Excess protein synthesis in Drosophila Fragile X mutants impairs long-term memory

François V. Bolduc 1,2, Kimberly Bell2, Hilary Cox2, Kendal Broadie 3 & Tim Tully1,2,4*

1Watson School of Biological Sciences
2Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York, USA 11724
3Vanderbilt University 1210 MRB III,VU Station B, Box 35-1634,Nashville, TN 37235-1634
4Dart Neuroscience, LLC, 7374 Lusk Blvd., San Diego CA  92121
Figure S1. Neurogenetic characterizations of disruptions of Fmr1. A) Western Blot analysis from adult heads of wild-type (WT) flies and Fmr1$^{3}$ [FMR1(3)] or Fmr1$^{B55}$ (FMR1B55) homozygous mutants. Using an anti-dFMRP 5A11 primary antibody, no FMRP was discernable in Fmr1$^{3}$ or Fmr1$^{B55}$, compared to that in wild-type flies (dark upper band). 50 ug of total protein was loaded per lane. Anti-actin immunostaining was used as a loading control (unmarked lower band). B) Immunohistochemistry of adult whole-brain using anti-dFMRP 5A11 antibody revealed widespread cytoplasmic expression of FMRP in wild-type flies, which was reduced in both Fmr1 mutants. C) Olfactory acuity to OCT and MCH was not different from wild-type (WT) flies, in Fmr1$^{3}$ [FMR1(3)] or Fmr1$^{B55}$ (FMR1B55) mutants (ANOVA, P = 0.7526). N = 4 PIs per group. D) Shock reactivity also did not differ among wild-type flies and the Fmr1 mutants (ANOVA, P = 0.122). N = 4 PIs per group. E) Western blot analysis and quantification from adult heads of wild-type flies (WT) or rutabaga$^{I}$ (Rut) mutants. Decreased level of FMRP in adult rutabaga mutants. 50 ug of total protein was loaded per lane. Anti-tubulin immunostaining was used as a loading control (unmarked lower band). F) Expression of UAS-FMRRNAi(1-7) spatially restricted to (i) the central complex, using the Feb170 GAL4 driver, or (ii) the antennal projection neurons, using the GH146 GAL4 driver, yielded similar one-day memory after spaced training among
wild-type flies (WT), Feb170/+;UAS-FmrRNAi(1-7)/+ [FEB170;FMRRNAI(1-7)] or GH146/+;UAS-FmrRNAi(1-7)/+ [GH146;FMRRNAI(1-7)] transgenic flies and the UAS-FmrRNAi(1-7)/+ [FMRRNAI(1-7)], Feb170/+ (FEB170/WT) or GH146/+ (GH146/WT) genetic controls (ANOVA, P = 0.857). N = 8 PIs per group. G) Western blot analysis from adult heads of wild-type flies (WT) (lane 1), two different transgenic strains, UAS-FmrRNAi(1-7)/+;elavGAL4/+ [FMRRNAI(1-7):Elav] (lane 2) or UAS-FmrRNAi(2-1)/+;elavGAL4/+ [FMRRNAI(2-1):Elav] (lane 3), heterozygous Fmr1B55 flies [FMR1B55/WT] (lane 4) and heterozygous Fmr13 flies [Fmr1(3)/WT] (lane 5). This revealed a significant reduction of FMRP in both transgenic flies with overexpression of FmrRNAi via the pan-neural GAL4 driver, elavGAL4. 50 ug of total protein was loaded per lane. Anti-Actin was used as a loading control. H) Quantification of the WB signal revealed a reduction of more than 50% for both FmrRNAi(1-7)/+;elavGAL4/+ [RNAI(1-7)] and UAS-FmrRNAi(2-1)/+;elavGAL4/+ [RNAI(2-1)] compared to heterozygous Fmr1 mutants (Fmr1B55 flies [FMR1B55/WT] (lane 4) and Fmr13 flies [Fmr1(3)/WT]).
Supplementary Figure 2

(a) Performance Index over time with different genotypes.

(b) Performance Index for different genotypes in learning phase.

(c) Performance Index for different genotypes in spaced and massed tests.

(e) α-FMRP expression in WT and 747 genotypes.

(f) α-FASII expression in WT and 747 genotypes.

(g) α-FMRP expression in Gal80tub-747 and FmrRNAi 1-7(18C) genotypes.

