The *Drosophila* Fragile X Gene Negatively Regulates Neuronal Elaboration and Synaptic Differentiation

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Summary

Fragile X Syndrome (FraX) is the most common form of inherited mental retardation. The disease is caused by the silencing of the *fragile X mental retardation 1* (*fmr1*) gene, which encodes the RNA binding translational regulator FMRP [1–4]. In FraX patients and *fmr1* knockout mice, loss of FMRP causes denser and morphologically altered postsynaptic dendritic spines [5–7]. Previously, we established a *Drosophila* FraX model and showed that dFMRP acts as a negative translational regulator of Futsch/MAP1B and negatively regulates synaptic branching and structural elaboration in the peripheral neuromuscular junction (NMJ) [8]. Here, we investigate the role of dFMRP in the central brain, focusing on the mushroom body (MB), the learning and memory center [9, 10]. In MB neurons, dFMRP bidirectionally regulates multiple levels of structural architecture, including process formation from the soma, dendritic elaboration, axonal branching, and synapticogenesis. *Drosophila fmr1* (dfmr) null mutant neurons display more complex architecture, including overgrowth, overbranching, and abnormal synapse formation. In contrast, dFMRP overexpression simplifies neuronal structure, causing undergrowth, underbranching, and loss of synapse differentiation. Studies of ultrastructural *dfmr* mutant neurons reveal enlarged and irregular synaptic boutons with dense accumulation of synaptic vesicles. Taken together, these data show that dFMRP is a potent negative regulator of neuronal architecture and synaptic differentiation in both peripheral and central nervous systems.

Results and Discussion

Mushroom Body Structure Is Largely Normal in *dfmr* Null Mutants

Both mammalian and *Drosophila* Fragile X Mental Retardation Protein (FMRP) is enriched in neuronal soma, undetectable in nuclei, and comprises only a small percentage of protein present in neuronal processes [11–14]. Similar to that found in the mushroom body (MB), dFMRP is prominently enriched in the soma of all neurons but is undetectable in nuclei (Figure 1A). The protein is present at high levels only within cell body cytoplasm, with little or no detectable dFMRP in dendrites or axonal projections. The mushroom body has four structural sections including cell bodies, dendrites (i.e., calyx), peduncle, and the axon lobes (Figure 1B). dFMRP is similarly expressed in the three classes of neurons: γ, α/β, and α/β’ neurons [15]. γ neurons project a single horizontal axon, whereas α/β’ and α/β neurons have two axon projections, one horizontal and one vertical (Figure 1B). We took advantage of the relative simplicity of these neurons to analyze the role of dFMRP in their morphological differentiation. Here, we used the MARCM (mosaic analysis with repressible cell marker) system [16], which uniquely labels single homozygous mutant neurons to reveal the whole projections of labeled neurons in the intact brain.

In FraX patients and *fmr1* knockout mice, brain morphology is grossly normal with no detectable abnormalities in specific brain regions [17, 18]. Similarly, null *dfmr* mutants display normal gross-brain morphology, including an architecturally normal mushroom body (Figure 1B, [19]). Mild overgrowth of the β lobe, resulting in the crossing of the brain midline, was observed in some mutants (see also [20]). In contrast, dFMRP overexpression (OE) caused dramatic structural defects in mushroom body axon lobes (Figure 1B), with the β lobe always overextended across the midline. The β’ lobe often displayed apparently random directions of axon projection often outside of the mushroom body domain (Figure 1B). The γ lobe always displayed a dramatic decrease in volume and was even completely lost (Figure 1B). Thus, *dfmr* null mutants display only subtle defects in gross mushroom body architecture, whereas *dfmr* overexpression causes dramatic defects. Because FraX is caused by loss of FMRP, we therefore turned our attention to the single-cell and subcellular level to examine neuronal architecture.

Loss of dFMRP Converts Unipolar Neurons into Multipolar Neurons

The MARCM technique provides a uniquely powerful approach for examining mutant neurons in situ at a single cell level of resolution [16]. All the analyses presented in this study represent quantification from single, isolated *dfmr* mutant mushroom body neurons in an otherwise nonmutant brain. To make sure all phenotypes arose from *dfmr* mutation, we used two independently generated *dfmr* null mutant alleles, which represent overlapping intragenic deficiencies (Figure 2A), *dfmr*mut [8] and *dfmr* [19]. In addition, dFMRP was overexpressed in single neurons to examine the consequence of excess protein. For controls, single-cell clones of GFP-expressing wild-type (WT) neurons were made (Figure 2B). In turn, we examined each region of the mutant neurons, progressing from the cell body through the dendrites, axons, and synapses.

