Fragile X mental retardation protein regulates trans-synaptic signaling in Drosophila

Samuel H. Friedman¹, Neil Dani¹, Emma Rushton¹ and Kendal Broadie¹,*

SUMMARY

Fragile X syndrome (FXS), the most common inherited determinant of intellectual disability and autism spectrum disorders, is caused by loss of the fragile X mental retardation 1 (FMR1) gene product (FMRP), an mRNA-binding translational repressor. A number of conserved FMRP targets have been identified in the well-characterized Drosophila FXS disease model, but FMRP is highly pleiotropic in function and the full spectrum of FMRP targets has yet to be revealed. In this study, screens for upregulated neural proteins in Drosophila fmr1 (dfmr1) null mutants reveal strong elevation of two synaptic heparan sulfate proteoglycans (HSPGs): GPI-anchored glypican Dally-like protein (Dlp) and transmembrane Syndecan (Sdc). Our recent work has shown that Dlp and Sdc act as co-receptors regulating extracellular ligands upstream of intracellular signal transduction in multiple trans-synaptic pathways that drive synaptogenesis. Consistently, dfmr1 null synapses exhibit altered WNT signaling, with changes in both Wingless (Wg) ligand abundance and downstream Frizzled-2 (Fz2) receptor C-terminal nuclear import. Similarly, a parallel anterograde signaling ligand, Jelly (Wit) and phosphorylation of the Mothers against decapentaplegic (MAD) transcription factor to similarly modulate synaptic structure.

INTRODUCTION

Fragile X syndrome (FXS) is caused solely by loss of fragile X mental retardation protein (FMRP), which binds mRNAs to mediate transcript stability and trafficking, and acts as a negative regulator of translation (Laggerbauer et al., 2001; Li et al., 2001; Zhang et al., 2001; Lu et al., 2004; Muddashetty et al., 2007; Tessier and Broadie, 2008). Both humans with FXS and animal models of the disease (murine and Drosophila) exhibit synaptogenesis defects characterized by overgrowth and supernumerary synaptic contacts (Rudeli et al., 1985; Hinton et al., 1991; Gatto and Broadie, 2011; Tessier and Broadie, 2012). FXS disease models also exhibit defects in synaptic function, including elevated neurotransmission and altered activity-dependent plasticity (Zhang et al., 2001; Repicky and Broadie, 2009; Callan and Zarnescu, 2011; Gross et al., 2012). In the Drosophila FXS model, synaptic defects are rescued by introduction of human FMRI, but not the closely related FXR1 or FXR2 (Coffee et al., 2010; Tessier and Broadie, 2012), showing functional conservation of FMRP-dependent synaptic mechanisms. The numerous presynaptic and postsynaptic defects in the FXS disease state, which have often been first characterized in the Drosophila FXS model (Zhang et al., 2001; Pan et al., 2004; Pan and Broadie, 2007; Tessier and Broadie, 2012), have established clear roles for FMRP on both sides of the synaptic cleft. Conserved FMRP targets that have been functionally evaluated include presynaptic microtubule-associated protein 1B (MAP1B) (Zhang et al., 2001; Lu et al., 2004) and membrane-associated scaffold postsynaptic density protein of 95 kDa (PSD-95) (Zalfa et al., 2007; Muddashetty et al., 2011). Yet, the full spectrum of FMRP targets is unknown, and Drosophila remains an excellent model in which to study this complex regulation. Importantly, although some synaptogenic defects are rescued cell-autonomously, others require FMRP in the synaptic partner, demonstrating non-cell-autonomous requirements (Gatto and Broadie, 2008; Tessier and Broadie, 2012). Thus, FMRP might influence synaptogenesis via trans-synaptic signaling, regulating the cooperative differentiation of both sides of the synapse.

Trans-synaptic signaling pathways have been particularly well characterized at the Drosophila neuromuscular junction (NMJ) model synapse (Bayat et al., 2011; Dani and Broadie, 2012; Koles and Budnik, 2012; Rohrbough et al., 2013). A classic WNT pathway involves presynaptic secretion of Wingless (Wg), anterograde activation of postsynaptic Frizzled-2 (Fz2) receptors, internalization and cleavage of the Fz2 C-terminus (Fz2-C), and finally Fz2-C nuclear import leading to modulation of synaptic structure and function (Packard et al., 2003; Salinas, 2003; Mathew et al., 2005; Koles and Budnik, 2012). Recent work has shown that Fz2-C localizes with translationally silenced ribonucleoprotein particles and aids in their trafficking outside the nucleus, facilitating local protein synthesis (Speese et al., 2012). A BMP pathway involves postsynaptic secretion of Glass bottom boat (Gbb), retrograde activation of presynaptic receptors containing Wishful thinking (Wit) and phosphorylation of the Mothers against decapentaplegic (MAD) transcription factor to similarly modulate synaptic structure.
Disease Models & Mechanisms

DMM

RESEARCH ARTICLE

proteoglycans (HSPGs), a class of membrane-bound proteins with characteristics such as structural abnormalities and altered neurotransmission, and defects in activity-dependent synaptic plasticity underlie the symptoms of FXS. Because trans-synaptic intercellular signaling is a key feature of synaptic development and plasticity — it controls the operant interplay orchestrating synaptic connectivity and communication strength — defects in trans-synaptic signaling might therefore be involved in the development of the coupled pre- and post-synaptic deficits seen in the FXS disease state.

Results

In this study, the authors use the well-characterized Drosophila FXS disease model (dfmr1 null) to test whether misregulation of known trans-synaptic signaling pathways forms a central feature of the FXS disease state. Using a candidate screening approach, they show that two synapse hepatic sulfate proteoglycans (HSPGs) that act as extracellular co-receptors for WNT and BMP intercellular signaling ligands are strongly upregulated in dfmr1 null flies. Examination of the anterograde WNT and retrograde BMP trans-synaptic signaling cascades and the recently identified anterograde Jeb-Alk signaling pathway at the neuromuscular junction model synapse indicates that both the WNT and the Jeb-Alk trans-synaptic pathways are strongly misregulated in dfmr1 null flies but that the BMP pathway is unaltered. Finally, the authors show that correction of HSPG upregulation is sufficient to fully restore both synaptic architecture and neurotransmission strength to the wild-type condition.

