Drosophila ORB protein in two mushroom body output neurons is necessary for long-term memory formation

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Memory is initially labile and gradually consolidated over time through new protein synthesis into a long-lasting stable form. Studies of odor-shock associative learning in *Drosophila* have established the mushroom body (MB) as a key brain structure involved in olfactory long-term memory (LTM) formation. Exactly how early neural activity encoded in thousands of MB neurons is consolidated into protein-synthesis–dependent LTM remains unclear. Here, several independent lines of evidence indicate that changes in two MB vertical lobe V3 (MB-V3) extrinsic neurons are required and contribute to an extended neural network involved in olfactory LTM: (*i*) inhibiting protein synthesis in MB-V3 neurons impairs LTM; (*ii*) MB-V3 neurons show enhanced neural activity after spaced but not massed training; (*iii*) MB-V3 dendrites, synapsing with hundreds of MB α/β neurons, exhibit dramatic structural plasticity after removal of olfactory inputs; (*iv*) neurotransmission from MB-V3 neurons is necessary for LTM retrieval; and (*v*) RNAi-mediated down-regulation of oo18 RNA-binding protein (involved in local regulation of protein translation) in MB-V3 neurons impairs LTM. Our results suggest a model of long-term memory formation that includes a systems-level consolidation process, wherein an early, labile olfactory memory represented by neural activity in a sparse subset of MB neurons is converted into a stable LTM through protein synthesis in dendrites of MB-V3 neurons synapsed onto MB α lobes.

CPEB | CREB | fragile X mental retardation | STAU | PUM

Long-term memory (LTM) and long-term synaptic plasticity require de novo protein synthesis, which is regulated at transcriptional and/or translational levels in a synapse-specific manner (1–3). Synapse-specific plasticity during LTM formation in some contexts may involve local regulation of protein translation by a family of RNA-binding proteins, the cytoplasmic polyadenylation element-binding proteins (CPEBs) (2). Neuronal CPEBs have two conformational states. The inactive state predominates at low levels of CPEB expression and represses translation from nascent mRNAs. The active state is achieved either via a self-perpetuating prion-like state when expression levels surpass a threshold or via Ca^{2+}/calmoduline-dependent protein kinase II (CaMKII)-mediated phosphorylation, and translation is initiated by elongation of an mRNA's poly-A tail (4–6). In other species, CPEB1 has been shown to contribute to long-term facilitation or potentiation (5, 7). In *Drosophila*, oo18 RNA-binding protein 2 (ORB2) appears required for long-term memory formation after courtship conditioning (8, 9). Any role for ORB in fruit fly memory formation, however, remains unclear.

Drosophila can learn to associate an odor (conditioned stimulus, CS) with foot-shock punishment (unconditioned stimulus, US). This odor–shock association initially is labile, lasting for only about a day after one training session. With repetitive, spaced training (ST) sessions (rest intervals between each session), a protein synthesis-dependent, LTM is formed. With repetitive, massed training (MT) sessions (no rest intervals between sessions), LTM is not formed. The consolidation of odor–shock memories presumably involves multiple nodes in the underlying neuronal network, including antenna lobes (10), mushroom body (MB) (11–13), ellipsoid body (14), and two dorsal anterior lateral (DAL) neurons (15). The MB in each hemisphere consists of ~2,500 intrinsic neurons that can be classified into at least five major types: γ, α′/β′, pioneer α/β, early α/β, and late α/β (16). Surprisingly, inhibition of protein synthesis or disruption of cAMP response element binding protein 2 (CREB2) activity in MB neurons does not impair LTM; rather, these hallmarks of memory consolidation are required in DAL neurons (15). Exactly how early labile memory encoded in the MB neurons is converted into a stable LTM in DAL neurons and whether such experience-dependent transcription and translation during LTM formation involves additional neurons remain unclear. Here, we show that LTM formation requires CREB2-independent protein synthesis and ORB-dependent translational regulation in two MB output neurons.

