Neural circuit architecture defects in a *Drosophila* model of Fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase

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**SUMMARY**
Fragile X syndrome (FXS), caused by loss of the fragile X mental retardation 1 (*FMR1*) product (FMRP), is the most common cause of inherited intellectual disability and autism spectrum disorders. FXS patients suffer multiple behavioral symptoms, including hyperactivity, disrupted circadian cycles, and learning and memory deficits. Recently, a study in the mouse FXS model showed that the tetracycline derivative minocycline effectively remediates the disease state via a proposed matrix metalloproteinase (MMP) inhibition mechanism. Here, we use the well-characterized *Drosophila* FXS model to assess the effects of minocycline treatment on multiple neural circuit morphological defects and to investigate the MMP hypothesis. We first treat *Drosophila* Fmr1 (*dfmr1*) null animals with minocycline to assay the effects on mutant synaptic architecture in three disparate locations: the neuromuscular junction (NMJ), clock neurons in the circadian activity circuit and Kenyon cells in the mushroom body learning and memory center. We find that minocycline effectively restores normal synaptic structure in all three circuits, promising therapeutic potential for FXS treatment. We next tested the MMP hypothesis by assaying the effects of overexpressing the sole *Drosophila* tissue inhibitor of MMP (TIMP) in *dfmr1* null mutants. We find that TIMP overexpression effectively prevents defects in the NMJ synaptic architecture in *dfmr1* mutants. Moreover, co-removal of *dfmr1* similarly rescues TIMP overexpression phenotypes, including cellular tracheal defects and lethality. To further test the MMP hypothesis, we generated *dfmr1;mmp1* double null mutants. Null *mmp1* mutants are 100% lethal and display cellular tracheal defects, but co-removal of *dfmr1* allows adult viability and prevents tracheal defects. Conversely, co-removal of *mmp1* ameliorates the NMJ synaptic architecture defects in *dfmr1* null mutants, despite the lack of detectable difference in MMP1 expression or gelatinase activity between the single *dfmr1* mutants and controls. These results support minocycline as a promising potential FXS treatment and suggest that it might act via MMP inhibition. We conclude that FMRP and TIMP pathways interact in a reciprocal, bidirectional manner.

**INTRODUCTION**
Fragile X syndrome (FXS), the most common genetic determinant of cognitive impairment and autism spectrum disorders (Koukoui and Chaudhuri, 2007; Penagarikano et al., 2007), is caused solely by the loss of the fragile X mental retardation 1 (*FMR1*) gene product (FMRP) (Pieretti et al., 1991). FMRP is an mRNA-binding protein known to regulate mRNA stability, mRNA trafficking and the translation of a number of neuronal transcripts (Laggerbauer et al., 2001; Li et al., 2001; Lu et al., 2004; Muddashetty et al., 2007; Tessier and Broadie, 2008; Zhang et al., 2001). Clinically, FXS is a wide-spectrum disorder, with patients displaying hyperactivity, disrupted sleep patterns and mild to severe intellectual disability, among other behavioral impairments (Einfeld et al., 1991; Elia et al., 2000; Hagerman et al., 2010; Hagerman et al., 2009; Levenga et al., 2010; Penagarikano et al., 2007). At a cellular level, defects in cortical dendritic spine morphology have been observed in FXS patient brain autopsies (Hinton et al., 1991; Rudelli et al., 1985), suggesting immature synaptic connections. A great deal of investigation in FXS disease models supports the conclusion that FMRP plays a predominant role in the activity-dependent regulation of synaptic development and plasticity (Antar and Bassell, 2003; Auerbach and Bear, 2010; Costa-Mattioli et al., 2009; Huber et al., 2002; Pan et al., 2008; Tessier and Broadie, 2008; Tessier and Broadie, 2010; Waung and Huber, 2009; Zhang and Broadie, 2005). Given the high prevalence of this devastating neurological condition, pharmacological treatments for FXS have long been sought. Many studies support the metabotropic glutamate receptor (mGluR) theory of FXS, which suggests that enhanced mGluR signaling causes defects in synaptogenesis, dendritic spine maturation, and long-term depression (LTD) and potentiation (LTP) (Antar et al., 2004; Antar and Bassell, 2003; Auerbach and Bear, 2010; Bear, 2005; Bear et al., 2008; Bear et al., 2004; Dolen and Bear, 2008; Dolen et al., 2010; Dolen et al., 2007; Huber et al., 2002; Meredith et al., 2010; Pan et al., 2008; Pan et al., 2004; Penagarikano et al., 2007; Repicky and Broadie, 2009; Waung and Huber, 2009). These findings have made mGluRs the primary

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target of FXS drug discovery (Levenga et al., 2010). Specifically, mGluR inhibitors, such as 2-methyl-6-phenylethynyl-pyridine (MPEP), have proven to be effective at rescuing FXS defects in both *Drosophila* and mouse models of the disease (Bolduc et al., 2008; Choi et al., 2010; Dolen and Bear, 2008; Dolen et al., 2010; McBride et al., 2005; Pan et al., 2008). In *Drosophila*, MPEP rescues cognitive impairments, synaptic plasticity, courtship and mushroom body learning defects in *Drosophila fmr1* (*dfmr1*) null animals. Genetic studies of mGluR mutants strongly support the mechanism (Bear, 2005; Bear et al., 2008; Bear et al., 2004; Dolen and Bear, 2008; Dolen et al., 2010; Dolen et al., 2007; McBride et al., 2005; Pan and Broaddie, 2007; Pan et al., 2008; Repicky and Broadie, 2009). Although MPEP cannot be used clinically owing to its toxicity, next generation mGluR antagonists are in clinical trials (Levenga et al., 2010; Wang et al., 2010). Lithium, an inhibitor of GSK3β, which is a downstream effector of mGluR signaling, has also been taken into FXS clinical trials and shown to have beneficial effects on the disease state (Berry-Kravis et al., 2008). Thus, the mGluR pathway is certainly one promising avenue for FXS treatment.

