Neuron, Vol. 45, 753–764, March 3, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.01.038

Pharmacological Rescue of Synaptic Plasticity, Courtship Behavior, and Mushroom Body Defects in a *Drosophila* Model of Fragile X Syndrome

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Summary

Fragile X syndrome is a leading heritable cause of mental retardation that results from the loss of *FMR1* gene function. A *Drosophila* model for Fragile X syndrome, based on the loss of *dfmr1* activity, exhibits phenotypes that bear similarity to Fragile X-related symptoms. Herein, we demonstrate that treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium can rescue courtship and mushroom body defects observed in these flies. Furthermore, we demonstrate that *dfmr1* mutants display cognitive deficits in experience-dependent modification of courtship behavior, and treatment with mGluR antagonists or lithium restores these memory defects. These findings implicate enhanced mGluR signaling as the underlying cause of the cognitive, as well as some of the behavioral and neuronal, phenotypes observed in the *Drosophila* Fragile X model. They also raise the possibility that compounds having similar effects on metabotropic glutamate receptors may ameliorate cognitive and behavioral defects observed in Fragile X patients.

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Introduction

Fragile X syndrome is the most common form of inherited mental retardation, affecting 1 in 6000 births. The degree of cognitive deficits observed in Fragile X patients ranges from mild learning disabilities to severe mental retardation, with progressive cognitive decline occurring with age. In addition, Fragile X syndrome is associated with several clinically relevant behaviors that include sleep disorders, attention deficit disorder, hyperactivity, aggression, and autistic behavior (Fisch et al., 1999; O'Donnell and Warren, 2002; Wright-Talamante et al., 1996). A significant neuroanatomical defect associated with Fragile X is abnormal dendritic spine morphology that has been identified in affected humans at autopsy (O'Donnell and Warren, 2002). This is consistent with the theory that dendritic spine dysgenesis is involved in mental retardation in humans (Purpura, 1974).

Fragile X syndrome is caused by loss of *FMR1* gene function (O'Donnell and Warren, 2002). A mouse knockout of the *Fmr1* gene recapitulates several aspects of Fragile X syndrome including subtle learning and memory deficits (Bakker and Oostra, 2003). Consistent with a defect in synaptic plasticity, several recent electrophysiological studies of synaptic plasticity in the *Fmr1* knockout mouse have suggested an imbalance between long-term depression (LTD) and long-term potentiation (LTP). *Fmr1* knockout mice have enhanced LTD in the hippocampus as a result of increased activity of the metabotropic glutamate receptor (mGluR) group I type 5 (Huber et al., 2002). Although LTP has been found to be unaffected in the hippocampus of the knockout mice (Huber et al., 2002; Li et al., 2002), Li et al. (2002) found a significant reduction in LTP in the cerebral cortex along with reduced expression of the AMPA receptor. Inappropriate mGluR signaling has been linked to several phenotypes in mice that are similar to symptoms of Fragile X patients, leading to the proposal that a significant portion of the disease phenotypes are due to mGluR misregulation (Bear et al., 2004).

Recently, a *Drosophila* model for Fragile X syndrome was developed that is based on loss-of-function mutants of *dfmr1*, the single homolog of the *FMR1* gene in the *Drosophila* genome (Wan et al., 2000). Studies of these mutants uncovered neuronal and behavioral phenotypes with parallels to symptoms observed in Fragile X patients. These include alterations in circadian rhythms, synaptic branching, and courtship behavior (Dockendorff et al., 2002; Lee et al., 2003; Morales et al., 2002; Zhang et al., 2001), but defects in cognitive function were not reported in these studies.

Given the enhanced mGluR activity in the brains of *Fmr1* knockout mice, we explored the possibility that similar mGluR misregulation might exist in the *dfmr1*

mutant flies and cause some of the observed phenotypes. Consistent with this hypothesis we have found that treatment with four independent mGluR antagonists or lithium can restore the naive courtship levels of the *dfmr1* mutants to those observed with control flies. We also examined the learning and memory capabilities of the *dfmr1* mutant flies, using the conditioned courtship paradigm. We found that although *dfmr1* mutants display normal learning during training with a previously mated female, they fail to display any memory of the training, thus identifying a cognitive deficit in these mutants. Moreover, *dfmr1* mutant flies display normal memory of conditioned courtship when treated with the above-mentioned agents. Previous studies have linked the mushroom bodies (MBs) to learning and memory (de Belle and Heisenberg, 1994; Joiner and Griffith, 1999; McBride et al., 1999; Pascual and Preat, 2001). Since a defect in the morphology of MBs has been described in *dfmr1* mutants (Michel et al., 2004), we examined the effect of our drug treatments on these malformations. We found that although certain treatments can rescue them, restoration of normal MB structure is not a prerequisite for restoring memory. In sum, our results indicate that enhanced mGluR activity is a conserved feature of the fly model for Fragile X and is causative of some of the behavioral and neuronal phenotypes. These findings also suggest that similar modulation of mGluR activity in Fragile X patients should be explored as an approach to ameliorate their cognitive defects and behavioral symptoms.

Results

Restoration of Naive Courtship Levels in *dfmr1* Mutant Flies with MPEP Treatment

To investigate the effect of modulating mGluR activity on the behavior of the *dfmr1* mutant flies, we examined the *Drosophila* genome to determine the complexity of mGluRs and their distribution, as well as looked for drugs that might be useful in antagonizing their activity. Only two potential mGluRs are present in the *Drosophila* genome, *DmGluRA* and *DmGluRB* (also called *DmXR*). Sequence comparison and pharmacological studies indicate that DmGluRA is most closely related to vertebrate group II and group III mGluRs (see Figure S1 in the Supplemental Data available with this article online; Parmentier et al., 1996). It is expressed throughout the brain with enhanced expression in the antennal lobes, optic lobes, mushroom bodies, central complex, and median bundle (Ramaekers et al., 2001). DmGluRB bears closest homology to group III and the next closest homology to group II mGluRs (Figure S1), but does not appear to bind glutamate (Mitri et al., 2004). Nonetheless, if the glutamate binding of DmGluRB is impaired, it could still be activated by glutamate if it is able to form dimers with DmGluRA (Mitri et al., 2004; Pommier et al., 2003).

Since in vivo pharmacological studies of the mGluRs in *Drosophila* have not been previously reported, we looked for mGluR antagonists that work in vivo in mammals and whose binding pockets have been well characterized. 2-methyl-6-(phenylethynyl)pyridine (MPEP) is an antagonist of mammalian group I subtype 5 mGluR that has a well-characterized binding pocket (Malherbe et al., 2003; Pagano et al., 2000). The appropriate putative secondary structure and residues critical for the efficient binding of MPEP are for the most part conserved in the *Drosophila* mGluR sequences (Figure S2). Thus it appeared that MPEP might be capable of antagonizing the *Drosophila* mGluRs. They also appeared to be the only targets, as a genomic database (BLAST) search for other protein sequences with primary sequence homology to the MPEP binding pocket region of human mGluR5 failed to reveal any other potential targets.

