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Supplementary Materials for

Requirement for Nuclear Calcium Signaling in *Drosophila* Long-Term Memory

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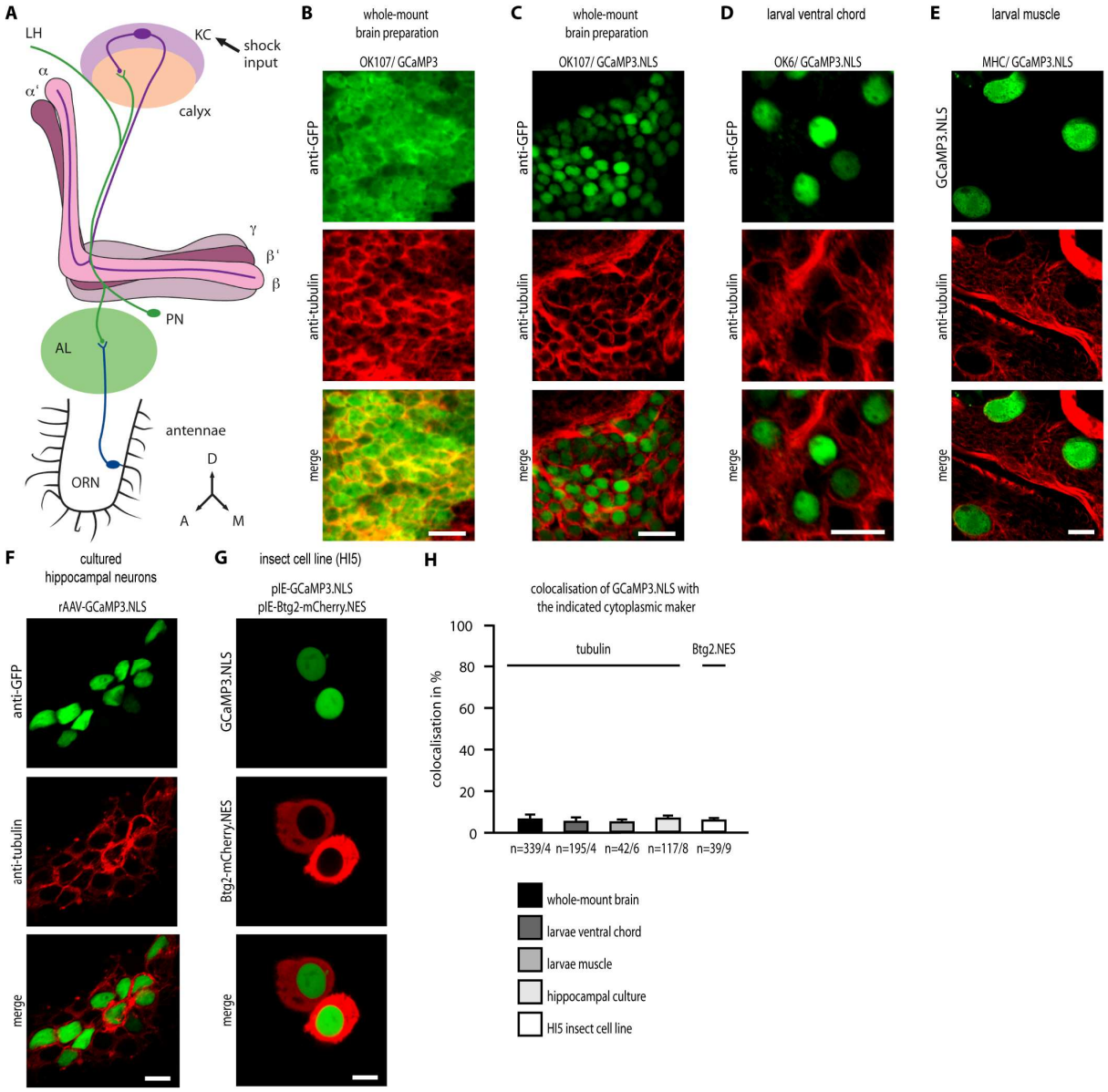
A
LH
KC
shock input
α
α'
calyx
γ
β'
β
PN
AL
antennae
ORN
D
A
M
B
whole-mount brain preparation
OK107/ GCaMP3
anti-GFP
anti-tubulin
merge
C
whole-mount brain preparation
OK107/ GCaMP3.NLS
anti-GFP
anti-tubulin
merge
D
larval ventral chord
OK6/ GCaMP3.NLS
anti-GFP
anti-tubulin
merge
E
larval muscle
MHC/ GCaMP3.NLS
GCaMP3.NLS
anti-tubulin
merge
F
cultured hippocampal neurons
rAAV-GCaMP3.NLS
anti-GFP
anti-tubulin
merge
G
insect cell line (HI5)
pIE-GCaMP3.NLS
pIE-Btg2-mCherry.NES
GCaMP3.NLS
Btg2-mCherry.NES
merge
H
colocalisation of GCaMP3.NLS with the indicated cytoplasmic maker
tubulin
Btg2.NES
colocalisation in %
100
80
60
40
20
0
n=339/4
n=195/4
n=42/6
n=117/8
n=39/9
whole-mount brain
larvae ventral chord
larvae muscle
hippocampal culture
HI5 insect cell line