In addition to mGluR inhibitors, a new possible FXS drug treatment is the tetracycline derivative minocycline, the potentially beneficial effects of which were recently revealed in a study involving a mouse model of FXS (Bilousova et al., 2009). Minocycline has previously been proven to be effective in treating a surprising range of neurological disorders, including multiple sclerosis, Huntington’s disease, Parkinson’s disease and Alzheimer’s disease (Choi et al., 2007; Kim and Suh, 2009; Popovic et al., 2002; Wang et al., 2003; Wu et al., 2002). In the mouse FXS model, minocycline promoted the maturation of hippocampal dendritic spines towards normal morphology, both in vitro and in vivo, and repressed anxiety and memory defects in an elevated plus-maze test (Bilousova et al., 2009). More recently, an open-label add-on minocycline trial of FXS patients reported a beneficial effect on five out of six measured behaviors and improved the score that was obtained on the ABC-C irritability scale (Paribello et al., 2010). Minocycline has several known targets, including, but not limited to, p38 MAPK, iNOS and caspases (Kim and Suh, 2009). However, the mouse FXS study suggested that minocycline functions specifically by inhibiting matrix metalloproteinase-9 (MMP9) (Bilousova et al., 2009). The 24 mammalian MMPs are a family of zinc-dependent extracellular proteases, which cleave membrane-associated and secreted proteins to remodel the extracellular matrix (Ethell and Ethell, 2007; Page-McCaw et al., 2007; Rivera et al., 2010). MMPs are involved in normal neuronal development, function and plasticity, and are also implicated in several neurological pathologies (Rivera et al., 2010). In *FMRI* knockout (KO) mouse hippocampus, MMP9 expression and activity were increased, and minocycline decreased this enhancement (Bilousova et al., 2009). MMP9 treatment of wild-type hippocampal neurons in culture produced dendritic spine profiles that were similar to *FMRI* KO neurons. Other MMPs (i.e. MMP2) were not detectably involved. Four endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) are known in mammals (Stetler-Stevenson, 2008).

In this study, we use the *Drosophila* FXS model to test the effects of minocycline on a broad range of neuronal architectural phenotypes and investigate the hypothesized MMP mechanism. We assay three neural cell types in *dfmr1* null mutants, including motor neurons, circadian clock neurons, and the learning and memory center neurons, and show in each case that minocycline treatment effectively restores synaptic connectivity architecture towards that of wild type. We next genetically mimic the proposed MMP inhibition effect of minocycline treatment with overexpression of the sole *Drosophila* TIMP in *dfmr1* null mutants (Page-McCaw et al., 2007; Page-McCaw et al., 2003). Consistent with the MMP hypothesis, we show that TIMP overexpression fully restores normal neuromuscular junction (NMJ) synaptic architecture in the *dfmr1* null mutant condition. Conversely, *dfmr1* removal similarly prevented TIMP overexpression phenotypes, including tracheal defects and lethality, suggesting that the TIMP and *Drosophila* FMRP (dFMRP) pathways are interdependent. In *Drosophila*, there are only two MMPs: secreted MMP1 and membrane-anchored MMP2 (Page-McCaw et al., 2007; Page-McCaw et al., 2003). *Drosophila* *mmp1* null mutants exhibited both the tracheal defects and lethality that had been shown to be dFMRP dependent. We therefore generated *dfmr1;mmp1* double null mutants and similarly found the same mutual repression of both classes of null mutant phenotypes, which is consistent with the TIMP overexpression analyses. We did not detect changes in MMP1 expression or enzymatic activity in *dfmr1* mutants compared with controls, but did demonstrate striking genetic interactions between dFMRFamide (dFRFamide) and MMP1. These data suggest that minocycline acts via MMP inhibition to alleviate FXS model defects, as previously reported (Bilousova et al., 2009), and provide the first direct proof for reciprocal, bidirectional interaction between the FMRP and TIMP molecular pathways.

**RESULTS**

Minocycline partially restores *dfmr1* null NMJ synapse morphology

The effects of dFMRP loss on NMJ architecture have been well documented (Coffee et al., 2010; Gatto and Broaddie, 2008; Zhang et al., 2001). The *dfmr1* null synapse displays an overgrowth defect with increased branch number, more mature synaptic boutons and an accumulation of developmentally arrested satellite (or mini) boutons (Beumer et al., 2002; Beumer et al., 1999; Coffee et al., 2010; Gatto and Broaddie, 2008). To explore the effect of minocycline treatment on these phenotypes, we assayed wandering third instar NMJs that were co-labeled with anti-horseradish-peroxidase (anti-HRP; to delineate the presynaptic terminal) and anti-discs-large (anti-DLG; to mark the postsynaptic compartment) (Fig. 1). The number of NMJ branches, defined as a process with ≥2 boutons, was significantly higher in the *dfmr1* null mutants compared with control (Fig. 1A). The number of mature type 1b boutons, defined as DLG-positive varicosities ≥2 μm in diameter, was likewise significantly increased in the *dfmr1* null synapse (Fig. 1A,C). The number of immature satellite boutons, defined as varicosities <2 μm in diameter and directly attached to a 1b bouton, was similarly elevated (Fig. 1B,D). We used these NMJ structural parameters as our first test of minocycline treatment effectiveness in the *Drosophila* FXS model.

We supplied minocycline by feeding at several dosages throughout development to both control and *dfmr1* null animals, and then assayed NMJ structure in the mature wandering third instar (Fig. 1). With treatment of 20 μM minocycline, the mature
synaptic bouton number in dfmr150M (see Zhang et al., 2001) null mutants was significantly restored towards control levels (control: 17.2±1.08, dfmr1: 31.4±1.71, P≤0.001; dfmr1 + minocycline: 24.9±2.20, n=12, P<0.05; Fig. 1A,C). Moreover, the accumulation of developmentally arrested satellite boutons at the dfmr150M null synapse was fully restored to control levels (control: 1.9±0.25, dfmr1: 4.1±0.39, P<0.001; dfmr1 + minocycline: 1.83±0.30, n=12), a highly significant effect (P=0.001; Fig. 1B,D). To ensure that this effect was not allele specific, we examined the effect of minocycline on an independent dfmr1 null allele (dfmr1F) (see Dockendorff et al., 2002). Minocycline treatment again significantly restored defects in both mature synaptic bouton number (dfmr1F: 26.2±1.41; dfmr1F + minocycline: 18.8±2.62; n=7; P<0.05) and satellite bouton accumulation (dfmr1F: 3.43±0.43; dfmr1F + minocycline: 1.36±0.52; n=7; P<0.05) towards control levels. For both phenotypes, lower dosages of minocycline were administered to determine dosage dependence. Clear evidence of a dose dependency was evident for both mature boutons [in dfmr1F nulls: 30.5±2.64 (5 μM), 29.7±1.04 (10 μM) and 24.9±2.20 (20 μM); n=12 each condition] and immature boutons [in dfmr1F nulls: 3.86±0.40 (2 μM), 2.85±0.33 (5 μM) and 1.83±0.30 (20 μM); n=12 each condition] (Fig. 1C,D). No greater restorative effects were observed in the dfmr1 null condition at minocycline levels >20 μM.

