Supplementary Information for

CREBA and CREBB in two identified neurons gate long-term memory formation in *Drosophila*

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This PDF file includes:

Supplementary text

Figures S1 to S5

Tables S1 to S2

SI References

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 12h:12h light:dark cycle. All fly strains used in experiments are listed in Table S2.

CRE-luciferase assay

We tested in vitro whether CREBA could serve as an activator at *cAMP response element* (*CRE*) sites using a reporter construct linking the firefly *luciferase* coding sequence downstream of the Δ(-71) *CRE* sequence of the rat *somatostatin* promoter. Undifferentiated F9 cells were maintained, harvested and transfected by the calcium phosphate method as described previously (1). We tested *creb* transgenes with or without co-transfection with the *protein kinase-A catalytic subunit* (*PKA*) cloned under the control of the mouse metallothionein-1 promoter (J. Yin, U. Wisconsin) in the F9 mouse teratocarcinoma cell line as described (1).

Behavior

Behavior protocols for analyzing associative memory with an aversive Pavlovian olfactory conditioning procedure are well established (2, 3). Memory performance was evaluated by training 6- to 7-day-old flies in a T-maze apparatus. 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 these two odors. Flies were exposed sequentially to two odors conducted through the training chamber in a current of air (odors were bubbled at 750 ml/min). A single training session (1x) is preceded by 90 s exposure to fresh air, followed by delivery of one odor (conditioned stimulus, CS) in the presence of electric shock (unconditioned stimulus,

US), consisting of 12 x 1.5 s pulses of 60V dc electric shocks with 5 s interpulse intervals for 60 s (CS^+). Flies are then exposed to fresh air for 45 seconds, followed by the second odor without shock for 60 s (CS^-). For 1-day memory experiments, flies receive either one training session as described above (1x), massed training (multiple sessions without rest intervals; *e.g.*, 3xM or 10xM) or spaced training (multiple sessions each separated by 15 min rest intervals; *e.g.*, 3xS or 10xS). For these training protocols, automated robotic trainers are used (see 2 for details).

Memory assessment testing was conducted using a T-maze device, in a dark environment-controlled room at specified temperatures and 70 % relative humidity. Flies were transported to a T-maze choose point where they are exposed to converging currents of air, each carrying either OCT or MCH, arriving from opposite arms of the T maze. Flies were allowed to choose between the CS^+ and CS^- for 120 s, at which time they were trapped inside their respective arms of the T maze (by sliding the elevator out of register), anesthetized and counted. Flies that chose to avoid the CS^+ ran into the T maze arm containing the CS^-, while flies that chose to avoid the CS^- ran into the T maze arm containing the CS^+. For each half experiment, a performance index (PI) was calculated as the normalized percent avoidance of the CS^+ odor. To account for possible biases (naïve odor preference, machine imbalance, or nonassociative changes in olfaction), an overall PI was calculated as the average of two sequential tests in which OCT and MCH were each used as the CS^+. Genetic backgrounds of all fly strains were equilibrated to the "Canton" wild-type background by five or more generations of backcrossing. All genotypes were trained and tested in parallel and rotated between all of the robotic trainers to ensure a balanced experiment. In *tub-Gal80^{ts}* experiments, flies raised at 18 °C were transferred to 30 °C for at least 3 days before experiments. For blocking protein synthesis, flies were fed with 35 mM cycloheximide (Sigma) in 5 %

glucose 1 day before training until just before the test (2).

KAEDE Measurement

To measure the amount of newly synthesized KAEDE in DAL neurons, we used the following procedures (see 4). (i) Pre-existing KAEDE proteins were photoconverted into red fluorescence proteins by 365-395 nm UV irradiation generated from a 120W mercury lamp. For the behavior assay, 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. (ii) Individual single neurons expressing KAEDE were directly visualized through an dissected fly brain. Living samples were used because the signal to noise ratio of green versus red KAEDE is greatly reduced after chemical fixation. (iii) KAEDE expressed 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. (iv) A single optical slice through cell body of DAL neuron at a resolution of 1024 x 1024 pixels was imaged under a confocal microscope with a 40X 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 setting maximized for green and red KAEDE immediately before and after photoconversion, respectively. (v) In all cases, both green KAEDE excited by a 488 nm laser and red KAEDE excited by 561 nm laser were measured. Using the amount of red KAEDE as an internal standard to calibrate individual variation, we calculated the increasing rate of green KAEDE synthesis after photoconversion with the formula $(\Delta F) = \% (Ft_1\text{-}\underline{\text{average}}\ Ft_0)/\underline{\text{average}}\ Ft_0$, where Ft_1 and Ft_0 are the ratio of averaged intensities between green (G) and red (R) KAEDE (Gt_0/Rt_0) immediately after photoconversion (t_0) and at a specific later time point (t_1), respectively. All experiments were performed by adult specific expression of transgenes, except the *crebA>transgenes*. Flies were raised at 18 ºC and then transferred