We first examined the ability of MPEP treatment to restore normal courtship behavior in the *dfmr1* mutant males. Courting *Drosophila* males perform a characteristic sequence of behaviors: orienting toward and following the female, tapping her with his forelegs, vibrating one wing, licking her genitalia, and attempting to copulate (Bastock and Manning, 1955; Bastock, 1956; Sturtevant, 1915). The percentage of time that the male spends performing any of these behaviors toward a target during a defined period of time is referred to as the courtship index (CI) (Siegel and Hall, 1979). In a previous study, we observed that *dfmr1* mutant males, or "FS" males (homozygous $dfmr1^3$ plus one transgenic copy of a genomic fragment containing a frameshift mutation in the *dfmr1* open reading frame; see Dockendorff et al., 2002) did not court virgin females as vigorously as did wild-type control males (*w*–) or "Rescue" males (homozygous $dfmr1^3$ plus one transgenic copy of a wild-type genomic *dfmr1* fragment; see Dockendorff et al., 2002). Because the Rescue and FS lines are genetically identical except for their ability to express wild-type *dfmr1* protein (Dockendorff et al., 2002), these two lines were used for most comparisons.

MPEP efficacy was tested by treating larvae during development and adult flies after eclosion to determine if restoration of normal behavior required reduction of mGluR activity during either or both of these periods. These groups are designated in the following text by a binary code in which the first two-letter acronym indicates the food type given larvae during development, and the second acronym denotes the food type given the adult flies upon eclosion and for 4 days thereafter. All flies were placed on control (CT) food the day before testing.

In testing decreasing concentrations of MPEP (M), we found that treatment with 86 μM MPEP resulted in a striking increase in the naive courtship levels of FS flies. When compared to the courtship activity of CT-CT FS flies, significantly more courtship activity was observed in FS flies that were treated with 86 μM MPEP as larvae, as adults, or at both stages (Figures 1A–1D). This increase in activity was also accompanied by an increase in courtship quality, but no detectable changes in locomotor activity, visual acuity, or olfaction capabilities (Figure S3). The fact that significant enhancement was obtained by drug treatment only during adulthood suggests that MPEP does not need to act to prevent developmental (i.e., in the period preceding eclosion) defects that are caused by the loss of *dfmr1* function to rescue the naive courtship defect. However, treatments that included drug exposure during development clearly provided the highest levels of recovery.

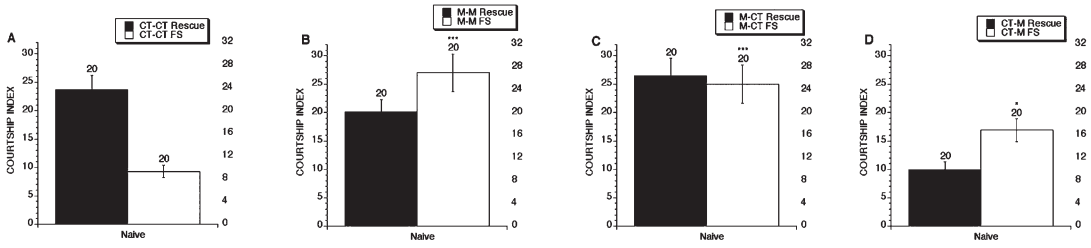

Figure 1. The Effect of MPEP on Naive Courtship in Flies Lacking *dfmr1* Activity

(A–D) Naive courtship of Rescue and FS flies exposed to MPEP. Filled bars indicate Rescue males (*dfmr1*^{3} + wild-type rescue fragment); open bars indicate FS males (*dfmr1*^{3} + frame-shifted rescue fragment). Mean CIs (±SEM) are plotted; Ns are indicated above each bar for all groups. For levels of significance, *p < 0.005; **p < 0.0005; ***p < 0.0001. Flies were raised on either control food (CT) or food supplemented with 86 μM MPEP (M). The first abbreviation indicates the food type that the larvae grew up on, and the second indicates the food type the adult males were placed on within 4 hr of eclosion. (A) Without drug treatment (CT-CT), FS males court virgin females less vigorously than do Rescue flies. Comparisons in panels (B)–(D) are made relative to the CT-CT mean of the same genotype in (A). (B) When raised on MPEP-containing food during both development and adulthood (M-M), FS flies court virgin females as well as do untreated Rescue males shown in (A). The naive courtship levels of M-M Rescue and CT-CT Rescue flies do not differ significantly. (C) Rescue and FS flies treated with MPEP as larvae and then placed on CT food as adults (M-CT) courted as vigorously as CT-CT Rescue flies. (D) When treated with MPEP only as adults (CT-M), FS flies displayed significantly greater courtship activity relative to CT-CT FS flies, whereas courtship activity of the Rescue flies was significantly depressed relative to CT-CT Rescue flies.

When Rescue flies were raised on CT food and then placed on MPEP-containing (M) food as adults, courtship activity was depressed relative to that in CT-CT Rescue flies, (Figures 1A and 1D). This decrease in courtship was not due to a detectable change in locomotor activity, visual acuity, or olfaction capabilities and did not result in a change in courtship quality (Figure S3). Thus, giving this drug to wild-type flies causes at least one adverse effect and provides further evidence that MPEP can act in the adult nervous system to modulate male courtship. However, Rescue flies that underwent MPEP treatment during larval development were unaffected. Both M-CT and M-M Rescue flies courted at levels similar to CT-CT Rescue flies in the naive courtship assay (Figures 1A–1C; p = 0.19 and p = 0.39, respectively). Thus, while MPEP treatment of wild-type adult males inhibits their courtship activity, flies that were exposed to MPEP as larvae appear to be less sensitive to the drug as adults.

Restoration of Naive Courtship Levels in the *dfmr1* Mutant Flies Occurs through Reduction of mGluR Activity

To verify that the results obtained by MPEP treatment were due to a reduction in mGluR activity, we performed several additional tests. First, we used a lower concentration of MPEP, as initial experiments were done with a concentration that has been shown to antagonize mammalian NMDA receptor activity to some degree (Spooren et al., 2001). We used MPEP at 8.6 μM, a concentration several-fold lower than that which affects NMDA receptor activity. Next, we used several different mGluR antagonists, including LY341495, MPPG [(RS)-α-methyl-4-phosphonophenylglycine], and MTPG [(RS)-α-methyl-4-tetrazolylphenylglycine]. LY341495 was chosen because it has no effect on NMDA receptor activity and has also been shown to be a potent mGluR antagonist in vivo (Rasmussen et al., 2004). Recently it has been shown to antagonize the DmGluRA in a heterologous expression system (Bogdanik et al., 2004). MPPG and MTPG are group II antagonists that were chosen because they also have been shown to antagonize the DmGluRA in a heterologous expression system (Parmentier et al., 1996). In contrast to MPEP, these antagonists act through a different mechanism. LY341495, MPPG, and MTPG are competitive antagonists that compete with glutamate for the glutamate binding pocket, whereas MPEP is a noncompetitive antagonist that binds in the seven-transmembrane region of the receptor (Spooren et al., 2001). The concentration of LY341495 used in these studies was 400 nM, as this was previously shown to selectively block the activity of only group II (types 2 and 3) and group III (type 8) mGluRs in mammalian studies (Kingston et al., 1998). Concentrations of MPPG at 573 μM and of MTPG at 348 μM were used because these concentrations are required to obtain an IC_{50} in the heterologous expression system (Parmentier et al., 1996). Finally, we used LiCl at concentrations of 5 mM and 50 mM. Previously, LiCl was shown to facilitate CREB DNA binding activity, inhibit GSK-3B activity, and inhibit inositol trisphosphate receptor-mediated calcium release (Berridge, 1993; Berridge et al., 1989; Grimes and Jope, 2001; Takei et al., 1998). Since group II mGluRs have been linked to pathways that diminish CREB DNA binding and enhance inositol trisphosphate receptor-mediated calcium release (see Figure S4 and legend), LiCl should act to decrease these mGluR effects. Therefore, even though lithium may have other effects, it affects signaling pathways overlapping with those affected by mGluR antagonists. It should be noted that although lithium treatment can have serious side effects in humans and its effects on cognition have not been well characterized, it is approved by the FDA for other uses, and there is anecdotal evidence indicating that it may have benefits with regard to mood stabilization and aggression in Fragile X patients (Hagerman and Hagerman, 2002).

