Published in final edited form as:

Sci Signal. ; 10(504): . doi:10.1126/scisignal.aan3181.

Neuronal activity drives FMRP- and HSPG-dependent matrix metalloproteinase function required for rapid synaptogenesis

Mary L. Dear¹, Jarrod Shilts¹, and Kendal Broadie^{1,2,3,†,*}

¹Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA

²Department of Cell & Developmental Biology, Vanderbilt Medical Center, Nashville, TN, USA

³Vanderbilt Brain Institute, Vanderbilt University and Medical Center, Nashville, TN, USA

Abstract

Matrix metalloproteinase (MMP) functions modulate synapse formation and activity-dependent plasticity, with MMP dysfunction implicated in Fragile X syndrome (FXS), a disease caused by the loss of the RNA binding protein FMRP and characterized by neurological dysfunction and intellectual disability. Gene expression studies in *Drosophila* suggest that Mmps cooperate with the heparan sulfate proteoglycan (HSPG) glypican co-receptor Dally-like protein (Dlp) to restrict *trans*-synaptic Wnt signaling, and that synaptogenic defects in the fly model of FXS are alleviated by both Mmp inhibition and Dlp genetic reduction. Here, we used the *Drosophila* neuromuscular junction (NMJ) glutamatergic synapse to test activity-dependent Dlp and Mmp intersections in the context of FXS. We found that rapid, activity-dependent synaptic bouton formation was dependent upon secreted Mmp1. Acute neuronal stimulation reduced Mmp2 abundance, but increased that of both Mmp1 and Dlp as well as Dlp and Mmp1 co-localization at the synapse. Dlp function bidirectionally controlled Mmp1 abundance, localization and proteolytic activity around synapses. Dlp glycosaminoglycan (GAG) chains mediated this functional interaction with Mmp1. In the FXS fly model, activity-dependent increases in Mmp1 abundance and activity were lost but were restored by reducing the amount of synaptic Dlp. These data indicate that neuronal activity-induced, HSPG-dependent Mmp regulation drives activity-dependent synaptogenesis, and that this is impaired in FXS. Thus exploring this mechanism further may reveal therapeutic targets with potential to restore synaptogenesis in FXS patients.

Introduction

Developing and maintaining proper synaptic connectivity requires tightly coordinated intercellular signaling across the synaptic cleft between pre- and postsynaptic cells. This feat is achieved through selective expression of a range of cell adhesion molecules and secreted ligands within the highly specialized extracellular synaptomatrix that separates synaptic partners (1, 2). This cellular interface is highly adaptable (plastic), sculpted by use (activity),

*Corresponding author. kendal.broadie@vanderbilt.edu.

#Current affiliation: Wellcome Trust Sanger Institute, Hinxton, CB10 1SA, UK

†Present address: 6270A Medical Research Building III, Vanderbilt University, 465 21st Avenue South Nashville, TN 37232, USA

The authors declare no conflicts of interest, financial or otherwise.

and constantly remodeled by secreted enzymes molding the dynamic environment (3–6). A principal challenge is to understand exactly how these extracellular forces are regulated to control activity-dependent synaptic development. A core family of secreted proteases, called matrix metalloproteinases (MMPs), occupy a key nexus of coordinated cell-cell and cell-matrix signaling to modulate synaptic architecture and neurotransmission strength (7–9). Conserved MMP domains include N-terminal secretory signal sequence, the cleavable pro-domain, zinc-binding catalytic site and the closely linked C-terminal hemopexin domain (10). Changes in MMP function regulate synaptogenesis and mediate activity-dependent remodeling, and MMP dysfunction is implicated in numerous neurological disorders including autism, epilepsy, addiction and schizophrenia (11–15). Thus, MMP-dependent control of the extracellular synaptomatrix interface appears critical for normal synaptic development, and is implicated in a wide range of synaptic disease states.

The *Drosophila* glutamatergic neuromuscular junction (NMJ) synapse has proven an excellent model to interrogate synaptomatrix questions, with conserved extracellular mechanisms and well defined activity-dependent synaptogenic processes (2, 16–18). Compared to challenges of testing the 24 MMPs in mammalian models, *Drosophila* has just 2 MMPs, secreted Mmp1 and glycosylphosphatidylinositol (GPI)-anchored Mmp2 (19–21); although one Mmp1 isoform is also GPI-anchored (22). Both Mmps regulate motor neuron axon defasciculation, with the matrix molecule Faulty Attraction (Frac) identified as an Mmp2 substrate promoting motor axon targeting (23, 24). Both Mmps also regulate synaptogenesis in multiple contexts, including reshaping dendritic arbors (25, 26) and axonal terminals (27). At the NMJ, these two Mmps coordinate structural and functional synaptogenesis by heparan sulfate proteoglycan (HSPG)-dependent regulation of Wnt Wingless (Wg) *trans*-synaptic signaling (7). In the *Drosophila* model of Fragile X syndrome (FXS), aberrant HSPG- and Mmp-dependent Wg signaling is causative for synaptogenic defects (28). FXS is an intellectual disability and autism spectrum disorder caused by loss of Fragile X Mental Retardation Protein (FMRP), an mRNA-binding translational regulator with roles in activity-dependent synaptogenesis (29–31). The mRNAs encoding HSPGs and Mmps are putative FMRP targets and the corresponding protein products are more abundant in the *Drosophila* FXS model (28, 29, 32, 33).

HSPGs are particularly intriguing extracellular regulators of activity-dependent MMP localization and function (34, 35). The diverse, multi-functional HSPGs can be fully secreted, membrane-tethered, or transmembrane, and consist of a core protein plus heparan sulfate (HS) glycosaminoglycan (GAG) chains. HSPGs are known to function as extracellular signaling platforms linking secreted ligands, receptors and proteases, and MMPs can also proteolytically cleave HSPGs in a reciprocal HSPG-Mmp relationship (34, 36–39). HSPGs are potent regulators of activity-dependent synaptic development and plasticity (40–43). The ~17 HSPGs in mammals present considerable experimental challenges, but the *Drosophila* genome encodes just five HSPGs in total, with only three synaptic HSPGs (38, 40, 41). At the NMJ, the secreted HSPG Perlecan mediates Wg signaling directionality, and serves to restrict synaptic structural growth (44), the transmembrane HSPG Syndecan conversely promotes synaptic development (41), whereas the GPI-anchored HSPG Dally-like Protein (Dlp) serves as a critical biphasic regulator of Wg signaling to modulate structural and functional synaptogenesis (40, 45, 46). *Trans*-

synaptic Wg signaling drives activity-dependent synaptogenesis, promoting both structural growth and functional strengthening in response to changes in neural activity (47–50). Activity-induced Wg signaling mediates rapid, new synaptic bouton formation (47), but a role for Dlp in this synaptogenic mechanism has not been explored.

Given the role of neuronal activity in controlling FMRP abundance and function (51–54), with known requirements in activity-dependent synaptogenesis (28, 55–60), we hypothesized a downstream mechanistic link between the membrane-anchored glypican Dlp and the Mmps driving activity-induced synapse modulation. In testing this hypothesis, we found that Mmp1, but not Mmp2, is required for rapid, activity-dependent synaptic bouton formation. Using both acute transgenic and ionic depolarization paradigms, we found that Mmp1 is increased in response to heightened activity, with both FMRP and Dlp required for this activity-dependent mechanism. Conversely, Mmp2 is reduced after neuronal stimulation. We found that Dlp co-localization with Mmp1 is enhanced by neuronal activity, and that Dlp induces Mmp1 synaptic localization with heightened co-localization in use-activated synapses. We found both FMRP and Dlp are required for this activity-dependent Mmp1 enhancement, but the FMRP requirement was by-passed by genetically reducing Dlp abundance in the FXS model. Overall, these results show that the HSPG glypican Dlp is a direct, positive regulator of basal and activity-dependent Mmp1 abundance at the synapse. Our study also reveals that inappropriate Mmp1 regulation in this FXS model can be rectified by reducing genetic expression of Dlp, suggesting future work should examine whether targeting the human glypicans, HS-modifying enzymes, —or another HSPG-MMP— paired mechanism— might correct neuropathological synaptic defects in FXS patients.

Results

Mmp1 is required for rapid activity-dependent synaptic bouton formation

At the *Drosophila* NMJ, presynaptic bouton formation occurs rapidly in response to acute neuronal stimulation (47). The initial, immature, transitional “ghost boutons” contain synaptic vesicles and presynaptic markers, such as membrane marker Horse Radish Peroxidase (HRP), but are largely devoid of presynaptic active zones and postsynaptic markers, such as Discs Large (DLG) (47, 61–63). Activity-dependent ghost boutons develop into mature boutons, and thus represent the immediate read-out for activity-dependent synaptogenesis (47, 63, 64). To acutely increase activity, we genetically expressed the temperature-dependent ion channel dTRPA1 (Transient receptor potential cation channel A1) in motor neurons (*vglut-Gal4>UAS-dTRPA1*) (65–69), and then assayed new ghost bouton formation after a transient 1-hour temperature increase (Fig. 1). We used the *mmp1*^{Q112*} genetic null allele to remove Mmp1 function, and the *mmp2*^{W307*} genetic null allele in *trans* to chromosomal deficiency *Df(2R)BSC132* to remove Mmp2 function (21, 70). In each *mmp* mutant condition (*mmp*^{−/−}), we compared four genotypes at both restrictive (18°C) and permissive (30°C) temperatures (8 conditions total): the driver controls (*vglut-Gal4/+*), experimental animals (*vglut-Gal4>UAS-dTRPA1*), *mmp* driver controls (*vglut-Gal4/+*, *mmp*^{−/−}) and experimental mutants (*vglut-Gal4>UAS-dTRPA1*, *mmp*^{−/−}).

Unstimulated controls (*vglut-Gal4/+*) or transgenic experimental animals reared at the dTRPA1-restrictive temperature (*vglut-Gal4>UAS-dTRPA1*) displayed very few ghost boutons (Fig. 1A,B; Fig. S1A,B; Fig. S2A,B), consistent with previous reports (47, 61–63). In contrast, shifting *vglut-Gal4>UAS-dTRPA1* animals to a dTRPA1-permissive temperature resulted in a significant increase in activity-dependent ghost bouton formation (Fig. 1A,B; Fig. S2B,C). We did not observe any difference in ghost bouton number after shifting *vglut-Gal4/+* driver controls to the dTRPA1-permissive temperature, confirming the temperature shift did not contribute to synaptogenesis. We next tested the Mmp1 requirement for ghost bouton formation. In all non-stimulated conditions, ghost bouton number was completely indistinguishable between *vglut-Gal4/+* control, *vglut-Gal4>UAS-dTRPA1*, *vglut-Gal4/+*, *mmp1^{Q112*}/mmp1^{Q112*}* null, and the *vglut-Gal4>UAS-dTRPA1*, *mmp1^{Q112*}/mmp1^{Q112*}* animals (Fig S1A,B). After acute neuronal stimulation, we did not detect any increase in ghost bouton formation in the absence of Mmp1 (Fig. 1A,B). There was no detectable difference between the *mmp1* null driver control and the *mmp1* null dTRPA1 stimulated conditions. As a result, the *mmp1* null stimulated condition (*vglut-Gal4>UAS-dTRPA1*, *mmp1^{Q112*}/mmp1^{Q112*}*) had significantly reduced bouton numbers compared with the wildtype stimulated condition (*vglut-Gal4>UAS-dTRPA1*; Fig. 1A,B).