to 30 ^{o}C to remove GAL4 inhibition by GAL80^{ts} for 3 days before training.

Immunohistochemistry

Brains were dissected in phosphate-buffered saline (PBS), heated with a commercial microwave oven in 4 % paraformaldehyde on ice for 60 s (repeated 3x), and then in 4 % paraformaldehyde with 0.25 % Triton X-100 for 60 s (repeated 3x). After being washed in PBS for 10 min at room temperature, brain samples were incubated in PBS containing 2 % Triton X-100 and 10 % normal goat serum and degassed in a vacuum chamber to expel tracheal air for 4 cycles (depressurize to -70 mmHg then hold for 10 min). Next, brain samples were blocked and penetrated in PBS-T at 4 °C overnight and then incubated in PBS-T containing one of the following primary antibodies: (i) 1:40 mouse 4F3 anti-DLG antibody (Developmental Studies Hybridoma Bank, Univ. of Iowa), (ii) 1:500 rabbit anti-CREBA antibody (CREBA Rbt-PC was deposited to the DSHB by Andrew, D.J. / Hanlon, C.D.) and (iii) 1:500 rat anti-DDC antibody (from J. Hirsh), at 4 °C for 1 day. After being washed in PBS-T three times, samples were incubated in PBS-T containing of the following secondary antibody: (i) 1:200 biotinylated goat anti-mouse IgG (Molecular Probes), (ii) 1:200 biotinylated goat anti-rabbit IgG (Sigma) and (iii) 1:200 biotinylated goat anti-rat IgG (Sigma) at 25 °C for 1 day. Next, brain samples were 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 in *FocusClear*, an aqueous solution that renders biological tissue transparent (5), for 5 min and mounted between two cover slips separated by a spacer ring of ～200 μm in thickness. Sample brains were imaged under a Zeiss LSM 780 confocal microscope with a 40X C-Apochromat water-immersion objective lens (N.A. value 1.2, working distance 220 μm).

Quantification of Antibody Labeling

For each targeted structure, a single optical section was taken under the same recording condition. In each sample, the mean intensity value (total intensity/total area) in DAL nuclear and that in cytosol were measured, and the ratio (Nuclear/Cytosol) was calculated (Fig. S2*C*). The mean intensity value (total intensity/total area) in DAL cytosol and that in ellipsoid body (EB) were measured, and the ratio (Cytosol/EB) was calculated (Fig. S2*D*).

Quantification and Statistical Analysis

All raw data were analyzed parametrically with Prism 9, SigmaPlot 10.0 and SigmaStat 3.5 statistical software. Comparisons were evaluated by either unpaired *t*-tests or one-way analysis of variance (ANOVA). Mann-Whitney Rank Sum tests were used when variances were found to be unequal. All data are presented as the mean ± SE.