We conclude that minocycline can significantly reverse, in a dosage-dependent manner, the increased synaptic bouton number that characterizes the FXS model condition without detectably altering synaptic architecture in the wild-type control.

Minocycline ameliorates dfmr1 null mutant circadian clock circuit defects

FXS is associated with central synaptic dysfunction, and central synapses might respond differently to minocycline treatment compared with the peripheral NMJ. We therefore next wanted to test drug treatments in behaviorally relevant CNS circuits. One characteristic behavioral manifestation in FXS patients is disrupted sleep patterns and associated hyperactivity (Elia et al., 2000; Penagarikano et al., 2007). Similarly, dfmr1 null animals exhibit circadian arrhythmicity and hyperactivity during normal sleep periods (Bushey et al., 2009; Dockendorff et al., 2002; Gatto and Broadie, 2009a; Gatto and Broadie, 2009b). In the underlying circadian clock circuit, a crucial subset of clock neurons expressing the neuropeptide Pigment dispersing factor (PDF) show well-documented synaptic overgrowth defects in the dfmr1 null condition (Dockendorff et al., 2002; Gatto and Broadie, 2009a; Gatto and Broadie, 2009b; Inoue et al., 2002; Morales et al., 2002). Anti-PDF staining of these small ventrolateral (sLNv) clock neurons in dfmr1 null brains revealed an increased number of synaptic boutons, with overextension of their spatial distribution extending from the dorsal horn bifurcation point (Fig. 2). We employed these clock neuron synaptic architecture defects as the second test of minocycline treatment effectiveness in the Drosophila FXS model.

We fed animals minocycline throughout larval development and adulthood. The same optimal minocycline concentration (20 μM) was used as above for larval treatment. Adults eclosed on apple juice plates with yeast paste, both containing minocycline (1 mM). A higher concentration was used after eclosion because the drug is better tolerated in adults than in larvae. Staged animals were aged to 3 days post-eclosion (d3) with brain dissections conducted at zeitgeber time 2-4 (2-4 hours after lights on). Consistent with earlier reports (Gatto and Broadie, 2009b), dfmr150M null brains showed increased numbers of sLNv, clock neuron PDF-reactive synaptic boutons in dorsal protocerebral projections (45±4.4
Minocycline restores dfmr1 null mushroom body neuron defects

The FXS hallmark is cognitive dysfunction in learning and memory (Penagarikano et al., 2007; Wang et al., 2010). Therefore, we next examined the central brain mushroom body (MB), the well-established olfactory learning and memory center of the Drosophila brain (Margulies et al., 2005; Zars et al., 2000). The dfmr1<sup>50M</sup> null mutant manifests well-documented defects in the MB α-, β- and γ-lobes; these defects are characterized by axonal overgrowth of Kenyon cell neurons (Pan et al., 2004; Tessier and Broaddie, 2008) and have been best characterized in the MB γ-lobe neurons, which show increased axonal length and excess synaptic branching in the dfmr1 null brain compared with controls (Fig. 3). As the third and final test of minocycline treatment effectiveness in the Drosophila FXS model, we assayed architectural changes in these MB learning and memory center neurons.

We employed the mosaic analysis of repressible cell marker (MARCM) genetic clonal technique to examine control and mutant Kenyon cell neurons in an otherwise wild-type brain (Lee and Luo, 1999; Pan et al., 2004; Tessier and Broaddie, 2008). γ-lobe neurons were chosen for their relatively simple morphology and striking dfmr1<sup>50M</sup> phenotypes (Fig. 3A). Clones of single cells were produced to examine cell-autonomous phenotypes by heat-shocking control and mutant animals, both non-treated and treated, at 24 hours after egg laying, to cause appropriate mitotic recombination events. Null dfmr1<sup>50M</sup> mutant and control neurons in the adult MB γ-lobe were visualized using anti-GFP antibodies to the membrane-targeted GFP (Fig. 3A), providing clear measurements of neuronal
architecture. In young adult brains (0-4 hours post-eclosion), dfmr1null null Kenyon cell axonal length and branch number were both significantly increased (P<0.05) compared with control neurons. The total axonal length was nearly twice as great in the mutant cells (control: 79.38±6.57 μm vs dfmr1null: 122.42±8.11 μm; n≥5 for each genotype; Fig. 3B). Likewise, the number of synaptic branches was nearly twice as great in the mutant cells (control: 3.8±0.37 vs dfmr1null: 6.6±0.6; n≥5 for each genotype; Fig. 3C).

Using the same minocycline feeding protocol as above, we next assayed dfmr1null null mutant Kenyon cell neurons from treated versus non-treated animals. The neuronal architecture of minocycline-treated mutant neurons was significantly rescued compared with non-treated mutant neurons (Fig. 3A). Axonal length was decreased in treated mutant MB γ-lobe cells by ~50% (treated dfmr1null: 90.61±6.48; n≥5; P<0.05; Fig. 3B). By comparison, treated control neurons showed no significant difference in axonal length compared with non-treated controls (control + minocycline: 74.91±7.48 μm). Synaptic branching was also significantly decreased (P<0.01) by ~50% in minocycline-treated mutant neurons (number of branches per neuron in treated dfmr1null: 2.8±0.37; n≥5; Fig. 3C). Again, treated controls showed no difference from non-treated controls (number of branches per neuron in control + minocycline: 2.6±0.4). Thus, we conclude that neuronal over-elongation on a single neuron level in the learning and memory center of the Drosophila brain is rescued by minocycline administration. Overall, minocycline treatment proved highly effective in reversing synaptic defects in three radically different circuit locations: motor neurons in the neuromusculature, clock neurons in the circadian circuit and Kenyon cells in the MB circuit.

TIMP overexpression fully rescues dfmr1 null synaptic morphological defects

In the mouse FXS model, the effect of minocycline in suppressing synaptic defects was attributed to inhibition of MMP9 (Bilousova et al., 2009). The Drosophila FXS model provides a simple system to test this proposed mechanism: Drosophila has only two MMPs, compared with the 24 mammalian MMPs, and a single TIMP, compared with the four mammalian TIMPs (Page-McCaw, 2008). The sole Drosophila TIMP is a particularly useful tool because it functions as an endogenous MMP inhibitor that is directly analogous to the proposed exogenous MMP inhibitor activity of minocycline. Thus, if the MMP hypothesis is correct, TIMP overexpression should phenocopy minocycline treatment. A UAS-TIMP transgene (Glasheen et al., 2009; Page-McCaw et al., 2003) was recombined onto the dfmr1null null chromosome, and then driven with ubiquitous UH1-Gal4 that was similarly recombined onto the dfmr1null null chromosome. NMJ synapse phenotypes were compared between the control (dfmr1null null mutants with the UH1-Gal4 driver alone; UH1-Gal4, dfmr1null) and the experimental line of ubiquitously driven TIMP in the homozygous dfmr1null null mutant (UH1-Gal4,dfmr1null/UAS-TIMP,dfmr1null) (Fig. 4).