Results

To address how early, neural activity in MB neurons is consolidated into a stable protein-synthesis–dependent LTM outside of MB, we searched individual MB output neurons for evidence of de novo protein synthesis after odor–shock training. Using targeted expression of a cold-sensitive $RICIN^{cs}$, a potent toxic protein that inactivates ribosomes at 30 °C but not 18 °C (15), we found that de novo protein synthesis in *E0067-*, *E1132-* and *G0239-Gal4* neurons after spaced training was necessary for LTM formation (Fig. 1 *A* and *B*). In control experiments, expression of inactivated $RICIN^{cs}$ after spaced training, or activated $RICIN^{cs}$ after massed training, did not affect 1-d memory (Fig. 1*B*). Two pairs of MB extrinsic neurons, called MB-V3 neurons (17), were common to the expression patterns of these three Gal4 lines (Fig. S1). In particular, *G0239-Gal4* expressed only in MB-V3 neurons (Fig. 1*A*) and, thus, was selected for further behavioral study.

Labeling MB-V3 dendrites with Down syndrome cell adhesion molecule fused with GFP (Dscam::GFP) established that they projected to the tip of the MB α lobe. Conversely, labeling MB-V3 axons with synaptotagmin fused with GFP (syt::GFP) showed that they projected to the superior dorsofrontal protocerebrum (Fig. 1*C*). To assess potential connections between MB-V3 dendrites and axons of MB neurons, we used the GFP reconstitution across synaptic partners (GRASP) technique (18). One half of the split-GFP GRASP reporter was expressed in the MB-V3 neurons, whereas the other half of the split-GFP

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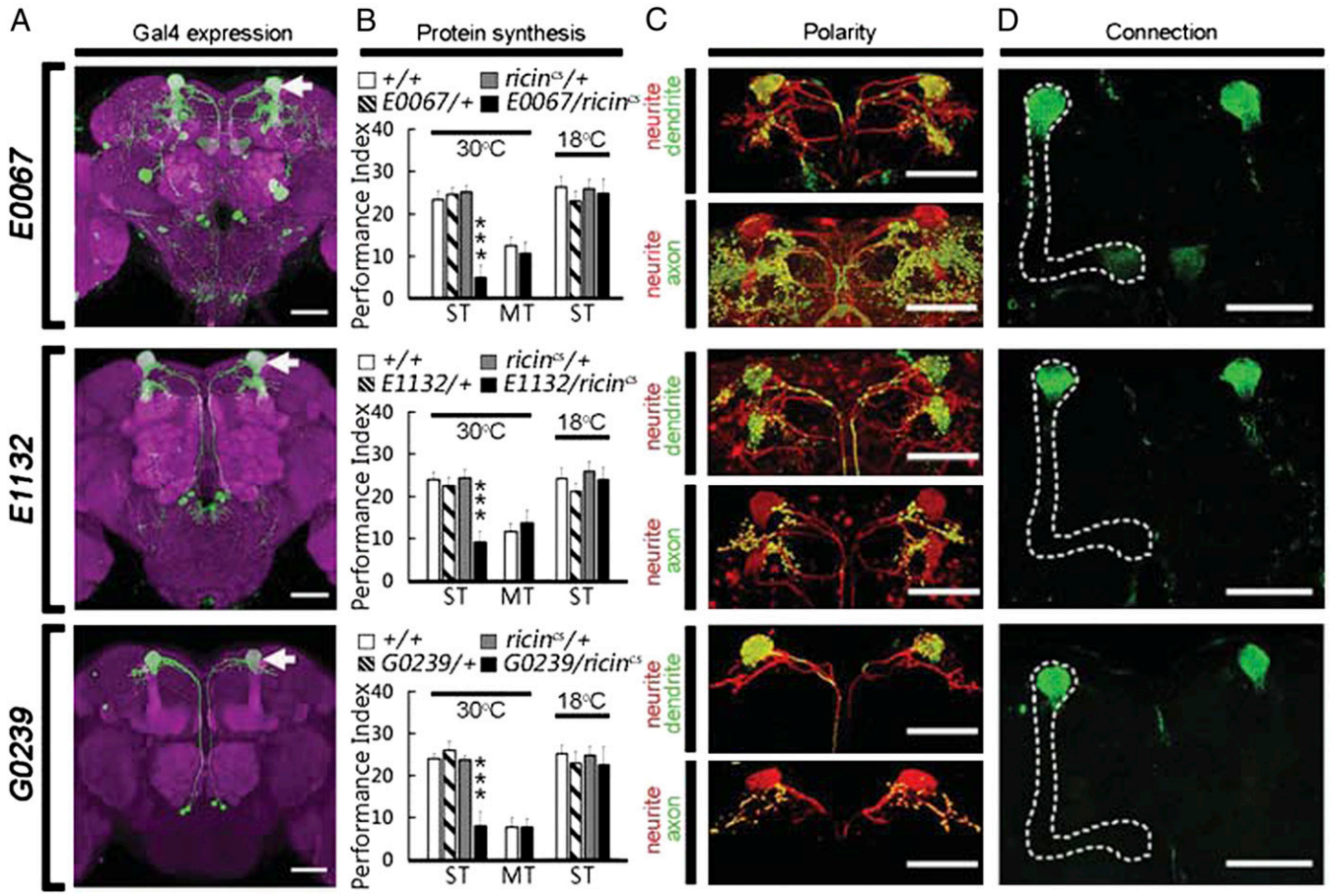

