Supplementary Information for

CREBB repression of protein synthesis in mushroom body gates long-term memory formation in *Drosophila*

Hsuan-Wen Lin[a,1], Chun-Chao Chen[a,1,2], Ruei-Yu Jhang[a,b], Linyi Chen[a,b], J. Steven de Belle[a,c,d,e,f], Tim Tully[a] and Ann-Shyn Chiang[a,g,h,i,j,k,2]

[a]Brain Research Center, National Tsing Hua University, Hsinchu 30013, Taiwan;

[b]Institute of Systems Neuroscience and Department of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan;

[c]School of Life Sciences, Arizona State University, Tempe, AZ 85281;

[d]Department of Psychological Sciences, University of San Diego, San Diego, CA 92110;

[e]School of Life Sciences, University of Nevada, Las Vegas, NV 89154;

[f]MnemOdyssey LLC, Escondido, CA 92027;

[g]Institute of Systems Neuroscience and Department of Life Science, National Tsing Hua University, Hsinchu 30013, Taiwan

[h]Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

[i]Institute of Molecular and Genomic Medicine, National Health Research Institutes, Zhunan 35053, Taiwan

[j]Graduate Institute of Clinical Medical Science, China Medical University, Taichung 40402, Taiwan;

[k]Kavli Institute for Brain and Mind, University of California at San Diego, La Jolla, CA

92093-0526, USA

[1]H.-W.L. and C.-C.C. contributed equally to this work.

[2]Correspondence: chenchunchao@gapp.nthu.edu.tw or aschiang@life.nthu.edu.tw

This PDF file includes:

Supplementary text

Figures S1 to S6

Tables S1 to S2

SI References

Materials and Methods

In this report we used an automated olfactory aversive learning task (1) and assessed LTM after RNAi knockdown or expression of transgenes in these temporally and spatially restricted domains to identify the subsets of neurons critical for this task. We evaluated training-responsive CREBB or 5-HT1A expression with confocal microscopy using a *Gal4*-targeted UV-sensitive KAEDE reporter system (2). In various experiments, flies were fed CXM to provide a systemic level of protein synthesis inhibition.

Fly Strains

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 12:12-h light:dark cycle. All genotypes and sources are listed in *SI Appendix,* Table S2.

Behavior

Olfactory associative learning was evaluated by training 6- to 7-day-old flies in a T-maze apparatus with a Pavlovian olfactory conditioning procedure (3) as described previously (2, 4, 5). All experiments were conducted in the dark in an environment-controlled room at the required temperatures and 70% relative humidity during ZT4 – 8 (10:00 AM – 14:00 PM). The odors used were 3-octanol (OCT) and 4-methylcyclohexanol (MCH). Each experiment consisted of two groups of approximately 100 flies, each of which was conditioned with one of the two odors. Flies were exposed sequentially to two odors that were carried through the training chamber in a current of air (odors were bubbled at 750 ml/min). In a single training session, flies first were exposed for 60 s to the conditioned stimulus (CS^+), during which time they received the unconditioned stimulus (US), which consisted of 12 1.5-s pulses of 60 V dc electric shock presented at 5-s interpulse intervals.

After the presentation of the CS^+ condition, the chamber was flushed with fresh air for 45 s. Then flies were exposed for 60 s to the unpaired CS^-. To evaluate memory retention immediately after single-session training (acquisition), flies were gently tapped into an elevator-like compartment immediately after training. After 90 s, the flies were transported to the choice point of a T-maze, in which they were exposed to two converging currents of air (one carrying OCT, the other MCH) from opposite arms of the maze. Flies were free to choose between and walk toward the CS^+ and CS^- for 120 s, at which time they were trapped inside the respective arms of the T-maze (by sliding the elevator out of the register), anesthetized and counted. Flies that chose to avoid the CS^+ ran into the T-maze arm containing the CS^-, whereas flies that chose to avoid the CS^- ran into the arm containing the CS^+. For each experiment, a performance index $(PI_{1,2}) = (N_{CS-} - N_{CS+})/(N_{CS-} + N_{CS+})$ was calculated and averaged over these two complementary experiments, with the final PI $= (PI_1 + PI_2)/2$. Averaging of the two reciprocal scores eliminated any potential biases originating from the machine, naïve odor preferences, or non-associative changes in olfaction. For 24-h memory experiments, flies were subjected to single-session training, training massed together without rest, or training spaced out with 15-min rest intervals. For these training protocols, robotic trainers were used. All genotypes were trained and tested in parallel and rotated among all of the robotic trainers to ensure a balanced experiment. The genetic backgrounds of all fly strains were equilibrated to the "Canton" wild-type background by five or more generations of backcrossing. In *tub-Gal80^{ts}* experiments, flies raised at 18 °C were transferred to 30 °C for at least five days before the experiments.