We next tested whether Mmp2 is similarly required for activity-dependent ghost bouton formation (Fig. S2). In all non-stimulated conditions, ghost bouton number was indistinguishable between *vglut-Gal4/+*, *vglut-Gal4>UAS-dTRPA1*, *vglut-Gal4/+*, *mmp2^{W307*}/Df(2R)BSC132* null and the *vglut-Gal4>UAS-dTRPA1*, *mmp2^{W307*}/Df(2R)BSC132* animals (Fig S2A,B). After acute neuronal stimulation, we saw a significant increase in ghost bouton formation in both the *vglut-Gal4>UAS-dTRPA1* (control) and *vglut-Gal4>UAS-dTRPA1*, *mmp2^{W307*}/Df(2R)BSC132* (mutant) conditions, with no significant difference detected between the groups (Fig. S2C,D). Consistently, activity-dependent ghost bouton formation in the *mmp2* null dTRPA1-stimulated condition was significantly increased relative to both the driver (*vglut-Gal4/+*) and the *mmp2* null (*vglut-Gal4/+*, *mmp2^{W307*}/Df(2R)BSC132*) stimulated controls (Fig. S2C,D). Together, these results show that Mmp2 is not detectably involved in ghost bouton formation, whereas Mmp1 is required for this activity-dependent synaptic bouton formation.

Co-localized Mmp1 and Dlp exhibit positive activity-dependent regulation

We next tested whether activity regulates the abundance of synaptic Mmps using both genetic and ionic stimulation paradigms (64, 65, 71, 72). We labeled Mmp1 with well-characterized antibodies, using detergent-free conditions to mark extracellular protein (7, 21, 73–75). Mmp1 abundance and spatial distribution were both increased by stimulation (Fig. 2). First, we again used dTRPA1 to transgenically increase activity, and found stimulation caused a significant >40% increase in the abundance of Mmp1 compared to that in matched controls (*vglut-Gal4/+*; Fig. 2A,B). We repeated tests with a more restricted Gal4 driver (*CcapR-Gal4*), which expresses dTRPA1 in only a subset of motor neurons to provide an additional internal control (Fig. S3). *CcapR-Gal4* drives in the RP3 motor neuron innervating muscle 6 and 7 (NMJ m6/7), but is excluded from the motor neuron innervating muscle 4 (NMJ m4). At the dTRPA1-restrictive temperature (18°C) we found no difference in Mmp1 between driver controls (*CcapR-Gal4/+*) and *CcapR-Gal4>UAS-dTRPA1*

conditions at either NMJ m6/7 or m4 (Fig. S3A,B). Mmp1 was significantly increased by >50% at NMJ m6/7 after the heat-induced dTRPA1 channel activation (30°C) in *CcapR-Gal4>UAS-dTRPA1* compared to the *CcapR-Gal4/+* driver control (Fig. S3C,D). However, there was no change in Mmp1 abundance at NMJ m4 after dTRPA1 neuronal stimulation, consistent with the restricted *CcapR-Gal4>UAS-dTRPA1* expression pattern (Fig. S3C,D).

Following the dTRPA1 transgenic trials, we tested Mmp1 abundance and distribution after depolarizing synaptic terminals with KCl (high [K⁺]) for varying time periods (2–60 mins). We found that synaptic Mmp1 was highly increased after just 10 minutes of acute stimulation (Fig. 2A,B), and therefore used this rapid stimulation paradigm for the remainder of our studies (64). Similar to the above dTRPA1 results, this acute depolarizing stimulation caused a highly significant >50% increase in Mmp1 at the synapse relative to unstimulated controls (Fig. 2A,B). Although Mmp2 had no detectable role in activity-dependent bouton formation (Fig. S2), we also tested whether Mmp2 is regulated by neuronal activity. We labeled extracellular Mmp2 under detergent-free conditions utilizing our previously characterized polyclonal antibody (7), and quantified Mmp2 abundance at the synapse before and after the ionic depolarization paradigm (Fig. S4). In contrast to Mmp1, synaptic Mmp2 was significantly decreased by ~35% following acute, high [K⁺] stimulation (Fig. S4A,B). Together, these results demonstrate that elevated neuronal activity causes concurrent strong Mmp1 increase and Mmp2 reduction at the synapse. Consistent with previous studies, the loss of Mmp2 may be a secondary consequence of increased Mmp1 function (7). The specific increase in Mmp1 supports its requirement in activity-dependent synaptic bouton formation.

We previously established a strong genetic interaction between Dlp and both Mmps at the NMJ (7). We therefore next tested whether Dlp is similarly regulated by activity (Fig. S5). Dlp was labeled with a well-characterized antibody (28, 41, 76), as well as a transgenic green fluorescent protein (GFP) tag in the endogenous *dlp* locus (*Dlp::GFP*) (77, 78). After an acute increase in activity, NMJs displayed an immediate and striking increase in synaptic Dlp intensity (Fig. S5A). With high [K⁺] depolarization, GFP labeling showed a significant >60% increase in *Dlp::GFP* and Dlp labeling consistently revealed a significant >60% increase compared to the unstimulated control condition (Fig. S5B). These results indicate that activity induces rapid upregulation of Dlp at the synapse. In unstimulated terminals, both Mmp1 and Dlp appeared in concurrent domains in a dynamic subset of synaptic boutons (Fig. 3A; arrows), with overlap but also with spatial separation of the two proteins. Upon acute stimulation, both proteins were significantly increased within the same synaptic subdomains (Fig. 3A, top panels; arrows), and the incidence of co-localization became much greater (Fig. 3A, bottom panels; asterisks). Given that HSPGs bind and localize MMPs in other contexts (34, 36, 39), we hypothesized that activity stimulates the co-localization of Dlp and Mmp1 at the synapse, with GPI-tethered Dlp serving to capture and localize the secreted Mmp1.

To test for this co-regulation, we triple-labeled unstimulated and high [K⁺] stimulated *Dlp::GFP* terminals with antibodies against GFP, Mmp1 and HRP. In parallel comparisons, we confirmed a significant ~50% increase in Dlp and Mmp1 after stimulation compared to unstimulated controls (Fig. 3A,B). Moreover, acute stimulation increased the spatial overlap

between Dlp and Mmp1, with specificity confirmed by multiple antibody and imaging controls (Fig. 3C and Fig. S6). We assessed co-occurrence with Manders' co-localization coefficient (MCC) measurements (79, 80). Upon stimulation, the Mmp1:Dlp MCC significantly increased over 2-fold compared to unstimulated controls (Fig. 3C). Likewise, the Dlp:Mmp1 MCC showed a similar significant increase in stimulated terminals (Fig. 3C). Thus, with acute stimulation both Dlp::GFP and Mmp1 MCC values were similarly increased compared to unstimulated controls, demonstrating a rapid activity-dependent recruitment of both proteins to co-localized synaptic domains. These results have four possible interpretations; 1) Dlp and Mmp1 have unrelated activity-dependent increases within the same dynamic synaptic domains, 2) secreted Mmp1 drives the activity-dependent Dlp increase, 3) membrane-tethered Dlp drives the activity-dependent Mmp1 increase, recruiting Mmp1 to synaptic subdomains, or 4) Dlp and Mmp1 activity-dependent increases are reciprocally co-dependent.

Dlp localizes Mmp1 in synaptic domains and promotes Mmp1 abundance

To first test the hypothesis that Dlp regulates Mmp1, we altered synaptic Dlp expression and quantified Mmp1 abundance. At the *Drosophila* NMJ, Dlp serves as a Wg co-receptor in a mechanism dependent on HS-GAG sulfation state, and Dlp also binds the receptor phosphatase dLAR through HS-GAG chains to modulate synaptogenesis (40, 41). Complete loss of *dlp* is embryonic lethal, so we used *dlp*^{A187/+} heterozygotes and *dlp*^{RNAi} (70, 81). To reduce Dlp at the NMJ, we targeted *dlp*^{RNAi} to both pre- (*elav-Gal4*) and postsynaptic (*24B-Gal4*) cells (*elav-Gal4*, *24B-Gal4*>*UAS-dlp*^{RNAi}). Both *dlp*^{A187/+} and *dlp*^{RNAi} significantly reduced synaptic Dlp abundance by >45% (Fig. S7A,B). In parallel, *dlp*^{A187/+} significantly reduced Mmp1 intensity by >30% compared to background controls (Fig. 4A,B), and *dlp*^{RNAi} likewise significantly decreased Mmp1 intensity by >20% compared to Gal4 driver controls (Fig. 4A,B). To conversely test whether Dlp overexpression reciprocally increases Mmp1, we postsynaptically overexpressed wild-type Dlp (*24B-Gal4*>*UAS-dlp*^{WT}; (82)). Excess Dlp caused a significant, >50% increase in synaptic Mmp1 (Fig. 4C,D). Moreover, the expanded Dlp domain caused a striking increase in perisynaptic Mmp1 localization (Fig. 4C). Taken together, these results indicate that Dlp positively regulates Mmp1 intensity and spatial distribution at the synapse.

HSPG interactions are often mediated through HS-GAG chain binding, which can anchor interactors in close proximity, influencing both diffusion and clustering (83, 84). In addition, however, HSPG core proteins also have well-characterized binding functions (38, 46, 85–87). Therefore, we next sought to test whether Dlp-dependent Mmp1 regulation is mediated through the Dlp core protein, HS-GAG chains, or both. To test Dlp HS-GAG chain requirements, we overexpressed a HS-deficient Dlp (*UAS-dlp*^{HS}), in which all 5 serine GAG attachment sites have been mutated to alanine (46). We reasoned if the Dlp-Mmp1 interaction is mediated by the Dlp core protein, then overexpressing HS-deficient Dlp should phenocopy the synaptic Mmp1 increase caused by overexpressing wild-type Dlp (Fig. 4C,D). We confirmed that the *24B-Gal4*>*UAS-dlp*^{HS} overexpression was comparable to the *24B-Gal4*>*UAS-dlp*^{WT} condition, with very high synaptic expression in both cases (Fig. S7C). However, in stark contrast to the striking Mmp1 increase with wild-type Dlp overexpression, Dlp^{HS} overexpression did not cause any change in synaptic Mmp1

abundance compared to Gal4 driver control (*24B-Gal4/+*, Fig. 4C,D). When the activity-induced Mmp1 increase caused by wild-type Dlp were compared to Dlp^{HS}, there was a significant decrease (Fig. 4C,D). These results strongly suggest that Dlp promotes synaptic Mmp1 localization through HS-GAG chain interaction.