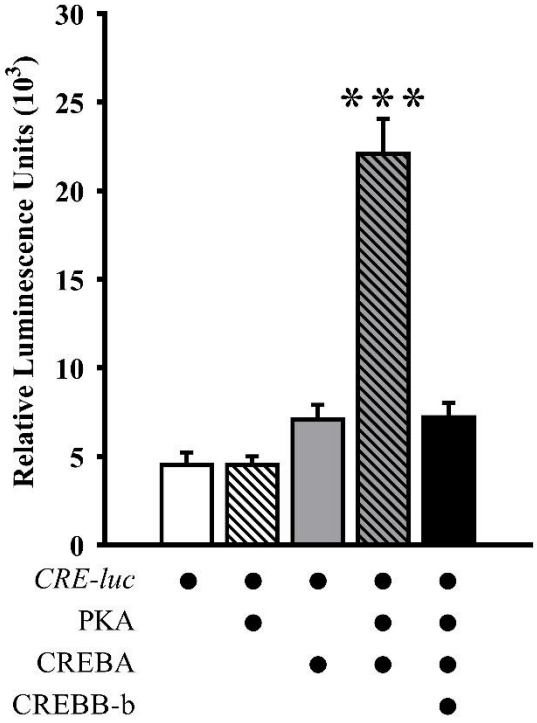

Fig. S1. CREBA behaves like an activator at *CRE* sites in vitro. *crebA* expression (CREBA) activates a *CRE-luciferase* reporter in a PKA-dependent manner, whereas co-expression of *crebB-b* (CREBB-b) represses this CREBA activation (1). F9 mouse teratocarcinoma cells were transiently transfected with *CRE-luciferase* reporter plasmid (a fusion of the Δ(-71) *CRE*-containing fragment of the rat *somatostatin* promoter and the firefly *luciferase* coding regions). Different groups received *PKA*, *crebA* or *crebB-b* expression vectors separately or in combination, as indicated. $n \geq 3$ wells/bar. ***, $P < 0.001$.

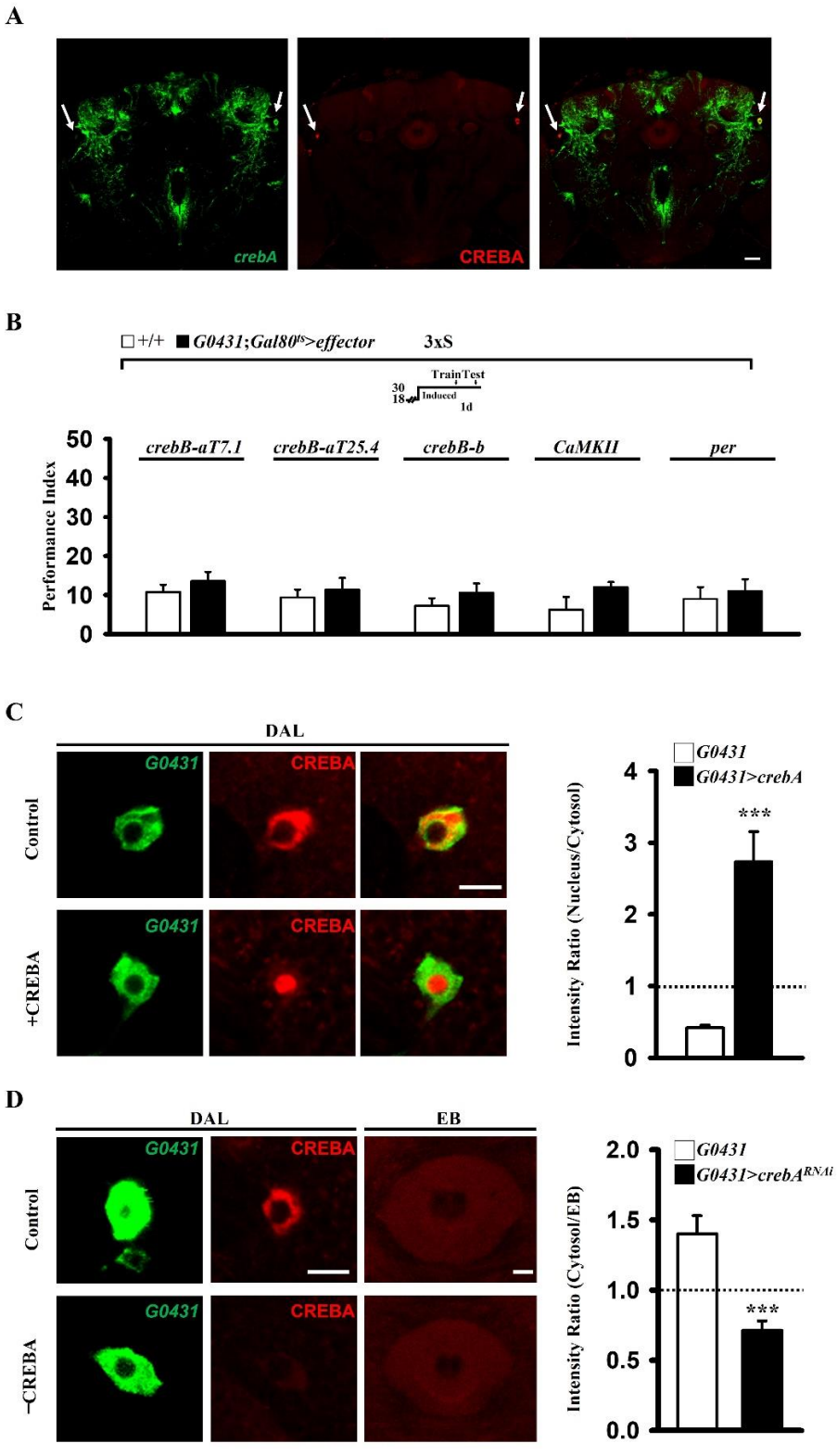
A
crebA
CREBA
B
+/+
G0431;Gal80ts>effector
3xS
30
18
Train Test
Induced
1d
crebB-aT7.1
crebB-aT25.4
crebB-b
CaMKII
per
Performance Index
50
40
30
20
10
0
C
DAL
G0431
CREBA
Control
+CREBA
G0431
G0431>crebA
***
Intensity Ratio (Nucleus/Cytosol)
4
3
2
1
0
D
DAL
EB
G0431
CREBA
Control
−CREBA
G0431
G0431>crebARNAi
***
Intensity Ratio (Cytosol/EB)
2.0
1.5
1.0
0.5
0.0

