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Using *Drosophila* as a tool to identify Pharmacological Therapies for Fragile X Syndrome

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Abstract

Despite obvious differences such as the ability to fly, the fruit fly *Drosophila melanogaster* is similar to humans at many different levels of complexity. Studies of development, cell growth and division, metabolism, and even cognition, have borne out these similarities. For example, *Drosophila* bearing mutations in the fly gene homologue of the known human disease Fragile X, are affected in fundamentally similar ways as affected humans. The ramification of this degree of similarity is that *Drosophila*, as a model organism, is a rich resource for learning about human cells, development and even human cognition and behavior. *Drosophila* has a short generation time of ten days, is cheap to propagate and maintain and has a vast array of genetic tools available to it; making *Drosophila* an extremely attractive organism for the study of human disease. Here, we summarize research from our lab and others using *Drosophila* to understand the human neurological disease, called Fragile X. We focus on the *Drosophila* model of fragile X, its characterization, and use as a tool to identify potential drugs for the treatment of Fragile X. Several clinical trials are in progress now that were motivated by this research.

Keywords

Disease Modeling; Fragile X; *Drosophila*; Pharmacological Treatments; Autism; Cognitive impairment

Fragile X

Fragile X syndrome (FXS) is the leading single gene cause of intellectual disability (ID) and autism [1]. Male patients with Fragile X typically have an IQ below 100 (with the average being close to 50), as well as memory, executive function and sleep deficits. They also have recognizable physical features such as large ears, an elongated face, a high-arched palate and macro-orchidism in post-pubertal males [2–5]. Co-morbid autism afflicts 25–67% of males

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with fragile X [1, 6, 7], with the severity of autism generally increasing with the severity of intellectual disability [6]. Far fewer fragile X females are diagnosed with autism. The autistic symptoms (communication, social skills, and repetitive behaviors) in Fragile X patients have historically worsened with age. As in autism, sleep problems are common in Fragile X patients. Examination of brain tissue from fragile X patients shows dendritic spine immaturity.

In most cases, the disease occurs when patients inherit an *FMR1* gene with an aberrant 5′ untranslated region (5′UTR) containing increases in the normal number of a CGG repeat sequence. The normal range of repeats is 5 to 30, but when the repeat length increases to above 200 a cellular response is triggered that increases local DNA methylation and histone modification changes, leading to transcriptional repression of the gene locus [5, 8]. The subsequent loss of the fragile X mental retardation protein, FMRP [9, 10] causes the disease symptoms.

Drosophila has a single highly conserved *FMR1* gene, called *dfmr1*, which is 35% identical and 60% similar to the human Fragile X gene, *FMR1* [11]. The majority of the gene sequence identity corresponds to known protein-protein interaction domains, and nucleotide-binding domains needed for FXS function. In addition, the developmental expression pattern of *dfmr1* is analogous to that of the mouse and human FMR1 proteins [11–13]. *Drosophila* mutants bearing either point mutations, or that lack all- or most of- the *dfmr1* gene-coding region lack detectable FMRP expression, providing the basis of a *Drosophila* fragile X model [13–16]. Studies using this *Drosophila* Fragile X model have uncovered behavioral, neuroanatomical and biochemical phenotypic similarities with human FXS patients (Table I) [17–19]. That this model is relevant to human FXS is underscored by the fact that pharmacological treatments that rescue *Drosophila* FXS phenotypes also rescue related mouse FXS phenotypes and more recently, human Fragile X patient symptoms [20–27] in clinical trials. We will now focus the rest of this review on our approaches and that of others to use the *Drosophila* model of FXS to identify potential treatments for Fragile X.

Social Defects in the Fragile X model

The *Drosophila* male is born with an innate ability to perform a stereotypic courtship ritual to entice a receptive female to mate. In courtship, male flies perform a characteristic sequence of behaviors (Figure 1) [28–30]. These behaviors are repeated with some variation until successful copulation occurs. This social behavior can be quantified by measuring the percentage of time a male engages in courtship activity during a 10-minute test interval. This percentage is referred to as the courtship index (CI) and gives a measure of overall courtship activity. The quality of courtship behavior can be measured by determining the percentage of time spent performing each of the steps in the courtship ritual (Figure 1).

