A cost of long-term memory in *Drosophila*

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Two distinct forms of consolidated associative memory are known in *Drosophila*: long-term memory (LTM) and anesthesia-resistant memory (ARM) (*1*-*2*). In the context of Pavlovian aversive olfactory learning, LTM will only form after repeated conditioning events separated by rest intervals (spaced protocol) whereas ARM will also form if consecutive conditioning events immediately follow one another (massed protocol) (*2*). LTM is more stable, but, in contrast to ARM, its formation requires protein synthesis (*2*). LTM is thus likely to be energetically costly, but it is not clear if the additional energy demand is of any significance for the animal's fitness.

We thus studied how induction of consolidated memory affects resistance of adult flies to extreme stress imposed by absence of food and water. We used an outbred wildtype *D. melanogaster* line which had been selected for improved memory and shows particularly good LTM (*3*). We trained the flies to associate an odor with an aversive mechanical shock (*associative conditioning*) (*4*). Five consecutive training sessions were either separated by 20 min intervals (spaced protocol) or followed one another immediately (massed protocol). Both protocols induce avoidance of the odor previously associated with the shock if assayed 24 h after conditioning (Fig. 1A). However, the response after the spaced protocol is protein synthesis-dependent, i.e., involves LTM, whereas the response after the massed protocol is not (Fig. 1A). In control experiments, other flies were either exposed to mechanical shocks without any odors (*shock only*), or exposed to both shocks and odors, but not concurrently (*non-associative conditioning*), which does not lead to formation of even short-term associative memory (red bars, Fig. 1A) (*4*).

We subjected flies to one of seven treatments (shock only, associative conditioning and non-associative conditioning × massed versus spaced protocol, plus untreated control), and directly afterwards assayed individually their desiccation and starvation resistance, measured as time till death without food and water (*4*). Despite exposure to the same shock and odors, flies conditioned in the associative spaced protocol died on average 4 hours (19 %) earlier than flies subjected to non-associative spaced conditioning ($F_{1,116} = 28.1$, $P < 10^{-3}$; Fig. 1B). This effect was the same for males and females (interaction $F_{1,116} = 0.1$, $P = 0.74$). This difference might have been due to greater activity induced by associative conditioning, but video tracking of individual flies indicated no difference in locomotor activity (Fig. S1) (*4*). No difference in time to death under stress was observed for flies subject to associative versus non associative conditioning in the massed protocol, in which LTM is not formed ($F_{1,116} = 0.7$, $P = 0.42$; Fig. 1B). The earlier death of flies subjected to associative spaced

conditioning relative to those subjected to other conditioning treatments is thus likely to reflect additional strain due to formation and maintenance of LTM. Given the importance of desiccation and starvation resistance in natural *Drosophila* populations (*5, 6*), this result suggests that long-term memory not only has benefits, but also ecologically relevant costs. Whether, and to what extent, natural selection favors improvements of memory will depend on the balance of the costs and benefits (*7*). Such costs may also help to explain why evolution has maintained ARM as another form of consolidated memory, distinct from LTM – while LTM is more stable than ARM, it is also apparently more expensive (*8*).

References and Notes:

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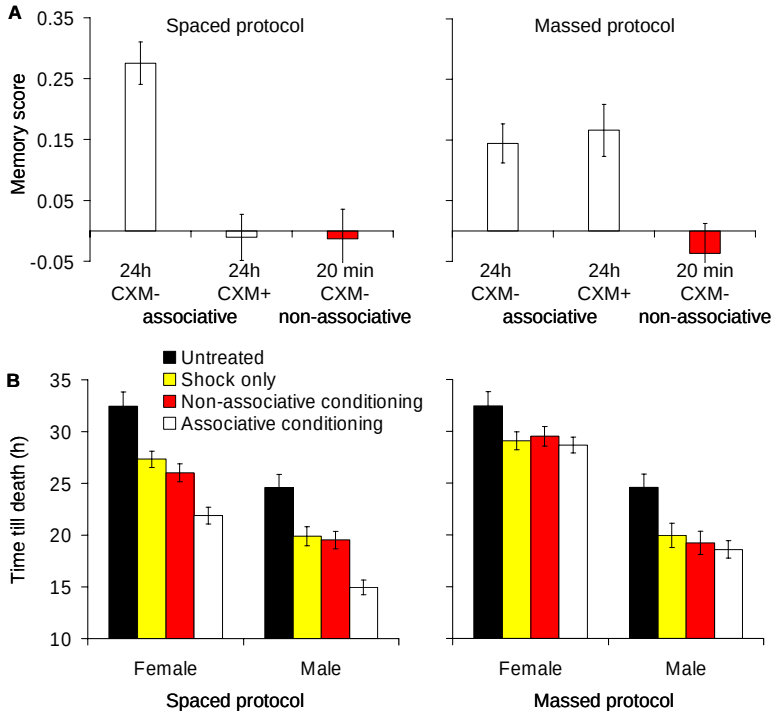

Fig. 1: (A) Olfactory memory (mean ± standard error; N = 8 replicate memory scores per treatment) induced by spaced and massed conditioning protocols. In both protocols associative conditioning induces 24 h memory in normal flies (CXM-), but treatment the protein synthesis inhibitor cycloheximide (CXM+) erases the response after the spaced protocol. No associative memory is detectable 20 min after non-associative conditioning (red bars). (B) Time till death (mean ± standard error) without food and water of flies subject to different conditioning treatments (N = 30 flies per treatment and sex).