Fig. S1. Confocal analysis of the nuclear localization of GCaMP3.NLS in vivo and in cultured cells. (**A**) Simplified schematic representation focusing on the *Drosophila* mushroom bodies (purple, gray, peach) and their olfactory inputs. See introduction for details. Abbreviations: Olfactory receptor neuron (ORN), projection neuron (PN), antennal lobe (AL), mushroom body Kenyon cell (KC) and lobes (α, β, α', β', γ), lateral horn (LH), anterior (A), dorsal (D), medial (M). (**B, C**) Confocal images of whole-mount preparations of fly brains expressing GCaMP3 **(**OK107/ GCaMP3) or GCaMP3.NLS **(**OK107/ GCaMP3.NLS) in mushroom body neurons. The upper row shows immunolabeling with an antibody against GFP (green) of the recombinant calcium indicators GCaMP3 (B) visible in the cytoplasm and the nucleus and GCaMP3.NLS (C) visible exclusively in the nucleus. Flies were counterstained with an antibody against the cytoplasmic marker tubulin (red). Merged images in the lower panels show colocalized signal in yellow. The scale bar is 10 µm. (**D**) Confocal images of motor neurons in the 3rd instar larval ventral chord expressing GCaMP3.NLS (OK6/ GCaMP3.NLS) and immunolabeled with an antibody against GFP (green) and an antibody against tubulin (red). Merged imaged shows the lack of colocalization. The scale bar is 10 µm. (**E**) Confocal images of muscle cells in the 3rd instar larvae expressing GCaMP3.NLS (MHC/ GCaMP3.NLS) detected with an antibody against GFP (green) and tubulin (red). Merged imaged shows the lack of colocalization. The scale bar is 10 µm. (**F**) Confocal images of cultured hippocampal neurons from mouse infected with AAV-GCaMP3.NLS. GCaMP3.NLS staining is green (detected with by immunolabeling with an antibody against GFP) and tubulin is red. Merged imaged shows the lack of colocalization. The scale bar is 10 µm. (**G**) Confocal images of the insect cell line HI5 cotransfected with pIE-GCaMP3.NLS (green) and pIE-Btg2-mCherry.NES (red). Merged imaged shows the lack of colocalization. The scale bar is 10 µm. (**H**) Quantitative analysis of the percentage of the GCaMP3.NLS fluorescence signal colocalizing with the cytoplasmic markers tubulin or Btg2.NES in each of the experiments shown in panels C to G. Costes technique for automatic thresholding was used. N values below each bar indicate the number of cells / the number of flies or larvae or coverslips analyzed.