(h) α-FASII expression in Gal80tub-747 and FmrRNAi 1-7(30C) genotypes.
Figure S2. Characterization of the acute role of FMRP. A) When hsp70GAL4/+; UAS-Fmr+/+ transgenic flies were subjected to one training session three hours (0) after heat-shock (HSGAL4; UAS-FMR+HS), immediate memory was similar to that produced from training in the absence of heat shock (HSGAL4; UAS-FMR-HS) (+hs vs. –hs, P = 0.195). Similar results were obtained for wild-type flies (WT) (+hs vs. –hs, P = 0.84) and for the hsp70GAL4/+ (HSGAL4/WT) (+hs vs. –hs, P = 0.564) and UAS-Fmr+/+ (UAS-FMR/WT) (+hs vs. –hs, P = 0.0719) genetic controls. These data indicate that induced overexpression of Fmr+ at the time of training did not affect learning or memory retrieval. When hsp70GAL4/+; UAS-Fmr+/+ transgenic flies were subjected to one training session 24 hours (24) after heat-shock (HSGAL4; UAS-FMR+HS), immediate memory was similar to that produced from training in the absence of heat shock (HSGAL4; UAS-FMR-HS) (+hs vs. –hs, P = 0.327). Immediate memory also was not affected 24 hours after heat shock in wild-type flies (WT) (+hs vs. –hs, P = 0.90) and in the hsp70GAL4/+ (HSGAL4/WT) (+hs vs. –hs, P = 0.34) and UAS-Fmr+/+ (UAS-FMR/WT) (+hs vs. –hs, P = 0.88) genetic controls. These data indicate that induced overexpression of Fmr+ at the time of testing (one-day memory) did not affect learning or memory retrieval. N = 4 PIs per group. B) Spatio-temporal knockdown of FMRP in MB does not affect learning. Overexpression of the UAS-FmrRNAi(1-7) transgene in MB was induced by placing tub-GAL80Δ/+; 747/+; UAS-FmrRNAi(1-7)/+ transgenic flies [GAL80; 747; FMRRNAI(1-7)] at 30°C for 3 days, which disables GAL80Δ thereby enabling 747- driven GAL4-induced expression of the UAS-FmrRNAi transgene. Memory immediately after one training session did not differ among wild-type (WT) flies, the UAS-FmrRNAi(1-7)/+ [FMRRNAI(1-7)/WT]] or tub-GAL80Δ/+; 747/+ (GAL80; 747/WT) genetic controls (ANOVA, P = 0.682). N = 2 PIs per group. C) One-day memory after spaced training is similar among wild-type flies (WT), transgenic
flies and their UAS-FmrRNAi(1-7)/+ [FMRNAI(1-7)] or tub-GAL80\textsuperscript{\textcircled{S}}/+;747/+ (GAL80;747) genetic controls, all of which were grown, trained and tested at 18\textdegree C (ANOVA, P = 0.684). N = 8 PIs per group. D) One-day memory after massed training also was similar among these genotypes raised at 18\textdegree C (ANOVA, P = 0.569). N = 4 PIs per group. E) Expression of the UAS-FmrRNAi 1-7 with the mushroom body specific Gal4 line 747 [747:FmrRNAI 1-7] leads to visible decreased level of FMRP in Kenyon cells (N=10 brains per genotype) compared to genetic control without the 747 driver [WT:FmrRNAI 1-7]. F) Expression of the UAS-FmrRNAI 1-7 with the mushroom body specific Gal4 line 747 [747:FmrRNAI 1-7] does not create \textbeta-lobe midline crossing defects (0/20 brains examines). G) Induced expression (30\textdegree C for 3 days in adults) of FmrRNAi 1-7 transgene, which also is spatially restricted to the adult MB caused significant decrease FMRP level in Kenyon cells selectively at restrictive temperature [Gal80tub-747:FmrRNAi 1-7 (30C)] compared to permissive temperature [Gal80tub-747:FmrRNAi 1-7 (18C)] (N=10 brains per genotype) H) but showed no midline crossing defect in the MB of tub-GAL80\textsuperscript{\textcircled{S}}/+;747/+;UAS-FmrRNAi(1-7)/+ [Gal80tub-747:FmrRNAi 1-7 (30C)] transgenic flies as well as in appropriate control [Gal80tub-747:FmrRNAi 1-7(18C)] flies. (Brains from a pair of these two genotypes were processed and imaged together; shown is a representative comparison from a sample size of N = 20 pairs (from two individual biological replicates of N=10 per genotype), each of which showed a similar effect).
Supplementary Figure 3

