Synaptic Orb2A Bridges Memory Acquisition and Late Memory Consolidation in Drosophila

Highlights

- *Drosophila* exhibits late long-term memory consolidation
- DA-aSP13 neurons mediate both memory acquisition and memory consolidation
- *Drosophila* CPEB, Orb2, acts downstream of DopR1 in the MB γ neurons
- Synaptic Orb2A is required during memory acquisition and Orb2B during consolidation

Authors

Sebastian Krüttner, Lisa Traunmüller, Ugur Dag, ..., Jasprina N. Noordermeer, Brett D. Mensh, Krystyna Keleman

Correspondence

kelemank@janelia.hhmi.org

In Brief

Krüttner et al. demonstrate that, similar to mammals, invertebrates exhibit late memory consolidation mediated by neural pathways engaged earlier during memory acquisition. Late reactivation of a dopaminergic neuron class in *Drosophila* recruits Orb2B to form complexes in synapses tagged by Orb2A upon memory acquisition; these complexes regulate CaMKII translation.

Krüttner et al., 2015, Cell Reports 11, 1953–1965

June 30, 2015 ©2015 The Authors

http://dx.doi.org/10.1016/j.celrep.2015.05.037
Synaptic Orb2A Bridges Memory Acquisition and Late Memory Consolidation in Drosophila

Sebastian Krüttner, Lisa Traunmüller, Ugur Dag, Katharina Jandrasits, Barbara Stepien, Nirmala Iyer, Lee G. Fradkin, Jasprina N. Noordermeer, Brett D. Mensh, and Krystyna Keleman

INTRODUCTION

The brain rapidly learns environmental associations and behavioral contingencies but is selective about which lessons it commits to long-term memory. In mammals, long-term memory 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 or how information is stored during the delay period. Here, we show that, during courtship suppression learning, Drosophila exhibits delayed long-term memory consolidation. We also show that the same class of dopaminergic neurons engaged earlier in memory acquisition is also both necessary and sufficient for delayed long-term memory consolidation. Furthermore, 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. Thus, our results provide evidence for the role of a neuromodulated, synapse-restricted molecule bridging memory acquisition and long-term memory consolidation in a learning animal.

SUMMARY

To adapt to an ever-changing environment, animals consolidate some, but not all, learning experiences to long-term memory. In mammals, long-term memory 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 or how information is stored during the delay period. Here, we show that, during courtship suppression learning, Drosophila exhibits delayed long-term memory consolidation. We also show that the same class of dopaminergic neurons engaged earlier in memory acquisition is also both necessary and sufficient for delayed long-term memory consolidation. Furthermore, 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. Thus, our results provide evidence for the role of a neuromodulated, synapse-restricted molecule bridging memory acquisition and long-term memory consolidation in a learning animal.

“Important” behavioral moments for memory acquisition and memory consolidation (Plaçais 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 naive males learn to preferentially court receptive virgin females after experiencing unsuccessful courtship of already mated (and therefore unresponsive) 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 long-term memory are not clear. Recently, it has been found that orchestrated activity of three clusters of dopaminergic neurons positively affect long-term memory formation during olfactory learning (Plaçais 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 can be mediated by a variety of protein-synthesis-independent mechanisms (Kandel, 2001). Long-term memories 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 long-term memory.
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 (L-LTP, an in vitro correlate of short-term memory) into L-LTP (a physiological correlate of long-term memory) 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 that elicits short-term memory can be consolidated into long-term memory 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 long-term memory 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 subfamilies. 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 (mcPCEB2–4; Richter, 2001) or long-term memory formation (Drosophila Orb2; Keleman et al., 2007; Majumdar et al., 2012). 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 (Krüttner et al., 2012; Majumdar et al., 2012). A protein network has been recently identified that 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 unresponsive mated female for 5–7 hr leads to a long-term suppression of his courtship preferentially toward a mated, but not a virgin, female for at least 24 hr. We were able to prevent the expression of this long-term memory 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 hr results in short-term memory, but not long-term memory; however, by stimulating the same class of dopaminergic neurons, aSP13, many hours after this exposure, we were able to transform short-term memory into long-term memory. Having revealed that long-term memory 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 long-term memory 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 long-term memory 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 Short-Term Memory into Long-Term Memory

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 short-term memory, but not long-term memory, 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 hr, trained them for 1 hr with mated female, and then activated dopamine pathways globally by feeding the animals with dopamine for 23 hr (Riemensperger et al., 2011). This resulted in robust long-term memory, in the form of a strong suppression of their courtship toward mated females in comparison to virgin females when tested 24 hr later. This memory was quantified as a learning index (LI), which measures the extent of the courtship suppression (Figure 1; Table S1). By contrast, training for 1 hr alone induced normal short-term memory, but not long-term memory, and dopaminergic activation alone did not induce long-term memory. Thus post-acquisition dopamine stimulation consolidates short-term memory into long-term memory.

Subset of PAM-DA Neurons, aSP13, Mediates Late Long-Term Memory 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, 2005). 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 memory into a long-lasting one, we asked whether delayed activation of PAM-DA neurons might be sufficient for consolidation of courtship short-term memory into long-term memory. To test whether and when activity of the PAM-DA neurons is sufficient for long-term memory consolidation, we thermogenetically 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 short-term memory.

Males expressing TrpA1 were incubated at 32°C for 2 hr at various time points after 1-hr training with a mated female. Flies, which were incubated at 32°C 8–10 hr after training fully consolidated short-term memory into long-term memory, 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 (Figures 2Ai and 2Aii; Table S2). Importantly, this consolidated form of memory was dependent on de novo protein synthesis, because feeding the males with the protein synthesis inhibitor cycloheximide prevented the PAM-DA-stimulation-induced long-lasting memory (Figure 2Aiii; Table S2). Given our previous results showing that PAM-DA neurons are necessary for short-term memory acquisition, these results suggest that delayed activation of the same PAM-DA neurons between 8 and 11 hr after initial learning for long-term memory consolidation. Thus, delayed activation of a specific set of PAM-DA neurons is also necessary for formation of courtship long-term memory.

To test whether late post-acquisition activity of the aSP13-DA neurons is required between 8 and 11 hr after initial learning for long-term memory consolidation, we focused on DopR1 and DopR2 because DD2R is not expressed in the MB and DopEcR does not act as a dopamine receptor in courtship-suppression learning (Ishimoto et al., 2005). To determine which dopamine receptor has a role in long-term courtship memory consolidation, we performed a complementary set of experiments. We trained males for 7 hr with recently mated females (sufficient to result in long-term memory) and silenced the same aSP13 neurons with a temperature-sensitive shibire mutant, LexAop-shiG2, between 8 and 11 hr after onset of training. Males expressing shiG2 under the control of VT5526-LexA and incubated at 32°C continued to court mated females vigorously, thus failing to display long-term memory at the 24-hr test point. In contrast, genetic control animals incubated at 32°C and males that remained at 22°C displayed normal long-term memory (Figure 2C; Table S4). These results indicate that activity of the aSP13-DA neurons is required between 8 and 11 hr after initial learning for long-term memory consolidation. Thus, delayed activation of a specific neural subset is both necessary and sufficient for long-term memory consolidation of courtship learning in Drosophila.