Mushroom body neurons are exclusively unipolar [15, 21]. Cell bodies extend only a single primary process, which subsequently branches to form distinctive dendrites and axon projection (Figure 2B). A small minority of wild-type cells display one to two tiny hair-like additional projections (arrow), but these processes are always very short and thin compared with the primary process. In wild-type, it is exceptionally rare for the

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additional processes to contain any branches or elaborations. In contrast, dfmr mutant neurons have a strong tendency to extend supernumerary processes, thus always converting the characteristic unipolar neurons in wild-type into multipolar neurons in mutants (Figure 2B). In addition, the additional processes in dfmr mutants are usually long and thick, branch extensively and differentiate varicosities characteristic of synapses (Figure 2B). In contrast, dFMRP overexpression results in even fewer processes than wild-type, generating an entirely smooth cell body profile. We quantified the average number of additional processes in single α/β’ neurons. Null mutants display a 3-fold increase in the number of cell body processes (WT, 1.27 ± 0.3 n = 11; dfmrOSM, 3.84 ± 0.53, n = 19; dfmrDR, 3.75 ± 0.72, n = 12), whereas dFMRP overexpression reduces the number of processes 2-fold (WT, 1.27 ± 0.3; dfmrOE, 0.64 ± 0.2, n = 22; Figure 2C). Similar phenotypes were also observed in γ and α/β neurons. These data show that dFMRP strongly negatively regulates the generation of processes from neuronal cell bodies.

dFMRP Negatively Regulates Dendritic Elaboration

Several common features of mushroom body neuron dendritic arbors aid in the description of dfmr mutant phenotypes. First, all three classes of neurons have three to four primary dendritic branches. Second, primary branches rarely contain higher-order branches along their initial length. Third, primary branches end with a fine dendritic terminal arbor that forms a highly characteristic “claw-like” structure (Figure 3; [22]). We used these conserved features as a basis to analyze the role of dFMRP in dendrite morphogenesis. Late-born α/β neurons (mitotic recombination was induced ~12 hr after pupa formation; [22]) were used for detailed morphometric studies.

Dendrites on dfmr mutant neurons are consistently abnormal in morphology and projection, owing to the increased complexity of several structural features (Figure 3). The number of primary dendritic branches are more variable in the mutant, ranging from three to six (compared to three to four in wild-type). In addition, nearly all mutant dendrite processes have clear supernumerary higher-order branches, and the clustered, fine dendritic arbors normally restricted to the extreme termini (the claw-like structure; [22]) spread aberrantly along the entire length of mutant dendritic branches (Figure 3). These excess fine dendritic processes convert the clear, orderly wild-type dendrites into disordered, “cotton wool-like” dendrites in dfmr mutants. On the other hand, dFMRP-overexpressing mutants lose the clustered fine dendritic arbors and show severe reduction or complete loss of the claw-like structure at the terminal of dendritic branches (Figure 3). Thus, removal of dFMRP increases the branching and structural complexity of dendritic processes, whereas overexpres-
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Figure 2. dFMRP Loss of Function Results in Aberrant Multipolar Neurons

(A) Genomic structure of the dfmr locus and the two independent dfmr intragenic deletion mutants used in this study; dfmr<sup>50M</sup> [8] and dfmr<sup>3</sup> [19]. Note that the dfmr<sup>3</sup> deletion reported here has verified breakpoints that differ from what was reported in Dockendorff, et al.

(B) Representative images of α/β neuronal soma and processes in single-cell MARCM clones. Wild-type (WT) neurons are typically unipolar, but, with rare frequency, there are 1–2 other short processes (arrow). These tiny processes lack secondary branches. Overexpression of dFMRP (OE) results in a cleaner cell body, with no excess processes. In contrast, dfmr mutant neurons always display multiple processes (arrows), becoming characteristically multipolar. These supernumerary processes are multibranched and contain varicosities resembling synaptic boutons. The scale bar represents 5 μm.