(a) \( \alpha \)-FMRP

(b) FMRP signal

(c) Performance Index

(d) Performance Index
**Figure S3. FMRP is acutely involved in LTM formation.** A) FMRP expression is increased after spaced training but not massed training. Anti-FMRP immunostaining was imaged from pairs of adult whole-brains from wild-type flies (i) before (PRE) or immediately after (POST) spaced training with MCH as the CS+ (SPACED) and (ii) before or after massed training with MCH as the CS+ (MASSED). (Brains from a pre/post pair were processed and imaged together; shown is a representative comparison from each training protocol). Comparison of FMRP level post spaced training was scored by a blind observer as increased (8/11 post spaced training brains) from untrained whereas none of the brain showed increased FMRP level in the post-massed training group compared to its untrained group (0/13 post massed training brains)). B) Quantification of the FMRP signal PRE and POST training showed a significant increased after spaced training (SPACED) (P<0.0001) but no changed after massed training (MASSED) (P=0.123). C) One-day memory after spaced training did not differ among wild-type (WT) flies, the Ago2414/+;Fmr13/+ double heterozygote [AGO2/WT;FMR(3)/WT] and the Ago2414/+ (AGO2/WT) or Fmr13/+ [FMR1(3)/WT] genetic controls (ANOVA, P = 0.698). N = 8 PIs per group. D) Induced overexpression of an Ago1+ transgene in hsGAL4/+;UAS-Ago1+ transgenic flies (HSGAL4;UAS-AGO1) subjected to heat shock three hours before spaced training (+HS) produces one-day memory significantly lower than in the same flies in the absence of heat shock (-HS) (P = 0.002). In UAS-Ago1+/+ genetic controls (UAS-AGO1/WT) performance with or without heat shock is similar (P = 0.22). N = 8 PIs per group.
SUPPLEMENTARY METHODS

*Drosophila strains*

Flies were raised and disposed of as per Cold Spring Harbor Laboratory regulations under the supervision of Dr. Tim Tully. The *Fmr1* mutant and the *Fmr1*;*gen-FMR* transgenic mutant were obtained from Dr. Jongens (U. Penn) \(^2\). The *Fmr1* mutant \(^3\) and *UAS-FMR* transgenic flies were obtained from the Broadie laboratory (Vanderbilt U). The *Argonaute* mutant was obtained from Bloomington Stock Center. *UAS-AGO1* transgenic flies were obtained from Dr. Uemura (Kyoto U.). The *staufen* mutant was obtained from Dr. Dubnau (CSHL) \(^4\). *UAS-FmrRNAi* transgenic flies were generated for this study. In each case, flies were outcrossed for six generations to *w* \(^{1118}(isoCJ1)\) control flies to equilibrate genetic backgrounds.

*Generation of UAS-FmrRNAi transgenic flies*

The first RNAi construct was synthesized from published sequence previously shown to work in Schneider2 cells \(^5\). It covers base pair 569 to 1069 of *Fmr1*. The first segment contained 500 bp corresponding to the beginning of the *Fmr1* coding region; the second contained a spacer region consisted of the second exon of *GFP*; the third segment consisted of the reverse-complement sequence from the first segment. This synthesized sequence (Retrogen) then was cloned into a “pBIMBO” vector \(^6\). Transgenic lines were established by BestGene, using *w* \(^{1118}(isoCJ1)\) as the parental stock. A second construct was designed after the Heidelberg RNAi probe ID# BKN27935, targeted region
corresponding to base pair 670 to 950 of the Fmr1 gene. Transgenic flies were generated as above. The sequence of each construct is provided below.

Construct #1

\(\text{EcoR1 site}\) GAATTC (FORWARD) ATGGAAAGATCTCTCTCTGGAAGTTCCGCTCGA CAATGGCGCCTACTACAAGGGAAGGGCAGTGACAGCTGTCGCGGATGATGGCATC TTTGTGGATGTGAGCGGCTGCGCCAGAGTATGAAATATCCAACTTGGTGAGCG TTTGCGCTGCGCGGCAGAACCAGTTCAGGAGTGCTCTCTCGAGGAGAGGAGAAGTTCGAGCTGGCCTCAATGGCGCCAGCGGACACCTTTACTGACGACGTCGACAGCTGTCACCTGGCC

T(\text{XBA 1 site}) TCTAGA
Construct #2

(EcoR1site)GAATTC(Forward)AGGTCTTTGGCTGTAATTCGGGATTCGGTTGGGCAGCGACCAATTCAAGATTTGCTGTAATTGGCGGATTCGAGTTT
TTGGGCAGCGACGAACCAATTCAACAGATTTCGGTGTAGGACGCTCTCAAAAC
CGATGTACGCGACCACATAGATTTCCGCTTTTGCGCATTTTAATAATGCCCACC
CACCAGCCGCAAGGTTCCTCGATCGTTCTGCGGCTAGTAACACCTCACTTTCAATGCCCTCCTCG
AAGATCCGTTGGGCAGCCACCTCAACCGGTTCCTCAGGCGAGGCAGGGCGAAGTTT
CACAAATGGATATTTTCACTACTCTCCGAGGCACGG(CAPER)CACGGGCCTGCACTTGCC
TTCAAGCCGCTACCCCCCGACCACATGAAGCGACGACGACTCTCTTTCAAGTCCCGCCA
TGCCCGAAGGCTACGTCCAGGACGCAACCACATCTTCTTTCAAGGACGACGGCA
CTACAAGCGCCGCCGAGGGTGAAGTTCCAGGGCGACCCCTGTCATGAACCGC
ATCGAGCTGAAGGGCATCGACTTTCAAGGAGGACGCAACCATCCTGGGGCACA
AAGCCTGAGTACAACCTACACAGCAGCCACCAACGTCTATATCATCGAGCCGACAAAGC
AGAAGAACGCGATCAAGGTAGATCTCAAGATCCCGCACACATCGAGGAC
GCAGCGGTGCAGCTC(REVERSE)CGTGCACCGAGAGTATGAAATATCCATTTGTG
AACGTTCGCTGCGCGCCCG
AGGAAACCGTTGGAGGTCGGGCACCCGATCTTCCAGGAGGACGTGAAGTTG
GAGGTGTTCAGCGCAGCAACGATCGAGGGAACCTGCGGCTGGTGTTGGTTG
CATTATTTAAATGCGCAAAAGCGGAAATCTATCGCTCGCGTACATCGGTT
TTGAGACGTCCTACACCGAAATCTGTAATTTGCGTCTGTTCGTCGCGCCAAA
AAGCTGAATCGGAAGACCTACAGGACCAAGACCT(Xba I)TCTAGA
Genetic crosses

