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Supplemental Information

A GABAergic Feedback Shapes Dopaminergic Input on the *Drosophila* Mushroom Body to Promote Appetitive Long-Term Memory

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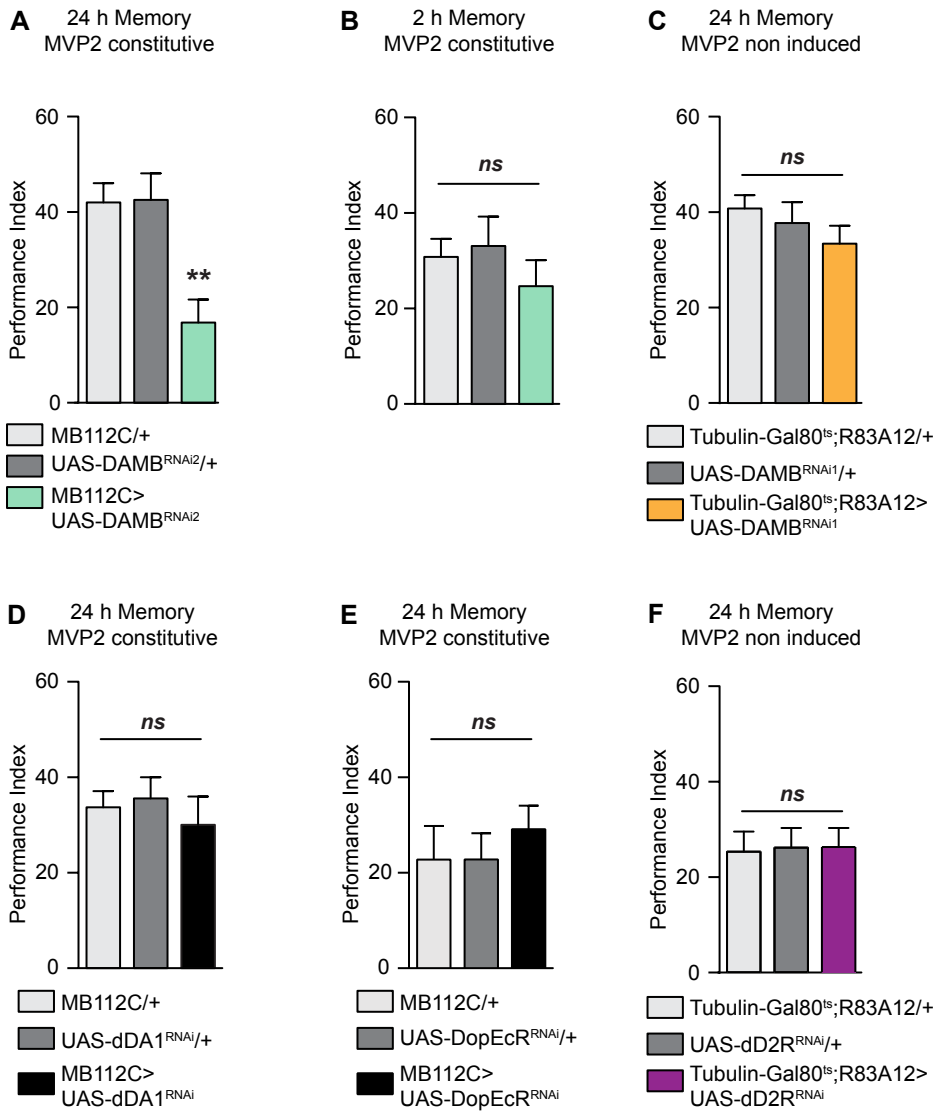
A
24 h Memory
MVP2 constitutive
Performance Index
60
40
20
0
**
MB112C/+
UAS-DAMB^RNAi2/+
MB112C>
UAS-DAMB^RNAi2
B
2 h Memory
MVP2 constitutive
Performance Index
60
40
20
0
ns
C
24 h Memory
MVP2 non induced
Performance Index
60
40
20
0
ns
Tubulin-Gal80^ts;R83A12/+
UAS-DAMB^RNAi1/+
Tubulin-Gal80^ts;R83A12>
UAS-DAMB^RNAi1
D
24 h Memory
MVP2 constitutive
Performance Index
60
40
20
0
ns
MB112C/+
UAS-dDA1^RNAi/+
MB112C>
UAS-dDA1^RNAi
E
24 h Memory
MVP2 constitutive
Performance Index
60
40
20
0
ns
MB112C/+
UAS-DopEcR^RNAi/+
MB112C>
UAS-DopEcR^RNAi
F
24 h Memory
MVP2 non induced
Performance Index
60
40
20
0
ns
Tubulin-Gal80^ts;R83A12/+
UAS-dD2R^RNAi/+
Tubulin-Gal80^ts;R83A12>
UAS-dD2R^RNAi