As shown in Fig. 4A,B, the wandering third instar NMJ was co-labeled with anti-HRP (presynaptic, red) and anti-DLG (postsynaptic, green) to visualize synaptic architecture. A representative comparison is shown for the UH1-Gal4 driver alone in the dfmr1null null control and the TIMP overexpression condition in the dfmr1null null. Mutant overgrowth and over-elongation defects were confirmed in the driver control genetic background, including increased arbor branch number (Fig. 4C), increased mature type 1b bouton number (Fig. 4D) and increased immature satellite bouton number (Fig. 4E). By contrast, TIMP overexpression (dfmr1null null mutants with the driver alone exhibited 2.95±0.21 branches, compared with 2.27±0.23 in the TIMP overexpression dfmr1null nulls, a significant change back to control levels (n=11; P<0.05; Fig. 4C). Moreover, the number of mature synaptic boutons was significantly restored towards normal by TIMP overexpression (dfmr1null null control: 28.3±1.35 vs TIMP overexpression dfmr1null null: 20.7±1.57; n=11; P<0.01; Fig. 4D). Finally, the developmentally arrested satellite bouton number was also significantly reduced by TIMP overexpression (dfmr1null null control: 4.08±0.59 vs TIMP overexpression dfmr1null null: 2.5±0.50; n=11; P<0.001; Figs 4B,E). Thus, TIMP overexpression prevents all dfmr1null null defects in NMJ synaptic development, mimicking minocycline effects, although the genetic intervention is more effective than the drug treatment (compare Figs 1 and 4). These results support the conclusion that minocycline is acting through an MMP inhibitory mechanism to rectify dfmr1 synaptic defects.

dFMRP removal reciprocally suppresses TIMP overexpression phenotypes

We noted in the above analyses that TIMP-overexpressing dfmr1null null animals were robust, healthy and fully viable. This came as a surprise because TIMP overexpression alone causes larvae to appear thin and sickly and results in 100% penetrant larval-pupal lethality (Glasheen et al., 2009; Page-McCaw et al., 2003). It therefore seemed that removal of dFMRP was suppressing the
negative consequences of TIMP overexpression. We tested this observation by examining viability and cellular phenotypes in four genotypes: the driver-only control (UH1-Gal4/+), the ubiquitous TIMP overexpression condition (UH1-Gal4/UAS-TIMP), TIMP overexpression in the dfmr150M heterozygous background (UH1-Gal4, dfmr1/UAS-TIMP, dfmr1) and TIMP overexpression in the dfmr150M homozygous background (UH1-Gal4, dfmr1/UAS-TIMP, dfmr1, dfmr1) (Fig. 5).

We first quantified the viability of these four genotypes (Fig. 5A). Control and experimental crosses were set to lay at 25°C at the same time and were analyzed for viability at two time intervals: (1) from embryo (day 0) to pupation (day 5); and (2) from pupation (day 5) to adulthood (day 10). TIMP overexpression caused reduced viability during the first phase (embryonic–larval development) and complete lethality during the second phase (pupal to adult development). Control animals showed 60% survival to pupation, whereas TIMP overexpression animals showed only 26% survival. Removal of dFMRP restored TIMP overexpression viability back to >50%. During pupation, nearly all control pupae eclosed to viable adults, whereas no adults overexpressing TIMP emerged (control: 95.2±2.2% viable vs TIMP overexpression: 0.2±0.2%; n=9 trials; P<0.001) (Fig. 5A). The dfmr150M heterozygote in the TIMP overexpression background only slightly ameliorated the lethal phenotype, although a few adults of this genotype did eclose (Fig. 5A). By sharp contrast, remarkable rescue of the TIMP overexpression lethality occurred with complete dFMRP loss: most pupae eclosed to adulthood similar to the controls (TIMP overexpression dfmr1 null: 77.8±11.8%; n=9 trials), a highly significant improvement (P<0.001; Fig. 5A). Thus, we conclude that...
removal of dFMRP can nearly completely restore the viability of TIMP-overexpressing animals.

TIMP overexpression is also known to cause tracheal stretching and deformation, usually resulting in dorsal tracheal breaks (Glasheen et al., 2009; Glasheen et al., 2010; Page-McCaw et al., 2003). Importantly, these tracheal defects phenocopy the mmp1 null mutant, but not the mmp2 mutant, suggesting that MMP1 is specifically involved in this mechanism. We therefore next tested the effects of dFMRP loss on these tracheal TIMP overexpression phenotypes (Fig. 5B). Animals overexpressing TIMP exhibited clear tracheal deformation, with these animals exhibiting 1.43±0.48 breaks per dorsal trachea ($n \geq 7$) and with ~85% of trachea exhibiting at least one prominent tracheal break. By contrast, control animals (UH1-Gal4/+ never showed any detectable breaks in the dorsal trachea (0 breaks; $n \geq 7$). Removal of one copy of $dfmr1^{50M}$ provided no significant change in the TIMP overexpression phenotype: these larvae had 1.14±0.34 breaks per dorsal trachea (UH1-Gal4/UAS-TIMP). By contrast, total dFMRP loss (homozygous $dfmr1^{50M}$) completely prevented TIMP overexpression tracheal defects (Fig. 5B). Animals overexpressing TIMP in the $dfmr1^{50M}$ homozygous null background never showed any detectable breaks in the dorsal trachea (0 breaks; $n \geq 7$). Thus, removal of dFMRP can compensate for TIMP overexpression defects at both the cellular level and at the level of whole animal viability. We conclude that TIMP and dFMRP must reciprocally regulate each other in overlapping pathways.