Double heterozygote, staufen$^{D3/+;Fmr1^3}$, was generated by mating staufen$^{D3}/CyO$ males to Fmr1$^3/Tm6b,Tb,Sb$ females. All progeny were trained and tested together; CyO$^+$,Sb$^+$ progeny then were used to calculate the performance index (PI). A similar approach was used to evaluate Ago1$^{08121/+;Fmr1^3/+}$ flies. The Gal 80 approach was adapted from 7. A tub-Gal80$^{ts}$;747 homozygous stock was bred by us from tub-Gal80$^{ts}$/tub-Gal80$^{ts}$;TM2/TM6B,Tb males (obtained from Dr. Y. Zhong at CSHL) and Sp/CyO;Sb/TM3 double balancer females (maintained as an outcrossed stock in the laboratory). For behavioral experiments, tub-Gal80$^{ts}$;747 females were mated to UAS-FmrRNAi/UAS-FmrRNAi males.

Pavlovian olfactory learning

In general, Drosophila were raised at 22°C and placed at 25°C overnight prior to behavioral experiments. Adult Drosophila less than 3 days old are trained and tested with the classical conditioning procedure of Tully & Quinn 8 and modified as in Tully et al 9. About 100 flies are trapped inside a training chamber, 95% of the inside of which is covered with an electrifiable copper grid. Flies are allowed 90 seconds to acclimate and then are exposed sequentially to two odors, 3-octanol (OCT) and 4-methylcyclohexanol (MCH), carried through the chamber in a current of air (750mL/min; relative concentrations of OCT and MCH are adjusted so that naïve flies distributed themselves 50:50 in the T-maze; see below). Flies first are exposed for 60 seconds to the
conditioned stimulus (CS+; either OCT or MCH), during which time they received the unconditioned stimulus (US; twelve 1.25 seconds pulses of 60V DC electric shock at 5 second interpulse intervals). After the CS+ presentation, the chamber is flushed with fresh air for 45 seconds. Then, flies are exposed for 60 seconds to a second, control stimulus (CS-; either MCH or OCT), which is not paired with electric shock. After the CS- presentation, the chamber is again flushed with fresh air for 45 seconds.

To test for conditioned odor avoidance after classical conditioning, flies are tapped gently from the training chamber into an elevator-like compartment that transports them to the choice point of the T-maze. Ninety seconds later, the flies are exposed to two converging current of air one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies are allowed to choose between the CS+ and CS- for 120 seconds, at which time they are trapped inside their respective arms of the T-maze (by sliding the elevator out of register), anesthetized and counted. Adult *Drosophila* less than 3 days old were either subjected to classical (Pavlovian) olfactory conditioning for (i) one training session (learning), (ii) 10 training sessions without a rest interval (massed training) or (iii) 10 training sessions with 15 minutes rest between each (spaced training). After training, flies were stored at 18°C and then conditioned responses were tested at a 24-hour retention interval.

Two groups of flies are trained and tested in one complete experiment. The CS+ is OCT and the CS- is MCH for one group; the CS+ is MCH and the CS- is OCT for the second group. The performance index (PI) is calculated as the average of the fraction of the
population avoiding the shock-associated odor minus the fraction avoiding the control odor for each group of flies trained in one experiment. In other words, the PI enumerates the distribution of flies in the T-maze as a normalized “percent correctly avoiding the shock-paired odor” and ranges from 0 for a 50:50 distribution to 100 for a 100:0 distribution. For learning experiments, flies are tested immediately after one training session.

Data from an experiment were subjected to a one-way ANOVA (JMP from SAS, Inc.), followed by planned pairwise comparisons as indicated in text and figure legend. An alpha = 0.05 was corrected for multiple comparisons using Bonferroni. Comparisons with one, two or three asterisks indicate significances of \( P < 0.05 \), 0.001 and 0.0001, respectively. All graphs depict mean +/- S.E.M.