Pharmacological treatment

To block protein synthesis, flies were fed 35 mM cycloheximide (Sigma) in 5% glucose 1 day before training until immediately before the test (1).

crebB and _5-HT1A_ promoter constructs

To engineer the *5-HT1A* promoter construct, polymerase chain reaction (PCR) was performed using genomic DNA from the wild-type *Canton-S* w^{1118} (iso1CJ) fly line as the template together with the forward primer 5′AGTGACGGCCGTATTTGATGCTCGACATGGC 3′ and the reverse primer 5′AGTGAGGTACCTTTGTGGATACTCGGTGTGTTTTTT 3′. A 5.2-kb PCR product was generated and inserted into the TA vector. Subsequent sub-cloning was performed to insert the 5.2-kb promoter region into a specific insertion vector, pBPGAL4.2Uw-2, via *Aat*II (5′) and *Kpn*I sites (3′). The promoter construct was injected into $attP^{40}$-containing fly strains to obtain the transgenic fly lines. To engineer the *creb2* promoter construct, polymerase chain reaction (PCR) was performed using genomic DNA from the wild-type *Canton-S* w^{1118} (iso1CJ) fly line as the template together with the forward primer 5′GAAAAGTGCCACCTGCTGCATGTCTACCAACAGTTCGAG 3′ and the reverse primer 5′CCGGATCTGCTAGCGGTTCCAGCTGCTGTCTGTATGAC 3′. A 11.6-kb PCR product was generated and inserted into the pBPGAL4.2Uw-2 vector which was digested with AatII and KpnI using In-Fusion® cloning system (Clontech). The promoter construct was injected into $attP^{40}$-containing fly strains to obtain the transgenic fly lines.

KAEDE measurement

KAEDE is a photoconvertible green fluorescent protein, irreversibly changing its structure to a red fluorescent protein upon ultraviolet irradiation (6). Taking advantage of circadian transcription and protein synthesis in the lateral clock neurons, we previously validated *de*

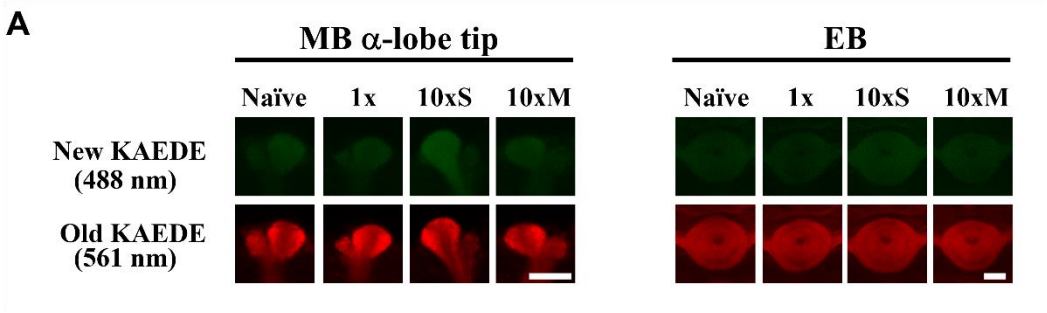
novo KAEDE synthesis in *per-Gal4>UAS-kaede* flies, in which it faithfully reports the cyclic transcriptions of the *period* gene. Feeding cycloheximide also suppressed green KAEDE synthesis, while not affecting the already-converted red KAEDE (2). To measure the amount of newly synthesised KAEDE in MB neurons, we used procedures adapted from a previous study (2). Briefly, pre-existing KAEDE proteins were photoconverted into red fluorescent proteins by 365–395 nm UV irradiation generated from a 120-W mercury lamp. For behavioral testing, approximately 15–20 flies kept in a clear plastic syringe were directly exposed to UV light at a distance of 5 cm for 1 h. Individual neurons expressing KAEDE were directly visualized through an open window in the fly's head capsule. Living samples were used because the signal-to-noise ratio of green to red KAEDE is greatly reduced after chemical fixation. KAEDE neurons were located in less than 5 s by a fast pre-scanning of red KAEDE excited by a 561-nm laser, to avoid unnecessary fluorescence quenching of green KAEDE during repeated scanning. A single optical slice through the MB α-lobe tip was imaged at a resolution of 1024×1024 pixels under a confocal microscope with a 40× C-Apochromat water-immersion objective lens (N.A. value 1.2, working distance 220 μm). All brain samples in the experiment were imaged with the same optical settings maximized for green and red KAEDE immediately before and after photoconversion, respectively. In all cases, both green KAEDE (excited by a 488-nm laser) and red KAEDE (excited by a 561-nm laser) were measured. By using the amount of red KAEDE as an internal standard to calibrate individual variation, we calculated the rate of increase in green KAEDE synthesis after photoconversion with the formula $(\Delta F) = \%(Ft_1 - \text{average } Ft_0)/\text{average } Ft_0$, where Ft_1 and Ft_0 are the ratios of the averaged intensities of green (G) to red (R) KAEDE (Gt_0/Rt_0) immediately after photoconversion (t_0) and at a later specific time point (t_1), respectively.