Fig. 1. Inhibiting protein synthesis in MB-V3 neurons impairs LTM formation. (*A*) Expression patterns of *E0067-*, *E1132-* and *G0239-Gal4s*. GFP labeled two pairs of MB-V3 neurons innervating α-lobe tips (arrow). Brain structures were immunostained with antidiscs large antibody (magenta). *E0067-* and *E1132-Gal4s* also expressed in brain surface glia (digitally removed). (Scale bars, 50 μm.) (*B*) Effects of RICIN^{cs} inhibition in *E0067-*, *E1132-*, and *G0239-Gal4* neurons. One-day memory is impaired by inhibiting protein synthesis after spaced training (ST), but not massed training (MT), with active RICIN^{cs} (30 °C). One-day memory after spaced training was normal with inactive RICIN^{cs} (18 °C). Values are means ± SEM (***$P < 0.001$; $n = 8$ for each group). (*C*) The MB-V3 neuron projects Dscam-positive dendrites (green) exclusively within the entire α-lobe tip and syt-positive axons (green) at superior dorsofrontal protcerebrum outside of the MB. Fibers are labeled by a monomeric Kusabira Orange (mKO) protein (red). (*D*) Structural connections between MB-V3 dendrites and axons of MB neurons (*L0124-LexA*, dotted line) were visualized by GRASP labeling (green, *Lower*). (Scale bars, 50 μm.) For more details, please see *SI Methods*.

GRASP reporter was expressed in distinct types of MB neurons using an appropriate LexA driver (Fig. S2). We found that MB-V3 dendrites are in close contact with axons of three types of MB α/β neurons, including pioneer, early, and late α/β neurons (Fig. 1*D* and Fig. S2). They do not make contact with MB α′/β′ neurons, however (Fig. 1*A*).

Next, we evaluated the role of neural activity from MB-V3 by temporally blocking neurotransmssion with *UAS-shi^{ts1}*, a temperature-sensitive mutant dynamin protein SHIBIRE that blocks neurotransmission at 30 °C but not at 18 °C (19). Blocking neurotransmssion from MB-V3 neurons during LTM retrieval impaired 1-d memory after spaced training but not after massed training (Fig. 2*A*). Intriguingly, normal 24-h memory retention required neurotransmission from MB-V3 neurons only during the second, but not the first or the third, 8-h period after spaced training. This requirement for synaptic transmission 8–16 h after spaced training was specific to LTM formation, because the same disruption did not affect 1-d memory after massed training (Fig. 2*A*) and was corroborated with an independent MB-V3–expressing Gal4 driver, *E0067-Gal4* (Fig. S3). Synpatic transmission from MB-V3 neurons also was not required for learning or for 3-h memory after a single training session (Fig. 2*B*). Together, these data establish that neural activity from MB-V3 is required specifically for LTM consolidation and retrieval.

The GRASP technology can signal only close proximity between neurons (18), so we confirmed direct synaptic connectivity using EM labeling. A membrane-fused horseradish peroxidase (HRP::CD2) was expressed in the pioneer α/β MB neurons and/or in MB-V3 neurons. We found that small HRP-positive MB-V3 dendrites synpased with HRP-positive MB neurons peripherally at the tip of the α lobe and with large HRP-negative boutons at the core α lobe (Fig. S4 *A*–*E*). These EM observations suggest a direct synaptic connection between MB-V3 dendrites and axonal terminals of several different types of α/β MB neurons. Blocking neurotransmission from the pioneer α/β neurons in *c708a-Gal4* impaired 24-h memory during retrieval, but not during consolidation (Fig. S4*F*). Also, neurotransmission outputs from core α/β neurons (20) and MB-V3 neurons are both necessary during a delayed time period after spaced training for LTM formation. Altogether, these data show direct structural connectivity and are suggestive of potential functional connectivity between α/β MB neurons and MB-V3 neurons.