Supporting Online Material to:

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Materials and Methods

Conditioning procedure:

Conditioning and memory tests were performed on samples of 50 adult flies (sexes mixed), raised in standard conditions and aged 3-5 days. The conditioning procedure consisted of 5 training sessions separated by 20 min intervals (spaced protocol) or immediately following one another (massed protocol).

(A) Associative conditioning: In each training session flies were first exposed for 30 s to one odorant simultaneously with mechanical shock (2000 rpm vibration pulses of 1 s duration, delivered every 5 s by a test tube shaker). This period was followed by a 60 s rest period (no odor and no shock). Then, for 30 s another odorant was delivered, without shock. The training session ended with a second rest period of 60 s. 3-octanol and 4-methylcyclohexanol (both 0.6ml/l of paraffin) were used as odorants.

(B) Non-associative conditioning was similar except that the conditioned stimulus (odor) and the unconditioned stimulus (shock) were not presented simultaneously. Instead, in each training session the flies first received for 30 s shock only (on odors). Then one odorant was provided for 30 s without shock, followed by 60 s rest period, 30 s of exposure to the second odorant, and 30 s of rest.

Memory assay:

We tested 24 h memory retention after associative conditioning in the spaced and massed protocols. In order to verify that only the spaced protocol induces LTM (which is protein synthesis dependent), flies were fed 35 mM protein synthesis inhibitor cycloheximide (Sigma) in 4% sucrose (CMX+) or 4% sucrose alone (CXM-) for 15 h before training and for 24 h between training and memory assay. We also assayed 20 min memory retention after non-associative conditioning (without cyclohexamide treatment).

For the assay 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 octanol and the other methylcyclohexanol, and allowed to choose between the two odors for 60s. The memory score was calculated as the difference in the proportion of individuals choosing

octanol between flies conditioned to avoid methylcyclohexanol and those conditioned to avoid octanol.

Desiccation and starvation resistance:

Three treatments were added to the four conditioning treatments described above (spaced versus massed protocol × associative versus non-associative conditioning). *Shock only*: flies were exposed to a shock treatment, but no odors; the shock treatment followed either the spaced or the massed protocol. *Untreated*: flies were not exposed to odors or mechanical shock, but otherwise handled in the same way as in the other treatments.

Fifty flies (sexes mixed) were placed in an empty vial and subject to one of those seven treatments (four vials per treatment). After the longest treatment was completed, 30 flies per sex and treatment were individually transferred to an empty Petri dish (diameter 5.4 cm) and kept at 25°C and 70% humidity. Dead flies were counted and sexed 7, 8, 10, 11, 13, 15, 17, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 35 and 39 h after being removed from food. At 39h all flies were dead. Time till death was analyzed with a two-way ANOVA with treatment and sex as factors.

Locomotor activity:

Flies were subjected to associative or non-associative conditioning in the spaced protocol and then individually transferred to empty Petri dishes. The locomotion of each fly was recorded 0-1 h, 2-3 h and 4-5 h after conditioning using cameras (capture rate 4 images/s). Sixteen flies per sex and treatment were assayed over four days. Using VideoScript 3.0.12 we estimated the total distance covered by the fly during recording. There was no difference in locomotion between sexes (repeated-measures ANOVA, $F_{1,123} = 1.6$; $P = 0.23$), and between flies subject to associative and non-associative conditioning ($F_{1,123} = 0.01$; $P = 0.93$; Fig. S1), indicating that associative conditioning does not induce increased locomotor activity.

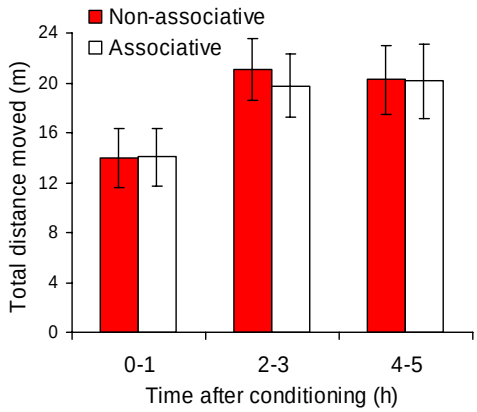

Fig. S1. Total distance moved by individual fly (mean ± standard error, sex pooled) 0-1 h, 2-3 h and 4-5 h after being subjected to non-associative or associative spaced conditioning.