Figure S1. Supplemental evidence of DAMB and dD2R requirement in MVP2 neurons for appetitive LTM (related to Figure 1)

(A-B) Similar results as in Figure 1A-B were obtained using a second non-overlapping DAMB RNAi (*UAS-DAMB*^{RNAi2}) to constitutively down-regulate DAMB in MVP2 neurons, revealing impaired appetitive LTM ((A) n=14, $F_{2,39}$=8.20, p<0.05) but normal 2 h memory ((B) n=15, $F_{2,42}$=2.17, p=0.13). (C) Non-induced controls for DAMB RNAi (*UAS-DAMB*^{RNAi1}) in adult MVP2 neurons displayed normal LTM (n=12, $F_{2,33}$=1.00, p=0.38). (D-E) Inhibition of any of the other D1-like dopamine receptor in MVP2 did not affect LTM: (D) neither dDA1 constitutive down-regulation in MVP2 affected LTM (n=17, $F_{2,48}$=0.36, p=0.70), (E) neither DopEcR constitutive down-regulation (n=14, $F_{2,39}$=0.26, p=0.77). (F) Non-induced controls for dD2R RNAi in adult MVP2 neurons displayed normal LTM ((E) n=12, $F_{2,33}$=0.02, p=0.98). Mean ± S.E.M. Statistical tests were performed using one-way ANOVA, **p<0.01 in post hoc comparison. See also Table S1 for sugar perception and olfactory acuity controls.

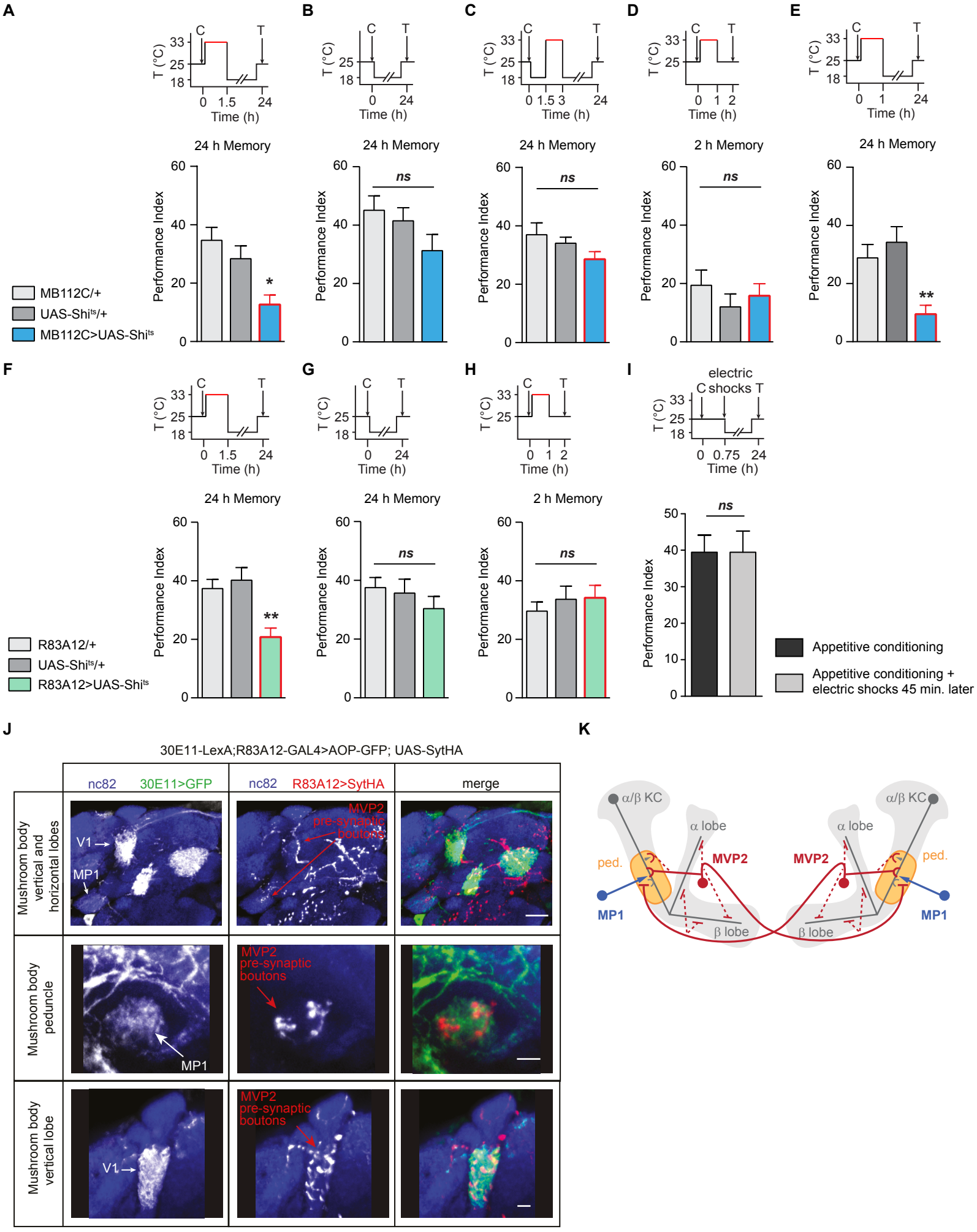
A
B
C
D
E
F
G
H
I
J
K
T (°C)
Time (h)
C
T
24 h Memory
2 h Memory
Performance Index
ns
MB112C/+
UAS-Shi^ts/+
MB112C>UAS-Shi^ts
R83A12/+
R83A12>UAS-Shi^ts
electric shocks
Appetitive conditioning
Appetitive conditioning + electric shocks 45 min. later
30E11-LexA;R83A12-GAL4>AOP-GFP; UAS-SytHA
nc82
30E11>GFP
R83A12>SytHA
merge
Mushroom body vertical and horizontal lobes
Mushroom body peduncle
Mushroom body vertical lobe
V1
MP1
MVP2 pre-synaptic boutons
α/β KC
α lobe
β lobe
ped.
MVP2
MP1