Despite the lack of Mmp2 involvement in this synaptogenic mechanism, we also tested whether synaptic Mmp2 may be regulated by Dlp, independently or downstream of the Mmp1 changes. Dlp is a direct Mmp2 proteolytic substrate (70) and Mmp1 and Mmp2 are reciprocally co-regulated at the NMJ synapse under both basal conditions and after neuronal stimulation (Fig. 2; Figs. S3 and S4; (7)). To test whether Dlp regulates synaptic Mmp2, we again used *dlp*^{A187/+} heterozygotes to reduce Dlp and postsynaptic Dlp overexpression (*24B-Gal4>UAS-dlp*^{WT}) to increase Dlp, respectively (Fig. S7). We quantified Mmp2 abundance at the NMJ compared to matched controls under both conditions (Fig. S8). We did not observe any change in synaptic Mmp2 in the *dlp*^{A187/+} heterozygous condition compared to *w*¹¹¹⁸ genetic background control (Fig. S8A,B). Postsynaptic Dlp overexpression caused a subtle ~10% decrease in Mmp2 abundance at the NMJ (Fig. S8A,B). These data show that Dlp has a minor influence on synaptic Mmp2, but functions as a strong, bidirectional regulator of Mmp1 at the synapse.

Dlp is required for the rapid, activity-dependent increase of synaptic Mmp1

Because Dlp and Mmp1 are both increased by acute neuronal activity, and Dlp regulates synaptic Mmp1 abundance, we next hypothesized that Dlp is required for the activity-induced Mmp1 increase at the synapse. To test this hypothesis, we genetically manipulated Dlp and quantified Mmp1 after acute high [K⁺]-induced depolarization (Fig. 5). Supporting the above results, we found an activity-dependent increase in Mmp1 abundance in both *w*¹¹¹⁸ background and Gal4 driver controls, with significant increases in all comparisons. Reducing Dlp expression abolished any detectable Mmp1 increase in response to the acute activity stimulation (Fig. 5A,B). Indeed, we found instead a small ~15% decrease in synaptic Mmp1 abundance in the stimulated *dlp*^{A187/+} heterozygotes relative to unstimulated *dlp*^{A187/+} controls (Fig. 5B). The *dlp*^{RNAi} condition yielded very similar results, with a small, non-significant ~12% reduction in synaptic Mmp1 after the high [K⁺] stimulation (Fig. 5B). Conversely, the activity-stimulated Mmp1 abundance was significantly increased a further ~65% with Dlp overexpression (Fig. 5C,D). The basal amount of Mmp1 at the synapse was increased by Dlp overexpression (Fig. 4), yet there was still a strong increase in synaptic Mmp1 abundance after acute depolarizing stimulation, consistent with Dlp promoting the activity-dependent increase in Mmp1. Taken together, these results show that Dlp is required for the activity-dependent Mmp1 increase at the synapse.

Previous work has established that HS-GAG chains often mediate Dlp functions and binding interactions (41, 46, 82). Therefore we next tested whether a HS-GAG chain-deficient Dlp (*UAS-dlp*^{HS}) interferes with the activity-dependent synaptic Mmp1 induction (Fig. 5C,D). Although the endogenous Dlp with intact HS-GAG chains was still present, we hypothesized that the HS-GAG chain deficient Dlp should dominantly dampen activity-induced Mmp1 elevation. After acute high [K⁺]-induced depolarization, we observed increased synaptic abundance of Mmp1 in both *24B-Gal4/+* driver control and *24B-Gal4>UAS-dlp*^{WT}

overexpression conditions, with a significant increase in both conditions (Fig. 5C,D). However, the synaptic Mmp1 abundance in the activity-stimulated *24B-Gal4>UAS-dlp^{HS}* synapses was only slightly increased (by 19%) compared to the matched unstimulated controls, a much weaker albeit still significant response (Fig. 5C,D). In sharp contrast, the activity-induced Mmp1 increase was significantly reduced at *24B-Gal4>UAS-dlp^{HS}* NMJ synapses compared with the wild-type Dlp overexpression condition (19% vs. ~65%, respectively). Taken together, these results support the conclusion that both basal and activity-induced Mmp1 regulation by Dlp at the synapse are directly mediated through HS-GAG chain-binding interactions in the extracellular space.

At NMJs lacking Mmp1 (*mmp1* null mutants), basal Dlp abundance is reduced and Dlp is more spatially restricted, thus Mmp1 reciprocally serves as a positive regulator of synaptic Dlp (7). We consistently found above that Dlp is required for the activity-dependent Mmp1 increase at the synapse (Fig. 5). However, there still might be a reciprocal Mmp1 requirement for the activity-dependent increase in Dlp abundance. To test for this putative co-regulation, we next genetically reduced Mmp1 abundance using two independent approaches and then quantified Dlp intensity after acute high [K⁺]-induced depolarization (Fig. S9). Consistent with above results, control animals displayed a significant >30% increase in synaptic Dlp following the acute stimulation (Fig. S9A,B). Using the combinatorial pre- and postsynaptic *mmp1^{RNAi}* knockdown (*elav-Gal4, 24B-Gal4>UAS-mmp1^{RNAi}*), synaptic Mmp1 was reduced by ~70%, yet we still observed the significant increase in Dlp following depolarizing stimulation (Fig. S9C,D). We validated these results in a *trans*-heteroallelic *mmp1* mutant condition (*mmp1^{Q112*}/mmp1^{Q273*}*) and again observed a similar, maintained >30% increase in synaptic Dlp following acute stimulation (Fig. S9A,B). These results indicate that Mmp1 is not reciprocally required for the activity-dependent Dlp increase at the synapse. Taken together, the data suggest that Mmp1 is downstream of Dlp in this activity-dependent mechanism.

Synaptic Dlp bidirectionally determines proteolytic function at the NMJ

After discovering that synaptic Mmp abundance and distribution are dependent on Dlp (Figs. 4, 5), we next assayed whether these changes correlate with synaptic proteolytic activity. We used in situ zymography at the NMJ to measure the metalloproteinase-dependent conversion of a dye-quenched fluorogenic gelatin (DQ-gelatin), and quantified fluorescence changes in both the *dlp* reduction and overexpression conditions used above (33, 88). Although this method cannot differentiate the contributions of different proteases, it provides a live readout of net enzymatic activity through quantifiable fluorescence changes. Consistent with above results, *dlp^{A187/+}* heterozygotes showed a significant ~35% reduction in proteolytic activity at the synapse compared to *w¹¹¹⁸* controls (Fig. 6A,B). Moreover, Dlp overexpression (*24B-Gal4>UAS-dlp^{WT}*) resulted in a significant ~50% increase in proteolytic activity at the synapse (Fig. 6A,C). We also tested whether the Dlp-mediated increase in proteolytic function required HS-GAG chains as above (*24B-Gal4>UAS-dlp^{HS}*). These NMJs still displayed a significant ~60% increase in proteolytic activity compared to the matched driver control (*24B-Gal4/+*), with no detectable difference between Dlp^{WT} and Dlp^{HS} overexpression conditions (Fig. 6A,C). Taken together, we conclude that Dlp abundance is a strong determinant of proteolytic gelatinase activity at the synapse, consistent with above

measurements of Dlp-dependent synaptic Mmp1 abundance, with the enzymatic function reliant upon the Dlp core protein.

FMRP regulation of activity-dependent synaptic Mmp1 requires Dlp function

Dlp is upregulated in the *Drosophila* model of FXS (*dfmr1* null mutant), Mmp1 and Dlp genetically interact with FMRP, and independently correcting either Dlp or Mmp1 in *dfmr1* mutants ameliorates FXS-associated synaptogenic defects (13, 28, 33). In conjunction with our above findings, this led us to hypothesize a coordinated FMRP-HSPG-Mmp axis converging in the synaptomatrix to enable activity-dependent synaptogenesis. To test this hypothesis, we first quantified synaptic Mmp1 in *dfmr1* null mutants (89). Mmp1 was significantly increased by >50% in *dfmr1* animals relative to controls (Fig. 7A,B). Consistently, we detected a parallel >40% increase in synaptic Dlp at *dfmr1* null synapses, a similar significant increase compared to controls (Fig. S10A,B). Our working model proposes Dlp positively regulates synaptic Mmp1, and we therefore hypothesized that increased Dlp is causative for the corresponding Mmp1 increase in the FXS model. To test this, we removed one copy of *dlp* in the *dfmr1* null background to reduce Dlp abundance, and then tested whether this restored normal synaptic Mmp1 intensity (Fig. 7A,B). As expected, Dlp expression was significantly reduced at *dlp*^{A187/+}, *dfmr1/dfmr1* synapses compared to the FXS model, with Dlp signal intensity comparable to wild-type controls (Fig. S10A,B). Moreover, the 50% Mmp1 increase observed in the FXS model was fully corrected in *dlp*^{A187/+}, *dfmr1/dfmr1* synapses, with Mmp1 signal intensity now indistinguishable from controls (Fig. 7A,B). Therefore, *dlp* co-removal in the FXS disease model is sufficient to restore normal synaptic Mmp1 abundance.

Finally, we asked whether FMRP is required for the activity-induced, Dlp-mediated synaptic Mmp1 enhancement. To test this hypothesis, we again stimulated synapses with high [K⁺] and quantified synaptic Mmp1 (Fig. 7C,D). After stimulation, wild-type synapses displayed a significant >50% increase in Mmp1 compared to unstimulated controls (Fig. 7C,D). Conversely, there was no change in synaptic Mmp1 abundance following stimulation in *dfmr1* null mutants (Fig. 7C,D). Incorporating this into our working model predicts that the FMRP-dependent activity-induced increase in Mmp1 is mediated through Dlp. Therefore, we hypothesized that Dlp reduction in *dfmr1* null animals would restore activity-induced Mmp1 regulation. Consistently, removing a single copy of *dlp* in the FXS model was sufficient to fully restore the activity-induced Mmp1 increase following activity stimulation (Fig. 7C,D). Similar to the stimulated controls (*w*¹¹¹⁸), synaptic Mmp1 was significantly increased by >50% in the stimulated *dlp*^{A187/+}, *dfmr1/dfmr1* condition compared to unstimulated *dlp*^{A187/+}, *dfmr1/dfmr1* synapses (Fig. 7C,D). Accordingly, stimulated *dlp*^{A187/+}, *dfmr1/dfmr1* synapses now displayed a significant increase compared to stimulated *dfmr1* homozygous null mutants (Fig. 7D). Thus, *dlp* co-removal in the FXS disease model restores activity-induced Mmp1 regulation back towards the wild-type condition. Collectively, the findings provide the previously missing mechanistic link in the FMRP-Dlp-Mmp1 pathway, leading us to propose Dlp misregulation mediates the disease-associated Mmp1 dysfunction causal for the FXS model's synaptogenic defects that characterize the disease.