**DopR1 Is Necessary for Long-Term Memory Consolidation**

In rodents, dopamine-mediated memory persistence requires the adenyl 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 on our analysis on DopR1 and DopR2 because 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<sup>tmp</sup> or DopR2<sup>tmp</sup>, in long-term memory and memory-consolidation paradigms (Keleman et al., 2012).

Males lacking DopR1 were unable to form long-term courtship memory after 7 hr of training. In contrast, DopR2 mutants displayed normal long-term memory (Figure 3A; Table S5). To confirm that long-term memory deficit in the DopR1 mutants is...
indeed due to loss of DopR1 function, we analyzed DopR1
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 long-term memory (Figure 3A; Table S5). However, given that DopR1 receptor is required for acquisition of the courtship short-term memory (Keleman et al., 2012), the impairment of long-term memory 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 long-term memory after acquisition,
we fed wild-type flies and DopR2** mutants (lacking DopR2, but not DopR1) after 7-hr training for long-term memory
and males fed with the antagonist (SCH23390) specific
for both receptors (Gotzes et al., 1994). These flies did not form long-term mem-
med 24 hr after being trained for 1 hr with a mated female and being warmed at 32°C for 2 hr at the
indicated time points (i); experimental control males, which stayed at 22°C all the time; and the
control animals, which were warmed at 32°C for 2 hr between 8 and 10 hr after learning (ii); and
males fed with the cycloheximide during activation with TrpA1 between 8 and 10 hr after 1 hr
training (iii). p values are for H0 LI = 0; ***p < 0.001. See Table S2.

(B) Subset of the aSP13-DA neurons is sufficient
for the long-term memory consolidation. Males of the indicated genotypes were tested in single-pair
assays with mated females 24 hr 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 and 10 hr after training. p values are for
H0 LI = 0; **p < 0.01; ***p < 0.001. See Table S3.

(C) Post-acquisition silencing of the aSP13-DA
neurons prevents long-term memory formation.
Males of the indicated genotypes were tested in
single-pair assays with mated females 24 hr after
training for 7 hr with a mated female and being warmed at 32°C (except control males, which
stayed at 22°C) between 8 and 11 hr after training. p values are for H0 LI = 0; ***p < 0.001. See
Table S4.
The plots indicate mean learning indices ± SEM.

To test both dopamine receptors in memory consolidation
paradigm, we tested DopR1 and DopR2 mutants for courtship
suppression after being trained for short-term memory in combination with dopamine feeding. Mutants for DopR1 were unable to consolidate long-term memory, whereas the DopR2 mutants
performed equally well as the wild-type animals (Figure 3B; Table S6). To dissociate the role of DopR1 in memory acquisition and memory consolidation, we fed wild-type males and
DopR2 mutants with the SCH23390 in addition to dopamine. These males, in contrast to animals fed only with dopamine, did not suppress their courtship toward mated females during test, thus failing to display long-term courtship memory (Figure 3B; Table S6). These results indicate that DopR1 has a role in the consolidation of short-term memory into long-term memory 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 long-term memory. 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 long-term memory consolidation (Figure 3C; Table S7).

**Orb2 Mediates Long-Term Memory 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 long-term memory (Krüttner et al., 2012; Majumdar et al., 2012). 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 (orb2DQ) and has been previously shown to be dispensable for short-term memory but critical for both Orb2 complex formation and the maintenance of the courtship memory after 6 hr (Keleman et al., 2007; Krüttner et al., 2012).

orb2DQ mutants were unable to consolidate short-term memory into a memory lasting 24 hr upon feeding with dopamine, in comparison to the animals bearing the wild-type allele (orb2+; Figure 4A; Table S8).

To investigate which type of dopamine receptor is mediating the Orb2 complex formation and hence long-term memory 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 (Orb2GFP) in immunoprecipitates from brains of the DopR1 or DopR2 mutant flies upon stimulation with dopamine. As predicted, Orb2GFP complex was not detected in brain extracts from the animals that were not fed with dopamine (both the wild-type and mutants). In contrast, Orb2GFP complex was detected in brain extracts from the wild-type 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 (Figures 4B and 4C; 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 (RNP) (Krüttner et al., 2012; Majumdar et al., 2012). In contrast, endogenous Orb2A was expressed at levels undetectable by confocal microscopy. When expressed with GFP-tagged genic transgene rescue, Orb2A was detected at very low levels (Majumdar et al., 2012). Consistent with the genetic data that revealed a functional requirement for Orb2A in long-term memory, 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\textsuperscript{Orb2AGFP} and orb\textsuperscript{Orb2BGF}, respectively, we determined their subcellular localization (Krüttner 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 orb\textsuperscript{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\textsuperscript{GFP} is absent from the neuronal cell bodies (arrow) of the Kenyon cells. (ii) Orb2A\textsuperscript{GFP} labeled by DAB precipitates is present in T-bars (asterisk) and active zones (double asterisk) in the MB γ lobe synapses. (iii) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is detected in the KC cell bodies including T-bars (asterisk) and active zones (double asterisk). (iv) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is present in the MB γ lobe including T-bars (asterisk). In all panels, scale bars represent 500 nm.

Figure 5. Orb2A Is Localized in Synapses of the MB γ Lobe

Immuno-EM of the orb\textsuperscript{Orb2AGFP} and orb\textsuperscript{Orb2BGF} 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\textsuperscript{GFP} is absent from the neuronal cell bodies (arrow) of the Kenyon cells. (ii) Orb2A\textsuperscript{GFP} labeled by DAB precipitates is present in T-bars (asterisk) and active zones (double asterisk) in the MB γ lobe synapses. (iii) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is detected in the KC cell bodies including T-bars (asterisk) and active zones (double asterisk). (iv) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is present in the MB γ lobe including T-bars (asterisk). In all panels, scale bars represent 500 nm.

