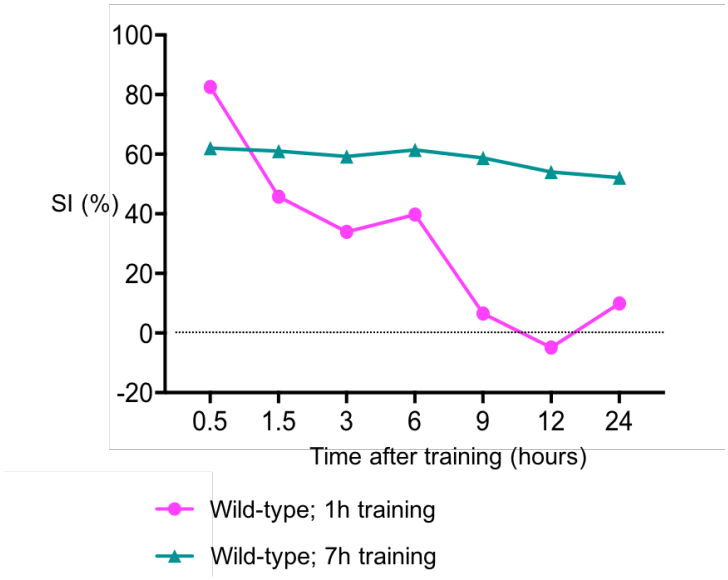

Figure 1.2: Flies are able to form short-term and long-term courtship memories. Flies which are trained with mated females for one hour have a significant Suppression Index (SI) when they are tested immediately after training. This memory starts to decay after few hours and become indistinguishable than 0 after 6 hours. However, training with mated female for seven hours induces more stable memory which persists for 24 hours or more (Keleman et al., 2007).

1.3. Mushroom Body: memory center in *Drosophila*

In fly's brain, mushroom body neuropil (MB) is essential for memory formation (Heisenberg et al., 1985). MB consists of intrinsic Kenyon Cells (KCs). KCs receive their sensory inputs in the dendritic region called calyx (Heisenberg, 2003). They send their axons in three bundles called MB lobes; αβ, α'β' and γ (Heisenberg, 2003). MB is composed of approximately 2200 cells per hemisphere and seven different cell types. γ neurons are further divided into dorsal and main subtypes while α'β' neurons consist of the middle and anterior-posterior layers and lastly αβ neurons are classified as posterior, core and surface neurons (Aso et al., 2014a).

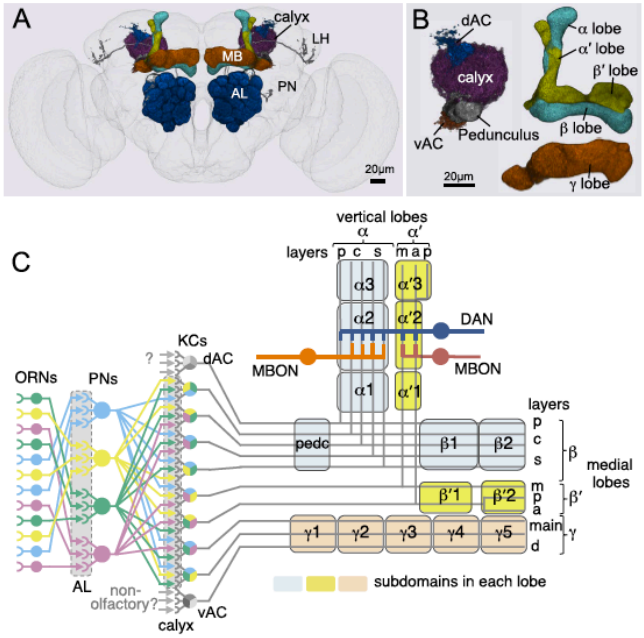

Figure 1.3: Anatomy of adult *Drosophila* brain and mushroom bodies. A) A general view of olfactory pathways in adult fly brain demonstrating mushroom bodies (MB), antennal lobe (AL), lateral horn (LH), projection neurons (PNs) and calyx. B) Sub-regions of MB showing the distinct lobes. C) A schematic representation of MB. Olfactory information is relayed to MB from olfactory receptor neurons (ORN) in antennal lobe (AL) via projection neurons (PNs). Axons of MB intrinsic Kenyon cells (KCs) form αβ, α'β' and γ lobes. They form MB compartments together with dopaminergic input (DAN) and mushroom body output neurons (MBON) (Aso et al., 2014a).

Anatomically, MBs are divided into distinct compartments (Figure 1.3). Each compartment consists of KCs, dopaminergic input (DANs) and corresponding mushroom body output neurons (MBONs).

Odor signals received by the antennal lobe (AL) are transmitted to Kenyon Cells (KCs) via projection neurons (PNs) (Tanaka et al., 2004). It was shown that the connections and convergence of PNs to KCs is probabilistic and random (Caron et al., 2013; Gruntman and Turner, 2013). KCs respond to given odor in a very sparse manner (Turner et al., 2008), however, they can predict the identity of the odor at very high accuracy (Hige et al., 2015). These properties of the MB neurons facilitate the high capacity of memory by minimizing the interference of irrelevant information (Hige, 2018)

KCs convey the information to other brain regions via MBONs. They can be distinguished into distinct classes based on the type of the neurotransmitters they release. Cholinergic MBONs send their dendrites to vertical lobes (α and α') while glutamatergic MBONs to horizontal lobes (β, β' and γ). Finally, GABAergic MBONs extend their dendrites into the region which resides at the intersection of vertical and horizontal lobes. There are 34 MBONs which fall into 21 types. Considering there are over 2000 KCs and high level convergence should occur between MB neurons and MBONs. Artificial activation of specific MBONs may induce either approaching or avoidance behavior thus promoting positive and negative valences accordingly (Aso et al., 2014b).

Each MB compartment also involves corresponding dopaminergic neurons (DANs). They have been shown to relay reward or punishment information (Aso et al., 2012; Mao and Davis, 2009). There are two main types of DANs sending axons to MBs, PPL1 and PAM DANs. PPL1 DANs send their axons mainly to vertical lobes (Aso et al., 2010) and PAM DANs innervate two horizontal lobes (Mao and Davis, 2009).

1.4. Different types of memories in *Drosophila*

Flies, as other animals, can form memory of different duration depending on the training regime. For example, one single training cycle with the odor that is paired with twelve of one-second electric shocks (CS^+), is sufficient to induce short-term memory and intermediate-term memory. These memories decay within hours after the training (Davis, 2011). However, if training cycles are repeated multiple times with resting periods in between, called spaced training, flies are able to form long-term memories which would last for up to 3-7 days.

Dopaminergic neurons have been shown to be involved in both short and long-term memories. PAM DANs are involved in both short- and long-term memories (Yamagata et al., 2015). DANs innervating the α1 compartment, PAM-α1 are necessary and sufficient for long-term appetitive memory (Yamagata et al., 2015). PAM-α1 neurons also form recurrent circuit with MBON-α1 and interfering with this feedback loop during memory acquisition and consolidation periods impairs long-term memory (Ichinose et al., 2015). It was also shown that, long-term memory training can induce slow oscillations in two pairs of PPL1 class of dopaminergic neurons (MV1 and MP1)

and their activity leads to long-term olfactory memory (Placais et al., 2012). Activation of MV1 and MP1 neurons after learning experience was sufficient to induce long-term aversive memory in starved flies (Musso et al., 2015), which is normally not possible since this kind of memory highly depends on the nutrient state (Placais and Preat, 2013). Dopaminergic MV1 and MP1 neurons are also implicated in forgetting (Berry et al., 2015; Berry et al., 2012). Activating of those neurons after training facilitated memory decay while silencing of them increased the duration and persistence of memory (Berry et al., 2012). Similarly, activity of another set of dopaminergic neurons, PAM-β'1 promotes forgetting (Shuai et al., 2015).

Similarly, flies are able to form short-term courtship memory when naïve males are trained with mated females for about 45-60 minutes. They exhibit a robust memory when tested after 15 minutes. This memory can persist up to 6 hours than decays completely when flies are tested 24 hours later. Pairing naïve males with unreceptive mated female for several hours results in long-term memory which persists for several days.

Previously our group demonstrated that PAM DANs innervating the MBγ5 compartments (DAN-aSP13) are required and sufficient to induce short-term courtship memory (Keleman et al., 2012). Chronic inhibition of DAN-aSP13 activity with the tetanus toxin light chain (TNT), leads to impairment of short-term courtship memory. Moreover, activation of same neurons with temperature sensitive cation channel, TrpA1, even in solitary males, produced a significant memory, reflected by courtship suppression. In parallel, with the involvement of DANs in short-term courtship memory, flies lacking their dopamine receptors, DopR1, have as well impaired

short-term courtship memory and expression of DopR1 in MB-γ neurons fully restores it (Keleman et al., 2012).

More recently our lab showed that MBONs that innervate the tip of the MB-γ lobe are also involved in short-term courtship memory (Zhao et al., 2018). These MBONs are called MBON-M6, also MBON-γ5/β2', and form a recurrent circuit with MB-γ and DAN-aSP13. Both DAN-aSP13 and MB-γ neurons are activated during courtship conditioning. Upon their activation, aSP13 neurons release dopamine to modulate the synapses between MB-γ neurons and MBON-M6 neurons, resulting in potentiation of MBON-M6 neurons. MBON-M6 neurons provide a feedback to DAN-aSP13 neurons to make them persistently active and release dopamine. This activity can sustain for 2-3 hours, highly overlapping with the duration of STM (Zhao et al., 2018).

We demonstrated that DAN-aSP13 neurons are also involved in long-term courtship memory consolidation. Temporal silencing of aSP13 neurons after long-term memory training, completely abolished 24-hour memory (Kruttner et al., 2015). DAN-aSP13 neurons are also sufficient to consolidate long-term courtship memory. Activation of DAN-aSP13 neurons after 1 hour training, which is sufficient to induce only short-term but not long-term memory, resulted in a robust and significant 24-hour memory (Kruttner et al., 2015). These results increased our understanding of short- and long-term courtship memory at the circuit level and showed that DAN-aSP13 might play a pivotal role in these processes as their activity is required and sufficient for both short-term and long-term courtship memory.

Molecular mechanisms underlying long-term courtship memory in Drosophila are also not fully understood yet. Our laboratory has shown that Drosophila CPEB, Orb2, or rather its Q-domain, is required specifically for long-term but not short-term courtship memory (Keleman et al., 2007; Kruttner et al., 2012; Kruttner et al., 2015).

Mutant flies lacking the Q domain, showed normal short-term but impaired long-term memory (Keleman et al., 2007). Mutant flies after training for several hours, which normally is sufficient to induce long-term memory have initially normal memory, which decays after about 6 hours which is similar to a time course of the short-term memory decay. These data suggest that Orb2 is not required for learning and short-term memory, but rather is necessary for consolidation of short-term to long-term memory.

Q-domains in many proteins are thought to facilitate prion-like protein complexes. Orb2A and Orb2B isoforms also form amyloid-like heteromeric complexes mediated by their Q-domains (Kruttner et al., 2012; Majumdar et al., 2012). Deletion of the Q-domain in Orb2A, not only results in severe reduction in complex formation but also impaired long-term memory as mentioned above. Moreover, Orb2 protein complexes are induced by neuronal stimulation only in the wild-type flies but not in flies lacking the Q-domain on Orb2A. Deletion of the Q-domain in Orb2B did not have such an effect on memory, demonstrating that protein-protein interaction mediated by the Q-domain of Orb2A are necessary for long-term memory. RBD domain was not involved in formation of protein complexes however deletion of RBD in Orb2B, not in Orb2A, also impaired long-term memory. To summarize, upon neuronal stimulation Orb2A recruits Orb2B to form

protein complexes through their Q-domains and these protein complexes regulate translation of target mRNAs which necessary for LTM consolidation.

1.5. Aim of my project

We previously showed that activity of DAN-aSP13 neurons at the specific time window after training sufficient to induce long-term memory is required for memory long-term memory consolidation. Interestingly, this time window is identical to the time when either short-term courtship memory or long-term memory in Orb2 mutant flies decays. Moreover, activation of DAN-aSP13 neurons in the same time window is sufficient to consolidate short-term courtship memory to long-term memory. Therefore, an obvious and intriguing question is: what triggers activation of DAN-aSP13 or how it remains active after the training when males stay alone after training? For example, in rodents it has been demonstrated that neurons, which were previously active during memory acquisition are reactivated during sleep for memory consolidation (Buzsaki, 1998). As reported in other animals, also in Drosophila, sleep is involved in LTM consolidation (Donlea et al., 2009; Donlea et al., 2011). Therefore, we hypothesized that DAN-aSP13 might be reactivated during sleep for long-term memory consolidation. My goal was to investigate whether and how is sleep is involved in DAN-aSP13 reactivation and hence long-term courtship consolidation using genetics, behavioral assays in combination with perturbation and monitoring neuronal activity of the specific neurons.

2. RESULTS

2.1. DAN-aSP13 neurons are more active after long-term training

Silencing DAN-aSP13 neurons between ZT08-ZT11 after training to induce long-term memory with a temperature-sensitive dynamin, which blocks synaptic transmission at higher temperatures, results in severe reduction of long-term courtship memory. Conversely, activation of DAN-aSP13 between 8-10 hours after training to induce short-term memory consolidated this memory to long-lasting (Kruttner et al., 2015). These data suggested that activity of DAN-aSP13 neurons is necessary and sufficient in this specific time window after learning however, there was no direct evidence of DAN-aSP13 neurons being active during memory consolidation in intact animals. To test this, I adapted a newly developed method to monitor neuronal activity in freely behaving flies (Guo et al., 2017). Specifically, I wanted to monitor activity of DAN-aSP13 neurons during sleep after a learning experience sufficient to induce long-term courtship memory. This method, based on the activity reporter, *Luciferase*, conjugated with calcium indicator, Calmodulin and transcription factor motif of Activity Regulated Genes, reports neuronal activity by measuring luminescence. I expressed this luciferase reporter specifically in DAN-aSP13 using Gal4/UAS system. Flies were starved overnight prior to training and then fed with a substrate, *Luciferin*, during training and monitoring periods. After 6.5 hours of training, both naïve and trained males were transferred to 96-well plate and luminescence readings were taken with 1-minute intervals over 5 hours. Results of this assay demonstrated that DAN-aSP13 activity was significantly higher in trained males during the same time window when their activity is required

for memory consolidation (Figure 2.1). Thus, these results suggested that DAN-aSP13 neurons are indeed active after training for long-term memory.

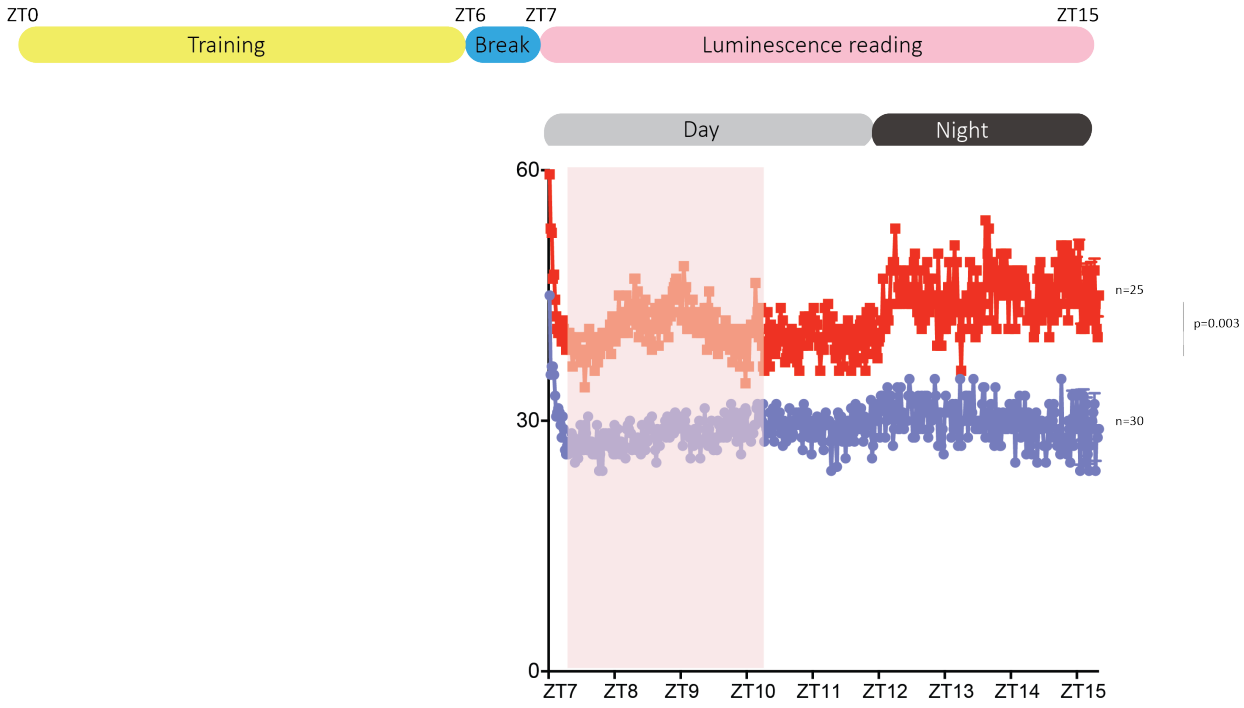

Figure 2.1 DAN-aSP13 neurons are more active after long-term training. Lola-Luc reporter was expressed in DAN-aSP13 neurons by using MB315b driver line. Flies were starved overnight prior to experiment and then fed with *Luciferin* dissolved in sucrose solution during training and monitoring. Luminescence signal was significantly higher in trained than naïve males. Activity of DAN-aSP13 neurons in trained animals was higher including the specific time window, marked in pink. (Permutation test with null hypothesis Luc_{exp}=Luc_{naive}

2.2. Experienced males sleep more after long-term training

Given that activity levels of DAN-aSP13 neurons are higher in experienced males than naïve animals in that specific time window after training, I was wondering whether sleep, as reported in rodents, is also involved during this time. It was previously shown that males which were trained for several hours slept more than naïve control males (Ganguly-Fitzgerald et al., 2006).

Therefore, I investigated whether experienced males sleep during that specific time window after training sufficient to induce long-term memory. I trained *Drosophila* males for 6.5 hours (ZT0-ZT6.5) and afterwards monitored their sleep till test after 24 hours (ZT7-ZT24). I observed that sleep levels in experienced males were higher during day time and there was no significant change in sleep amount during nighttime. The biggest change was observed after 1 to 3 hours after the training, ZT8 and ZT10 respectively (Figure 2.2A and 2.2B).

To test whether the increased sleep after training is caused by the fatigue due to long training with mated females, I tested in parallel a memory mutant flies. I tested males that are missing the Q-domain in Orb2, Orb2ΔQ, which our lab showed previously has a normal short-term memory but are not able to consolidate it to long-term memory (Keleman et al., 2007; Kruttner et al., 2012; Kruttner et al., 2015). Importantly, during training they court trainer females with the intensity comparable to the wild type animals. Sleep monitoring of Orb2ΔQ flies showed that there was no change in sleep amount between the naïve and experienced males; neither during the day nor during the night periods (Figure 2.2A and 2.2B). As expected, their memory reflected suppression index at 24h was indistinguishable than 0. These results suggested that, the increased levels of sleep after training in wild-type males depends on a learning experience rather than decreased locomotion caused by the long-term training (Figure 2.2C).

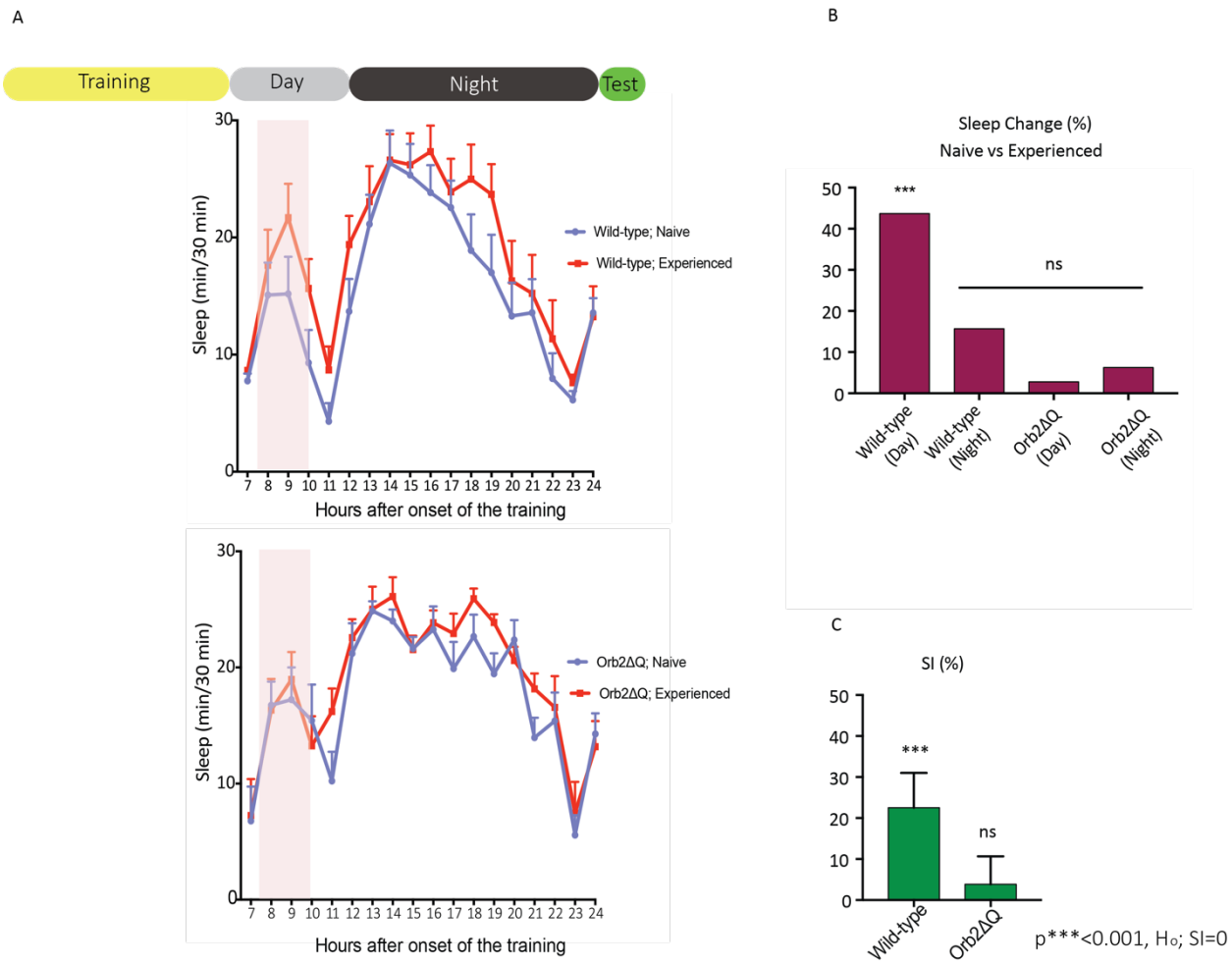

Figure 2.2: Experienced males sleep more after training. **A**) After 6.5 hours training, flies were placed in tubes to monitor their sleep with *Drosophila* Activity Monitor system. Experienced wild type males slept more tan naïve males after training. Naïve and experienced mutant males for the Q-domain in Orb2 had the same amount of sleep. **B**) Quantification of **A**. **C)** Wild type males formed a robust long-term memory while Orb2ΔQ flies did not.

2.3. Day-time sleep and activity of DAN-aSP13 are required for long-term memory consolidation

The observation of the increased sleep in experienced animals raised the question of whether sleep during this period is required for memory consolidation. To test this, I deprived flies of sleep mechanically at different time periods after 6.5 hours of training sufficient to induce long-term memory, until the night-time. Sleep deprivation was strong enough to prevent flies from sleeping but gentle enough for the behavioral performance. Sleep deprivation between the ZT7-ZT9 and ZT8-ZT10 impaired the long-term memory similar to males deprived of sleep longer (ZT7-ZT11 and ZT8-ZT12). However, flies that received the same treatment between ZT9-ZT11 and ZT10-ZT12 but were not deprived of sleep had a robust and significant memory reflected by significantly higher (SI%) in comparison to the control group which were allowed to sleep. These results demonstrated that the sleep during the first 3 hours after the training is critical for long-term memory consolidation. (Figure: 2.3)

In a similar set of experiments, I silenced DAN-aSP13 with the temperature sensitive Shi^{ts} which blocks synaptic transmission at 32°C, after training sufficient to induce long-term memory. Incubating flies at 32°C for 2-hour time periods after training between ZT7-ZT9 and ZT8-ZT10 but not between ZT9-ZT11 and ZT10-ZT12 severely impaired long-term memory reflected by SI(%) indistinguishable from 0. In other words, sleep and activity of DAN-aSP13 neurons are required for memory consolidation during the same time windows after the onset of the training. These

results suggested that DAN-aSP13 may be activated by sleep after training for courtship long-term memory consolidation in *Drosophila* males.

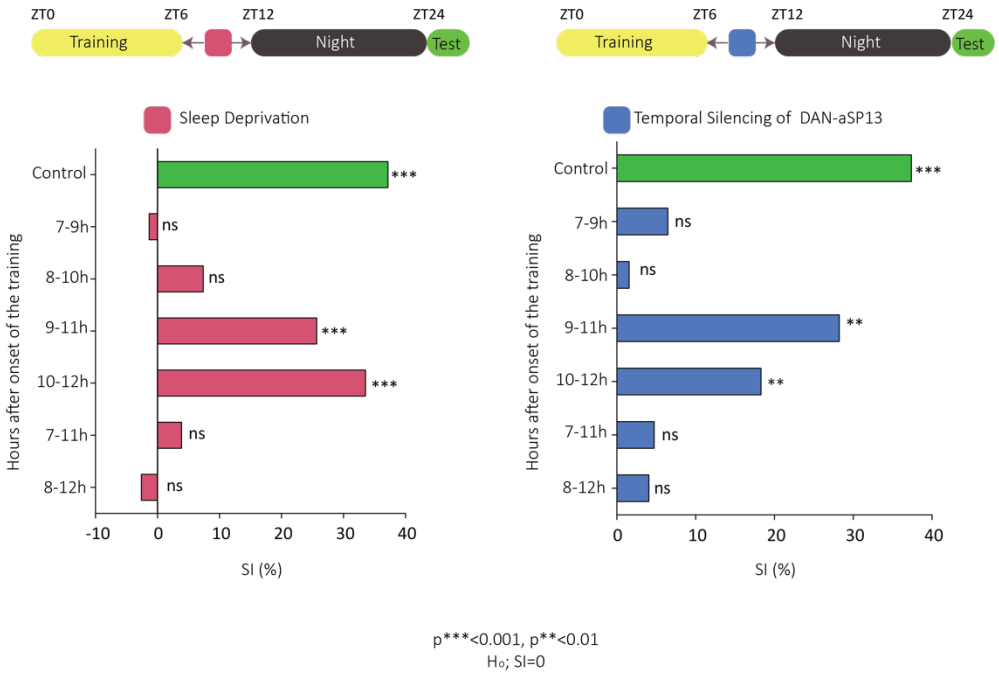

Figure 2.3: Sleep and DAN-aSP13 activity at the same time window after long-term training is required for memory consolidation. After long-term training, flies were mechanically deprived of sleep in 2-hour time intervals during day period (left panel). Similarly, DAN-aSP13 activity was silenced thermogenetically, (*VT005526LexA>LexAop-TrpA1*) (right panel). Both sleep and DAN-aSP13 activity is essential between 7-10 hours from the onset of training.