Discussion

We report here that Mmp1, but not Mmp2, is required for rapid, activity-dependent synapse development (ghost bouton formation) in *Drosophila* (47, 64). Although both *Drosophila* Mmps act to restrict NMJ growth over developmental time (7), there is a clear differential requirement in fast activity-dependent de novo synaptic bouton formation. Thus, distinct Mmp-dependent pathways control basal versus activity-induced synaptogenesis. Similarly separable roles have been shown for parallel molecular mechanisms. For example, the actin regulator Cortactin is required for fast activity-dependent ghost bouton formation, but has no detectable role in basal synaptic bouton maturation (90). Moreover, high $[K^+]$ depolarizing activity stimulation quickly restricts miRNA functions with exclusively activity-dependent roles (91, 92). Our results suggest that Mmps (including proteolytic substrates or effectors) differentially control synaptic bouton formation in distinct time frames, revealing context-specific roles in long-term development versus acute, activity-dependent synaptogenesis. Consistent with the selective Mmp1 requirement, we also find that neuronal stimulation only increased the extracellular localization and abundance of Mmp1, whereas that of Mmp2 was reciprocally suppressed by acutely increased activity. This bidirectional co-regulation by neuronal activity may reflect Mmp class interactions, given that Mmp2 restricts synaptic Mmp1 abundance, spatial distribution and synaptogenesis requirements (7). This likely represents an important extracellular regulatory mechanism at the synapse, controlling the function of Mmp-dependent synaptomatrix outputs.

Both thermogenic dTRPA1 stimulation and the much more acute high $[K^+]$ -induced depolarization induce a rapid increase in synaptic Mmp1 abundance and Mmp1-dependent new bouton formation, suggesting activity-dependent Mmp1 function enables synaptogenesis. Given the relatively rapid responses to high $[K^+]$ stimulation (10 minutes), the synaptic Mmp1 increase is most likely a post-transcriptional mechanism occurring directly at the synapse. Multiple molecular changes induced by acute high $[K^+]$ are cycloheximide-independent (64). We hypothesized the Mmp1 increase occurs through extracellular regulation, and identified a key interaction with the membrane-tethered HSPG Dlp. We observed that synaptic abundance of Dlp rapidly increased with acute neuronal stimulation, and tight spatial co-localization with Mmp1 in synaptic subdomains was heightened by increased activity. Our data demonstrates that Dlp is a strong, positive regulator of synaptic Mmp1, with Dlp bidirectionally determining Mmp1 abundance at the synapse. As a GPI-anchored glypican, Dlp can interact with many extracellular molecules to bring Mmp1 substrates within close proximity. In parallel, Mmp2 proteolytically cleaves Dlp to regulate Wg signaling in the *Drosophila* ovary (70), and a similar role likely occurs at the synapse, where Mmp2 spatially confines Dlp in the synaptic domain (7). Thus, Mmp2-dependent Dlp processing may antagonize the Dlp-Mmp1 interaction, which would necessitate reciprocal co-regulation of Mmp1 and Mmp2.

We found that Dlp functions are context-dependent, with synaptic Dlp coordinating signaling mechanisms converging in the synaptomatrix. Consistently, synaptic defects observed in mutant conditions that also concurrently display misregulated Dlp are remediated by restoring Dlp expression back towards wild-type (7, 28). HSPGs consist of a core protein linked to sulfated GAG chains, with both components contributing to ligand

interactions (38, 46, 83–87). We found that an HS-GAG chain-deficient Dlp was unable to efficiently retain Mmp1 at the synapse or enhance activity-dependent control of Mmp1 abundance. We suggest this defect arises from loss of direct binding between Mmp1 and Dlp HS-GAG chains. Protease-HSPG interactions are bidirectional, with HS-GAG chains regulating protease localization, activity and inhibition, whereas proteases conversely mediate HSPG proteolytic processing and turnover through cleavage of core proteins (34–39, 93). We suggest the Dlp-Mmp1 interaction is mediated through HS-GAG chains through a non-proteolytic mechanism (7). We further suggest that the synaptic co-localization of Dlp and Mmp1 is mediated through Dlp HS-GAG chains, with Dlp recruiting essential Mmp1 function driving activity-dependent bouton formation. With in situ zymography proteolytic function tests at the synapse (33, 88), we consistently found that reduced Mmp function correlated with Dlp depletion and increased function with Dlp overexpression. However, enzymatic activity appears primarily dependent upon interaction with the Dlp core protein. Note that this assay reflects net proteolytic function, with likely context-dependent contributions by a variety of synaptic proteases.

Our data from the *Drosophila* disease model of FXS indicate that Mmp1 is constitutively increased and that activity-dependent Mmp1 enhancement is lost, but that both defects are prevented when Dlp is suppressed. The mouse FXS model mirrors effects observed in *Drosophila*: mouse MMP-9 is similarly upregulated, and pharmacological and genetic inhibition of MMP-9 restores synaptic development (13, 94, 95). This suggests the HSPG-Mmp mechanism identified here may be conserved in mammals as well. The FMRP mRNA-binding translational repressor is required for activity-dependent synaptogenesis and is itself directly upregulated by neuronal activity (51–54). Moreover, HSPGs are predicted FMRP targets (32), and synaptic HSPGs are increased in the *Drosophila* FXS model (28). Thus, FMRP might directly regulate *mmp1* and/or *dlp* translation in an activity-dependent mechanism. HSPG Dlp functions as a bifunctional Wg co-receptor during normal synaptogenesis, with impaired Wg *trans*-synaptic signaling causative in FXS disease-associated synaptogenic defects (28, 40). Mmp1, Dlp and Wg are at least partially interdependent, and all three are disrupted in the FXS disease state, which could therefore collectively impede the activity-induced Mmp1 enhancement characterizing the FXS condition, dependent on the Dlp interaction. It will be of great interest in our future studies to dissect the differential activity requirements of this FMRP-Wg-Dlp-Mmp1 mechanism.

How do FMRP, Dlp and Mmp1 intersect in activity-dependent synaptogenesis? Dlp is a positive regulator of Mmp1, and is required for activity-dependent increases in Mmp1 abundance. Why then is the activity-induced Mmp1 increase not present in the FXS disease model despite the more abundant synaptic Dlp? It seems counterintuitive that *dfmr1* mutants display increased Dlp, yet the activity-induced Mmp1 increase was completely abolished, and this defect could be corrected by reducing Dlp. One possibility is a ceiling effect in the FXS condition, in which maximal amounts of Dlp or Mmp1 no longer permit changes in response to activity. We feel this unlikely because Mmp1 was significantly increased by Dlp overexpression, yet an activity-induced Mmp1 increase persisted. Another possibility is the relative abundance of Dlp and Mmp1 control their interactions. Consistently, Dlp has biphasic functions as both a positive and a negative regulator of Wg *trans*-synaptic signaling, depending on relative abundance of Wg ligand, Frizzled-2 receptor and Dlp co-receptor (40,

46). Is an unknown effector(s) regulated by FMRP modulating the Dlp-Mmp1 intersection? We feel this most likely, as numerous activity-dependent changes can increase Dlp in the synaptomatrix. As a core signaling platform, Dlp could interact with an unknown protein(s) in Mmp1 secretion or sequestration. In any case, it is apparent that FMRP loss impedes Dlp and Mmp1 interaction in response to neuronal activity, and that reducing Dlp in the FXS model restores this core activity-dependent intersection, enabling appropriate control of Mmp1 induction at the synapse.

Materials and Methods

Drosophila genetics

All stocks were maintained on standard medium at 25°C, except for lines used to manipulate neuronal activity with dTRPA1 (see below). The following Gal4 driver lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC; Bloomington, Indiana): pan-neuronal driver *elav-Gal4* (#8760), selective neuronal driver *CcapR-Gal4* (#39292), glutamatergic neuron driver *vglut-Gal4* (#26160) and muscle-specific driver *24B-Gal4* (#1767). The dual neuron and muscle driver *elav-Gal4*, *24B-Gal4* was created using standard genetic recombination (96). For the dTRPA1 studies, *vglut-Gal4* or *CcapR-Gal4* were crossed to the warmth-activated *UAS-dTRPA1* (BDSC, #26263) cation channel transgene to stimulate activity within motor neurons (65–69). Genetic lines used to manipulate Mmps include; 1) *mmp1^{Q112*}* (BDSC #59380) genetic null loss of function allele, 2) *mmp1^{Q273*}* hypomorphic allele caused by a point mutation early stop codon, 3) *UAS-mmp1^{RNAi}* (97), 4) *mmp2^{W307*}* genetic null loss of function allele, and 5) chromosomal deficiency *Df(2R)BSC132* (BDSC #9410) that removes the entire *mmp2* gene (21, 70). Standard recombination was used to create *vglut-Gal4*, *mmp1^{Q112*}* and *vglut-Gal4*, *mmp2^{W307*}*, and *UAS-dTRPA1*, *mmp1^{Q112*}* and *UAS-dTRPA1*, *Df(2R)BSC132* lines, which were then crossed to generate *vglut-Gal4>UAS-dTRPA1*, *mmp1^{Q112*}/mmp1^{Q112*}* and *vglut-Gal4>UAS-dTRPA1*, *mmp2^{W307*}/Df(2R)BSC132*, respectively. The genetic lines used to manipulate Dlp expression include; 1) *dlp^{A187}*, a 26-nucleotide reading frame shift deleting the GAG attachment domain, GPI-anchor and part of the cysteine-rich region (81), 2) *UAS-dlp^{RNAi}* (Vienna *Drosophila* Resource Center (VDRC), #10299), 3) wildtype *UAS-dlp^{WT}* (BDSC, #9160; (82), and 4) *UAS-dlp^{HS}*, with all 5 GAG attachment sites (Ser⁶²⁵, Ser⁶²⁹, Ser⁶³¹, Ser⁶⁴³ and Ser⁶⁸⁶) mutated to alanines (46, 87). The Dlp::GFP line used was *y¹, w^{*}; dlp^{MI04217-GFSTE1}* (BDSC, #60540; (77, 78) containing an *EGFP-FIAsH-StrepII-TEV-3xFlag* insertion within a *dlp* coding intron, shared by both annotated transcripts, resulting in expression of Dlp tagged with internal GFP tag incorporated into the endogenous *dlp* locus. The *dfmr1^{50M}* deletion allele is a full loss of function null mutant (89). Standard genetic recombination was used to generate *dlp^{A187}*, *dfmr1^{50M}/TM6*, Hu-GFP (28), which was subsequently crossed to *dfmr1^{50M}/TM6*, Tb to produce *dlp^{A187/+}*, *dfmr1^{50M/50M}* animals. Genetic controls included the *w¹¹¹⁸* genetic background, as well as *Gal4* drivers alone crossed into the *w¹¹¹⁸* background.

Activity manipulations

A high [K⁺] depolarization paradigm was performed for 10 minutes using 90mM KCl in 1.8 mM CaCl₂ saline (64). Unstimulated controls received a 10-minute mock treatment with

standard physiological saline (see below). For dTRPA1 studies, the pan-motor neuron driver *vglut-Gal4* (BDSC #26160, *OK371*) (98–100) or selective neuronal driver *CcapR-Gal4* (BDSC #39292 (*101–103*)), were crossed to warmth-activated *UAS-dTRPA1* cation channel transgene (BDSC, #26263) to stimulate activity (65–69). Animals were raised at the dTRPA1-restrictive temperature (18°C) until the wandering 3rd instar stage, then transferred to pre-temperature treated apple juice agar plates for 1-hour at the dTRPA1-permissive temperature (30°C). Controls included *vglut-Gal4/+* or *CcapR-Gal4/+* (+/- temperature shift), *vglut-Gal4/+*, *mmp1^{Q112*}/mmp1^{Q112*}* or *vglut-Gal4/+*, *mmp2^{w307*}/Df(2R)BSC132* (+/- temperature shift), *vglut-Gal4>UAS-dTRPA1* and *CcapR-Gal4>UAS-dTRPA1* (no temperature shift), and *vglut-Gal4>UAS-dTRPA1*, *mmp1^{Q112*}/mmp1^{Q112*}* or *vglut-Gal4>UAS-dTRPA1*, *mmp2^{w307*}/Df(2R)BSC132* (no temperature shift).