**Task-relevant sensori-motor responses**

Olfactory acuity and shock reactivity were assessed as in Boynton & Tully\(^\text{10}\) and Dura et al\(^\text{11}\), respectively. Briefly, odor avoidance at the concentrations used for the conditioning experiments was quantified in mutant and control flies. Naïve flies were placed in the T maze and given a choice between an odor (OCT or MCH) and air. The odor is naturally aversive, and flies usually avoided the T-maze arm containing the odor by running into the opposite arm. After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted. For shock reactivity, flies were given a choice between an electrified grid in one T-maze arm and an unconnected grid in the
other. After the flies distributed themselves for 2 minutes, they were trapped, anesthetized and counted.

**Conditional transgene expression**

Acute heat shock was performed as in Tully et al. Briefly, flies were grown at 18°C to minimize leaky expression the hsp70-Gal4 transgene and then placed as newly eclosed adults at 25°C overnight on the day prior to training. Approximately 100 flies were placed in a foam-stoppered glass vial containing a strip of filter paper (to avoid excess humidity) and submerged in a circulating water bath at 37°C for 35 minutes. Flies then were transferred back to food vials preheated to 25°C. Heat shock was performed either 3 hours prior to training (Fig. 2A) or testing (Fig. 2B). For experiments using the Gal80ts method, flies were raised at 18°C, then 1 day-old adult flies were placed at 31°C (restrictive temperature, which disables GAL80ts repression of GAL4) or at 18°C (permissive temperature for GAL80ts repression of GAL4) for 3 days in glass bottles with filter paper. Flies then were trained and tested at 31°C or 18°C, respectively.

**Drug feeding**

Approximately 100 flies were placed in plastic vials with Whatman filter paper containing 200 uL of solution. Flies were fed overnight prior to training at 25°C and 70% humidity with either cycloheximide or puromycin. Cycloheximide was dissolved in 3% ethanol/5% glucose, while a ready-made solution of puromycin (in water) was diluted with glucose and water to a final concentration of 5% glucose (treatment). Vehicle treatment consisted of 3% ethanol/5% glucose alone (vehicle) for CXM experiment or
5% glucose alone (vehicle) for Puromycin experiment. MPEP was obtained from Dr Mike Tranfaglia (FRAXA). Flies were fed either 8.6 mM MPEP in 3% ethanol/5% glucose/3% DMSO (treatment) or 3% ethanol /5% glucose/3% DMSO alone (vehicle).

**Western blot analysis**

About 50 ug of protein extracted from adult heads were loaded per lane with extraction buffer. Electrophoresis was conducted as suggested (Invitrogen) for 55 minutes using a 3-8% gradient gel. Blotting was conducted for 1 hour at room temperature. Anti-FMRP 5A11 antibody 1:500 (Developmental Studies Hybridoma Bank, University of Iowa) was used in combination with the WesternBreeze kit (Invitrogen) for mouse antibody detection. Loading control was obtained either with mouse anti-actin (Sigma) (1:5000) (Fig. S1A) or with mouse anti-tubulin 7E (DSHB)(1:10) (Fig. S1E). Quantification was done on 6 measurements per lane and obtained using ImageJ software.

**Whole brain imaging**

Two-to-5 day old adult flies were dissected and processed as described previously in 12. Females only were selected for consistency. Briefly, flies were dissected in PBS on day 1 and then transferred to 4% PA for fixation at room temperature for 10 minutes. Then, brains were placed in vacuum for 15 minutes in 0.2% Triton in 4% PA, followed by penetration/blocking buffer for 2 hours at 4°C. Brains were transferred to dilution buffer containing the primary antibody and placed overnight at 4°C. On day2, brains were washed with wash buffer 4 times (10 minutes/time) and then transferred to secondary antibody and incubated overnight at 4°C in the dark. On day 3, brains were washed again 4 times (10 minutes/time) and then mounted in a well made of 2 stacked reinforcer O-
rings. The well was filled with approximately 7 μL of Focus-Clear solution (Cedar Lane Laboratories) and covered with a cover slip. Imaging used a 20x dry objective with Zeiss LSM file version 3.5.0.376 Zeiss LSM data server. The average thickness of Z-stack is approximately 100μm. Both anterior and posterior orientations were collected.