**dFMRP removal similarly prevents mmp1 null phenotypes**

In *Drosophila*, there are only two MMPs: secreted MMP1 and membrane-anchored MMP2 (Page-McCaw et al., 2007; Page-McCaw et al., 2003). *Drosophila mmp1* mutants exhibit both tracheal defects and lethality that mimic the TIMP overexpression condition. We therefore next generated $dfmr1/mmp1$ double null mutants (Fig. 6). We confirmed the genotype with western blots for dFMRP and MMP1, showing both proteins to be completely absent in the analyzed condition (Fig. 6A). As it did in TIMP overexpression animals, dFMRP removal rescued adult viability in mmp1 mutants, which alone showed 100% penetrant lethality (Fig. 6B). Null mmp1 mutants had reduced viability during larval development compared with controls ($w^{118}$: 60%; mmp1: 17%). By sharp contrast, double mutants were almost as viable as controls, with >50% during larval survival to pupation. During pupation, the remnant mmp1 mutants all died, whereas control animals almost all survived into adulthood ($w^{118}$: 94.3±3.32% viable vs mmp1: 33.3±0.29% viable; $n=8$ independent trials; $P<0.001$; Fig. 6B). Similar to TIMP overexpression in the $dfmr1^{50M}$ null background, the majority of $dfmr1/mmp1$ double null animals survived through pupation and eclosed at levels much more comparable to the control than the mmp1 single mutant ($dfmr1/mmp1$: 77.6±9.23% viable; $n=8$ trials; $P<0.001$; Fig. 6B). Thus, we conclude that co-removal of dFMRP restores viability of mmp1 null mutants, similar to the TIMP overexpression result.

We next tested whether removal of dFMRP could similarly restore mmp1 tracheal deformation and breakage phenotypes. In mmp1 nulls, ~75% of dorsal trachea exhibit gross deformation with an obvious tracheal break (Fig. 6C). On average, mmp1 mutants had 1.17±0.27 breaks per animal ($n \geq 7$), whereas control animals exhibited no detectable tracheal deformations or breaks (compare far left and middle panels in Fig. 6C). Upon examination of $dfmr1/mmp1$ double mutants, we found remarkable rescue of the dorsal tracheal deformation and breakage that characterizes mmp1 mutants, with double mutants never showing any detectable breaks in the dorsal trachea (0 breaks; $n \geq 7$; Fig. 6C). We conclude that dFMRP removal totally suppresses the tracheal deformation and breakage phenotypes of the mmp1 mutant condition. Thus, dFMRP removal prevents both the developmental lethality and tracheal cellular defects otherwise caused by loss of MMP1, indicating a clear interaction between dFMRP and MMP1.
MMP1 removal rescues dfmr1 null synaptic morphological defects

Western blots show that, in the hippocampus of FMR1 KO mice (P7), active MMP9 expression levels were increased compared with controls, and MMP9 gelatinase activity was increased, as measured by gel zymography (Bilousova et al., 2009). Upon minocycline treatment, MMP9 expression and activity were both reduced in the FMR1 KO mice compared with controls. Additionally, MMP9 treatment of wild-type hippocampal neurons resulted in hippocampal dendritic spine profiles similar to FMR1 KO spines. Thus, we wanted to first assay for biochemical differences in MMP1 expression and activity in the dfmr1 null nervous system. We first examined MMP1 expression in the nervous system by western blot using anti-MMP1 antibodies for the catalytic domain (Glasheen et al., 2009). We found clear expression of MMP1 in the nervous system, but no detectable difference between control, dfmr150M heterozygous and dfmr150M null brains (supplementary material Fig. S1). Likewise, MMP1 immunolabeling indicated the clear presence of secreted MMP1 in the extracellular space surrounding NMJ synaptic boutons (supplementary material Fig. S2B), but no detectable change in expression in controls and dfmr150M nulls upon quantification (supplementary material Fig. S2C). As a measure of gelatinase activity, we then employed a recently devised in situ technique using a fluorescein-conjugated DQ-gelatin that fluoresces when cleaved (Bajenaru et al., 2010; Vidal et al., 2010). We found clear indication of MMP-dependent enzymatic function at the NMJ that was markedly decreased upon TIMP overexpression (supplementary material Fig. S3A) and colocalized with MMP1 protein (compare to supplementary material Fig. S2B). We did not detect any obvious difference in gelatinase activity levels between control and dfmr150M null synapses (supplementary material Fig. S3B). The enzymatic readout of fluorescence intensity quantification was not significantly different between genotypes (supplementary material Fig. S3C). Thus, we conclude that MMP1 is appropriately positioned to interact with dFMRP at the synapse, but we failed to detect changes in MMP1 expression or activity in the dfmr1 null condition.

With clear evidence of MMP1 at the NMJ, we next employed our genetic double mutants to test the effect of MMP1 removal on dfmr1 null synapse architecture defects (Fig. 7). Representative NMJ images show w1118 controls, dfmr150M null mutants and dfmr150M;mp1Q1112 double mutants co-stained with anti-HRP (red, presynaptic) and anti-DLG (green, postsynaptic) (Fig. 7A). We again found increases in synaptic bouton number (control: 15.69±0.87; dfmr1: 28.55±1.29; n≥21; P<0.001) and branch number (control: 2.02±0.14; dfmr1: 3.11±0.22; n≥21; P<0.001) in the dfmr150M mutant condition. In double mutants, synaptic bouton number was strongly rescued back towards control levels by MMP1 removal (dfmr1;mp1Q1112: 19.3±1.3; n=15 animals; Fig. 7B), a highly significant effect (P<0.001). The level of reduction in the double mutant was comparable to rescue provided by TIMP overexpression. MMP1 removal also prevented the increased number of synaptic branches that characterizes the dfmr1 null condition (dfmr1;mp1Q1112: 2.38±0.20; n=17; P<0.05). Thus, we conclude that removal of MMP1 ameliorates dfmr1 null synaptic defects, in a manner similar to both minocycline treatment and TIMP overexpression. These data show that MMP1 and dFMRP pathways interact at the synapse and provide support for the hypothesis that MMP inhibition might be the mechanism of minocycline action in the FXS disease state.

**DISCUSSION**

A recent report showed that minocycline treatment caused significant improvements in the mouse FXS model (Bilousova et al., 2009). To extend this foundation and to test the proposed mechanism, we conducted a follow-up minocycline trial in our Drosophila FXS model, examining different peripheral nervous system (PNS) and CNS neural circuits. We report here that minocycline treatment prevents both structural over-elaboration and synaptic developmental defects caused by dFMRP loss in a wide range of circuits, extending the results of the mouse study. Our
previous work, among others, has characterized dfmr1 null synaptic architecture defects in motor neurons at the NMJ (Gatto and Broadie, 2008; Repicky and Broadie, 2009; Zhang et al., 2001), in clock neurons of the circadian activity circuit (Gatto and Broadie, 2009b), and in Kenyon cells of the mushroom body learning and memory circuit (Pan et al., 2004; Tessier and Broadie, 2008). All of these defects were responsive to some degree to minocycline treatment. Interestingly, CNS circuit defects were more completely rescued compared with the peripheral NMJ, although NMJ defects were prevented in a dosage-dependent manner. Surprisingly, in all three circuits, wild-type neuron morphology was not detectably affected by minocycline treatment, showing that loss of dFMRP sensitizes the cell structure to minocycline activity. By contrast, mouse studies have shown that minocycline alters wild-type neuron architecture (Bilousova et al., 2009), suggesting that a difference must be present between Drosophila and mammalian systems. We conclude that the Drosophila FXS model supports the mouse FXS model in suggesting that minocycline should be an effective treatment for FXS patients.