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 (RNP) (Krüttner et al., 2012; Majumdar et al., 2012). In contrast, endogenous Orb2A was expressed at levels undetectable by confocal microscopy. When expressed with GFP-tagged genic transgene rescue, Orb2A was detected at very low levels (Majumdar et al., 2012). Consistent with the genetic data that revealed a functional requirement for Orb2A in long-term memory, 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\textsuperscript{Orb2AGFP} and orb\textsuperscript{Orb2BGF}, respectively, we determined their subcellular localization (Krüttner 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 orb\textsuperscript{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\textsuperscript{GFP} is absent from the neuronal cell bodies (arrow) of the Kenyon cells. (ii) Orb2A\textsuperscript{GFP} labeled by DAB precipitates is present in T-bars (asterisk) and active zones (double asterisk) in the MB γ lobe synapses. (iii) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is detected in the KC cell bodies including T-bars (asterisk) and active zones (double asterisk). (iv) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is present in the MB γ lobe including T-bars (asterisk). In all panels, scale bars represent 500 nm.

Figure 5. Orb2A Is Localized in Synapses of the MB γ Lobe

Immuno-EM of the orb\textsuperscript{Orb2AGFP} and orb\textsuperscript{Orb2BGF} 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\textsuperscript{GFP} is absent from the neuronal cell bodies (arrow) of the Kenyon cells. (ii) Orb2A\textsuperscript{GFP} labeled by DAB precipitates is present in T-bars (asterisk) and active zones (double asterisk) in the MB γ lobe synapses. (iii) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is detected in the KC cell bodies including T-bars (asterisk) and active zones (double asterisk). (iv) Orb2B\textsuperscript{GFP} labeled by DAB precipitates is present in the MB γ lobe including T-bars (asterisk). In all panels, scale bars represent 500 nm.
kept at 27°C throughout adulthood and when shifted to 27°C ~2 hr before the end of training. They did not form long-term memory 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 long-term memory in any condition (Figure 6; Table S10). Therefore, we conclude that presence of Orb2B is dispensable during the training and shortly after but is necessary continuously about 2 hr 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 long-term memory 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 7A; 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-CaMKII-3'UTR), in the α, β, γ MB neurons using MB247-Gal4. 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 hr 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 Orb2 isoform (orb2ΔA; Figures 7B and 7C; Table S12), whose Q domain is required for Orb2 complex formation (Krüttner et al., 2012). Thus, these results suggest that Orb2

Figure 7. 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 Fluc fluorescence in the presence of wt Orb2 to the Fluc 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 (orb2ΔA; right panel) stimulated by feeding with dopamine at the indicated time intervals. p values are for H0 ftx = ft0. See Table S12.
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 long-term memory 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, whereas 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 (Buzsáki, 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 ripples, may be critical for memory consolidation. Our results that the synaptically localized Orb2A isoform and its Q domain are required during memory acquisition in MB neurons for subsequent long-term memory 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; Krüttner et al., 2012) into translationally active protein complexes (Si et al., 2003b) to regulate synthesis of proteins essential for the long-term memory, such as CaMKII.

In this work, we provide evidence that the late activation of the same neural and molecular pathways that are necessary and sufficient for early memory acquisition is 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 adaptive 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 hr light/dark cycle at 25 °C and 60% relative humidity. The Canton-S strain was used as the wild-type D. melanogaster flies. The following fly stocks, H09-Gal4, TH-Gal4, c739-Gal4, c305-Gal4, Y201-Gal4, MB247-Gal4, UAS-Trp, UAS-DopR1, DopR1ras, DopR2ras, and DopR1ras were previously described by Kelemen et al. (2007) and Kelemen et al. (2012). The Orb2O2, Orb2~, and Orb2~CPEBRR flies were generated by Krüttner et al. (2012). The VT5526-LexA driver and LexAop-Trp flies are unpublished reagents from B.J. Dickson (personal communication). The UAS-EYFP-CaMKII3'UTR flies were generated by 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 (Kelemen et al., 2007; Siwicky and Ladewski, 2003). Details can be found in the Supplemental Information.

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 testing, flies were starved on a wet filter paper for 16 hr. After short-term memory training (1 hr), at indicated time points, flies were transferred to chambers containing filter paper soaked with 80 µl of 2% sucrose solution supplemented with either dopamine, ciclohexamide, or 1 mM 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 or 7 days. First, they were trained for 1 hr at 22 °C, shifted to 32 °C at indicated time points for 2 hr, and thereafter placed at 22 °C until the test at 25 °C (24 hr after training).

For silencing with Shn~, males were collected and aged as described above. They were trained for 1 hr at 22 °C, shifted to 32 °C at indicated time points, and thereafter placed at 22 °C until the test at 25 °C (24 hr 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 were: w1: tub-GAL80ts, UAS-orb2A (UAS-orb2AAS); orb2~O2, MB247-Gal4 and w1: tub-GAL80ts, UAS-orb2B (UAS-orb2B); orb2~CPEBRR, MB247-Gal4.

Immunohistochemistry

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

Immunoprecipitation and Western Blot

IP and WB were carried out as described previously (Krüttner et al., 2012) on adult brains of w1: tub-GAL80ts, UAS-orb2Arasp and w1: orb2~O2rasp to investigate Orb2~O2rasp complex formation. To determine on/off kinetics of Orb2 expression in TARGET experiment, IP and WB were performed on brain extract from w1: tub-Gal80 ts, UAS-orb2B:Orb2~CPEBRR MB247-Gal4. Details can be found in the Supplemental Information.

ImmuNo-EM

The heads of heterozygous viable orb2~CPEBRR and orb2~CPEBRR 6– or 7-day-old flies were fixed in 4% paraformalde, 0.1% glutaraldehyde, and 0.07 M phosphate buffer (pH 7.3) for 3 hr at 40 °C and prepared for immuno-EM as described (Krüttner et al., 2012). Details can be found in the Supplemental Information.

Statistical Analysis

Lis were calculated using a custom MATLAB script based on the algorithm described in Kamysh et al. (1999) and implemented in Kelemen et al. (2007). Briefly, the entire set of courtship indices for both the naive and trained flies was pooled and then randomly assorted into simulated naive and trained sets of the same size as in the original data. A Li was calculated for each of 100,000 randomly permuted data sets, and p values were estimated as the fraction for which Li > Li (to test H0, Li = 0) or Li > Li – Li, (to test H0, Li = Li – Li). p values are for Hc: Li = Li (permutation test) and p < 0.05, **p < 0.01, and ***p < 0.001 for Hc: 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; supplemental tables show values derived from both mean ± SD and median CIs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and twelve tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.05.037.

AUTHOR CONTRIBUTIONS

S.K. and K.K. conceived the project and designed the experiments. S.K. and L.T. performed all the experiments with the exception of the memory-consolidation assay using virgin females as testers, silencing of the aSP13 neurons after long-term memory training, and post-acquisition inactivation of DopPs in memory consolidation and long-term memory assays, which were performed by U.D. S.K. and L.T. performed data analysis with help from U.D. K.J. helped S.K. and L.T. with behavioral experiments. B.S. performed CaMKII translation-repression assay. N.I. performed EM analysis. J.N.N. and L.G.F. made an initial observation of Orb2A localization. K.K. supervised the project and wrote the manuscript with help of B.D.M.

