Supplementary information

Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*

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Supplementary Figure 1: Behavioral controls for experiments with *Th-GAL4*.

(a) *Th-GAL4/UAS-shit\(^{22}\)* flies showed a normal 3 h performance at permissive temperature (20°C) after single cycle conditioning (F\((2,41) = 5.350, p = 0.0086, n \geq 14\). Post hoc pairwise comparisons: *Th-GAL4/UAS-shit\(^{22}\)* vs. *Th-GAL4/+*: p < 0.01, *Th-GAL4/UAS-shit\(^{22}\)* vs. +/UAS-shit\(^{22}\) : p > 0.05). (b) Olfactory acuity of *Th-GAL4/UAS-shit\(^{22}\)* flies was similar to that of the genetic controls at restrictive temperature (31°C) both for 3-octanol (O; F\((2,21) = 0.6754, p = 0.5197, n = 8\), and 4-methylcyclohexanol (M; F\((2,21) = 1.156, p = 0.3340, n = 8\). (c) *Th-GAL4/UAS-shit\(^{22}\)* flies showed a normal 24 h performance at permissive temperature (20°C) after massed conditioning (F \((2,35) = 0.35, p = 0.71, n = 12\). (d) Blocking dopaminergic neurons output during memory retrieval after massed training did not alter 24-hour memory performance (F\((2,45) = 0.5691, p = 0.54, n \geq 15\). (e) *Th-GAL4/+; +/UAS-TrpA1* flies showed a normal 3h performance at permissive temperature (20 °C) after single cycle conditioning (F\((2,21) = 0.22, p = 0.802, n = 8\).
Supplementary Figure 2: Behavioral controls for experiments with NP0047-GAL4 and cold shock experiment.

(a) NP0047-GAL4/UAS-shi<sup>av</sup> flies showed a normal 24 h performance at permissive temperature (20°C) after massed conditioning (F<sub>(2,20)</sub> = 0.25, p = 0.78, n = 7). (b) One-minute activation of NP0047-GAL4 neurons straight after single cycle conditioning impairs 3 h memory (p = 0.0003, n ≥ 14). The memory drop persisted when a 2 min cold-shock anesthesia was performed 2 h after training (p = 0.0084, n ≥ 18), showing that ARM is decreased. (c) NP0047-GAL4/+; +/-UAS-TrpA1 flies showed a normal 3 h performance at permissive temperature (20°C) after single cycle conditioning (F<sub>(2,20)</sub> = 4.27, p = 0.03, n = 7. Post hoc pairwise comparisons: NP0047-GAL4/+; +/-UAS-TrpA1 vs. NP0047-GAL4/+; +/-UAS-TrpA1 vs. +/-UAS-TrpA1: p < 0.05).
Supplementary Figure 3: Spontaneous activity in MV1 and MP1 neurons are indistinguishable.

(a) Recordings were obtained for 6 flies at different times in regions where labeling could be unequivocally attributed to either MV1 or MP1 neurons. Both types of neurons show large amplitude spontaneous calcium changes compared with control structures ($p = 0.0028$, $n = 6$). (b–d): Correlation is high between signals from MV1 and MP1 neurons, considering amplitude (b) ($R^2 = 0.90$), frequency (c) ($R^2 = 0.99$) and quality factor (d) ($R^2 = 0.95$).
Supplementary Figure 4: No activity was detectable in V1 neurons after any type of conditioning.

V1 neurons and neighbouring structures chosen as controls produced noisy signals of similar amplitude, after one cycle of conditioning ($p = 0.13$, $n = 8$ flies), five massed cycles ($p = 0.46$, $n = 11$) and five spaced cycles ($p = 0.08$, $n = 8$).
Supplementary Figure 5: Activity of MV1 and MP1 neurons is enhanced after TrpA1 activation.

(a) Co-expression of GCaMP3 and TrpA1 in NP0047-GAL4 neurons led to a defect in 3 h memory at permissive temperature (F(2,20) = 13.20; p < 0.0001; n ≥ 9). (b) From top to bottom, three examples illustrating the triggering of sustained, and sometimes oscillatory (bottom two examples), activity in MV1 and MP1 neurons by thermal activation in flies carrying GCaMP3 and TrpA1 with initially silent MV1 and MP1 neurons. (c) Time course of the long-lasting amplitude change in MV1 and MP1 neurons' activity triggered by a brief thermal activation to 30 °C, averaged over 7 flies (left). Plotting the maximal amplitude reached before and after activation reveals a significant increase (right, p = 0.019 , paired t-test, n = 7). (d) By contrast, amplitude remained constant in flies not carrying UAS-TrpA1, and no significant increase could be measured (p = 0.52, paired t-test, n = 8). In this series of experiments in which agarose could not be applied on the brain to restrain its movement, baseline amplitude was higher than in other experiments. Thus baseline amplitude reached ~20% even in flies expressing both GCaMP3 and TrpA1, whose MV1 and MP1 neurons had very little spontaneous activity.
Supplementary Figure 6: Dopaminergic neurons' control over ARM levels is independent from the radish gene.