(C) Quantification of the number of processes projecting from cell bodies. The bars show the mean ± SEM. Significance: 0.001 < p < 0.05 (*); 0.0001 < p < 0.001 (**).

Overexpression of dFMRP decreases dendritic branching and simplifies the arbor. These data show that dFMRP functions as a negative regulator of dendritic elaboration.

dFMRP Negatively Regulates Axonal Branching

Progressing from the cell body and through the dendritic arbor, we next consider the role of dFMRP on axonal projection and structure. The initial focus was the γ neuron because it has only one axonal branch and a particularly simple elaborative pattern in the mushroom body. The wild-type γ axon enters the horizontal axon lobe from the ventral edge, bends upwards to enter the γ lobe, and terminates near the dorsal boundary of the horizontal lobe (Figure 4A). The wild-type γ neuron never branches prior to entering the γ lobe but typically has several specific small branches along the main process [15].

The axons of dfmr mutant γ neurons are much more structurally elaborate than those of wild-type. Mutant neurons always display significantly increased axonal branching and always have significantly more and longer axonal branches (Figure 4A). These large, supernumerary branches do not follow the main axon trajectory, but rather they extend in apparently random directions to invade inappropriate territory (Figure 4A). Some of the excess branches in mutants are so large that the γ neuron appears to possess duplicate main-axonal processes (Figure 4A). The number of these large branches (>5 μm in length) increased on average by >50% (WT, 3.74 ± 0.28, n = 19; dfmr<sup>3</sup>, 6.46 ± 0.8, n = 13; dfmr<sup>50M</sup>, 5.33 ± 0.33, n = 21; Figure 4B), and the total length of axon branches also significantly increased (WT, 65.68 ± 5.07 μm; dfmr<sup>3</sup>, 103.33 ± 8.5 μm; dfmr<sup>50M</sup>, 84.29 ± 5 μm; Figure 4C). There were also dramatic reductions in the number of processes projecting from cell bodies (Figure 4C).

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dFMRP Regulates Synapse Differentiation

Loss of dFMRP results in striking defects in neuronal architecture (Figures 2–4), suggesting that synaptic connectivity might be impaired. Recent work in mouse fmr1 mutants has indicated defects in synaptic plasticity [23]. Likewise, our previous work on dfmr mutants in the...
**Figure 3. dFMRP Negatively Regulates Dendrite Elaboration**

Representative images of late-born α/β neuron dendritic arbors in single-cell MARCM clones. Wild-type (WT) dendrites typically display three primary branches. There are no higher-order branches but only a single, well-defined claw-like structure of fine processes at the termini (arrows). Three images of dfmr mutant neurons show more complex and disordered dendritic structure. Primary dendrites display clear secondary branches. The fine dendritic processes, normally restricted to the termini, spread aberrantly along the primary branches. These defects convert the orderly wild-type dendrites into disordered, cotton wool-like structures. dFMRP overexpression results in loss of the claw-like termini. Arrows show the reduced or absent claw-like structures. The scale bar represents 10 μm.

**Drosophila** neuromuscular junction (NMJ) and eye has strongly suggested specific defects at the synapse [8]. Therefore, we next examined synaptic differentiation in dfmr mutant MB clones at both the light and electron microscopy levels.

In wild-type animals, mushroom body axonal processes display a relatively smooth profile, with only subtle varicosities indicative of synaptic connections [24]. In contrast, dfmr mutant axons show a markedly discontinuous profile, with enlarged varicosities fairly evenly distributed along axons, producing a highly characteristic “beads on a string” appearance (Figures 4A and 4D). In contrast, dFMRP overexpression resulted in a smoother axon profile than wild-type (Figures 4A and 4D). The position and spacing of these varicosities strongly suggests that they represent the altered features of presynaptic boutons.

To examine in detail synaptic differentiation in dfmr mutants, we next employed electron microscopy to examine MARCM mutant clones. For labeling, we used a peroxidase-conjugated anti-CD8 antibody followed by a Ni²⁺-enhanced DAB reaction to produce an electron-dense, membrane-associated signal clearly marking the dfmr mutant neurons (Figure 5A and see Experimental Procedures). We focused our analysis on α/β' axons in the horizontal axon lobe (Figure 5B); they showed an extensive overbranching phenotype and prominently discontinuous axon profiles at the light microscope level.