To visualize functional responses to conditioned odors in MB-V3 dendrites synapsing at the tip of α lobe, we used the calcium-sensitive fluorescent protein, UAS-GCaMP1.6 (21). In naïve flies, MB-V3 neurons (Fig. 2*C*), but not ellipsoid body neurons (Fig. S5), showed strong responses to eight different odors [3-octanol (OCT), 4-methylcyclohexanol (MCH), benzaldehyde (BA), ethyl

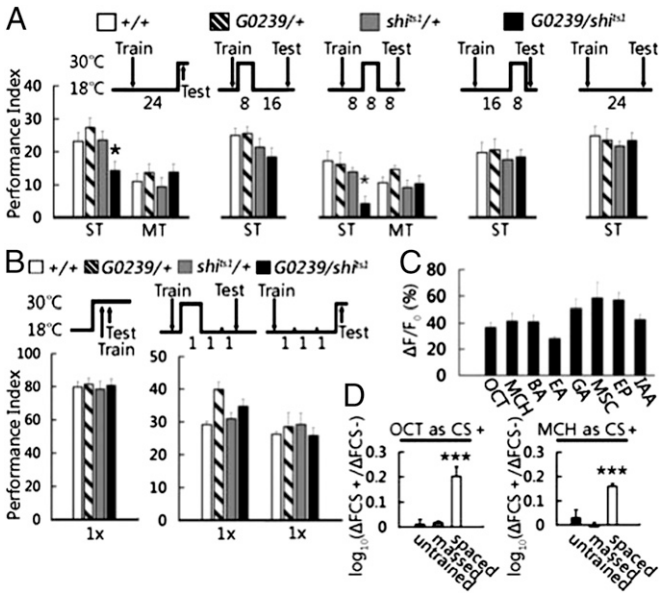

Fig. 2. Neurotransmission and functional response of MB-V3 neurons during LTM formation. (*A*) Roles of MB-V3 neurotransmission on LTM. Blocking neurotransmission from MB-V3 neurons with temperature sensitive shi^{ts1} protein (30 °C) during retrieval ($P = 0.015$; $n = 12$) or 8–16 h after training ($P = 0.012$; $n = 8$) impaired 1-d memory after spaced training, but not after massed training. Blocking neurotransmission during 0–8 or 16–24 h after spaced training or keeping the shi^{ts1} flies in permissive temperature (18 °C) had no effect on 1-d memory. (*B*) Blocking neurotransmission from MB-V3 neurons during acquisition or 3 h after a single training session had no effects on memory retention. Values are means ± SEM ($n = 8$ for each group). (*C*) Neural activity in MB-V3 neurons in response to eight different odors (OCT, MCH, BA, EA, GA, MSC, EP, and IAA). Values are means ± SEM (n ≧ 8 for each odor). (*D*) Enhanced neural activity in MB-V3 neurons in response to conditioned odors after spaced, but not massed, training. Values are means ± SEM ($P < 0.001$; n ≧ 8 for each group). For more details, please see *SI Methods*.

acetate (EA), geranyl acetate (GA), methyl salicylate (MSC), ethyl propionate (EP), and isoamyl acetate (IAA)]. Thus, the MB-V3 neurons appear to be odor generalists. In response to conditioned odors (using either OCT or MCH), MB-V3 neurons exhibited an elevated GCaMP intensity 24 h after spaced but not after massed training compared with naïve (untrained) flies (Fig. 2*D*).

