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

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Abstract

We used Drosophila olfactory memory in order to understand in vivo the molecular basis of cognitive defect in Fragile X syndrome. We observed that Fragile X protein (FMRP) was required acutely and interacted with argonaute1 and staufen in long-term memory (LTM). Occlusion of long-term memory formation in Fragile X mutants could be rescued by protein synthesis inhibitors, suggesting that excess baseline protein synthesis could impact negatively on cognition.

Keywords

Long-term memory; Fragile X; Argonaute 1; RNAi; Drosophila

INTRODUCTION

Fragile X mental retardation syndrome, caused by the absence of FMRP, is the most frequent cause of heritable mental retardation in males. Lack of FMRP has been associated with increases in general protein synthesis 1 downstream of metabotropic glutamate receptor (mGluR) activation 2. FMRP is associated with elements of LTM such as protein synthesis, staufen 3, 4 and the RNAi pathway 5, 6 but the link between excess protein synthesis and cognition remains unclear.

RESULTS

Fmr1 is involved in olfactory learning and long-term memory formation

We took advantage of two null alleles of *Fmr1*, $Fmr1^{3}$ and $Fmr1^{B55}$ to investigate memory formation after Pavlovian olfactory conditioning 7 in Drosophila . We confirmed that FMRP

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AUTHOR CONTRIBUTION

FB conceptualized the project,conducted experiments, wrote the manuscript; KB performed behavior experiments;HC performed imaging experiments; KB help with conceptualization; TT conceptualized and supervised the project and wrote the manuscript.

is not detected in brains of these *Fmr1* mutants (Fig. S1a,b). Nonetheless, these mutants respond normally to the odors and footshock (Fig. S1c,d).

After repetitive training sessions, two distinct forms of long-lasting memory can be distinguished 7. Massed training (MT) (ten training sessions without rest interval) produces a decremental, cycloheximide-insensitive memory (ARM), while spaced training (ST) (ten training sessions with a 15-min rest interval) produces both ARM and a nondecremental cycloheximide-sensitive (protein synthesis-dependent) long-term memory (LTM). Mutants homozygous for either the $Fmr1^{3}$ or $Fmr1^{B55}$ allele showed defects in one-day memory after ST but not after MT, and this LTM defect is rescued in $Fmr1^{3}$ mutants expressing a genomic transgene containing $Fmr1^{+}$ driven by its endogenous promoter (Fig. 1 a,b). Mutants heteroallelic for $Fmr1^{3}$ and $Fmr1^{B55}$ were also defective for one-day memory after ST but not after MT. (Fig. 1 c,d).

We determined a spatial requirement for FMRP by evaluating LTM formation using RNA interference. Western blot analysis from adult brain after pan-neuronally restricted expression of the *UAS-FmrRNAi* construct with *Elav-GAL4* revealed a significant decrease (40% of baseline) in FMRP level (Fig. S1g,h). Furthermore, we replicated the ß-lobe midline crossing defect (classified as in 8 : 4 severe, 3 moderate and 2 mild in 20 brains for transgenic flies, compared to 0 for each category in 20 brains from wild-type flies) (Fig. 1h). We then evaluated the effects on LTM formation of FMRP knockdowns restricted to the MB, because of its previously demonstrated role in olfactory LTM formation 9. Using two different MB PGAL4 drivers and two different *UAS-FmrRNAi* constructs, knockdown of FMRP yielded defects in one-day memory after ST but not after MT (Fig. 1 e,f). Interestingly, we observed decreased FMRP level in Kenyon cells (Fig. S2e) but no ß-lobe midline crossing when expression of *UAS-Fmr-RNAi* was restricted to the MB (using either *OK107-Gal4* or *747-Gal4*; N=20 flies per genotype; Fig. S2f). In contrast to these results, knockdown of FMRP in the central complex (*Feb170*) or projection neurons from antennal lobe (*GH146*) did not yield defects in one-day memory after ST (Fig. S1f).