If DAN-aSP13 neurons are really activated during sleep, I hypothesized that inducing sleep artificially should also have similar effect on memory consolidation. Previously we showed that, artificial activation of DAN-aSP13 neurons after 1-hour training was sufficient to consolidate short-term memory to long-term, even though 1-hour training by its own is not able to induce long-term memory. It has been reported that, activation of Fan-Shaped body (FB) neurons promote sleep (Donlea et al., 2011). Particularly, the neurons labeled by 104y Gal4 line (Figure 2.4A) induce sleep within minutes upon activation with temperature sensitive cation channel TrpA1, which depolarizes the neurons at 32°C (Figure 2.4B).

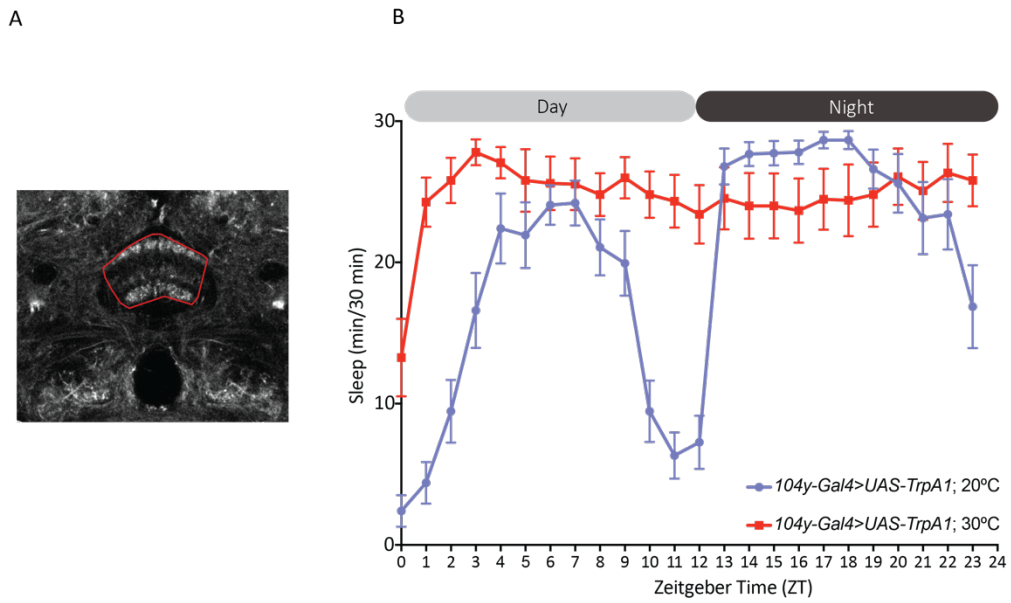

Figure 2.4: Activation of fan-shaped body neurons labeled by 104y-Gal4 driver line promotes sleep. **A)** Expression pattern of 104y-Gal4 line (Donlea et al., 2014; Donlea et al., 2011). Fan-shaped body marked in red. B) Flies expressing *104y-Gal4>UAS-TrpA1* sleep more upon activation in 32°C than flies which remained at 20°C.

Both naïve males and males trained with mated females for 1-hour were incubated at 32°C at 2-hour intervals after the onset of the training. The memory test on the following day demonstrated that, activation of 104y-FB neurons between ZT5-ZT7 was sufficient to consolidate short-term memory reflected by a robust SI%. Warming males at any other time periods did not produce any significant SI% compared to the control group which remained at 20°C all the time after the training. Similarly, when DAN-aSP13 neurons were activated with *CsChrimson* by red light, the most significant effect was also observed between ZT5-ZT7 (Figure 2.5). Altogether, the periods of DAN-aSP13 activity and sleep induction which are sufficient to consolidate short-term memory into long-term memory are highly overlapping.

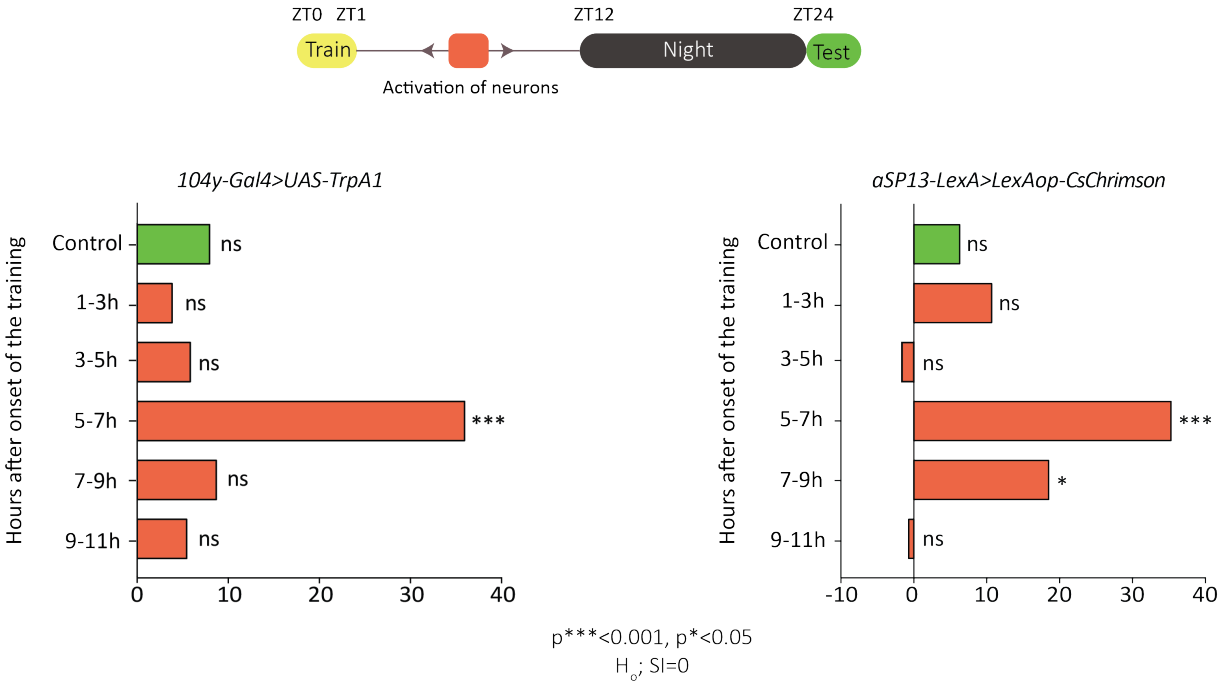

Figure 2.5: Sleep induction and DAN-aSP13 activation is sufficient for memory consolidation. Temporal activation of sleep promoting FB neurons (left panel) or DAN-aSP13 (right panel) between 5-7 hours after training for short-term memory resulted in a robust long-term memory when tested at 24-hour. Activation in different time windows did not consolidate long-term memory.

To test whether activity of 104y-FB neurons and thus sleep is also required for long-term memory consolidation I silenced them with temperature sensitive Shi^{ts} which blocks synaptic transmission in higher temperature, after training sufficient to induce long-term memory. The long-term memory was significantly reduced upon 104-FB silencing compared to the control flies suggesting that sleep induced by 104-FB neurons after learning experience is necessary for memory consolidation. Interestingly, activity of 104y-FB neurons and DAN-aSP13 neurons is necessary in the same time window after training (Figure 2.6).

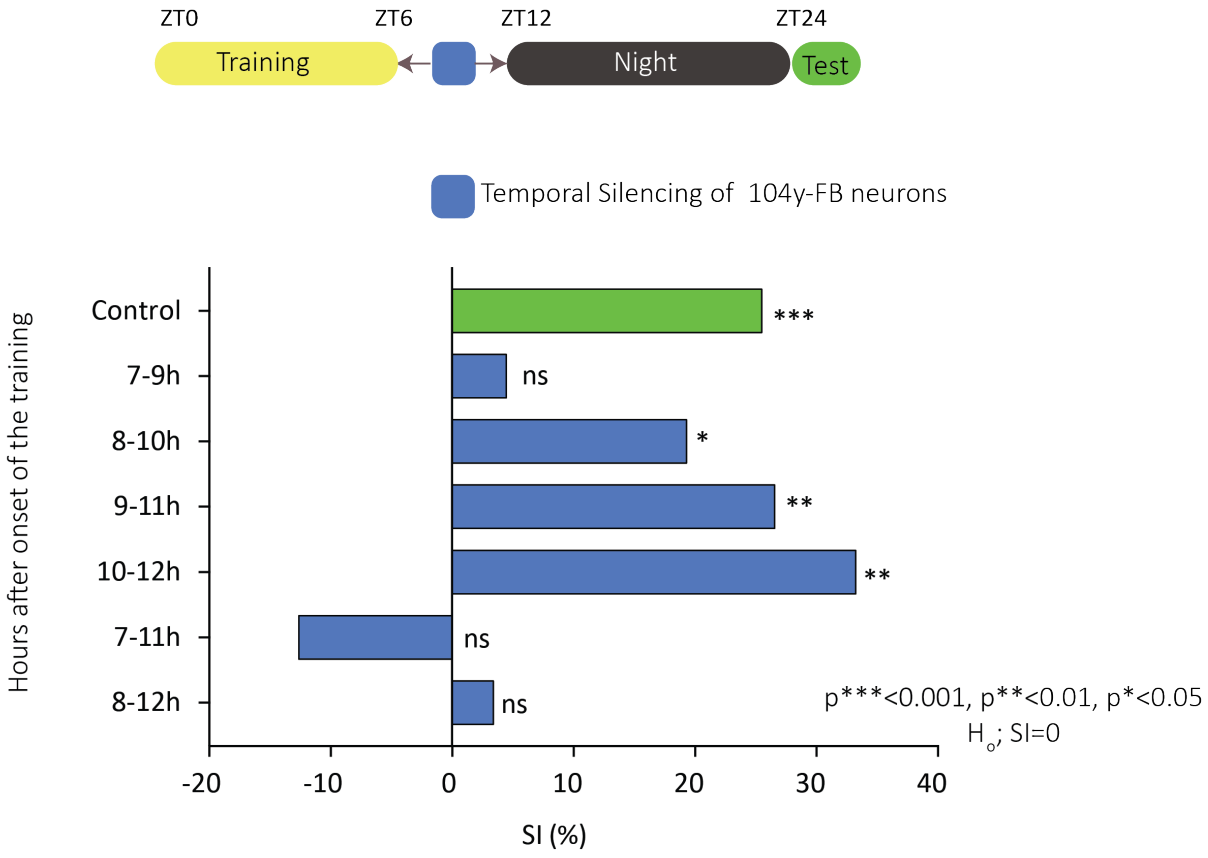

Figure 2.6: Sleep induced by 104-FB neurons is necessary for memory consolidation. Inhibition of FB neurons between 7-9h from onset of training severely reduced 24- memory performance.

These results suggested that activation of 104 FB neurons and thus sleep induction activates DAN-aSP13. To test it I expressed TrpA1 in 104y-Gal4 to activate FB neurons and promote sleep and Shi^{TS} in DAN-aSP13 neurons to simultaneously activate 104 FB neurons and silence DAN-aSP13 neurons. After 1-hour of training flies were warmed to higher temperature between the ZT5-ZT7. As expected the control flies expressing TrpA1 in FB neurons but not ShiTS in aSP13 had a significant SI when tested after 24 hours. However, the flies expressing both TrpA1 and Shi^{ts} were not able to consolidate their memory (Figure 2.7). These results suggest that activity of sleep induced by activation 104y-FB neurons consolidates memory by activation of DAN-aSP13.

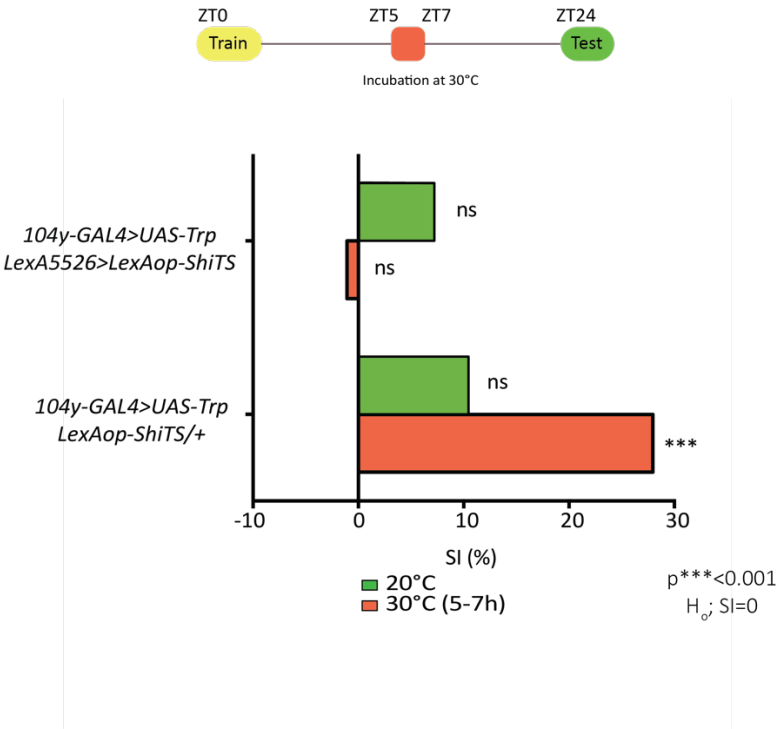

Figure 2.7: Sleep induced memory consolidation requires DAN-aSP13 activity. Activation of 104y-FB neurons is sufficient to consolidate memory (lower orange bar). However silencing DAN-aSP13 while activating FB neurons at same time does not consolidate memory (upper orange bar).

2.4. 104y-FB neurons are functionally connected to aSP13 neurons

Our data suggested that 104y-FB neurons and DAN-aSP13 neurons are functionally connected. So, the next step was to check if 104y-FB neurons provide an excitatory input onto DAN-aSP13 neurons. To test this, we expressed optogenetic activator *CsChrimson* in 104y-FB neurons and Ca^{2+} indicator in PAM-DANs, including DAN-aSP13 neurons. We activated 104y-FB with red light and monitored changes in calcium levels in DAN-aSP13 as a proxy for its activation. Activation of all 104y-FB neurons elicited excitatory response in DAN-aSP13 neurons suggesting that these neurons are functionally connected, most likely indirectly (Figure 2.8).

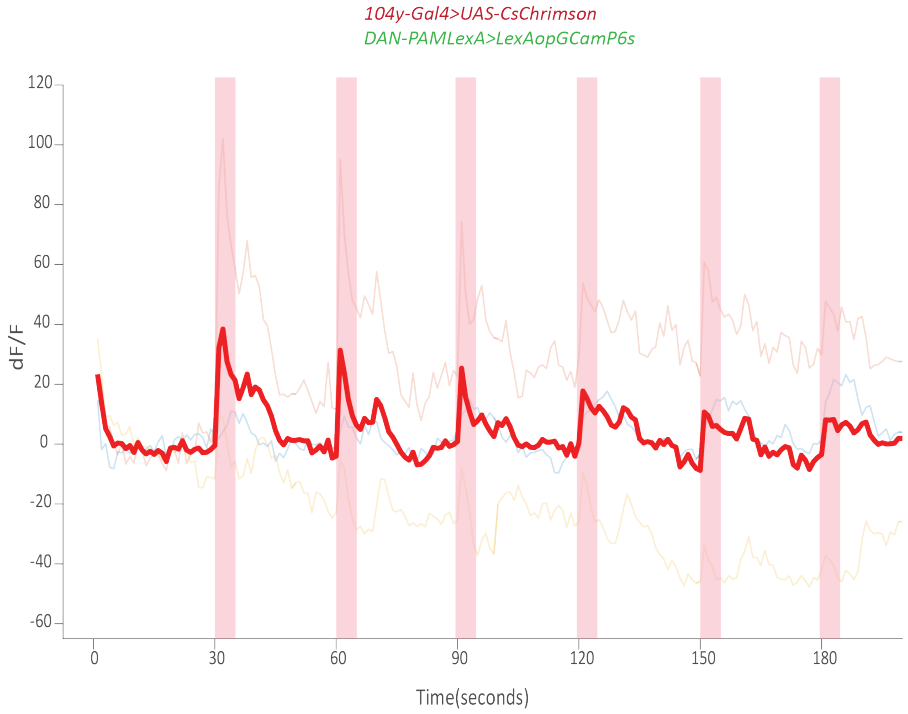

Figure 2.8: Activation of 104y-FB neurons elicits excitation in DAN-aSP13 neurons. Optogenetic activation of 104y neurons evokes calcium responses in DAN-aSP13.

Since 104y-Gal4 line is not exclusive for FB to activate specific FB neurons we employed Digital Mirror Device (DMD). This tool allowed us to target and activate specific regions of interest (ROI). Before proceeding to the actual experiments, the specificity of DMD was tested by expressing both *CsChrimson* and *GCamp6s* in 104y neurons. Then, individual ROIs in 104y-FB neurons (either ventral or dorsal layer of FB neurons) were activated in the presence of Tetrodotoxin, which inhibits voltage gated sodium channels thus inhibiting the propagation of action potential. Only neurons in specific ROIs were activated, meaning that activation by DMD is specific to relevant ROI (Figure 2.9).

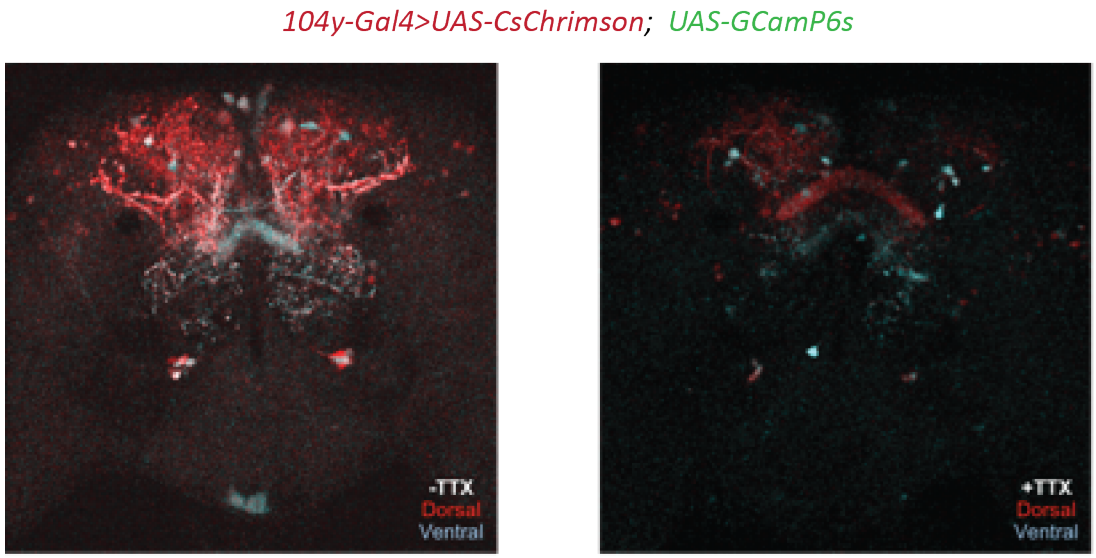

Figure 2.9: Digital Mirror Device (DMD) allows to specifically activate neurons of interest.
Distinct layers of FB are activated with DMD. Local activation of either dorsal (red) or ventral layer (blue) in presence of Tetrodotoxin (TTX) activates only neurons of interest (right panel).

Having established that we can specifically activate distinct layers of FB, we asked whether FB neurons as a whole activate DAN-aSP13 and if so what specific layer of FB provides an excitatory input onto DAN-aSP13?

Activation of all FB neurons with *CsChrimson* labeled by 104y-Gal4 elicited a robust excitatory response in DAN-aSP13 neurons monitored by changes in calcium level (Figure 2.10A). Similar effect on DAN-aSP13 we observed upon activation of the ventral layer of FB (vFB) but not upon activation of dFB neurons which occasionally inhibited activity of DAN-aSP13 (Figure 2.10B and Figure 2.10C).

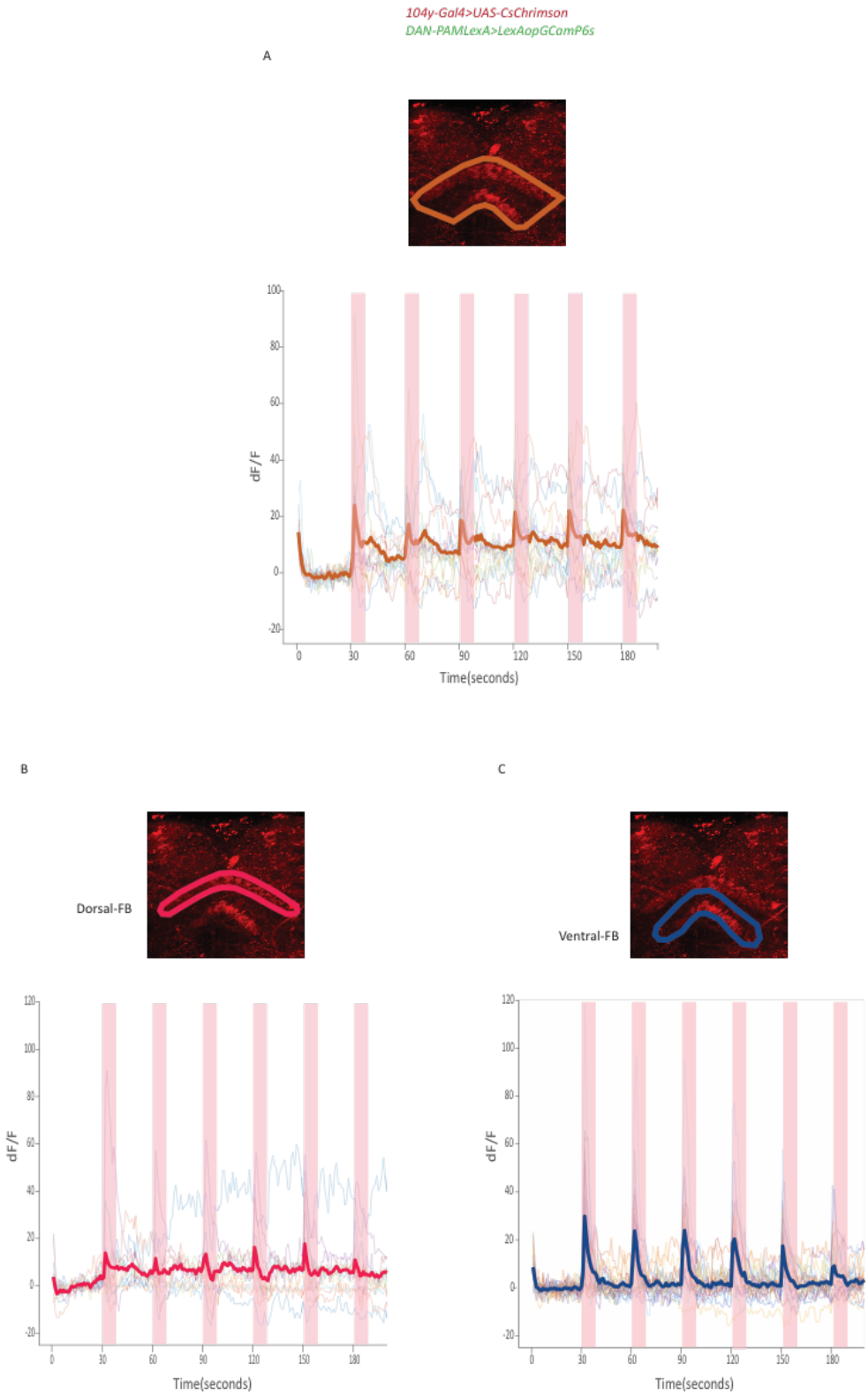

Figure 2.10: vFB neurons elicit excitatory response in DAN-aSP13 neurons. Using DMD, activation **A)** of all layers of FB labeled by 104y-Gal4 elicited excitatory responses in DAN-aSP13 neurons (cont'd on the next page).

B) Activation of dorsal layer of 104 FB (dorsal FBFB) and **C)** ventral layer of 104 FB (ventral-FB) elicited small not significant excitatory and inhibitory responses and robust excitatory responses in DAN-aSP13, respectively.

104y-Gal4 line labels multiple FB neuronal layers. Multicolor Flip-out revealed that there are three major cell types constituting the FB region of the 104y-Gal4 line. The axons of those neurons form the dorsal, medial and ventral FB layers (Figure 2.11).

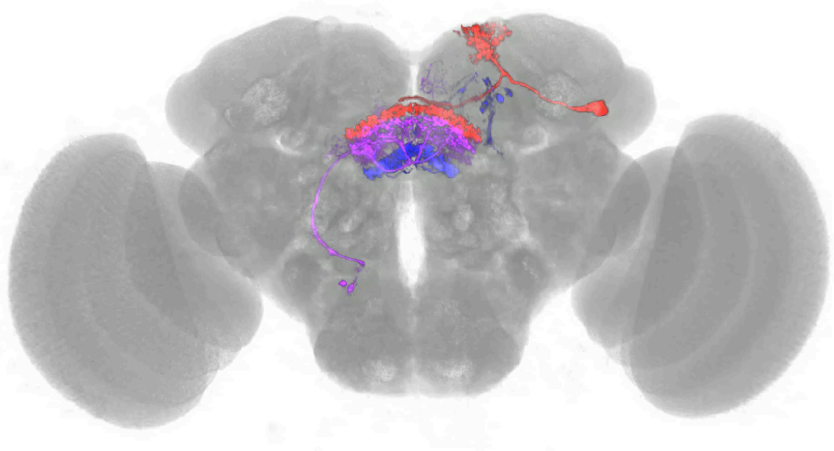

Figure 2.11: Multicolor Flip-out image of 104y-Gal4. 104y-Gal4 line labels 3 major classes of neurons in fan-shaped body: dorsal (red), medial (purple) and ventral (blue).