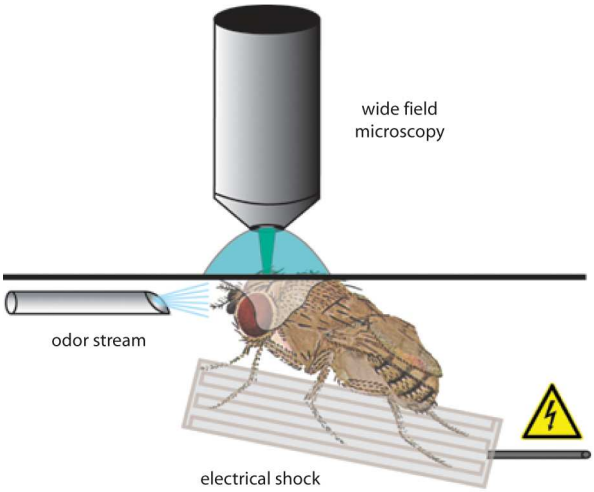

Fig. S2. Schematic diagram of the in vivo calcium imaging setup. In vivo calcium imaging was performed with a wide field fluorescence microscope through an opening in the head cuticle of flies whose heads were glued to a coverslip and whose feet were in contact with a copper grid that delivered the electric shocks. Foot shocks consisted of repeated electric pulses (13 × 1.5 sec 10 μA pulses at 0.2 Hz). The fly's antennae received a constant stream of air passed through a vial containing either mineral oil alone or 10% OCT or MCH in mineral oil.