Prior to current clinical trials of mGluR inhibitors, pilot studies were conducted in both Drosophila and mouse FXS models on lithium and MPEP, among other drugs (Berry-Kravis et al., 2008; Choi et al., 2010; Levena et al., 2010; McBride et al., 2005; Meredith et al., 2010; Pan et al., 2008). Similarly, a very recent open-label add-on minocycline FXS treatment trial has been performed (Paribello et al., 2010). Encouragingly, the 19 FXS patients that completed the study showed substantial improvement in four out of five measures on the Aberrant Behavior Checklist-Community (ABC-C) scales and a similar gain on the Clinical Global Improvement (CGI) scale. The trial reported a wide variety of FXS symptoms being improved by minocycline treatment, including irritability, stereotypy, hyperactivity and inappropriate speech subscales (Paribello et al., 2010). Even though this trial was not double-blind or placebo-controlled, and was done on a small scale, these preliminary findings are highly promising. The widespread behavioral improvement in this trial is echoed in the present study by the rescue upon minocycline treatment of three very different neural circuits that function in locomotion, circadian activity and cognitive learning and memory. Another recent study showed that the most common side effect of minocycline treatment on FXS patients was limited to gastrointestinal problems, including loss of appetite (Utari et al., 2010). Taken together, these data support moving forward to full clinical trials of minocycline for FXS patients was limited to gastrointestinal problems, including loss of appetite. The fact that minocycline has long been an established drug should greatly facilitate its development as a new FXS treatment.

Minocycline has been proposed to function as an MMP inhibitor in alleviating defects in the mouse FXS model (Bilousova et al., 2009). In FMR1 KO hippocampal lysates, both the active form of MMP9 and its gelatinase activity were reportedly increased, and minocycline reduced MMP9 expression and, to a lesser extent, gelatinase activity. Additionally, MMP9 treatment of wild-type cultured hippocampal neurons caused dendritic spine defects resembling FMR1 KO spines, mimicking a state of enhanced mGluR signaling and increased LTD, consistent with the mGluR theory of FXS pathogenesis (Bilousova et al., 2009). It was therefore postulated that enhanced mGluR signaling could be affecting increased MMP expression and/or activity. To test this hypothesis of MMP inhibition, we genetically overexpressed the sole Drosophila TIMP in a dfmr1 null animal, a direct genetic means to inhibit MMP activity. The accessibility of the particularly well-characterized NMJ made it ideal for these mechanistic studies. Consistent with the hypothesis, we show complete prevention of dfmr1 null defects in synaptic development. Moreover, to our astonishment, we found reciprocal suppression of TIMP overexpression phenotypes with regards to both cellular defects and loss of viability. These data suggest that MMP inhibition might be responsible for the alleviative effects of minocycline on FXS defects. We further tested genetic double mutants that were null for both mmp1 and dfmr1. Consistent with the TIMP overexpression studies, co-removal of dFMRP suppressed both the developmental lethality and cellular defects of mmp1 mutants. Although no detectable change was observed in MMP1 expression or enzymatic activity at the dfmr1 null NMJ, co-removal of mmp1 significantly rescued dfmr1 synaptic architecture defects in a manner phenocopied by minocycline treatment. This independently suggests that MMP1 inhibition might be the minocycline mechanism of action in the Drosophila FXS model.

The general reciprocal suppression of dfmr1 null with TIMP-overexpression and mmp1 null phenotypes suggests a strong relationship between these pathways. The obvious question is to determine how the two pathways interact, whether through direct or indirect means, or at the level of mRNA or protein. The more plausible explanation is probably indirect interaction, perhaps in an mGluR-dependent mechanism at the synapse. In the mGluR theory, receptor signaling drives elevated mRNA translation regulated by FMRP, which in turn controls levels of synaptic glutamate receptors (GluRs) mediating LTD and LTP synaptic plasticity (Bear et al., 2004). Mammalian MMP9 is secreted in response to GluR activation to mediate MMP-dependent LTD and LTP synaptic plasticity (Bear et al., 2004). Mammalian MMP9 is secreted in response to GluR activation to mediate MMP-dependent LTD and LTP synaptic plasticity (Michaluk and Kaczmarek, 2007; Nagy et al., 2006; Tian et al., 2007). Like the Drosophila MMP1 expression and activity reported here at the glutamatergic NMJ, MMP9 is localized to mammalian glutamatergic synapses (Gawlik et al., 2009; Konopacki et al., 2007), although the mechanism of MMP9 action in plasticity is still unclear. However, MMP synaptic localization and involvement with mGluR-regulated synaptic events provide a common foundation with FMRP function, from which mechanistic studies can be launched. Potentially, FMRP could control many steps in the regulation of MMP release, localization or activity regulation, and this control would not necessarily be reflected in gross changes in MMP expression or even basal enzymatic activity, consistent with the biochemical data in this study. MMPs could reciprocally ameliorate FMRP activity by directly or indirectly modulating mGluR signaling. Although the ‘mGluR theory’ provides a possible explanation for the interaction between MMP and FMRP at the synapse, the suppression of non-neuronal MMP phenotypes suggests that there also might be other ways in which these two pathways collide.

In closing, we stress that these studies provide strong correlation between the effects of minocycline treatment, TIMP overexpression and MMP1 loss of function on the Drosophila FXS model, but a causal link in a common pathway has yet to be proven. Beyond MMPs, minocycline could also be affecting other...
targets to exert its effects. For example, minocycline is a known p38 MAPK inhibitor (Kim and Suh, 2009). p38 MAPK has been implicated in mGluR-dependent LTD, which requires activation of p38 MAPK and protein tyrosine phosphatases in the CA1 region of the adult rat hippocampus (Moult et al., 2008; Pi et al., 2004). The mGluR theory of FXS would allow for increased p38 MAPK activation and increased tyrosine phosphatase activity to cause enhanced LTD, for example, which could be alleviated by minocycline inhibition. TIMPs also regulate this pathway. Both mammalian TIMP-1 and TIMP-2 have been implicated in causing increases in tyrosine kinase activity, the antagonist to the second requirement for p38-dependent enhanced LTD (Yamashita et al., 1996). Thus, the reciprocal suppression of phenotypes presented here could also support this type of model for minocycline activity. Additionally, in both amyotrophic lateral sclerosis and Huntington's disease models, minocycline has been found to be effective by inhibiting caspases and iNOS (Chen et al., 2000; Zhu et al., 2002). Thus, these two targets could also be viable mechanisms for minocycline action. Future studies will focus on investigating the links between minocycline activity and the TIMP pathway in the modulation of dFMRP requirements at the synapse and in other tissues.