The observations that (*i*) MB-V3 neurons respond generally to odors and (*ii*) show an experience-dependent increase in neural activity prompted us to ascertain whether these neurons were capable of experience-dependent structural changes, a cellular mechanism often attributed to LTM formation (22–25). In naïve flies, MB-V3 neurons showed intact morphology (Fig. S6 *A*, *E*, and *I*). About 3 wk after removal of both antennae from newly eclosed adults, in contrast, some MB-V3 neurons showed dramatic changes in morphology, including unrestricted dendrite arborization (Fig. S6 *B* and *G*), cell body displacement (Fig. S6 *C* and *H*), extended dendrite innervation further into the α lobe (Fig. S6*C*) and extra branching to innervate the tip of α lobe (Fig. S6*D*), whereas structural changes in axons appeared relatively minor (Fig. S6 *F–H*). In the *alpha-lobe-absence* (*ala*) mutant, MB-V3 neurons failed to arborize in the absence of MB vertical lobes but grew normally in the absence of MB horizontal lobes (Fig. S6 *J–L*). Thus, a previous conclusion that LTM must reside within the MB because LTM was impaired in the *ala* mutant (11) now can be reinterpreted: Disconnection of MB-V3 neurons from the α lobes prevents the proper induction of protein synthesis within MB-V3 neurons (Fig. 1) during LTM formation. We also tried to investigate if any morphological change in MB-V3 neurons could be detected after conditioning, but as expected any putative dendritic plasticity induced by spaced training was too subtle to visualize (Fig. S6 *M* and *N*). Together, these results indicate that MB-V3 neurons are specialized for olfactory information processing, exhibit experience-dependent structural plasticity, and are required for LTM consolidation and retrieval.

LTM formation requires the transcription factor, CREB, and the *N*-methyl-D-aspartate (NMDA) receptor in many animals, including *Drosophila* (12, 14, 26). Recently, we showed that down-regulation of either CREB2 or NMDA receptor in two DAL neurons is sufficient to impair LTM, which is consistent with our finding that LTM is impaired when protein synthesis is inhibited in DAL neurons (15). Unexpectedly, we found that (*i*) over-expressing a CREB repressor protein (*UAS-dcreb2-b*; Fig. 3*A*) or (*ii*) knockdown of CREB2 with *UAS-creb2*^{RNAi} (Fig. 3*B*) or (*iii*) of NMDA receptor with *UAS-dsNR2*;*UAS-dsNR1* in the MB-V3 neurons did not impair 1-d memory after spaced training (Fig. 3*C*). Also, 1-d memory after spaced training remains intact after RNAi-mediated knockdown of *Drosophila* ORB2 in MB-V3 neurons (Fig. 3*D*), even though inhibition of protein synthesis in these neurons impairs LTM (Fig. 1). In contrast, RNAi-mediated knockdown in the MB-V3 neurons of either *Drosophila* ORB (Fig. 3 *E* and *F* and Figs. S7 and S8), or CaMKII (Fig. 3*G* and Fig. S8), which regulates CPEB phosphorylation bidirectionally (6), impaired 1-d memory after spaced, but not massed, training.

We have previously shown that in vivo disruptions of fragile X mental retardation (FMR), STAUFEN or PUMULIO proteins, additional molecular machinery involved in local control of mRNA translation, also yield defective LTM (27, 28). Using targeted RNAi-mediated knockdown in the MB-V3 neurons, we found that disruptions of FMR (Fig. 3 *H* and *I*), STAUFEN (Fig. 3*J*), or PUMULIO (Fig. 3*K*) protein again impaired 1-d memory after spaced training, but not after massed training. These transgenic flies nonetheless exhibited normal learning (Fig. S9), implying that acquisition and the sensorimotor responses that subserve it are normal. The efficiency of RNAi knockdown was determined via immunostaining; protein expressions were significantly decreased in the targeted MB-V3 neurons but not in other brain regions (Fig. S8). The specificity of RNAi knockdowns was addressed by misexpressing the RNAi transgenes in mushroom body M3 (MB-M3) neurons, a different set of MB extrinsic neurons required for 3-h memory (29); 1-d memory after spaced training was normal in these cases (Fig. S10). Together, these results suggest that ORB-dependent protein synthesis might be regulated by mRNA translation in MB-V3 neurons, a process quite different from the CREB-dependent regulation of transcription in DAL neurons.