ACKNOWLEDGMENTS

We thank U. Heberlein, B. Dickson, and V. Jayaraman for critical comments on the manuscript; R. Fetter for supervising the EM studies; C. Kent for advice on statistics; and B. Koster, Anja de Jong, Jos Onderwater, Erik Bos, and Christina Avramut for help with the initial EM experiments. The work was supported by the grants from Austrian Science Funds to K.K. as well as FWF P21854-B09 and WWTF P21854.

Received: October 2, 2014
Revised: April 14, 2015
Accepted: May 21, 2015
Published: June 18, 2015

REFERENCES

Ashraf, S.I., McLoon, A.L., Sciarici, S.M., and Kunes, S. (2006). Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell 124, 191–205.

Berke, J.D., Paletzki, R.F., Aronson, G.J., Hyman, S.E., and Gerfen, C.R. (1998). A complex program of striatal gene expression induced by dopaminergic stimulation. J. Neurosci. 18, 5301–5310.

Buzsáki, G. (1998). Memory consolidation during sleep: a neurophysiological perspective. J. Sleep Res. 7 (1), 17–23.

Buzsáki, G., Horváth, Z., Urioste, R., Hetke, J., and Wise, K. (1992). High-frequency network oscillation in the hippocampus. Science 256, 1025–1027.

Carr, M.F., Jadhav, S.P., and Frank, L.M. (2011). Hippocampal replay in the awake state: a potential substrate for memory consolidation and retrieval. Nat. Neurosci. 14, 147–153.
Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. Cell 139, 405–415.

Coultrap, S.J., and Bayer, K.U. (2012). CaMKII regulation in information processing and storage. Trends Neurosci. 35, 607–618.

Dickson, B.J. (2008). Wired for sex: the neurobiology of Drosophila mating decisions. Science 322, 904–909.

Draper, I., Kurnshjan, P.T., McBride, E., Jackson, F.R., and Kopin, A.S. (2007). Locomotor activity is regulated by D2-like receptors in Drosophila: an anatomical and functional analysis. Dev. Neurobiol. 67, 378–393.

Foster, D.J., and Wilson, M.A. (2006). Reverse replay of behavioural sequences in hippocampal place cells during the awake state. Nature 440, 680–683.

Frey, U., and Morris, R.G. (1997). Synaptic tagging and long-term potentiation. Nature 385, 533–536.

Gotzès, F., Balfanz, S., and Baumann, A. (1994). Primary structure and functional characterization of a Drosophila dopamine receptor with high homology to human D1/D5 receptors. Receptors Channels 2, 131–141.

Guo, A., Li, L., Xia, S.Z., Feng, C.H., Wolf, R., and, Heisenberg, M. (1996). Conditioned visual flight orientation in Drosophila: dependence on age, practice, and diet. Learn. Mem. 3, 49–59.

Hafer, N., Xu, S., Bhat, K.M., and Schedl, P. (2011). The Drosophila CPEB protein Orb2 has a novel expression pattern and is important for asymmetric cell division and nervous system function. Genetics 189, 907–921.

Heam, M.G., Ren, Y., McBride, E.W., Reveillaud, I., Beinborn, M., and Kopin, A.S. (2002). A Drosophila dopamine 2-like receptor: Molecular characterization and identification of multiple alternatively spliced variants. Proc. Natl. Acad. Sci. USA 99, 14554–14559.

Ishimoto, H., Sakai, T., and Kitamoto, T. (2009). Ecdysone signaling regulates the formation of long-term courtship memory in adult Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 106, 6381–6386.

Jadhav, S.P., Kemere, C., German, P.W., and Frank, L.M. (2012). Awake hippocampal sharp-wave ripples support spatial memory. Science 336, 1454–1458.

Kamyashev, N.G., Iladi, K.G., and Bragina, J.V. (1999). Drosophila conditioned courtship: two ways of testing memory. Learn. Mem. 6, 1–20.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science 294, 1030–1038.

Keleman, K., Krüttner, S., Alenius, M., and Dickson, B.J. (2007). Function of the Drosophila CPEB protein Orb2 in long-term courtship memory. Nat. Neurosci. 10, 1587–1593.

Keleman, K., Vrontou, E., Krüttner, S., Yu, J.Y., Kurtovic-Kozaric, A., and Dickson, B.J. (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship learning. Nature 489, 145–149.

Kim, Y.C., Lee, H.G., Seong, C.S., and Han, K.A. (2003). Expression of a D1 dopamine receptor DDA1/DmDOP1 in the central nervous system of Drosophila melanogaster. Gene Expr. Patterns 3, 237–245.

Krüttner, S., Stepben, B., Noordermeer, J.N., Mommaas, M.A., Mechtler, K., Dickson, B.J., and Keleman, K. (2012). Drosophila CPEB Orb2A mediates memory independent of its RNA-binding domain. Nature 486, 393–395.

Lucchesi, W., Mizuno, K., and Giese, K.P. (2011). Novel insights into CaMKII function and regulation during memory formation. Brain Res. Bull. 85, 2–8.

Majumdàr, A., Cesario, W.C., White-Grindley, E., Jiang, H., Ren, F., Khan, M.R., Li, L., Choi, E.M., Kannan, K., Guo, F., et al. (2012). Critical role of amyloid-like oligomers of Drosophila Orb2 in the persistence of memory. Cell 148, 515–529.

Mao, Z., and Davis, R.L. (2009). Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits 3, 5.

Martin, S.J., Grimwood, P.D., and Morris, R.G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. Annu. Rev. Neurosci. 23, 649–711.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron 24, 967–977.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765–1768.

McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Sci. STKE 2004, p16.

Mendez, R., and Richter, J.D. (2001). Translational control by CPEB: a means to the end. Nat. Rev. Mol. Cell Biol. 2, 521–529.

Moncada, D., and Viola, H. (2007). Induction of long-term memory by exposure to novelty requires protein synthesis: evidence for a behavioral tagging. J. Neurosci. 27, 7476–7481.

Musso, P.Y., Tchenio, P., and Pretz, T. (2015). Delayed dopamine signaling of energy level builds appetitive long-term memory in Drosophila. Cell Rep. 10, 1023–1031.

Nicoll, R.A., and Malenkova, R.C. (1999). Expression mechanisms underlying NMDA receptor-dependent long-term potentiation. Ann. N Y Acad. Sci. 868, 515–525.

Ostaf, T.A., Zuker, C.S., and Reiser, M.B. (2011). Visual place learning in Drosophila melanogaster. Nature 474, 204–207.

Piácais, P.Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Bellati-Guerin, G., Vernier, P., Birman, S., Tanimoto, H., and Pretz, T. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in Drosophila. Nat. Neurosci. 15, 592–599.