Immunocytochemistry confocal imaging

Imaging was performed on wandering 3rd instars at muscle 4 and 6/7 NMJs. Staged larvae were dissected in physiological saline (in mM: 128 NaCl, 2 KCl, 4 MgCl₂, 70 sucrose, 5 HEPES, and 0 CaCl₂ pH 7.2), fixed with 4% paraformaldehyde (4% PFA + 4% sucrose in phosphate buffered saline (PBS)) for 15 minutes, washed 3X with PBS, and then incubated with primary antibodies diluted in PBS overnight at 4°C. Preparations were then washed 3X with PBS, incubated with secondary antibodies diluted in PBS for 2 hours at room temperature, washed 3X with PBS, and then mounted in Fluoromount-G (Electron Microscopy Sciences). For co-localization studies, a 1-hour blocking step (0.5% Bovine Serum Albumin (BSA) in PBS) was used, with primary and secondary antibody incubations containing 0.5% BSA in PBS. Primary antibodies included: Alexa-Fluor 488 conjugated goat α-HRP (1:200; Jackson Laboratories 123-545-021), Cy3-conjugated goat α-HRP (1:250; Jackson Laboratories 123-165-021), Cy5-conjugated goat α-HRP (1:200; Jackson Laboratories 123-605-021), rabbit α-GFP (Abcam, ab290), rabbit α-Mmp2 (1:500), mouse α-Mmp1 (1:1:1 at 1:10; DSHB, 3B8D12, 3A6B4, 5H7B11), mouse α-DLG (1:200; DSHB, DLG1) and mouse α-Dlp (1:5; DSHB, 13G8). Secondary antibodies included (all 1:500; Invitrogen): goat α-mouse (488, 568), donkey α-mouse 555, donkey α-rabbit 488 and goat α-rabbit (488, 568). All labeling was performed detergent-free (extracellular labeling only (73, 74)), except for structural studies using mouse α-DLG, which required addition of 0.1% Triton X-100 to both primary and secondary antibody incubations. NMJ Z-stacks were acquired with a Zeiss LSM 510 META laser-scanning confocal using 40×/1.4 (ghost boutons) and 63×/1.4 (all other experiments) Plan Apochromat oil immersion objectives.

Synaptic in situ zymography

NMJ in situ zymography assays were performed as previously described (33, 88). Briefly, live larval preparations were dissected exposing the neuromusculature in physiological saline, and then immediately submerged in the zymography buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl₂, 0.2 mM NaN₃, pH 7.6) containing 500 µg/ml of fluorescein-conjugate DQ porcine gelatin (Molecular Probes by Life Technologies, D-12054). Preparations were incubated for 45 minutes at room temperature, kept stationary and protected from light. Preparations were then briefly rinsed 3X in PBS, fixed in 4% PFA in PBS for 30 minutes, and washed again 3X in PBS. Preparations were then incubated with Cy3-conjugated goat α-HRP (1:250; Jackson Laboratories 123-165-021) for 1 hour at room

temperature. Preparations were finally washed again 3X in PBS and mounted in Fluoromount-G (Electron Microscopy Sciences). NMJ Z-stacks were acquired with a Zeiss LSM 510 META laser-scanning confocal microscope using a 488 nm argon laser to excite the fluorescein substrate, and imaged with a 63x/1.4 Plan Apo oil immersion objective.

Quantification and statistical analyses

All experiments were independently performed at least 3 times, with all comparisons performed blind. All analyses were done on staged and sized-matched animals. For each trial, 3–5 animals per condition were assayed, with NMJs from both left and right segments (A3–A4) analyzed. For all intensity comparisons, images were obtained with the same confocal settings and quantified in parallel using NIH ImageJ software (104). Intensity measurements were made with the HRP signal delineated from Z-stack areas of maximum projections. Muscle intensity was taken from areas adjacent to the HRP-marked NMJ, and subsequently subtracted from the NMJ intensity measurement. NMJ and background region selection excluded regions that overlapped with obstructions, such as trachea. For high $[K^+]$ stimulation studies involving multiple genotypes, each stimulated condition was normalized to its own unstimulated genotype control. Confocal settings were maintained constant for unstimulated and stimulated conditions within each genotype. All cross-compared genotypes within each experiment were processed together and imaged at the same time. Ghost boutons were quantified as HRP-positive and DLG-negative varicosities emanating from the main NMJ arbor. DLG-negative was defined as <2 standard deviations below the mean. Contrast and brightness were applied uniformly using ImageJ. Co-localization analyses were performed using ZEN image processing software (Zeiss). Single slices ($<1 \mu\text{m}$) from the middle of a Z-stack were analyzed for each NMJ. Measurements of full width at half maximum (FWHM) for axial resolution were FWHM_{488} : 840nm and FWHM_{555} : 880nm. HRP was used as a guide to create an ROI. Thresholds were manually set based on background intensity values. Manders' overlap coefficients and weighted Manders' co-localization coefficients were used (80). All images were filtered and processed in parallel in ImageJ before being exported to Adobe Photoshop. ImageJ versions used were 1.46r, 1.51h and 1.51p (104, 105). All reported statistical comparisons were always performed using Instat3 software (GraphPad Software). Mann-Whitney U tests and ANOVAs (Kruskal-Wallis) were used for all the nonparametric comparisons. ANOVA tests were used for all data sets of 3 comparisons, followed by appropriate post hoc analyses as stated in the figure legends. All data are presented in figures as $\text{mean} \pm \text{SEM}$, and N represents the total number of NMJs analyzed from at least 3 independent replicates. Significance is indicated in figures by $*p<0.05$, $**p<0.01$ and $***p<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Bloomington *Drosophila* Stock Center (BDSC, Indiana University) and the Vienna *Drosophila* RNAi Center (VDRC, www.vdrc.at; Vienna, Austria) for essential stocks used in this study. We thank the Developmental Studies Hybridoma Bank (DSHB, University of Iowa) for providing critical antibodies used in this study. We are particularly grateful to Dr. Xinhua Lin (Fudan University, China and Cincinnati Children's Hospital Medical

Center, Ohio USA) for the *UAS-dlp^{HS}* mutant transgene and Dr. Dirk Bohmann (University of Rochester Medical Central, New York) for the *UAS-mmp1^{RNAi}* line. We are grateful to Caleb Doll and Tyler Kennedy for manuscript input, and the entire Broadie lab for helpful insights. This work was supported by NIH grants MH096832 and MH084989 to KB. MLD performed all the experiments except for the zymography assays, which were performed by JS. KB and MLD jointly conceived the experiments, and KB oversaw all the studies. The manuscript was co-written by MLD and KB.

References

1. Barros CS, Franco SJ, Müller U. Extracellular Matrix: Functions in the Nervous System. *Cold Spring Harb Perspect Biol.* 2011; 3:a005108. [PubMed: 21123393]
2. Dani N, Broadie K. Glycosylated synaptomatrix regulation of trans-synaptic signaling. *Dev Neurobiol.* 2012; 72:2–21. [PubMed: 21509945]
3. Benson DL, Huntley GW. Building and remodeling synapses. *Hippocampus.* 2012; 22:954–968. [PubMed: 20882551]
4. Conant K, Allen M, Lim ST. Activity dependent CAM cleavage and neurotransmission. *Front Cell Neurosci.* 2015; 9:305. [PubMed: 26321910]
5. Huntley GW. Synaptic circuit remodelling by matrix metalloproteinases in health and disease. *Nat Rev Neurosci.* 2012; 13:743–57. [PubMed: 23047773]
6. Shinoo T, Goda Y. Tuning synapses by proteolytic remodeling of the adhesive surface. *Curr Opin Neurobiol.* Dec 2.2015 :148–155.
7. Dear ML, Dani N, Parkinson W, Zhou S, Broadie K. Two matrix metalloproteinase classes reciprocally regulate synaptogenesis. *Development.* 2016; 143:75–87. [PubMed: 26603384]
8. Ethell IM, Ethell DW. Matrix metalloproteinases in brain development and remodeling: Synaptic functions and targets. *J Neurosci Res.* 2007; 85:2813–2823. [PubMed: 17387691]
9. Wlodarczyk J, Mukhina I, Kaczmarek L, Dityatev A. Extracellular matrix molecules, their receptors, and secreted proteases in synaptic plasticity. *Dev Neurobiol.* 2011; 71:1040–1053. [PubMed: 21793226]
10. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res.* 2006; 69:562–573. [PubMed: 16405877]
11. Nagappan-Chettiar S, Johnson-Venkatesh EM, Umemori H. Activity-dependent proteolytic cleavage of cell adhesion molecules regulates excitatory synaptic development and function. *Neurosci Res.* 2017; 116:60–69. [PubMed: 27965136]
12. Reinhard SM, Razak K, Ethell IM. A delicate balance: role of MMP-9 in brain development and pathophysiology of neurodevelopmental disorders. *Front Cell Neurosci.* 2015; 9doi: 10.3389/fncel.2015.00280
13. Sidhu H, Dansie LE, Hickmott PW, Ethell DW, Ethell IM. Genetic removal of matrix metalloproteinase 9 rescues the symptoms of fragile X syndrome in a mouse model. *J Neurosci.* 2014; 34:9867–9879. [PubMed: 25057190]
14. Tsilibary, E., Tzinia, A., Radenovic, L., Stamenkovic, V., Lebitko, T., Mucha, M., Pawlak, R., Frischknecht, R., Kaczmarek, L. *Progress in Brain Research.* Dityatev, A. Wehrle-Haller, B., Pitkanen, A., editors. Vol. 214. Elsevier; 2014. p. 135-157.
15. Vafadari B, Salamian A, Kaczmarek L. MMP-9 in translation: from molecule to brain physiology, pathology, and therapy. *J Neurochem.* 2016; 139:91–114.
16. Broadie K, Baumgartner S, Prokop A. Extracellular matrix and its receptors in Drosophila neural development. *Dev Neurobiol.* 2011; 71:1102–1130. [PubMed: 21688401]
17. Harris KP, Littleton JT. Transmission, development, and plasticity of synapses. *Genetics.* 2015; 201:345–375. [PubMed: 26447126]
18. Menon K, Carrillo R, Zinn K. Development and Plasticity of the Drosophila Larval Neuromuscular Junction. *Wiley Interdiscip Rev Dev Biol.* 2013; 2:647–670. [PubMed: 24014452]
19. Llano E, Pendas AM, Aza-Blanc P, Kornberg TB, Lopez-Otin C. Dm1-MMP, a matrix metalloproteinase from Drosophila with a potential role in extracellular matrix remodeling during neural development. *J Biol Chem.* 2000; 275:35978–35985. [PubMed: 10964925]