More recently, it was shown that activation of a small subset of neurons in the dorsal layer of FB (dFB), labeled by R23E10-Gal4 line (Figure 2.11A), promotes sleep (Donlea et al., 2014). dFB neurons regulate homeostatic sleep as they receive inputs from Ellipsoid Body neurons which encode the sleep demand after waking experience and eventually initiate the sleep state of the animal (Liu et al., 2016). Interestingly, activation of dFB was not sufficient for long-term courtship memory consolidation suggesting that some other sleep neurons present in the 104y-Gal4 line might be involved in long-term courtship memory consolidation (Figure 2.11C). Accordingly,

silencing of dFB neurons after training sufficient to induce long-term memory did not impair the memory (Figure 2.11D). All together, these data suggested that, experienced-dependent sleep is regulated by different subset of FB neurons than dFB neurons.

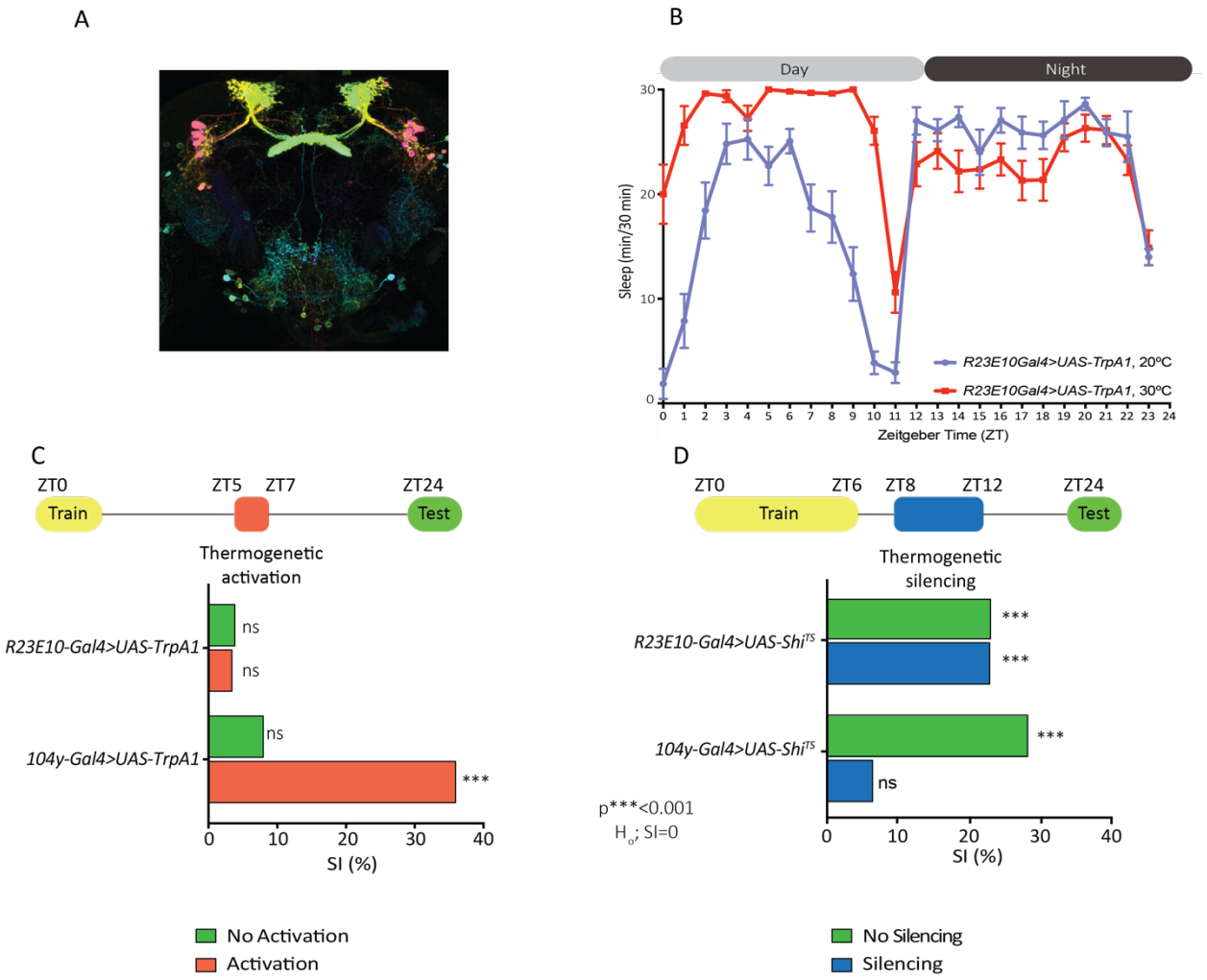

Figure 2.12: dFB neurons are not involved in experience dependent sleep and memory consolidation **A)** Expression pattern of R23E10-Gal4. **B)** Activation of dFB neurons labeled by R23E10 promotes sleep. **C)** Thermogenetic activation of R23E10 neurons was not sufficient to consolidate memory (upper orange bar) **D)** Temporal silencing dFB neurons did not impair long-term memory consolidation (upper blue bar).

A similar set of functional connectivity experiments as done with 104y-Gal4 line was carried out with the R23E10-Gal4 line, instead of 104y-Gal4. Activation of R23E10 neurons did not elicit any response in DAN-aSP13 (Figure 2.12). These results supporting the hypothesis that experience-dependent sleep might be controlled by neuronal pathways other than R23E10 neurons involved in homeostatic sleep regulation.

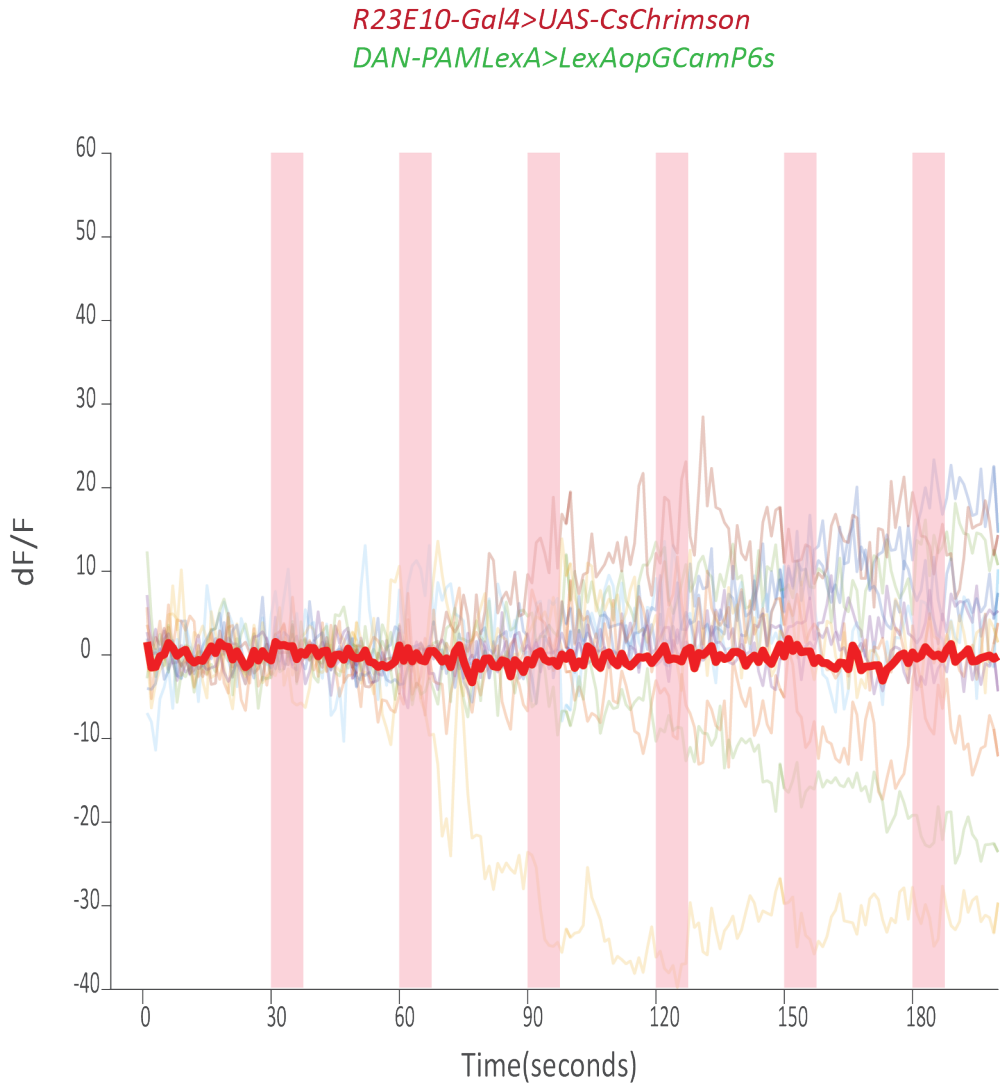

Figure 2.12: dFB neurons are not functionally connected to DAN-aSP13 neurons. Activation of dFB neurons labeled by R23E10-Gal4 line did not elicit any excitatory responses in DAN-aSP13 neurons.

2.5. Neurons in the vFB regulate experience dependent sleep for memory consolidation

Given that neurons in the ventral layer of FB defined by 104y-Gal4 line activate DAN-aSP13 we hypothesized that these neurons might be involved in the experience dependent sleep regulation and thus long-term memory consolidation. We searched the extensive Janelia Gal4 line collection for the Gal4 lines with the expression in the vFB. We started with 3 lines from the collection (Figure 2.13A)

All three lines, with an overlapping expression in the vFB neurons defined by 104yGal4 induced a robust sleep upon activation with TrpA1 (Figure 2.13B). Moreover, local activation of the ventral in all three lines with *CsChrimson* elicited increased calcium levels monitored with *GCamP6s* in the DAN-aSP13 neurons (Figure 2.13C). These results suggest that vFB neurons might be involved in courtship long-term memory consolidation however, further studies are needed to test whether activity of these neurons is both necessary and sufficient for consolidation of the long-term courtship memory.

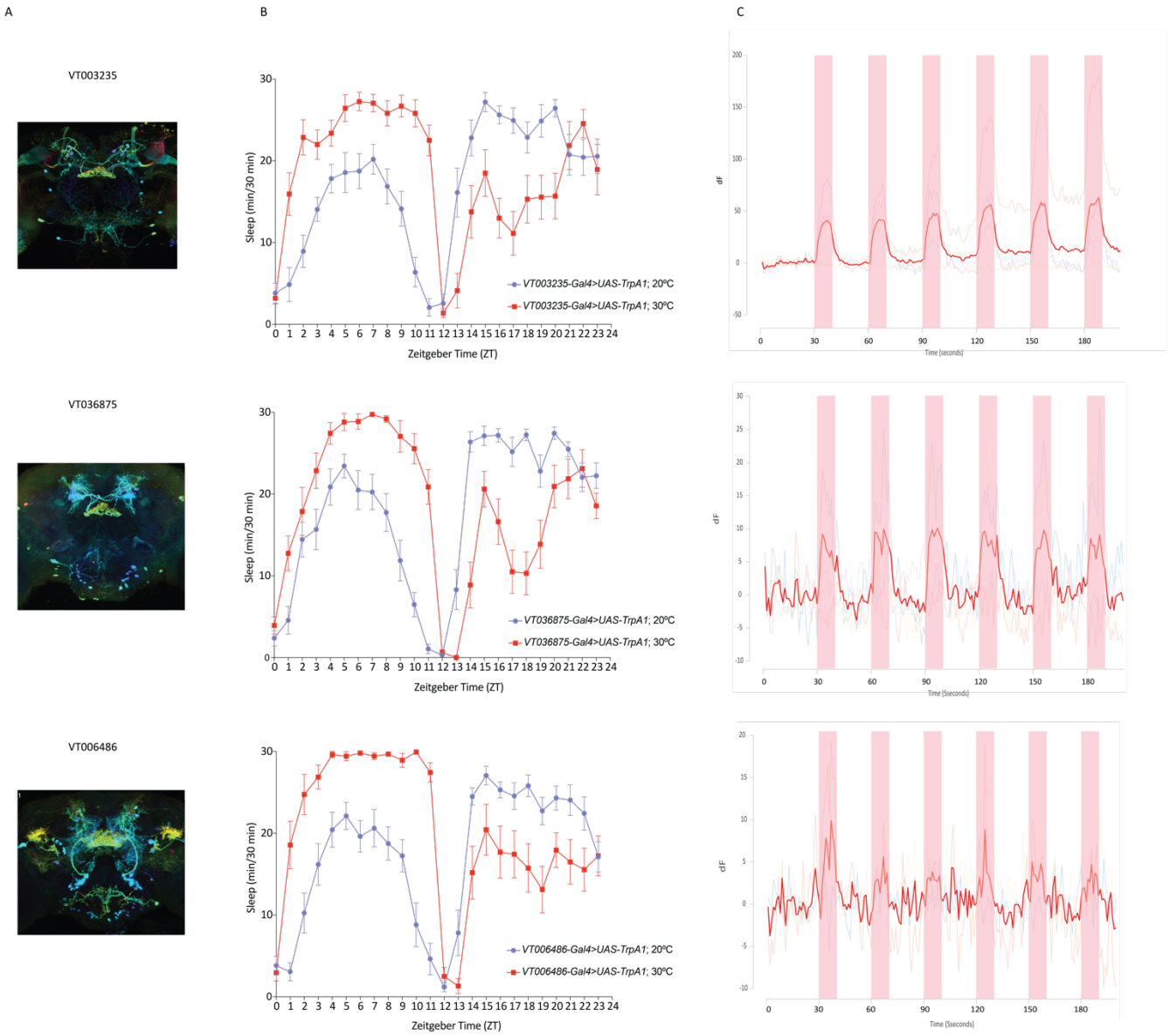

Figure 2.13: vFB might be involved in memory consolidation by activating DAN-aSP13 neurons. **A)** Expression patterns VT003235, VT036875, VT006486. **B)** Activation of each line by TrpA1 promoted sleep during day time, although some decrease in sleep was observed during night time. **C)** Optogenetic activation of vFB neurons defined by VT003235, VT036875 and VT006486 lines evoked excitatory responses in DAN-aSP13 neurons.

3. **DISCUSSION**

Our group previously showed that activity of DAN-aSP13 neurons is both sufficient and required for short-term courtship memory formation (Keleman et al., 2012; Zhao et al., 2018). During my doctoral research, me and my colleagues demonstrated that activity of the same DAN-aSP13 neurons is also necessary and sufficient for long-term courtship memory consolidation during a specific time window after learning (Kruttner et al., 2015). These data suggested that DAN-aSP13 neurons might be activated in this time window in freely behaving animals after learning. Accordingly, I demonstrated that DAN-aSP13 neurons are indeed more active after experience sufficient to generate long-term courtship memory than in animals that were not trained. Reminiscent of results in rodents, that spontaneous reactivation during sleep of neurons previously engaged in memory acquisition is sufficient to consolidate spatial memory (Lee and Wilson, 2002). I hypothesized that DAN-aSP13 neurons might be activated during sleep. Correspondingly, I demonstrated that trained males have an increased day-time sleep in that specific time window after long-term memory training suggesting that sleep after learning activate DAN-aSP13. To test the role of sleep in DAN-aSP13 activation, I systemically examined requirement and sufficiency of both DAN-aSP13 and sleep for memory consolidation. Interestingly, sleep and activity of DAN-aSP13 neurons are sufficient and necessary in the same time window after learning experience suggesting that sleep activates DAN-aSP13. Accordingly, I identified a novel class of sleep promoting neurons in the ventral fan-shaped body (vFB) to be not only necessary and sufficient for long-term memory consolidation but as well providing an excitatory input onto DAN-aSP13. These data suggested that sleep after courtship experience,

sufficient to induce long-term memory, triggers post-learning activation of DAN-aSP13 neurons which promotes memory consolidation.

A large number of studies demonstrated the importance of sleep for mnemonic performance of animals (Saletin and Walker, 2012). Animals that are allowed to sleep after learning experience have an enhanced memory (Payne et al., 2012; Plihal and Born, 1997; Tucker et al., 2006; Walker et al., 2002; Wamsley, 2014). Moreover, studies in rodents have revealed that reactivation of learning activity pattern in neuronal networks during sleep is essential for spatial memory consolidation (Euston et al., 2007). In rats, for example, hippocampal place cells which were activated during spatial learning exhibit the same pattern of activity during sleep (Lee and Wilson, 2002). This post-learning reactivation occurs during slow-wave sleep (SWS) (Kudrimoti et al., 1999) which is thought to regulate synaptic homeostasis, critical for memory consolidation (Cirelli, 2013). However, the direct link between sleep promoting neurons and reactivation has not been firmly established nor the mechanisms underlying this specific reactivation are known.

It is well established now that invertebrates, including *Drosophila*, exhibit most of the hallmarks of the mammalian sleep (Shaw, 2000) (van Alphen et al., 2013). Sleep in *Drosophila* has been implicated in regulation of molecular processes related to synaptic function (Bushey et al., 2011). Also, the role of *Drosophila* sleep in memory has been well documented. Sleep deprivation following learning impairs (Ganguly-Fitzgerald et al., 2006) while sleep induction enhances memory (Donlea et al., 2011). During course of my studies I identified a specific class of sleep promoting neurons in vFB that reactivates DAN-aSP13 neurons for memory consolidation.

FB neurons in fly brain have been shown to be involved in sleep behavior (Donlea et al., 2011). Recently it has been demonstrated that dorsal fan-shaped body (dFB) neurons regulate sleep homeostasis (Donlea et al., 2014; Pimentel et al., 2016). dFB neurons sense the sleep drive generated by R2 ring neurons of ellipsoid body and induces sleep (Donlea et al., 2018; Liu et al., 2016). My behavioral experiments showed that dFB neurons are neither sufficient nor required for courtship long-term memory consolidation suggesting that newly identified sleep promoting, vFB neurons, might induce a distinct type of sleep, perhaps experience dependent. Therefore, I hypothesized that vFB sense an experience dependent sleep drive, potentially form the MB neurons.

Multiple studies have implicated MB in regulation of sleep (Joiner et al., 2006; Pitman et al., 2006). Recently, specific MB neurons have been implicated in sleep regulation (Sitaraman et al., 2015a). Specifically, MB-γ neurons (Sitaraman et al., 2015a) and MBON-$\gamma 2\alpha'1$ neurons (Aso et al., 2014b) were shown to promote sleep upon their activation. So, it is possible that information of a learning experience is encoded in MB-γ Kenyon cells and relayed to vFB through MBON--γ cells. This might result in activation of vFB and thus experience-dependent sleep which in turn activates DAN-aSP13 for LTM consolidation. However, this will require further studies of connectivity between the MB and FB neurons.

I have shown that DAN-aSP13 neurons are significantly more active in intact experienced males. However, imaging experiments showed that sleep promoting vFB neurons have a potential to

activate almost all DANs. These results posit a question whether all DANs are indeed reactivated during sleep or only DAN-aSP13 or subset of DANs are active. To answer this question, further experiments to monitor DANs activity in intact animals are needed. However, in case if only DAN-aSP13 or subset of DANs is active during sleep I hypothesize that potential mechanism of this selective activation might be due to their increased baseline after being active during training. Another potential mechanism might be up-regulation of specific neurotransmitter receptors or ion channels. Results of all DANs being active during sleep after learning in intact animals would suggest that either all DANs and thus MB compartments are necessary for courtship LTM consolidation or only those which are necessary are competent for LTM consolidation. For example, they may form Orb2 complexes after training (Kruttner et al., 2015; Majumdar et al., 2012).

Curiously, DAN-aSP13 have been recently shown to be wake promoting (Sitaraman et al., 2015b) neurons, which is counterintuitive with them being activated during sleep. It has been postulated that there are two different modes of dopamine release: tonic single spike activity and burst spike firing (Goto et al., 2007; Grace and Bunney, 1984a, b). In tonic firing, baseline activity of dopaminergic neurons is spontaneously occurring and these neurons receive inhibitory inputs to keep their activity levels low (Grace and Bunney, 1979). Here we thought that such an activation mode might exist upon their activation during sleep thus keeping them active under sleep conditions.

Finally, based on our data, we propose a following model of how DAN-aSP13 activity is regulated for memory consolidation during experience dependent sleep (Figure 3.1). During learning experience, which is sufficient to induce long-term memory, MB neurons and DAN-aSP13 are activated by olfactory and behavioral cues presented by unreceptive mated female, respectively. Synaptic plasticity induced in MBγ neurons and MBON-M6 is conveyed somehow to vFB. As a result, they exhibit an increased sleep drive and activate DAN-aSP13 neurons. As vFB have a potential to activate all DANs, we hypothesize that specificity of this reactivation or consolidation is installed by molecular processes taking place either in relevant DANs (increased baseline) or MB neurons (formation of Ob2 complexes).

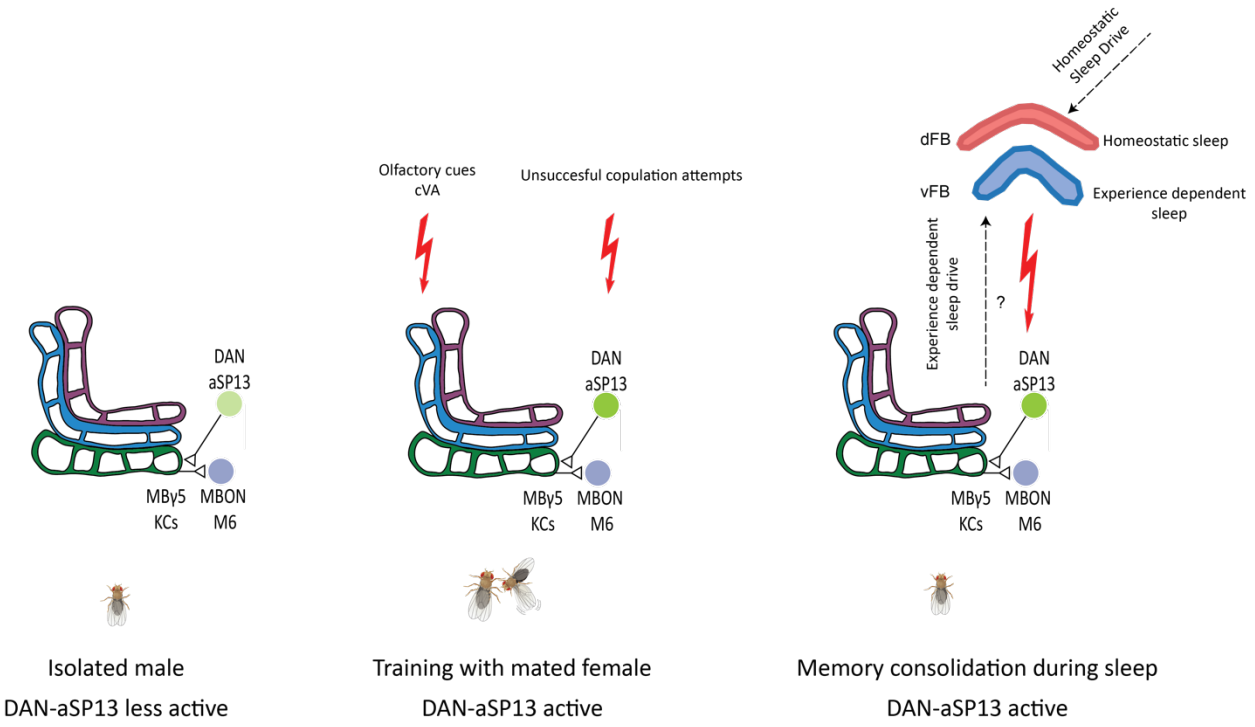

Figure 3.1 Working model of sleep dependent memory consolidation. Naïve isolated male doesn't receive any external sensory signals and DAN-aSP13 activity is kept at low levels. However, during training with mated female, male is subjected to multimodal sensory inputs and DAN-aSP13 neurons become more active. Higher activity of DAN-aSP13 neurons regulates synaptic plasticity between MB-γ neurons and MBON-M6 which are necessary for memory formation. After long-term training, males are separated from female and stay alone. Ventral fan-shaped body neurons (vFB) are potentiated, likely through mushroom body network and promote sleep and activate DAN-aSP13 neurons for memory consolidation.

4. Materials & Methods

4. Materials & Methods

4.1. Fly stocks

Flies were fed with cornmeal-agar mix and were maintained in incubator with 12-hour light/dark cycle at 25°C and 60% relative humidity. 20XUAS-TTS-Shibire-ts1- (Pfeiffer et al., 2012), UAS-dTrpA1, CSMH (Hamada et al., 2008), 20XUAS-CsChrimson-mVenus trafficked in attP18 (Klapoetke et al., 2014), VT-lines (Tirian and Dickson, 2017) and GMR-lines (Jenett et al., 2012) were supplied by Janelia Fly Facility. Fly line *UAS-FLP, Lola>stop>Luc* was a gift from Michael Rosbash. 20XUAS-CsChrimson, LexAop-GCamp6s; R58E02LexA flies were prepared by Allan Wong with Zhengchang Lei and used for functional connectivity experiments.

4.2. Courtship conditioning assay

Wild-type flies and the crosses not involving temperature sensitive effectors were kept in the incubators with 25°C temperature and 60% humidity. In case of the crosses of driver lines to *TrpA1* and *Shibire* , flies were housed in the 22°C and 20°C incubators respectively. Males were collected after eclosion and isolated as individuals in 96-well chambers, also called as fly hotels, containing fly food enriched with yeast. They were aged for 4 days at 25°C and 7-10 days at lower temperatures.

In courtship conditioning, flies were trained either for 1 hour or 7 hours. For 1 hour, short-term training, single males were placed in courtship chambers with arenas of 10 mm diameter and paired with mated females. After each male has the female partner, the chamber was placed under the camera (brand) and it was video-taped for 10 minutes. Their courtship levels during the first 10 minutes are considered as Courtship index of naive males. Flies were left to court for additional 50 minutes. After total of 1-hour training, males were transferred to a new chamber and were waited for about 15 minutes before being tested with fresh mated females for another 10 minutes. The extent of courtship during this time is regarded as Courtship Index of experienced males.

In 7-hours, long-term training, males were paired with mated females in the 96-well chambers with food. During training, flies were watched closely for every 10-20 minutes to see if any copulation event occurs. If males achieve copulation at any time of the training, they were excluded from further processing. At the end of the training, males were removed from the females and replaced in a new 96-well chamber containing food. They were tested on the following day. Naïve males were undergone same treatments except they kept being in isolation during the training time in contrast to being paired with mated females.