The courtship index of naïve *dfmr1* mutant males paired with virgin females, was significantly reduced, compared to naive control males (Figure 2) [15]. More specifically, fragile X flies failed to sustain courtship, resulting in a lower percent of fragile X flies that progressed to later steps of courtship (genital licking and copulation). These results suggested a social deficit in the *Drosophila* model for fragile X [15].

Another behavior that can be monitored in *Drosophila*, is grooming behavior. This behavior is repetitive in nature, but brief; normally lasting for a few seconds. In contrast to wild type flies, *dfmr1* mutant *Drosophila* engage in excessively long time periods of grooming behavior, suggesting that these males are more prone to repetitive behaviors, as is the case in autistic patients [31].

Learning and memory in Drosophila

Several learning and memory paradigms have been developed in *Drosophila*. The two most popular are a classical avoidance conditioning paradigm (an associative memory paradigm also known as the odor-shock paradigm) and a conditioned courtship paradigm (an associative memory paradigm also referred to as the courtship conditioning paradigm).

In the odor-shock paradigm, *Drosophila* memory is measured by the rate at which flies learn to distinguish between an odor associated with an adverse event (electric shock to the foot [32–38]), and one associated with a neutral event (no shock). After a single training session, 0–2 minutes after training is referred to as immediate or immediate-recall memory and is measured behaviorally by the percentage of flies that move to the chamber lacking a shock. This form of memory had been previously referred to as learning, with the terminology changing in the mids 1990s, although to this day is still sometimes referred to as learning. Short-term memory is measured at 60 minutes after training and medium term memory 2–7 hours after training. There are two components of long-term memory, anesthesia resistant memory (ARM) and long-term memory (LTM). ARM lasts for up to 48 hours after training and is not dependent on de novo protein synthesis and is typically tested 1 day after massed training. LTM is de novo protein synthesis dependent, can last at least 8 days but is typically tested 1–4 days after spaced training (for review see Skoulakis EM, Grammenoudi S., 2006). Memory is tested after the delay interval by giving the flies a choice between the two odors, in a T-maze. The flies that have learned to pair the correct odor with the shock, choose the part of the T-maze with the other odor.

In the conditioned courtship paradigm, a male fly is paired for an hour with a previously-mated female (unreceptive female), and tested to see if the male remembers the female cues and suppresses his courtship behavior when subsequently paired with a virgin (receptive) female [28–30, 38–42]. This memory requires the male to pair complex female avoidance behaviors with associated sensory signals [43–49]. In this assay, learning-during-training (LDT) can be measured by comparing the CI during the first ten minutes, with the CI in the last ten-minutes of the pairing. This is sometimes simply referred to as learning, but is more related to working memory since it happens while the environmental stimulus is still present. Generally a 40% reduction or more in courtship activity is observed during the learning-during-training assay.

Memory is assessed in the next step of the paradigm. Males with typical memory will exhibit depressed levels of courtship behavior for 2–3 hours after the learning experience/training [40]. This is evaluated by comparing the behavior of the trained versus an untrained male, when each is paired with a receptive (virgin) female. A trained male with normal memory should show relatively depressed levels of courtship. A modified version of the conditioned courtship paradigm can be utilized to establish and measure long-term memory lasting out to 9 days after training [50].

dfmr1 mutants had learning and memory deficits by both the odor-shock and conditioned courtship memory paradigms, and in a fashion consistent with the cognitive deficits of patients with Fragile X syndrome. Specifically, *dfmr1* mutants had impairments in learning (immediate memory) with less choosing the shock-free chamber compared to control flies, and forgot what they learned within one day [39]. In the courtship-based assay *dfmr1* mutants showed both immediate-recall memory and short-term memory deficits. First, learning-during-training was initially normal at 5 days of age, but no longer detectable at 20 days of age, perhaps due to cognitive decline with age in the fragile X model [51]. Second, although learning-during-training was normal at 5 days of age, *dfmr1* mutants had immediate-recall memory deficits at 0–2 minutes after training (Figure 2), short-term memory deficits at 60 minutes after training, and long-term memory deficits after one day of

training. In *Drosophila,* each of these time points corresponds to a different, genetically separable, form of memory (immediate-recall, short-term memory, and long-term memory, respectively) [17, 52]. Finally, in addition to learning-during-training decaying with age, repetitive-type behaviors increased with age in *dfmr1* mutants: The percentage of time *dfmr1* mutants groomed themselves is elevated over controls, and increases with age [31].