Qin, H., Cressy, M., Li, W., Coravos, J.S., Izzì, S.A., and DUBnau, J. (2012). Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in Drosophila. Curr. Biol. 22, 608–614.

Quinn, W.G., Harris, W.A., and Benzer, S. (1974). Conditioned behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 71, 708–712.

Redondo, R.L., Okuno, H., Spooner, P.A., Frenguelli, B.G., Bito, H., and Morris, R.G. (2010). Synthetic tagging and capture: differential role of distinct calcium/calmodulin kinases in protein synthesis-dependent long-term potentiation. J. Neurosci. 30, 4981–4989.

Richter, J.D. (2001). Think globally, translate locally: what mitotic spindles and neuronal synapses have in common. Proc. Natl. Acad. Sci. USA 98, 7069–7071.

Remensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., Iché-Torres, M., Casar, M., Strauss, R., Pretz, T., et al. (2011). Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. Proc. Natl. Acad. Sci. USA 108, 834–839.

Rosenzweig, M., Brennan, K.M., Taylor, T.D., Phelps, P.O., Patapoutian, A., and Garrity, P.A. (2005). The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis. Genes Dev. 19, 419–424.

Rossato, J.I., Bevilaqua, L.R., Izquierdo, I., Medina, J.H., and Cammarota, M. (2009). Dopamine controls persistence of long-term memory storage. Science 325, 1017–1020.

Schwaerzel, M., Monastirioti, M., Scholtz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J. Neurosci. 23, 10495–10502.

Si, K., Giustetto, M., Etkin, A., Hsu, R., Janisiewicz, A.M., Miniaci, M.C., Kim, J.H., Zhu, H., and Kandel, E.R. (2003a). A neuronal isofrom of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell 115, 893–904.

Si, K., Lindquist, S., and Kandel, E.R. (2003b). A neuronal isofrom of the aplysia CPEB has prion-like properties. Cell 115, 879–891.

Siegel, R.W., and Hall, J.C. (1979). Conditioned responses in courtship behavior of normal and mutant Drosophila. Proc. Natl. Acad. Sci. USA 76, 3430–3434.
Siwicki, K.K., and Ladewski, L. (2003). Associative learning and memory in Drosophila: beyond olfactory conditioning. Behav. Processes 64, 225–238.

Smith, W.B., Starck, S.R., Roberts, R.W., and Schuman, E.M. (2005). Dopaminergic stimulation of local protein synthesis enhances surface expression of GluR1 and synaptic transmission in hippocampal neurons. Neuron 45, 765–779.

Srivastava, D.P., Yu, E.J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T., and Evans, P.D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. J. Neurosci. 25, 6145–6155.

Sutton, M.A., and Schuman, E.M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. Cell 127, 49–58.

Tempel, B.L., Livingstone, M.S., and Quinn, W.G. (1984). Mutations in the dopa decarboxylase gene affect learning in Drosophila. Proc. Natl. Acad. Sci. USA 81, 3577–3581.

Theis, M., Si, K., and Kandel, E.R. (2003). Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus. Proc. Natl. Acad. Sci. USA 100, 9602–9607.

Waddell, S. (2010). Dopamine reveals neural circuit mechanisms of fly memory. Trends Neurosci. 33, 457–464.

Wang, X.P., and Cooper, N.G. (2009). Characterization of the transcripts and protein isoforms for cytoplasmic polyadenylation element binding protein-3 (CPEB3) in the mouse retina. BMC Mol. Biol. 10, 109.

Wang, H., Hu, Y., and Tsien, J.Z. (2006). Molecular and systems mechanisms of memory consolidation and storage. Prog. Neurobiol. 79, 123–135.

White-Grindley, E., Li, L., Mohammad Khan, R., Ren, F., Saraf, A., Florens, L., and Si, K. (2014). Contribution of Orb2A stability in regulated amyloid-like oligomerization of Drosophila Orb2. PLoS Biol. 12, e1001786.

Wilson, M.A., and McNaughton, B.L. (1994). Reactivation of hippocampal ensemble memories during sleep. Science 265, 676–679.

Wise, R.A. (2004). Dopamine, learning and motivation. Nat. Rev. Neurosci. 5, 483–494.

Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M.A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J.R., and Richter, J.D. (1998). CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. Neuron 21, 1129–1139.

Wustmann, G., Rein, K., Wolf, R., and Heisenberg, M. (1996). A new paradigm for operant conditioning of Drosophila melanogaster. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 179, 429–436.

Yu, J.Y., Kanai, M.I., Demir, E., Jefferys, G.S., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr. Biol. 20, 1602–1614.
Synaptic Orb2A Bridges Memory Acquisition and Late Memory Consolidation in *Drosophila*

Sebastian Krüttner, Lisa Traunmüller, Ugur Dag, Katharina Jandrasits, Barbara Stepień, Nirmala Iyer, Lee G. Fradkin, Jasprina N. Noordermeer, Brett D. Mensh, and Krystyna Keleman
Supplemental Information

Figure S1 VT005526-LexA drives expression exclusively in the aSP13 dopaminergic neurons (Figure 2B and 2C)

Expression pattern of the VT005526-LexA line used for the activation and silencing experiments with LexAop-TrpA1 and LexAop-shi respectively, shown in the Figure 2B and 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 (Figure 6)

Head extracts from w+; tubGal80ts, UAS-orb2BGFP; orb2^{2RP_EBRBD} 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 (Figure 1)

| Genotype | Test | DA  | Train | n  | CI (%) mean±sd | 10%-ile 90%-ile | LI (%) mean±sd | P | P L1=L1n |
|----------|------|-----|-------|----|----------------|-----------------|----------------|---|-----------|
| 1        | Canton-S | 20 min | - | - | 57 | 52.3±26.3 | 50.0 | 0.00 | 0.000 | 0.000 |
| 2        | Canton-S | 20 min | - | + | 57 | 13.9±18.9 | 5.0 | 0.00 | 0.000 | 0.000 |
| 3        | Canton-S | 24 hrs | - | - | 54 | 53.4±25.4 | 60.0 | 12.50 | 0.000 | 0.000 |
| 4        | Canton-S | 24 hrs | - | + | 61 | 51.1±25.3 | 55.0 | 15.00 | 0.000 | 0.000 |
| 5        | Canton-S | 24 hrs | - | - | 54 | 53.4±25.4 | 60.0 | 12.50 | 0.000 | 0.000 |
| 6        | Canton-S | 24 hrs | + | - | 66 | 57.1±29.7 | 65.0 | 13.50 | 0.000 | 0.000 |
| 7        | Canton-S | 24 hrs | + | - | 66 | 57.2±29.7 | 65.0 | 13.50 | 0.000 | 0.000 |
| 8        | Canton-S | 24 hrs | + | + | 67 | 30.1±24.8 | 25.0 | 0.00 | 0.000 | 0.000 |
| 9        | Canton-S | 24 hrs | (w/ virgin) | + | - | 45 | 79.3±28.4 | 90.0 | 31.00 | 0.000 | 0.000 |
| 10       | Canton-S | 24 hrs | (w/ virgin) | + | + | 57 | 66.1±32.5 | 75.0 | 5.00 | 0.000 | 0.000 |

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 (H0: LI = 0) or for the null hypothesis that specific experimental and control males learn equally well (H0: LIx = LIy).