Discussion

Drosophila MB-V3 neurons are similar to honey bee pedunculus extrinsic (PE1) neurons, which are considered to act as the conditioned response neurons when training increases activity in the output synapses of simultaneously activated Kenyon cells (30). Structurally, MB-V3 and PE1 neurons both are MB efferent neurons connecting many Kenyon cells in the α lobe, which is crucial for LTM formation, to the superior dorsofrontal protocerebrum. Functionally, both types of neurons are responsive to multiple odors and show enhanced neural activity to conditioned odor after repetitive training. During olfactory conditioning, an odor CS signal is encoded in sparse subsets of MB neurons (31), whereas the aversive US signal is delivered to most, if not all, Kenyon cells via dopaminergic neurons (29, 32, 33). The striking dendritic plasticity observed after antennal removal (Fig. S6) suggests that MB-V3 neurons are integrally involved in processing information from antennal sensory inputs. A previous model suggests that MB-V3 neurons act as odor generalists that receive synapses from all three types of α/β Kenyon cells (Fig. S2) and respond to all tested odors (Fig. 2*C*). Conditioning to

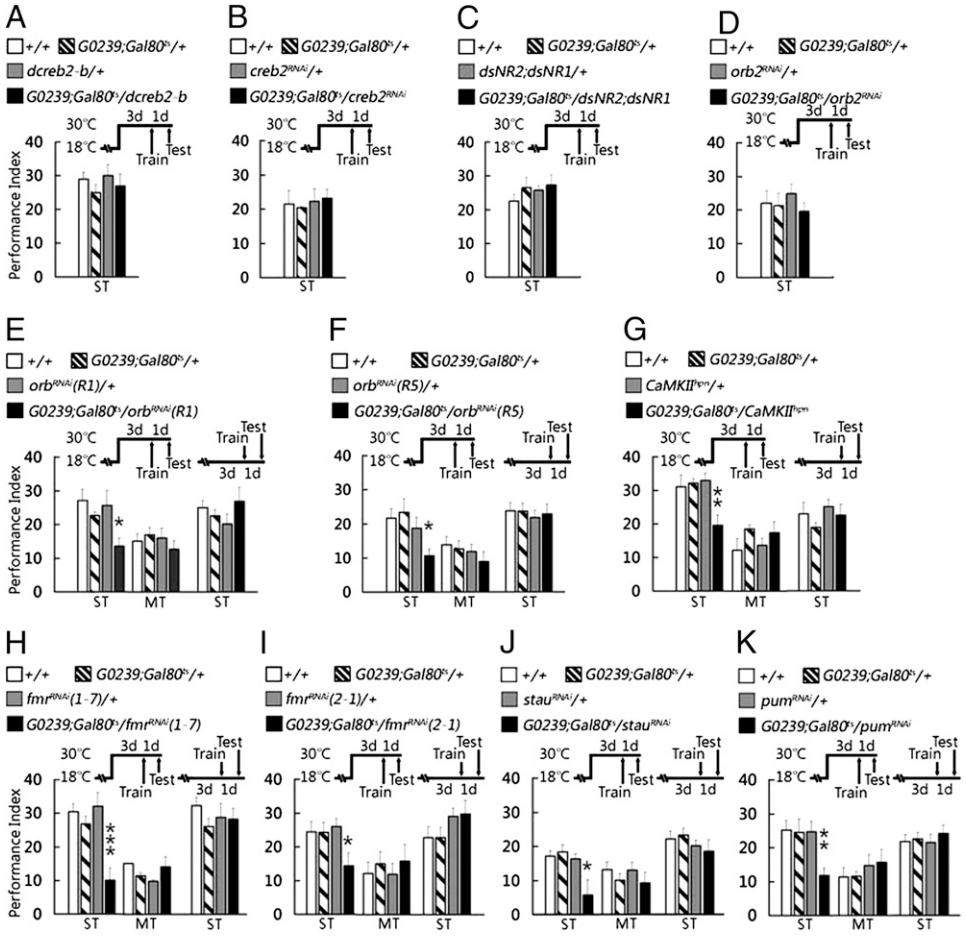

Fig. 3. Molecular machinery for LTM formation in the MB-V3 neurons. The *G0239-Gal4*-driven *UAS-RNAi* constructs (or CREB repressor) were inhibited by activated Gal80^{ts} (18 °C) throughout development and then were expressed by inactivating Gal80^{ts} (30 °C) 3 d before training. Control flies were kept constantly at 18 °C (activated Gal80^{ts}). (*A–D*) Induced knockdown of CREB with either *UAS-dcreb2-b* transgenic overexpression (*A*) or *UAS-creb2^{RNAi}* (*B*), of NMDAR1 and NMDAR2 with *UAS-dsNR2;UAS-dsNR1* (*C*), or of ORB2 (*D*) with *UAS-orb2^{RNAi}* did not affect 1-d memory after spaced training. (*E–K*) In contrast, 1-d memory was impaired after spaced, but not massed, training with induced knockdown of ORB with *UAS-orb^{RNAi}(R1)* ($P = 0.013$) (*E*) or *UAS-orb^{RNAi}(R5)* ($P = 0.01$) (*F*), of CaMKII with *UAS-CaMKII^{hpn}* ($P = 0.003$) (*G*), of FMR with *UAS-fmr^{RNAi} (1–7)* ($P < 0.001$) (*H*) or *UAS-fmr^{RNAi} (2–1)* ($P = 0.011$) (*I*), of STUAFEN with *UAS-stau^{RNAi}* ($P = 0.015$) (*J*) or of PUMILIO with *UAS-pum^{RNAi}* ($P = 0.009$) (*K*). Values are means ± SEM ($n \geqq 8$ for each group). For more details, please see *SI Methods*.

specific odors then induces increased neural activity and de novo protein synthesis (Fig. 1), rendering MB-V3s as "conditioned response neurons" that anticipate the US (30).