Neuroanatomical defects in the Fragile X model

Despite significant symptoms, the brains of patients with Fragile X look quite healthy; however closer examination shows some localized size variation, in addition to reliable differences at the level of neurons [53]. The overall brain size and structure of the *dfmr1* mutant brain also appears normal, however more detailed analysis has identified consistent defects in select sets of neurons in the central and peripheral nervous systems. For example, examination of the neuromuscular junction in *dfmr1* mutant larvae, reveals an over elaboration of “bou-tons”; the sites of synapse formation [13] (Table I). Additional gross neuranatomical defects are also observed in the fruitfly: in *dfmr1 mutants the* mushroom bodies required for short- and long-term memory formation exhibit neuron based structures indicative of inappropriate midline crossing of neurons, compared to control flies [17, 54, 55] (Figure 3).

Drug treatments in the *Drosophila* Fragile X model

The *Drosophila* reproductive cycle is 10–14 days long; which makes orally-delivered drug testing quick and simple. We have successfully used our *Drosophila* model for Fragile X to identify drug candidates for the treatment of this disorder. These drugs are currently in different phases of clinical trials.

The appropriate balance of mGluR signaling pathways relative to GABA signaling pathways is required for maximum learning and memory in *Drosophila*, and in other mammals [17, 51] [56–65]. Our earlier studies indicated that a shift in this balance in favor of mGluR activity was causing the learning and memory defect in our model for Fragile X (see [17] for details). Furthermore results from cells from human patients indicated impaired cAMP signaling [57, 61] and mouse studies indicated that there was enhanced mGluR signaling in the hippocampus of the FXS model brain[66], motivating us to test the effect of decreasing mGluR signaling via pharmacological treatment[17].

The mammalian genomes contain eight different metabotropic glutamate receptors (mGluRs), which are subdivided into three groups (I, II and III), based on downstream signaling events. In contrast, the *Drosophila* genome contains a *single* mGluR called *DmGluRA*, which in neurons is connected to the *Drosophila* homologues of mammalian Group I and Group II mGluR receptor signaling pathways [17, 67–69]. We added several mGluR antagonists, and lithium, (which acts downstream of mGluR, but in the same pathways) to the fly food to reduce mGluR signaling and increase cAMP signaling [17]. The drug was added to the food at different time periods, including during the larval growth period (development), adulthood, or during both time periods. Flies were tested in adulthood for fragile X-related symptoms. Interestingly all drug treatments, but not vehicle containing food, rescued the naïve courtship (social interaction) phenotype, immediate-recall and short-term memory (Figure 4). In contrast, drug needed to be added during development to rescue the mushroom body phenotype of neurons [17]. These results demonstrated that inhibiting the mGluR pathway rescued relevant FXS phenotypes.

These studies were the first to indicate that drug treatment after the bulk of brain development could rescue a developmental brain disorder. These studies demonstrated that social and cognitive impairments were not set in stone by immutable developmental

circuitry, but that adulthood signaling was important in social behavior and memory and that modulating adulthood signaling could ameliorate social impairments. [70–76] Interestingly the administration of the specific group II antagonist LY341495 or lithium in full adult mice at eight weeks of age has recently been shown to reverse phenotypes in adult FXS mice [77, 78] as has treatment with the group I mGluR antagonist CTEP started soon after weening but before adulthood [78].

In another study, a relatively high-throughput screen has also been employed to identify drugs that rescue relevant *Drosophila* FXS mutant phenotypes. By taking advantage of the observation that elevated levels of glutamate in fly food is toxic to *dfmr1* mutants, Chang et al., 2008, performed a drug screen to identify compounds that rescued the lethality of *dfmr1* mutants. They screened the Spectrum collection of 2,000 FDA approved drugs collection and identified a few that not only rescued the lethality but also rescued the naïve courtship and mushroom body cross-over defects. Three of the identified compounds have the commonality in that they act to promote γ-aminobutyric acid (GABA) receptor activity [79]. Interestingly the identification of these compounds matches findings that there are deficiencies of GABA(A) receptor signaling in the *Drosophila* and mouse fragile X models [80].