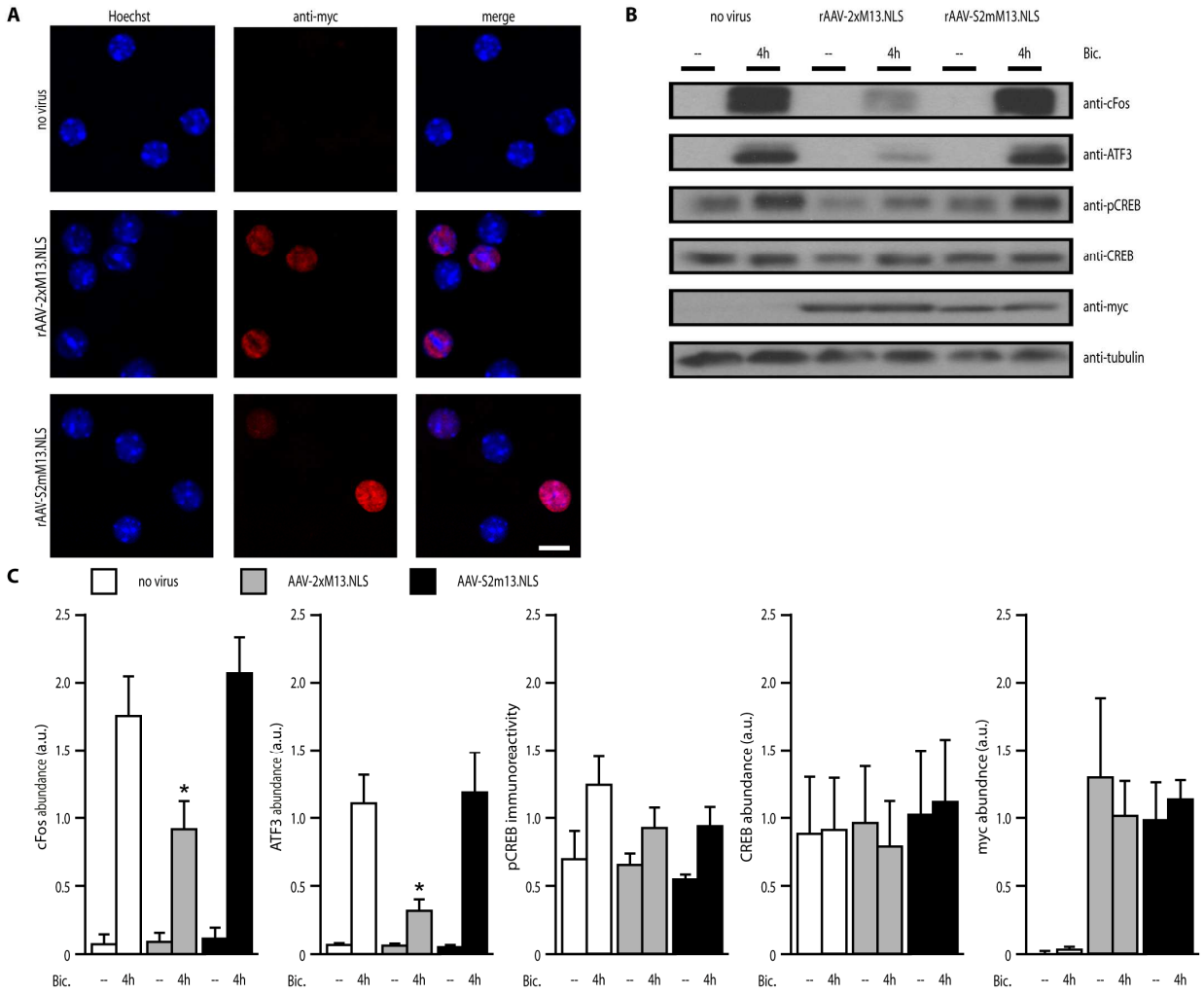

Fig. S3. Characterization of the nuclear Ca^{2+}/CaM inhibitor rAAV-2xM13.NLS and the scrambled control rAAV-S2mM13.NLS in cultured mouse hippocampal neurons. (**A**) Representative confocal images of hippocampal neurons uninfected (no virus) or infected with rAAVs expressing 2xM13.NLS or S2mM13.NLS, which contained a myc tag for detection with an antibody against myc (red). Nuclei of cells were counterstained with Hoechst (blue). Scale bar is 15 μm. (**B**) Immunoblot analysis of hippocampal neurons uninfected (no virus) or infected with rAAVs expressing 2xM13.NLS or S2mM13.NLS. 2xM13.NLS and S2mM13.NLS were detected with an antibody against the myc tag. Neurons were unstimulated (--) or exposed to bicuculline (Bic, 50 μM) for 4 hr to induce action potential bursting. The effect of 2xM13.NLS and S2mM13.NLS on the bicuculline-induced increase in the abundance of cFos and ATF3 is shown. The effect on the abundance of phosphorylated CREB (pCREB) and total CREB is also shown. (**C**) Quantitative analysis of the immunoblots of cFos, ATF3, pCREB, CREB, 2xM13.NLS, and S2mM13.NLS. All results were normalized to tubulin abundance. a.u. arbitrary units (n = 3, * $p < 0.05$).