20. Llano E, Adam G, Pendás AM, Quesada V, Sánchez LM, Santamaría I, Noselli S, López-Otín C. Structural and enzymatic characterization of *Drosophila* Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue-specific expression. *J Biol Chem*. 2002; 277:23321–23329. [PubMed: 11967260]
21. Page-McCaw A, Serano J, Sante JM, Rubin GM. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. *Dev Cell*. 2003; 4:95–106. [PubMed: 12530966]
22. LaFever K, Wang X, Page-McCaw P, Bhawe G, Page-McCaw A. Both *Drosophila* matrix metalloproteinases have released and membrane-tethered forms but have different substrates. *Sci Rep*. 2017; 7doi: 10.1038/srep44560
23. Miller CM, Page-McCaw A, Broihier HT. Matrix metalloproteinases promote motor axon fasciculation in the *Drosophila* embryo. *Development*. 2008; 135:95–109. [PubMed: 18045838]
24. Miller CM, Liu N, Page-McCaw A, Broihier HT. *Drosophila* Mmp2 Regulates the Matrix Molecule Faulty Attraction (Frac) to Promote Motor Axon Targeting in *Drosophila*. *J Neurosci*. 2011; 31:5335–5347. [PubMed: 21471368]
25. Kuo CT, Jan LY, Jan YN. Dendrite-specific remodeling of *Drosophila* sensory neurons requires matrix metalloproteases, ubiquitin-proteasome, and ecdysone signaling. *Proc Natl Acad Sci U S A*. 2005; 102:15230–15235. [PubMed: 16210248]
26. Yasunaga KI, Kanamori T, Morikawa R, Suzuki E, Emoto K. Dendrite Reshaping of Adult *Drosophila* Sensory Neurons Requires Matrix Metalloproteinase-Mediated Modification of the Basement Membranes. *Dev Cell*. 2010; 18:621–632. [PubMed: 20412776]
27. Depetris-Chauvin A, Fernández-Gamba Á, Gorostiza EA, Herrero A, Castaño EM, Ceriani MF. Mmp1 Processing of the PDF Neuropeptide Regulates Circadian Structural Plasticity of Pacemaker Neurons. *PLoS Genet*. 2014; 10:e1004700. [PubMed: 25356918]
28. Friedman SH, Dani N, Rushton E, Broadie K. Fragile X mental retardation protein regulates trans-synaptic signaling in *Drosophila*. *Dis Model Mech*. 2013; 6:1400–1413. [PubMed: 24046358]
29. Bassell GJ, Warren ST. Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. *Neuron*. 2008; 60:201–214. [PubMed: 18957214]
30. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. *Eur J Hum Genet*. 2008; 16:666–672. [PubMed: 18398441]
31. Verkerk AJ, Pieretti M, Surtcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GJB, Bionden LA, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, et al. Identification of a Gene (FMR-1) Containing a CGG Repeat Coincident with a Breakpoint Cluster Region Exhibiting Length Variation in Fragile X Syndrome. *Cell*. 1991; 65:905–914. [PubMed: 1710175]
32. Darnell JC, Van Driesche SJ, Zhang C, Hung KYS, Mele A, Fraser CE, Stone EF, Chen C, Fak JJ, Chi SW, Licatalosi DD, Richter JD, Darnell RB. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell*. 2011; 146:247–261. [PubMed: 21784246]
33. Siller SS, Broadie K. Neural circuit architecture defects in a *Drosophila* model of Fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase. *Dis Model Mech*. 2011; 4:673–685. [PubMed: 21669931]
34. Georges S, Heymann D, Padrines M. Modulatory effects of proteoglycans on proteinase activities. *Methods Mol Biol*. 2012:307–322.
35. Tocchi A, Parks WC. Functional interactions between matrix metalloproteinases and glycosaminoglycans. *FEBS J*. 2013; 280:2332–2341. [PubMed: 23421805]
36. Murphy G, Nagase H. Localizing matrix metalloproteinase activities in the pericellular environment. *FEBS J*. 2011; 278:2–15. [PubMed: 21087456]
37. Ramani VC, Pruett PS, Thompson CA, DeLucas LD, Sanderson RD. Heparan sulfate chains of syndecan-1 regulate ectodomain shedding. *J Biol Chem*. 2012; 287:9952–9961. [PubMed: 22298773]
38. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 2011; 3:a004952. [PubMed: 21690215]

39. Theocharis A, Gialeli C, Bouris P, Giannopoulou E, Skandalis S, Aletras A, Iozzo R, Karamanos N. Cell-matrix interactions: focus on proteoglycan-proteinase interplays and pharmacological targeting in cancer. *FEBS J.* 2014; 281:5023–5042. [PubMed: 25333340]
40. Dani N, Nahm M, Lee S, Broadie K. A Targeted Glycan-Related Gene Screen Reveals Heparan Sulfate Proteoglycan Sulfation Regulates WNT and BMP Trans-Synaptic Signaling. *PLoS Genet.* 2012; 8:e1003031. [PubMed: 23144627]
41. Johnson KG, Tenney AP, Ghose A, Duckworth AM, Higashi ME, Parfitt K, Marcu O, Heslip TR, Marsh JL, Schwarz TL, Flanagan JG, Van Vactor D. The HSPGs Syndecan and dallylike bind the receptor phosphatase LAR and exert distinct effects on synaptic development. *Neuron.* 2006; 49:517–531. [PubMed: 16476662]
42. Minge D, Senkov O, Kaushik R, Herde MK, Tikhobrazova O, Wulff AB, Mironov A, van Kuppevelt TH, Oosterhof A, Kochlamazashvili G, Dityatev A, Henneberger C. Heparan Sulfates Support Pyramidal Cell Excitability, Synaptic Plasticity, and Context Discrimination. *Cereb Cortex.* 2017; 27:903–918. [PubMed: 28119345]
43. Yamaguchi, Y., Inatani, M., Matsumoto, Y., Ogawa, J., Irie, F. Roles of heparan sulfate in mammalian brain development Current views based on the findings from ext1 conditional knockout studies. Vol. 93. Elsevier Inc; 2010.
44. Kamimura K, Ueno K, Nakagawa J, Hamada R, Saitoe M, Maeda N. Perlecan regulates bidirectional Wnt signaling at the Drosophila neuromuscular junction. *J Cell Biol.* 2013; 200:219–233. [PubMed: 23319599]
45. Wu, Y., Belenkaya, TY., Lin, X. *Methods in Enzymology.* Fukuda, M., editor. Vol. 480. Elsevier Inc; 2010. p. 33-50.
46. Yan D, Wu Y, Feng Y, Lin SC, Lin X. The Core Protein of Glypican Daily-Like Determines Its Biphasic Activity in Wingless Morphogen Signaling. *Dev Cell.* 2009; 17:470–481. [PubMed: 19853561]
47. Ataman B, Ashley J, Gorczyca M, Ramachandran P, Fouquet W, Sigrist SJ, Budnik V. Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. *Neuron.* 2008; 57:705–718. [PubMed: 18341991]
48. Kerr KS, Fuentes-Medel Y, Brewer C, Barria R, Ashley J, Abruzzi KC, Sheehan A, Tasdemir-Yilmaz OE, Freeman MR, Budnik V. Glial wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the Drosophila neuromuscular junction. *J Neurosci.* 2014; 34:2910–20. [PubMed: 24553932]
49. Miech C, Pauer HU, He X, Schwarz TL. Presynaptic Local Signaling by a Canonical Wingless Pathway Regulates Development of the Drosophila Neuromuscular Junction. *J Neurosci.* 2008; 28:10875–10884. [PubMed: 18945895]
50. Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V. The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell.* 2002; 111:319–330. [PubMed: 12419243]
51. Antar LN, Afroz R, Dichtenberg JB, Carroll RC, Bassell GJ. Metabotropic Glutamate Receptor Activation Regulates Fragile X Mental Retardation Protein and FMR1 mRNA Localization Differentially in Dendrites and at Synapses. *J Neurosci.* 2004; 24:2648–2655. [PubMed: 15028757]
52. Irwin SA, Swain R, Christmon CA, Chakravarti A, Weiler IJ, Greenough WT. Evidence for altered Fragile-X mental retardation protein expression in response to behavioral stimulation. *Neurobiol Learn Mem.* 2000; 73:87–93. [PubMed: 10686126]
53. Irwin SA, Christmon CA, Grossman AW, Galvez R, Kim SH, DeGrush BJ, Weiler IJ, Greenough WT. Fragile X mental retardation protein levels increase following complex environment exposure in rat brain regions undergoing active synaptogenesis. *Neurobiol Learn Mem.* 2005; 83:180–187. [PubMed: 15820853]
54. Weiler IJ, Irwin SA, Klintsova AY, Spencer CM, Brazelton AD, Miyashiro K, Comery TA, Patel B, Eberwine J, Greenough WT. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc Natl Acad Sci U S A.* 1997; 94:5395–5400. [PubMed: 9144248]