Implications and future directions

These findings show that key synaptogenic HSPGs are upregulated and trans-synaptic signaling pathways are strongly impaired in the absence of FMRP, thereby identifying trans-synaptic signaling misregulation as a central feature of the FXS disease state, at least in the Drosophila genetic model. Recent promising clinical trials on potential treatments for FXS that are founded on insights from animal disease models have focused attention on extracellular synaptic components and the mechanisms of trans-synaptic signaling. Moreover, current FDA-approved therapies for FXS, such as lithium, have non-synaptic effects on a translational repressor. We then tested each of the above three trans-synaptic pathways (Wg and Gbb) and found that both anterograde pathways (Wg and Jeb) were strongly dysregulated at dfmr1 null synapses, but there was no change in the retrograde pathway (Gbb). The ‘exchange-factor model’ predicts that downstream signaling is dependent on the ratio of Wg ligand, Dlp co-receptor and Fz2 receptor, such that excess Dlp causes Wg to be sequestered away from Fz-2 receptors, causing Wg accumulation without activating downstream signaling. Consistent with this prediction, the changes in the downstream signaling of Fz2-C (Wg pathway) and dPERK (Jeb pathway) occurred proportionally in the ratio of Wg ligand and Jeb versus HSPG co-receptor (Dlp and Sdc) at dfmr1 null synapses, whereas pMAD signaling (Gbb pathway) was not altered. Mechanistically, HSPG overexpression mimicked dfmr1 null synaptic phenotypes, and genetically correcting HSPG elevation in the dfmr1 null background restored both excess synaptic structure and elevated neurotransmission strength back to wild-type levels. Importantly, genetically correcting HSPG elevation in the dfmr1 null background was sufficient to restore normal trans-synaptic signaling. Taken together, these results suggest that FMRP repression of synaptic HSPG co-receptors normally regulates trans-synaptic signaling to two or three glycosaminoglycan chains composed of a repeating sulfated disaccharide. These repeats, which are in close proximity to the cell surface, bind protein signaling ligands (Dani and Brodie, 2012). First, it was recently shown in both Drosophila and mouse FXS models that FMRP regulates extracellular matrix metalloproteinases (MMPs) in a pathway that is central to the control of synaptic properties (Bilousova et al., 2009; Siller and Brodie, 2011). In Drosophila, we showed that dfmr1 null synaptogenic defects are effectively prevented by pharmacological inhibition of MMP activity, transgenic overexpression of the endogenous tissue inhibitor of MMPs (TIMP), or co-removal of secreted MMP-1 (Siller and Brodie, 2011; Siller and Brodie, 2012). Importantly, HSPGs are well-established MMP proteolytic targets (d’Ortho et al., 1997; Yu and Woolston, 2000; Egeblad and Werb, 2002; Choi et al., 2012). Moreover, two synaptic HSPGs at the Drosophila NMJ, GPI-anchored Dally-like protein (Dlp) and transmembrane Syndecan (Sdc), play key roles in the modulation of synaptic structure and function (Johnson et al., 2006). Second, we have recently shown that misregulation of Dlp and/or Sdc HSPGs is causative in multiple trans-synaptic signaling defects at the Drosophila NMJ (Dani et al., 2012). Specifically, we demonstrated that Dlp and Sdc act as co-receptors for extracellular ligands to regulate their abundance and mediate downstream signaling. Additionally, we revealed that regulation of the extracellular ligands is interdependent on the relative abundance of both cognate receptor and HSPG co-receptor, creating the tiered system of regulation postulated in the ‘exchange-factor model’ (Yan et al., 2009; Dani et al., 2012). Finally, HSPG RNA transcripts are direct binding targets of FMRP as recently shown by high-throughput sequencing of RNA isolated via crosslinking immunoprecipitation (HITs-CLIP) (Darnell et al., 2011). Taken together, these lines of evidence support the hypothesis that synaptic levels might be altered in the dfmr1 null disease state to cause impaired trans-synaptic signaling and thus defects in synaptic structure and function.

To test this hypothesis, we first assayed the synaptic expression of Dlp and Sdc HSPGs, and found that both were highly elevated in dfmr1 null NMJ synapses, consistent with FMRP functioning as a translational repressor. We then tested each of the above three trans-synaptic pathways (Wg, Gbb and Jeb), and found that both anterograde pathways (Wg and Jeb) were strongly dysregulated at dfmr1 null synapses, but there was no change in the retrograde pathway (Gbb). The ‘exchange-factor model’ predicts that downstream signaling is dependent on the ratio of Wg ligand, Dlp co-receptor and Fz2 receptor, such that excess Dlp causes Wg to be sequestered away from Fz2 receptors, causing Wg accumulation without activating downstream signaling. Consistent with this prediction, the changes in the downstream signaling of Fz2-C (Wg pathway) and dPERK (Jeb pathway) occurred proportionally to changes in the ratio of ligand (Wg and Jeb) versus HSPG co-receptor (Dlp and Sdc) at dfmr1 null synapses, whereas pMAD signaling (Gbb pathway) was not altered. Mechanistically, HSPG overexpression mimicked dfmr1 null synaptic phenotypes, and genetically correcting HSPG elevation in the dfmr1 null background restored both excess synaptic structure and elevated neurotransmission strength back to wild-type levels. Importantly, genetically correcting HSPG elevation in the dfmr1 null background was sufficient to restore normal trans-synaptic signaling. Taken together, these results suggest that FMRP repression of synaptic HSPG co-receptors normally regulates trans-synaptic signaling to...
modulate synaptic structure and function, and that disruption of this mechanism is causal in synaptogenesis defects in the Drosophila disease model of fragile X syndrome.