Another compound that has efficacy in the fly fragile X model is the drug minocycline. This derivative of tetracycline was tested in the fly model as it was shown to rescue defects in the neuronal morphology displayed by the mouse FXS model [25]. In the fly study the effects of minocycline treatment were examined in three different classes of neurons: motor neurons, circadian neurons, and mushroom body neurons. In all three neurons the synaptic connectivity defects were rescued by minocycline treatment and the results were validated by genetic manipulations [24, 81]. Interestingly in the mouse, minocycline treatment has been shown to inhibit MMP9 activity, which is also inhibited by cAMP signaling [82, 83]. The overall data from both the fly and mouse models indicate that minocycline treatment should be examined as an approach to treat FXS symptoms.

Pharmacological treatments for the excessive grooming phenotype of the *dfmr1* mutants have also been identified. Unlike other phenotypes, such as naïve courtship, memory and the mushroom body defects, this phenotype was exacerbated by treatment with mGluR antagonists, but interestingly was rescued by treatment with reserpine. Basic research into the grooming behavior of *Drosophila* has demonstrated that the addition of monoamines dopamine, octopamine and serotonin to decapitated flies increases grooming behavior. Interestingly monoamine synthesis has been found to be elevated in *dfmr1* mutants [84] and over-expression of *Drosophila* vesicular monoamine transporter (VMAT) transporter that loads monoamines into synaptic vesicles also increases grooming behavior. Examination of *dfmr1* mutants revealed elevated levels of VMAT mRNA and protein [31]. Reserpine is a known antagonist of VMAT and treatment with this drug was found to suppress the excessive grooming behavior [31]. As excessive grooming is a phenotype displayed by the mouse FXS model, it clearly important to explore this therapeutic route in the mouse model. Also as reserpine has broad effects on monoamine transport more selective inhibitors of the specific monoamines should be explored in the fly and mouse models as a route to suppress excessive grooming behavior which might relate to the repetitive behaviors displayed by fragile X patients.

As discussed in this review, the development of a *Drosophila* fragile X model and its initial characterization has led to the realization that it displays several seemingly relevant phenotypes. The relevance of these phenotypes is highlighted by the fact that they have been useful in combination with basic research and with findings from studies using the mouse fragile X model to suggest routes to pursue for pharmacological testing in Fragile X patients.

However the final validation from any model comes from the ability of the model to guide the identification of treatments that have efficacy in human patients. The *Drosophila* model has reached this benchmark. Clinical trials, utilizing lithium, mGluR antagonists and minocycline have all indicated promising results, suggesting further focus on such compounds in full placebo controlled trials is warranted [20, 21, 85](Table I). The *Drosophila* model has provided initial data in the cases of lithium and mGluR antagonists and GABA agonists to pursue. It has also demonstrated great utility in how it can be used from hypothesis testing based on basic research findings to unbiased drug screening. Thus its utilization in the study of other human diseases affecting cognition and behavior should be considered.

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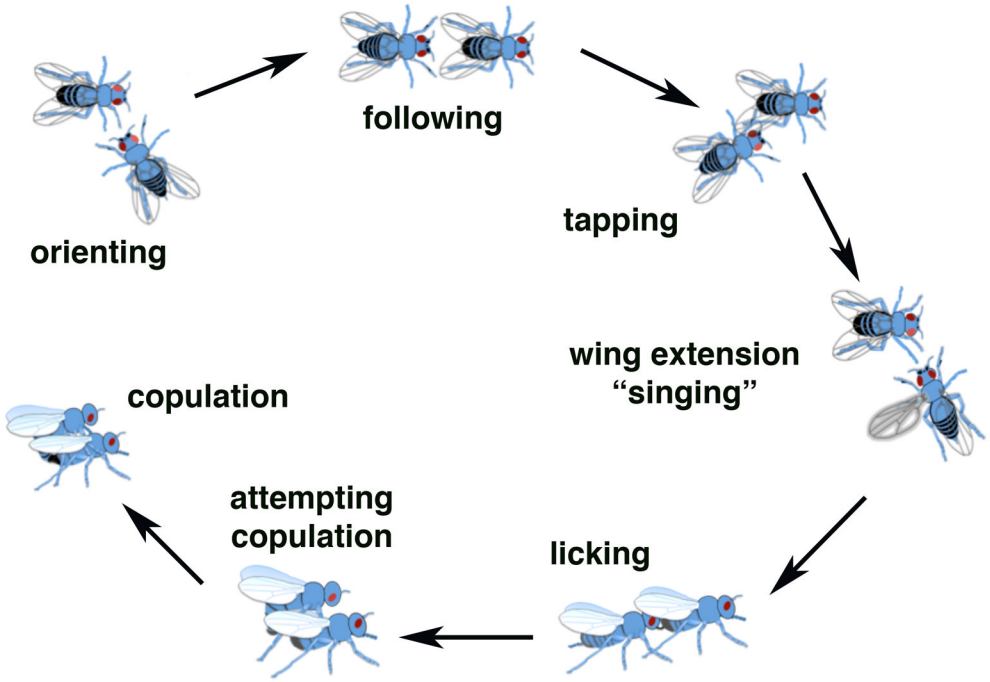