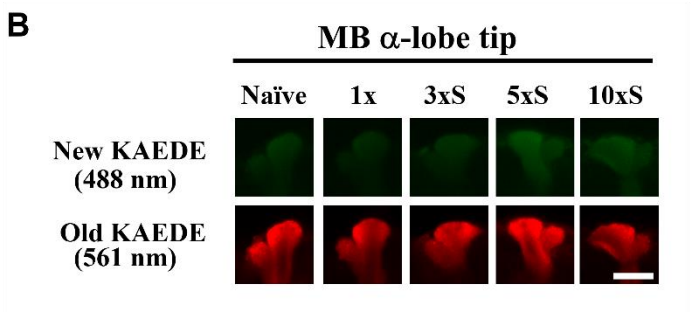
Immunohistochemistry

Brains were dissected in phosphate-buffered saline (PBS), fixed with a commercial microwave oven (2,450 MHz, 1100 Watts) in 4% paraformaldehyde on ice for 60 s three times, and then immersed in 4% paraformaldehyde with 0.25% Triton X-100 for 60 s three times. After being washed in PBS for 10 min at room temperature, brain samples were incubated in PBS containing 2% Triton X-100 (PBS-T) and 10% normal goat serum, and then degassed in a vacuum chamber to expel tracheal air for four cycles (depressurizing to –70 mmHg and then holding for 10 min). Next, brain samples were blocked and penetrated in PBS-T at 4 °C overnight, and then incubated in PBS-T containing 1:40 mouse 4F3 anti-DLG antibody (Developmental Studies Hybridoma Bank, University of Iowa) to label Disc large proteins at 4 °C for 1 day. Samples were subsequently washed in PBS-T three times and incubated in PBS-T containing 1:200 biotinylated goat anti-mouse IgG (Molecular Probes) as the secondary antibody at 25 °C for 1 day. Brain samples were then washed and incubated with 1:500 Alexa Fluor 635 streptavidin (Molecular Probes) at 25 °C for 1 day. Finally, after extensive washing, immunolabeled brain samples were directly cleared for 5 min in *FocusClear*, an aqueous solution that renders biological tissue transparent (7), and mounted between two cover slips separated by a spacer ring with a thickness of ~200 μm. Sample brains were imaged under a Zeiss LSM 780 or 880 confocal microscope with a 40× C-Apochromat water-immersion objective lens (N.A. value 1.2, working distance 220 μm).