Figure S2. Supplemental evidence for the requirement of MVP2 activity at a specific time period after conditioning for LTM formation, and anatomical data supporting an MVP2/MP1 synaptic connection (related to Figure 2)

(A) Using the *MB112C-split-Gal4* driver in combination with *UAS-Shi^{ts}* to block MVP2 output immediately after training for 1h30 impaired LTM (n=12, $F_{2,33}$=7,71, p<0.01). (B) LTM was normal at the permissive temperature (n=14, $F_{2,39}$=2.04, p=0.14). (C) 1.5 h blockade of MVP2 activity 1.5h after conditioning did not impaired LTM (n=22, $F_{2,63}$=1.98, p=0.15). (D) One-hour MVP2 activity blockade after training did not impair 2 h memory (n=16, $F_{2,45}$=0.64, p=0.53). (E) One-hour MVP2 activity blockade after training impaired LTM (n=12, $F_{2,33}$=8.55, p<0.01). (F-H) Similar results were obtained using another MVP2 driver (*R83A12-Gal4*): (F) MVP2 blockade immediately after conditioning impaired LTM (n=17, $F_{2,48}$=8.68, p<0.01), while (G) LTM in permissive controls was normal (n=18, $F_{2,51}$=0.79, p=0.46) and (H) a 1 h block of MVP2 activity did not affect 2 h memory (n=10, $F_{2,27}$=0.38, p=0.68). (I) Wild-type flies were trained with appetitive conditioning. An experimental group was then delivered 12 pulses of 60V-electric shocks 45 minutes after conditioning, while a control group was kept untreated. Appetitive memory performance was tested 24 h later. There was no difference between the two groups (n=12, t-test, t_{22}=0.00; p=1.00). (J) Immuno-histochemistry of *30E11-LexA;R83A12-Gal4>UAS-Syt-HA;;AOP-mCD8::GFP* females flies brain showing GFP tagged MP1 neurons (in green) and MVP2 neurons pre-synapses tagged with Syt-HA (in red) and neuronal marker nc82 (in blue). Each image displayed is a maximum projection of 5 consecutive images from the original confocal stack, which were spaced by 1µm. The top row display a global view of the MB lobes showing projection from V1 and MP1 dopaminergic neurons on the vertical lobes and the γ1 compartment, respectively, as well as the extended presynaptic projections of MVP2 neurons on the MB lobes, and in other areas surrounding the MB (see text). The middle row focuses on the MB peduncle compartment, where MP1 neurons show a dense projection area. MVP2 pre-synapses are present in the same area. The bottom row is a similar zoom-in on the vertical lobes showing MVP2 pre-synaptic projections, which were already described (see text). Scale bar is 15µm on the top row, and 5µm on the middle and bottom rows. (K) Schematic diagram of the MP1-MVP2 recurrent anatomical circuit on the α/β neurons of the MB. There is one MP1 neuron (in blue) per hemisphere, which projects to the peduncle MB compartment ('ped.' in yellow) where it makes direct synaptic contact with MVP2 (in red). There is one MVP2 neuron per hemisphere, which sends its axonal projection to the contralateral peduncle compartment. The other MVP2 axonal projections on the α and β lobes are represented in dashed lines. For clarity, projections from MP1 and MVP2 neurons in the γ1 compartment and outside MB are not illustrated here.