For FMRP imaging before or after training, wild-type flies were placed at 25°C overnight prior to training and then trained at 25°C with MCH as the CS+. Flies were assigned randomly to the “pre” or “post” groups. For training, flies were assigned randomly to the spaced or massed groups and then were processed for imaging in parallel with the experimenter blind to treatment. Anti-Fmr1 5A11 (supernatant) was used at a concentration of 1:100 (DSHB). The setting for image acquisition was red gain at 724. The secondary antibody Cy3 anti-mouse was obtained from Jackson ImmunoLaboratory and used at a concentration of 1:200. A blind observer was then asked to score all the pictures of the untrained versus trained flies for each treatment group. Each image for post-spaced or post-massed training was presented successively and compared to their paired untrained controls. For each image from the post-training group, the examiner was asked if the FMRP level was increased or not in the post-training image compared to all the images from their untrained control group. The total number of images increased was added for spaced and massed training. For quantification, the software imageJ was used. The red channel was converted into black and white and the signal intensity was then measured for each brain from the series. The data were then analyzed using a two-tailed t-test. FMRP immunostaining and Western blotting used anti-Fmr1 antibody, 5A11 (DSHB; 3). Mushroom body structure was assessed using anti-FasII antibody, 1D4.
We used the classification system for midline crossing established in Michel et al.\textsuperscript{13}. We did not observe any accessory lobe phenotype. The secondary antibody Cy3 anti-mouse was obtained from Jackson ImmunoLaboratory and used at a concentration of 1:200.
SUPPLEMENTARY NOTE

Figure 1. *Drosophila* Fragile X mental retardation protein is required for learning and 1-day memory after spaced training. A) One-day memory after spaced training is defective in *Fmr1*3 [*FMR1(3)*] (vs. WT, P < 0.0001) and *Fmr1*B55 [*FMR B55*] (vs. WT, P < 0.0001) mutants and is rescued with an FMR+ transgene carrying the endogenous promoter of *Fmr1* [*FMR1(3)+GENOMIC*] (vs. *Fmr1*3, P < 0.0001; vs. WT, P = 0.715). N = 8 PIs per group. B) In contrast, one-day memory after massed training did not differ among wild-type (WT), *Fmr1*3 [*FMR1(3)*] and *Fmr1*B55 [*FMR B55*] mutants and transgenic mutants carrying the genomic-FMR+ construct [*FMR1(3)+GENOMIC*] (ANOVA, P = 0.597). N = 8 PIs per group. C) One-day memory after spaced training was defective in heteroallelic *Fmr1*3/*Fmr1*B55 mutants [*FMR B55/FMR 1(3)*] (vs. WT, P = 0.0001) but not in *Fmr3*/+ [*FMR1(3)/+*] (vs. WT, P = 0.891) or *Fmr*B55*/+ [*FMR B55/+*] (vs. WT, P = 0.796) heterozygotes. N = 8 PIs per group. D) In contrast, one-day memory after massed training did not differ significantly among these same four genotypes (ANOVA, P = 0.191). N = 8 PIs per group. E) Spatially restricted, RNAi-mediated knockdown of FMRP expression in adult mushroom body (MB) throughout development and adulthood disrupts one-day memory after spaced training. To rule out driver-specific artifacts, we used two different GAL4 drivers, 747 and *OK107*, with preferential expression in the mushroom body to express *UAS-FmrRNAi 1-7*. To rule out construct-specific off-site artifacts, we used two different FmrRNAi constructs [(1-7)
and (2-1)] expressed under the same Gal4 line 747. And, to rule out any insertion site-specific artifacts, we used two transgenic lines from UAS-FmrRNAi construct 1, [(1-7) and (1-10)], with different genomic insertion sites. One-day memory after spaced training was significantly lower than normal (WT) in OK107/+;UAS-FmrRNAi(1-7)/+ [OK107;FMRRNAi(1-7)] (vs. WT, P < 0.0001), in OK107/+;UAS-FmrRNAi(1-10)/+ [OK107;FMRRNAi(1-10)] (vs. WT, P = 0.001), in 747/+;UAS-FmrRNAi(1-7)/+ [747;FMRRNAi(1-7)] (vs. WT, P = 0.0046) and in 747/+;UAS-FmrRNAi(2-1)/+ [747;FMRRNAi(2-1)] (vs. WT, P = 0.001) flies. In contrast, one-day memory after spaced training was normal in the control genotypes, OK107/+ [OK107/WT] (vs. WT, P = 0.192), UAS-FmrRNAi(107)/+ [FMRRNAi(1-7)/WT] (vs. WT, P = 0.597), 747/+ [747/WT] (vs. WT, P = 0.249) and UAS-FmrRNAi(2-1)/+ [FMRRNAi(2-1)/WT] (vs. WT, P = 0.705). N = 8, 8, 4, 8, 8, 8 and 8 PIs, respectively, for the genotypes listed. F) Spatially restricted, RNAi-mediated knockdown of FMRP expression in adult mushroom body (MB) throughout development does not disrupt one-day memory after massed training for any of the groups in E (ANOVA, P = 0.817) N = 4-8 PIs for each genotype. G) Learning is significantly impaired when Fmr1 is disrupted or when FMRP is knocked down in MB throughout development and adulthood. Memory retention immediately after one training session was significantly lower than normal (WT) in Fmr1B55 (FMR B55) (vs. WT, P = 0.0062) or in Fmr1B55/Fmr1B55 mutant [FMR B55/FMR B55] (vs. WT, P = 0.0045). RNAi-mediated knockdown of FMRP in MB of OK107/+;UAS-FmrRNAi(1-7)/+ transgenic flies [OK107;FMR-RNAi(1-7)] also
yielded a significant learning defect (vs. WT, P = 0.034). N = 4 PIs per group. H) RNAi-mediated knockdown of FMRP pan-neuronally in *elavGAL4/+;UAS-FmrRNAi(1-7)/+* flies recapitulates a previously reported mushroom body midline crossing defect (see text). MB lobes were visualized using anti-Fas II1D4 immunostaining of adult whole-mount brains (a total of N = 20 brains per genotype were immunostained; see Methods).