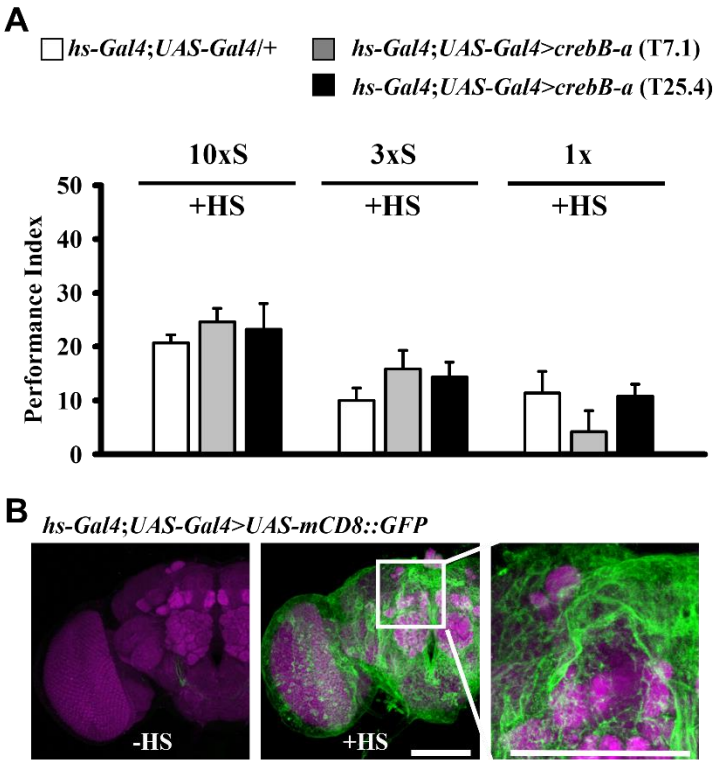
Statistics

Raw data were analyzed parametrically with SigmaPlot 10.0 and SigmaStat 3.5 statistical software. All the data including the behavior Performance Index (PI) or KAEDE image (ΔF) were evaluated via unpaired *t*-test (two groups) or one-way analysis of variance (ANOVA) (> two groups). Data were evaluated with the Mann-Whitney Rank Sum Test in cases of unequal variances. Data in all figures are presented as the mean ± SE. Experiments were replicated using multiple *Gal4* drivers with equivalent expression patterns, and multiple effector genes and reagents that impact shared cellular functions.