The first objective was to confirm the light microscope impression of enlarged presynaptic bouton size. We therefore serial sectioned labeled α/β' axons and measured the cross-section area of all profiles containing T-bar active zones, which define presynaptic neurotransmitter release sites (Figures 5D and 5F, arrows). The average cross-section, bouton area of dfmr mutant neurons was significantly (P = 0.05) enlarged compared to labeled wild-type neurons (WT, mean = 0.19 ± 0.01 μm², n = 97; dfmrOE, mean = 0.24 ± 0.02, n = 108; Figures 5D and 5E). In addition, dfmr mutant boutons display a markedly more variable size. In the 200 ± boutons measured, the largest and smallest bouton ranges both occurred in mutant neurons (Figure 5E); the area of the largest dfmr bouton was >50% larger than the largest wild-type bouton, and the area of smallest dfmr bouton was ~35% decreased compared to the smallest wild-type bouton. These results suggest that dFMRP negatively regulates the morphological differentiation of synaptic boutons, and also increases the fidelity of bouton size.
Figure 4. dFMRP Negatively Regulates Axonal Branching

(A) Representative γ neuron axons in single-cell MARCM clones. The γ neuron axon normally enters the horizontal axon lobe from the ventral edge, bends upwards to enter the γ lobe, and finally projects to the dorsal boundary of the horizontal lobe. In wild-type (WT) neurons, the axon projection has only one main axonal branch with some specific small branches (arrows) along the main process. dFMRP overexpression causes dramatic under-branching of the axon. The γ neurons maintain their processes along the ventral edge of the horizontal lobe and aberrantly invade the β lobe. The dfmr mutants display the opposite phenotype of axonal overbranching. Null mutant neurons always have more and longer axonal branches. These large branches don’t follow the main axon direction but rather extend in an apparently random direction (arrows). The scale bar represents 10 μm.

(B) Quantification of the large branches (>5 μm) for a single γ axon.

(C) Quantification of total branch length of a single γ axon. WT, n = 19; dfmr50M, n = 21; dfmr1, n = 13; OE, n = 24. Bars show mean ± SEM. Significance 0.001 < p < 0.05 (*); 0.0001 < p < 0.001 (**); p < 0.0001 (***).

(D) Representative α/β neuron axon morphologies in single-cell MARCM clones. WT neurons characteristically have one small side branch in proximal quarter region and branches clustered at the terminal of the horizontal lobes (arrowheads). Note that GFP distribution is relatively even along the axon, with only small varicosities. dfmr mutant neuron axons display overbranching. Axons have longer side branches in the proximal quarter and terminal branching region. The GFP distribution is strikingly altered, with enlarged puncta (arrows) distributed along the axons. In the overexpression neuron, arrowheads indicate the positions of branches in the WT axon. The scale bar represents 20 μm.

The immediately striking observation was that dfmr mutant presynaptic boutons were almost filled with evenly sized, electron-lucent synaptic vesicles, clearly several orders of magnitude more vesicles than in control boutons (Figures 5C, 5D, and 5F). Indeed, vesicle density within mutant boutons was so high that it often precluded the ability to resolve other features of the active zone and effectively prevented any ability to clearly resolve individual vesicles for quantification. Therefore, to partially quantify the vesicle accumulation phenotype, we measured the bouton area fully occupied by vesicles as a percentage of the total bouton area (Figure 5G). In control neurons, slightly less than 50% of the bouton area is normally occupied by synaptic vesicles, whereas nearly 75% of the bouton is occupied by vesicles in dfmr mutant neurons. Thus, the average area occupied by vesicles is increased 50% (p < 0.001) in dfmr boutons compared to internal control boutons (WT, mean = 49% ± 3%, n = 27; dfmr50M mean = 73% ± 5%, n = 25; Figures 5F and 5G). In fact, the actual vesicle
Figure 5. dFMRP Regulates Synaptic Differentiation

(A) DAB-stained brain revealing a large MARCM clone within the mushroom body. The scale bar represents 100 μm.

(B) Confocal image of the whole mushroom body MARCM clone. Quadrangle represents the plane of section through the horizontal lobe, where all electron micrographs were taken. The scale bar represents 10 μm.

(C) Electron micrograph (11 500×) of the mushroom body horizontal lobe; labeled MARCM clone cells (L) and unlabeled cells (U). The scale bar represents 500 nm.