### Table S2 Post-learning thermogenetic activation of PAM-DA neurons (Figure 2A)

| Genotype | Time at 32°C | Train | n  | CI (%) mean±sd | 10%-ile 90%-ile | LI (%) mean±sd | P | P L1=L1n |
|----------|--------------|-------|----|----------------|-----------------|----------------|---|-----------|
| 1        | HL09-Gal4, UAS-TrpA1, 32°C | 2-4 | - | 34 | 82.8±29.5 | 100.0 | 20.00 | 0.000 | 0.000 |
| 2        | HL09-Gal4, UAS-TrpA1, 32°C | 2-4 | + | 36 | 71.6±34.3 | 90.0 | 8.50 | 0.000 | 0.000 |
| 3        | HL09-Gal4, UAS-TrpA1, 32°C | 4-6 | - | 34 | 82.5±27.5 | 95.0 | 29.00 | 0.000 | 0.000 |
| 4        | HL09-Gal4, UAS-TrpA1, 32°C | 4-6 | + | 36 | 71.4±30.1 | 75.0 | 25.00 | 0.000 | 0.000 |
| 5        | HL09-Gal4, UAS-TrpA1, 32°C | 6-8 | - | 36 | 76.0±24.0 | 80.0 | 33.50 | 0.000 | 0.000 |
| 6        | HL09-Gal4, UAS-TrpA1, 32°C | 6-8 | + | 34 | 65.7±30.2 | 70.0 | 25.00 | 0.000 | 0.000 |
| 7        | HL09-Gal4, UAS-TrpA1, 32°C | 8-10 | - | 35 | 83.1±21.9 | 95.0 | 52.50 | 0.000 | 0.000 |
| 8        | HL09-Gal4, UAS-TrpA1, 32°C | 8-10 | + | 36 | 44.2±33.5 | 40.0 | 5.00 | 0.000 | 0.000 |
| 9        | HL09-Gal4, UAS-TrpA1, 32°C | 10-12 | - | 34 | 58.5±29.6 | 55.0 | 22.50 | 0.000 | 0.000 |
| 10       | HL09-Gal4, UAS-TrpA1, 32°C | 10-12 | + | 34 | 64.7±36.0 | 80.0 | 7.50 | 0.000 | 0.000 |
Table S3 Post-learning thermogenetic activation of aSP13-DA neurons (Figure 2B)

| Genotype | Time at 32 °C | Train | n | CI (%) mean±sd | 10%-%ile 90%-%ile | LI (%) mean±sd | P LI=0 | P LI=LI |
|----------|---------------|-------|---|---------------|-----------------|----------------|---------|---------|
| 1       | TH-Gal4, FF, UAS-TrpAl, 32 °C | 8-10 | 36 | 67.4±27.2 | 77.5 | 20.00±96.50 | 0.001 | 0.000 |
| 2       | TH-Gal4, FF, UAS-TrpAl, 32 °C | 8-10 | 36 | 45.8±28.1 | 40.0 | 8.50±90.00 | 0.005 | 0.003 |
| 3       | VT005526-LexA, LexAop-TrpAl, 32 °C | 8-10 | 34 | 60.2±35.0 | 50.0 | 7.50±100.00 | 0.005 | 0.001 |
| 4       | VT005526-LexA, LexAop-TrpAl, 32 °C | 8-10 | 34 | 60.9±33.9 | 50.0 | 12.50±100.00 | 0.005 | 0.003 |
| 5       | VT005526-LexA, LexAop-TrpAl, 22 °C | 8-10 | 34 | 71.0±25.1 | 77.5 | 27.50±100.00 | 0.015 | 0.862 |
| 6       | VT005526-LexA, LexAop-TrpAl, 32 °C | 8-10 | 34 | 80.3±20.1 | 87.5 | 46.00±100.00 | 0.036 | 0.272 |
| 7       | TH-Gal4, 32 °C | 8-10 | 36 | 55.0±23.1 | 45.0 | 30.00±95.00 | 0.057 | 0.771 |
| 8       | TH-Gal4, 32 °C | 8-10 | 36 | 60.6±22.6 | 25.0 | -10.1±10.5 | 0.015 | 0.862 |
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_e = LI)

**Table S4 Post-learning thermogenetic silencing of aSP13-DA neurons (Figure 2C)**

| Genotype | Train | n  | CI (%) mean±sd | 10%-%ile | LI (%) mean±sem | P LI=0 | P LI_e=LI_e |
|----------|-------|----|----------------|-----------|-----------------|-------|-------------|
| 1        | VT005526-LexA, LexAop-shi°, 22°C | -  | 88.3±18.0 95.0  | 62.00 100  | 34.1±5.0 36.8  | 0.000 | 0.000       |
| 2        | VT005526-LexA, LexAop-shi°, 22°C | +  | 58.2±26.5 62.5  | 12.00 91.5  | 8.1±4.5 10.0  | 0.052 | 0.023       |
| 3        | VT005526-LexA, LexAop-shi°, 32°C | -  | 92.5±18.5 100  | 76.00 100  | 32.1±8.0 40.0  | 0.001 | 0.831       |
| 4        | VT005526-LexA, LexAop-shi°, 32°C | +  | 85.0±17.5 90.0  | 60.00 100  | 8.1±4.5 10.0  | 0.052 | 0.023       |
| 5        | LexAop-shi°, 32°C | -  | 68.7±25.3 75.0  | 33.00 95.0  | 32.1±8.0 40.0  | 0.001 | 0.831       |
| 6        | LexAop-shi°, 32°C | +  | 46.7±26.9 45.0  | 5.00 85.0  | 32.1±8.0 40.0  | 0.001 | 0.831       |
| 7        | VT005526-LexA, 32°C | -  | 60.9±17.5 65.0  | 35.00 85.0  | 32.1±8.0 40.0  | 0.001 | 0.831       |
| 8        | VT005526-LexA, 32°C | +  | 41.9±23.4 45.0  | 4.00 73.0  | 31.1±7.6 30.8  | 0.009 | 0.767       |

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_e = LI)