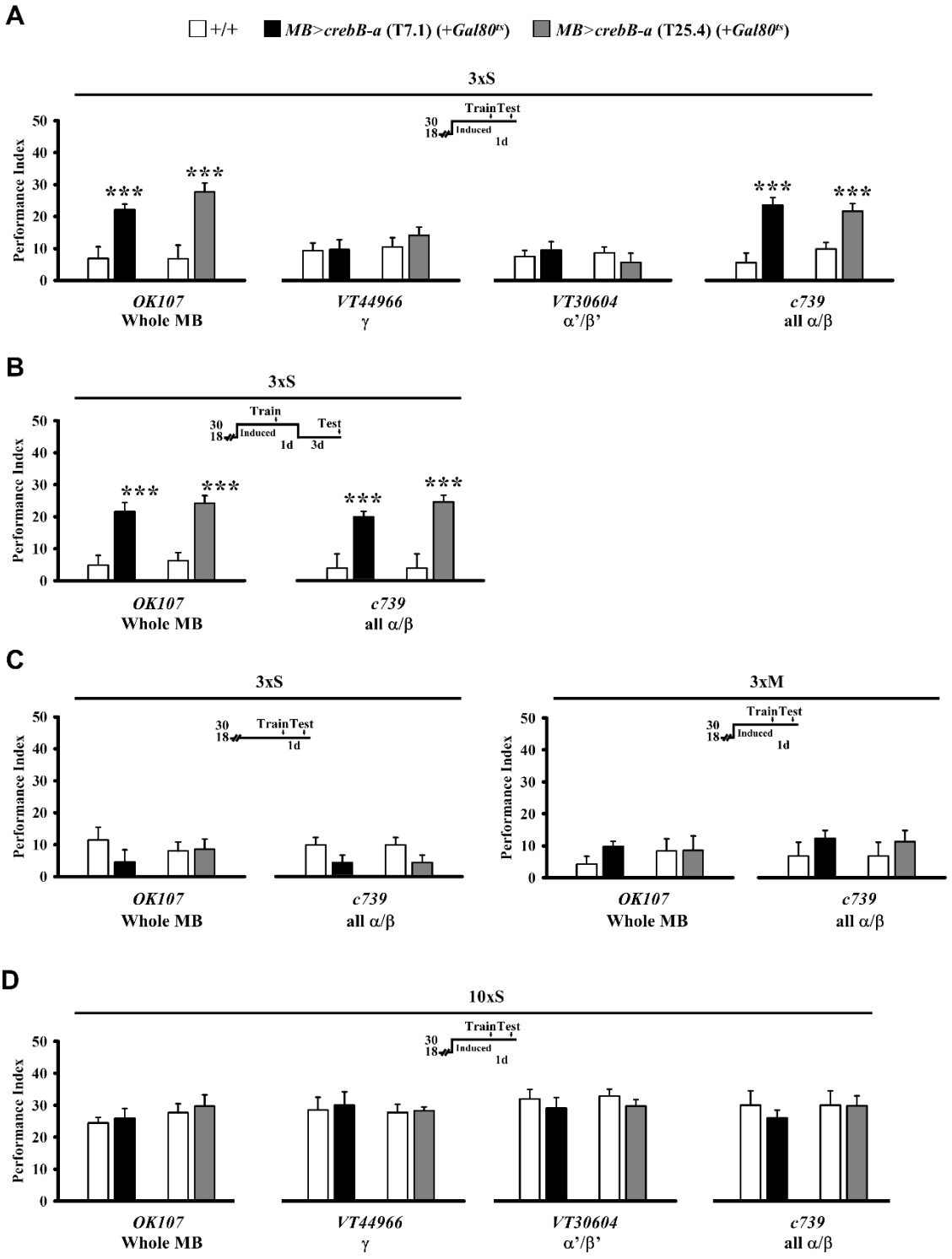


SI Appendix Fig. S1. Representative images of new KAEDE synthesis in response to different learning protocols. Spaced training activates *crebB* transcription in the MB−α lobe tip. (*A*) *crebB* promoter activity reported by *de novo* KAEDE synthesis after 1x, 10xS and 10xM training compared to naïve control flies, estimated by the ratio of new (green, 488 nm) and preexisting (red, 561 nm) proteins (% $\Delta F/ \bar{F}_0$). For each brain, single optical slices through the MB α-lobe tip or ellipsoid body (EB) were imaged under identical conditions. (*B*) *de novo* KAEDE synthesis after 1x, 3xS, 5xS and 10xS training compared to naïve control flies. A minimum of 5xS training cycles are necessary to observe KAEDE synthesis reflecting *crebB* activity. Scale bar = 10 μm. Flies carry the same transgenes as in **Fig. 1B**.