Considering the high prevalence of learning disability in Fragile X patients , defects in MBs of *Fmr1* mutants 8 and their role in learning 10, we asked whether olfactory learning was impaired in *Fmr1* mutants and observed significant defects in $Fmr1^{3}$, $Fmr1^{B55}$ homozygotes, the $Fmr1^{3}/Fmr1^{B55}$ heteroallelic mutant and in flies expressing *UAS-FmrRNAi* in MB (Fig. 1g).

FMRP is required physiologically in adult for LTM

To dissect the involvement of FMRP in brain development (see Fig. 1h) from its physiological role during adult LTM formation, we modulated FMRP expression using conditional transgenic tools. Acute adult heat shock-induced overexpression of wild-type FMRP produced a significant decrement in one-day memory after ST but not after MT (Fig. 2 a,c, d). In contrast, overexpression of FMRP after ST beyond memory consolidation (Fig. 2b) did not affect one-day memory (Fig. 2c). Interestingly, learning appeared normal at 3 or 24 hours after overexpression of $Fmr1^{+}$ in adult (Fig. S2a).

We confirmed these results with complementary experiments using spatio-temporal control of RNAi-mediate knockdown of FMRP. We used the temperature-sensitive, *tubulin-driven GAL80^{ts}* transgene, along with *MB (747) GAL4*-driven expression of *UAS-FmrRNAi* to disrupt FMRP specifically in adult MB. At restrictive temperature (30°C), when GAL80^{ts} no longer represses GAL4-driven expression of *UAS-FmrRNAi*, we observed a significant defect in one-day memory after ST but not after MT (Fig. 2e,f). Learning was normal (Fig. S2B). At permissive temperature (18°C), when GAL80^{ts} is capable to inhibit GAL4-driven expression of *UAS-FmrRNAi*, one-day memory after spaced or massed training was normal (Fig. S2c,d). Despite a significantly decreased FMRP level in Kenyon cells after incubation at restrictive temperature (30C; Fig. S2g), no MB midline defect was observed (0/20 brains) (Fig. S2h).

We observed a significant increase in FMRP expression acutely after ST ($P<0.0001$) but not after MT (P=0,123) (Fig. S3a,b). Interestingly, we observed that *Drosophila* adenylyl cyclase mutants, *rutabaga^{1}*, had lower FMRP level (Fig. S1e).

Staufen and Argonaute 1 interact with FMRP during memory formation

Staufen has been co-localized with FMRP in neural granules and has been implicated in LTM formation 4. Thus, we asked whether *Fmr1* and *stau* interact functionally, using a classic test for genetic interaction. Heterozygous *stau^{D3}*/+ or *Fmr1^{3}*/+ flies displayed normal one-day memory after spaced or massed training; in contrast, double heterozygous *stau^{D3}*/+;*Fmr1^{3}*/+ flies showed defective one-day memory after ST but not after MT (Fig. 2g,h). This result suggests that FMRP and Staufen interact genetically during LTM formation specifically.

FMRP also has been implicated as a molecular component of the RISC (RNAi) complex with Ago1. *So* we decided to evaluate whether *Fmr1* and *Ago1* interacted during memory formation. Heterozygous *Fmr1^{3}*/+ or *Ago1^{08121}*/+ flies displayed normal one-day memory after spaced or massed training; in contrast, the double heterozygote, *Ago1^{08121}*/+; *Fmr1^{3}*/+, showed defective one-day memory after ST but not after massed training (Fig. 2i, j). No such transdominant effect was observed in *Ago2^{414}*/+;*Fmr1^{3}*/+ flies (Fig. S3c). We also were able to demonstrate an acute adult effect of *Ago1 overexpression on* LTM formation (Fig. S3d). Together with the transdominant interaction between FMRP and Ago1, this observation suggests that FMRP regulates LTM formation via the miRNA pathway.