**Table S5 Post-acquisition inactivation of DopR1 after training for LTM (Figure 3A)**

| Genotype | Train | n  | CI (%) mean±sd | 10%-%ile | LI (%) mean±sem | P LI=0 | P LI_e=LI_e |
|----------|-------|----|----------------|-----------|-----------------|-------|-------------|
| 1        | Canton-S | -  | 78.7±21.5 85.0  | 45.00 100  | 36.8±5.0 47.1  | 0.000 | 0.000       |
| 2        | Canton-S | +  | 49.8±30.8 45.0  | 10.00 100  | 10.6±6.2 11.8  | 0.051 | 0.074       |
| 3        | DopR1°° | -  | 78.7±22.8 85.0  | 42.50 100  | 10.6±6.2 11.8  | 0.051 | 0.074       |
| 4        | DopR1°° | +  | 70.4±28.6 75.0  | 21.00 97.0  | 38.4±6.5 60.0  | 0.000 | 0.857       |
| 5        | DopR2°° | -  | 67.0±25.9 75.0  | 35.00 95.0  | 38.4±6.5 60.0  | 0.000 | 0.857       |
| 6        | DopR2°° | +  | 41.3±32.2 30.0  | 5.00 91.0  | 38.4±6.5 60.0  | 0.000 | 0.381       |
| 7        | DopR1°° | -  | 65.5±28.8 65.0  | 20.00 100  | 30.3±9.5 30.8  | 0.004 | 0.521       |
| 8        | DopR1°° | +  | 45.7±27.9 6.00  | 6.00 30.8  | 30.3±9.5 30.8  | 0.004 | 0.521       |
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 \( (H_0: LI_\alpha = LI_\beta) \)

**Table S6 Post-acquisition inactivation of DopR1 (Figure 3B)**

| Genotype       | DA | Train | n  | CI (%) mean±sd     | 10%-ile 90%-ile | LI (%) mean±sem median | \( P \) LI=0 | \( P \) LI\_\alpha=LI\_\beta |
|----------------|----|-------|----|--------------------|-----------------|------------------------|-------------|-----------------------------|
| 1 Canton-S     | +  | -     | 72 | 63.4±29.4 75.0     | 16.50           | 98.50                  | 0.177       | 0.632                       |
| 2 Canton-S     | +  | +     | 70 | 39.4±28.6 35.0     | 0.50            | 89.50                  | 0.000       | 0.000                       |
| 3 DopR1\_\text{imp} | +  | -     | 36 | 70.9±27.7 75.0     | 27.50           | 100                    | 0.572       | 0.632                       |
| 4 DopR1\_\text{imp} | +  | +     | 33 | 72.1±22.8 75.0     | 37.00           | 100                    | 0.000       | 0.000                       |
| 5 DopR2\_\text{imp} | +  | -     | 53 | 58.6±27.9 60.0     | 15.00           | 95.00                  | 0.000       | 0.000                       |
| 6 DopR2\_\text{imp} | +  | +     | 67 | 34.6±30.0 25.0     | 0.00            | 82.00                  | 0.000       | 0.893                       |
| 7 Canton-S     | +  | -     | 35 | 57.1±23.4 60.0     | 30.00           | 90.00                  | 0.000       | 0.675                       |
| 8 DopR2\_\text{imp} | +  | -     | 36 | 44.4±31.0 40.0     | 1.50            | 85.00                  | 0.000       | 0.000                       |
| 10 DopR2\_\text{imp} | +  | +     | 32 | 37.5±29.7 30.0     | 3.50            | 81.50                  | 0.181       | 0.000                       |

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 \( (H_0: LI_\alpha = LI_\beta) \)

**Table S7 LTM rescue with DopR1 in subsets of MB neurons (Figure 3C)**

| Genotype       | Train | n  | CI (%) mean±sd median | 10%-ile 90%-ile | LI (%) mean±sem median | \( P \) LI=0 | \( P \) LI\_\alpha=LI\_\beta |
|----------------|-------|----|-----------------------|-----------------|------------------------|-------------|-----------------------------|
| 1 UAS-DopR1; DopR1\_\text{imp} | -     | 31 | 82.0±23.4 90.0        | 55.00           | 100                    | 0.000       | 0.675                       |
| 2 UAS-DopR1; DopR1\_\text{imp} | +     | 33 | 77.1±22.0             | 42.50           | 60.0±5.0               | 0.177       | 0.000                       |

5
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-assay 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$_1$).

Table S8 orb2 mutant in courtship memory consolidation assay (Figure 4A)

| Genotype               | Train | DA | n  | CI (%) mean±sem | 10% -ile | 90% -ile | LI (%) mean±sem | P LI=0 | P LI$_n$=LI$_1$ |
|------------------------|-------|----|----|-----------------|----------|----------|-----------------|--------|-----------------|
| 1 Canton-S             | -     | +  | 36 | 58.5±2.1 | 55.0     | 10.00    | 100             | 0.000  | 0.261           |
| 2 Canton-S             | +     | +  | 35 | 27.9±25.2 | 25.0     | 0.00     | 62.00           | 52.3±8.5 | 0.000            |
| 3 orb20231O           | -     | +  | 36 | 76.0±23.0 | 80.0     | 33.50    | 100             | 3.2±6.8 | 0.330            |
| 4 orb20231O           | +     | +  | 33 | 73.5±19.3 | 75.0     | 45.00    | 98.00           | 6.2     | 0.209            |

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$_1$).

Table S9 Quantification of the Orb2 oligomers (Figure 4C)

| Genotype | Wt (-DA) | DopR1$^{Np}$ (-DA) | DopR2$^{Np}$ (-DA) | Wt (+DA) | DopR1$^{Np}$ (+DA) | DopR2$^{Np}$ (+DA) |
|----------|----------|-------------------|-------------------|----------|-------------------|-------------------|
| Mean Intensity | 358.83 | 316.12 | 229.34 | 6523.18 | 204.30 | 5546.77 |
| MI$_n$/MI$_{DA}$ | 1 | 0.88 | 0.64 | 18.18 | 0.79 | 15.46 |
WB signal corresponding to the Orb2-GFP oligomers (Fig. 4B) has been quantified using Fiji-ImageJ (Fig. 4C). 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 (Figure 6)**  