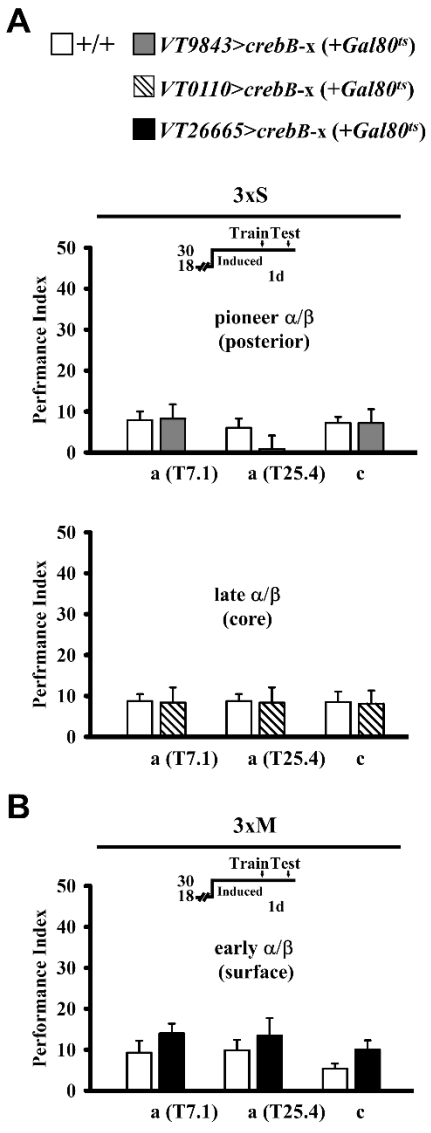
163

164 **SI Appendix Fig. S2.** Effects of *hs-Gal4>UAS-crebB-a* expression on 1-day memory
165 formation. (*A*) Over-expressing two independent lines (T7.1 and T25.4) of *crebB-a* with
166 heat-shock induced Gal4 did not affect 1-day memory after 10xS or 3xS, or 1x training. (*B*)
167 In *hs-Gal4>UAS-GFP* flies, heat-shock induced Gal4 drives GFP expression at low level
168 in the MB neurons. All brains were counterstained for DLG (magenta). Scale bar = 50 μm.



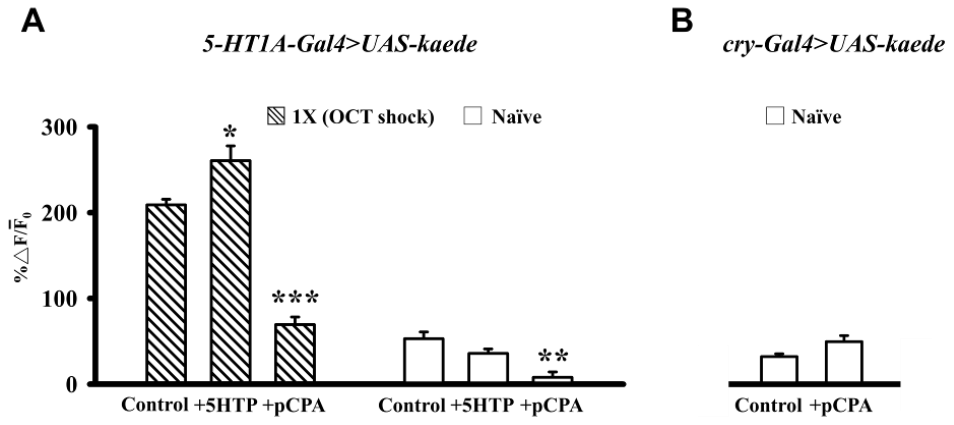
SI Appendix Fig. S3. Effects of over-expressing CREBB-a in subsets of MB neurons on 1-day memory formation. (*A*) Effects of adult-stage specific overexpressing *crebB-a* (with

two independent lines) in whole MB, γ, α'/β', or α/β neurons on LTM formation after 3xS. *Gal4*-targeted transgene overexpression is induced at the restrictive temperature for *tub-Gal80^{ts}* (30 °C) from at least five days before training until testing. (*B*) The enhanced memory after 3xS lasted at least 4 days. (*C*) Memory is unaffected in these flies held at the permissive temperature for *tub-Gal80^{ts}* (18 °C) after 3xS (left) and at 30 °C after 3xM (right). (*D*) One-day memory is also unaffected in these flies at 30 °C after 10xS.



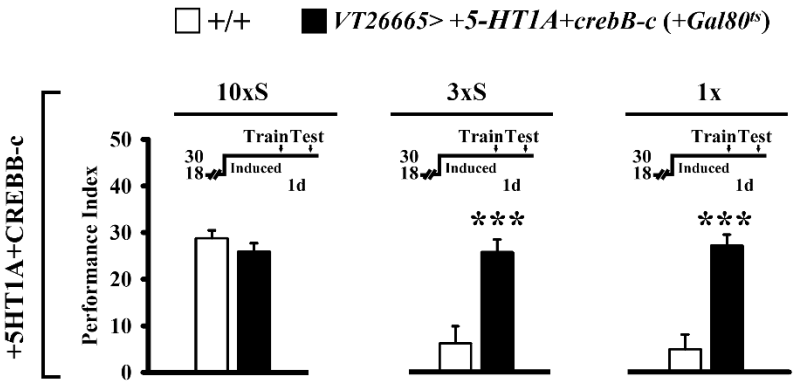
178

179 **SI Appendix Fig. S4.** Effects of over-expressing CREBB-a and -c in subpopulations of
180 α/β MB neurons on 1-day memory formation. (*A*) Overexpressing CREBB-a (with two
181 independent lines: T7.1 or T25.4) or CREBB-c in pioneer or late α/β neurons does not
182 affect 1-day memory after 3xS. *Gal4*-targeted transgenes overexpression is induced at the
183 restrictive temperature for *tub-Gal80*^{ts} (30 °C) from five days before training until testing.
184 (*B*) Overexpressing these transgenes in early α/β neurons does not affect 1-day memory
185 after 3xM.