Inhibition of protein synthesis ameliorates the LTM defect in *Fmr1* mutants

Several reports of increased global protein synthesis have led to the hypothesis that FMRP inhibits protein synthesis. So, could this FMRP-mediated misregulation of protein synthesis affect LTM formation? We tested this question by feeding *wild-type* and *Fmr1* mutants flies protein synthesis inhibitors, cycloheximide (CXM) or puromycin (PURO). We used variations around concentration established for memory inhibition for CXM 7 and measurement of protein synthesis inhibition in *Drosophila* brain for Puromycin (Tully, unpublished). At relatively low concentrations of CXM or PURO, one-day memory after ST was not affected in *wild-type* flies but significantly ameliorated in the *Fmr1^{B55}* and *Fmr1^{3}* mutants (Fig. 3a,c), while no effects on one-day memory after MT were observed (Fig.

3b,d). We also observed a significant improvement of LTM with metabotropic glutamate receptor antagonist, MPEP, for the *Fmr1*^{B55} and *Fmr1*^{3} mutants (Fig. 3e; cf. 11).

The genetic interaction of *Fmr1* with *staufen* and *Ago1* (above) led us to consider that excess protein synthesis may occur via a deregulation of the miRNA pathway in Fragile X mutants. To test this hypothesis, we assessed the effects of CXM on the *Ago1*^{08121}/+;*Fmr1*^{3}/+ and *Ago1*^{08121}/+;*Fmr1*^{B55}/+ double heterozygotes. The memory defect one day after ST of the double heterozygote was significantly ameliorated in the drug-treated group compared to the vehicle-treated group (Fig. 3f). Together, these data reveal an ongoing requirement for FMRP, protein synthesis and miRNA processing during LTM formation.

DISCUSSION

Our behavioral study is the first to show an acute requirement for FMRP during long-term memory formation. Our results suggest that LTM dysfunction in *Fmr1* mutants occurs via a miRNA-dependent misregulation of protein synthesis involving *Ago1* and *staufen*. A general excess of protein synthesis possibly occludes the synthesis of subset of proteins required for LTM. This molecular mechanism may ramify more generally, as inhibition of protein synthesis reverses epileptic activity in the Fragile X knockout mouse 12. Our data also extend to the behavioral level the observation that dendritic elongation in response to the metabotropic glutamate receptor agonist, DHPG, could be blocked by the protein synthesis inhibitor, puromycin 13. In addition, the occlusion hypothesis could explain why the Fragile X knockout mouse failed to assemble polyribosomes and synthesize PSD95 and AMPAR in response to DHPG treatment 14, 15. More generally, the therapeutic use of inhibitors of protein synthesis may extend to other disorders, such as tuberous sclerosis, where deregulation of baseline protein synthesis also occurs or dampen other behavioral defects associated with Fragile X disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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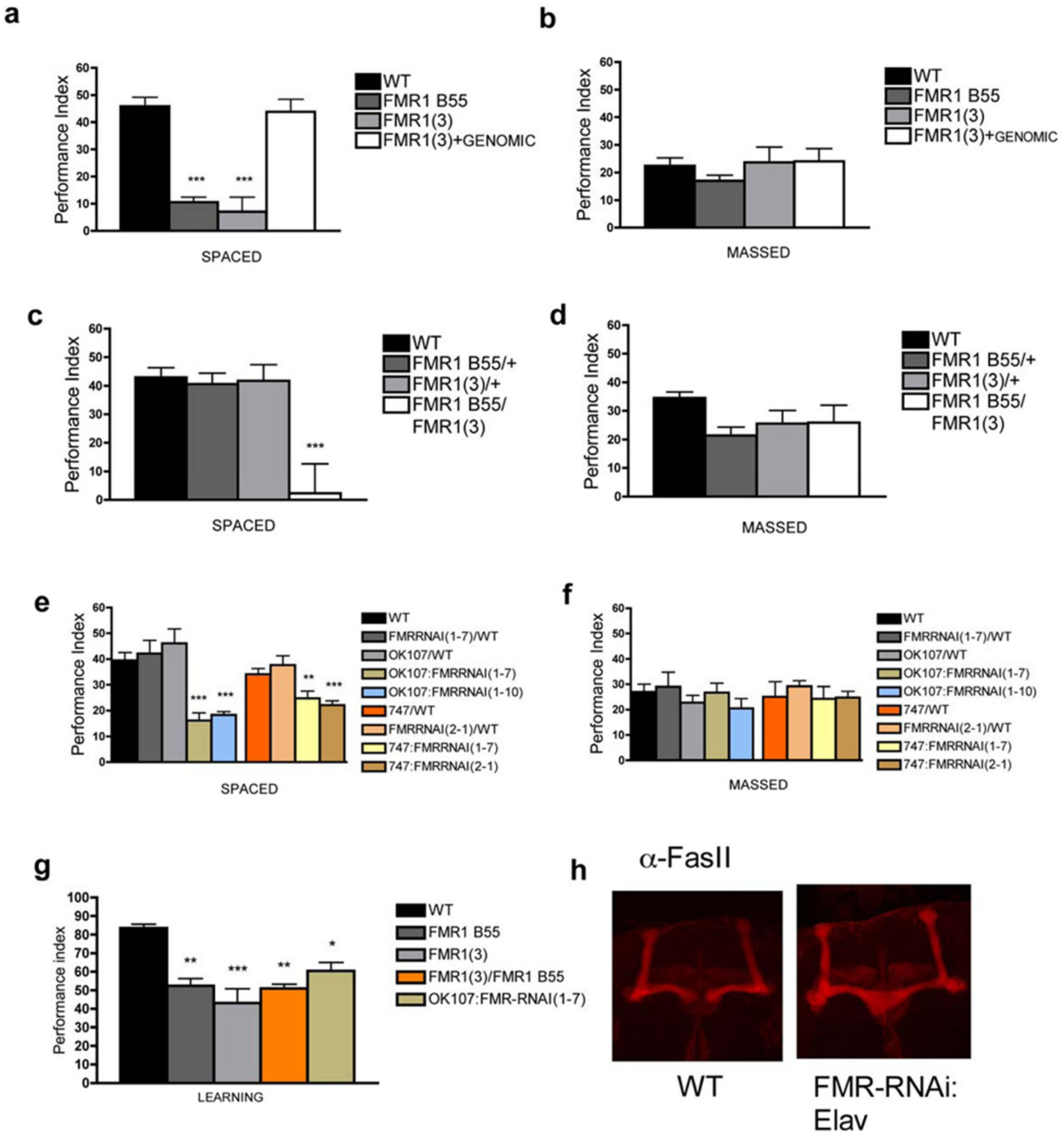