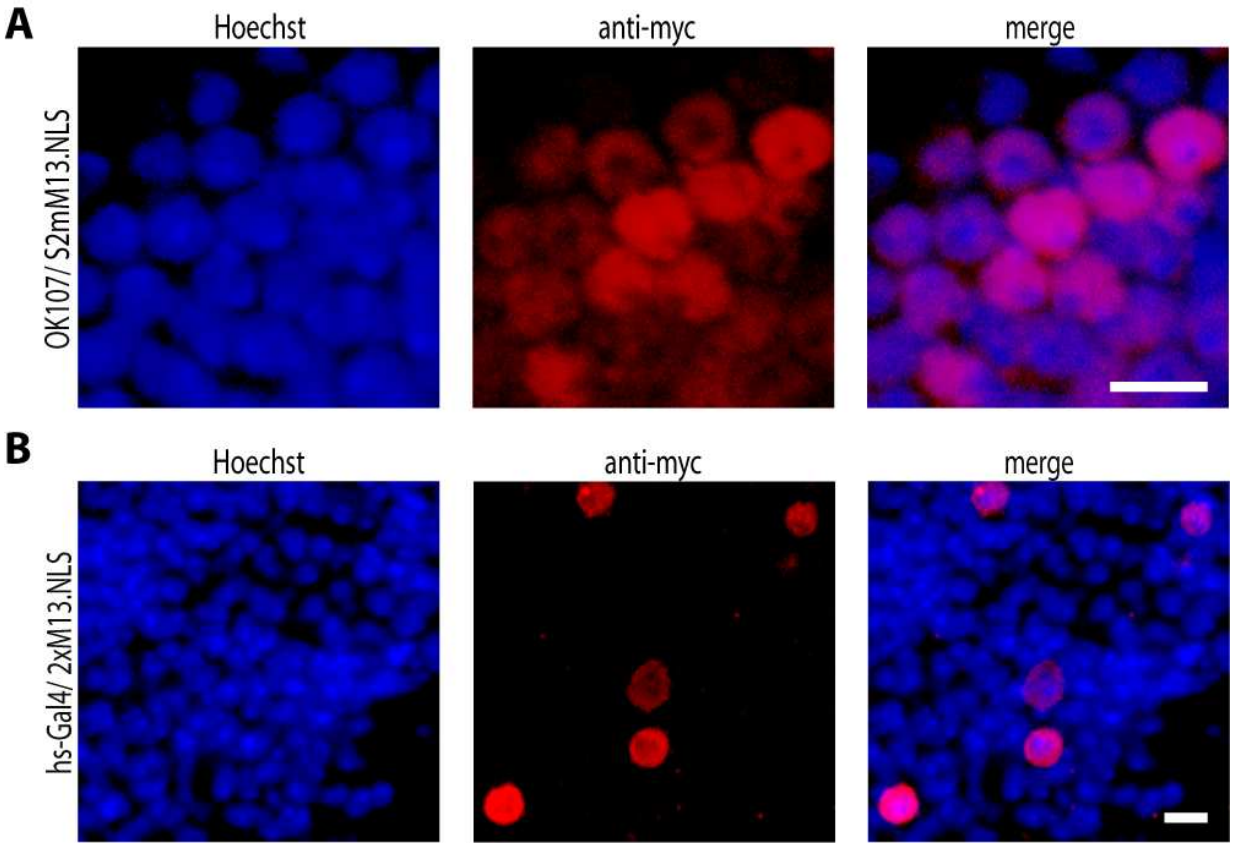

Fig. S4. Confocal images of fly brains expressing S2mM13.NLS and 2xM13.NLS. Images show anti-myc (red) and nuclear chromatin labeled with Hoechst (blue). Non-linear scaling of Hoechst images was necessary to visualize entire nuclei. The scale bars are 7.5 µm.

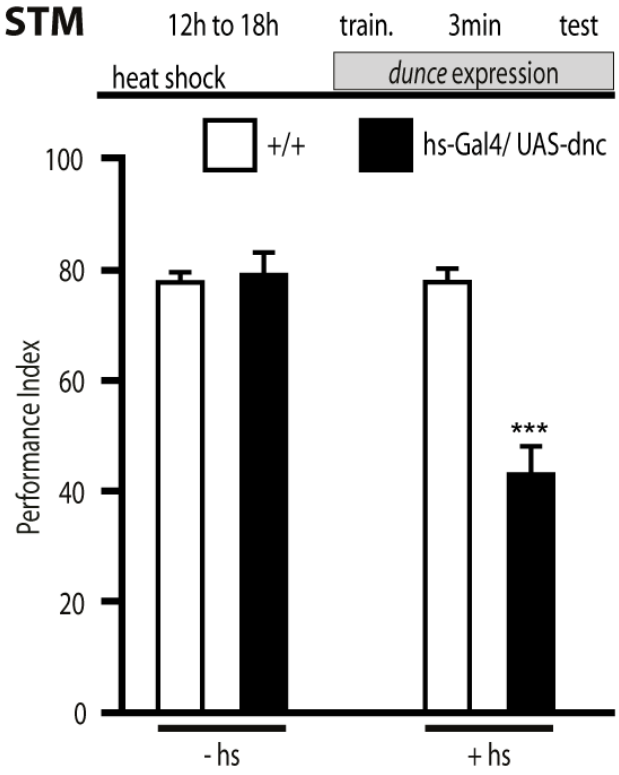

Fig. S5. Flies overexpressing the cAMP phosphodiesterase–encoding gene (*dunce*) under the control of a heat shock promoter show impaired STM. Flies were subjected to heat shock for 30 min at 37ºC (+hs) or not (-hs) and were trained and tested for STM. Significant reductions (*** $p < 0.001$) are indicated between the heat-shocked dunce-expressing flies and the heat-shocked wild-type control flies (n = 6 independent experiments with on average 2 x 80-100 flies in each experiment)