4.3. Temporal Silencing with *Shibere^{TS}*

In order to silence the neurons of interest, temperature sensitive *Shibire* was expressed with the corresponding driver lines. In this system, a mutant form of dynamin protein inhibits the synaptic

vesicle recycling at restrictive temperatures, higher than 29°C but not at permissive temperatures, 20-22°C (McGuire et al., 2001). Flies expressing *Shibire* with UAS or LexAop system were kept at 20°C all the time except the period in which acute silencing our neurons were desired. For silencing, the fly hotels were simply transferred to warmer incubator at 32°C from 20°C incubator.

4.4. Neuronal Activation with *TrpA1* and *CsChrimson*

For activating neurons temporally, either *TrpA1* or *CsChrimson* was used. TrpA1 is a temperature sensitive, non-specific cation channel and reversibly depolarizes the neurons where it is expressed at higher temperatures, 28-3°C (Rosenzweig et al., 2005). Flies carrying one allele of the *TrpA1* were kept at 22°C until the time of the activation.

CsChrimson is a red shifted channelrhodopsin, light-gated ion channel allows ion transfer with red light stimulation resulting in neuronal activation(Klapoetke et al., 2014). Flies carrying one allele of *CsChrimson* were prevented from day light and kept under the blue light with intensity lower than 1.0 $\mu W/mm^2$. For activation purpose, testing chambers with the flies placed on a rig, designed by Instrument Design&Fabrication at Janelia Research Campus, and red light was delivered (Frequency: 20 Hertz, Wavelength: 625 nm).

4.5. Recording and Analysis of Courtship Behavior Assays

Videos of the behavioral assays were recorded by Prosilica GT cameras (Allied Vison Technologies) for 10 minutes. A software, Matebook (Machacek, C., Schusterreiter, C. and Dickson, B.J., unpublished) was used for deriving Courtship index (CI) which was calculated by measuring the percentage time spent by a male for courting with the test female. The suppression index was calculated by the formula $SI=100*(CI_{naive}-CI_{exp})/ CI_{naive}$ as measuring the difference as percentage with the courtship indices of naïve and experienced males. The values and significance levels of SI was determined by a MATLAB script and permutation test (Kamyshev et al., 1999). With this method, each suppression index was calculated by generating 100.000 random permutations and P values by determined for the null hypothesis that the learning is 0 (H_0: SI = 0) and the null hypothesis that experimental and control males learn equally well (H_0: SI = SI_c).

4.6. Sleep Monitoring

Drosophila Activity Monitor (DAM) system was used for tracking the locomotion and sleep of the flies (Donelson et al., 2012). This system detects locomotion as the flies cross the infrared beams at the midline of the monitor. The sleep of Drosophila is defined as the 5 minutes of the quiescence, therefore if the individual fly does not move along the infrared beam for 5 minutes, the system attributes this as sleep. For sleep deprivation assays, flies were deprived of sleep

mechanically. Monitors were placed on a shaker and vibrations applied at a random frequency to prevent the flies to get habituated to the stress condition and fall asleep.

4.7. Luminescence Assays

Luminescence experiments for detecting neuronal activity was modified from previous studies (Guo et al., 2017). Flies were starved on filter water soaked in water overnight prior to the training. They were transferred to fresh chambers with filter paper containing 40 mM Luciferin (GOLDBIO) in 2% sucrose solution. For monitoring after training, flies were placed into 96-well plates (Greiner Bio-one) with 40 mM Luciferin in 2% sucrose solution. CLARIOstar microplate reader (BMG Labtech) was used for luminescence detection.

4.8. Calcium Imaging for Functional Connectivity

Flies were anesthetized on ice and their brains were dissected with forceps under the light microscope. The brain was kept in saline solution (103 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 8 mM trehalose, 10 mM glucose, 5 mM TES and bubbled with 95% O_2/5% CO_2) (Strother et al., 2018; Wilson et al., 2004). The brains were imaged by using a two-photon microscope (Prairie Ultima) with near-infrared excitation at 920 nm (Coherent Chamelon Ultra II). 25x objective was used. Data were analyzed by custom generated MATLAB (Mathworks) code. Imaging experiments were carried out in collaboration with Zhengchang Lei (Janelia Research Campus).

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SUPPLEMENTAL INFORMATION

Figure S1: Sleep traces for genetic control with one copy of TrpA1

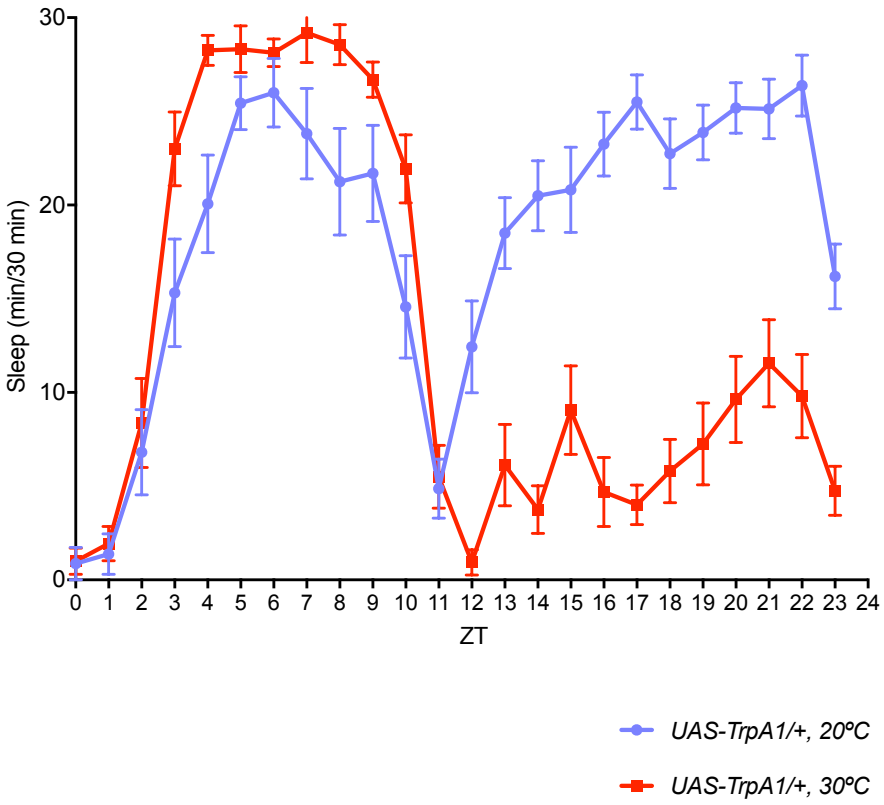

Table S1 Long-term memory in wild-type and Orb2ΔQ mutant flies

	Genotype	Test	Train	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*Canton-S*	24hrs	-	73	40.78		
2	*Canton-S*	24hrs	+	72	30.16	29.36	0
3	*Orb2ΔQ*	24hrs	-	40	30.56		
4	*Orb2ΔQ*	24hrs	+	40	29.56	3.26	0.54

Table S2 Sleep levels in naïve and experienced males

	Wild-type, naïve		Wild-type, trained		Orb2DQ, naïve		Orb2DQ, trained	
ZT	*Average*	*SEM*	*Average*	*SEM*	*Average*	*SEM*	*Average*	*SEM*
	7.081933333	1.1591	7.083333333	1.0104	5.7014	2.3979	5.9107	2.035
7	7.758333333	0.625	8.645833333	0	17.38193333	2.6684	16.0863	3.4521
	11.48333333	1.6246	13.54166667	1.8436	24.81943333	2.1655	21.56546667	1.347
8	15.09306667	2.7711	17.625	3.0407	25.85416667	2.415	25.77083333	1.3659
	14.4111	3.1185	20.9375	2.9151	25.97916667	1.1397	26.2113	0.62895
9	15.19583333	3.1629	21.6875	2.901	25.29166667	2.9453	24.24703333	1.7035
	12.06806667	2.8561	18.66666667	3.2912	23.47916667	2.5645	22.30953333	2.4052
10	9.2986	2.8075	15.64583333	2.5131	20.5139	2.7935	22.0387	2.8665
	6.7125	2.0308	10.52083333	2.4744	16.25	2.0653	18.0744	2.5552
11	4.304166667	1.5511	8.6875	2.0143	17.1736	1.3196	15.61903333	1.5838
	12.85416667	2.4529	18.60416667	2.1975	20.75693333	3.4386	21.11903333	2.7692
12	13.6986	2.7792	19.39583333	2.4544	23.80556667	3.4057	24.3512	2.7906
	16.82776667	3.0856	21.5	2.878	26.02083333	3.0551	24.5982	3.1953
13	21.14443333	2.4965	23.0625	3.0286	27.22916667	2.9437	25.3869	2.7088
	24.22223333	2.5458	23.72916667	2.625	28.52083333	2.5503	25.53273333	2.0021
14	26.35276667	2.7846	26.60416667	2.2246	28.30556667	1.6805	27.4137	1.8708
	25.6486	2.8427	27	1.8526	28.16666667	1.5	27.64583333	0.81378
15	25.3375	2.6615	26.20833333	2.6815	29.0764	0.19365	27.30953333	0.54677
	24.01806667	2.7824	25.83333333	2.7232	28.27776667	0.75829	27.47023333	0.0625
16	23.83333333	2.3385	27.33333333	2.2243	28.58333333	0.085391	26.62796667	0.0625
	22.64583333	2.6266	25.02083333	2.831	27.5	0.60467	25.90773333	1.4317
17	22.5514	2.3035	23.89583333	2.8236	27.84723333	0.44605	25.98513333	1.0886
	19.33193333	2.9887	24	3.0138	26.84026667	1.8686	26.47916667	1.9116
18	18.9014	3.0744	24.97916667	2.9622	27.14583333	2.7996	26.0893	2.4202
	19.1264	3.3478	24.45833333	2.5214	25.70833333	3.137	25.85713333	2.9522
19	17	3.2255	23.6875	2.5815	25.68056667	2.9958	24.6607	2.8947
	15.56806667	3.2794	18.16666667	3.1378	26.18056667	2.9395	23.98513333	3.1185
20	13.3	2.8446	16.29166667	3.4196	24.69443333	3.2749	24.38393333	3.1989
	15.22083333	2.7999	17.9375	3.6158	25.08333333	3.3191	24.26486667	3.7002
21	13.57776667	2.8744	15.22916667	3.2866	23.52776667	3.141	21.15476667	3.5531
	10.94166667	2.5699	11.4375	2.5815	22.35416667	3.314	20.70236667	3.4483
22	7.968066667	2.1426	11.35416667	3.2857	19.99306667	2.138	19.84226667	2.7807
	8.25	1.8209	11.04166667	2.4914	16.65276667	2.0243	17.8988	2.9791
23	6.143066667	0.75	7.604166667	0.68617	14.77776667	2.8431	13.81546667	2.7771
	8.9111	1.7865	9.5	1.6162	18.9861	2.9352	18.34523333	2.4057
24	13.56666667	1.2546	13.22916667	2.6149	19.75	2.8634	20.8869	2.8769

Table S3 Post-learning thermogenetic silencing of DAN- aSP13 neurons

	Genotype	Train	Time of silencing at 32°C	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*VT005526LexA>LexAop-shi^{ts}*	-	-	53	59.11		
2	*VT005526LexA>LexAop-shi^{ts}*	+	-	53	37.01	37.39	0
3	*VT005526LexA>LexAop-shi^{ts}*	-	7-9h	36	57.35		
4	*VT005526LexA>LexAop-shi^{ts}*	+	7-9h	35	53.63	6.49	0.23
5	*VT005526LexA>LexAop-shi^{ts}*	-	8-10h	53	50.04		
6	*VT005526LexA>LexAop-shi^{ts}*	+	8-10h	54	49.26	1.57	0.46
7	*VT005526LexA>LexAop-shi^{ts}*	-	9-11h	34	43.36		
8	*VT005526LexA>LexAop-shi^{ts}*	+	9-11h	32	31.12	28.23	0.003
9	*VT005526LexA>LexAop-shi^{ts}*	-	10-12h	53	44.37		
10	*VT005526LexA>LexAop-shi^{ts}*	+	10-12h	54	36.25	18.30	0.03
11	*VT005526LexA>LexAop-shi^{ts}*	-	7-11h	53	45.48		
12	*VT005526LexA>LexAop-shi^{ts}*	+	7-11h	51	43.32	4.74	0.34
13	*VT005526LexA>LexAop-shi^{ts}*	-	8-12h	54	43.93		
14	*VT005526LexA>LexAop-shi^{ts}*	+	8-12h	54	42.14	4.07	0.26

Table S4 Post-learning sleep deprivation on wild-type flies

	Genotype	Train	Time of silencing at 32°C	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*Wild-type*	-	-	35	54.58		
2	*Wild-type*	+	-	34	34.28	37.19	0
3	*Wild-type*	-	7-9h	31	42.74		
4	*Wild-type*	+	7-9h	27	43.32	-1.34	0.56
5	*Wild-type*	-	8-10h	35	34.87		
6	*Wild-type*	+	8-10h	34	32.29	7.39	0.36
7	*Wild-type*	-	9-11h	23	45.63		
8	*Wild-type*	+	9-11h	26	33.91	25.69	0.004
9	*Wild-type*	-	10-12h	34	29.65		
10	*Wild-type*	+	10-12h	36	19.70	33.56	0.001
11	*Wild-type*	-	7-11h	28	34.69		
12	*Wild-type*	+	7-11h	29	33.34	3.89	0.31
13	*Wild-type*	-	8-12h	34	28.11		
14	*Wild-type*	+	8-12h	36	28.84	-2.59	0.61

Table S5 Sleep behavior upon activation of fan-shaped body neurons

	***104y-Gal4>UAS-TrpA1*, 20ºC**		***104y-Gal4>UAS-TrpA1*, 30ºC**	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	2.4	1.1288	13.2667	2.749
	4.1333	1.1249	14.8	2.9491
1	4.4	1.4729	24.2667	1.7443
	6.8	2.1116	26.6667	1.0541
2	9.4667	2.2208	25.8	1.6042
	11	2.6886	27.3333	0.9595
3	16.6	2.6526	27.8	0.91652
	19.4	1.7588	26.0667	1.3817
4	22.4	2.4781	27.0667	1.1104
	24	1.8491	26.9333	1.5598
5	21.9333	2.3309	25.8	2.2172
	22.5333	2.1753	26.5333	1.5146
6	24.0667	1.4023	25.6	1.8893
	22.5333	2.1511	25.6	2.0232
7	24.2	1.5892	25.5333	1.8306
	23.2	2.0637	24.6667	2.3698
8	21.0667	1.9772	24.8	1.5156
	18.3333	1.9777	21.5333	2.7669
9	19.9333	2.2959	26	1.4671
	14.5333	2.7253	25.4	1.9243
10	9.4667	2.1688	24.8	1.6365
	8.4667	1.9949	24.8	2.0753
11	6.3333	1.6465	24.3333	1.8662
	4.6	1.961	24.3333	2.0946
12	7.2667	1.8758	23.4	2.0766
	25.6667	1.3475	22.9333	2.3593
13	26.8	1.2769	24.5333	2.2014
	28.3333	0.97427	22	2.8217
14	27.6667	0.86005	24	2.3197
	28	0.71714	25	2.0166
15	27.7333	0.86996	24	2.2991
	28.4	0.74194	23.6667	2.3004
16	27.8	0.82347	23.6667	2.2713
	27.0667	1.7981	22.9333	2.5042

17	28.6667	0.59094	24.4667	2.1666
	28.4667	0.59255	24.2667	2.1235
18	28.6667	0.64488	24.4	2.5407
	26.9333	1.1971	25.8667	2.1244
19	26.6	1.3863	24.8	2.2745
	24.9333	2.3674	26.6	2.0256
20	25.6	2.0651	26.0667	2.0035
	22	2.8082	26.6667	1.7118
21	23.1333	2.5502	25.0667	2.0435
	18.7333	3.1554	25.7333	2.1122
22	23.4	2.4972	26.3333	2.0625
	20.8	2.4186	24.8	2.1229
23	16.8667	2.9324	25.8	1.8341
	11.2	2.9491	26.5333	2.0491

Table S6 Post-learning thermogenetic activation of fan-shaped body neurons

	Genotype	**Trai**	**Time at 32°C**	**n**	**CI (%)** median	**LI (%)** median	**P LI=0**
1	*104y-Gal4>UAS-TrpA1*	-	-	51	36.32		
2	*104y-Gal4>UAS-TrpA1*	+	-	42	33.78	8.29	0.25
3	*104y-Gal4>UAS-TrpA1*	-	1-3h	36	27.49		
4	*104y-Gal4>UAS-TrpA1*	+	1-3h	32	25.88	5.85	0.33
5	*104y-Gal4>UAS-TrpA1*	-	3-5h	36	26.89		
6	*104y-Gal4>UAS-TrpA1*	+	3-5h	32	25	7.03	0.33
7	*104y-Gal4>UAS-TrpA1*	-	5-7h	34	30.75		
8	*104y-Gal4>UAS-TrpA1*	+	5-7h	34	19.70	35.94	0
9	*104y-Gal4>UAS-TrpA1*	-	7-9h	35	24.77		
10	*104y-Gal4>UAS-TrpA1*	+	7-9h	34	22.74	8.20	0.11
11	*104y-Gal4>UAS-TrpA1*	-	9-11h	52	26.15		
12	*104y-Gal4>UAS-TrpA1*	+	9-11h	52	24.73	5.43	0.19

Table S7 Post-learning optogenetic activation of DAN-asP13 neurons

	Genotype	Train	Activation time	n	CI (%) median	LI (%) median	P LI=0
1	*VT005526LexA >LexAop-CsChrimson*	-	-	33	44.17		
2	*VT005526LexA >LexAop-CsChrimson*	+	-	31	41.39	6.03	
3	*VT005526LexA >LexAop-CsChrimson*	-	1-3h	34	30.74		
4	*VT005526LexA >LexAop-CsChrimson*	+	1-3h	33	26.61	13.45	0.17
5	*VT005526LexA >LexAop-CsChrimson*	-	3-5h	34	39.48		
6	*VT005526LexA >LexAop-CsChrimson*	+	3-5h	26	41.00	-3.85	0.65
7	*VT005526LexA >LexAop-CsChrimson*	-	5-7h	31	41.40		
8	*VT005526LexA >LexAop-CsChrimson*	+	5-7h	26	26.78	35.03	0.0002
9	*VT005526LexA >LexAop-CsChrimson*	-	7-9h	31	41.39		
10	*VT005526LexA >LexAop-CsChrimson*	+	7-9h	31	33.69	18.59	0.03
11	*VT005526LexA >LexAop-CsChrimson*	-	9-11h	32	35.45		
12	*VT005526LexA >LexAop-CsChrimson*	+	9-11h	34	35.57	-0.34	0.57

Table S8 Post-learning thermogenetic silencing of fan-shaped body neurons

	Genotype	Train	Time of silencing at 32°C	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*104y-Gal4>UAS- shi^{ts}*	-	-	75	47.06		
2	*104y-Gal4>UAS- shi^{ts}*	+	-	77	35.41	24.75	0.0006
3	*104y-Gal4>UAS- shi^{ts}*	-	7-9h	47	27.81		
4	*104y-Gal4>UAS- shi^{ts}*	+	7-9h	53	26.56	4.47	0.42
5	*104y-Gal4>UAS- shi^{ts}*	-	8-10h	34	31.97		
6	*104y-Gal4>UAS- shi^{ts}*	+	8-10h	34	25.80	19.29	0.008
7	*104y-Gal4>UAS- shi^{ts}*	-	9-11h	46	27.75		
8	*104y-Gal4>UAS- shi^{ts}*	+	9-11h	46	20.23	27.08	0.004
9	*104y-Gal4>UAS- shi^{ts}*	-	10-12h	30	35.23		
10	*104y-Gal4>UAS- shi^{ts}*	+	10-12h	27	23.52	33.23	0.0003
11	*104y-Gal4>UAS- shi^{ts}*	-	7-11h	69	28.93		
12	*104y-Gal4>UAS- shi^{ts}*	+	7-11h	70	30.84	-6.62	0.71
13	*104y-Gal4>UAS- shi^{ts}*	-	8-12h	63	30.68		
14	*104y-Gal4>UAS- shi^{ts}*	+	8-12h	65	29.65	3.38	0.30

Table S9 Post-learning thermogenetic activation of fan-shaped body neurons and thermogenetic silencing of DAN-aSP13 neurons

	Genotype	Train	Incubation at 32°C	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*104y-Gal4>UAS- TrpA1; VT005526-LexA>LexAop- shi^{ts}*	-	-	50	46.79		
2	*104y-Gal4>UAS- TrpA1; VT005526-LexA>LexAop- shi^{ts}*	+	-	48	43.41	7.21	0.12
3	*104y-Gal4>UAS- TrpA1; VT005526-LexA>LexAop- shi^{ts}*	-	5-7h	50	47.57		
4	*104y-Gal4>UAS- TrpA1; VT005526-LexA>LexAop- shi^{ts}*	+	5-7h	46	48.09	-1.08	0.56
5	*104y-Gal4>UAS- TrpA1; LexAop- shi^{ts}/+*	-	-	47	43.34		
6	*104y-Gal4>UAS- TrpA1; LexAop- shi^{ts}/+*	+	-	49	38.81	10.45	0.11
7	*104y-Gal4>UAS- TrpA1; LexAop- shi^{ts}/+*	-	5-7h	51	49.42		
8	*104y-Gal4>UAS- TrpA1; LexAop- shi^{ts}/+*	+	5-7h	49	35.60	27.96	0

Table S10 Sleep behavior upon activation of dorsal fan-shaped body neurons

	***R23E10-Gal4>UAS-TrpA1*, 20ºC**		***R23E10-Gal4>UAS-TrpA1*, 30ºC**	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	1.875	1.1288	20	2.8387
	3.5625	1.1249	25.625	1.8323
1	7.875	1.4729	26.5625	1.8529
	13.4375	2.1116	28.4375	0.81634
2	18.4375	2.2208	29.625	0.27195
	23.1875	2.6886	28.5	0.7188
3	24.8125	2.6526	29.375	0.56181
	22.6875	1.7588	29.9375	0.0625
4	25.25	2.4781	27.25	1.2128
	25.25	1.8491	30	0
5	22.6875	2.3309	30	0
	22.3125	2.1753	29.25	0.56642
6	25.0625	1.4023	29.8125	0.1875
	22.9375	2.1511	29.0625	0.76086
7	18.6875	1.5892	29.6875	0.25362
	16.5625	2.0637	29.375	0.43661
8	17.8125	1.9772	29.625	0.25617
	14.125	1.9777	29.625	0.375
9	12.375	2.2959	30	0
	8.25	2.7253	28	1.0042
10	3.875	2.1688	26.0625	1.3338
	0.6875	1.9949	22.125	2.2947
11	2.9375	1.6465	10.625	1.9576
	23.0625	1.961	12.6875	2.8907
12	27	1.8758	22.875	2.1171
	25.8125	1.3475	20.1875	2.3224
13	26.125	1.2769	24.125	1.7002
	24.4375	0.97427	23.6875	1.8568
14	27.375	0.86005	22.1875	1.9963
	26.1875	0.71714	23.75	1.2264
15	24	0.86996	22.375	1.8368
	26.0625	0.74194	23.375	1.372

16	27.0625	0.82347	23.3125	1.5213
	27.875	1.7981	22.375	1.8002
17	25.875	0.59094	21.3125	1.8835
	27.6875	0.59255	24.3125	1.4193
18	25.625	0.64488	21.375	1.9914
	26.4375	1.1971	21.875	1.7269
19	27.125	1.3863	25.4375	1.3384
	29.6875	2.3674	25.875	1.4168
20	28.625	2.0651	26.3125	1.2965
	28.5625	2.8082	25.9375	1.286
21	25.875	2.5502	26.125	1.3475
	26.8125	3.1554	25.6875	1.264
22	25.5	2.4972	23.25	1.4186
	25.0625	2.4186	21.9375	1.7547
23	14	2.9324	14.875	1.6829
	6.5625	2.9491	1.1875	0.54175

Table S11 Post-learning thermogenetic activation of dorsal fan-shaped body neurons

	Genotype	Time at 32°C	Train	n	CI (%) median	LI (%) median	P LI=0
1	*R23E10-Gal4>UAS-TrpA1*	-	-	32	55.57		
2	*R23E10-Gal4>UAS-TrpA1*	-	+	34	52.98	4.66	0.15
3	*R23E10-Gal4>UAS-TrpA1*	5-7h	-	33	52.52		
4	*R23E10-Gal4>UAS-TrpA1*	5-7h	+	32	50.72	3.41	0.40
5	*104y-Gal4>UAS-TrpA1*	-	-	36	31.51		
6	*104y-Gal4>UAS-TrpA1*	-	+	31	29.10	7.62	0.25
7	*104y-Gal4>UAS-TrpA1*	5-7h	-	33	42.16		
8	*104y-Gal4>UAS-TrpA1*	5-7h	+	35	29.76	29.41	0.0005

Table S12 Post-learning thermogenetic silencing of fan-shaped body neurons

	Genotype	Train	Time of silencing at 32°C	*n*	CI (%) median	LI (%) median	*P LI=0*
1	*R23E10-Gal4>UAS- shi^{ts}*	-	-	36	63.27		
2	*R23E10-Gal4>UAS- shi^{ts}*	+	-	36	48.75	22.95	0
3	*R23E10-Gal4>UAS- shi^{ts}*	-	8-12h	35	62.12		
4	*R23E10-Gal4>UAS- shi^{ts}*	+	8-12h	34	50.52	22.84	0
5	*104y-Gal4>UAS- shi^{ts}*	-	-	26	59.85		
6	*104y-Gal4>UAS- shi^{ts}*	+	-	26	42.94	28.25	0
7	*104y-Gal4>UAS- shi^{ts}*	-	8-12h	18	50.25		
8	*104y-Gal4>UAS- shi^{ts}*	+	8-12h	23	48.14	4.19	0.29