METHODS

**Drosophila genetics**

All stocks were maintained at 25°C on standard medium in a 12:12 hour light-dark cycling humidified incubator. The genetic background strain was w^1118. Two dfmr1 null allele stocks were used: dfmr1^{1GM}/TM6GFP and dfmr1^{1T}/TM6c (Zhang et al., 2001; Dockendorff et al., 2002). For MARCM analyses, the control was obtained by crossing heatshock-FLP, mCD8-GFP; FRT82B, Tabulin P-Gal80; Gal4-OK107 with y, w; FRT82B, and the null mutant condition was obtained by crossing with FRT82B, dfmr1^{1GM}/TM6GFP. Recombinant lines were generated with UAS-TIMP (Glasehen et al., 2009; Page-McCaw et al., 2003), with UH1-Gal4 introduced into the dfmr1^{1GM} null background using standard genetic techniques. G418 resistance and anti-dFMRP immunoblotting were used to confirm the dfmr1^{1GM} null background. For synaptic structure analyses on the recombinant animals, the negative control was obtained by crossing UH1-Gal4, dfmr1^{1GM}/TM6GFP with dfmr1^{1GM}/TM6GFP, and the experimental animals (kept strictly at 25°C) were obtained through crossing UH1-Gal4, dfmr1^{1GM}/TM6GFP with UAS-TIMP, dfmr1^{1GM}/TM6GFP. Double mutants were generated from mmp1^{Q12} null and dfmr1^{1GM} null stocks using standard genetic techniques (Glasehen et al., 2009; Page-McCaw et al., 2003). Genotype was confirmed by western blots for dFMRP and MMP1 (Fig. 6A).

**Minocycline administration**

Minocycline (Sigma-Aldrich, St Louis, MO) was made as a 10 mM stock solution stored at 4°C. Genetic control and dfmr1 null strains were maintained on apple juice agar plates with yeast paste in which minocycline had been diluted at the specified concentrations in both the agar and the yeast paste. Apple juice plates with minocycline were replaced every other day throughout development with yeast paste containing minocycline made fresh each time. The same larval administration protocol was used for NMJ, clock neuron and MARCM assays. Adults were allowed to eclose on fresh apple juice agar plates with yeast paste that both contained 1 mM minocycline.

**Viability assays**

*Drosophila* crosses were maintained on apple juice agar plates with yeast paste from egg laying (embryo) through adult eclosion, at 25°C. Egg number was counted on the first laying day (day 0) and the number of animals counted on each subsequent day. To determine viability until pupation (% viability = [(pupae)/embryos] × 100), the number of animals alive on day 5 was used for calculations. For viability until adulthood (% viability = [(adults)/(pupae)] × 100), the number of adults on day 10 was used.

**Immunocytochemistry**

Antibody labeling was performed on third instar larval and adult brains as described previously (Coffee et al., 2010; Gatto and Broadie, 2008; Gatto and Broadie, 2009b; Tessier and Broadie, 2008). Briefly, samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) for 40 minutes. Preparations were rinsed with 3× PBS, and then blocked and permeabilized with 1% bovine serum albumin (BSA) and 0.5% normal goat serum (NGS) in 0.2% Triton X-100 in PBS (PBST) for 1 hour at room temperature. Primary and secondary antibodies were diluted in PBST with 0.2% BSA and 0.1% NGS, and incubated overnight at 4°C and for 2 hours at room temperature, respectively. Primary antibodies used included: anti-DLG [1:200; 4F3 monoclonal, Developmental Studies Hybridoma Bank (DSHB)], anti-HRP (1:250; rabbit, Sigma), anti-PDF (1:5; C7 monoclonal, DSHB), anti-GFP (1:500; clone 6662, HYBRIDOMA BANK [DSHB]), anti-mouse IgG (all from Invitrogen-Molecular Probes). Preparations were mounted in FluoroMount G (EMS, Hatfield, PA) and fluorescent images collected using a ZEISS LSM 510 META laser scanning confocal microscope. ImageJ (http://rsb.info.nih.gov/ij/) was used for fluorescence intensity quantifications.

**NMJ structure analyses**

NMJs from wandering third instar larvae were quantified for synaptic structure as previously described (Coffee et al., 2010; Gatto and Broadie, 2008). Briefly, the muscle 4 NMJ of abdominal segment 3 was used for all quantification. Values were determined for both left and right hemisegments and averaged for each animal (n=1). A synaptic branch was defined as an axonal projection with at least two synaptic boutons. Two populations of synaptic boutons were defined for quantification: (1) type Ib (>2 μm diameter) and (2) satellite or mini (≤2 μm diameter and directly attached to a type Ib bouton). Each class of bouton is reported as number per terminal.

**PDF clock neuron analyses**

slN, clock neuron structure was quantified as described previously (Coffee et al., 2010; Gatto and Broadie, 2009b). Briefly, a PDF-
adapted Sholl array analysis was employed using a series of 10-μm-spaced concentric rings centered on the dorsal horn bifurcation of the sLN, terminal arbor and extending for 100 μm. Assays were conducted at zeitgeber time 2-4. Both total number of PDF-positive synaptic boutons (≥1 μm in diameter) and the number of boutons within each concentric ring throughout the arbor were counted. Values were determined for each arbor in both left and right hemispheres of the brain and averaged for each animal (n=1).

MARC M analyses
Single-cell MARCM clones were generated and quantified as previously described (Lee and Luo, 1999; Pan et al., 2004; Tessier and Broadie, 2008). Briefly, to generate Kenyon cell clones in the MB γ-lobe, embryos were laid for a 4 hour period at 25°C and then heat-shocked at 24 hours at 37°C for 1 hour. All quantification was done on single-cell γ-neuron MARCM clones. The axonal length and branch number parameters were determined with LSM software on 3D confocal z-stacks for each individual neuron. For γ-neuron structural quantification, the primary axonal branch was identified first, with all other processes extending from the primary axon counted as branches. Axonal length was determined based on the length of the primary axonal process.

Tracheal analysis
Trachea were imaged in living animals using a Nikon 90i upright compound microscope connected to a Photometrics CoolSNAP HQ2 camera. Breaks in the dorsal trachea were counted per animal for each assayed genotype.