186

187 **SI Appendix Fig. S5.** Activation of the *5-HT1A* gene is regulate by serotonin level. (*A*) *5-*
188 *HT1A* promoter activity in the MB α-lobe tip 24 h after no training (naïve) or 1x in flies
189 fed 5% glucose alone (control) or with 5HTP to increase 5HT, or pCPA to decrease 5HT.
190 (*B*) *cry* promoter activity in MB α-lobe tip of naïve flies fed 5% glucose alone (control) or
191 pCPA.



192

193 **SI Appendix Fig. S6.** CREBB is sufficient to fully rescue LTM impairment caused by
194 over-expressing 5-HT1A. Co-overexpressing 5-HT1A and CREBB-c proteins in early α/β
195 neurons does not impair 1-day memory after 10xS (left), but enhances 1-day memory after
196 3xS (center) or 1x (right). *Gal4*-targeted 5-HT1A and CREBB-c overexpression is induced
197 by relieving *tub-Gal80*^{ts} inhibition at the restrictive temperature (30 °C) from five days
198 before training until testing.

SI Appendix Table S1. Total number of MB neurons containing in specific *MB-Gal4* lines in each hemisphere

***Gal4* driver**	**Number of neurons**
5-HT1A (early α/β neurons)	421±21 (N = 4)
VT26665 (early α/β neurons)	482±12 (N = 4)
VT26665 + 5-HT1A	495±11 (N = 4)
G0391 (early α/β neurons)	478±32 (N = 7)
OK107 (whole MB neurons)	2316±181 (N = 8)

The number of GFP-labeled nuclei in the MB was determined by the total count in *MB-Gal4>UAS-GFP::lacZ.nls* flies. Each nucleus was manually marked with a landmark sphere in Amira software, and the total number of spheres was automatically determined. Values are means ± SEM.

SI Appendix Table S2. Reagents and Genotypes.