Fig. S2. CREBA manipulation in DAL neurons. (*A*) *crebA* expression visualized with *crebA-Gal4* driven *UAS-mCD8::GFP* (green) (left) and CREBA immunoreactivity (red) (middle) co-localize in DAL neurons (arrows) (right). Scale bar = 20 μm. (*B*) Overexpressing *crebB-aT7.1*, *crebB-aT25.4*, *crebB-b*, *CaMKII* and *per* in DAL neurons does not influence 1-day memory after 3xS training. (*C*) *crebA* overexpression (+CREBA) in DAL neurons using *G0431-Gal4* driven *UAS-crebA* (left, bottom). DAL neurons visualized with *G0431-Gal4* driven *UAS-GFP* (green) (left). CREBA immunoreactivity (red) (middle left) and merged images (middle right) show that overexpressed CREBA translocates from the cytosol to the nucleus, as quantified by immunostaining intensity ratios between the nucleus and cytosol in DAL neurons (right). (*D*) CREBA down-regulation (–CREBA) in DAL neurons using *G0431-Gal4* driven *UAS-crebA*^{RNAi} (left, bottom). DAL neurons visualized as in (C) (left). CREBA immunoreactivity in DAL neurons (red) shows near complete absence of protein (middle left) as verified by a similar absence of protein in the ellipsoid body (EB) (middle right), and quantified by immunostaining intensity ratios between DAL cytosol and the EB (right).

In all supplemental figures, temperature control schedules are indicated (top). Flies were raised at 18 ºC and then transferred to 30 ºC to remove GAL4 inhibition by GAL80^{ts} for 3 days prior to training, thereby enabling manipulation of target genes. Memory PIs are calculated as the normalized percent avoidance of shock-paired odor. All images are single optical slices of dissected brains and are compared with controls under identical conditions. Scale bars = 10 μm unless stated otherwise. Bars represent mean ± SE, $n \geq 8$/bar. ***, $P < 0.001$. All genotypes are listed in Table S2.