In testing the effectiveness of 8.6 μM MPEP, 400 nM LY341495, 573 μM MPPG, 348 μM MTPG, and LiCl at

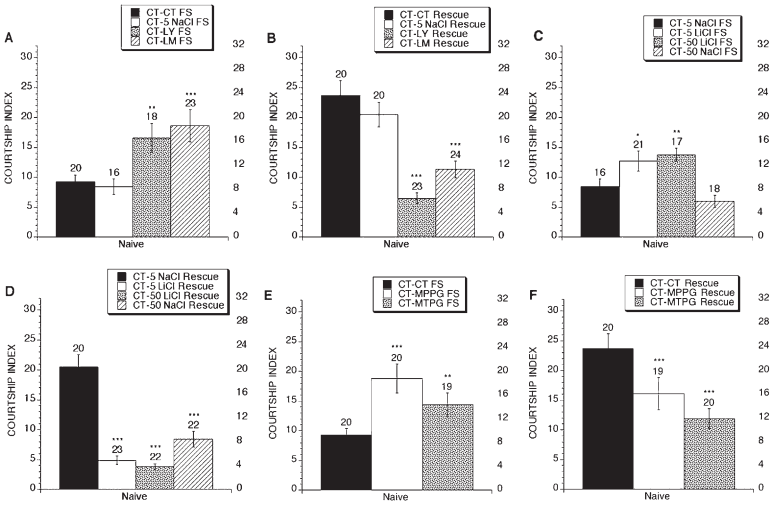

Figure 2. The Naive Courtship Levels of Flies Lacking *dfmr1* Activity and Treated with Low Doses of MPEP, LY341495, Lithium, MPPG, or MTPG

(A–F) The naive courtship levels of FS flies (A, C, and E) and Rescue flies (B, D, and F) were tested after a diet of CT food during development and then food containing either NaCl or a test drug for 4 days during adulthood. All flies were placed on CT food 24 hr before measurement of naive courtship levels. Naive courtship levels were plotted as described in the legend of Figure 1, except that the levels of significance are indicated as follows: *p < 0.05; **p < 0.005; ***p < 0.001. (A and B) Adult male flies were fed food containing 8.6 μM MPEP (CT-LM), 400 nM LY341495 (CT-LY), or 5 mM NaCl (CT-5 NaCl). (C and D) Adult male flies were fed food containing 5 mM NaCl (CT-5 NaCl), 50 mM NaCl (CT-50 NaCl), 5 mM LiCl (CT-5 LiCl), or 50 mM LiCl (CT-50 LiCl). The naive courtship levels shown for CT-CT FS flies and CT-CT Rescue flies in Figures 2A, 2B, 2E, and 2F are replicated from Figure 1A as a reference point to compare with the CT-5 NaCl, CT-MPPG, and CT-MPTG groups. In (A)–(D), comparisons are made relative to the 5 mM NaCl control treatment group of the same genotype. In previous experiments we determined that this treatment did not affect naive courtship levels for both genotypes. (A) Statistically significant increases in naive courtship levels were obtained for the CT-LY FS and CT-LM FS flies when compared to the levels obtained for the CT-5 NaCl FS flies. (B) The naive courtship levels of Rescue flies are reduced by treatment with food containing a lower dose of MPEP or LY341495. (C) Both LiCl treatments increase naive courtship levels of FS flies, while treatment with 50 mM NaCl had no positive effect on courtship levels. (D) Treatment of adult Rescue flies with 5 mM LiCl, 50 mM LiCl, or 50 mM NaCl results in a significant reduction in naive courtship levels. In (E) and (F), comparisons are made relative to the CT-CT group. (E and F) the levels of significance are indicated as follows: **p < 0.005; ***p < 0.0001. FS males (E) and Rescue males (F) raised on control food and then fed either control food (CT-CT) or food containing 573 μM MPPG (CT-MPPG) or 348 μM MTPG (CT-MTPG). (E) Treatment of adult FS flies with 573 μM MPPG or 348 μM MTPG results in a significant increase in courtship activity. (F) Treatment of adult Rescue flies with 573μM MPPG or 348 μM MTPG results in a significant decrease in courtship activity.

dosages of both 5 mM and 50 mM, we observed that naive courtship was significantly restored (Figures 2A, 2C, and 2E), whereas no increase was observed in CT (5 mM and 50 mM NaCl) FS flies (Figure 2C). Thus, the treatment with these five independent drugs leads to a similar significant rescue of naive courtship, indicating that reduction of mGluR activity in *dfmr1* mutant flies leads to the restoration of this behavior. Also noteworthy is the consistent effect that these drugs had on the Rescue flies as they all led to a similar depression in naive courtship activity (Figures 2B, 2D, and 2F). The consistency of these results argues that all of these drug treatments are affecting the same target.

Learning and Memory Phenotypes of *dfmr1* Mutants Assessed by Courtship Conditioning

Since mGluR activity has been linked to learning and memory processes and our results presented above indicate that mGluR signaling is altered in the *dfmr1* mutant flies, we investigated whether learning and memory defects could be detected in flies lacking *dfmr1* activity. Learning and memory can be examined in *Drosophila* by utilizing conditioned courtship behavior. In conditioned courtship, a male fly learns to modify his courtship behavior after experience with an unreceptive female (Hall, 1994). This is a complex associative learning paradigm that potentially involves multiple sensory inputs (Ackerman and Siegel, 1986; Tompkins, 1984; Tompkins et al., 1982, 1983). Courting male flies perform a sequence of behaviors (as described above). Virgin females generally respond by mating; however, recently mated females are unreceptive, display different behaviors, and will not allow copulation to occur (Greenspan and Ferveur, 2000). They also have an altered pheromonal profile that the naive male finds less provocative (Cobb and Ferveur, 1996). A naive male paired with a mated female will initially court her, but his courtship activity soon decreases; after 1 hr of experience with the mated female, his courtship, when subsequently paired with a virgin female, remains depressed for 2 to 3 hr (Siegel and Hall, 1979). This effect is not a general suppression of all courtship activity, since a male's tendency to court an immature male is not suppressed (Gailey et al., 1984). Also, experience with a virgin female does not depress courtship toward a subsequent virgin female, as long as copulation is not prevented (Gailey et al., 1984). After training with a previously mated female, a decrease in CI toward virgin females is indicative of behavioral plasticity in the form of memory.