(D) Electron micrograph (25 000×) of a mushroom body horizontal lobe with wild-type (WT, left) and dfmr50M (right) MARCM clones. Arrows indicate the electron-dense, labeled bouton membrane. The scale bar represents 500 nm.

(E) Quantification of labeled bouton area in both WT and dfmr50M clones. The dfmr null mutant neurons display a significantly enlarged average bouton area and more variable distribution of bouton sizes than wild-type (WT, n = 97; dfmr50M, n = 108, p = 0.05).

(F) High magnification of a single synaptic bouton from WT (left) and dfmr50M null mutant (right) neurons. The null mutant boutons show a dramatically increased density of synaptic vesicles (SV) throughout the bouton interior and at presynaptic active zones (arrows). The scale bar represents 100 nm.

(G) Quantification of synaptic vesicle density. Bars indicate the percentage of the total bouton area occupied by synaptic vesicles. (WT, n = 25; dfmr50M, n = 27, p < 0.001).

accumulation in mutant boutons is much greater than is reflected in these numbers because vesicles in the mutant are much more densely accumulated to the extent that normally prevents clear resolution of individual vesicles. A vesicle accumulation defect of this severity has not been previously reported for Drosophila. This defect could be due to hyperactive vesicle biogenesis or an arrest in vesicular exocytosis, either one resulting in increased vesicle density. Our previous studies have revealed synaptic vesicle accumulation only in mutants with severely impaired vesicular exocytosis and neurotransmitter release, such as dUNC-13 and dCAPS mutants [25, 26]. Thus, dfmr mutant synapses of mushroom body neurons display aberrant ultrastructural profiles...
consistent with defective synaptic function and impaired neurotransmitter release.

Conclusions
Here, we examined the role of dFMRP in the structural differentiation of the three neuronal classes ($\gamma$, $\alpha'/\beta'$, and $\alpha/\beta$ neurons) comprising the mushroom body, the insect brain's learning and memory center suggested to functionally correspond to the mammalian hippocampus [9, 10, 27]. In all neuronal classes, loss of dFMRP causes increased structural complexity throughout the entire neuron, including the extension of additional processes from neuronal soma, overbranching and overgrowth of both the dendritic field and axon processes, and consequent defects in projection. Correspondingly, dFMRP overexpression causes decreased structural complexity throughout the entire neuron, including reduction of cell body processes, undergrowth of dendritic arbor, underbranching and undergrowth of axonal processes, and consequent defects in connectivity. Thus, the level of dFMRP bidirectionally regulates growth and architectural elaboration throughout these neurons, altering the availability of both synaptic input and output sites. Ultrastructure analysis of dfmr mutants shows enlarged synaptic boutons, irregular bouton size, and abnormal synaptic vesicle accumulation. These defects indicate impaired synaptogenesis and suggest arrested synaptic function, including either abnormal vesicle biogenesis or impaired synaptic vesicle exocytosis and consequent loss of neurotransmitter release.

In mammals, FMRP has been clearly implicated in synaptic mechanisms, although with a predominantly postsynaptic association. FMRP mRNA and protein are both found localized in dendritic spines in the mouse brain [13, 28], and FMRP is locally translated in an activity-dependent mechanism that requires activation of metabotropic glutamate receptors [29, 30]. FMRP is, in turn, required for mGluR-dependent translation [29, 31]. Mouse fmr1 mutants have reduced GluR1 subunits at cortical synapses but not in the hippocampus or cerebellum, and similarly, long-term potentiation is reduced in the cortex but not in the hippocampus [32]. Huber et al. [23] showed that FMRP is also required for mGluR-dependent long-term depression in the hippocampus. Although these results suggest primarily postsynaptic roles for FMRP, identification of FMRP mRNA targets has conversely suggested mostly presynaptic functions. Among presynaptic targets identified, there have been MUNC13, NAP-22, SEC-7, and RAB-5 [1, 33]. These putative FMRP targets provide possible mechanistic explanations for the presynaptic defects in Drosophila reported here and previously [8].