Table S13 Sleep behavior upon activation of neurons labeled by *VT003235-Gal4*

	***VT003235Gal4>UAS-TrpA1*, 20ºC**		***VT003235Gal4>UAS-TrpA1, 30ºC***	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	3.8125	1.2152	3.1875	0.69052
	1.3125	0.9024	6	1.4888
1	4.875	2.0734	15.9375	2.5972
	4	1.1328	18.875	2.6924
2	8.9375	1.9842	22.875	2.1639
	8.9375	1.4067	23.375	1.678
3	14.0625	1.4619	22	1.8051
	17.9375	2.0544	23.9375	1.7138
4	17.8125	1.7302	23.375	1.5887
	20.125	2.3556	25.125	1.5965
5	18.5625	2.4596	26.4375	1.6406
	19.4375	2.2211	27.25	1.0428
6	18.75	2.1243	27.25	1.142
	20.6875	2.2337	28.25	0.59512
7	20.1875	1.8217	27.0625	1.0703
	20.875	2.3289	27.1875	1.1076
8	16.875	2.1445	25.8125	1.5444
	17.9375	1.9546	28.1875	0.87187
9	14.125	2.1289	26.6875	1.3345
	12.8125	2.4448	25.5	1.1972
10	6.375	1.7979	25.8125	1.6463
	3.1875	0.76496	24.5	1.4916
11	2.0625	1.0781	22.5	1.8797
	1.1875	0.62061	15.5625	2.463
12	2.5625	1.1654	1.375	0.53131
	10.5625	3.1131	0.4375	0.4375
13	16.125	2.9957	4.125	2.0994
	22.125	2.7956	9.5	2.7704
14	22.8125	2.214	13.75	3.2035
	26.4375	1.0723	16.625	2.8706
15	27.1875	1.1805	18.5	2.8665
	26.8125	1.198	15.1875	2.8726
16	25.625	1.0912	13	2.4341

	26.75	1.1885	14.5625	2.7988
17	24.9375	1.5638	11.125	2.6846
	24.5	1.96	13.25	3.0297
18	22.875	1.8839	15.3125	2.9094
	25.125	1.5596	9.0625	2.9713
19	24.875	1.983	15.5625	2.6863
	25.25	1.199	12.25	2.5257
20	26.4375	1.0367	15.6875	2.7954
	21.5	2.4444	17.1875	3.1094
21	20.75	2.4958	21.875	2.9494
	24	1.8348	21.625	2.7124
22	20.4375	2.1946	24.5625	1.7464
	20.5625	1.732	26.25	1.2433
23	20.5625	2.1291	18.9375	3.0869
	14.75	2.1937	12.375	2.7232

Table S14 Sleep behavior upon activation of neurons labeled by *VT036875-Gal4*

	***VT036875Gal4>UAS-TrpA1*, 20ºC**		***VT036875Gal4>UAS-TrpA1, 30ºC***	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	2.375	0.9393	3.9375	1.0742
	2.0625	1.2893	5.6875	0.70545
1	4.5625	1.6906	12.75	2.134
	6.125	1.8025	16.6875	2.588
2	14.4375	2.1271	17.875	2.9536
	17.1875	2.5482	20.375	2.5214
3	15.6875	2.4828	22.875	2.1792
	18.875	2.4356	25	2.0936
4	20.875	2.2265	27.4375	1.3353
	20.75	2.0135	27.1875	1.6361
5	23.4375	1.4548	28.8125	1.0576
	22.3125	2.3695	29.75	0.1118
6	20.5	2.384	28.875	0.93486
	20.9375	2.032	29.0625	0.65491
7	20.25	2.1593	29.75	0.1118
	21.1875	1.8624	28.75	1.25
8	17.75	2.3085	29.1875	0.41047
	17.3125	2.4524	28.0625	1.7428
9	11.875	2.4831	27.0625	1.9375
	8.5625	1.9832	27.625	1.3689
10	6.5	1.4972	25.5625	1.8506
	2.625	1.125	24.75	2.3121
11	1.0625	0.58785	20.875	2.1348
	2.0625	1.1743	12.4375	2.7156
12	0.3125	0.2183	0.625	0.34004
	1.5625	1.0761	0	0
13	8.3125	2.4319	0	0
	22.5625	1.6708	0.9375	0.9375
14	26.375	1.2446	8.875	2.8223
	26.875	1.3536	19	2.9011
15	27.125	1.1863	20.625	2.1658
	27.625	1.0036	21.75	2.25
16	27.1875	0.82774	16.625	2.7822

	26.4375	0.96163	15.5625	2.7477
17	25.1875	1.787	10.5	2.641
	25.875	1.0912	9.8125	2.4887
18	27.25	0.71589	10.3125	2.612
	24.75	1.1493	11.625	2.921
19	22.8125	1.9647	13.875	2.9395
	25.4375	1.6227	12.3125	2.7045
20	27.4375	0.7526	20.9375	2.5988
	24.375	1.4574	18.9375	3.031
21	25.5	0.89907	21.875	2.4373
	24.1875	1.2084	21.875	2.3271
22	22.0625	1.7284	23.125	2.2838
	20.5625	1.873	20.9375	1.8962
23	22.25	1.5559	18.5625	1.5678
	22.75	1.606	15	1.6758

Table S15 Sleep behavior upon activation of neurons labeled by *VT006486-Gal4*

	***VT006486Gal4>UAS-TrpA1*, 20ºC**		***VT006486Gal4>UAS-TrpA1, 30ºC***	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	3.8125	1.1519	2.9375	0.99778
	1.625	0.75208	7.875	2.234
1	3.0625	1.1123	18.5625	2.9154
	4.5	1.1832	22.4375	2.4916
2	10.25	2.4435	24.75	2.474
	14.4375	2.5739	24.875	2.2096
3	16.1875	2.5499	26.875	1.4688
	20.375	1.9168	29.5625	0.24098
4	20.4375	2.1213	29.625	0.375
	21.1875	2.0761	29.3125	0.47186
5	22.125	1.653	29.4375	0.5625
	22.0625	1.657	28.8125	0.95402
6	19.625	1.9341	29.8125	0.1875
	22.875	1.8861	30	0
7	20.625	2.2911	29.4375	0.4375
	23.5625	1.9832	29.25	0.62915
8	18.75	2.0176	29.6875	0.17604
	16.9375	2.0585	29.4375	0.30233
9	17.25	1.9885	28.9375	0.83401
	14.6875	2.7321	29.6875	0.3125
10	8.8125	2.6712	29.9375	0.0625
	8.375	2.4527	29.125	0.50724
11	4.625	1.9233	27.4375	1.1901
	2.4375	0.83151	17.4375	2.6645
12	1.1875	0.58608	2.5	1.0992
	6.9375	2.9332	1.0625	1.0625
13	7.8125	2.7722	1.3125	0.9252
	10.6875	2.6216	6.25	2.6101
14	24.5	1.0448	15.1875	3.2303
	24.625	1.4602	20.125	3.1923
15	27.0625	1.1492	20.4375	3.1265
	24.5625	1.08	22.0625	2.573
16	25.3125	0.96488	17.6875	3.1816

	24.75	1.6034	14.1875	3.0594
17	24.5625	1.6456	17.4375	2.8693
	25.4375	1.5491	16.375	3.1009
18	25.8125	1.333	15.75	2.946
	25.625	1.2711	10.9375	2.2088
19	22.75	1.6721	13.125	2.8444
	23.5	1.8303	11.25	2.8889
20	24.3125	1.4824	17.9375	2.1203
	26.6875	1.3029	12.75	2.5306
21	24.0625	1.8674	16.5	2.7356
	23.6875	1.5048	15.1875	2.3456
22	22.4375	2.0165	15.5625	2.606
	19.625	1.5272	20.875	2.0081
23	17.125	1.8571	17.25	2.4571
	11.25	1.8495	11.125	1.9102

Table S16 Sleep behavior for genetic control with flies carrying one copy of TrpA1

	***UAS-TrpA1/+*, 20ºC**		***UAS-TrpA1/+, 30ºC***	
ZT	*Average*	*SEM*	*Average*	*SEM*
0	0.875	0.875	1	0.70711
	0.875	0.625	0.3125	0.3125
1	1.375	1.0873	1.9375	0.91044
	3.4375	1.3873	3.3125	1.6195
2	6.8125	2.2716	8.375	2.3803
	9.6875	2.3536	15.3125	2.7909
3	15.3125	2.869	23	1.9727
	16.9375	2.9304	26.5	1.103
4	20.0625	2.602	28.25	0.80364
	22.25	2.2976	28.0625	1.0703
5	25.4375	1.4171	28.3125	1.2474
	26	1.5492	28.5	0.7692
6	26	1.8348	28.125	0.74092
	25.1875	1.8353	29.1875	0.43987
7	23.8125	2.4139	29.1875	1.5808
	23.3125	1.543	29.6875	0.3125
8	21.25	2.8453	28.5625	1.0723
	23.9375	2.0746	26.6875	1.4768
9	21.6875	2.567	26.6875	0.94745
	19.3125	2.7865	24.8125	1.4469
10	14.5625	2.7294	21.9375	1.8222
	10.6875	2.6024	16.75	2.4774
11	4.875	1.5782	5.5	1.6733
	5.4375	1.7888	0	0
12	12.4375	2.4494	0.9375	0.67988
	18.6875	1.5349	4.4375	1.5491
13	18.5	1.8974	6.125	2.1754
	19	1.9558	5.625	1.7769
14	20.5	1.8664	3.75	1.2698
	18.875	2.1735	5.75	1.75
15	20.8125	2.2789	9.0625	2.3708

	20.625	2.1811	6.25	1.6944
16	23.25	1.709	4.6875	1.8478
	23.375	2.2396	7.375	2.2228
17	25.5	1.4491	4	1.0607
	25.3125	1.4163	6.625	1.5515
18	22.75	1.854	5.8125	1.6937
	22.5625	2.0083	7.4375	2.1832
19	23.875	1.4631	7.25	2.1956
	21.8125	1.4178	7.1875	2.288
20	25.1875	1.3516	9.625	2.3001
	21.9375	2.3883	10.0625	2.0072
21	25.125	1.5913	11.5625	2.3291
	21.5625	2.1505	9.75	2.207
22	26.375	1.6301	9.8125	2.2215
	21.5	1.9408	6	1.6708
23	16.1875	1.7326	4.75	1.3181
	0	0	6.0625	1.1382

ZUSAMMENFASSUNG

Drosophila melanogaster (Fruchtfliegen) können aversive oder appetitive Reize mit Düften assoziieren und so ein aversives oder entsprechend appetitives Duftgedächtnis formen. Zusätzlich zu dieser einfachen Form des assoziativen Lernens sind männliche Fliegen in der Lage, eine komplexere Form des Lernens zu bilden, nämlich das Balzgedächtnis. Bei dieser Lernform wird das Balzverhalten zu einem paarungs-unwilligen und bereits verpaarten Weibchen unterdrückt, nachdem das Männchen mehrfach vom Weibchen abgelehnt wurde. Ältere Arbeiten haben gezeigt, dass die Aktivität der dopaminergen aSP13 (DAN-aSP13) Neurone, welche die Spitze der Pilzkörper Gamma Neurone innervieren, notwendig und hinreichend für die Bildung des Kurzzeit Balzgedächtnis sind. Kürzlich konnte außerdem gezeigt werden, dass Aktivität der gleichen DAN-aSP13 Neurone notwendig und hinreichend ist, um die so gespeicherten Erinnerungen in das Langzeit-Gedächtnis zu überführen. Angelehnt an Ergebnisse aus Arbeiten an Nagern, die zeigen, dass Neuronen die vormals in Gedächtnisbildung involviert waren, räumliches Gedächtnis bilden können, wenn sie spontan während des Schlafens reaktiviert worden waren, stelle ich die These auf, dass DAN-aSP13 während des Schlafens aktiviert werden könnten. Dazu habe ich systematisch die Voraussetzungen und Hinlänglichkeit von Schlaf und DAN-aSP13 Neurone in der Gedächtnisbildung untersucht. Nach der Erfahrung des Balzens sind DAN-aSP13 Neuronen und Schlaf tatsächlich im gleichen Zeitfenster aktiviert und somit hinreichend und notwendig für Gedächtnis Konsolidierung. Das Ergebnis deutet darauf hin, dass Schlaf DAN-aSP13 Neurone aktiviert. Dementsprechend habe ich eine neue Klasse von Schlaf-fördernden Neurone im ventralen fächerartigen Körper (fan-shaped body, vFB) des

Zentralkomplex identifiziert, welche nicht nur hinreichend und notwendig für Langzeitgedächtnis Konsolidierung sind, sondern die auch einen exzitatorischen Eingang auf DAN-aSP13 Neurone bilden. Tatsächlich deuten meine Daten darauf hin, dass Schlaf nach der Balzerfahrung, welches Langzeitgedächtnis induziert, die Aktivität der DAN-aSP13 Neurone nach dem Lernen erhöht.

APPENDIX A: Synaptic Orb2A bridges memory acquisition and late memory consolidation in *Drosophila*

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Summary

To adapt to an ever-changing environment, animals consolidate some but not all learning experiences to long-term memory (LTM). In mammals, LTM consolidation often involves neural pathway reactivation hours after memory acquisition. It is not known whether this delayed-reactivation schema is common across the animal kingdom, nor how information is stored during the delay period. We show here that during courtship suppression learning, *Drosophila* exhibits delayed LTM consolidation. We also show that the same class of dopaminergic neurons engaged earlier in memory acquisition is also both necessary and sufficient for delayed LTM consolidation. Further, we present evidence that, during learning, the translational regulator Orb2A tags specific synapses of mushroom body neurons for later consolidation. Consolidation involves the subsequent recruitment of Orb2B and the activity-dependent synthesis of CaMKII. Our results thus provide evidence for the role of a neuromodulated, synapse-restricted molecule bridging memory acquisition and LTM consolidation in a learning animal.

Introduction

The brain rapidly learns environmental associations and behavioral contingencies, but is selective about which lessons it commits to long-term memory. Evidence from multiple approaches has provided clues to how something learned becomes something remembered, as well as how and where memories are stored. Neuromodulatory systems are thought to exert substantial control over whether a learning experience is memorable. Neuromodulatory pathways are activated during and/or after "important" behavioral moments for memory acquisition and memory consolidation (Placais et al., 2012; Rossato et al., 2009; Schwaerzel et al., 2003; Wise, 2004) and blocking them inhibits the formation of memories (Schwaerzel et al., 2003; Wise, 2004). Intriguingly, the process of memory consolidation often involves a critical period, sometimes many hours after the initial learning period, during which a reactivation of brain activity is required. In some cases, this reactivation involves a literal replay of a learning experience, either during the awake (Carr et al., 2011) or sleeping (Wilson and McNaughton, 1994) states.

Adult *Drosophila* flies exhibit remarkable behavioral complexity that can be modified by experience. They can learn to avoid or approach odors that were previously associated with an electric shock (Quinn et al., 1974) or with sugar reward (Tempel et al., 1984), respectively. Flies can also learn visual, tactile and even spatial cues (Guo et al., 1996;

Ofstad et al., 2011; Wustmann et al., 1996). A robust form of memory in flies is courtship conditioning, whereby naïve males learn to preferentially court receptive virgin females after experiencing unsuccessful courtship of already mated (and therefore unreceptive) females. Depending on the learning experience, flies can form memories lasting from minutes to hours to several days (McBride et al., 1999; Siegel and Hall, 1979). However, the mechanisms that trigger production of LTM are not clear. Recently, it has been found that orchestrated activity of three clusters of dopaminergic neurons positively affect LTM formation during olfactory learning (Placais et al., 2012) and delayed activity of the specific dopaminergic neurons is critical for consolidation of the long-term appetitive memory (Musso et al., 2015).

Short-term memory (STM) can be mediated by a variety of protein-synthesis-independent mechanisms (Kandel, 2001). LTMs are thought to reflect protein synthesis-dependent morphological and biochemical changes taking place in specific synapses within neuronal networks (Sutton and Schuman, 2006). Because more synapses in the brain are activated during memory acquisition than eventually might become consolidated, there must be mechanisms for determining which particular synapses will ultimately encode a given LTM. Furthermore, these mechanisms must be capable of maintaining such specificity during the interval between memory acquisition and its consolidation.

The synaptic tag and capture hypothesis (Frey and Morris, 1997; Martin et al., 2000) proposes how specific synapses come to store a given memory. The original experimental evidence in support of this hypothesis came from *in vitro* electrophysiological studies in hippocampal slices. Conversion of early long-term potentiation (E-LTP, an *in vitro* correlate of STM) into L-LTP (a physiological correlate of LTM) at synapses activated by a strong stimulation after a weak one suggested that synapses activated during behavioral memory acquisition might be tagged for a protein synthesis-dependent long-term consolidation. Behavioral studies in rodents demonstrated that training which elicits STM can be consolidated into LTM by a novel experience capable of inducing protein synthesis (Moncada and Viola, 2007). Moreover, activation of the dopaminergic ventral tegmental area in rats after learning induces protein synthesis, which is required for LTM consolidation (Rossato et al., 2009). Nonetheless, the molecular mechanisms of synaptic

tagging have not been identified within the contexts of specific neuronal pathways and learning animals.

Candidates for such a synaptic tag are members of the Cytoplasmic Polyadenylation Element Binding family of proteins (CPEB) (Si et al., 2003a). CPEB proteins can be divided into two sub-families. The CPEB-I subfamily includes the *Xenopus* CPEB1 and its *Drosophila* ortholog Orb1, which both regulate mRNA translation during oogenesis (Mendez and Richter, 2001). Members of the CPEB-II subfamily have been found to function in synaptic plasticity (mCPEB2-4) (Richter, 2001) or long-term memory formation (*Drosophila* Orb2) (Keleman et al., 2007; Majumdar et al., 2012a). Almost all CPEBs exist in multiple variants generated by alternative mRNA splicing (Theis et al., 2003; Wang and Cooper, 2009). Orb2 has two isoforms, which contain a glutamine-rich domain (Q-domain), present also in some but not all CPEBs in other species (Hafer et al., 2011; Si et al., 2003a). Orb2A recruits Orb2B into complexes, essential for memory persistence, through its Q-domain upon neuronal activation. After being recruited into complexes, Orb2B regulates translation through its RNA-binding domain (Kruttner et al., 2012; Majumdar et al., 2012a). A protein network has been recently identified which links neuronal activity and the reactivity of Orb2A (White-Grindley et al., 2014). Together these data suggest that Orb2 and its CPEB homologs are promising candidates to serve as a molecular bridge between memory acquisition and consolidation in a spatio-temporally specific manner upon dopaminergic modulation. However, this hypothesis has not been directly tested in behaving animals.

To investigate the mechanisms of long-term memory consolidation in *Drosophila* we employed a courtship memory consolidation paradigm. Courtship learning can induce either short- or long-lived courtship memories depending on the duration of the learning experience (McBride et al., 1999; Siegel and Hall, 1979). For example, exposing a *Drosophila* male to an unreceptive mated female for 5-7 hours leads to a long-term suppression of his courtship preferentially towards a mated, but not a virgin, female for at least 24 hours. We were able to prevent the expression of this LTM by blocking the aSP13 dopaminergic neurons several hours after their initial involvement during learning

experience with a mated female (Keleman et al., 2012). Further, exposure to a mated female for only 1 hour results in STM but not LTM; however, by stimulating the same class of dopaminergic neurons, aSP13, many hours after this exposure, we were able to transform STM into LTM. Having revealed that LTM consolidation requires late activation of the same class of dopaminergic neuron, aSP13, that hours earlier is necessary for memory acquisition, we explored it further using genetic, molecular and behavioral analyses. We established that the DopR1 type of receptor, shown previously to be necessary for memory acquisition, is also required for late LTM consolidation in the mushroom body (MB) γ neurons. We determined that Orb2A is localized at synapses in the MB neurons and functions during memory acquisition to mark potentially specific MB neurons and specific synapses for eventual LTM consolidation. Upon subsequent late dopaminergic pathway activation, Orb2A recruits Orb2B into complex to regulate translation of CaMKII, a key protein involved in triggering memory persistence (Ashraf et al., 2006; Coultrap and Bayer, 2012).

Results

Dopaminergic stimulation after learning is sufficient to consolidate STM into LTM

We employed a paradigm for courtship memory consolidation to investigate the molecular basis and spatiotemporal relationships between the two key processes of long-term memory formation, memory acquisition and its consolidation, in the *Drosophila* male. We combined a learning experience sufficient to establish courtship STM but not LTM, with a subsequent stimulation of the dopaminergic pathways, which is thought to induce local protein synthesis at synapses (Smith et al., 2005). We starved naïve males for 16 hours, trained them for 1 hour with mated female, and then activated dopamine pathways globally by feeding the animals with dopamine for 23 hours (Riemensperger et al., 2011). This resulted in robust LTM, in the form of a strong suppression of their courtship towards mated females in comparison to virgin females when tested 24 hours later. This memory was quantified as a learning index (LI), which measures the extent of the courtship suppression (Figure 1 and Table S1). By contrast, training for 1 hour alone

induced normal STM but not LTM, and dopaminergic activation alone did not induce LTM. Thus post-acquisition dopamine stimulation consolidates STM into LTM.

Subset of PAM-DA neurons, aSP13, mediates late LTM consolidation in a protein-synthesis-dependent manner

DA neurons are organized in the fly brain into 15 clusters, with the PPL1-DA and PAM-DA cluster innervating the MB lobes, a neuropil consisting of the axonal projections of the intrinsic MB cells called Kenyon Cells (KC) (Mao and Davis, 2009). A subset of the PAM-DA neurons was previously implicated in courtship memory encoding (Keleman et al., 2012). Given that global post-acquisition stimulation of dopamine pathways was sufficient to consolidate short-term courtship memory into a long lasting one, we asked whether delayed activation of PAM-DA neurons might be sufficient for consolidation of courtship STM into LTM. To test whether and when activity of the PAM-DA neurons is sufficient for LTM consolidation we thermo-genetically manipulated their activity by a temperature gated cation channel, *TrpA1* (Rosenzweig et al., 2005). We expressed *UAS-TrpA1* with *HL09-Gal4* (Claridge-Chang et al., 2009) in a large population of DA neurons, including PAM-DA cluster and few neurons of PPL1-DA cluster, in combination with training for STM.

Males expressing TrpA1 were incubated at 32°C for two hours at various time points after one-hour training with a mated female. Flies, which were incubated at 32°C 8-10 hours after training fully consolidated STM into LTM, in contrast to appropriate genetic control flies or flies that were switched to 32°C at other time points, or control flies that remained at 22°C throughout (Figure 2Ai, ii and Table S2). Importantly, this consolidated form of memory was dependent on *de novo* protein synthesis, since feeding the males with the protein synthesis inhibitor cycloheximide prevented the PAM-DA stimulation-induced long lasting memory (Figure 2Aiii and Table S2). Given our previous results showing that PAM-DA neurons are necessary for STM acquisition, these results suggest that delayed activation of the same PAM-DA neurons between 8-10 hours after training is sufficient to consolidate STM into a protein-synthesis-dependent LTM.

The PAM-DA cluster consists of over 100 neurons, a subset of which expresses *fruitless (fru)*, a gene causally linked with multiple aspects of male courtship behavior (Dickson, 2008). The specific *fru*$^{+}$ class of PAM-DA neurons innervating MB γ neuropil, aSP13, was implicated recently in courtship memory encoding (Keleman et al., 2012). To investigate whether activity of the *fru*$^{+}$ set of PAM-DA neurons is sufficient for memory consolidation after learning, we restricted expression of *UAS-TrpA1* to two *fru*$^{+}$ neurons using *TH-Gal4, UAS>stop> TrpA1*, and *fru*^{FLP} (where ">" represents an FRT site, the target of the Flp recombinase) (Yu et al., 2010). Males expressing *TrpA1* in *fru*$^{+}$ *TH-Gal4* neurons displayed suppressed courtship towards mated females at 24 hours post-training when incubated at 32°C between 8-10 hours after learning, indicating that they robustly formed LTM (Figure 2B and Table S3). When we restricted expression of *TrpA1* exclusively to a single class of aSP13 neurons, using *VT5526-LexA, LexAop-TrpA1* (Figure S1) (B. J. Dickson, unpublished) males incubated at 32°C from 8-10 hours post-training fully consolidated LTM in contrast to genetic control under the same conditions, or control flies that remained at 22°C throughout (Figure 2B and Table S3). These results suggest that post-training activation of the dopaminergic neurons, aSP13, is sufficient for consolidation STM into LTM.

To test whether late post-acquisition activity of the aSP13-DA neurons is also necessary for formation of courtship LTM, we performed a complementary set of experiments. We trained males for 7 hours with recently mated females (sufficient to result in LTM) and silenced the same aSP13 neurons with a temperature-sensitive shibire mutant, *LexAop-shi*^{ts} between 8-11 hours after onset of training. Males expressing *shi*^{ts} under the control of *VT5526-LexA* and incubated at 32°C continued to court mated females vigorously thus failing to display LTM at the 24 hours test point. In contrast, genetic control animals incubated at 32°C and males that remained at 22°C displayed normal LTM (Figure 2C and Table S4). These results indicate that activity of the aSP13-DA neurons is required between 8-11 hours after initial learning for LTM consolidation. Thus, delayed activation of specific neural subset is both necessary and sufficient for long-term memory consolidation of courtship learning in *Drosophila*.

DopR1 is necessary for LTM consolidation

In rodents, dopamine-mediated memory persistence requires the adenylyl cyclase stimulatory D1-like type of dopamine receptors (Rossato et al., 2009). There are four dopamine receptors in *Drosophila*: two D1-like dopamine receptors, DopR1 and DopR2 (Hearn et al., 2002; Kim et al., 2003), one D2-like type receptor, DD2R (Draper et al., 2007) and recently identified DopEcR (Srivastava et al., 2005). To determine which dopamine receptor has a role in long-term courtship memory consolidation we focused our analysis on DopR1 and DopR2 since DD2R is not expressed in the MB and DopEcR does not act as a dopamine receptor in courtship suppression learning (Ishimoto et al., 2009). We tested null mutants for either type of receptor, *DopR1*^{attp} and *DopR2*^{attp} in LTM and memory consolidation paradigms (Keleman et al., 2012).

Males lacking *DopR1* were unable to form long-term courtship memory after 7 hours of training. In contrast, *DopR2* mutants displayed normal LTM (Figure 3A, and Table S5). To confirm that LTM deficit in the *DopR1* mutants is indeed due to loss of *DopR1* function, we analyzed *DopR1*^{res} flies where the deleted genomic region was reintegrated by site-specific transgenesis (Keleman et al., 2012). These flies performed just as well as wild type animals suggesting that DopR1 but not DopR2 is required for LTM (Figure 3A, and Table S5). However, given that DopR1 receptor is required for acquisition of the courtship STM (Keleman et al., 2012)**,** the impairment of LTM in *DopR1* mutants might be due to its involvement in this early phase of memory formation: thus these results alone do not prove DopR1's role in memory consolidation. To address explicitly the requirement of DopR1 in LTM after memory acquisition we fed wild type flies and *DopR2*^{attp} mutants (lacking DopR2 but not DopR1) after seven hour training for LTM with the antagonist (SCH23390) specific for both receptors (Gotzes et al., 1994). These flies did not form LTM in contrast to animals that were not fed with the antagonist, suggesting that DopR1 has a post-acquisition role in LTM (Figure 3A, and Table S5).