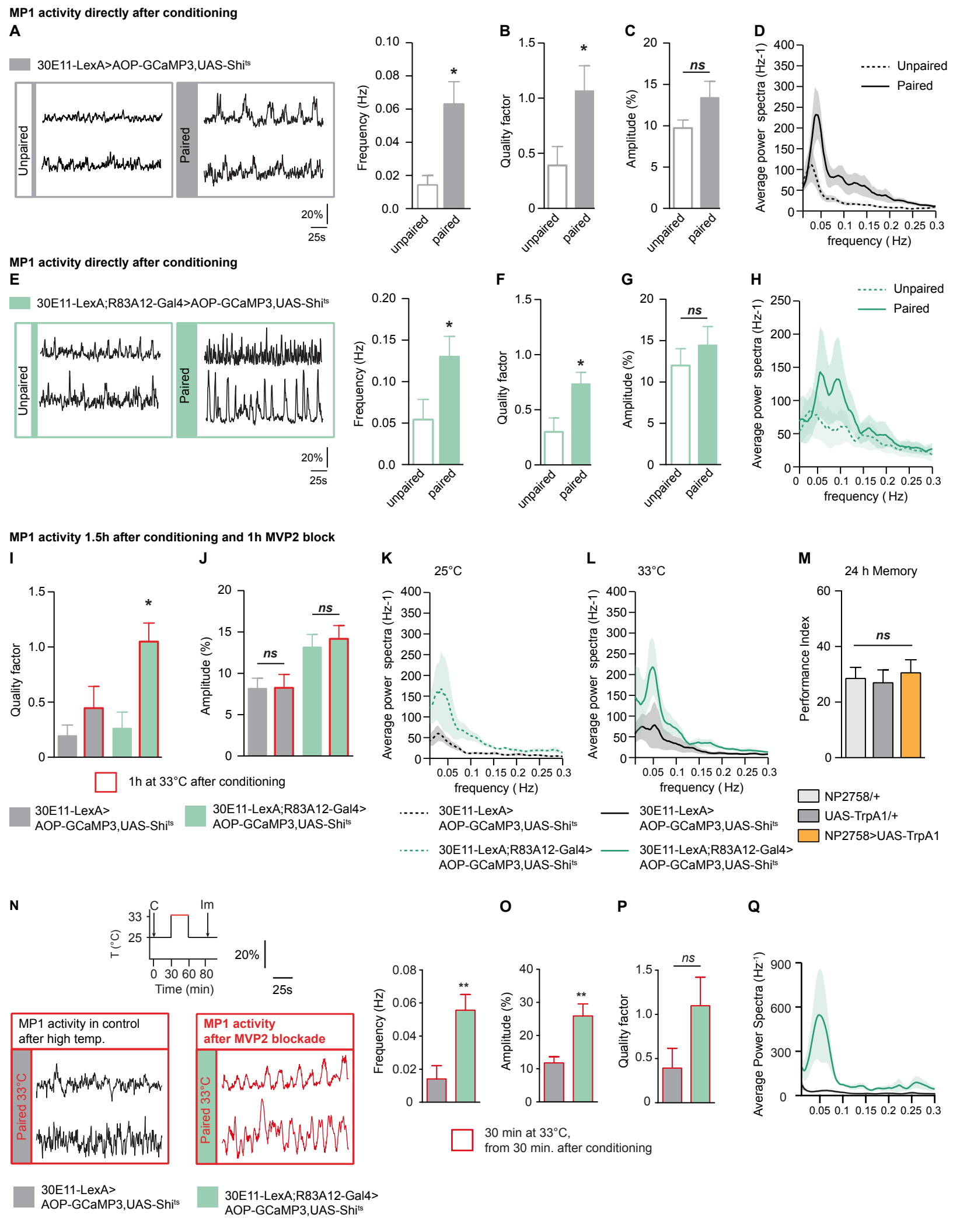
MP1 activity directly after conditioning
A
30E11-LexA>AOP-GCaMP3,UAS-Shits
Unpaired
Paired
20%
25s
Frequency (Hz)
unpaired
paired
B
Quality factor
C
Amplitude (%)
ns
D
Average power spectra (Hz-1)
Unpaired
Paired
frequency (Hz)
MP1 activity directly after conditioning
E
30E11-LexA;R83A12-Gal4>AOP-GCaMP3,UAS-Shits
F
G
H
MP1 activity 1.5h after conditioning and 1h MVP2 block
I
J
1h at 33°C after conditioning
30E11-LexA>AOP-GCaMP3,UAS-Shits
30E11-LexA;R83A12-Gal4>AOP-GCaMP3,UAS-Shits
K
25°C
L
33°C
M
24 h Memory
Performance Index
NP2758/+
UAS-TrpA1/+
NP2758>UAS-TrpA1
N
C
Im
T (°C)
Time (min)
MP1 activity in control after high temp.
MP1 activity after MVP2 blockade
Paired 33°C
O
P
Q
30 min at 33°C, from 30 min. after conditioning
Average Power Spectra (Hz-1)