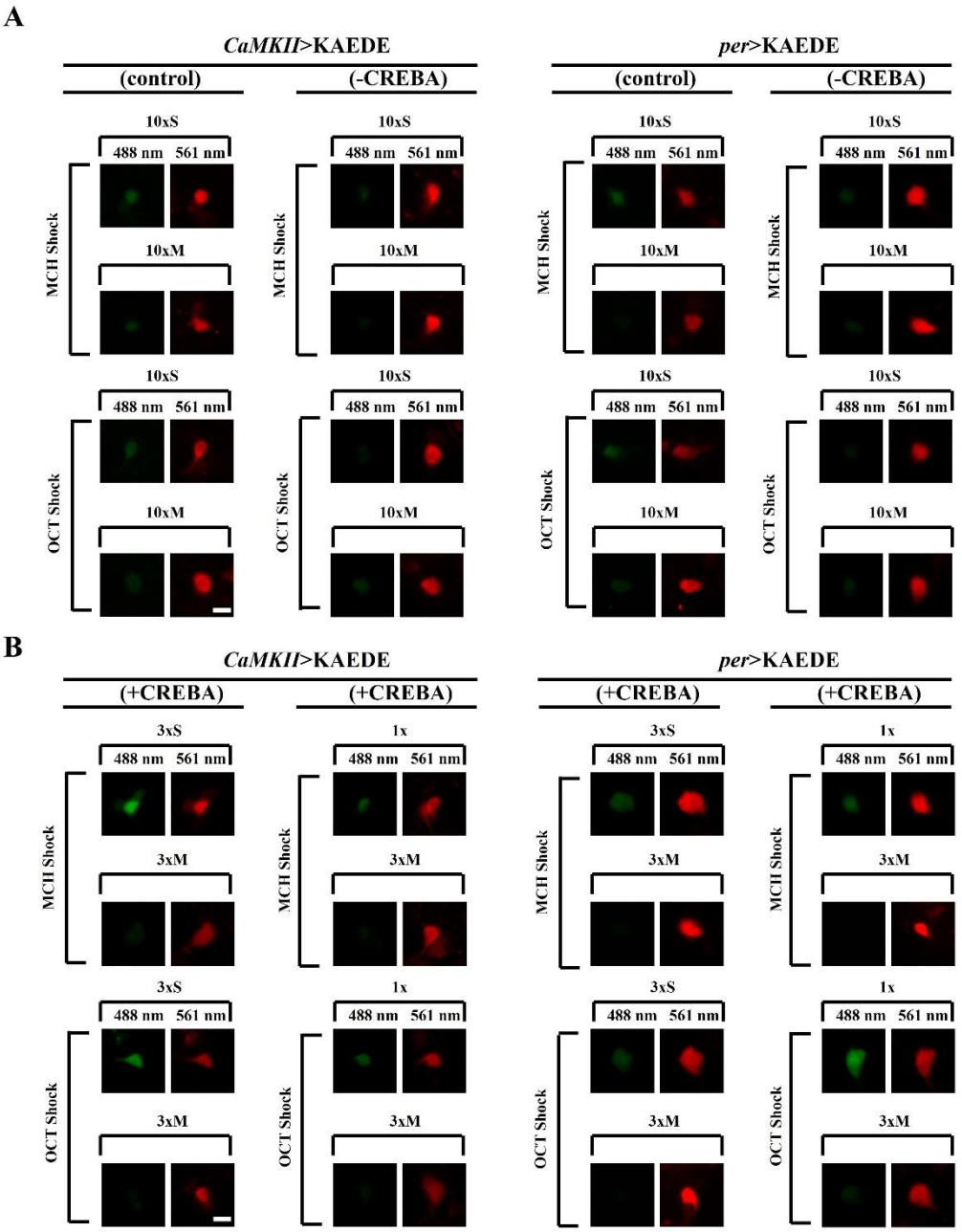
A
CaMKII>KAEDE
per>KAEDE
(control)
(-CREBA)
(control)
(-CREBA)
10xS
10xM
488 nm
561 nm
MCH Shock
OCT Shock
B
CaMKII>KAEDE
per>KAEDE
(+CREBA)
(+CREBA)
(+CREBA)
(+CREBA)
3xS
1x
3xM

Fig. S3. CREBA regulation of *CaMKII* and *per* transcriptional activity in DAL neurons. (*A*) CREBA down-regulation (–CREBA) influences on *CaMKII* (left) and *per* (right) promoter activities in DAL neurons after 10xS (top) and 10xM (bottom) training. (*B*) *crebA* overexpression (+CREBA) influences on *CaMKII* (left) and *per* (right) promoter activities in DAL neurons after 3xS (top left), 1x (top right) and 3xM (bottom) training. See Fig. 3 for quantification and comparison of these data.