RESULTS

Two synaptic HSPGs are strongly upregulated in the absence of FMRP

The extracellular synaptomatrix plays crucial roles in shaping Drosophila NMJ synaptic development and modulation (Broadie et al., 2011), with conserved functions in mammalian synapse structural and functional maturation (Dityatev and Schachner, 2006; Vautrin, 2010; Barros et al., 2011). For example, two membrane-anchored HSPGs regulate structural and functional differentiation of the Drosophila NMJ; GPI-anchored Dlp and transmembrane Sdc (Johnson et al., 2006). In other cellular contexts, these same HSPGs act as crucial regulators of intercellular communication (Yan and Lin, 2009; Kleinschmit et al., 2010; Dejima et al., 2011), and we have recently shown that these two HSPGs function as co-receptors controlling secreted ligand abundance during trans-synaptic signaling at the Drosophila NMJ (Dani et al., 2012). Given that HSPG transcripts were recently identified as FMRP direct-binding targets by HTS-CLIP (Darnell et al., 2011), we hypothesized that changes in HSPG protein levels at the synapse could provide a candidate mechanism to explain synaptogenesis defects in the dfmr1 null disease model (Zhang et al., 2001; Pan et al., 2004; Gatto and Brodie, 2008). To begin to test this hypothesis, we first probed Dlp and Sdc expression at the NMJ using well-characterized antibodies (Fig. 1).

In genetic controls (w1118) and two dfmr1 null mutants, we analyzed NMJs of mature larvae for Dlp and Sdc, co-labeling with anti-horseradish peroxidase (HRP) to mark neuronal membranes (Fig. 1). In controls, Dlp exhibited punctate expression surrounding each HRP-marked presynaptic bouton (Fig. 1A). Dlp seemed to be specifically localized at boutons, with axonal expression largely undetectable. In contrast, dfmr1 null NMJs showed a clear increase in Dlp expression intensity, with a divergence from control punctate expression towards a pattern that covers most of the bouton (Fig. 1A). Dlp expression was quantified by measuring fluorescence intensity within the HRP-marked region to define the synaptic domain. Comparative measurements show that Dlp expression was elevated by ~90% in two dfmr1 nulls compared with controls (fluorescence intensity: normalized w1118, 1.0±0.044; dfmr150M, 1.90±0.074, P<0.001; dfmr12, 1.88±0.070, P<0.001; Fig. 1B). Similarly, in control NMJs, Sdc exhibited a halo-like expression pattern surrounding HRP-marked synaptic boutons (Fig. 1C). Likewise, this HSPG was significantly elevated in abundance at dfmr1 null synapses compared with in controls, exhibiting a more intense expression closely overlapping with the HRP signal (Fig. 1C). In quantifying intensity, Sdc expression was found to be increased by ~50% over control levels (fluorescence intensity: w1118, 1.0±0.037; dfmr150M, 1.49±0.076, P<0.001; dfmr12, 0.18±0.06, P<0.01; Fig. 1D). These results show that both Dlp and Sdc HSPGs are strongly upregulated at the NMJ synapse in the absence of FMRP.

Elevated abundance of the WNT ligand Wg in the synaptomatrix signaling domain

We have recently shown that these membrane HSPGs act as co-receptors for the WNT ligand Wg at the Drosophila NMJ (Dani et al., 2012). They act to trap the Wg ligand at the synaptic interface, with regulation of HSPG sulfation state determining synaptic Wg levels (Dani et al., 2012). Wg is the best-characterized trans-synaptic signal in this system; it is secreted from the presynaptic terminal to activate postsynaptic receptors in an anterograde signaling pathway, as well as activating autocrine presynaptic receptors, to modulate both structural and functional synaptogenesis (Salinas, 2003; Koles and Budnik, 2012). Multiple studies show that Wg is regulated in both absolute abundance and spatial distribution by the Dlp HSPG in other Drosophila cellular contexts (Khare and
RESEARCH ARTICLE

All studies were performed in detergent-free, unpermeabilized conditions, to selectively visualize only extracellular Wg (Rushton et al., 2009; Dani et al., 2012). As discovered previously, we observed that Wg exhibits dynamic expression at the *Drosophila* NMJ, with strong expression in a fluctuating subset of synaptic boutons (Fig. 2A). In control *w*1118 synapses, Wg appeared as a punctate pattern across the surface of HRP-marked boutons, with different boutons expressing different amounts of the signal. At dfmr1 null NMJs, there was a clearly increased intensity of Wg expression, and a broader spatial distribution compared with controls (Fig. 2A). In quantifying Wg intensity, comparative measurements showed that dfmr1 nulls displayed a significantly elevated overall abundance compared with controls (fluorescence intensity: normalized *w*1118, 1.0±0.062; dfmr150M, 1.28±0.103; dfmr12, 1.39±0.14, *P*<0.05; Fig. 2B). In addition, dfmr1 nulls showed an increase in the spatial distribution of Wg, both as the fraction of NMJ area expressing Wg and as the number of synaptic boutons expressing Wg. We quantified these parameters by measuring Wg expression area and dividing by HRP-marked area, to normalize for NMJ size. Quantification showed that Wg area is ~65% higher in dfmr1 nulls compared with controls (fraction of NMJ area expressing Wg: *w*1118, 1.0±0.15; dfmr150M, 1.65±0.23; dfmr12, 1.60±0.22, *P*<0.05; Fig. 2C). We finally measured the number of Wg-positive synaptic boutons as a fraction of total boutons, showing that controls exhibit ~50% Wg-positive boutons and dfmr1 nulls ~75% Wg-positive boutons (fraction of boutons expressing Wg: *w*1118, 1.0±0.076; dfmr150M, 1.45±0.077; dfmr12, 1.65±0.13, *P*<0.001; Fig. 2D). Taken together, these data show that dfmr1 synapses exhibit increased Wg ligand abundance and spatial distribution.