Figure S3. Pairing a sugar reward and an odorant increases MP1 frequency oscillations immediately after training and Supplementary analyses of MP1 oscillations during consolidation after MVP2 blockade (related to Figure 3)

(A-D) At the permissive temperature, *30E11-LexA>AOP-GCaMP3;UAS-Shi^{ts}* flies exhibited an increased frequency of MP1 calcium oscillations 0.5 h after training with a paired protocol, as compared to an unpaired protocol ((A) n=9, t_{16}=3.34, p<0.05). At the permissive temperature, *30E11-LexA>AOP-GCamP3;UAS-Shi^{ts}* flies displayed an increased quality factor of MP1 oscillations 0.5 h after training with a paired protocol as compared to an unpaired protocol ((B) n=9, t_{16}=2.50, p<0.05) whereas the amplitude of MP1 calcium oscillations was not changed significantly ((C) n=9, t_{16}=1.67, p=0.11). (D) Average power spectra of MP1 activity across all imaged flies, *30E11-LexA >AOP-GCaMP3;UAS-Shi^{t}* trained flies exhibit a peak revealing an oscillatory activity. (E-H) At the permissive temperature, *30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts}* flies displayed an increased frequency of MP1 oscillations 0.5 h after training with a paired protocol as compared to an unpaired protocol ((E) n=9, t_{16}=2.23, p<0.05) as well as an increased quality factor of MP1 oscillations ((F) n=9, t_{16}=2.66, p<0.05). (G) No significant change in the amplitude of MP1 calcium oscillations was observed 0.5 h after training with a paired protocol as compared to an unpaired protocol (n=9, t_{16}=0.81, p=0.43). (H) Average power spectra of MP1 activity across all imaged flies, *30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts}* trained flies exhibited a peak revealing an oscillatory activity. Mean ± S.E.M. Statistical tests were performed t-test, *p<0.05. (I-L) When MVP2 activity was blocked for 1 h after paired training (*30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts}* flies at 33°C), the quality factor of MP1 oscillations was significantly higher than in the permissive (paired 25°C *30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts}* flies) and temperature controls (paired 33°C *30E11-LexA>AOP-GCaMP3;UAS-Shi^{ts}* flies) ((I) n=9, $F_{3,32}$=6.12, p<0.01). (J) When MVP2 activity was blocked for 1 h after paired training (*30E11-LexA;R83A12-Gal4>AOP-GCamP3;UAS-Shi^{ts}* flies at 33°C) the amplitude of MP1 oscillations was not significantly different from the permissive control (paired 25°C *30E11-LexA;R83A12-Gal4>AOP-GCamP3;UAS-Shi^{ts}* flies). However, compared to flies without the MVP2 *R83A12-Gal4* driver (*30E11-LexA>AOP-GCamP3;UAS-Shi^{ts}* flies at either 25°C or 33°C), the oscillation seemed to be higher; this effect was independent of the temperature and consequently of MVP2 blockade (n=9, $F_{3,32}$=4,43, p<0.05). (K-L) Average power spectra of MP1 activity across all imaged flies at permissive temperature (K) and at 33°C (L) showing that only when MVP2 neurons are blocked during 1h (*30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts} at 33°C)* trained flies exhibited a peak revealing an oscillatory activity. As for the amplitude of the oscillations, we can note a difference in the power spectra between the two genotypes. (M) Expression of *TrpA1* in MP1 neurons using the *NP2758* driver did not affect LTM at permissive temperature (n=13, $F_{2,36}$=0.16, p=0.85). (N-Q) After paired training, flies were stored at 25°C for 0.5 h and then transferred at 33°C for 0.5h. Flies were prepared immediately after for

imaging, so that recordings were performed 80 minutes after conditioning. When MVP2 activity was blocked using this protocol (*30E11-LexA;R83A12-Gal4>AOP-GCaMP3;UAS-Shi^{ts}* flies, n = 8), the frequency and amplitude of MP1 oscillations were significantly higher than in genotypic controls (paired 33°C *30E11-LexA;+>AOP-GCaMP3;UAS-Shi^{ts}* flies, n = 9) ((N) frequency: t_{15}=3,38, p<0.005; (O) amplitude: t_{15}=3,65, p<0.005). (P) The quality factor also tended to increase, although not reaching significance (p=t_{15}=1.84, p=0.085). (Q) Average power spectra of MP1 activity across all imaged flies showing that only when MVP2 neurons are blocked during 0.5h after 0.5h at permissive temperature, trained flies exhibited a peak revealing an oscillatory activity. Time courses of temperature shifts are displayed above the MP1 recordings (C: conditioning, Im: imaging). Illustrative examples of MP1 neuron recordings are displayed for all conditions. Mean ± S.E.M. Statistical tests were performed using the t-test (A-C, E-G, N-P) or one-way ANOVA (I-J, M). Pairwise post hoc comparisons are indicated only if significant (*p<0.05).