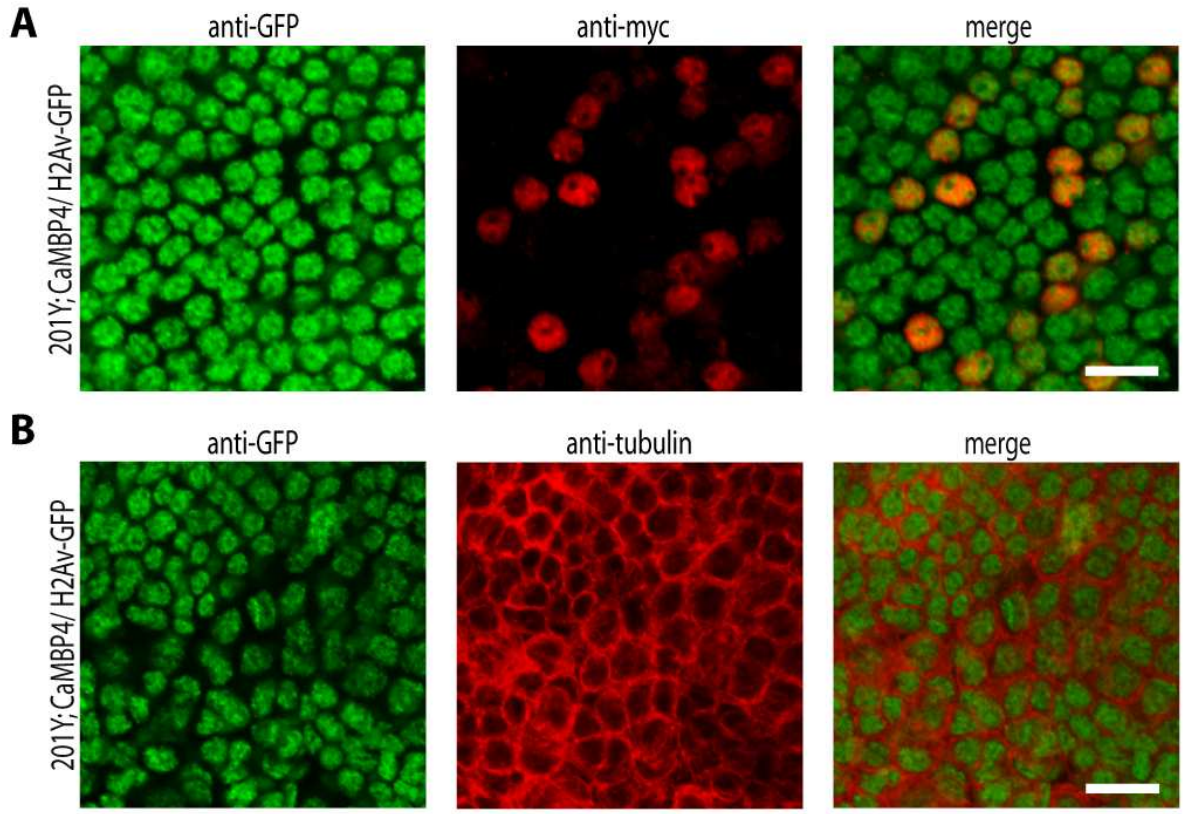

Fig. S6. Confocal images of fly brains expressing CaMBP4 and H2Av-GFP in the γ neurons of the mushroom bodies. The images show 201Y; CaMBP4/ H2Av-GFP fly brains expressing histone2Av-GFP (H2Av-GFP) under control of histon2Av promoter immunolabeled with anti-GFP (green) and either CaMBP4 immunolabeled with anti-myc (red, A) or the cytoplasmic marker tubulin immunolabeled with anti-tubulin (red, B). Scale bars are 10 µm.