To test both dopamine receptors in memory consolidation paradigm we tested *DopR1* and *DopR2* mutants for courtship suppression after being trained for STM in combination

with dopamine feeding. Mutants for *DopR1* were unable to consolidate LTM, while the *DopR2* mutants performed equally well as the wild type animals (Figure 3B and Table S6). To dissociate the role of DopR1 in memory acquisition and memory consolidation we fed wild type males and *DopR2*^{attp} mutants with the SCH23390 in addition to dopamine. These males, in contrast to animals fed only with dopamine did not suppress their courtship towards mated females during test thus failing to display long-term courtship memory (Figure 3B and Table S6). These results indicate that DopR1 has a role in the consolidation of STM into LTM upon post-acquisition dopamine stimulation.

DopR1 is required in the MB γ neurons for short-term courtship memory encoding (Keleman et al., 2012). To investigate in which MB neurons DopR1 is required for memory persistence, we expressed *UAS-DopR1* transgene in the *DopR1* mutant background using MB lobe-specific Gal4s (Keleman et al., 2012) and tested them for LTM. Memory was fully rescued when DopR1 was provided back in the γ but not α, β and α', β' MB neurons indicating MB γ neurons as a likely site of LTM consolidation (Figure 3C and Table S7).

Orb2 mediates LTM consolidation downstream of DopR1

Feeding animals with dopamine activates dopaminergic pathways globally, which leads to formation of Orb2 protein complexes consisting of Orb2A and Orb2B. The Orb2 complex correlates strictly with the ability of males to form courtship LTM (Kruttner et al., 2012; Majumdar et al., 2012a). To test whether Orb2 complexes are required for dopamine mediated memory consolidation we used an endogenously modified *orb2* mutant allele. This mutant lacks the Q-domain (*orb2*$^{\Delta Q}$) and has been previously shown to be dispensable for STM but critical for both Orb2 complex formation and the maintenance of the courtship memory after 6 hours (Keleman et al., 2007; Kruttner et al., 2012). *orb2*$^{\Delta Q}$ mutants were unable to consolidate STM into a memory lasting 24 hours upon feeding with dopamine, in comparison to the animals bearing the wild type allele (*orb2*$^{+}$) (Figure 4A and Table S8).

To investigate which type of dopamine receptor is mediating the Orb2 complex formation and hence LTM consolidation, we examined whether the propensity of Orb2 to form complexes depends on either DopR1 or DopR2 receptor. We investigated the endogenous Orb2 protein tagged with the GFP tag ($Orb2^{GFP}$) in immunoprecipitates from brains of the *DopR1* or *DopR2* mutant flies upon stimulation with dopamine. As predicted, $Orb2^{GFP}$ complex was not detected in brain extracts from the animals that were not fed with dopamine (both the wild type and mutants). In contrast, $Orb2^{GFP}$ complex was detected in brain extracts from the animals fed with dopamine, but not in *DopR1* mutants. Although levels of the Orb2 protein were lower in both mutants in comparison to the wild type animals, particularly in the animals lacking *DopR2,* the propensity to form Orb2 complexes seems not to be affected (Figure 4B, C and Table S9). These results suggest that DopR1 functions upstream of Orb2 complex formation and hence memory consolidation.

Orb2A is localized mainly to synapses in MB neurons

Light microscopy studies using antibodies against the GFP tag fused to the endogenous Orb2 protein determined that Orb2A and Orb2B isoforms are localized in the nervous system in distinct patterns. Orb2B appears to be widely distributed throughout various regions of the nervous system, including the lobes, calyces, and soma of the MB. In neurons, Orb2B is expressed very broadly, including in ribonucleoprotein transport granules (RNPs) (Kruttner et al., 2012; Majumdar et al., 2012a). In contrast, endogenous Orb2A was expressed at levels undetectable by confocal microscopy. When expressed using GFP-tagged genomic transgene rescue, Orb2A was detected at very low levels (Majumdar et al., 2012b). Consistent with the genetic data that revealed a functional requirement for Orb2A in LTM, we hypothesized that Orb2A is expressed in the adult brain at very low levels or/and in very few cells, and only in a specific cellular compartment, at the synapses, and therefore undetectable by light microscopy.

Using immuno-electron microscopy against the GFP tag on the endogenous Orb2A and Orb2B proteins encoded by $orb^{Orb2AGFP}$ and $orb^{Orb2BGFP}$ respectively, we determined their

subcellular localization (Kruttner et al., 2012). We examined KC somata and the output region of the MB, tip of the γ lobe, innervated by the aSP13 neurons in the brains of viable heterozygous *orb*^{Orb2AGFP} and *orb*^{Orb2BGFP} flies. We detected Orb2A protein present in a pattern distinct to that of the Orb2B protein. Orb2B is broadly expressed in the KC cell bodies and axons of the γ neurons, including synapses, while Orb2A is excluded from the neuronal cell bodies and is almost exclusively present in synapses of the MB γ lobe (Figure 5A). These results imply that Orb2 isoforms, which function in LTM through distinct mechanisms (Kruttner et al., 2012) likely play distinct roles in LTM.

Orb2A is required during memory acquisition and Orb2B during memory consolidation

To investigate the temporal requirement of Orb2 isoforms in LTM we manipulated in a temporal manner expression of either Orb2 isoform using TARGET expression system. TARGET uses ubiquitous expression of *Gal80*^{ts} to conditionally suppress a Gal4-driven transgene; at 18°C, Gal80 inhibits Gal4 activity and expression of the transgene, but at 27°C *Gal80*^{ts} is inactive and the transgene is expressed (McGuire et al., 2004). Since it is thought that Orb2 functions in the MB γ neurons for LTM (Keleman et al., 2007) we used TARGET in combination with *MB247-Gal4*, which drives expression in the MB γ and αβ neurons as this genetic combination resulted in the healthy progeny able to perform courtship learning assays.

To examine temporal requirement of Orb2A and its Q-domain we expressed in the MB neurons either wild type Orb2A (*UAS-orb2AGFP*) or Orb2A with the Q-domain deleted (*UAS-orb2ΔQGFP*) under control of *MB247-Gal4* and temperature gated *tub-Gal80*^{ts}, in the flies lacking the A isoform (*orb2B*$^{\Delta Q}$) and hence unable to form Orb2 complexes and LTM. We performed the IP and WB against the GFP tag to analyze the on/off kinetics of Orb2 protein expression using TARGET system. Orb2 protein is expressed at high levels within 3-4 hours after temperature shift to 27°C and is not detectable at the time of memory retrieval (Figure S2). Flies had fully rescued LTM both when kept at 27°C throughout adulthood and when shifted to 27°C for the duration of the training (Figure

5B and Table S10), and only when a wild type but not the Q-domain deleted Orb2A was present. These results suggested that the Orb2A isoform and its Q-domain are required in the MB neurons during memory acquisition for LTM formation.

Because of the role Orb2B plays during development, animals lacking this isoform do not survive to adulthood, thus we could not directly assess its temporal requirement in LTM. Therefore, we employed mutant flies in which the RNA-binding domain of Orb2B was substituted with the RNA-binding domain of the mouse homolog mCPEB2 (*orb2*^{mCPEB2RBD}) (Kruttner et al., 2012). These viable (but unable to form LTM) mutant flies allowed us to assess the temporal requirement of Orb2B and its RNA-binding domain in memory independently of its role during development. Flies expressing wild type Orb2B (*UAS-orb2BRBD)* had normal LTM when kept at 27°C throughout adulthood and when shifted to 27°C ~ 2 hours before the end of training. They did not form LTM when kept at 27°C during training only and when switched to 27°C right after training. Importantly, this memory was dependent on the RNA-binding domain because the males with the RNA-binding domain mutated (*UAS-orb2BRBD**) could not form LTM in any condition (Figure 5B and Table S10). Therefore, we conclude that presence of Orb2B is dispensable during the training and shortly after but is necessary continuously about 2 hours after training. Together these results suggest that the Orb2A isoform, which is localized to MB synapses, is necessary during memory acquisition, whereas the Orb2B isoform (recruited into complexes with Orb2A) is necessary during LTM consolidation.

Orb2 regulates translation of CamKII in MB neurons

Dopamine regulates the expression of proteins essential for long-lasting memories (Berke et al., 1998), such as Calcium/Calmodulin dependent kinase (CaMKII). CaMKII translation at synapses is dependent on neuronal activity both in mouse and *Drosophila* (Ashraf et al., 2006; Coultrap and Bayer, 2012) and is conferred by its 3'UTR, which is recognized by CPEB proteins in mouse (Wu et al., 1998). *Drosophila* CaMKII has been identified as an Orb2 mRNA target (our unpublished results) and Orb2 regulates its translation by binding to the specific sequence in the 3'UTR (Figure 6A and Table S11).

Given that CaMKII is a key molecule implicated in memory persistence and Orb2 regulates translation of CaMKII, we investigated whether Orb2 might function in memory consolidation by regulating synthesis of CaMKII. We expressed a fluorescent reporter of CaMKII translation, CaMKII 3'UTR appended to the EYFP coding region (*UAS-EYFP*$^{3'UTR}$), in the α, β, γ MB neurons using *MB247-Gal4* (Ashraf et al., 2006). We monitored the change in intensity of the EYFP signal in the MB γ neurons upon neuronal stimulation with dopamine in comparison to unstimulated control brains.

We observed a striking increase of the EYFP signal after stimulation with dopamine. The EYFP signal was highest at 6 and 12 hours post-dopamine stimulation in comparison to unstimulated control brains at baseline. Importantly, we did not observe a dopamine-induced EYFP increase in an Orb2 mutant lacking the Orb2A isoform (*orb2*$^{\Delta A}$) (Figure 6B, C and Table S12), whose Q-domain is required for Orb2 complex formation (Kruttner et al., 2012). Thus, these results suggest that Orb2 complexes induced upon dopamine stimulation regulate translation of CaMKII and possibly other molecules essential for synaptic plasticity.

Discussion

Our results demonstrate that the process of LTM consolidation in *Drosophila* requires activation of the same neural pathway that, hours earlier, is required for memory acquisition. Specifically, we identified a subset of PAM-DA neurons (aSP13) whose activation mediates both memory acquisition and late memory consolidation. This permitted us to examine how memory is maintained during the interval between memory acquisition and memory consolidation. First, we established that aSP13 neurons mediate both memory acquisition and memory consolidation through the activation of the DopR1 type of receptor and through *Drosophila* CPEB, Orb2, in the MB γ neurons. Then, we determined that the Orb2A isoform is localized mainly to synapses in the MB neurons and is required during memory acquisition, tagging the relevant neurons and potentially their synapses for subsequent memory consolidation, while Orb2B, recruited into

complexes with Orb2A, is required during memory consolidation. Finally, we show that together they regulate the activity-dependent synthesis of CaMKII, a key protein involved in the molecular basis of memory persistence (Coultrap and Bayer, 2012; Lucchesi et al., 2011; Redondo et al., 2010).

Delayed post-learning reactivation of neural pathways has been shown to exist in vertebrates (Buzsaki, 1998; Foster and Wilson, 2006; Wilson and McNaughton, 1994). Spontaneous neuronal replay after learning occurs both in the awake and sleeping states (Carr et al., 2011; Wilson and McNaughton, 1994) but the causal link between replay and memory consolidation has not been firmly established. Interfering with sharp wave ripples (SWRs), which are temporally correlated with neuronal replay in awake animals (Buzsaki et al., 1992), impaired spatial memory formation in rats (Jadhav et al., 2012). These results suggested that replay might mediate memory consolidation, however they could not rule out that other effects of SWRs may be critical for memory consolidation. Our results that courtship memory acquisition and consolidation in *Drosophila* are mediated by activation of the same neuronal pathway provides further evidence that reactivation might play a key role in memory consolidation. An emerging view is that multiple types of neural signals are involved in memory formation, including neural representations of the specific content to be stored, along with signals pertaining to the importance or valence of an event, which may influence whether the content becomes consolidated. The aSP13 pathway implicated in the present work may be of the latter type, given that it is a neuromodulatory pathway. Prior work in rodents has implicated both types of signals in memory formation: for example, SWRs are thought to carry the content of a spatial trajectory (Carr et al., 2011) while the neuromodulatory pathways are thought to convey the salience of the content (Musso et al., 2015; Waddell, 2010).

There are emerging clues regarding the molecular bases underlying delayed neuronal reactivation-dependent memory consolidation. Recently, a requirement of NMDA receptor reactivation for memory consolidation has been explored in rodents (Wang et al., 2006). This led to the formulation of the synaptic reentry reinforcement hypothesis (SRR), which posits that memory consolidation requires delayed reactivation of the

NMDA receptor to convert STM into LTM. Interestingly, one of the signaling molecules downstream of NMDA receptor is CamKII, which is believed to be responsible for potentiating the synapses involved in learning (Nicoll and Malenka, 1999). Our results are consistent with the SRR, but involving DopR1 instead of NMDAR, as we found that the *Drosophila* DopR1, which is required during memory acquisition (Keleman et al., 2012) seems to be also necessary for late memory consolidation. This is consistent with a recent finding that DopR1 expression in the MB γ neurons is sufficient to fully support both short- and consolidated long-term memory in *Drosophila* (Qin et al., 2012). If the SRR hypothesis is correct (i.e., synaptic reentry leads to reinforcement), what molecular change signifies that an "initial entry" has previously occurred in a synapse, such that another synaptic event counts as "re-entry"?

The synaptic tagging and capture hypothesis has provided a conceptual framework for how relevant information might be stored in the intervening time period between memory acquisition and memory consolidation and how specific synapses eventually come to store a given memory. The ability to temporally dissociate memory acquisition and its consolidation in the courtship memory consolidation paradigm allowed us to investigate the molecular basis of this hypothesis in learning *Drosophila*. Our results that the synaptically-localized Orb2A isoform and its Q-domain are required during memory acquisition in MB neurons for subsequent LTM consolidation support the likelihood that synaptic Orb2A might act to tag the specific synapses for later memory consolidation. At present we cannot distinguish between the possibilities that this tagging is an effect of a synapse-specific post-translational modification of Orb2A or its mere presence at a synapse (White-Grindley et al., 2014). Thus during memory acquisition, Orb2A or a modification thereof, might mark activated synapses as potential sites for subsequent memory storage. Only in those synapses where the delayed activation occurred, would Orb2A recruit Orb2B (and possibly its associated mRNAs) (Kruttner et al., 2012) into translationally active protein complexes (Si et al., 2003b) to regulate synthesis of proteins essential for the LTM, such as CaMKII.

In this work we provide evidence that the late activation of the same neuronal and molecular pathways that are necessary and sufficient for early memory acquisition, are also necessary and sufficient for late memory consolidation in *Drosophila*. These findings confirm principles that were strongly implied by work in mammals (Carr et al., 2011; Wang et al., 2006; Wilson and McNaughton, 1994), and extend this paradigm to invertebrates. Taking advantage of the tools available for the molecular and circuit analysis in *Drosophila* we provide a functional link between occurrence of the delayed neural pathway activation and memory consolidation and start to identify the molecular and circuit mechanisms underlying this consolidation. The occurrence of these phenomena in evolutionarily distinct species implies that delayed activation might serve a key algorithmic role in adaptive learning. Moreover, a high degree of conservation of the involved molecules (Theis et al., 2003) suggests that the molecular mechanisms uncovered in flies might be broadly utilized in the animal kingdom.

Experimental Procedures

Fly stocks

Flies were maintained on conventional cornmeal-agar medium under a 12-h light/dark cycle at 25°C and 60% relative humidity. The Canton-S strain was used as the wild type *D. melanogaster* flies. *HL09-Gal4, TH-Gal4, c739-Gal4, c305-Gal4, Y201-Gal4, MB247-Gal4, UAS-Trp, UAS-DopR1, DopR1^{attp}, DopR2^{attp}, DopR1^{res}* (Keleman et al., 2007; Keleman et al., 2012). *Orb2^{DQ}*, *Orb2^{+}*, *Orb2^{mCPEBRBD}* (Kruttner et al., 2012). *VT5526-LexA, LexAop-Trp* (B.J.Dickson unpublished). *UAS-EYFP-CaMKII3'UTR* (Ashraf et al., 2006). All mutant flies were backcrossed to the Canton-S for four generations before being used for behavioral assays.

Behavioral Assays

Behavioral assays were executed at variable phases of the circadian clock of the flies. Courtship conditioning assays were carried out as described previously (Keleman et al., 2007; Siwicki and Ladewski, 2003). Details can be found in SI.

Memory consolidation assay by dopamine feeding was performed as follows. Freshly hatched males were collected and aged individually in food chambers as for courtship conditioning. Prior to training flies were starved on a wet filter paper for 16 hrs. After short-term memory training (1 hr) at indicated time points flies were transferred to chambers containing filter paper soaked with 80 ul of 2% sucrose solution supplemented with either dopamine, cyclohexamide or SCH23390 as indicated (concentrations used: 20mM dopamine, 35mM cyclohexamide, 1uM SCH23390).

Memory consolidation assay by thermogenetic activation with TrpA1 was performed as follows. Freshly hatched males were collected and aged individually in food chambers at 22°C for 6-7 days. First, they were trained for 1hr at 22°C and shifted to 32°C at indicated time points for 2 hrs and thereafter placed at 22°C until the test at 25°C (24 hrs after training).

For silencing with Shi^{ts} males were collected and aged as described above. They were trained for 7 hrs at 22°C and shifted to 32°C at indicated time points and thereafter placed at 22°C until the test at 25°C (24 hrs after training).

TARGET experiments were conducted as described (McGuire et al., 2003). For the experiment all flies were raised and kept at 18°C and shifted to 27°C at indicated time intervals. Test was performed at 25°C. Genotypes of the experimental flies: *w+*; *tub-GAL80*^{ts}, *UAS-orb2A (UAS-orb2A*$^{\Delta Q}$); *orb2B*$^{\Delta Q}$,*MB247-Gal4* and *w+*; *tub-GAL80*^{ts}, *UAS-orb2B (UAS-orb2B*)*; *orb2*^{mCPEB2RBD}, *MB247-Gal4*

Immunohistochemistry
Immunohistochemistry on adult brains was performed as described (Yu et al., 2010). Details on antibodies used can be found in the SI.

Immunoprecipitation and Western Blot
IP and WB were carried out as described previously (Kruttner et al., 2012) on adult brains of: *w+;+; orb2*^{GFP}, *DopR1*^{attp} and *w+;+; orb2*^{GFP}, *DopR2*^{attp} to investigate Orb2^{GFP} complex formation. To determine on/off kinetics of Orb2 expression in TARGET experiment, IP and WB were performed on brain extract from *w+;tubGal80, UAS-orb2B;Orb2*^{mCPEBRBD},*MB247-Gal4*. Details can be found in the SI.

Immuno-EM
The heads of heterozygous viable *orb2*^{Orb2AGFP} and *orb2*^{Orb2BGFP} 6-7 days old flies were fixed in 4% paraformalde, 0.1% glutaraldehyde, 0.07M phosphate buffer pH 7.3 for 3 hrs at 40°C and prepared for immuno-EM as described (Kruttner et al., 2012). Details can be found in the SI.

Statistical Analysis
LIs were calculated using a custom MATLAB script based on the algorithm described in (Kamyshev et al., 1999) and implemented in (Keleman et al., 2007). Briefly, the entire set of courtship indices for both the naive and trained flies were pooled and then randomly assorted into simulated naive and trained sets of the same size as in the original data. A LI_p was calculated for each of 100,000 randomly permuted data sets, and p values were estimated as the fraction for which $LI_p > LI$ (to test H_0, LI = 0) or $LI_p > LI - LI_0$ (to test H_0, $LI = LI_0$). p values are for H_0: LI = LI_1 (permutation test) and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for H_0, LI = 0 (permutation test). Figures in the main text show LIs +/-sem calculated using the propagation of error formula and p values calculated from mean CIs; supplementary tables show values derived from both mean ± sd and median CIs.

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Figure Legends

Figure 1. Post-learning global activation of dopamine pathways is sufficient to consolidate STM into LTM.
Learning indices of the wild type *Canton-S* males tested in single pair assays with mated or virgin females, either 24 hrs after being starved for 16 hrs, trained with mated female for 1 hr and fed with dopamine for 23 hrs (if indicated) (LTM) or immediately after training (STM) (light green bar). *P* values are for H_0 LI=0, * P<0.05, *** P<0.001. See Table S1.

Figure 2. Subset of the PAM-DA neurons, aSP13, consolidates STM into LTM in a protein synthesis dependent manner.
A. Post-acquisition activation of the DA neurons mediates protein synthesis dependent memory consolidation.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after being trained for 1 hr with a mated female and warmed at 32°C for 2 hrs at the indicated time points (i), experimental control males, which stayed at 22°C all the time and the genetic control animals, which were warmed at 32°C for 2 hrs between 8-10 hrs after learning (ii). Learning index of the males fed with

the cycloheximide during activation with TrpA1 between 8-10 hrs after 1 hr training (iii). *P* values are for H_0 LI=0, *** P<0.001. See Table S2.
B. Subset of the aSP13-DA neurons is sufficient for the LTM consolidation.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after training for 1 hr with a mated female and being warmed at 32°C (except control males which stayed at 22°C) between 8-10 hrs after training. *P* values are for H_0 LI=0, ** P<0.01, *** P<0.001. See Table S3.
C. Post-acquisition silencing of the aSP13-DA neurons prevents LTM formation.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after training for 7 hrs with a mated female and being warmed at 32°C (except control males which stayed at 22°C) between 8 and 11 hrs after training. *P* values are for H_0 LI=0, *** P<0.001. See Table S4.

Figure 3. DopR1 is necessary in the MB γ neurons for LTM consolidation.
A. Post-acquisition inactivation of DopR1 impairs LTM.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after being trained for 7 hrs with a mated female and fed with SCH23390 for 6 hrs if indicated. *P* values are for H_0 LI=0, *** P<0.001, ** P<0.01. See Table S5.
B. DopR1 is required for dopamine mediated memory consolidation.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after being starved for 16 hrs, trained for 1 hr with a mated female and fed with dopamine and SCH23390 as indicated between training and test. *P* values are for H_0 LI=0, *** P<0.001. Table S6.
C. DopR1 functions in LTM in the MB γ neurons.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after being trained for 7 hrs with a mated female. *P* values are for H_0 LI=0, *** P<0.001. See Table S7.

Figure 4. Orb2 mediates LTM consolidation downstream of DopR1.
A. Orb2 is required for dopamine mediated memory consolidation.
Learning indices (green bars) of males of the indicated genotypes tested in single pair assays with mated females 24 hrs after being starved for 16 hrs, trained with a mated female for 1 hr and fed with dopamine. *P* values are for H_0 LI=0, *** P<0.001. See Table S8.
B. DopR1 mediates Orb2 oligomer formation.
Head extracts from adult flies of the indicated genotypes were analyzed by IP and WB for presence of the $Orb2^{GFP}$ complexes, after being starved for 16 hrs and fed with dopamine as indicated.
C. WB signal (from panel C) corresponding to the Orb2-GFP oligomers has been quantified using Fiji-ImageJ. The values on Y axis represent the mean intensity normalized to the wild type not treated with dopamine (wt - dopamine). See Table S9.

Figure 5. Orb2A is required during memory acquisition and Orb2B during memory consolidation
A. Orb2A is localized in synapses of the MB γ lobe.

Immuno-EM of the *orb2*^{Orb2AGFP} and *orb2*^{Orb2BGFP} heterozygous brains. The sagittal sections of the brain in the region of the KC somata and the tip of the MB γ lobe were analyzed.
(**i**) Orb2A^{GFP} is absent from the neuronal cell bodies (arrow) of the Kenyon cells.
(**ii**) Orb2A^{GFP} labeled by DAB precipitates is present in T-bars (asterisk) and active zones (double asterisk) in the MB γ lobe synapses.
(**iii**) Orb2B^{GFP} labeled by DAB precipitates is detected in the KC cell bodies including T-bars (asterisk) and active zones (double asterisk).
(**iv**) Orb2B^{GFP} labeled by DAB precipitates is present in the MB γ lobe including T-bars (asterisk).
In all panels scale bars are 500 nm.
B. Orb2A and its Q-domain functions during memory encoding while Orb2B and its RNA-binding domain during memory consolidation.
(Upper panel) Schematic of the rescue experiment with depicted domain organization of the proteins involved.
(Lower panel) Learning indices (green bars) of the temporal rescue of the *orb2*$^{Orb2B\Delta Q}$ and *orb2*^{mCPEB2RBD} mutants with either *UAS-orb2A*/*UAS-orb2AΔQ* or *UAS-orb2B*/*UAS-orb2BRBD** under control of *MB247-Gal4* and *tubGal80ts*, trained and tested in single pair assays with mated females for long-term memory and treated according to the regime outlined in the center of the panel. *P* values are for H_0 LI=0, *** P<0.001, ** P<0.01. See Table S10.

Figure 6. Orb2 regulates synthesis of CaMKII in MB neurons.
A. Orb2 regulates translation of CaMKII through its 3'UTR. Translation of the Firefly luciferase (Fluc) reporter tethered to the CaMKII 3'UTR is suppressed by Orb2 but not when tethered to the control 3'UTR, which does not contain Orb2 specific binding sequence. The values on Y axis represent the ratio of the normalized Fluc to Rluc fluorescence in the presence of wt Orb2 to the Fluc/Rluc fluorescence in the presence of Orb2RBD* with RBD mutated. * P<0.05. See Table S11.
B. Representative confocal projections of the MB (lobes) expressing *UAS-EYFP - CaMKII-3'UTR* with the *MB247-Gal4* and stained with the anti GFP antibodies. The adult brains were either unstimulated (left panel) or stimulated (right panel) by feeding with dopamine.
C. *UAS-EYFP-CaMKII-3'UTR* was expressed in the MB neurons with the *MB247-Gal4*. Intensity of fluorescence was measured in the MB γ lobe of the adult brains either wild type (*orb2*$^{+}$) (left panel) or orb2 mutant (*orb2*$^{\Delta A}$) (right panel) stimulated by feeding with dopamine at the indicated time intervals. *P* values are for H_0 ftx=ft0. See Table S12.

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Authors contribution: KK, SK conceived the project and designed the experiments. SK, LT, UD performed the experiments with help of KJ. BS performed CaMKII translation repression assay. NI performed EM analysis. JNN and LGF made an initial observation

of Orb2A localization. KK supervised the project and wrote the manuscript with help of BM.