In the conditioned courtship paradigm, learning during training can be assayed by comparing the CI of the first 10 min after the male is paired with an unreceptive female with the CI of the last 10 min period of the pairing. Wild-type flies typically show a 40% or more decrease in courtship activity (Joiner and Griffith, 1997; Kane et al., 1997). Hence, learning during training is a form of behavioral plasticity, but it may be a mix of associative and nonassociative memories and is distinct from memory as assayed posttraining, which is an associative memory. Additionally, intact memory can occur without learning during training, and learning during

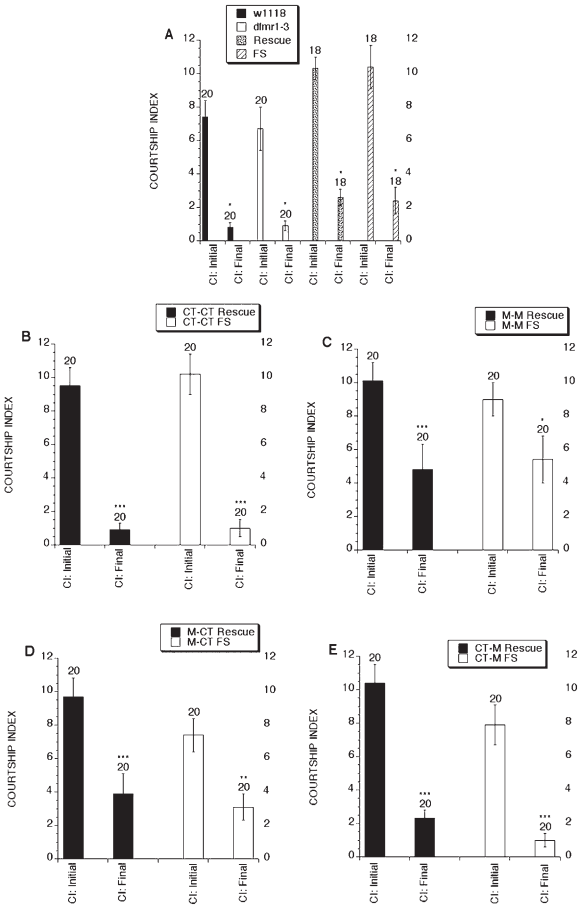

Figure 3. Learning during the Training Phase of Conditioned Courtship Is Normal for Flies Lacking *dfmr1* Activity and Is Unaffected by MPEP Treatment

(A–E) Courtship conditioning of *dfmr1* mutant or control flies that were either untreated or given food containing MPEP. Mean CIs (±SEM) and levels of significance are plotted as described in the legend of Figure 1. (A) The initial and final courtship levels of w^{1118} (black bars) and $dfmr1^{3}$ (open bars), Rescue (speckled bars), and FS (striped bars) flies. For (B)–(E), filled bars indicate CI values for Rescue flies; open bars indicate CI values for FS flies. In these experiments, MPEP was used at a concentration of 86 μM. (B) Rescue and FS flies given CT food both during development and as adults. (C) Rescue and FS flies on M food both during development and as adults. (D) Rescue and FS flies on M food during development and CT food as adults. (E) Rescue and FS flies on CT food during development and M food as adults. All groups of flies, regardless of treatment, exhibited intact learning during training as demonstrated by a significant depression of courtship activity from the initial to the final interval of the training session.

training can occur without posttraining memory (Joiner and Griffith, 1997; Kane et al., 1997).

To assess learning during training, male flies were placed in a training chamber with a previously mated female for 1 hr, and the amount of time that the male spent actively courting in the initial 10 min interval was compared to the amount of courtship activity observed in the final 10 min interval (Figure 3A). The courtship levels of *w*–, *dfmr1*, Rescue, and FS were all similar and showed significant depression from the initial to the final intervals, indicating that all groups demonstrated learning during training (Figure 3A). It is important to note that the level of courtship behavior toward the previously mated female was similar among all four genotypes. This indicated that although naive courtship toward a virgin female had previously been shown to be depressed for *dfmr1* and FS flies (Dockendorff et al., 2002), there is enough courtship activity toward an unreceptive female to adequately train each of the two mutant groups. This is important to note, because without active courting, the male fly cannot be trained (Tompkins et al., 1982, 1983).

Since MPEP treatment had positive effects in FS flies and negative effects in Rescue flies on naive courtship capabilities, we examined if MPEP treatment had any effects on these flies' learning capabilities during training. We found that treatment by MPEP either in development or in adulthood, or at both times, did not prevent learning during training (Figures 3B–3E). Also, treatment of adults with 400 nM LY341495, 573 μM MPPG, or 348 μM MTPG had no effect on learning during training (data not shown). It is noteworthy that this was also true for the CT-M Rescue and CT-CT FS flies. These two groups displayed similarly low CIs in the naive courtship assay, but during the courtship conditioning training session displayed levels of courtship similar to those of all of the other groups, indicating that enough courtship activity was present to adequately train each of these two groups.

Analysis of Immediate Recall Memory in *dfmr1* Mutant Flies Trained by Courtship Conditioning

In *Drosophila* there are five phases of memory that have been identified and categorized in several genetic and pharmacological studies. There is an immediate recall at 0–2 min posttraining; short-term memory out to 1 hr; medium-term memory out to 6 hr; anesthesia-resistant memory out to two days; and long-term memory, which lasts up to 9 days posttraining and appears to be protein synthesis-dependent (Greenspan, 1995). Since the *dfmr1* and FS flies display normal behavioral plasticity with regard to learning during training, we next investigated their memory. To examine the immediate recall memory, we took males that had just completed a 1 hr training session with a previously mated female and immediately placed them in a new chamber with a virgin target female for a 10 min interval. This CI was then compared to the courtship level of naive males that had been placed in a training chamber for 1 hr with no female before being introduced to a virgin target female. In Figure 4A, the *w*– and Rescue flies showed significant depression of courtship activity after training compared to that of naive males. However, *dfmr1* and FS mutant flies courted just as vigorously after training with a previously mated female as did naive males. This implicates a deficit in behavioral plasticity for *dfmr1* and FS mutant flies at immediate recall (0–2 min memory).

Rescue of Memory with mGluR Antagonists and LiCl Treatment

Since naive courtship was rescued and the normal learning during training displayed by the FS flies was

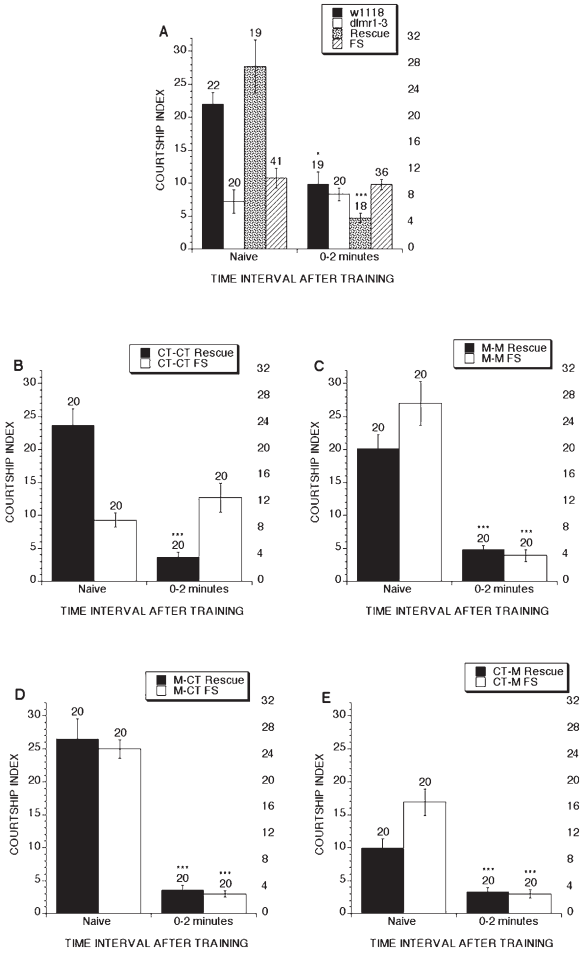

Figure 4. *dfmr1* Mutant Flies Lack Detectable Immediate Recall Memory, and Treatment with MPEP Rescues This Deficit