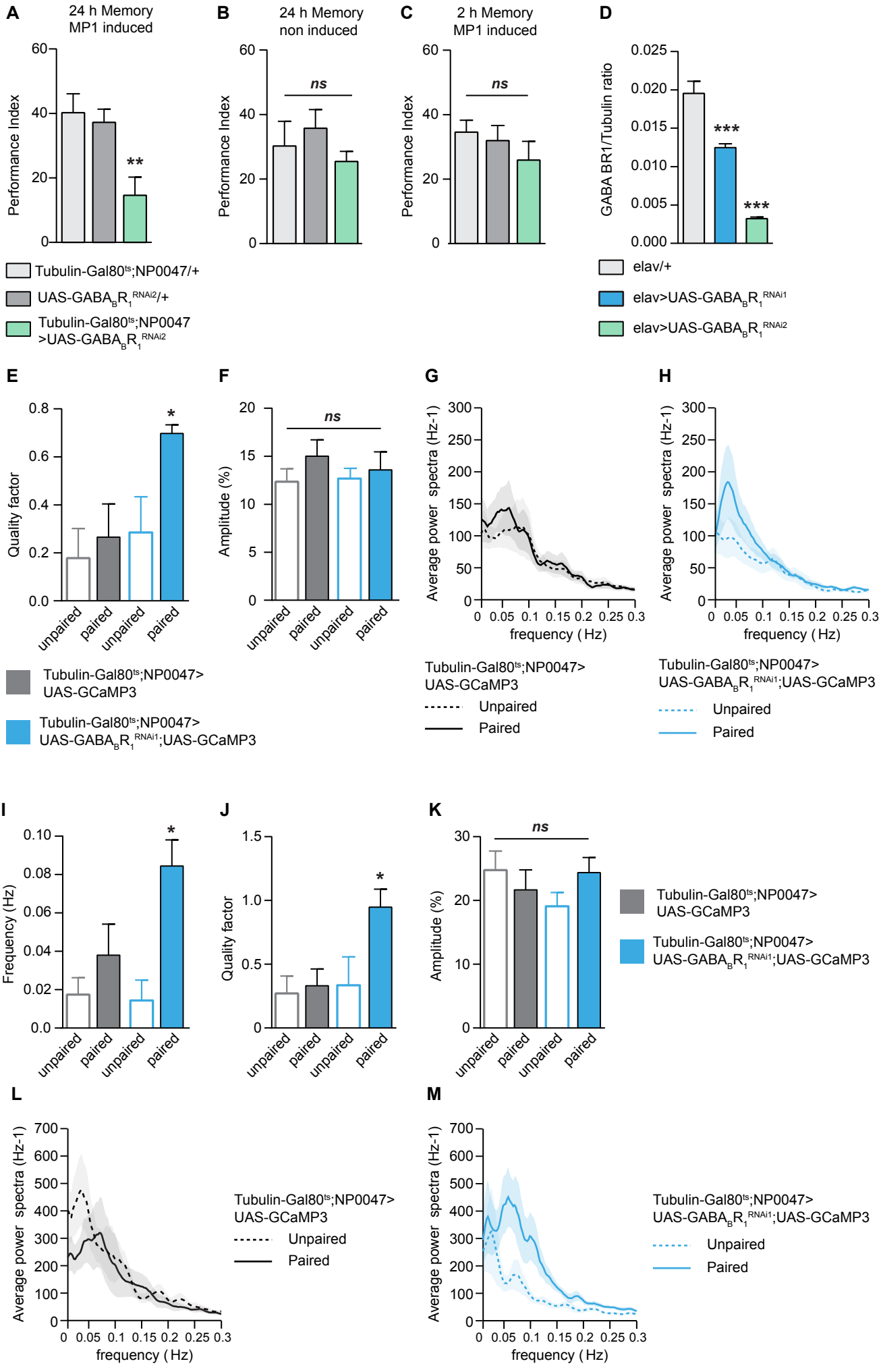
A
24 h Memory
MP1 induced
Performance Index
60
40
20
0
**
B
24 h Memory
non induced
ns
C
2 h Memory
MP1 induced
D
GABA BR1/Tubulin ratio
0.025
0.020
0.015
0.010
0.005
0.000
***
***
Tubulin-Gal80ts;NP0047/+
UAS-GABABR1RNAi2/+
Tubulin-Gal80ts;NP0047
>UAS-GABABR1RNAi2
elav/+
elav>UAS-GABABR1RNAi1
elav>UAS-GABABR1RNAi2
E
Quality factor
0.8
0.6
0.4
0.2
0.0
*
unpaired
paired
unpaired
paired
F
Amplitude (%)
20
15
10
5
0
ns
G
Average power spectra (Hz-1)
300
250
200
150
100
50
0
0 0.05 0.1 0.15 0.2 0.25 0.3
frequency (Hz)
H
Tubulin-Gal80ts;NP0047>
UAS-GCaMP3
Unpaired
Paired
Tubulin-Gal80ts;NP0047>
UAS-GABABR1RNAi1;UAS-GCaMP3
I
Frequency (Hz)
0.10
0.08
0.06
0.04
0.02
0.0
J
Quality factor
1.5
1.0
0.5
0
K
Amplitude (%)
30
20
10
0
L
M