An increased ratio of HSPG co-receptor to Wg ligand depresses Fz2-C signaling

Wg acts through the Fz2 nuclear import pathway (Mathew et al., 2005): Wg binding to postsynaptic Fz2 receptors stimulates receptor internalization and cleavage of the C-terminus (Fz2-C), which translocates to postsynaptic nuclei to control ribonucleoprotein (RNP) export and local translation (Fig. 3) (Speese et al., 2012). Regulation of this signaling pathway is quantified by counting the number of Fz2-C RNP granule puncta in muscle nuclei (Mathew et al., 2005; Speese et al., 2012). Using this measure, we have recently shown that HSPG modulation regulates Wg abundance at the NMJ synapse to control Fz2-C nuclear translocation (Dani et al., 2012). The exchange-factor model states that downstream signaling is dependent on the ratio of Wg ligand, Dlp co-receptor and Fz2 receptor, such that excess Dlp causes Wg to be sequestered away from Fz-2 receptors, causing Wg accumulation without activating downstream signaling (Yan et al., 2009). Based on this foundation, it is possible that there could be a similar downstream impact on the Fz2-C nuclear import pathway in the dfmr1 null condition. We tested this hypothesis by examining Fz2-C expression in postsynaptic muscle nuclei (Fig. 3).

Wandering third instar muscles were co-labeled with anti-Fz2-C and the nuclear marker propidium iodide (PI; Fig. 3A). Strikingly, in low-magnification images of muscle, Fz2-C nuclear accumulation was clearly detectable in genetic controls (Fig. 3A, arrows), yet obviously absent in dfmr1 null mutants (Fig. 3A). High-magnification images of control nuclei show clear punctate domains...
within the PI-marked nucleus, indicating an accumulation of Fz2-C RNP s (Fig. 3B). There is also a build-up of Fz2-C puncta directly surrounding the nucleus in controls, presumably representing Fz2-C being trafficked to/from the nucleus (Speese et al., 2012). In dfmr1 nulls, the amounts of both the intra-nuclear and extra-nuclear puncta were very markedly reduced (Fig. 3B), denoting a decrease in the activity of the intracellular signaling cascade (Fig. 3C). To quantify this pathway, we counted the number of Fz2-C puncta in nuclei for both control and dfmr1 null mutants, and then calculated an average number of puncta per nucleus normalized for each muscle (Fig. 3D). These analyses revealed a stark decrease in Fz2-C nuclear localization in dfmr1 nulls compared with controls (normalized w^{1118}, 1.0±0.16; dfmr1^{50M}, 0.55±0.09, \( P<0.05; \) dfmr1^2, 0.43±0.06, \( P<0.01; \) Fig. 3D). These results show that, despite increased Wg abundance in the extracellular synaptomatrix, the Fz2 nuclear import signaling pathway is downregulated in the absence of FMRP. Our interpretation of this result, as we have recently shown (Dani et al., 2012), is that dramatic overexpression of HSPG co-receptors sequesters Wg ligand away from Fz2 receptors, and thereby depresses overall Wg trans-synaptic signaling in the dfmr1 null condition.

**A second anterograde trans-synaptic pathway is depressed at dfmr1 null NMJs**

We have recently established Jeb as another presynaptically secreted signaling ligand, which binds postsynaptic Alk receptors to activate an anterograde MAPK pathway of ERK phosphorylation (dpERK) and nuclear import (Rohrbough and Broadie, 2010; Rohrbough et al., 2013). Although Jeb has not been shown to interact with an HSPG co-receptor, it is known to be regulated by the endogenous lectin Mind-the-gap (MTG) (Rohrbough and Broadie, 2010). Given the strong effect on Wg trans-synaptic signaling in the dfmr1 null disease state, we wished to next test specificity by determining whether this separate anterograde pathway might also be impacted. We therefore performed similar experiments, probing the NMJ synapse with well-characterized antibodies to Jeb (Englund et al., 2003) and activated dpERK (Yung et al., 1997), with the NMJ co-labeled with HRP and the PI nuclear markers, respectively. We assayed Jeb expression with detergent-free labeling, as above using unpermeabilized conditions to selectively visualize the extracellular ligand (Rushton et al., 2009). A summary of these data are shown in Fig. 4.

In genetic controls (w^{1118}), Jeb expression was strongly associated with NMJ boutons, closely overlapping with the HRP-marked neuronal membrane, with little or no detectable expression associated with axonal regions (Fig. 4A). In dfmr1 null NMJs, Jeb expression was very strongly reduced. In mutants, Jeb seems to maintain bouton-specific localization, yet the boutons secrete less detectable ligand (Fig. 4A). Fluorescence intensity quantification shows a highly significant loss of Jeb in dfmr1 nulls compared with controls (normalized w^{1118}, 1.0±0.049; dfmr1^{50M}, 0.73±0.071, \( P<0.01; \) dfmr1^2, 0.67±0.056, \( P<0.001; \) Fig. 4B). Jeb on the extrasynaptic muscle surface, away from the NMJ terminal, was similarly reduced (w^{1118}, 1.0±0.049; dfmr1^{50M}, 0.73±0.071, \( P<0.01; \) dfmr1^2, 0.67±0.056, \( P<0.001; \) supplementary material Fig. S1). In parallel, we analyzed downstream dpERK signaling in muscle nuclei. In controls, dpERK was expressed in NMJs and within postsynaptic muscle nuclei (Fig. 4C). In dfmr1 nulls, the amount of activated dpERK trafficked to muscle nuclei was obviously reduced. In mutants, dpERK spatial localization was similar to controls, but fluorescence intensity is clearly diminished. We quantified dpERK labeling within PI-marked muscle nuclei (Fig. 4D). In the dfmr1 null condition, dpERK nuclear localization was significantly reduced compared with controls (fluorescence intensity: w^{1118}, 1.0±0.04; dfmr1^{50M}, 0.84±0.03, \( P<0.05; \) dfmr1^2, 0.80±0.04, \( P<0.05); \) These results show that both Wg–Fz2-C and
Jeb-dpERK anterograde signaling is decreased in the absence of FMRP.