(A–E) Immediate recall memory was examined in untreated *dfmr1* mutant and control flies and in response to 86.0 μM MPEP treatment. Mean CIs (±SEMs) and levels of significance are plotted as described in the legend of Figure 1. (A) The w^{1118} (black bars) and Rescue (speckled bars) lines show depression of courtship activity after training compared to naive trained males. $dfmr1^{3}$ (open bars) and FS flies (striped bars) display no detectable depression relative to naive trained males. (B–E) Filled bars, CI values for Rescue flies; open bars, CI values for FS flies. (B) Examination of immediate recall memory in Rescue and FS flies that have been fed control food. As observed in (A), Rescue flies display a significant reduction in courtship activity toward a virgin female immediately after the 1 hr training session, whereas no detectable difference is observed in similarly treated FS flies. (C–E) Rescue and FS flies given food containing MPEP during development and as adults (M-M Rescue and M-M FS) (C); during development only (M-CT Rescue and M-CT FS) (D); or during adulthood only (CT-M Rescue and CT-M FS) (E) display significant reduction in courtship activity toward a virgin female immediately after training.

not significantly affected by MPEP treatment, we examined the ability of MPEP to restore immediate recall (0–2 min) memory. As observed above, the CT-CT FS flies failed to exhibit any change in their courtship activity as a result of training (Figure 4B). Importantly, all of the FS groups that were treated with MPEP displayed significant experience-dependent reduction of courtship activity immediately after training when compared to the naive courtship levels obtained for each treatment protocol (Figures 4C–4E). Therefore, treatment of FS flies with MPEP during development, or as adults alone, is sufficient to restore behavioral plasticity in flies that lack *dfmr1* function.

In this experiment, we also observed that all Rescue groups demonstrated significant depression of courtship activity immediately after training relative to group-matched naive flies (Figures 4B–4E). This indicates that the administration of M food during development, adulthood, or at both times, does not adversely affect immediate recall in these control groups, although naive courtship was depressed in the CT-M Rescue line (Figure 4E).

Since immediate recall was not detected in flies lacking *dfmr1* activity, we explored whether short-term memory was also affected, and, if so, determined whether it could be rescued by MPEP. To assay short-term memory, the trained male was placed in a holding chamber for 60 min and subsequently placed in a testing chamber with a virgin female target (Figures 5A–5D). The results obtained parallel those of immediate recall after MPEP treatment. All of the FS flies treated with MPEP, during development, as adults, or at both times, displayed significant experience-dependent reduction of courtship activity at 60 min after training (Figures 5B–5D). In contrast, the CT-CT FS flies courted just as vigorously at 60 min after training as did naive CT-CT FS flies, indicating an absence of short-term memory in these mutant flies (Figure 5A). As observed with immediate recall memory, none of the MPEP treatments affected the short-term memory displayed by the Rescue flies (Figures 5A–5D).

To verify that the restoration of memory observed with the MPEP-treated FS flies was occurring through the downregulation of mGluR activity levels, we determined if treatment of mutant flies during adulthood with 8.6 μM MPEP, 400 nM LY341495, or 5 mM or 50 mM LiCl (see above) would also restore memory. The 8.6 μM MPEP and 400 nM LY341495 treatments restored short-term memory that was indistinguishable from that observed for these flies treated with 86 μM MPEP (Figures 5E, 5F, and 4D). Short-term memory was also restored in the FS flies treated with either 5 mM or 50 mM LiCl; however, no restoration of short-term memory was observed with either control NaCl treatment (Figures 5H and 5G). Short-term memory was disrupted in Rescue flies treated with 5 mM or 50 mM LiCl, but remained intact in Rescue flies treated with either concentration of NaCl (Figures 5H and 5G). These results imply that LiCl can increase naive courtship and restore short-term memory in FS flies, possibly by modulating signaling in a manner similar to the downstream effects of mGluR antagonists.

The memory assays as performed above utilized a virgin female target to test for memory. Since flies lacking *dfmr1* activity display reduced courtship activity toward these targets, we wanted to verify that the observed memory deficit was not due to a problem with recognizing or processing the appropriate cues from the virgin female target. Therefore, we used a modified version of the conditioned courtship paradigm in which the male is paired with a mated female target subse-

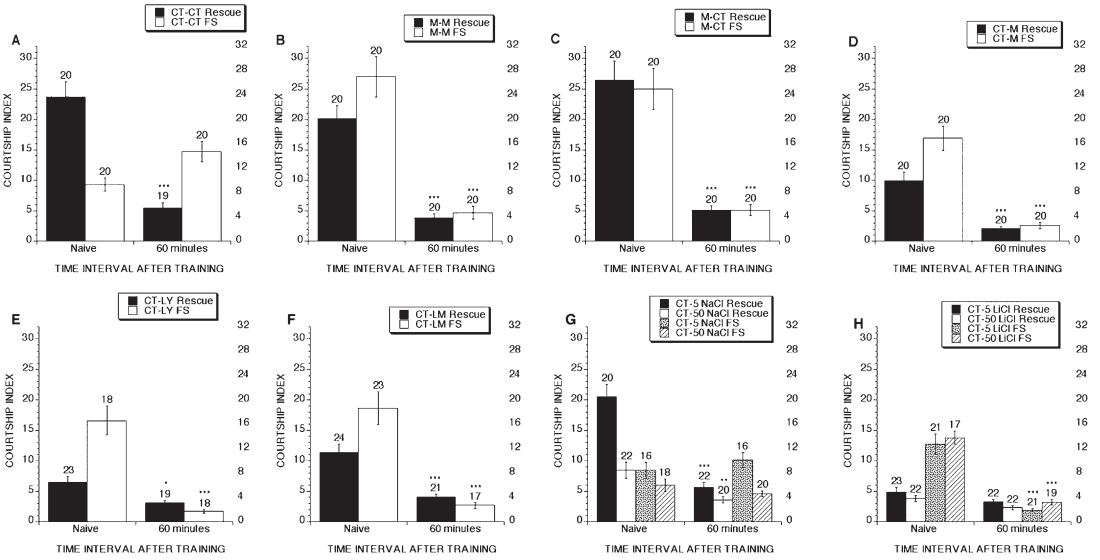

Figure 5. Effects of Drug Treatment on Short-Term Memory in Flies Lacking *dfmr1* Activity