Figure 1. ***Drosophila*** **FMRP is required for olfactory learning and 1-day memory after ST**

A) One-day memory after ST is defective in ***FMR1(3)*** (vs. WT, $P < 0.0001$) and ***FMR B55*** (vs. WT, $P < 0.0001$) and rescued with ***FMR1(3)+GENOMIC*** (vs. $Fmr1^3$, $P < 0.0001$; vs. WT, $P = 0.715$). **B)** In contrast, one-day memory after MT was normal. N = 8 PIs per group. **C)** Defective one-day memory after ST in ***FMR B55/FMR 1(3)*** (vs. WT, $P = 0.0001$), **D)** but normal after MT. N = 8 PIs per group. **E)** One-day memory after ST was significantly lower than ***WT*** in ***OK107;FMRRNAi(1-7)*** (vs. WT, $P < 0.0001$), ***OK107;FMRRNAi(1-10)*** (vs. WT, $P = 0.001$), ***747;FMRRNAi(1-7)*** (vs. WT, $P = 0.0046$) and in ***747;FMRRNAi(2-1)***

(vs. WT, P = 0.001) flies, but normal in the control genotypes: ***OK107/WT*** , ***FMRRNAi(1-7)/WT***, ***747/WT*** and ***FMRRNAi(2-1)/WT***. N = 8, 8, 4, 8, 8, 8, 8 and 8 PIs. **F)** One-day memory after MT for any of the groups in **E)** was normal. N = 4–8 PIs for each genotype. **G)** Learning was significantly lower than ***WT*** in ***FMR B55*** (vs. WT, P = 0.0062), ***FMR 1(3)*** (vs. WT, P = 0.0002), ***FMR B55/FMR 1(3)*** (vs. WT, P = 0.0045) or ***OK107;FMR-RNAi(1-7)*** (vs. WT, P = 0.034). N = 4 PIs per group. All graphs depict mean +/− S.E.M. H) *elavGAL4*/+;*UAS-FmrRNAi (1-7)*/+ present MB midline crossing defect (N=20).