| Genotype | n | CI naïve (%) mean±sd | 10%-ile 90%-ile n | CI exp (%) mean±sd | 10%-ile 90%-ile n | LL (%) mean±sem | P  LI=0 | P  Li0=LI  |
|----------|---|-----------------------|-------------------|-------------------|-------------------|----------------|--------|-------------|
| 1 TubG80ts;orb2Q9Δ;UAS-Orb2A; MB247-Gal4 34 | 26.8±29.5 10.0 | 0 77.50 | 36 | 24.9±21.8 15.0 | 0.00 | 7.1±5.0 | 0.382 | 0.783 |
| 1 TubG80ts;orb2Q9Δ;UAS-Orb2AΔQ; MB247-Gal4 34 | 20.4±19.2 15.0 | 0 46.5 | 36 | 23.1±19.5 20.0 | 0.00 | -12.8±2.3 | -3.3 | 0.716 | 0.852 | 0.931 |
| 2 TubG80ts;orb2Q9Δ;UAS-Orb2A; MB247-Gal4 35 | 71.4±17.5 75.0 | 45.00 90.00 | 36 | 48.6±28.9 45.0 | 3.50 | 31.9±7.2 | 40.0 | 0.000 | 0.000 |
| 2 TubG80ts;orb2Q9Δ;UAS-Orb2AΔQ; MB247-Gal4 33 | 62.1±27.6 70.00 | 50.00 95.00 | 43 | 57.2±28.2 65.0 | 10.00 | 7.9±9.9 | 7.1 | 0.222 | 0.499 | 0.109 |
| 3 TubG80ts;orb2Q9Δ;UAS-Orb2A; MB247-Gal4 33 | 48.3±27.4 45.00 | 12.00 91.00 | 34 | 31.6±28.8 25.0 | 0.00 | 34.6±12.3 | 44.4 | 0.008 | 0.015 |
| 3 TubG80ts;orb2Q9Δ;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 | 0.046 | 0.049 |
| 4 TubG80ts;orb2Q9Δ;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 | 0.760 | 0.843 |
| 5 TubG80ts;orb2Q9Δ;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 | 0.744 |
| 5 TubG80ts;orb2Q9Δ;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;orb2Q9Δ;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;orb2Q9Δ;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;orb2Q9Δ;UAS-Orb2BRD; MB247-Gal4 18 | 54.2±25.8 50.0 | 19.50 95.00 | 36 | 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;orb2Q9Δ;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;orb2Q9Δ;UAS-Orb2BŘRD; MB247-Gal4 33 | 81.5±15.9 85.0 | 62.50 97.50 | 34 | 75.6±21.6 80.0 | 45.00 | 97.50 | 7.2±5.5 | 5.9 | 0.037 | 0.890 | 0.033 |
| 3’ TubG80ts;orb2Q9Δ;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;orb2Q9Δ;UAS-Orb2BŘRD; 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;orb2Q9Δ;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 | 0.296 | 0.099 |
| 4’ TubG80ts;orb2Q9Δ;UAS-Orb2BŘRD; 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 | 0.711 | 0.721 |
| 5’ TubG80ts;orb2Q9Δ;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 | 0.000 |
| 5’ TubG80ts;orb2Q9Δ;UAS-Orb2BŘRD; 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 | 0.277 | 0.076 |

Courtship indices of males of the indicated genotypes either trained for 7 hrs with a mated female (CI exp) or remaining alone (CI naïve), treated as indicated in Fig. 6 and tested in single-pair assays with mated females. P values determined by permutation test for the null hypothesis that learning equals 0 (H0: LI = 0) or for the null hypothesis that rescue flies with the wild type isofom learns equally well as rescue flies with the mutated isofom in the same conditions (H0: LIe = LI)
Table S11 Orb2 regulates translation of CaMKII (Figure 7A)

| Genotype   | 3'UTR       | Npc2a-3'UTR-RA (control) | CaMKII-3'UTR-RH |
|------------|-------------|--------------------------|-----------------|
|            | Orb2 wt     | Orb2RRM*                 | Ratio wt/RRM*   |
| FLuc/Rluc  | 16.183      | 14.680                   | 1.1023          |
|            | 16.272      | 15.319                   | 1.0622          |
|            | 45.179      | 43.740                   | 1.0329          |
|            | 42.370      | 44.147                   | 0.9597          |
|            | 6.380       | 4.599                    | 1.3992          |
|            | 7.629       | 7.367                    | 1.0355          |

| Genotype   | Ratio wt/RRM* | Mean intensity | SEM     | P(ftx=ft0) | P(ftx=ft24) |
|------------|---------------|----------------|--------|------------|-------------|
| FLuc/Rluc  | 1.1023        | 11.995         | 12.896 | 0.931      | 0.997       |
| FLuc/Rluc  | 1.0622        | 12.388         | 12.413 | 0.997      | 0.997       |
| FLuc/Rluc  | 1.0329        | 23.614         | 26.492 | 0.891      | 0.891       |
| FLuc/Rluc  | 0.9597        | 46.313         | 52.440 | 0.883      | 0.883       |
| FLuc/Rluc  | 1.3992        | 29.167         | 29.608 | 0.985      | 0.985       |
| FLuc/Rluc  | 1.0355        | 30.007         | 33.264 | 0.902      | 0.902       |
| Mean       | 1.098         | 14.852         | 21.392 | 0.694      | 0.694       |
| SEM        | 0.0630        | 0.0225         | 0.0164 | 0.0061     | 0.0164      |

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. 7A). 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 (Figure 7C)

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

| Genotype               | DA (hrs) | n  | Mean intensity | SEM | P(ftx=ft0) |
|------------------------|----------|----|----------------|-----|------------|
| + CamKII 3’UTR, Orb2ΔA | 0        | 6  | 21.96          | 4.3 |            |
| + CamKII 3’UTR, Orb2ΔA | 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. 7C. $P$ 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} × 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 63x 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 CaCl$_2$, 3mM MgCl$_2$, 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$_2$O$_2$. The brains were then rinsed with 0.1 M Na-cacodylate buffer, followed by OTOT enhancement (10 min cycles of 0.01% OsO$_4$ 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% OsO₄ 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 mm/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-Orb2 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 casAWM1/casAWMr primers and cloned into the pAMW-MCS linearised with Nhel/ApaI (Roche) to obtain pAMW-cassette. For pAMW-RLuc-cassette destination vector the Firefly luciferase PCR product obtained by amplifying pAC-Fluc-6XS with Fluc/Fluc primers was digested with SpeI/Nhel and cloned into Nhel 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 Rluc/Rluc primers and cloned SpeI/Nhel into Nhel cut vector. To create final pAMW-RLuc-SV403’UTR-polyA vector SV40 3’UTR was PCR amplified from pAMW with SV403UTR/SV403UTR 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% CO₂ 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 1ml/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 lystate 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.

Mastushita-Sakai, T., White-Grindley, E., Samuelson, J., Seidel, C., and Si, K. (2010). Drosophila Orb2 targets genes involved in neuronal growth, synapse formation, and protein turnover. Proc Natl Acad Sci U S A 107, 11987-11992.
Siwicki, K.K., and Ladewski, L. (2003). Associative learning and memory in Drosophila: beyond olfactory conditioning. Behav Processes 64, 225-238.
Yu, J.Y., Kanai, M.I., Demir, E., Jefferis, G.S., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr Biol 20, 1602-1614.