Reagent or Fly Genotypes		Source	Identifier
Antibodies			
Mouse 4F3 anti-DLG antibody		Developmental Studies Hybridoma Bank, University of Iowa	RRID: AB_528203
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary antibody, Biotin-XX		Molecular Probes	Catalog #: B-2763
Streptavidin, Alexa Fluor™ 635 conjugate		Molecular Probes	Catalog #: S32364
Chemicals			
Mineral Oil		Fisher Scientific	Catalog #: O122
4-methylcyclohexanol (98%)		Sigma-Aldrich	Catalog #: 153095
3-octanol (99%)		Sigma-Aldrich	Catalog #: 218405
Glucose		Fisher Scientific	Catalog #: D16
Cycloheximide (CXM)		Sigma-Aldrich	Catalog #: 01810
L-5-hydroxytryptophan (5HTP)		Alfa Aesar	
DL-p-chlorophenylalanine (pCPA)		Sigma-Aldrich	
Experimental Models: Organisms/Strains			
+/+	*Canton-S w1118* (isoCJ1)	(1)	
tub-Gal80^{ts}	*P{tubP-GAL80^{ts}}20* on Chr 2; *P{tubP-GAL80^{ts}}2* on Chr3	Bloomington *Drosophila* Stock Center	RRID:BDSC: 7019, 7017
VT44966	*P{VT44966-Gal4}attP2* on Chr 3	Vienna *Drosophila* Resource Center	N/A
VT30604	*P{VT30604-Gal4}attP2* on Chr 3	Vienna *Drosophila* Resource Center	N/A
VT9843	*P{VT9843-Gal4}attP2* on Chr 3	Vienna *Drosophila* Resource Center	N/A
VT26665	*P{VT26665-Gal4}attP2* on Chr 3	Vienna *Drosophila* Resource Center	N/A
VT0110	*P{VT0110-Gal4}attP2* on Chr 3	Vienna *Drosophila* Resource Center	N/A
OK107	*P{GawB}OK107* on Chr 4	Bloomington *Drosophila* Stock Center (2)	RRID:BDSC: 854
c739	*P{GawB}c739* on Chr 3	Bloomington *Drosophila* Stock Center (2)	RRID:BDSC: 7362
hs-Gal4	N/A	from Y. Zhong	N/A
GFP	*P{UAS-mCD8::GFP}LL5*, *P{UAS-mCD8::GFP}2 on* Chr 2; *P{UAS-mCD8::GFP.L}LL6 on* Chr 3	Bloomington *Drosophila* Stock Center	RRID:BDSC: 5137; 5130
CREBB RNA interference	*P{TRiP.JF02494}attP2* on Chr 3	Bloomington *Drosophila* Stock Center	RRID:BDSC: 29322
CREBB RNA interference	*P{KK108927}VIE-260B* on Chr 2	Vienna *Drosophila* Resource Center	RRID:VDRC: v101512
5-HT1A RNA interference	*P{TRiP.JF01852}attP2* on Chr 3	Bloomington *Drosophila* Stock Center	RRID:BDSC: 25834
5-HT1A RNA interference	on Chr 2 and 3	NIG-Fly Stock Center (8)	RRID:NIG: 16720
crebB-aT7.1	*P{UAS-CrebB-17A-a.cor}T7.1* on Chr 1	(9)	RRID:BDSC: 9232
crebB-aT25.4	*P{UAS-CrebB-17A-a.cor}T25.4* on Chr 1	(9)	RRID:BDSC: 9233
crebB-b	*P{UAS-crebB-b}* on Chr 1	(10)	N/A
crebB-c	*P{dCREB-B-UAS}88* on Chr 2; *P{dCREB-B-UAS}94* on Chr 3	Bloomington *Drosophila* Stock Center (11)	RRID:BDSC: 7220, 7219
5-HT1A	*P{UAS-5-HT1A.R}2* on Chr 2*; P{UAS-5-HT1A.R}3* on Chr 2	Bloomington *Drosophila* Stock Center	RRID:BDSC: 27630, 27631
kaede	*P{UAS-kaede}* on Chr 3	(2)	N/A

SI Appendix References

1. T. Tully, T. Preat, S. C. Boynton, M. Del Vecchio, Genetic dissection of consolidated memory in *Drosophila*. Cell 79, 35-47 (1994).
2. C. C. Chen et al., Visualizing long-term memory formation in two neurons of the *Drosophila* brain. Science 335, 678-685 (2012).
3. T. Tully, W. G. Quinn, Classical conditioning and retention in normal and mutant *Drosophila* melanogaster. J Comp Physiol A 157, 263-277 (1985).
4. T. P. Pai et al., *Drosophila* ORB protein in two mushroom body output neurons is necessary for long-term memory formation. Proc Natl Acad Sci U S A 110, 7898-7903 (2013).
5. J. K. Wu et al., Long-term memory requires sequential protein synthesis in three subsets of mushroom body output neurons in *Drosophila*. Sci Rep 7, 7112 (2017).
6. R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno, A. Miyawaki, An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc Natl Acad Sci U S A 99, 12651-12656 (2002).
7. A. S. Chiang et al., Three-dimensional mapping of brain neuropils in the cockroach, *Diploptera punctata*. J Comp Neurol 440, 1-11 (2001).
8. P. T. Lee et al., Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. Proc Natl Acad Sci U S A 108, 13794-13799 (2011).
9. B. Perazzona, G. Isabel, T. Preat, R. L. Davis, The role of cAMP response element-binding protein in *Drosophila* long-term memory. J Neurosci 24, 8823-8828 (2004).
10. D. Yu, D. B. Akalal, R. L. Davis, *Drosophila* alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning.

231 Neuron 52, 845-855 (2006).
232 11. S. Eresh, J. Riese, D. B. Jackson, D. Bohmann, M. Bienz, A CREB-binding site as a
233 target for decapentaplegic signaling during *Drosophila* endoderm induction. EMBO J
234 16, 2014-2022 (1997).