(A–H) Short-term (60 min) memory was measured in Rescue and FS flies that were either given control food or administered various drug treatments as described below. Short-term memory was measured by placing a trained male in a holding chamber for 60 min, then subsequently placing him in a testing chamber with a virgin female target for a 10 min courtship interval. The resulting CI is compared to the CI obtained for naive courtship. For (A)–(D), the mean CIs (±SEM) and levels of significance are plotted as described in the legend of Figure 1. (A–F) Black bars, CIs of Rescue flies; open bars, CIs of FS flies. (A) The courtship activity of Rescue flies is significantly reduced 60 min posttraining when compared to the level of naive courtship. FS flies 60 min posttraining fail to display any reduction in courtship levels when compared to naive courtship levels, thus demonstrating lack of any detectable short-term memory in this assay. (B–D) Rescue and FS flies whether fed 86 μM MPEP during development and adulthood (M-M Rescue and M-M FS) (B); during development alone (M-CT Rescue and M-CT FS) (C); or during adulthood alone (CT-M Rescue and CT-M FS) (D) all display a significant reduction in courtship activity toward a virgin female 60 min after training when compared to the naive courtship levels obtained for similarly treated flies. For (E–H), mean CIs (±SEMs) and the levels of significance are plotted as described in the legend of Figure 2 (A–D). (E and F) Examination of short-term memory in Rescue and FS flies that were either fed food containing 400 nM LY341495 (CT-LY Rescue or CT-LY FS) (E) or 8.6 μM MPEP as adults (CT-LM Rescue or CT-LM FS) (F). All treatment groups displayed significant reduction in courtship activity toward a virgin female 60 min after training when compared with naive courtship levels obtained with similarly fed flies of the same genotype. (G and H) Examination of short-term memory in Rescue and FS flies that were either fed food containing 5 mM NaCl (CT-5 NaCl Rescue and CT-5 NaCl FS) or 50 mM NaCl (CT-50 NaCl Rescue and CT-50 NaCl FS) as adults (G) or were treated with 5 mM LiCl (CT-5 LiCl Rescue and CT-5 LiCl FS) or 50 mM LiCl (CT-50 LiCl Rescue and CT-50 LiCl FS) as adults (H). (G) Rescue flies given food containing 5 mM NaCl (black bar) or 50 mM NaCl (open bar) as adults display significant short-term memory, whereas no such memory is detected in FS flies given food containing 5 mM NaCl (speckled bar) or 50 mM NaCl (striped bar) as adults. (H) Rescue flies given food containing 5 mM LiCl (black bar) or 50 mM LiCl (open bar) as adults do not display detectable short-term memory. However, short-term memory was restored in FS flies fed food containing 5 mM LiCl (speckled bar) or 50 mM LiCl (striped bar) as adults.

quent to training (Kane et al., 1997; Joiner and Griffith, 1997, 1999; Kamyshev et al., 1999). Since the FS and Rescue flies respond similarly to these targets with regard to learning during training, any deficit in memory could not be attributed to sensory defects. We found that CT-CT FS flies failed to demonstrate memory at immediate recall and short-term memory, whereas CT-CT Rescue flies demonstrated memory of training at either time point (Figure 6A). As observed above, treatment of FS flies with mGluR antagonists—in this case 86 μM MPEP, 400 nM LY341495, 573 μM MPPG, or 348 μM MTPG, as adults—led to restoration of short-term memory, and short-term memory remained intact in Rescue flies (Figures 6B–6E). Thus, the memory deficit observed in the mutant flies is not due to a sensory processing impairment, but is definitively a memory impairment, and this impairment can be rescued by treatment with four independent mGluR antagonists or LiCl. It is important to note that the robust deficit in synaptic plasticity in FS flies, with regard to memory, is a critical extension of the previous animal models of Fragile X syndrome in other organisms, since memory deficit is one of the most prominent aspects of the human disorder.

Rescue of β-Lobe Fusions with mGluR Antagonists

Mushroom bodies are involved in learning and memory in the conditioned courtship and the odor-shock classical conditioning paradigms in *Drosophila* (de Belle and Heisenberg, 1994; Joiner and Griffith, 1999; McBride et al., 1999; Pascual and Preat, 2001). Recent studies of *dfmr1* mutants have revealed that the β-lobes of the mushroom bodies (MBs) cross over the midline and fuse at a fairly high frequency (Michel et al., 2004). Since memory defects in the *dfmr1* mutants were restored by treatment with mGluR antagonists, we wanted to determine if similar treatments could also restore the MB defect. With anti-FasII labeling, we observed frequent β-lobe fusion defects in brains derived from 0- to 2-day-old FS mutant flies, but not Rescue flies (Figures

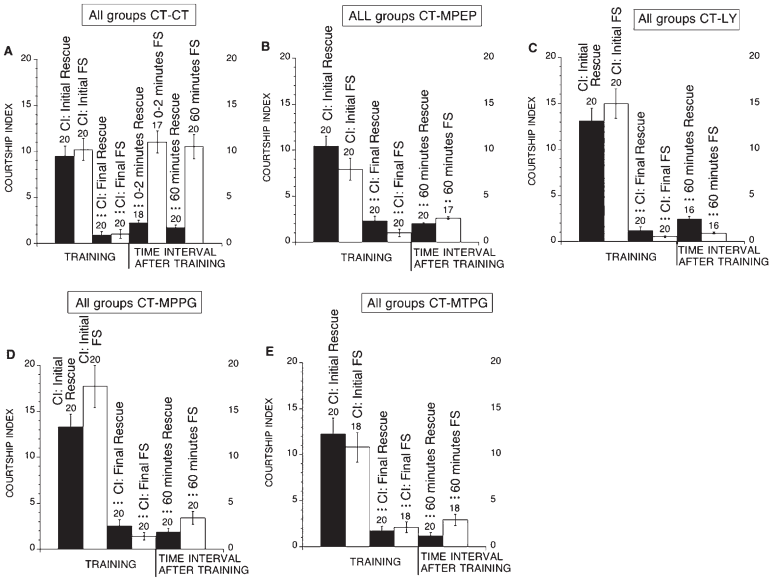

Figure 6. *dfmr1* Mutant Flies Lack Detectable Short-Term Memory when Tested with Mated Female Targets, a Deficit Which Is Restored by Treatment with mGluR Antagonists

(A–E) Short-term (60 min) memory was measured in Rescue and FS flies that were either given control food or administered various drug treatments as described below. Short-term memory was measured by placing a trained male in a holding chamber for 60 min (after a 1 hr training with a previously mated female), then subsequently placing him in a testing chamber with a mated female target for a 10 min courtship interval. This CI is compared to the CI obtained for naive courtship of a previously mated female, i.e., the CI during the first 10 min of the training session with a previously mated female. Additionally, for reference the CI during the last 10 min of the training period is also shown. For (A–E), the mean CIs (±SEM) are plotted as in Figure 1. The levels of significance are indicated as follows: *p <0.05; **p <0.005; ***p < 0.0001. (A) Rescue flies kept on control food alone demonstrate memory at 0–2 min and 60 min after training. In contrast, FS flies kept on control food alone do not demonstrate memory at either time point. (B) Rescue and FS flies treated with 86 μM MPEP demonstrate memory at 60 min posttraining. (C) Rescue and FS flies treated with 400 nM LY341495 demonstrate memory at 60 min posttraining. (D) Rescue and FS flies treated with 573 μM MPPG demonstrate memory at 60 min posttraining. (E) Rescue and FS flies treated with 348 μM MTPG demonstrate memory at 60 min posttraining.

7A–7F). Using the scoring method described by Michel et al. (2004), we observed defects ranging from mild to severe in roughly 70% of the FS mutant brains, whereas only 10% of Rescue fly brains displayed defects, all of which were mild (Figure 7F). Interestingly, the penetrance of this phenotype was greatly reduced when FS flies were raised on food containing 8.6 μM MPEP, 400 nM LY341495, or 348 μM MTPG (Figure 7F). No effect was observed when these drugs were fed to Rescue flies during development (data not shown).

This rescue of the β-lobe fusion defect suggests that prevention of this defect is key to rescuing the memory defects observed in FS mutant flies. If this is true, then we would expect that treating FS mutant flies with mGluR antagonists during adulthood alone would lead to similar morphological rescue. To test this hypothesis, we treated FS mutant flies with 8.6 μM MPEP for 4 days, starting at eclosion, and then transferred them to normal food for 24 hr before examining the morphology of their MBs. For comparison we also examined the MBs of flies on control food for 5 days. Contrary to the results obtained when the drug treatments were performed during development, we did not observe any rescue of the β-lobe fusion defects with the treatment during adulthood (Figure 7G). Thus, it appears that rescue of this morphological defect is not absolutely required for the rescue of the memory defects observed in the *dfmr1* mutant flies.