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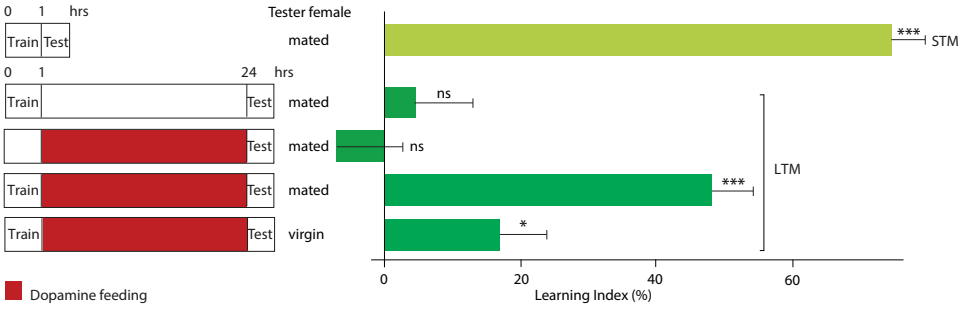

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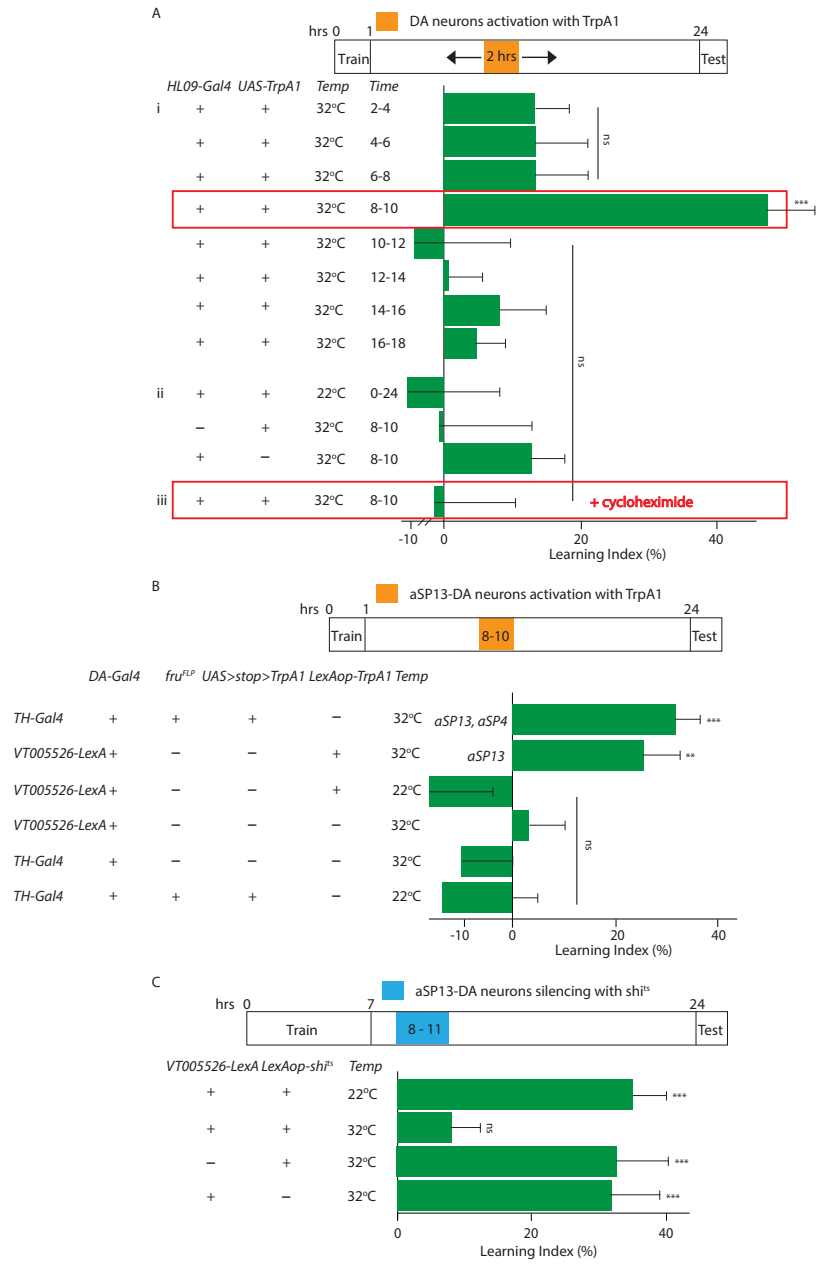

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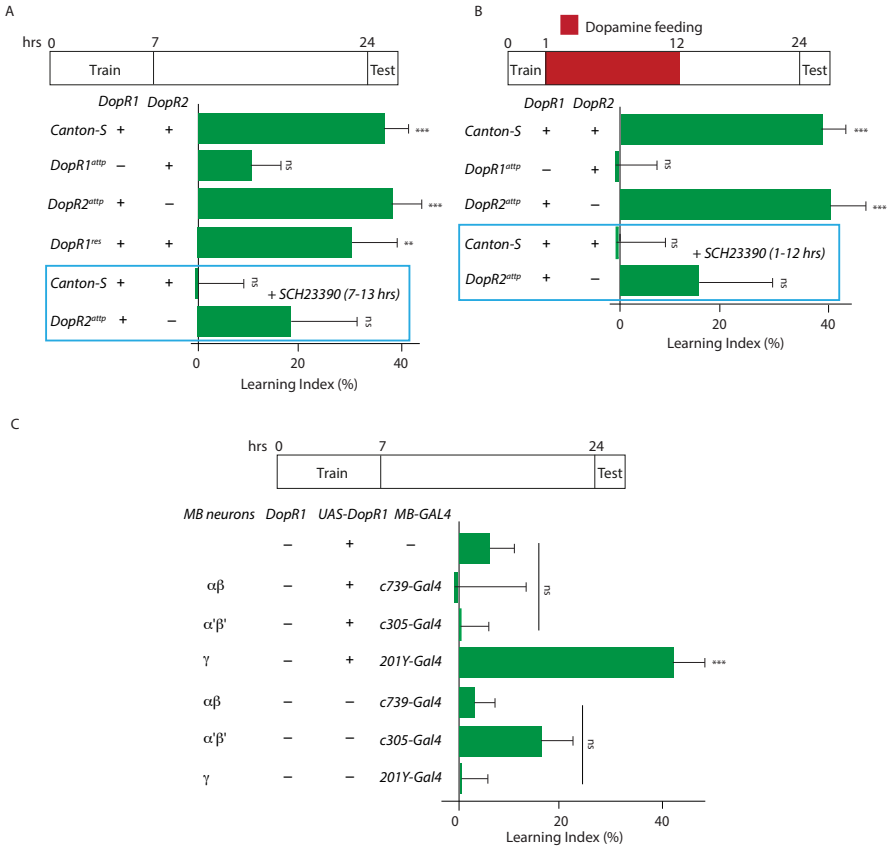

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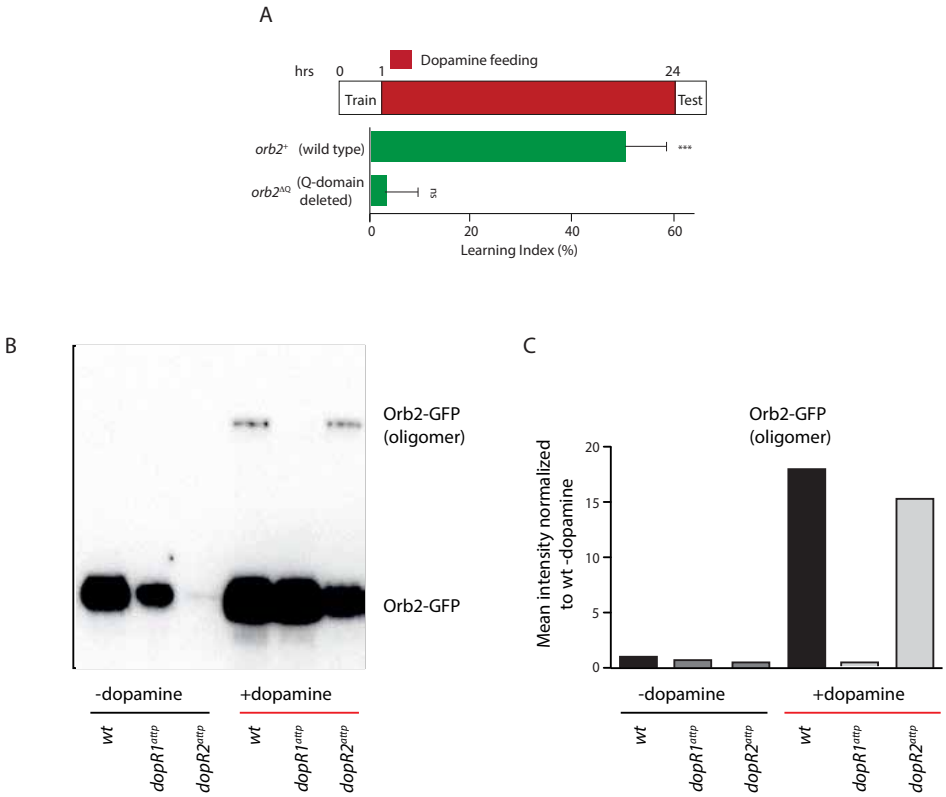

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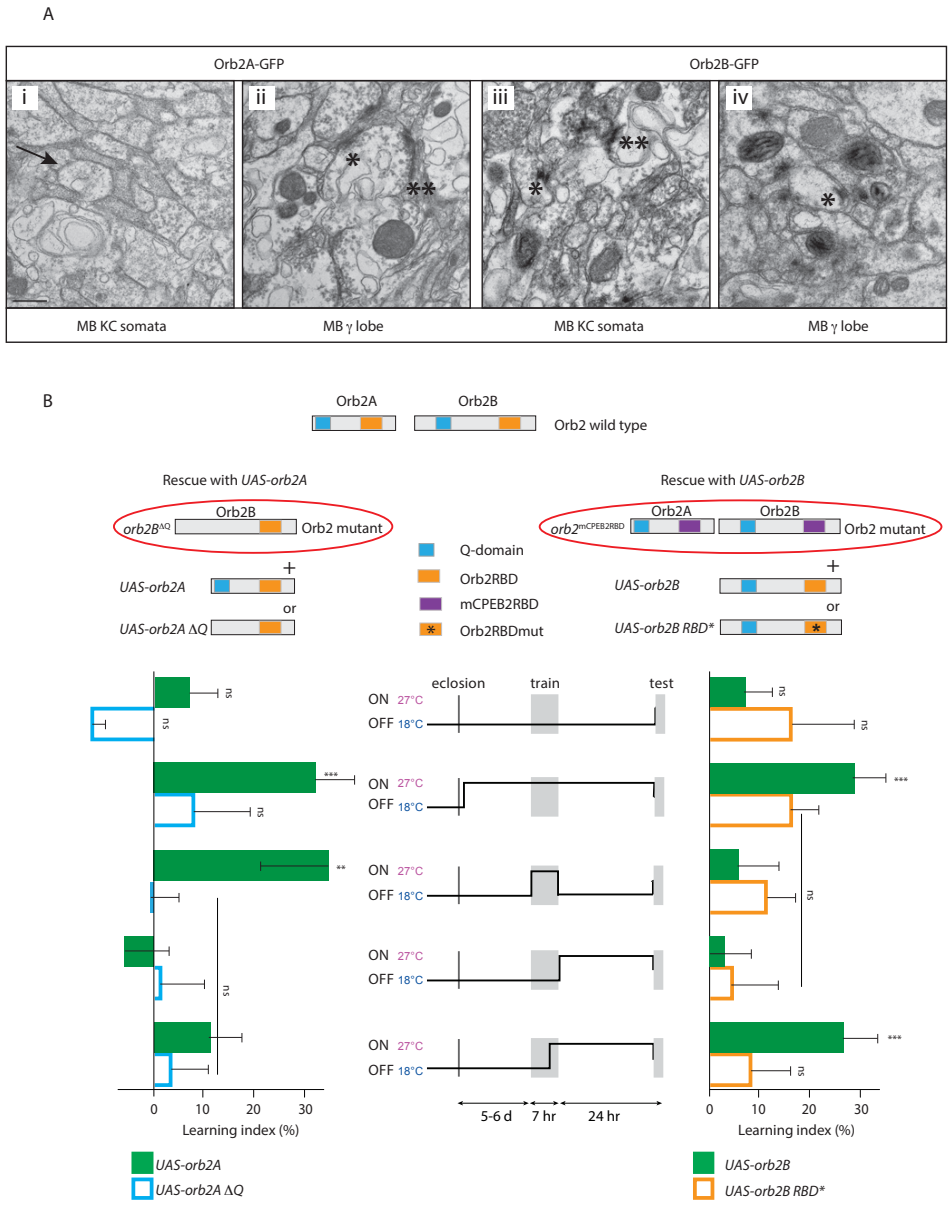

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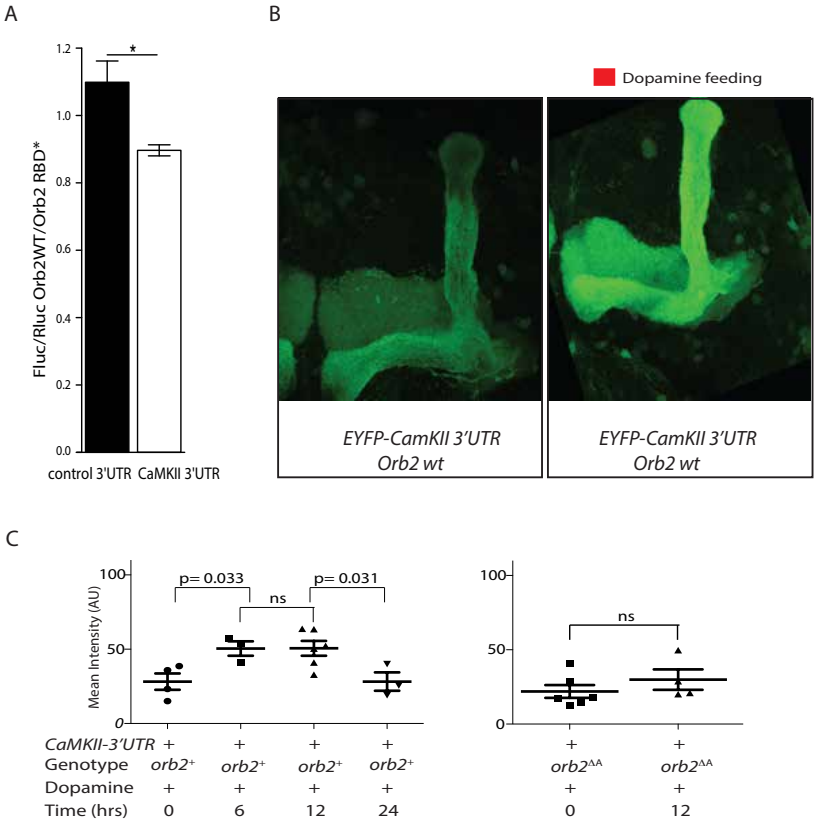
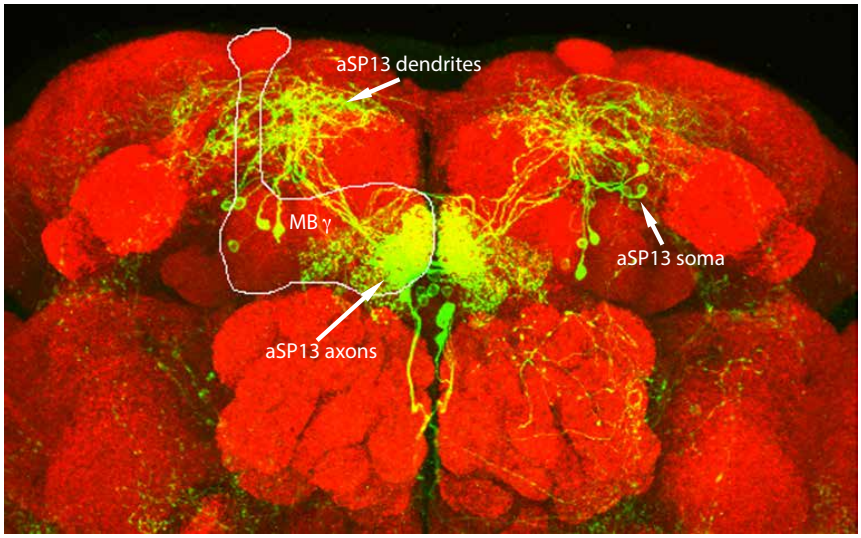


Figure-6 (Kruettner)

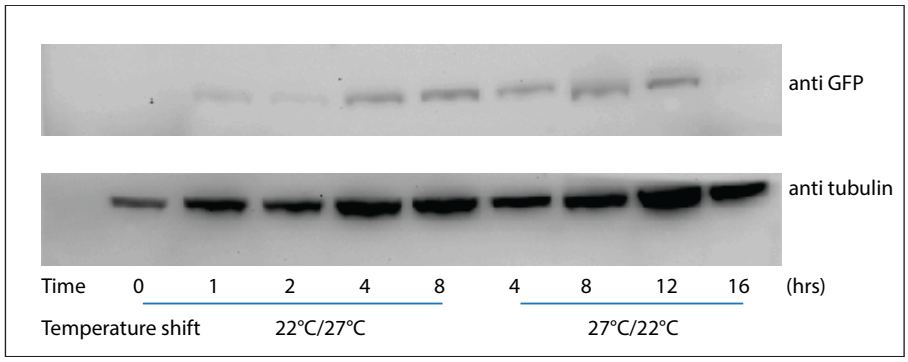
Supplemental Information

Figure S1 VT5526-LexA drives expression exclusively in the aSP13 dopaminergic neurons



Expression pattern of the VT5526-LexA line used for activation experiment with TrpA1 illustrated in the Figure 2C. There are typically 2-6 aSP13 neurons per hemisphere. The presynaptic termini of aSP13 neurons (axons) are located at the tip to the MB γ lobe. The postsynaptic termini (dendrites) are located in the medial protocerebrum.

Figure S2 On/Off kinetics of Orb2 in TARGET experiment



Head extracts from *w+;tubGal80ts, UAS-orb2BGFP;orb2*^{mCPEBRBD} adult flies were analyzed by IP and WB with Abs against the GFP tag at indicated time points after temperature shift either from 22°C to 27°C or back to 22°C after 7 hour induction at 27°C.

Table S1 Post-learning global activation of dopamine pathways

	Genotype	Test	DA	Train	*n*	CI (%) mean±sd median	10%-ile 90%-ile	LI (%) mean±sem median	*P* *LI=0*	*P* $LI_n=LI_4$
1	*Canton-S*	20'	-	-	57	52.3±26.3 50.0	15.00 90.00			
2	*Canton-S*	20'	-	+	57	13.9±18.9 5.0	0.00 31.00	73.5±5.0 90.0	0.000 0.000	0.000 0.000
3	*Canton-S*	24hrs	-	-	54	53.4±25.4 60.0	12.50 85.00			
4	*Canton-S*	24hrs	-	+	61	51.1±25.3 55.0	15.00 85.00	4.4±8.7 8.3	0.319 0.473	
5	*Canton-S*	24hrs	-	-	54	53.4±25.4 60.0	12.50 85.00			
6	*Canton-S*	24hrs	+	-	66	57.1±29.7 65.0	13.50 91.50	-7.1±9.7 -8.3	0.770 0.878	0.386 0.486
7	*Canton-S*	24hrs	+	-	66	57.2±29.7 65.0	13.50 91.50			
8	*Canton-S*	24hrs	+	+	67	30.1±24.8 25.0	0.00 66.00	47.3±6.3 61.5	0.000 0.000	0.000 0.002
9	*Canton-S*	24hrs (w/ virgin)	+	-	45	79.3±28.4 90.0	31.00 100			
10	*Canton-S*)	24hrs (w/ virgin)	+	+	57	66.1±32.5 75.00	5.00 100	16.6±7.0 16.7	0.017 0.018	0.304 0.512

Courtship indices of the *n* Canton-S males fed with sucrose only (DA-) or supplemented with dopamine (DA+) after being trained with a mated female for 1 hr (Train +) or staying alone (Train-) as indicated in Fig. 1, and tested in single-pair assays with mated or virgin females when indicated. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S2 Post-learning thermogenetic activation of PAM-DA neurons

	Genotype	Time at 32°C	Train	*n*	CI (%) mean±sd median	10%-ile 90%-ile	LI (%) mean±sem median	*P* *LI=0*	*P* $LI_n=LI_{18}$
1	*HL09-Gal4, TrpA*, 32°C	2-4	-	34	82.8±29.5 100.0	20.00 100			
2	*HL09-Gal4, TrpA*, 32°C	2-4	+	36	71.6±34.3 90.0	8.50 100	13.5±5.0 10.0	0.067 0.131	0.091 0.028
3	*HL09-Gal4, TrpA*, 32°C	4-6	-	34	82.5±27.5 95.0	29.00 100			
4	*HL09-Gal4, TrpA*, 32°C	4-6	+	36	71.4±30.1 75.0	25.00 100	13.5±8.0 21.05	0.052 0.064	0.073 0.028
5	*HL09-Gal4, TrpA*, 32°C	6-8	-	36	76.0±24.0 80.0	33.50 100			
6	*HL09-Gal4, TrpA*, 32°C	6-8	+	34	65.7±30.2 70.0	25.00 100	13.5±8.2 12.5	0.061 0.244	0.075 0.120
7	*HL09-Gal4, TrpA*, 32°C	8-10	-	35	83.1±21.9 95.0	52.50 100			
8	*HL09-Gal4, TrpA*, 32°C	8-10	+	36	44.2±33.5 40.0	5.00 97.00	46.9±7.2 57.9	0.000 0.000	0.000 0.000
9	*HL09-Gal4, TrpA*, 32°C	10-12	-	34	58.5±29.6 55.0	22.50 100			
10	*HL09-Gal4, TrpA*, 32°C	10-12	+	34	64.7±36.0 80.0	7.50 100	-10.6±14.2 -45.4	0.772 0.937	0.922 0.680
11	*HL09-Gal4, TrpA*, 32°C	12-14	-	36	59.3±27.9	23.50			

					60.00	96.50			
12	*HL09-Gal4, TrpA*, 32˚C	12-14	+	36	59.0±33.2 55.00	13.50 100	0.7±5.0 8.3	0.485 0.434	0.478 0.491
13	*HL09-Gal4, TrpA*, 32˚C	14-16	-	33	87.9±20.5 95.00	67.00 100			
14	*HL09-Gal4, TrpA*, 32˚C	14-16	+	36	80.7±30.4 95.0	15.50 100	8.2±6.9 0.0	0.134 0.964	0.141 0.009
15	*HL09-Gal4, TrpA*, 32˚C	16-18	-	33	92.6±13.1 95.0	77.00 100			
16	*HL09-Gal4, TrpA*, 32˚C	16-18	+	36	87.9±22.2 95.0	61.00 100	5.0±4.6 0.0	0.149 0.970	0.166 0.001
17	*HL09-Gal4, TrpA*, 22˚C	-	-	30	66.7±34.7 75.0	5.50 100			
18	*HL09-Gal4, TrpA*, 22˚C	-	+	34	75.0±33.2 95.0	10.00 100	-12.5±13.7 -26.6	0.835 0.877	
19	*UAS-TRP*/+, 32˚C	8-10	-	35	54.9±33.1 55.0	10.00 95.00			
20	*UAS-TRP*/+, 32˚C	8-10	+	34	55.0±30.2 55.0	10.00 92.50	-0.2±13.9 0.0	0.511 0.636	0.533 0.572
21	*HL09-Gal4*/+, 32˚C	8-10	-	35	50.9±29.9 40.0	15.00 100			
22	*HL09-Gal4*/+, 32˚C	8-10	+	35	44.3±27.6 35.0	15.00 92.00	12.9±5.0 12.5	0.169 0.242	0.208 0.433
23	*HL09-Gal4, TrpA*, 32˚C +PSI	8-10	-	36	63.9±34.4 70.00	15.00 100			
24	*HL09-Gal4, TrpA*, 32˚C +PSI	8-10	+	34	66.8±29.1 70.0	17.50 100	-4.5±12.2 0.0	0.642 0.657	0.665 0.573

Courtship indices of males of the indicated genotypes, retained at 22˚C or warmed to 32˚C for 2 hrs at the time points according to Fig. 2A after either 1 hour training with a mated female (Train+) or staying alone (Train-) as indicated above and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S3 Post-learning thermogenetic activation of aSP13-DA neurons

	Genotype	**Time at 32˚C**	**Train**	**n**	**CI (%)** mean±sd median	**10%-ile** **90%-ile**	**LI (%)** mean±sem median	**P LI=0**	**P LI_n=LI_6**
1	*TH-Gal4,FF,TrpA*, 32˚C	8-10	-	36	67.4±27.2 77.5	20.00 96.50			
2	*TH-Gal4,FF,TrpA*, 32˚C	8-10	+	36	45.8±28.1 40.0	8.50 90.00	32.0±5.0 46.7	0.001 0.006	0.000 0.097
3	*VT5526-LexA,LexAopTRPA1*, 32˚C	8-10	-	28	79.8±18.4 85.0	48.00 100			
4	*VT5526-LexA, LexAopTRPA*, 32˚C	8-10	+	34	60.2±35.0 50.00	7.50 100	24.6±7.6 41.2	0.005 0.001	0.006 0.003
5	*VT5526-LexA, LexAopTRPA1, 22˚C*	-	-	34	60.9±33.9 65.0	12.50 100			
6	*VT5526-LexA, LexAopTRPA1, 22˚C*	-	+	34	71.0±25.1 77.5	27.50 100	-16.7±13.2 -25.0	0.915 0.862	
7	*VT5526-LexA/+,32˚C*	8-10	-	17	82.9±13.4 85.0	64.00 105			
8	*VT5526-LexA/+,32˚C*	8-10	+	16	80.3±20.1 87.5	46.00 100	3.2±7.1 0.0	0.356 0.891	0.272 0.269
9	*TH-Gal4*, 32˚C	8-10	-	36	55.0±23.1 45.0	30.00 95.00			
10	*TH-Gal4*, 32˚C	8-10	+	36	60.6±22.6 57.5	25.00 90.00	-10.1±10.3 -22.2	0.857 0.906	0.771 0.407

11	*TH-Gal4,FF,TrpA,22°C*	-	-	33	63.9±25.1 75.0	25.00 95.00			
12	*TH-Gal4,FF,TrpA,22°C*	-	+	29	73.1±19.1 75.0	45.00 100	-14.3±5.0 0.0	0.944 0.769	

Courtship indices of males of the indicated genotypes, retained at 22°C or warmed to 32°C for 2 hrs according to Fig. 2B after either 1 hour training with a mated female (Train+) or staying alone (Train-) as indicated above and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S4 Post-learning thermogenetic silencing of aSP13-DA neurons