**Retrograde BMP signaling is not altered at dfmr1 null NMJs**

Given that two anterograde trans-synaptic pathways are misregulated in parallel in the absence of FMRP, we next set out to test whether retrograde signaling is similarly impacted. A well-characterized retrograde pathway involves postsynaptic secretion of the BMP Gbb, which binds presynaptic receptors to stimulate downstream phosphorylation of the transcription factor MAD (pMAD), which translocates to motor neuron nuclei in the CNS (McCabe et al., 2003; Keshishian and Kim, 2004; Kim and Marqués, 2010). Using an anti-Gbb antibody that we recently characterized (Dani et al., 2012), Gbb levels were compared between genetic control (w1118) and dfmr1 null synapses. In parallel, we used a widely employed anti-pMAD antibody (Persson et al., 1998) to assay the downstream signal transduction pathway. A summary of these studies is shown in Fig. 5.

Detergent-free conditions were again employed to visualize only secreted Gbb at HRP-labeled NMJs (Fig. 5A). In controls, Gbb exhibited consistent high expression surrounding synaptic terminals, with Gbb expression surrounding and extending beyond HRP-marked synaptic boutons. In qualitative comparisons, dfmr1 null Gbb expression seemed very similar to controls (Fig. 5A). Quantified Gbb intensity analyses similarly revealed no detectable changes in Gbb levels at dfmr1 null NMJs compared with controls (normalized w1118, 1.0±0.087; dfmr150M, 0.99±0.16, P>0.05; dfmr12, 1.0±0.11, P>0.05; Fig. 5B). Although the Gbb abundance was not detectably changed in dfmr1 nulls, we still investigated downstream pMAD signaling to test for possible functional misregulation. Because activated pMAD translocates into presynaptic nuclei, we measured signaling by imaging motor neuron nuclei in the CNS (Fig. 5C). PI was again used to mark nuclei (Fig. 5C, red), showing non-pMAD interneuron nuclei adjacent to pMAD-positive motor neuron nuclei (Fig. 5C, green; yellow overlap). We quantified pMAD expression intensity by outlining motor neuron nuclei in the PI red channel and measuring pMAD intensity in the green channel. This quantification revealed no detectable pMAD changes in dfmr1 nulls compared with controls (w1118, 1.0±0.052; dfmr150M, 1.17±0.077, P>0.05; dfmr12, 1.04±0.061, P>0.05; Fig. 5D). Thus, we conclude
that the retrograde BMP pathway is independent of FMRP regulation, suggesting that FMRP selectively regulates anterograde trans-synaptic signaling.

**Genetically reducing HSPG levels in dfmr1 nulls restores synaptic architecture**

The overelaboration of NMJ architecture caused by dfmr1 loss has been well documented, including observations of increased axonal branching and supernumerary bouton formation (Zhang et al., 2001; Gatto and Broadie, 2008). Likewise, Wg and Jeb trans-synaptic signals have previously been well documented to similarly regulate synaptic architecture (McCabe et al., 2003; Korkut and Budnik, 2009; Ball et al., 2010; Budnik and Salinas, 2011; Rohrbough et al., 2013). We therefore hypothesized that FMRP-dependent regulation of trans-synaptic signaling could contribute to dfmr1 structural phenotypes. Moreover, we hypothesized the causal link between FMRP and trans-synaptic signaling to be the HSPG co-receptors, which we have recently shown control trans-synaptic signaling to modulate NMJ structure (Dani et al., 2012). If this model is correct, then correcting HSPG levels in dfmr1 nulls should restore synaptic architecture back towards wild-type levels. To test this hypothesis, we crossed heterozygous dlp/+ and sdc/+ null mutations into dfmr1 homozygous null backgrounds, both singly and in combination, and assayed synaptic branching and bouton number in the resulting triple-mutant animals. A summary of these studies is shown in Fig. 6.

We assayed NMJ structure by co-labeling with anti-HRP and anti-DLG (Discs-large) to delineate the pre- and postsynaptic terminals (Fig. 6A). Type 1 synaptic boutons were defined as ≥2 μm in minimal diameter and DLG positive, and branches were defined as HRP-positive processes with ≥ two type 1 boutons. As reported previously, dfmr1 null branch number was significantly increased compared with genetic controls (normalized w1118, 1.0±0.067; dfmr150M, 1.64±0.086, P<0.01; Fig. 6A,B). Likewise, the number of type 1 synaptic boutons was similarly increased in the dfmr1 nulls compared with controls (w1118, 1.0±0.069; dfmr150M, 1.72±0.078, P<0.01; Fig. 6A,C). We first attempted to mimic dfmr1 null phenotypes by inducing Dlp elevation at otherwise wild-type
NMJs using postsynaptic driver 24B-GAL4, in order to assign causality to synaptic architecture defects. We confirmed Dlp overexpression by analyzing Dlp fluorescence intensity at the NMJ (normalized w^{1118}, 1.0±0.10; 24B × UAS-Dlp, 3.76±0.37, P<0.001; supplementary material Fig. S2A,B). Supporting our hypothesis, Dlp overexpression mirrored dfmr1 null synaptic structure phenotypes, including both increased branch number (normalized 24B × w^{1118}, 1.0±0.05; 24B × UAS-Dlp, 1.62±0.09, P<0.001; Fig. 6A,B) and synaptic bouton number (24B × w^{1118}, 1.0±0.044; 24B × UAS-Dlp, 1.41±0.056, P<0.001; Fig. 6A,C). Although we did not analyze Sdc overexpression, a previous study has shown that it causes a similar change in NMJ architecture (Johnson et al., 2006). These data suggest that HSPG overexpression is sufficient to produce the synapse overelaboration characterizing dfmr1 null NMJs.