Free Running Rest:Activity Rhythm Defects Are Not Rescued in the *dfmr1* Mutant Flies by MPEP Treatment

In the initial characterization of the *dfmr1* mutants, several groups identified a defect in free running rest:activity rhythms. This failure to maintain normal circadian regulation of locomotor activity was attributed to defects in circadian output, rather than a defect in the clock (Dockendorff et al., 2002; Morales et al., 2002). We investigated whether circadian regulation in the *dfmr1* mutant flies could also be rescued by inhibition of mGluR activity. Toward this goal, we gave *dfmr1*, FS, and Rescue flies food containing several different concentrations of MPEP, including 86 μM. The mutant and control flies were fed the MPEP-containing food during development and/or as adults and/or during the monitoring of locomotor activity. In all of our trials, we failed to detect any rescue of circadian behavior (Table S1 and data not shown). Therefore, the failure of *dfmr1* flies to maintain free running rest:activity rhythms does not appear to be due to the same defect that causes reduced naive courtship, MB fusions, and a lack of memory in these flies.

Discussion

Previous studies of *dfmr1* mutants have revealed phenotypes with parallels to those observed in the *Fmr1* knockout mouse and Fragile X patients. In this study we have expanded the behavioral analysis of the *Drosophila* Fragile X model to include learning during training and memory as measured by the conditioned courtship paradigm with both virgin and mated female targets. In this assay, we found that *dfmr1* mutants display normal behavioral plasticity of learning during training, but lack any detectable memory of this training, even when tested immediately thereafter. These results indicate that cognitive dysfunction, the hallmark feature of Fragile X syndrome, is also a phenotype of the *Drosophila* Fragile X model.

Recent progress in the study of the mouse model of

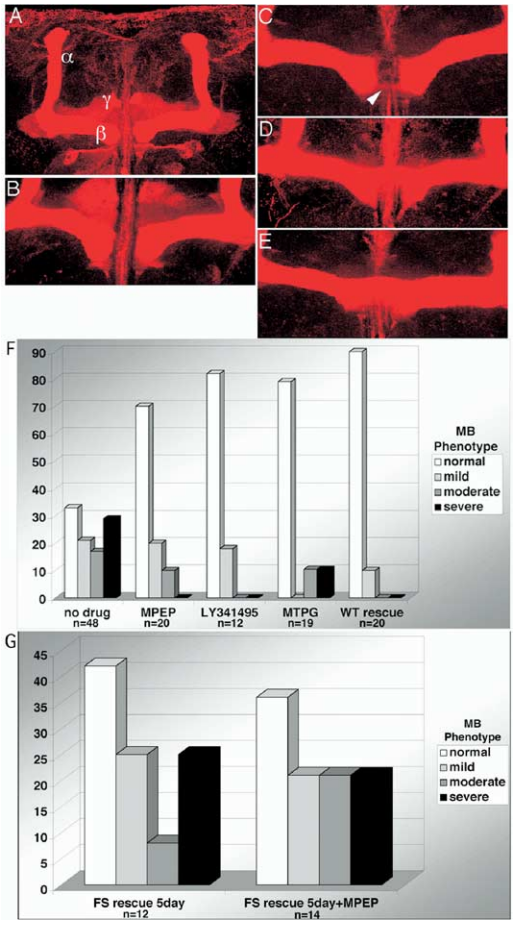

Figure 7. Fusion of MB β lobes and Rescue of These Fusions with mGluR Antagonists

(A–E) Brains from 0- to 2-day-old FS flies, stained with α-fasicillin II (ID4) and a rhodamine-coupled secondary antibody. (A) The α, β, and γ lobes of the MBs are clearly labeled with this antibody and appear normal in this control brain. (B–E) Higher magnification of the β lobes at the midline. (B) A *dfmr1* mutant brain with normal β lobes. (C–E) Mutant brains displaying a "mild" ([C], arrowhead), "moderate" (D), and "severe" (E) level of midline crossing by the β lobes. (F) Bar graphs revealing the penetrance of the β lobe fusion detected in untreated "no drug" (0- to 2-day-old) FS mutant brains, or those given food containing 8.6 μM MPEP, 400 nM LY341495, or 348 μM MTPG. W-T rescue flies are *dfmr1* mutants containing one copy of the *dfmr1* genomic rescue fragment. The number of brains that were examined is listed below each group. (G) *dfmr1* mutant brains from 5-day-old adults that were either given control food the entire time or were given food containing 8.6 μM MPEP for 5 days starting immediately after eclosion (FS rescue 5 day + MPEP).

Fragile X has led to a theory that the absence of FMRP leads to misregulation of protein synthesis at the synapse that occurs in response to mGluR activity (Huber et al., 2002; Bear et al., 2004). An extension of this theory is that modulation of mGluR activity with antagonists should reverse phenotypes attributable to loss of FMRP function (Huber et al., 2002; Bear et al., 2004). Since so many similarities exist between symptoms observed in Fragile X patients and phenotypes observed in the fly and mouse models, we speculated that *dfmr1* in the fly and FMRP in mouse and humans regulate conserved pathways important for cognition and behavioral activities. This reasoning led us to test the previously mentioned "mGluR Theory" of Fragile X (Bear et al., 2004), using the robust behavioral phenotypes observed in the fly Fragile X model. In support of the mGluR theory, we have found that treatment of *dfmr1* mutants with mGluR antagonists or LiCl leads to rescue of the courtship and memory defects in mutant flies. In addition, we have also observed that these treatments can rescue β-lobe defects in the MBs of *dfmr1* mutant flies. These results indicate that misregulation of mGluR activity is an evolutionarily conserved feature of the mouse and *Drosophila* models for Fragile X syndrome and that in the fly model this misregulation is likely to be the underlying cause of the naive courtship and cognitive defects. These results suggest modulation of mGluR activity as a potential treatment to ameliorate the symptoms of Fragile X syndrome. For further discussion, refer to Figure S4.

In light of the observed rescue of naive courtship and memory, it is interesting to consider from a behavioral standpoint what the drug treatments are correcting. In this study and as observed previously (Dockendorff et al., 2002), we found that the *dfmr1* mutant males display reduced courtship activity toward a virgin female. Although they progress beyond the initial stages of courtship, indicating an ability to recognize at least some of the cues of the virgin females, the *dfmr1* mutant males fail to advance to the more complex stages of courtship. Previously we have shown that *dfmr1* mutant males also court immature males with reduced vigor (Dockendorff et al., 2002). The low courtship levels of *dfmr1* mutant males could be explained by a lack of fine motor skill, an inability to maintain courtship interest, or an inability to efficiently process the multiple cues presented by virgin females and integrate them into efficient courtship activity.