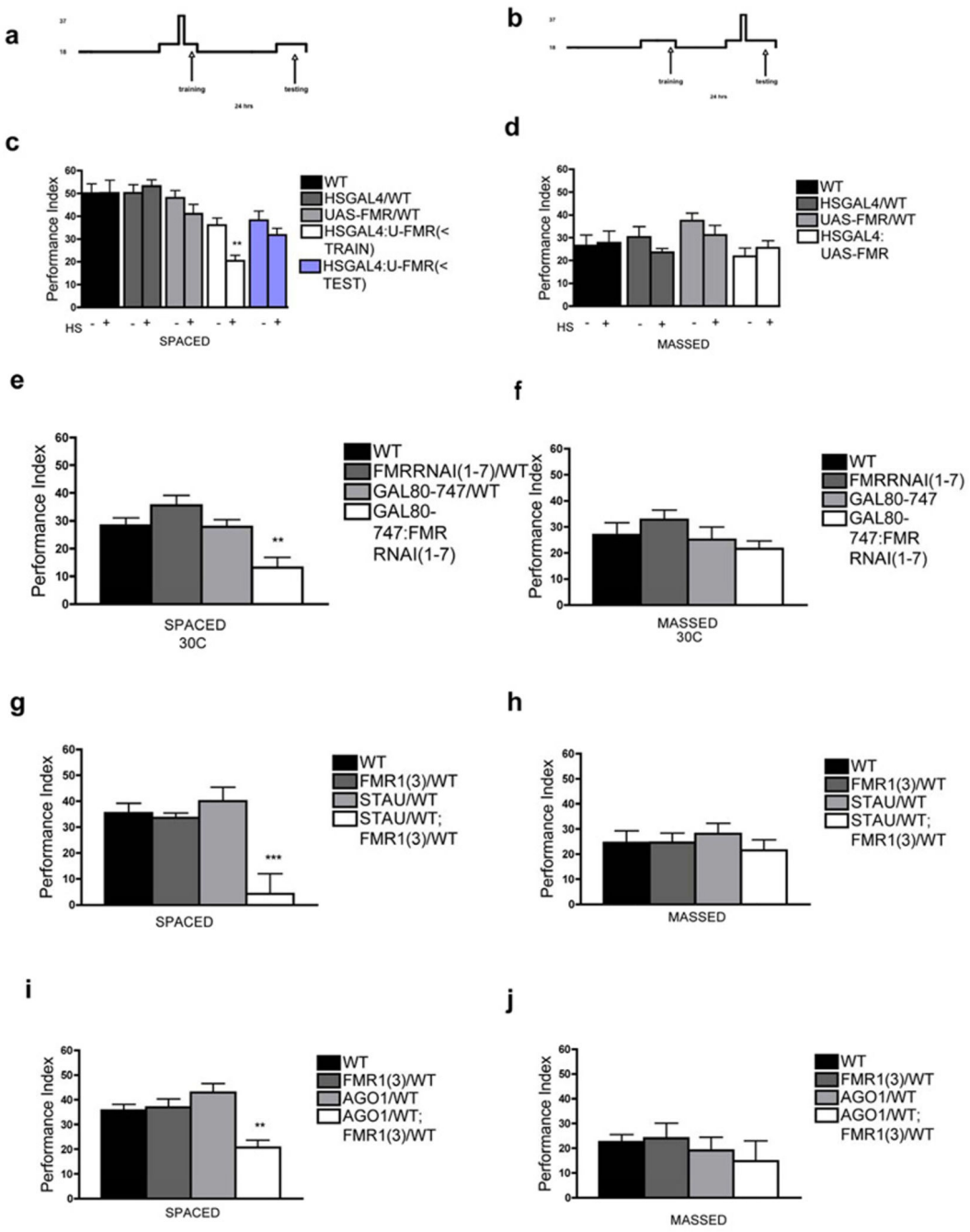

Figure 2. *Drosophila* FMRP is required acutely for LTM formation and interacts with Staufen and Argonaute 1