We next analyzed the consequences of heterozygous genetic reduction of dlp alone and in tandem with sdc in the dfmr1 null background. We first confirmed that the heterozygotes express reduced Dlp protein levels (normalized w^{1118}, 1.0±0.048; dlp^{A187}/+, 0.65±0.054, P<0.001; supplementary material Fig. S2C,D). As predicted by the above Dlp overexpression results, genetic reduction of Dlp alone significantly reduced synaptic overelaboration in the dfmr1 null as normalized to w^{1118} genetic control, both in branch number (dfmr1^{50M}, 1.64±0.081; dlp^{A187}, dfmr1^{50M}/dfmr1^{50M}, 1.17±0.066, P<0.01; Fig. 6A,B) and bouton number (dfmr1^{50M}, 1.72±0.078; dlp^{A187}, dfmr1^{50M}/dfmr1^{50M}, 1.23±0.049, P<0.01; Fig. 6A,C). Likewise, genetic reduction of both Dlp and Sdc HSPGs in tandem similarly reduced synaptic branch number (dfmr1^{50M}, 1.64±0.081; sdc^{23}/+, dlp^{A187}, dfmr1^{50M}/dfmr1^{50M}, 1.26±0.08, P<0.01; Fig. 6A,B) and bouton number (dfmr1^{50M}, 1.72±0.078; sdc^{23}/+, dlp^{A187}, dfmr1^{50M}/dfmr1^{50M}, 1.24±0.062, P<0.01; Fig. 6A,C). The restored synaptic architecture of the double heterozygous mutants in the dfmr1 null background was not significantly different from...
the w^{118} genetic control. Additional controls are shown in supplementary material Fig. S3, for branching (normalized w^{118}, 1.0±0.067; UAS-Dlp/+, 1.0±0.064; dlp A187/+, 1.08±0.09; sdc 23/+, dlp A187/+, 1.04±0.11, $P>0.05$; supplementary material Fig. S3A,B) and bouton number (normalized w^{118}, 1.0±0.069; UAS-Dlp/+, 1.0±0.064; dlp A187/+, 1.04±0.11, $P>0.05$; supplementary material Fig. S3C). These results show that Dlp elevation in the dfmr1 null accounts for synapse architecture defects in the FXS disease state condition.

Genetically reducing HSPG levels in dfmr1 nulls restores synaptic function

We have previously shown that NMJ synaptic transmission strength is significantly elevated in dfmr1 null mutants (Zhang et al., 2001). Consistently, we have recently shown that HSPG co-receptors acting as potent regulators of trans-synaptic signaling also strongly modulate synaptic function (Dani et al., 2012). We therefore hypothesized that the observed functional change in dfmr1 nulls might also be driven by HSPG elevation (Fig. 1). To test this hypothesis, we assayed animals with two-electrode voltage-clamp (TEVC) electrophysiology to compare excitatory junctional current (EJC) amplitudes. Representative records show ten averaged nerve-stimulation-evoked EJC responses (1.0 mM extracellular Ca$^2^+$) for all tested genotypes shown in Fig. 7A, with amplitude quantification shown in Fig. 7B. Consistent with previous reports, EJC amplitudes were significantly elevated by ~20% in dfmr1 nulls compared with controls (w^{118}, 215.38±8.31 nA; dfmr1 S0M, 252.89±7.91 nA, $P<0.05$; Fig. 7A,B). Dlp overexpression with the postsynaptic 24B-GAL4 driver resulted in a significant elevation in synaptic transmission strength as compared with the driver-alone control (w^{118}×24B-GAL4, 215.36±12.04 nA; UAS-Dlp/+, 213.95±8.84 nA; UAS-Dlp × 24B-GAL4, 276.47±8.18 nA, $P<0.01$; Fig. 7A,B). These data suggest that Dlp overexpression is sufficient to produce the synapse strengthening that is observed in dfmr1 null NMJs.

We next examined the consequences of heterozygous genetic reduction of Dlp alone, as well as of Dlp and Sdc in combination, in the dfmr1 null background. Compared with the elevated EJC amplitude of dfmr1 nulls (252.89±7.91 nA, $P<0.01$, n=9; Fig. 7A,B), genetic reduction of Dlp alone (dlp A187, dfmr1 S0M/dfmr1 S0M) did not effectively restore transmission, as compared with genetic controls (w^{118}, 215.38±8.31 nA; dlp A187/+, 224.81±7.53 nA; dlp A187, dfmr1 S0M/dfmr1 S0M, 273.08±11.29 nA, $P<0.01$; Fig. 7A,B).
Disease Models & Mechanisms

likewise reduced in the two condition, synaptic Jeb expression was restored back towards normal (Fig. S5A,B). Conversely, in the two dfmr1 receptor elevation in material Fig. S6C,D). These findings suggest that the HSPG co-architecture and function in this FXS disease model.