55. Doll CA, Broadie K. Neuron class-specific requirements for Fragile X Mental Retardation Protein in critical period development of calcium signaling in learning and memory circuitry. *Neurobiol Dis.* 2016; 89:76–87. [PubMed: 26851502]
56. Doll CA, Broadie K. Impaired activity-dependent neural circuit assembly and refinement in autism spectrum disorder genetic models. *Front Cell Neurosci.* 2014; 8:30. [PubMed: 24570656]
57. Doll CA, Broadie K. Activity-dependent FMRP requirements in development of the neural circuitry of learning and memory. *Development.* 2015; 142:1346–1356. [PubMed: 25804740]
58. Gatto CL, Broadie K. Temporal requirements of the fragile X mental retardation protein in the regulation of synaptic structure. *Development.* 2008; 135:2637–2648. [PubMed: 18579676]
59. Tessier CR, Broadie K. *Drosophila* fragile X mental retardation protein developmentally regulates activity-dependent axon pruning. *Development.* 2008; 135:1547–1557. [PubMed: 18321984]
60. Cavolo S, Bulgari D, Deitcher D, Levitan E. Activity Induces Fmr1-Sensitive Synaptic Capture of Anterograde Circulating Neuropeptide Vesicles. *J Neurosci.* 2016; 36:11781–11787. [PubMed: 27852784]
61. Ataman B, Ashley J, Gorczyca D, Gorczyca M, Mathew D, Wichmann C, Sigrist SJ, Budnik V. Nuclear trafficking of *Drosophila* Frizzled-2 during synapse development requires the PDZ protein dGRIP. *Proc Natl Acad Sci U S A.* 2006; 103:7841–7846. [PubMed: 16682643]
62. Piccioli ZD, Littleton JT. Retrograde BMP signaling modulates rapid activity-dependent synaptic growth via presynaptic LIM kinase regulation of cofilin. *J Neurosci.* 2014; 34:4371–81. [PubMed: 24647957]
63. Vasin A, Zueva L, Torrez C, Volfson D, Littleton JT, Bykhovskaia M. Synapsin regulates activity-dependent outgrowth of synaptic boutons at the *Drosophila* neuromuscular junction. *J Neurosci.* 2014; 34:10554–63. [PubMed: 25100589]
64. Lee J, Geng J, Lee J, Wang A, Chang K. Activity-induced synaptic structural modifications by an activator of integrin signaling at the *Drosophila* neuromuscular junction. *J Neurosci.* 2017; 37:3246–3263. [PubMed: 28219985]
65. Chen X, Rahman R, Guo F, Rosbash M. Genome-wide identification of neuronal activity-regulated genes in *Drosophila*. *Elife.* 2016; 5:1–21.
66. Fukui A, Inaki M, Tonoe G, Hamatani H, Homma M, Morimoto T, Aburatani H, Nose A. Lola regulates glutamate receptor expression at the *Drosophila* neuromuscular junction. *Biol Open.* 2012; 1:362–75. [PubMed: 23213426]
67. Gillespie JM, Hodge JLL. CaMKII autophosphorylation in neuronal growth, calcium signaling, and learning. *Front Mol Neurosci.* 2013; 6doi: 10.3389/fnmol.2013.00027
68. Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, Garrity Pa. An internal thermal sensor controlling temperature preference in *Drosophila*. *Nature.* 2008; 454:217–220. [PubMed: 18548007]
69. Pulver SR, Pashkovski SL, Hornstein NJ, Garrity Pa, Griffith LC. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in *Drosophila* larvae. *J Neurophysiol.* 2009; 101:3075–88. [PubMed: 19339465]
70. Wang X, Page-McCaw A. A matrix metalloproteinase mediates long-distance attenuation of stem cell proliferation. *J Cell Biol.* 2014; 206:923–936. [PubMed: 25267296]
71. Dudu V, Bittig T, Entchev E, Kicheva A, Julicher F, Gonzalez-Gaitan M. Postsynaptic mad signaling at the *Drosophila* neuromuscular junction. *Curr Biol.* 2006; 16:625–635. [PubMed: 16581507]
72. Levitan ES, Lanni F, Shakiryanova D. In vivo imaging of vesicle motion and release at the *Drosophila* neuromuscular junction. *Nat Protoc.* 2007; 2:1117–25. [PubMed: 17546002]
73. Rohrbough J, Rushton E, Woodruff E, Fergestad T, Vigneswaran K, Broadie K. Presynaptic establishment of the synaptic cleft extracellular matrix is required for post-synaptic differentiation. *Genes Dev.* 2007; 21:2607–2628. [PubMed: 17901219]
74. Rushton E, Rohrbough J, Broadie K. Presynaptic secretion of mind-the-gap organizes the synaptic extracellular matrix-integrin interface and postsynaptic environments. *Dev Dyn.* 2009; 238:554–571. [PubMed: 19235718]

75. Glasheen BM, Kabra AT, Page-McCaw A. Distinct functions for the catalytic and hemopexin domains of a *Drosophila* matrix metalloproteinase. *Proc Natl Acad Sci U S A*. 2009; 106:2659–2664. [PubMed: 19196956]
76. Lum L, Yao S, Mozer B, Rovescalli A, Von Kessler D, Nirenberg M, Beachy P. Identification of Hedgehog Pathway Components by RNAi in *Drosophila* Cultured Cells. *Science* (80-). 2003; 299:2039–2045.
77. Nagarkar-Jaiswal S, Lee PT, Campbell ME, Chen K, Anguiano-Zarate S, Gutierrez MC, Busby T, Lin WW, He Y, Schulze KL, Booth BW, Evans-Holm M, Venken KJT, Levis RW, Spradling AC, Hoskins RA, Bellen HJ. A library of MiMICs allows tagging of genes and reversible, spatial and temporal knockdown of proteins in *Drosophila*. *Elife*. 2015; 2015:1–28.
78. Venken KJT, Schulze KL, Haelterman Na, Pan H, He Y, Evans-Holm M, Carlson JW, Levis RW, Spradling AC, Hoskins Ra, Bellen HJ. MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes. *Nat Methods*. 2011; 8:737–743. [PubMed: 21985007]
79. Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol*. 2011; 300:C723–C742. [PubMed: 21209361]
80. Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localization of objects in dual-colour confocal images. *J Microsc*. 1993; 169:375–382.
81. Han C, Belenkaya TY, Wang B, Lin X. *Drosophila* glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process. *Development*. 2004; 131:601–11. [PubMed: 14729575]
82. Baeg GH, Lin X, Khare N, Baumgartner S, Perrimon N. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development*. 2001; 128:87–94. [PubMed: 11092814]
83. Poulain FE, Yost HJ. Heparan sulfate proteoglycans: a sugar code for vertebrate development? *Development*. 2015; 142:3456–67. [PubMed: 26487777]
84. Smith PD, Coulson-Thomas VJ, Foscarin S, Kwok JCF, Fawcett JW. GAG-ing with the neuron”: The role of glycosaminoglycan patterning in the central nervous system. *Exp Neurol*. 2015; 274:100–114. [PubMed: 26277685]
85. Herndon M, Stipps C, Lander A. Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding. *Glycobiology*. 1999; 9:143–55. [PubMed: 9949192]
86. Kirkpatrick CA, Knox SM, Staatz WD, Fox B, Lercher DM, Selleck SB. The function of a *Drosophila* glypican does not depend entirely on heparan sulfate modification. *Dev Biol*. 2006; 300:570–582. [PubMed: 17055473]
87. Yan D, Wu Y, Belenkaya TY, Tang X, Lin X. The cell-surface proteins Dally-like and Ihog differentially regulate Hedgehog signaling strength and range during development. *Development*. 2010; 137:2033–2044. [PubMed: 20501592]
88. Shilts J, Broadie K. Secreted tissue inhibitor of matrix metalloproteinase restricts trans -synaptic signaling to coordinate synaptogenesis. *J Cell Sci*. 2017; 130:2344–2358. [PubMed: 28576972]
89. Zhang YQ, Bailey AM, Matthies HJG, Renden RB, Smith MA, Speese SD, Rubin GM, Broadie K. *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell*. 2001; 107:591–603. [PubMed: 11733059]
90. Alicea D, Perez M, Maldonado C, Dominicci-Cotto C, Marie B. Cortactin is a regulator of activity-dependent synaptic plasticity controlled by Wingless. *J Neurosci*. 2017; 37:2203–2215. [PubMed: 28123080]
91. Nesler KR, Sand RI, Symmes BA, Pradhan SJ, Boin NG, Laun AE, Barbee SA. The miRNA Pathway Controls Rapid Changes in Activity-Dependent Synaptic Structure at the *Drosophila* melanogaster Neuromuscular Junction. *PLoS One*. 2013; :8.doi: 10.1371/journal.pone.0068385
92. Jin P, Warren S. New insights into fragile X syndrome: from molecules to neurobehaviors. *Trends Biochem Sci*. 2003; 28:152–158. [PubMed: 12633995]
93. Kainulainen V, Wang HM, Schick C, Bernfield M. Syndecans, heparan sulfate proteoglycans, maintain the proteolytic balance of acute wound fluids. *J Biol Chem*. 1998; 273:11563–11569. [PubMed: 9565572]

94. Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW, Ethell IM. Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet.* 2009; 46:94–102. [PubMed: 18835858]
95. Janusz A, Milek J, Perycz M, Pacini L, Bagni C, Kaczmarek L, Dziembowska M. The Fragile X mental retardation protein regulates matrix metalloproteinase 9 mRNA at synapses. *J Neurosci.* 2013; 33:18234–41. [PubMed: 24227732]
96. Jumbo-Lucioni PP, Parkinson WM, Kopke DL, Broadie KS. Coordinated movement, neuromuscular synaptogenesis and trans-synaptic signaling defects in *Drosophila* galactosemia models. *Hum Mol Genet.* 2016; 25:3699–3714. [PubMed: 27466186]
97. Uhlirova M, Bohmann D. JNK- and Fos-regulated *Mmp1* expression cooperates with Ras to induce invasive tumors in *Drosophila*. *EMBO J.* 2006; 25:5294–5304. [PubMed: 17082773]
98. Baek M, Mann RS. Lineage and birth date specify motor neuron targeting and dendritic architecture in adult *Drosophila*. *J Neurosci.* 2009; 29:6904–6916. [PubMed: 19474317]
99. Kolodziejczyk A, Sun X, Meinertzhagen IA, Nässel DR. Glutamate, GABA and acetylcholine signaling components in the lamina of the *Drosophila* visual system. *PLoS One.* 2008; 3doi: 10.1371/journal.pone.0002110
100. Mahr A, Aberle H. The expression pattern of the *Drosophila* vesicular glutamate transporter: A marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expr Patterns.* 2006; 6:299–309. [PubMed: 16378756]
101. Li HH, Kroll JR, Lennox SM, Ogundeyi O, Jeter J, Depasquale G, Truman JW. A GAL4 driver resource for developmental and behavioral studies on the larval CNS of *Drosophila*. *Cell Rep.* 2014; 8:897–908. [PubMed: 25088417]
102. Pfeiffer BD, Jenett A, Hammonds AS, Ngo TTB, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Lavery TR, Mungall C, Svirskas R, Kadonaga JT, Doe CQ, Eisen MB, Celniker SE, Rubin GM. Tools for neuroanatomy and neurogenetics in *Drosophila*. *Proc Natl Acad Sci U S A.* 2008; 105:9715–9720. [PubMed: 18621688]
103. Jenett A, Rubin GM, Ngo TTB, Shepherd D, Murphy C, Dionne H, Pfeiffer BD, Cavallaro A, Hall D, Jeter J, Iyer N, Fetter D, Hausenfluck JH, Peng H, Trautman ET, Svirskas RR, Myers EW, Iwinski ZR, Aso Y, et al. A GAL4-Driver Line Resource for *Drosophila* Neurobiology. *Cell Rep.* 2012; 2:991–1001. [PubMed: 23063364]
104. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012; 9:676–82. [PubMed: 22743772]
105. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012; 9:671–675. [PubMed: 22930834]

One Sentence Summary

Secreted MMP protease localized by surface proteoglycan mediates use-dependent synaptogenesis.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Mmp1 mediates rapid activity-dependent synaptic bouton formation

(A) NMJs co-labeled for HRP and DLG after dTRPA1 stimulation in the indicated genotypes. White stars mark ghost boutons. Scale bar: 5 μ m. Higher magnification images of synaptic boutons are shown below; scale bar: 2 μ m. (B) Quantified ghost bouton number per terminal after dTRPA1 stimulation: *vglut-Gal4*^{+/+} (N=28, 1.54 $\pm$ 0.30), *vglut-Gal4*^{>UAS-dTRPA1} (N=20, 7.4 $\pm$ 0.97), *vglut-Gal4, mmp1*^{Q112*/mmp1}Q112* (N=24, 1.54 $\pm$ 0.34) and *vglut-Gal4*^{>UAS-dTRPA1, mmp1}Q112*/mmp1Q112* (N=24, 1.38 $\pm$ 0.33). Data are mean $\pm$ SEM from 3 replicates; N = number of NMJs. ***p<0.001 by nonparametric ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons post-test. Non-significant (p>0.05) comparisons are not represented for 1) *vglut-Gal4*^{+/+} vs. *vglut-Gal4, mmp1*^{Q112*/mmp1}Q112* and 2) *vglut-Gal4*^{+/+} vs. *vglut-Gal4*^{>UAS-dTRPA1, mmp1}Q112*/mmp1Q112* and 3) *vglut*-