A) Protocol used to overexpress *UAS-Fmr+* before training or **B)** after training. **C)** One-day memory in ***HSGAL4;U-FMR*** flies heat-shocked (+) before ST (<**TRAIN**) was significantly reduced (P = 0.0015) as opposed to those heat-shocked after ST (<**TEST**) (P = 0.242). No effect was observed in the genetic controls, ***HSGAL4/WT*** or ***UAS-FMR/WT***. N = 8 PIs per group. **D)** One-day memory after MT did not differ. N = 8 PIs per group. **E)** Adult *UAS-FmrRNAi(1-7)* expression in MB of ***GAL80;747;FMRRNAI(1-7)*** *flies* for 3 days caused

defect in one-day memory after ST compared to ***WT*** (P = 0.0021), ***FMRRNAI(1-7)/WT)*** (P < 0.0001) or ***GAL80;747/WT*** (P = 0.0028) genetic controls. N = 8 PIs per group. **F)** One-day memory after MT is not affected. N = 8 PIs per group. **G)** One-day memory after ST is reduced in ***STAU/WT;FMR1(3)/WT*** compared to wild-type flies ***WT*** (P = 0.0002), ***FMR1(3)/WT*** (P = 0.0004) or ***STAU/WT*** (P < 0.0001). N = 8 PIs per group. **H)** One-day memory after MT was normal in these genotypes. N = 8 PIs per group. **I)** One-day memory after ST is reduced in ***AGO1/WT;FMR1(3)/WT*** compared to controls ***WT*** (P = 0.0016), ***FMR1(3)/WT*** (P = 0.0007) or ***AGO1/WT*** (P < 0.0001). N = 16 PIs per group. **J)** One-day memory after MT was normal in these same genotypes. N = 8 PIs per group. All graphs depict mean +/− S.E.M.