In testing this hypothesis, we discovered dramatic upregulation of GPI-anchored glypanic Dlp and transmembrane Sdc HSPGs at dfmr1 null NMJ synapses. Indeed, these are among the largest synaptic molecular changes reported in the Drosophila FXS disease model (Tessier and Broadie, 2012). Importantly, HSPGs have been shown to play key roles in synaptic development. For example, the mammalian HSPG Agrin has long been known to regulate acetylcholine receptors (Parkhomovskiy et al., 2000), interconnected with a glycan network modulating trans-synaptic signaling (Klee and Schachner, 2004). In Drosophila, Dlp, Sdc and Perlec HSPGs mediate axon guidance, synapse formation and trans-synaptic signaling (Yamaguchi, 2001; Lee and Chien, 2004; Van Vactor et al., 2006; Dani et al., 2012; Kamimura et al., 2013). Previous work on dlp mutants has reported elevated neurotransmission (Johnson et al., 2006), paradoxically similar to the Dlp overexpression phenotype shown here. However, the previous study did not show Dlp overexpression electrophysiological data, although it did show increased active zone areas consistent with strengthened neurotransmission. The same study reported that Dlp overexpression decreased bouton number on muscle 6/7, which differs from our finding of increased bouton number on muscle 4. Because HSPG co-receptors regulate trans-synaptic signaling (Dani et al., 2012), we next tested dfmr1 mutants for changes in three established pathways at the Drosophila NMJ. We found there to be strong alterations in both Wg and Jeb signaling, with anterograde signaling being downregulated in both cases. In contrast, we found no change in the retrograde BMP Gbb pathway, suggesting that FMRP plays specific roles in modulating anterograde trans-synaptic signaling during synaptogenesis. The defect in Jeb signaling seems to be simple to understand, with decreased symaptomatrix ligand abundance coupled to
Disease Models & Mechanisms

FMRP regulates trans-synaptic signaling

Disease Models & Mechanisms ● DMM

FMRP regulates trans-synaptic signaling

Disease Models & Mechanisms

MATERIALS AND METHODS

Drosophila genetics

Two dfmr1 null alleles (dfmr150M and dfmr17) (Zhang et al., 2001; Dockendorff et al., 2002) were used, with homozygous mutants being selected using GFP balancer chromosomes and compared...
with the w1118 genetic background control. For overexpression studies, UAS-dlp and 24B-GAL4 driver lines (Brand and Perrimon, 1993) were crossed for elevated postsynaptic expression. Double mutants were generated by recombining the dlpA187 null allele (Han et al., 2004) onto the dfmr150M null chromosome (dlpA187, dfmr150M/TM6Huh-GFP). This line was crossed to dfmr150M/TM6Tb-GFP, to generate dlpA187, dfmr150M/dfmr150M (50% reduced Dlp in the dfmr1 null background). The sdc23 null allele (Steigemann et al., 2004) was crossed to dfmr150M/TM6Tb-GFP to generate sdc23/Cyo-GFP, dfmr150M/TM6Tb-GFP. The two lines were crossed to create sdc23/+; dlpA187, dfmr150M/dfmr150M flies (50% reduced Sdc and Dlp in the dfmr1 null background).

Immunocytochemistry
Wandering third instars were fixed using Bouin’s fixative for 30 minutes (Dlp) or 4% paraformaldehyde for 10 minutes (everything else). Preparations were rinsed 3x with PBS and processed without detergent for extracellular labeling (Dlp, Sdc, Wg, Gbb, Jeb), or with 0.2% Triton X-100 detergent for intracellular labeling (dFz-C, pMad, dpErk, DLG), with anti-HRP labeling neuronal membranes (Rushton et al., 2009; Dani et al., 2012). Preparations were incubated with primary antibodies overnight at 4°C, followed by secondary antibodies for 4 hours at room temperature (RT). Primary antibodies included: mouse anti-Dlp (13G8, 1:4; Developmental Studies Hybridoma Bank (DSHB)), rabbit anti-Sdc (1:200) (Spring et al., 1994), mouse anti-Wg (4D4, 1:2; DSHB), rabbit anti-Gbb (1:100) (Dani et al., 2012), guinea pig anti-Jeb (1:1000) (Rohrbough and Broadie, 2010), rabbit anti-dFz-C (1:500) (Mathew et al., 2005), rabbit anti-pMad (1:1000) (Persson et al., 1998), mouse anti-dpERK (M8159, 1:1000; Sigma-Aldrich) (Rohrbough et al., 2013), mouse anti-DLG (DLG1, 1:200; DSHB).

Secondary antibodies included: Alexa-Fluor-488-conjugated goat anti-mouse IgG (1:200), Alexa-Fluor-488-conjugated goat anti-rabbit IgG (1:250) and Alexa-Fluor-488-conjugated goat anti-guinea pig IgG (all from Invitrogen-Molecular Probes). Neuronal presynaptic terminals were co-labeled using anti-HRP directly conjugated to Cy3 (1:100; Jackson ImmunoResearch Laboratories, Inc.). Nuclei were labeled using propidium iodide (PI; 1:1000; Sigma-Aldrich) or DRAQ5 (1:1000; Cell Signaling Technology) for each; muscle NMJs (4, 6/7) and corresponding motor neuron cell bodies in abdominal segment 3 (combined left and right hemisegments for each; n=1). All expression analyses were done using NIH ImageJ software with the threshold function outlining HRP-labeled NMJs and PI-labeled nuclei. All figure images are maximum projection Z-stacks, with intensity measured as maximum pixel intensity. Intensities and areas were measured as the outlined signal of interest area over the total HRP-labeled NMJ area to normalize for synaptic terminal size. A threshold of two standard deviations below the area over the total HRP-labeled NMJ area to normalize for synaptic boutons. ImageJ software was used to threshold fluorescence intensity and count synaptic boutons and branches.

Electrophysiology
TEVC records were made from the wandering third instar as previously described (Beumer et al., 1999; Dani and Broadie, 2012). Briefly, dissected insects were secured on sylgard-coated coverslips with surgical glue (liquid suture), and segmental nerves were cut near the ventral nerve cord. Recording was performed in 128 mM NaCl, 2 mM KCl, 4 mM MgCl2, 1 mM CaCl2, 70 mM sucrose and 5 mM Hepes. Recording electrodes were filled with 3 M KCl and had resistances of >10 MΩ. Evoked EJC recordings were made from the voltage-clamped (Vclamped=−60 mV) muscle 6 in segment A3 with a TEVC amplifier (Axoclamp 200B; MDS Analytical Technologies). The cut segmental nerve was stimulated with a glass suction electrode at a suprathreshold voltage level (50% above baseline threshold value) for 0.2-0.5 ms duration. Records were made with 0.2 Hz nerve stimulation with episodic acquisition and analyzed with Clampex software (version 7.0; Axon Instruments).