**Figure 2. Drosophila Fragile X mental retardation protein is required acutely for LTM formation and interacts with Staufen and Ago1.** A) Protocol used to overexpress UAS-Fmr+ before training. B) Protocol used to overexpress *UAS-Fmr*+ after training. C) One-day memory after spaced training is impaired when FMRP is overexpressed before LTM consolidation. When heat-shocked (+) before spaced training, one-day memory in *hsp70GAL4/+;UAS-Fmr+/+ [HSGAL4;U-FMR(<TRAIN)]* transgenic flies was significantly reduced from such memory produced in the absence of heat shock (-). (+hs vs. –hs, P = 0.0015). No such heat-shock effect was observed in the genetic controls, *hsp70GAL4/+ (HSGAL4/WT)* (+hs vs. –hs, P = 0.521) or *UAS-Fmr+/+ (UAS-FMR/WT)* (+hs vs. –hs, P = 0.218). When heat-shocked after spaced training three hours before testing, one-day memory in *hsp70GAL4/+;UAS-Fmr+/+ [HSGAL4;U-FMR(<TEST)]* transgenic flies was normal (vs. WT, P = 0.242). N = 8 PIs per group. D) One-day memory after massed training, in contrast, did not differ among these genotypes with (+) or without (-) heat shock (ANOVA, P= 0.159). N = 8 PIs per group. E) Spatio-temporal knockdown of FMRP in MB disrupts one-day memory after spaced training. Overexpression of the
UAS-FmrRNAi(1-7) transgene in MB was induced by placing *tub-GAL80*^{ts}/+;747/++;UAS-FmrRNAi(1-7)/+ transgenic flies

\[ \text{GAL80;747;FMRRNAI(1-7)} \] at 30°C for 3 days, which disables GAL80*^{ts} thereby enabling 747- driven GAL4-induced expression of the UAS-FmrRNAi transgene.