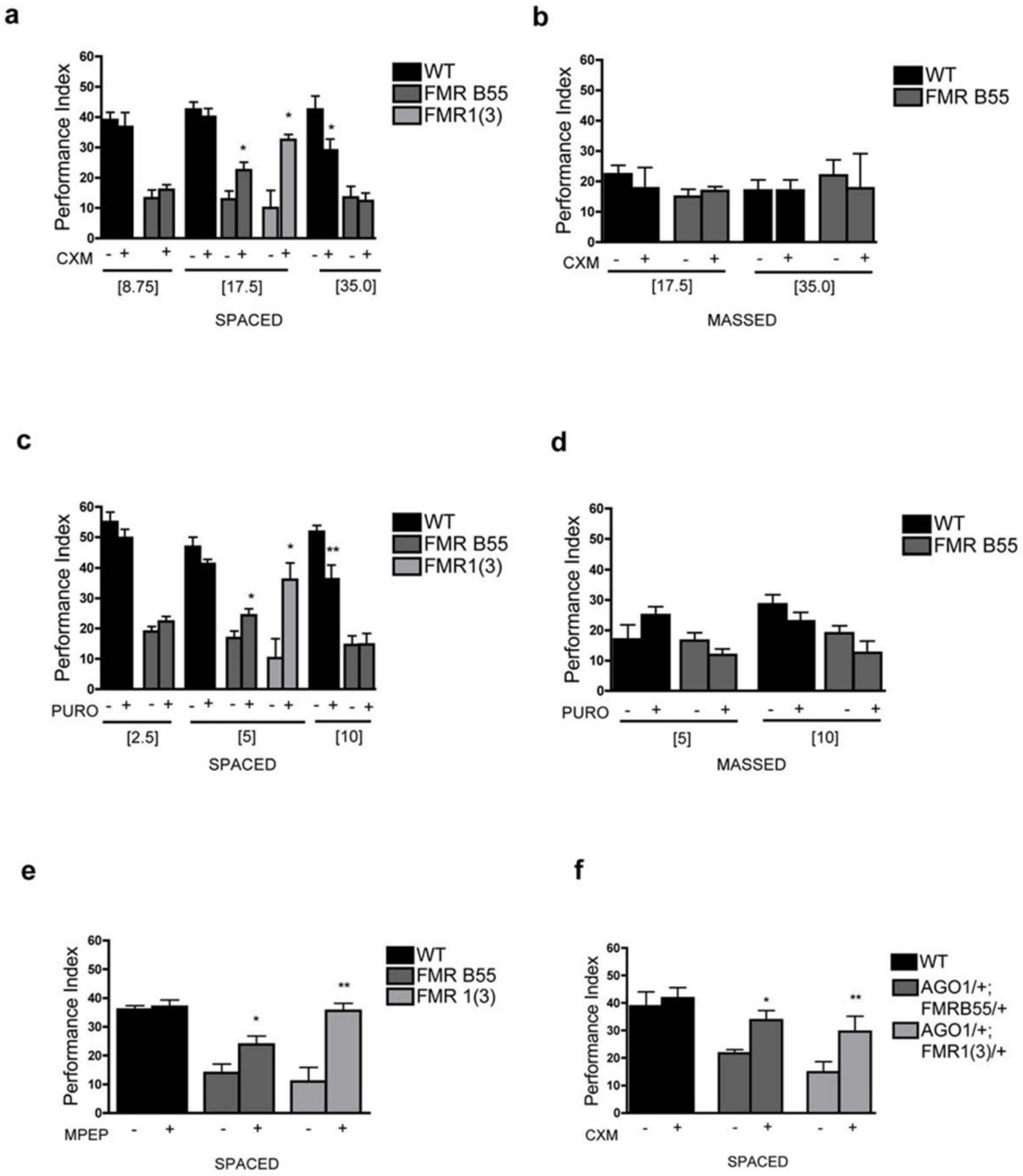

Figure 3. Inhibition of protein synthesis ameliorates the LTM defect of *Fmr1* mutants

A) At the low cycloheximide dose (8.75mM or 17.5mM), one day memory after ST in *wild-type* flies was not affected (P=0.682, P = 0.977). ***FMR B55*** performance was not modified at 8.75mM (P=0.4229) but was ameliorated at 17.5mM (P = 0.0244), as was ***FMR1(3)*** (P=0.0196). At the 35mM, performance in wild-type flies was reduced (P = 0.018), while ***FMR B55*** was unaffected (P = 0.806). N = 8 PIs per group. **B)** One-day memory after MT was unaffected. N = 8 PIs per group. **C)** Puromycin at low dose (2.5mM and 5mM) had no effect in *wild-type* (P = 0.278, P = 0.133). No effect for ***FMR B55*** with 2.5mM (P = 0.174)

but 5mM Puromycin ameliorated performance of both ***FMR B55*** (P = 0.0233) and ***FMR1(3)*** (P = 0.0173), while puromycin 10mM disrupted memory in *wild-type* (P = 0.0059) but had no effect on ***FMR B55*** (P = 0.979). N = 8 – 12 PIs per group. **D)** One-day memory after MT was unaffected by puromycin in *wild-type* or ***FMR B55***. N = 8 PIs per group. **E) MPEP**, ameliorated one-day memory after ST in *Fmr1* mutants ($Fmr1^{B55}$: P =0.0355; $Fmr1^{3}$: P =0.0024). N = 8 PIs per group. **F)** CXM (17.5mM) ameliorated the deficit in one-day memory after ST in ***AGO1*/+; *FMR1B55*/+** (P=0.0452) and ***AGO1*/+;*FMR1(3)*/+** (P = 0.0058). N = 8 PIs per group. All graphs depict mean +/− S.E.M.