Figure S4. Supplementary evidence of D-GABA$_B$R$_1$ requirement in MP1 neurons for appetitive LTM and supplemental analyses of D-GABA$_B$R$_1$ down-regulation effect on MP1 oscillations (related to Figure 4)

(A) Similar results as in Figure 4A-C were obtained using a second non-overlapping D-GABA$_B$R$_1$ RNAi (*UAS-GABA$_B$R$_1^{RNAi2}$*), revealing that down-regulation of D-GABA$_B$R$_1$ in MP1 adult neurons impaired LTM (n=14; $F_{2,39}$=7.07, p<0.05). (B) No LTM defect was observed in the non-induced control (n=9, $F_{2,24}$=0.77, p=0.74). (C) Expression of D-GABA$_B$R$_1$ RNAi in MP1 adult neurons did not impair 2 h memory (n=11; $F_{2,30}$=0.43, p=0.66). See also Table S2 for sugar perception and olfactory acuity controls. (D) qPCR analysis of *GABA$_B$R$_1$* mRNA targeting by the RNAi-GABA$_B$R$_1$ constructs. Total RNA was extracted from *elav/+* and *elav/RNAi-Nep1C* fly heads, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using tubulin (Tub) expression as a reference. Results are shown as ratios to the reference (*Mann-Whitney test*, Bonferroni correction significance 0.025 ***p < 0.001, n = 4). (E-F) In flies co-expressing D-GABA$_B$R$_1$ RNAi1 and GCaMP3 in MP1 adult neurons, the quality factor of MP1 calcium oscillations was significantly increased 1.5 h after appetitive training, as compared to unpaired controls and both paired and unpaired flies that did not express D-GABA$_B$R$_1$ RNAi ((E) n=9; $F_{3,32}$=3.73, p<0.05) whereas the amplitude of MP1 calcium oscillations was not significantly changed 1.5 h after appetitive training, as compared to unpaired controls and both paired and unpaired flies that did not express D-GABA$_B$R$_1$ RNAi ((F) n=9; $F_{3,32}$=0.35, p=0.79). (G-H) Average power spectra of MP1 activity across all imaged flies in control genotype (G) and in flies co-expressing D-GABA$_B$R$_1$ RNAi1 and GCaMP3 in MP1 adult neurons (H) showing that only when D-GABA$_B$R$_1$ is downregulated paired trained flies exhibited a peak revealing an oscillatory activity compared to unpaired flies. (I-M) MP1 activity after Methyl-cyclohexanol conditioning instead of Octanol conditioning showing the same effect. (I-J) Both the frequency (I) and the quality factor (J) of MP1 calcium oscillations was significantly higher 1.5 h after appetitive training in flies co-expressing D-GABA$_B$R$_1$ RNAi and GCamP3 in adult MP1 neurons, than in unpaired controls and both paired and unpaired flies that do not express the D-GABA$_B$R$_1$ RNAi ((I) n=8; $F_{3,28}$=6.57, p<0.05) and (J) n=8; $F_{3,28}$=3.85, p<0.01). (K) As for octanol conditioning, in paired flies co-expressing D-GABA$_B$R$_1$ RNAi and GCamP3 in adult MP1 neurons the amplitude of MP1 oscillation after Methylcyclohexanol was not different from controls (n=8; $F_{3,28}$=0.96, p=0.43). (L-M) Average power spectra of MP1 activity across all imaged flies in control genotype (L) and in flies co-expressing D-GABA$_B$R$_1$ RNAi1 and GCaMP3 in MP1 adult neurons (M) showing that similarly to O-pairing only when D-GABA$_B$R$_1$ is downregulated M-paired trained flies exhibited a peak revealing an oscillatory activity compared to unpaired flies. Mean ± S.E.M. Statistical tests were performed using one-way ANOVA; pairwise post hoc comparisons are indicated only if significant (*p<0.05; **p<0.01).