Despite the defects in naive courtship, no defects were detected in learning during training in the conditioned courtship paradigm. This indicates that *dfmr1* mutants are capable of sensing, interpreting, and responding properly to the negative cues of the previously mated female. However, *dfmr1* mutants lacked any detectable memory of this training. This memory deficit could be due to abnormal synaptic plasticity, to some inability to recognize female target cues, or to disruption in some type of output mechanism required for suppression of courtship. Although we cannot rule out the latter two possibilities completely, we have several reasons to think that the deficit is a specific impairment of memory. First, the *dfmr1* mutant flies respond normally to the previously mated female during training. Additionally, mutants have a high enough courtship level during training to expect a reduction in courtship activity after training, which is key since courtship is required for the associative memory to be formed (Tompkins et al., 1982, 1983). In fact, the *dfmr1* mutant flies do show a reduction of courtship during the training session that matches the levels displayed by the control flies. Thus, the mutant flies can suppress courtship activity to a level low enough for a memory phenotype to be seen if their memory was intact. Therefore, a generalized inability to suppress courtship due to an output problem seems unlikely. This is particularly clear

when it is realized that the degree of suppression of courtship behavior in FS flies treated with mGluR antagonists is similar to that seen in untreated FS flies during the last 10 min period of the training session with a previously mated female.

Our results also indicate that the lack of behavioral plasticity of the *dfmr1* mutant flies is not due to an artifact of low naive courtship masking the memory. With respect to this point, it is interesting to note that the control flies fed MPEP as adults (CT-M Rescue) have very similar naive courtship levels as the mutants, but CT-M Rescue flies display memory. Also we have observed that restoration of memory is not dependent on complete rescue of naive courtship. For example, treatment of *dfmr1* mutant flies with 5 mM LiCl resulted in minimal rescue of naive courtship, but does restore memory. Perhaps the most striking observation is that the mutants display no memory even when paired with a mated female target. This clearly demonstrates that the memory defect is not the result of an impairment in processing sensory cues. Therefore, we feel that the lack of behavioral plasticity is most likely due to defects in the ability to integrate complex environmental stimuli into an associative memory, and this ability is what is restored by treatment with the mGluR antagonists MPEP, MPPG, MTPG, and LY341495, or with lithium.

Previously, short-term memory and long-term memory have been shown to require the MBs in the conditioned courtship paradigm and olfaction association paradigm (McBride et al., 1999; Joiner and Griffith, 1999; Heisenberg, 1980; de Belle and Heisenberg, 1994; Dubnau et al., 2001; McGuire et al., 2001; Pascual and Preat, 2001). Recent studies identifying a morphological defect in the MBs would seem to provide an explanation for the memory defects we have observed in the *dfmr1* mutant flies (Michel et al., 2004). More importantly, though, we have found that treatment with mGluR antagonists during development dramatically rescues this defect. Two observations, however, preclude us from accepting this defect as the sole explanation of the memory deficit. First the penetrance of the β-lobe fusion phenotype does not fully correlate with the penetrance of the memory defect. Although 70% of the *dfmr1* mutant brains show some detectable level of fusion, 30% have normal morphology. This is in contrast to the memory defect, for all of the mutant flies display a lack of detectable memory. Also, the treatment of mutant flies with mGluR antagonists only during adulthood clearly rescues the memory defect, but does not rescue the β-lobe fusion defect. Thus, there must be additional unidentified defects that cause the memory deficits.

The finding that the fusion of the β-lobes can be ameliorated by treatment with mGluR antagonists during development indicates that this neuronal defect is due to misregulation of mGluR signaling. Studies by Michel et al. (2004) indicated that in wild-type brains, the neurons of the β-lobes stop growing prior to reaching the midline crossing. Thus, the fusion of the β-lobes observed in the *dfmr1* mutants is most likely due to a failure to respond to midline cues that signal these neurons to stop their growth, rather than to defects in pruning processes that cross the midline. This hypothesis fits well with recent findings by Kreibich et al. (2004) that demonstrate that enhanced mGluR1 signaling can reduce the responsiveness of growth cones to repellent cues.

In conclusion, we have extended the *Drosophila* model of Fragile X Syndrome to include a phenotype of memory impairment in an ethologically relevant memory paradigm. We have demonstrated that treatment of *dfmr1* mutant flies with drugs that antagonize mGluR activity results in the restoration of naive courtship activity, MB fusion, and memory as assayed in the conditioned courtship paradigm. These findings are consistent with recent studies of the mouse model of Fragile X Syndrome and suggest modulation of mGluR activity as a therapeutic approach to ameliorate cognitive dysfunction in individuals afflicted with Fragile X Syndrome.

Experimental Procedures

Drosophila Strains

Drosophila strains used in this study are described in Dockendorff et al. (2002). The *Drosophila* strains were cultured at 25°C in 50%–70% humidity in a 12 hr:12 hr light:dark (LD) cycle, on cornmeal-sucrose-yeast medium that was supplemented with the mold inhibitor methylparaben and autoclaved (Villella and Hall, 1996). Drugs were obtained from Tocris-Cookson (UK), solubilized according to manufacturer's instructions, and added to the fly food after cooling to the appropriate concentration.

Behavioral Training and Testing

Virgin male flies were collected under ether anesthesia within 4 hr of eclosion. Males were placed in individual small food tubes (15 × 75 mm plastic tubes containing 10–15 mm of food). Virgin XX, *yf* females were collected on the day of eclosion and kept in food vials in groups of 10 to 15. Flies were aged for 5 days in a 12:12 LD cycle at 25°C before behavioral training and testing. All testing was performed during the relative light phase. Mated females were 5 days old and observed to mate with a male the night before training. The virgin females that were used as targets were 4 days old. All male subjects were transferred to fresh control food the day before testing. Male flies were assigned to random groups for behavioral training and testing, which was performed blind (Siegel and Hall, 1979; Kane et al., 1997; McBride et al., 1999). The total amount of time that a male was engaged in courtship activity while paired with an unanesthetized target female, either during a test period of 10 min or until successful copulation, was scored. The CI was calculated as the percentage of total observation time spent courting (Siegel and Hall, 1979).

Staining and Analysis of Mushroom Body Morphology

Brains from 0- to-2-day-old adults or 5-day-old adults were dissected, fixed, and stained as described in Dockendorff et al. (2002). Confocal microscopy was performed using a Leica Scanning laser confocal microscope. β-lobe fusions were analyzed and scored by obtaining optical stacks of the β-lobes as described by Michel et al. (2004).

Statistical Analyses

Courtship indices of tested males were subjected to arcsin square root transformations to approximate normal distributions (Sokal and Rolfe, 1995; van Swinderen and Hall, 1995; Villella and Hall, 1996; Joiner and Griffith, 1997). ANOVAs were performed on pairwise comparisons of arcsin transformed data to obtain critical p values. All statistics were performed using Statview 3.0, including χ^2 analysis of the binning of courtship, olfactory acuity, visual acuity, and locomotion assays.

Supplemental Data

Supplemental Data include four figures, a table, Supplemental Experimental Procedures, and Supplemental References and can be

found with this article online at http://www.neuron.org/cgi/content/full/45/5/753/DC1/.

Acknowledgments

Thanks to J.B. Jenkins, J. Hinchey, and S. Campbell for critical reading of the manuscript and scientific discussion. Thanks to R.J. Hagerman, L.C. Griffith, and M. Tranfaglia for helpful comments in addressing specific aspects of the manuscript. Thanks to L. Restifo for discussing results prior to publication and for helpful tips for the analysis of mushroom body morphology. Thanks to R.C. McBride, G.M. McBride, P.J. McBride, R.J. Choi, E. Carlin, S. Chiorean, R. Rudominer, and B. Schoenfeld for technical assistance. We thank the FRAXA Research Foundation for support of Y. Wang and partial support of S. Chiorean, as well as the gift of MPEP. This work was also supported in part by an NIH grant to T.A.J.

Received: July 21, 2004
Revised: December 7, 2004
Accepted: January 25, 2005
Published: March 2, 2005

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