	Genotype	Train	*n*	CI (%) mean±sd median	10%-ile 90%-ile	LI (%) mean±sem median	*P* *LI=0*	*P* $LI_n=LI_6$
1	*5526LexA, LexAop-shi^{ts}*, 22°C	-	33	88.3±18.0 95.0	62.00 100			
2	*5526LexA, LexAop-shi^{ts}*, 22°C	+	36	58.2±26.5 62.5	12.00 91.50	34.1±5.0 36.8	0.000 0.000	
3	*5526LexA, LexAop-shi^{ts}*, 32°C	-	36	92.5±18.5 100	76.00 100			
4	*5526LexA, LexAop-shi^{ts}*, 32°C	+	33	85.0±17.5 90.0	60.00 100	8.1±4.5 10.0	0.052 0.023	0.001 0.001
5	*LexAop-shi^{ts}*, 32°C	-	35	68.7±25.3 75.0	33.00 95.00			
6	*LexAop-shi^{ts}*, 32°C	+	33	46.7±26.9 45.0	5.00 85.00	32.1±8.0 40.0	0.001 0.001	0.831 0.883
7	*5526LexA*, 32°C	-	29	60.9±17.5 65.0	35.00 85.00			
8	*5526LexA*, 32°C	+	33	41.9±23.4 45.0	4.00 73.00	31.1±7.6 30.8	0.000 0.009	0.767 0.718

Courtship indices of males of the indicated genotypes, retained at 22°C or warmed to 32°C according to Fig. 2C after 7 hour training with a mated female (Train+) or staying alone (Train-) as indicated above and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S5 Post-acquisition inactivation of DopR1 after training for LTM

	Genotype	Train	n	CI (%) mean±sd median	10%-ile 90%-ile	LI (%) mean±sem median	P LI=0	P $LI_n=LI_2$
1	*Canton-S*	-	59	78.7±21.5 85.0	45.00 100			
2	*Canton-S*	+	60	49.8±30.8 45.0	10.00 100	36.8±5.0 47.1	0.000 0.000	
3	*DopR1^{attp}*	-	54	78.7±22.8 85.0	42.50 100			
4	*DopR1 ^{attp}*	+	51	70.4±28.6 75.0	21.00 97.00	10.6±6.2 11.8	0.051 0.074	0.002 0.006
5	*DopR2 ^{attp}*	-	69	67.0±25.9 75.0	35.00 95.00			
6	*DopR2 ^{attp}*	+	67	41.3±32.2 30.0	5.00 91.00	38.4±6.5 60.0	0.000 0.000	0.857 0.381
7	*DopR1^{res}*	-	30	65.5±28.8 65.0	20.00 100			
8	*DopR1^{res}*	+	31	45.7±27.9 45.0	6.00 89.00	30.3±9.5 30.8	0.004 0.051	0.521 0.142

9	*Canton-S*+ SCH23390	-	36	63.0±26.9 65.0	23.50 96.50			
10	*Canton-S*+ SCH23390	+	36	63.1±24.5 60.0	32.00 95.00	-0.4±9.7 7.7	0.519 0.402	0.000 0.003
11	*DopR2*^{attp}+SCH23390	-	36	40.0±24.9 35.0	85.50 78.00			
12	*DopR2*^{attp}+SCH23390	+	32	32.7±24.3 25.0	50.00 78.50	18.4±13.6 28.6	0.108 0.230	0.127 0.323 P LI_n=LI_{10}

Courtship indices of males of the indicated genotypes either trained for 7 hrs with a mated female (Train+) or remaining alone (Train-) as indicated in Fig. 3A and tested in single-pair assays with mated females. Indicated males were fed for 6 hrs with DopR1&2 antagonist, SCH23390, after 7 hrs training on water only. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: LI_n = LI_c)

Table S6 Post-acquisition inactivation of DopR1

	Genotype	**DA**	**Train**	**n**	**CI (%)** mean±sd median	**10%-ile** **90%-ile**	**LI (%)** mean±sem median	**P LI=0**	**P LI_n=LI_2**
1	*Canton-S*	+	-	72	63.4±29.4 75.0	16.50 98.50			
2	*Canton-S*	+	+	70	39.4±28.6 35.00	0.50 89.50	39.7±5.0 53.3	0.000 0.000	
3	*DopR1*^{attp}	+	-	36	70.9±27.7 75.0	27.50 100			
4	*DopR1*^{attp}	+	+	33	72.1±22.8 75.0	37.00 100	-1.6±8.7 0.0	0.572 0.632	0.000 0.001
5	*DopR2*^{attp}	+	-	53	58.6±27.9 60.0	15.00 95.00			
6	*DopR2*^{attp}	+	+	67	34.6±30.0 25.0	0.00 82.00	40.9±7.4 58.3	0.000 0.000	0.893 0.675
7	*Canton-S*	+ SCH23390	-	35	57.1±23.4 60.0	30.00 90.00			
8	*Canton-S*	+ SCH23390	+	35	58.0±23.9 65.0	23.00 87.00	-1.5±10.0 -8.3	0.561 0.762	0.001 0.002
9	*DopR2*^{attp}	+ SCH23390	-	36	44.4±31.0 40.0	1.50 85.00			
10	*DopR2*^{attp}	+ SCH23390	+	32	37.5±29.7 30.0	3.50 81.50	15.5±15.3 25.0	0.181 0.177	0.355 0.303 P LI_n=LI_8

Courtship indices of males of the indicated genotypes fed with sucrose supplemented with dopamine (DA+) after being starved for 16 hrs and trained with a mated female for 1 hr (Train+) or remaining alone (Train-) as indicated in Fig. 3B and tested in single-pair assays with mated females. Indicated males were fed with dopamine supplemented with DopR1&2 antagonist SCH23390. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: LI_n = LI_c)

Table S7 LTM rescue with DopR1 in subsets of MB neurons

	Genotype	**Train**	**n**	**CI (%)** mean±sd median	**10%-ile** **90%-ile**	**LI (%)** mean±sem median	**P LI=0**	**P LI_n=LI_2**
1	*UAS-DopR1; DopR1*^{attp}	-	31	82.0±23.4 90.0	55.00 100			
2	*UAS-DopR1; DopR1*^{attp}	+	33	77.1±22.0 85.0	42.50 97.50	6.0±5.0 5.6	0.177 0.263	

3	*UAS-DopR1;c739-Gal4; DopR1^{attp}*	-	29	53.7±25.4 60.0	15.00 85.00			
4	*UAS-DopR1;c739-Gal4; DopR1^{attp}*	+	34	54.0±30.3 50.0	10.00 90.00	-0.6±13.0 16.7	0.518 0.402	0.644 0.681
5	*UAS-DopR1;305-Gal4; DopR1^{attp}*	-	20	84.5±15.7 90.0	60.00 100			
6	*UAS-DopR1;305-Gal4; DopR1^{attp}*	+	19	84.5±14.9 90.0	55.00 100	0.03±5.8 0.0	0.519 0.553	0.501 0.711
7	*UAS-DopR1;Y201-Gal4; DopR1^{attp}*	-	34	74.5±21.9 80.0	41.00 95.00			
8	*UAS-DopR1;Y201-Gal4; DopR1^{attp}*	+	36	44.9±26.0 42.5	10.00 80.00	39.8±6.6 50.0	0.000 0.000	0.001 0.009
9	*c739-Gal4; DopR1^{attp}*	-	31	89.7±14.5 95.0	67.00 100			
10	*c739-Gal4; DopR1^{attp}*	+	33	86.9±17.9 80.0	60.00 100	3.1±4.2 0.00	0.244 0.922	0.692 0.347
11	*305-Gal4; DopR1^{attp}*	-	32	77.8±21.0 75.0	45.00 98.50			
12	*305-Gal4; DopR1^{attp}*	+	36	65.1±24.5 67.5	28.50 100	16.3±6.6 23.5	0.014 0.004	0.261 0.087
13	*201-Gal4; DopR1^{attp}*	-	34	82.2±18.7 87.5	57.50 100			
14	*201-Gal4; DopR1^{attp}*	+	34	81.8±19.1 90.0	55.00 100	0.5±5.6 -5.9	0.484 0.868	0.500 0.339

Courtship indices of males of the indicated genotypes either trained for 7 hrs with a mated female (Train+) or remaining alone (Train-) as indicated in Fig. 3C and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S8 *orb2* mutant in courtship memory consolidation assay

	Genotype	**Train**	**DA**	**n**	**CI (%)** mean±sd median	**10%-ile** **90%-ile**	**LI (%)** mean±sem median	**P LI=0**	**P LI_n=LI_2**
1	*Canton-S*	-	+	36	58.5±32.1 55.0	10.00 100			
2	*Canton-S*	+	+	35	27.9±25.2 25.0	0.00 62.00	52.3±8.5 54.5	0.000 0.000	
3	*orb2*$^{orb2\Delta Q}$	-	+	36	76.0±23.0 80.0	33.50 100			
4	*orb2*$^{orb2\Delta Q}$	+	+	33	73.5±19.3 75.0	45.00 98.00	3.2±6.8 6.2	0.330 0.209	0.000 0.022

Courtship indices of males of the indicated genotype fed with sucrose supplemented with dopamine (+ DA) after being trained with a mated female for 1 hr (Train+) or remaining alone (Train-) as indicated in Fig. 4A and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H_0: $LI_n = LI_c$)

Table S9 Quantification of the Orb2 oligomers from Figure 4C

Genotyp	***Wt (-DA)***	***DopR1^{attp}(-DA)***	***DopR2^{attp} (-DA)***	***Wt (+DA)***	***DopR1^{att} (+DA)***	***DopR2^{attp} (+DA)***
Mean Int	358.83	316.12	229.34	6523.18	204.30	5546.77
MI_N /MI	1	0.88	0.64	18.18	0.79	15.46

WB signal corresponding to the Orb2-GFP oligomers (Fig. 4C) has been quantified using Fiji-ImageJ (Fig. 4D). Mean intensity was normalized to the wild type not treated with dopamine (wt - DA).

Table S10 Temporal rescue of LTM with Orb2A and Orb2B isoforms in MB

	Genotype	*n*	CI naïve (%) mean±sd median	10%-ile 90%-ile	*n*	CI exp (%) mean±sd median	10%-ile 90%-ile	LI (%) mean±sem median	*P LI=0*	*P* $LI_{wt}=LI_{*}$
1	*TubG80^{ts};orb2$^{\Delta Q\Delta A}$;UAS-Orb2A;MB247-Gal4*	34	26.8±29.5 10.0	0 77.50	36	24.9±21.8 15.0	0.00 58.00	7.1±5.0 -50.0	0.382 0.783	
1	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2AΔQ;MB247-Gal4*	34	20.4±19.2 15.0	0 46.5	36	23.1±19.5 20.0	0.00 46.5	-12.8±2.3 -33.3	0.716 0.852	0.545 0.931
2	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2A;MB247-Gal4*	35	71.4±17.5 75.0	45.00 90.00	36	48.6±28.9 45.0	3.50 81.50	31.9±7.2 40.0	0.000 0.000	
2	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2AΔQ;MB247- Gal4*	33	62.1±27.6 70.00	50.00 95.00	43	57.2±28.2 65.0	10.00 95.00	7.9±9.9 7.1	0.222 0.499	0.039 0.109
3	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2A;MB247-Gal4*	33	48.3±27.4 45.00	12.00 91.00	34	31.6±28.8 25.0	0.00 80.00	34.6±12.3 44.4	0.008 0.015	
3	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2AΔQ;MB247-Gal4*	39	54.1±26.2 60.0	9.00 85.55	31	54.2±26.1 60.0	10.01 0.00	-0.2±5.0 0.0	0.498 0.571	0.046 0.049
4	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2A;MB247-Gal4*	31	68.4±28.8 75.0	10.00 95.00	36	72.5±18.8 75.0	42.00 95.00	-6.0±9.1 0.0	0.760 0.843	
4	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2AΔQ;MB247-Gal4*	36	67.6±27.5 75.0	20.50 95.00	34	66.9±24.5 70.0	27.50 95.00	1.1±9.0 6.7	0.463 0.421	0.578 0.744
5	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2A;MB247-Gal4*	33	74.4±20.9 85.0	35.00 95.00	36	66.1±23.0 70.0	28.50 91.50	11.1±6.8 17.6	0.062 0.026	
5	*TubG80ts; orb2$^{\Delta Q\Delta A}$;UAS-Orb2AΔQ;MB247-Gal4*	34	77.7±22.8 85.0	57.50 100	34	75.1±27.9 85.0	27.50 100	3.2±7.6 0.0	0.343 0.674	0.442 0.102
1'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2B;MB247-Gal4*	32	57.0±30.7 60.0	15.00 95.00	36	52.9±28.5 45.0	13.50 95.00	7.2±5.0 25.0	0.285 0.245	
1'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2BRBD*;MB247-Gal4*	18	54.2±25.8 50.0	19.50 95.50	18	45.6±17.8 45.0	9.50 76.00	15.9±13.0 10.0	0.136 0.374	0.650 0.645
2'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2B;MB247-Gal4*	35	79.9±21.9 85.0	45.00 100	34	57.2±27.1 65.0	7.50 85.00	28.4±6.8 23.52	0.000 0.000	
2'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2BRBD*;MB247-Gal4*	33	81.52±15.93 85.0	62.50 97.50	34	75.6±21.6 80.0	45.00 97.50	7.27±5.5 5.9	0.111 0.890	0.021 0.033
3'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2B;MB247-Gal4*	35	66.6±22.5 70.0	30.00 92.00	34	62.7±26.9 65.0	23.00 95.00	5.9±8.6 7.1	0.263 0.550	
3'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2BRBD*;MB247-Gal4*	26	52.9±33.0 60.0	3.00 95.00	27	46.9±33.7 40.0	5.00 91.00	11.4±5.0 33.3	0.273 0.134	0.747 0.268
4'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2B;MB247- Gal4*	36	69.7±26.9 80.0	27.00 95.00	36	67.8±21.6 65.0	33.50 95.00	2.8±8.1 18.8	0.365 0.096	
4'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2BRBD*;MB247-Gal4*	32	63.9±25.4 75.0	19.50 93.50	36	61.3±26.6 65.0	18.50 95.00	4.2±9.6 13.3	0.336 0.195	0.911 0.721
5'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2B;MB247-Gal4*	36	79.0±23.3 85.0	41.00 100	36	57.5±28.1 65.0	5.00 85.00	27.2±7.1 23.5	0.000 0.007	
5'	*TubG80ts;orb2^{mCPEB2RBD};UAS-Orb2BRBD*;MB247-Gal4*	25	79.6±24.2 90.0	38.00 97.00	32	73.3±26.8 85.0	31.00 100	7.9±8.1 5.6	0.188 0.277	0.074 0.266

Courtship indices of males of the indicated genotypes either trained for 7 hrs with a mated female (CI exp) or remaining alone (CI naive), treated as indicated in Fig. 5B and tested in single-pair assays with mated females. *P* values determined by permutation test for the null hypothesis that learning equals 0 (H_0: LI = 0) or for the null hypothesis that rescue flies with the wild type isoform learns equally well as rescue flies with the mutated isoform in the same conditions (H_0: $LI_n = LI_*$)

Table S11 Orb2 regulates translation of CaMKII

3'UTR	Npc2a-3'UTR-RA (control)			CaMKII-3'UTR-RH		
Genotype	**Orb2 wt**	**Orb2RRM***	**Ratio wt/RRM***	**Orb2 wt**	**Orb2RRM***	**Ratio wt/RRM***
FLuc/Rluc	16.183	14.680	1.1023	11.995	12.896	0.931
	16.272	15.319	1.0622	12.388	12.413	0.997
	45.179	43.740	1.0329	23.614	26.492	0.891
	42.370	44.147	0.9597	46.313	52.440	0.883
	6.380	4.599	1.3992	29.167	29.608	0.985
	7.629	7.367	1.0355	30.007	33.264	0.902
				14.852	21.392	0.694
Mean			1.098			0.896
SEM			0.0630			0.0164
P (T-test)			0.0225			

Dual luciferase reporter assay is S2 cells co-expressing either Firefly luciferase tethered to the CaMKII 3'UTR or control Npc2a-3'UTR (does not contain Orb2 specific binding sequence) and Renilla luciferase tethered to the SV40 3'UTR (Fig. 6A). The values represent Firefly luciferase signal normalized to Renilla luciferase fluorescence in S2 cells expressing either Orb2 wt or Orb2 with the RBD mutated, Orb2RRM*.

Table S12 Mean intensity of the *EYFP-CaMKII-3'UTR* in the MB gamma neurons

	Genotype	DA (hrs)	n	Mean intensity	SEM	P(ftx=ft0)	P (ftx=ft24)
1	+ *CamKII 3'UTR, wt Orb2*	0	4	28.21	5.5		0.99
2	+ *CamKII 3'UTR, wt Orb2*	6	3	50.50	4.8	0.03	0.04
3	+ *CamKII 3'UTR, wt Orb2*	12	6	50.63	4.9	0.02	0.03
4	+ *CamKII 3'UTR, wt Orb2*	24	3	28.23	6.2	0.99	

	Genotype	DA (hrs)	*n*	Mean intensity	SEM	*P*(ftx=ft0)
1	+ *CamKII 3'UTR*, $Orb2^{\Delta A}$,	0	6	21.96	4.3	
2	+ *CamKII 3'UTR*, $Orb2^{\Delta A}$	6	4	29.92	6.89	0.33

Medium intensity of the fluorescence measured in the gamma lobe of the MB of the indicated genotype according to Fig. 6B (Fig. 6C). *P* values determined by 2-sided t-test for the null hypothesis that the fluorescence intensity at time xhr equals the intensity at 0hr (H_0: ftx=ft0) or 24 hrs (H_0: ftx=ft24)

Material and methods

Courtship Conditioning Paradigm

Flies were maintained on conventional cornmeal-agar medium under a 12 hrs light: dark cycle at 25°C and 60% relative humidity. Courtship assays were performed at variable circadian clock of the flies. Males were assayed for courtship conditioning as described (Siwicki and Ladewski, 2003). For training, individual males were placed in food chambers either with (trained) or without (naive) a single premated female. After training, each male was recovered, transferred to a fresh food vial and kept in isolation until testing. For long-term memory, males were trained for 6–7 hrs and tested after 24 hrs. For short-term memory, the training period was 1 hr and the test was performed after 30 min. Tests were performed in a 10 mm diameter courtship chamber and

videotaped for 10 min (JVC handycam, 30 GB HD). Videos were scored manually and blind to the genotype for CI, which is the percentage of time each male spent courting during the test. Courtship index (CI) was used to calculate the Learning Index (LI): CI_{naive}-$CI_{trained}/CI_{naive} \times 100$.

Immunohistochemistry

Immunohistochemistry on adult brains was performed as described (Yu et al., 2010). Fly brains were dissected (between 5 to 8 days after eclosion) in PBS and fixed using 4% paraformaldehyde in PBST (PBS with 0.3% Triton X-100) for 20 min at 24°C. After washing in PBST, the tissue was blocked in 5% normal goat serum in PBST for at least 2 hrs. The primary antibody and secondary antibody were incubated for 48 hrs at 4°C. The brains were washed with PBST 3 × 10 min and then overnight at 4°C between the primary and secondary antibody incubations. After the secondary antibody incubation, samples were washed 3 × 10 min and overnight at 4°C before mounting in Vectashield (VectorLabs). Antibodies used: rabbit polyclonal anti-GFP (1:5,000, Torri Pines); secondary Alexa-488 antibodies (1:1,000, Invitrogen).

Confocal Microscopy

For imaging and measurement of the fluorescence intensity of the EYFP+/- CaMKII-3'UTR, the fly brains immunostained as described above, were scanned using a Zeiss LSM 710 with a Zeiss Multi Immersion Plan NeoFluar 63× objective. Scanning parameters were set to image the entire mushroom body. Images were taken at 785 × 785 pixels. Images were processed in Imaris for fluorescence quantification. Briefly a cuboid of similar size was set as surface into each MB gamma lobe and the mean YFP fluorescence quantified.

Immunoprecipitation and Western Blot

Adult heads of the indicated genotype were lysed in homogenization buffer (PBS,150mM NaCl, 0.1mM CaCl2, 3mM MgCl2, 5% Glycerol, 1mM DTT, 0.1% TritonX100, 0.1% NP40, EDTA free protease inhibitor cocktail from Roche). The lysate was cleared by centrifugation prior to incubation with Chromotek GFPtrap beads (according to the manufacturer protocol). The proteins were transferred to a PVDF membrane (Millipore) overnight in the cold room at 35mv. Membrane was blocked in 5% milk prior to incubation for 1 hr with a primary antibody. After 3 washes in PBST (PBS+ 0,05%Tween20) membrane was incubated for 1 hr in a secondary antibody. The membrane was developed using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoScientific). Antibodies used: anti-GFP (Abcam 6556 rabbit polyclonal, 1:2,000).

Immuno-EM on adult brains

The brains of 6-7 day old adult flies were dissected in cold fly saline and fixed for 3 hours on ice with 0.1% glutaraldehyde/4% formaldehyde in 0.07M sodium phosphate buffer, pH 7.4, rinsed with 0.1 M phosphate buffer containing 0.1% saponin, and incubated overnight with an HRP-conjugated rabbit anti-GFP polyclonal antibody (Life Technologies, A10260, anti-GFP, rabbit IgG fraction, horseradish peroxidase conjugate) at 1:200 dilution in 0.1 M phosphate buffer containing 5% normal goat serum/1% BSA at 4° C. The brains were then rinsed with 0.1 M phosphate/0.1% saponin buffer and reacted with 0.5 mg/ml DAB in 0.1 M phosphate buffer containing 0.1% saponin for 45 minutes following the addition of 10 μl 0.03% H_2O_2. The brains were then rinsed with 0.1 M Na-cacodylate buffer, followed by OTOT enhancement (10 min cycles of 0.01% OsO4 in 0.1M Na-cacodylate buffer followed by 0.1% thiocarbohydrazid1M Na-

cacodylate buffer with 0.1M Na-cacodylate buffer rinses between steps), and a final 1% OsO_4 in 0.1 M Na-cacodylate buffer step for 1 hour at room temperature. Following osmication, the samples were rinsed with 0.1 M Na-cacodylate buffer, water and then dehydrated in ethanol followed by propylene oxide and embedded in Eponate 12 resin (Ted Pella, Redding, CA). Embedded brains were imaged using a Zeiss Versa 510 X-ray microscope operated at 40kV and 0.7 μm/pixel resolution. The computed tomograms were used to provide coordinates of the cell bodies of the Kenyon cells and gamma lobes of the mushroom body in each sample. A Leica Ultracut 6 ultramicrotome was used to cut 90 nm sections at the level of the Kenyon cells somata and gamma lobes of the mushroom body. Unstained sections were imaged with an FEI Spirit BioTWIN TEM operated at 80kV.

CaMKII translation suppression assay

Luciferase reporter assay was done essentially as described (Mastushita-Sakai et al., 2010). In short, Orb2 plasmids were prepared by amplifying Orb2 CDS using primers containing attB sites. For Orb2 RRM1&2* site directed mutagenesis was used. All primers are listed below. Products were cloned into pDONR221 and recombined to obtain pAWM-Orb2B WT or RRM1&2* vectors. To create control (*Renilla*) and test (Firefly) luciferase expressing constructs pAMW (The Drosophila Gateway Vector Collection) was cut with BamHI (Fermentas) and the backbone fragment was ligated with MCS (multiple cloning site). Next, the Gateway expression cassette was PCR amplified from pAMW with casAWMf/casAWMr primers and cloned into the pAMW-MCS linearised with NheI/Asp718 (Roche) to obtain pAMW-cassette. For pAMW-Fluc-cassette destination vector the Firefly luciferase PCR product obtained by amplifying pAC-Fluc-6xBS with Flucf/Flucr primers was digested with SpeI/NheI and cloned into NheI cut pAMW-cassette vector. For pAMW-Rluc-cassette-polyA first the polyA signal sequence was amplified from pAMW with polAf/polAr primers and cloned with Asp718/XhoI. Subsequently, *Renilla* luciferase was PCR amplified from pAC-Rluc with Rlucf/Rlucr primers and cloned SpeI/NheI into NheI cut vector. To create final pAMW-Rluc-SV403'UTR-polyA vector SV40 3'UTR was PCR amplified from pAMW with SV403UTRf/SV403UTRr primers and cloned first into pDONR221 and then recombined with pAMW-Rluc-cassette-polyA. CaMKII 3'UTR and Npc2a-RA 3'UTR were PCR amplified using the following primers containing attB sites. PCR product was sub-cloned into pDONR221 vector using Gateway technology (Life Technologies). Resulting entry clones were recombined with pAMW-Fluc-cassette to obtain pAMW-Fluc-3'UTR plasmids. All constructs were confirmed by sequencing.
S2 cells were grown in semi-adhering liquid cultures at 27°C in water-jacketed incubator, with 5% CO_2 in liquid Schneider's Drosophila Medium (Invitrogen) supplemented with 10% fetal calf serum and PenStrep (Invitrogen) without agitation. S2 cells were split 1:10, grown overnight and diluted in Schneider's Drosophila Medium to 1mln/ml. Cells were transferred to 96-well culture plates 100μl per well. 120ng of DNA was used per transfection containing 10ng of pAMW-Rluc-SV40-polyA, 10ng of pAMW-Fluc-3'UTR reporter plasmid and 5ng pAWM-protein filled up to final DNA amount by inert bacterial plasmid pGEX-2T (GE Healthcare). Cells were transfected by adding 4.7μl of DNA/FuGENE (Promega FuGENE® HD Transfection Reagent) mix per 100ul cells and pipetting up and down. After 48h cells were transferred to deep well plates in 600μl 1xPBS, harvested by spinning 5 minutes at 800g, washed twice with 400μl 1xPBS and lysed in 30μl 1xPassive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega). For dual luciferase assay 10μl of lysate was pipetted onto 96-well plate and luciferase signals were measured in Synergy Plate Reader (BioTek) by adding 20μl Luciferase Assay Substrate in Luciferase Assay Buffer II, shaking, incubating 2 minutes and measure and 20μl Stop & Glo Substrate in Stop & Glo Buffer, shaking, incubating 2 minutes and measuring. Firefly luciferase signal was normalised to *Renilla* luciferase and ratio between signal from cells expressing Orb2B WT to Orb2B RRM1&2* mutant protein was calculated from the means for 3 replicates from the

same transfection. Each reaction was repeated in at least 6 independent experiments. Mean ratios between WT and mutant protein signals were calculated and compared to negative control (Npc2a-RA 3'UTR) using unpaired Student *t* test.

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