Figure 1. ***Drosophila*** **courtship behavior**

Adult males will perform an innate stereotypic behavior to entice receptive females to mate. This courtship ritual involves seven basic steps that are generally performed in the order presented in the figure. The courtship starts by the male orienting toward the female and following her. The male then taps the female to pick up pheromonal cues and then initiates "singing" by extending and vibrating a wing. The male then licks the female abdomen and if the female displays receptive behavior he will attempt and if successful initiate copulation.

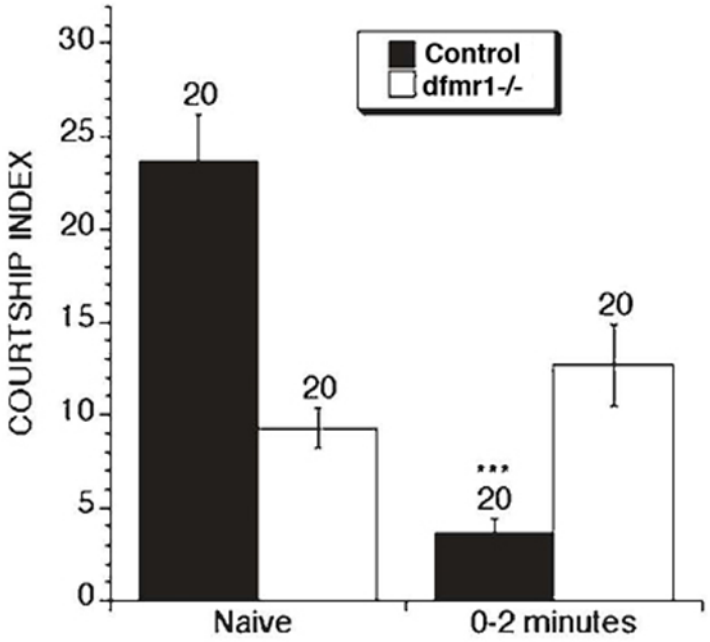

Figure 2. Naïve courtship and memory phenotypes displayed by the *Drosophila* fragile X model

Measuring the total amount of time a male courts in a 10 min test interval can quantitate the level of naïve courtship activity of a particular strain of fly. The total courtship time is divided by 10 mins to derive a courtship index (C.I.). *Dfmr1* mutant males (white bar) display reduced naïve courtship relative to controls (black bar). This deficit in courtship activity is not due to sensory or locomotor defects [15]. Learning can be tested in the courtship paradigm by placing a male in a courtship chamber with an unreceptive female. A normal male will learn not to court the female within a one-hour training session. *Dfmr1* mutants display a normal learning profile with respect to controls (not shown, see [17]). Once trained, males remember the negative experience of the training and fail to court even receptive females for up to three hours after training. The right two bars in this figure show immediate recall memory that is tested within 2 mins of training, by placing a freshly trained male with a receptive female. Control males displays significant reduction in courtship (indicated by asterisks, $p<0.001$), whereas no difference is observed between the level of naïve courtship and the level of courtship at 2 mins post-training displayed by the *dfmr1* mutants.