A wealth of evidence suggests that aversive olfactory memory exists as a persistent neural activity ("trace") in a sparse subset of MB neurons—initially in the γ lobes lasting for several minutes and then spreading to α/β lobes for several hours (34–36). This physiological trace drives a protein-synthesis–dependent process in postsynaptic MB-V3 neurons, yielding structural change at MB::MB-V3 synapses (Fig. 4*A*). Recent advances in several animal models have suggested two distinct components of synaptic capture that stabilize long-term functional and structural changes at specific synapse: one requires CREB-regulated transcription and NMDA receptor/PKA activities, whereas the other requires CPEB-regulated translation and local protein synthesis (3). Our results suggest that the latter molecular mechanism might occur in MB-V3 neurons (Fig. 4*B*). LTM was normal after disruption of CREB2 or NMDA but was impaired after down-regulation of ORB (a CPEB variant that lacks the "prion domain") and several RNA-binding proteins (RBPs), including FMR, STAUFEN, and PUMULIO that suppress and stabilize mRNA distributed among synapses (Fig. 3). LTM also was impaired by down-regulation of CaMKII (Fig. 3*G*), which contains in its 3′ UTR *cis*-acting localization elements for RBPs and prevents their degradation and transportation (2, 10, 27, 37, 38). Consequently, local protein synthesis at the MB-V3 dendrites is capable of providing a mechanism permitting rapid synaptic changes in response to presynaptic CS/US signals. Interestingly, despite the role for ORB2 (a CPEB variant that contains the prion domain) in long-term courtship memory (8, 9), our results showed that down-regulation of ORB2 in MB-V3 neurons did not affect olfactory LTM (Fig. 3*D*). These observations imply that different CPEB proteins (i.e., ORB and ORB2) may function in different neurons underlying different forms of long-term memory, although our negative results cannot completely exclude ORB2 function in MB-V3 neurons.

Our results suggest that spaced training induces coincident neural activity between subsets of MB axons and MB-V3 dendrites. This coincident synaptic activity disinhibits translation of nascent mRNAs, presumably contained in neural granules along with ORB, FMR, PUMILIO, and STAUFEN, in a CaMKII-dependent fashion (38). A new memory trace then emerges from the functional and structural changes induced at these coincident MB::MB-V3 synapses. Thus, a single MB-V3 neuron is capable of performing selective synaptic plasticity similar to the glomerulus-specific plasticity of a single olfactory local neuron in the antenna lobe (39). Our model also predicts that a different subset of MB-V3 dendrites will be used to encode conditioned