One-day memory after spaced training in these flies is significantly lower than that in wild-type (WT) (P = 0.0021) flies or in the UAS-FmrRNAi(1-7)/+ [FMRRNAI(1-7)/WT] (P < 0.0001) or *tub-GAL80*^{ts}/+;747/+ (GAL80;747/WT) (P = 0.0028) genetic controls. N = 8 PIs per group. F) One-day memory after massed training is not affected in these same genotypes under the same temperature conditions. (ANOVA, P = 0.311). N = 8 PIs per group. G) One-day memory after spaced training is significantly reduced in *stauD3/+;Fmr13/+* double heterozygotes [STAU/WT;FMR1(3)/WT] compared to wild-type flies (WT) (P = 0.0002) or *Fmr13/+* [FMR1(3)/WT] (P = 0.0004) or *stauD3/+* (STAU/WT) (P < 0.0001) heterozygotes. N = 8 PIs per group. H) One-day memory after massed training was normal in these same genotypes (ANOVA, P = 0.75). N = 8 PIs per group. I) One-day memory after spaced training is significantly reduced in *Ago108121/+;Fmr13/+* double heterozygotes [AGO1/WT;FMR1(3)/WT] compared to wild-type flies (WT) (P = 0.0016), *Fmr13/+* [FMR1(3)/WT] (P = 0.0007) or *Ago108121/+* (AGO1/WT) (P < 0.0001) heterozygotes. N = 16 PIs per group. J) One-day memory after massed training was normal in these same genotypes (ANOVA, P = 0.698). N = 8 PIs per group.
Figure 3. Inhibition of protein synthesis ameliorates the LTM defect of Fmr1 mutants. A) One-day memory in wild-type (WT) or the Fmr1B55 mutant (FMR B55) or Fmr13 (FMR1(3)) fed cycloheximide (CXM; +) or vehicle (-) before spaced training. Three different doses of CXM, 8.75 mM ([8,75]), 17.5 mM ([17.5]) and 35 mM ([35.0]) were assessed. At the lower dose (8.75mM or 17.5mM), memory in wild-type flies was not affected (vs. WT vehicle, P=0.682, P = 0.977 respectively). Memory deficit of the Fmr1B55 mutant (FMR B55) was not different at 8.75mM (P=0.4229) but ameliorated significantly at 17.5mM (vs. mutant vehicle, P = 0.0244). Similar results were observed for Fmr13 (FMR1(3)) (vs. mutant vehicle, P=0.0196). At the higher dose, memory in wild-type flies was reduced significantly (vs. WT vehicle, P = 0.018; replicating earlier reports), while the memory deficit of the Fmr1B55 mutant was unaffected (vs. mutant vehicle, P = 0.806). N = 8 PIs per group. B) One-day memory after massed training was unaffected by either of these CXM doses in wild-type flies or the Fmr1B55 mutant (17.5mM vs. vehicle, P = 0.51; 35mM vs. vehicle, P=0.746). N = 8 PIs per group. C) To generalize this novel observation to another protein synthesis inhibitor, one-day memory after spaced training in wild-type (WT) flies or the the Fmr1B55 (FMR B55) and Fmr13 (FMR1(3)) mutants was evaluated using puromycin (PURO). Here again, three doses, 2.5 mM ([2.5]), 5 mM ([5]) and 10 mM ([10]), were used, and similar results on memory were observed: the lower dose (2.5mM and 5mM) had no effect in wild-type flies (drug vs. vehicle, P = 0.278, P = 0.133 respectively). There was also no effect for Fmr1B55 mutants with 2.5mM (drug vs. vehicle,P = 0.174) but 5mM Puromycin ameliorated the
memory deficits of both the \textit{Fmr1}^{B55} (drug vs vehicle, P = 0.0233) and \textit{Fmr1}^3 (drug vs vehicle, P = 0.0173) mutants, while the higher dose disrupted memory in wild-type flies (drug vs. vehicle, P = 0.0059) but had no effect on the memory deficit of the \textit{Fmr1} mutant (drug vs. vehicle, P = 0.979). N = 8 - 12 PIs per group. D) One-day memory after massed training was unaffected by either low or high dose of PURO in wild-type flies (drug vs. vehicle, P = 0.17 and 0.21, respectively) or the \textit{Fmr1}^{B55} mutant (drug vs. vehicle, P = 0.163 and 0.18, respectively). N = 8 PIs per group. E) The metabotropic glutamate receptor antagonist, MPEP, also ameliorated the \textit{Fmr1} mutant deficit in one-day memory after spaced training (cf. 1) (\textit{Fmr1}^{B55}; drug vs. vehicle, P = 0.0355; \textit{Fmr1}^3; drug vs. vehicle, 0.0024). N = 8 PIs per group. F) The low dose of CXM (17.5mM) also ameliorated the deficit in one-day memory after spaced training in the \textit{Ago1}^{08121}/+;\textit{Fmr1}^{B55}/+ (\textit{AGO1}/+; \textit{FMR1}^{B55}/+) (drug vs. vehicle, P=0.0452) and the \textit{Ago1}^{08121}/+;\textit{Fmr1}^3/+ double heterozygote (\textit{AGO1}/+;\textit{FMR1}(3)/+) (drug vs. vehicle, P = 0.0058). N = 8 PIs per group.
SUPPLEMENTAL REFERENCES

1. McBride, S. M. et al. Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. Neuron 45, 753-64 (2005).
2. Dockendorff, T. C. et al. Drosophila lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. Neuron 34, 973-84 (2002).
3. Inoue, S. et al. A role for the Drosophila fragile X-related gene in circadian output. Curr Biol 12, 1331-5 (2002).
4. Dubnau, J. et al. The stau/en/pumilio pathway is involved in Drosophila long-term memory. Curr Biol 13, 286-96 (2003).
5. Caudy, A. A., Myers, M., Hannon, G. J. & Hammond, S. M. Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev 16, 2491-6 (2002).
6. Piccin, A. et al. Efficient and heritable functional knock-out of an adult phenotype in Drosophila using a GAL4-driven hairpin RNA incorporating a heterologous spacer. Nucleic Acids Res 29, E55-5 (2001).
7. McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K. & Davis, R. L. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765-8 (2003).
8. Tully, T. & Quinn, W. G. Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol [A] 157, 263-77 (1985).
9. Tully, T., Preat, T., Boynton, S. C. & Del Vecchio, M. Genetic dissection of consolidated memory in Drosophila. Cell 79, 35-47 (1994).
10. Boynton, S. & Tully, T. latheo, a new gene involved in associative learning and memory in Drosophila melanogaster, identified from P element mutagenesis. Genetics 131, 655-72 (1992).
11. Dura, J. M., Preat, T. & Tully, T. Identification of linotte, a new gene affecting learning and memory in Drosophila melanogaster. J Neurogenet 9, 1-14 (1993).
12. Xia, S. et al. NMDA receptors mediate olfactory learning and memory in Drosophila. Curr Biol 15, 603-15 (2005).
13. Michel, C. I., Kraft, R. & Restifo, L. L. Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. J Neurosci 24, 5798-809 (2004).