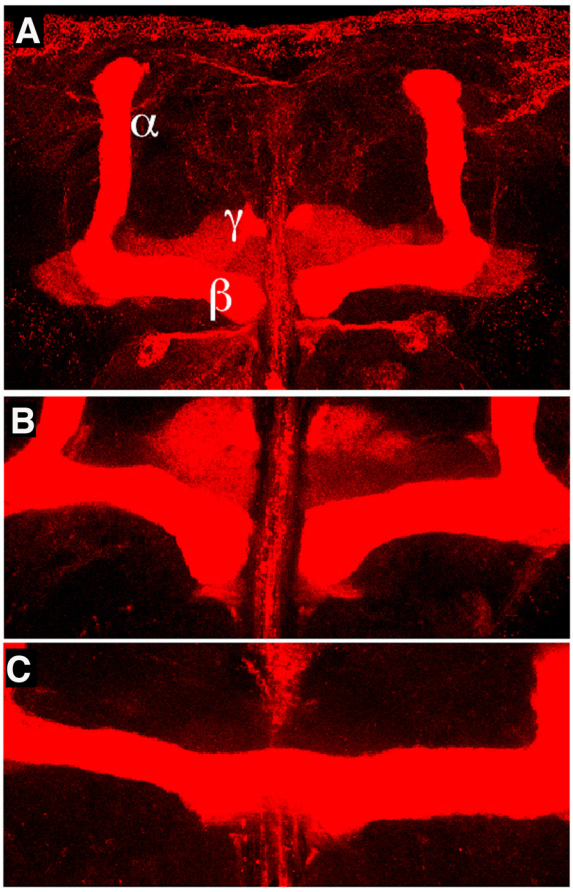

Figure 3. Mushroom body phenotype of *dfmr1* mutants

A) Whole mount immunostaining of a *Drosophila* brain with anti-fasicillin II reveals the mushroom body (MB) of the fly brain, which contains three bilaterally symmetric lobes, α, β, and γ. The MB is the major learning and memory center of the fly brain and is thought to be analogous to the vertebrate hippocampus. B) An image of the β–lobes of a control MB shows that the β–lobes grow toward, but do not cross the mid-line of the brain. C) An image of the β–lobes of a *dfmr1* mutant MB shows a severe cross-over phenotype that is observed in some of the mutant brains.