(a–b) Spontaneous activity of MV1 and MP1 neurons was similar in wild-type and rsh naive males. In both cases signals spanned a wide frequency range, resulting in monotonically decreasing power spectra (a) (+/Y: n = 7, 4 oscillating flies; rsh/Y: n = 6, 3 oscillating flies). The average amplitudes of MV1 and MP1 neurons' activity were also similar (b) (p = 0.15). (c) Consistent with imaging results, carrying the radish mutation did not prevent flies from forming additional memory when their dopaminergic neurons were blocked after conditioning (Females: F(3,66) = 20.59; p < 0.0001; n ≥ 16; Males: F(3,62) = 40.93; p < 0.0001; n ≥ 15; # symbols indicate the level of significance of the post-hoc test between rsh/+ (respectively rsh/Y) and CS.)
Supplementary Figure 7: Activation or blockade of dopaminergic neurons before single-cycle training does not impair learning.

(a) 1-minute activation of Th-GAL4 neurons 5 minutes before single-cycle training did not impair learning \([F(2,29) = 0.34; p = 0.37; n = 10]\). (b) 8-minute blockade of Th-GAL4 neurons 15 minutes before single-cycle training had no effect on learning \([F(2,23) = 0.20; p = 0.82; n = 8]\). (c) 8-minute blockade of NP0047-GAL4 neurons 15 minutes before single-cycle training had no effect on learning \([F(2,23) = 0.984; p = 0.39; n = 8]\).
Supplementary Figure 8: ARM-regulating neurons gate LTM formation during the inter-trial intervals.

The three panels illustrate the role of oscillatory dopaminergic neurons after single cycle (a), massed (b) and spaced training (c), and their effect on the two independent pathways leading to the formation of consolidated ARM or LTM, respectively. (a) After single-cycle training, oscillatory modulation on the mushroom body from MV1 and MP1 neurons prevents excessive ARM formation and prepares the brain for a potential multiple and spaced experience, though sufficient activity of the ARM pathway remains which prevents LTM from being formed after this single cycle. (b) Massed training reduces the activity of MV1 and MP1 neurons, which leads to robust ARM consolidation and represses the LTM pathway. (c) Only during the rest intervals of a spaced training can oscillatory neurons repeatedly and fully inhibit ARM consolidation, letting the brain engage into the energetically costly LTM pathway. Alternatively to this model, dopaminergic neurons oscillations could independently inhibit the ARM pathway and gate the LTM pathway, enabling the latter only when maintained all along spaced training.
**Supplementary Movie 1:** Confocal stack showing the *NP4700-GAL4* expression pattern visualized by *mCD8::GFP* (white). Neuropils are counterlabeled with an anti-synapsin antibody (orange).

**Supplementary Movie 2:** Confocal stack showing the *NP0047-GAL4* expression pattern visualized by *mCD8::GFP* (white). *TH* immunoreactive cells are labeled in magenta.

**Supplementary Movie 3:** Confocal stack showing the *NP0047-GAL4* expression pattern, in combination with *Th-GAL80*, visualized by *mCD8::GFP* (white). Neuropils are counterlabeled with an anti-synapsin antibody (orange).

**Supplementary Movie 4:** Confocal stack showing the *NP0047-GAL4* expression pattern, in combination with *Th-GAL80*, visualized by *mCD8::GFP* (white). *TH* immunoreactive cells are labeled in magenta.

**Supplementary Movie 5:** Spontaneous activity oscillations in MB projections from MV1 and MP1 neurons. This movie is accelerated 10 times; the real duration of the recording was 330 s. Oscillation characteristics were: left hemisphere: $f_0 = 0.11$ Hz, $Q = 2.1$, amplitude 29% and right hemisphere: $f_0 = 0.105$ Hz, $Q = 1.6$ amplitude 32%. Raw 8-bit grayscale images were smoothed with a 2-pixel radius Gaussian filter, a constant value of 30 was subtracted from the resulting whole images, and contrast was then enhanced by rescaling intensity to reach 1.5% saturated pixels on one oscillation peak image (image processing performed with ImageJ).