Experimental Procedures

Genetics
The following Drosophila strains were generated by standard genetic methods: (1) heatshock-FLP, mouse CD8-GFP; FRT82B, tubulin P-GAL80(TM3); GAL4-OK107. (2) y, w; FRT82B/TM3. (3) FRT82B, dfmr$^{100M}$/TM6. (4) FRT82B, dfmr$^{100M}$/TM6. (5) FRT82B, UAS-dfmr. GAL4-OK107 was used to drive the visualizer mushroom body clones in all cases [34]. Mouse CD8-GFP was used to label clones. All dfmr$^{100M}$, dfmr$^{100M}$, and UAS-dfmr insertions were recombined to the FRT82B chromosome by standard methods, based on the presence of FRT82B carrying the G418 resistance [35] and PCR confirmation of all the genetic elements.

Immunohistochemistry
The following antibodies were used: rat anti-mouse CD8a, 1:100 (Caltag); mouse anti-Drosophila Fasciclin II, 1:10 (Developmental Studies Hybridoma Bank); mouse anti-dFMRP, 1:1000 (Developmental Studies Hybridoma Bank); FITC-conjugated goat anti-rat IgG, 1:100 (Jackson); goat Cy3-conjugated goat anti-mouse IgG, 1:100 (Jackson); rat anti-Drosophila DLG, 1:100 (V. Budnik). All fluorescent images were collected using a ZEISS LSM510 META Laser Scanning Microscope and image collection software. All image processing was done with Adobe Photoshop 7.0.

Mosaic Analysis with a Repressible Cell Maker (MARC)
The MARC technique was employed as described in Lee and Luo [16]. To generate MARCM clones in the $\gamma$-neuron, embryos were collected within a 5 h window and cultured at 25°C. Twenty-hour-old embryos were heat shocked at 37°C for 1 hr. To generate $\alpha'/\beta'$- and $\alpha/\beta$-MARCM clones, the same heat shock was done to 5-day-old larvae and 8-day-old pupa, respectively. Adult brains were dissected in 1× PBS, fixed in 4% paraformaldehyde for 30 min, and processed with immunostaining.

Morphological Quantification
All quantification was done on single-cell MARCM clones. For $\gamma$-neuron axonal quantification, the primary axon branch was identified first, and all other processes extended from this main trunk were counted as branches. The length of each branch was measured based on 3D images from confocal microscopy. All branch lengths of single-axon branches were added together to get the total cumulative length. For $\alpha'/\beta'$-neuron cell body process quantification, all processes except the main process were counted. One branch was counted as one process. In mutants, branch numbers >10 (always difficult to tell apart) were counted as 10.

Electron Microscopy
Ultrastructural analyses of Drosophila brains were done with standard protocols [36, 37]. Briefly, 1–3-day-old adult brains with MARCM clones were dissected in 1× PBS buffer and immediately fixed for 30 min in 2% paraformaldehyde. The samples were subsequently rinsed with PBS-BSA buffer for 3 hr at 4°C, stained with rat anti-mouse CD8 antibody (1:100) for 12 hr at 4°C, washed with PBS horse serum buffer for 3 hr at 4°C, stained with biotinylated anti-rat IgG (1:50) for 12 hr at 4°C, and washed overnight in 1× PBS at 4°C. Brains were sequentially treated with Vectastain ABC kit (Vector Laboratories), stained with a Vector peroxydase substrate DAB kit, washed with PBS for 3 hr at 4°C, and incubated overnight in 1% glutaraldehyde at 4°C. Samples were transferred to 2% glutaraldehyde for 45 min, transferred to 1% osmium tetroxide in ddH2O for 1 hr, and stained en bloc in 2% aqueous uranyl acetate for 1 hr. Brains were dehydrated and embedded in araldite. Ribbons of thin (~50 nm) sections were obtained with a Leica Ultracut UCT5 Ultramicrotome and examined on a Phillips CM 12 TEM.

Ultrastructural Quantification
To assay bouton area, 25,000 images were used for comparing wild-type and dfmr mutant MARCM mushroom body clones. Areas were measured with National Institutes of Health (NIH) ImageJ software. To assay synaptic vesicle density, 25000 images were used. In dfmr mutant MARCM mushroom body clones, 2 μm circles were centered over the labeled cell and assays made of labeled bouton (dfmr mutant) and unlabeled boutons (control) in a nearest-neighbor comparison. Total bouton area and synaptic vesicle-occupied bouton area were measured with NIH ImageJ software.

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