ACKNOWLEDGEMENTS
We are grateful to Merton Bernfield for anti-Sdc antibody, Vivian Budnik for anti-Frizzled-2C antibody and Ruth Palmer for anti-Jeb antibody. We thank the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa for numerous antibodies and the Drosophila Bloomington Stock Center at Indiana University for numerous genetic stocks. We are particularly grateful to Dr Cheryl Gatto for exceptional guidance and mentorship of S.H.F.

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

AUTHOR CONTRIBUTIONS
K.B conceived and designed experiments, which were performed by S.H.F. and N.D. All data was analyzed by S.H.F. and N.D. The paper was written by S.H.F. and K.B. Gatto for exceptional guidance and mentorship of S.H.F.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/doi/10.1242/dmm.012229/-/DC1

REFERENCES


Supplementary material Fig. S1. Reduced Jeb levels in the dfmr1 null muscle. (A) Representative NMJ images co-labeled with presynaptic marker (HRP, red) and anti-Jelly Belly (Jeb, green) in control (w^{1118}) and dfmr1 null (dfmr1^{50M}) wandering third instar muscle 4. Scale bar: 15μm. (B) Quantification of Jeb muscle surface intensity away from the NMJ domain in two dfmr1 null alleles (dfmr1^{50M}, dfmr1^{F}) normalized to the genetic control (w^{1118}). Sample sizes are ≥9 animals and ≥18 muscles for each of the three genotypes. Statistical significance shown as ***P<0.001.
Supplementary material Fig. S2. Quantification of Dlp levels with genetic manipulations. (A) Representative NMJ images co-labeled with presynaptic marker anti-horseradish peroxidase (HRP, red) and anti-Dally-like Protein (Dlp, green) in control (w^{1118}) and postsynaptic Dlp overexpression (24B x UAS-Dlp) conditions. Scale bars: 15μm and 5 μm. (B) Quantification of Dlp intensity normalized to genetic control (w^{1118}). Sample size is ≥3 animals and ≥6 NMJs for each genotype. (C) Representative NMJ images in control and double heterozygous dlp^{1087}/+; sdc^{23}/+ conditions. Scale bars: 15μm and 5 μm. (D) Quantification of Dlp intensity normalized to genetic control (w^{1118}). Sample size is ≥8 animals and ≥16 NMJs for each genotype. Statistical significance shown as ***p<0.001.
Supplementary material Fig. S3. Genetic controls for NMJ synaptic architecture studies. (A) Representative muscle 4 NMJ images co-labeled with presynaptic marker anti-horseradish peroxidase (HRP, red) and postsynaptic marker anti-Discs Large (DLG, green) in control (w^{1118}), w^{1118} crossed to UAS-Dlp, heterozygous dlp^{A187/+}, and double heterozygous dlp^{A187/+}; sdc^{23}+. Scale bar: 15μm. Quantification of NMJ branch (B) and type 1 bouton (C) numbers. Sample size is ≥6 animals and ≥12 NMJs for each genotype. Statistical significance shown as N.S. (P>0.05).
Supplementary material Fig. S4. Synaptic HSPG levels in the triple mutant condition. (A) Representative NMJ images co-labeled with anti-HRP (red) and anti-Dlp (green) in genetic control (w^{1118}) and sdc23/+; dlpA187, dfmr150M/dfmr1^{50M} triple mutant condition. Scale bars: 25μm and 5μm. (B) Quantification of Dlp intensity normalized to control. Sample size is ≥8 animals and ≥16 NMJs for genotype. (C) Representative NMJ images co-labeled with anti-HRP (red) and anti-Syndecan (Sdc, green) in control and triple mutant. Scale bars: 25μm and 5μm. (D) Quantification of Sdc intensity normalized to control. Sample sizes are ≥8 animals and ≥16 NMJs for each genotype. Statistical significance shown as N.S. (P>0.05).
Supplementary material Fig. S5. Wg and Jeb ligand levels in the triple mutant condition. (A) Representative NMJ images co-labeled with anti-HRP (red) and anti-Wingless (Wg, green) in control (w1118) and sdc23/++; dlpA187, dfmr150M/dfmr150M triple mutant conditions. Scale bars: 15μm and 5 μm. (B) Quantification of Wg intensity normalized to control. Sample size is ≥8 animals and ≥15 NMJs for each genotype. (C) Representative NMJ images co-labeled with anti-HRP (red) and anti-Jelly Belly (Jeb, green) in w1118 and triple mutant conditions. Scale bars: 15μm and 5 μm. (D) Quantification of Jeb intensity normalized to control. Sample sizes are ≥8 animals and ≥16 NMJs for each genotype. Statistical significance shown as N.S. (P>0.05).
Supplementary material Fig. S6. Fz2C- and dpERK nuclear localization in the triple mutant condition. (A) Representative images of wandering third instar muscle 4 nuclei co-labeled with nuclear marker Draq5 (red) and anti-Frizzled C-terminus (Fz2-C, green) in control (w1118) and sdc23/+; dlpA187, dfmr150M/dfmr150M triple mutant conditions. Scale bar: 5μm. (B) Quantification of Fz2-C nuclear localization, measured as Fz2-C puncta number, normalized to genetic control. Sample sizes are ≥3 animals and ≥6 muscles for each genotype. (C) Representative images of muscle nuclei co-labeled with nuclear marker Draq5 (red) and anti-diphosphorylated extracellular signal regulated kinase (dpERK, green) in control and triple mutant. Scale bar: 5μm. (D) Quantification of dpERK nuclear intensity levels normalized to genetic control. Sample sizes are ≥8 animals and ≥15 muscles for each genotype. Statistical significance shown as N.S. (P>0.05).