In all supplemental figures, promoter activity was reported by KAEDE fluorescent protein synthesis. Living flies were subjected to UV irradiation to convert pre-existing green KAEDE into red KAEDE prior to training. Newly synthesized KAEDE was quantified 24 h after aversive electric shock conditioning of 4-methylcyclohexanol (MCH) (top) or 3-octanol (OCT) (bottom). KAEDE synthesis was estimated as the ratio between new (green, 488 nm excitation) and preexisting (red, 561 nm excitation) KAEDE ($\% \Delta F / \bar{F}_0$). Flies were raised at 18 ºC and then transferred to 30 ºC to remove GAL4 inhibition by $GAL80^{ts}$ for 3 days prior to training.

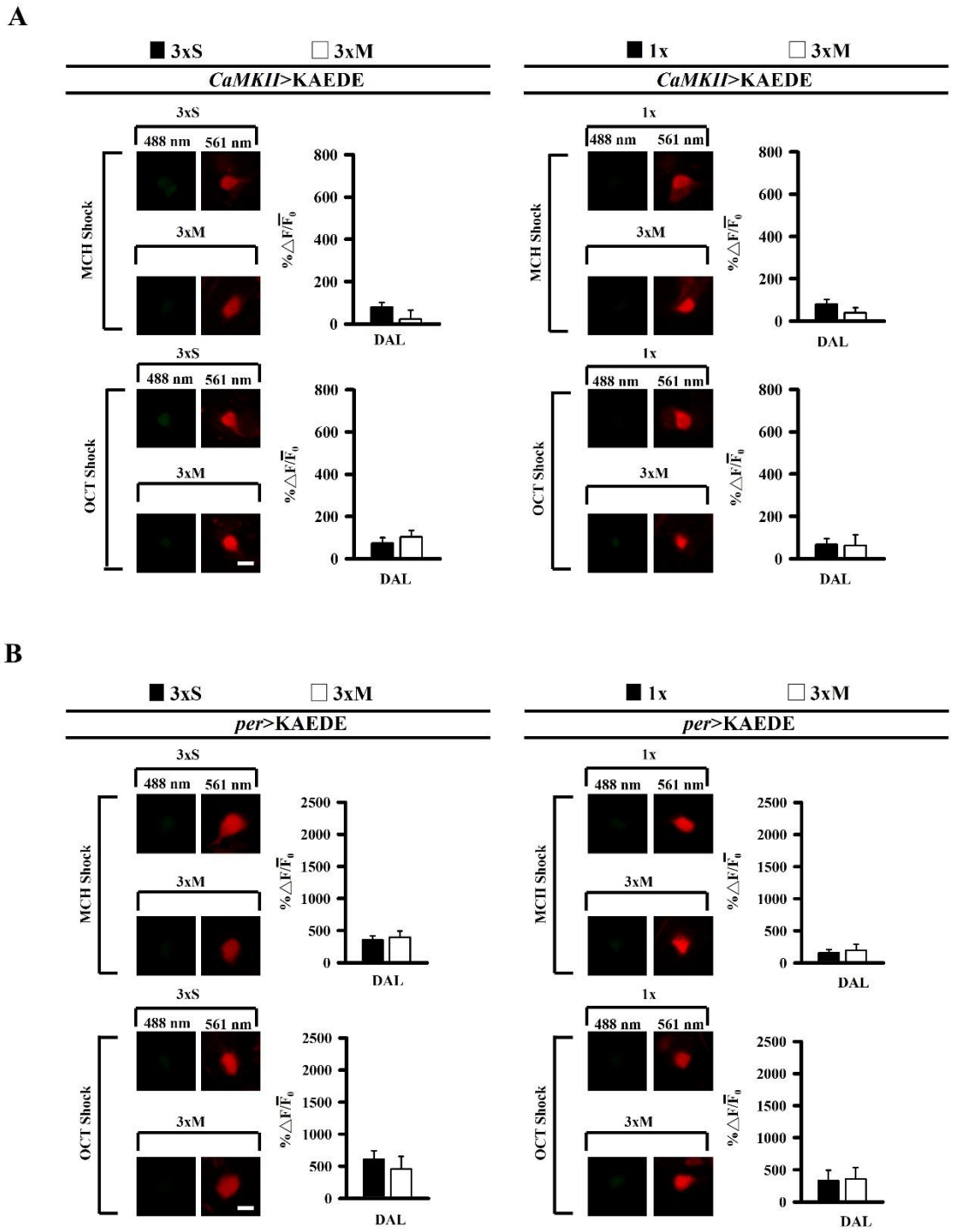

Fig. S4. Sub-threshold training does not induce (*A*) *CaMKII* or (*B*) *per* transcriptional activity in DAL neurons. For both genes, training effects of 3xS (top) compared with 3xM (bottom) (left) and effects of 1x (top) compared with 3xM (bottom) (right) were not significant. Promoter activity in DAL neurons was estimated and compared as described in Fig. 3 and Fig. S3.