Genotypes	Sugar response		Naive odor avoidance			
			Octanol		Methylcyclohexanol	
	Mean± S.E.M	Statistics	Mean± S.E.M	Statistics	Mean± S.E.M	Statistics
MB112C/+	0.36±0.04	$F_{2,39}$=1.41 p=0.26 n=14	0.60±0.05	$F_{2,57}$=1.16 p=0.32 n=20	0.46±0.07	$F_{2,57}$=0.97 p=0.38 n=20
UAS-DAMB^{RNAi1}/+	0.24±0.04		0.51±0.07		0.38±0.06	
MB112C>UAS-DAMB^{RNAi1}	0.32±0.07		0.47±0.06		0.51±0.07	
Tubulin-Gal80^{ts};83A12/+	0,48±0.07	$F_{2,45}$=0.81 p=0.45 n=16	0.46±0.04	$F_{2,51}$=1.59 p=0.21 n=18	0.59±0.04	$F_{2,51}$=0.45 p=0.64 n=18
UAS-DAMB^{RNAi1}/+	0,47±0.09		0.42±0.05		0.55±0.06	
Tubulin-Gal80^{ts};83A12> UAS-DAMB^{RNAi1}	0,59±0.06		0.54±0.05		0.62±0.05	
MB112C/+	0.33±0.08	$F_{2,45}$=0.27 p=0.76 n=16	0.47±0.08	$F_{2,45}$=1.19 p=0.32 n=16	0.62±0.06	$F_{2,45}$=0.31 p=0.74 n=16
UAS-DAMB^{RNAi2}/+	0.28±0.07		0.58±0.07		0.62±0.06	
MB112C>UAS-DAMB^{RNAi2}	0.25±0.06		0.43±0.07		0.57±0.06	
MB112C/+	0.26±0.10	$F_{2,63}$=0.06 p=94 n=22	0.44 ±0.14	$F_{2,45}$=0.25 p=0.78 n=16	0.55±0.10	$F_{2,45}$=0.26 p=0.77 n=16
UAS-dD2R^{RNAi}/+	0.28±0.07		0.54±0.14		0.45±0.11	
MB112C>UAS-dD2R^{RNAi}	0.24±0.08		0.42±0.12		0.47±0.11	
Tubulin-Gal80^{ts};83A12/+	0.42±0.08	$F_{2,69}$=0.70 p=0.50 n=24	0.31.±0.12	$F_{2,27}$=0.17 p=0.84 n=10	0.56±0.10	$F_{2,39}$=1.60 p=0.22 n=13-15
UAS-dD2R^{RNAi}/+	0.40±0.08		0.38±0.09		0.48±0.07	
Tubulin-Gal80^{ts};83A12 >UAS-dD2R^{RNAi}	0.30±0.08		0.39±0.11		0.32±0.12	

Table S1. Sugar response and olfactory acuity (Related to Figures 1 and S1)

Genotypes	Sugar response		Naive odor avoidance			
			Octanol		Methylcyclohexanol	
	Mean± S.E.M	Statistics	Mean± S.E.M	Statistics	Mean± S.E.M	Statistics
Tubulin-Gal80^{ts};NP0047/+	0.24±0.06	$F_{2,45}$=0.12 p=0.89 n=16	0.42±0.06	$F_{2,45}$=0.30 p=0.74 n=16	0.52±0.08	$F_{2,45}$=1.35 p=0.27 n=16
UAS-GABA$_B$R$_1^{RNAi1}$/+	0.30±0.10		0.45±0.06		0.54±0.05	
Tubulin-Gal80^{ts};NP0047> UAS-GABA$_B$R$_1^{RNAi1}$	0.24±0.10		0.49±0.06		0.65±0.05	
Tubulin-Gal80^{ts};NP0047/+	0.29±0.08	$F_{2,51}$=0.32 p=0.73 n=18	0.53±0.06	$F_{2,33}$=0.97 p=0.39 n=12	0.66±0.06	$F_{2,27}$=2.31 p=0.12 n=10
UAS-GABA$_B$R$_1^{RNAi2}$/+	0.22±0.07		0.52±0.08		0.51±0.08	
Tubulin-Gal80^{ts};NP0047> UAS-GABA$_B$R$_1^{RNAi2}$	0.27±0.06		0.62±0.04		0.47±0.06	
NP2758/+;Tubulin-Gal80^{ts}	0.42±0.07	$F_{2,33}$=0.62 p=0.54 n=12	0.56±0.08	$F_{2,33}$=0.32 p=0.73 n=12	0.60±0.04	$F_{2,33}$=0.75 p=0.48 n=12
UAS-GABA$_B$R$_1^{RNAi1}$/+	0.34±0.07		0.47±0.08		0.66±0.05	
NP2758;Tubulin-Gal80^{ts}> UAS-GABA$_B$R$_1^{RNAi1}$	0.45±0.08		0.53±0.08		0.58±0.04	

Table S2. Sugar response and olfactory acuity (Related to Figures 4 and S4)