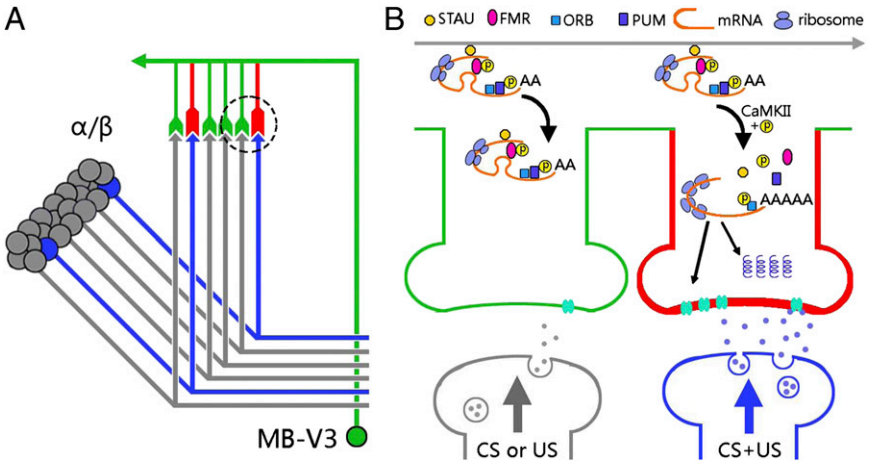

Fig. 4. A model of local mRNA translation in *Drosophila* olfactory memory. (*A*) Schematic representation of memory transformation from physiological coding in sparse α/β Kenyon cells (blue) to structural coding (red) in preexisting postsynaptic components of a MB-V3 neuron. (*B*) At activated MB-V3 synapses (red), presynaptic CS+US signals (blue), but not CS alone or US alone (gray), an CREB-independent mechanism relieves the translational block of RNA granules, which involves (*i*) STAUFEN detachment (38, 41), (*ii*) FMR and PUMILIO dephosphorylations (38, 42, 43), and (*iii*) ORB phosphorylation by activated CaMKII, leading to the poly-A tail elongation (4–6). Consequently, these events recruit additional ribosomes to up-regulate mRNA translation of LTM-related proteins (42), which eventually lead to an enhanced response to the CS+. Note that the subcellular locations of proteins and their cofunctions in the model have not been experimentally determined.

responses for different odors. Several obvious gaps remain: (*i*) Are these translation-related molecules located in postsynaptic compartment in MB-V3 neurons, and do they function together during LTM formation? (*ii*) What effector proteins are synthesized during LTM consolidation? (*iii*) Are they causally related to the observed increase in neural activity in MB-V3 neurons during LTM retrieval? Elucidation in MB-V3 neurons of this differential synaptic coding—and the neurotransmitters involved—promises to reveal further how synaptic plasticity encodes in memory consolidation and retrieval at a systems level (Fig. 2).

By identifying specific neuronal types involved in olfactory long-term memory formation in *Drosophila*, we have begun to deconvolute two molecular mechanisms, regulation of transcription and regulation of translation, both of which have been implicated in several model systems. Previously, we have shown that CREB-dependent transcription is involved with LTM formation in DAL neurons, whereas here we show instead that ORB-dependent translation is involved with LTM formation in MB-V3 neurons. Thus, two molecular mechanisms of protein synthesis may be partitioned into different neurons outside of MB but nonetheless within a common memory circuit.

MB-V3 and DAL neurons also appear to play different roles at the systems level. The former encodes odor specificity among the synapses connecting to MB (Fig. 4), whereas the latter is recruited to the memory circuit presumably to strengthen a conditioned response. Interestingly, this notion predicts that LTM is likely to be enhanced by activation of DAL but not MB-V3 neurons. It remains to be addressed whether MB-V3 neurons connect directly or indirectly to DAL neurons (15), which then might serve to form a neural circuit loop with MB during LTM retrieval. Alternatively, MB-V3 neurons may modulate presynaptic MB activity directly through retrograde feedback, as in the *Aplysia* model system (40).

Methods

Fly Strains and Behavior. Fly stocks were maintained on standard corn meal/yeast/agar medium at 25 ± 1 °C or 18 ± 1 °C and 70% relative humidity on a 12h:12h light:dark cycle. Memory behaviors were performed as previously described (15). For detailed fly strains, please see *SI Methods*.

In Vivo GCaMP Imaging. In vivo GCaMP imaging is as previously described in Wang et al. (1). Please see *SI Methods* for more details.

Statistics. Raw data were analyzed with SigmaPlot 10.0 and SigmaStat 3.5. The data were evaluated via one-way ANOVAs followed by planned comparisons among the relevant groups with a Tukey's honestly significant difference test. The *P* values were evaluated via one-way ANOVAs.

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