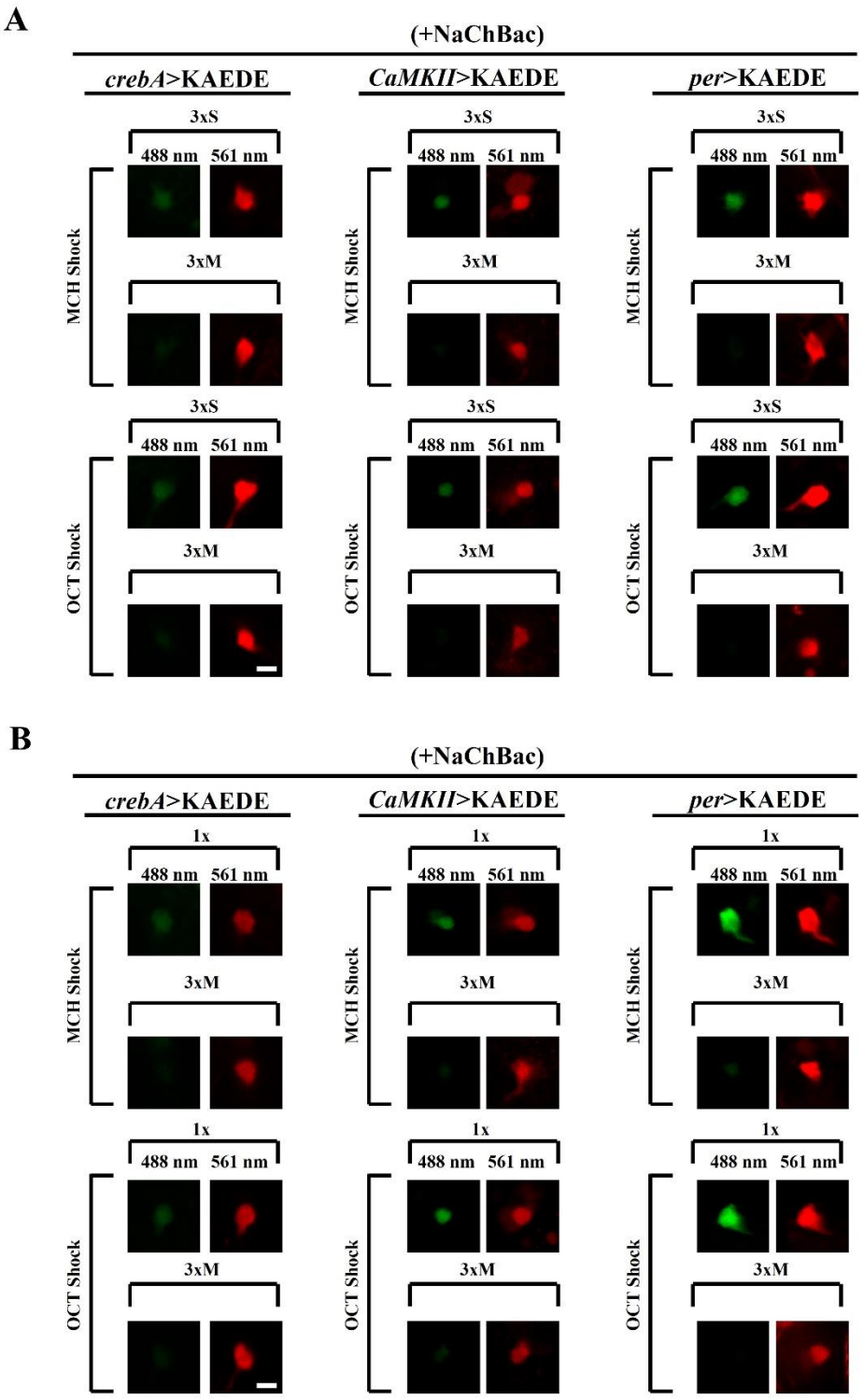
A
(+NaChBac)
crebA>KAEDE
CaMKII>KAEDE
per>KAEDE
3xS
488 nm
561 nm
3xM
MCH Shock
OCT Shock
B
(+NaChBac)
crebA>KAEDE
CaMKII>KAEDE
per>KAEDE
1x
488 nm
561 nm
3xM
MCH Shock
OCT Shock