Gal4, mmp1^{Q112}/mmp1^{Q112*}* vs. *vglut-Gal4>UAS-dTRPA1, mmp1^{Q112*}/mmp1^{Q112*}*.
Temperature controls are shown in fig. S1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. Synaptic Mmp1 is rapidly increased after acute neuronal stimulation

(**A**) Images of NMJs following the denoted stimulation, co-labeled with HRP and Mmp1 in the indicated genotypes. Heat map shows Mmp1 alone (scale below); white outline: HRP bouton. Scale bar: 2 μ m. (**B**) Quantification of synaptic Mmp1 intensity normalized to matched controls in: *vglut-Gal4*^{+/+} (N=10, 1.0 $\pm$ 0.07) vs. *vglut-Gal4*^{>UAS-dTRPA1} (N=13, 1.42 $\pm$ 0.1) and *w*¹¹¹⁸ control (unstimulated, N=56, 1.0 $\pm$ 0.03) vs. *w*¹¹¹⁸ stimulated (high [K⁺], N=57, 1.54 $\pm$ 0.07). **p<0.01 and ***p<0.001 by unpaired t-test with Welch correction (dTRPA1) or Mann-Whitney U-Test (High [K⁺]). Data are mean $\pm$ SEM from 3 replicates for each experiment; N = number of NMJs. Additional controls shown in fig. S3.

Fig. 3. Mmp1 and Dlp co-localization in synaptic subdomains is increased by acute activity
(A) Images of Dlp::GFP NMJs labeled with HRP, GFP and Mmp1 under basal conditions or following high $[K^+]$ stimulation. Dlp::GFP and Mmp1 signals are shown as a heat map with HRP synaptic outlines in white. Arrows indicate overlapping Dlp::GFP and Mmp1 signals; scale bar: 5 μ m. Higher magnification images below show single optical sections; scale bar: 1 μ m. Asterisks denote overlapping Dlp::GFP and Mmp1, shown pseudo-colored in white in the far right images. **(B)** Quantification of both Dlp::GFP and Mmp1 fluorescence intensities normalized to unstimulated controls. “Dlp::GFP”: control (N=20, 1.0 ± 0.06) vs. stimulated (N=19, 1.48 ± 0.09); and “Mmp1”: control (N=20, 1.0 ± 0.05) vs. stimulated (N=19,

1.56±0.14). ***p<0.001 by unpaired t-test with Welch correction,. (C) Quantification of Manders' co-localization coefficients. M_A (Mmp1/Dlp::GFP) in basal control (N=27, 0.33±0.03) and after stimulation (N=27, 0.72±0.03). M_B (Dlp::GFP/Mmp1) in basal control (N=27, 0.37±0.03) and after stimulation (N=27, 0.59±0.03). ***p<0.001 by unpaired t-test (M_A) or Mann-Whitney U-Test (M_B). Data are mean ± SEM from 3 replicates; N = number of NMJs. Additional controls shown in figs. S5 and S6.

Fig. 4. Synaptic Dlp positively and bidirectionally regulates secreted Mmp1 abundance
(A and B) Images of NMJs (A) and quantification of Mmp1 fluorescence intensity (B) from the denoted *dlp* reduction condition compared to matched controls co-labeled with HRP and Mmp1. Mmp1 signal intensity is shown as a heat map with HRP synaptic outlines in white. Scale bars: 2 μ m. Fluorescence intensity was normalized to that from matched genetic controls: *w¹¹¹⁸* (N=34, 1.0±0.04) vs. *dlp^{A187/+}* (N=37, 0.67±0.04), and *elav-Gal4,24B-Gal4/+* control (N=28, 1.0±0.07) vs. *elav-Gal4,24B-Gal4>UAS-dlp^{RNAi}* knockdown (N=32, 0.77±0.05). *p<0.05 and ***p<0.001 by Mann-Whitney U-Test. **(C and D)** As in (A and B), from *dlp* overexpression conditions. *24B-Gal4/+* (N=51, 1.0±0.03), *24B-Gal4>UAS-dlp^{WT}* (N=42, 1.55±0.09), and *24B-Gal4>UAS-dlp^{HS}* (N=34, 1.13±0.06). ***p<0.001 by nonparametric ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons post-test. The non-significant (p>0.05) comparison for *24B-Gal4/+* vs. *24B-Gal4>UAS-dlp^{HS}* is not shown. Data are mean ± SEM from at least 3 independent replicates for each experiment; N = number of NMJs. Abundance of Dlp in each of the genetic manipulations is shown in fig. S7.

Fig. 5. Dlp mediates activity-dependent regulation of synaptic Mmp1

(A and B) Images of NMJs (A) and quantification of Mmp1 fluorescence intensity (B) from the indicated *dlp* reduction condition treated with or without high $[K^+]$ and co-labeled for HRP and Mmp1. Mmp1 signal intensity is shown as a heat map with HRP synaptic outlines in white. Scale bars: 2 μ m. Quantified Mmp1 fluorescence intensity from stimulated conditions (bar graphs) was normalized to unstimulated controls (red lines). *w¹¹¹⁸* control (N=17, 1.0 ± 0.08) vs. *w¹¹¹⁸* stimulated (N=19, 1.34 ± 0.06); *dlp^{A187/+}* control (N=19, 1.0 ± 0.03) vs. *dlp^{A187/+}* stimulated (N=21, 0.85 ± 0.06); *elav-Gal4,24B-Gal4/+* control (N=26, 1.0 ± 0.04) vs. *elav-Gal4,24B-Gal4/+* stimulated (N=24, 1.34 ± 0.06) and *elav-Gal4,24B-Gal4>UAS-dlp^{RNAi}* control (N=25, 1.0 ± 0.06) vs. *elav-Gal4,24B-Gal4>UAS-dlp^{RNAi}* stimulated (N=18, 0.88 ± 0.06). (C and D) As in (A and B), from *dlp* overexpression conditions. *24B-Gal4/+* control (N=21, 1.0 ± 0.06) vs. *24B-Gal4/+* stimulated (N=18, 1.39 ± 0.07); *24B-Gal4>UAS-dlp^{WT}* control (N=21, 1.0 ± 0.03) vs. *24B-Gal4>UAS-dlp^{WT}* stimulated (N=16, 1.64 ± 0.16); and *24B-Gal4>UAS-dlp^{HS}* control (N=23, 1.0 ± 0.05) vs. *24B-Gal4>UAS-dlp^{HS}* stimulated (N=13, 1.19 ± 0.08). (B,D) For stimulated vs. unstimulated pair-wise comparisons (red asterisks), significance determined by Mann-

Whitney U-Tests and across genotypes (black) by Unpaired t-tests (B) or nonparametric ANOVA (Kruskal-Wallis; D) with Dunn's multiple comparisons post-test, indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Non-significant ($p > 0.05$) comparisons: stimulated vs. unstimulated *elav-Gal4, 24B-Gal4 > UAS-dlp^{RNAi}* (B), stimulated *24B-Gal4/+* vs. stimulated *24B-Gal4 > UAS-dlp^{WT}* (D), and stimulated *24B-Gal4/+* vs. stimulated *24B-Gal4 > UAS-dlp^{HS}* (D) are not shown. Data are mean $\pm$ SEM from 3 independent replicates; N = number of NMJs.

Fig. 6. Dlp positively and bidirectionally regulates proteolytic activity at the synapse
(A) Images of NMJs from the indicated *dlp* reduction and overexpression co-labeled for HRP (red) and in situ zymography activity (green) compared to matched genetic controls. Gelatinase activity shown as a heat map with HRP synaptic outlines in white. Scale bar: 2 μ m. **(B)** Quantified in situ zymography fluorescent intensity normalized to controls for *dlp* reduction: *w¹¹¹⁸* control (N=35, 1.0 $\pm$ 0.06) and *dlp^{A187/+}* heterozygote (N=33, 0.66 $\pm$ 0.04). Significance determined by Unpaired t-test with Welch correction, indicated by ***p<0.0001. **(C)** Quantified in situ zymography fluorescent intensity normalized to controls for *dlp* overexpression conditions: *24B-Gal4/+* control (N=35, 1.0 $\pm$ 0.05), *24B-Gal4>UAS-dlp^{WT}* (N=18, 1.5 $\pm$ 0.09) and *24B-Gal4>UAS-dlp^{HS}* (N=20, 1.6 $\pm$ 0.18). **p<0.01 by nonparametric ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons post-test. Data shown as mean $\pm$ SEM from 3 independent replicates; N = number of NMJs.

Fig. 7. FMRP regulation of the activity-dependent Mmp1 enhancement requires Dlp
(A and B) Images of NMJs (A) and quantification of Mmp1 fluorescence intensity (B) from the denoted genotypes, co-labeled with HRP and Mmp1. Mmp1 intensity shown as a heat map; white outlines mark synaptic HRP. Scale bars: 2 μ m. Quantification of Mmp1 fluorescence intensity was normalized to *w¹¹¹⁸* control (n=49, 1.0±0.03), *dfmr1^{50M/50M}* (n=35, 1.57±0.12) and *dlp^{A187/+} dfmr1^{50M/50M}* (n=22, 1.07±0.07). **(C and D)** As in (A), treated with or without high $[K^+]$. Red line, unstimulated control. *w¹¹¹⁸* control (unstimulated, n=13, 1.0±0.04) vs. *w¹¹¹⁸* stimulated (n=15, 1.51±0.1); *dfmr1^{50M/50M}* control (unstimulated, n=15, 1.0±0.07) vs. *dfmr1^{50M/50M}* stimulated (n=13, 1.02±0.11); and *dlp^{A187/+} dfmr1^{50M/50M}* control (unstimulated, n=14, 1.0±0.04) vs. *dlp^{A187/+} dfmr1^{50M/50M}* stimulated (n=17, 1.55±0.16). For stimulated vs. unstimulated pair-wise comparisons, significance determined by Mann-Whitney U-Tests, indicated by **p<0.01 and ***p<0.001 (red asterisks). In (B) and across stimulated genotypes in (D), significance determined by nonparametric ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons post-test, indicated by *p<0.05, **p<0.01, and ***p<0.001 (black asterisks). Non-significant (p>0.05) comparisons for *w¹¹¹⁸* vs. *dlp^{A187/+} dfmr1^{50M/50M}* (B), stimulated vs. unstimulated *dfmr1^{50M/50M}* (D) and stimulated *w¹¹¹⁸* vs. stimulated *dlp^{A187/+} dfmr1^{50M/50M}* (D) are not shown. Data are mean ± SEM from at least 3 independent replicates; N = number of NMJs.