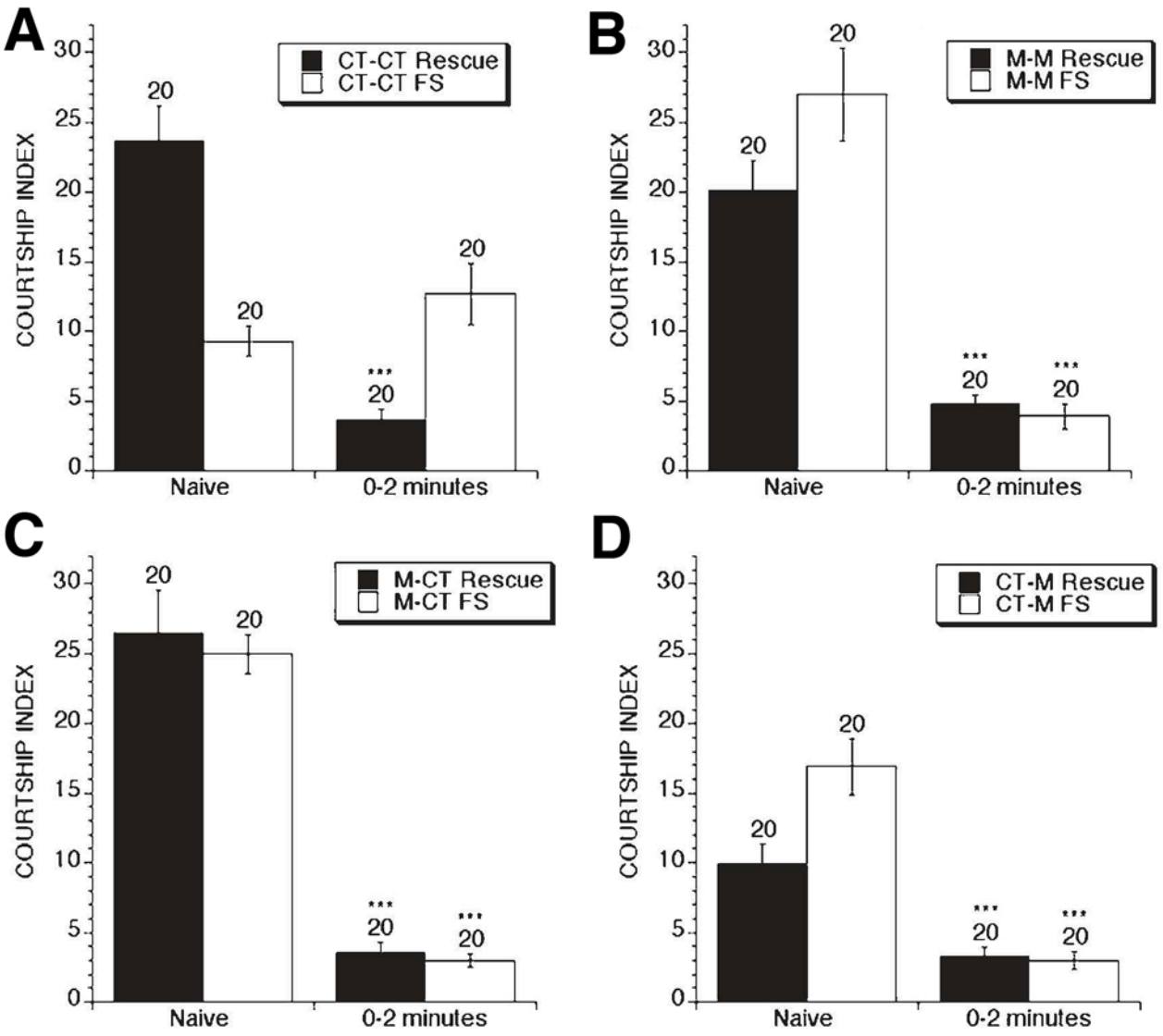

Figure 4. Rescue of naïve courtship and memory by treatment with the mGluR antagonist MPEP

Control (Rescue) and *dfmr1* mutants (FS) were: A) raised in control food during development and adults (CT-CT); B) raised in food containing the mGluR antagonist MPEP (drug name) during development and as adults before testing (M-M); C) raised in MPEP containing food during development and then put on control food as adults before testing (M-CT); D) raised on control food during development and then placed on MPEP containing food during adulthood before testing. A) Treatment with only control food reveals the same *dfmr1* phenotypes displayed in Figure 2, i.e. reduced naïve courtship and no detectable immediate recall memory. A–C) Treatment of the *dfmr1* mutants with MPEP containing food during development and adulthood, development alone or adulthood alone, leads to significant rescue of the naïve courtship and memory deficits, indicated with asterisks, $p<0.001$.

Table I

Dfmr1 mutant phenotypes and effective pharmacological treatments that rescue them.

Analysis	Phenotype	Drug Rescue	Reference
Neuronal Anatomy	**Central Neuron targeting defects** (mushroom body cross-over)	**mGluR antagonist** Lithium,MPEP,MPPG, MTPG and LY341495	[19]
		GABA agonist GABA, Creatinine and Nipecotic acid	[79]
		Muscarinic ACH antagonist Pilocarpine nitrate, Aminobenztropine	[79]
		Antibiotic Minocycline	[26]
	Peripheral Synaptic Defects (over-elaboration of NMJ structure)	**mGluR antagonist** MPEP and genetic reduction of DmGluRA	[71]
		Antibiotic Minocycline	[26]
	Central Synaptic Defects (Over-elaboration of sLNv neurons)	**Antibiotic** Minocycline	[26]
	Neurotransmitter- containing vesicle Defects (elevated presynaptic vesicle pool)	**mGluR antagonist** MPEP and genetic reduction of DmGluRA	[71]
Behavior	**Repetitive Behaviors** (Excessive grooming behavior)	**Neurotransmitter transport (into vesicles) antagonist** Reserpine	[35]
Behavior	**Social Behaviors** (naïve courtship)	**mGluR antagonist** Lithium, MPEP,MPPG, MTPG, LY341495	[19]
		GABA agonist GABA, Creatinine, Nipecotic acid	[79]
		Muscarinic ACH antagonist Pilocarpine nitrate and Aminobenztropine	[79]
Cognition	**2 minute memory** (*Drosophila* "Immediate recall memory")	**mGluR antagonist rescue** Lithium, MPEP, MPPG, MTPG. LY341495	[19]
	60 minute memory (*Drosophila* "short-term memory")	**mGluR antagonist** Lithium, MPEP, MPPG, MTPG, LY341495	[19]
	One day memory (*Drosophila* "long-term memory"-protein synthesis-dependent)	**mGluR antagonist** MPEP	[43]
	Age-dependent learning decline	**mGluR antagonist** Lithium, MPEP,MPPG, MTPG, LY341495	[19]