Western immunoblotting
Western blots were performed as previously described (Coffee et al., 2010; Tessier and Broadie, 2008). Briefly, five brains were dissected per genotype and placed in 1× NuPage (Invitrogen, Carlsbad, CA) sample buffer with 2-mercaptoethanol. Samples were centrifuged for 1 minute at 12,000 g, boiled for 10 minutes and centrifuged again for 1 minute. Samples were then loaded onto a 4–12% Bis-Tris gel, electrophoresed at 200 V for 50 minutes, and transferred at 100 V for 1 hour to nitrocellulose. Membranes were rinsed with NanoPure water, blocked in Odyssey blocking buffer (Li-Cor, Lincoln, NE) for 1 hour, and probed overnight with primary antibody at 4°C. Blots were then washed three times in 0.1% PBST for 5 minutes, incubated with secondary antibody at 25°C for 1 hour, and washed in a similar manner for 5 minutes three times each. Primary antibodies used were: mouse monoclonal cocktail anti-MMP1 to the catalytic domain (DSHB) (Glaseen et al., 2009; Page-McCaw et al., 2003), mouse monoclonal anti-dFMRP (1:2500, DSHB) and mouse monoclonal anti-actin (JLA20, 1:50; DSHB). Secondary antibodies were: Alexa-Fluor-680-conjugated goat anti-mouse (1:10,000) and IRDye-800 goat anti-mouse (1:1000). Odyssey software was used for image capture and analysis.

In vivo zymography
The in situ zymography technique was adapted from recent studies in mice and Drosophila (Bajenaru et al., 2010; Vidal et al., 2010). Briefly, wandering third instars were dissected and incubated immediately in 500 μg/ml of DQ-gelatin (Fluorescein conjugated; Molecular Probes) for 40 minutes in 1× reaction buffer (Molecular Probes) at 25°C. Preparations were rinsed in PBS three times and then fixed for 30 minutes in 4% paraformaldehyde/4% sucrose in PBS at 25°C. After three PBS washes, preparations were incubated with Cy3-conjugated anti-HRP (1:200, goat; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at 25°C. Samples were washed three times in PBS and mounted in FluoroMount G (EMS, Hatfield, PA). All fluorescent images were done on single-cell γ-neuron MARCM clones. For γ-neuron structural quantification, the primary axonal branch was identified first, with all other processes extending from the primary axon counted as branches. Axonal length was determined based on the length of the primary axonal process.

Clinical issue
Fragile X syndrome (FXS), which occurs in ~1 in 4000 males and ~1 in 6000 females, causes a variety of behavioral symptoms, including hyperactivity, disrupted circadian patterns, and cognitive learning and memory deficits. FXS results from a loss of the fragile X mental retardation 1 (FMR1) protein product (FMRP), which is an mRNA-binding translational regulator. Loss of FMRP is proposed to cause enhanced metabotropic glutamate receptor (mGluR) signaling, which leads to increased long-term potentiation (LTP) and depression (LTD) (affecting synaptic plasticity). Tests in mouse and Drosophila models of FXS suggest that mGluR inhibitors are an effective means by which to prevent or rescind FXS phenotypes, but developing other methods of treatment is also desired. A recent mouse study indicated that the tetracycline derivative minocycline might be an effective therapy for FXS through its ability to inhibit upregulated matrix metalloproteinase-9 (MMP9), a secreted gelatinase that cleaves extracellular proteins to remodel the extracellular matrix.

Results
In this study, the authors use the well-characterized Drosophila FXS disease model (dfmr1 null) to test the effects of minocycline in three different classes of neural circuits: the neuromusculature, the circadian clock circuit, and the brain learning and memory center. In all three locations, they find that mutant synaptic connectivity defects are rescued by minocycline treatment. To test the MMP hypothesis, they overexpress the sole Drosophila tissue inhibitor of matrix metalloproteinases (TIMP) in dfmr1 null mutant flies and assay for rescue of synaptic defects. Similarly to minocycline, TIMP overexpression also restores normal synaptic connectivity in the Drosophila FXS model. Moreover, removal of Drosophila FMRP (dfMRP) reciprocally prevents defects induced by TIMP overexpression, including cellular phenotypes and pre-adult lethality, showing that the TIMP and dfMRP pathways bidirectionally overlap. Similarly, removal of dfMRP suppresses both lethality and tracheal breaking defects observed in mmpl1 null flies. Despite the fact that MMP1 expression and gelatinase activity in the nervous system are found to be the same in control and dfmr1 null flies, removal of MMP1 from dfmr1 null flies partially rescues FXS synaptic structure defects. These data are consistent with the MMP hypothesis and explain how minocycline suppresses defects in animal models of FXS.

Implications and future directions
The results of the recent mouse study and this Drosophila study concur that minocycline is a highly effective treatment in FXS disease models. Moreover, a very recent open-label, add-on clinical trial on FXS patients also strongly supports the therapeutic potential of minocycline. Together, these studies mandate that minocycline should be tested in a double-blind, placebo-controlled clinical trial in patients with FXS. Additionally, the reciprocal regulation of the FMRP and TIMP pathways found here suggest that more work is necessary to further elucidate the interplay between these pathways and the common components of each that might be targeted in novel treatment strategies for FXS. Conversely, this study also suggests that investigating FMRP and its targets in cancer biology and therapeutics is warranted, given the central role of MMPs in cancer progression.
collected using a ZEISS LSM 510 META laser scanning confocal microscope. ImageJ was used for fluorescence intensity quantification.

**Statistics**

Statistical analyses were performed using GraphPad InStat 3 (GraphPad Software, San Diego, CA). The more conservative unpaired, nonparametric, two-tailed Mann-Whitney tests were applied to determine significance. For drug treatment assays, means of control versus dfmr1 nulls were compared, and means of dfmr1 nulls versus each drug-treated condition were compared. For genetic analyses, the control animal was compared with the mutant condition, which was then compared with rescue animals. All other comparisons were control to dfmr1 nulls. Significance is represented in the figures as P<0.05 (*), P<0.01 (**) and P<0.001 (***)

**REFERENCES**


**SUPPLEMENTARY MATERIAL**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.008045/-/DC1

**COMPETING INTERESTS**

The authors declare that they do not have any competing or financial interests.

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**AUTHOR CONTRIBUTIONS**

S.S.S. performed all experiments, analyzed all data and co-wrote the manuscript. K.B. conceived the study, designed experiments, supervised experimental work and co-wrote the manuscript.

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Drosophila Fragile X minocycline trial


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