Fig. S5. Elevated neuronal excitability influences on *crebA*, *CaMKII* and *per* transcription activity in DAL neurons. *NaChBac* overexpression (+NaChBac) influences on *crebA* (left), *CaMKII* (middle) and *per* (right) promoter activity in DAL neurons in response to aversive olfactory training. (*A*) 3xS (top) or 3xM (bottom) training. (*B*) 1x (top) and 3xM (bottom) training. Promoter activity in DAL neurons was estimated and compared as described in Fig. 1*C* and Fig. S4. Refer to Fig. 4*D* and *E* for quantification and comparison of these data.

Table S1. BG00224 insertion information.

Strain	Arm	Range	Strand	Cytology	Gene(s)
BG00224	3L	15529174:15529174	-1	71E1	CrebA(FBgn0004396)

Flank: BG00224-3'
GTTCTGGGTGATGACTGTTGGTTGCTTCTTTATAACACTTATTAAGGGGA
AACCTGTTGCTTCCAGAGAATGTTTTCACTTGACATTGTTATAGTCTGCG
TGTGTGTGTGTGTGTGTGACTTTTTCGTTTGTATGTGTATACCGGGCATT
CATTCGTATTGGTTGTGGTATTTGGGGTGTATCTGTGTACGGATCTAACT
TGGTTTACAACTGTCGGCTGGATTTAACTTGTTGCATGCGC

Baylor gene trap line BG00224 obtained from H. Bellen (Baylor College of Medicine) contains a *Gal4* (*P{GT1}* element) insertion in the *crebA* gene.

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
Rabbit anti-CREBA antibody		Developmental Studies Hybridoma Bank, University of Iowa	RRID: AB_10805295
Rat anti-DDC antibody		(6)	N/A
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary antibody, Biotin-XX		Molecular Probes	Catalog #: B-2763
Anti-Rabbit IgG (whole molecule)-Biotin antibody produced in goat		Sigma	Catalog #: B8895
Anti-Rat IgG (whole molecule)–Biotin antibody produced in goat		Sigma	Catalog #: B7139
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		Sigma-Aldrich	Catalog #: 01810
Experimental Models: Organisms/Strains			
+/+	*Canton-S w1118 (iso1CJ)*	(2)	
tub-Gal80^{ts}	*P{tubP-GAL80^{ts}}20* on *Chr 2*	Bloomington *Drosophila* Stock Center	RRID:BDSC: 7019
G0431-Gal4 (12837)	*P{GT1}BG02822* on *Chr 2* specific in DAL neurons	Bloomington *Drosophila* Stock Center	RRID:BDSC: 12837
CaMKII-Gal4	*Gal4* driver, *on Chr 3*	(7)	N/A
per-Gal4	*Gal4* driver, *on Chr 2*	(8)	N/A
crebA-Gal4	*Gal4* driver, *on Chr 3*	(9)	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
crebA	*P{UAS-crebA.R} on Chr 2*	Bloomington *Drosophila* Stock Center	RRID:BDSC: 32572
v110650	*P{KK*108357*}VIE-260B on Chr 2* CREBA RNA interference	Vienna *Drosophila* Resource Center	RRID:VDRC: v110650
crebB-aT7.1	*P{UAS-crebB-aT7.1} on Chr 1*	(10)	
crebB-aT25.4	*P{UAS-crebB-aT25.4} on Chr 1*	(10)	
crebB-b	*P{UAS-crebB-b} on Chr 1*	(11)	N/A
CaMKII	*P{UAS-CaMKII.R3} on Chr 2*	Bloomington *Drosophila* Stock Center	RRID:BDSC: 29662
per	*P{UAS-per} on Chr 3*	(8)	N/A
KAEDE	*P{UAS-KAEDE} on Chr 3*	(4)	N/A
NaChBac	*P{UAS-NaChBac} on Chr 2*	(12)	N/A

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